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Characterising the novel activation of \textit{wt1b} in the notochord damage response of zebrafish larvae

Juan Carlos López-Báez

PhD by Research
University of Edinburgh
2014
Declaration

This thesis has been entirely composed by me and I have carried out all experiments, except where otherwise stated. This work has not been submitted for any other degree or professional qualification.

Juan Carlos Lopez-Baez, July 2014
Abstract

The notochord is the defining structure of all chordates. A semi-flexible elongated tube of cells, it forms along the central axis of the embryo and provides axial support during development. It also acts as a signalling centre during early embryogenesis, controlling the patterning of a number of tissues and establishing the early body axis of the embryo.

In vertebrates, the function of the notochord expands beyond early development. It creates morphogenic gradients for the patterned formation of the vertebral bodies and, in adults, the remnants of the notochord form the nucleus pulposus, a gel-like structure with an integral role in the distribution of vertebral pressure in the intervertebral disc. Little is known about how the notochord copes with damage during embryogenesis, but degeneration of the nucleus pulposus can lead to debilitating spinal disorders.

In this thesis, I use a zebrafish model system to present new data that describes the cellular behaviours associated with how the notochord copes with external damage and how this damage can influence the future development of the vertebrae. I have uncovered a novel damage response in the notochord of zebrafish larvae and characterised the morphogenetic changes involved in the process using transgenic fluorescent lines. I have explored the damage in the context of the Wilms’ Tumour 1 (Wt1) gene, a vertebrate-conserved transcription factor, which has recently been associated with several regenerative responses, and discovered that one of its zebrafish orthologues, wt1b, becomes upregulated in the notochord damage response.

I have used fluorescent confocal imaging and immunohistochemistry to present new evidence that shows that upon injury, the outer notochord sheath cells upregulate the expression of wt1b. Additionally, I have used time-lapse microscopy
to show that damage to the notochord induces novel morphological changes in the injured organ, which include the loss of cellularity of the inner vacuolated cells and the movement of the wt1b-positive outer sheath cells into the injured lumen.

Long-term imaging experiments have also demonstrated the capacity of the notochord to heal the damage over time, which ultimately leads to the formation of an extra, smaller vertebra in the wounded area. Skeletal staining of these fish has revealed a previously unknown putative cartilage switch at the site of damage, which leads to the formation of the new vertebral body. This finding has been supported by the microarray analysis of the injured area, which shows the unexpected de-novo expression of cartilage markers at the site of damage.

The work in this thesis identifies for the first time an endogenous repair mechanism in the notochord of zebrafish larvae and describes the cellular, genetic and molecular processes controlling this novel wt1b-associated damage response.
Acknowledgements

First and foremost I would like to thank my supervisors, E. Elizabeth Patton and Nicholas Hastie, who have supported me for the last (slightly more than) 4 years. They first accepted me into their labs in the final rotation of my master and then agreed to let me continue into a PhD with them. Their ideas, suggestions and their belief in me have helped me throughout the PhD and have been pivotal to the success of this project. I want to thank them for all those countless meetings and the interesting science (and life) talks that underwent in them. I also want to thank the MRC for their sponsorship and the whole of the IGMM for providing the best environment possible to carry out a PhD.

I would also like to thank the Patton lab, past and present, especially Zhiqiang, who has shared his wisdom on molecular biology with me in numerous occasions and always patiently explained things. Also Witek, who with his injection wizardry has helped me create several of the lines in this thesis and, in the final stages of the PhD, has helped me with experiments. He has been a great friend and comrade and listened to me and shared jokes from day one. Also a big mention goes to Karthika, who has skilfully and methodically cared for the fish and always been there whenever I have needed any advice or help with them.

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This thesis is dedicated to my late grandmother Pepi. She fuelled my passion for science from an early age and has been one of the biggest influences in my life. Abuela, this thesis wouldn’t have happened if it weren’t for you.

*Te quiero mucho Oma Pepi*
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>BCV</td>
<td>before caudal vein</td>
</tr>
<tr>
<td>BOP</td>
<td>basioccipital articulatory process</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>col2a1a</td>
<td>collagen type 2, alpha 1a</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenyindole</td>
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<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleotide</td>
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<tr>
<td>dpa</td>
<td>days post amputation</td>
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<tr>
<td>dpi</td>
<td>days post injury</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
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<td>green fluorescent protein</td>
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<tr>
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<td>hours post injury</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>IVD</td>
<td>intervertebral disc</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>real time qPCR</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBSTw</td>
<td>TBS tween</td>
</tr>
<tr>
<td>TF</td>
<td>tail fin</td>
</tr>
<tr>
<td>TN</td>
<td>tip of notochord</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activation sequence</td>
</tr>
<tr>
<td>YS</td>
<td>yolk sac</td>
</tr>
<tr>
<td>wt1a/b</td>
<td>Wilms tumour 1a and b</td>
</tr>
</tbody>
</table>
CHAPTER 1:

Introduction
1.1. Wilms tumour 1: A multifaceted gene with opposing roles in development and disease

Wilms tumour 1 (WT1) is quite a unique gene. Discovered almost a quarter of a century ago, it was first identified as a mutated tumour suppressor in its eponymous paediatric cancer (Call et al., 1990; Gessler et al., 1990). However, since its identification, new insights have emerged into its cellular and physiological functions inside the body, with its roles expanding beyond tumor protection into development, cancer progression and adult homeostasis (Figure 1.1B).

Human pathologies and animal studies have helped to uncover the essential function of WT1 in development. Soon after its discovery, WT1 mutations were identified in a variety of other congenital disorders, which resulted in kidney malformations and gonadal dysgenesis (reviewed in Miller-Hodges & Hohenstein, 2011). These discoveries highlighted the fundamental roles of the WT1 gene in the formation of the urogenital system and were complemented with RNA identification analysis that validated the expression of the gene in these organs during development (Pritchard-Jones et al., 1990).

Animal model studies have extended the knowledge of its developmental functions and shown that WT1 has prominent roles in early kidney formation, nephrogenesis, gonadal survival and gender determination (Kreidberg et al., 1993; Hammes et al., 2001; Davies et al., 2004; Wilhem & Englert, 2002). Additionally, other studies have also uncovered new functions beyond urogenital development, with WT1 been shown to be required for the formation of several other tissues, including the heart, the spleen, the adrenal glands, the liver and the olfactory system (Zhou et al., 2008; Herzer et al., 2009; Moore et al., 1999; Ijpenberg et al., 2007; Wagner et al., 2005).

Outside development, a growing body of literature has begun to associate the expression of WT1 with the tumorigenic progression of certain cancers, including leukaemia, breast cancer and other solid tumours (Miller-Hodges & Hohenstein,
2011; Huff et al., 2011). In these, the de-novo activation of WT1 has been hypothesised to help cancerous cells to bypass senescence and induce metastasis, defining it as a marker for poor disease prognosis (Miller-Hodges & Hohenstein, 2011). The tumorigenic role of WT1 in these cancers has underlined its likely oncogenic function in adult tumours, resulting in its identification as a potential novel target for immunotherapeutic treatment (Sugiyama, 2010).

More recently, the role of WT1 in post-embryonic tissues has also been explored (Chau et al., 2011). It is known that only a small percentage of cells in the body continuously express Wt1 into adulthood, including the glomerular cells of the podocytes, the Sertoli and granulosa cells of the male and female reproductive systems, the mesothelium of visceral organs and a small percentage of cells in the bone marrow of long bones (Chau & Hastie, 2012). Our lab has used a tamoxifen-induced conditional deletion system to systematically abrogate the expression of WT1 in adult mice (Chau et al., 2011). This genetic ablation caused an unexpected significant phenotype in the injected animals. These showed a pronounced loss of fat and a reduction in bone integrity, as well as severe glomerulosclerosis, spleen hypertrophy, pancreatic degeneration and defects in erythropoiesis (Chau et al., 2011; Chau et al., 2013). These results uncovered previously unidentified roles for Wt1 in adult tissue homeostasis and revealed new primary functions for the gene beyond its essential role in embryonic development.

The functional heterogeneity of WT1 could intrinsically be related to the complexity of its locus. The gene encodes a vertebrate-conserved C2H2 zinc finger transcription factor, which, in mammals, has been predicted to code for over 36 different isoforms (Hohenstein & Hastie, 2006; Figure 1.1A). Its protein diversity stems from the number of alternative start codons, splicing acceptor sites and RNA editing sequences that are present within its 10 exons locus (Figure 1.1A). In reality, however, only two of its isoforms stand out as the most prominently expressed (Haber et al., 1991). These are the ±KTS isoforms, which utilise an alternative splice
acceptor between exon 9 and 10 to either insert or exclude a lysine-threonine-serine (±KTS) sequence from the translated peptide (Figure 1.1A).

The last 4 exons of the Wt1 gene encode the most distinctive feature of the WT1 protein, its 4 zinc finger domains (Figure 1.1A). These are essential for its function, as they mediate the DNA and protein interactions of the WT1 peptide (Hastie, 2001). The insertion of the KTS sequence disrupts the conformational structure of the third and fourth zinc fingers of the WT1 protein, which substantially affect the DNA binding capabilities of the +KTS variant (Laity et al., 2000; Figure 1.1A). This inclusion/exclusion interchange means that -KTS protein acts as the main transcriptional regulator of its targets, while the +KTS variant has been proposed to act beyond its transcriptional regulatory functions (Hastie, 2000). In fact, the cellular distribution of this variant inside the cell and its in-vitro association with RNA splicing factors has suggested that this variant may act in the post-transcriptional control of RNA processing (Larsson et al., 1995; Davies et al., 1998; Hastie, 2000).

The importance of both splice isoforms has been validated with the use of knockout animals. Homozygous mutants for each variant have uncovered the individual requirement of both ±KTS forms in renal development and gonadal differentiation. They have shown that the -KTS form predominantly inhibits early kidney formation, glomerulogenesis and gonadal cell survival, while the +KTS form has a more predominant role in the formation of the podocytes and in the acquisition of a male sex phenotype (Hammes et al., 2001). Additionally, separate knockout studies have shown that other variants, such as those using the exon 5 splice site or the alternative upstream start codon CTG, do not display any overt developmental phenotypes and their adults are viable and fertile (Natoli et al., 2002; Miles et al., 2003). Evolutionary studies of the WT1 gene have shown that only the ±KTS
Figure 1.1. The Wilms tumour 1 gene. (A) The genetic locus of the mammalian Wt1 gene contains 10 exons with a number of splicing acceptor sites, alternative start codons and RNA editing sites that confer the gene with the ability to putatively transcribe over 36 different protein isoforms (red boxes). Green boxes highlight the key interacting domains of the protein, including its Zn finger region coded by the last 4 exons of the gene. Picture extracted from Hohenstein & Hastie, 2006. (B) Boxes highlighting the variety of functions of the Wt1 gene in development, paediatric malignancies, adult homoestasis and adult cancers and pathologies.
isoforms are conserved amongst vertebrates, which demonstrates the dominance of these variants in the regulation of WT1 function (Kent et al., 1995).

1.1.1. Wt1 in development: A tissue specific control of the EMT/MET process

The functions of WT1 in development were first uncovered through its pathological mutation in congenital malignancies. As mentioned above, apart from its role in the development of Wilms tumour, mutations in the WT1 gene have also been associated with other childhood urogenital disorders and gender reversal pathologies, including Denys-Drash, Fraser and WAGR syndromes (reviewed in depth in Miller-Hodges & Hohenstein, 2011).

Mouse model studies have uncovered the conserved role of the gene in mammalian development and have revealed new insights into its upregulation in other non-genitourinary organs, including the heart, the spleen, the mesothelial layer of visceral organs, the diaphragm, certain areas of the brain, the spinal cord and the limbs (Armstrong et al., 1993; Moore et al., 1998a; Moore et al., 1999). The importance of Wt1 in these tissues has also been explored using knockout animals, with homozygous Wt1 mutants showing renal agenesis, disturbed gonadal development and dying at mid-gestation due to pericardial bleeding from defects in the vascular development of the heart (Kreidberg et al., 1993; Moore et al., 1999). Additionally, new Wt1 mutants created to bypass the heart phenotype have uncovered the pivotal roles of the Wt1 gene in late embryogenesis, with homozygous knockout animals showing specific disruptions in the development of the adrenal glands, the spleen, the ganglion cells of the eye, the olfactory system and the liver (Moore et al., 1999; Herzer et al., 1999; Wagner et al., 2002; Ijpenberg et al., 2007).

The role of Wt1 in the kidneys and the heart has been studied in more detail than in any of the other organs. In these, mutants and transgenics have uncovered one of
the most striking features of the Wt1 gene; its ability to regulate two mutually opposing processes in a tissue-specific manner: a mesenchymal to epithelial transition (MET) in the kidneys, and an epithelial to mesenchymal transition (EMT) in the heart.

During renal development, the Wt1 gene expresses in a multistep manner. It first expresses in the early kidney, where it is thought to act as an anti-apoptotic signal for the establishment of the intermediate mesoderm and its derivative, the metanephric blastema, and then increases its expression in the later stages, at the onset of nephrogenesis and the late formation of the glomerulus (Pritchard-Jones et al., 1990; Armstrong et al., 1993; Krediberg et al., 1993).

The nephrons form via the recurrent interaction between the mesenchymal metanephric blastema and the epithelial uretic bud (Chau & Hastie, 2012; Little & McMahon, 2012). During the early stages of nephrogenesis, the uretic bud invades the mesenchyme of the blastema and, as it does this, it is induced to branch by signals coming from the mesenchymal cells (Chau & Hastie, 2012; Little & McMahon, 2012). The branching ducts then reciprocate the signal and direct the condensation of the metanephric blastema around the ends of their ducts, forming the cap mesenchyme, which will provide the lineage-committed progenitors required for nephrogenesis (Little & McMahon, 2012).

At the onset of nephron formation, mesenchymal cells of the cap mesenchyme drastically increase their levels of Wt1, leading to the initiation of an MET process that will lead to the formation of the epithelial renal vesicle and its subsequent comma-shaped and S-shaped bodies (Chau & Hastie, 2012). The increase in Wt1 expression leads to the upregulation of Wnt4, the master regulator of nephrogenesis, which has been shown to be directly downstream of Wt1 during renal development (Sim et al., 2002; Davies et al., 2004; Essafi et al., 2011).
In the later stages of nephrogenesis, the expression of the \textit{Wt1} gene becomes localised to the most distal part of the forming nephron, where it guides the morphogenetic development of the podocytes (Chau \textit{et al.}, 2011; Little & McMahon, 2012). It then remains active in these cells into adulthood, where it regulates the homeostatic balance of the podocytic cells (Chau \textit{et al.}, 2011).

In the heart, the role of \textit{Wt1} is contrastingly different to that of the kidney. During its early development, \textit{Wt1} is expressed in the epicardium, the thin layer of mesothelial cells that forms around the heart (Moore \textit{et al.}, 1999). These cells envelop the organ and have a prominent role in its development, providing the progenitor cell population necessary for the formation of the vascular smooth muscle, cardiac fibroblasts, endothelial cells and a subset of cardiomyocyte cells (Zhou \textit{et al.}, 2008; Cai \textit{et al.}, 2008).

These progenitor cells (known as epicardium-derived progenitor cells or EDPC) are created via the activation of an EMT in a subset of cells of the epicardium. These \textit{Wt1}-positive cells then detach from the mesothelium and invade the forming heart, where they contribute to its development. \textit{Wt1} becomes upregulated in these epicardial cells prior to the EMT and its expression is maintained in the migrating EDPCs, only to be switched off in terminally differentiated cells (Martinez-Estrada \textit{et al.}, 2010). The Hastie lab and others have shown that \textit{Wt1} is required for the cardiac EMT and its targeted deletion leads to a corrupted heart development, causing thinner ventricular walls and a lack of vascularity in the heart (Wagner \textit{et al.}, 2005; Zhou \textit{et al.}, 2008; Martinez-Estrada \textit{et al.}, 2010; von Gise \textit{et al.}, 2011).

The Hastie lab has also explored the specific function of \textit{Wt1} in heart development and identified that it regulates EMT by directly causing the upregulation of the mesenchymal marker \textit{Snai1} and downregulating the epithelial marker \textit{Cdh1} (E-cadherin; Martinez-Estrada \textit{et al.}, 2010). Interestingly, another recent paper from our lab discovered that this \textit{Wt1} activation of EMT was achieved via the direct
downregulation of Wnt4 in the epithelial cells, strengthening the concept of a dichotomous role for Wt1 in MET and EMT processes (Essafi et al., 2012).

Another prospective developmental EMT regulated by Wt1 has been uncovered during liver development (Ijpenberg et al., 2007). Homozygous Wt1 mutants that live past midgestation (Moore et al., 1999) display an aberrant hypoplasia in the liver, which leads to the development of a reduced organ and the inability to fully form its lobes (Ijpenberg et al., 2007). Wt1 is known to express in the coelomic epithelium of the liver, a thin layer of cells that covers the visceral organ and is akin to the epicardial mesothelium (Moore et al., 1998). By using LacZ Wt1 transgenics, Ijpenberg and colleagues showed that the Wt1-positive cells of the coelomic epithelium were able to migrate into the forming hepatic organ and contribute to the establishment of the stellate cell population (Ijpenberg et al., 2007). They also showed that homozygous mutants had considerably reduced numbers of stellate cells and exhibited defects in hepatoblast proliferation. This led them to postulate that these migrating Wt1-expressing cells could be providing progenitor stem cells for the stellate cell development of the organ. This concept has been further strengthened by lineage tracing analysis, which has shown that Wt1 expressing cells of the mesothelium give rise to the hepatic stellate cells and the perivascular mesenchymal cells of the liver during its development (Asahina et al., 2011).

1.1.2. Wt1 and its prospect role in regeneration

There is an intensive search for organ-specific stem cell populations that could be used for the therapeutic treatment of pathologically injured organs. The prominent role of Wt1 in the developmental generation of progenitor stem cells in the heart and the liver, offers the tantalizing possibility that the gene could have a similar function in the provision of multipotent stem cells beyond embryogenesis (Zhou et al., 2008; Ijpenberg et al., 2007). Most specifically, it may have a function in the
activation of potential reparative or regenerative responses in each organ after damage. Indeed, it has been shown that Wt1 becomes upregulated in the regenerative responses of both organs in mammals and other vertebrates (Zhou et al., Kikuchi et al., 2011; Li et al., 2013) and that Wt1-derived cells can contribute to the rebuilding of each organ (Smart et al., 2011; van Wijk et al., 2012; Li et al., 2013).

In the mammalian heart, the expression of Wt1 is maintained throughout embryogenesis, but decreases to almost non-existent levels post development (Zhou et al., 2011; van Wijk et al., 2012). Cardiac damage causes the re-induction of embryonic markers in the epicardium, including the upregulation of Wt1, which has been viewed as a possible stem cell source required for heart repair. This feature has been explored by several studies, but the results have been partly inconclusive.

All studies concur that upon injury, the epicardial layer of the heart is lost around the damaged area and the injury leads to the re-upregulation of Wt1, and other developmental markers, in the intact epicardium, especially at the border zone between the injured and healthy tissue (Zhou et al., 2011; van Wijk et al., 2012). Within days of the injury, the Wt1-positive mesothelial layer then envelops the site of damage and induces the strong proliferation of its cells, which leads to the thickening of the epicardium and the subepicardial mesenchyme in the injured area (Zhou et al., 2011; van Wijk et al., 2012; Smart et al., 2011). It is at this point, where the studies differ in establishing the contribution of these cells to the cardiac regenerate. Some studies have shown that the epicardial Wt1-expressing cells can then invade the myocardium and generate both endothelial and cardiomyocyte cells in the regenerating organ (van Wijk et al., 2012; Smart et al, 2011). However, a similar study has challenged this concept and suggested that these cells release paracrine signals that regulate the regenerate, but themselves do not contribute to the cardiomyocyte lineage (Zhou et al., 2011). This last option has also been proposed in zebrafish, where the epicardial cells have been shown to lead to the
formation of new vasculature, but do not contribute to the formation of new cardiomyocytes during cardiac regeneration (Lepilina et al., 2006; Schnabel et al., 2011).

The effect of the mesothelium in the regeneration of the liver has been less studied. A recent paper has shown that new cells originate from the liver mesothelium upon injury, which unlike the adult epicardium maintains its Wt1 expression into adulthood. These contribute to the hepatic regenerate after chemical or mechanically induced fibrosis, but cannot be induced following hepatic lobe resectioning, highlighting the injury-specificity of the response (Li et al., 2012). The newly generated mesothelial cells were shown to contribute to the regeneration of the stellate cell population and the myofibroblasts of the liver, acting as putative progenitor/stem cells during this process (Li et al., 2012).

The central role of the Wt1 gene in the generation of progenitor/stem cells during development and its potentially functional conservation during tissue regeneration, encouraged the idea that the gene could have more widespread roles in other regenerative or reparative responses. Additionally, its vertebrate conservation made it an ideal candidate for cross-species investigation.
1.2. Tissue regeneration and repair: two similar responses to damage with different outcomes

Tissue regeneration is the process by which organs and tissues can reconstitute their original structure after it has been lost or damaged. It exists as two interlinked forms, a physiological process, necessary for the maintenance of the high cellular turnover of certain organs, and a reparative epimorphic process, acting in response to pathological damage.

As mammals, we are limited in the amount of regeneration that we can achieve. During embryogenesis and shortly after birth, mammals have the ability to regenerate complex tissues, but this ability is progressively lost after birth and as we age (Kumar et al., 2010). For example, human and mouse foetus in the first two thirds of development have the remarkable ability to heal their skin without scarring, whilst mouse embryos and neonates display an unusual capacity to regenerate their hearts after damage (Yanas, 2005; Wulf et al., 2011; Porrello et al., 2011). Other examples include the ability of children to regrow their fingertips after amputation, a feat also observed in fetal mice (Poss et al., 2010; Han et al., 2003). However, as adults, only a handful of our tissues have the ability to truly regenerate and in most cases do not show the same regenerative capacity seen in lower animals (Poss et al., 2010).

Our regenerative ability depends on the proliferative capacity of the parenchymal cells of our organs and the presence of dividing or quiescent stem cells within them. Considering this, all tissues in the body can be categorised into 3 different groups, depending on the proliferative potential that they exhibit. Highly proliferative tissues are known as continuously dividing or labile tissues; rarely dividing tissues that can rapidly respond to stimuli are known as quiescent or stable tissues; and tissues with terminally differentiated cells that are removed from the cell cycle are known as non-dividing or permanent tissues (Kumar et al., 2010).
Labile tissues undergo life-long physiological regeneration, which is required for the maintenance of a tissue’s biological integrity. Examples of labile tissues are the skin epidermis or the haematopoietic system, which harbour highly proliferating stem cell populations. These tissues exhibit the strongest regenerative capacity and are the most responsive to external damage due to their biological nature.

Stable tissues represent organs with quiescent stem or parenchymal cell populations that rarely replicate under physiological conditions, but can be primed into accelerated divisions in response to stimuli, such as tissue damage. This group includes tissues such as the liver or the kidney and organs with a high number of fibroblast, endothelial or skeletal cells. These tissues can aptly respond to moderate external damage and heal with a significant amount of regeneration depending on the insult.

Probably the most notable example of this group is the liver, which has the ability to reform after the surgical resection of up to 70% of its tissue (Fausto, 2000). However its regeneration is not entirely conventional. The loss of tissue does not generate the regrowth of the missing section, but instead triggers a compensatory growth in its remaining lobes. Additionally, studies have shown that the growth is achieved via the induced proliferation of its differentiated parenchymal cells, without the need for the expression of multipotent progenitors (Fausto & Campbell, 2003).

Lastly, the non-dividing/permanent tissues are organs with mostly terminally differentiated cells that are removed from the cell cycle and are unable to divide further. These include tissues that may contain a population of low proliferating stem cells, such as the central nervous system (Conover & Notti, 2008), and organs with a reduced ability to regenerate after injury, such as the heart (Zhou et al., 2011; van Dijk et al., 2012).
The level of regeneration of a tissue is not only dependent on its proliferative capacity, but also in the amount of damage incurred (Kumar et al., 2010). For example, labile tissues would normally regenerate an injury as long as their resident stem cell population have not been compromised or their extracellular matrix (ECM) has not been affected (Kumar et al., 2010; Figure 1.2). These are paramount to the mammalian regenerative process as they provide the source for new cells to be generated and the structural scaffold for the new cells to align, respectively. Additionally, the ECM contains some of the signals, growth factor and chemokines necessary for the proper arrangements of the cellular layers and the acquisition of specific cellular fates.

It is during this destructive damage that a reparative response is activated in combination with the regenerative signal. This leads to the abnormal regeneration of the affected tissues, causing deposition of scar tissue and the dysfunctional regrowth of the injured area (Figure 1.2). This kind of wound healing response is the more common mechanism of less proliferative tissues, where regeneration and repair act simultaneously on the damage.

Tissue repair is the most common damage response in mammals and it is normally coupled with a limited amount of regeneration. The hallmark of repair is the deposition of collagenous fibres at the site of injury, which replaces the cellular structures affected by the damage and leads to the formation of scar tissue as the wound heals (Yannas, 2005; Figure 1.2). The deposition of collagenous tissue is coupled to an orchestrated array of cellular responses (immune, fibroblast, myoblast and endothelial cells), molecular signals and active ECM remodelling (eg. wound contraction) that leads to the repair of the affected area (Figure 1.2; reviewed in Kumar et al., 2010).
Figure 1.2. Physiological differences between regeneration and repair. Tissue injury can lead to regeneration or repair depending on the type of tissue and the kind of insult received by the organ. Chart adapted from Kumar et al., 2010.

<table>
<thead>
<tr>
<th>Tissues used and available tools</th>
<th>Hydra</th>
<th>Planarians</th>
<th>Zebrafish</th>
<th>Xenopus laevis</th>
<th>Axolotl</th>
<th>Newt</th>
<th>Mouse</th>
<th>Drosophila melanogaster/midgut, germ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key tissues assessed in regeneration studies</td>
<td>Whole animal</td>
<td>Whole animal, germ cells, nervous system</td>
<td>Fins, heart, retina, spinal cord, hair cells</td>
<td>Tail, leg, eye, heart, skin, gut</td>
<td>Tail, limb, heart, retina</td>
<td>Limbs, face, heart, tail, spinal cord, retina</td>
<td>Blood, skeletal muscle, liver, pancreas, peripheral nerve, skin, gut epithelium, germ cells</td>
<td>Micronet, germ cells</td>
</tr>
<tr>
<td>Genome sequencing finished or ongoing</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>–</td>
<td>–</td>
<td>✓</td>
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<tr>
<td>Knockout by homologous recombination</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>RNAi</td>
<td>✓</td>
<td>✓</td>
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<td>–</td>
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<td>✓</td>
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<td>Transgenesis</td>
<td>✓</td>
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<td>✓</td>
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<tr>
<td>Recombinase-driven lineage tracing</td>
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<td>–</td>
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<td>–</td>
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<td>–</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Cell transplantation or tissue grafting</td>
<td>✓</td>
<td>✓</td>
<td>–</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Forward genetics</td>
<td>–</td>
<td>–</td>
<td>✓</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>✓</td>
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<tr>
<td>Potential for real-time imaging of regeneration</td>
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<td>✓</td>
<td>✓ (in vitro)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>✓</td>
</tr>
<tr>
<td>Stage- or age-dependent regenerative capacity</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>✓</td>
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<td>–</td>
<td>✓</td>
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</table>
It is this preference for repair rather than regeneration that has encouraged the search for the key elements that control this skewed balance in tissue healing. For this, scientists have looked at lower animals with stronger predisposition for regenerative responses, in order to assess the genetic, molecular and cellular controls that regulate both regeneration and repair. It is widely assumed that our inability to regenerate is due to a gain of function event associated with our survival, which made us more prone to repair (Bedelbaeva et al., 2010). This means that the key to regeneration could be within our genomes and just needs to be reactivated to confer us with the potential to faithfully reconstruct our lost or damaged organs.

1.2.1. Animal models for the study of regeneration

The study of regeneration in animal models dates back to the 18th century, when Abraham Trembley first noticed the regenerative ability of a small fresh water cnidarian polypop, the Hydra (Tanaka & Reddien, 2011). He discovered how this small distant metazoan had the capacity to regrow its head and foot poles after it had been sectioned in half, effectively creating 2 new individual from each subdivision (Tanaka & Reddien, 2011).

Since then, new regenerative systems have emerged for the study of regeneration. From pluripotent small fresh water invertebrates, such as the aforementioned Hydra or the planarian worm, to more defined vertebrate models, such as salamanders, zebrafish, Xenopus tadpoles or even mice (Tanaka & Reddien, 2011; Table 1.1).

The use of these models has allowed the dissection of the cellular, molecular and genetic factors guiding the regenerative process and helped to develop most of the current knowledge that exists on tissue regeneration (Poss et al., 2010; Tanaka &
Reddien, 2011; Table 1.1). For instance, the use of animal models has helped to identify the modes in which tissues can respond to external damage and trigger stem cell activation and tissue regrowth (Figure 1.3A). These include the identification of new organ-specific resident stem cell populations; the ability of certain tissues to induce compensatory proliferative growth; the discovery of novel dedifferentiation processes for the generation of multipotent progenitors; and the transdifferentiation of differentiated and dedifferentiated cells to other cellular fates (Figure 1.3A; Tanaka & Reddien, 2011). These discoveries in lower animals have complemented human studies and, in some cases, proposed new investigative leads or prospective therapeutic regenerative approaches.

The Hydra and the planarian worm have helped in the study of the most basic understanding of adult cell pluripotency and organism-wide regeneration. These animals have the unequal ability to recreate whole individuals from small clumps of cells, with the Hydra relying on cell to cell interactions for the development of its pluripotent potential and the planarian worm displaying single-cell based pluripotent capacity (Tanaka & Reddien, 2011; Table 1.1). Examples of this are highlighted by the Hydra’s ability to form new polyps from the reaggregation of disassociated cells or the ability of single pluripotent cells to restore regenerative competence and contribute to all the cell lineages of an irradiated worm in single cell transplantation experiments in the planarian worm (Technau et al., 2000; Wagner et al., 2011). Additionally, the Hydra has been used as a model for the study of transdifferentiation, as it can reprogram its epithelial cells to acquire new cellular fates after amputation without the need for cell proliferation (Sanchez-Alvarado, 2006; Holstein et al., 1991)

Vertebrate models, on the other hand, have been used for the study of tissue-specific regeneration and repair (Poss et al., 2010). The urodele amphibians (which include the Axolotl and the newt) have become classic systems for the study of appendage regeneration, as they can fully regrow their limbs after full or partial
amputation (Tanaka & Reddien, 2011). However, their regenerative capacity is not limited to the limb, as they exhibit some of the most dramatic regenerative responses seen in vertebrates, with the ability to regenerate their eyes, their hearts, their brains and their spines, as well as other tissues (Sanchez-Alvarado, 2006; Table 1.1).

Limb regeneration, however, has become the most studied process in these animals and has been used as an experimental paradigm for the study of regeneration in complex tissues. The number of tissues involved, the induction of cellular dedifferentiation, the formation of a regeneration blastema and the preservation of positional memory in the cells of the regenerate has attracted extensive research into the process. It is known that upon amputation, the salamander limb forms an epithelial layer over the wound, which closes the injury and releases signals that induce the dedifferentiation of the cells in the stump (Poss et al., 2010; Figure 1.3A and 1.3B). These dedifferentiating cells will form the multipotent progenitor blastema, which is essential for the regeneration response and will drive the growth of the new tissue (Figure 1.3B).

The development of new transgenic tools has greatly helped to dissect the genetic and cellular interactions controlling these events (Tanaka & Reddien, 2011; Table 1.1). This has resulted in the recent identification of the lineage restrictive nature of the response, which has demystified the idea that one multipotent cell population could give rise to all the different cells in the limb (Kragl et al., 2009). In fact, Kragl and colleagues discovered that the blastema was made up of a heterogenous population of cells that arose from the dedifferentiation of a multitude of cells from the tissues in the limbs of adult Axolots (Kragl et al., 2009). Using cell fate analysis and transplantation experiments they showed that the specific progenitor cells formed via the dedifferentiation of a certain tissue, mostly led to the formation of that tissue in the regenerate in a lineage-restricted manner (Kragl et al., 2009). This
is a feat that has similarly been observed in zebrafish and *Xenopus* larvae (see below).

Similarly to the urodeles, the adult zebrafish has begun to be used in the study of appendage regeneration. Their tail fins observe a similar degree of complexity as the urodele limbs and regenerate in a phenotypically similar manner, with the formation of a lineage-restricted blastema at the site of damage (Knopf *et al.*, 2011; Tu & Johnson, 2011). Moreover, both zebrafish adults and larvae show a regenerative competence similar to that of urodele amphibians in several of their tissues (Gemberling *et al.*, 2013) and have the added advantage that are amenable to forward genetics, Cre-LoxP conditional manipulation and real-time imaging, unlike other vertebrate models (Poss *et al.*, 2010; Table 1.1). These advantages and more details on the benefits of using zebrafish as a model system are further discussed in the next section.

Lastly, two age-restricted animal systems have also been utilised for the study of regeneration. These are the *Xenopus* frog and the mammalian mouse (Poss *et al.*, 2010; Table 1.1). Unlike urodeles or zebrafish, their regenerative capabilities are restricted to the early stages of their development, with *Xenopus* frogs losing their regenerative capacity after larval metamorphosis and mice showing predisposition for regeneration during foetal development and neonatal stages (Poss *et al.*, 2010). They have, however, shown a variety of regenerative responses at these stages with *Xenopus* tadpoles regenerating their tails, limbs and retinas in much the same way as urodele amphibians (Gargioli & Slack, 2004; Slack & Chen, 2008) and mice showing scarless wound healing, heart regeneration and digit regrowth after injury or amputation (Muneoka *et al.*, 2008). Additionally, the mouse model has been used to study possible therapeutic treatments for tissue regeneration, such as the chemical induction of heart regeneration in models of myocardial infarction (Smart *et al.*, 2011).
Figure 1.3. Types of regenerative responses.
Finally, it is worth considering that any reparative and regenerative responses that could be identified in lower vertebrates would have to be validated and refined in mammals, before they could serve as prospective human therapeutic treatments. This means that the mouse system has an uttermost importance in regeneration studies and will play a significant role in the development of the new assays that will shape the future of regenerative medicine.

1.2.2. Zebrafish: A vertebrate system for the study of tissue regeneration

Zebrafish have emerged as an increasingly popular model for the study of tissue regeneration. They exhibit a number of tissue-specific regenerative responses and are susceptible to a variety of genetic and molecular approaches, including forward and reverse genetics, chemical screens and transgenesis (Table 1.1; reviewed in depth in Lawson & Wolfe, 2011). Moreover, their semi-transparent bodies make them suitable for real-time in vivo fluorescent imaging, which can be complemented with the growing range of experimental protocols that can be used in gene and protein expression studies (Gemberling et al., 2013).

Logistically, zebrafish have proven to be advantageous because of their small size, their fast external embryonic development, their ease to breed under laboratory conditions and the high number of fertilised eggs that can be acquired from a single
mating (Gemberling et al., 2013). Additionally, the zebrafish genome has been fully decoded and shown to have a high degree of genetic and sequence homology with the mammalian genome (Kettleborough et al., 2013).

Zebrafish can also regenerate their appendages, which exhibit a similar tissue complexity as the urodele limb (Johnson & Weston, 1995; Poss et al., 2002a; Gemberling et al., 2013). Their tail fins are epithelial structures composed of innervated and vascularised rays of dermal bone, interspaced by a similarly vascularised mesenchyme of connective tissue (Becerra et al., 1993; Figure 1.4). Each dermal ray is made of two opposing C-shaped hemirays surrounded by layers of osteoblast, which encase a matrix of fibroblasts, pigment cells, neurones and blood vessels within them (Gemberling et al., 2013; Figure 1.4). Studies have shown that fin amputation leads to the fast epithelisation of the open wound and the induction of a regeneration blastema at the end of each ray, which drives the regrowth of the fin tissues and the formation of a morphologically identical fin 2 weeks after the initial damage (Tanaka & Reddien, 2011).

Zebrafish also have the capacity to regenerate other tissues, including their spinal cords (Becker et al., 1997), their livers (Sadler et al., 2007), their kidneys (Diep et al., 2011), their optic nerves (Bernhardt et al., 1996) and their hearts (Poss et al., 2002b). The heart, in particular, has attracted a high amount of attention, due to its dumbfounding capacity to regenerate after surgical resectioning, cryoinjury and cardiomyocyte ablation (Poss et al., 2002b; Leipilina et al., 2006; Jopling et al., 2010; Schnabel et al., 2011; Wang et al., 2011).

The experimental amenability of zebrafish has been used in the exploration of its regenerative processes, allowing researchers to uncover some of the genetic and cellular interactions behind their epimorphic regrowth (Gemberling et al., 2013). For example, forward genetic screens, such as those using random N-ethyl-N-
**Figure 1.4.** The structure and cellular composition of the adult zebrafish caudal fin. The tail fin is divided into metameric bone segments (bone rays) interspaced by mesenchyme. Each bone ray is made of two opposing concave dermal bones surrounded by a population of osteoblast and encasing a enervated and vascularised mesenchyme of pigment cells (melanocyte, iridophores, xanthophores) and dermal fibroblasts. Picture taken from Tu & Johnson, 2011. Scale bars = 0.2 mm.

**Figure 1.5.** Wt1 in zebrafish. The zebrafish paralogues, wt1a and wt1b (orange and green respectively) show a high degree of protein sequence conservation with their human parologue and the wt1 gene of other teleost fish species. Tree created by Philippe Gautier (MRC Human Genetics Unit). (B) The wt1a and wt1b fluorescent transgenic lines show a high degree of homology with the endogenous RNA expression pattern of each gene in the pronephros over the first 65 hours of life. Abbreviations: im = intermediate mesoderm; glo = glomerulus; tu = tubules; ep = exocrine pancreas. Picture adapted from Bollig et al., 2009.
nitrosourea (ENU) mutagenesis (Haffter et al., 1996), and positional cloning have helped to dissect the genetic networks controlling the time-restricted stages of fin regeneration, including its epithelialisation, blastema formation and tissue outgrowth (Poss et al., 2002a; Nechiporuk et al., 2003; Makino et al., 2005; Whitehead et al., 2005). These have been combined with other approaches, such as candidate screening (using inhibitory compounds or morpholino knockdowns) and microarray analysis, which have identified new genetic regulators of fin regeneration (Poss et al., 2000; Kawakami et al., 2006; Thummel et al., 2006; Jazwinska et al., 2007; Yin et al., 2008). Some of these have also been tested in other regenerating vertebrate models, where they have shown to have a similar conserved role (Kawakami et al. 2006).

Additionally, more recently, fate map analysis has helped to dissect the cellular component of fin regeneration. These studies have uncovered the heterogeneous origin of the dedifferentiated blastemal cells, suggesting that similarly to urodeles, the tail fin blastema is composed of dedifferentiated cell from all cell lineages (Tu & Johnson, 2011). Moreover, these multifated progenitors contribute in a fate-restrictive manner to the regenerate, with only endothelial and pigment cell progenitors contributing to more than one cell lineage (Tu & Johnson, 2011; Knopf et al., 2011; Sousa et al., 2011).

However, new research has challenged this assumption and shown that bone-forming osteoblasts can be generated in the absence of an osteoblast source (Singh et al., 2012). These authors combined lineage tracing analysis and cell specific genetic ablations (using a nitroreductase-prodrug conditional system; Curado et al., 2007) to erase the osteoblasts of the tail fin before amputation. They discovered that even in the absence of an endogenous osteoblast population, new osteoblasts populated the regenerate and contributed to the regeneration of the dermal rays (Singh et al., 2012). Interestingly, no osteoblasts were observed in the rest of the fin even after regeneration had occurred and there was no difference in the
regenerative capacity of the ablated and non-ablated tails, as both tails regenerated at a similar rate (Singh et al., 2012). These findings strongly suggested that transdifferentiation could be occurring in the regenerating zebrafish fin and, at least in the case of osteoblast, a second source of cells could be contributing to the new population of bone forming cells in a non-fate restrictive manner.

Similar experimental techniques have been employed to identify the cellular, molecular and genetic contributions needed for proper heart regeneration (Gemberling et al., 2013). The zebrafish’s cardiac regenerative capacity was uncovered 12 years ago in a seminal paper published by the Keating group (Poss et al., 2002b). They discovered that upon the resection of 20% of the heart ventricle, the adult zebrafish was able to recreate its damaged heart after just 60 days post surgery (Poss et al., 2002b). They suggested that increased cardiomyocyte proliferation was behind the regrowth of the heart tissue and showed that the loss of the mitotic checkpoint kinase msp1, identified in a ENU forward genetic screen, abrogated the progression of heart regeneration, leading to the accumulation of fibrotic tissue (Poss et al., 2002b). Interestingly, in a parallel study, the authors also used this mutant to show that msp1 is similarly required for the establishment of a blastemal population during tail fin regeneration, therefore genetically linking both regenerative processes (Poss et al., 2002a).

Since then, a wealth of research has emerged in the study of zebrafish heart regeneration (Gemberling et al., 2013; Poss et al., 2010). More recent papers have utilised lineage-tracing analysis (Jopling et al., 2010; Kikuchi et al., 2010; Kikuchi et al., 2011), alternative mechanical and genetic ablation procedures (Wang et al., 2011; Schnabel et al., 2011; Gonzalez-Rosa et al., 2012), transgenics (Lepilina et al., 2006; Wang et al., 2013) and microarray analysis (Lien et al., 2006) to define the heart signals and the cellular mechanisms controlling the cardiac regrowth. They have uncovered that heart damage triggers the dedifferentiation and consecutive proliferation of terminally differentiated cardiomyocytes, which are the primary
providers of new cells in the regenerating tissue (Jopling et al., 2010; Kikuchi et al., 2010). Similar fate mapping experiments have uncovered a role for the epicardial cells in the regeneration process, showing how they contribute to the novel vasculogenesis of the regenerate (Lepilina et al., 2006; Kikuchi et al., 2011). Moreover, the epicardium has been proposed to provide an environment for the growth of the regenerate and it has additionally been shown to upregulate early developmental markers upon injury, suggesting that heart regeneration could be guided by the reactivation of embryogenesis (Gemberling et al., 2013).

Tail fin and heart regeneration are not the only two regenerative responses observed in zebrafish, but are the ones that have been mostly studied (others reviewed in Gemberling et al., 2013 and Tanaka & Reddien, 2011). What is special about these processes is that they occur in tissues voided of somatic stem cell populations, which open new avenues to explore the genetic activations required to turn terminally differentiated cells into progenitors capable of regeneration. As such, they offer a better prospective therapeutic potential, raising the possibility of understanding the genetic requirements necessary to switch on regeneration in tissues, such as the heart or the limbs, where limited regeneration occurs in response to pathological damage.

1.2.3. Wt1 in zebrafish

As already mentioned, Wt1 is highly conserved amongst vertebrate species (Kent et al., 1995). In zebrafish, wt1 exists as two duplicated orthologues, namely wt1a and wt1b, which are thought to have arisen after the genome duplication of ray-fin fish (Postlethwaith et al., 2000; Bollig et al., 2006). These paralogues show high sequence homology between them and their mammalian counterpart, with an especially high conservation amongst the C2H2 zinc finger
Figure 1.6. Wt1 fluorescent transgenic lines. (A) Fluorescent transgenic lines have been created from each wt1 zebrafish parologue, using the intergenic regions between each gene and their respective upstream genes (blue and orange arrows). Each locus shows a degree of synteny with the WT1 locus in humans. (B) The wt1b:GFP zebrafish line highlights most of the zebrafish pronephros in the first 48 hpf. (C) The fluorescent line also highlight other tissues aside of the pronephros (pr), including the brain (br), the retina (re), the gill arches (ga) and the heart sac (hs). Abbreviations: tu = tubule; du = duct; glo = glomerulus. Scale bar = 100 μm.
region, where both genes preserve the ±KTS sequence that is characteristic of the
Wt1 gene (Bollig et al., 2006; Figure 1.5A). Additionally both zebrafish genes show
synteny with the human genomic locus, with upstream and downstream elements
conserved amongst the two orthologues (Bollig et al., 2009; Figure 1.6A).

Similarly to mammals, their expression is central to renal development. Both genes
show spatiotemporal overlapping expression in the early zebrafish kidney, the
pronephros, but are not entirely confined to the same regions (Serluca & Fishman,
2001; Bollig et al., 2006 Perner et al., 2007; Figure 1.5B). It is generally assumed that
wt1a controls early renal development, the subsequent nephrogenesis and
podocyte differentiation, while wt1b is required for the late development of the
podocytes and their maintenance (Perner et al., 2007; Figure 1.5B).

This is especially highlighted by their knockdown patterns. Wt1a morphants show
an unstructured pronephros by 24hpf and are unable to form a glomerulus or
podocytic extensions in later development (Perner et al., 2007; Hsu et al., 2003). On
the other hand, larvae treated with wt1b morpholinos present a generally normal
pronephric development up until 24 hpf, but develop cysts 24 hours later in the
glomerular edges (Perner et al., 2007). Additionally, unlike the wt1a morphants,
they appear to form the podocyte fingers of the renal filtrate, as observed by the
expression of nephrin and podocin markers, which are absent in the wt1a
morpholino-treated larvae (Perner et al., 2007).

Both genes have been used as markers for podocyte development and have been
extensively used in kidney developmental studies (Wingert et al., 2007; Winger &
Davidson, 2011; Zhou et al., 2010; Diep et al., 2011), which have also helped to
further unravel the role of wt1a in glomerular and podocytic formation (O’Brien et
al., 2011). However, both genes have shown to be expressed and required in the
formation of other tissues (Bollig et al., 2006; Perner et al., 2007). Already, early RT-
PCR experiments revealed that both genes were expressed in the heart, spleen,
muscle, skin, ovary and testis of adult zebrafish, with \(\text{wt1a}\) solely expressing in the liver and \(\text{wt1b}\) having a unique expression in the eyes and the gills (Bollig et al., 2006). These findings were subsequently extended with the use of morpholinos and the creation of transgenic fluorescent lines, which highlighted their involvement in the organogenesis of other non-renal organs (Perner et al., 2007; Bollig et al., 2009; see next section).

As such, morpholino injections against \(\text{wt1a}\) not only resulted in a disrupted pronephros, but also caused pericardial and yolk sac edema (Perner et al., 2007). Interestingly, however, these were not observed until 4 dpf, a phenotype that was also echoed by separate independent experiments (Perner et al., 2007; Serluca, 2008). Similarly, \(\text{wt1b}\) morphants also showed heart sac defects, with edemas forming in these larvae at around the same time (Perner et al., 2007). These fish, however, also showed a curved tail phenotype early on development, with most injected larvae displaying a bent body axis by 24 hpf (Perner et al., 2007). These findings showed that both \(\text{wt1}\) genes have mutual and separate roles in development and can be used to explore organ formation outside the renal tissue.

In fact, this has been the case, with both genes been used to study the formation of the proepicardium, the interrenal glands (the fish’s homologous adrenal glands) and progenitor germ cell migration (Serluca, 2008; Kikuchi et al., 2011; Hsu et al., 2003; Chai et al., 2003; Weidinger et al., 2002). Additionally, new lines of inquest have focused on the role of \(\text{wt1b}\) in heart regeneration, as it uniquely shows to be re-upregulated in the epicardium after both ventricle resection and cryoinjury (Schnabel et al., 2011; Gonzalez-Rosa et al., 2011; Itou et al., 2012; Gonzalez-Rosa et al., 2012).

It is this later role in regeneration that has given prominence to the \(\text{wt1b}\) gene. It is interesting to see that even though both genes are expressed in the epicardium and the myocardium of the developing zebrafish heart, only \(\text{wt1b}\) appears to be strongly
re-expressed after injury and could denote a preference for this gene in regenerative responses.

1.2.4. Wt1 fluorescent zebrafish lines that highlight endogenous gene expression

Perner and colleagues have created fluorescent transgenic lines for each zebrafish paralogue (Perner et al., 2007; Bollig et al., 2009). They have used the upstream intergenic regions of the wt1 genes to drive GFP cassettes in a tissue specific manner (Figure 1.6A). In the case of the wt1a:GFP line, the driver region is made of a 35.1 Kb sequence, which includes an unknown Ensembl-predicted gene and the last 2 exons of the ccdc73 gene, a gene that has no known functional role in the genome (Figure 1.6A; blue arrow). On the other hand, the wt1b:GFP line is driven by a 25.9 Kb sequence between the 3’ to 5’ transcribed ga17 gene (also known as eif3m) and wt1b (Figure 1.6A; orange arrow).

It has been shown that each line generally recapitulates the expression of the endogenous gene that they represent, with both lines fluorescing the kidneys, as well as other tissues (Figures 1.5B and 1.6C; Perner et al., 2007; Bollig et al., 2009). However, even though the wt1a:GFP line appears to show wt1a-specific restricted expression, the wt1b line has also shown additional ectopic expression beyond its prescribed tissues (Figure 1.5B). These new foci of expression appear in the exocrine pancreas and the gut at 65 hpf, two tissues thought to be voided of wt1b expression (Figure 1.5B; Perner et al., 2007). Interestingly, wt1b’s upstream gene, ga17, has been shown to express in the pancreas, therefore strongly suggesting that the wt1b:GFP construct could contain promoters that control the expression of this inversely transcribed gene (Perner et al., 2007). Additionally, GFP is also seen in the ducts and the tubules of the forming pronephros, two other sites that do not appear to express wt1b during development (Figure 1.6B and 1.7B). This, however, has been hypothesised to be due to the possible cellular stability of the GFP protein.
after it has been activated and the existence of a potential common progenitor population between the cells in these regions and the cell of the podocytes, where the \( \text{wt1b} \) gene expresses (Perner et al., 2007).

Remarkably, the extensive fluorescent expression of the \( \text{wt1b:GFP} \) line has made it the more popular transgenic line of the two. Its pronephros-wide fluorescence has led to its use in kidney developmental studies, to account for the effects of morpholinos in pronephric development (Perner et al., 2007; Bollig et al., 2009; Westhoff et al., 2013) and to outline the morphogenesis and regeneration of the mesonephros in juvenile and adult zebrafish (Zhou et al., 2010; Diep et al., 2011). Moreover, it was initially used to identify the reupregulation of the \( \text{wt1b} \) gene in the epicardium of regenerating zebrafish hearts (Schnabel et al., 2011; Gonzalez-Rosa et al., 2011), where it has been used to uncover the differential morphology of \( \text{wt1b} \)-positive epicardial cells and to study the contribution of EDPCs to the regenerating cardiac tissue (Gonzalez-Rosa et al., 2012).

In this thesis, I have utilised the \( \text{wt1b:GFP} \) zebrafish line for most of my experiments. In order to improve their visual capacity and therefore achieve more detailed imaging, I crossed the \( \text{wt1b:GFP} \) zebrafish to a pigment-free \textit{casper} transgenic line (Figure 1.7A; White et al., 2008 - see Chapter 3 for more details). These crosses created virtually transparent larvae that exhibited the same GFP patterns as normal \( \text{wt1b:GFP} \) individuals (Figure 1.7B). Additionally, these larvae gave rise to translucent \( \text{wt1b:GFP} \) adults, which were more suitable for the fluorescent imaging of juvenile and adult fish (Figure 1.7C).

\textbf{Figure 1.7. A transparent \textit{wt1b:GFP} line.} (A) Pigmented \( \text{wt1b:GFP} \) line was crossed to transparent \textit{casper} line (White et al., 2008) and their offsprings incrossed. F\(_2\) generation was then selected according the GFP expression and presence of pigment. (B) \( \text{wt1b:GFP} \); casper line has no pigment and expresses the \( \text{wt1b} \) fluorescent transgene. (C) Six month adults display a translucent body and express the GFP transgene in the olfactory pit (O), pharyngeal cartilage (PhC), retina (Re), forebrain (Fb), gut (Gut), kidney (Ki), inner ear (Ie), end of kidney duct (KD) and interray space(ISR). Other abbreviations: Br = brain; Ga = gill arches; Hs = heart sac. Scale bars = 100 μm and 5 mm respectively.
Figure 1.7. A transparent wt1b:GFP line.
1.3. The vertebrate notochord

In my work, I have focused on the characterisation of a novel repair mechanism in the zebrafish notochord. The notochord is an evolutionarily conserved tubular structure that is characteristic of all chordates, including vertebrates (Satoh et al. 2012; Stemple, 2005). It is one of the first organs to form and provides important structural and signalling cues during embryogenesis, acting as a scaffold for development and providing paracrine signals that induce the patterning of its surrounding organs (Stemple, 2005).

1.3.1. Notochord formation and its developmental functions

The way in which the notochord forms is highly conserved. The notochord originates from the dorsal organiser, an early developmental tissue with the capacity to form a second body axis in transplantation experiments (Spemann & Mangold, 1924; De Robertis, 2006). The dorsal organiser (also known as the “node” in mammals and the “embryonic shield” in zebrafish) induces the formation of the axial chordamesoderm, the lineage precursor of the notochord, which aligns along the midline of the embryo and extends along its body creating the anterior-posterior (A/P) axis (Adams et al., 1990; Koehl et al., 2000; Glickman et al., 2003; Imuta et al., 2014). This extension is fundamental for the elongation of the embryo and homozygous and heterozygous mutants that do not properly extend their notochord display a severe or moderate shortening of their body length (Beddington et al., 1992; Halpern et al., 1993; Talbot et al., 1995).

As the chordamesodermal cells align along the midline, they vacuolate and become encased in a tough semi-flexible sheath of collagens and glycoproteins known as the perinotochordal basement membrane (Stemple, 2005). The formation of this sheath and the vacuolation of the cells are key to the notochord’s structural
function, as they act together to create the turgor pressure necessary to provide the hydrostatic skeleton needed to support embryogenesis (Adams et al., 1990; Koehl et al., 2000; Parsons et al., 2002; Coutinho et al., 2004; Ellis et al., 2013). Moreover, the disruption of either process also affects the notochord’s post-embryonic functions, with their disturbance affecting the proper formation of the vertebral column (see below; Aszodi et al., 1998; Smits & Lefebvre, 2003; Choi & Harfe, 2011; Ellis et al., 2013).

The early formation of the notochord is also modulated by a number of highly conserved key regulatory genes, which include Foxa2, Brachyuri (T) and Noto (Stemple, 2005; Chan et al., 2014). These are important for the acquisition of a notochordal fate and their deletion either abrogates notochord formation (Ang & Rossant, 1994; Stemple et al., 1996) or vastly impairs notochord development (Beddington et al., 1992; Schulte-Merker et al., 1994; Talbot et al., 1995; Abdelkhalek et al., 2004). Additionally, other genes, such as sonic hedgehog (Shh) have been shown to be required for the maintenance of the notochord, playing important roles in its survival, as well as in the patterning of its surrounding tissues (Roelink et al., 1994; Chiang et al., 1996; Chan et al., 2014).

As already mentioned, the structural role of the notochord is complemented with its paracrine functions. It provides the signals necessary for the patterning of its surrounding organs, leading to the acquisition of a dorso-ventral (D/V) axis and left-right asymmetry (Stemple, 2005; Chan et al., 2014). As such, its patterning activities influence the development of the somites, the neural tube, the blood vessels, the heart and the pancreas (Pourquié et al., 1993; Yamada et al., 1991; Fouquet et al., 1997; Ericson et al., 1996; Kim et al., 1997). One of its key signals is the morphogenetic release of Shh, which apart from modulating notochord cell survival, it also induces the formation of the sclerotome and the floor plate and stimulates the acquisition of a ventral cell fate in the motor neurons of the neural tube (Figure
1.8A and B; Johnson et al., 1994; Chiang et al., 1996; Ericson et al., 1997; Fleming et al., 2001).

Additionally, the notochord is crucial for the development of the vertebral bodies (Chiang et al., 1996; Fleming et al., 2001). In higher vertebrates, the vertebrae arise from the sclerotome portion of the somites, which is induced to migrate, proliferate and condense around the notochord (Figure 1.8B and C; Christ & Wilting, 1992; Chan et al., 2014). These movements are coordinated by signals emitted from the notochord and include the release of Shh, which, aside from its sclerotome-inducing function, also works together with other signals to trigger the migration of the sclerotome cells and their subsequent condensation around the notochord (Monsoro-Burq, 2005; Chan et al., 2014). The condensed sclerotome forms the perichordal tube, which acquires a metameric pattern of condensed and less condensed areas that will give rise to the vertebral bodies and the intervertebral disc respectively (Figure 1.8C; Monsoro-Burq, 2005).

It should be noted that in zebrafish and other teleosts, however, the notochord has a more direct role in vertebral formation, with vertebral bodies (chordacentra) first forming from the metameric mineralisation of the perinotochordal sheath and then forming a second layer of bone (autocentra) from surrounding sclerotome-derived cells (Figure 1.9B; Grotmol et al., 2003; Fleming et al., 2004; Bensimon-Brito et al., 2012).
Figure 1.8. Notochord development and its role in the intervertebral disc. (A) The notochord (blue) lies ventral to the neural tube and is flanked by metameric units of somites (pink). (B) The notochord releases signals that pattern the somites and induce the formation of the sclerotome, which then migrates and proliferates covering the notochord. (C) The migrating cells condense around the notochord and form dense (red) and less dense (orange) segments, which will give rise to the intervertebral disc and vertebral bodies respectively. (D) As the vertebral bodies form, the notochord is sequestered into the intervertebral discs, where the notochord cells accumulate. (E) In the developed intervertebral disc (IVD), the notochord forms the nucleus pulposus (NP), while the condensed sclerotome cells form the different layers of the annulus fibrosus (inner and outer layers; IAF and OAF), which are joined at each end to each vertebrae via the cartilage endplate (CEP). (F) Histologically stained section showing the physiology of the mouse IVD. Other abbreviations: VB = vertebral body; SOC = secondary ossification centre; GP = growth plate; POC = primary ossification centre. Diagram taken from Chan et al., 2014.
1.3.2. The post-embryonic functions of the notochord

Even though in most vertebrates the notochord expresses transiently during development, its roles are not limited to embryogenesis. It has been shown that as the vertebral bodies form, the notochord regresses and accumulates in the intervertebral discs (IVDs), the fibrocartilaginous structure that interspaces the metameric vertebrae (Figure 1.8D and E; Smits & Lefebvre, 2003; Choi et al., 2008; Haga et al., 2009; McCann et al., 2012; Chan et al., 2014).

The IVDs are essential for the proper functioning of the spine, acting as shock absorbers and conferring a higher degree of mobility to the backbone (Chan et al., 2014). They are composed of 3 different structures: an inner gel-filled cushion known as the nucleus pulposus (NP), a surrounding layer of chondrocytic and fibroblastic cells known as the annulus fibrosus (AF) and a final layer of cartilage known as the cartilage endplate (CEP) which joins the IVD with the surrounding vertebrae (Figure 1.8E; Chan et al., 2014).

Within the IVD, the notochord cells will give rise to the NP, which is central to the function of the structure (Figure 1.8E; Chan et al., 2014; Choi et al., 2008; McCann et al., 2012). Its functionality arises from its high proteoglycan content, which osmotically attracts water into the NP and confer it with the turgor necessary to resist compressing forces (Adams & Roughley, 2006; Chan et al., 2014).

In mammals, the NP is made of two cell populations, a population of large vacuolated notochord-like cells and another population of smaller chondrocyte-like cells (Chan et al., 2014). Even though each population appears to be morphologically different, recent lineage tracing analysis has shown that both populations descend from the notochord and even share common genetic markers (Choi et al., 2008; McCann et al., 2012; Minogue et al., 2010). Studies have shown that the cellular balance of the NP changes over time, with the notochord-like cells
disappearing from the NP as we age. This cellular shift has been proposed to be behind the age-related pathological degeneration of the NP, which compromises the functions of the IVD and leads to chronic back pain and other spine-related malignancies (Chan et al., 2014). Due to the clinical and economical impact of these pathologies, a high amount of research is being undertaken to understand the chronological degeneration of the NP, with an eye on trying to develop novel therapeutic treatments that could restore its homeostatic functionality (Makarand et al., 2011; Chan et al., 2014).

In other animals, IVD formation has not been as extensively investigated, with only a handful of studies looking at zebrafish IVD formation using transgenics and histological staining (Haga et al., 2009; Dale & Topczewski, 2011; Bensimon-Brito et al., 2012). These have confirmed that the same notochord cell accumulation occurs in the IVDs of zebrafish, which leads to the formation of a vacuolated NP in these regions. However, no study has looked any deeper into this structure and its exact cellular and molecular profile remains to be elucidated.

1.3.3. The zebrafish notochord

The zebrafish notochord forms in much the same way as the notochord forms in other vertebrates. It arises from the embryonic shield and forms via the extension, convergence and mediolateral intercalation of the chordamesoderm (Glickman et al., 2003; Stemple, 2005). As one of the first organs to form, it is the predominant structure in the early embryo and serves as a hydrostatic skeleton for the free-swimming zebrafish larvae until the vertebral column forms (Figure 1.9; Stemple, 2005).

The zebrafish notochord is composed of two populations of cells encased in a thick perinotochordal sheath (Figure 1.9A). A population of inner vacuolated cells (IVC or
chordocytes; Ellis et al., 2012) which form the body of the organ and maintain the turgor pressure inside the notochord, and a second population of epithelial-like cells known as notochord sheath cells (NSC or chordoblasts; Dale & Topczewski, 2011; Ellis et al., 2012), which envelop the inner cells and secrete the thick basement membrane sheath that surrounds the notochord (Figure 1.9A; Kimmel et al., 1995; Stemple, 2005; Yamamoto et al., 2010). This perinotochordal basement membrane is important for the biology of the notochord and it is made of three different layers of collagens and proteoglycans, which act as a physical barrier upon which the inner vacuolated cells can exert their force (Parsons et al., 2002; Coutinho et al., 2004; Gansner & Gitlin, 2008; Yamamoto et al., 2010; Corallo et al., 2013).

Each notochord cell population is established in the first 24 hours of development and both derive from progenitors of the midline-aligned chordamesoderm (Yamamoto et al., 2010; Dale & Topczewski, 2011). Recent studies have explored the acquisition of each cellular fate and revealed that the activation of notch signalling via a mib-jag1 pathway induces the differentiation of NSC in the cells of the chordamesoderm (Yamamoto et al., 2010). Furthermore, they have shown that
Figure 1.9. The zebrafish notochord and vertebral formation.
inhibition of this signalling cascade leads to an increase in the number of vacuolated cells, therefore suggesting that the chordamesoderm is normally predisposed to form vacuolated inner cells in the absence of the notch signal (Yamamoto *et al*., 2010).

Other studies have also looked at the genes driving the vacuolation of the inner notochord cells and investigated the origin of the intracellular vacuoles (Coutinho *et al*., 2004; Ellis *et al*., 2012). They have revealed that the activation of genes involved in the coatamer complex assembly and late endosomal trafficking are responsible for the vacuolation of the inner notochord cells, with their disruption respectively leading to a generalised cell death in the notochord and a defective axial skeletogenesis in the later larvae (Coutinho *et al*., 2004; Stemple, 2005; Ellis *et al*., 2012). Additionally, Ellis and colleagues showed that the vacuoles are lysosome-related organelles (LRO), which form via the activation of an alternative vesicle forming mechanism in the Golgi complex (Ellis *et al*., 2012).

These studies have also shown that the aberrant acquisition of each cell fate can affect the establishment of the perinotochondal sheath (Coutinho *et al*., 2004; Yamamoto *et al*., 2010). These combine well with other findings that demonstrate that the proper acquisition of a perinotochondal sheath is fundamental for the proper differentiation of the notochordal cells (Parsons *et al*., 2002; Gansner *et al*., 2007; Pagnot-Minot *et al*., 2008; Corallo *et al*., 2013). This means that a tight relationship

**Figure 1.9. The zebrafish notochord and vertebral formation.** (A) The zebrafish notochord (black arrow) is made of two cell populations, an inner vacuolated cell (IVC) population, which makes the body of the notochord, and an outer sheath cell (OSC) population that surrounds it in a monolayer. Both cells are encased in a thick perinotochondal sheath (PNS). (B) The zebrafish notochord mineralises over time to form the vertebral bodies. Bone formation starts in an anterior to posterior manner with the first vertebrae (centra) forming at a body length of 4.4 mm (i), with centra 3, 4 and 5 forming first (C3-5). At 5.5 mm body length (ii) the whole notochord is patterned by rings of mineralising chordacentra. At this point the first cartilaginous arches (grey; arrow head) begin to form in the most anterior end of the notochord, the Weberian apparatus, and the first cartilage hypurals can be observed in the tail fin end. By 6.0 mm, all vertebrae have formed, with the Weberian apparatus fully differentiated (anterior end) and the hypurals fully formed at the caudal fin. Diagram taken from Bensimon-Brito *et al*., 2012; abbreviations in paper. Black represents bone and grey represents cartilage. Scale bar = 1 mm.
exists in the notochord between its parenchymal and extracellular components, with their balance being necessary for the proper differentiation and function of the organ.

Moreover, similarly to higher vertebrates, the zebrafish notochord is also involved in the mineralisation of the axial skeleton (Stemple, 2005; Fleming et al., 2004; Bensimon-Brito et al., 2012). However, unlike mammals and birds, vertebral formation is, at least initially, devoid of sclerotome involvement and does not require a cartilage anlage for the formation of bone (Javidan et al., 2004; Fleming et al., 2004; Bensimon-Brito et al., 2012).

In fact, the primitive vertebral bodies, also known as chordacentra, form from the patterned calcification of the notochord sheath, which creates metameric mineralised rings around the notochord in an anterior to posterior fashion (Figure 1.9B; Fleming et al., 2004; Haga et al., 2009; Bensimon-Brito et al., 2012).

The formation of the chordacentra is followed by the deposition of a second layer of bone (the autocentrum) on each ring (Figure 1.9B; Bensimon-Brito et al., 2012; Grotmol et al., 2003). This is done by sclerotome-derived osteoblasts, which accumulate outside of the notochord in the centres of ossification and lay intramembranous bone on the rostral and caudal end of each ring (Bensimon-Brito et al., 2012; Grotmol et al., 2003). The deposition of the autocentrum helps the vertebra to expand and acquire a full vertebral body (Bensimon-Brito et al., 2012). Lastly, the final components to form are the neural and haemal arches, which begin to form after the deposition of the autocentra has been completed and are arranged in a similar sclerotome-induced intramembranous fashion (Fleming et al., 2004; Bensimon-Brito et al., 2012).

As the vertebral bodies form, the notochord cells regress towards the IVD and similarly to other vertebrates, appear to assemble into a nucleus pulposus-like...
structure (Haga et al., 2009; Bensimon-Brito et al., 2012). Interestingly, however, these appear to contain both vacuolated cells and sheath cells within them, making them more heterogenous than those of higher vertebrates (Haga et al., 2009; Dale & Topcezswki, 2010; Bensimon-Brito et al., 2012). As already mentioned, however, the zebrafish IVD have not been properly studied and it is not known whether these cells populate the structure throughout the life span of the fish.
1.4. Summary

In this thesis I have aimed to combine the regenerative capacity of zebrafish with their powerful genetics and imaging adaptability, in order to uncover new *wt1*-associated regenerative or reparative responses.

My initial work was based on preliminary results by Kerrie Marie-Taylor, a former PhD student in the Patton lab, who used the fluorescent *wt1b*:GFP zebrafish line to observe for novel GFP upregulations in the regenerating melanocytes of tail amputated larvae. She had observed a slight rise of fluorescence after the tail amputations, but had not characterised the response further, as the signal was independent of melanocyte regeneration.

In the next three results chapters I follow on from her findings and describe this response in more detail. I identify its triggers, define its physiological movements and cellular identities, and uncover its effects on notochord and vertebral development. The first chapter (Chapter 3) describes the tail amputations and alternative needle injuries that I used to identify the tissue specificity of the response and to define its cellular dynamics. The second chapter (Chapter 4) deepens into the cellular identities of the response and analyses the contribution of the notochord cell populations and the immune system to the process. Lastly, the final chapter (Chapter 5) looks at the genetic upregulations and repressions induced by the notochord damage, validates the expression of *wt1b* in the response and focuses on the bone and cartilage interplay that is triggered by the injury in the notochord.

Overall, these experiments help me identify a previously uncharacterised damage response in the notochords of zebrafish larvae and reveal that the notochord can activate a repair mechanism in response to external insult.
CHAPTER 2:

Materials and Methods
2.1. Materials

2.1.1. Chemicals and Reagents
(N.B.: Organised in alphabetical order)

**Acros Organics, USA:** Eosin Y, high purity

**Agilent Technologies, USA:** Gene Expression Hybridisation Kit
Low Input Quick Amp Labelling Kit

**Dako, UK:** Protein block, serum free
Antibody Diluent
HRP Rabbit/Mouse (DAB+)
EnVision™ FLEX DAB+ Chromagen
EnVision™ FLEX Substrate Buffer

**Invitrogen, UK:** Acridine Orange
Agarose
Glycoblué®
ProLong® Gold (+DAPI)
RNaseOUT Recombinant Ribonuclease Inhibit
Trizol®

**MRC HGU Core Facility, UK:** Absolute Ethanol
Acetic Acid
Chloroform
0.5M EDTA pH 7.5
HCl
Methanol
MilliQ dH2O
Paraffin
Phosphate Buffered Saline (PBS)
Xylene
1M Tris pH 9
Sigma-Aldrich, UK:  
- Alcian Blue 8GX powder  
- Alizarin Red S  
- Calcium Chloride dehydrate  
- Citric Acid  
- Donkey Serum  
- DPX Mount  
- 30% Hydrogen Peroxide  
- Lithium Carbonate  
- Low Melting Point Agarose  
- Magnesium Sulfate Heptahydrate  
- Potassium Chloride  
- Potassium Hydroxide  
- RT-PCR Primers  
- Sodium Chloride  
- Sodium Citrate  
- Sodium Hypochlorite  
- Tricaine methanesulfonate

Thermo-Scientific, UK:  
- Harris Haematoxylin

Electron Microscopy Sciences, USA:  
- 16% Paraformaldehyde (PFA)

2.1.2. Solutions  
(N.B.: Organised in alphabetical order)

Acid/Alcohol:  
- HCl  
- 100% EtOH  
- MilliQ H2O

Acridine Orange:  
- Acridine Orange Powder  

Make up to 1 litre with E3
<table>
<thead>
<tr>
<th><strong>1% Low Melting Point Agarose:</strong> (Imaging)</th>
<th>Low Melting Point Agarose</th>
<th>50mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Make up to 50ml with E3</strong></td>
<td><strong>N.B.: Heat up the mix until it goes into solution and then add 10 drops of tricaine</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>2% Agarose:</strong> (Embedding for sectioning)</th>
<th>Agarose</th>
<th>2g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Make up to 100 ml in 1x PBS</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>0.2% Alcian Blue:</strong> (Stock solution)</th>
<th>Alcian blue 8GX</th>
<th>0.2 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% EtOH</td>
<td>11.2 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Lightly heat up and swirl until all dissolves</strong></td>
<td><strong>then add 95% EtOH to 100 ml of volume</strong></td>
<td><strong>N.B.: Ensure that there are no precipitates</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>0.04% Alcian Blue:</strong> (Working solution)</th>
<th>0.2% Alcian blue</th>
<th>10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 7.5</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td>1M MgCl$_2$</td>
<td>500 μl</td>
<td></td>
</tr>
<tr>
<td>95% EtOH</td>
<td>32.6 ml</td>
<td></td>
</tr>
<tr>
<td>MilliQ dH$_2$O</td>
<td>1.9 ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>0.5% Alizarin Red S:</strong> (Stock solution)</th>
<th>Alizarin Red S powder</th>
<th>0.25 g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Make up to 50 ml in MilliQ dH$_2$O</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>0.01% Alizarin Red stain:</strong></th>
<th>0.5% Alizarined Red stock</th>
<th>1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% glycerol</td>
<td>12.5 ml</td>
<td></td>
</tr>
<tr>
<td>1M Tris pH 7.5</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td>MilliQ dH$_2$O</td>
<td>31.5 ml</td>
<td></td>
</tr>
</tbody>
</table>
1% Aqueous Eosin: Eosin 1 g  
*Make up to 100 ml in MilliQ dH2O*

**Bleach Solution:** Sodium hypochlorite 180 μl  
(For growing up embryos) MilliQ dH2O 500 ml

**Bleach Solution:** Potassium Hydroxide 3 g  
(Immunohistochemistry) 30% Hydrogen Peroxide 10 ml  
*Make up to 100ml in MilliQ dH2O*

**Citrate Buffer (0.01M; pH 6):** 0.1M Citric Acid 82 ml  
0.1M Sodium Citrate 18 ml  
*Make up to 1 litre in MilliQ dH2O*

**Citric Acid (0.1M):** Citric Acid 10.5 g  
*Make up to 500 ml in MilliQ dH2O*

**Heat inactivated Donkey Serum:** Donkey serum was divided into 1 ml aliquots and heat inactivated at 65°C for 30 minutes.

**10% Donkey Serum:** Heat inactivated Donkey Serum 1ml  
(Blocking Solution) TBSTw 9ml

**E3 Embryo Media (60x):**  
Sodium Chloride 17.2 g  
Magnesium Sulfate Heptahydrate 4.9 g  
Calcium Chloride dihydrate 2.9 g  
Potassium Chloride 0.76 g  
*Make up to 1 litre in MilliQ dH2O*
**E3 Embryo Media (1x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>60x Embryo Medium (E3)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

*Make up to 6 litres in MilliQ dH2O*

**Eosin stain**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Aqueous Eosin</td>
<td>300 ml</td>
</tr>
<tr>
<td>1% Ethanol</td>
<td>100 ml</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

*N.B.: The eosin stain was filtered before use*

**1% Ethanol**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Ethanol</td>
<td>1 ml</td>
</tr>
<tr>
<td>MilliQ dH2O</td>
<td>99 ml</td>
</tr>
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</table>

**70% Ethanol:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Ethanol</td>
<td>35 ml</td>
</tr>
<tr>
<td>MilliQ dH2O</td>
<td>15 ml</td>
</tr>
</tbody>
</table>

**25, 50 and 75 MeOH:**

Methanol was mixed with PBS (v/v) to a total volume of 50 ml according to percentages.

**2%/4% PFA:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>16% PFA</td>
<td>1.5/3 ml</td>
</tr>
<tr>
<td>PBS</td>
<td>10.5/9 ml</td>
</tr>
</tbody>
</table>

*N.B.: Once made, keep at 4°C and discard after 1 week*

**0.1M Sodium Citrate:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate powder</td>
<td>12.9 g</td>
</tr>
</tbody>
</table>

*Make up to 500 ml in MilliQ dH2O*

**10x TBS:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>13.9 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>87.66 g</td>
</tr>
</tbody>
</table>

*Make up to 1 litre with MilliQ H2O*
1x TBS: 10x TBS 50 ml
MilliQ H₂O 450 ml

0.1% 1x TBSTw: Tween-20 500 μl
1x TBS 500 ml

N.B.: Stir mix until it all goes into solution

Tricaine, pH7: Tricaine methanesulfonate 2g
1M Tris, pH9 10.5 ml

Make up to 500ml in MilliQ dH₂O

2.1.3. Instruments

Agilent Technologies, USA: Whole Zebrafish (V3) Genome Oligo microarray
Hybridisation chamber
Oven
Microarray Scanner System
Feature Extraction Software

BD Medical, USA: BD Microlance 25G 5/8 0.5 mm x 16 mm needles
BD Plastipak 1 ml Syringes

Bioconductor, USA: Limma R Package

Biostad, Canada: Miles Scientific Tissue TEK VIP automated processor
<table>
<thead>
<tr>
<th>Company</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carl Zeiss, UK</td>
<td>Axioplan II fluorescence microscope</td>
</tr>
<tr>
<td>Invitrogen, UK</td>
<td>RNAse-free Microcentrifuge tubes</td>
</tr>
<tr>
<td>Iwaki Glass, Japan</td>
<td>Iwaki glass plates</td>
</tr>
<tr>
<td>Nikon, UK</td>
<td>Nikon AZ100 Macrope</td>
</tr>
<tr>
<td></td>
<td>Nikon Inverted Light Microscope</td>
</tr>
<tr>
<td></td>
<td>Nikon UV G Filter Cube</td>
</tr>
<tr>
<td></td>
<td>Nikon A1R Confocal Microscope System</td>
</tr>
<tr>
<td></td>
<td>Nikon Eclipse TiE inverted microscope</td>
</tr>
<tr>
<td></td>
<td>NIS Elements Software</td>
</tr>
<tr>
<td>Leica Microsystems, UK</td>
<td>Aluminium embedding molds</td>
</tr>
<tr>
<td></td>
<td>Leica SP5 confocal microscope</td>
</tr>
<tr>
<td></td>
<td>MZFLIII fluorescence stereomicroscope</td>
</tr>
<tr>
<td></td>
<td>100W Hg light source</td>
</tr>
<tr>
<td></td>
<td>RM2235 rotary microtome</td>
</tr>
<tr>
<td>Mai Tai, Spectra Physics, USA</td>
<td>Pulsed Ti:Sapphire multiphoton laser</td>
</tr>
<tr>
<td>Marzhauser Wetzlar, Germany</td>
<td>3 axis motorised stage</td>
</tr>
<tr>
<td>MRC HGU Core Facility, UK</td>
<td>Glass inserts</td>
</tr>
<tr>
<td></td>
<td>Glass vials</td>
</tr>
<tr>
<td></td>
<td>Cold plate</td>
</tr>
<tr>
<td>Prior Scientific Instruments, UK</td>
<td>Lumen 200 Pro light source</td>
</tr>
<tr>
<td></td>
<td>ScPrior H101 motorised stage</td>
</tr>
<tr>
<td>Company</td>
<td>Product/Service</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Qiamaging, Canada</td>
<td>Qimaging Retiga Exi Cam</td>
</tr>
<tr>
<td></td>
<td>Micropublisher 3.3mp cooled colour CCD Cam</td>
</tr>
<tr>
<td>Photometrics Ltd, USA</td>
<td>Photometrics Coolsnap HQ2 camera</td>
</tr>
<tr>
<td>Rosetta Biosoftware, USA</td>
<td>Resolver® gene expression data analysis system</td>
</tr>
<tr>
<td>Sakura, Netherlands</td>
<td>Miles Scientific Tissue TEK VIP automated processor</td>
</tr>
<tr>
<td>Solent Scientific, UK</td>
<td>CO2 and temperature control environmental chamber</td>
</tr>
<tr>
<td>Scanalytics, Canada</td>
<td>IPLab Spectrum Software</td>
</tr>
<tr>
<td>Simport, Canada</td>
<td>Unisette™ processing/embedding cassettes</td>
</tr>
<tr>
<td>Solent Scientific, UK</td>
<td>Incubation chamber</td>
</tr>
<tr>
<td>Swann Morton, UK</td>
<td>Sterile Long Handle Scalpel Blades</td>
</tr>
<tr>
<td>TPP, Switzerland</td>
<td>Tissue culture plates</td>
</tr>
<tr>
<td>University of California, USA</td>
<td>Micromanager Software</td>
</tr>
</tbody>
</table>
2.2. Fish Stocks

2.2.1. Animal husbandry

2.2.1.1. Zebrafish Facility

All zebrafish were raised in a re-circulating closed water system (Aquatic Habitats, UK) at 28°C with a pH of 7.4 in a 14 hours/10 hours day/night light cycle. All lines were established from bleached embryos collected from adult matings (pairs or marbles). The embryos were bleached as soon as they were collected (at approximately 3 hpf) in alternating washes of bleach solution and MilliQ dH2O (5 minutes each wash for a total of 20 minutes). The embryos were placed in E3 media for 5 minutes and transferred into new E3 before being grown in a tissue culture petri dish in an incubator at 28°C.

After 5 days, the bleached embryos (now larvae) were transferred to static tanks at room temperature (22-23 °C) and grown to 14 dpf. During this period, the larvae were fed a mixture of paramecium and ZM000 dry food (ZM Systems, UK). At 14 dpf, the larvae were transferred into the system and raised to adulthood. While in the system, the larvae were fed a mixture of granular dry food (ZM Systems) and brine shrimp.

2.2.1.2. Experimental Procedures

All experimental procedures were approved by the UK Home Office and were in accordance with the UK Animals (Scientific Procedures) Act 1986. Permission to perform all of the experiments was granted to Personal Project License (PPL) number 60/3992 (updated to 70/8000) and Personal Individual License (PIL) number 60/12873.
2.2.2. Transgenic Lines

2.2.2.1. entpd5:RFP

The transgenic line recapitulates the expression of the ectonucleoside triphosphate diphosphohydrolase 5 (entpd5) enzyme, a enzyme that was recently identified in a forward screen to be a novel key player in zebrafish skeletogenesis (Huitema et al., 2012). This enzyme has been associated with bone forming osteoblasts and highlights bone and forming chordacentra where it controls phosphate homeostasis. The line was kindly provided by Stefan Schulte-Merker (Utrecht, Holland) and details of the line can be found in Huitema et al., 2012.

2.2.2.2. SAGFF214:GFP

The transgenic line highlights the vacuolated notochord cells, utilising a Gal4:UAS system, driven by the expression of the NADH-cytochrome b5 reductase gene, to express a cell surface GFP marker in these cells. The line was kindly provided by Stefan Schulte-Merker and was originally created by Yamamoto and colleagues in Japan (for details see Yamamoto et al., 2010).

2.2.2.3. shh:GFP

The transgenic line recapitulates the expression of the developmental morphogen sonic hedgehog (shh), highlighting the notochord in the early embryo and the floor plate in the later larvae. The line was kindly provided by Patricia Yeyati (MRC Human Genetics Unit - Edinburgh, UK). The line was originally created by Raymond Ertzer and colleagues in Strasbourg (France) and the details of the line can be found in Ertzer et al., 2007.
2.2.2.4. wt1a:GFP

The transgenic line recapitulates the expression of the first discovered zebrafish Wilms tumour 1 gene, wt1a. The line expresses GFP in the glomerulus of the developing pronephros and in the larval hindbrain (see Figure 1.5B in Chapter 1 - Introduction). The line was originally created by Frank Bollig and was kindly provided by Christoph Englert (Jena, Germany). The details of the line can be found in Bollig et al., 2009.

2.2.2.5. wt1b:GFP

The transgenic line recapitulates the expression of the second zebrafish Wilms tumour 1 gene, wt1b. The line expresses GFP in the developing pronephros, highlighting the glomerulus, the pronephric tubules and the pronephric duct of the zebrafish embryo (see Figures 1.5B and 1.6B in Chapter 1 - Introduction). Other sites of expression include wt1b-expressing tissues, such as the heart sac, the hindbrain, the gill arches and the retina (see Figure 1.6C in Chapter 1 - Introduction), as well as wt1b non-expressing tissues, such as the gut and the endocrine pancreas, where the fluorescence is driven by the expression of its upstream gene, ga17 (see Figure 1.5B and Perner et al., 2007). The line was created by Birgit Perner and was kindly provided by Christoph Englert (Jena, Germany). Details of the line can be found in Perner et al., 2007 and Bollig et al., 2009.
2.2.2.6. Other wt1b:GFP lines

As part of the project, new lines were created in order to analyse the injury response and identify its cellular origins, the fate of its cells and the processes governing the response.

2.2.2.6.1. wt1b:GFP; casper

The transgenic line was created in order to trace the response in a pigment-free system. This line allowed for the response to be followed into adulthood, without the possibility of pigment masking the response. The line was created by crossing homozygous wt1b:GFP fish to homozygous pigment-free transparent casper fish (White et al., 2009) over two generations (see Figure 1.7 in Chapter 1 - Introduction).

2.2.2.6.2. wt1b:GFP; col2a1a:RFP

The transgenic line was created in order to study how notochord sheath cells behave in response to notochord damage. The line was created by co-injecting an R2-col2a1a:mCherry construct (Dale & Topczewski, 2011) with a Tol2 transposase (Kawakami, 2007) into a 1 to 2-cell stage wt1b:GFP fertilised eggs. The transgene highlighted the outer epithelial notochord sheath cells with a mCherry fluorophore and the wt1b:GFP background allowed for the observation of these cells in the context of the GFP response. The construct was kindly provided by Rodney Dale (Chicago, USA) and the details of the construct are published and can be found in Dale & Topczewski, 2011.
2.2.2.6.3. *wt1b:GFP; entpd5:RFP*

The line was created by crossing homozygous *wt1b:GFP* adults to heterozygous *entpd5:RFP* adults and then selecting for double positive GFP⁺RFP⁺ embryos.
2.3. Tail amputations and notochord needle injury

2.3.1. Tail amputations

Transgenic embryos were grown in E3 media to 3 dpf and anaesthetised by the addition of tricaine to a final concentration of 15mM. Tail amputations were carried out once no movement could be observed. The larvae were placed sagittally on the petri dish and a sterile scalpel blade was used to amputate the tails of the larvae.

Different amputation sites were used depending on the experiment been performed (see Chapter 3 – Results for details). The injured larvae were then transferred into fresh E3, allowed to recover and placed at 28°C to be grown to the desired stage. Non-amputated age-matched larvae were grown as non-amputated controls.

2.3.2. Notochord Needle Injury

Similarly to tail amputated larvae, 3 dpf larvae were anaesthetised in tricaine and observed for cessation of movement. The larvae were then placed sagittally on the petri dish and using an electrolysis-sharpened tungsten wire (Brady, 1965; made by Alyson Ross, MRC HGU, UK) the notochords of the larvae were targeted at different locations (see Chapter 3 – Results for details). The injured larvae were transferred to fresh E3, allowed to recover and placed at 28°C to be grown to the desired stage. Non-injured age-matched larvae were grown as non-injured controls.
2.4. Imaging

2.4.1. Live microscopy

2.4.1.1. Leica MZFLIII Stereomicroscope

A Leica MZFLIII fluorescence stereomicroscope with a 100W Hg source and a Leica GFP1 and RFP filter was used for the fluorescent selection of transgenic larvae.

2.4.1.2. Nikon AZ100 Macroscope

For time-course experiments, larvae were imaged using a Nikon AZ100 upright macroscope with a 2x and a 5x objective. Brightfield and fluorescent images were captured using a Qimaging Retiga Exi camera and an Intensilight 130W Hg light source for brightfield imaging. Fluorescence was taken using a Nikon UV GFP filter cube. Images were analyzed and processed using the IPLab Spectrum software.

For time-lapse imaging a different AZ100 setting was used, comprising a Nikon AZ100 macroscope with 2x and 5x objectives and a Lumen 200 Pro light source. Fluorescence was taken using Nikon GFP filter cubes with a Photometrics Coolsnap HQ2 camera. A Prior H101 motorised stage with focus motor and encoders was used to move the stage during multi-position time-lapse experiments. To maintain a 28°C temperature, all time-lapses were done using an environmental chamber fitted with CO2 and temperature control. The Micro-Manager software was used to capture the images, control the filter wheel and analyse the data.

2.4.1.2.1 Time-course Experiments

In time-course experiments, larvae were injured and imaged at pre-determined time-points. At each time-point the larvae were anaesthetised with 15mM tricaine.
(as described above), positioned sagittally and imaged in the anaesthetic using the Nikon AZ100 system (see above). After imaging, the larvae were transferred back into E3 media, allowed to recover and placed back in the incubator at 28°C until the next time point.

2.4.1.2.2. Time-lapse

Time-lapse experiments were designed to span over a 48 hours period with brightfield and GFP fluorescent images taken at 30 to 60 minutes intervals depending on the experiment. Due to the upright nature of the macroscope, larvae were injured and embedded sagittally on a drop of 1% low-melting point agarose on a specially designed insert containing a glass cover slip. Once the agarose solidified, the insert was inverted into an Iwaki plate containing a small volume of anaesthetic. This allowed for the larvae to be imaged through a glass interface, improving imaging. The larvae were imaged under anaesthetic for 48 hours and were terminated in an irreversible dose of tricaine at the end of the experiment.

2.4.1.3. Nikon A1R confocal

A Nikon A1R confocal system was used for time-course and time-lapse experiments. The system was composed of a Nikon Eclipse TiE inverted microscope with Perfect Focus System and four laser modules: 405 nm (laser diode), 457 nm, 488 nm, 514 nm (multi-line Argon) 561 nm (diode-pumped solid-state), 638nm (laser diode). The microscope contained emission filters for DAPI, GFP, RFP and Cy5. Imaging was captured using Nikon Nis-Elements C software. The environmental temperature was maintained during imaging with a Solent Scientific incubation chamber incorporating temperature and humidified CO2 control.
2.4.1.3.1. Time-course Experiments

Confocal time-course experiments were designed in a similar manner to AZ100 macroscope time-courses, with anaesthetised larvae being imaged at pre-determined time-points. However, unlike the macroscope time-courses, the larvae were embedded in agarose prior to the imaging.

In brief, the larvae were embedded sagittally in a drop of 1% low melting point agarose containing tricaine on a glass-bottomed Iwaki plate. Once solidified, a second layer of agarose was added to cover the droplet and prevent the agarose from detaching from the glass surface. E3 containing tricaine (same E3 used to anaesthetise the larvae) was added to the well once the second layer had hardened and the plate was taken to image.

The larvae were imaged using x20 or x40 lenses with a 480nm laser (GFP) and/or a 520nm laser (RFP) being used to capture fluorescence, depending on the experiment. A Z-plane range of 80-10 μm was used in each experiment (approximate width of the notochord) with a Z-step of 2.85 μm.

After imaging, the larvae were carefully rescued from the agarose using surgical forceps and placed in fresh E3 media to recover. Once recovered, larvae were placed in an incubator at 28°C until the next time-point. At the end of the experiment, larvae were terminated in an overdose of tricaine.

2.4.1.3.2. Time-lapse Experiments

Time-lapse experiments were designed in a similar manner to the AZ100 time-lapses, with minor modifications to account for laser phototoxicity.
The larvae were set up and imaged as described above (Nikon A1R confocal - Time-Course Experiments), with injured and non-injured larvae being embedded sagittally in agarose on glass-bottomed Iwaki plates. The larvae were imaged over a period of 48 hours using a x20 lens with a Z-plane range of 80-100 μm. To reduce exposure, Z-steps were increased to 8 μm steps and the frequency of the imaging was reduced to 1-hour intervals. As with other time-lapses, a constant temperature of 28°C was maintained throughout the experiment and the larvae were terminated in an overdose of tricaine at the end of the experiment.

2.4.1.4. Leica Multiphoton – time lapse experiments

A Leica SP5 confocal microscope was also used for time-lapse analysis. The microscope was equipped with a pulsed Ti:Sapphire multiphoton laser and a 3 axis motorised stage for 3 dimensional multipoint imaging. GFP and RFP fluorescence was produced at a wavelength of 890 nm and the fluorescence output was collected using GaAsP hybrid detectors at GFP wavelength of 495-558 nm and RFP wavelengths of 573-684 nm.

For the time-lapses, larvae were injured, anaesthetised and embedded in low melting point agarose as described above. Similarly to other time-lapse experiments, larvae were terminated at the end of the experiment in an over-dose of tricaine.

2.4.2. Imaging sections

2.4.2.1. Brightfield Imaging – Discovery

A Zeiss Axioplan II fluorescence microscope with a Plan Apochromat objective was used for brightfield imaging of tissue sections. Images were captured using a
Qimaging Micropublisher 3.3mp cooled CCD camera and analysed using the IPLab Spectrum software.

2.4.2.2. Fluorescence Imaging – Limelight

A Zeiss Axioplan II fluorescence microscope with Pan-neofluar objectives was used for the fluorescent imaging of sections. Images were captured using a Coolsnap HQ CCD camera through a Chroma #83000 triple band pass excitation filter set with excitation filters installed in a motorised filter wheel. Image analysis was performed using in-house scripts in IPLab Spectrum.
2.5. Fixing and Paraffin Embedding

Two protocols were designed for PFA fixing and paraffin embedding injured and non-injured fish. The protocols took in consideration tissue penetrance and bone formation that was proportional to the age of the fish.

As a general rule, larvae younger than 20 dpf were fixed overnight and quickly embedded in paraffin after xylene treatment. Older larvae and juvenile fish, however, required a longer fixation, an additional decalcifying step (adapted from Moore et al., 2002) and a longer embedding process.

2.5.1. Larvae younger than 20 dpf

2.5.1.1. PFA Fixation

Injured and non-injured transgenic zebrafish were grown to their required stage, culled in anaesthetic and fixed overnight in 4% PFA at 4°C.

The next day, the larvae were washed away from the fixative in 2 x 5 minutes washes in PBS and then dehydrated in a series of rising methanol:PBS concentrations (25:75, 50:50, 75:25 and 100% methanol) for 2 minutes each step before being stored in a final wash of 100% methanol at -20°C for at least 24 hours before being processed (see below).

2.5.1.2. Paraffin embedding

Dehydrated larvae were washed out of the methanol in 2 x 10 minutes washes of 100% EtOH and transferred to glass vials to be cleared in xylene in 2 x 10 minutes washes at room temperature. While waiting, tubes of xylene and a 1:1
xylene:paraffin mix were heated up to 65°C. The third 10 minutes xylene wash was done in the pre-heated xylene at 65°C to get the samples into temperature and paraffin was gradually introduced with another 10 minutes wash at 65°C in the pre-heated 1:1 xylene:paraffin. Two subsequent 10 minutes washes at 65°C in 100% paraffin prepared the larvae for the embedding.

The larvae were transferred onto an aluminium embedding mold and shallowly covered in paraffin, where they were re-positioned onto the required plane for sectioning (either sagitally or coronally). The paraffin was allowed to solidify around the samples before a plastic processing cassette was introduced. The aluminium mold was transferred to a cold plate where it was allowed to cool at -4°C overnight.

2.5.2. Larvae older than 20dpf and adult fish

2.5.2.1. PFA Fixation and EDTA decalcification

Before fixing, late larvae, juveniles and adults were culled in an overdose of anaesthetic for 5 minutes. Dead fish were taken out of the anaesthetic and using forceps, their bellies were ruptured to facilitate the introduction of the fixative into the body. The fish were fixed in 4% PFA, rocking, at 4°C for 3 days.

After 3 days, the fish were washed twice in 0.5M EDTA (pH 7.5) and decalcified in a final wash of EDTA for 5 days in a rocker at 4°C. The decalcified fish were then washed twice in 70% ethanol and kept at 4°C until processed (see below).

2.5.2.2. Embedding

The embedding was outsourced to the Histology Department in the Breakthrough Cancer Unit at the Western General Hospital and was carried out by Bob Morris and
In brief, the fixed fish were embedded in 2% agarose in PBS before being taken to an embedding cassette for processing. Processing was performed in a Miles Scientific Tissue TEK VIP automated processor where the fish were taken through 2 hours washes at 50%, 70%, 95% IMS and 3 x 2 hours washes in 100% IMS, with a final 3 hours wash in 100% IMS.

The fish were then cleared in 2 x 2 hours xylene washes and a final 3 hours xylene wash before they were prepared for embedding. The samples were taken through 2 x 2 hours washes in paraffin with a final 3 hours paraffin wash, before being processed in paraffin in a Leica EG1160 embedding centre.

2.5.3. Sectioning

For sectioning all embedded samples were sectioned using a Leica RM2235 rotary microtome to a width of 5 μm. The sections were placed on warm water and mounted on frosted microscopes slides with 4-5 sections per slide. The sections were placed in an oven at 50°C and allowed to dry overnight before they were used for immunohistochemistry.
2.6. Immunohistochemistry

2.6.1. Haematoxylin & Eosin staining

A number of slides were selected and pre-stained in haematoxylin and eosin in order to visualize the sectioned tissues. In brief, the slides were de-waxed in 3 x 5 minutes washes of xylene and 3 x 5 minutes washes of 100% ethanol and rehydrated in 5 minutes washes of 90%, 70% and 50% Ethanol (in MilliQ dH2O) before a final 5 minutes wash in tap water.

The slides were over-stained in haematoxylin for 4 minutes to highlight the nuclear bodies and washed in running tap water for a few minutes to remove all excess stain. The slides were then washed for a few seconds in an acid/alcohol solution to remove non-specific background staining, washed in running tap water, blued up in saturated lithium carbonate for a few seconds and washed again in running tap water for at least 5 minutes. To highlight the cytoplasm of the cells, the slides were transferred into eosin stain for 3 minutes and cleared from excess stain in running tap water. The slides were taken into a 100% ethanol wash for a few seconds, before being immersed into a second 100% ethanol wash for 1 minute.

The slides were prepared for mounting by placing them in 3x5 minutes 100% ethanol washes, followed by 3x5 minutes xylene washes. Importantly, carry over ethanol was tapped away onto a cloth before placing the slides into the first xylene wash, as ethanol reacts with xylene to form a white solid that could disrupt the visualization of the slides.

The slides were kept in xylene and mounted using DPX mounting media and cover slips, ensuring that no bubbles formed between the sections and the cover slip. The slides were allowed to dry inside a hood for a few hours before being imaged in an inverted light microscope (see above).
2.6.2. DAB staining

As with the H&E staining, the slides were de-waxed in 3 x 5 minutes washes in xylene and 3 x 5 minutes washes in 100% ethanol, before being taken through decreasing ethanol concentrations for 5 minutes each step to rehydrate the tissues (90% to 50% Ethanol).

After the rehydration, the slides were taken through 2 x 5 minutes washes in MilliQ water before being incubated in bleach solution for 10-15 minutes to remove any pigment from the sections.

The slides were washed away from the bleach in 3 x 5 minutes MilliQ dH2O washes and prepared for the antigen-unmasking step. For the unmasking, freshly made 0.01M citrate buffer was microwaved inside an open pressure cooker for 12 minutes until it reached boiling point. Importantly, this step was done concomitantly with the MilliQ dH2O washes and timed to end with the end of the final MilliQ dH2O wash.

During the antigen unmasking, the slides were placed into the boiling citrate buffer and the pressure cooker was shut tight behind them. The cooker was microwaved for 2 minutes, until the yellow piston on the cooker’s lid went up and stayed up even when pushed down (if not, the cooker was microwave further until it happened). With the piston up, the cooker was microwaved for an extra 5 minutes to increase the pressure inside.

At the end of the 5 minutes, the cooker was taken out of the microwave and its pressure released until the piston could no longer hold itself up. The lid was opened and the open cooker was allowed to cool down at room temperature for 20 minutes.
The slides were then washed twice in MilliQ dH2O for 5 minutes each step and then in 1 x TBS for another 5 minutes. The slides were blocked with Dako serum-free blocking solution for 30 minutes and incubated overnight with a primary antibody diluted in Dako diluent in a humidifying chamber at 4°C (for antibodies and dilutions see Table 1).

The following day, the slides were taken through 3 x 5 minutes washes in TBSTw to remove excess primary antibody and were then incubated in DAKO HRP Rabbit/Mouse (DAB+) secondary antibody for an hour. The slides were subsequently washed in 3 x 5 minutes washes in TBSTw and incubated for 10 minutes in a 1:50 mix of EnVision™ FLEX DAB+ Chromagen in EnVision™ FLEX Substrate Buffer.

The chromagen was removed in 3 x 5 minutes TBSTw washes and the slides were counterstained in haematoxylin for 4 minutes. After the 4 minutes, the slides were placed in running tap water and then differentiated in acid/alcohol for a few seconds before being placed again in running tap water for 5 minutes. The slides were taken through 3 x 5 minutes 100% alcohol washes and 3 x 5 minutes xylene washes to prepare them to be mounted.

The slides were mounted in DPX media using cover slips and allowed to dry under the hood for a few hours before being imaged under a light microscope.

2.6.3.2. Immunofluorescence

The slides were de-waxed, washed and bleached as described above (DAB staining). The slides were then washed off the bleach and prepared for the antigen retrieval step by washing them in 3 x 5 minutes TBSTw. The antigen was subsequently retrieved in citrate buffer as shown above (DAB staining).
The slides were cooled and washed in 3 x 5 minutes washes in TBSTw before being blocked in 10% heat inactivated donkey serum for 2 hours. On the mean time, the chosen primary antibody was diluted in 1% heat inactivated donkey serum in TBSTw (for details see Table 1) and incubated with the slides overnight at 4°C in a humidifying chamber.

The next day, the primary antibody was washed off in 3 x 5 minutes TBSTw washes to remove the non-bound primary antibody of the slides. A secondary antibody was added. In all cases, the secondary antibody used was an anti-rabbit AlexaFluor 488 secondary antibody raised in donkey at a concentration of 1:800 in 1% heat inactivated donkey serum. The slides were incubated for 1 hour and the secondary antibody was washed off in 3 x 5 minutes TBSTw washes in the dark (N.B.: The slides were kept in the dark after the secondary antibody incubation).

The slides were mounted in ProLong Gold mounting media containing DAPI and covered with cover slips. They were curated overnight before being imaged in a fluorescent stereomicroscope.
2.7. Tissue staining

2.7.1. Cell death - Acridine Orange

Wild type AB larvae were injured and grown with age-matched non-injured controls to the desired time-points (0 hpi, 24 hpi or 72 hpi). At each time-point the larvae were placed in 2 μg/ml acridine orange (as specified in Westerfield, 2000) for an hour before being washed three times in fresh E3, anaesthetised in tricaine and imaged under brightfield and fluorescent conditions in a Nikon AZ100 Macroscope.

2.7.2. Bone Staining

2.7.2.1. Calcein

Wild type AB larvae were injured and grown together with their age-matched non-injured controls for 30 days. The fish were immersed in 0.2% (w/v) calcein (as specified in Du et al., 2001) for 10 minutes at their required time-points, washed and imaged under anaesthetic using a Nikon AZ100 Macroscope.

2.7.2.2. Alizarin Red

Transgenic wt1b:GFP larvae were injured and grown together with their age-matched non-injured controls for 30 days. At the each time-point (same as above) the fish were incubated in 50 μg/ml alizarin red (as described in Kimmel et al., 2010), washed and imaged under anaesthetic using a Nikon AZ100 Macroscope.

For confocal imaging, another set of larvae were injured and grown together with their age-matched controls. The fish were treated as described above and then irreversibly anaesthetised at each time-point for 5 minutes until the cessation of the
heartbeat. The dead fish were embedded in 1% low-melting point agarose on Iwaki plates (as described above) containing an excess of tricaine. The solidified agarose was covered in more tricaine and the fish were imaged using the A1R Nikon confocal microscope.

2.7.3. Cartilage and Bone – Alcian Blue and Alizarin Red

Needle-injured larvae were grown to the desired stages, culled under anaesthetic, fixed in PFA and placed in alcian blue and alizarin red following the protocol outlined in Walker & Kimmel 2007 with modifications from www.zfin.org.

In brief, larvae were fixed in 2% PFA for 1 hour at room temperature. The larvae were then washed in 100 mM Tris (pH 7.5)/10 mM MgCl₂ for 10 minutes and placed in 0.04% alcian blue stain/50 mM over night in a rocker at room temperature.

The larvae were washed in 80% EtOH/100 mM Tris (pH 7.5)/50 mM MgCl₂ for 5 minutes, followed by similar washes at 50% and 25% EtOH/100 mM Tris (pH 7.5). The larvae were then bleached in open tubes in 1 ml 3% H₂O₂/0.5% KOH for 10 minutes, washed with 1 ml of 25% glycerol/0.1% KOH twice for 10 minutes each wash or until no more bubbles could be seen in the fixed larvae.

The larvae were then stained in 1 ml of 0.01% alizarin red stain (pH 7.5) at room temperature on a rocker for 30 minutes and the stain was washed off using 1 ml 50% glycerol/0.1% KOH for 10 minutes on a rocker. The larvae were placed in a second 50% glycerol/0.1% KOH solution and imaged using a Nikon AZ100 macroscope.
2.8. RNA Extraction

For RNA extraction, the larvae were needle injured at a site more caudal than normal in order to avoid dissecting the wt1b:GFP expressing anal opening when collecting the tissues (see Chapter 5 - Results for details).

2.8.1. Collection of tissue samples

Fifty to a hundred zebrafish larvae were needle injured and grown to 72 hpi with age-matched non-injured controls. The fish were anaesthetised in 15mM tricaine until no movement could be observed. While waiting, 1 ml of RNA-later was aliquoted into two eppendorf tubes (one for injured and the other for the non-injured larvae) and chilled in ice.

Using a sterile scalpel blade the area around the site of injury was dissected (see Chapter 5 – Results for details) and collected with a P10 pipette to minimise the E3 carry over. The tissue was transferred to the chilled RNA-later and the remaining of the larvae (head, torso and tail) were placed in pure tricaine.

The procedure was repeated with every larva, with the injured larvae always done first to ensure that the same number of injured and control larvae were used.

2.8.2. RNA Extraction

To overcome its viscosity, one third of the RNA later was removed and replaced with MilliQ dH₂O before the centrifugation of the samples. The diluted samples were centrifuged at 6000 G for 5 minutes at 4°C until all the tissue had pelleted to the bottom.
The diluted RNA-later was discarded and replaced with 500 μl of Trizol®. A 1ml syringe with a 25G 5/8 needle was used to add the Trizol® and later used to mascerate the samples. The samples were passed several times through the needle until all the tissue had ruptured and dissolved into the solution. RNA was extracted following Trizol® manufacturer’s instructions. During the extraction, 1 μl of Glycoblue was added to the isopropanol to help pellet and visualise the RNA. The final RNA was eluted and collected in 15 μl of MilliQ dH₂O.

The quality of the RNA and its concentration was measured using an Agilent 2100 bioanalyzer. A Thermo Scientific Nanodrop™ 2000 spectrophotometer was used to calculate the level of solvent or organic contamination of the sample.
2.9. Microarray Analysis

2.9.1. Preparation of RNA for microarray analysis

RNA was extracted from injured and non-injured larvae as described above and sent to Myltenyi Biotec (Germany) who conducted the microarray analysis.

The samples were sent in triplicates (3x injured RNA and 3x non-injured RNA) and were quality checked (QC) and measured upon arrival with an Agilent 2100 bioanalyzer.

The RNA was amplified and Cy3-labelled using Agilent’s Low Input Quick Amp Labelling Kit, following manufacturer’s instructions. The cRNA yield and dye incorporation were measured using a ND-100 Spectrophotometer.

2.9.2. Microarray platform and hybridisation

The labelled cRNA was hybridised against a 4x44K Agilent Whole Zebrafish (V3) Genome Oligo Microarray. Hybridisation was performed following the Agilent 60-mer oligo microarray processing protocol with Agilent’s Gene Expression Hybridisation Kit (Agilent Technologies) using Agilent’s recommended hybridisation chamber and oven.

In brief, 1.65 μg of Cy3-labelled fragmented cRNA was hybridised overnight to the microarray platform in hybridisation buffer (17 hours at 65°C). The microarrays were washed once with Agilent Gene Expression Wash Buffer 1 for 1 minute at room temperature and a second time for 1 minute with pre-heated (37°C) Agilent Gene Expression Wash Buffer 2.
The fluorescent output from the hybridised arrays were detected using Agilent’s Microarray Scanner System (Agilent Technologies).

2.9.3. Analysis of Microarray data

The microarray images were read out and processed using Agilent’s Feature Extraction Software (FES – Agilent Technologies), which determined feature intensities, rejected outliers and calculated statistical confidences.

Differential gene expression was determined using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware). The raw data for each experiment was compiled and normalised by dividing the intensity values by their median. As all samples were labelled with Cy3, the data was compared in triplicates (injured vs. non-injured control) and the ratios calculated by dividing the sample signal intensity by the control signal intensity.

The normalised data was analysed by Graeme Grimes at the MRC Human Genetics Unit (Edinburgh) and using the R Biocondutor package Limma, he found the differentially expressed genes of the list. Probes that did not give results for two or more replicates were discarded.
2.10. Fluorescence-Activated Cell Sorting (FACS)

2.10.1. Collection of tissue samples

2.10.1.1. Collecting tissues for FACS of wt1b:GFP injured larvae

Forty six wt1b:GFP larvae were needle injured and grown to 3 dpi. Their injured torsos were dissected as described above (RNA Extraction - Collection of tissue samples). Controls tissue was dissected from a downstream non-injured area of the torso of the injured animal that was similar in size (see Chapter 3 – Results for details). Additionally, twenty non-fluorescent injured wild types were also grown to 3 dpi and were used to equilibrate the FACS machine and establish an autofluorescence threshold.

Tissue collection was performed as described above (RNA Extraction - Collection of tissue samples) except that the tissues were collected in cold PBS supplemented with 2% FCS, instead of RNA-later, to avoid the destruction of the endogenous fluorescence of the cells (Zaitoun et al., 2010)

2.10.1.2. Collecting tissues for FACS of wt1b:GFP; R2-col2a1a:RFP injured larvae

Fifty wt1b:GFP; R2-col2a1a:RFP larvae were needle injured and grown to 3dpi with age-matched non-injured controls. Similarly to the wt1b:GFP tissue collection, twenty non-fluorescent wild type larvae were needle injured and grown to the same stage to be used to equilibrate the FACS machine and establish the autofluorescence threshold.

Tissue collection was performed as described above (RNA Extraction - Collection of tissue samples), except for the fact that whole tails were used for the FAC sorting, instead of a small torso section, to enrich the number of RFP cells in the sort (see
Chapter 4 – Results for details). The dissected tissues were collected in cold PBS supplemented with 2% FCS.

2.10.2. Tissue disassociation

The protocol for tissue disassociation was adapted from Manoli & Driever, 2012. In brief, the tails were washed once in 1% FBS in 0.5x Danieau’s solution followed by 4 washes in 0.5x Danieau’s solution only.

The samples were centrifuged at 310g for 1 minute at 4°C, the supernatant discarded and re-suspended in 0.5x Danieau’s solution, before being centrifuged again at 310g for another minute at the same temperature. The supernatant was removed and the pellet re-suspended in 1x trypsin-EDTA solution. The sample was mixed up and down several times and then incubated for 7 minutes in ice.

The reaction was stopped by adding FCS to a final concentration of 5%. The mix was centrifuged at 200g for 7 mins at 4°C and the supernatant discarded at the end of it. FACSMAX cell disassociation solution was added to the pellet and the sample was centrifuged at 310g for 1 minute at 4°C. The supernatant was discarded and the pellet resuspended in 200 μl of FACSMAX solution in ice.

FACSMAX was added to a 40 μm cell strainer to calibrate it, placed on an agar-coated petri dish on ice and then the cell suspension was transferred onto the strainer. Using the plunger from a 1 ml syringe, the tissues were squished against the strainer until all tissue disappears, collecting the flow through on the petri dish.

The flow through was transferred into a new eppendorf tube and 100 ul of FACSMAX solution was used to collect any remaining cells that might have been left on the petri dish.
2.10.3. FAC Sorting

The flow cytometry analysis of the cells was performed using a FACSria2 SORP instrument (BD) equipped with a 405nm, a 488nm and a 561nm laser. Green fluorescence was detected using GFP filters 525/50BP and 488nm laser, red fluorescence was detected using 585/15BP filter and 561nm laser. Data was analyzed using FACSDiva software (BD) Version6.1.3.
CHAPTER 3: Results

The *de-novo* activation of *wt1b*:GFP initiates a novel response to damage in the zebrafish notochord
3.1. Introduction

The essential role of WT1 as a regulator of the cardiac EMT needed for heart development (Martinez-Estrada et al., 2010) raised the question of whether WT1 could have a similar conserved role in other EMT processes. Guided by my supervisors at the beginning of my PhD, I decided to study whether WT1 could be involved in tissue regeneration. For this, I exploited the regenerative capabilities of zebrafish and explored whether wt1 could be involved in their fin regeneration response.

Tail fin regeneration is one of the most widely characterised processes in zebrafish. The capacity of zebrafish to regenerate its tail tissue has long fascinated researchers and has led to a multitude of studies that have identified some of the most important genetic and cellular components that mediate the process (see references in Introduction above).

To examine whether any of the two zebrafish wt1 paralogues are expressed in the tail fin regeneration response, I used the two fluorescent transgenic wt1 zebrafish lines, which faithfully recapitulate the spatiotemporal expression patterns of the wt1a and wt1b gene (Bollig et al., 2006; Perner et al., 2007). These lines were used to visually assess if either gene could be fluorescently upregulated in the tail upon injury and to reveal their expression pattern in the regenerative response.

For these experiments I tail amputated 3 dpf zebrafish larvae using a scalpel blade and imaged their responses in time-course and time-lapse experiments. I also used targeted tail amputations to identify the tissue specificity of the responses and created a novel, less invasive, injury method to explore the fate of the injured larvae over time.
3.2. Specific tail amputations uncover the novel activation of wt1b:GFP in the notochord in response to injury

3.2.1. Tail amputations trigger a change of cellularity in the notochord that is coupled to a de-novo activation of wt1b:GFP

To explore the possible involvement of wt1 in the tail regeneration response, I initially tail amputated wt1b:GFP transgenic zebrafish and followed them over a period of 24 hours.

During this time, I recorded their response to the damage at 0, 5 and 24 hours post amputation (hpa). The images were captured under brightfield and fluorescence and the larvae were assessed for any morphological changes or de-novo fluorescent activation that could be triggered by the amputation (Figure 3.1A).

Immediately after the amputation, no significant changes could be observed in the tails of the amputated larvae (Figure 3.1B – 0 hpa). However, by 5 hpa, a change in cellular morphology was evident in the notochords of the damaged larvae, where the vacuolated cells appeared to be losing their cellularity (Figure 3.1B – 5 hpa; orange arrow). Interestingly, this cellular change was happening at an area some distance away from the site of damage, creating a loss of uniformity in the otherwise tightly packed row of cells (Figure 3.1B – 5 hpa; orange arrow). Notably, no novel GFP upregulation could be appreciated at this time in the amputated tails (Figure 3.1B – 5 hpa merged).

The fluorescent profile of the larvae changed by 24 hpa. At this point a hint of GFP upregulation could be distinguished in the tails of the amputated wt1b:GFP larvae (Figure 3.1B – 24 hpa; yellow arrow head). When inspected further, the de-novo upregulation could be seen to be centrally located inside the area that had previously undergone the loss of cellularity (Figure 3.1B – 24 hpa; yellow arrow).
Figure 3.1. Tail amputations trigger the de-novo upregulation of wt1b:GFP in the notochord of injured larvae (A) Three dpf wt1b:GFP larvae were tail amputated and imaged at 0, 5 and 24 hours post amputation (hpa). (B) At 5 hpa, the amputated larvae exhibited a loss of cellularity in the notochord that arose some distance away from the site of damage (orange arrow). The cellularity loss led to the de-novo upregulation of the wt1b:GFP marker by 24 hpa (yellow arrow head and yellow arrow). Scale bar = 100 μm.
By this point, the gap had widened and no vacuolated notochord cells could be observed within the space (Figure 3.1B – 24 hpa; black box). However, a closer inspection revealed that the gap was not empty and in fact was populated by a group of smaller cells from where the novel wt1b:GFP expression originated (Figure 3.1B – 24 hpa; inset). These cells did not resemble their neighbouring vacuolated cells and appeared to have been recruited to the site in response to the damage (Figure 3.1B – 24 hpa; inset).

3.2.2. Evaluating the role of wt1a in the notochord damage response

Considering this result, I wanted to explore the role of the second wt1 zebrafish orthologue, wt1a, in the damage response. For this I used the wt1a:GFP transgenic line, amputating their larvae in parallel with wt1b:GFP larvae and following both lines over time. With this, I wanted to see whether the same result could be phenocopied in the wt1a fluorescent line.

Additionally, I decided to follow the damaged larvae for a longer period of time. I wanted to examine what happened to these cells over time and if they could contribute to the rebuilding of the lost tissue at the amputation site.

I tail amputated 3 dpf larvae from each wt1 fluorescent transgenic line and followed their response over a 72 hours period. I imaged the larvae at 0, 24 and 72 hpa under brightfield and fluorescent conditions to evaluate the progression of the response (Figure 3.2A).

The tail amputations faithfully recapitulated the morphology changes previously observed in the injured larvae (Figure 3.1D – 24 hpa; blue arrows). Interestingly, however, while the amputation triggered a de-novo GFP upregulation in the
Figure 3.2. The notochord response is wt1b:GFP specific and does not induce a novel GFP upregulation in wt1a:GFP larvae (A) Three dpf wt1a:GFP and wt1b:GFP were tail amputated and imaged at 0, 24 and 72 hpa. (B) The tail amputations triggered the creation of a “regeneration blastema” at the site of amputation by 24 hpa (blue arrows). The amputation also caused the upregulation of GFP in the notochords of wt1b:GFP larvae (yellow arrow heads), a feat that was not observed in the injured wt1a:GFP larvae. The GFP upregulation appeared stronger in the wt1b:GFP larvae by 72 hpa (yellow arrows). The wt1a:GFP larvae never showed an upregulation of their fluorescence, even though a loss of cellularity could be observed in their notochords (orange arrow). Scale bar = 100 μm
wt1b:GFP larvae, no fluorescence upregulation could be observed in the wt1a:GFP amputated tails (Figure 3.2B – 24 hpa; yellow arrow heads).

This result was more evident by 72 hpa. At this point, the notochords of all transgenic larvae had initiated the injury response and the change of cellularity could be observed along the caudal end of their tails (Figure 3.2B – 72 hpa). However, while the expansion of the response led to a similar amplification of the GFP response in the wt1b:GFP larvae (Figure 3.2B – 72 hpa; yellow arrows), no GFP response could be seen in the wt1a:GFP larvae (Figure 3.2B – 72 hpa; orange arrow).

The amputations also revealed how the newly developed GFP expressing cells tended to congregate at the caudal most end of the tail, with the number of cells accumulating increasing with the passage of time (Figure 3.2B – 24hpa vs 72 hpa). Most cells accumulated inside a protruding circular mass of cells that had formed at the end of each amputated tail (Figure 3.2B – 72 hpa; orange and yellow arrows). This circular mass is a common feature of tail amputated larvae and has been previously described by Rojas-Muñoz and colleagues, who call it the “larval regeneration blastema”, as its genetic profile resembles that found in the regenerating blastema of amputated adult zebrafish tails (Rojas-Muñoz et al., 2009).

3.2.3. Time-lapse experiments help to visualise the cellular changes triggered in response to the damage

To record and dissect the cellular processes arising in response to the amputation, I designed a live in vivo time-lapse imaging experiment with the amputated zebrafish larvae. Wt1b:GFP larvae were tail amputated, embedded in 1% low melting point
Figure 3.3. Time-lapse analysis reveals the dynamic development of the tail amputation response. (A) *Wt1b:GFP* larvae were tail amputated and imaged for 48 hours in 30 minutes intervals. (B) The time-lapse showed that the first onset of GFP expression appeared in the notochord by 5 hpa (red arrow). The fluorescent response developed and extended along the notochord via the induction of new losses of cellularity in the inner cells of the notochord (yellow arrow head). The GFP response reached the amputation site by 22 hpa, entering the “regeneration blastema” that had formed at the end of the notochord (blue arrow), accumulating inside over time. Interestingly, a strong tail fin regeneration could be observed in the amputated larvae (red arrow head).
agarose and imaged over 48 hours in 30 minutes intervals under brightfield and fluorescent conditions (Figure 3.3A and Figure 2.2 in Materials and Methods).

The time-lapse showed that within a few hours, the notochord reacted to the amputation and triggered the loss of cellularity I had seen in the time-course experiments (Figure 3.3B; see video 1 in Appendix). Similarly, this change arose some distance away from the site of damage and, interestingly, led to the first expression of GFP at 7 hpa (Figure 3.3B – 7 hpa; red arrow).

The initial pocket of wt1b:GFP positive cells quickly expanded and, surprisingly, appeared to travel down the notochord towards the amputation site (Figure 3.3B – 7 hpa to 22 hpa). As it did, additional cellularity changes in the notochord were observed, coupled with increased GFP upregulation that led to the amplification of the response (Figure 3.3B – 17 hpa; yellow arrow head).

By 22 hpa, the GFP response arrived at the site of damage, entering the regeneration blastema at the amputation site, where they accumulated over time (Figure 3.3B – 22 hpa to 47 hpa). Interestingly, as these events unfolded, the tail could be seen to be regenerating, regrowing its fin tissue (Figure 3.3B – 47 hpa; red arrow head). This opened the question of whether the wt1b:GFP response could be directly influencing this process or even be providing the factors necessary for the proper development of the tail regeneration response.

3.2.4. The wt1b:GFP response is triggered by notochord damage and is independent of the tail fin regeneration response

In order to examine the link between tail fin regeneration and the novel wt1b:GFP-coupled response to damage (from hereon in, collectively known as the GFP
response), I designed a series of tail amputations and used them to injure the \textit{wt1b}:GFP larvae.

These amputations were designed to cause selective damage to the tail, targeting the tail fin, as well as different parts of the notochord. The reason for this was that I wanted to explore the triggers of the response and challenge the link between the GFP response and tail fin regeneration. These amputations were named according to the area of the tail that they affected and where the cut was being performed (Figure 3.4A).

As such, the “tail fin” (TF) amputation cut through the caudal tail fin of the larvae and left the notochord undamaged. The “tip of the notochord” (TN) amputation cut through the most posterior end of the notochord, causing minor damage to the organ. The “before the caudal vein” (BCV) amputation used the caudal vein as reference and cut the tail at a site just before the posterior end of the caudal vein, causing a more extensive notochord damage. Finally, the “past caudal vein” (PCV) amputation went deeper into the notochord, cutting the tail at a site just past the posterior loop of the caudal vein, affecting the notochord and one of the primary blood vessels of the larvae (Figure 3.4A).

I tail amputated \textit{wt1b}:GFP larvae at the sites described above and followed them for 72 hours, imaging them at 0, 24 and 72 hpa under brightfield and fluorescent conditions (Figure 3.4A).

The first thing I noticed with the amputations was that only notochord-damaged tails resulted in the upregulation of a GFP response (Figure 3.4B and 3.4C). In fact, TF amputations did not trigger a GFP response or a loss of cellularity in the notochord, even though they led to marked fin regeneration in the tails of the TN amputated larvae (Figure 3.4B – TF; orange arrow head). This result indicated that
there was no link between caudal fin regeneration and the GFP response. Thus, tail fin regeneration was independent of the GFP response.

Notochord damaging amputations (TN, BCV and PCV) showed an “anteriority” pattern to their GFP responses, with the strength and the frequency of the responses being proportional to the depth of the cut. As such, TN amputations resulted in a modest GFP response in the injured notochords (data not shown), which was only observed in 25% of all injured larvae (Figure 3.4B and Figure 3.4C – TN).

In contrast, the deeper BCV and PCV amputations triggered stronger GFP responses in their larvae. BCV amputated larvae responded to the damage with a visible GFP response in over 70% of all amputations (Figure 3.4C – BCV). In general, these larvae showed a faint GFP expression in their notochords by 24 hpa that translated into a stronger GFP upregulation by 72 hpa (Figure 3.4B –BCV; blue arrow).

PCV amputated larvae showed the strongest response to injury. The notochords of these larvae normally displayed a stronger GFP upregulation by 24 hpa, which was not seen in other injured larvae (Figure 3.4B – 24 hpa PCV; yellow arrow head). By 72 hpa, they also expressed a more intense GFP up-regulation, which was coupled to a more extensive GFP response (Figure 3.4B –72 hpa PCV; yellow arrow). Additionally, unlike the other amputations, all PCV amputations resulted in a successful GFP response to injury (Figure 3.4C – PCV).

These results highlighted the notochord specificity of the response, exempting it from having a role in the tail fin regeneration process, and uncovered an anteriority pattern to the way the notochord responds to damage.
Figure 3.4. Selected tail amputations uncover the notochord specificity of the response.
3.3. A novel needle injury approach and a new transparent wt1b:GFP transgenic line for the long-term analysis of the response

3.3.1. Designing a novel needle injury approach to target the notochord

Tail amputation experiments had helped me uncover a previously unknown notochord response to damage in the zebrafish larvae. However, the tail amputations were highly invasive and created extensive secondary damage to other tissues.

This damage jeopardised the survival of the larvae and meant that tail amputations were unsuitable for the long-term analysis of the response. Additionally, tail amputations also limited the area of the notochord that could be injured and excluded the possibility of injuring the notochord at more anterior sites.

To overcome these limitations, I had to consider alternative methods of damage that could (i) target the notochord effectively with minimum disruption to surrounding tissues; (ii) allow for the possibility to target other more anterior areas of the notochord that could otherwise be inaccessible; and (iii) increase the survival rate of the larvae after injury.
I trialled several approaches to specifically target the notochord, including the use of lasers, that could cause individual cell ablations (similar to Fleming et al., 2004), and the use of chemicals, such as the copper chelating compound neocuprin (Gansner et al., 2007) that could cause a more generalised notochord disruption. Unfortunately, these approaches were either slow to set up or caused too much unspecific damage to the notochord. For these reasons, I designed a novel manual approach that could specifically target the notochord and reduce the amount of time required for the injury.

This approach used an electrolytically sharpened tungsten wire (Brady, 1965), which was fine enough to be used as a needle to damage the notochord without majorly affecting its surrounding tissues (Figure 3.5A). The method proved very successful and I chose it as my preferred method due to its reliability, reproducibility and the speed of the approach, which allowed for tens of larvae to be injured in a short period of time (see below).

3.3.2. Creating a novel transparent wt1b:GFP line to visualise the development of the response over time

The aim of using an alternative method to injure the larvae was to follow the damage response over time. I needed to clarify the role of the GFP cells in the damage response and the effect that these cells could have in the normal morphogenesis of the notochord.

To explore this, I needed a pigment-free system that would offer the clarity necessary to follow the response over a long period of time. Unfortunately, zebrafish develop pigment as they age, with three distinct chromatophores
contributing to their pigmentation: black melanophores, yellow xanthophores and iridescent iridophores (Quigley and Parichy, 2002).

Zebrafish initially develop as translucent animals, but by 24 hpf, they begin to develop pigment in clusters around the head, yolk sac and dorsal, ventral and lateral lines. As they age, these clusters extend, covering their bodies and forming intricate patterns that will give rise to the fish’s common yellow and grey colouring and characteristic black stripes. As the fish develop their pigment, they lose their translucency and become extensively more difficult to image, negating the proper analysis of the response.

Recently, a new transparent zebrafish line has been created that bears recessive mutations in two pigment forming genes, roy and mitfa (White et al., 2008). The line, also known as casper, is virtually transparent throughout its life span, with the fish failing to develop any chromatophores in their bodies except for in their eyes (see Figure 1.7B and C in Introduction chapter).

In order to create a transparent wt1b:GFP zebrafish line, I crossed the transparent casper line to the homozygous wt1b:GFP line and incrossed their offspring (F1 generation) in sibling-sibling matings (see Figure 1.7A in Introduction chapter). The resulting F2 fish were selected for transparency and wt1b:GFP expression (Figure 1.7B and C in Introduction chapter).

3.3.3. Using the transparent wt1b:GFP; casper line in needle injury experiments

Having developed a less invasive injury approach and a pigment-free system to follow the response over time, I wanted to combine them together to explore the response in more detail. I wanted to observe whether the needle injury could trigger the same morphology changes that I had observed in the notochord with the
tail amputations and whether, in combination, they could help me clarify the role of the GFP cells in the damage response and its effect in normal notochord morphogenesis.

For this, I initially targeted the notochords of the transparent\textit{wt1b}:GFP; casper larvae with the needle at the PCV amputation site and followed the larvae for the first 72 hours of the response (Figure 3.5A). I chose the PCV amputation site because it had been the most anterior site that I had used in the tail amputation experiments and, as such, it had given the strongest pattern of response.

I needle injured 3 dpf transparent \textit{wt1b}:GFP:casper larvae at the PCV site and assessed their response during the initial 72 hpi. To better detail the morphological changes arising from the response, the larvae were imaged every 24 hours at 0, 24, 48 and 72 hpi under brightfield and fluorescent conditions (Figure 3.5A).

Immediately after the injury, at 0 hpi, the advantage of the needle approach was evident. The needle created a cleaner, notochord specific damage to the tail with minor disruption to the surrounding tissues (Figure 3.5B – 0 hpi).

By 24 hpi, the injury had already undergone a loss of cellularity at the site of damage (Figure 3.5B – 24 hpi; blue arrow and inset). This loss was accompanied by the novel upregulation of \textit{wt1b}:GFP (Figure 3.5B – 24 hpi; blue arrow head). Unlike in the tail amputations, however, the GFP response did not appear to initiate some distance away from the site of injury and instead, it was localised to the site of injury itself.

By 48 hpi, the wound had expanded, appearing larger than 24 hours earlier (Figure 3.5B – 48 hpi; orange arrow and inset). Interestingly, the increase in wound size correlated with an increase in the GFP response and a higher amount of cellularity.
Figure 3.5. Novel needle injuries specifically target the notochord and trigger localised \textit{wt1b:GFP} responses.
Figure 3.5. Novel needle injuries specifically target the notochord and trigger localised \textit{wt1b}:GFP responses. (A) Three dpf \textit{wt1b}:GFP; casper larvae were needle injured at two sites: PCV and YS (end of yolk sac site). The injured larvae were followed for 72 hours and imaged at 0, 24, 48 ad 72 hpi. (C) The PCV injury specifically targeted the notochord and triggered a loss of cellularity at the site of damage (blue, orange and yellow arrow) that was accompanied by the expression of \textit{wt1b}:GFP (blue, orange and yellow arrow heads). (D) The YS injury triggered a larger loss of cellularity in the notochords of the injured larvae (blue, orange and yellow arrows) that resulted in a stronger GFP response (blue, orange and yellow arrow heads). Scale bar = 100 μm.

Loss at the site of damage (Figure 3.5B – 48 hpi; orange arrow head), strengthening the link between the cellularity loss and the GFP upregulation. Notably, unlike the responses observed in the tail amputation experiments, the GFP response in needle injured larvae decreased in strength by 72 hpi (Figure 3.5B – 72 hpi; yellow arrow head). The cells became more confined as new notochord formed around the wound, closing the gap the needle had created (Figure 3.5B – 72 hpi; yellow arrow and inset). This strongly hinted towards a potential repair process and excitingly suggested, for the first time, that the notochord was able to respond to insults and repair the wound induced by the external damage (Figure 3.7B – 72 hpi; yellow arrow).

3.3.4. Targeting more anterior areas of the notochord

The notochord specificity of the needle injury and the reduced damage that it generated in the surrounding tissues meant that this method was ideal to be used to target more anterior areas of the notochord.

With this in mind, I targeted an area of the notochord that was upstream of the PCV site, in the middle of the body, just above the end of the yolk sac (YS; Figure 3.5A). I choose this area for several reasons: (i) Similar to the caudal vein in the amputation
experiments, the end of the yolk sac served as a visual landmark to effectively replicate the wounding, which allowed me to injure approximately the same area of the notochord with every larva; (ii) the droplet shape of the larval body made it difficult to effectively target any areas past this site, as the curvature limited the possibility to pierce through the skin and muscles to reach the notochord; (iii) lastly, wt1b:GFP larvae have a strong GFP expression along their pronephros. The pronephros runs parallel to the notochord and only diverts around the YS area. By injuring this area, I avoided the strong wt1b:GFP expression of the pronephros that could potentially mask the notochord GFP response.

I needle injured the transparent wt1b:GFP; casper larvae at the YS site and followed the larvae for 72 hours, imaging them at 0 hpi, 24 hpi, 48 hpi and 72 hpi under brightfield and fluorescent conditions (Figure 3.5A).

At 0 hpi, similarly to the PCV needle injuries, the needle could be seen to have efficiently targeted the notochord, creating damage specifically inside the organ (Figure 3.5C – 0 hpi).

By 24 hpi, a very strong GFP response had engulfed the injured area, where the aftermath of the injury could still be seen as a black hole within the notochord (Figure 3.5C – 24 hpi; blue arrow head and blue arrow respectively). Amongst the debris, the notochord could be seen to be undergoing the loss of cellularity previously observed with other injuries, which, similarly to PCV needle injuries, appeared to initiate inside the site of damage (Figure 3.5C – 24 hpi; blue arrow and inset).

At 48 hpi, the loss of cellularity had progressed, opening the wound in the notochord (Figure 3.5C – 48 hpi; orange arrow and inset). Unlike the previous PCV needle injuries, the GFP expression completely engulfed the site of damage, appearing as an uninterrupted GFP band along the notochord (Figure 3.5C – 48 hpi;
orange arrow head). The stronger response correlated with the anterior pattern that I had previously observed in the tail amputations, where more anterior injuries yielded stronger GFP responses.

By 72 hpi, the site of injury appeared more defined, with cells conglomerating inside the damaged area and forming structures that hinted towards the repair seen in the PCV needle damaged notochord (Figure 3.5C – 72 hpi; yellow arrow and inset). The GFP response appeared weaker but more spread, with the GFP response forming amongst the new notochordal structures at the site of injury (Figure 3.5C – 72 hpi; yellow arrow head).

3.3.5. Using time-lapse experiments to understand the cellular changes in the notochord triggered by the YS needle injury

As with the tail amputation experiments, I decided to design a time-lapse experiment that could help me study the way in which the notochord responded to the needle injury over the first 48 hours of the response. With this, I wanted to explore the cellular dynamics triggered by the needle injury and determine the morphological changes undergone by the notochord in response to the damage.

Three dpf transparent wt1b:GFP; casper larvae were needle injured at the YS site and imaged in brightfield and fluorescence over 48 hours at 60 minutes intervals (Figure 3.6A; Video 2).

The time-lapse analysis showed that the needle injury was able to trigger the change of cellularity observed in previous tail amputation experiments (Figure 3.6B – blue arrow heads and blue arrows). Similarly, the change of cellularity also led to the de-novo expression of wt1b:GFP, which could be seen in the notochord for the first time 5 hours after the injury (Figure 3.6B – 5 hpi; orange arrow). Interestingly,
Figure 3.6. Time-lapse imaging reveals how the notochord reacts to the needle injury. (A) Three dpf wt1b:GFP; casper larvae were needle injured at the YS site and imaged for 48 hours at 60 minutes intervals. (B) The time-lapse revealed that the needle injury caused a vast loss of cellularity at the site of damage and other more upstream areas of the notochord (blue arrow heads). The first onset of the response was observed at 5 hpi (orange arrow) and was shortly followed by novel upregulations in other areas, including the site of damage (blue arrows and red asterisk). As the response developed, new fragmentation events could be seen in the inner vacuolated cells of the notochord, enhancing the response (red arrows). Scale bar = 50 μm.
its upregulation was initiated some distance away from the injured area, unlike what I had observed in the time-course experiments. This peculiarity was probably due to the combination of the needle injury and the agarose embedding, which could have exacerbated the damage. Similarly, the same phenotype was also observed in the time-lapses of the PCV needle damaged larvae (Video 3).

By 10 hpi, two new pockets of GFP positive cells could be seen in the damaged notochord, this time, closer to the site of damage (Figure 3.6B – 10 hpi; blue arrows). At this point, the first onset of GFP expression was also readily visible at the site of injury amongst all the cellular debris created by the needle (Figure 3.6B – 10 hpi; red asterisk).

In the following 15 hours, more vacuolated cells underwent a change of cellularity, which contributed to the amplification of the GFP profile of the response (Figure 3.6B – 15 and 20 hpi ; red arrows). Notably, no new cellular changes were observed in the notochord after the initial 25 hours of the response (Figure 3.6B – 25 hpi to 40 hpi). This contrasted with the marked intensification of the GFP response, which gradually increased in time. This increase in expression could indicate that the wt1b:GFP cells were proliferating inside the notochord, although, alternatively, it could also signify that there is an increase in the transcriptional rate of the wt1b gene in the injured notochords. Either way, this increase in fluorescence suggested that the response enhanced and strengthened over time.

3.3.6. Assessing the incidence of cell death at the site of injury

Time-lapse and time-course experiments had revealed how the some of the vacuolated notochord cells around the site of injury underwent a change of cellularity in response to the damage. Similarly, they also showed that the change of cellularity was preceded by the onset of wt1b:GFP fluorescence and that these
reshaping cells morphologically resembled cells undergoing apoptosis. The activation of Wt1 has previously been associated with the onset of cellular death (Englert et al., 1995; Perner et al., 2007; Bourkoula et al., 2014), which could mean that the upregulation of the wt1b fluorophore could indicate the cell’s terminal fate.

In order to test this hypothesis, I decided to explore the prevalence of cell death at the site of injury and uncover whether it could have an active role in driving the damage response and contributing to the fluorescent signal after the injury. For this, I live stained injured embryos in acridine orange. Acridine orange is a potent fluorescent dye that actively penetrates dying cells or cell with a weakened plasma membrane and intercalates their DNA (Westerfield 2000; Tucker & Lardelli, 2007).

I needle injured 3 dpf non-fluorescent wild type AB zebrafish larvae and raised the injured larvae in separate groups to 0, 24 and 72 hpi. At each time-point, I incubated the injured larvae in acridine orange and washed out the dye in several E3 washes before imaging them under brightfield and fluorescent lighting (Figure 3.7A; see Materials and Methods for details).

At 0 hpi, all injured larvae showed a pronounced green fluorescence around the site of injury that was not present in non-injured controls (Figure 3.7B – injured; yellow dotted box). A closer look at this area revealed the presence of cellular debris created by the piercing of the notochord around the site of damage (Figure 3.7C – 0 hpi brightfield). The debris was recognised by the acridine orange stain and highlighted the amount of cell death and the tissue damage created by the needle immediately after the injury (Figure 3.7C – 0 hpi; white arrow).

By 24 hpi, the acridine orange stain showed that dead cells still populated the damaged area (Figure 3.7C – 24 hpi fluorescence; red arrow). Importantly, by then, the notochord had already undergone its characteristic change of cellularity in
Figure 3.7. Cell death does not drive the damage response in the needle injured larvae. (A) AB larvae were needle injured and placed in acridine orange at 0, 24 and 72 hpi. (B) Acridine orange staining (green) highlighted areas of cell death in the injured larvae (yellow box and yellow arrow head) and certain organs, such as the olfactory placode and the anal exit (white arrow head and white arrow; asterisk denotes autofluorescence from the yolk sac). (C) A closer look at the injured area revealed that the needle triggered a strong cell death signal at the site of damage shortly after the injury (white arrow). By 24 hpi, a more confined pocket of dead cells could be seen in the needle injured side of the larva, but was absent from the non-penetrated side (red arrows). The cells were solely located at the site of damage and did not overlap with the areas of the notochord that had undergone the loss of cellularity (red arrow head). By 72 hpi, no more dead cells could be observed at the injured site (yellow arrow), even though a clear gap had formed at the site of damage, which normally would be filled with wt1b:GFP positive cells (yellow arrow head). Scale bar = 100 μm.
response to the injury (Figure 3.7C – 24 hpi brightfield; red arrow head). The presence of both events at the same time potentially suggested that the cell death could be driving the change of cellularity observed in the injured notochord. This rationale, however, was dismissed when I turned the larvae around onto their opposite side and observed the site of injury through the side that had not been penetrated by the needle. From here, no acridine orange positive cells could be seen around the damaged area, indicating that the dead cells were found outside of the notochord, probably in the tissues that had been damaged by the introduction of the needle (Figure 3.7C – 24 hpi; needle entry side vs. opposite side). This result was in accordance with what I had observed by eye under the microscope, as the dead cells appeared to be above the injured notochord but not in the notochord itself (data not shown).

By 72 hpi, no dead cells could be seen around the site of damage (Figure 3.7C – 72 hpi fluorescence; yellow arrow). The acridine orange stain did not highlight the present of any cellular debris within the injury, even though a significant gap had formed inside the injured notochord, which would normally be populated by the wt1b:GFP positive cells (Figure 3.7C – 72 hpi; yellow arrow head).

3.3.7. Growing needle-injured transparent wt1b:GFP; casper zebrafish to adulthood to understand the long-term effect of the injury in the notochord

Having investigated the first 72 hours of the response, I wanted to explore the fate of the wt1b:GFP cells in the notochord response and evaluate how the notochord responded to the damage. I had already seen how in the course of the first 72 hours, the site of injury had shown the capacity to repair and heal part of the induced damage. I wanted to see whether the notochord was able to heal the wound completely and if this capacity was directly linked to the presence of wt1b:GFP cells at the site of injury.
For this, I used the transparent wt1b:GFP zebrafish line and the needle approach (defined above), as, in combination, they will provide me with a long-term viable pigment free system to study the response over time. I needle injured 3 dpf transparent wt1b:GFP; casper larvae and followed their response to damage over time to 28 dpi, imaging them at 0, 3, 14, 21 and 28 dpi (Figure 3.8A). However, it must be noted that because our rearing methods (which include the growth of larvae at room temperature from 5 to 14 dpf; see Materials & Methods for details), our larvae are slightly more developmentally delayed than similarly aged larvae from previous publications. For this reason, I complement the dpi measurement with the standard length (SL) of the larvae at the time, which gives a true indication of their developmental stage (based on measurements from Parichy et al., 2009).

The needle injury triggered the previously observed strong wt1b:GFP response at 72 hpi, creating extensive changes of cellularity in the vacuolated cells of the damaged notochord (Figure 3.8B – 3 dpf and 3 dpi; white arrow and white arrow head respectively).

By 14 dpi, the site of injury had undergone a significant transformation. The gap created by the needle had reduced in size and the strength of the wt1b:GFP response had vastly decreased, with a small portion of the GFP response localised to the site of injury and some neighbouring areas within the notochord (Figure 3.8B – 14 dpi; yellow arrow and yellow arrow head). Additionally, the affected vacuolated cells around these areas appeared to have condensed, forming tighter structures around the site of damage (Figure 3.8B – 14 dpi; yellow arrow head).

By 21 dpi, the wound had almost completely sealed. A scar was noticeable at the site of damage and a small pocket of GFP-positive cells could still be seen within it (Figure 3.8B – 21 dpi; blue arrow). The fragmented area within the notochord that surrounded the site of injury looked more defined and even resembled the areas of
Figure 3.8. Injured larvae heal their notochords over time. (A) Three dpf wt1b:GFP;casper larvae were needle injured and imaged at 0, 3, 14, 21 and 28 dpi to assert the fate of the injury. (B) The needle injury triggered an extensive GFP upregulation in the notochords of the wt1b:GFP larvae at 3 dpi (white arrow and white arrow head). By 14 dpi, the GFP response appeared to have become more confined, with a more concise GFP expression at the site of injury and a reduced number of GFP cells in other more upstream areas (yellow arrow and yellow arrow head). By 21 dpi, wt1b:GFP positive cells could be seen at the injured area, where they had become localised to a specific strip of the notochord (blue arrow). Its upstream area, appeared to have condensed and re-gained a more notochord-like look (blue arrow head). By 28 dpi, only a few wt1b:GFP positive cells remained at the site of injury (red arrow head). The injury appeared to have formed a scar at the site of damage (red arrow and inset), which dissected the notochord in two. By this point, the notochord had also began to ossify, with neural arches and centra forming along its structure (orange arrows and green arrows respectively).
the notochord that have not been injured by the needle (Figure 3.8B – 21 dpi; blue arrow head). Intriguingly, the injured site underwent a new change of morphology by 28 dpi. By this point, the vertebral bodies had started to form along the length of the notochord, with the most anterior vertebrae exhibiting the first neural arches (Figure 3.8B – 28 dpi; green arrows and orange arrows respectively). At the site of injury, the scar formed by the needle damage had transformed into a blackened area that sectioned the notochord in two (Figure 3.8B – 28 dpi; red arrow). Interestingly, at the edge of the hole, a pocket of GFP cells appeared to exhibit a stronger fluorescent mark than 7 days earlier. These cells were localised to a small area of the notochord, which coincided with a developing vertebral body (Figure 3.8B – 28 dpi; red arrow head and inset). The co-localisation of the GFP signal with the vertebra did not appear to be a random event and potentially meant that the \textit{wt1b}:GFP response could be directly involved in the mineralization of the vertebral column after the injury (n=10; see Chapter 4 below).
3.4. Histological analysis highlights the changes of cellularity in the notochord and reveals the presence of small GFP expressing cells at the site of injury

3.4.1. Histological analysis and immunostaining of the notochord at 72 hpi

The live imaging of the injured larvae had helped me capture the cellular dynamics of the response. However, I wanted to examine the cellularity changes triggered by the needle and get an insight into the spatial distribution of the GFP cells at the site of damage. To examine this, I histologically stained the injured and non-injured larvae and used a cohort of antibodies to determine the location of the GFP cells and their activity inside the wound.

As I had observed the strongest GFP activation at 72 hpi, I YS injured 3 dpf wt1b:GFP larvae and grew them to 72 hpi, imaging them and processing for sectioning at this time-point (Figure 3.9A). All larvae were sectioned sagittally and processed in haematoxylin and eosin (H&E) before being immuno-stained against GFP and the cell division marker phospho-histone 3 (pH3) (Figure 3.9A).

The H&E staining revealed how smaller vacuolated cells had replaced the large inner vacuolated cells around the site of injury (Figure 3.9B – H&E; double asterisk and asterisk). These cells looked like they were the product of the cellularity changes I had seen in my previous time-lapse experiments and, as such, confirmed that the vacuolated cells underwent a change of cellularity in response to the injury.

Additionally, within the damaged area, a multitude of smaller cells could be seen populating the gap created by the needle (Figure 3.9B – H&E). These cells looked very different to the large vacuolated cells of the notochord and when observed closely, they appeared to be more mesenchymal in nature (Figure 3.9B – H&E; inset).
Figure 3.9. Immunohistochemistry highlights the change of cellularity in the notochord and identifies the localisation and the proliferative profile of the GFP cells.
Figure 3.9. Immunohistochemistry highlights the change of cellularity in the notochord and identifies the localisation and the proliferative profile of the GFP cells. (A) Three dpf $wt1b$:GFP;casper larvae were needle injured and grown to 72 hpi, when they were imaged and fixed for sectioning and histology. (B) The histological analysis of the injured site revealed the extensive change of cellularity in the area in comparison with the normal physiology of the notochord (orange asterisk vs. double orange asterisk). It also uncovered the presence of smaller mesenchymal-like cells at the site of damage (inset). GFP immunostaining of the area identified these cells $wt1b$:GFP cells of the response (orange arrow and orange arrow heads). Staining for pH3, a marker of cell division, demonstrated the proliferative capacity of some of the cells that occupied the injured site (green arrow). Scale bar = 50 μm.

Unsurprisingly, staining with the GFP antibody revealed that these mesenchymal-like cells were responsible for the strong fluorescence observed at the site of injury (Figure 3.9B – α-GFP). The GFP immunostaining also highlighted the varied compartmentalisation of the GFP protein, with nuclear and cytoplasmic expression found alike within the cell population (Figure 3.9B – α-GFP; orange arrows and orange arrow heads respectively).

Staining with the pH3 staining showed a more variable result. At 3 dpi, not many cells could be observed undergoing cell divisions at the site of damage, though dividing cells could be seen amongst these cells (Figure 3.9B – α-pH3; green arrow). The presence of a mitotic marker within these cells suggested that cell proliferation could be one of the reasons why the response increased in strength over time; even after no more cellularity changes were evident in the notochord.

3.4.2. Histologically defining the first 24 hours of the response

Time-lapse imaging had shown the importance of the initial 24 hours in the development and establishment of the GFP response. During this time, the cells of the notochord reacted to the injury, initiated the response and amplified it over time.
Similarly to what I had done with the 72 hpi larvae, I also wanted to explore the changes of cellularity undergone by the notochord during the first 24 hours of the process. I wanted to define the response immunohistochemically to determine the location of the first onset of GFP and validate the dynamic changes I had observed during my imaging experiments.

To do this, I YS needle injured wt1b:GFP larvae and grew them separately to 6, 12 and 24 hpi. At each time-point the larvae were fixed, embedded and section for histology (Figure 3.10A). At the same time, I also needle-injured a second group of larvae and grew them in parallel. These larvae were used to show case the development of the response and were imaged under brightfield and fluorescent lighting at each time-point.

At 6 hpi, the damage in the notochords of the injured larvae could be easily distinguished amongst the row of cells, even though no observable GFP upregulation could be distinguished at this point (Figure 3.10A – 6 hpi merged).

The H&E staining phenocopied the imaged larvae, as the sections the notochord could be seen parted by the needle injury, which created a division in the row of vacuolated cells (Figure 3.10B – 6 hpi H&E). The lack of GFP was consistent with the in-situ staining of the tissue with the GFP antibody, which did not identify any GFP positive cells in the area at this point (Figure 3.10B – 6 hpi α-GFP). Interestingly, staining with a pH3 antibody showed the presence of proliferating cells at the edge of the notochord, in an area populated by the cells from the surrounding notochord sheath (Figure 3.10B – 6 hpi α-pH3; orange arrow).

At 12 hpi, the first expression of GFP could be seen developing inside the wound of the injured larvae (Figure 3.10B – 12 hpi merged; red arrow). At this point, H&E staining showed that the first cells had begun to populate the site of damage, a sign of the initiation of the response (Figure 3.10B – 12 hpi H&E; red arrow heads).
Figure 3.10. The histological analysis of the first 24 hours of the response. (A) Three dpf transparent \textit{wt1b:GFP; casper} larvae were needle injured and grown to 6, 12 and 24 hpi, when they were fixed and sectioned for histology. (B) H&E staining of the injured larvae revealed the cellularity changes undergone by the notochord after the damage and highlighted the presence of cells into the site of injury (red arrow and red arrow heads). GFP immunostaining unveiled the fast population of the injured area with GFP expressing cells (blue arrow and blue arrow head) and the presence of proliferating cells in the notochord, both at the site of injury and annexed areas (orange and yellow arrows). Scale bar = 50 μm.
The low GFP upregulation could not yet be detected immunohistochemically in the sectioned tissue (Figure 3.10B – α-GFP). Similarly, no pH3 was observed in the sections of the injured notochord at this point (Figure 3.10B – α-pH3).

By 24 hpi, the response had undergone a broad change. The wound had extended and could be seen filled with a homogeneous GFP signal (Figure 3.10B – 24 hpi merged). H&E staining highlighted the change of cellularity that had started to occur at the site of injury and the appearance of a large number of cells at the site of damage (Figure 3.10B – 24 hpi H&E). GFP staining of the area yielded a strong result, showing the presence of GFP positive cells at the wound and, interestingly, at the edges of fragmenting vacuolated cells (Figure 3.10B – 24 hpi α-GFP; blue arrow head and blue arrow respectively). pH3 staining also revealed that there was a proliferative signal in a number of these cells inside the site of damage (Figure 3.10B – 24 hpi α-pH3), though there weren’t enough numbers to conclude with certainty that these cell divisions were behind the increase in GFP signal.

3.4.3. FAC sorting GFP cells from the injured notochord

The experiments highlighted in this chapter have shown how a new population of wt1b:GFP positive cells were created in response to notochord damage. To further support their existence and quantify the numbers of GFP cells that were created in response to the injury, I carried out a FAC sort analysis of the injured notochords.

As I had observed the strongest GFP upregulation at 72 hpi, I needle injured wt1b:GFP larvae and grew them for 72 hours, before dissecting out the part of the torso containing the injured notochords (Figure 3.11A; red box). The torsos were dissected to avoid the strong GFP expression of the kidneys, heart sac and forebrain of the wt1b:GFP larvae (Figure 3.11A; arrow heads). As a control, tissue was taken
from similar sized section downstream of the injured area in the same larvae (Figure 3.11A; blue box).

Forty-six larvae were used for the FACS experiment. The injured larvae were dissected and their tissues were collected in cold PBS complemented with 2% FCS (see Chapter 2 - Materials & Methods for details). The tissues were disassociated using a mixture of collagenases and trypsin, as described in Manoli & Driever, 2012 and passed through a 40 μm cell strainer to disassociate the cells. The cells were then sorted inside a FACSaria2 SORP machine and selected for GFP expression.

The injured torsos yielded 875 GFP positive cells (4.1% of all sorted cells), against 73 positive cells (0.4% of all sorted cells) from the non-injured sections of the larvae. It should be noted, however, that cells are normally lost during the isolation procedure, therefore the total number of cells is only partly representative of the total amount of cells found in each group of embryos. That said, this result further validated the presence of GFP expressing cells in the injured notochords and demonstrated that the injury triggers a significance increase in GFP cells.

It was also interesting to see that there was also a small population of GFP expressing cells in the non-injured larvae. This could suggest that there are other non-notochord GFP positive cells in the wt1b:GFP larvae.
Figure 3.11. FAC sorting the wt1b:GFP cells. (A) Three dpf transparent wt1b:GFP; casper larvae were needle injured and grown to 72 hpi. The injured site and a downstream homologous areas (red and blue boxes) were dissected from each larva, pooled and their cells were disassociated and FAC sorted. (B) The FACS analysis showed a notable increase in the number of fluorescent cells in the injured area in comparison to the non-injured areas revealing the presence of almost 20 GFP positive cells at the site of damage per injured larvae. Y-axis represents GFP expression and X-axis represents RFP expression.
3.5. Discussion

3.5.1. The notochord reacts to external damage via the activation of a novel \textit{wt1b}:GFP cellular response

In my search for new \textit{wt1}-associated processes, I uncovered a previously unidentified damage response in the notochord of zebrafish larvae. Using fluorescent transgenic lines, I identified the genetic markers of the response and in combination with selective tail amputations and a novel needle injury approach I characterised the physiological features of the process.

I discovered that, upon injury, the inner cells of the notochord undergo a loss of cellularity that creates a gap in the tight row of vacuolated notochord cells, which becomes populated with \textit{de-novo} expressing of \textit{wt1b}:GFP positive cells. The different injury approaches also helped me to define the biology of the response. They revealed that the process is independent of tail fin regeneration and it is governed by an anteriority effect that patterns the strength of the response to damage.

Time-lapse analysis helped me establish the chronological development of the response and highlighted key episodes of the process. For example, in both tail amputated and needle injured larvae the responses were initiated at around 5 to 7 hpi. Interestingly, the site of initiation differed between the amputated and the needle ablated larvae. In the former, the response usually started some distance away from the amputation plane, whilst in the needle-injured notochords the response tended to be more injury centric.

The disparity in the initiation of the responses could potentially be due to the amount of damage introduced to the notochord and its surrounding tissues by each injury method. The severity of the tail amputation could be inhibiting the initiation
of the response around the damage area, hence triggering signals for its initiation further upstream. Additionally, it could also be that more anterior sites could contain a higher concentration of the cells needed for the successful initiation of the response, a presumption supported by the anteriority effect that I had found to control the process.

The time-lapses also highlighted the way in which the GFP response developed. Over the first 24 hours, the response extended along the notochord, amplifying its fluorescent signal inside the organ. The amplification of the response was due to the rapid appearance of other GFP coupled changes of cellularity close to the initial site of expression. The proximity between the events and the rapid development of the response, could potentially highlight the existence of an underlying interaction between the wt1b:GFP positive cells and its environment, which could promote new morphology changes in the inner vacuolated cells. This hypothesis particularly held true in the tail-amputated notochords, where the initial onset of GFP could be seen triggering changes of cellularity in the downstream vacuolated cells.

Interestingly, the response developed differently after the initial 24 hours. No more changes of cellularity could be observed in the injured notochord after this stage, even though there was a notable increase in the fluorescent signal of the response. Potentially, this could be explained by the assumption that the wt1b:GFP cells could be proliferating inside the injured areas of the notochord, a possibility that was supported by the pH3 staining of the injured area (see below). Alternatively, higher transcriptional activity in the cells could be driving the increase in the signal.

The wt1b:GFP specificity of the response is also one of its most remarkable features. Notably, this is not the first time that the wt1b:GFP reporter line has been associated with a tissue damage response. Recently, two labs uncovered the de-novo expression of wt1b:GFP in the epicardium of cryoinjured adult hearts and showed how the GFP cells could penetrate the regenerate and contribute to the
regrowth of the heart (Schnabel et al., 2011; Gonzalez-Rosa et al., 2011). Moreover, one of the labs also showed that the fluorescent signal was concomitant with the *de-novo* upregulation of *wt1b*, linking the expression of the reporter line with the endogenous gene (Gonzalez-Rosa et al., 2011).

Similarly to the heart, the notochord has a thin layer of epithelial-like cells enveloping the main body of the organ (Stemple, 2005; Dale & Topczewski, 2011; Ellis et al., 2013). These cells are known as the outer sheath cells or chordoblasts and are in charge of secreting the different layers of perinotochordal sheath that holds the notochord together (Stemple, 2005; Yamamoto et al., 2010; Ellis et al., 2013). It would be interesting to study the role of these cells in the needle injury response and see whether they can directly or indirectly affect the development of the *wt1b*:GFP response.

Lastly, it is also worth mentioning the striking cellular movements observed in the tail-amputated larvae. These movements allow the *wt1b*:GFP cells that arise some distance away from the site of the amputation to move towards the injured end of the notochord, filling the regeneration blastema. The movements could potentially be attributed to the sudden drop in pressure in the amputated notochord, which could be driving the notochord content out of its shell. However, a recent paper highlighting the same process showed that the formation of a regenerating blastema and the movement of the notochord cells could be partially inhibited using PI3K and ErbB specific inhibitors, which caused a partial delay to its development (Rojas-Munoz et al., 2009). It would be interesting to evaluate this further and try to conclude whether there is a link between this migration and the upregulation of the *wt1b*:GFP cells.
3.5.2. The wt1b:GFP cells become more localised and reduce their expression at the site of injury as the wound heals over time

I also aimed to follow the progression of the wt1b:GFP response, to uncover the fate of the GFP cells and visualise the way in which the notochord reacts to the injury. I took advantage of my newly created transparent wt1b:GFP; casper line to explore the development of the response in a pigment-free system and used the less invasive needle injury approach to successfully grow the injured larvae to adulthood.

I found that the needle injury triggered a widespread response in the notochord during the first 72 hours of the response. However, the response reduced in strength over the following 25 days, as highlighted by a clear decrease in the signal strength and a confinement of the GFP cells to certain areas of the injury. In parallel to this, the wound decreased in size. Over the course of the 25 days, the gap created by the needle closed down, with the creation of new inner notochord cell-like structures and the condensation of cells at the site of damage. By the end of the time-course, the affected area looked very similar morphologically to its surrounding non-injured notochord, though the injury had led to the formation of a scar at the area penetrated by the needle.

These results suggested that the notochord was able to heal and repair the damage caused by the injury, but could not faithfully regenerate the tissue to its original state. The fact that the wound healed as the fluorescent output of the response decreased also tentatively suggested that there could be a direct correlation between the repair of the damage and wt1b:GFP cells, whereby the GFP cells could have an active part in the replenishing of the lost notochord.

It would be worth looking individually at the population of the vacuolated cells to explore whether the closure of the gap was due to the creation of new vacuolated
cells or the movement of cells into the gap by the growth of the notochord (see next Chapter). The initial evidence, however, indicates that the axial movement of the notochord does not fill the wound. The position of the wound in relation to the caudal end of the gut and the developing somites showed that the location of the wound was conserved to the 16th somite of the larvae throughout the length of the experiment. Moreover, the wound and its affected upstream area seemed to span across the same three somites at each time-point.

The appearance of the mineralizing chordacentra towards the end of the time-course indicated that there was a need to explore the interaction between the wt1b:GFP response and the forming vertebrae. The imaging of the larvae at 28 dpi had shown that centra formed around the injured area and even overlapped with the location of the wt1b:GFP expressing cells. In its normal morphogenesis, the notochord cells would ultimately regress to the intervertebral disc, leaving way to the formation of the vertebral bodies (Bensimon-Brito et al., 2012; Haga et al., 2009). In my next chapter I studied the effect of the needle injury on the vertebral biogenesis and established how the notochord damage affected the formation of the vertebral column.

3.5.3. Histology highlights the cellular processes that shape the response and reveal the proliferation and possible mesenchymal identity of the GFP positive cells

The immunohistochemical analysis of the response helped me validate the morphological changes endured by the notochord after the damage. H&E stains and GFP and pH3 antibodies highlighted the cellular heterogeneity of the wound and defined the spatial distribution and proliferative profile of the GFP cells.

I discovered that the injured area was heavily populated with mesenchymal-like cells, which shared the space with smaller vacuolated cells created by the change of
cellularity. GFP staining identified the mesenchymal-like cells as the *wt1b*:GFP expressing cells of the response, whilst pH3 antibodies demonstrated that some of these cells were actively dividing inside the damaged notochord.

Using these methods, I also tracked the first 24 hours of the response. The H&E staining validated the cellular rearrangements observed in the live imaging and showed how the site of injury progressively widened in time, heavily influenced by the change of cellularity in the vacuolated cells. The H&E staining also highlighted the presence of the first cells inside the wound at 12 hpi and showed how their numbers increased with the widening of the wound over time.

The low fluorescence found at the site of injury at 12 hpi meant that the GFP staining could not validate the fluorescent character of the cells inside the wound early on the response. The antibody, however, strongly highlighted the presence of the *wt1b*:GFP cells at 24 hpi, once their numbers had increased. PH3 staining of the sections also revealed the mitotic profile of some of the GFP expressing cells, which could potentially explain the increase in the GFP signal after the first 24 hours of the response. Interestingly, the pH3 antibodies also identified the presence of mitotic cells in the layer of notochord sheath cells early in the response, which putatively indicated that these cells also responded to external insult.

Overall, these results confirmed the presence of a population of *wt1b*:GFP mesenchymal-like cells at the site of injury and unveiled that some of these proliferated inside the wound as part of the response. Intriguingly, the ubity of these cells amongst what looked like “mid-transitioning” inner vacuolated cells suggested that there was a possibility that the mesenchymal-like cells could originate from the notochord’s vacuolated cells. This hypothesis was strengthened by the lack of cellular death observed inside the notochord in the acridine orange experiments, which meant that the loss of cellularity observed in these cells at the beginning of the response was not due to apoptosis.
However, the number of cells present at the injured site was substantially higher than the number of vacuolated cells expected to have undergone a cellularity change in the injured area. This and the GFP staining of notochord sheath cells close to the site of injury, did not exclude the possibility that these cells could have a notochord sheath origin. Alternatively, there could be a dual contribution from each notochord cell population. This last premise was partly supported by the incomplete staining of all the mesenchymal-like cells with the GFP antibody. If this was to be the case, it would partly resemble the regenerative response seen in the damaged heart, where epicardial and cardiomyocyte cells dually contribute to the regeneration of the wound (Jopling et al., 2010; Smart et al., 2011; Schnabel et al., 2011; Gonzalez-Rosa et al., 2011).

Lastly, the identification of the mesenchymal-like cells as the source of the \textit{wt1b}:GFP signal strongly argued in favour of the existence of an EMT response in the notochord. This, however, needs to be validated with the identification of EMT associated markers and the confirmation of the expression of the endogenous \textit{wt1b} gene in the damage response.
CHAPTER 4: Results

The needle injury triggers a \textit{de-novo} expression of \textit{wt1b}:GFP in the outer notochord sheath cells and a double vertebrae phenotype in adults.
4.1. Introduction

Using tail amputations and a novel needle injury approach I had visually identified for the first time the existence of an innate damage response in the notochords of zebrafish larvae. I had used fluorescent transgenic lines, time-lapse imaging and immunohistochemical analysis to characterise the timings of the response, observe the cellular changes triggered by the injury and decipher some of the genetic markers governing the process.

Next, I wanted to determine the cellular origin of the response. As mentioned earlier, the notochord is made of two distinct cell populations: a population of large vacuolated inner cells that makes the body of the notochord and a population of outer epithelial-like cells that form a cellular layer around it (Stemple, 2005). I used pre-existing cell-specific fluorescent transgenic lines to individually highlight each cell population and study their responses to the injury in the context of the wt1b:GFP response (Yamamoto et al., 2010; Dale & Topczewski, 2011). With this, I aimed to decipher their role in the initiation of the response and establish their cellular behaviour in response to the damage.

Secondly, I wanted to study how the immune system reacted to the needle damage. The innate immune cells have been shown to be important for the regulation of wound healing and regeneration in zebrafish and other vertebrates (Park & Barbul 2004; Tidball and Wehling-Hendricks et al., 2007; Eming et al., 2009; Niethammer et al., 2009; Li et al., 2012; Godwing et al., 2013). I wanted to discern how the two immune cell populations of the early larvae, the macrophages and neutrophils, were drafted into the damaged area and how they interacted with the environment of the wound. For this, I made use of two fluorescent transgenic lines that specifically highlighted each cell population (Hall et al., 2007; Gray et al., 2011) and crossed them to wt1b:GFP fish to explore their interaction with the nascent GFP response.
Additionally, I also looked at the hydrogen peroxide signal. Hydrogen peroxide is one member of the ROS family, which is linked to tissue damage and wound formation (Niethammer et al., 2009). Previously, reports have linked its expression with the regenerative capacity of the zebrafish tail and the recruitment of the immune cells to the site of damage (Niethammer et al., 2009; Li et al., 2012). Using a fluorescent detection system on a wt1b:GFP background, I reported the presence of hydrogen peroxide at the wound and established its chronological development at the site of injury.

Lastly, I decided to look at skeletal mineralization of the notochord. In zebrafish, as well as other vertebrates, the notochord provides the scaffold for the development of the vertebral column and has been shown to be essential for its proper formation (Fleming et al., 2001; Fleming et al., 2004; Christiansen et al., 2009; Haga et al., 2009; Ellis et al., 2012; Bensimon-Brito et al., 2012; Gray et al., 2014). Mineralizing rings, known as chordacentra, form along the length of the notochord in an anterior to posterior manner and establish the site of development of the future vertebrae in vertebrates (Haga et al., 2009; Bensimon-Brito et al. 2012).

In collaboration with Stefan Schulte-Merker at the Hubrecht Institute (Utrecht, The Netherlands), I used a novel chordacentra-specific fluorescent transgenic zebrafish line (Huijtema et al., 2012; unpublished data) to explore how notochord damage affected the mineralization patterning of the notochord in the context of the wt1b:GFP response. Additionally, I examined the long-term effect of the notochord injury in the forming vertebrae using a series of calcium binding vital dyes to analyse the ossification of the notochord over time. With these experiments I aimed to understand how the vertebrae formed along the injured notochord and investigate whether the wt1b:GFP response could have a role in vertebrae formation.
4.2. Notochord injury triggers the activation of \textit{wt1b}:GFP in the outer notochord sheath cells that surround the site of damage

4.2.1. \textit{Fluorescent zebrafish lines highlight the two distinct cell populations in the notochord}

The zebrafish notochord is a structurally simple organ. It is composed of two discrete cell populations that are surrounded by a tough but flexible extracellular basement membrane that keeps the notochord under pressure (Stemple, 2005; Figure 4.1A). In its core, a row of large vacuolated cells forms the main body of the organ. These cells occupy the inner space of the notochord and push against the extracellular matrix to confer the notochord with the necessary turgor pressure for its structural and mechanical functions (Adams \textit{et al.}, 1990; Stemple, 2005; Yamamoto \textit{et al.}, 2010; Ellis \textit{et al.}, 2012). In between the inner cells and the basement membrane (also known as perinotochordal sheath) lies a layer of epithelial-like cells that envelope the inner cells and secrete some of the components of the extracellular sheath. Unlike the inner cells, these cells are highly endoplasmic and play an important role in the development and maintenance of the middle and outer layers of the perinotochordal sheath (Coutinho \textit{et al.}, 2004; Stemple, 2005; Yamamoto \textit{et al.}, 2010).

Two fluorescent zebrafish lines exist that can identify and highlight each cell populations inside the notochord. The first of the lines, the R2-\textit{col2a1a}:mCherry zebrafish line was created by Rodney Dale at the Northwestern University and uses a teleost conserved regulatory sequence of the collagen type II alpha 1 (\textit{col2a1a}) gene (Dale & Topczewski, 2012; known as \textit{col2a1a}:RFP from hereon in). This sequence specifically drives \textit{col2a1a} expression in the outer notochord sheath cells (Figure 4.1 and 4.2B) and certain craniofacial cartilages (Dale & Topczewski, 2012; data not shown).
Figure 4.1. Fluorescent transgenic lines highlight the cell populations of the notochord. The notochord is composed of two cell populations, an epithelial-like notochord sheath cell population (outer cells) and a large vacuolated notochord cell population (inner cells), which are tightly wrapped by a thick but elastic extracellular basement membrane (peri-notochordal sheath). Two zebrafish fluorescent lines were used to study each cell population separately: SAGFF214A; UAS:GFP, which highlighted the plasma membrane of the inner vacuolated cells and the R2-col2a1:RFP, which highlighted the monolayer of outer sheath cells.
The second fluorescent line, SAGFF214A; UAS:GFP (from hereon in known as SAGFF214A:GFP), specifically highlighted the inner vacuolated cells of the notochord, outlining their plasma membrane (Yamamoto et al., 2010; Figure 4.1 and 4.4B). The line was created by Mai Yamamoto (Nagoya University, Japan) and it uses a Gal4 insertion in the NADH-cytochrome b5 reductase2 gene to fluorescently highlight the cell membrane of the inner vacuolated cells when crossed to a fluorescent UAS line (Yamamoto et al., 2010).

I used these transgenic lines to study how the two cell populations reacted to the notochord damage and, in the case of the notochord sheath-specific col2a1a:RFP line, I combined it with the wt1b:GFP transgene, to uncover the involvement of the outer sheath cells in the GFP response.

4.2.2. The de-novo GFP response initiates in the surrounding sheath cells and propagates into the lumen of the injured notochord

To evaluate how the outer notochord sheath cells behaved during the wt1b:GFP response, I created a transparent wt1b:GFP; R2-col2a1a:mCherry zebrafish line. Together with Witold Ribsky in my lab, we injected the R2-col2a1a:mCherry construct (provided by Rodney Dale, Northwestern University) into wt1b:GFP; casper 1 cell stage zebrafish embryos and screened the embryos at 3 dpf for mosaic expression of the construct in the notochord sheath cells. Positive embryos were grown to adulthood and mated against either AB wild types or casper fish to determine germ-line transmission (see Materials and Methods for more details).

The F1 generation of the injected fish were used for my needle injury assays. Similarly to previous experiments, 3 dpf wt1b:GFP; col2a1a:RFP larvae were YS needle injured and followed for 72 hours after the injury. The larvae were imaged at 0, 24 and 72 hpi under brightfield and fluorescent conditions (Figure 4.2A).
Figure 4.2. The *col2a1a:RFP* line highlights the notochord sheath nature of the *wt1b:GFP* response. (A) Three dpf *wt1b:GFP; col2a1a:RFP* larvae were YS needle injured and imaged at 0, 24 and 72 hpi. (B) The transgenic animals showed that the needle injury created an acellular gap in the outer sheath layer of *col2a1a:RFP* positive cells immediately after the damage (0 hpi; white circle). By 24 hpi, RFP positive cells had covered the gap and *wt1b:GFP* cells could be seen populating the damaged area (24 hpi; orange and blue arrows respectively). Transverse sections revealed that the *wt1b:GFP* signal and the RFP signal co-localised in the outer sheath cells at the site of injury (24 hpi; orange arrow heads). Additionally, *wt1b:GFP* cells simultaneously appeared to “bud off” from the outer sheath layer of the notochord (24 hpi; blue arrow heads). At 72 hpi, *wt1b:GFP* cells and *col2a1a:RFP* cells could be seen populating the lumen of the injured notochord (72 hpi; orange arrow head). Scale bar = 50 μm.
The first thing I noticed was that immediately after the injury the sheath of outer notochord cells appeared disrupted at the site of damage (Figure R7C – 0 hpi; injured vs. non-injured control). The needle had pierced through the otherwise evenly scattered notochord cell layer, creating a cell-less gap around the site of damage (Figure 4.2B – 0 hpi; dotted line).

By 24hpi, however, the gap had been partially repopulated by other notochord sheath cells (Figure 4.2B – 24 hpi; orange arrow and dotted line). Interestingly, as the gap repopulated, the wt1b:GFP signal could be seen overlapping with the red fluorescence of the outer notochord cells (Figure 4.2B – 24 hpi; blue arrow). Cross-sectional views of the injured area validated this observation and revealed that the GFP signal emanated from the RFP positive cells (Figure 4.2B – 24 hpi; orange arrow heads). Additionally, at this stage, wt1b:GFP-only positive cells could be seen protruding from the RFP layer of outer notochord cells and pointing towards the injured lumen of the notochord (Figure 4.2B – 24 hpi; blue arrowhead).

By 72 hpi, the damage response had intensified (Figure 4.2B – 72 hpi). The amount of GFP signal at the site of damaged had increased and cross-sections of the injured area revealed that the surge in signal strength was due to the invasion of a large number of fluorescent cells of the site of injury (Figure 4.2B – 72 hpi; orange arrow head). Importantly, these cells expressed a variety of fluorescent profiles, with wt1b:GFP and wt1b:GFP-mCherry double positive cells equally populating the lumen of the notochord (Figure 4.2B – 72 hpi; orange arrowhead).

This data strongly suggested that the notochord sheath cells were the cellular initiators of the wt1b:GFP response. It revealed that upon injury the outer notochord cells were able to upregulate the wt1b fluorescent reporter and move to the injured area to replenish the void created by the injury in the cellular membrane. Additionally, the time-course had shown that the increase in the wt1b:GFP signal was due to an increase in the number of GFP positive cells, which
appeared to “bud off” from outer cell layer of the notochord and invade the injured lumen. Taken together these results show that the outer cells can react to the notochord damage and migrate into the centre of the notochord after the endogenous upregulation of the wt1b:GFP marker.

4.2.3. FAC sorting confirms the notochord sheath nature of the wt1b:GFP cells of the damage response

Even though fluorescent microscopy had revealed the notochord sheath cell origin of the wt1b:GFP response, I wanted to validate this observation by carrying out a FAC sort analysis of the injured area.

Three dpf wt1b:GFP; col2a1a:RFP larvae (n=35) were needle injured and grown to 72 hpi. The larvae were grown to this stage because it was the time at which I had seen the highest number of GFP and RFP positive cells. At 72 hpi, the injured and non-injured larvae were anaesthetised in tricaine and their tails were sectioned at a site just anterior to the YS injury (Figure 4.3; blue box). The sectioned tails from each group were pooled together and disassociated using a cocktail of collagenases, before being fed to the a FACS machine and sorted in accordance to their fluorescent profile (protocol adapted from Manoli et al., 2012; see Materials and Methods for details).

The FACS results confirmed the notochord sheath nature of the wt1b:GFP-positive cells. The results showed that the injured larvae had 100 times more GFP-RFP double positive cells than the non-injured controls, where the number of double positive cells was almost non-existent (289 cells vs. 3 cells; Figure 4.3 - GFP-RFP double positive).
Figure 4.3. FACS analysis validates the col2a1a:RFP profile of the wt1b:GFP response. The tails from 72 hpi wt1b:GFP; col2a1a:RFP larvae and their non-injured siblings were sectioned, collected and their cells dissociated for FAC sorting (blue rectangles; n=35 larvae per group). FACS analysis showed an almost 10-fold difference in the number of “GFP and RFP” cells between injured and non-injured samples. The analysis also showed an increase in the number of GFP (“All GFP”) cells and identified a small population of wt1b:GFP only cells in non-injured larvae (GFP only). The total number of cells sorted was consistent between injured and non-injured larvae (“Neg” and “Total number”).

### Table 4.3.1

<table>
<thead>
<tr>
<th></th>
<th>injured</th>
<th></th>
<th>uninjured</th>
</tr>
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<tbody>
<tr>
<td>Neg</td>
<td>36,570</td>
<td>cells</td>
<td>36,188</td>
</tr>
<tr>
<td>RFP only</td>
<td>632</td>
<td>cells</td>
<td>735</td>
</tr>
<tr>
<td>All GFP</td>
<td>553</td>
<td>cells</td>
<td>161</td>
</tr>
<tr>
<td>GFP and RFP</td>
<td>289</td>
<td>cells</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>149,692</td>
<td>cells</td>
<td>154,838</td>
</tr>
</tbody>
</table>

![Image of FACS analysis](image)
The numbers also differed when looking at the GFP positive cells. The injured larvae had 5 times more wt1b:GFP expressing cells than their non-injured controls (553 cells vs. 161 cells; Figure 4.3 – GFP). Interestingly, the FACS analysis indicated that there was a basal number of GFP cells in non-injured larvae, opening the possibility that other tissues could have a number of low GFP expressing cells. Additionally, the analysis not only accounted for the difference in the number of GFP cells, but similarly to the previous wt1b:GFP FAC sort (see Chapter 3), it also identified a small population of GFP expressing cells in the non-injured control larvae.

Importantly, when looking at the control cell populations, both injured and non-injured larvae had similar number of non-positive cells (36,188 cells for the non-injured larvae vs. 36,570 cells for the injured larvae; Figure 4.3) and RFP-only expressing cells (735 cells for the non-injured larvae vs. 632 cells for the injured larvae; Figure 4.3).

Lastly, the FAC sort also revealed an interesting pattern. It showed that the needle injury also triggered an increase in the number of RFP positive cells in the notochord. This was shown by considering the amount of RFP and GFP-RFP double positive cells in the injured and non-injured notochord (920 cells for the injured larvae vs. 735 cells for the non-injured larvae; Figure 4.3), which suggested that there was also an increase in the number of RFP positive cells at the site of damage and could explain the presence of a higher number of RFP-positive cells in the imaged wounds. However, the FACS analysis would need to be repeated in order to test the statistical significance of these results.

4.2.4. Time-lapse imaging highlights the wt1b:GFP activation of the surrounding outer sheath cells and the 4D progression of the response
The time-course experiment and FAC sort analysis helped me determine the notochord sheath nature of the wt1b:GFP response. They had shown how the first expression of wt1b:GFP is localised to the outer sheath cells and how, in time, the number of wt1b:GFP and wt1b:GFP-RFP double positive cells increased and invaded the injured area, populating the site of damage.

I wanted to explore how the response arose after the injury and track its development and its progression in the 3D structure of the notochord. For this, I designed time-lapse experiments with the wt1b:GFP; col2a1a:RFP zebrafish line, using a laser confocal imaging system that could let me analyse the 4D development of the response in real time (See Materials and Methods for details).

Thee dpf wt1b:GFP; col2a1a:RFP larvae were YS needle injured and followed for 48 hours. The larvae were imaged at 60 minutes intervals under brightfield and fluorescent conditions over a 120 μm Z-plane (Figure 4.4A). To minimise the exposure to the laser and reduce phototoxicity, the Z-plane images were taken in 10 μm steps, which caused a considerable loss of resolution in the images but increased the overall survival of the larvae (see Figure 4.4B and Video 4A and 4B).

As reported in my previous time-lapses, the first GFP upregulation was observed in the notochord at around 6 hpi (Figure 4.4B – 6 hpi; yellow arrow). The initial signal arose some distance away from the damaged area but was soon followed by new wt1b:GFP upregulations in cells closer to the site of damage (Figure 4.4B – 11 hpi; yellow arrow and Video 4). Transverse imaging confirmed the notochord sheath cell origin of the response, showing how the outer layer of RFP expressing cells upregulated the GFP marker at the site of damage (Figure 4.4B – 11 hpi; yellow arrow head).

From hereon, the response gradually increased via the amplification of the initial wt1b:GFP cells, which invaded the lumen of the notochord and eventually
populated the whole injured area (Figure 4.4B - 16 hpi to 41 hpi; white arrow head). The cross-section of the notochord also highlighted the emergence of new pockets of *wt1b*:GFP expressing cells in other areas of the outer notochord cell layer, which

Figure 4.4. Time-lapse imaging reveals the spatiotemporal movement of the cells into the lumen of the injured notochord. (A) Three dpf *wt1b*:GFP; *col2a1a*:RFP larvae were YS needle injured and imaged for 48 hours at 60 minutes intervals. (B) The time-lapse highlighted the movements of the *wt1b*:GFP in the injured notochords. The onset of the *wt1b*:GFP signal can be observed at around 6 hpi (6hpi; yellow arrow). New *wt1b*:GFP cells appear close to the site of damage and co-localise with the *col2a1a*:RFP signal (11 hpi; yellow arrow and yellow arrow head). By 16 hpi, *wt1b*:GFP cells can be seen budding off the outer sheath cell layer and filling the notochord space over time (16 hpi – 41 hpi; white arrow head). New *wt1b*:GFP sites appear in the RFP positive outer sheath cells throughout the time-lapse (21 hpi; orange arrow heads).
also contributed to the accumulation of \textit{wt1b}:GFP cells at the wound (Figure 4.4B – 21 hpi; orange arrow heads).

By 41 hpi, the damaged area had been completely engulfed by a mixture of \textit{wt1b}:GFP expressing cells and \textit{wt1b}:GFP; \textit{col2a1a}:RFP double positive cells (Figure 4.4B – 41 hpi). Unfortunately, the long z-steps used in the time-lapse imaging did not provide the cellular resolution and structural detail necessary to render 3D images that could convincingly emphasise the co-localisation of the fluorescent signals at the beginning and at the end of the experiment. For this reason, we decided to employ a more sensitive imaging system to image the larvae in the time-lapse experiments.

\textit{4.2.5. Multiphoton microscopy improves the 4D analysis of the response and highlights the cellular transformations of the outer sheath cells post injury}

The 4D analysis of the \textit{wt1b}:GFP response had identified the genetic characterisation and dynamic movements of the outer notochord sheath cells following notochord damage. It had uncovered how the notochord sheath cells began to express the \textit{wt1b}:GFP reporter after the injury and shown how these cells could subsequently “bud off” from the outer membrane and invade the wound.

However, the analysis was limited by the low quality three-dimensional imaging of the process. The high phototoxicity of the confocal lasers meant that during the long-term analysis of the response, the exposure to the larvae had to be reduced to preserve the sample and ensure its survival. The limited exposure affected the clarity of the image and its resolution, which led to a loss of cellular detail in the time-lapse experiments.
To circumvent the laser phototoxicity and improve the cellular imaging, I time-lapse imaged the injured larvae using a multiphoton laser confocal system (see details in Materials and Methods). The imaging was done at the Wellcome Trust Centre of Cell Biology in Edinburgh and carried out by Dr. David Kelly at the Centre of Optical Instrumentation Laboratory (COIL) following my instructions.

Three dpf \textit{wt1b}:GFP; \textit{col2a1a}:RFP larvae were YS needle injured and followed for a 48 hours period. As with previous confocal time-lapse experiments, the larvae were imaged at 60 minutes intervals over a Z-plane distance of 120 μm (Figure 4.5A). Unlike the previous time-lapses, however, the larvae were imaged in 2 μm z-steps to increase the cellular resolution of the response.

The smaller z-steps and the higher exposure to the lasers considerately improved the imaging of the response (Figure 4.5B and Video 5). In this time-lapse, the first onset of the process was seen at around 8 hpi, when an RFP expressing outer notochord sheath cells could clearly be seen upregulating the \textit{wt1b}:GFP transgene (Figure 4.5B – 8 hpi; white arrow). Unfortunately, the imaging did not fully render the complete 3D tube structure of the notochord and meant that one of its sides was not displayed during the whole time-lapse. This could explain the slightly delayed onset of the response, as the expression was initiated in the side of the notochord that was missing in the picture (Figure 4.5B – 8hpi).

By 16 hpi, the initial \textit{wt1b}:GFP expression could be seen expanding and increasing its fluorescent signal. This appeared to be creating a new \textit{wt1b}:GFP; \textit{col2a1a}:RFP cell that moved into the injured lumen (Figure 4.5B – 16 hpi; blue arrow head). At 16 hpi, another \textit{col2a1a}:RFP cell could seen initiating the transcription of the \textit{wt1b} reporter and fully expressing it by 24 hpi (Figure 4.5B – 16 hpi and 24 hpi; yellow arrow and yellow arrow head respectively – see Video 5).
Figure 4.5. Multiphoton imaging identifies cell-specific \textit{wt1b}:GFP upregulations in the \textit{col2a1a}:RFP outer cell layer. (A) Three dpf \textit{wt1b}:GFP; \textit{col2a1a}:RFP larvae were YS needle injured and imaged for 48 hours at 60 minutes intervals using a multiphoton imaging system. (B) The time-lapse detailed the cell-specific timed upregulations of the \textit{wt1b}:GFP reporter in the \textit{col2a1a}:RFP outer notochord cells. The first expression of the marker was observed at 8 hpi (white arrow) and was soon followed by new foci of expression at 16 hpi (yellow arrow and yellow arrow head) and at 24 hpi (green arrows). The signal exacerbated at each site of expression and converged in the middle the wound (white and yellow arrow heads), engulfing the lumen of the notochord over time (24 hpi – 48 hpi).
As this unfolded, the initial GFP response could be seen expanding into the lumen of the notochord, whilst new sites of expression appeared in other RFP positive cells around the outer cell layer (Figure 4.5B – 24 hpi; white arrow head and green arrows respectively). By 32 hpi, $\text{wt1b}:\text{GFP}$ positive cells from all foci began to assemble in the lumen of the notochord, almost engulfing the whole area (Figure 4.5B – 32 hpi).

In the following hours, the GFP signal eventually covered the damaged site and could be seen as a homogenous ball of GFP and GFP-RFP (yellow) cells (Figure 4.5B – 40 hpi to 48 hpi). The presence of the RFP cells in the injured lumen was confirmed by assessing the output of the RFP channel alone (data not shown).

This data verified my previous observations and showed how individual $\text{col2a1a}:\text{RFP}$ cells gradually upregulated the $\text{wt1b}$ GFP marker and invaded the injured site. Unfortunately, this system was not sensitive enough to distinguish specific cell divisions in the site of injury (see Chapter 3 – pH3 immunohistochemistry) but the broad changes of cellularity of the newly expressing GFP cells (Figure 4.5B – 8 hpi to 24 hpi; white arrow and white arrow head) and the gradual invasion of the injured space suggested that cell proliferation could be driving the expansion of the response inside the wound.

4.2.6. The needle injury create a gap in the row of vacuolated cells of the notochord that repopulates with new “vacuolated cells” over time

Having unfolded the behaviour of the notochord sheath cells in response to the injury and discovered their role in the $\text{wt1b}:\text{GFP}$ damage response, I decided to use the second transgenic zebrafish line to explore how the inner vacuolated cells reacted to the damage and how they progressed after it.
The SAGFF214A:GFP transgenic line was kindly donated by Stefan Schulte-Merker at the Hubrecht Institute (Utrecht, The Netherlands; Yamamoto et al., 2010). The line contained a membrane-bound Gal4 construct that highlighted the plasma membrane of the notochord inner cells in the presence of a fluorescent UAS reporter. The experiments were done in collaboration with the Schulte-Merker lab and were performed by me at the Hubrecht Institute. The different geographical location meant that the transgenic line could not be crossed to the wt1b:GFP line and, as such, the experiments were performed on SAGFF214A; UAS:GFP line in the absence of a wt1b:GFP background.

Three dpf SAGFF214A:GFP larvae were YS needle injured and followed for 144 hours. The injured larvae were imaged at 48, 72 and 144 hpi under brightfield and fluorescent conditions (Figure 4.6A). The discovery of a new phenotype in the inner cells of the notochord meant that the injured larvae were grown for a longer period of time than other larvae in previous experiments, as to allow for the full unfolding of the response (see below).

In accordance with my previous results, the first thing that I noticed on the injured larvae was the gap that the needle injury had created in the continuous row of inner vacuolated cells of the notochord (Figure 4.6B – 48 hpi). One thing that I had not previously noticed, however, was the presence of two structural extensions at the site of injury, which protruded into the empty space from each side of the wound (Figure 4.6B – 48 hpi; yellow arrows).

These protrusions appeared to be important for the later stages of the response and, in fact, by 72 hpi, it appeared as if a SAGFF214A:GFP signal was starting to form along these structures (Figure 4.6B – 72 hpi; orange arrows).

Indeed, by 144 hpi, new SAGFF214A positive cells could be seen at the sites where the initial signals were expressed (Figure 4.6B – 144 hpi; orange arrow heads).
Figure 4.6. The needle damage creates a gap in the row of vacuolated cells that becomes repopulated with new cells over time. (A) Three dpf SAGFF214; UAS:GFP larvae were YS needle injured and imaged at 48, 72 and 144 hpi. (B) The needle injury formed a gap in the continuous row of vacuolated cells of the notochord. Inside the gap two elongated protrusions were observed extending from each side of the wound at 48 hpi (yellow arrows). Upon these, a GFP signal could seen building inside the gap by 72 hpi (orange arrows), which led to the formation of small SAGFF214A; UAS:GFP cells at the site of damage by 144 hpi (orange arrow heads).
These cells were smaller than the surrounding vacuolated cells, but the expression of the SAGFF214A marker in them suggested that they had the same genetic background. The three dimensional rendering of the injured area highlighted the high number of these cells that had occupied the “empty” space and showed how they accumulated along the extending structures of the wound, closing the gap created by the needle.

It should be noted that unlike in the non-injured controls, the position and the shape of the vacuolated cells surrounding the site injury did not change (Figure 4.6B – injured vs. control). This meant that the creation of the new SAGFF214A cells was not due to the natural morphogenetic growth of the notochord but was due to a *de-novo* growth signal at the wound. This tightly correlated with what I had observed in my previous experiments (Chapter 3 – Long-term analysis of the response).

Overall, these results showed that even though the injury created a gap in the row of vacuolated cells, it was also able to trigger the release of cell growth signals at the site of damage. It would be interesting to decipher whether the invasion of the *wt1b*:GFP cells could be directly associated with the creation of the new SAGFF214A positive cells at the site of injury or whether these cells could be coming directly from the potential transdifferentiation of the invading notochord sheath cells.
4.3. Immune cells are recruited to the site of damage and may be involved in the progression of the wt1b:GFP response

4.3.1. \( \text{H}_2\text{O}_2 \) is released from the site of damage after needle injury

Having identified the cellular origin of the notochord damage response and explored the behaviour of each subpopulation of notochord cells, I wanted to focus on the contribution of the immune cells to the process. Immune cells are key to the protection of organisms and are recruited to injured sites via signals released by the tissue damage. Recently, macrophages have been associated with the regenerative response of certain tissues in several organisms, including mice, salamanders and zebrafish (Tidball & Wehling-Henricks, 2007; Li et al., 2012; Godwin et al., 2013).

I had previously observed a population of free-moving wt1b:GFP positive cells in my time-lapse experiments (data not shown). These cells could be seen moving outside of the notochord and being recruited to the damage, where they potentially contributed to the wt1b:GFP response. The existence of these cells was additionally validated by fluorescent sorting experiments. FAC sorts had highlighted a small population of GFP expressing cells in the tails of the non-injured wt1b:GFP larvae. However, the existence of these cells had not been validated visually and no GFP cells had ever been seen past the caudal end of the pronephros in non-injured larvae.

For this reason and for their role in the development of other regenerative and wound healing processes, I wanted to investigate the behaviour of the immune cells in the notochord damage response. Before doing this, however, I wanted to evaluate whether the needle injury triggered the necessary signals to recruit the cells to the wound.
To examine this, I used a chemical detection system that could detect the presence of hydrogen peroxide ($H_2O_2$) in *in-vivo* systems. Hydrogen peroxide is one of several reactive oxygen species (ROS) that are released by the cells of damaged tissues and is linked to several processes during injury, including inflammation and pathogen protection (Nathan & Cunningham-Bussel 2013). Additionally, recent publications have shown that hydrogen peroxide is also necessary for the recruitment of neutrophils and macrophages to the site of damage and for some of the regenerative responses seen in *Xenopus* tadpoles and zebrafish (Niethammer *et al.*, 2009; Rieger & Sagasti, 2011; Gauron *et al.*, 2013; Love *et al.*, 2013).

Hydrogen peroxide was detected using the fluorescent chemical sensor pentafluorebenzenesulfonyl fluorescein, which selectively binds hydrogen peroxide and fluoresces in its presence (Niethammer *et al.*, 2009; Rieger & Sagasti, 2011).

Three dpf transparent *wt1b:GFP; col2a1a:RFP*; casper larvae were needle injured in the presence of the hydrogen peroxide chemical sensor and immediately embedded in agarose and imaged over a 12 hours period at 15 minutes intervals (Figure 4.7A; Video 6A and 6B). The larvae were treated with the pentafluorebenzenesulfonyl fluorescein as outlined in Niethammer *et al.*, 2009 (see Materials and Methods for details).

Immediately after the needle damage, a strong fluorescence could be observed all around the body of the larvae (Figure 4.7B – 1hpi; injured and control). Amongst the strong background, however, a clear rectangular area could be distinguished above the site of damage, covering the hole created by the needle in the RFP layer of outer notochord cells (Figure 4.7B – 1hpi; white arrow head).

The overall intensity of the fluorescence quickly decreased and by 2 hpi, only a faint basal expression of hydrogen peroxide could be seen above the injured area (Figure 4.7B – 2 hpi; white arrow head and Video 6A). Detailed cross-sectional images of
Figure 4.7. The notochord releases a short burst of hydrogen peroxide in response to the needle damage. (A) Three dpf *wt1b*; *col2a1a*:RFP were incubated in pentafluorobenzyl sulfonyl fluorescein, YS needle-injured and time-lapse imaged for 12 hours at 15 minutes intervals. (B) The time-lapse showed that the needle injury triggered the upregulation of the fluorescent sensor shortly after the damage (1 hpi; white arrow head). The signal decreased over time, with a basal expression remaining at the site of injury throughout the time-lapse (white arrow head). The signal decreased long before the onset of the *wt1b*:GFP response (blue arrow). (C) Cross sections of the site of injury identified a short burst of expression inside the tube structure of the notochord in the first hour of the time-lapse, which rapidly engulfed the notochord lumen and then disappeared by 2 hpi (yellow arrow and yellow arrow head). As this occurred, the fluorescent expression on the outside of the notochord remained stable (white arrow). Sagittal images showed how during this time the notochord sheath cells closed the gap created by the injury. Asterisk denotes yolk sac autofluorescence and white line contours the gap created by the needle in the layer of outer sheath cells. Scale bar = 50 μm.
the wound throughout this period highlighted the short-lived activity of the sensor inside the notochord (Figure 4.7C). The fluorescence appeared as a burst of signal that built up shortly after the start of the imaging and engulfed the inside the hollowed structure within 30 minutes (Figure 4.7C; yellow arrow and yellow arrow head). The signal eventually receded as fast as it built up and could only be detected outside the tube of the notochord at 2 hpi (Figure 4.7C; white arrow head).

The basal expression outside of the notochord was maintained for the remainder of the time-lapse, but became weaker over time (Figure 4.7B; white arrow head). Interestingly, the time-lapse also highlighted the fast movement of the col2a1a:RFP cells of the outer sheath layer. Within an hour of the start of the imaging they had moved into the wound and covered the gap created by the needle (Figure 4.7B – 2 hpi; yellow arrow and Figure 4.7C - sagittal). This fast movement could explain the transient expression of the hydrogen peroxide signal and, if so, it would signify that the notochord is able to seal the wound effectively. As previously reported, the onset of wt1b:GFP was observed in the RFP outer notochord sheath cells by 6 hpi (Figure 4.7B – 6 hpi; blue arrow).

Contrastingly, no noticeable GFP signal was observed in the non-injured larvae, even though, similarly to the injured larvae, the earliest images showed a strong background signal (Figure 4.7B – non-injured).

4.3.2. Neutrophils are recruited to the site of damage but leave before the initiation of the wt1b:GFP response

The hydrogen peroxide sensor had identified the presence of a short-lived strong hydrogen peroxide signal in the injured tissues. The strength of the signal quickly
died down, but appeared to be maintained at very low levels throughout the first 12 hours after the injury.

In order to test whether this signal could be enough to attract the immune cells to the site of damage, I made use of two fluorescent immune cell transgenic zebrafish lines. These lines highlighted the neutrophils (lysc:dsRED; Hall et al., 2007) and the macrophages (fms:Gal4; UAS:mCherry; Gray et al., 2011) of the zebrafish larvae in red fluorescence and allowed me to crossed both lines to a wt1b:GFP fish. Three dpf lyc:dsRED; wt1b:GFP larvae were needle injured and imaged for 24 hours at 60 minutes intervals. The injured larvae were recorded under brightfield and fluorescent conditions to assess the behaviour of the neutrophil cells in response to the needle damage (Figure 4.8A – see video 7).

The time-lapse showed that already by 1 hpi, a strong presence of RFP positive neutrophil cells could be observed around the wounded area (Figure 4.8B – 1 hpi; white arrow). As previously published, these cells responded rapidly to damage and migrated to the site of injury to provide the first line of defence against the invading pathogens (Li et al., 2012).

The immune cells remained at the site of damage for another 3 hours but were gone by 5 hpi, leaving the injured area and reverting back to their default position in the ventral side of the larval tail (Figure 4.8B – 3 hpi and 5 hpi; white arrow and video 7).

The wt1b:GFP response did not start until 2 hours later, long after the last neutrophils had left the site of injury (Figure 4.8B – 7 hpi; yellow arrow). This meant that the neutrophils did not interact with the nascent wt1b:GFP cells and potentially did not have a major role in the initiation of the notochord damage response.
Figure 4.8. Macrophages conglomerate around the tube structure of the injured notochord.
Figure 4.8. Macrophages conglomerate around the tube structure of the injured notochord. (A) Three dpf lsc:dsRED x wt1b:GFP and fms:Gal4; UAS:merry x wt1b:GFP (neutrophils and macrophages respectively) were needle injured and imaged for 24 hours at 60 minutes intervals. (B) Neutrophils were already present at the site injury at the beginning of the time-lapse (2 hpi; white arrow). They remained at the wound for a few hours, leaving the damaged area before the initiation of the wt1b:GFP response (yellow arrow). GFP expressing neutrophils were observed during the time-lapse, but the expression was not wt1b:GFP specific (white arrow heads; see text for details). (C) Only a few macrophages could be observed at the site of damage at the beginning of the time-lapse (2 hpi; white arrows and white arrow heads). By 5 hpi a higher number of macrophages had been recruited to the site of injury (5 hpi; white arrows) and accumulated around the tube structure of the notochord (5 hpi; white arrow heads). The wt1b:GFP expression was born close to the congregating macrophages (8 hpi; yellow and white arrows and arrow heads respectively) and new areas of expression appeared close to the macrophages throughout the time-lapse (white and yellow arrows). The white line delineates the contour of the notochord and asterisks marks yolk sac autofluorescence. Scale bar = 50 μm.

It should also be mentioned that throughout the time-lapse, several RFP expressing neutrophil cells could be seen expressing GFP (Figure 4.8B; white arrow heads). This GFP expression, however, was not specifically coming from the wt1b promoter and was due to a background Kaede expression that was found in the neutrophil line.

4.3.3. Macrophages respond to the tissue damage and invade the injured area interacting with the nascent wt1b:GFP response

Having explored how the neutrophils act against the needle damage, I next wanted to use the second fluorescent transgenic immune line to look at the behaviour of macrophages in the injury response.

Macrophages are known to remain at the site of damage longer than neutrophils, engulfing dead cells and interacting with their surroundings using their filopodia extensions (Li et al., 2012). The recent associations between macrophages and the regenerative responses in zebrafish and other organisms (Tidball & Wehling-Henricks, 2007; Li et al., 2012; Godwin et al., 2013) make them an interesting subset of cells to investigate for the notochord injury response.
Three dpf *fms*Gal4; UAS:mCherry; *wt1b*:GFP were needle injured and imaged under the same conditions as the neutrophil transgenic line (Figure 4.8A; video 8A and 8B).

The time-lapse showed that the macrophages were slower to respond to the damage (Video 8A). By 2 hpi, only a couple of macrophages had managed to gather around the site of injury (Figure 4.8C – 2 hpi; white arrows and white arrow heads), which contrasted with the fast recruitment of neutrophils observed in the previous time-lapses.

The number of macrophages that were drawn to the site of damage increased in the following hours. By 5 hpi, new macrophage cells could be seen gathering around the injured area (Figure 4.8C – 5 hpi; white arrows). Interestingly, they appeared to accumulate around the tube structure of the notochord, with some of them even appearing to protrude into the broken sheath (Figure 4.8C – 5 hpi; white arrow heads).

At 8 hpi, the first GFP upregulation was observed in the notochord (Figure 4.8C – 8 hpi; yellow arrow and yellow arrow head). By this time, a fixed number of macrophages had accumulated around the injured area and could be seen in close proximity to the area where the GFP response had begun (Figure 4.8C – 8 hpi; white arrows and white arrow heads). Using the time-lapse alone, it was difficult to determine whether there was any direct interaction between the RFP expressing macrophages and the GFP notochord sheath cells, but it is worth noting that the GFP response initiated very close to the protruding macrophages.

By 11 hpi, the strength of the GFP response had increased and new GFP cells could be seen arising from other areas of the notochord sheath (Figure 4.8C – 11 hpi; yellow arrow). Interestingly, the macrophages did not move from their positions
around the notochord, remaining static in the same location as 3 hours earlier (Figure 4.8C – 11 hpi – white arrow).

At 13 hpi, new foci of GFP cells could be seen appearing around the notochord in areas close to the surrounding macrophages (Figure 4.8C - 14 hpi; yellow arrows). Finally by 17 hpi, the GFP response began to amplify, almost covering the needle-entry side of the injured notochord sheath (Figure 4.8C – 17 hpi; yellow arrow and yellow arrow head). This side was heavily populated by the RFP positive macrophages and areas of overlap could be appreciated between the GFP cells and the RFP positive macrophages (Figure 4.8C – 17 hpi; white arrow). This result highlighted the possible existence of an interaction between the wt1b:GFP positive sheath cells and the macrophages and potentially meaning that the macrophages could have a direct influence on the developing damage response. However, this should be experimentally addressed using other methods (see Discussion below).
4.4. The needle injury triggers the expression of a de-novo calcification signal at the site of damage that leads to a double vertebrae phenotype in the adult fish

4.4.1. Notochord Injury triggers the de-novo upregulation of entpd5:RFP at the site of damage

The notochord is essential for the proper formation of the vertebrae in mammals and teleost fish (Fleming et al., 2001; Stemple et al., 2005). It serves as a scaffold around which the spinal column can form and together with the sclerotome releases the mineralisation signals required for the patterning and ossification of its vertebrae (Fleming et al., 2004; Bensimon-Brito et al., 2012; Ellis et al., 2013). Additionally, its contribution to the spinal column transcends the embryonic and larval stages of vertebrates. The remnants of the notochord are known to form the nucleus pulposus, a glycoprotein-rich jelly like structure that forms the core of the intervertebral disc (Choi et al., 2008; McCann et al., 2012; Bensimon-Brito et al., 2012). Recent fate-map studies have outlined the sole contribution of the notochord to the cell populations of the nucleus pulposus and even shown that notochord-like cells inside the structure are directly responsible for its homoestasis and limited repair (Choi et al., 2008; Minogue et al., 2010; Choi et al., 2011; McCann et al., 2012; Chan et al., 2014).

In zebrafish, a row of metameric calcifying rings, known as chordacentra, form around the notochord in an anterior to posterior fashion before the development of the vertebrae (see Figure 1.9 in Chapter 1 - Introduction). These rings delineate the future sites where the vertebrae will form and ossify as the larva grows.

In collaboration with Stephan Schulte-Merker at the Hubrecht Institute (Utrecht, Netherlands), I used a chordacentra specific fluorescent reporter line developed by his lab in order to study the effect of the needle injury in the formation of the chordacentra and the future vertebrae (Huijtema et al., 2012 and unpublished data).
The fluorescent transgenic line highlights the pockets of expression of the ectonucleoside triphosphate diphosphohydrolase 5 (entpd5) enzyme, an E-type NTPase that is found in bone mineralizing environments and has been shown to be essential for skeletal morphogenesis (Figure 4.9B and Video 9; Huitema et al., 2012).

I crossed the entpd5:RFP line to wt1b:GFP zebrafish and selected their offspring for wt1b:GFP and entpd5:RFP expression. I used double positive larvae and the entpd5:RFP only larvae (data not shown) in needle injury experiments to assess how the notochord damage affected the pattern of expression of the entpd5:RFP line and observe if there was an overlap between the wt1b:GFP and entpd5:RFP signals.

Three dpf wt1b:GFP; entpd5:RFP larvae were YS needle injured and followed over time. The wt1b:GFP; entpd5:RFP larvae were grown for 72 hours and imaged at 0, 24 and 72 hpi with a confocal microscope under brightfield and fluorescent conditions (Figure 4.9A).

It should be noted that apart from highlighting the forming chodacentra in the notochord, the entpd5:RFP larvae also show a yet uncharacterised basal RFP expression along the ventral axis of the notochord (Figure 4.9B; blue arrow; Stefan Schulte-Merker - personal communication). This is important because immediately after the injury, the first thing that I noticed was that notochord damage triggered the expression of a stronger ventral fluorescence at the wound (Figure 4.9C – 0 hpi; blue arrow heads).

By 24 hpi, as the wound opened, the entpd5:RFP expression could be seen completely engulfing the site of damage (Figure 4.9C – 24 hpi Brightfield & RFP). At this point, wt1b:GFP cells could also be seen gathering around the damaged area, but appeared to be located around the wound (Figure 4.9C – 24 hpi RFP & GFP). This was confirmed by the 3D rendering of the injured area, which revealed that
Figure 4.9. Needle injury triggers the de-novo upregulation of entpd5:RFP at the site of damage.
Figure 4.9. Needle injury triggers the de-novo upregulation of entpd5:RFP at the site of damage. (A) Three dpf entpd5:RFP; wt1b:GFP larvae were needle injured and imaged for 0, 24 and 72 hpi. (B) The entpd5:RFP reporter highlights the forming chordacentra in the notochord (yellow arrow). The forming chordacentra could be seen forming some distance away from the site of damage (orange arrow). The green arrow head highlights the basal expression of entpd5:RFP in the notochord. (C) The needle injury triggered an immediate increase in fluorescence in the areas close to the site of damage (blue arrow heads). The entpd5:RFP signal was seen highly upregulated at the site of damage by 24 hpi, sharing the space with the first wt1b:GFP cells. By 72 hpi, the GFP cells at the site of injury had multiplied and interlinked with the RFP signal. A reduction in the basal expression of the fluorophore could be seen in injured and non-injured larvae at this point (yellow arrow heads). Scale bar = 50 μm.

whilst the wt1b:GFP cells were still concentrating in the surrounding notochord sheath layer, the entpd5:RFP signal and RFP positive cells could be found both inside and outside of the site of damage (Video 10). The imaging also showed that there was no overlap between the two fluorophores at the injured area, which suggested that there could be two distinct populations of cells invading the wound.

The expression of the entpd5:RFP signal at the site of injury should also be considered in the context of the forming chordacentra. Wider imaging of the injured larvae showed that, at this stage, the metameric mineralising structures were still some distance away from the site of damage, forming at more anterior areas of the notochord (Figure 4.9B; yellow arrow vs. orange arrow). This meant that the RFP signal triggered by the injury was not part of the normal mineralization pattern of the notochord and showed that the needle injury was potentially able to trigger the early onset of mineralisation in the notochord.

By 72 hpi, the number of wt1b:GFP cells had increased and could be seen populating the whole wound (Figure 4.9C – 72 hpi RFP & GFP and Video 11). The de-novo entpd5:RFP response still resided within the site of injury, but appeared lower than 48 hours earlier (Figure 4.9 – 72 hpi Brightfield & RFP). The lower RFP intensity coincided with a lower RFP expression in the ventral axis of the notochord, which could suggest that the reduction in strength could be due to a natural decrease in the ventral expression of the reporter in the whole notochord (Figure 4.9 – 72 hpi RFP & GFP; yellow arrow heads in Injured and non-injured control).
The 3D rendering of the area showed that even with the increase GFP cells there did not seem to be a clear overlap between the two populations of cells (Video 11). However, RFP positive and GFP positive cells were, in some cases, quite close to each other, therefore a FAC sort of the area would help to validate the distinct fluorescent nature of the two populations of cells (Figure 4.9 – 72 hpi and Video 11).

4.4.2. Bone staining of injured larvae over time highlights a defect in vertebrae formation at the site of damage that leads to a double vertebra phenotype

Given that the needle injury had triggered the de-novo upregulation of entpd5:RFP, a chordacentra marker that has been associated with mineralizing environments and bone formation, I wanted to explore whether the injury could alter the pattern of ossification in the zebrafish notochord.

For this I used two bone staining vital dyes, calcein and alizarin red S. These dyes highlight calcifying structures and can be used to follow bone formation in the growing zebrafish. I used these dyes to follow the injured larvae from their early larval stage, when the chordacentra begin to form, to their juvenile stage (approx. 30 dpf), visualizing how the notochord ossifies the damaged and undamaged areas.

To begin with, I used the calcein dye on injured non-fluorescent wild type AB fish. I used these fish as calcein emits green fluorescence in the presence of calcium ions (Du et al., 2001; Haga et al., 2009). I used the protocols specified in Wesfield 2001 and Haga et al., 2009 (see Materials & Methods for details).

Three dpf zebrafish larvae were YS needle injured and grown to 35 dpi (Figure 4.10A). The larvae were immersed in the calcein stain and were imaged at 18 dpf,
Figure 4.10. *Injured larvae form a extra vertebra at the site of damage* (A) Three dpf AB larvae were YS needle-injured, grown to 18, 24 and 38 dpi, incubated in the calcein stain at each stage and imaged. (B) Calcein staining of 18 dpi larvae highlighted the metameric development of the chordacentra along the notochord of the injured and non-injured larvae (yellow arrow heads). The orange circle denotes the site of damage in the injured larvae. By 24 dpi, all chordacentra had formed and a new mineralised body could be observed at the site of damage (yellow asterisk and white arrow head) surrounded by two thinner chordacentra (14 and 15). At 38 dpi, the vertebral column of all fish had formed. Injured fished showed the expression of smaller fully formed vertebra at the site of injury (green arrows). Scale bar = 500 μm.
24 dpf and 38 dpf to account for the chordacentra formation and subsequent vertebrae development. The larvae were grown at room temperature from 5 dpf, which meant that vertebrae development was delayed in comparison to previously stipulated times (Haga et al., 2009). To account for the delayed development at the lower temperatures, the body length of the larvae is shown besides the dpi, to give a more precise reference of the age of the fish.

At 18 dpi, injured and non-injured larvae appeared to have a similar segmented pattern of chordacentra development (Figure 4.10B – 18 dpi; injured vs. non-injured). The centra formed in an anterior to posterior manner, with each centra first forming at the dorsal and ventral edges of the notochord before merging to form its particular ring structure (Figure 4.10B – 18 dpi; yellow arrow heads). At this point the forming chordacentra had yet to arrive to the site of needle damage (Figure 4.10B – 18 dpi; orange dotted circle).

By 24 dpi, the row of chordacentra had extended along the length of the notochord and could be seen forming the centra of the vertebrae and, in the most anterior areas, even forming the first neural arches of the vertebrae (Figure 4.10B – 24 dpi; vertebrae 2 - 7). At the site of needle injury, the metameric patterning of the vertebrae appeared to have been lost. The needle injury had created what looked like a vestigial vertebra that appeared to have formed in the middle of another vertebra, splitting it in half (Figure 4.10B – 24 dpi; asterisk between vertebrae 14 and 15 and white arrow head in inset).

Two weeks later, the vertebrae of the injured and non-injured larvae appeared to have developed and ossified without major complications to the spinal column (Figure 4.10B – 38 dpi). At the site of needle damage, however, an interesting outcome had occurred. The three vestigial vertebral bodies created by the injury had formed three almost perfect smaller vertebrae that, similarly to the neighbouring vertebrae, had neural and hemal arches (Figure 4.10B – 38 dpi; green
arrows). This phenotype was readily phenocopied in all injured larvae and demonstrated that the needle damage, even though invasive, could be repaired without affecting the formation of the spinal column (n= 5 larvae).

4.4.3. Exploring vertebrae formation in the injured wt1b:GFP larvae

Having identified the double vertebrate phenotype triggered by the notochord injury, I wanted to understand how the phenotype related to the GFP response. I wanted to explore whether the wt1b:GFP cells could have any any direct involvement in the irregular vertebral outcome.

I had previously followed the wt1b:GFP response over time and discovered that it persisted at the site of injury until the juvenile stages, even though it became more localised as the site of injury decreased and healed over time.

I wanted to use the signal continuum of the GFP response to explore the interactions between the wt1b:GFP expressing cells and the forming vertebrae. For this, I used the second of the two bone-specific vital dyes, alizarin red S. With this dye I wanted to highlight the forming bone under red fluorescence and observe for any RFP-GFP overlap against the wt1b:GFP background.

Three dpf transparent wt1b:GFP:casper larvae were YS needle injured and grown to 38 dpi (Figure 4.11A). The injured larvae were incubated in the dye and imaged at 3 time-points: 18, 24 and 38 dpi; under brightfield and fluorescent conditions. Similarly to the previous experiment, the larvae were raised at room temperature from 5 dpf, therefore the measurement of their body lengths appears next to their dpi value.
Figure 4.11. The wt1b:GFP signal persists in the injured notochord and populates the inside of the newly formed vertebra (A) Three dpf wt1b:GFP; casper larvae were YS needle-injured, grown to 18, 24 and 38 dpi, incubated in alizarin red at each stage and imaged. (B) At 18 dpi, the alizarin red staining on the wt1b:GFP larvae showed that no visible centra formed at the site of injury and instead a pocket of wt1b:GFP cells populated the wound (18 hpi – Zoom). By 24 dpi, the GFP cells could still be seen at the site of damage (orange arrow head). The cells shared the site with two thin developing mineralising chordacentra (24 dpi – Zoom; blue and white arrow heads and arrows). Both mineralising structures were smaller than the other non-damaged surrounding chordacentra (24 dpi – Zoom; yellow arrow head and arrow). The GFP expression persisted to 38 dpi (38 dpi; orange arrow head) with the chordacentra at the site of injury (38 dpi – Zoom; blue and white arrow heads and arrows) and the other non-damaged chordacentra (yellow arrow) almost fully formed. Scale bars = 200 μm.
At 18 dpi, the row of chordacentra could be seen running down the axis of the larvae (Figure 4.11B – 18 dpi). The metameric structures appeared uninterrupted throughout the notochord, except at the site of damage, where a strong GFP upregulation could be observed (Figure 4.11B – 18 dpi; blue and yellow arrow heads). A closer look at the area revealed that no chordacentra had formed at the injury and, instead, the site was solely populated by \textit{wt1b}:GFP cells (Figure 4.11B – 18 dpi; inset).

By 24 dpi, the notochord had mineralised, forming the bodies of the vertebrae (Figure 4.11B – 24 dpi). A strong GFP expression could be readily observed at the site of damage and what was a gap at 18 dpi, had begun to form what looked like a vertebra (Figure 4.11B – 24 hpi; yellow arrow head). A zoom into the injured area revealed how two thinner vertebrae had formed at the site of damage, one of which was directly above the pocket of \textit{wt1b}:GFP cells (Figure 4.11B – 24 dpi; blue, white and yellow arrow heads and arrows). The GFP signal appeared to amalgamate with the calcifying structure and be located inside the forming chordacentra (Figure 4.11B – 24 dpi merged; white arrow head).

At this point, brightfield imaging also revealed that the new vertebra was forming directly at the gap where the injury had happened (Figure 4.11B – 24 dpi; blue arrow head and yellow arrow head). It meant that the darker tonality that I had previously reported did not represent the formation of a scar, but instead highlighted the site where the new vertebra was forming (see Chapter 3 – Growing injured larvae).

By 38 dpi, the vertebral column had completely formed, with most vertebral bodies beginning to acquire their \textit{amphicoelous} shape and their characteristic neural and hemal arches (Figure 4.11B – 38 dpi). A look at the site of damage revealed that the two thinner vertebrae had grown and thickened, developing a similar but shorter structure than the surrounding vertebrae (Figure 4.11B – 38 dpi RFP; blue and white
arrow heads and arrows vs. yellow arrow head and arrows; n=3/3). Under red and green fluorescence, the area of damage revealed that GFP cells still populated the shorter vertebra, exhibiting a relatively strong expression within the formed vertebrae (Figure 4.11B – 38 dpi; white arrow head). It was difficult to determine the exact location of these cells or whether they interacted with the forming bone, but they appeared to inhabit the hollow inside of the vertebra (Figure 4.11B – white arrow head).

4.4.4. Defining the interactions between the wt1b:GFP cells and the forming vertebrae in the 3D structure of the notochord

The imaging of the developing vertebrae in the injured wt1b:GFP larvae showed that a new vertebra formed around the wt1b:GFP cells in response to the damage. The merging of the fluorescent channels suggested that the GFP cells could be interacting with the forming vertebrae, though the 2D nature of the images was not sufficient to assert this.

In order to define the exact location of the wt1b:GFP expressing cells in the 3D structure of the notochord and reveal their interactions with the forming vertebra, I used a confocal imaging system that could image through the z-plane of the mineralizing structure.

Three dpf wt1b:GFP larvae were needle injured and grown to 18, 21 and 28 dpi (Figure 4.12A). At each specified time-point, the larvae were live stained in alizarin red S and then culled in an overdose of tricaine. The larvae were assessed for the cessation of the heartbeat and the suppression of the blood flow before the beginning of the imaging. The dead larvae were then embedded in 1% low melting-point agarose and imaged with a confocal microscope under fluorescence (see Materials and Methods for details).
Figure 4.12. The $wt1b$:GFP signal overlaps with the forming alizarin red positive chordacentra.
Figure 4.12. The wt1b:GFP signal overlaps with the forming alizarin red positive chordacentra. (A) Three dpf wt1b:GFP;casper larvae were YS needle-injured, grown to 18, 21 and 28 dpi. At each stage the larvae were incubated in alizarin red, culled and imaged using a high-resolution confocal system. (B) At 18 dpi, a strong wt1b:GFP expression could be seen at the site of damage and, amongst this signal, an alizarin red positive structure could be observed forming inside the wound (18 dpi; yellow arrow heads and arrow). Larvae observed at 21 dpi showed a primordial vertebral structure forming amongst the wt1b:GFP cells at the site of damage with co-localising signals between the vertebra and the cells of the response (21 dpi; white arrow heads). Larvae at 28 dpi showed a mostly fully formed vertebral body that was akin to the surrounding non-injured units. An overlap in the signals could still be observed at the site of damage (blue arrow head). (C) A red alizarin structure formed at the site of injury even in the absence of nearby chordacentra (white arrow). (D) Two phenotypes were observed at 28 dpi, fused, where a whole vertebra formed at the wound, and, in some instances, non-fused, where parts of the forming vertebra had not yet fused (white arrows). In both instances, overlapping signals could be observed at the site of damage (white arrow heads). Abbreviations: Sag = sagittal; x<sup>o</sup> = angled view; tr = transverse view.

As seen in my previous experiments, by 18 dpi, the chordacentra had begun to form along the length of the notochord (Figure 4.12B – 18 dpi). At the site of injury, a strong pocket of GFP expressing cells could be observed amongst the forming chordacentra (Figure 4.12B – 18 dpi sag; yellow arrow head). When observed at an angle and cross-sectionally, the cells could be seen forming in the centre of the chordacentra rings, occupying the lumen of the notochord (Figure 4.12B – 18dpi; x<sup>o</sup> and CS).

The more detailed imaging achieved with the confocal system additionally revealed that the GFP cells overlapped with an alizarin red signal at the site of damage (Figure 4.12B – 18 dpi; yellow arrow heads and yellow arrow; n=5/5). Even though the presence of the alizarin signal could be confused with the signal from a forming chordacentra, the notochords from other less developed injured siblings showed that the RFP signal formed at the site of injury even without any nearby forming chordacentra (Figure 4.12C; white arrow). This meant that the needle injury was able to trigger an early calcification signal at the site of damage, which could tightly link with the recruitment of entpd5:RFP cells to the wound at earlier stages.

At 21 dpi, the first vestigial vertebral centra could be seen forming around the wt1b:GFP cells (Figure 4.12B – 21 dpi; white arrow head). Angled views and transverse sections of the mineralizing notochord, revealed how most of the
wt1b:GFP cells were found in the middle of the forming rings, though a subpopulation of these cells appeared to be located within the forming vertebra at the site of damage (Figure 4.12B – 21 dpi; white arrow heads; n= 2/3).

Lastly, by 28 dpi, all centra could be seen fully formed along the notochord (Figure 4.12B – 28 dpi). At the site of damage, however, there was a heterogenous phenotype in response to the injury. All observed larvae showed the persistent expression of wt1b:GFP cells inside the forming centrum and their co-localisation with the calcified structure (Figure 4.12B – 28 dpi and Figure 4.12D; blue and white arrow heads; n=4/4). However, the degree of fusion of the vertebra varied between the injured larvae. Some larvae showed complete fusion and a shortened centrum by this point, whilst others could be seen to be in the process of fusing (Figure 4.12D – fused vs. non-fused; white arrows n=2/4 for each group). Interestingly, the degree of fusion appeared to correlate with the strength of the response (Figure 4.12D – fused vs. non-fused; white arrow heads), which could suggest that the wt1b:GFP cells may have an active role in the formation of the chordacentrum at the site of damage.
4.5. Discussion

4.5.1. The notochord injury triggers the upregulation of wt1b:GFP in the outer notochord sheath cells and the creation of new SAGFF214 positive cells at the wound

The use of cell-specific fluorescent transgenic lines and high-resolution imaging allowed me to visually analyse the individual spatiotemporal rearrangements triggered by the injury in the cell populations of the notochord. The experiments uncovered a new degree of cellular complexity in the damage response, highlighting the behaviour of each cell subpopulation in response to the damage and simultaneously identifying the cellular origin of the wt1b:GFP response.

The notochord sheath specific \textit{col2a1a}:RFP transgenic line (Dale & Topczewski, 2011) helped me visualise the way in which the outer notochord cells responded to the damage. Time-course and time-lapse experiments identified the central role of these cells in the notochord damage response, where they were responsible for the closure of the wound and the initiation of the wt1b:GFP signal. Moreover, 3D imaging revealed how these newly expressing wt1b:GFP outer notochord cells were able to detach from their cell layer and invade the injured lumen of the notochord, populating the gap created by the needle.

The imaging also highlighted the cellular heterogeneity of the population of cells at the site of damage, revealing the presence of a mixture of GFP-RFP and GFP-only positive cells inside the wound. This diversity was validated by the use of FACS analysis, which showed the needle damaged caused a surge in the number of GFP and RFP-positive cells, as well as the novel expression of a population of double positive GFP-RFP cells in the injured wt1b:GFP; \textit{col2a1a}:RFP larvae. This result also confirmed the notochord sheath nature of the GFP cells, showing that both reporters co-localised to the same cells at the site of damage.
The wt1b:GFP activation of these cells and their migration towards the lumen of the notochord, phenotypically resembled the developmental and regenerative migration of progenitor stem cells from the epicardium to the myocardium of the heart (Lepilina et al., 2006; Martinez-Estrada et al., 2010; Smart et al., 2011; van Wijk et al., 2012). Additionally, previous studies have revealed that heart injury triggers the de-novo upregulation of wt1b:GFP in the epicardium of the regenerating adult zebrafish heart, a feat that was demonstrated to be due to the specific novel expression of the endogenous wt1b gene (Schnabel et al., 2011; Gonzalez-Rosa et al., 2011).

These observations made it plausible that a similar mechanism could be in place in the notochord during the damage response. For this reason, the putative expression of the endogenous wt1b in the GFP response should be evaluated, as well as its role in the process. The 3D movement of the wt1b:GFP cells potentially suggested that wt1b could be behind the detaching of the GFP positive cells from the sheath layer and their movement to the injured space. It also raised the hypothesis of whether it could be doing this through the same EMT pathway used during cardiac development/regeneration.

The presence of the endogenous wt1b gene in the response would be addressed in my next chapter, whilst its role in the process is currently being studied using a notochord sheath specific wt1b dominant negative line, created in collaboration with Christoph Englert at the Leibniz Institute for Age Research (Jena, Germany). Due to the on-going status of the wt1b dominant negative experiment, its result would not be presented in this thesis but its details would be discussed in the final chapter of this manuscript.

On the other hand, the SAGFF214A fluorescent transgenic line had helped me visualise how the inner vacuolated cells of the notochord reacted to the needle
damage. The line highlighted the creation of a cell-less gap in the otherwise continuous row of vacuolated cells. This result agreed with what I had previously observed in my time-lapse experiments, where the needle damage triggered a loss of cellularity in the inner vacuolated notochord cells.

Intriguingly, the fluorescent line also revealed a second behaviour in these cells. At 72 hpi, a new fluorescent signal began to emerge at the site of injury. This signal translated into the creation of new non-vacuolated SAGFF214A positive cells by 144 hpi. These cells appeared to re-populate the wound, narrowing the gap created by the needle. Even though they were morphologically different to their surrounding vacuolated cells, the fact that they expressed the same cellular marker suggested that they shared a common genetic origin.

The fluorescent line also revealed the presence of SAGFF214A-positive finger-like structures at the site of damage. These elongated from the vacuolated cells at either side of the wound and, interestingly, appeared to form a platform for the creation of the new cells. It would be interesting to identify the biological profile of these structures and determine their role in the repopulation of the injured notochord.

The closure of the wound was in accordance with what I had previously observed in the long-term analysis of the response. Unfortunately, due to the fluorescent profiles of the SAGFF214A and wt1b:GFP larvae, I was not able to study the behaviour of the inner vacuolated cells in the context of the GFP response. This would have allowed me to decipher the potential link between the invading wt1b:GFP cells and the newly forming inner cells. Two alternatives to this, however, would be to either incubate the injured wt1b:GFP larvae in the vital fluorescent dye BODIPY TR methyl ester, which highlights all cells in the body with a red fluorophore (Cooper et al., 2004; Ellis et al., 2012) or to use the already constructed wt1b:mCherry line for this experiment (Diep et al., 2011).
4.5.2. Macrophages surround the site of injury and interact with the growing wt1b:GFP signal

I also visually explored the role of the immune cells in the notochord damage response. Using vital dyes, fluorescent transgenic lines and high resolution imaging, I assessed the presence of hydrogen peroxide at the site of injury and tracked the behaviour of the innate immune cells in response to the damage.

I observed that upon damage, the injured area became engulfed by hydrogen peroxide. Its expression appeared fast, within the first hour of injury and could be seen outside and inside of the notochord. Inside the notochord, the expression was transient and appeared as a burst of signal in the injured lumen. Outside of the notochord, the expression was maintained for a longer period of time and even though it continuously faded throughout the length of the time-lapse, its expression did not completely disappear and could be seen as a basally low fluorescent glow.

Hydrogen peroxide expression has been linked to the recruitment of leukocytes to the site of damage (Niethammer et al., 2009). This suggested that the sudden burst of hydrogen peroxide could be used to recruit the immune cells into the injured area. However, the expression of hydrogen peroxide has also been associated with the regenerative and wound healing responses in urodele amphibians and zebrafish (Love et al., 2013; Gauron et al., 2013). In fact, Gauron and colleagues denoted that the length of hydrogen peroxide expression could indicate the nature of an injury process and distinguish between regeneration and wound repair in a damaged tissue. They showed that a shorter 2 hours expression signified a wound healing response, whilst a maintained 18 hours expression hallmarked a regenerative process.

The transient expression of hydrogen peroxide in my notochord injury response suggested that the notochord might be undergoing a reparative process in response
to the needle damage. This result tightly agreed with what I had observed in my previous experiments, where the notochord does not appear to be able to fully recreate the lost tissue in the long-term analysis of the response. Suggestively, the basal presence of hydrogen peroxide in the injured somite above the notochord could be indicative of muscle regeneration at the site of injury, but that was a hypothesis that was not tested in this thesis.

It should also be taken in consideration the fact that the pentafluorobenzenesulfonyl fluorescein signal could be lost in time. Looking at the non-injured controls, one could see that a ubiquitously expressed fluorescent signal tainted all larvae over the first 2 hours after the injury. This background signal eventually disappeared in injured and non-injured larvae, which could suggest that the fluorescent signal could be systematically lost as the chemical was used up. However, the strong presence of background fluorescence in the yolk sac remnant of the larvae and previously published reports indicated otherwise (Niethammer et al., 2009; Feng et al., 2010)

Using transgenic lines and time-lapse imaging, I had also explored the behaviour of the innate immune cells in the injury response. The time-lapses had shown that, upon injury, the neutrophils were the first cells to be drawn to the damaged area. Once there, they erratically hovered around the injured site for several hours and in most cases (4/7) left before the commencement of the wt1b:GFP response. However, even when they were still present at the start of the GFP response, they left shortly after its initiation and did not appear to interact with the forming response (data not shown). Their short-lived presence at the injured area and their fast recruitment to the damage was concomitant with what had previously been documented (Niethammer et al., 2009; Li et al., 2012) and could suggest that they may not play a part in the initiation or the development of the notochord damage response.
On the other hand, the macrophages were slower to respond to the damage. Unlike the neutrophils, they arrived several hours after the needle injury and instead of hovering above the injured area they congregated forming static clusters around the tube structure of the notochord, remaining at these positions throughout the course of the time-lapse. Their presence in relation to the approximate 3D structure of the notochord showed that they interlaced with the damaged sheath at the injured area and even appeared to interact with the forming GFP response.

This result putatively suggested that there could be a direct interaction between the macrophages and the damaged notochord and may mean that the presence of the phagocytes in the wt1b:GFP response could be important for its development. Macrophages are key components of the wound healing response and have even been linked to the reparative and regenerative responses seen in vertebrates, including humans (Park & Barbul, 2004; Tidball & Wehling-Henricks, 2007; Li et al., 2012; Godwin et al., 2013; Davies et al., 2013; Sanclier et al., 2013 and references therein). Their role in these processes putatively agrees with this hypothesis, however, the chemical or genetic ablation of the cells will shed more information on their role in the response. Previous reports have used pu.1 morpholinos and membrane enclosed toxins such as clodronate liposomes (Feng et al., 2010; van Rooijen & Hendrikx, 2010) for the ablation of macrophages, so it would be interesting to use either approach to study how the wt1b:GFP response develops in the absence of the macrophages.

Taken together, these experiments showed that the injury triggered a short-lived release of hydrogen peroxide that preceded the recruitment of the innate immune cells to the site of damage. According to recent studies, this release could be identifying the nature of the response and reaffirming the wound healing character of the process. Time-lapse analysis allowed me to track the movement of the immune cells and showed that each immune cell population behaved differently at the site of damage. The neutrophils moved rapidly and hovered frantically around
the injury before exiting the area prior to the start of the wt1b:GFP response. The macrophages, on the other hand, formed clusters around the notochord and persisted at the site of injury throughout time, interconnecting with the sheath of the notochord and interacting with the GFP process.

Even though, these experiments did not assess the role of the immune system in the notochord damage response, they exposed the behaviour of the innate immune cells in the process and highlighted the macrophage population as a potential target for future experiments.

4.5.3. Needle injury triggers a calcification signal that leads to a delayed vertebral development and the formation of an additional vertebra at the site of damage

Lastly, I wanted to understand the way in which the needle injury affected the mineralization of the notochord and its effect in the development of the vertebrae. For this, I used a newly developed chordacentra specific transgenic zebrafish line (Hiutema et al., 2012) and bone specific fluorescent dyes, which allowed me to visually analyse the effect of the injury in the early formation of the vertebrae and assess how the damage affected the proper development of the spinal column. The experiments were analysed in the context of the wt1b:GFP response, using high resolution imaging to interpret the link between bone formation and the GFP cells.

Using the chordacentra-specific entpd5:RFP line, I revealed how the notochord injury triggered the de-novo upregulation of the mineralization marker at the wound, long before its normal expression in the forming chordacentra rings reached the site of damage. Time-course experiments showed how the signal became highly upregulated in the first 24 hours of the response, developing concomitantly with the wt1b:GFP cells inside the wound. Interestingly, unlike the
wt1b:GFP response, the entpd5:RFP signal decreased in time, with the expression at 72 hpi appearing significantly reduced and more localised than 48 hours earlier.

The 3D analysis of the injury at 24 and 72 hpi also revealed that the entpd5:RFP signal did not co-localise to the wt1b:GFP cells but instead appeared to highlight the presence of a second type of cell at the wound. This was especially evident at 24 hpi, when the wt1b:GFP cells could be seen populating the circumference of the notochord, whilst entpd5:RFP positive cells could be seen populating the ventral side of the injured lumen.

In their paper, Huitema and colleagues demonstrated that the fluorescent entpd5 marker specifically highlighted the population of osteoblast in the maxillofacial skeletal tissues (Huitema et al., 2012), therefore suggesting that these RFP-positive cells could be similarly osteoblastic in nature. Their presence strengthened the possibility that damage to the notochord could be triggering an early calcification event at the site of injury. However, the presence of these osteoblasts at the wound should be clarified, either by the use of other osteoblast specific transgenic lines or the in-situ detection of osteoblast markers (Huitema et al., 2012; Singh et al., 2012; Bensimon-Brito et al., 2012).

The potential existence of a second population of cells at the wound, suggested that the needle injury could be triggering the upregulation of two independent processes at the site of damage. However, their close interlinked arrangement could similarly mean that both populations may interact with each other and act as part of the same process. To validate the presence of the two independent populations at the wound, FACS analysis should be undertaken in the injured notochord to assess for any overlap in fluorescence. However, if the entpd5:RFP marker is truly highlighting the presence of osteoblasts at the wound, the expression of the col2a1a cartilage marker in the wt1b:GFP cells could explain the lack of co-expression between the two fluorescent signals.
The *de-novo* expression of calcifying signals at the site of injury was also corroborated by the use of bone specific fluorescent dyes in the later stages of development. Calcein and alizarin red dyes highlighted the potential early formation of a mineralising structure at the site of damage at around 18 dpi. This was seen both in the presence and absence of surrounding chordacentra, which determined that the process was independent of the signals driving vertebral morphogenesis in the notochord. The long-term analysis of the response also showed that the injury led to the creation of an extra vertebra in the grown injured larvae (n=5/5; a phenotype termed “double vertebrae”). In all the injured notochords the new vertebra was smaller and, in some cases, it formed in conjunction with a second similar sized vertebra.

The long-term analysis of the injured larvae highlighted the slower formation of these vertebrae in comparison to the other vertebra of the backbone. Their development appeared to be independent of the anterior-posterior mineralisation signals driving the patterned formation of the chordacentra along the notochord (Du *et al.*, 2001; Haga *et al.*, 2009; Bensimon-Brito *et al.*, 2012). This was confirmed by the absence of chordacentra rings at the site of injury in the fully patterned notochord, which suggested that their development was either halted by inhibitory signals released by the cells at the site of injury or it was triggered by the loss of the structural scaffold necessary for the formation of the mineralizing rings. Interestingly, this phenotype has previously been reported in laser-ablated notochords (Fleming *et al.*, 2004), which indicated that the inhibition could be due to the release of non-mineralising signals in the affected area. Importantly, in all instances, the vertebra/vertebrae at the wound fully developed and phenotypically resembled the other vertebrae, albeit shorter in size and, in some cases, displaying some defects in the formation of their arches.
Lastly, I used the alizarin red S stain to explore the effect of the needle damage in the developing vertebra in the context of the $wt1b$:GFP response. Interestingly, I discovered that the GFP expression was always found at the site where the extra vertebra was forming. High resolution imaging of the 3D structure of the notochord showed that, in most cases, the $wt1b$:GFP signal co-localised with the vestigial vertebral structure forming at the site of damage. Remarkably, the degree $wt1b$:GFP expression appeared to correlate with the level of development of the forming vertebrae, with less developed vertebrae showing a stronger upregulation at the site of injury (as shown in Figure 4.12D). This observation, in combination with the fluorescent imaging of the site of damage, strongly implicated the $wt1b$:GFP cells in the formation of the vertebral body at the wound. However, GFP staining analysis would need to be carried out in sections of injured juvenile larvae to confirm the co-localisation of the GFP cells with the forming skeletal structure.

Taken together, these results suggested that the needle injury could be triggering the upregulation of a separate non-canonical calcification pathway in the YS area of the notochord. This calcification would be tightly linked to the de-novo expression of $wt1b$:GFP cells at the site of damage, which might be needed for the proper development of the process.

A recent descriptive paper by Bensimon-Brito and colleagues, elegantly dissected the formation of the axial skeleton in the zebrafish notochord (Bensimon-Brito et al., 2012). They discovered that the pattern of mineralisation varied between the different areas of the organ, depending on whether the skeletogenesis required the formation of a cartilaginous anlage prior to the development of the vertebral centrum.

In most of the notochord (the anterior, abdominal and caudal areas) the centrum formed via the direct mineralization of the enveloping notochord sheath and did not require a cartilage precursor. In the caudal fin, however, the pattern was
different, with centra forming only after the establishment of the ventrally located cartilaginous hypurals (Figure 1.9B in Chapter 1 – Introduction; Bensimon-Brito et al., 2012). These appeared to trigger a build up of ventrally deposited minerals at the sheath of the notochord, which led to the formation of the caudal fin chordacentra (Bensimon-Brito et al., 2012).

The existence of an alternative cartilage-mediated ossification process in the notochord provided a possible method by which the delayed vertebra at the site of injury could be forming. The presence of wt1b:GFP; col2a1a:RFP cells at the site of injury and their interaction with the forming vertebrae supported the viability of this hypothesis. The hypurals are highly populated by chondrocytes, which drive the ossification process (Bensimon-Brito et al., 2012). For this reason, it would be important to determine the nature of the wt1b:GFP cells and their function at the site of damage. Alcian blue staining of the injured larvae and immunohistochemical detection of chondrocytes markers at the site of injury would help to consolidate this theory.

The tissue expression profile of the wt1b:GFP fish also strengthened the possibility of this hypothesis. In fact, during the long-term analysis of the injured and non-injured larvae, I had observed the presence of scattered wt1b:GFP positive cells around the hypurals and forming caudal fin vertebrae (data not shown), which could suggest that the wt1b:GFP fluorescent cells may be involved in their development. Moreover, the adult wt1b:GFP zebrafish also shows a strong GFP expression in the pharyngeal arches of the maxillofacial skeleton (see Figure 1.7C in Chapter 1 - Introduction). Both the hypurals and the pharyngeal arches ossify through a cartilage-mediated chondral mineralisation (Gavaia et al., 2006) and are highlighted by the col2a1a:RFP fluorescent line (Dale & Topczewski, 2011). This could indicate a possible link between the presence of wt1b:GFP cells and cartilage-mediated bone development, however, this is a hypothesis that would need to be explored further.
CHAPTER 5: Results

Exploring the transcriptomic profile of the notochord injury response in the zebrafish larvae
5.1. Introduction

In my previous chapters I had uncovered and described some of the cellular interactions, molecular signals and dynamic rearrangements governing the notochord damage response. The accumulation of these events indicated that the injury was triggering the activation of a complex network of gene activations and repressions at the site of damage. I needed to explore and identify the genetic pathways that were controlling this series of changes and uncover how they influenced the future fate of the injured notochord.

The use of fluorescent transgenic lines had already highlighted some of the potential genetic markers of the process. These included the putative involvement of \( wt1b \), which had been the central to the discovery of notochord response, as well as other markers such as \( col2a1a \), which highlighted the origin and nature of the newly formed cells.

Unfortunately, fluorescent transgenic lines do not always recapitulate the endogenous expression of the gene that they are representing. Their fluorescent signal could be affected by the presence of endogenous promoter sequences at their integration site or by the existence of uncharacterised alternative promoter regions within the construct itself.

Perner and colleagues had noted that the \( wt1b:GFP \) line showed a mismatched expression of fluorescence past 65 hpf (Perner et al., 2007). At this point, the larvae showed new foci of expression in the intestines and endocrine pancreas, two sites that are voided of \( wt1b \) expression (Perner et al., 2007; Bollig et al., 2009). Using RNA in-situ hybridisation, it was revealed that the misexpression of the GFP construct was due to the existence of promoter sites for the 3’ to 5’ transcribed \( ga17 \) gene, which lays upstream of the \( wt1b \) gene.
I needed to elucidate whether the GFP expression of the notochord damage response was been driven by the *de-novo* upregulation of the *wt1b* gene or *ga17*. I designed a novel zebrafish *wt1* antibody and immunohistochemically analysed the expression of the *wt1* protein in the injured and non-injured larvae. In collaboration with Witold Rybski, I supported the results with whole-mount RNA *in-situ* hybridisations (WISH) against *wt1b*, to evaluate the expression of the gene at the site of damage and confirm the specificity of the antibody. Moreover, I carried out a microarray analysis of 72 hpi larvae, in order to understand the genetic processes been activated during the notochord damage response. It unveiled a surprising genetic profile that highlighted new processes driving the broad phenotypic changes triggered by the injury.
5.2. Immunohistochemistry and WISH validate the expression of wt1b at the site of damage and highlight other novel areas of expression

5.2.1. Designing a new wt1 zebrafish antibody

In order to validate the expression of the wt1b gene in the notochord damage response, I decided to immunohistochemically identify the expression of the endogenous protein in the wt1b:GFP cells. Unfortunately, there are no commercial antibodies that can detect either of the two zebrafish wt1 orthologues. With this in mind, I synthesised a new rabbit polyclonal antibody using Cambridge Research Biochemicals (CRB) antibody production services (http://www.crbdiscovery.com/home/).

CRB carried out an antigen prediction analysis and highlighted the third zinc finger domain of wt1b as the strongest epitope for the binding of the antibody (Figure 5.1A; red box). Unfortunately, the strong protein sequence conservation between wt1a and wt1b meant that the antibody recognised the expression of both wt1 paralogues (Figure 5.1A). However, the needle injury experiments had not shown any wt1a:GFP upregulation in the damaged notochord (Figure 3.2). This strongly excluded the presence of the endogenous wt1a gene in the process, as the expression of the wt1a:GFP transgene had been shown to specifically match that of the endogenous wt1a transcript (Perner et al., 2007; Bollig et al., 2009).

The antibody was created as specified in the TARGET antibodies production protocol in the CRB website (http://www.crbdiscovery.com/services/antibody-production/target-antibodies.php). In short, a small peptide with the chosen sequence was synthesised and purified by HPLC. Two rabbits were inoculated with the peptide during the immunization process and serum was collected from the rabbits at three different time intervals. At each stage the serum was tested for the presence of wt1 antibodies using EMSA and on the final collection day, the antibody
content of the serum was extracted and purified. The purified polyclonal wt1 antibody was delivered to us after 5 months.

I used the antibody for the immohistochemical identification of the endogenous wt1 protein at the site of damage, comparing it to the pattern of binding that I had previously observed using the GFP antibody, in order to validate the specificity of the novel immunoglobulin in the injured larvae.

5.2.2. The wt1 antibody binds wt1b:GFP cells in the kidney and at the site of damage

I trialled the newly synthesised wt1 antibody on paraffin-embedded sections and established the optimal dilutions for chromagen and fluorescent immunohistochemistry (1:25,000 and 1:33,000 respectively).

Similarly to previous experiments, I needle-injured 3 dpf wt1b:GFP; casper larvae and raised them to 72 hpi. At this point the larvae were anaesthetised, fixed in 4% PFA, methanol dehydrated and embedded in paraffin to be sagitally cut into 5 μm sections (Figure 5.1B; see Materials & Methods for details).

DAB immunohistochemistry revealed that the wt1 antibody was able to bind and recognise a peptide in the cells at the site of injury (Figure 5.1C - injured α-WT1; blue arrow). The staining resembled the previous patterns observed in the GFP staining of the injured site (see Chapter 3) and, importantly, it did not show specific binding in the notochords of non-injured controls (Figure 5.1C - α-WT1 non-injured; red arrow head).

Interestingly, the antibody also recognised a number of cells in the neural tube of both injured and non-injured larvae (Figure 5.1C; α-WT1 injured and non-injured; orange arrows). This was a site of expression that I had not previously observed in my GFP antibody staining or the fluorescent transgenic animals, but it is not a site
Figure 5.1. A novel wt1 antibody binds to the \textit{wt1b}\textit{:GFP} expressing cells of the injured notochord and the pronephros. (A) Amino acid sequence comparison between \textit{wt1b} and \textit{wt1a} proteins. Red box highlights antibody binding epitope. (B) Three dpf \textit{wt1b}\textit{:GFP}; casper larvae were YS needle injured and grown to 72 hpi. The larvae were fixed, embedded and sectioned to be used in DAB staining experiments with the wt1 antibody. (C) The wt1 antibody recognised cells inside the wound (blue arrow) and in the neural tube (orange arrows). No staining was seen in non-injured notochords (blue arrow head). Scale bar = 30 μm.
unfamiliar to the expression of wt1, as previous studies had shown that wt1 can become upregulated in the lateral motor neurons of developing mouse embryos (Armstrong et al., 1992; Moore et al., 1998).

5.2.3. Fluorescent immunohistochemistry validates wt1 antibody binding and uncovers new sites of wt1 expression

The experiment was repeated using a secondary fluorescent antibody (see Materials & Methods for details). The reasons for doing this was that I wanted to validate the DAB experiments and achieve a better visual output of the binding. Additionally, the use of fluorescence would allow me to separate the different fluorescent channels and analyse them individually.

Fluorescent cells were seen at the head of the pronephros and at the site of injury, with the latter showing a weaker signal (Figure 5.2A - α-WT1 and α-WT1 DAPI). The difference in strength correlated with what I had seen in my transgenic experiments, where all animals that I imaged showed a stronger fluorescent expression at the kidney than at the site of damage.

The imaging showed that only the cells inside the wound and the cells in the surrounding notochord sheath layer stained positive for the wt1 antibody (Figure 5.2A - α-WT1 and α-WT1 DAPI; yellow and white arrow heads respectively). No cells outside the notochord showed any antibody staining, confirming the specificity of the immunoglobulin at highlighting the cells inside the wound (Figure 5.2A - α-WT1 and α-WT1 DAPI; red arrow heads). It is worth noting that several cells and tissues in the sections autofluoresced under the fluorescent light (Figure 5.2A - α-WT1 DAPI; white asterisk). These appear as yellow-green tissues, as the sections were imaged through the red (antibody) and green (no antibody) fluorescent channels.
Figure 5.2. Immunofluorescence validates DAB staining at the injury and uncovers novel areas of wt1 expression in the zebrafish head
Figure 5.2. Immunofluorescence validates DAB staining at the injury and uncovers novel areas of wt1 expression in the zebrafish head. (A) Fluorescent immunochemistry using the wt1 antibody on 3 dpi larvae shows staining at the pronephros and at the site of damage. Wt1 antibody highlights cells inside the wound (yellow arrow heads) and in the outer notochord sheath layer (white arrow heads). Red arrow heads highlight cells outside the notochord (B) Zoomed out image of the injured notochord highlights the staining pattern in the wound and in the flanking non-injured notochord areas. Yellow arrows denote antibody binding, while red arrows and red arrow head show cells that do not show antibody staining. (C) Head staining with the wt1 antibody. Zoomed insets show the kidney (yellow arrow; K1), the chondracranium (red arrow; Ch), the notochord (No) and the brain (white arrow and arrow head; Br). In all images anterior is left, posterior is right, dorsal is up and ventral is down. Scale bars = 30 μm.

To validate the specificity of the antibody and uncover other possible expression sites, I took wider images of the notochord and the head of the larvae (Figure 5.2B and 5.2C). In the notochord, the wider view showed that only the cells at the injury displayed specificity to the antibody (Figure 5.2B; yellow arrows). No other visible cells inside or outside of the notochord showed antibody specificity, even though a weak background signal could be observed in all tissues (Figure 5.2B –red arrows and red arrow heads). Similarly to previous imaging, the asterisk denote the autofluorescent expression.

In the head and torso of the larvae, the antibody unveiled a different pattern of expression. It strongly bound to the cells of the pronephros (Figure 5.2C; yellow arrow) and also showed other sites of expression around the renal structure. These sites included the skeletal chondrocranium and the brain (Figure 5.2C; red arrow and green arrow/arrow head). Additionally, a washed expression could be seen in the lining of the intestine, but its specificity was difficult to determine. The chondrocranium compromises the early cartilaginous skeleton of the head and extends from the basioccipital articulatory process (BOP), which connects the notochord with the head structure, to the mandible of the fish. The antibody showed specificity for the chondrogenic cells of the BOP, which populate the inside of the tissue (Figure 5.2C; red arrow). This pattern was similarly seen in other cartilage of the head, including the pharyngeal arches, which are also fluorescently highlighted in wt1b:GFP animals in later stages (data not shown).
On the other hand, the hindbrain showed a high number of scattered cells with specificity for the wt1 antibody (Figure 5.2C – hindbrain; green arrow head). Some of those cells could be seen in midbrain-hindbrain boundary, which is a site of wt1b:GFP cells in the fluorescent transgenic animals and has shown GFP antibody specificity in previous immunohistochemical experiments (Figure 5.2C – hindbrain; white arrow and data not shown).

Overall, the chromagen and fluorescent immunostaining showed that the wt1 antibody was able to recognise the same sites of expression as the GFP antibody. These sites included the notochord injury, the kidney and the hindbrain-midbrain boundary. Additionally, the wt1 antibody also recognised other sites of expression that I had not previously observed. These should be validated in order to attest for the expression of the wt1b gene at these sites.

5.2.4. Whole mount in-situ hybridisation confirms the upregulation of wt1b in the notochord damage response and in the other sites recognised by the antibody

The antibody staining revealed the potential upregulation of the endogenous wt1b protein in the notochord damage response. Additionally, it also highlighted other pockets of expression in the larvae, which had not been previously reported. In order to confirm the validity of the antibody results, I wanted to explore the RNA expression pattern of the wt1b gene to make sure that the immunoglobulin was recognising the right peptide.

I had previously attempted to verify the specificity of the antibody using Western blots on morpholino injected 24 hpf embryos. However, technical difficulties and the mosaic effect of the morpholinos had meant that its validation had been difficult. The existence of a previously published wt1b RNA probe meant that I could
use it to verify the antibody results and reveal other sites of expression for the *wt1b* gene in the injured and non-injured larvae (Perner *et al.*, 2007; Bollig *et al.*, 2009).

Unfortunately, previous reports have highlighted the difficulty of staining the notochord in zebrafish larvae after 48 hpf (Thisse & Thisse, 2008). This meant that I could not use the RNA probes in whole mount *in-situ* hybridisation experiments with needle-injured larvae. In order to circumvent this, I decided to explore the expression of the *wt1b* RNA transcript in tail amputation experiments. Unlike needle injuries, the tail amputations cause a large opening in the caudal end of the notochord and lead to the exogenous accumulation of *wt1b*:GFP positive cells in the forming regeneration blastema, which made the area of damage more accessible to the probes (see Chapter 3).

PTU-treated 3 dpf *wt1b*:GFP larvae were tail amputated at the PCV area and grown to 48 hpa with their non-injured controls. At this point, all larvae were PFA fixed overnight and progressively methanol dehydrated (Figure 5.3A). The dehydrated larvae were then used for the WISH experiment as outlined in Thisse & Thisse, 2008 (see Materials & Methods for details). The sense and antisense RNA probes were synthesised from a construct provided by Christoph Englert (Jena, Germany). Witold Ribsky in our lab carried out the synthesis of the probe and completed the RNA staining of the injured fish. The larvae were imaged using a Nikon AZ100 Macrooscope system.

The experiment revealed that the *wt1b* antisense probe was able to identify the expression of the gene in several cells of the regeneration blastema (Figure 5.3B – antisense; green arrow; n=8 out of 9 larvae). This expression matched the normal GFP expression seen in the tail-amputated *wt1b*:GFP larvae and together with the antibody data confirmed the novel upregulation of *wt1b* in the notochord damage response. Additionally no probe binding was observed in the sense probe or in the
tails of the non-injured anti-sense treated controls (Figure 5.3B – sense and non-injured antisense; green arrow head).

The probe also highlighted other areas of expression that show similarity with the antibody staining. In the head, the probe recognised the expression of the gene in the brain and parts of the chondrocranium, including Meckel’s cartilage and the pharyngeal arches (Figure 5.3B – antisense injured and non-injured; purple, purple and blue arrows respectively). No head staining was observed in the injured and non-injured embryos incubated with the sense probe (Figure 5.3B – sense; arrow heads).

The antisense probe also identified a notable site of expression in the heart and potentially the kidney and the BOP of the larvae (Figure 5.3B – antisense; red, orange and blue arrows). The expression in the heart, however, was not always recapitulated in all samples and hence should be addressed with more experiments (Figure 5.5B – antisense; red arrow vs. black arrow head). On the other hand, the putative expression of the probe in the deeper tissues, such as the BOP and the kidneys, should be investigated further with sections of the wt1b stained larvae.

Taken together with the antibody staining, this data corroborated the specific upregulation of the wt1b gene in the notochord damage response and uncovered new sites of expression for the gene in the maxillofacial cartilage.
Figure 5.3. Whole mount RNA staining highlights areas of wt1b transcription (A) Three dpf wt1b:GFP; col2a1a:RFP larvae were YS needle injured and grown to 48 hpi. The injured larvae were fixed and dehydrated before the RNA staining experiment (B) Injured and non-injured larvae were stained with antisense and sense wt1b probes. The arrows and arrow heads highlight the regeneration blastema (green arrow and arrow head), the brain (Br; purple arrow and arrow head), parts of the chondrocranium (Ch; blue arrows and arrow heads), the heart (He; red arrow and arrow head and black arrow head) and the kidneys (Ki; orange arrow and arrow head).
5.3. Microarray analysis of the response reveals some of the genetic changes triggered by the notochord injury in the larvae

5.3.1. Collecting tissue samples for the microarray analysis

Having uncovered the presence of *wt1b* in the notochord damage response, next, I wanted to explore the genetic profile driving the progression of the process. For this, I decided to carry out a microarray analysis of 3 dpi zebrafish larvae.

I YS injured 3 dpf *wt1b*:GFP; casper zebrafish and grew them together with non-injured controls to the specified time (n=50 larvae per population; 3 replicates of each population). At this point, I dissected the damaged area of the injured larvae and their homologous area in the non-injured controls (Figure 5.4A). The dissected tissue for each population was collected and RNA was extracted from the pooled samples. The RNA was sent to Miltenyi Biotec (Germany) where it was quality checked, amplified, labelled with Cy3 dye and hybridised against a Whole Zebrafish (V3) Genome Oligo Microarray (see Materials & Methods for details).

Each population was analysed independently and the single fluorescence output of each gene was determined. The raw data was analysed by Graeme Grimes (MRC HGU), who pooled replicates together and compared the injured populations vs. the non-injured controls. The microarray resulted in the identification of 36,688 gene changes in the injured vs. non-injured larvae.

5.3.2. Identifying statistically significant genes by their q-value

I sorted the gene list provided by Graeme according to their p-values and adjusted p-values (q-values), identifying the most significant genes using a 5% cut off point for each parameter. I discovered that using the p-value alone, 2,845 genes of the
Figure 5.4. Microarray analysis of injured and non-injured larvae  (A) Three dpf wt1b:GFP; casper larvae were YS needle injured and grown to 72 hpi. Injured area was dissected (blue square) and its RNA extracted and sent for microarray. (B) Volcano plot showing upregulated (green dots) and downregulated (red dots) hits of injured vs. non-injured control samples. Coloured dots above the blue broken line show q-value significant hits. Green and red outlined dots above the green broken line show p-value significant genes.
Table 5.1. Q-value significant genes. Table showing the names, functions, q-values and expression change (log₂ and fold change) of the q-value significant genes of the microarray analysis. Green boxes show upregulated genes, red box shows downregulated gene.

<table>
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<th>Gene</th>
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<th>Log₂</th>
<th>Fold Change</th>
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</table>

Table 5.2. P-value significant genes associated with chondrogenesis. Table showing the names, functions, p-values and expression change (log₂ and fold change) of the genes in the microarray associated with cartilage development.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
<th>P-value</th>
<th>Log₂</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>col2a1a</td>
<td>Collagen type 2 alpha 1a</td>
<td>Main extracellular component of cartilage</td>
<td>1.14 x 10⁻²</td>
<td>2.31</td>
<td>4.97</td>
</tr>
<tr>
<td>col2a1b</td>
<td>Collagen type 2 alpha 1b</td>
<td>Main extracellular component of cartilage</td>
<td>3.16 x 10⁻²</td>
<td>0.60</td>
<td>1.52</td>
</tr>
<tr>
<td>col11a2</td>
<td>Collagen type 11 alpha 2</td>
<td>Main component of cartilage</td>
<td>1.88 x 10⁻²</td>
<td>1.39</td>
<td>2.63</td>
</tr>
<tr>
<td>col9a1</td>
<td>Collagen type 9 alpha 1</td>
<td>Main component of cartilage</td>
<td>1.9 x 10⁻²</td>
<td>1.63</td>
<td>3.10</td>
</tr>
<tr>
<td>col9a2</td>
<td>Collagen type 9 alpha 2</td>
<td>Main component of cartilage</td>
<td>1.54 x 10⁻²</td>
<td>1.18</td>
<td>2.26</td>
</tr>
<tr>
<td>LOC559593</td>
<td>Aggrecan-core like protein</td>
<td>Main component of cartilage</td>
<td>9.41 x 10⁻⁵</td>
<td>1.98</td>
<td>3.95</td>
</tr>
<tr>
<td>sox9b</td>
<td>SRY-box 9b</td>
<td>Key mediator of chondrogenesis</td>
<td>2.65 x 10⁻²</td>
<td>3.23</td>
<td>9.39</td>
</tr>
<tr>
<td>chad</td>
<td>Chondroadherin</td>
<td>Chondrocyte adhesion protein</td>
<td>7.12 x 10⁻⁴</td>
<td>1.32</td>
<td>2.49</td>
</tr>
<tr>
<td>mir-140</td>
<td>microRNA 140</td>
<td>Sox9 regulated cartilage gene</td>
<td>4.34 x 10⁻³</td>
<td>1.26</td>
<td>2.40</td>
</tr>
</tbody>
</table>
36,688 were statistically significant in the microarray list (Figure 5.4B; green and red dots). However, when the same threshold was applied using the more stringent q-value, only 9 genes of the 36,688 showed a statistically significant change (Figure 5.4B; blue dots).

The q-value is considered the standard for microarray analysis, as it takes into account the false discovery rate (FDR) present when analysing large amounts of data (Aubert et al., 2004). Even though my microarray was ultimately exploratory, I also wanted to identify genes that were undeniably differentially expressed in response to the needle damage.

The FDR treatment yielded 9 genes that were significantly expressed in the injured vs. non-injured population. From these, 8 genes were upregulated and 1 gene was downregulated (Figure 5.4B; red and green full coloured dots above broken green line). Unfortunately, the zebrafish genome has not been fully annotated and for this reason only 4 of the 9 genes could be characterised, 3 were not identified and the characterisation of one, zgc:92041, had a predicted entry, based on its homology to a vertebrate gene. Additionally, one of the genes, fibronectin 1b (fn1b) appears twice in the list, as it is recognised by two different probes in the hybridisation array (Table 5.1).

These statistically significant genes highlighted bone/cartilage regulatory genes, immunological response genes and components of the extracellular matrix (ECM; Table 5.1). They were, in order of significance: matrix gla protein (mgp), fibronectin 1b (fn1b), a gene similar to elastase 2 (ela2), coagulation factor XIII A1 polypeptide b (f13a1b) and complement component 6 (c6).

The most significant gene, mgp (q-value = 5.0 x 10⁻³), codes for a vitamin K-dependent small extracellular matrix protein that binds calcium and phosphate ions and inhibits hydroxyapatite formation during ectopic calcification and bone
development (Price & Williamson, 1985; Luo et al., 1997; Yamagi et al., 1999; Schurgers et al., 2013). Initially isolated from bone, mgp has also been shown to express in immature, proliferating and hypertrophic chondrocytes, as well as blood vessels and certain soft tissues, such as the kidney, the heart and the lungs (Price et al., 1983; Hale et al., 1988; Fraser & Price, 1988; Yamagi et al., 1999; Barone et al., 2004; Gavaia et al., 2006). Its bone inhibiting properties are due to the post-translational gamma-carboxylation of its glutamate residues, which give it the ability to bind and repress the accumulation of hydroxyapatite in tissues and inhibit the bone-inducing expression of BMP2 in the mineralising extracellular matrices (Zebboudj et al., 2002 Sweatt et al., 2003; Schurgers et al., 2013). In zebrafish, its expression has been identified in the cartilage of the developing and adult zebrafish, specifically in the pre-hypertrophic and hypertrophic chondrocytes, as well as in mineralising tissues undergoing chondral ossification (Gavaia et al., 2006).

The high degree of statistical significance of the mgp gene was also coupled with a high degree of expression (Table 5.1). The microarray analysis showed a log₂ change of 7.04 (131.56-fold) between the injured and non-injured populations, making the mgp gene the most upregulated gene in my cohort. Its expression in the non-injured samples showed that under normal circumstances the physiological expression of the gene ranged between 0.6-1.2, increasing its expression to a range of 94.4-168.2 3 days after the needle injury (see Supplementary data).

The high upregulation of this calcification inhibitor could explain the delayed vertebra formation that I see in the injured notochords over time. The gene could become activated and carboxylated in the local area, thus halting the formation of the vertebra around the wound.

The second most significant gene was fn1b (q-value = 1.3 x 10⁻²), one of two zebrafish orthologues of the fibronectin 1 gene (Sun et al., 2005). A widely conserved extracellular cell adhesion protein of the basement membrane,
fibronectin is essential for the developmental movements required during late gastrulation, as well as the acquisition of basement membrane integrity and the progression of wound healing (George et al., 1993; Winklbauer & Keller, 1996; Kumar et al., 2010; Hynes & Naba, 2011). In zebrafish, fn1b has only recently begun to be studied and it has been shown to have important roles in somite integrity, interrenal vesicle movements and more recently in tail and heart regeneration (Jülich et al., 2005; Yoshinari et al., 2008; Chiu et al., 2012; Wang et al., 2013). Previous studies have highlighted the role of its mouse and human orthologues in developmental and pathological EMTs, where it has been proposed to be a mesenchymal marker of the process (Zeisberg & Neilson, 2009; Balanis et al., 2013).

In the microarray, the fn1b gene appeared twice in the list of significant genes by q-value. In both cases it showed a very high upregulation in the injured samples, with a 4.5 and 4.1 log2 fold change respectively (22.63 and 17.09 fold changes).

The heterogeneous nature of the fn1b gene could explain the number of different probes found in the microarray, as fn1b has been shown to code for different protein isoforms due to the presence of a number of splice sites in its gene (Sun et al., 2005). It should also be noted that 2 other fn1b probes were also present in the microarray data. These showed a high degree of p-value significance and were close to the 5 x 10^-2 threshold that determined statistical significance in the adjusted p-value (q-values = 5.5 x 10^-2 and 5.7 x 10^-2 respectively; Supplementary Data). In both cases, the needle injury induced a similar high transcriptional upregulation as seen in the more significant probes (see Supplementary Data). It is worth mentioning that some of the non-injured controls showed a high basal expression of the fn1b probes, which could highlight the high expression of the gene in normal development (see Supplementary Data).

Two immune response genes were also found within the list of most significant hits. These were zgc:92041, a gene that has been predicted to code for a novel protein
similar to elastase-2 (elα2), and complement component 6 (c6), an important constituent of the complement cascade (Table 5.1).

Elastase-2 is a serine protease found in the azurophil granules of neutrophil cells (Pham, 2006). Together with cathepsin G and other proteases, it acts in the non-oxidative antimicrobial arm of the neutrophil response, killing the engulfed bacteria inside phagocyte (Belaaouaj et al., 1998; Weinrauch et al., 2002, Benabid et al., 2012). Its role in immunity does not only extend to its antimicrobial properties, as it can also act extracellularly to activate pro-inflammatory signals that regulate the second wave of the immune response (Pham, 2006). It is this extracellular plasma membrane-bound elastase that has additionally been associated with the pathological degradation of the extracellular matrix, leading to the digestion of elastin, collagens and fibrillins of the basement membrane during wound healing, sun-induced aging and disease (Grinnell & Zhu, 1995; Labat-Robert et al., 2000; Rijken & Bruijnzeel, 2009).

The microarray data highlighted the elα2 gene as the third most significant gene, with a q-value of 1.9 x 10^{-2} and a 4.14 log₂ fold upregulation (17.6 fold; Table 5.1). The high upregulation could potentially acknowledge the presence of a high amount of neutrophils in the collected sectioned tissue around the injured area. However, previous literature has shown that elα2 mRNA is only transcribed during early myeloblast development and not in the mature neutrophil (Fouret et al., 1989; Pham, 2006). It is well understood that in zebrafish, erythromyeloid development happens in the posterior blood island of the zebrafish, which is located ventrally from the site of damage, just posterior to the end of the yolk sac (Bertrand et al. 2007). Considering the way in which the tissue was collected for the RNA extraction, it is highly probable that the high elastase-2 expression could be due to the enhanced neutrophil production in this area in response to the needle damage.
On the other hand, complement component 6 (c6) is an essential member of the membrane attack complex (MAC), an effector of the immune complement cascade. The assembly of the MAC is central to the innate and adaptive immune system. It is initiated via the formation of the c5b-C7 complex, which anchors to the bacterial plasma membrane and leads to the lysis of microbial cells through the sequestering of the c8 and c9 peptides (Dunkelberger & Song, 2010; Aleshin et al., 2012). C6 is an essential component of the initial anchoring complex and has been shown to be required for its proper antimicrobial function (Orren et al., 1987; Dulkenberg & Song, 2010). The c6 gene showed a high upregulation in the needle-injured population with a 2.95 log₂ fold expression over the non-injured controls (7.75 fold; Table 5.1). Additionally, similarly to the other genes, it also showed high q-value significance, with a 2.85 x 10⁻² value (p-value = 6.21 x 10⁻⁶). Three other microarray probes also recognised the expression of the c6 gene, with near significant q-values and highly significant p-values (see Supplemetary Data for details).

The last of the significant genes was f13a1b, which codes for one of the orthologues of the A1 subunit of factor XIIIa. Factor XIIIa (FXIIIa) is a highly prolific calcium-dependent transglutaminase enzyme involved in blood clotting, cell-matrix adhesion, wound healing, angiogenesis, bone formation and matrix stabilisation (Iismaa et al., 2009; Muszbek et al., 2011). The enzyme exists as an extracellular heterotetrameric zymogen in blood plasma and as an activated homodimer inside cells. Extracellularly, FXIIIa combines with its B-subunits to form an inactive FXIIIa₂B₂, which is cleaved into an active FXIIIa homodimer by thrombin in the last step of thrombogenesis where it stabilises the fibrin clot (Iismaa et al., 2009).

Intracellularly, the enzyme exists as a FXIIIa homodimer in platelets, monocytes, fibroblasts and osteochondrogenic cells (Iismaa et al., 2009; Muszbek et al., 2011). Even though its canonical function is in the coagulation cascade, FXIIIa has also been shown to induce the proliferation, migration and survival of monocytes, fibroblasts and endothelial cells (Dardik et al., 2007; Dardik et al., 2005).
Additionally, it also mediates the alternative activation of monocytes and trigger angiogenic signals in vitro and in-vivo (Muszbek et al., 2011).

The role of FXIIIA in bone formation stems from its expression in pre-hypertrophic and hypertrophic chondrocytes during endochondral ossification (Iismaa et al., 2009). Here, it acts concomitantly with another transglutaminase, transglutaminase 2 (TG2), to induce the formation of the mineralised matrix that is required for later bone development (Nurminskaya et al., 1998; Johnson et al., 2008). Its expression, however, is not only limited to chondrocytes during this process and the gene has additionally been identified in the surrounding osteoblasts, which secrete the calcified matrix that forms the endochondral bone (Al-Jallad et al., 2011; lisma et al., 2009). In zebrafish, its expression has only recently been defined and the f13a1b gene appears to be exclusively expressed in the developing pectoral fin bud during larval stages (Deasey et al., 2012; initially named zFXIIIa-87).

In the injured population, f13a1b was significantly upregulated with a q-value of 2.65 x10^{-2} and a log2 fold change of 3.23 (9.39 fold). The f13a1b gene was also recognised by a slightly less significant (q-value = 5.54 x 10^{-2}) microarray probe, which showed a similar level of upregulation of the gene (see Supplementary data for details).

Taken together, the activation of these 5 genes indicated that the needle injury triggered some important response activations in the damaged area: from immune processes to broad extracellular matrix re-organisations. It was especially interesting to see the high upregulation of both mgp and f13a1b in the injured population, as their expression could be denoting de-novo activation of osteochondrogenic lineages at the site of damage.

Unfortunately, the partial annotation of the zebrafish genome meant that almost half of the q-value significant genes could not be defined. Their identification could
bring new insights into the responses triggered by the needle and help establish more concrete assumptions about the pathways being activated. It would be of special interest to determine which one is the gene that becomes significantly downregulated in response to the damage, as it could highlight the inhibition of an important signal in the process.

5.3.3. P-value significant genes: Cartilage

The high upregulations of the \textit{mgp} and \textit{f13a1b} genes hinted towards the \textit{de-novo} acquisition of chondrogenic features in the injured tissue after the needle damage. Gavaia and colleagues had shown that the endogenous \textit{mgp} gene expresses in the chondrogenic populations of the endochondral tissues of the craniofacial, fin bud and axial skeletons (Gavaia \textit{et al.}, 2006). On the other hand, FXIIA has been implicated in osteochondrogenic modulation of bone formation in birds and mammals (Iisma \textit{et al.}, 2009) and its expression in zebrafish has been located to the developing chondrogenic mesenchyme of the pectoral fin bud (Deasey \textit{et al.}, 2012). Taking into account the enriched expression of both genes in my microarray and their known physiological roles in bone development, I decided to look for other genes in the list associated with chondrogenic development.

It is worth highlighting that vertebral calcification in the zebrafish notochord occurs via the activation of intramembranous mineralisation signals in the notochord sheath that cause the formation of metameric calcifying rings around the tube-like organ (Flemming \textit{et al.}, 2004; Bensimon-Brito \textit{et al.}, 2012). Intramembranous ossification does not require the establishment of cartilage anlagen and thus arises from the osteoblastic maturation of mesenchymal cells at the site of bone formation (Lefebvre & Bhattaram, 2010). If cartilage were to be forming, it would mean that the notochord damage would be inducing the activation of an alternative
bone development process for the formation of the delayed vertebra/vetebrae at the site of damage.

To validate this, I looked for the expression of other cartilage markers in my microarray data. I scrutinized the top 100 most significant genes in my microarray list and then also arbitrarily searched for known chondrogenic markers. I found a list of genes that are strongly associated with cartilaginous tissues, both during normal chondrogenic development and chondral bone formation.

The first genes that I found were collagen genes. It is known that bone and cartilage have different collagen biological profiles depending on their stage of development (Zhang et al., 2009). As such, I found 5 collagens gene associated with chondrogenic tissues, these were: col2a1a, col2a1b, col11a2, col9a1 and col9a2 (Kessels et al., 2014; Zhang et al., 2009). From these, col2a1a and col2a1b are two orthologues of collagen type 2, the main fibrillar collagen found in cartilage and main markers of articular and hyaline cartilage (Zhang et al., 2009; Lefebvre & Bhattaram 2010). Whilst col2a1a appeared highly upregulated, with a 4.97 fold change in expression, col2a1b was more modestly expressed in the injured population with a 1.52 fold upregulation (Table 5.2). Both genes have been involved in early cartilage formation in the zebrafish head, with very similar expressions during the initial 48 hours of development (Dale & Topczewski, 2011). Col2a1a has also been highlighted as the only one of the two to be expressed in the chondrogenic mesenchyme of the developing pectoral fin bud, as well as being a marker of the early notochord and the outer sheath cells of the later notochordal stages (Dale & Topczewski, 2011; see Chapter 4).

Col11a2, col9a1 and col9a2 are all minor fibrilar collagens that interact with collagen 2 in cartilage and are involved in its fibrilogenesis (Zhang et al., 2009). Their expression in the injured population was markedly upregulated with col11a2,
col9a1 and col9a2 showing a 2.63, 3.10 and 2.26 increase in expression in the injured tissue (Table 5.2).

Aggrecan is another cartilage-specific extracellular structural protein that was highly upregulated in my microarray list. Aggrecan is known to be the most abundant proteoglycan expressed in chondrogenic tissues (Zhang et al., 2009; Kessler et al., 2014). The expression of aggrecan (identified in the array as aggrecan core protein-like or LOC559593) was highly upregulated in the injured samples showing almost a 4-fold change of expression after injury in comparison with the non-injured control (Table 5.2). Additionally, unlike most of the collagen genes, its expression was almost non-existent in the non-injured population, which suggested that the gene had been activated in response to the needle damage.

The neural crest marker sox9b was also upregulated in my microarray list. Its mammalian counterpart, Sox9, is considered the master regulator of chondrogenesis, inducing a cartilage fate in the mesenchymal cells of the early skeleton and triggering the upregulation of collagen 2 and other cartilage markers (Bi et al., 1999; Bell et al., 1997; Ng et al., 1997; Bridgewater et al., 1998). In zebrafish Sox9 exists as two orthologues, sox9a and sox9b, which synergistically recapitulate the actions of the mammalian gene (Chiang et al., 2001; Yan et al., 2002). The genes have overlapping but spatially distinct expression patterns and have been shown to both cooperate and be independent of each other during the osteochondrogenic development of the early skeleton (Yan et al., 2002; Yan et al., 2005).

Sox9b was moderately upregulated in my data, showing a 2.22 fold change in the injured population (Table 5.2). The sole upregulation of this gene could suggest that, if cartilage was to be forming, it could be due to the expression of this gene. However, sox9b has also been shown to express in the early notochord and its
deletion leads to the acquisition of a curved notochord phenotype in the affected mutants (Yan *et al.*, 2005).

Two other cartilage-specific genes were similarly upregulated in my microarray. MicroRNA 140 (*mir140*), a highly conserved regulator of chondrogenesis, and chondroadherin (*chad*), a matrix-cell anchor protein found in cartilage and other skeletal tissues (He *et al.*, 2011; Hessle *et al.*, 2013). The highly conserved *mir140* is a cartilage-specific downstream effector of Sox9 that was first identified in a microRNA specific RNA screen in zebrafish (Wienholds *et al.*, 2005; Miyaki *et al.*, 2010). Its expression and functional roles have been explored in mice, chicken and zebrafish, where it has been found to be expressed in the proliferating chondrocytes of developing cartilage and in mature articular cartilage (Darnell *et al.*, 2006; Eberhart *et al.*, 2008; Miyaki *et al.*, 2010). MiR-140 is required for the proper development and homoestasis of chondrogenic tissues and the appropriate progression of endochondral bone (Miyaki *et al.*, 2010; Nakamura *et al.*, 2011; Papaioannou *et al.*, 2013). Additionally, the downregulation of miR-140 has been linked to osteoarthritic cartilage in humans and mice, with its overexpression been proposed as a potential treatment for the disease (Miyaki *et al.*, 2010).

*Chad*, on the other hand, is a leucine-rich repeat proteoglycan known to mediate the interactions between cells and the extracellular matrix in skeletal tissues (Shen *et al.*, 1998). Unlike miR-140, no functional studies have been undertaken to identify the role of *chad* in zebrafish, though a high-throughput expression analysis has identified its expression in the developing notochord of 24hpf to 48hpf larvae (Thisse & Thisse, 2004). Additionally, RNA-seq data has highlighted its expression during swimbladder development, where it was identified as one of the top enriched genes in the process (Zheng *et al.*, 2011). Contrastingly, its function in humans, mice and other vertebrates has been more broadly investigated, where it has been shown to be highly expressed in chondrogenic tissues and have a lower expression profile in bone, connective tissue and the eye (Shen *et al.*, 1998; Tasheva
et al., 2004). In cartilage it expresses close to chondrogenic cells, binding their alpha-2 beta-1 integrin receptors and mediating their attachment to the fibrillar collagens of the extracellular matrix (Camper et al., 1997; Mansson et al., 2001). The protein mostly expresses in proliferating chondrocytes and a knock out mice of the gene showed a mild skeletal phenotype in the cartilage growth plate of endochondral bones (Shen et al., 1998; Hesle et al., 2013).

Both genes showed a 2.5 fold upregulation in the needle-injured animals. The increase in expression of these genes and the other cartilage markers strongly indicated that the needle was triggering the de-novo expression of cartilage after damage, however, further confirmation of this was required (see below). Additionally, it should also be said that even though it is interesting to see that a number of chondrogenic markers were statistically upregulated in the injured larvae, these were purposely identified due to their association with cartilage formation and their expression should be confirmed using other statistically significant analysis.

5.3.4. P-value significant genes: Notochord development and immune response

My previous imaging experiments had shown that the needle injury caused extensive morphological rearrangements in the notochords of the damaged larvae. These changes included the loss of cellularity of the vacuolated cells and the activation and movement of the wt1b:GFP positive cells from the outer notochord sheath layer to the injured lumen.

Initially, I wanted to find out whether the damage could be triggering a repair or regenerative response inside the notochord. I had seen that even though the notochord was able to grow back and repopulate the damaged space, the regrowth did not faithfully resemble the original tissue. This suggested that the notochord
was able to repair the damage caused by the needle but did not trigger a complete regenerative response at the injured area. This was further highlighted by the fact that the forming delayed vertebra did not completely resemble the other vertebral units, appearing smaller and, in some cases, showing defective neural and hemal arches (see Chapter 4).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
<th>P-value</th>
<th>Log2</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>co5a3a</td>
<td>Collagen type V alpha 3a</td>
<td>Notochord development</td>
<td>5.00 x 10^-2</td>
<td>1.22</td>
<td>2.33</td>
</tr>
<tr>
<td>co8a1a</td>
<td>Collagen type VIII alpha 1a</td>
<td>Notochord sheath development</td>
<td>7.17 x 10^-3</td>
<td>2.08</td>
<td>4.22</td>
</tr>
<tr>
<td>col15a1a</td>
<td>Collagen type XV alpha 1a</td>
<td>Notochord sheath development</td>
<td>4.58 x 10^-3</td>
<td>1.03</td>
<td>2.04</td>
</tr>
<tr>
<td>ntla</td>
<td>Notall a (brachyuni)</td>
<td>Early notochord development</td>
<td>4.87 x 10^-3</td>
<td>1.10</td>
<td>2.29</td>
</tr>
<tr>
<td>shha</td>
<td>Sonic hedgehog a</td>
<td>Early notochord development</td>
<td>3.35 x 10^-3</td>
<td>1.09</td>
<td>2.13</td>
</tr>
<tr>
<td>twist2</td>
<td>Twist 2</td>
<td>Axial mesoderm marker</td>
<td>3.14 x 10^-2</td>
<td>0.81</td>
<td>1.76</td>
</tr>
<tr>
<td>nmb</td>
<td>Indian hedgehog b (echiota)</td>
<td>Axial mesoderm marker</td>
<td>5.57 x 10^-3</td>
<td>1.17</td>
<td>2.25</td>
</tr>
<tr>
<td>lox15b</td>
<td>Lysyl oxidase-like 5b</td>
<td>Notochord sheath development</td>
<td>1.24 x 10^-3</td>
<td>3.51</td>
<td>11.41</td>
</tr>
<tr>
<td>mpeg1</td>
<td>Macrophage expressed 1</td>
<td>Macrophage differentiation</td>
<td>4.26 x 10^-4</td>
<td>1.99</td>
<td>3.06</td>
</tr>
<tr>
<td>sp1la</td>
<td>SPV proviral integration oncoprote 1a</td>
<td>Macrophage differentiation</td>
<td>4.06 x 10^-3</td>
<td>1.29</td>
<td>2.45</td>
</tr>
<tr>
<td>sp1lb</td>
<td>SPV proviral integration oncoprote 1b</td>
<td>Macrophage differentiation</td>
<td>5.43 x 10^-4</td>
<td>1.41</td>
<td>2.66</td>
</tr>
<tr>
<td>ccr3.2</td>
<td>Chemokine (C-X-C motif receptor 3, tandem duplicate 2)</td>
<td>Macrophage recruiting cytokine</td>
<td>1.07 x 10^-2</td>
<td>1.36</td>
<td>2.57</td>
</tr>
</tbody>
</table>

Table 5.3. P-value significant genes associated with notochord development and macrophage differentiation. Table showing the names, functions, p-values and expression change (log2 and fold change) of the genes in the microarray associated with notochord development (purple boxes) and macrophage differentiation and recruitment (orange boxes).
In order to find out the signals being activated inside the damaged notochord that caused the repopulation of the injured space, I looked at the microarray for hints on the re-expression of known notochord developmental genes. I had already seen that the injury triggered the upregulation of *col2a1a* in my microarray data, which was consistent with what I had seen in the transgenic work (Table 5.2; see Chapter 4 for details). *Col2a1a* is highly expressed during the initial stages of notochord development, where it highlights both vacuolated and non-vacuolated cells before their commitment into two different populations (Mangos *et al*., 2010; Dale & Topczewski, 2011).

As I browsed through the data, I discovered other notochord-associated collagens in the list of upregulated hits. These included some of the cartilage genes that I mentioned above, such as *col2a1b, col11a2, col9a1* and *col9a2* (Table 5.2; Dale & Topczewski 2011; Fang *et al*., 2010; Thisse *et al*., 2001; Mangos *et al*., 2010) and notochord specific genes, such as *col5a3a, col8a1a* and *col15a1a* (Fang *et al*., 2010; Gansner *et al*., 2008; Gray *et al*., 2013; Pagnon-Minot *et al*., 2008). It should be said, though, that even though all of these genes are expressed at the early stages of notochord development, only *col2a1a, col2a1b, col8a1a* and *col15a1a* are known to affect its morphogenesis (Table 5.3; Stemple *et al*., 1996; Mangos *et al*., 2010; Duran *et al*., 2011; Pagnon-Mignot *et al*., 2008; Gansner *et al*., 2008). Additionally, while the knock down of most of these genes causes a disrupted notochord sheath and curved body axis, *col15a1a* morpholino injected larvae do not develop curved bodies but instead fail to differentiate their notochord cells, revealing the essential role of this collagen fibre in early notochord development (Pagnon-Minot *et al*., 2008).

Both the *col5a3a* and *col15a1b* genes showed very similar 2 fold upregulation after the damage, whilst the *col8a1a* gene showed a much higher 4-fold upregulation in comparison with the non-injured contros (Table 5.3). However, while *col8a1a* and
col15a1b showed strong p-values, col5a3a was right at the border of significance, with a value of 5.0 x 10^{-2} (Table 5.3).

Aside of the collagen genes, I also searched for genes involved in early notochord morphogenesis, especially those involved in axial chordamesoderm development. Surprisingly, I found an array of early notochord markers were upregulated in the injured population. These included brachyuri/no tail (ntla; Schulte-Merker et al., 1994; Wilkinson et al., 1990), twist2 (Germanguz et al., 2007), indian hedgehog b/echidna hedgehog (ihhb; Currie & Ingham 1996) and sonic hedgehog a (shha, Krauss et al., 1993). All these genes were upregulated at least 2 fold in the injured samples, with twist2 being the only one with an upregulation below that mark (Table 5.3). Additionally, the microarray data also revealed the high expression of the late notochord gene, lysyl oxydase 5b (loxl5b), which is required for notochord sheath integrity (Gansner et al., 2007). This copper-dependent enzyme was highly upregulated with an 11.41 fold change (3.51 log2 change).

Apart from the notochord genes, I also observed a wide cohort of immune response genes, which were mostly associated with the macrophage response (Table 5.3). My previous imaging data had already shown how macrophages formed a semi-ring-like structure around the site of injury. The presence of these genes helped to reaffirm those experiments (see Chapter 4). Some of the most significant genes were the macrophage marker macrophage expressed 1 (mpeg1; Zakrzewska et al., 2010; Ellet et al., 2011) as well as the zebrafish orthologues for the myeloid differentiation marker spi1, spi1a and spi1b (Zakrzewska et al., 2010). The data showed that mpeg1 was upregulated almost 4 fold in the injured tissue, while the spi1a and spi1b showed approximately a 2.5 fold change each (Table 5.3)

Moreover, the results also showed a wide range of chemokines being upregulated in the injured tissue, concomitant with the expected immune response (see Supplementary Data). These included the macrophage migration chemokine
receptor C-X-C motif receptor 3.2 (cxcr3.2; Zawkrzewska et al., 2010), which showed a 2.57 fold upregulation.

These genes validated the presence of the phagocytes in the injured tissues and were further corroborated by the expression of other genes such as zgc:110349 and zgc:92049, that code for different parts of the major histocompatibility complex class II, the major presenting complex in macrophages and other antigen presenting cells (Lang et al., 1994). These showed a 3.3 and 2.1 fold expression increment respectively (see Supplementary Data).

Overall, these genetic upregulations showed that the needle injury was able to trigger the re-expression of early developmental markers in the notochord and validated the presence of activated macrophages at the site of damage. It remains to be tested whether the expression of these genes could be linked to the repair response of the notochord and whether the presence of the macrophages could have as of yet uncharacterised role in the whole process.

5.3.5. Network enrichment analysis: GOrilla highlights pathway activations in the injured sample

The manual characterisation of the genes in the microarray data could only yield restricted assumptions on the processes being induced by the damage. In order to explore the presumptive pathways and networks been activated by the injury, I decided to use the gene ontology (GO) enrichment analysis software GOrilla on my microarray data (Eden et al., 2009).

The software is based on the Gene Ontology (GO) project, which was established to provide a universal nomenclature that could be used to define, annotate and group together the attributes of all the genes in the genome (Ashburner et al., 2000). These annotations are known as gene ontology (GO) terms and are differentiated
into 3 hierarchical groups depending on whether they highlight “biological processes”, “molecular functions” or “cellular components” (Eden et al., 2009). The GORilla software works by determining the GO terms of the genes in a list of hits ranked by their individual significance. Once the GO terms for the list are defined, their significance and enrichment are calculated. The calculation takes into consideration the number of genes found for each GO term in the ranked list, the number of total genes that could potentially be recognised by a specific GO term and the position that each gene occupies in the list (Eden et al., 2009). Apart from calculating the significance and enrichment of the GO terms, the software also represents the data in a hierarchical tree graph, which delineates the association between the different GO terms.

I sorted my list of genes by their statistical significance, ranking the most significant genes at the top and the least significant genes at the bottom. I then inputted the sorted microarray list (36,688 genes; gene names only) into the software, which identified the GO terms associated with each gene. After the removal of duplicated genes, the software produced a list of GO terms from 35.11% of the genes from my microarray list (12,880 from 36,688 gene entries).

The results identified 10 GO terms associated with “biological processes”, 4 GO terms associated with “functional processes” and 13 GO terms highlighting “cellular components” that were enriched in my microarray (Tables 5.4 to 5.6).

The “biological processes” highlighted pathways associated with morphological changes, developmental differentiation, the immune response, wound healing, and cellular metabolism (Table 5.4; Figure 5.4B). The most significant of the GO terms identified 37 genes in my list associated with the “regulation of multicellular organismal process”, which showed an enrichment value of 2.07 and a p-value significance of $1.34 \times 10^{-4}$ (Table 5.4).
<table>
<thead>
<tr>
<th>GO TERM DESCRIPTION</th>
<th>P-VALUE</th>
<th>Q-VALUE</th>
<th>ENRICHMENT</th>
<th>NUMBER OF GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of multicellular organismal process</td>
<td>1.34 x 10^{-4}</td>
<td>7.11 x 10^{-1}</td>
<td>2.07</td>
<td>37/292</td>
</tr>
<tr>
<td>Immune system process</td>
<td>3.14 x 10^{-4}</td>
<td>8.36 x 10^{-1}</td>
<td>2.27</td>
<td>26/158</td>
</tr>
<tr>
<td>Muscle cell development</td>
<td>3.21 x 10^{-4}</td>
<td>5.70 x 10^{-1}</td>
<td>5.48</td>
<td>9/54</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid biosynthetic process</td>
<td>3.22 x 10^{-4}</td>
<td>4.29 x 10^{-1}</td>
<td>76.67</td>
<td>2/2</td>
</tr>
<tr>
<td>Regulation of organ morphogenesis</td>
<td>4.11 x 10^{-4}</td>
<td>4.37 x 10^{-1}</td>
<td>5.51</td>
<td>8/26</td>
</tr>
<tr>
<td>Regulation of heart morphogenesis</td>
<td>4.84 x 10^{-4}</td>
<td>4.29 x 10^{-1}</td>
<td>8.96</td>
<td>5/10</td>
</tr>
<tr>
<td>Striated muscle cell development</td>
<td>8.01 x 10^{-4}</td>
<td>6.09 x 10^{-1}</td>
<td>5.48</td>
<td>8/48</td>
</tr>
<tr>
<td>Notochord cell differentiation</td>
<td>8.15 x 10^{-4}</td>
<td>5.42 x 10^{-1}</td>
<td>23.45</td>
<td>3/8</td>
</tr>
<tr>
<td>Cell development</td>
<td>8.16 x 10^{-4}</td>
<td>4.82 x 10^{-1}</td>
<td>2.56</td>
<td>19/201</td>
</tr>
<tr>
<td>Regulation of wound healing</td>
<td>8.25 x 10^{-4}</td>
<td>4.39 x 10^{-1}</td>
<td>65.05</td>
<td>2/3</td>
</tr>
</tbody>
</table>

Table 5.4. List of biological processes associated with the microarray data. Table showing the GO description, p-value, q-value, enrichment and number of genes (genes present/total number of genes for GO term) in the biological processes enriched in the microarray analysis.

<table>
<thead>
<tr>
<th>GO TERM DESCRIPTION</th>
<th>P-VALUE</th>
<th>Q-VALUE</th>
<th>ENRICHMENT</th>
<th>NUMBER OF GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ion binding</td>
<td>1.12 x 10^{-4}</td>
<td>2.32 x 10^{-1}</td>
<td>1.81</td>
<td>54/421</td>
</tr>
<tr>
<td>Hormone activity</td>
<td>1.70 x 10^{-4}</td>
<td>1.76 x 10^{-1}</td>
<td>2.71</td>
<td>20/58</td>
</tr>
<tr>
<td>Peptidyl-lysine 5-dioxigenase activity</td>
<td>2.47 x 10^{-4}</td>
<td>1.70 x 10^{-1}</td>
<td>10.34</td>
<td>4/4</td>
</tr>
<tr>
<td>Glutamate decarboxylase activity</td>
<td>3.22 x 10^{-4}</td>
<td>1.66 x 10^{-1}</td>
<td>76.67</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Table 5.5. List of biological functions associated with the microarray data. Table showing the GO description, p-value, q-value, enrichment and number of genes (genes present/total number of genes for GO term) in the biological functions enriched in the microarray analysis.
<table>
<thead>
<tr>
<th>GO TERM DESCRIPTION</th>
<th>P-VALUE</th>
<th>Q-VALUE</th>
<th>ENRICHMENT</th>
<th>NUMBER OF GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular region</td>
<td>4.36 x 10^{-10}</td>
<td>3.15 x 10^{-7}</td>
<td>1.91</td>
<td>101/414</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.42 x 10^{-6}</td>
<td>5.13 x 10^{-4}</td>
<td>3.85</td>
<td>19/51</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>1.98 x 10^{-5}</td>
<td>4.76 x 10^{-3}</td>
<td>6.92</td>
<td>10/71</td>
</tr>
<tr>
<td>Cell junction</td>
<td>2.89 x 10^{-5}</td>
<td>5.22 x 10^{-3}</td>
<td>3.05</td>
<td>21/174</td>
</tr>
<tr>
<td>Troponin complex</td>
<td>1.51 x 10^{-4}</td>
<td>2.17 x 10^{-2}</td>
<td>4.35</td>
<td>10/19</td>
</tr>
<tr>
<td>Occluding junction</td>
<td>1.75 x 10^{-4}</td>
<td>2.10 x 10^{-2}</td>
<td>5.85</td>
<td>9/46</td>
</tr>
<tr>
<td>Tight junction</td>
<td>1.75 x 10^{-4}</td>
<td>1.80 x 10^{-2}</td>
<td>5.85</td>
<td>9/46</td>
</tr>
<tr>
<td>Cell-cell junction</td>
<td>1.93 x 10^{-4}</td>
<td>1.74 x 10^{-2}</td>
<td>4.37</td>
<td>12/82</td>
</tr>
<tr>
<td>Anchored component of membrane</td>
<td>2.63 x 10^{-4}</td>
<td>2.11 x 10^{-2}</td>
<td>10.91</td>
<td>5/12</td>
</tr>
<tr>
<td>Cortical cytoskeleton</td>
<td>9.96 x 10^{-4}</td>
<td>7.18 x 10^{-2}</td>
<td>42.93</td>
<td>2/2</td>
</tr>
<tr>
<td>Cortical actin cytoskeleton</td>
<td>9.96 x 10^{-4}</td>
<td>6.53 x 10^{-2}</td>
<td>42.93</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Table S.6. List of components associated with the microarray data. Table showing the GO description, p-value, q-value, enrichment and number of genes (genes present/total number of genes for GO term) in the list of components enriched in the microarray analysis.
This was followed by GO terms associated with “immune system processes”, “muscle cell development”, “gamma-aminobutyric acid biosynthetic process”, “regulation of organ morphogenesis”, “regulation of heart morphogenesis”, “striated muscle cell development”, “notochord differentiation”, “cell development” and “regulation of wound healing” (Table 5.4). From these, the “gamma-aminobutyric acid biosynthetic process”, “regulation of wound healing” and “notochord cell differentiation” appeared as the most enriched processes, with values of 76.67, 65.05 and 23.45 respectively (Table 5.4).

The “molecular function” group was more restricted and only produced 4 GO terms that were significantly associated with my microarray list (Table 5.5). These were (in order of significance) “calcium ion binding”, “hormone activity”, “peptidyl-lysine 5-dioxygenase activity” and “glutamate decarboxylase activity”. The last two terms showed the highest level of enrichment, with values of 10.34 and 76.67 respectively.

Lastly, the “cellular component” group showed the largest amount of associated GO terms of the three groups. It also showed the highest amount of significance, with all GO terms but the last 2 showing q-value significance below 5% (Table 5.6). The list showed a highly significant enrichment of extracellular components, with the top 3 most significant hits highlighting the “extracellular region”, “collagen” and the “extracellular matrix” (Table 5.6). These were followed by GO terms associated with cell-cell interactions, which included (in order of significance) genes involved in “cell junction”, “troponin complex”, “occluding junction”, “tight junction”, “cell-cell junction”, “anchored component of membrane”, “cortical cytoskeleton” and “cortical actin cytoskeleton”. Once more, the last three components showed the highest enrichment values, with 10.91, 42.93 and 42.93 respectively.

The overall data highlighted some of the processes that I had observed by manually searching the microarray list. It showed a highly significance enrichment of the
immune response, as well as an enrichment of notochord cell differentiation, organ morphogenesis and wound healing. It emphasised the significant upregulation of an array of calcium binding proteins, including mgp and the osteochondrogenic regulator bmp1b (see Discussion below). It also underlined the high presence of extracellular components and collagen-associated proteins in the injured larvae, which are linked to several processes including wound healing, extracellular rearrangements, cell differentiation and cellular movements.

Additionally, the GOrilla results showed high significance enrichment for muscle development and muscle differentiation components, which was to be expected from the injury response. The results also highlighted the prominence of gamma-aminobutyric acid biosynthesis pathway, which showed the strongest enrichment of all GO terms. This, however, was due to the presence of the 2 components of each GO term in my microarray list.

Overall, the network enrichment analysis helped to validate the prospective pathway alterations that I had observed with the manual search of the data and highlighted other non-obvious hits related with the damage response.

5.3.6. Validating the expression of chondrogenic genes: Alizarin red and alcian blue staining uncover cartilage formations at the site of injury

The genetic analysis of the injured larvae had unexpectedly highlighted the expression of a number of chondrogenic genes 3 days after the needle damage. I wanted to understand the reason behind the activation of these genes and determine whether they could be forming cartilage in response to the injury.

In order to address this, I used a cartilage staining protocol, which uses the positively charged basic dye alcian blue in order to recognise the negatively charged
mucopolysaccharides of cartilage, staining them in a pale blue colour (Walker & Kimmel, 2007). I combined the alcian blue stain with alizarin red, in order to visualise the formation of cartilage in relation to bone development. I used a previously described method, applying minor modifications retrieved from the ZFIN (www.zfin.org) website (Walker & Kimmel, 2007; see Materials and Methods for details).

I needle injured 3 dpf wt1b:GFP; casper larvae and grew them with non-injured controls to 3, 10 and 18 dpi. At each time point, the larvae were fixed, dehydrated and stained in alcian blue and alizarin red dyes (Figure 5.5A).

The staining revealed the presence of an alcian blue deposition in the wounds of the injured larvae at 3 dpi, a pattern that was not replicated in the non-injured controls (Figure 5.5B – 3 dpi; green arrow). At this point, the only alcian blue that could be seen in the developing larvae was that of the early skeleton of the head (Figure 5.5B – 3 dpi; purple arrow head). The larvae also showed areas of alizarin red staining, which highlighted the first ossifying structures of the fish, including the first forming chordacentra at the rostral end of the notochord (Figure 5.5B – 3 dpi; black arrows). Interestingly, a mild accumulation of alcian blue could also be seen in between the forming chordacentra.

At 10 dpi, the wound still showed a strong alcian blue staining (Figure 5.5B – 10 dpi; green arrow). By now, however, evenly spaced strips of alcian blue could be seen running down the length of the notochord (Figure 5.5B – 10 dpi; orange arrows). At its most anterior end, these strips interspaced with the forming ossifying chordacentra exactly where I had previously seen the accumulation of alcian blue at 3 dpi (Figure 5.5B – 10 dpi; orange arrows vs. black arrows).

By 18 dpi, bone development had markedly increased in the larvae, with chordacentra rings metamERICALLY segmenting the notochord (Figure 5.5B – 18 dpi).
A 3 days post fertilization; wtIβ:GFP; casper

B Alcian blue & alizarin red stained

C

<table>
<thead>
<tr>
<th>AB &amp; AR</th>
<th>GFP &amp; AR</th>
<th>GFP &amp; AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 dpi</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At this point, broad centra occupied the anterior torso, with strong rings of alcian blue interspacing the mineralised structures in the larvae (Figure 5.5B – 18 dpi; black arrows and orange arrows respectively). However, in some of the more developed animals, these cartilaginous rings had disappeared, with the centra appearing more defined and displaying the first cartilaginous structures of the Weberian apparatus (Figure 5.5B – 18 dpi; black arrow heads and orange arrow heads respectively).

At the site of injury, a strong alizarin red staining could be seen covering the wound (Figure 5.5B – 18 dpi; green arrow). Its presence indicated that a mineralised matrix had formed in response to the injury and caused the heavy accumulation of bone tissue. This was especially noticeable when compared with the surrounding chordacentra rings, which could only be seen under fluorescent light due to the stringent bleaching conditions used during the staining procedure (Figure 5.5B and 5.5C).

Fluorescent imaging of the larvae before fixation also revealed the presence of wt1b:GFP cells at the site of damage (Figure 5.5C – GFP & AR). Side by side comparison of the injured area before and after the staining revealed that the wt1b:GFP cells were found inside the deposited bone, with the cartilage rings forming in between the centra in a similar manner as in the more anterior areas of the notochord (Figure 5.5C – AB & AR vs. GFP & AR vs. AR).
Overall, these results showed that the needle injury triggered the *de-novo* accumulation of cartilage tissue inside the wound. This in turn led to the development of a bone matrix in the injured area and the concomitant existence of both osteochondrogenic structures at the site of injury. This was especially noticeable in other less developed stained larvae, where the dual presence of cartilage and bone could be seen co-existing inside the wound (data not shown). Even though no direct connection can be drawn from this data, it does suggest that the calcifying vertebral body could be forming through an alternative chondral ossification process, whereby a deposition of cartilage stimulates bone formation.

Moreover, it was surprising to see that metameric rings of alcian blue and alizarin red interlinked along the notochord in the developing larvae. This pattern has not been previously described, though *col2a1a* expression has been observed in transgenic animals and in antibody staining experiments (Dale & Topczewski, 2011; Bensimon-Brito *et al.*, 2012). These rings could be marking the intervertebral disc of the vertebral column and could form in response to the accumulation of the notochord sheath cells in these areas. However, the fact that they appear long before the calcifying chordacentra form along the notochord and before the onset of notochord cell movement could indicate that they may have a different independent role.
5.4. Discussion

5.4.1. Wt1 is upregulated in the wt1b:GFP cells of the notochord response after the needle damage and in the chondrogenic cells of ossifying cartilage

The combination of immunohistochemistry and whole mount in-situ hybridisation had helped me validate the expression of the endogenous wt1b gene in the notochord damage response. The new wt1 antibody identified the endogenous expression of wt1 in the cells inside the notochord injury, while the RNA probe confirmed the wt1b specificity of the response in tail-amputated larvae.

Both methods also identified new areas of expression for the gene, which included chondrogenic structures of the maxillofacial skeleton and a myriad of neuronal cells in the brain. These sites of expression presented the possibility of new potential roles for the wt1b gene. It was particularly interesting to see the expression of the Wt1 protein in the pharyngeal arches, the BOP and the jaws before the onset of bone formation. In these structures, bone forms via the perichondral mineralisation of cartilage, which could suggest that wt1b may be involved in the establishment of this process. This hypothesis would interlink well with my transgenic animal data and could provide an explanation for the de-novo activation of wt1b:GFP cells in the notochord response and the delayed formation of the vertebra. The number of chondrogenic genes in my microarray data and the deposition of alcian blue at the site of damage also supported this notion and could lay the question of whether the wt1b:GFP cells could be acting as chondrogenic precursors inside the wound.

Even though no direct link has been established between Wt1 and cartilage, recent work from Nick Hastie’s lab has shown that the general deletion of Wt1 in adult mice affects the growth plate of long bones (Chau et al., 2011). This could hint towards a possible involvement of Wt1 in the establishment or maintenance of the chondrogenic cells in these tissues. However, immunohistochemical analysis of
mouse bones has not provided conclusive evidence for the expression of the Wt1 protein in the chondrocytes of these growth plates (Sophie McHaffie – personal communication).

Additionally, no chondrogenic aberrations have been reported in the morpholino knockdowns of the wt1b gene (Perner et al., 2007). However, the wt1b:GFP larvae highlight the upregulation of wt1b in the pharyngeal arches of the early larva. In fact, a close inspection of the reported morphants in the literature (Perner et al., 2007) revealed a reduced head structure in the injected animals, not seen in the wt1a or control morpholino fish (Perner et al., 2007). It would be important to address this situation in order to validate the role of the wt1b gene in the development of the skeletal maxillofacial structures of the zebrafish larvae.

We are currently working on two projects to decipher the role of the wt1b:GFP cells in the notochord damage response. In the first project we would utilise a dominant negative peptide to specifically abrogate the expression of the endogenous wt1b protein in the notochord outer sheath cells (in collaboration with Uta Neumann in Christoph Englert’s lab - Jena, Germany). The second project would create a ubiquitously active fluorescent reporter line using a tamoxifen inducible wt1b:Cre (created by Dr. Zhiqiang Zeng in our lab) and a previously reported ubiquitously activated ubi:LoxP-dsRED-LoxP:GFP line (Mosimann et al., 2011). This will enable us to carry out lineage tracing analysis of the activated wt1b:GFP cells after notochord damage.

These experiments would help us uncover the function of the wt1b gene in the injury process and specify the long-term role or fate of the activated wt1b positive cells in the notochord response.
5.4.2. Microarray analysis of the response highlights genes involved in chondrogenic development, wound healing, the immune response and notochord differentiation

The microarray analysis provided a tool to explore the genetic profile of the needle damage response. It highlighted 2,845 probes that were significantly altered in the injured larvae, with 9 of those probes showing strong q-value significance. From these 9 probes, only 5 of them recognised genes with defined identities in the zebrafish genome. These genes helped me to initially determine some of the varied processes governing the damage response, as they highlighted the expression of genes involved in immune response processes (ela-2 and c6), wound healing (fn1b) and, surprisingly, osteochondrogenic development (mgp and f13a1b).

These hits were validated by gene ontology enrichment analysis, which not only confirmed the significant upregulation of these pathways, but also uncovered other enriched processes in the injured population. The analysis found a variety of genes associated with muscle cell development, notochord cell differentiation, hormone activity and neuropeptide synthesis that were altered after the needle damage. Additionally, it also highlighted some of the components enriched in the injured larvae, which included extracellular matrix peptides, collagens and cell junction proteins.

By combining these results with manual searches of the data, I discovered genes that are central to cartilage formation, notochord differentiation and the immune response. These included chondrogenic transcriptional regulators, such as sox9b and mir140, collagen and other extracellular matrix proteins, early notochord genes and macrophage development and differentiation genes.

The microarray analysis also served as a platform to validate some of my previous results, such as the enhancement of the col2a1a expression and the presence of macrophages around the site of damage. However, it did not recognise the
significant expression of the \textit{wt1b} gene in the injured tissue, showing a low p-value result for the expression of the gene (1.61 \times 10^{-2}; see Supplementary Data). Importantly, it also did not show a significant change of expression in the \textit{ga17} gene, which showed an even lower p-value result of 9.98 \times 10^{-1} (see Supplementary Data; gene found as \textit{eif3m}). Interestingly, it did highlight the expression of the \textit{wt1a} gene in the injured tissue, showing that it was moderately downregulated after the needle damage (1.87 fold; p-value = 6.04 \times 10^{-3}; see Supplementary data).

The inability of the microarray analysis to recognise the expression of the \textit{wt1b} gene was not entirely surprising. In the injured larvae, the gene is expressed in approximately 4-5\% of the cells of the collected tissue (as observed in the FAC sort analysis; see Chapter 3) and both antibody and RNA \textit{in-situ} analysis had shown that its expression levels were not particularly high in the injured notochord. I had already validated the expression of the gene in the injured area, so the main purpose of the analysis was to uncover new gene expression changes linked to the needle injury and the notochord damage response.

It was surprising to see that only 9 out of over 36,000 probes recognised by the microarray analysis showed q-value significance in the injured population. The lack of q-value significance was similarly replicated in the gene ontology analysis, with no biological or functional process showing significant q-value expression after the false discovery rate (FDR) evaluation. These results suggested that there were limitations in the acquisition of the RNA for the microarray. One reason for this could be the localised effect that the needle damage has in the injured larvae, which is diminished by the volume of tissue used for the microarray analysis.

I had previously attempted to carry RNA extractions on FAC sorted GFP cells in order to make the results more specific. However, the limited number of GFP positive cells activated in the notochord response meant that it was technically difficult and highly laborious to retrieve enough number of cells to successfully
extract a good quantity of RNA to be used in the microarray. This could be circumvented by using more sensitive techniques, such as RNA-seq or even single cell RNA-seq (Mortazavi et al., 2008). Alternatively, decreasing the number of cells used in the analysis, either by extracting the notochord or using methods that can specifically select the fluorescent cells, such as laser micro-dissections, would help to increase the specificity of the results to the notochord process.

Another alternative could be to insert boundaries in the analysis of the data, in order to decrease the genetic output of the microarray. This could see the removal of any genes that show a fold change value lower than 1.5, which would remove genes from the list that do not become majorly altered after the needle damage, such as housekeeping genes. A boundary like this will bring down the number of recognised probes in the microarray, which would seemingly decrease the q-values of the remaining probes, increasing the number of probes with a q-value below the 5% threshold. This approach, however, could result in the removal of important genes from the list and may only give a partial read of the significant gene expression patterns at the site of damage.

Nonetheless, the microarray analysis was merely exploratory and the q-value and p-value significance of the probes only served as a way to highlight the best candidates to validate for future experiments. RT-qPCR experiments would need to be carried out in order to confirm the expression of the genes in my candidate list. These should be complemented with RNA and protein in-situ detection experiments, in order to verify the cellular expression of each gene and confirm that they are translated into protein.
5.4.3. Notochord injury upregulates genes involved in chondrogenesis and leads to the formation of perichondrial bone at the site of damage

The microarray featured some strong chondrogenic hits. Within the q-value significant list of genes, the osteogenesis regulator *mgp* showed the strongest and most significant upregulation in my cohort. Additionally, *f13a1b*, a gene that has been implicated in the modulation of osteochondrogenic balance, was similarly strongly upregulated within this list.

The strong upregulation of both genes prompted me to look for chondrogenic markers in my microarray list. I discovered several genes with well-established roles in the regulation of cartilage development, including transcriptional activators of chondrogenesis, such as *sox9b* and *mir140*; collagen components of cartilage, such as *col2a1a*, *col2a1b*, *col11a2*, *col9a1* and *col9a2*; and extracellular proteins associated with chondrogenic tissues, such as aggrecan and chondroadherin (*chad*).

The expression of these genes hinted towards the development of a chondrogenic response in the injured larvae, a notion that was further validated by the deposition of alcian blue at the site of damage. Unsurprisingly, these weren’t the only cartilage genes upregulated in the injured samples, as other genes such as *fgf20b* (2.20 fold change) and *nkx3.2* (2.37 fold change) were also found in my microarray. These genes have been associated with the early development of chondrogenesis in the zebrafish head, with *fgf20b* required for the differentiation of the cranial neural crest cells into an ectomesenchymal fate and *nkx3.2* restricting these cells into the chondrogenic lineage (Yamauchi *et al.*, 2011; Miller *et al.*, 2003; Goldring *et al.*, 2005; see Supplementary Data). Additionally, *Fgf20* has recently been shown to be a downstream target of *Wt1* in the developing kidney (Motamedi *et al.*, 2014), therefore potentially suggesting that its expression could be due to the *de-novo* upregulation of the *wt1* gene.
The expression of *sox9b* instead of *sox9a* suggested that cartilage could be forming by the activation of this orthologue. *Sox9a* has been shown to play a major role in the general development of the chondrocranium, with mutants and morpholino knockdowns showing a widespread inability to form most of the head cartilage (Yan *et al.*, 2002). *Sox9b* mutations and morpholino downregulations, on the other hand, have been shown to have a more concentrated effect in the head skeleton, with its inactivation affecting the development of the pharyngeal cartilage, ceratobranchials and the neurocranium only (Yan *et al.*, 2005). However, *sox9b* has a stronger effect in the fin fold and the later development of the head, with mutants and morphants showing a reduced endochondral disc at the pectoral fin and an inability to form both chondral and intramembranous bone in the head (Yan *et al.*, 2005; Dalq *et al.*, 2012).

Previous reports in mice have shown that the *Sox9* gene is found downstream of Wt1 in the developing testis and its expression is positively modulated by the upregulation of the *Wt1* gene (Gao *et al.*, 2006). This could indicate that a similar regulatory pathway may be in place in the damage response. Transgenic experiments have already shown that *wt1b:GFP* and *col2a1a:RFP* transgenes co-localise to the same cells. As *col2a1a* is a target of *sox9b*, it wouldn’t be surprising to imagine that *sox9b* could also be upregulated in these cells. This expression, however, would need to be validated, in order to identify the cellular localisation of the *sox9b* gene in the notochord damage response.

Cartilage and notochord have very similar gene expression profiles (Stemple, 2005). The collagen markers that I had uncovered in my microarray are similarly expressed in the developing notochord as they are in hyaline and articular cartilage (Eyre, 2002). Even *sox9b* has been shown to be transiently expressed in the notochord at 20 hpf and exhibit an effect in its morphogenesis, with mutants and morphants displaying curved body axis characteristic of notochord malformations (Chiang *et
However, the expression of the cartilage markers aggrecan, mir140 and chondroadherin, strongly supported the idea that the notochord damage was triggering the onset of chondrogenesis in the needle-injured larvae.

The alcian blue and alizarin red staining experiments also validated this theory. They showed that an alcian blue deposition accumulated at the site of damage over time and, eventually, led to the concomitant expression of both cartilage and bone at the wound. The presence of both skeletal elements strongly indicated that the delayed vertebra was forming as a result of the accumulation of the chondrogenic tissue at the injury, a feature that is characteristic of perichondrial bone formation. This offers the possibility that in response to the damage the notochord halts intramembranous bone development at the site of injury and then activates an alternative ossification pathway to reconstruct the missing vertebra/vertebrae.

Lastly, the staining also uncovered the presence of alcian blue rings along the notochord. It would be important to elucidate whether these cartilage rings could have a role in normal vertebral development or whether they might just highlight the areas where the notochord sheath cell will accumulate to form the intervertebral disc. Bensimon-Brito and colleagues recently explored the mineralisation patterns behind the forming vertebrae and found that after a chordacentra ring has formed, an external autocentrum forms, which expands the vertebra sideways (both anteriorly and posteriorly) to widen its body (Bensimon-Brito et al., 2012). It would be worth exploring whether these cartilage rings have a role in the expansion of the developing chordacentra. It might signify that the formation of cartilage is required for vertebral expansion; a finding that would contradict previous reports suggesting that the notochord in zebrafish calcifies in an acartilaginous intramembranous fashion (Flemming et al., 2004; Bensimon-Brito et al., 2012).
5.4.4. Markers for early notochord development in the needle-injured larvae hint towards a developmental regression in the notochord after damage

The microarray highlighted the expression of a number of important notochord developmental markers in the injured larvae. These included early axial chordamesoderm markers such as *ntla*, *ihhb*, *shha* and *twist2*, and later developmental genes, such as *col5a3a*, *col8a1a* and *loxl5b*. The expression of these genes suggested that the notochord was responding to the damage by activating an early developmental state inside the wound. This notion is concomitant with the potential activation of a repair/regenerative response, which would help to explain the formation of new notochord tissue at the site of injury that I had seen in my imaging experiments.

The experiments had shown that within 5 days after the injury, a number of SAGFF214A cells began to form inside the wound, closing the gap created by the injury and slowly repopulating and repairing the damaged area (see Chapter 4). The expression of these early notochord genes potentially suggested that a dedifferentiation could be occurring at the site of injury in response to the damage, which could be behind the repopulation process.

These genes weren’t the only notochord markers that I observed in the microarray list. Four other key early notochord genes were differentially expressed in my list, which validated the profile of the notochord response. These were laminin γ1 (*lamc1*), *hps5*, glypican 4 (*gpc4*) and nodal-related 2 (*ndr2*), which have all been associated with mutations affecting notochord development (Stemple *et al.*, 1996). They all showed moderate change in expression, with *lamc1*, *hps5* and *gpc4* showing slight upregulations (fold changes of 1.51, 1.57 and 2.71 respectively; see Supplementary Data) and *ndr2* showing a downregulation in its signal (fold change of 1.79; see Supplementary Data). The latter was especially interesting as mutations
in the ndr2 gene have been associated with a permanent activation of the col2a1a gene inside the notochord of zebrafish embryos (Stemple et al., 1996).

Overall the microarray data showed that there was a good indication that the notochord was upregulating several of its early developmental markers in response to the damage. It remains to be uncovered whether the expression of these genes is linked to the activation of the wt1b gene and whether the wt1b:GFP play an important role in the repair and regeneration of the notochord. For this, the expression of each gene should be examined in more detail and their upregulations should be confirmed using other RNA and protein detection methods.

5.4.5. Additional pathways associated with the notochord damage response

The microarray highlighted the differential expression of thousands of genes after the injury. The GOrilla software helped me to delineate some of the most significant responses and pathways activations that resulted from the needle damage. Apart from the aforementioned processes, two other terms from the “biological responses” list associated with muscle formation were significantly expressed in my list. These were “muscle development” and “striated muscle development”.

The GOrilla software recognised 9 out of 54 genes associated with muscle development in my array and 8 out of 48 associated with striated muscle development, with both showing significant enrichments with a value of 5.48 (Table 5.4). The list of genes included muscle specific genes such as myosin-associated mybpc1 (myosin binding protein c1) and myl7 (myosin, light polypeptide 7), as well as signalling genes such as ihhb and shha. The latter were included in the list due to their function as an important signalling centre for muscle development and their role in the establishment of muscle cell progenitors (Borycki et al., 1999; Currie & Ingham, 1996). Their exo-notochordal roles in muscle development may suggest
that the activation of these genes could be associated with a repair response in the injured muscle.

Additionally, several muscle developmental markers were also highly expressed in the top 100 most significant genes in my microarray (see Supplementary Data). These included fn1b, which was found twice in my q-value list, and was amongst the most highly upregulated genes. Fn1b has been shown to express in the paraxial mesoderm during somitogenesis and morphants of the gene affect somite development, highlighting the importance of fn1b in the process (Jülich et al., 2005; shown as fn3 in the paper).

Even though I did not explore the effect of the needle injury on somite morphogenesis, my microarray analysis could be used as a base to study this process and could add a wealth of understanding to somite and muscle repair. Additionally, it would be interesting to understand the notochord – somites interplay during the muscle damage response and see whether similar notochord signals may become upregulated when only the somatic muscle is targeted.
CHAPTER 6:

Summary

& Future Directions
6.1. Summary

My experiments in this thesis have identified and characterised a novel injury-triggered response in the notochords of zebrafish larvae. I have shown that, when damaged, the notochord induces a repair response that involves the *de-novo* upregulation of *wt1b*, the accumulation of chondrogenic tissues inside the area of damage and the late formation of the vertebral body around the wound. Using an array of methods, I have explored the response, examining the cellular movements that are triggered by the injury, the effects that the damage has on the structural integrity of the notochord and the future vertebral column, and the genetic oscillations that arise as a consequence of the damage.

Using an array of fluorescent transgenic lines, live microscopy, histology and immunohistochemistry, I have found that injury to the notochord induces a set of morphogenetic changes in the organ. These include the loss of cellularity of its inner vacuolated cells around the site of damage and the inner migration of *wt1b*-activated sheath cells from the surrounding monolayer into the injured space. I have also used real-time microscopy to uncover the rates of the response and discovered that *wt1b* becomes rapidly activated in the sheath cells, only 5-7 hours after the initial damage. This activation then leads to the migration of the *wt1b*-expressing sheath cells into the lumen of the notochord 10 hours after the activation, where the migrating cells then accumulate, strengthen the response and eventually engulf the site of damage just 72 hours after the injury has happened.

By growing injured *wt1b*:GFP larvae to their juvenile stage, I was also able to evaluate the progression of the response and explore the long-term effects of the damage on the notochord and the vertebral column. I discovered that the notochord eventually repairs itself, repopulating the gap created by the loss of cellularity and forming a notochord-like appendage in the damaged area. Interestingly, my imaging also showed that the strength of the response weakened
over time, with \textit{wt1b}:GFP cells reducing in number and becoming more localised at the site of injury as the notochord repaired itself. I complemented these findings with the live skeletal staining of the injured larvae, which showed that the damage disrupted the metameric ossification of the chordacentra rings around the injured area. However, the damage also led to the activation of an alternative mineralisation pathway at the wound, which induced the formation of a delayed vertebra that, in several cases, developed as two.

Lastly, I aimed to dissect these results with a microarray analysis of the response at 72 hpi. The analysis recognised the presence of strong bone inhibitory signals in the damaged area, which helped to explain the halted chordacentra formation around the wound. Additionally, it highlighted the upregulation of several cartilage and early notochord markers at the site of injury. By using alcian blue and alizarin red stains, I probed the expression of the chondrogenic markers in the damaged notochords and saw that cartilage tissue accumulated inside the wound as early as 72 hpi, with its expression growing stronger in later stages. Remarkably, the dual staining also showed that both bone and cartilage matrices co-existed at the site of injury as the vertebra formed, strongly indicating that bone could be forming via the prior deposition of chondrogenic tissue at the site of damage and highlighting the potential existence of an alternative ossification process in the injured notochord.

Other findings have also added interesting insights into the response. For example, staining with acridine orange has shown that cell death does not drive the loss of cellularity, while cell specific fluorescent lines have helped to identify the nature of the new cells that form at the site of injury and highlighted the actions of the immune system during the damage response. These events, however, need to be explored further with new experiments in order to understand their involvement and contributions to the notochord process.
In summary, I have found a novel damage response in the notochord of zebrafish larvae and used transgenics, immunohistochemistry, fluorescent imaging, RNA analysis and chemical stains to describe the process and define some of its more important characteristics. However, more experiments are required to fully understand the dynamics of the process and the roles of the genetic, cellular and molecular signals that define it.


6.2. Future directions

6.2.1. Current experiments

The experiments outlined in this thesis have helped me define some of the main features of the damage response. However, there are still certain unknowns that need to be examined in order to fully understand the characteristics of the process. One of those is the understanding of the role of the \( wt1b \)-expressing cells in the response. Do they contribute to the healing of the notochord or are they instead involved in the delayed formation of the vertebra/vertebrae at the site of damage?

To determine this, we are currently working on a lineage-tracing experiment that would help us assess the contribution of the \( wt1b \)-positive cells to the process. Dr. Zhiqiang Zeng, a senior postdoc in my lab, has created a tamoxifen-induced Cre recombinase construct (CreERT) that uses the \( wt1b \) promoter from the \( wt1b:GFP \) transgenic line to drive the expression of the recombinase enzyme in a \( wt1b \) specific manner (Perner et al., 2007; Figure 1.7A). We have already injected the construct into AB and “\( ubi:switch \)” (\( ubi:LoxP-GFP-LoxP:mCherry; \) Mosimann et al., 2011) embryos and we are currently awaiting to select founders with germ line transmission.

The advantage of this line is that it would allow us to induce the “genetic switch” in \( wt1b \)-expressing cells at the time of damage, avoiding the strong \( wt1b \) expression that is induced in the kidney and other organs during early development. We have already tested the efficiency of the construct using mosaic \( wt1b:CreERT; ubi:switch \) animals and seen that scattered cells inside the wound can undergo the fluorescent switch. Witold Ribsky in my lab is currently assessing the contribution of these mosaic cells to the injured notochord, growing the injected animals to their juvenile stage and then using microscopy and immunohistochemistry to define the location and nature of the mCherry cells inside the wound.
Another important aspect of the response that we need to assess is the function of the \textit{wt1b} gene in the process. Our results show that the gene is upregulated prior to the inwards migration of the sheath cells, which could suggest that \textit{wt1b} may be involved in facilitating this process. In order to examine this hypothesis, we have established a collaboration with Dr. Uta Neumann and Dr. Christoph Englert (Jena, Germany), in order to use a \textit{wt1b} dominant negative construct that they have developed. This construct has been tested \textit{in vitro} and shown to both inhibit the expression of the \textit{wt1a} and \textit{wt1b} peptides. With the help of Dr. Zhiqiang Zeng, I have cloned the R2 promoter of the \textit{col2a1a} gene (Dale & Topczewski, 2011) in front of the \textit{wt1b} dominant negative sequence, in order to create a construct that can induce the expression of the \textit{wt1b} antagonist in a notochord sheath specific manner.

We have already injected the construct into \textit{wt1b}:GFP; \textit{col2a1a}:RFP and \textit{col2a1a}:RFP embryos, which have been selected, grown and examined in order to establish founders. Currently, I am working on making homozygous mutant of each line to test whether the dominant negative construct can abrogate the endogenous expression of the \textit{wt1b} gene, which would help us determine the role of the gene in the notochord damage response.

Lastly, I also want to examine the cellular profile of the injury in grown juvenile larvae and understand the interactions that exist between the \textit{wt1b}:GFP cells and the forming vertebra/vertebrae at the site of damage. My fluorescent imaging experiments had shown that the GFP cells are found very close to the mineralising body of the forming centra and, in many cases, even interact with the mineralising matrix. This could suggest that there is a possible link between the \textit{wt1b}:GFP cells and the forming bone. I want to use histology to look at the cellular heterogeneity of the wound and then use a GFP antibody to identify the GFP expressing cells at this later stage and assess their interaction with the mineralising matrix. I would
supplement this with cryosectioning and bone staining, which would also help me explore this interaction, using the endogenous fluorescence of the wt1b:GFP larvae and the fluorogenic output of the alizarin red dye to assess their overlap.

These experiments would be central to the understanding of the main aspects of the notochord damage response and would help define the repair process, adding important insights into the role of the response in the notochord.

6.2.2. Other prospective experiments

Other experiments would need to be undertaken in order to complement our current knowledge of the response and to explore new aspects of the process that have not been covered in this thesis. These experiments should look at certain areas, such as the presence of specific biological markers and the contribution of external cells to the process, as well as validate some of the experimental candidates highlighted by the microarray analysis and examine other possible biological pathways that could be influencing the response.

6.2.2.1. Biological markers of the response

My experiments have already identified two central markers of the notochord damage response: the upregulation of the wt1b gene in the notochord sheath cells and the enhancement of the col2a1a expression at the site of damage. Both markers have served to identify the origin of the response and to track the movements of the activated sheath cells inside the wound. However, their individual expressions could also serve as indicators of other processes.
For example, the expression of the *wt1b* gene could tentatively indicate that an EMT, similar to that seen in the heart and liver of mice, could be driving the progression of the response. This possibility is strengthened by the fact that *wt1b* is upregulated prior to the movement of the activated sheath cells into the notochord and also because, as it appears, only *wt1b*-positive cells are primed to invade the wound.

To explore the possibility of an EMT, mesenchymal markers, such as snai1 and snai2, N-cadherin or vimentin, and epithelial markers, such as E-cadherin, would have to be examined in injured and non-injured larvae. These markers have previously been associated with the expression of Wt1 in the murine heart system and therefore would be good indicators to assess the presence of an EMT in the notochord process (Martinez-Estrada et al., 2010). Additionally, if the dominant negative construct is successful at repressing the actions of the wt1b protein, a similar comparison should be done between injured wild type and mutant larvae to determine the difference in expression levels of each marker in the absence or presence of the *wt1b* gene.

On the other hand, the *col2a1a* profile of the invading cells, as denoted by the *col2a1a*:RFP expression, may not only indicate the origin of the cells but could also be highlighting their chondrogenic nature. This possibility could explain the build up of cartilage tissue inside the site of damage and, if it were to be the case, it would indicate that the sheath cells could be providing cartilage precursors to the wound.

Several chondrogenic markers, such as sox9b, aggregcan, chondroadherin or even *col2a1a*, could be used to examine this hypothesis, using immunohistochemistry or RNA *in situ* hybridisation to define their expressions inside the injured notochord. As shown in previous chapters, all of these markers are upregulated in the microarray analysis of the response, therefore it would be interesting to see which are the cells responsible for their expression. If these detection methods highlight
the wt1b-positive cells as the cell behind the transcription of these genes, it will provide strong evidence for the chondrogenic nature of these cells and it would indicate that they might be behind the build up of cartilage inside the wound.

6.2.2.2. Contribution of external cells to the notochord damage response

In my analysis of the notochord damage response, I also explored the behaviour of the immune system. Using fluorescent transgenic lines I had seen that the neutrophils were rapidly recruited to the site of damage, but then left shortly before the beginning of the response. On the other hand, the macrophages were slower to migrate towards the wound, but once there, they accumulated around the injured notochord and even appeared to protrude into the damaged sheath. It would be important to evaluate the purpose of the macrophage response, in order to define whether their presence is required for the progression of the injury process.

One way to do this is by using tools that could systematically ablate the population of macrophages in the zebrafish larvae. This could be done by using macrophage specific toxins such as clodronate liposomes, which are artificially made lipid vesicles with a clodronate toxin core. These vesicles can be injected into the body and are stable until they are engulfed by the macrophages, which break down their lipid bilayer releasing the clodronate toxin inside their cells (Rooijen & Hendrikx, 2010). This approach has recently been used successfully in zebrafish for the first time, where it has shown to achieve a systematic ablation of all macrophages inside an injected 24 hpf embryo (Bernut et al., 2014).

Additionally, the interaction between the macrophages and the damaged notochord could be explored in more detail using antibodies against macrophage specific markers, such as mpeg1, or even antibodies against the RFP signal emitted
by the fluorescent macrophages around the wound. These would help to determine the extent to which the macrophages interact with the nascent \textit{wt1b}:GFP response and it would detail how these cells assemble around the injured notochord.

Moreover, future experiments should also address the presence of osteoblasts at the site of damage. This is because of the \textit{entpd5}:RFP positive cells that accumulated inside the wound during the first 3 days of the notochord damage response. It would be worth determining whether these cells are truly osteoblastic, by using antibodies or RNA probes against osteoblast markers such as osteocalcin and osterix (Huijema \textit{et al.}, 2012; Li \textit{et al.}, 2009; Simes \textit{et al.}, 2004).

Additionally the role of these cells in the notochord process could be evaluated in order to determine whether they are necessary for any stage of the response. A way to this could be by using the genetic ablation line used in Singh \textit{et al.}, 2013, which relies on the enzymatic reaction between the nitroreductase enzyme and its prodrug substrate to specifically ablate osteoblasts in zebrafish (Sing \textit{et al.}, 2013). However, before we can utilise this approach, we need to make sure that these \textit{entpd5}:RFP cells are osterix expressing cells, due to the osterix-specific expression of the construct.

My imaging had also suggested that the \textit{entpd5}:RFP cells were independent of the \textit{wt1b}:GFP response. A FACS analysis of the injured \textit{entpd5}:RFP; \textit{wt1b}:GFP would need to be undertaken in order to confirm this possibility and validate the mutually exclusivity of the two cell population. It is interesting to note that my microarray analysis had highlighted the presence of several osteogenic markers in the injured larvae, therefore strongly supporting the notion that osteoblastic cells are present in or around the notochord wound early on the response.

Lastly, it would be worth analysing how the injured \textit{entpd5}:RFP; \textit{wt1b}:GFP develop over time. It has been shown that the \textit{entpd5}:RFP cells accumulate in rings around
the ossifying chordacentra, highlighting the centres of mineralisation as the bone matrix forms. It would be interesting to identify whether these cells still arrange in the same manner around the wound of the injured larvae or whether this pattern is affected in the damaged tissue. This would help to define the effects of the needle injury and see whether the loss of forming chordacentra at the wound is due to a loss of the structural support needed for their alignment around the centra or whether it is due to bone suppressing signals coming from the wound.

6.2.2.3. Validating microarray candidates of the notochord response

The microarray analysis identified over 2,000 candidate genes with statistically significant expression in the notochord injury response. From these only a small subset showed q-value significance, with the identified genes highlighting some of the prospective signals that could be acting on the injured notochord, including the expression of bone inhibitors (mgp) and tissue repair genes (fn1b and f13a1b), the activation of immune response processes (ela2 and c6) and the potential presence of chondrogenic markers (mgp and f13a1b).

Future experiments would need to validate the expression of these genes using RT-qPCR methods and, wherever possible, utilise immunohistochemistry or RNA in situ analysis to identify the cells transcribing these genes. For example, one especially interesting hit would be mgp, the top candidate gene in my list. To uncover the cellular expression of the gene in the injured larvae, I would be using an antibody synthesised by Simes and colleagues that specifically recognises the mgp protein in zebrafish larvae (Simes et al., 2004; Gavaia et al., 2006).

Additionally, some of the candidate genes that I had identified in my microarray would need to be explored further. These include the chondrogenic hits, as well as the notochord-related markers. It would be especially important to use antibody or
RNA *in situ* staining to define the cellular expression of several of these genes, as they will allow us to understand the notochord response better. These include the expression of *ntlA*, due to its function as an early notochord marker; *col2a1a*, in order to validate the genetic profile of the invading sheath cells; *aggrecan*, due to its important chondrogenic nature; and *sox9b*, which is my highest chondrogenic hit and could serve to identify the potential chondrogenic precursors of the response.

The microarray also uncovered other hits that could help define the molecular interactions that shape the notochord damage process. As already mentioned above, amongst my list, there are genes that suggest that bone-forming cells could be present at the site of damage, including osteocalcin (*bgn*; 3.15 fold upregulation), osteopontin (*spp1*; 4.32 fold upregulation) and asporin (*aspn*; 6.51). However, similarly, I have found several genes involved in the release of bone inhibiting signals, as well as identified a number of downregulated mineralisation genes, which could suggest that a complex network of signals inside the wound could potentially be regulating these cells.

It would be important to ascertain the expressions of these genes with RT-qPCR analysis, in order to understand the cues shaping the damage response in the injured notochord. Moreover, as already mentioned above, *in situ* staining would also help to define the cellular culprits in charge of the upregulation of these genes.

Aside of cartilage and bone forming markers, the microarray also consistently highlighted the significant downregulation of light-induced circadian rhythm genes in the injured larvae (data not shown). It would be interesting to explore this pattern of expression further, as it could indicate that the injury may trigger a temporal arrest in the development of the larvae. This would be consistent with the unconfirmed observation that injured larvae tend to be more developmentally delayed than their non-injured sibling controls.
Lastly, we are also considering carrying out RNA-seq experiments in FAC sorted \textit{wt1b}:GFP cells, as it would give a more concise cell-specific insight into the genetic profile of the response. It would help to define the genes expressed in the activated sheath cells, highlighting their biological markers and helping to determine the nature of these cells. Unfortunately, there are some caveats to this experiments, as the needle injury to the notochord only triggers the expression of a low number of \textit{wt1b}:GFP cells per larvae. This strongly limits the acquisition of enough cells to extract sufficient RNA to carry out the experiment. However, as already mentioned in my previous chapters, other methods, such as single cell RNA-seq could help to overcome this limitation.

6.2.2.4. Searching for alternative biological pathways

One of the benefits of using zebrafish as a model for research is its amenability for large-scale chemical screens, which can identify new candidate genes or pathways regulating developmental and cellular responses (Lawson & Wolfe, 2011). I would use the \textit{wt1b}:GFP larvae against a wide range of pre-existing small molecules libraries to find new players controlling the notochord damage response and see whether any of them could exacerbate or, ideally, halt the progression of the response after injury.

Also, I would like to explore the function of specific pathways in the response. One of those pathways would be the retinoic acid synthesis pathway, which has been linked to several regenerative responses in zebrafish, including heart repair and tail fin regeneration (Kikuchi \textit{et al}., 2011; Gemberling \textit{et al}., 2013; Blum & Begemann, 2014). Another reason for focusing on this pathway is that previous reports have shown that the retinoic acid synthesising enzyme Raldh2 is directly downstream of \textit{Wt1} in the developing heart of mouse embryos (Guadix \textit{et al}., 2011; Chau & Hastie, 2013). This means that there is a possibility that a similar interaction may exist in
the notochord damage response upon the upregulation of \textit{wt1b}. To test this possibility, I will incubate injured and non-injured larvae in all trans-retinoic acid, as well as in retinoic acid inhibitors and see how they affect the notochord and the development of the response.
6.3. Concluding Remarks

The work that I have undertaken in this thesis has identified a novel damage response paradigm in the notochords of zebrafish larvae. It has highlighted how the notochord copes with external insult and defined the cellular movements that are triggered inside the notochord, with an emphasis on the potential role of the notochord sheath cells in driving the response.

My experiments have also recognised the upregulation of *wt1b* as a strong feature of the process, with its transgenic fluorescent line additionally acting as a visual marker of the response. Long-term analysis has unveiled the repair capabilities of the notochord, which has the ability to repopulate the damaged area over time. These long-term experiments have also uncovered the presence of cartilage tissue inside the wound and shown that a delayed vertebra forms in the presence of the cartilaginous anlage. All of these findings have been strengthened with the microarray analysis of the process, which has identified the expression of bone inhibiting signals in the wounded area and highlighted the expression of potential chondrogenic markers early on the notochord response.

New research would have to establish the links between all of these processes and uncover the function of the *wt1b*-positive cells in the repair response. Additionally the biological profile of the activated sheath cells would need to be elucidated, as my findings seem to indicate that they play a central role in the notochord process. It wouldn’t be far fetched to think that they may act as stem cell progenitors to the response and therefore could have important implications in repair and regeneration research. Currently, there are a number of on-going studies that are trying to find novel cellular, genetic or molecular treatments to reverse or treat intervertebral disc degeneration (Chan *et al.*, 2014), therefore, if this theory is proven right, it could mean that the sheath cells may provide a source of stem cells that could be used as a cellular therapy for treatment.
However, it is not clear whether the mammalian notochord has a layer of outer sheath cells surrounding its vacuolated core and, even if they do, they may not respond similarly to external damage. This could be a path worth investigating, in order to develop a more concise knowledge of the structure of the mammalian notochord and to determine whether it is capable of reacting to external damage.

Similarly, the intervertebral disc (IVD) of zebrafish and its formation has not been extensively studied, so the possibility that notochord sheath cells provide a source of latent progenitor cells should be explored first in this system. It is interesting to note, that transgenic animals and histology seems to suggest that the zebrafish IVD is made of both inner vacuolated cells and notochord sheath cells (Yaga et al., 2009; Dale & Topczewski, 2011; Bensimon-Brito et al., 2012), therefore providing a good example to study this hypothesis further.

My findings could potentially pave the way to important notochord and IVD studies in zebrafish and mammals, however, in the context of this thesis, my experiments have demonstrated that the notochord can respond to external insult and does so by inducing a notochord sheath cell specific wt1b-associated process.
CHAPTER 7:

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7.1. References


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