Quantitative Proteomic Approaches in a Zebrafish Model of Granulocytic Inflammation

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

Fakultät für Chemie und Biowissenschaften
Karlsruher Institut für Technologie (KIT) – Universitätsbereich

Genehmigte

DISSERTATION

Von

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Indian

2015

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Tag der mündlichen Prüfung: 10.02.2015

I hereby declare that this dissertation is my own independent work. I have only used the given sources and materials. I have cited others work appropriately.

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02.01.2015

Abstract

Neutrophils represent the most abundant type of leukocytes in vertebrates and are the first cells migrating to sites of trauma. Despite their fundamental importance, our understanding of the molecular changes occurring within neutrophils upon inflammation in a living animal remains fairly limited. In this study, the zebrafish model was used to investigate the in vivo orchestration of a neutrophil inflammatory response at the protein level.

Firstly, the proteome of resting whole kidney marrow (WKM) neutrophils was investigated. Resting WKM neutrophils express more than 1500 proteins. Of these, 75% are enriched in neutrophils compared to neutrophil free brain tissue. In addition, the zebrafish neutrophil proteome shows partial conservation of immune related proteins between zebrafish and human. Secondly, a chemically induced inflammation assay in adult zebrafish was established in order to investigate proteome changes within activated neutrophils. To this end, a highly accurate and precise quantitative proteomics approach was required allowing for relative quantification of protein levels between samples. Two proteomics approaches, the label free and the SILAC (stable isotope labeling by amino acids in cell culture) method were thus applied to investigate the proteome dynamics within neutrophils upon inflammation. Through label-free proteomics 48 differentially regulated proteins were identified during inflammation. Gene ontology analysis revealed that these proteins are associated with cell cycle, nitric oxide signaling, regulation of cytoskeleton rearrangement and intermediate filaments as well as immune-related processes such as antigen presentation, leucocyte chemotaxis, and IL-6 signaling. Comparison of protein expression dynamics with transcript expression dynamics suggests the existence of regulatory mechanisms confined to the protein level for some genes. The SILAC approach identified 61 differentially regulated proteins during different stages of inflammation associated with motility, leukocyte and macrophage chemotaxis as well as localization pathways. Furthermore, comparison between label free and SILAC approach revealed that SILAC is more accurate and reproducible compared to the label free method. This is largely due to the availability of internal controls via spike in of SILAC proteins into neutrophil proteins at an early stage of sample preparation thereby reducing variation between replicates. In summary, this thesis provides the first a complementary analysis of zebrafish neutrophil proteomes, identified a suitable proteomics approach for zebrafish immune

cells and will thereby add to the understanding of physiological immune responses and potentially support the development of therapeutics for immunological disorders.

Zusammenfassung

Neutrophile Granulozyten bilden die größte Leukozytenpopulation in Wirbeltieren und sind oft die ersten Zellen, die auf Gewebeschäden reagieren. Trotzdem ist unser Verständnis der molekularen Veränderungen innerhalb Neutrophiler während Entzündungen im lebenden Tier noch unvollständig. In dieser Dissertation wurde daher am Modell des Zebrabärblings die komplexe Dynamik der intrazellulären Veränderungen auf Proteinebene während einer neutrophilen Entzündungsreaktion in lebenden Fischen untersucht.

Dazu wurde zuerst das Proteom von nativen Neutrophilen im Nierenmark von adulten Zebrabärblingen analysiert. Native Neutrophile exprimieren mehr als 1500 Proteine. Davon waren 75% ausschließlich in Neutrophilen präsent, nicht aber in Vergeichsproben von grundsätzlich neutrophilfreiem Hirngewebe. Darüber hinaus zeigt das Proteom von Neutrophilen im Zebrabärbling eine teilweise Konservierung von immunassoziierten Proteinen zwischen Fisch und Mensch. Des Weiteren wurde in dieser Arbeit ein chemisch induzierter Entzündungsassay in adulten Zebrabärblingen etabliert, der es ermöglichte Veränderungen im Proteom von aktivierten Neutrophilen während der Entzündung zu untersuchen. Da zu diesem Zweck eine präzise quantitative Analysemethode eingesetzt werden muß, die eine relative Quantifizierung der Proteinmengen zwischen zwei oder mehreren Proben erlaubt, wurden die markierungsfreie massenspektrometrische Quantifizierung sowie die SILAC Methode (stable isotope labeling by amino acids in cell culture) getestet und miteinander verglichen. Mit der markierungsfreien Proteomik konnten 48 unterschiedlich regulierte Proteine während der Entzündung identifiziert werden. *Gene Ontology* (GO) Analyse ergab, daß diese Proteine mit Zellzyklus, der Stickoxid-Signalgebung, der Regulierung des Zytokeletts sowie Immun-Prozessen wie der Antigenpräsentation, Leukozyten-Chemotaxis, und IL-6 Signalgebung assoziiert sind. Ein Vergleich der Proteinexpression mit der Analyse von Transkriptionsdaten zeigte, daß für einige Gene regulatorischen Mechanismen verstärkt auf der Proteinebene stattfinden. Über den SILAC Ansatz gelang es 61 differentiell regulierte Proteine während verschiedener Stadien der Entzündung zu identifizieren. Hierbei ergab die GO Analyse zusätzliche Assoziierung mit Zellmotilität, Leukozyten und Makrophagen-Chemotaxis sowie Lokalisierungswegen. Der direkte Vergleich zwischen markierungsfreier Methode und der SILAC Methode ergab einen deutlichen Vorteil der Isotopenmarkierung in Bezug auf Präzision und Reproduzierbarkeit. Dies ist vor allem auf die Verfügbarkeit von

internen Kontrollen über Zumischung von Kontroll-SILAC Proteinen in die Proben von Neutrophilproteinen zurückzuführen, die in einem frühen Stadium der Probenvorbereitung erfolgt und dadurch die Variabilität zwischen einzelnen Proben reduziert. Zusammenfassend stellt die vorliegende Arbeit die erste umfangreiche Analyse des Proteoms von neutrophilen Granulozyten des Zebrabärblings dar und identifiziert die SILAC Methode als geeigneten Proteomik Ansatz für Zebrabärbling Immunzellen. Damit wird das Zebrabärblingsmodell in Zukunft weiter zum Verständnis der physiologischen Immunreaktionen beisteuern können und möglicherweise die Entwicklung von Therapeutika für Immunstörungen beschleunigen.

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Abbreviations

µg	Microgram
µM	Micromolar
AGM	Aorta-gonad mesonephrons
ATP	Adenosine triphosphate
BiNGO	Biological networks gene ontology tool
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
ChIn	Chemically-induced inflammation
CHT	Caudal hematopoietic tissue
$CuSO_4$	Copper sulphate
Da	Dalton
DAMPs	Damage-associated molecular patterns
DAVID	Database for annotation visualization and integrated discovery
DTT	Dithiothreitol
EMPs	Erythromyeloid precursors
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FSC	Forward scatters
FT-ICR	Fourier transform ion cyclotron resonance
GO	Gene Ontology
g	Grams
HCD	Higher energy collisional dissociation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hr	Hour
HSC	Hematopoietic stem cells
LC	Liquid-based chromatographic
LC-MS-MS	Liquid chromatography-mass spectrometry
LIT	Linear ion trap
LTQ	Linear trap quadrupole
M	Molar
m/z	Mass/charge
MALDI	Matrix assisted laser desorption/ ionization

mg/ml	Milligram per milliliter
Min	Minutes
mM	Millimolar
MS/MS	Mass spectrometry
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information
Nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PMF	Peptide mass fingerprinting
QIT	Quadrupole ion trap
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
sec	Second
SILAC	Stable isotope labeling by amino acids in cell culture
SSC	Side scatters
STRAP	Software tool for rapid annotation of proteins software program
Tris-HCl	Tris-(hydroxylmethyl)aminomethane-HCL
UHPLC	Ultra pressure liquid chromatography
WKM	Whole kidney marrow

1. Introduction

1.1. Zebrafish model system

Danio rerio (Zebrafish) vertebrate model system was first introduced in late 1960 by George Streisinger (Streisinger *et al.*, 1981). It is a freshwater animal and provides a unique model organism for investigating developmental and disease processes (Meeker & Trede, 2008; White *et al.*, 2013). Over the last few decades zebrafish have been extensively adopted into a variety of research areas, including: developmental biology, immunology, and drug or chemical screening due to several advantages compared to other animal models. Female zebrafish produce several hundred eggs each week. Given the fact that fertilization and development occur externally, the tracking or visualization of development and function of the immune system is greatly facilitated by various microscopy techniques. Moreover, embryos are transparent, allowing the direct visualization of the developmental process. This process is very rapid with most of the organ systems fully developed by five days of post fertilization. Adult zebrafish are small in size, which allows a large number of fish to be maintained in a relatively small facility. These aspects of zebrafish biology have made it an ideal animal model for chemical screening (Hertog, 2005; Ou *et al.*, 2012; Zon & Peterson, 2005). Chemical screens can be performed easily, quickly, and at a low cost. This is done by dissolving a small amount of chemical into water and large-scale chemical screening can be carried out in multi well plates (Wittmann *et al.*, 2011).

Zebrafish are a genetically tractable model organism. The entire genome has been sequenced, assembled, and large numbers of genetic markers have been mapped. Zebrafish genetic maps show higher conservation between the zebrafish and human genome. Moreover, zebrafish also hold several genetic and experimental tools to generate various disease models (Huang *et al.*, 2012; Lawson & Wolfe, 2011; Lieschke & Currie, 2007). Firstly, most of the zebrafish disease models are created by a forward genetics approach; such as ethyl-nitroso-urea (ENU) or non-targeted retroviral and transposon-mediated insertional mutagenesis. Forward genetics screening in the zebrafish have been instrumental in identifying gene mutations that affect the development of the fish (Meeker & Trede, 2008). These genetic approaches enabled the identification of genes involved in patterning, regeneration, and development of organs; including: the heart, eye, and blood.

Table 1: List of transgenic zebrafish reporter line available for immune cells

Tissue	Transgene	Publication
Neutrophils	*lyz:EGFP, lyz:DsRed, mpx:EGFP*	(Hall *et al.*, 2007), (Renshaw *et al.*, 2006)
Embroyonic macrophage	*mpeg1:EGFP*	(Ellett *et al.*, 2011)
Red blood cells	*gata1a:DsRed and gata1:GFP*	(Long *et al.*, 1997), (Traver *et al.*, 2003b)
Pan- leukocytes	*Ptprc:DsRed*	(Bertrand *et al.*, 2008)
Eosinophils	*gata2a:EGFP*	(Balla *et al.*, 2010)
B cells	*ighm1:EGFP*	(Page *et al.*, 2013)
Immature B and T cells	*rag 2:EGFP*	(Langenau *et al.*, 2003)
Mature T cells	*lck:EGFP*	(Langenau *et al.*, 2004)
Lymphoid precursors, T cells	*il7r:mCherry*	(Stachura & Traver, 2011)
B cells, macrophages and dendritic cells	*mhcII:GFP, mhcII:AmCyan*	(Wittamer *et al.*, 2011)
Lymphoid precursors	*ccr9a:cfp*	(Stachura & Traver, 2011)

In addition, zebrafish rely on three main reverse genetic techniques including targeting induced local lesions in genome (TILLING), gene knockdown using morpholinos, and transgenics for gene knockdown as conventional knockdown method were not established in zebrafish (Ablain & Zon, 2013; Meeker & Trede, 2008). However, recently CRISPR-Cas genome editing technique was established to facilitate RNA guided site-specific DNA cleavage (Hwang *et al.*, 2013). Finally, transgenic lines can be generated in which the target transgene can lead to the expression of fluorescent proteins under control of tissue or cell specific promoters. There are various zebrafish transgenic lines relevant to immunological studies that have been established (Table1) (Stachura & Traver, 2011). All of these powerful genetic manipulations, pharmacological screens, and transgenic lines have made zebrafish an ideal model system to study the immune system (Renshaw & Trede, 2012).

1.2. Immune system

The immune system protects against threats like parasites, bacteria, and viruses. Two fundamental branches of the immune system include innate and adaptive immunity. The

innate immune system is believed to have predated the adaptive immune response, which is based on several pieces of evidence. Firstly, innate host defenses are found in all multicellular organisms, whereas adaptive immunity is found only in vertebrates. Secondly, innate immune recognition distinguishes self from non-self perfectly, a condition not satisfied by adaptive immune response. Thirdly, the innate immune system uses receptors that are an ancestral in their lineage, whereas adaptive immunity appears to use the same effector mechanism guided by clonally specific antibodies. Unlike the adaptive immune system, innate immune system does not confer long lasting or protective immunity to the host, but only confers immediate non-specific protection. The responses of the innate immune cells are molecularly driven by a diverse array of pattern recognition receptors (PRRs) that bind prevalent biomolecules of pathogens known as 'pathogen-associated molecular patterns' (PAMPs) or can be activated by non-microbial 'damage-associated molecular patterns' (DAMPs) (Chen & Nuñez, 2010).

1.3. Zebrafish immune system

Zebrafish have become a powerful vertebrate model system to study hematopoiesis and immunity (LeBert & Huttenlocher, 2014; Renshaw & Trede, 2012; Traver *et al.*, 2003a). The zebrafish immune system is similar to the mammalian immune system, but studying human immune system *in-vivo* is difficult because of its complex development. Therefore, in recent years the uses of zebrafish have gradually extended to the study of human diseases; which include cancer and immunological related disorders. This is due to particular advantages inherent to the zebrafish (Lieschke & Trede, 2009; Trede *et al.*, 2004). These advantages lie in the chronological separation of innate and adaptive immunity during embryonic and larval development. Only the innate immune system is active during larval stages, whereas the development of a functional adaptive immune system requires several weeks. This temporal separation enables the study of the vertebrate innate immune response *in vivo* without the confounding aspects of adaptive immunity (Novoa & Figueras, 2012). Moreover, the zebrafish's rapid development, transparent embryo, and availability of various transgenic lines (neutrophil, macrophage, eosinophil) have provided new insights towards the understanding of innate immunity including immune cell migration, regeneration, and host-pathogen interactions in the developing vertebrate embryo (Keightley *et al.*, 2014).

Additionally, the zebrafish immune system contains almost the full repertoire of lymphoid organs and immune cells found in mammals (Traver *et al.*, 2003a; Yoder *et al.*, 2002).

1.3.1. Hematopoiesis in Zebrafish

Immune cell development in vertebrate embryos occurs in a two-step process; primitive hematopoiesis and definitive hematopoiesis (Bertrand *et al.*, 2007; Davidson & Zon, 2004; de Jong & Zon, 2005). Primitive hematopoiesis is the first wave of blood development that occurs from 12 to 24 hr post fertilization (hpf). Contrary to mammals, zebrafish lack yolk sac blood islands, this thusly leads to primitive hematopoiesis to occur anatomically at two locations. The Inner cell mass primarily produces cells of erythroid lineages and the rostral blood island in the anterior portion of the embryo, produces a primitive macrophage population. In addition to macrophages, the next types of immune cells that arise during primitive hematopoiesis are neutrophils and thrombocytes. Immature neutrophils are first detected at 48hpf. Similar to other vertebrates, zebrafish erythropoiesis also requires various genes including scl, gata and lmo2 during primitive hematopoiesis (Figure 1a) (Carroll & North, 2014; Paik & Zon, 2010).

Similarly to primitive hematopoiesis, definitive hematopoiesis has also been investigated. Bertrand *et al*., 2007 were the first to notice a transient wave of definitive hematopoiesis, termed erythromyeloid precursors (EMPs) develop at posterior blood island. EMPs provide the initial innate immune cells, as it is present in the embryo, before the appearance of hematopoietic stem cells (HSCs) (Bertrand *et al.*, 2007; Carroll & North, 2014). These EMPs produce both definitive erythroid as well as distinct myeloid cell (neutrophilic granulocyte, monocyte, and macrophage) groups. Previous studies have shown that EMPs have limited cell differentiation, giving rise only to erythroid and myeloid population and have a transient population because of their limited self-renewal capabilities. Definitive HSCs arises from hemogenic endothelium shortly after the beginning of the blood cell circulation. These cells arise in the aorta-gonad mesonephrons (AGM) by 30 hpf in zebrafish. Subsequently, HSCs migrate through the blood to the caudal hematopoietic tissue (CHT) pro-nephrons. Similar to fetal liver in mammals, CHT represents a midway site of hematopoietic differentiation.

HSC cells differentiate into erythroid, myeloid, and thromboid cells at approximately 3dpf (Figure 1a) (Bertrand *et al.*, 2010; Carroll & North, 2014; Kissa & Herbomel, 2010).

Like mammals, HSCs and the blood cells developmental process is regulated by various genetic factors. A common signaling cascade including vascular endothelial growth factors (Vegf), bone morphogenic protein (Bmp), Notch followed by Hedgehog, prostaglandin E2, runt-related transcription factor (Runx) pathways control the immune cell as well as blood cell development (Carroll & North, 2014; Chen & Zon, 2009; Ellett & Lieschke, 2010; Paik & Zon, 2010). Definitive HSCs particular gene signature including c-myb and runx1, start expressing in the AGM after 26hpf (Davidson & Zon, 2004).

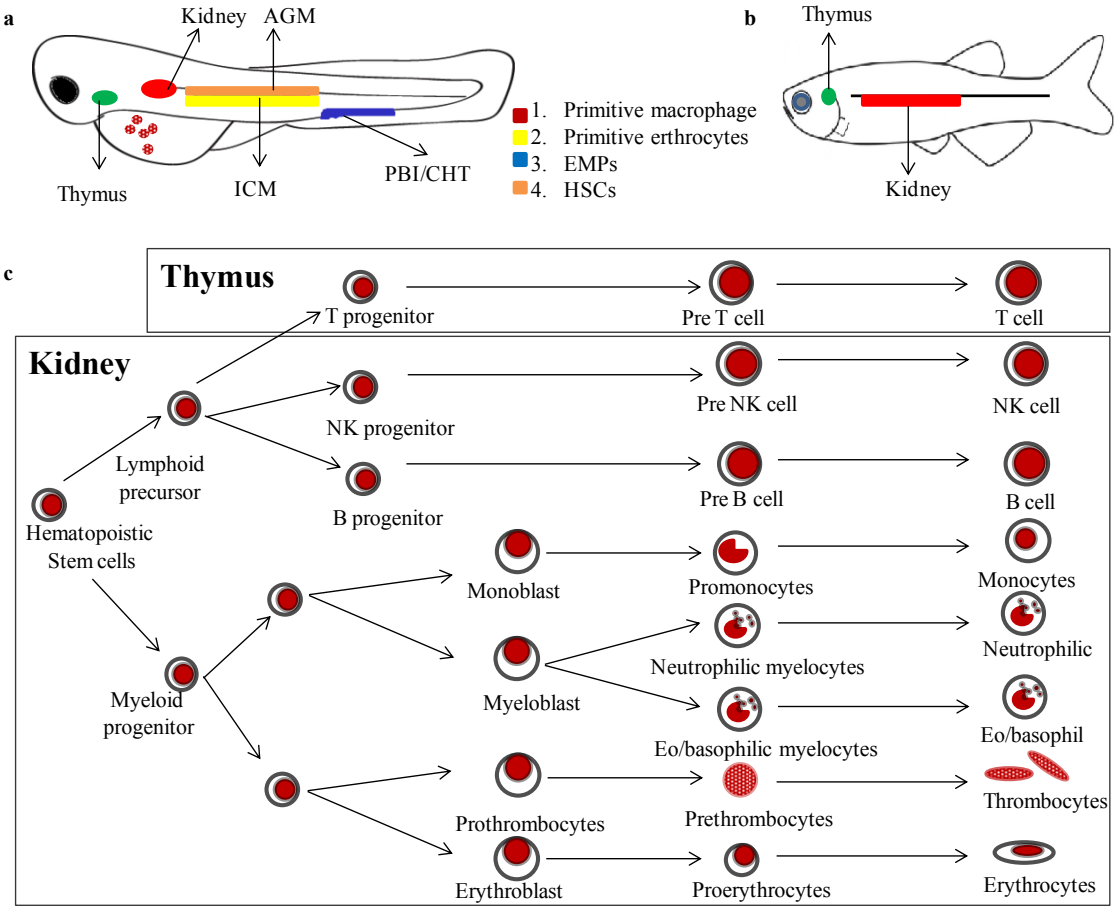


Figure 1: Model of hematopoietic ontogeny in the zebrafish embryo and adult zebrafish. *(a) Four independent waves of precursor production. First, primitive macrophages arise in cephalic mesoderm, migrate onto the yolk ball, and spread*

throughout the embryo (1). Then, primitive erythrocytes develop in the intermediate cell mass (2). The first definitive progenitors are EMPs, which develop in the posterior blood island (PBI; orange, 3). Later, HSCs arise in the AGM region (teal, 4), migrate to the CHT (b) Hematopoiesis in adult zebrafish occurs in kidney and thymus (c) Hematopoiesis in the kidney. The HSCs give rise to two distinct types of cell lineages including myeloid and lymphoid progenitor cells. The myeloid cells develop into monocytes, granulocytes, thrombocytes, and erythrocytes. On other side, lymphoid lineages give rise to B, T cells and NK cells. However, T cells migrate and developed into the thymus

1.3.2. Whole Kidney Marrow as a hematopoietic organ

CHT is intermediate hematopoietic organ of zebrafish larvae. Erythropoiesis, myelopoiesis, and thrombopoiesis shift to the kidney marrow at around 5 dpf. Zebrafish whole kidney marrow is the primary site for hematopoiesis from larval stages to the adult stage (Ellett & Lieschke, 2010). However, some of the hematopoietic processes may also occur in the developed spleen of zebrafish. Hematopoiesis occurs in the kidney, in the vestiges of the pronephrons and between the tubules of the mesonephrons. Zebrafish kidney starts producing mature myeloid and lymphoid cells within two weeks of initial development (Figure 1b and c) (Boatman *et al.*, 2013; Davidson & Zon, 2004; Murayama *et al.*, 2006).

1.3.2.1. Zebrafish immune cells

1.3.2.1.1. Neutrophils

Neutrophils (Heterophil) are one of the most abundant granulocytes of zebrafish. Like human, zebrafish neutrophils have multi-lobe and segmented nuclei. However, zebrafish neutrophils nuclei are 2 to 3 lobed compared to 5 lobed nuclei in human neutrophils. Zebrafish neutrophils have pale cytoplasm that contains two distinct type of granules: azurophilic and non-azurophilic. Zebrafish neutrophils are well equipped with antimicrobial defenses. For example, zebrafish neutrophils exhibit higher expression of myeloperoxidase enzyme, which form a primary defense in the cell (Crowhurst *et al.*, 2002; Renshaw *et al.*, 2006).

1.3.2.1.2. Eosinophils

Morphological appearance of zebrafish eosinophils is significantly different compared to their mammalian counterpart (Crowhurst *et al.*, 2002). Zebrafish eosinophils are small, have a non-segmented peripherally located nucleus, and are characterized by an eosinophilic cytoplasm. Eosinophil cytoplasm is granular like a neutrophil; however, eosinophil granules are larger as well as spherical. Contrary to neutrophils, eosinophil granules do not stain with a respective stain. Like neutrophils, eosinophils also reside in the kidney and blood circulation. In addition, developmental stages of eosinophils could be observed in adult zebrafish whole kidney marrow. Mammalian eosinophils play a crucial role in host defense including asthma and allergic diseases, although the role of zebrafish eosinophils still needs to be investigated. Recently, an eosinophil specific zebrafish transgenic line (*gata2*) has been generated that could help to dissect the functional characterization of eosinophils in the zebrafish immune system. (Balla *et al.*, 2010).

1.3.2.1.3. Mast cell

Mast cells play key roles in inflammation and allergic reaction. Like their mammalian counterparts, morphological characteristics of mast cell in zebrafish have been recently identified. Expression of mast cell specific enzyme carboxypeptidase A5 in blood cells at 24hpf has been noticed. Mast cells are expresses in the zebrafish gill and intestine (Dobson *et al.*, 2008).

1.3.2.1.4. Macrophage

Zebrafish macrophages are one of the first immune cells to arise during hematopoiesis. They are large cells with phagosomes, as well as more cytoplasm content compared to nuclei. In addition, their cytoplasm is vacuolated and agranular (Crowhurst *et al.*, 2002). They are one of the key phagocytic innate immune cells essential for host defense. Macrophages are activated by as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) during sterile or non-sterile inflammation. Activated macrophages phagocytize the noxious materials and are involved in the clearance of a large number of dying neutrophils, erythrocytes, and

cellular debris. Additionally, macrophage activate other immune cells such as lymphoid cells (Benard *et al.*, 2014; Ellett *et al.*, 2011).

1.3.2.1.5. Lymphoid cells

Fluorescence activated cell sorting (FACS) analysis of whole kidney marrow and thymus has shown the existence of diverse populations of lymphocytes. Additionally, it has been also noted that lymphoid lineage cells are smaller in size and less granular as compare to myeloid lineage cells (Traver *et al.*, 2003b).

1.3.2.1.6. B cells

B cells are an important type of lymphocytes involved with adaptive immunity. B cell receptors (BCRs) enable antigen recognition and discrimination of B cells from other lymphocytes such as natural killer cells and T cells. Zebrafish B cells develop within the WKM at around 2-3 weeks post fertilization. B cell development within the kidney marrow hinders direct developmental visualization. Contrary to the five immunoglobulins (Igs) of mammals, zebrafish exhibits only three Igs; IgM, IgD, and IgZ. However, mammals do not express IgZ. Recently, a B cell specific marker gene and zebrafish transgenic line (*igm:eGFP*) has been generated. Moreover, they have also observed that B cells have phagocytic capacity to phagocytize the pathogens (Page *et al.*, 2013).

1.3.2.1.7. T cells

T cell exhibits T cell receptors and plays a crucial role in cell-mediated immunity. In zebrafish, a definitive hematopoietic cell migrates to CHT, then to the kidney, and finally the thymus for development and selection. T cells ultimately differentiate and populate in the thymus after three days of post fertilization. All four TCR chains of the mammalian system are also found in zebrafish. T cells of zebrafish also express CD4 and CD8 marker genes (Langenau *et al.*, 2004; Langenau & Zon, 2005).

1.3.2.1.8. Dendritic cells (DC)

Dendritic cells are crucial link between the innate and the adaptive immune system. Zebrafish DCs are presents in various adult tissues, which resemble mammalian DCs.

Zebrafish DCs can be enriched by their affinity for lectin peanut agglutinin. Similar to mammalian DCs, zebrafish DCs exhibit expression of il12, MHC class II, iclp1 and csf1r, which are associated with DC function and antigen presentation. It has been shown that zebrafish DCs could activate T lymphocytes in an antigen dependent manner (Lugo-Villarino *et al.*, 2010).

1.3.2.1.9. Natural killer (NK) cells

NK cells are derived from lymphoid lineages and are classified as innate lymphoid cells. They form an important component of the innate immune system. NK cells main roles are to discriminate self from non-self and to provide a first line of defense against foreign or transformed cells, such as virally infected cells. Paralleling mammals, zebrafish also exhibit the existence of NK cells. Yoder *et al*., have shown that zebrafish express novel immune type receptors (NITRs) orthologous to mammalian NK cell receptors. Mammalian NK cell receptors display both inhibitory and activating forms. In zebrafish, 39 types of NITRs have been identified. Of the 39 NITR genes, only nitr9 showed similarity to mammalian NK cell activating receptor (Yoder *et al.*, 2010).

1.4. Inflammation

Inflammation is an immediate response of the innate immune system to maintain homeostasis in response to infection or injury. Macroscopic inflammatory symptoms include redness, swelling, heat, and pain, resulting from increased blood flow, increased permeability across the blood capillaries, which allows leukocytes and large molecules (cytokine, antibody) to leave the blood stream and cross the endothelium wall towards the site of injury or infection (Rocha e Silva, 1978).

1.4.1. Sterile inflammation

Inflammation that occurs in the absence of pathogen like injury, chemically induced and dying cells is known as sterile inflammation. Similarly to infection mediated inflammation, sterile inflammation induces immune cell recruitment at the site of inflammation (Figure 2) (Chen & Nuñez, 2010; Menezes *et al.*, 2011; Rock *et al.*, 2010). Most of the time cell death occurs in two ways: apoptosis and necrosis. Apoptosis is a natural process of cellular death. However, during extreme cellular

damage normal apoptosis does not occur but rather necrotic cell death which occurs under extreme cellular stress or trauma. These dying cells lose plasma membrane integrity rapidly, and consequently intracellular materials spill into the extracellular environment and triggers sterile inflammation (Kono & Rock, 2008; Shen *et al.*, 2013). The inflammatory process is controlled or coordinated at various stages including inducers, sensors, mediators, and effectors of inflammation.

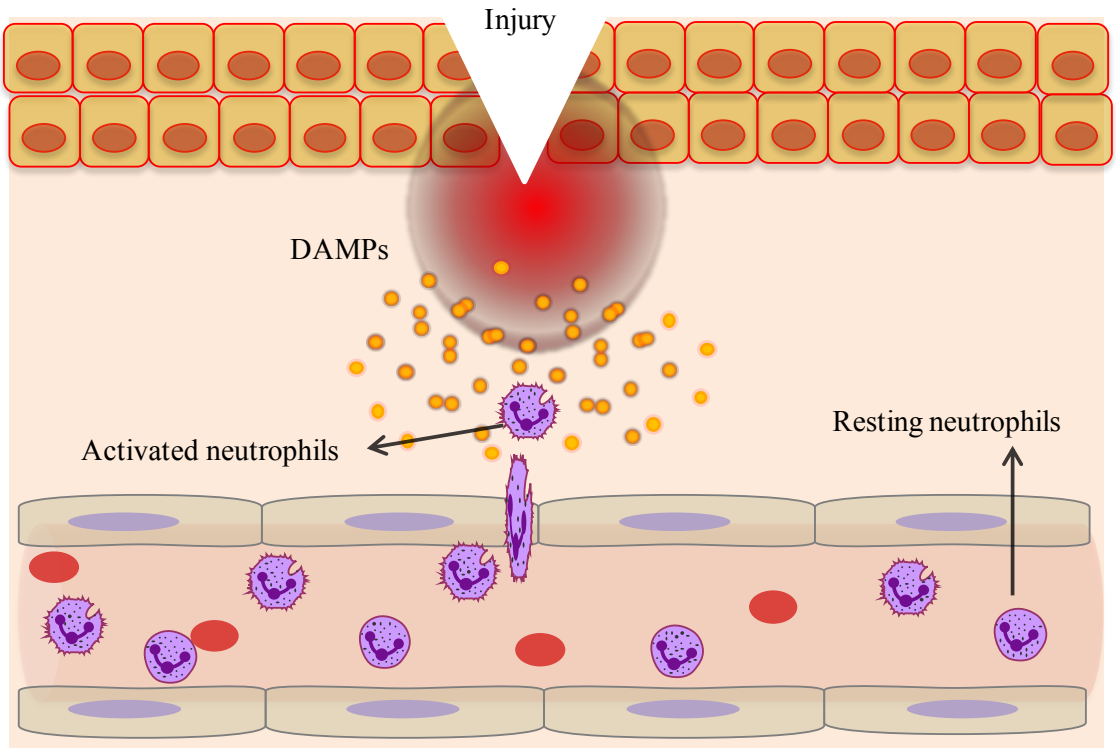


Figure 2: Schematic illustration of sterile inflammation. *Tissue injury in the absence of infection releases danger signals that stimulate neutrophils recruitment to the site of sterile inflammation*

1.4.1.1. Inflammatory inducers and sensors

Inflammation inducers are the signals that initiate an inflammatory response during sterile or non-sterile inflammation. They activate inducer specific sensors in order to produce a specific sort of mediator (McDonald & Kubes, 2011). There are two types of inflammatory inducers: exogenous and endogenous inducers. Exogenous inducers are categorized as microbial inducers and non-microbial inducers. Each microorganism exhibits uniquely conserved moieties known as PAMPs. These molecular patterns are sensed by pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs)

and NOD-like receptors (NLRs) in order to stimulate pro-inflammatory responses. TLRs are transmembrane proteins located on the cell surface and endosomes. Immune cell TLRs sense the microbial inducers and initiate production of inflammatory mediators such as prostaglandins, chemokines, and cytokines (Chen & Nuñez, 2010; Medzhitov, 2008).

Endogenous inducers are signals produced by tissue injury or stress in the absence of pathogen, releases intracellular molecule inducers known as DAMPs. These DAMPs (HSPs, ATP, S100, HMGB1, uric acid, extracellular matrix and endogenous nucleic acid) are recognized by PRRs and play a similar function as PAMPs. For example, TLR 2 and TLR4 sense the extracellular matrix as DAMPs and activate the production of cytokine and chemokines (Chen & Nuñez, 2010; Kono & Rock, 2008; McDonald & Kubes, 2011).

1.4.1.2. Inflammatory mediators

Inflammatory inducer molecules stimulate the production of a certain number of inflammatory mediators to attract leukocytes (Coussens & Werb, 2002; Medzhitov, 2010; Sadik *et al.*, 2011). Based on their biochemical properties inflammatory mediators have been characterized into seven groups including vasoactive amines, vasoactive peptides, complement fragments, lipid mediators, cytokines, chemokines, and proteolytic enzymes (Medzhitov, 2008). During the inflammatory process mast cells and platelets release vasoactive amines such as histamine and serotonin, which ease the leukocyte's recruitment by increasing vascular permeability and vasodilation. Second, vasoactive peptides are generated by proteolytic process and extracellular fluid, resulting in mast cell degranulation. Third, the complement system (C3a, C4a and C5a) circulates in the blood as inactive forms. During cellular stress, inflammatory inducers activate complement system, including C5a, to promote granulocyte and monocyte recruitment (Iadecola & Anrather, 2011; Mayadas *et al.*, 2009). Fourth, lipid meditators (eicosanoids) also play an important role in inflammation and inflammation resolution. Eicosanoids including prostaglandins, leukotrienes, lipoxins are mostly produced from arachidonic acid by neutrophils, macrophages, and dendritic cells via three pathways: lipoxygenase, P-450 epoxygnase, and cyclooxygenase mediated production (Harizi *et al.*, 2008). These lipid molecules regulate wide variety of physiological and

pathological process. For example; prostaglandins including PGE2 and PGI2 induce vasculature permeability and vasodilation in order to attract immune cells such as neutrophil and macrophages. On the other hand, lipoxins mediate inflammation resolution and regeneration by inhibiting neutrophil filtration to inflammation site (Hirata & Narumiya, 2012; Levy *et al.*, 2001; Serhan, 2007; Serhan & Savill, 2005). Fifth, cytokines including TNF alpha, IL-1, IL-6 and others are also important inflammation mediators, which are produced mostly by macrophages and mast cells. These cytokine mediators play a vital role in endothelial activation and leukocyte recruitment during the inflammatory response (Feghali &Wright, 1997; Medzhitov, 2010; Sanjabi *et al.*, 2009). In addition to cytokines, chemokines are key mediators of inflammation produced by many cell types. They are specialized to control leukocytes extravasation and chemotaxis towards the affected tissue (Moser, 2004; Sallusto & Baggiolini, 2008; Thelen & Stein, 2008). Next, and finally, inflammatory mediators can be proteolytic enzymes such as elastin, cathepsins, and matrix metalloproteinases (MMPs). These proteolytic enzymes play a crucial role in host defense, tissue remodeling, and immune cell migration. Mmps are cell secreted soluble and membrane bound enzymes that degrade extracellular matrix to facilitate neutrophil migration in tissue (Coussens & Werb, 2002; Hall *et al.*, 2014; LeBert & Huttenlocher, 2014; Wolf & Friedl, 2011).

1.4.1.3. Inflammation effectors cells

1.4.1.3.1. Neutrophils attraction

Cells that respond to inflammatory mediators are known as effectors of inflammation such as neutrophils, macrophages, and mast cells. Neutrophils are short-lived and the most abundant leukocytes in mammals and zebrafish. They provide a first line of host defense against sterile (tissue damage) or non-sterile (pathogen infection) inflammation. Mature neutrophils are released into the blood stream and circulate for next 1-2 days. After 1-2 days they undergo apoptosis and are cleared by dendritic or macrophages. In response to wounding, infection or other inflammatory stimuli, neutrophils are the first immune cell recruited at the inflammatory site and perform well defined effector functions, such as phagocytosis and activation of other immune system components (Henry *et al.*, 2013; Renshaw *et al.*, 2006). At the site of inflammation, inflammatory mediators activate the circulating resting neutrophils. Neutrophils exit from the blood

vessels and migrate to the inflamed site by various processes such as rolling, adhesion, crawling and transmigration (Kolaczkowska & Kubes, 2013; Nathan, 2006; Phillipson & Kubes, 2011).

In the initial phase of the inflammatory response, inducers and mediators activate the surrounding endothelial cells and subsequently up-regulate the expression of adhesion molecules, such as P and E selectins. Neutrophils exhibit expression of glycosylated ligands for these selectins, including P-selectin glycoprotein ligand 1 (PSGL-1). These adhesive molecules (P and E selectins) tether circulating neutrophils to the vessel wall. Neutrophils roll along the endothelium under shear stress generated by blood flow (Phillipson & Kubes, 2011; Sundd *et al.*, 2012; Wang & Arase, 2014). In addition, involvement of selectin ligands (PSGL1) on neutrophils also activates various kinases such as Src family kinasem and Syk, phosphoinositide 3- kinase (P13K). These kinases subsequently activate the integrin molecules expressed by neutrophils (Mueller *et al.*, 2010; Yago *et al.*, 2010). Rolling neutrophils further interact with integrins and induce conformational changes and clustering of β_2 integrins. Conformational changes of β_2 integrin expose its ligand to intracellular adhesion molecule 1(ICAM1) expressed by inflamed endothelial cells. Activated integrins enable neutrophil adhesion by inhibiting and stopping neutrophil rolling (Constantin *et al.*, 2000; Luo, 2012; Petri *et al.*, 2008). Once neutrophils are adhered to the endothelium, neutrophils might migrate through endothelial cell barrier by two ways; either paracellularly (passing between endothelial cells) or transcellularly (passing through pores or passages that traverse the endothelial cytoplasm). Transmigration of neutrophils depends on adhesion molecules including PECAM-1 and CD99 expressed by the endothelium and leukocytes. PECAM-1 endothelium transmembrane junction molecules participate in pre-cellular transmigration. Pre-cellular transmigration is the migration of leukocytes from the luminal side of capillaries through endothelial cell junctions to the abluminal side. However, the trans-cellular pathways are a key route for a small subset of the neutrophil population for rapid emission (Carman & Springer, 2008; Wang & Arase, 2014).

Once neutrophils are recruited to the inflammatory site, neutrophils directly recognize PAMPs with the help of PRRs and destroy them. Neutrophils are involved in pathogen killing, through release of their toxic granule contents packed with proteolytic enzymes, antimicrobial proteins, and reactive oxygen species. Moreover, neutrophil granules

release enzymes such as NADPH dependent oxidase and MPO, which contribute in the production of antimicrobial molecules as reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) (Kobayashi *et al.*, 2005). Moreover, highly activated neutrophils expel nuclear extracellular traps (NETs) including histones and DNA in order to eliminate extracellular pathogens. Phagocytosis prevents inflammation from spreading and aids in resolution (Lu *et al.*, 2012).

1.4.1.4. Inflammation resolution

Once the initiating noxious materials are removed via phagocytosis, the inflammatory reaction must be resolved in order to prevent the inflammation from spreading becoming chronic or causing diseases. Reduction or removal of leukocytes or cell debris from the inflamed site is called catabasis or resolution of inflammation. The resolution process is rapidly initiated by cellular pathways that actively biosynthesize locally, as well as dual-acting, anti-inflammatory cytokines, such as IL-10, TGF-β and dual acting anti-inflammatory (Sultani *et al.*, 2012) and pro-resolution lipid mediators, i.e. the lipoxins, resolvins, and protectins (Ariel, 2012; Bannenberg & Serhan, 2010; Bannenberg *et al.*, 2005; Serhan *et al.*, 2008). Resolution of inflammation is distinct from anti-inflammatory processes because pro-resolution mediators actively promote clearance of microorganisms and apoptotic cells. So mediators of the resolution of inflammation are different from immunosuppressors of inflammation as they activate mechanisms that bring about the restoration of homeostasis in inflamed tissue. Once neutrophils arrive at inflamed site and phagocytized noxious materials. Furthermore, neutrophils induce the production of anti-inflammatory or pro-resolution lipid mediators including protectins, resolvins, and lipoxins to resolve the inflammation. Lipoxins play a dual role in inflammatory resolution by selectively stopping neutrophil and eosinophil infiltration and activating macrophage recruitment at inflamed site (Levy *et al.*, 2001; Schwab & Serhan, 2006; Serhan *et al.*, 2008; Uddin & Levy, 2011). Recruited macrophages increase phagocytosis of microorganisms and apoptotic cells, and increase the exit of phagocytes from the inflamed site through the lymphatics.

1.5. Neutrophil inflammatory response in zebrafish model system

Neutrophilic inflammation is an essential immune response to maintain the zebrafish primary immune system. Tissue damage or infection stimulates leukocyte recruitment to

the wound site through immediate release of 'danger signals', such as ATP, uric acid, lipids, DNA and nuclear proteins (Škoberne *et al.*, 2004). In the case of injury, neutrophils migrate to the injury site by sensing a hydrogen peroxide gradient produced by damaged epithelial cell (Niethammer *et al.*, 2009). Yoo *et al*., has further revealed that H_2O_2 produced by injury oxidizes cysteine of Lyn, a member of Src family kinases (SFKs) present in neutrophils. Lyn kinase is an important molecule of neutrophils; it controls the neutrophil endothelium adhesion and directional migration. Oxidation of the cysteine amino acid stimulates Lyn kinase, which in turn activates the phosphotidylinositol (3, 4, and 5) triphosphate (PI3K) (Yoo *et al.*, 2011). Activated PI3K further activates Rho GTPase (Rac2) at the leading edge to induce cytoskeleton reorganization that lead neutrophil directional migration. Moreover, Rac2 can also activate PI3K for neutrophil cytoskeleton reorganization and directional migration (Deng & Huttenlocher, 2012; Deng *et al.*, 2011).

Once neutrophils have reached the inflamed site, neutrophil gets involved in phagocytosis, removing microbes and damaged tissue to initiate resolution. Resolution of inflammation or reduction of neutrophils at a site of tissue injury occurs in numerous ways (Deng & Huttenlocher, 2012). These include apoptosis, removal of dead neutrophils and noxious materials, and neutrophil reverse migration (Mathias *et al.*, 2006). Previous studies have shown that only a small number of cells at the injured site undergo apoptosis and the rest of the neutrophil cell reverse migrates from the site of inflammation (Starnes & Huttenlocher, 2012).

1.6. Challenges

Various powerful studies of granulocytic inflammation have been carried out in zebrafish model system to ascertain the roles of that particular inflammatory gene. So far most of the zebrafish research on inflammation has been done based on imaging (Enyedi *et al.*, 2013), chemical screening (Zon & Peterson, 2005), and FACS (Mathias *et al.*, 2009) and gene expression analysis. Gene expression analysis is derived from large-scale transcriptomics including microarrays (van der Vaart *et al.*, 2013) and RNA seq (Ordas *et al.*, 2011). Most likely, protein abundance is calculated or predicted based on the gene expression analysis. However, previous comparative studies in various organisms have shown fairly limited correlation between protein and mRNA.

Researchers have suggested four basic arguments behind this poor correlation. First, the level of transcription of a gene gives only a rough estimate of its level of expression into protein. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Secondly, many proteins experience post-translational modifications that profoundly affect their activities. For instance, a protein may not be active until it becomes phosphorylated. Thirdly, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Finally, many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules(Muers, 2011).

Traditionally, zebrafish researchers rely on antibodies or immunohistochemistry for protein analysis. However, each of these methods only opens small window to understand the complexity of multilayer cellular proteins because of inaccessibility of zebrafish specific antibodies. Therefore, researchers have introduced proteomics techniques to define global analysis of proteins in a protein complex of cell, tissue or complete organism.

1.7. Proteomics

Proteomics study includes protein identification, quantification, interactions as well as determination of their localization, and most importantly investigation of their function (Nilsson *et al.*, 2010). Mass spectrometry based proteomics have become the primary technology to study the proteins in complex mixtures. Over the years, different mass spectrometry techniques have been developed to perform proteomics analysis (Micallef *et al.*, 2010; Roe & Griffin, 2006).

1.7.1. Mass spectrometry based proteomics

As the name implies, a mass spectrometer measures the mass to charge ratio of electrically charged molecules (proteins or peptides). A mass spectrometer contains three important parts including an ion source, a mass analyzer, and ion detector. In the first instance, liquid samples are ionized into charged particles and detectors detect the mass to charge ratio (Catherman *et al.*, 2014). The two most commonly used sources include matrix assisted laser desorption/ ionization (MALDI) and electrospray

ionization (ESI) (Chen, 2008; Singh *et al.*, 2010a; 2011). MALDI and ESI produce charged ions with very low internal energy. MALDI ionizes co-crystalized predigested peptide samples from a target metal plate (Medzihradszky *et al.*, 2000). While ESI ionize the molecules from a liquid phase and is therefore readily coupled to liquid-based chromatographic (LC) separation techniques. Compared to MALDI, liquid chromatography (LC) coupled ESI-MS enables analysis of complex peptide mixtures. Once the peptide mixture is ionized, charged particle reaches the mass analyzer, which is one of the crucial modules of mass spectrometer. MALDI mostly consists time of flight (TOF) analyzer that enables the separation of ion according to time of flight. Mass spectrometry has key parameters include sensitivity, resolution, mass accuracy, and reproducibility to perform proteomics (Medzihradszky *et al.*, 2000). To date, three types of mass analyzers are used for proteomics analysis such as time of flight, quadruple, and ion trap instruments (quadrupole ion trap -QIT, linear ion trap -LIT, Fourier transform ion cyclotron resonance -FT-ICR). All mass analyzers differ noticeably in sensitivity, resolution, and mass accuracy and hold some weaknesses and strength (Aebersold & Mann, 2003a).

The ion trap instruments or methods are robust, sensitive, and relatively inexpensive. They are extensively used in proteomics research (Figeys & Aebersold, 1997). In this dissertation, ion trap (Orbitrap) instrumentation was used for proteomics analysis. Therefore, a brief introduction will be given to this particular method. The Orbitrap mass analyzer consists of a small electrostatic device into which ion packets are injected at energies to orbit around a central, spindle shaped electrode. The image current of the axial motion of the ions is picked up by the detector. Currently in proteomics research, LTQ Orbitrp is a widely used instrument, which is a hybrid of low-resolution linear ion trap and orbitrap analyzer (Michalski *et al.*, 2011). First LTQ-Orbitrap in MS mode traps the ion population in the center for high resolution analysis based on Orbitrap analyzer. In MS/MS mode the linear ion trap only retains a chosen mass window, which is activated by a supplemental radio frequency (RF) field leading to fragmentation of the trapped precursor ions, and records the signal of a mass dependent scan at low resolution. This method was further developed for more efficient ion entrapment for fragmentation, induced by Higher energy Collisional Dissociation (HCD) (Shao *et al.*, 2014). This energy causes the peptide ion to fragment at different points, commonly at the peptide bonds. HCD fragmentation is similar to fragmentation in triple quadrupole

or quadrupole TOF. HCD based fragmentation provides higher mass accuracy in the Orbitrap analyzer (Michalski *et al.*, 2011). The recorded ions represents tandem mass spectrum (MS/MS) that contains information of the amino acid sequence (Aebersold & Mann, 2003a).

Further, this peptide mass fingerprinting (PMF) is used for protein identification. Several software packages or search engines including Mascot, Sequest, MaxQuant are available to assist in the protein identification (Cox & Mann, 2008; Cox *et al.*, 2009). These software programs help to compare the experimental PMF mass (ion spectra) with theoretical mass value derived from DNA or protein databases including Uniprot, NCBI, and International protein index (IPI).

1.7.2. Quantitative proteomics

Quantitative proteomics is an approach to measure the amount of protein change in samples during an altered state. Quantitative proteomics are two types including relative and absolute quantitative proteomics. Absolute quantification is the measurement of exact amount of protein such as concentrations of plasma marker proteins in units or the copy number of a protein per cell. Relative quantification depends on the comparison of protein expression in two samples. There are various methods for relative protein quantification including 2D (DIGE) (Saxena *et al.*, 2012), label-free (Andreev *et al.*, 2012), and stable isotope labeling (Tao & Aebersold, 2003) but the best method is defined based on three aspects including rapidity, efficiency, and reproducibility (Altelaar *et al.*, 2013b; Ong & Mann, 2005a).

Two-dimensional gel electrophoresis is traditional quantitative proteomics approach, allows the separation of the proteins based on molecular weight and isoelectric point. Further, this method has been upgraded in which proteins were labeled with different fluorescent dyes and separated on gels to visualize differentially regulated proteins. Although, this method provided good sensitivity, linearity, and dynamic range compared to traditional antibody based analysis, this method stills have some limitations. First, this method requires many more replicates and image analyses for significant results. Secondly, this approach can visualize only few hundred proteins and, most importantly, this method has certain limitations in the identification of small

molecular weight and low abundant proteins (Abdallah *et al.*, 2012; Deracinois *et al.*, 2013).

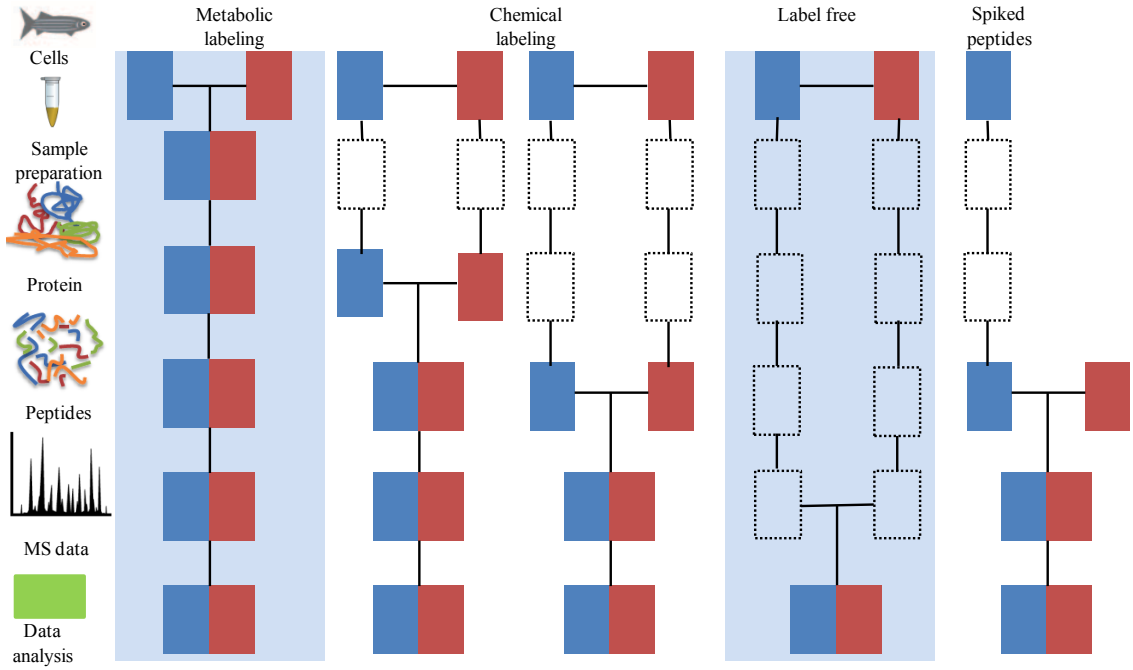


Figure 3: Workflows of commonly used quantitative proteomics approaches. *Boxes in blue and red symbolize two experimental conditions. Horizontal lines indicate when samples are combined. Dashed lines indicate points at which experimental variation and thus quantification errors can occur. This figure is adopted from Ong et al., 2005 and Bantscheff et al., 2007*

With time and demand, mass spectrometry based quantitative proteomics has gained a lot of popularity and various developments have been made in quantitative proteomics approaches. To date, two quantitative proteomics analysis have been applied for complex protein samples such as label-free and labeling approaches (Figure 3) (Blagoev *et al.*, 2003; Ong & Mann, 2005a).

1.7.2.1. Label free proteomics

Label free quantitative proteomic methods allow for the quantification of relative expression changes in two samples without the use of labeling. This is one of the rapidly growing proteomics methods because of its speed, low cost, and easy and multiple sample analysis (Wasinger *et al.*, 2013; Zhu *et al.*, 2010). There are two

strategies for label free quantification including spectral counting and peptide peak intensity measurement. In the first case, spectral counting implies the abundance of protein, by means of higher spectra denoting more highly abundant proteins (Wang *et al.*, 2008b). Therefore, relative protein quantification is calculated by comparing the number of spectra from the same protein of two samples. However, spectral counting is protein centric and it is less reliable for small and less abundant proteins. In addition, the spectral counting method does not hold much promise to identify proteins with less expression change (Old *et al.*, 2005; Wasinger *et al.*, 2013). Secondly, relative quantification using peptide peak intensity measurement involves comparing the MS peptide ion intensities belonging to a given protein. This method provides better identification of differentially regulated proteins. The label free protein quantification implies that samples are prepared and compared separately and individually analyzed by MS/MS. However, a major limitation of label free quantitative method is systemic and non-systemic variation between replicates.

Label free quantification has been applied in various biological research fields. Professor Mann's group applied label free quantitative proteomic strategies to understand the proteome of mouse dendritic cells upon viral infection. They further suggested that a label free quantitative strategy is one of the better choices for limited amount of tissue or cell material (Luber *et al.*, 2010). Hogl *et al*., 2013 had for the first time applied a label free quantification approach to understand the membrane protein dynamics of zebrafish upon Bace 1 protease knockdown. They successfully identified 4500 membrane proteins in the zebarfish brain. Moreover, they have also reported that the expression of 24 proteins was altered due to Bace 1 knockdown (Hogl *et al.*, 2013).

1.7.2.2. Labeling method

As the name implies, labeling quantitative proteomics method in MS quantify the proteins based on specific stable isotope labeling. Stable isotope labeling techniques are based on the introduction of a differential mass tag (internal standard) that affects only the mass of protein without changing chemical properties of proteins and subsequently, protein quantification performed by comparing the intensities of labeled mass tags. There are three well-known stable isotope labeling methods; enzymatic, chemica, and metabolic. The stable isotope labeling method is more reliable and reproducible

compared to label free quantitative proteomic approaches (Bantscheff *et al.*, 2007; 2012).

1.7.2.3. Enzymatic labeling

This method is performed during proteolytic digestion or after proteolysis in a second incubation step with proteases. In this method, ^{18}O incorporated into C-terminal of peptides resulting in a mass shift of 2 Dalton per ^{18}O atom. While Trypsin and Glu-C labeling introduce two oxygen atoms resulting in 4 Da mass shifts; which are, generally, sufficient for differentiation of isotopemers (Heller *et al.*, 2003). However, this method could not achieve full labeling. Therefore, each peptide has different rates of labeling which hinder data analyses (Bantscheff *et al.*, 2007; Goshe & Smith, 2003).

1.7.2.4. Chemical labeling

Chemical isotopes bind to reactive amino acid side chains of proteins or peptides are known as chemical labeling. Numerous chemical labeling methods including isotope coded affinity tags (ICAT), isotope coded protein labels (ICPL), tandem mass tags (TMT), and isobaric tags for relative and absolute quantification (iTRQ) are available to perform quantitative proteomics analysis. ICAT chemical label binds to cysteine amino acids. Therefore, this method is not suitable for the identification and quantification of proteins that lack cysteine amino acids (Wasinger *et al.*, 2013).

However, current chemical labeling methods (TMT and iTRQ) target the protein or peptide N terminus and epsilon amino group of lysine residue. These labeling reagents are also known as isobaric tags because they primarily target amines (Thompson *et al.*, 2003; Wiese *et al.*, 2007). iTRQ labeling allows identification of comparison of up to eight samples in parallel. iTRQ is incorporated in different protein samples after trypsin digestion and subsequently digested and labeled samples (treated and control) combined together for mass spectrometry analyses. iTRQ labeled different states peptide sample shows similar mass because labeled peptides from different states are isobaric. However, differential behavior of peptides can be observed during mass spectrometry fragmentation of peptide spectra. Nevertheless, this labeling method depends on the side chain reaction of amino acid that could lead to unexpected products that can produce a bias in protein quantification (Aggarwal *et al.*, 2006; Wasinger *et al.*, 2013; Wiese *et*

al., 2007). Additionally, as mentioned earlier that chemical labeling is introduced after protein digestion of different samples and therefore can create inconsistent protein digestion thus leading to labeling variation. In addition, these chemical labeling reagents are very costly, which limit larger numbers of data analyses (Elliott *et al.*, 2009).

1.7.2.5. Metabolic labeling

In metabolic labeling strategies, the labeled isotope analog is synthesized into each protein during cell growth or cell division. Therefore, this method provides an alternative strategy for accurate quantitative proteome analysis. Metabolic labeling was first established in bacteria and yeast by growing them into ^{15}N enriched media. However, ^{15}N labeling was not feasible for most mammalian cells. There are a number of essential amino acids that cannot be synthesized by mammalian cells. Therefore, these essential amino acids should be supplied to cell in cell culture medium (as food supplements) for cell survival. Ong *et al.,* 2002 were the first to report this method by introducing $^{13}C_6$-arginine and $^{13}C_6$-lysine into mammalian cell culture (Ong *et al.*, 2002). As the metabolic labeled analog is supplied to cell culture this metabolic labeling method has been named as stable isotope labeling by amino acid in cell culture (SILAC). These isotopically labeled amino acid cell culture media are available on the market. Once labeled analogs of an amino acid is supplied to cell culture instead of natural amino acids, labeled amino acids get incorporated into proteins during normal protein synthesis. Fully SILAC labeled cells can be achieved after 3-5 exchanges of media. Once isotopic labeled amino acids are incorporated into proteins, it induces a 6 Dalton mass differences between labeled and non- labeled amino acids. As there are no chemical differences between natural (light) and labeled amino acid (heavy) SILAC labeled cells behave as normal cells would. For accurate quantitative proteomics analysis, light cells can be treated or infected and heavy cells can be kept as a control cells. In mass spectrometry analysis, each peptide appears in a pair with 6 Dalton mass differences, and relative expression of the same protein in two samples can be measured based on the peptide intensity (Mann, 2014; Ong & Mann, 2005b; Ong *et al.*, 2002). The main advantage of SILAC is that treated and non-treated samples can be pooled prior to protein extraction. Therefore, manual errors get eliminated during sample preparation resulting in higher quantification accuracy, more precession, and higher reproducibility. Moreover, this method also enables the quantification of small changes

in protein levels, as well as, protein modifications. Although, metabolic labeling is one of the most highly sensitive methods for protein quantification, protein quantification based isotopic labeling of arginine residues has limitations due to metabolic conversion of arginine to proline residues (Ong & Mann, 2005a; Ong *et al.*, 2003).

1.7.2.5.1. In vivo metabolic labeling

Animal models including fruit flies, rat, zebrafish and mice serve as tools to mimic human physiology and disease. Therefore, extension of quantitative proteomics using SILAC creates an opportunity to study a wide range of diseases that effects tissues or organ proteomes. To date, SILAC has been expanded by labeling entire organism including mouse (Kruger *et al.*, 2008), drosophila (Sury *et al.*, 2010), rat (Rauniyar *et al.*, 2013), and zebrafish (Konzer *et al.*, 2013). SILAC labeling of whole organisms requires preparation of food that contains the SILAC amino acid as the sole nutrient source and that is compatible with growth of the organism. McClatchy *et al., 2007* achieved approximately 90% SILAC labeling by feeding ^{15}N enriched spirulina diet to rats for 44 days. Similar to cell lines, the SILAC labeled rat did not produce any phenotypic discrepancies compared to non-labeled control rats. Organs including brain, heart, and muscles did not achieve more than 75 % SILAC labeling, even though the nitrogen source from the diet is the same. This disparity is due to certain tissues having slower protein turnover rates than others (McClatchy *et al.*, 2007). To mitigate the inconsistent labeling rate, Kruger *et al.* 2008 selected $^{13}C_6$ lysine to establish a metabolically labeled mouse (Kruger *et al.*, 2008). They prepared a mouse diet by replacing natural lysine amino acid with isotopic labeled lysine variant and feed the mice with labeled chow regularly. Similar to the SILAC labeled rat, mice showed similar food consumption, normal fertility, and normal weight gain compared to non-labeled mice. After two generations of continuous feeding with the isotopic labeled diet, mice were fully labeled with isotopic lysine residue. These techniques enable accurate protein quantification of proteins in various organs and tissue without any chemical labeling, which might lead to chemical modification and inaccurate protein quantification (Mann, 2006).

Despite its various advantages, heterogeneity and complexity of tissue samples limit its direct comparison with non-labeled tissue. Therefore, Geiger *et al.,* 2013 recommend

using the SILAC model organisms as standards rather than as the experimental system themselves, since the SILAC food might have metabolic effects. Moreover, with the spike-in standard approach the same SILAC organism can be used for experiments with different age groups, incubation, and strains of various genetic backgrounds (Geiger *et al.*, 2011; Gilmore *et al.*, 2013).

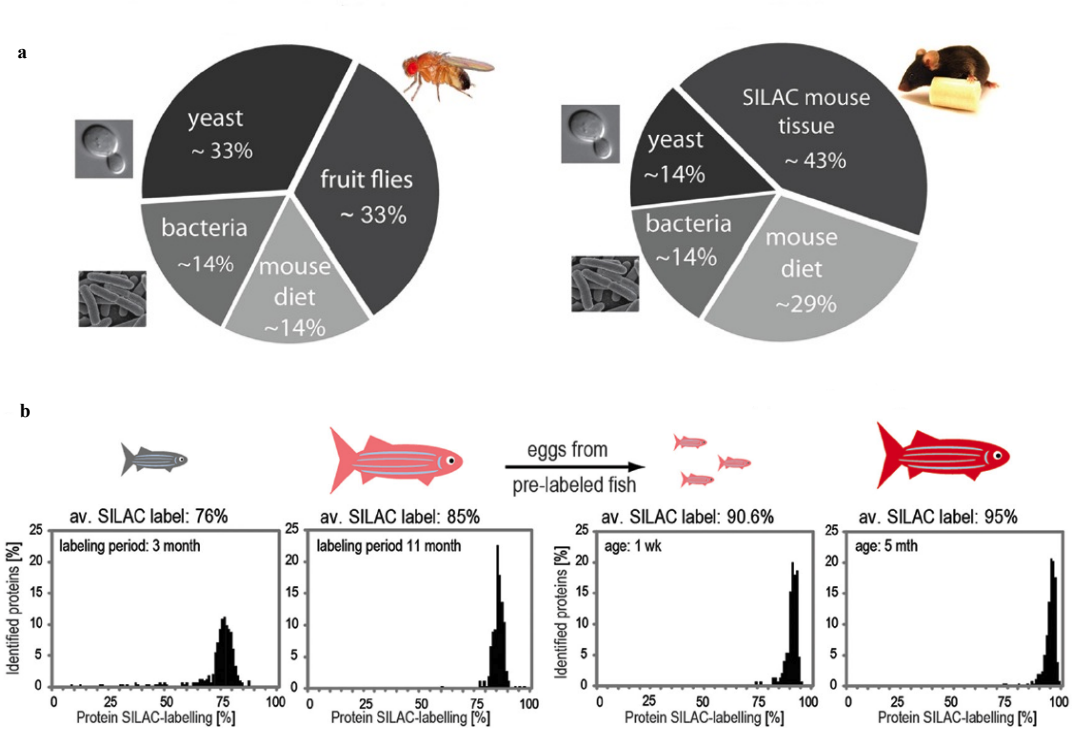


Figure 4: In-vivo metabolic labeling in zebrafish*. (a) In-house developed SILAC diet for adult zebrafish consists of heavy labeled cells of E. coli, S. cerevisiae, mouse tissue and SILAC mouse diet (b) Lys-6 diet feeding to nonlabled zebrafish upto 3-11 months labeled on average 85% proteins of zebarfish. This plot was achieved by analysis of 200 proteins. Whereas, SILAC incorporation rate were further increased after one week and five months of F1 generation Knozer et al., 2013*

In continuation to mouse SILAC labeling, a Swedish research group has established SILAC labeled zebrafish. They used the $^{13}C_6$ lysine mouse diet technique to label zebrafish proteins and checked the labeling efficiency in various organs. Notably, the incorporation of metabolic isotopic lysine residues into zebrafish was low in compared to other SILAC labeled organism and contrary to the other SILAC labeled organisms, they found physiological abnormalities (inability to breed and development) in SILAC

labeled zebrafish. They hypothesized that the rodent diet might not contain sufficient nutrition required for fish (Westman-Brinkmalm *et al.*, 2011). Therefore, the Kruger group had to first establish a Lys-6 containing universal fish food for larval and adult zebrafish and feed the fish up to one generation. They achieved a 98% SILAC incorporation in brain, blood, heart, muscle, gills, spleen, skin, and liver (Figure 4). Interestingly, they did not observe any physiological abnormalities in SILAC labeled zebrafish (Konzer *et al.*, 2013; Nolte *et al.*, 2014).

Although, labeling efficiency of various organs of zebrafish have been checked, SILAC labeling in the whole organisms cells including immune and epithelial cell are still lacking.

1.8. Aim of the study

Neutrophils are the first immune cells respond to sterile or non-sterile inflammation. To date, little is known about molecular changes in neutrophils upon inflammation. The zebrafish is increasingly used as a model to genetically address immunological problems due to several advantages including availability of transgenic lines of fluorescently labeled neutrophils and macrophages. Moreover, recently an interesting chemically-induced inflammation (ChIn) assay, using copper sulphate ($CuSO_4$) to trigger robust sterile inflammation was established in zebrafish larvae. Wounding of zebrafish lateral line neuromasts is inflicted chemically by adding micromolar concentrations of $CuSO_4$ to the bathing water, resulting in rapid recruitment of leukocytes to injured neuromasts. Hence, ChIn assay enables automated screening procedures towards the identification of immune-modulatory activities of candidate compounds in zebrafish larvae. However, it was currently unknown whether the ChIn assay could also be used in adult zebrafish. Therefore, the first aim of my thesis was to establish chemically induced inflammation in adult zebrafish to understand the molecular changes in neutrophils. In past, microarray and RNA sequencing analysis of adult and embryo zebrafish infected with various pathogens has been performed. However, proteo-genomics studies showed clear differences in transcript and protein expression levels in various biological systems. Thus, second and central aim of my thesis was to select and optimize suitable quantitative proteomics method to determine inflammatory proteome of adult zebrafish WKM neutrophils

2. Methods and materials

2.1. Materials

2.1.1. Reagents and consumables

Table 2: List of reagents/plastic ware

Name	Supplier
Copper II sulphate anhydrous ($CuSO_4$)	Carl Roth GmbH & Co KG
PeqGold Trifast reagent	Peqlab
QuantiTect Reverse TranscriptionQiagen	Qiagen
GoTaq qPCR master mix	Promega
SBS v3 kits	Illumina
Bradford reagents	Bio-Rad
Nu-PAGE gradient gels	Invitrogen
Colloidal Blue Staining Kit	Invitrogen
MSS222	Sigma-Aldrich
L-15 media	Sigma-Aldrich
Fetal bovine serum gold	PAA laboratories Gmbh
Acetone	AppliChem
Acetonitrile	Sigma Aldrich
Acetic acid	AppliChem
Formic	Thermo Scientific
Ethanol	Merck kGaA
SDS	Carl Roth GmbH
Tris/HCL	Carkl Roth GmbH
HEPES	Sigma Aldrich
Urea	Sigma Aldrich
Thiouria	Sigma Aldrich
DTT	Sigma Aldrich
Iodoacetamide	Sigma Aldrich
Microloader pipette tips	Eppendorf

2.1.2. Instruments

Table 3: List of instruments used in the study

Instrument	Manufacturer
FACS Aria II flow cytometer	BD Biosciences
StepOnePlus Thermo Cycler	ABI
Agilent Bioanalyzer 2100	Agilent
ChemiDoc MP Imaging System	Bio-Rad
LTQ Orbitrap XL mass spectrometer	Thermo Scientific
Lyophilizer	Martin Christ freeze dryers
Quadrupole-based mass spectrometer QExactive	Thermo Scientific
Leica MZFLIII Stereoscope	Leica
Stereoscope Olympus SZX7	Leica
DM5500 fluorescent microscope	Leica
Sonication	Branson Sonifier 250

2.1.3. Primers

Primer name	**Sequence**
myd88-fwd	CGAACACAGGAGAGAGAAGGAGTC
myd88-rev	TCAAAGGTCTCAGGTGTCAGTCC
il6-fwd	GATGACAGTGAAGCTCTTGGACAC
il6-rev	CCGATTCAGTCTGACCGGAGATTG
tnfa-fwd	CGTCTGCTTCACGCTCCATAAGAC
tnfa-rev	ATGGATGGCAGCCTTGGAAGTG
mmp9-fwd	TGATGCAGCTTTCGGTGGAGTG
mmp9-rev	TCCCGGCAGAAGTAGAAGAATCCC
tgfb1a-fwd	TGGGAAGGCAACACAAGGTG
tgfb1a-rev	TGAGAAATCGAGCCATGAACCAC
il8-fwd	TTTTCCTGGCATTTCTGACC
il8-rev	CGTCGGCTTTCTGTTTCAAT
bactin1-fwd	CCGGTTTTGCTGGAGATGA
bactin1-rev	CACATAGGAGTCTTTCTGTCCCATG
il1b-fw	ACGGATCCAGCTACAGATGCGACATGCA

il1b-rev	ACGAATTCCTTGAGTACGAGATGTGGAGA
cox2-fwd	TGAAAGTCCACCGACGTACAA
cox2-rev	CGGAGCCAAATGGTAGCATAC

2.1.4. Software

BD FACS Diva vs 8.1

Illumina RTAv1.13

CASAVA (Version 1.9) and Eland (Illumina)

Tophat (version 1.4.1)

Bowtie (version 0.12.7)

MaxQunat (Version 1.3.7.4)

Perseus (version 1.3.7.1)

STRAP (Software Tool for Rapid Annotation of Proteins software program)

DAVID (Database for Annotation Visualization and Integrated Discovery)

GeneGo software (www.genego.com)

Cytoscape (Version 3.1)

2.2. Methods

2.2.1. Zebrafish lines

Neutrophil-specific zebrafish reporter line *Tg(lyzC:DsRed)*^{nz50} was used in this dissertation (Hall *et al.*, 2007). All zebrafish husbandry and experimental procedures were performed in accordance with the German animal protection standards [Animal Protection Law, BGBl. I, 1934 (2010)] and were approved by the Local Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (License number: Proteome analyses of adult zebrafish: Az.: 35-9185.81/G-170/12 and general license for fish maintenance and breeding: Az.: 35-9185.64).

2.2.2. SILAC zebrafish

SILAC zebrafish were obtained from the Krüger lab at the MPI Bad Nauheim. SILAC zebrafish were established by supplementing diet containing $^{13}C_6$ lysine (Lys-6), and were maintained under standard laboratory conditions (Konzer *et al.*, 2013).

2.2.3. Induction of inflammation in adult fish

Healthy transgenic zebrafish (4-8 months old) were treated with 25μM $CuSO_4$ for 1hr. Following incubation, inflammation was confirmed visually by consideration of leukocyte influx to the gills.

2.2.4. Inflammation kinetics assay in adult zebrafish

Healthy transgenic zebrafish were incubated with 25 μM $CuSO_4$ and protein samples were collected at different time points. First, fish were incubated with $CuSO_4$ 10 min to 1hr (inflammation initiation t=10, t=30 and t=1hr), and subsequently, fish were transferred into fish water for 2hr (inflammation progression t=1.5hr and t=2hr). Finally, fish were incubated up to 8hr (inflammation resolution t=4hr and t=8hr), and further collected for cell sorting.

2.2.5. Isolation of marrow neutrophils

Adult *Tg(lyzC:DsRed)* zebrafish were anesthetized with 0.2 mg/ml of Tricane and killed in an ice bath. After decapitation and ventral incision the organs were removed to expose the whole kidney marrow (WKM). WKM was then removed and placed in L-15 media with 5% fetal bovine serum (FBS). The WKM was triturated and subsequently passed through a 40 micron filter and centrifuged at 450 g. Dissociated cells were suspended in media and filtered again. The single cell suspension was analyzed and sorted on a FACS Aria II flow cytometer. The WKM cell suspension was gated based on size and granularity using forward and side scatter characteristics, respectively. The fluorescently labeled cells from the DsRed reporter line were visualized using the PE filter set (582/15nm) upon excitation by a 488 nm laser line. DsRed positive cells were back-gated to confirm their myeloid characteristics and collected. Purity was verified by both, FACS reanalysis and visual inspection on a DM5500 fluorescent microscope. Cell viability was determined by Tryphan blue staining and cell numbers were determined

using a hematocytometer. Subsequently, cells were snap-frozen and stored at -80°C for later proteomic analysis.

2.2.6. Real time PCR analysis

RNA was extracted from inflamed and control neutrophils using PeqGold Trifast reagent according to the manufacturer's protocol, and cDNA was prepared using QuantiTect Reverse Transcription Kit. qPCR was performed on an ABI StepOnePlus Thermo Cycler with CRX96 real-time system and using GoTaq qPCR master mix. PCR was performed in triplicate using the following standard program: one cycle of 95° for 15 min, and 40 cycles of 95° for 15 sec plus 60° for 30 sec with known pro-inflammatory (*tnf-alpha, myd88, mmp9, il6, il8,*) and anti-inflammatory (*tgf-beta,*) marker genes. In addition*, il1 and cox2* genes were used to investigate the inflammation kinetics assay in adult zebrafish. Additionally, a melting curve was generated based on the following condition: 95° for 15 sec, 60° for 30 sec and 95° for 15 sec. Normalization was performed against zebrafish *bactin1*.

2.2.7. RNA sequencing

Total RNA was extracted with PeqGold Trifast reagent and quality of total RNA was checked using Agilent Bioanalyzer 2100 total RNA nano chip. Subsequently, sequencing libraries were generated from total RNA samples using the Truseq RNA protocol and paired end reads (2 x 50 nucleotides) were obtained with a Hiseq1000 using SBS v3 kits by multiplexing 2 samples on a single lane of sequencing. Moreover, cluster detection and base calling were performed using RTAv1.13 and quality of reads was assessed with CASAVA v1.9 and Eland using the zebrafish (zv9) genome. For transcript quantification, reads were mapped with the exon-exon junctions compatible mapper Tophat (Trapnell *et al.*, 2009) and Bowtie against the zebrafish genome using known exon junctions (Ensembl, release 67) and the options butterfly-search, coverage-search, microexon-search, min-anchor-length 5. The mean distance between read pairs were obtained from CASAVA analysis. Additionally, quantification of gene expression was performed with HT-Seq and differential gene expression made with DESeq R packages (Anders & Huber, 2010).

2.2.8. Label free in-gel LC-MS/MS analysis

2.2.8.1. 1D Electrophoresis

Sorted neutrophil cells (in duplicate) were lysed in protein lysis buffer containing 4% SDS, 100 mM Tris/HCl, pH 7.6 (Sigma) and heated at 99°C for 10 min. Subsequently, protein samples were homogenized by ultra-sonication (3 times, 30 second pulses with an interval of 60 seconds). The protein supernatant was collected after centrifugation at 15000 g for 15 min at room temperature, and total protein concentration was measured using the Bradford method. Fifty µg of protein were subjected in duplicate to gel electrophoresis using precast 4–12% Nu-PAGE gradient gels and separated on the basis of their molecular weight. The gel was stained with Colloidal Blue Staining Kit overnight. Subsequently, the gel was de-stained and documented. Gel lanes were cut into 9 slices, and each slice was de-stained by washing with 50 mM ammonium bicarbonate/50% ethanol followed by absolute ethanol. This was followed by reduction and alkylation with DTT and iodacetamide, respectively. Subsequently, gel slices were digested with mass-spectrometry grade trypsin with an enzyme to protein ration of 1:100. Peptides were eluted from the gel pieces using acetonitrile and desalted using homemade C18 columns (stage tips) (Kruger *et al.*, 2008).

2.2.8.2. Mass spectrometry analysis

Each trypsin-digested sample, representing the peptide content of one gel piece was eluted from stage tips, subjected to an automatic sampler and further analyzed by nano-reversed phase chromatography using an Agilent 1100 nanoflow system, online-coupled via in house packed fused silica capillary column emitters (length 15cm; ID 75µM; resin ReproSil-Pur C18-AQ, 3µm), and a nanoelectrospray source (Proxeon) to a LTQ Orbitrap XL mass spectrometer. Linear gradients from 5–35% buffer B (80% acetonitrile, 0.5% acetic acid) over 150 min at 200 nl/min were applied to elute peptides from the C18 column. The whole mass spectrometry process was operated in data-dependent mode, collecting collision-induced MS/MS spectra from LTQ-FT full scans from m/z 300 to m/z 1800; resolution r = 60,000; LTQ isolation and fragmentation at a target value of 10000. AGC target MS 30000 and 100ms and 300-750ms maximum injection time were applied for ion trap and Orbitrap, respectively. Subsequently, a 1.2 Dalton ion selection window for MS/MS was applied. The five most intense peaks from

full MS scan were fragmented in a linear ion trap using colloidal induced dissociation (CID) (35% normalized collision energy) and for LTQ Orbitrap measurements (MS/MS), the 15 most intense peaks were selected for fragmentation in the linear ion trap.

2.2.8.3. Label free method data analysis

The acquired mass spectrometry raw data was further analyzed by MaxQuant software. Peptide identification was performed by searching the peak list against the international protein index sequence database (zebrafish IPI, version 3.54) supplemented with commonly observed contaminants (embedded in MaxQuant) and concatenated with reversed versions of all sequences. Carbomidomethylation of cysteine was set as fixed modification; oxidation of methionine was kept as variable modification. Additionally, the search parameters included use of proteolytic enzyme and up to a maximum of 2 missed cleavages. Peptide mass tolerance was 6 ppm for precursor ion and 0.5 Dalton for fragment ion. A false discovery rate of 1% was applied for protein and peptide identification and proteins identified with at least 2 peptides or a single unique peptide were incorporated for data analysis.

2.2.9. Labeling approach (SILAC labeling)

2.2.9.1. Sample preparation for shotgun approach

Zebrafish WKM myeloid and neutrophil control cells and neutrophils from different time points of inflammation were homogenized in SDS lysis buffer containing 4% SDS in 100 mM Tris buffer (pH 7.6). Further, all samples were heated at 95 °C for 5 min and subsequently sonicated for DNA sharing. Supernatant of all the samples were collected by centrifugation at 16000 g for 10 min. Protein concentrations of the samples were measured by Biorad assay. Moreover, to generate a SILAC protein standard, equal amounts of myeloid cell population proteins from the SILAC zebrafish were combined and mixed with corresponding non-labeled neutrophil proteins (5 µg of labeled SILAC standard + 5 µg of non-labeled neutrophil proteins). These spike-in samples (5X2) were precipitated with ice cold acetone and protein pellets were collected by centrifugation and pellets were dried. The pellets were dissolved in 6M urea, 2M thiourea and 10mM HEPES, pH 8. Subsequently, protein pellets were reduced and alkylated with 1mM dithiothreitol (DTT) and 5mM iodoacetamide, respectively. Alkylated proteins samples

were digested with the endopeptidase Lys-C in Tris buffer (pH 8.5). Digested peptides were purified by stop and go extraction (STAGE) tips.

2.2.9.2. Mass spectrometry analysis of in-solution digest of SILAC labeled neutrophil samples

Digested peptides were analysed with ultra-high presure liqued chromatography and mass spectrometry. First, peptides were separated using a dual buffer method of A (0.1% (v/v) formic acid in H_2O) and B (0.1% (v/v) formic acid in 80% acetonitrile) on an easy nanoflow HPLC system. A linear gradient was applied from 7 to 35% B for 220 min followed by 95% B for 10 min and then re- equilibration to 5% B for 10 min on a 50 cm column (75 μm ID) packed in-house with 1.9 μm diameter C18 resin. Temperature of column was controlled in a custom-made column oven at 40 °C. The UHPLC system was coupled via a nanoelectrospray ionization source to the quadruple-based mass spectrometer QExactive. MS spectra were acquired using 3e6 as AGC target at a resolution of 70000 (200 m/z) in a mass range of 350−1650 m/z. For ion accumulation, 60 ms a maximum injection time was used and MS/MS events was measured in the data-dependent mode for the 10 most abundant peaks (Top10 method) in the high mass accuracy Orbitrap after Higher energy C-Trap Dissociation (HCD) fragmentation at 25 collision energy in a 100−1650 m/z mass range. Additionally, the resolution was set to 17 500 at 200 m/z combined with an injection time of 60 ms.

2.2.9.3. Data Analysis

Raw data were analysed using MaxQuant. Andromeda search engine was used for peptide searches against a Uniprot database of zebrafish including a list of common contaminants. MaxQuant was used to quantify the SILAC peptide pairs using the following parameters: LysC as the digesting enzyme, maximum missed cleavages of two, Lys6 as the SILAC labelling, carbamidomethylation as fixed modification, and oxidation of methionine and acetylation of protein N-terminus as variable modifications. The cutoff for mass deviation was set to a maximum value of 7 ppm for peptide mass and 0.5 Da for MS/MS ions. A minimum length of 6 amino acids and a false discovery rate of 1% were used for peptide identification. A minimum ratio count of two was used for SILAC quantification, and a protein identified with at least two

peptides and one unique peptide was used for further data analysis. Statistical analyses were performed using Perseus.

2.2.10. Gene ontology analysis

2.2.10.1. STRAP

Identified neutrophil proteins were further analyzed for their cellular localization, biological processes and molecular functions using STRAP analysis (Bhatia *et al.*, 2009) .

2.2.10.2. GeneGo-Metacore

Additionally, gene IDs of identified proteins were obtained using DAVID analysis (Huang *et al.*, 2009). Subsequently, the GeneGo software (www.genego.com) was applied to establish process, network and pathways maps with identified proteins.

2.2.10.3. Cytoscape

Moreover, neutrophil proteins were also analyzed by Biological Networks Gene Ontology tool (BiNGO) supported by Cytoscape. Cytoscape is an open source platform to investigate or visualize complex protein or transcript data for their molecular function and localization.

3. Results

3.1. Proteome mapping of adult zebrafish marrow neutrophils reveals partial cross species conservation to human peripheral neutrophils *(This chapter is taken from Singh et al., 2013)*

Modern mass spectrometry allows for cell type, or tissue-specific identification and quantification of proteins (Aebersold & Mann, 2003b) and several studies have been performed to explore the proteome of neutrophils from different animal model systems (Luerman *et al.*, 2010). Recently, various proteome analyses have also been done on zebrafish tissue. For example, the protein content of adult zebrafish brain (Singh *et al.*, 2010b), caudal fin (Saxena *et al.*, 2012; Singh *et al.*, 2011; Westman-Brinkmalm *et al.*, 2011), kidney (Saxena *et al.*, 2011), liver (Wang *et al.*, 2007) and gill (De Souza *et al.*, 2009) as well of developing zebrafish embryos (Link *et al.*, 2006) has been explored. Zebrafish whole kidney marrow is the main hematopoietic site in adult zebrafish analogous of mammalian bone marrow. However, although the zebrafish is increasingly applied for immunity-related research, a resource for the protein composition of neutrophils or other immune cells of zebrafish was still lacking. Therefore, the objective of the study described in this section was to establish a method to separate resting neutrophils from adult zebrafish and subsequently acquire the proteome map of marrow neutrophils.

3.1.1. Separation of neutrophil granulocytic cells from adult zebrafish whole kidney marrow

To elucidate the proteome of whole kidney marrow derived neutrophils, a transgenic neutrophil reporter line (lyzC:dsRed) of zebrafish was obtained and raised. Kidney was removed from adult fish and physically dissociated by trituration. Single suspension cells were separated based on forward scatters (FSC) and side scatters (SSC) by flow cytometer analysis. Forward Scatters is directly proportional to cell size, and side scatter is proportional to cell granularity. Flow cytometer analysis of WKM showed distinctive cell populations including lymphoid, myeloid and precursors cells based on cell size (FSC) and granularity (SSC). Myeloid cell population consists of neutrophils, eosinophils and monocytes. DsRed positive neutrophils from the WKM of transgenic zebrafish were resolved based on red fluorescence (PE filter). Further, cells were sorted and reanalyzed based on cell size and granularity. FACS reanalysis of sorted cells confirmed the myeloid characteristics based on

cell size and cell granularity. In addition, purity of cells was also validated microscopically. An average $3x10^6$ cells per fish were obtained. After sorting and purity assessment (>90%), the cells were spun down and subsequently proteins were extracted (Figure 5)

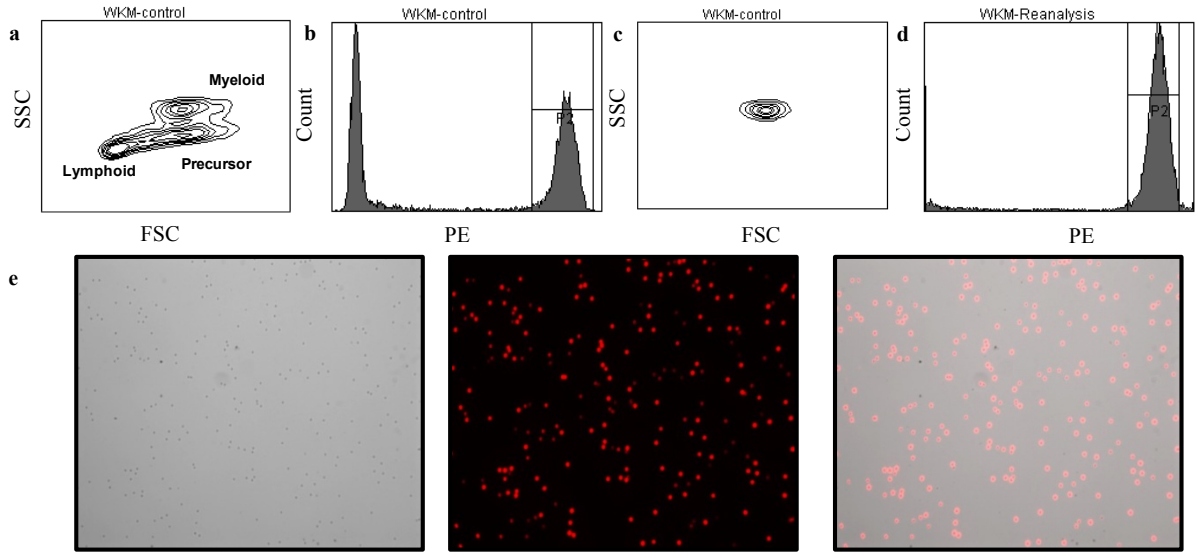


Figure 5: Separation of neutrophils from zebrafish whole kidney marrow based on FACS. *(a) Separation of major cell lineages of zebrafish WKM based on cell size (FSC) and cellular granularity (SSC). (b) Analysis of WKM cells based on red fluorescence of the neutrophil-specific lyzC:DsRed transgene. PE^+ cells were gated and collected (P2). (C) PE^+ cells (P2) gate back to the mature myeloid cell compartment based on cell size and granularity (d) Purity of collected cells was reanalyzed by FACS. More than 90% cells were PE^+ cells (e) In addition; purity of sorted cells was also examined under the microscope (brighfield channel, fluorescence channel, merged)*

3.1.2. Zebrafish resting neutrophils proteome

To elucidate the proteome profile of adult zebrafish neutrophils gel based proteomic analysis was performed. Upon analysis of total protein extract (50µg) from adult zebrafish marrow neutrophils by 1DE and LTQ Orbitrap XL MS, a total of 1544 proteins were identified from 9 trypsin-digested fractions (Appendix Table S1a & 1b). About 78% (1204/1544) of the proteins were identified upon the presence of multiple peptides. The remaining 340 proteins were identified on the basis of unique single peptides (Appendix Table S1c). Protein abundance ranged from ion intensities of 10^{10} to 10^4, with most abundant proteins represented by Mpx, Actin, Histone 2B and L-plastin (ion intensity $1x10^{10}$ – $4.9x10^9$) and the least

abundant protein was Dhrs7 (ion intensity 10^4). Out of the 1544 proteins identified, 201 were largely uncharacterized proteins (Appendix Table S1d) (with zgc or loc identifiers), while 195 proteins lacked annotated gene symbols and were thus identified through IPI IDs (Appendix Table S1e).

3.1.2.1. Neutrophil specific proteins in adult zebrafish marrow neutrophils

The protein content of a cell largely determines its functional state and may even provide a better indication of cellular function than the transcriptome. Nevertheless, highly abundant proteins often hinder the identification of other, less abundant proteins that may be more specific to the tissue- or cell type under investigation. To narrow down the protein content that may be specific to neutrophils versus other tissues neutrophil data set was compared with a protein data set from other tissue devoid of neutrophils. Hence, the adult zebrafish brain proteome was compared (Singh *et al.*, 2010a) with the current set of 1349 proteins with annotated gene symbols from neutrophils (Appendix Table 1f). Comparison of the neutrophil proteome with that of adult zebrafish brain revealed that 25% (340/1349) of proteins identified in neutrophils were also present in tissue from adult brain (Figure 6 & Appendix Table S2a). These common proteins included many cytoskeletal, enzymatic and metabolic proteins. The remaining 75% were enriched in neutrophils versus adult zebrafish brain tissue (Appendix Table S2b). A list of the most abundant neutrophil-specific proteins is shown in Table 4.

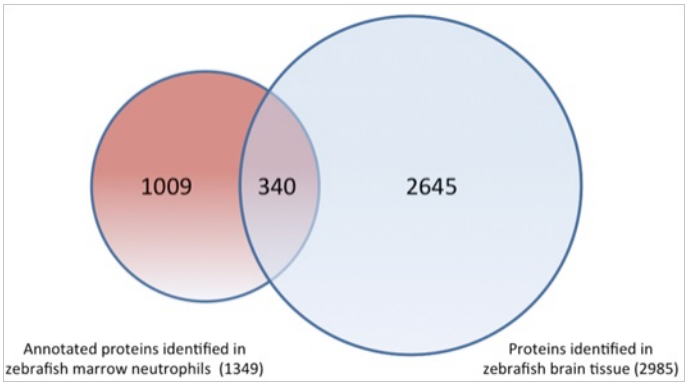


Figure 6: Zebrafish brain and neutrophil proteome comparison. *Venn diagram comparing zebrafish whole brain and zebrafish neutrophil proteome data sets*

Table 4: Most abundant proteins in zebrafish neutrophils

Gene Name	Protein name	Uniprot Id	Intensity	No. of peptides
npsn	Nephrosin	Q503K7	6974900000	13
lcp1;pls2	Plastin-2	Q6P698	4951600000	45
h2afx	similar to histone cluster 2	Q7ZUY3	4560900000	9
lyz	Lysozyme C	Q24JW2	3172600000	17
lect2	Leukocyte cell-derived chemotaxin 2 like	Q0H0R9	2321700000	14
nccrp1	nonspecific cytotoxic cell receptor protein 1	A1L1Z5	1100500000	14
si:dkeyp-46h3.6	Histone H3	Q4QRF4	1019300000	7
prdx5	Peroxiredoxin 5	Q502C8	988140000	9
cap1	Adenylyl cyclase-associated protein	A7E2H8	811930000	22
coro1a	Coronin, actin binding protein, 1A	Q7SX58	626720000	18
rdx	Radixin;67 kDa protein	Q66I42	565640000	31
anxa3b	annexin A3b	A8E5E5	463900000	26
wdr1	WD repeat domain 1	Q6NY25	342040000	24
calrl	Calreticulin like	Q6DI13	301730000	25
h1fx	H1 histone family, member X	Q802U8	240540000	4
rab1a	RAB1A	Q7ZSZ0	238780000	8
clic1	Chloride intracellular channel 1	Q6NYF2	237250000	13
rps13	Ribosomal protein S13	Q6IMW6	234040000	9
arpc3	Actin related protein	Q6ZM62	224530000	9
nme2	Nucleoside diphosphate kinase	Q7SXG5	213480000	7

List of the 20 most abundant neutrophil-specific proteins identified in zebrafish marrow neutrophils. Protein abundance is shown as ion intensity. Number of peptides depicts the number of individual peptides identified for each protein.

3.1.2.2. Process and Pathway Analysis

To determine annotated functional roles of the proteins identified from neutrophils, 1009 proteins with recognized gene symbols were submitted to Gene-Go (Metacore) for functional

and network pathways analysis. Oxidative phosphorylation and immune response, including alternative complement pathways, chemotaxis and CXCR4 signaling, together with cytoskeletal remodeling, apoptosis and survival, and transport were among the 20 most prominently associated pathways (Appendix 2).

For example, C3, C3a, iC3b, C3c, C3dg, C3b, C5 convertase as well as factor B, Bp, Ba were among the identified proteins, which were significantly associated with the humoral branch of the innate immune system acting to protect the host from microorganisms (Figure 7) (Thurman & Holers, 2006).

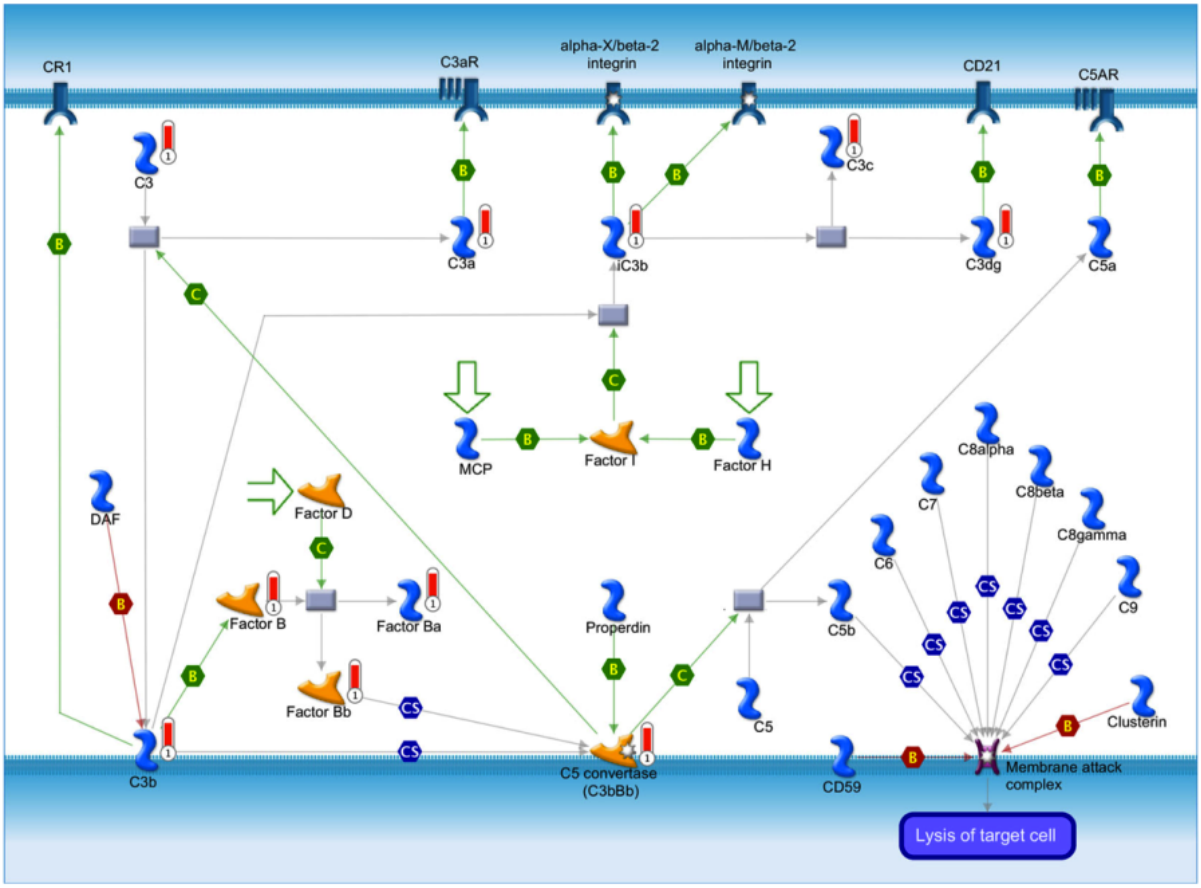


Figure 7: Complement pathway map analysis of zebrafish neutrophil proteins. *Alternative complement pathway map identified from the zebrafish neutrophil proteome. 10 different zebrafish neutrophil proteins were found associated with the alternative complement pathway. Proteins with red color thermometer bar represent the zebrafish neutrophil proteins identified in this study*

In addition, global process network analysis revealed a significant association of identified proteins with 12 different process networks (Appendix 3). A total of 67 proteins were associated with translation (initiation, elongation, termination) and 28 proteins with transcription (mRNA processing). 48 proteins were associated with immune response (30 with phagosome antigen presentation and 18 with phagocytosis). Phagocytosis is a key process of the innate immune system, in which cells engulf foreign particles or cell debris. For example, iC3b, C3, Myosin, Shp -1, Syk, Crkl, CDC42, Slp 76 and Hck and others are commonly associated with phagocytosis (Figure 8) (Flannagan *et al.*, 2012).

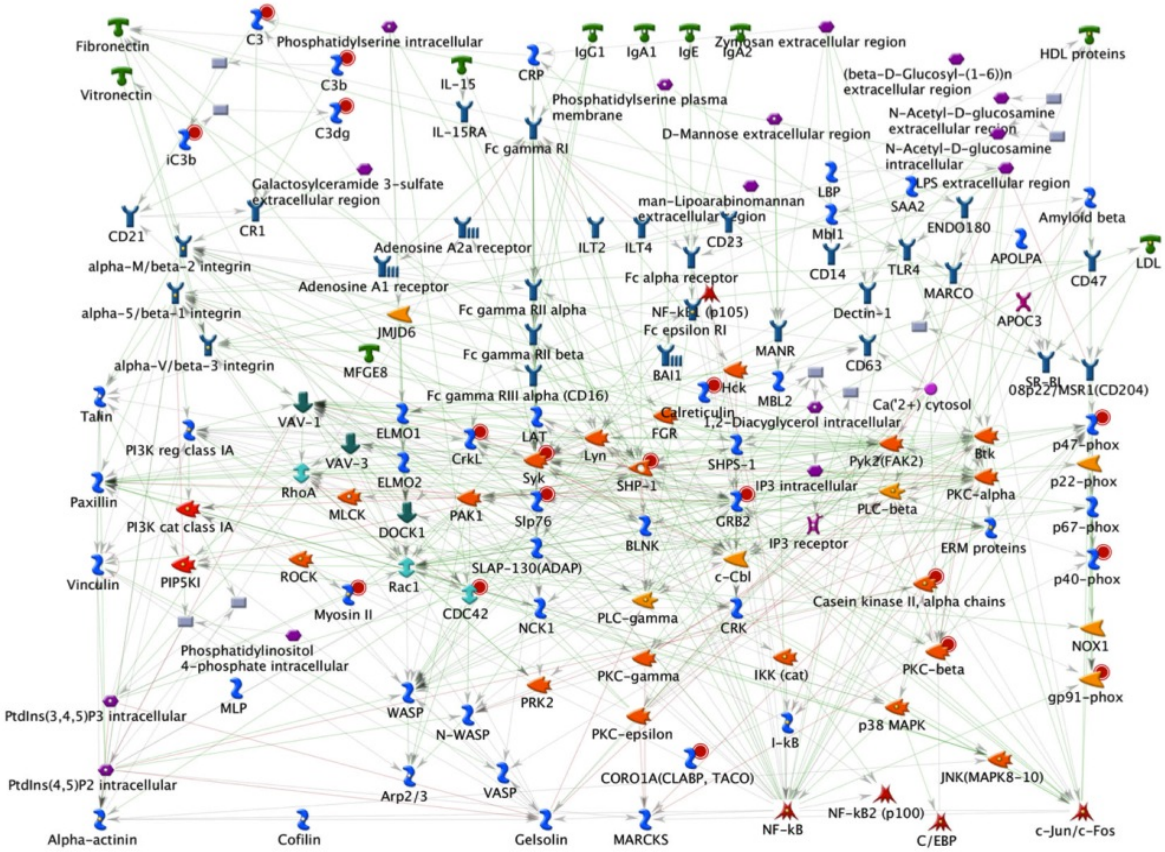


Figure 8: Phagocytosis network process analysis of zebrafish neutrophil proteins. *Phagocytosis process network identified from the neutrophil proteome dataset. 18 neutrophil proteins were found associated in this pathway. Proteins represented with a red circle represent the proteins identified in this study*

Further, enrichment by protein function analysis mapped the identified proteins into six different functional groups including enzymatic proteins, proteases, kinases, transcription factors, ligands and receptors (Table 5).

Table 5: Functional categories of zebrafish neutrophil proteins	
Classification	**Gene symbol**
Proteases	Acp1, Ppp3cc,Ptpn6, Mtmr6,Ppp1cb
Transcripation factors	Drap1, Pa2g4, Gabpa, Hmgb1, Hcfc1
Receptors	Atrn, M6pr, Ptgrc, CD41, CD82
Ligands	Lect2, Mif, Stoml2, B2m, Manf
Enzymes	Arsa, Asah1, Bdh1, Hexb, Glb1
Kinases	Csnk1a1i, Cpne3, Prkib, Slk
Phosphatases	Ppap2a, Minpp1, Nudt5, Pgaam1, Fbp1
List of the five most abundant proteins of different functional categories in zebrafish neutrophils	

3.1.2.3. Cross-species correlation between human and zebrafish resting neutrophils proteome

In lieu of protein data from human marrow neutrophils, comparison between zebrafish neutrophil dataset and a recently reported data of a whole cell lysate proteome of neutrophils isolated from peripheral blood were performed (Tomazella *et al.*, 2010). Of the 1544 proteins identified from neutrophils of adult zebrafish, only 1148 proteins were annotated with unique gene symbols (Appendix Table S2c). To maximize the number of proteins available for comparison the zebrafish proteins without unique gene symbols (395) were blasted against the human protein database at NCBI to identify orthologous proteins. Out of the 395 proteins, 89 produced hits with greater than 80% identity, 102 with 60-80% identity and 133 proteins produced hits with 40-60% identity to human proteins (Appendix Table S3a). Including the proteins identified through BLAST analysis (Appendix Table S3b), only 1472 zebrafish proteins were comparable with the human dataset. Tomazella *et al*. identified 1249 proteins, where 430 proteins were identified only from detergent soluble extracts and 240 proteins were identified only from detergent insoluble extracts of human neutrophils. 579 proteins were identified from both sample preparations. The proteome data of the here discussed zebrafish neutrophils are based on detergent soluble extracts. Therefore, the detergent soluble human and detergent soluble zebrafish neutrophil proteome data sets based on gene symbol were compared. More than 47% (471) of all proteins identified in the detergent soluble human neutrophil proteome were also found in the zebrafish neutrophil proteome (Figure 9a & Appendix Table S3c). Surprisingly, comparison of the detergent insoluble proteome of human

neutrophils and the zebrafish detergent soluble neutrophil proteome also revealed a more than 48% (386) overlap between the human and zebrafish sample preparations (Figure 9b).

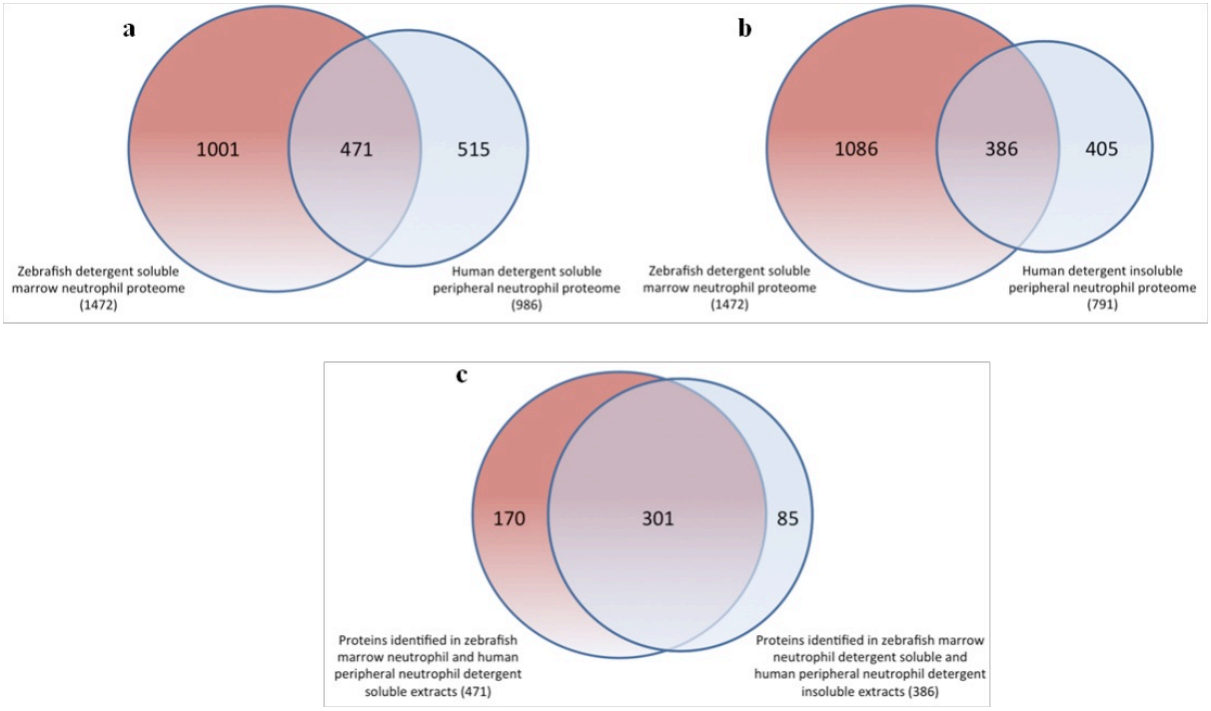


Figure 9: Cross species comparison of zebrafish and human neutrophil proteomes. *Venn diagrams comparing the zebrafish and human neutrophil proteomes (a) Comparison of zebrafish and human detergent soluble proteomes (b) Comparison of zebrafish detergent soluble and human detergent insoluble proteomes (c) A majority of proteins common between zebrafish detergent soluble and human detergent insoluble are also present in the human detergent soluble data set*

However, of 386, 78% (301) proteins were found in both, the detergent soluble and detergent insoluble human neutrophil proteome data set (Figure 9c). To perform cross-species comparison in gene ontology, the 471 proteins common between human and zebrafish detergent soluble extracts and 1002 (Appendix Table S3d) proteins found in zebrafish only but not in human neutrophils were submitted to Gene Go Metacore for functional, pathway and network analysis. Network process analysis revealed that commonly identified proteins significantly associate with 47 different network pathways, where six immune-related pathways were among the top 20 pathways (Figure 10). These include phagosome in antigen presentation, antigen presentation, neutrophil activation, amphoterin signaling, TCR signaling, TREM 1 signaling.

Figure 10: Cross-species neutrophil Gene-Go network process pathways. *Most prominent Gene-Go network process pathways associated with proteins identified in both zebrafish and human neutrophils*

In contrast, non-common proteins were significantly associated with only 20 different network pathways, with high ranking pathways such as translation, transcription, cytoskeleton regulation, cell cycle and muscle contraction, including only two immune–related pathways (rank 10 and 11) (Figure 11).

Figure 11: Gene-Go network process pathways of non-conserved neutrophil protein. *Most prominent Gene-Go network process pathways associated with proteins that were not conserved between zebrafish and human neutrophils*

Additionally, to investigate the conservation of immune related proteins between zebrafish and human neutrophils, Gene-Go Metacore analysis of both data sets was performed individually. Subsequently, the proteins specifically associated with immune system pathways were extracted and compared, revealing 54% similarity between zebrafish marrow neutrophils and human peripheral blood neutrophils. Although, proteins in both, human and zebrafish neutrophils were identified from all major compartments, the distribution to various compartments differed between the samples. In zebrafish neutrophils the percentage of nuclear proteins and proteins involved in macromolecular processes was higher than in human neutrophils. On other hand proteins from human neutrophils contained a higher proportion of proteins from plasma membrane, endosome, cytoplasm and mitochondria (Table 6).

Table 6: Comparison of human and zebrafish neutrophil proteomes based on subcellular localization

Compartment	Human	Zebrafish
Cytoplasm	19%	19%
Macromolecular complex	5%	10%
Intracellular organelles	10%	3%
Extracellular	6%	2%
Cell surface	1%	-
Plasma membrane	11%	3%
Cytoskeleton	6%	7%
Peroxisome	1%	-
Nucleus	13%	17%
Mitochondria	7%	5%
ER	4%	4%
Ribosome	-	8%
Chromosome	2%	3%
Endosome	4%	1%
Others	11%	19%

Furthermore, characterization based on biological processes revealed that in zebrafish almost twice as many proteins were associated with cellular processes compared to human neutrophils. Similarly, the fraction of proteins associated with metabolic processes was higher in zebrafish than in human neutrophils. Whereas, the numbers of proteins involved in regulation and development were similar in both species. However, human neutrophils from peripheral blood contained more proteins that are specifically associated with response to stimulus immune system processes and interaction with cells and organism than marrow neutrophils from zebrafish (Table 7).

Table 7: Comparison of human and zebrafish neutrophil proteomes based on association with biological process

Process	Human	Zebrafish
Regulation	21%	19%
Reproduction	3%	-

Response to stimulus	7%	3%
Cellular process	27%	44%
Developmental process	6%	7%
Growth	2%	-
Immune system process	5%	1%
Interaction with cells and organism	7%	2%
Localization	6%	6%
Metabolic process	8%	11%
Other	12%	8%

3.2. Proteome dynamics in neutrophils of adult zebrafish upon chemically-induced inflammation *(This chapter is taken from Singh et al., 2014, copyright (2014), with permission from Elsevier 2014)*

Result 3.1 showed that most of the neutrophil proteins were enriched in neutrophils and similar to human, zebrafish neutrophils also express certain number of immune related proteins. However, the expressions of these proteins during inflammation or disease state are not yet demonstrated. This chapter aimed to perform the proteome of zebrafish neutrophils upon sterile inflammation. Laser damage and tail fin amputation methods are routinely used to induce sterile inflammation in zebrafish larvae and adult zebrafish. However, these methods require extensive manual manipulation prone to large variability when applied at large scale. *d'Alencon, et al.,* established an interesting assay employing chemically-induced inflammation (ChIn), using copper sulphate to trigger robust sterile inflammation. Copper sulphate induces cell death of the sensory hair cells in the lateral line system of zebrafish larvae, which eventually regenerate within 24 hr after removal of the damaging agent (Hernández *et al.*, 2006; 2007). Cell death of hair cells induces rapid granulocyte recruitment to the affected tissue, a process that can be quantified using appropriate transgenic lines (Wittmann *et al.*, 2011). The ChIn assay hence, enables automated screening procedures towards identification of immune-modulatory activities of candidate compounds using zebrafish larvae. However, it is currently unknown whether the ChIn assay could also be applied in adult zebrafish. Here the applicability of chemically-induced inflammation in adult zebrafish was established and investigated the protein dynamics within neutrophils upon inflammation using label free proteomics approach. The label free method is an easy, cost

efficient and straightforward approach and it does not require labeling to determine relative amount of proteins.

3.2.1. Establishment of chemically induced inflammation in adult zebrafish

In order to assess the applicability of the ChIn assay to induce inflammation in adult zebrafish, fish of a transgenic neutrophil reporter line (lyzC:dsRed) were incubated with 25µM copper sulphate for 1hr. Subsequently, inflammation was confirmed by evaluation of neutrophil influx to the gills as fish gills are the first target organ for abiotic stress (Figure 12) (Lü *et al.*, 2013). Microscopic examination of zebrafish gill revealed that chemically induced inflammation has increased neutrophil influxes into zebrafish gill in comparison to non-treated zebrafish gill.

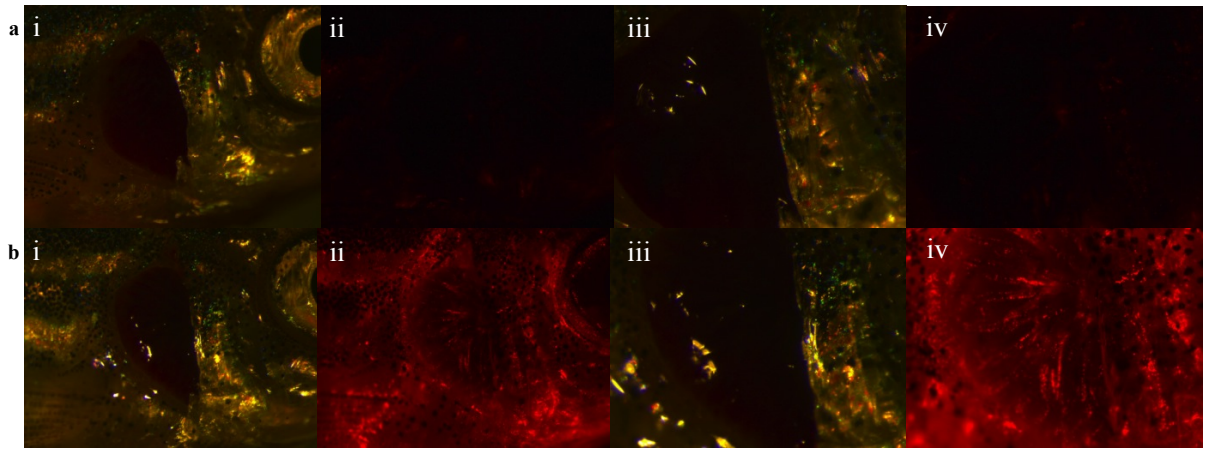


***Figure 12**: **Chemically-induced inflammation induces neutrophil influx into adult zebrafish gills.** (a) Control (untreated) gill: (i) Brightfield gill image, (ii) fluorescent image of zebrafish fish gill, (iii) close-up of (i) and (iv) close-up of (ii). (b) Copper sulphate treated gill: (i) Brightfield image of treated gill, (ii) fluorescent gill picture shows strong neutrophil influx into zebrafish fish gill after copper sulphate treatment, (iii) close-up of (i) and (iv) close-up of (ii)*

Importantly, this experiment confirmed that copper sulphate is capable to induce inflammation in adult zebrafish similar to zebrafish larvae. However, cells recruited to the gills were insufficient in number to perform further proteome or transcriptome analysis.

3.2.1.1. Chemically-induced inflammation activates neutrophils in the whole kidney marrow of adult zebrafish

As shown in chapter 3.1, a large population of granulocytes is present in adult whole kidney marrow. Therefore, I evaluated whether neutrophils within the kidney were activated upon copper sulphate treatment. To this end, neutrophil cells were sorted from inflamed and non-inflamed adult zebrafish whole kidney marrow for RNA extraction. qPCR analysis were performed to evaluate the inflammatory status of kidney marrow neutrophils. qPCR analysis showed that expression of the pro-inflammatory genes *tnfa, il6, il8, myd88, mmp9* was increased after copper sulphate treatment, while expression of the anti-inflammatory gene *tgfb* was reduced (Figure 13).

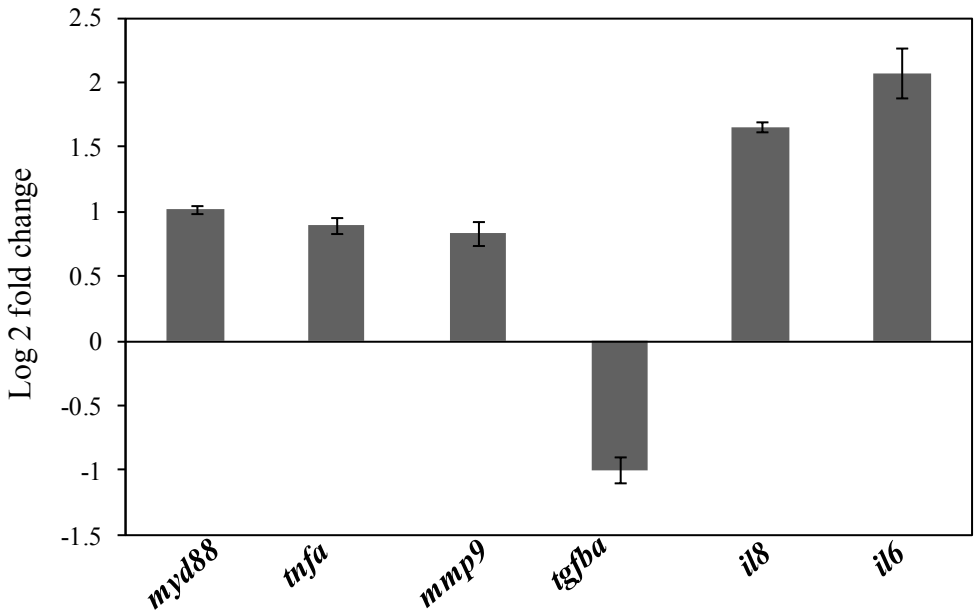


Figure 13: Chemically-induced inflammation activates the neutrophils of adult zebrafish kidney marrow*. qPCR analysis shows that chemically-induced inflammation significally increased the expression of pro-inflammatory genes (il6, il8, mmp9, myd88, tnfa) and reduced the expression of the anti-inflammatory gene tgfba*

These results indicated that chemically induced inflammation activates neutrophils residing in the whole kidney marrow of adult zebrafish enabling the use of this abundant cell population for further analysis.

3.2.2. Label free proteomics analysis to investigate chemically induced inflammation in adult zebrafish neutrophils

Transcript level analysis further validates the WKM neutrophils activation upon chemically induced inflammation. This study aimed to select suitable proteomics approach to perform quantitative proteomics. Various types of quantitative proteomics are presently used in proteomics research; advantages and disadvantages of these methods have been discussed in the introduction. Label free quantification is a method that aims to determine the relative amount of proteins in two or more samples. Contrary to other quantification method, this method does not rely on protein labeling. Therefore, it is an open method for any kind or number of protein quantification analysis. Here, label free proteomics approach was selected to investigate the effect of chemically induced inflammation on neutrophils proteome.

3.2.2.1. Inflammation-induced activation triggers changes of the protein content of neutrophils in the whole kidney marrow

To investigate the protein dynamics in whole kidney marrow neutrophils upon chemically-induced inflammation, a mass-spectrometry approach was applied. Inflamed and healthy zebrafish kidney marrows were isolated and proteins from neutrophil cells were extracted and quantified. Subsequently, 45 µg of total protein extract was separated by one-dimensional electrophoresis followed by LC-MS-MS analysis of in gel based tryptic digested peptides in triplicate. In total, more than 1500 proteins were identified with a false discovery rate of 1% at peptide level. Protein LFQ (label free quantification) intensities of inflamed and non-inflamed neutrophil samples were compared for protein quantification. A total 21 proteins detected in both, inflamed and control samples, were significantly differentially regulated between inflamed and control neutrophils ($p<0.05$). Out of 21 proteins, 14 proteins such as Ran, Rab27, Anxa2a, Psma1, Scinlb, Uqcrc2b, Serpinb1l1, Cathepsin D, Rac2, Aga, Gnai2l, Dnl and Mitochondrial ATP synthase subunit a, Mapk1 were up-regulated and 7 proteins, Tcp1, Fmo5, Gltp, Hsp90b, Ywhaqa, 60s Ribosomal protein L37a and Tubulin beta 2a, were significantly down regulated. Proteasome subunit alpha (Psma1) and Tubulin beta 2a proteins were highly influenced by chemically-induced inflammation. Their expression levels changed 2.29 fold and 0.53 fold, respectively (Figure 14, Table 8).

Table 8: Differentially regulated proteins in adult zebrafish neutrophils upon

chemically-induced inflammation

Gene symbol	Uniprot ID	Protein[a] fold change	Mol. wt	RNA[b] fold change
Psma1	Q6DGX8	2.29	29.248	3.71
Dnl	Q6P4U8	2.10	37.632	1.62
Uqcrc2b	Q6IQ59	1.72	48.122	2.40
Serpinb1l1	F1R9A9	1.51	43.457	1.93
Anxa2a	Q6P603	1.48	38.138	2.88
zgc:163069	F1R2V7	1.47	56.474	2.13
Ctsd	F1QDA2	1.45	45.28	3.40
Ran	P79735	1.42	24.46	2.7
Scinlb	A5PMZ3	1.41	79.466	1.24
Rab27	Q4V8U8	1.39	25.082	2.0
Mapk1	Q6P023	1.31	42.17	1.34
Rac2	A2BHI1	1.31	24.64	2.43
Gnai2l	Q6TNT8	1.28	40.83	1.26
Aga	A8KC00	1.28	36.20	2.24
Hsp90b	Q7T3L3	0.73	91.281	1.59
Fmo5	Q7T1D7	0.72	63.818	-
Tcp1	Q803P2	0.71	60.282	0.41
Gltp	A2BG43	0.71	23.94	-
Ywhaqa	Q7ZUM0	0.69	27.764	0.38
Rpl37a	A7YY10	0.67	10.205	0.29
Tubb2a	F1R6Y8	0.53	52.657	0.45

[a] The protein fold changes are calculated by dividing intensities of proteins from inflamed sample by intensities of proteins from control samples. With a p value < 0.05, a ratio of higher than 1.28 represents significantly up regulated and a ratio lower than 0.73 represents significantly down regulated

[b] Changes in corresponding gene expression are determined by RNA sequencing

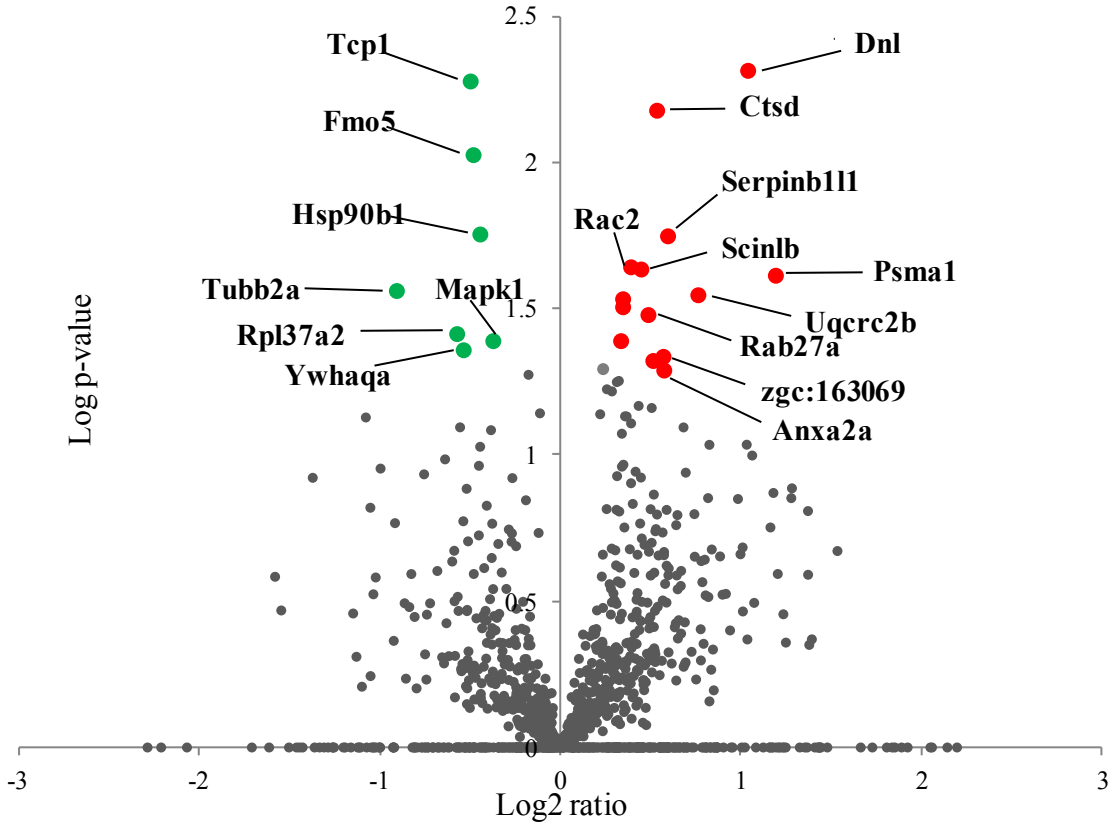


Figure 14: Differentially regulated proteins in neutrophils upon inflammation. *Green dots represent proteins significantly down regulated and red dots represent proteins significantly upregulated upon chemically-induced inflammation. P=0.05; Cut-off value 1.28*

In addition, qualitative analysis of the zebrafish inflammatory proteome was performed. To do this, certain numbers of proteins were identified, which were not detected in either the control neutrophils or the inflamed neutrophils sample. Qualitative data analysis revealed that 27 proteins were not detectable in either one of the samples in triplicate. Out of 27 proteins, 23 were only detected in neutrophils of treated fish indicating induction of their expression upon inflammation, while 4 proteins were only detected in neutrophils of control but not treated fish indicating a strong reduction of their expression level (Table 9).

Table 9: List of proteins, detected only in control or inflamed neutrophils

Gene symbol	I/C[a] 1	I/C[a] 2	I/C[a] 3	I/I[b] 1	I/I[b] 2	I/I[b] 3	RNA fold[c] change
Ca6	1429300	-	1608600	-	-	-	1.69
Hnrpkl	1433600	-	3204900	-	-	-	1.09

Mri1	2619100	-	3262200	-	-	-	0.125
Rdh12l	3222900	7135800	-	-	-	-	1.99
Ssrp1a	-	-	-	1180900	-	1106200	1.49
Phpt1	-	-	-	2022500	1577900	-	2.51
si:ch211-147j13.3	-	-	-	4218900	3364800	2503700	1.48
Acadsb	-	-	-	947540	-	1848000	1.76
Ddb1	-	-	-	2963200	-	2146200	3.18
Nipsnap3	-	-	-	2797600	2549200	-	-
zgc:153073	-	-	-	-	1191300	2104500	1.25
Arl8bb	-	-	-	12267000	-	7728200	0.96
Arl8ba	-	-	-	1095900	-	1087900	1.77
Ap2s1	-	-	-	1971900	-	3560800	-
Ubl5	-	-	-	-	3978800	2809200	-
Tia1l	-	-	-	2109800	2353800	1524200	1.15
Stk38a	-	-	-	-	2184400	1012500	0.25
Mt-atp8	-	-	-	5218300	5716400	-	0.72
Supt16h	-	-	-	1916300	-	2564000	0.95
Prkar2aa	-	-	-	-	2645900	1578700	1.37
Dbnlb	-	-	-	4656300	7060200	6870800	1.20
Zgc:101723	-	-	-	2748000	-	4767500	2.89
Si:ch211-175g6.7	-	-	-	1734500	1552100	1285400	1.08
Ppp5c	-	-	-	-	3513600	1850200	1.28
LOC560139	-	-	-	6319100	7045900	5808300	-
Nt5c1bb	-	-	-	1859500	2079400	-	0.24
Lactb2	-	-	-	2103100	1462900	-	-

[a] Intensities of proteins only identified in inflamed but not in control sample considered as up-regulated proteins

[b] Intensities of proteins only detected in control but not detected in inflamed considered as down regulated proteins

[c] Changes in corresponding gene expression are determined by RNA sequencing

To predict the biological consequence of differentially regulated neutrophil proteins, proteins were mapped for their cellular localization, biological processes and molecular functions based on STRAP gene ontology analysis. Gene ontology analysis of differentially expressed proteins revealed that the majority (48%) of proteins were localized to the cytoplasm, nucleus and cytoskeleton (Figure 15).

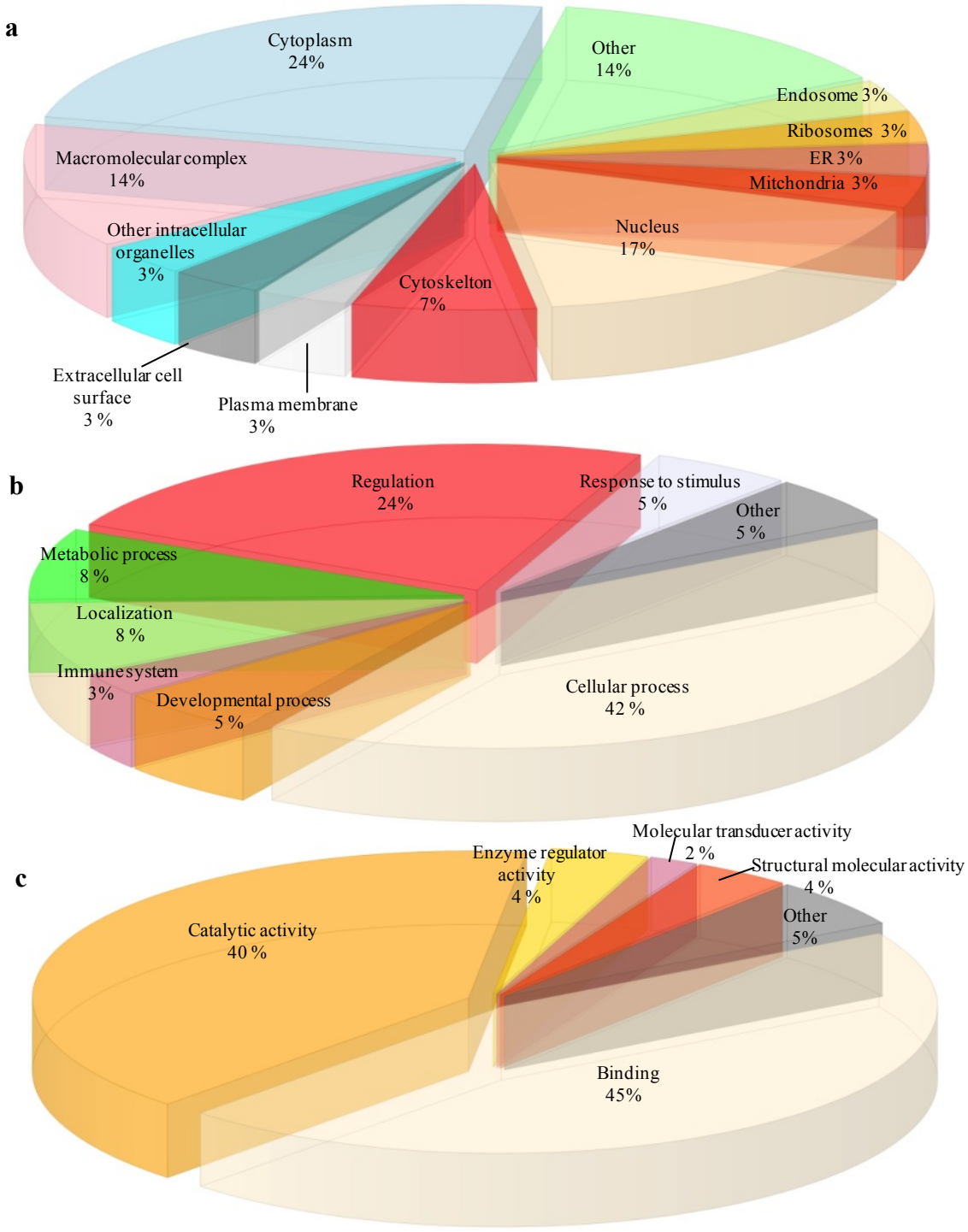


Figure 15: Gene ontology analysis of differentially expressed neutrophil proteins. *Distribution of 48 differentially regulated inflammatory proteins based on (a) localization, (b) biological process and (c) molecular function*

Most of these proteins are involved in cellular process (42%), regulation (24%), metabolic processes (8%), localization (8%), developmental processes (5%) as well as response to stimulus (5%), the immune system (3%) and other (5%). Moreover, differentially expressed

proteins are mainly associated with the molecular function of binding (40%) and catalytic activity (45%), while the remaining 15% of the proteins were associated with enzymatic regulation, structural molecular activity, molecular transducer activity and others.

Gene-Go Metacore analysis was performed to identify the regulatory pathways that differentially expressed neutrophil proteins are associated with (Figure 16).

Figure 16: List of network process pathways associated with identified differentially regulated neutrophil proteins. *Metacore analysis revealed that chemically-induced sterile inflammation in adult zebrafish neutrophils affects a broad range of biological pathways including cell cycle ($p=1.498\times10^{-4}$), nitric oxide signalling ($p=2.831\times10^{-4}$) as well as regulation of cytoskeleton rearrangement ($p=1.197\times10^{-3}$) and intermediate filaments ($p=1.196\times10^{-3}$). Moreover, differentially regulated proteins were significantly associated*

with immune system pathways such as phagosome antigen presentation ($p=3.389\times10^{-3}$), antigen presentation ($p=1.447\times10^{-3}$), leukocyte chemotaxis ($p=1.610\times10^{-3}$), and IL-6 signalling ($p=3.916\times10^{-3}$)

3.2.2.2. Comparison of inflammatory transcriptome and proteome suggests post-transcriptional gene regulation in neutrophils upon inflammation

Previous proteome and transcriptome studies showed week positive correlation between these two omes, which emphasizes the role of post-transcriptional regulation in protein translation (Jüschke *et al.*, 2013; Maier *et al.*, 2009). Therefore; in the current study, effects of chemically induced inflammation was also illustrated at transcript level and compared with proteome data set. Transcriptome analysis revealed a partial correlation to expression changes on the protein level (Table 8 and 9). For example, expression of proteasome subunit alpha increased 3.71 fold and Rpl37a changed 0.29 fold in agreement with changes observed on the protein level. However, hsp 90b was up regulated on the transcript level while down regulated on the protein level. The 27 proteins that showed no detectable signals in either one of the samples were also compared for their transcript level expression change. Comparison of transcriptome and proteome data revealed that only 44% of these genes (phpt1, srsp1a, acadsb, prkar2aa, dbnlb, sich 211-147j 13.3, arl8ba, zgc63840, zgc153073, Ppp5c, zgc101723, mri1 showed similar changes at the transcript level. 38% of these genes showed no changes of their corresponding transcripts (sich.211.175g6.7, suptl6h, tiall, arl8bb, hnrpkl, niplsnap3, ap2s1, ubl5, loc560139 and lactb2) indicating that these genes are mainly regulated on the protein level in this context. However, 18% genes (rdh12l, hnrpkl, ca6, nt5c1bb, stk38a) showed a reciprocal change of expression on the transcript and protein level.

3.3. Quantitative proteome analysis of an entire inflammatory reaction in adult zebrafish WKM neutrophils based on in vivo incorporation of stable isotopes

Inflammation plays a leading role in containing and resolving infection and also occurs under sterile conditions. Inflammation must be resolved in due time in order to prevent the inflammation to become chronic. In chapter 3.2, have shown that copper sulfate treatment to adult zebrafish activated whole kidney marrow neutrophils similar to zebrafish larvae and altered the expression of 48 genes on protein and RNA levels. However, there are no reports of inflammation kinetics or inflammation resolution in adult zebrafish. Copper sulfate treatment to zebrafish larvae induces granulocytes recruitment to the affected tissue

(mechano-sensory hair cells) within one hour (initiation phase). Resolution starts three hr post inflammation and the inflammation is almost resolved after 6 hr. In order to perform proteome analysis over the duration of an inflammatory reaction in adult zebrafish I here investigated the kinetics of inflammation in adult zebrafish and defined different time points of inflammation in adult zebrafish neutrophils.

In chapter 3.2, it has been explained that the label free method is an easy, cost efficient and straightforward approach to investigate inflammatory proteome analysis of whole kidney marrow neutrophils. The primary limitation, however, is that every sample needs to be handled separately from sample preparation to mass spectrometry analysis. Therefore, all steps starting from sample preparation to PMF data acquisition are prone to variations in protein quantification. Thus, protein quantification using the label free method frequently required several replicates and very consistent sample preparation. Furthermore, in the absence of highly sensitive mass spectrometry it would be very challenging to apply this method to investigate the inflammatory proteomics analysis of WKM neutrophils at different stages of inflammation.

Stable isotope labeling with amino acid in cell culture (SILAC) is a simple and robust method for accurate protein quantification. The SILAC method is widely adopted in other model organisms including mice, newts, and nematodes. SILAC is a precise and accurate quantification method in comparison to the label free approach because it can be spiked in each sample as an internal control at early stages of sample preparation. This reduces variable sample variations from each experiment step. Therefore, this section describes the establishment of SILAC labeling in adult zebrafish to investigate the proteome of an entire inflammatory reaction.

3.3.1. Chemically induced inflammation starts resolving two hr post-inflammation

Results of chapter 3.2 showed that chemically induced inflammation activates neutrophils residing in the whole kidney marrow of adult zebrafish and alters the expressions of various genes at protein and RNA level. It was investigated whether the inflammation kinetics in adults would be similar to the known kinetics in zebrafish larvae upon chemically induced inflammation in adult zebrafish WKM neutrophils based on qPCR analysis.

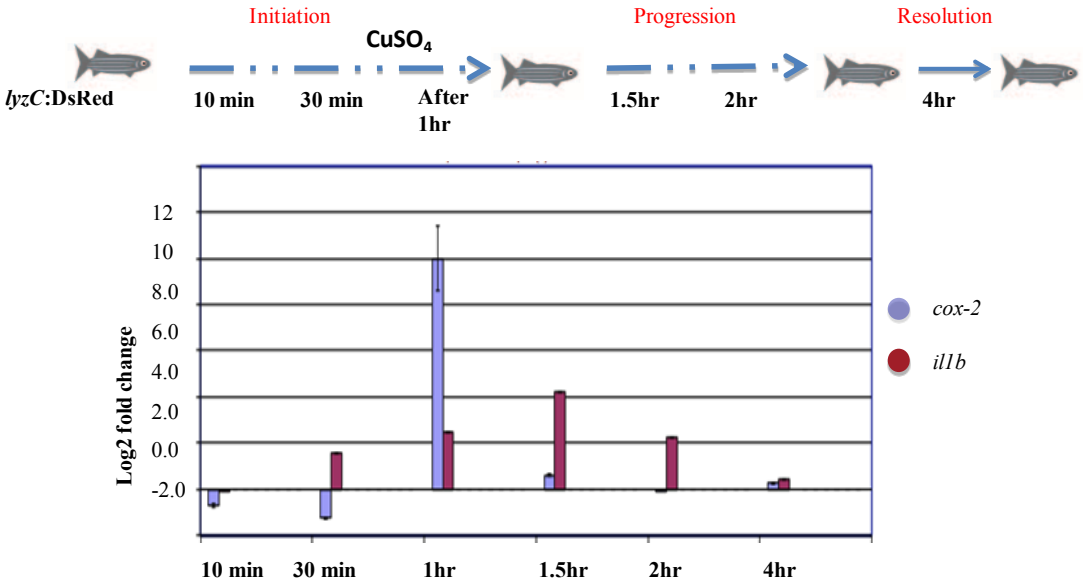


Figure 17: Chemically induced inflammation gradually starts resolving after four hr of inflammation. *qPCR analysis shows that chemically-induced inflammation activates neutrophils 30 minutes after treatment and 4hr after treatment inflammation gradually resolved*

To perform this, a neutrophil reporter line of adult zebrafish was treated with copper sulfate for 1 hour and subsequently the fish were transferred into normal fish water for up to 8 hr. The fish were then anesthetized and the kidney marrow was extracted. Neutrophil cells were separated FACS sorting. To obtain an overview of the inflammation kinetics, two genes were selected for qPCR analysis. The qPCR analysis of inflammatory marker genes revealed that four hr after copper sulfate treatment the level of inflammation decreasing almost to base levels indicating resolution of granulocytic inflammation (Figure 17). On the basis of these observations, five stages of inflammation including initiation (t=0 and t=1 hr), progression (t= 2hr) and resolution (t=4hr and 8hr) of were selected in order to perform quantitative proteomics of adult zebrafish neutrophils.

3.3.2. SILAC based proteomics analysis of chemically induced inflammation in adult zebrafish neutrophils

SILAC is a simple and robust in vivo labeling method that can be applied to study and analyze cells at multiple time points. Conventionally, SILAC has been used for two or three

different states from the same tissue protein. Here, whole myeloid population proteins were spiked-in into single cell neutrophil proteins and analyzed inflammatory proteome changes.

3.3.2.1. SILAC diet administration fully labels WKM proteins of adult zebrafish

SILAC labeling in Zebrafish has been elaborated in introduction. Knozer *et al*., had established SILAC labeled fish and achieved an average more than 95% labeling. However, they did not investigate the labeling efficiency in zebrafish whole kindey. Zebrafish whole kidney marrow is a major hematopoietic organ of zebrafish where majority of neutrophils reside. Therefore, in collaboration with Kruger lab, SILAC labeling efficiency in adult zebrafish WKM was analyzed. To test the SILAC based quantification method in zebrafish, equal amount of labeled (heavy) whole kidney marrow protein lysate was mixed into unlabeled whole kidney marrow protein lysate. Samples were digested in solution with the protease LysC and analyzed with LC-MS/MS on a hybrid quadrupole Orbitrap instrument (QExacative). As SILAC tissue was used as an internal standard, the direct ratio between labeled and non-labeled tissue was calculated. Direct comparison of SILAC ratios between replicates revealed Pearson correlations (r) more than 0.94. Whole kidney marrow proteomics analysis has revealed that similar to other organs zebrafish whole kidney marrow has also achieved more than 94% labeling. Therefore, SILAC quantification in zebrafish provides the same statistical accuracy as demonstrated for cell culture and other living animals.

3.3.3. Quantitative proteome analysis of inflammation kinetics

Once more than 94% SILAC labeling in zebrafish whole kidney marrow was achieved. My aim was to adopt this method to compare proteome dynamics of inflammation kinetics from initiation (1-hr), progression (2-hr) to resolution (4-hr, 8-hr) of adult zebrafish whole kidney marrow neutrophils. To this end, (*Tg(lyzC:DsRED2)nz50*) zebrafish neutrophil reporter line, which express the red fluorescence protein under the control of the lyzC promoter was employed. Neutrophil reporter lines of adult zebrafish were treated with 25 µM copper sulfate for 1hr and fish were transferred to normal fish water up to 8 hr. Subsequently, FACS analysis was performed to separate the fluorescent neutrophils cells. Significantly, more than 95% pure neutrophils proteins were extracted from all the time points. However, zebrafish SILAC labeled neutrophils reporter line was not available. Therefore, this study aimed to sort the whole myeloid population of the SILAC zebrafish. As it has been previously described, a

myeloid lineage consists of monocytes, neutrophils, eosinophils. Neutrophils are one of the most abundant polymorphonuclear leukocytes.

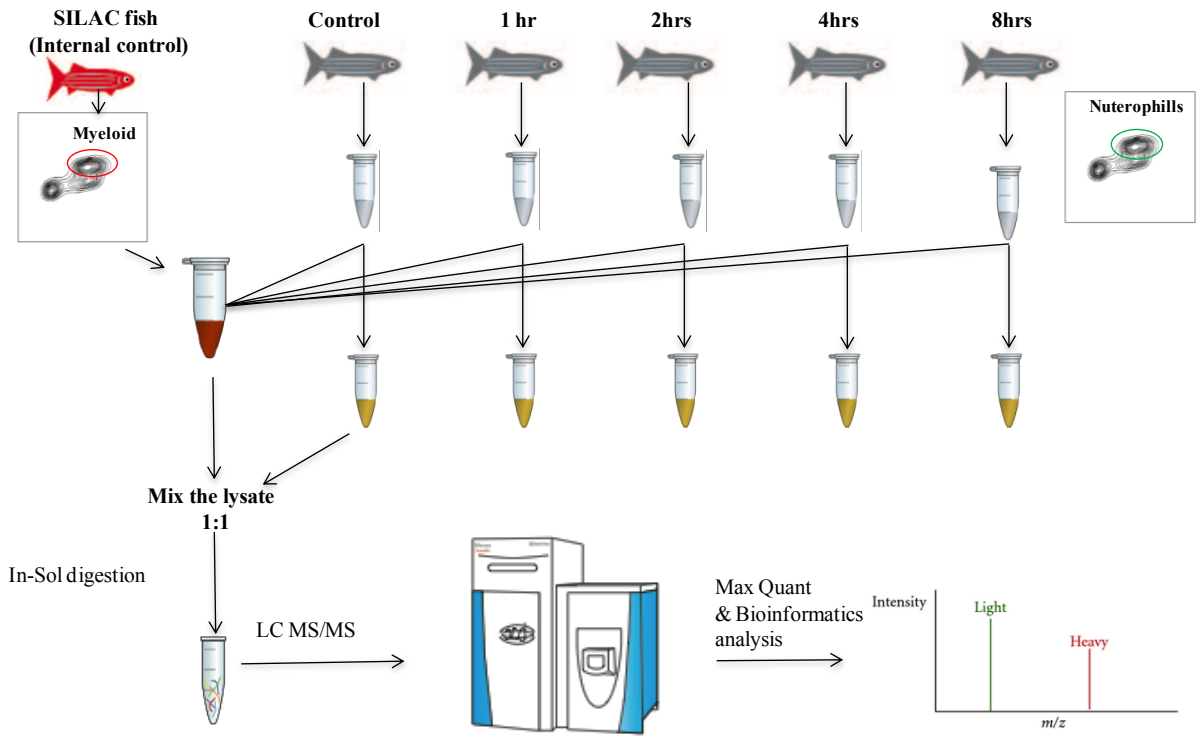


Figure 18: Experiment procedure for SILAC Spike-in into control and various stages of inflamed neutrophils samples and mass spectrometry analysis. *Proteomic workflow using SILAC labeling and mass spectrometry analysis. First fish was incubated with copper sulphate for 1hr and subsequently fish were transferred into fish water (upto 2hr, 4hr and 8hr) WKM were extracted and neutrophil cells were sorted based on FACS. For SILAC fish, whole myeloid cells population was sorted. Equal amount of SILAC proteins were mixed in to different time point of neutrophils proteins and subsequently proteins were digested and UHPLC-QExcative mass spectrometry was performed*

Approximately one million myeloid cells from SILAC fish were isolated and subsequently proteins were extracted. After protein extraction, equal amounts of SILAC standard myeloid cells proteins were mixed into each (0hr, 1hr, 2hr, 4hr, 8hr) non-labeled neutrophil proteins sample, which was obtained from transgenic neutrophil reporter line of adult zebrafish whole kidney marrow. Further samples were digested in solution with protease LysC and analyzed with LC-MS/MS on a hybrid quadrupole Orbitrap instrument (QExacative) (Figure 18). A total of 10 samples were measured and each sample was measured with four-hour gradient. Subsequently, PMF data were submitted to MaxQuant software tool against protein database

of zebrafish to identify proteins. In total more than 2000 proteins were identified with false discovery rate of 1%. As neutrophils proteins were spiked-in with SILAC proteins from zebrafish myeloid cells as internal standard, direct fold change of proteins were computed by comparing SILAC ratio of control vs SILAC ratio of neutrophil inflamed proteins (Figure 19). The data analysis with MaxQuant and Perseus allowed to quantify a total 692 proteins, of these, 61 proteins were significantly (p=0.05) differentially regulated.

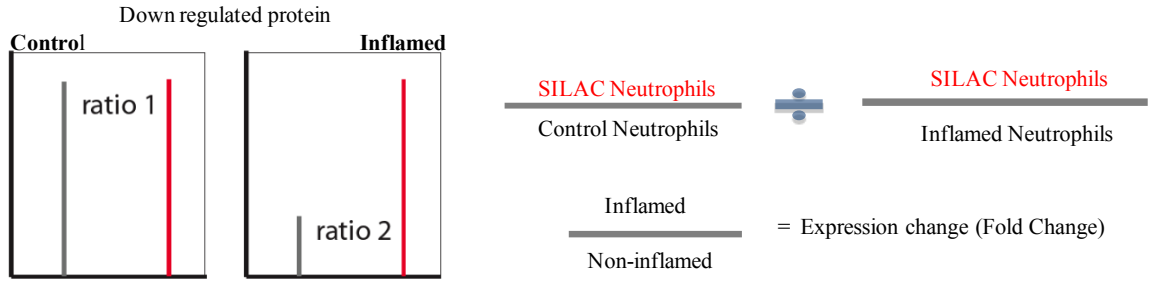


Figure 19: Determination of proteins expression based on SILAC mixture. *This is an example for calculating expression change between two samples based on SILAC (internal standard).* ***.*** *Grey peak represents the intensity of neutrophil proteins, whereas red peak denotes the intensity of SILAC labeled proteins*

3.3.3.1. Differentially regulated proteins in neutrophils upon chemically induced inflammation

In total, 61 proteins were detected that were up or down regulated with fold change of 1.5. Out of the 61 proteins, 20 proteins showed more than 1.5 fold up-regulation and 15 proteins showed 1.5 down regulation at each time point from 2hr to 8hr of post chemically induced inflammation. This observation indicates that inflammation progresses significantly during intermediate stage of inflammation kinetics. Among the 61 proteins differentially quantified from neutrophils, 12 proteins were always up or down regulated.

3.3.3.1.1. Chemically induced inflammation increases the expression of various neutrophils proteins

A heat map demonstrating the dynamics of protein expression in the neutrophils upon inflammation at different time points are shown in figure 20. There were three major expression patterns observed in up regulated proteins, these include proteins that always stays up regulated during inflammation initiation, progression and resolution such as; Si:dkey-

32n7.4, Stx11a, Si:ch211-15d5.5 and Zgc:110425 and Ckbb. On the other hand, there were group of proteins including Doc8, Ctsb and Cand1, whose expression increase initially and gradually decline 4 hr of post inflammation. This trend of expressions showed that these proteins might be involved in inflammation initiation. Finally, certain numbers of protein were also identified; those expressions were increased after 4hr and 8hr of inflammation (Table 10).

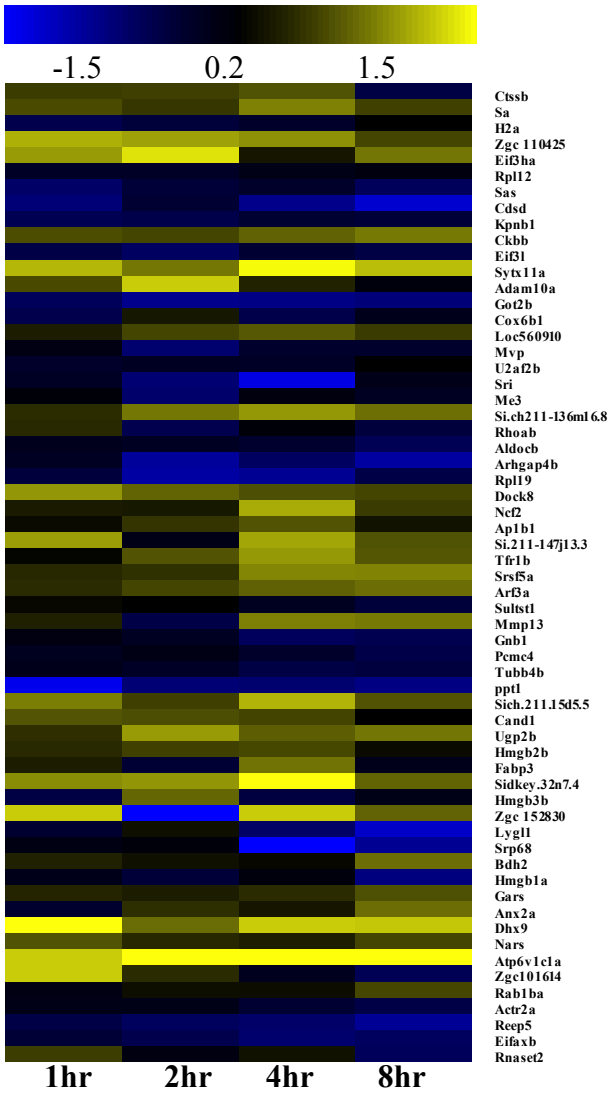


Figure 20: Chemically induced inflammation altered the expression of various neutrophil proteins. *Differentially regulated proteins were mapped in to multiple experiment viewers (MEV) tool to visualize the expression of same proteins at different time points. Blue represents down regulated protein and yellow represents up regulated proteins. This analysis showed that chemically induced inflammation induced the expression change of various proteins*

3.3.3.1.2. Chemically induced inflammation reduces the expression of neutrophils proteins

In addition to up-regulated proteins, I have also observed three different expression patterns of down regulated proteins. There were five proteins in neutrophils including Got2b, Rpl19, ppt1, Reep5 and Eif1axb; exhibiting a consistently reduced expression during inflammation initiation, progression and resolution. Furthermore, there were a group of proteins such as Sri, Mvp, Cox6b1, Elf3l, Me3, whose expression gradually decreased from 2 hr to 4 hr and later steadily start towards resolution. Lastly, It has been observed that maximum 22 proteins expression were reduced 8hr of post inflammation. These trends of protein expression from 4 hr and 8 hr demonstrate that these proteins might be key molecule players for inflammation resolution (Figure 20 and Table 10).

Table 10: Differentially regulate neutrophil proteins upon chemically induced inflammation:

Protein IDs	Gene names	Fold change[a]			
		1hr	2hr	4hr	8hr
Q502A6	Ctssb.1	0.365	0.377	0.474	-0.408
E7FAW6	LOC100331639	0.438	0.338	0.689	0.389
Q4V914	H2afy2	-0.453	-0.361	-0.264	0.010
F1R314	Zgc:110425	0.889	0.826	0.753	0.414
Q6AXJ2	Eif3ha	0.800	1.084	0.133	0.637
Q6DRE6	Rpl12	-0.248	-0.243	-0.139	-0.086
E9QGG4	LOC100000469	-0.583	-0.347	-0.270	-0.518
Q8AWD9	Ctsd	0.647	-0.311	-0.738	-1.021
F8W2W2	Kpnb1	-0.477	-0.437	-0.304	-0.352
Q8AY63	Ckbb	0.451	0.412	0.547	0.656
Q7T2A5	Eif3l	-0.421	-0.542	-0.319	-0.413
F1QX22	Stx11a	0.916	0.649	1.172	0.941
F1QCG3	Adam10a	0.436	NaN	0.214	-0.069

F1QCD4	Got2b	-0.513	-0.750	-0.715	-0.662
Q6DH63	Cox6b1	-0.453	0.142	-0.441	-0.160
E7F5A4	Loc560910	0.184	0.406	0.501	0.360
E9QEQ6	Mvp	-0.108	-0.608	-0.282	-0.282
Q6P022	U2af2b	-0.266	-0.220	-0.237	0.014
F1R4I7	Sri	-0.230	-0.628	-1.098	-0.159
A4QPA0	Me3	-0.038	-0.589	-0.086	-0.219
E7FDL3	Si:ch211-136m16.8	0.277	0.648	0.786	0.607
Q6DHM9	Rhoab	0.257	-0.461	-0.038	-0.365
Q8JH70	Aldocb	-0.186	-0.225	-0.293	-0.493
E7FEI6	Arhgap4b	-0.223	-0.809	-0.548	-0.830
Q6P5L3	Rpl19	-0.362	-0.839	-0.775	-0.421
F1QJ79	Dock8	0.779	0.559	0.460	0.414
F1QBW0	Ncf2	0.169	0.153	0.868	0.358
A5PMS9	Ap1b1	0.060	0.316	0.481	0.114
F1QES8	Si:ch211-147j13.3	0.812	-0.141	0.845	0.479
E7F131	Tfr1b	0.027	0.483	0.793	0.493
Q6TGT9	Srsf5a	0.251	0.306	0.708	0.700
Q6DHP3	Arf3a	0.272	0.406	0.544	0.604
F1QBS7	Sult2st1	0.036	-0.004	-0.209	-0.354
F1QCX8	Mmp13a	0.207	-0.417	0.685	0.655
Q6PH57	Gnb1	-0.101	-0.216	-0.525	-0.472
B0R1D0	Psmc4	-0.194	-0.136	-0.264	-0.425
Q6IQJ2	Tubb4b	-0.169	-0.236	-0.412	-0.373
F1R8J6	Ppt1	-1.138	-0.639	-0.615	-0.704
Q1LYB6	Si:ch211-15d5.5	0.676	0.380	0.906	0.484

Q5TYZ5	Cand1	0.487	0.455	0.403	0.009
F1QVK3	Ugp2b	0.288	0.798	0.535	0.638
Q66IB6	Hmgb2b	0.256	0.387	0.428	0.055
Q8UVG7	Fabp3	0.183	-0.323	0.627	-0.165
F1QSJ9	Si:dkey-32n7.4	0.738	0.776	1.292	0.559
F1Q895	Hmgb3b	-0.413	0.565	-0.390	-0.144
F1QPX6	Zgc:152830	NaN	-1.381	NaN	0.552
Q6DH80	Lygl1	-0.298	0.090	-0.571	-0.994
F1QI99	Srp68	-0.114	-0.044	-1.678	-0.773
Q561X9	Bdh2	0.208	0.100	0.036	0.603
Q6NX86	Hmgb1a	-0.159	-0.346	-0.069	-0.687
F1QUV7	Gars	0.232	0.184	0.273	0.463
Q6P603	Anxa2a	-0.289	0.295	0.135	0.604
B0S6Z1	Dhx9	1.609	0.598	NaN	0.977
F1R2L4	Nars	0.473	0.252	0.192	0.411
Q7T385	Atp6v1c1a	1.123	1.343	1.621	1.782
Q5U3U8	Zgc:101614	NaN	0.272	-0.191	-0.490
F1R541	Rab1ba	-0.109	0.077	0.074	0.415
F1QF15	Actr2a	-0.144	-0.140	-0.314	-0.422
Q6PBX9	Reep5	-0.415	-0.530	-0.586	-0.782
E9QJN5	Eif1axb	-0.338	-0.451	-0.604	-0.537
B8XY56	Rnaset2	0.370	-0.104	0.106	-0.515

[a] The protein fold changes are calculated by dividing SILAC ratios of proteins from inflamed sample by SILAC ratios of proteins from control samples. With a *p value* < 0.05, a log ratio of higher than 0,30 represents significantly up regulated and a log ratio more than -0,30 represents significantly down regulated

NaN means not identified in duplicates

3.3.3.2. Bioinformatics analysis reveals that differentially regulated proteins are enriched in various GO terms

As the zebrafish data base had poor annotation compared to other mammalian databases and because various identified differentially regulated proteins were unassigned or uncharacterized, Uniprot ID of identified differentially regulated proteins were converted to NCBI accession id and BLAST against human protein data base. BLAST analysis revealed that most of the differentially regulated proteins show more than 60% similarity with human proteins. Only few identified differentially regulated zebrafish neutrophils proteins showed less than 60% similarity against human protein database.

To obtain a better in-sight into differentially regulated proteins of neutrophils upon chemically induced inflammation, GO terms analysis was performed. Based on STRAP GO analysis tool the differentially regulated proteins were analyzed for their localization, process and molecular function. Localization of the 61 identified differentially regulated neutrophils proteins was examined. GO term analysis showed that approximately 60% of the differentially regulated proteins were localized to nucleus (21%), cytoplasm (16%), macromolecular process (11%) and others. The rest of the differentially expressed proteins were approximately equally localized to other organelles such as, mitochondria, ER chromosome, cytoskeleton, extracellular and intracellular organelles and plasma membrane. In addition, GO analysis of the differentially regulated proteins for their molecular function revealed that majorly differentially expressed proteins were associated with cellular processes (33%), regulation (24%), developmental processes (11%), location and immune system and interaction with cells and organism (4%). Important molecular functions associated with differentially regulated proteins were catalytic activity (47%), binding (35%) and structural molecular activity (11%) (Figure 21).

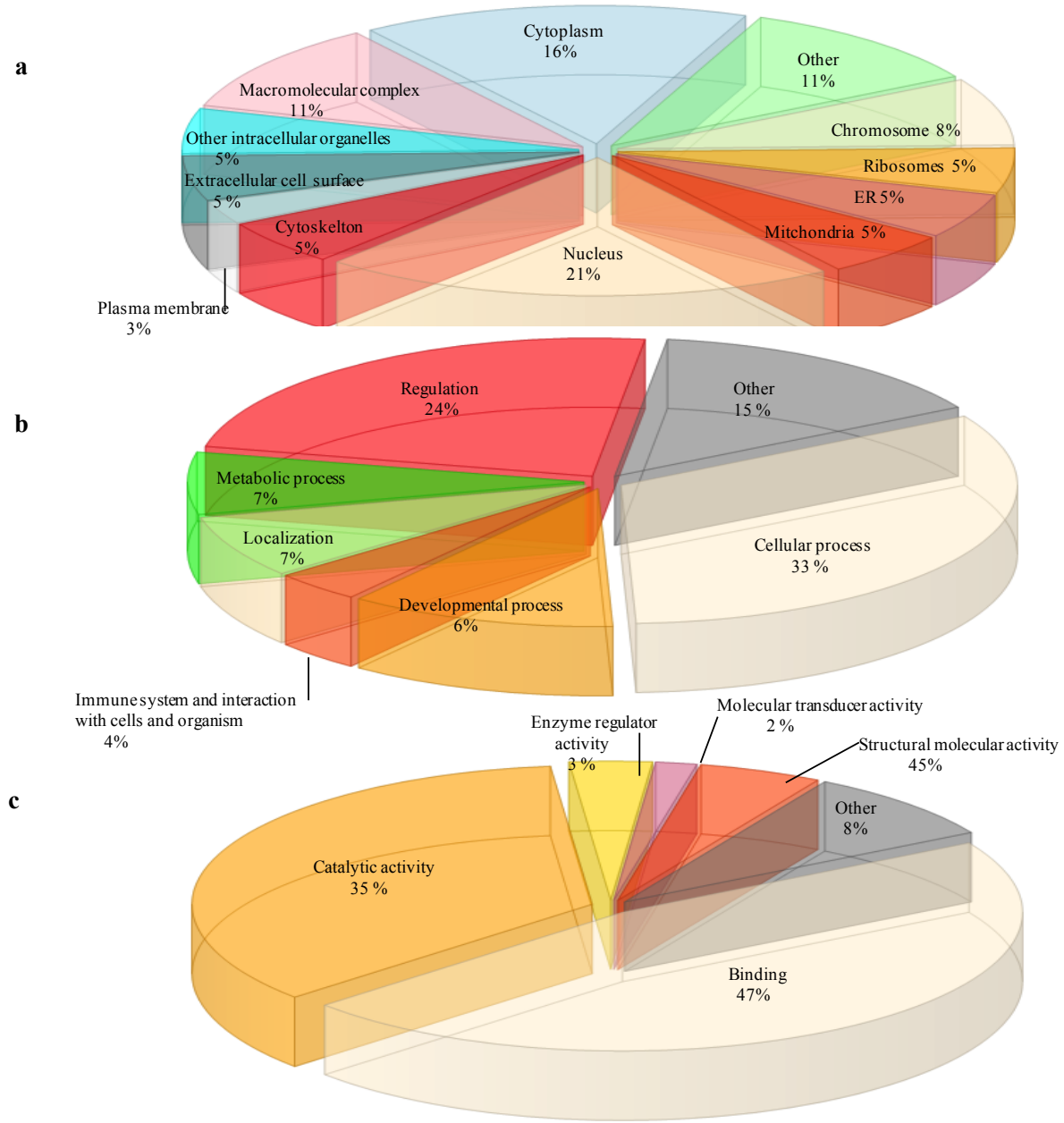


Figure 21: Majority of differentially regulated neutrophils proteins show homology with humans. *Distribution of 58 differentially regulated inflammatory proteins based on (a) localization, (b) biological process and (c) molecular function*

3.3.3.3. Functional characterization of differentially regulated identified neutrophils based on *In-silico* analysis

Of the 61 proteins, 58 proteins showed more than 60% similarity with human protein database. To explore the possible biological relevance of the identified 58 significant differentially regulated proteins, Cytoscape based *In-silico* analysis was performed. Genes

name of differentially regulated proteins were submitted into BiNGO supported by Cytoscape. Differentially regulated inflammatory proteins data were analyzed for their molecular and cellular function. BiNGO analysis showed that differentially regulated proteins were associated with various biological pathways such as: cellular molecular assembly (p= 0.0000035), cellular compartment assembly (0.00004), microtubule based movement (p=0.00042), cellular process (p=0.0028), cell migration (p=0.0029) as well as cell motility (p=0.0037) macrophage chemotaxis (p=0.0043) and proteins metabolic process (p=0.0042) determined by significant p=0.05 value.

A maximum 18 proteins including Me3, U2af2b, Ap1b1, Zgc:153264, Zgc:110425, Zgc:55461, Got2b, Actr2a, Ppt1, Stx11a, Tfr1b, H2afy2, Eif1axb, Zgc:123292, Rpl12, Rhoab, Kpnb1 and Mmp13a were most significantly (p=0.00284) associated with cell process pathway. In addition, Psmc4, Eif1axb, Ctsd, Ppt1, Actr2a, Ctssb.1, Rpl12, Tfr1b, Mmp13a differentially regulated inflammatory proteins were associated with protein metabolic process. Interestingly, many proteins play an important role in numerous pathways such as: cell motility, leukocytes and macrophage chemotaxis as well as localization of cell**.**

3.3.4. Comparison between label free and SILAC quantitative proteomics approach

To select the best-suited method to investigate an inflammatory proteome of limited number of neutrophils cells sorted from zebrafish whole kidney marrow two proteomics method were applied. . Both methods have showed that chemically induced inflammation altered the neutrophils proteome. However, comparison between label free and the SILAC approach revealed that label free method allowed quantifying less number of proteins in compare to SILAC method. Approximately, 60% proteins were commonly identified in the both methods, and overlaps of differentially regulated proteins were very less (20%) between two methods. In addition, methodology comparison of both approach revealed that label free approach take 5 days to analyze sample in triplicate, which was just analyzed within 24 hr by SILAC approach. Comparison of the both methods has been elaborated in discussion section.

4. Discussion

Zebrafish offers several advantages as an inflammatory model system compared to the mammalian inflammatory model system. As in humans, neutrophils are the most abundant innate immune cells also in adult zebrafish. However, it was not clear whether the zebrafish neutrophil is comparable to higher vertebrate neutrophils, and whether zebrafish neutrophils have similar defense mechanisms to those known from mammals. The application of proteomics for the characterization of neutrophils bears strong potential towards a better understanding of neutrophil biology.

4.1. Proteome profile of resting neutrophils

In this thesis, a comprehensive analysis of the proteome of neutrophils from the whole kidney marrow, the major hematopoietic compartment, of adult zebrafish was performed. A gel-based LC-MS/MS approach was employed to explore the proteome of FACS sorted neutrophils and identified 1544 proteins expressed in neutrophils. Subsequently, to identify proteins that may be particularly enriched in neutrophils, comparison between neutrophil proteomedata with the only publicly available proteome data of healthy brain of zebrafish was carried out. The healthy brain is expected to be devoid of neutrophils and the immune component present in the brain is comprised of microglia only (Svahn *et al.*, 2012). The proteome composition of wild zebrafish may not be entirely identical to the laboratory strain used in this study. However, the comparison with non-granulocytic tissue revealed that more than 75% of the proteins were enriched in neutrophils versus zebrafish brain tissue. Further, functional characterization of these proteins revealed that the majority of these proteins belong to various immune-related pathways such as alternative complement pathways, chemotaxis and CXCR4 signaling and inflammation.

4.1.1. Cross species conservation of neutrophils proteins and functions

In order to evaluate the degree of cross-species conservation, a comparative analysis between the identified neutrophil proteome of zebrafish and a recently published data set of human neutrophils was performed (Tomazella *et al.*, 2010). Of 986 proteins identified from the detergent soluble fraction of the human neutrophil proteome, only 47% (471) proteins were also present in the zebrafish neutrophil data set evaluated in this study. As expected, proteins common between human and zebrafish display significant association with various distinctly

immune-related pathways including neutrophil activation, antigen presentation and phagocytosis. Interestingly, 386 proteins were also found to be common between the zebrafish neutrophil proteome and the human detergent insoluble neutrophil proteome. However, only 85 proteins were actually specific to the human detergent insoluble neutrophil proteome, while the majority of proteins identified in the detergent insoluble fraction were also present in the detergent soluble fraction of human neutrophils. Analysis of the annotated subcellular localization of these proteins showed that only about 2% are membrane associated proteins, while the majorities were cytoplasmic or nuclear proteins indicating technical rather than biological reasons for the overlap of proteins between detergent soluble and detergent insoluble fractions. Among proteins conserved in zebrafish and human were many with well-characterized roles in the immune system such as L-plastin, Ferritin and S100. For example, Ferritin plays a pivotal role in iron homeostasis (Arosio & Levi, 2002) and restricts iron availability to microorganisms (Xiong *et al.*, 2011). The S100 protein is a calcium-binding protein (Anton Hermann, 2012) involved in various processes of the immune system such as leukocyte chemotaxis and adhesion (Foell & Roth, 2004; Ryckman *et al.*, 2003). Moreover, several members of the Rab protein family, heat shock proteins and histones were also identified. Rab proteins are known to regulate membrane trafficking. For example, Rab 5 plays a significant role in chemoattractant receptor endocytosis and fusion of intracellular granules with phagosomes in human neutrophils (Perskvist *et al.*, 2002). Similarly, some members of the HSP family such as HSP60, HSP70, HSP90 stimulate the cells of the innate immune system and thus act as danger signaling molecules during inflammation (Wallin *et al.*, 2002). Particular histones, along with DNA and antimicrobial proteins, are an important component of neutrophil extracellular trap (NETs) (Lu *et al.*, 2012). Moreover, WD repeat domain 1 (Wdr1), sorting nexin (Snx5), sorcin (Sri) proteins were also found in zebrafish resting neutrophils, whose functions are not well established in immune defense but may nevertheless be critical. For example, sorcin is a calcium binding protein involved in intracellular Ca^{2+} homeostasis and is expressed in leukocytes and lymphocytes (Rebhan *et al.*, 1997). Sorcin may thus be important for inflammation through its calcium regulatory function as calcium is an important mediator in innate immunity (Clapham, 2007). Similarly, Sorting nexin 5 is a intracellular trafficking protein and may play a vital role in modulation of secretory pathways for controlling cytokines and inflammation (Stow & Murray, 2013). The Wdr 1 protein promotes cofilin-mediated actin filament disassembly (Kato *et al.*, 2008).

Hence, Wdr1 may be important for immune cell migration during inflammation (Bravo-Cordero *et al.*, 2013).

4.1.2. Non-conserved proteins and technical differences between two data sets

The non-conserved proteins on the other hand were typically associated with translation, transcription, cytoskeletal remodeling and cell cycle. Neutrophils are cells of the myeloid lineage originating from marrow tissue and residing as immature or inactive cells in bone marrow (mammalian) or kidney marrow (zebrafish). The human proteome data used in this study is derived from peripheral blood neutrophils, while the zebrafish proteome studied here represents marrow neutrophils. Therefore, the limited degree of conservation between the human and fish proteomes analyzed here may in part be due to the differing nature of the samples. Kidney marrow neutrophils consist of developing and mature neutrophils, while peripheral blood neutrophils consist of functionally mature neutrophils only. In addition, although many aspects of the human and zebrafish hematopoietic systems are similar, differences do exist. For example the human hematopoietic system contains three types of granulocytes, neutrophils, eosinophils and basophils. This distinction is less clear in zebrafish, where a distinct basophil type has not been characterized yet (Crowhurst *et al.*, 2002). Thus it is conceivable that zebrafish neutrophils may combine functional repertoires that may be separated into multiple cell types in humans. Moreover, although there is no evidence that the $lyzC^{+}$ granulocyte population used in this study contains other cell types than neutrophils, different maturation states may be present. Protein identification strongly depends on subcellular protein distribution and the extraction method as well as sensitivity and precision of the mass-spectrometry approach used. This data suggest that extraction method used in this study or MS approach yielded more nuclear proteins at the expense of membrane- and cell surfaces proteins as compared to the human data set. However, because similar extraction and buffer conditions as well as a similar MS approach were applied for both, human and zebrafish samples, the observed variations in subcellular protein distribution and content are likely due to differences in neutrophil differentiation, maturation or activation. Thus, deeper analyses of compartment-specific proteomes of matched sample types will likely result in higher yields and better comparability regarding cross-species conservation between zebrafish and human neutrophil proteomes.

Nevertheless, cross comparison of immune related proteins of zebrafish and human revealed a conservation of more than 54% between zebrafish and human proteome data sets. In light of the sample differences mentioned above, this degree of similarity suggests a considerable conservation between the two species and warrants more detailed analyses in the future. To further investigate the similarity between zebrafish and human innate immune systems on a functional level, it is interesting to compare protein dynamics upon activation of zebrafish neutrophils through infection or upon sterile inflammation.

4.2. Proteome dynamics in neutrophils of adult zebrafish upon chemically induced inflammation

In chapter 3.2, it has been showed that chemically induced inflammation activates whole kidney marrow neutrophils in adult zebrafish, significantly changing the expression levels of several proteins. Interestingly, comparison of protein and transcript data revealed only partial correlation between changes in protein and mRNA levels. Such inconsistency indicates differences in genetic regulation of protein and transcript levels of certain genes in neutrophils upon inflammation that may be accounted for by inflammation-induced changes in translation efficiency, alternative splicing, folding, transport, localization, secretion or protein and RNA degradation (Ghazalpour *et al.*, 2011; Gry *et al.*, 2009). The identification of such inconsistencies triggers further investigation into the regulatory mechanisms and interactions between transcript abundance and protein level of a given gene.

4.2.1. Immune system associated differentially regulated inflammatory neutrophils proteins

The zebrafish kidney marrow is the main haematopoietic organ in adult fish containing a mixture of both immature and mature neutrophils. Changes in protein content identified in this study therefore likely reflect alterations in mature as well as immature neutrophils upon chemically-induced inflammation. The proteins that were responsive to inflammation are related to various biological functions, including immune system, cell cycle and cytoskeleton remodelling. HSPs are ubiquitously expressed and are essential for numerous processes including cell cycle control and signal transduction. Previous studies reported high expression levels of heat shock proteins (HSP70, HSP60, HSP90) during microbial infections (Multhoff, 2006; Pockley, 2003; Wallin *et al.*, 2002). However, it has been noted that Hsp90b expression was up regulated on the transcript level while down regulated on the protein level upon

chemically-induced inflammation in zebrafish. This could be due to biological differences mentioned earlier or indicate a negative feedback loop.

Additionally, inflammation induced the expression of several cytoskeletal proteins including the Rho family, small GTP binding protein (Rac2), tubulin (Tubb) and debrein-like protein (Dbnlb) in activated neutrophils of the adult zebrafish. Rac proteins are members of Rho family of GTPases and are key regulators of actin cytoskeleton and NADPH oxidase (Courjal *et al.*, 1997; Fenteany & Glogauer, 2004) and recognized for mediating the cytoskeletal dynamics required for various cell adhesion processes during cell migration and cell-cell adhesion (Alcaide *et al.*, 2009; Williams *et al.*, 2011). In vivo studies of neutrophil migration in zebrafish previously reported that Rac2 signalling is necessary for neutrophil motility and CXCR4-mediated neutrophil retention in hematopoietic tissue (Deng *et al.*, 2011; Shelef *et al.*, 2013). Moreover, upregulation of member of mammalian actin binding proteins (mAbp) such as a debrein-like protein (Dbnlb) was also observed, which is a major component of the beta 2 integrin mediated signalling cascade during complement-mediated phagocytosis and adhesion of neutrophils under physiological shear stress conditions. On the other hand, down regulation or genetic inhibition of mAbp1 in neutrophils results in defective cell migration under flow condition (Hepper *et al.*, 2012; Schymeinsky *et al.*, 2009). Therefore, our observation supports other reports implicating roles for Rac2 and mAbp in neutrophils upon inflammation. However, the role of Dbnlb has not been investigated in inflammation or neutrophil migration. Thus, investigation of the function of Dbnlb during leukocyte trafficking may provide additional insight into the regulation of leukocyte trafficking during acute inflammation.

Unfortunately, systematic comparison to other proteome analyses in zebrafish are currently not feasible due to substantial variability in the tissues analysed and the technologies applied. However, previous studies on infection-induced inflammation adult zebrafish tissues revealed that the expression of several proteins were similarly affected (Encinas *et al.*, 2010; Lü *et al.*, 2013; Xiong *et al.*, 2011). For example, Annexin A2a, is a calcium-dependent phospholipid binding protein associated with several biological and physiological processes and plays a crucial role in response to bacterial stimuli during host defence as an anti-inflammatory mediator (Yeh & Klesius, 2010). The observed increase of Annexin A2a also in chemically induced sterile inflammation confirms the vital role of this protein in inflammatory responses to various stimuli. Moreover, a significant increase in expression of the serine peptidase

inhibitor Serpin and the F-actin severing protein Scinderin b was noticed after chemically induced inflammation. Serpins are proteinase inhibitors which play a role in complement cascade, blood coagulation (Wang *et al.*, 2008a), and modulation of inflammatory response by suppressing protease dependent cell apoptosis (Lu *et al.*, 2011). Serpins are also involved in innate immunity providing bacteriostatic activity against Bacillus subtilis (Donpudsa *et al.*, 2009) Scinderin b is a calcium dependent F-actin severing protein, a vital component of the exocytotic machinery (Zhang *et al.*, 1996). Encinas *et al*. recently observed an increased expression of Scinderin b in zebrafish fin tissue after infection with viral haemorrhagic septicaemia virus. Present finding thus further indicates that there are groups of proteins that are similarly affected by sterile or infection-induced inflammation.

Chemically-induced inflammation also resulted in upregulation of cathepsin D in neutrophils. Cathepsin D is an aspartic protease, released by azurophilic granules in neutrophils. Studies in mice implicated Cathepsin D as an important protein for cytoskeleton remodelling and cell migration (Koch *et al.*, 2013). Additionally, elevated level of the ATP synthase subunit beta, a subunit of the V-ATPase complex was noticed. V-ATPases pump protons from the cytoplasm to the lumen (Hinton *et al.*, 2007) and maintain the cytoplasmic pH of neutrophils and macrophages (Nanda *et al.*, 1996). Previous studies have shown that inhibition of V-ATPase proteins attenuate the migration, adhesion and reactive oxygen production in neutrophils (Oliveira *et al.*, 2007).

4.2.2. Non-immune related function of differentially regulated inflammatory neutrophil proteins

In this study, various proteins were also identified such as tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide (Ywhaq) and adaptor related protein complex 2, sigma 1 (Ap2s1), which are differentially regulated during chemically induced inflammation. The functions of these proteins are not well characterized in immune system and inflammation. For example, Ywhaq belongs to a group of highly conserved proteins involved in several cellular processes such as cell-cell contact inhibition, signal transduction, apoptosis and cell adhesion and is highly expressed in leukocytes and lymphocytes (Aitken, 2006). Ywhaq may play important role in neutrophil adhesion and cell migration during inflammation. Similarly, adaptor related protein complex 2, sigma is involve in protein transport via transport vesicle in different membrane trafficking

pathways, clathrin dependent endocytosis as well as extracellular calcium homeostasis (Nesbit *et al.*, 2012). Calcium is an important mediator in innate immunity. Therefore, overexpression of Ap2s1 may regulate the endocytosis as well as calcium homeostasis mechanism in inflammation. In addition, up regulation of Arf-like family of small G proteins, ADP ribosylation factor like 8a and 8b (Arl8a and Arl8b) was noticed. Arl8b plays important role in regulating spatial distribution of lysosomes by controlling their motility on microtubule tracks. Lysosomes move towards the cell periphery and fuse with plasma membrane to release their content in order to degrade the extracellular matrix to promote cell migration (Tuli *et al.*, 2013). Hence, up regulation of Arl8b may promote the extracellular matrix degradation and subsequently neutrophil migration during inflammation.

4.2.3. Advantage and disadvantage of label free proteomics approach

So far label free proteomics analysis was used to investigate inflammatory proteome analysis of neutrophils, because a label free approach is a very simple, cost effective and reproducible quantitative proteomics approach. Label free quantification relies on measurement of spectral peak intestines or spectral counting (Asara *et al.*, 2008; Megger *et al.*, 2013). Labeling strategies such as iTRAQ (Patel *et al.*, 2009; Pereira *et al.*, 2011) and TMT require more sensitive and special mass spectrometers. Contrarily, the label free method can be performed using low-resolution mass spectrometry based on spectral counting quantification. Although this method appears straightforward, label free proteomics has inherent drawbacks. To perform label free proteomic analysis, control and treated samples are handled separately. Therefore, each sample experiences variability during preparation. Furthermore, the digested peptides are separated based on their hydrophobicity and charge. Later separated peptides are ionized in an ion source, where some peptides may naturally ionize more efficiently than others because the amino acid composition of every peptide differs and subsequently the ionized peptides detected in mass spectrometer also differ. Thus, protein quantification is performed based on either spectral counting or spectral peak intensities. Therefore, this method requires several replicates as well as precise sample preparation, highly sensitive mass spectrometry analysis and extensive raw data processing and bioinformatics analysis (Boehmer *et al.*, 2010; Wasinger *et al.*, 2013; Zhou *et al.*, 2012). In previous studies, comparison between label free and ITRQ and TMT showed that label free method provided best proteome coverage. However, quantification and reproducibility were very poor compared to labeling methods (Altelaar *et al.*, 2013a; Li *et al.*, 2011).

In summary, firstly chemically induced inflammation assay was established in adult zebrafish and subsequently; the protein dynamics in adult zebrafish whole kidney marrow neutrophils upon inflammation was investigated. Based on label free proteomics analysis several differentially regulated proteins were identified those are known to be involved in inflammatory processes. Several proteins were hitherto not described in the context of neutrophils and inflammation. Therefore, present study provides a valuable resource for further evaluation of inflammatory proteins over different time points of neutrophilic inflammation. However, proteomics analysis based on label free proteomics showed, high variation between replicates. Therefore, It has been thought that SILAC *in-vivo* labelling approach could improve the protein identification and reproducibility.

4.3. SILAC based proteomics analysis of chemically induced inflammation in adult zebrafish neutrophils

SILAC has been rapidly adopted as an approach in MS based proteomics. It is a simple, robust and accurate proteomics approach. Using a comparative SILAC proteomics approach focused on inflammatory proteins, 61 differentially regulated proteins in zebrafish WKM neutrophils were identified. Furthermore, comparison between results of label free and SILAC proteomics approaches were carried out.

4.3.1. SILAC: suitable quantitative proteomics approach for accurate protein quantification

For a comparison of protein quantification approaches, application of a single type of mass spectrometer is favorable. Gel based proteins fractionation and Orbitrap mass spectrometers were adopted to perform label free proteomics analysis. Nonetheless, in-solution protein digestion and highly sensitive Q-Exqutive mass spectrometry was selected to perform SILAC proteomics analysis. Comparison between SILAC and label free approach revealed that shotgun SILAC proteomics approach allowed us to identify and quantify a higher number of neutrophil proteins. Comparison of differentially regulated proteins reveled that only 20% differentially regulated proteins were commonly identified between both approaches. Low correlation between label free and SILAC methods might be due to different experimental procedures and mass spectrometers. For example: the label free proteomics experiment was carried out as follows: gel based proteins separation, independent experiment and on less sensitive mass spectrometer in comparison to SILAC spike in method. While SILAC

proteomics was performed by in-solution digestion and mass spectrometry analysis. Therefore, SILAC analysis did not go through multiple step sample preparation, which leads to less variation between replicates. SILAC spike in method is very economical, robust, and in principle applicable to various cell and tissue type (Zanivan *et al.*, 2013). Here, myeloid cell proteins were used to label neutrophil proteins due to lack of SILAC labeled neutrophil reporter line. The myeloid population gate in zebrafish WKM contains numerous cell types including neutrophils, monocytes and eosinophils. Therefore, highly abundant proteins of monocytes and eosinophils might be obscuring the identification of low abundant neutrophil proteins.

Protein reproducibility and accuracy were investigated for each quantification method by comparing quantitative results obtained from two replicate measurements (Lau *et al.*, 2014; Zanivan *et al.*, 2012). SILAC approach showed better reproducibility by correlation analyses of protein ratios by means of linear regression compared to label free method. With respect to accuracy, however, the two investigated methods of quantification showed clear differences (Li *et al.*, 2011). SILAC method was found to be considerably more accurate compared to label free methods. This higher precision of SILAC method allows the detection of even minute protein expression changes, which makes SILAC approach more favorable than the ion intensity based label free proteomics (Collier *et al.*, 2011; Lau *et al.*, 2014; Megger *et al.*, 2014).

4.3.2. Advantage and Drawbacks of SILAC quantitative proteomics method

Application of SILAC method has provided new insight to protein biology over the past decade. The robustness, low cost, and ease of implementation make SILAC an obvious choice, particularly when working with cultured cells or SILAC-compatible organisms. The ultimate benefit of SILAC over label free method is that sample can be spiked in at an early stage during the sample preparation. Theoretically, the SILAC method increases the precision and reproducibility by reducing both the sample loss and variability from sample preparation and mass spectrometry analysis (Bantscheff *et al.*, 2012; Choudhary & Mann, 2010; Mann, 2014; Ong, 2012). By comparing SILAC myeloid sample spike-in into different stages of the neutrophil inflammatory proteomic analysis, I showed that sample mixing at an early experiment step resulted in higher precision and repeatability in protein quantification. In addition, shotgun mass spectrometry (in-solution digestion) method was performed for

SILAC sample measurements, therefore, it saves mass spectrometry run time and fewer technical replicates are required compared to label free gel based mass spectrometry analysis. However, un-availability of SILAC labeled zebrafish transgenic reporter line for individual immune cells limits the application of SILAC proteomics analysis at cellular level.

4.3.3. Biological relevance of identified differentially regulated inflammatory proteins

SILAC quantitative proteomics method allowed us to identity a large number of differentially regulated proteins in adult zebrafish neutrophil cells upon chemically induced inflammation. Stress or injured cells release alarm signals or DAMP (Damage associated molecular pattern) including heat shock proteins (Hsps), cytokines and Hmgb1 that regulate the ongoing immune response (Chen & Nuñez, 2010; Hohne *et al.*, 2013; Kono & Rock, 2008; McDonald *et al.*, 2010; Škoberne *et al.*, 2004). For instance, Hmgb2b protein was up-regulated during inflammation progression and started resolving after 8 hr of inflammation. Hmgb2b and Hmgb3b show approximately 70% homology similarity with human HMGB1 and HMGB3 proteins, respetively. Hmgb2b play an important role in neutrophil recruitment at necrotic or damage sites. Hmgb protein inhibition significantly reduces cytokine production and neutrophil recruitments at wound sites (Wang *et al.*, 2013). In addition, Zgc: 110425 and H2afy2 show more than 60% and 73% homology similarity to human histone protein H1.1 and H2A.2 respectively. Endogenous histones play crucial functions as DAMP. Here, expression of H1.1 was gradually increased after 1hr of copper treatments. Furthermore, it expression started declining after 8 hr copper sulfate treatment. On the other hand, H2afy2 was down regulated at 1hr and 2hr of inflammation, but resolved latter to base point. Endogenous histone activates innate immune cells through DNA mediated TLR 9 activation during mice liver injury (Huang *et al.*, 2011).

Furthermore, sterile inflammation also resulted in over expression of Dock8 protein in neutrophils. Dock8 is an important activator of Rho GTPases and Rho GTPase activation is required for immune cell migration at injury or infection site (Yang *et al.*, 2009). It has been observed that chemically induced inflammation induces expression of Dock8 at the initial stage of inflammation and after 4hr of inflammation expression of Doc8 gradually started decreasing towards resolution. Additionally, elevated level of neutrophil cytosolic factor (Ncf2) protein at 4hr after copper sulfate treatment was observed. Nacf2 is one of the important protein subunits, which form the NADPH enzyme complex. NADPH plays a

crucial role in immune cell activation and phagocytosis (Bokoch & Zhao, 2006). Up-regulation of Ncf2 at late stage of inflammation may be required for phagocytosis during the resolution phase of inflammation.

The functions of other proteins identified here are not well characterized in immune system and inflammation. For example, Sorcin (Sri) is calcium binding protein and regulate intracellular calcium homeostasis (Ilari *et al.*, 2002). Sri had more than 75% homology similarity to human SRI B. Expression of Sri was down regulated at 2hr and 4hr of inflammation. However, expression of sorcin was resolved after 8hr of inflammation. A similar finding was reported by Hu *et al* in mice (Hu *et al.*, 2013). They showed that knock down of CXCR chemokine in mice reduced the expression of sorcin. CXC chemokines play a central role in neutrophil activation and chemotaxis. Phagocytosis of neutrophils down regulated the expression of chemokine receptor CXCR1 and CXCR2 (Doroshenko *et al.*, 2002). SILAC based result further support the notion that sorcin might be involved in inflammation resolution through phagocytosis.

Expression of Tfr receptor protein gradually increased up to 4hr of inflammation and after 8hr of inflammation its expression dropped towards resolution. Transferrin receptor protein (Tfr) is a carrier of transferrin proteins. Tfr imports the iron into the cell in a vesicle by receptor mediated endocytosis, which gets releases in to cell due to acidification of vesicle through hydrogen pump ions (Arosio & Levi, 2002; Lafourcade *et al.*, 2008; Ponka & Lok, 1999; van Dam *et al.*, 2002). Tfr protein might thus be involved in hydrogen ion pump activation required for neutrophils phagocytosis and inflammation resolution.

In summary, this analysis has provided a large data set of proteins differentially regulated in the course of inflammation. This provides a basis for follow-up experiments on the mechanism by which novel proteins are involved in the inflammation initiation, progression and resolution of chemically induced inflammation.

5. Conclusion and outlook

This study provides the first proteomics study of adult zebrafish whole kidney marrow neutrophils during resting and activated state. Initially, 1500 proteins were identified in resting adult zebrafish neutrophil. Of these 1500 proteins, 75% proteins were enriched in neutrophils compared to non-neutrophil tissue. These proteins were compared with human peripheral resting neutrophil proteins, and despite sample differences, 54% of immune-related proteins were found to be conserved between zebrafish and human resting neutrophils. Moreover, chemically induced inflammation assay in adult zebrafish was successfully established. Copper sulphate treatment resulted in activation of WKM neutrophils enabling application of this assay for further investigation of neutrophil biology in the zebrafish model system.

To investigate global proteome dynamics of neutrophils upon inflammation, two quantitative proteomics approaches were applied. First, a label free proteomics method was used to examine the proteome of neutrophils after 1h of chemically-induced inflammation. A total of 48 significantly-regulated proteins were identified. Comparison of protein expression levels with transcriptome data revealed partial correlation but also significant differences between transcript and protein expression for several genes. This finding confirms the notion that substantial post-transcriptional regulation occurs in vivo and underscores the importance of proteome studies to decipher the actual gene expression status of cell populations. As a proof of the principal validity of the label free proteomics approach, a number of proteins that were reportedly linked to inflammation and neutrophil migration was successfully identified. However, the reproducibility of this method remained a major challenge.

Instead, the quantitative SILAC method was used to elucidate the protein content in zebrafish granulocytes upon inflammation and this proved to be more accurate and reproducible. In this analysis, 61 proteins were found to be significantly, differentially-regulated during the course of inflammation. In conclusion, the SILAC technique identified and quantified considerably more proteins in comparison to label free technique and showed overall a greater sensitivity in detecting changes in protein expression. Taken together, this study demonstrates the value of proteomics in zebrafish to study immune response in individual leukocyte populations and provides new insights into the dynamics of granulocytic proteomes upon inflammation. It thereby paves the way for quantitative proteomics approaches aiming at the evaluation of

protein expression in physiological or disease states using zebrafish.

Outlook

The SILAC method has proven to be a valuable resource for quantitative proteomics analysis of granulocytic inflammation in adult zebrafish. However, as reported in chapter 3.3, transgenic SILAC reporter lines are not yet available. Therefore here the entire myeloid population of SILAC fish proteins was used to spiked-in neutrophil samples for quantitative proteomic analysis. This may preferentially yield quantification of proteins that are either highly abundant or commonly present in all myeloid cell populations. In the future, it will thus be of advantage to generate SILAC labeled transgenic reporter lines for individual myeloid populations such as granulocytes, macrophages, mast cells or dendritic cells. This would then allow for the use of specific internal controls in order to perform extensive quantitative proteomics. In addition, this SILAC based quantitative proteomics approach could then be further extended into zebrafish larvae. Chemically induced inflammation and small molecule screening assays are well established in zebrafish larvae to identify bioactive compounds with therapeutic potential. However, the identification of molecular targets of these compounds is currently an unresolved challenge. Therefore, the proteomics approach suggested in this study would represent a viable approach to identify target molecules in these screening assays.

Moreover, here a considerable number of proteins were identified that currently have no annotated function at all or no function associated with inflammatory reactions. Functional evaluation of these proteins in the context of inflammation will thus be the logical next step. Such studies will ideally be performed in zebrafish larvae to exploit the advantages of the zebrafish system using high end in vivo microscopy, pharmacological intervention or genetic manipulation via the CRISPR/Cas technology.

References

Abdallah, C., Dumas-Gaudot, E., Renaut, J. & Sergeant, K. (2012). Gel-Based and Gel-Free Quantitative Proteomics Approaches at a Glance. *International Journal of Plant Genomics* **2012**, 1–17.

Ablain, J. & Zon, L. I. (2013). Of fish and men: using zebrafish to fight human diseases. *Trends in Cell Biology* **23**, 584–586. Elsevier Ltd.

Aebersold, R. & Mann, M. (2003a). Mass spectrometry-based proteomics. *Nature* **422**, 198–207. Nature Publishing Group.

Aebersold, R. & Mann, M. (2003b). Mass spectrometry-based proteomics. **422**, 198–207.

Aggarwal, K., Choe, L. H. & Lee, K. H. (2006). Shotgun proteomics using the iTRAQ isobaric tags. *Briefings in Functional Genomics and Proteomics* **5**, 112–120.

Aitken, A. (2006). 14-3-3 proteins: a historic overview **16**, 162–172. Elsevier.

Alcaide, P., Auerbach, S. & Luscinskas, F. W. (2009). Neutrophil recruitment under shear flow: it's all about endothelial cell rings and gaps. *Microcirculation* **16**, 43–57.

Altelaar, A. F. M., Frese, C. K., Preisinger, C., Hennrich, M. L., Schram, A. W., Timmers, H. T. M., Heck, A. J. R. & Mohammed, S. (2013a). Benchmarking stable isotope labeling based quantitative proteomics. *Journal of Proteomics* **88**, 14–26. Elsevier B.V.

Altelaar, A. F. M., Munoz, J. & Heck, A. J. R. (2013b). Next-generation proteomics: towards an integrative view of proteome dynamics. *Nat Rev Genet* **14**, 35–48.

Anders, S. & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol* **11**, R106.

Andreev, V. P., Petyuk, V. A., Brewer, H. M., Karpievitch, Y. V., Xie, F., Clarke, J., Camp, D., Smith, R. D., Lieberman, A. P. & other authors. **(2012).** Label-Free Quantitative LC-MS Proteomics of Alzheimer's Disease and Normally Aged Human Brains. *J Proteome Res*.

Ariel, A. (2012). New lives given by cell death: macrophage differentiation following their encounter with apoptotic leukocytes during the resolution of inflammation 1–6.

Arosio, P. & Levi, S. (2002). Ferritin, iron homeostasis, and oxidative damage. *Free Radical Biology and Medicine* **33**, 457–463.

Asara, J. M., Christofk, H. R., Freimark, L. M. & Cantley, L. C. (2008). A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. *Proteomics* **8**, 994–999.

Balla, K. M., Lugo-Villarino, G., Spitsbergen, J. M., Stachura, D. L., Hu, Y., Bañuelos, K., Romo-Fewell, O., Aroian, R. V. & Traver, D. (2010). Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. *Blood* **116**, 3944–3954.

Bannenberg, G. L., Chiang, N., Ariel, A., Arita, M., Tjonahen, E., Gotlinger, K. H., Hong, S. & Serhan, C. N. (2005). Molecular circuits of resolution: formation and actions of resolvins and protectins. *J Immunol* **174**, 4345–4355.

Bannenberg, G. & Serhan, C. N. (2010). Specialized pro-resolving lipid mediators in the inflammatory response: An update. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1801**, 1260–1273.

Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Raida, M. & other authors. **(2007).** Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol* **25**, 1035–1044.

Bantscheff, M., Lemeer, S., Savitski, M. M. & Kuster, B. (2012). Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal Bioanal Chem* **404**, 939–965.

Benard, E. L., Racz, P. I., Rougeot, J., Nezhinsky, A. E., Verbeek, F. J., Spaink, H. P. & Meijer, A. H. (2014). Macrophage-Expressed Perforins Mpeg1 and Mpeg1.2 Have an Anti-Bacterial Function in Zebrafish. *J Innate Immun*.

Bertrand, J. Y., Kim, A. D., Teng, S. & Traver, D. (2008). CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. *Development* **135**, 1853–1862.

Bertrand, J. Y., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y. R. & Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111.

Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L. & Traver, D. (2007). Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development* **134**, 4147–4156.

Bhatia, V. N., Perlman, D. H., Costello, C. E. & McComb, M. E. (2009). Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Anal Chem* **81**, 9819–9823.

Blagoev, B., Kratchmarova, I., Ong, S.-E., Nielsen, M., Foster, L. J. & Mann, M. (2003).

A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* **21**, 315–318.

Boatman, S., Barrett, F., Satishchandran, S., Jing, L., Shestopalov, I. & Zon, L. I. (2013). Assaying hematopoiesis using zebrafish. *Blood Cells, Molecules, and Diseases* **51**, 271–276. Elsevier Inc.

Boehmer, J. L., DeGrasse, J. A., McFarland, M. A., Tall, E. A., Shefcheck, K. J., Ward, J. L. & Bannerman, D. D. (2010). The proteomic advantage: Label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis. *Vet Immunol Immunopathol* **138**, 252–266. Elsevier B.V.

Bokoch, G. M. & Zhao, T. (2006). Regulation of the phagocyte NADPH oxidase by Rac GTPase. *Antioxid Redox Signal* **8**, 1533–1548.

Bravo-Cordero, J. J., Magalhaes, M. A. O., Eddy, R. J., Hodgson, L. & Condeelis, J. (2013). Functions of cofilin in cell locomotion. *Nature Publishing Group* **14**, 405–415. Nature Publishing Group.

Carman, C. V. & Springer, T. A. (2008). Trans-cellular migration: cell–cell contacts get intimate. *Curr Opin Cell Biol* **20**, 533–540.

Carroll, K. J. & North, T. E. (2014). Oceans of opportunity: Exploring vertebrate hematopoiesis in zebrafish. *Experimental Hematology* **42**, 684–696. ISEH - International Society for Experimental Hematology.

Catherman, A. D., Skinner, O. S. & Kelleher, N. L. (2014). Top Down proteomics: Facts and perspectives. *Biochem Biophys Res Commun* **445**, 683–693. Elsevier Inc.

Chen, A. T. & Zon, L. I. (2009). Zebrafish blood stem cells. *J Cell Biochem* **108**, 35–42.

Chen, C.-H. W. (2008). Review of a current role of mass spectrometry for proteome research. *Anal Chim Acta* **624**, 16–36.

Chen, G. Y. & Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* **10**, 826–837.

Choudhary, C. & Mann, M. (2010). Decoding signalling networks by mass spectrometry-based proteomics. *Nat Rev Mol Cell Biol* **11**, 427–439.

Clapham, D. E. (2007). Calcium signaling. *Cell* **131**, 1047–1058.

Collier, T. S., Randall, S. M., Sarkar, P., Rao, B. M., Dean, R. A. & Muddiman, D. C. (2011). Comparison of stable-isotope labeling with amino acids in cell culture and spectral counting for relative quantification of protein expression. *Rapid Commun Mass Spectrom* **25**, 2524–2532.

Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C. & Laudanna, C. (2000). Chemokines trigger immediate beta 2 integrin affinity and mobility changes: Differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13**, 759–769.

Courjal, F., Chuchana, P., Theillet, C. & Fort, P. (1997). Structure and chromosomal assignment to 22q12 and 17qter of the ras-related Rac2 and Rac3 human genes. *Genomics* **44**, 242–246.

Coussens, L. M. & Werb, Z. (2002). Inflammation and cancer. *Nature* **420**, 860–867.

Cox, J. & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* **26**, 1367–1372.

Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J. V. & Mann, M. (2009). A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nature Protocols* **4**, 698–705.

Crowhurst, M. O., Layton, J. E. & Lieschke, G. J. (2002). Developmental biology of zebrafish myeloid cells. *Int J Dev Biol* **46**, 483–492.

Davidson, A. J. & Zon, L. I. (2004). The 'definitive' (and ‘primitive’) guide to zebrafish hematopoiesis. *Oncogene* **23**, 7233–7246.

de Jong, J. & Zon, L. I. (2005). Use of the zebrafish system to study primitive and definitive hematopoiesis. *Annu Rev Genet* **39**, 481–501.

De Souza, A. G., MacCormack, T. J., Wang, N., Li, L. & Goss, G. G. (2009). Large-Scale Proteome Profile of the Zebrafish (Danio rerio) Gill for Physiological and Biomarker Discovery Studies. *Zebrafish* **6**, 229–238.

Deng, Q. & Huttenlocher, A. (2012). Leukocyte migration from a fish eye's view. *Journal of Cell Science* **125**, 3949–3956.

Deng, Q., Yoo, S. K., Cavnar, P. J., Green, J. M. & Huttenlocher, A. (2011). Dual roles for Rac2 in neutrophil motility and active retention in zebrafish hematopoietic tissue. *Dev Cell* **21**, 735–745.

Deracinois, B., Flahaut, C., Duban-Deweer, S. & Karamanos, Y. (2013). Comparative and Quantitative Global Proteomics Approaches: An Overview. *Proteomes* **1**, 180–218. Multidisciplinary Digital Publishing Institute.

Dobson, J. T., Seibert, J., Teh, E. M., Da'as, S., Fraser, R. B., Paw, B. H., Lin, T. J. & Berman, J. N. (2008). Carboxypeptidase A5 identifies a novel mast cell lineage in the

zebrafish providing new insight into mast cell fate determination. *Blood* **112**, 2969–2972.

Donpudsa, S., Tassanakajon, A. & Rimphanitchayakit, V. (2009). Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp Penaeus monodon. *Developmental & Comparative Immunology* **33**, 481–488.

Doroshenko, T., Chaly, Y., Savitskiy, V., Maslakova, O., Portyanko, A., Gorudko, I. & Voitenok, N. N. (2002). Phagocytosing neutrophils down-regulate the expression of chemokine receptors CXCR1 and CXCR2. *Blood* **100**, 2668–2671.

Ellett, F. & Lieschke, G. J. (2010). Zebrafish as a model for vertebrate hematopoiesis. *Current Opinion in Pharmacology* **10**, 563–570. Elsevier Ltd.

Ellett, F., Pase, L., Hayman, J. W., Andrianopoulos, A. & Lieschke, G. J. (2011). mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* **117**, e49–56.

Elliott, M. H., Smith, D. S., Parker, C. E. & Borchers, C. (2009). Current trends in quantitative proteomics. *J Mass Spectrom* n/a–n/a.

Encinas, P., Rodriguez-Milla, M. A., Novoa, B., Estepa, A., Figueras, A. & Coll, J. (2010). Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicemia rhabdovirus. A proteomic and transcriptomic approach. *BMC Genomics* **11**, 518.

Enyedi, B., Kala, S., Nikolich-Zugich, T. & Niethammer, P. (2013). Tissue damage detection by osmotic surveillance. *Nat Cell Biol* **15**, 1123–1130.

Feghali, C. A., & Wright, T. M. (1997). Cytokines in acute and chronic inflammation. Front Biosci **2**, 12-26.

Fenteany, G. & Glogauer, M. (2004). Cytoskeletal remodeling in leukocyte function. *Curr Opin Hematol* **11**, 15–24.

Figeys, D. & Aebersold, R. (1997). High sensitivity identification of proteins by electrospray ionization tandem mass spectrometry: initial comparison between an ion trap mass spectrometer and a triple quadrupole mass spectrometer. *Electrophoresis* **18**, 360–368.

Flannagan, R. S., Jaumouillé, V. & Grinstein, S. (2012). The cell biology of phagocytosis. *Annu Rev Pathol Mech Dis* **7**, 61–98.

Foell, D. & Roth, J. (2004). Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheum* **50**, 3762–3771.

Geiger, T., Wisniewski, J. R., Cox, J., Zanivan, S., Kruger, M., Ishihama, Y. & Mann,

M. (2011). Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. *Nature Protocols* **6**, 147–157.

Ghazalpour, A., Bennett, B., Petyuk, V. A., Orozco, L., Hagopian, R., Mungrue, I. N., Farber, C. R., Sinsheimer, J., Kang, H. M. & other authors. **(2011).** Comparative Analysis of Proteome and Transcriptome Variation in Mouse. *PLoS Genet* **7**, e1001393 (M. Snyder, Ed.).

Gilmore, J. M., Milloy, J. A. & Gerber, S. A. (2013). SILAC surrogates: rescue of quantitative information for orphan analytes in spike-in SILAC experiments. *Anal Chem* **85**, 10812–10819.

Goshe, M. B. & Smith, R. D. (2003). Stable isotope-coded proteomic mass spectrometry. *Current Opinion in Biotechnology* **14**, 101–109.

Gry, M., Rimini, R., Strömberg, S., Asplund, A., Pontén, F., Uhlén, M. & Nilsson, P. (2009). Correlations between RNA and protein expression profiles in 23 human cell lines. *BMC Genomics* **10**, 365.

Hall, C., Flores, M. V., Storm, T., Crosier, K. & Crosier, P. (2007). The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC Dev Biol* **7**, 42.

Hall, C. J., Boyle, R. H., Sun, X., Wicker, S. M., Misa, J. P., Krissansen, G. W., Print, C. G., Crosier, K. E. & Crosier, P. S. (2014). Epidermal cells help coordinate leukocyte migration during inflammation through fatty acid-fuelled matrix metalloproteinase production. *Nat Commun* **5**, 3880.

Harizi, H., Corcuff, J.-B. & Gualde, N. (2008). Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in Molecular Medicine* **14**, 461–469.

Heller, M., Mattou, H., Menzel, C. & Yao, X. (2003). Trypsin catalyzed 16O-to-18O exchange for comparative proteomics: Tandem mass spectrometry comparison using MALDI-TOF, ESI-QTOF, and ESI-ion trap mass spectrometers. *J Am Soc Mass Spectrom* **14**, 704–718.

Henry, K. M., Loynes, C. A., Whyte, M. K. B. & Renshaw, S. A. (2013). Zebrafish as a model for the study of neutrophil biology. *Journal of Leukocyte Biology* **94**, 633–642.

Hepper, I., Schymeinsky, J., Weckbach, L. T., Jakob, S. M., Frommhold, D., Sixt, M., Laschinger, M., Sperandio, M. & Walzog, B. (2012). The mammalian actin-binding protein 1 is critical for spreading and intraluminal crawling of neutrophils under flow conditions. *The Journal of Immunology* **188**, 4590–4601.

Hermann, A., Donato, R., Weiger, T. M., & Chazin, W. J. (2012). S100 calcium binding proteins and ion channels. *Frontiers in pharmacology*, **3** Frontiers Media SA.

Hernández, P. P., Moreno, V., Olivari, F. A. & Allende, M. L. (2006). Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio). *Hear Res* **213**, 1–10.

Hernández, P. P., Olivari, F. A., Sarrazin, A. F., Sandoval, P. C. & Allende, M. L. (2007). Regeneration in zebrafish lateral line neuromasts: Expression of the neural progenitor cell marker sox2 and proliferation-dependent and-independent mechanisms of hair cell renewal. *Devel Neurobio* **67**, 637–654.

Hertog, J. (2005). Chemical Genetics: Drug Screens in Zebrafish. *Biosci Rep* **25**, 289–297.

Hinton, A., Bond, S. & Forgac, M. (2007). V-ATPase functions in normal and disease processes. *Pflugers Arch - Eur J Physiol* **457**, 589–598.

Hirata, T. & Narumiya, S. (2012). Prostanoids as regulators of innate and adaptive immunity. *Adv Immunol* **116**, 143–174.

Hogl, S., van Bebber, F., Dislich, B., Kuhn, P.-H., Haass, C., Schmid, B. & Lichtenthaler, S. F. (2013). Label-free quantitative analysis of the membrane proteome of Bace1 protease knock-out zebrafish brains. *Proteomics* **13**, 1519–1527.

Hohne, C., Wenzel, M., Angele, B., Hammerschmidt, S., Hacker, H., Klein, M., Bierhaus, A., Sperandio, M., Pfister, H. W. & Koedel, U. (2013). High mobility group box 1 prolongs inflammation and worsens disease in pneumococcal meningitis. *Brain* **136**, 1746–1759.

Hu, W., Wang, J., Luo, G., Luo, B., Wu, C., Wang, W., Xiao, Y. & Li, J. (2013). Proteomics-based analysis of differentially expressed proteins in the CXCR1-knockdown gastric carcinoma MKN45 cell line and its parental cell. *Acta Biochimica et Biophysica Sinica* **45**, 857–866.

Huang, D. W., Sherman, B. T. & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**, 44–57.

Huang, H., Evankovich, J., Yan, W., Nace, G., Zhang, L., Ross, M., Liao, X., Billiar, T., Xu, J. & other authors. **(2011).** Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology* **54**, 999–1008.

Huang, P., Zhu, Z., Lin, S. & Zhang, B. (2012). Reverse Genetic Approaches in Zebrafish. *Journal of Genetics and Genomics* **39**, 421–433. Elsevier Limited and Science Press.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J.-R. J. & Joung, J. K. (2013). nbt.2501. *Nature Biotechnology* **31**, 227–229. Nature Publishing Group.

Iadecola, C. & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nature Publishing Group* **17**, 796–808.

Ilari, A., Johnson, K. A., Nastopoulos, V., Verzili, D., Zamparelli, C., Colotti, G., Tsernoglou, D. & Chiancone, E. (2002). The crystal structure of the sorcin calcium binding domain provides a model of Ca2+-dependent processes in the full-length protein. *J Mol Biol* **317**, 447–458.

Jüschke, C., Dohnal, I., Pichler, P., Harzer, H., Swart, R., Ammerer, G., Mechtler, K. & Knoblich, J. A. (2013). Transcriptome and proteome quantification of a tumor model provides novel insights into post-transcriptional gene regulation. *Genome Biol* **14**, r133.

Kato, A., Kurita, S., Hayashi, A., Kaji, N., Ohashi, K. & Mizuno, K. (2008). Critical roles of actin-interacting protein 1 in cytokinesis and chemotactic migration of mammalian cells. *Biochem J* **414**, 261–270.

Keightley, M.-C., Wang, C.-H., Pazhakh, V. & Lieschke, G. J. (2014). Delineating the roles of neutrophils and macrophages in zebrafish regeneration models. *International Journal of Biochemistry and Cell Biology* **56**, 92–106. Elsevier Ltd.

Kissa, K. & Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–U125.

Kobayashi, S. D., Voyich, J. M., Burlak, C. & DeLeo, F. R. (2005). Neutrophils in the innate immune response. *Arch Immunol Ther Exp (Warsz)* **53**, 505–517.

Koch, S., Scifo, E., Rokka, A., Trippner, P., Lindfors, M., Korhonen, R., Corthals, G. L., Virtanen, I., Lalowski, M. & Tyynelä, J. (2013). Cathepsin D deficiency induces cytoskeletal changes and affects cell migration pathways in the brain. *Neurobiol Dis* **50**, 107–119.

Kolaczkowska, E. & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* **13**, 159–175.

Kono, H. & Rock, K. L. (2008). How dying cells alert the immune system to danger. *Nat Rev Immunol* **8**, 279–289.

Konzer, A., Ruhs, A., Braun, H., Jungblut, B., Braun, T. & Kruger, M. (2013). Stable isotope labeling in zebrafish allows in vivo monitoring of cardiac morphogenesis. *Mol Cell Proteomics* **12**, 1502–1512.

Kruger, M., Moser, M., Ussar, S., Thievessen, I., Luber, C. A., Forner, F., Schmidt, S., Zanivan, S., Fässler, R. & Mann, M. (2008). SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* **134**, 353–364.

Lafourcade, C., Sobo, K., Kieffer-Jaquinod, S., Garin, J. & van der Goot, F. G. (2008). Regulation of the V-ATPase along the Endocytic Pathway Occurs through Reversible Subunit Association and Membrane Localization. *PLoS ONE* **3**, e2758 (E. Joly, Ed.).

Langenau, D. M., Ferrando, A. A., Traver, D., Kutok, J. L., Hezel, J., Kanki, J. P., Zon, L. I., Look, A. T. & Trede, N. S. (2004). In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc Natl Acad Sci USA* **101**, 7369–7374.

Langenau, D. M., Traver, D., Ferrando, A. A., Kutok, J. L., Aster, J. C., Kanki, J. P., Lin, S., Prochownik, E., Trede, N. S. & other authors. **(2003).** Myc-induced T cell leukemia in transgenic zebrafish. *Science* **299**, 887–890.

Langenau, D. M. & Zon, L. I. (2005). The zebrafish: a new model of T-cell and thymic development. *Nat Rev Immunol* **5**, 307–317.

Lau, H.-T., Suh, H. W., Golkowski, M. & Ong, S.-E. (2014). Comparing SILAC- and stable isotope dimethyl-labeling approaches for quantitative proteomics. *J Proteome Res* **13**, 4164–4174.

Lawson, N. D. & Wolfe, S. A. (2011). Forward and Reverse Genetic Approaches for the Analysis of Vertebrate Development in the Zebrafish. *Dev Cell* **21**, 48–64. Elsevier Inc.

LeBert, D. C. & Huttenlocher, A. (2014). Inflammation and wound repair. *Semin Immunol*.

Levy, B. D., Clish, C. B., Schmidt, B., Gronert, K. & Serhan, C. N. (2001). Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol* **2**, 612–619.

Li, Z., Adams, R. M., Chourey, K., Hurst, G. B., Hettich, R. L. & Pan, C. (2011). Systematic Comparison of Label-Free, Metabolic Labeling, and Isobaric Chemical Labeling for Quantitative Proteomics on LTQ Orbitrap Velos. *J Proteome Res*.

Lieschke, G. J. & Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* **8**, 353–367.

Lieschke, G. J. & Trede, N. S. (2009). Fish immunology. *Current Biology* **19**, R678–R682.

Link, V., Shevchenko, A. & Heisenberg, C.-P. (2006). Proteomics of early zebrafish embryos. *BMC Dev Biol* **6**, 1.

Long, Q., Meng, A., Wang, H., Jessen, J. R., Farrell, M. J. & Lin, S. (1997). GATA-1

expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development* **124**, 4105–4111.

Lu, F., Lamontagne, J., Sun, A., Pinkerton, M., Block, T. & Lu, X. (2011). Role of the inflammatory protein serine protease inhibitor Kazal in preventing cytolytic granule granzyme A-mediated apoptosis. *Immunology* **134**, 398–408.

Lu, T., Kobayashi, S. D., Quinn, M. T. & Deleo, F. R. (2012). A NET Outcome. *Front Immunol* **3**, 365.

Luber, C. A., Cox, J., Lauterbach, H., Ben Fancke, Selbach, M., Tschopp, J., Akira, S., Wiegand, M., Hochrein, H. & other authors. **(2010).** Quantitative Proteomics Reveals Subset-Specific Viral Recognition in Dendritic Cells. *Immunity* **32**, 279–289. Elsevier Ltd.

Luerman, G. C., Uriarte, S. M., Rane, M. J. & McLeish, K. R. (2010). Application of proteomics to neutrophil biology. *Journal of Proteomics* **73**, 552–561.

Lugo-Villarino, G., Balla, K. M., Stachura, D. L., Bañuelos, K., Werneck, M. B. F. & Traver, D. (2010). Identification of dendritic antigen-presenting cells in the zebrafish. *Proc Natl Acad Sci USA* **107**, 15850–15855.

Luo, M. (2012). Current Chemical Biology Approaches to Interrogate Protein Methyltransferases. *ACS Chem Biol*.

Lü, A., Hu, X., Wang, Y., Shen, X., Li, X., Zhu, A., Tian, J., Ming, Q. & Feng, Z. (2013). iTRAQ analysis of gill proteins from the zebrafish (Danio rerio) infected with Aeromonas hydrophila. *Fish Shellfish Immunol*.

Maier, T., Güell, M. & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Letters* **583**, 3966–3973. Federation of European Biochemical Societies.

Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol* **7**, 952–958.

Mann, M. (2014). Fifteen years of Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC). *Methods Mol Biol* **1188**, 1–7.

Mathias, J. R., Dodd, M. E., Walters, K. B., Yoo, S. K., Ranheim, E. A. & Huttenlocher, A. (2009). Characterization of zebrafish larval inflammatory macrophages. *Developmental & Comparative Immunology* **33**, 1212–1217.

Mathias, J. R., Perrin, B. J., Liu, T.-X., Kanki, J., Look, A. T. & Huttenlocher, A. (2006). Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic

zebrafish. *Journal of Leukocyte Biology* **80**, 1281–1288.

Mayadas, T. N., Tsokos, G. C. & Tsuboi, N. (2009). Mechanisms of Immune Complex–Mediated Neutrophil Recruitment and Tissue Injury. *Circulation* **120**, 2012–2024. Am Heart Assoc.

McClatchy, D. B., Dong, M.-Q., Wu, C. C., Venable, J. D. & Yates, J. R. (2007). 15N Metabolic Labeling of Mammalian Tissue with Slow Protein Turnover. *J Proteome Res* **6**, 2005–2010.

McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. C. M., Beck, P. L., Muruve, D. A. & Kubes, P. (2010). Intravascular Danger Signals Guide Neutrophils to Sites of Sterile Inflammation. *Science* **330**, 362–366.

McDonald, B. B. & Kubes, P. P. (2011). Cellular and molecular choreography of neutrophil recruitment to sites of sterile inflammation. *J Mol Med (Berl)* **89**, 1079–1088.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature* **454**, 428–435.

Medzhitov, R. (2010). Inflammation 2010: New Adventures of an Old Flame. *Cell* **140**, 771–776.

Medzihradszky, K. F., Campbell, J. M., Baldwin, M. A., Falick, A. M., Juhasz, P., Vestal, M. L. & Burlingame, A. L. (2000). The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. *Anal Chem* **72**, 552–558. ACS Publications.

Meeker, N. D. & Trede, N. S. (2008). Immunology and zebrafish: Spawning new models of human disease. *Developmental & Comparative Immunology* **32**, 745–757.

Megger, D. A., Bracht, T., Meyer, H. E. & Sitek, B. (2013). Label-free quantification in clinical proteomics. *BBA - Proteins and Proteomics* **1834**, 1581–1590. Elsevier B.V.

Megger, D. A., Pott, L. L., Ahrens, M., Padden, J., Bracht, T., Kuhlmann, K., Eisenacher, M., Meyer, H. E. & Sitek, B. (2014). Comparison of label-free and label-based strategies for proteome analysis of hepatoma cell lines. *BBA - Proteins and Proteomics* **1844**, 967–976. Elsevier B.V.

Menezes, G. B., Mansur, D. S., McDonald, B., Kubes, P. & Teixeira, M. M. (2011). Sensing sterile injury: opportunities for pharmacological control. *Pharmacol Ther* **132**, 204–214.

Micallef, J., Dharsee, M., Chen, J., Ackloo, S., Evans, K., Qiu, L. & Chang, H. (2010). Applying mass spectrometry based proteomic technology to advance the understanding of multiple myeloma. *J Hematol Oncol* **3**, 13.

Michalski, A., Damoc, E., Hauschild, J.-P., Lange, O., Wieghaus, A., Makarov, A., Nagaraj, N., Cox, J., Mann, M. & Horning, S. (2011). Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. *Molecular & Cellular Proteomics* **10**, M111–011015.

Moser, B. (2004). Chemokines: role in inflammation and immune surveillance. *Annals of the Rheumatic Diseases* **63**, ii84–ii89.

Mueller, H., Stadtmann, A., Van Aken, H., Hirsch, E., Wang, D., Ley, K. & Zarbock, A. (2010). Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) gamma 2 and PI3K gamma pathways. *Blood* **115**, 3118–3127.

Muers, M. (2011). Gene expression: Transcriptome to proteome and back to genome. *Nat Rev Genet* **12**, 518.

Multhoff, G. (2006). Heat shock proteins in immunity. *Handb Exp Pharmacol* 279–304.

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H.-F., Handin, R. I. & Herbomel, P. (2006). Tracing Hematopoietic Precursor Migration to Successive Hematopoietic Organs during Zebrafish Development. *Immunity* **25**, 963–975.

Nanda, A., Brumell, J. H., Nordström, T., Kjeldsen, L., Sengelov, H., Borregaard, N., Rotstein, O. D. & Grinstein, S. (1996). Activation of proton pumping in human neutrophils occurs by exocytosis of vesicles bearing vacuolar-type H+-ATPases. *J Biol Chem* **271**, 15963–15970.

Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173–182.

Nesbit, M. A., Hannan, F. M., Howles, S. A., Reed, A. A. C., Cranston, T., Thakker, C. E., Gregory, L., Rimmer, A. J., Rust, N. & other authors. **(2012).** Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3. *Nat Genet* **45**, 93–97. Nature Publishing Group.

Niethammer, P., Grabher, C., Look, A. T. & Mitchison, T. J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* **459**, 996–999.

Nilsson, T., Mann, M., Aebersold, R., Yates, J. R., Bairoch, A. & Bergeron, J. J. M. (2010). Mass spectrometry in high-throughput proteomics: ready for the big time. *Nature Publishing Group* **7**, 681–685. Nature Publishing Group.

Nolte, H., Konzer, A., Ruhs, A., Jungblut, B., Braun, T. & Kruger, M. (2014). Global

Protein Expression Profiling of Zebrafish Organs Based on in Vivo Incorporation of Stable Isotopes. *J Proteome Res* **13**, 2162–2174.

Novoa, B. & Figueras, A. (2012). Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. *Adv Exp Med Biol* **946**, 253–275.

Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G., Mendoza, A., Sevinsky, J. R., Resing, K. A. & Ahn, N. G. (2005). Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Molecular & Cellular Proteomics* **4**, 1487–1502.

Oliveira, R. M. de, Antunes, E., Pedrazzoli, J. & Gambero, A. (2007). The inhibitory effects of H+K+ATPase inhibitors on human neutrophils in vitro: Restoration by a K+ ionophore. *Inflamm res* **56**, 105–111.

Ong, S.-E. (2012). The expanding field of SILAC. *Anal Bioanal Chem* **404**, 967–976.

Ong, S.-E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A. & Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular & Cellular Proteomics* **1**, 376–386.

Ong, S.-E., Foster, L. J. & Mann, M. (2003). Mass spectrometric-based approaches in quantitative proteomics. *Methods* **29**, 124–130.

Ong, S.-E. & Mann, M. (2005a). Mass spectrometry–based proteomics turns quantitative. *Nat Chem Biol* **1**, 252–262.

Ong, S.-E. & Mann, M. (2005b). A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protocols* **1**, 2650–2660.

Ordas, A., Hegedus, Z., Henkel, C. V., Stockhammer, O. W., Butler, D., Jansen, H. J., Racz, P., Mink, M., Spaink, H. P. & Meijer, A. H. (2011). Deep sequencing of the innate immune transcriptomic response of zebrafish embryos to Salmonella infection. *Fish Shellfish Immunol* **31**, 716–724. Elsevier Ltd.

Ou, H., Simon, J. A., Rubel, E. W. & Raible, D. W. (2012). Screening for chemicals that affect hair cell death and survival in the zebrafish lateral line. *Hear Res* **288**, 58–66. Elsevier B.V.

Page, D. M., Wittamer, V., Bertrand, J. Y., Lewis, K. L., Pratt, D. N., Delgado, N., Schale, S. E., McGue, C., Jacobsen, B. H. & other authors. **(2013).** An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood* **122**, e1–e11.

Paik, E. J. & Zon, L. I. (2010). Hematopoietic development in the zebrafish. *Int J Dev Biol*

54, 1127–1137.

Patel, V. J., Thalassinos, K., Slade, S. E., Connolly, J. B., Crombie, A., Murrell, J. C. & Scrivens, J. H. (2009). A Comparison of Labeling and Label-Free Mass Spectrometry-Based Proteomics Approaches. *J Proteome Res* **8**, 3752–3759.

Pereira, S. B., Ow, S. Y., Barrios-Llerena, M. E., Wright, P. C., Moradas-Ferreira, P. & Tamagnini, P. (2011). iTRAQ-based quantitative proteomic analysis of Gloeothece sp. PCC 6909: Comparison with its sheathless mutant and adaptations to nitrate deficiency and sulfur limitation. *Journal of Proteomics* **75**, 270–283.

Perskvist, N., Roberg, K., Kulyté, A. & Stendahl, O. (2002). *Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils. J Cell Sci*.

Petri, B., Phillipson, M. & Kubes, P. (2008). The Physiology of Leukocyte Recruitment: An In Vivo Perspective. *The Journal of Immunology* **180**, 6439–6446.

Phillipson, M. & Kubes, P. (2011). The neutrophil in vascular inflammation. *Nature Publishing Group* **17**, 1381–1390.

Pockley, A. G. (2003). Heat shock proteins as regulators of the immune response. *Lancet* **362**, 469–476.

Ponka, P. & Lok, C. N. (1999). The transferrin receptor: role in health and disease. *International Journal of Biochemistry and Cell Biology* **31**, 1111–1137.

Rauniyar, N., McClatchy, D. B. & Yates, J. R., III. (2013). Stable isotope labeling of mammals (SILAM) for in vivo quantitative proteomic analysis. *Methods* **61**, 260–268. Elsevier Inc.

Rebhan, M., Chalifa-Caspi, V., Prilusky, J. & Lancet, D. (1997). GeneCards: integrating information about genes, proteins and diseases. *Trends Genet* **13**, 163.

Renshaw, S. A., Loynes, C. A., Trushell, D. M. I., Elworthy, S., Ingham, P. W. & Whyte, M. K. B. (2006). A transgenic zebrafish model of neutrophilic inflammation. *Blood* **108**, 3976–3978.

Renshaw, S. A. & Trede, N. S. (2012). A model 450 million years in the making: zebrafish and vertebrate immunity. *Disease Models & Mechanisms* **5**, 38–47.

Rocha e Silva, M. (1978). A brief survey of the history of inflammation. *Inflamm res* **8**, 45–49. Springer.

Rock, K. L., Latz, E., Ontiveros, F. & Kono, H. (2010). The Sterile Inflammatory Response. *Annu Rev Immunol* **28**, 321–342.

Roe, M. R. & Griffin, T. J. (2006). Gel-free mass spectrometry-based high throughput proteomics: Tools for studying biological response of proteins and proteomes. *Proteomics* **6**, 4678–4687.

Ryckman, C. C., Vandal, K. K., Rouleau, P. P., Talbot, M. M. & Tessier, P. A. P. (2003). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol* **170**, 3233–3242.

Sadik, C. D., Kim, N. D. & Luster, A. D. (2011). Neutrophils cascading their way to inflammation. *Trends Immunol* **32**, 452–460.

Sallusto, F. & Baggiolini, M. (2008). Chemokines and leukocyte traffic. *Nat Immunol* **9**, 949–952.

Sanjabi, S., Zenewicz, L. A., Kamanaka, M. & Flavell, R. A. (2009). Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. *Current Opinion in Pharmacology* **9**, 447–453.

Saxena, S., Singh, S. K., Lakshmi, M. G. M., Meghah, V., Bhatti, B., Swamy, C. V. B., Sundaram, C. S. & Idris, M. M. (2012). Proteomic analysis of zebrafish caudal fin regeneration. *Mol Cell Proteomics* **11**, M111.014118.

Saxena, S., Singh, S. K., Lakshmi, M. G. M., Meghah, V., Sundaram, C. S., Swamy, C. V. B. & Idris, M. M. (2011). Proteome profile of zebrafish kidney. *Journal of Proteomics* **74**, 2937–2947.

Schwab, J. & Serhan, C. (2006). Lipoxins and new lipid mediators in the resolution of inflammation. *Current Opinion in Pharmacology* **6**, 414–420.

Schymeinsky, J., Gerstl, R., Mannigel, I., Niedung, K., Frommhold, D., Panthel, K., Heesemann, J., Sixt, M., Quast, T. & other authors. **(2009).** A fundamental role of mAbp1 in neutrophils: impact on beta(2) integrin-mediated phagocytosis and adhesion in vivo. *Blood* **114**, 4209–4220.

Serhan, C. N. (2007). Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu Rev Immunol* **25**, 101–137.

Serhan, C. N., Chiang, N. & Van Dyke, T. E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* **8**, 349–361.

Serhan, C. N. & Savill, J. (2005). Resolution of inflammation: the beginning programs the end. *Nat Immunol* **6**, 1191–1197.

Shao, C., Zhang, Y. & Sun, W. (2014). Statistical characterization of HCD fragmentation

patterns of tryptic peptides on an LTQ Orbitrap Velos mass spectrometer. *Journal of Proteomics* **109**, 26–37.

Shelef, M. A., Tauzin, S. & Huttenlocher, A. (2013). Neutrophil migration: moving from zebrafish models to human autoimmunity. *Immunol Rev* **256**, 269–281.

Shen, H., Kreisel, D. & Goldstein, D. R. (2013). Processes of Sterile Inflammation. *The Journal of Immunology* **191**, 2857–2863.

Singh, S. K., Aravamudhan, S., Armant, O., Kruger, M. & Grabher, C. (2014). Proteome dynamics in neutrophils of adult zebrafish upon chemically-induced inflammation. *Fish Shellfish Immunol*. **40**, 217–224.

Singh, S. K., Rakesh, K. S., Ramamoorthy, K., Pardha Saradhi, A. & Idris, M. M. (2010a). Proteome Profile of Zebrafish Brain Based on Gel LC-ESI MS/MS Analysis. *J Proteomics Bioinform* **3**, 135–142.

Singh, S. K., Lakshmi, M. G. M., Saxena, S., Swamy, C. V. B. & Idris, M. M. (2011). Proteome profile of zebrafish caudal fin based on one-dimensional gel electrophoresis LCMS/MS and two-dimensional gel electrophoresis MALDI MS/MS analysis. *J Sep Sci* **34**, 225–232.

Singh, S. K., Sethi, S., Aravamudhan, S. & Kruger, M. (2013). Proteome Mapping of Adult Zebrafish Marrow Neutrophils Reveals Partial Cross Species Conservation to Human Peripheral Neutrophils. *PLoS ONE* doi:10.1371/journal.pone.0073998.s007.

Singh, S. K., Sundaram, C. S., Shanbhag, S. & Idris, M. M. (2010b). Proteomic Profile of Zebrafish Brain Based on Two-Dimensional Gel Electrophoresis Matrix-Assisted Laser Desorption/Ionization MS/MS Analysis. *Zebrafish* **7**, 169–177.

Stachura, D. L. & Traver, D. (2011). Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol* **101**, 75–110.

Starnes, T. W. & Huttenlocher, A. (2012). Neutrophil Reverse Migration Becomes Transparent with Zebrafish. *Adv Hematol* **2012**, 1–11.

Stow, J. L. & Murray, R. Z. (2013). Intracellular trafficking and secretion of inflammatory cytokines. *Cytokine Growth Factor Rev* **24**, 227–239.

Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature* **291**, 293–296.

Sultani, M., Stringer, A. M., Bowen, J. M. & Gibson, R. J. (2012). Anti-Inflammatory Cytokines: Important Immunoregulatory Factors Contributing to Chemotherapy-Induced Gastrointestinal Mucositis. *Chemotherapy Research and Practice* **2012**, 1–11.

Sundd, P., Gutierrez, E., Koltsova, E. K., Kuwano, Y., Fukuda, S., Pospieszalska, M. K., Groisman, A. & Ley, K. (2012). 'Slings' enable neutrophil rolling at high shear. *Nature* **488**, 399–403.

Sury, M. D. M., Chen, J.-X. J. & Selbach, M. M. (2010). The SILAC fly allows for accurate protein quantification in vivo. *Molecular & Cellular Proteomics* **9**, 2173–2183.

Svahn, A. J., Graeber, M. B., Ellett, F., Lieschke, G. J., Rinkwitz, S., Bennett, M. R. & Becker, T. S. (2012). Development of ramified microglia from early macrophages in the zebrafish optic tectum. *Devel Neurobio* **73**, 60–71.

Škoberne, M., Beignon, A.-S. & Bhardwaj, N. (2004). Danger signals: a time and space continuum. *Trends in Molecular Medicine* **10**, 251–257.

Tao, W. A. & Aebersold, R. (2003). Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. *Current Opinion in Biotechnology* **14**, 110–118.

Thelen, M. & Stein, J. V. (2008). How chemokines invite leukocytes to dance. *Nat Immunol* **9**, 953–959.

Thompson, A., Schäfer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A. K. A. & Hamon, C. (2003). Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem* **75**, 1895–1904.

Thurman, J. M. & Holers, V. M. (2006). The central role of the alternative complement pathway in human disease. *J Immunol* **176**, 1305–1310.

Tomazella, G. G., daSilva, I., Thomé, C. H., Greene, L. J., Koehler, C. J., Thiede, B., Wiker, H. G. & de Souza, G. A. (2010). Analysis of detergent-insoluble and whole cell lysate fractions of resting neutrophils using high-resolution mass spectrometry. *J Proteome Res* **9**, 2030–2036.

Trapnell, C., Pachter, L. & Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105–1111.

Traver, D., Herbomel, P., Patton, E. E., Murphey, R. D., Yoder, J. A., Litman, G. W., Catic, A., Amemiya, C. T., Zon, L. I. & Trede, N. S. (2003a). The zebrafish as a model organism to study development of the immune system. *Adv Immunol* **81**, 253–330.

Traver, D., Paw, B. H., Poss, K. D., Penberthy, W. T., Lin, S. & Zon, L. I. (2003b). Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol* **4**, 1238–1246.

Trede, N. S., Langenau, D. M., Traver, D., Look, A. T. & Zon, L. I. (2004). The use of

zebrafish to understand immunity. *Immunity* **20**, 367–379.

Tuli, A., Thiery, J., James, A. M., Michelet, X., Sharma, M., Garg, S., Sanborn, K. B., Orange, J. S., Lieberman, J. & Brenner, M. B. (2013). Arf-like GTPase Arl8b regulates lytic granule polarization and natural killer cell-mediated cytotoxicity. *Mol Biol Cell* **24**, 3721–3735.

Uddin, M. & Levy, B. D. (2011). Resolvins: Natural agonists for resolution of pulmonary inflammation. *Progress in Lipid Research* **50**, 75–88.

van Dam, E. M., Broeke, Ten, T., Jansen, K., Spijkers, P. & Stoorvogel, W. (2002). Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem* **277**, 48876–48883.

van der Vaart, M., van Soest, J. J., Spaink, H. P. & Meijer, A. H. (2013). Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. *Disease Models & Mechanisms* **6**, 841–854.

Wallin, R. P. A., Lundqvist, A., Moré, S. H., Bonin, von, A., Kiessling, R. & Ljunggren, H.-G. (2002). Heat-shock proteins as activators of the innate immune system. *Trends Immunol* **23**, 130–135.

Wang, B., Zhao, J., Song, L., Zhang, H., Wang, L., Li, C., Zheng, P., Zhu, L., Qiu, L. & Xing, K. (2008a). Molecular cloning and expression of a novel Kazal-type serine proteinase inhibitor gene from Zhikong scallop Chlamys farreri, and the inhibitory activity of its recombinant domain. *Fish Shellfish Immunol* **24**, 629–637.

Wang, J. & Arase, H. (2014). Regulation of immune responses by neutrophils. *Ann NY Acad Sci* **1319**, 66–81 (N. R. Rose, Ed.).

Wang, M., You, J., Bemis, K. G., Tegeler, T. J. & Brown, D. P. G. (2008b). Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. *Briefings in Functional Genomics and Proteomics* **7**, 329–339.

Wang, N., MacKenzie, L., De Souza, A. G., Zhong, H., Goss, G. & Li, L. (2007). Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. *J Proteome Res* **6**, 263–272.

Wang, X., Sun, R., Wei, H. & Tian, Z. (2013). High-mobility group box 1 (HMGB1)-toll-like receptor (TLR)4-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: Interaction of γδ T cells with macrophages. *Hepatology* **57**, 373–384.

Wasinger, V. C., Zeng, M. & Yau, Y. (2013). Current Status and Advances in Quantitative

Proteomic Mass Spectrometry. *International Journal of Proteomics* **2013**, 1–12.

Westman-Brinkmalm, A. A., Abramsson, A. A., Pannee, J. J., Gang, C. C., Gustavsson, M. K. M., Otter, von, M. M., Blennow, K. K., Brinkmalm, G. G., Heumann, H. H. & Zetterberg, H. H. (2011). SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. *Journal of Proteomics* **75**, 425–434.

White, R., Rose, K. & Zon, L. (2013). Zebrafish cancer: the state of the art and the path forward. *Nat Rev Cancer* **13**, 624–636.

Wiese, S., Reidegeld, K. A., Meyer, H. E. & Warscheid, B. (2007). Protein labeling by iTRAQ: A new tool for quantitative mass spectrometry in proteome research. *Proteomics* **7**, 340–350.

Williams, M. R., Azcutia, V., Newton, G., Alcaide, P. & Luscinskas, F. W. (2011). Emerging mechanisms of neutrophil recruitment across endothelium. *Trends Immunol* **32**, 461–469.

Wittamer, V., Bertrand, J. Y., Gutschow, P. W. & Traver, D. (2011). Characterization of the mononuclear phagocyte system in zebrafish. *Blood* **117**, 7126–7135.

Wittmann, C. C., Reischl, M. M., Shah, A. H. A., Mikut, R. R., Liebel, U. U. & Grabher, C. C. (2011). Facilitating drug discovery: an automated high-content inflammation assay in zebrafish. *J Vis Exp* e4203–e4203.

Wolf, K. & Friedl, P. (2011). Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. *Trends in Cell Biology* **21**, 736–744.

Xiong, X.-P., Dong, C.-F., Xu, X., Weng, S.-P., Liu, Z.-Y. & He, J.-G. (2011). Proteomic analysis of zebrafish (Danio rerio) infected with infectious spleen and kidney necrosis virus. *Developmental & Comparative Immunology* **35**, 431–440.

Yago, T., Shao, B., Miner, J. J., Yao, L., Klopocki, A. G., Maeda, K., Coggeshall, K. M. & McEver, R. P. (2010). E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin L 2-mediated slow leukocyte rolling. *Blood* **116**, 485–494.

Yang, J., Zhang, Z., Roe, S. M., Marshall, C. J. & Barford, D. (2009). Activation of Rho GTPases by DOCK Exchange Factors Is Mediated by a Nucleotide Sensor. *Science* **325**, 1398–1402.

Yeh, H.-Y. & Klesius, P. H. (2010). Identification, phylogenetic relationships, characterization and gene expression patterns of six different annexins of channel catfish (Ictalurus punctatus Rafinesque, 1818). *Vet Immunol Immunopathol* **136**, 176–183.

Yoder, J. A., Nielsen, M. E., Amemiya, C. T. & Litman, G. W. (2002). Zebrafish as an immunological model system. *Microbes Infect* **4**, 1469–1478.

Yoder, J. A., Turner, P. M., Wright, P. D., Wittamer, V., Bertrand, J. Y., Traver, D. & Litman, G. W. (2010). Developmental and tissue-specific expression of NITRs. *Immunogenetics* **62**, 117–122.

Yoo, S. K., Starnes, T. W., Deng, Q. & Huttenlocher, A. (2011). Lyn is a redox sensor that mediates leukocyte wound attraction in vivo. *Nature* **480**, 109–112.

Zanivan, S., Krueger, M. & Mann, M. (2012). In vivo quantitative proteomics: the SILAC mouse. *Methods Mol Biol* **757**, 435–450.

Zanivan, S., Meves, A., Behrendt, K., Schoof, E. M., Neilson, L. J., Cox, J., Tang, H. R., Kalna, G., van Ree, J. H. & other authors. **(2013).** In vivo SILAC-based proteomics reveals phosphoproteome changes during mouse skin carcinogenesis. *Cell Rep* **3**, 552–566.

Zhang, L., Marcu, M. G., Nau-Staudt, K. & Trifaró, J. M. (1996). Recombinant scinderin enhances exocytosis, an effect blocked by two scinderin-derived actin-binding peptides and PIP2. *Neuron* **17**, 287–296.

Zhou, W., Liotta, L. A. & Petricoin, E. F. (2012). The Spectra Count Label-free Quantitation in Cancer Proteomics. *Cancer Genomics & Proteomics* **9**, 135–142.

Zhu, W., Smith, J. W. & Huang, C.-M. (2010). Mass Spectrometry-Based Label-Free Quantitative Proteomics. *Journal of Biomedicine and Biotechnology* **2010**, 1–6.

Zon, L. I. & Peterson, R. T. (2005). In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* **4**, 35–44.

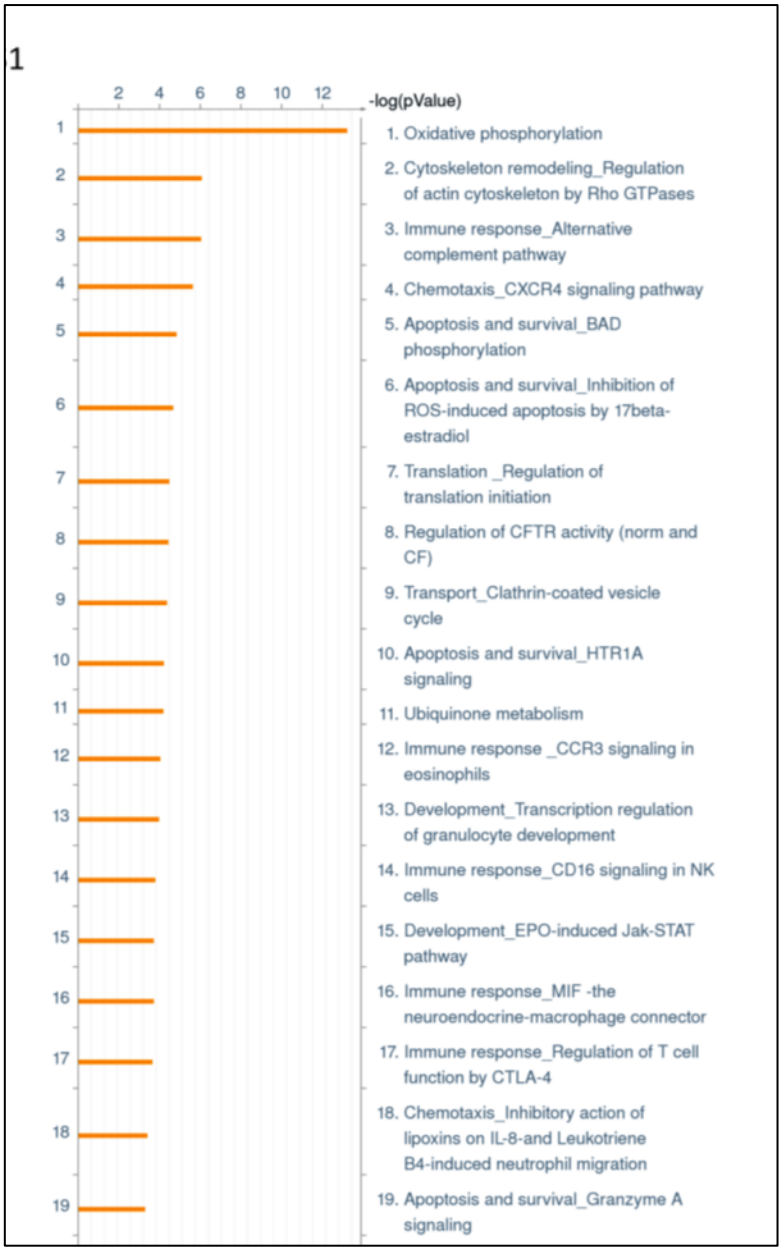
Appendices

Appendix 1

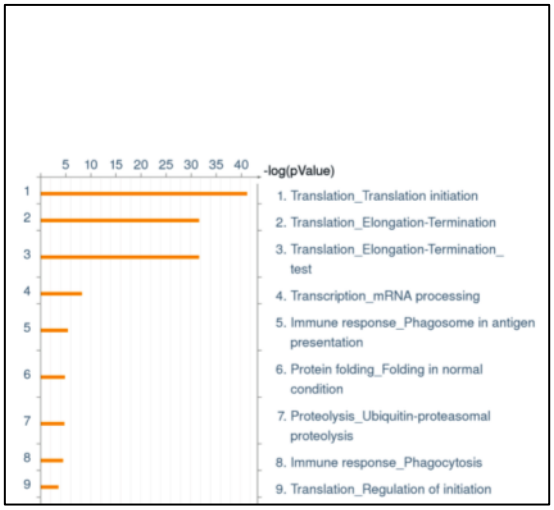
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0073998#s5 Table S1-S3

Singh SK, Sethi S, Aravamudhan S, Kruger M, Grabher C (2013) Proteome Mapping of Adult Zebrafish Marrow Neutrophils Reveals Partial Cross Species Conservation to Human Peripheral Neutrophils. PLoS ONE 8(9): e73998. doi:10.1371/journal.pone.0073998

Appendix 2



Appendix 2: Gene-Go pathway map analysis of zebrafish neutrophil proteins. *Most prominent Gene-Go pathway maps associated with identified neutrophil-specific proteins*



Appendix 3: Zebrafish neutrophil Gene-Go network process pathways. *Most prominent Gene-Go network process pathways associated with identified neutrophil-specific proteins*

Acknowledgement

Though only my name appears on the cover of this thesis, a great many people have contributed to its production. I owe my gratitude to all those people who have made this thesis possible and because of whom my Ph.D. experience has been one that I will cherish forever.

My deepest gratitude is to my research supervisor, Dr. Clemens Grabher for providing me the opportunity work on this project. I have been amazingly fortunate to have a guide who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered. His patience and support helped me overcome many crisis situations and finish this thesis.

I would also like to thank Prof. Cato, for his supervision and accepting to be first referee during my thesis.

I feel privileged in expressing my sincere regards and utmost gratitude to my Collaborator Dr. Kruger at MPI Bad Nauheim for allowing me to pursue mass spectrometry analysis in his lab. I would also like to thank his group specially Sriram and Anna for their help in data analysis.

I would also like thanking Dr. Armant, Ravi, Wilko, for their help during my Ph.D. thesis.

I want to thank my lab member Christine, Luke, and Tanja for their suggestion and help. I am very thankful to Peter for his help and support during FACS training and Sachin Sethi for bioinformatics analysis.

Apart from lab members, I would like to thank my friends Abbas, Sunil, Vasudha, Neeraj and Manvendra, who made my journey easy with their long lasting support and encouragements.

It's my fortune to gratefully acknowledge the support of some one special Aarti. Reaching up to here was not possible without her. She was always supportive and savior during personal and professional problems.

I would like to express my devotion to my parents and family for all the sacrifices they have made in the way of my education. They have never given up on me even at the times when it was very difficult for me go on.

Curriculum Vitae

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Education

1. **Ph.D. Thesis Title/Guide: -** "Quantitative Proteomic Approaches in a Zebrafish Model of Granulocytic Inflammation", under the supervision of Dr. C. Grabher, Institute of Toxicology and Genetics at the Karlsruhe Institute of Technology, Germany
2. **Master, M.Sc.** (Biochemistry) from Allahabad Agricultural Institute (Deemed) University, Allahabad, India, 2007

Publications from PhD work

1. Wacker I, Chockley P, Bartels C, Spomer W, Hofmann A, Gengenbach U, **Singh S**, Thaler M, Grabher C, Schroder R, Characterizing FAC-sorted populations of zebrafish immune cells by their 3D structure. **J. Microscopy (accepted)**
2. **Singh SK**, Sethi S, Aravamudhan S, Armant O, Krüger M, Grabher C**.** Inflammatory proteome and transcriptome analysis of adult zebrafish kidney marrow neutrophils **Fish Shellfish Immunol.** 2014 Sep;40(1):217-24
3. **Singh SK**, Sethi S, Aravamudhan S, Krüger M, Grabher C (2013) Proteome Mapping of Adult Zebrafish Marrow Neutrophils Reveals Partial Cross Species Conservation to Human Peripheral Neutrophils. **PLoSONE** (2013) 8(9): e73998. doi:10.1371/journal.pone.0073998
4. Wittmann C, Chockley P, **Singh SK**, Pase L, Lieschke GJ, and Grabher C,. Hydrogen peroxide in inflammation: messenger, guide, and assassin. **Adv Hematol.** (2012), 541471

Other publications

5. Purushothaman S., Saxena S, Vupalapathy M, Lakshmi MG, **Singh SK**, Brahmendra Swamy CV, Idris MM, Understanding the effect of disturbed light-dark cycle in zebrafish circadian rhythm. **J. Proteomics (Under revision)**
6. Saxena S, Dupont S, Vuppalapaty M, Lakshmi MG, **Singh SK,** Brahmendra Swamy CV, and Idris MM. Proteome map of the neural complex of the tunicate Ciona intestinalis, the closest living relative to vertebrates. **Proteomics** (2013) 13**,** 860–865.

7. Saxena S*, **Singh SK***, Lakshmi MG, Meghah V, Bhatti B, Brahmendra Swamy CV, Sundaram CS, Idris MM, Proteomic analysis of zebrafish caudal fin regeneration. **Mol. Cell. Proteomics**. (2012)Jan 25. **(* Equal contribution)**
8. Saxena S*, **Singh SK*,** Lakshmi MG, Meghah V, Sundaram CS, Swamy CV, Idris MM, Proteome profile of zebrafish kidney. **J. Proteomics** (2011) Nov 18; 74 (12): 2937-47. **(* Equal contribution)**
9. **Singh SK**, Lakshmi MG, Saxena S, Saxena P, Idris MM, Proteome profile of zebrafish olfactory lobe, **Zebrafish** (2011), 8(4): 183-189
10. **Singh SK,** Lakshmi MG, Saxena S, Swamy CV, Idris MM, Proteome profile of zebrafish caudal fin based on LC MSMS and 2DE MALDI MSMS analysis. **Journal of Separation Science J. Sep. Sci.** (2010), 33, 1–8
11. **Singh SK**, Sundaram CS, Shanbhag S, Idris MM, Proteomic profile of Zebrafish brain based on 2DE MALDI MS/MS analysis, **Zebrafish** (2010) Jun; 7(2): 169-77
12. **Singh SK,** K V Rakesh, Kalidas Ramamorthy, AVP Sardhi Idris MM, Proteomic profile of Zebrafish brain based on gel LC-ESI MS/MS analysis. **Journal of Proteomics and Bioinformatics** (2010) 3; 135-142

Published in conferences and Symposia

1. FishForPharma Genomics and Bioinformatics Workshop, 2014 Leiden Netherland
2. Zebrafish Lens proteome based on 1DE LCMS-MS and 2DE MALDI in 5th AOHUPO congress, 14 ADNAT convention 1st PSI conference new perspectives in proteome research, 2010 CCMB Hyderabad India.

Date: December 2014
Place: Karlsruhe, Germany

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