
THE UNIVERSITY *of* EDINBURGH


# Investigating the role of Endoplasmic Reticulum stress in neutrophils during tumour initiation

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The thesis submitted for the degree of
**Master of Science by Research**
2024

# Declaration

I, Peiyi Yu, hereby declare that the thesis presented is my own work, except where indicated. The work has not been submitted for any other degree or professional qualification.

Peiyi Yu

03.2024

## Acknowledgements

I am profoundly thankful to my supervisor, Dr. Yi Feng, for entrusting me with the invaluable opportunity to contribute to this project. Her exceptional guidance, efficiency, and many insightful suggestions have provided unwavering support and propelled my research endeavours forward from diverse angles. Under her mentorship, I have grown substantially professionally, acquiring invaluable experience and expertise along the journey.

I extend my heartfelt appreciation to the dedicated members of my laboratory, including Owen Lo, Kai Yee Chang, Eva Zhao, and the esteemed previous lab members, Dr. Jeanette Johansson and Dr. Abigail Elliot. Their unwavering support, collaborative spirit, and collective effort have been instrumental in the success and progress of this project. Together, we have tackled challenges, exchanged knowledge, and cultivated a collaborative and stimulating research environment that fosters growth and innovation.

Furthermore, I am grateful for the exceptional support and resources provided by the Biological Research and Veterinary Services (BVS) Aquatics Facility at Queen's Medical Research Institute, as well as the expertise and assistance offered by the staff at the IRR Flow Cytometry & Cell Sorting Facility. Their dedication, professionalism, and technical expertise have been invaluable in facilitating the execution of experiments and data analysis, contributing significantly to our research advancement.

I am particularly indebted to individuals such as Dr. Carl Tucker, Ailsa Laird, and Dr. Anna Popravko for their invaluable technical expertise and unwavering support throughout various stages of this project. Additionally, I extend my gratitude to my dear friends Dr. Elva Chang and Julia Wang, whose encouragement, insightful discussions, and assistance in troubleshooting technical issues have been precious and deeply appreciated.

Lastly, I want to express my deepest gratitude to my family and friends for their unwavering support, encouragement, and understanding throughout my academic journey. Their unconditional love, encouragement, and belief in me have been a constant source of strength and motivation, enabling me to navigate the challenges of graduate studies with confidence, resilience, and determination.

# Abbreviations

| | |
|---|---|
| atf6 | activating Transcription Factor 6 |
| eGFP | Enhanced green fluorescent protein |
| BFP | Blue fluorescent protein |
| NLS | Nuclear localization sequence |
| Krt19 | keratin 19 |
| LysC/LyzC | Lysozyme C |
| 4-OHT | 4-Hydroxy-Tamoxifen |
| DMSO | Dimethyl Sulfoxide |
| MS-222 | Tricaine Mesylate |
| HPF | Hours Post Fertilisation |
| HPI | Hours Post Induction |
| HPT | Hours Post Treatment |
| DPF | Day Post Fertilization |
| cfos | FBJ Osteosarcoma Oncogene |
| LMP | Low Melting Point |

## Lay Abstract

Neutrophils are a type of white blood cell typically associated with fighting infections. Surprisingly, these same cells appear to promote tumour growth.

Zebrafish, with their remarkable transparency and genetic resemblance to humans, have become indispensable tools in cancer research. Scientists can look into them through a microscope, observing biological processes in real time. Zebrafish models of tumour development led to the observation that neutrophils promote early tumour cell growth.

To understand the unexpected tumour-promoting function of neutrophils, we studied gene expression in neutrophils from zebrafish carrying early tumours, and we pinpointed a list of more active genes in neutrophils. Among these genes, we noticed a group of genes commonly increase together when cells respond to stress by a sudden need to make lots of proteins. We think this might hold clues to how neutrophils support tumour growth. To visualise such a response in neutrophils, zebrafish are engineered to express a fluorescent protein when cells are responding to an overdrive of making proteins, and this can be examined under a special microscope. Using this engineered zebrafish, I examined neutrophils near tumours; surprisingly, I did not see any fluorescent signal in neutrophils.

However, there are other ways to detect such stress, which I will need to test in future studies to confirm my current results. Further investigation is needed to unravel this complex interplay and uncover the full story. Understanding the intricate dynamics between neutrophils and tumours in zebrafish could offer valuable insights into cancer biology and potentially lead to innovative therapeutic approaches.

## Abstract

Neutrophils are pivotal innate immune cells conventionally recognised for their rapid response to infection sites. In recent years, immune suppressive and tumour-promoting neutrophils were found enriched in the tumour microenvironment, suggesting their role in driving the development of cancer. However, the role of neutrophils during tumour initiation is less clear. Studies in zebrafish tumour initiation models suggest that neutrophils might play a trophic role in promoting early tumour cell proliferation. However, the underlying mechanism remains to be determined.

To elucidate the mechanisms associated with this trophic function during tumour initiation, the Feng lab employed single-cell RNA sequencing analysis to capture all neutrophils in preneoplastic cell-carrying larvae to compare that with neutrophils from control larvae. This revealed a list of upregulated genes linked to endoplasmic reticulum (ER) stress. I hypothesize a potential connection in mechanisms between ER stress and the trophic function of neutrophils associated with PNCs.

In this project, I established an ER stress reporter zebrafish line Tg(*5XATF6RE*:eGFP) to visualise ER stress. I confirmed the functionality of the reporter fish, as previously described by Clark et al. (2020). However, utilising a time window of 24 hours post-induction (hpi) induced by 4-hydroxytamoxifen (4-OHT), I didn't visualise PNCs and PNC-associated neutrophils and proceeded with time-lapse confocal live imaging from 24 to 36 hpi to monitor ER stress in these cells. Surprisingly, my results indicated subtle intensity changes indicative of ER stress in PNC-associated neutrophils, as confirmed by FACS analysis.

I also generated a neutrophil-specific CAS9 expression transgenic zebrafish Tg(lyzC:CAS9), which paved the way for future neutrophil-specific modulation of ER Stress to study its functionality.

In summary, although I did not detect elevated ER stress in neutrophils in the PNC development model using the Tg(*5XATF6RE*:eGFP), I did not have enough time to employ alternative methods to evaluate ER stress in neutrophils. Therefore, whether ER stress is involved in modulating neutrophil function in the PNC development model remains to be determined. However, I have generated tools to facilitate future studies into ER stress and functional studies in neutrophils in zebrafish models.

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# 1 Introduction

## 1.1 Introduction to cancer

Cancer is a condition where certain cells in the body undergo uncontrolled growth and can spread to other areas. Usually, cells in the human body follow certain ways to grow, multiply, and eventually die off to be replaced by new ones as needed. However, the process can go awry, leading to abnormal or damaged cells proliferating inappropriately. These cells may form tumours, which can be either cancerous (malignant) or non-cancerous (benign)[1]. Approximately half of all individuals will experience the development of some cancer at some point in their lifetime. In the UK, breast cancer, lung cancer, prostate cancer and bowel cancer are the most common types of cancer[2]. The reasons of causing cancer are varied. Risk factors like genetic predispositions, environmental exposures, and lifestyle choices collectively contribute to the development of the disease, encompassing inherited mutations, age-related factors, carcinogenic substances, infectious agents, and unhealthy behaviours[3].

## 1.2 Heterogeneity of cancer and hallmarks

Cancer is a heterogeneous disease revealed by variability among individual tumour cells. The Variations are evident not just within a single tumour but also among patients with cancers originating from the same organ. The presence of intra- and inter-tumour heterogeneity poses challenges for predicting the outcome of cancer therapy[4].

Tumour is formed by the abnormal proliferation of cells, which can be either benign or malignant, carrying mutations and increasing proliferation, survival, invasion, and metastasis abilities[5].

The previous research revealed that the oncogenic process entails a dynamic transformation whereby a genetically modified cell undergoes malignant changes, culminating in the formation of a neoplastic cell mass[6]. The progression and acquired capabilities were concluded into “hallmarks of cancer”.

The concept of the hallmarks of cancer was initially introduced in 2000, outlining six hallmark capabilities that cancer cells acquire during their development, including evasion of apoptosis, insensitivity to antigrowth signals, self-sufficiency in growth signals, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis. This framework provided an understanding of the multistep transformation of normal cells into a neoplastic state[7]. In 2011, four additional hallmarks—reprogramming cellular metabolism, avoiding immune destruction, tumour-promoting inflammation, and genome instability and mutation—were introduced, expanding our understanding of the capabilities required for tumour growth and progression[8]. Over the past decade, researchers have gained a deeper understanding of the tumour microenvironment, composed of cancer cells and cancer stem cells, and associated various recruited stromal cell types. This expanded knowledge has led to the identification of new parameters, including unlocking phenotypic plasticity, non-mutational epigenetic reprogramming,

polymorphic microbiomes, and senescent cells, which contribute to the newly recognised hallmarks of cancer[9].

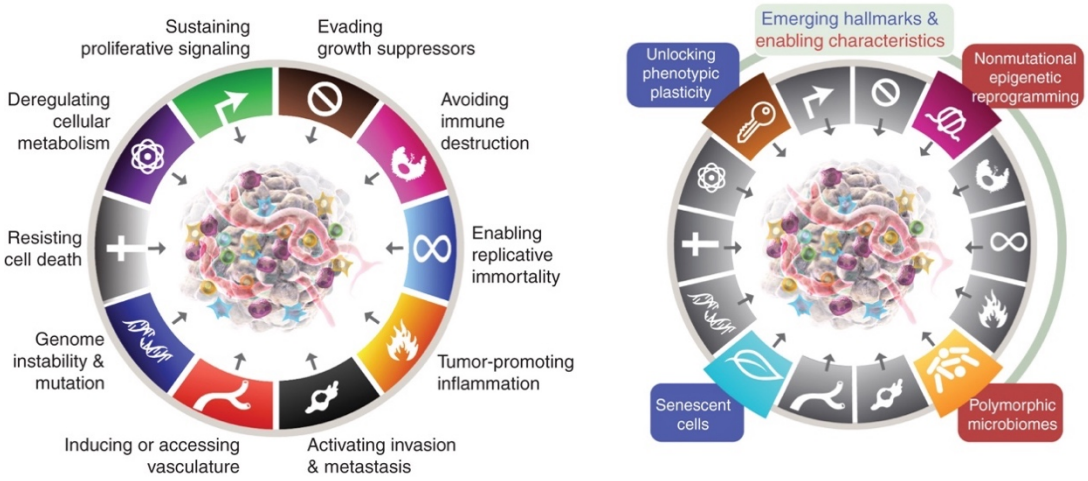


Figure 1.2.1 **Hallmarks of Cancer.** Figure reused from Douglas Hanahan; Hallmarks of Cancer: New Dimensions. Cancer Discov 1 January 2022; 12 (1): 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

## 1.3 Ras protein and the role in Cancer

Ras proteins are small GTPases which function as cell switches controlling cell proliferation, differentiation, migration, and apoptosis[10]. The activation of the RAS pathway is facilitated by guanine nucleotide exchange factors (GEFs). Upon binding an extracellular signal to membrane receptors, a signalling cascade is initiated, leading to the activation of GEFs, which catalyse the dissociation of guanine dinucleotides (GDP) from the RAS protein. This allows for the subsequent binding of guanine trinucleotides (GTP), thereby activating the pathway. Following activation, GTPase activating proteins (GAPs) play a critical role in promoting the hydrolysis of GTP back into GDP, leading to the inactivation of RAS and resetting the pathway to its inactive state[11].

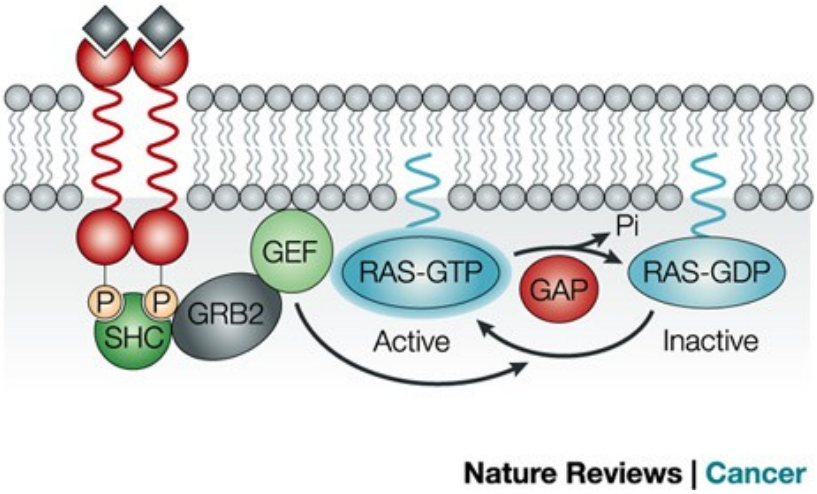


Figure 1.3.1 **RAS signalling pathways.** Figure reused from Downward, J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3, 11–22 (2003). https://doi.org/10.1038/nrc969) showing the switch function of the RAS protein.

However, oncogenic Ras proteins, characterised by gain-of-function missense mutations, drive oncogenesis by enhancing the rapid exchange between GTP and GDP binding or by impairing the binding process of GAPs, thereby sustaining the pathway in an activated state[12].

Mutations occurring within Ras genes have been identified in approximately 20% of human cancer cases, underscoring the profound impact of these genetic alterations in oncogenesis. This family of genes comprises HRAS, NRAS, KRAS4A, and KRAS4B, which are encoded by three Ras genes: HRAS, KRAS, and NRAS[13]. Among RAS-mutated cancer patients, KRAS mutation accounts for 76%, and HRAS accounts for 7%.

In the spectrum of human cancers, HRAS mutations, although less prevalent overall, are distinguished by their specialised contribution to the transformation of squamous epithelial cells in tissues such as the skin, lung, head and neck, and bladder[14,15]. According to previous studies, Val-12 exhibits the most minor GTPase activity compared to other amino acid substitutions at codon 12. $HRas^{G12V}$ demonstrates the highest transformation potential[16].

## 1.4 Inflammation and tumour-associated innate immunity

Inflammation is concluded as a response to injury, infection, or tissue stress and dysfunction. The initiation of the acute inflammatory response is prompted by infections, stimulating the activation and recruitment of neutrophils to initiate a cascade aimed at depleting infectious agents. Upon activation, neutrophils release various cytotoxic granule constituents, including reactive oxygen species (ROS), reactive nitrogen species, proteinase 3, cathepsin G, and elastase. Acute inflammation triggers cancer cell death through the induction of an immune response that is anti-tumorigenic[17].

However, if the initial clearance process is unsuccessful, macrophages and T cells within the human body will take over from the infiltrated neutrophils. Inadequate levels of these cells may result in the tissue transitioning into a chronic inflammatory state[18]. Chronic inflammation, mainly when it is uncontrolled, persistent, and unresolved, has been linked to an elevated likelihood of developing malignancies and facilitating the malignant advancement of cancer across various cancer types[19].

The recent study summarises cancer-associated chronic inflammation into two pathways: the intrinsic pathway involves the activation of diverse classes of oncogenes, which drive inflammation-related programs and contribute to creating an inflammatory environment; the extrinsic pathway involves inflammatory conditions that facilitate the development of cancer[17]. In more recent findings, there has been an emergence of evidence indicating that inflammation also plays a significant role during the initiation of tumours[20].

## 1.5 Neutrophil function with associated tumour

Neutrophils are known as one of the most abundant innate immune cells in the human body and one of the earliest immune cells recruited to infection and inflammatory response. They develop in the bone marrow and are derived from myeloid progenitors[21]. Once activated, their longevity increases by several folds to normal conditions, making them primed neutrophils showing to the infection site[22]. "Hallmarks of Cancer" pointed out that inflammatory cell types release chemicals like reactive oxygen species, promoting cancer cells' mutagenic and malignancy[8]. Tumour-associated neutrophils (TANs), a subset of inflammatory cells found in the vicinity of tumours, have been shown to negatively influence anti-tumour immunity and promote tumour progression and metastasis through the secretion of growth factors, chemokines, and inflammatory mediators such as matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), high mobility group box 1 (HMGB1), and interleukin-17 (IL-17)[23]. Additionally, neutrophils release the protease neutrophil elastase (NE), which enhances tumour proliferation by infiltrating the endosomal compartment of tumour cells and interacting with proteins through crosstalk[24]. Neutrophils with pro-tumour characteristics are classified as anti-tumorigenic N1, while those exhibiting an anti-tumour phenotype are referred to as pro-tumorigenic N2[25]. Yet, neutrophils have been demonstrated to facilitate adaptive anti-cancer immune responses within lymph nodes during the initial phase of cancer development, compared with the late phase of cancer in mammals[26]. The neutrophil response to transformed cells is conservative among species. In zebrafish melanoma models, tumour-associated neutrophils (TANs) were found to possess tumour-promoting capabilities through their facilitation of angiogenesis and suppression of cellular apoptosis[27].

## 1.6 Neutrophils' trophic function at the preneoplastic stage

Tumorigenesis encompasses a stepwise progression from preneoplastic to premalignant and malignant lesions, characterised by genomic instability and subsequent alterations in oncogenes, tumour suppressor genes, DNA repair genes, and signalling pathways. Preneoplastic cells are those that have undergone genetic and phenotypic changes, rendering them more prone to progressing towards malignancy compared to normal cells[28]. Early in 2010, host leukocytes were proved to be activated and recruited upon oncogenic HRAS mutation expressed in early-stage development in the zebrafish model. The study revealed that hydrogen peroxide ($H_2O_2$), a pro-inflammatory signal, was locally synthesised by transformed cells which, the similar findings were observed in the wound model. Interestingly, the inflammatory response amplified in the post-transformed cell and went towards a chronic inflammatory state, meaning "wounds do not heal"[29].

On the other hand, inhabiting the generation of hydrogen peroxide led to leukocyte recruitment, shown in both transformed cell models, and the wounding and growth of neoplastic cells number decreased[30]. Due to the difficulties of observing early tumourigenesis in mammalian models and human patients, the zebrafish model has been widely used to mimic pre-neoplastic cell development[20]. Later research has revealed that neutrophils exhibit a pro-tumorigenic role by suppressing NFκB activity, which is essential for the inflammatory response[31].

## 1.7 Endoplasmic reticulum stress mechanism

The Endoplasmic reticulum, the largest organelle in the cell, is known as the significant site for protein synthesis, transport, folding, lipid and steroid synthesis, carbohydrate metabolism, and calcium storage[32]. Since the endoplasmic reticulum

undertakes such important roles, strict regulation is necessary to help it maintain proper function.

Endoplasmic reticulum stress serves as a monitoring mechanism, safeguarding normal cellular functions and triggering cell death programming when cells are unable to recover from internal and external disturbances such as unfolded and misfolded proteins, redox stress, infections, and other perturbations[33]. The critical feature of ER stress is the activation of the unfolded protein response (UPR). In mammalian cells, the UPR operates through three transmembrane proteins: inositol-requiring enzyme 1 alpha (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6).[34] These transmembrane protein-mediated pathways play distinctive functions under UPR activation conditions. The PERK pathway mainly helps with initiating cell apoptosis programming, while ATF6 and IRE1$\alpha$ pathways help release ER membrane stress by increasing protein folding and processing capacity, which helps cell survival[35].

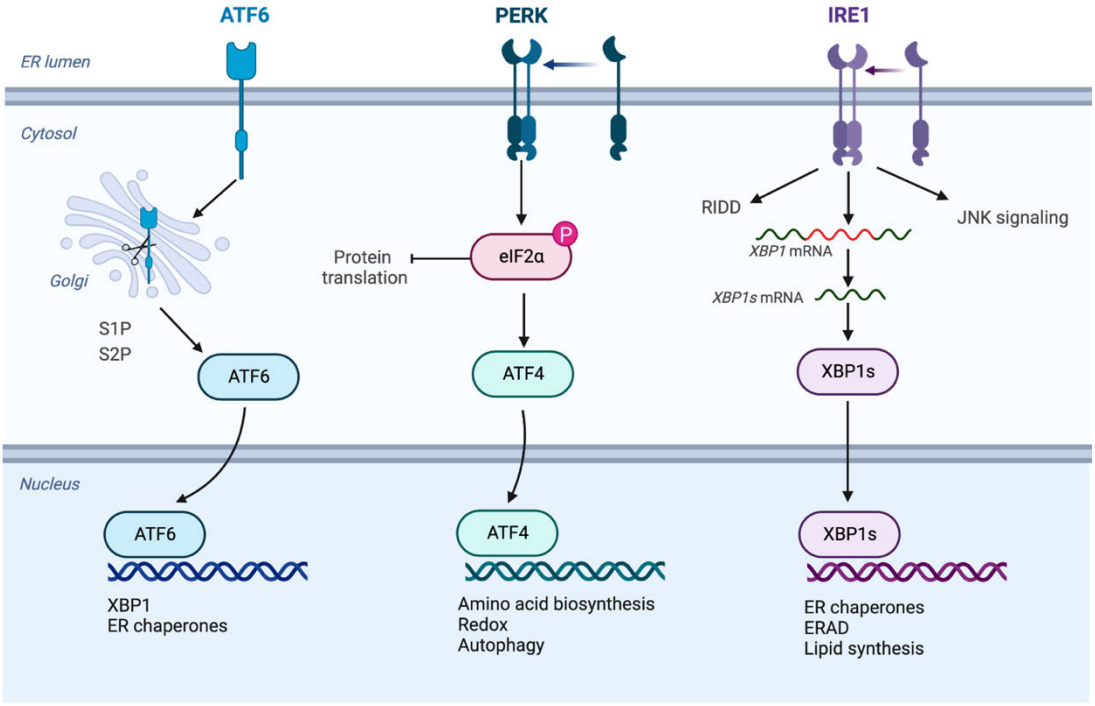


Figure 1.7.1 BioRender (2019). **UPR Signaling (ATF6, PERK, IRE1).** https://app.biorender.com/biorender-templates/figures/all/t-5dd9a632e8baa5007f62f161-upr-signaling-atf6-perk-ire1

The mechanism activation of UPR is to mediate the translational and transcriptional controls to eliminate such “wrongly formed proteins” while it will alter the cell fate towards cell apoptosis if the fixation step, restoration of ER homeostasis, fails[36,37]. Research from previous studies suggests that increased levels of endoplasmic reticulum (ER) stress and the resulting activation of the unfolded protein response (UPR) are associated with the onset and progression of diverse diseases, including myelin disorders like multiple sclerosis and Charcot-Marie-Tooth disease[38]. Not surprisingly, ER stress had been reported that upon undergoing oncogenic transformation, cancer cells enter a state of sustained, non-lethal endoplasmic reticulum (ER) stress response orchestrated by the activation of IRE1α and PERK. This response facilitates cellular adaptation and promotes tumour growth. On the other hand, the persistent activation of ER stress sensors, particularly IRE1α and

PERK, in tumour-associated myeloid cells plays a role in promoting cancer progression by shaping a tumour microenvironment (TME) that is tolerant and immunosuppressive [39].

## 1.8 Endoplasmic reticulum stress working in the immune system

The UPR activation plays a critical role in immune cell development and differentiation. ER stress has been proven to strongly influence innate and adaptive immune responses by mediating cytokine production in B cells, macrophages, and dendritic cells(DCs)[40]. Endogenous ER stress and the unfolded protein response (UPR) play crucial roles in regulating the function, expansion, and differentiation of suppressive myeloid cells within the tumour microenvironment of cancer hosts. Moreover, continuous activation of ER stress responses enables malignant cells to adapt to oncogenic and environmental challenges while facilitating diverse immunomodulatory mechanisms that promote malignant progression[39].

In macrophages, the main transmembrane proteins XBP1s and ATF6 were proven to work as positive regulators of inflammatory cytokine expression at post-toll-like receptor (TLR) stimulations [40]. However, there is a lack of research investigating ER stress and UPR activation, specifically in neutrophils, particularly in association with pre-neoplastic stage transformed cells.

## 1.9 Modelling tumour initiation in zebrafish

Zebrafish was first introduced as a model organism by Dr. George Streisinger in the 1970s due to the simplicity of working and the ease of genetic manipulation in this model organism. The adoption of zebrafish as a model organism surged in the 1990s, notably propelled by the creation of two significant genetic mutants.

Identifying mutants is a pivotal strategy in exploring diverse biological domains, thereby emphasising the importance of zebrafish as a valuable research tool. They became widely utilised due to their transparent bodies throughout development, sharing approximately 70% of genes with humans and harbouring a large number of genes associated with human diseases[41]. Moreover, their external embryo development provides an opportunity for convenient gene manipulation.

Zebrafish has emerged as a pertinent cancer model due to its possession of highly conserved immune systems and cancer-related genes that are homologous to those found in humans[20,42]. Mutations in RAS genes exhibit different patterns that vary across various types of cancer. Unlike oncogenic KRAS, which is most commonly altered in pancreatic cancer, colorectal cancer, and lung cancer, mutated HRAS is predominantly observed in dermatological cancers, as well as head and neck cancers[43]. The zebrafish model carrying oncogenic HRAS mutation, derived from human patients (HRASG12V), was established in 2009[44,45]. To achieve conditional expression of oncogenic HRAS and help with visualisation of the early tumour development in zebrafish skin cells, 4-Hydroxytamoxifen (4-OHT) inducible KalTA4-ER$^{T2}$/UAS expression system was created. The KalTA4-ER$^{T2}$/UAS system offers both inducible and reversible properties, enabling the induction of cell transformation with precise temporal and spatial resolution in vivo [46].

Our lab had already used this model to demonstrate enhanced proliferation in PNC at 18 hpi and increased apoptosis at 24 hpi. Using our zebrafish larvae model, a previous lab member conducted a single-cell RNA sequence data analysis. This analysis focuses on the PNC-associated neutrophils in zebrafish larvae upon overexpressing the oncogene HRAS in the basal layer skin cell model. This revealed the high upregulation at 24 hours post induction of PNCs compared with neutrophils

under normal conditions (Dr. Abigail Elliot). This suggests the upregulation of ER stress-related genes, especially atf6, may play a role in neutrophil driving the PNCs growth. The tumour microenvironment is known to be exposed to both intrinsic and extrinsic factors, leading to the adaptation of PNCs to stressful conditions. To further investigate how AT6-related ER stress regulates neutrophils in our PNC model, I used the Tg(5XATF6RE:eGFP) construct to develop the ATF6 reporter line and in vivo imaging of ATF6 signal with our inducible zebrafish PNC model.

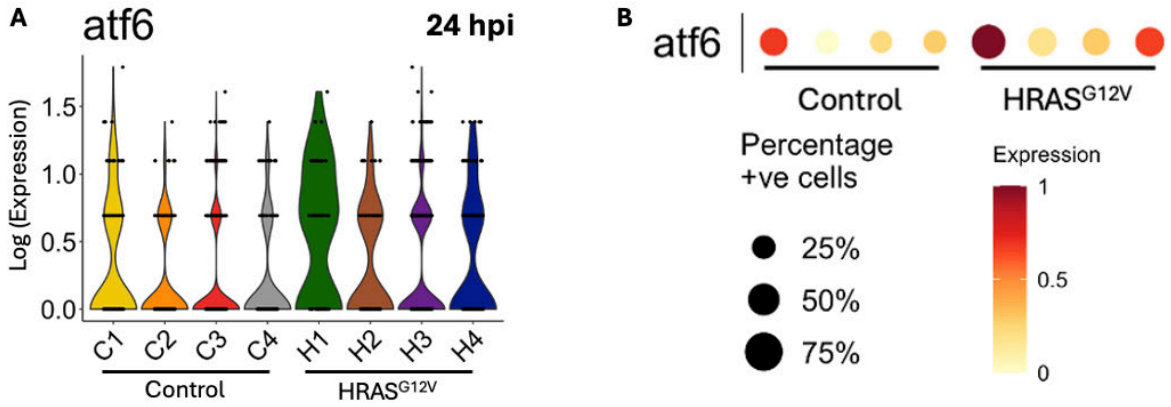


Figure 1.9.1 **atf6 upregulation in neutrophils upon HRAS$^{G12V}$ expression.** Figure **A** The violin plots indicating atf6 gene upregulation in PNCs-associated neutrophils at 24 hours post-induction. The clusters C1-C4 (control) and H1-H4 (HRAS) indicate different stages of neutrophil maturity (cluster 1 is early stage and 4 is late stage of maturation). Figure B is listed atf6 expression in neutrophils populations. Left 1-4 dots (control) matched with left clusters (C1-C4) in Figure A; Right 1-4 dots (HRAS$^{G12V}$) matched with right clusters (H1-H4) in Figure A. (Credit: Dr. Abigail Elliot)

# 2 Hypothesis

ER stress expressed in neutrophils plays a trophic function promoting tumour growth.

# 3 Aims

Aim 1: Establish ATF6RE:eGFP reporter fish line

Aim 2: Validate ATF6RE:eGFP reporter fish line reporting atf6 activity upon ER stress drug induction

Aim 3: Validate single-cell RNA sequencing analysis data upregulating atf6 in neutrophils upon HRAS$^{G12V}$ expression in zebrafish larvae

Aim 4: Generate tools which will facilitate future studies into ER stress and functional studies in neutrophils in zebrafish models

# 4 Material and Methods

## 4.1 Transgenic zebrafish lines

The list of transgenic zebrafish lines used in this project Table 1.

| In-text abbreviation | Full Transgenic line name | Markers | Place of birth |
|---|---|---|---|
| *5XATF6RE*:eGFP | Tg(Has.ATF6RE:eGFP)$^{mw}$ [84 47] | eGFP express in skeletal muscle and lens region | Animals born in the UK at a licensed establishment |
| K19;UAS:TagBFP-HRAS$^{G12V}$; lysC:NLS-mScarlet | Tg(krtt1c19e:KalTA4-ERT2; UAS:TagBFP-HRAS$^{G12V}$;lysC:NLS-mScarlet) | eGFP heart: K19;TagBFP and CFP lens for | Animals born in the UK at a licensed establishment |

| | | | |
|---|---|---|---|
| | | HRAS$^{G12V}$; mScarlet: neutrophils nucleus | |
| LyzC:cas9; cry:GFP | Tg(lyzC:Cas9, cry:GFP)$^{pu26}$ [48] | eGFP lens | Animals born in the UK at a licensed establishment |

Table 1 The transgenic zebrafish lines and relevant selection markers used in all experiments.

All of the adult fish were maintained in tanks in a 14-hr light-dark cycle. Transgenic zebrafish (Table 1) were used for the experiments. They were kept at approximately 28.5 ° C, with constant filtration, aeration, and daily feeding. BVS Aquatic helped with the growth and feeding of fish embryos and adult fish.

For breeding, male and female fish will be taken out the day before from the original tank and isolated in a 500ml tank containing a removable divider to ensure the breeding time is the same among experiments. Eggs were collected and grown in a 90mm diameter petri dish at 28.5°C in clean fish water (< 50 eggs per dish promising well development); egg water was changed daily.

## 4.2 Media and solution

All reagents were acquired from Sigma-Aldrich unless otherwise stated.

| Reagents | Ingredient |
|---|---|
| Embryo Medium | 0.5 µM NaCl |
| | 0.17 µM KCl |
| | 0.33 µM CaCl |
| | 0.33 µM $MgSO_4$ |
| | 0.1% Methylene Blue |
| 0.3x Danieau's | 58mM NaCl |
| Solution | 0.7mM KCl |
| | 0.4mM MgSO4 |
| | 0.6mM $Ca(NO_3)_2$ |
| | 5.0mM HEPES Adjust pH to 7.6 |
| Induction Solution | 0.3x Danieau's Solution |
| | 0.5%(v/v) DMSO |
| | 5 µM 4-Hydroxyamoxifen (4-OHT) (10mM stock in EtOH) |
| 4-Hydroxytamoxifen | Dissolve 4-Hydroxytamoxifen (Sigma, #H7904) particle in |
| 10 mM stock | ml 98% ethanol |
| LB Broth | 10g Tryptone |
| | 5g Yeast Extract |
| | 5g NaCl |
| | 1mL 1M NaOH |
| | 1L $H_2O$ Adjust pH to 7.0 |

| | |
|---|---|
| LB Agar | LB Broth |
| | 15 g/L Bacto Agar |
| PBS solution | 137mM NaCl |
| | 2.7mM KCl |
| | 10mM $Na_2HPO_4$ |
| | 1.8mM $KH_2PO_4$ |
| | Adjust pH to 7.4 |
| PBST | PBS |
| | 0.5%(v/v) Tween 20 |
| TAE Buffer 50x Stock | 242 g tris base in double-distilled H2O. |
| | 57.1 ml glacial acetic acid |
| | 100 ml 0.5 M EDTA solution (pH 8.0) |
| | Adjust volume to 1 L |
| | Dilute 1:50 using ultra-pure water |
| Tunicamycin 0.5mg/mL | 1mg Tunicamycin (Sigma-Aldrich #T7765) |
| stock | from *Streptomyces* sp. |
| | 2mL DMSO solution |
| | Dilute to target concentration with satisfying |
| | maximum DMSO usage. |
| | The concentration of tunicamycin in the experiment is |
| | 1μg/mL |
| Fish water | 60 μg/mL methylene blue salt dissolved in distilled |
| | water |
| Media A (FACS) | 1X HBSS (Gibco #14175095) |

| | |
|---|---|
| | 15 mM HEPES (Fisher bioreagents #BP299)<br>25 mM D-Glucose (Sigma#G8644)<br>2% sterile goat serum (Life Technologies #16210064) |
| The collagenase IV | 250 mg/mL Collagenase Type IV powder (Gibco #17104019)<br>Dissolve power in 1X HBSS (Gibco #14175095) |

## 4.3 Microinjection and generating of transgenic ATF6 reporter zebrafish line

Transposase mRNA tol2 was coinjected with *ATF6RE:*eGFP plasmid to generate transgenic fish lines. The injected embryos were selected via the selection marker at late 4dpf. The positive marker carriers were fed till raised to breeding age and crossed with wildtype to generate F1 generations. F1 generations were selected by their transgenic markers and raised to breeding age. F2 generations were selected from F1 male fish crossed with wildtype female fish, which contains equal selection marker expression level with the number of embryos ratio as 50% positive and 50% negative to ensure single transgene insertion for the future experiment.

## 4.4 5XATF6RE:eGFP reporter fish validation via drug treatment

This experiment is a repeat experiment published in 2020[47] to validate reporter function using drugs. The F1 paternal transgene carrier larvae were selected at around 24hpf (1dpf) by the eGFP expression in the skeletal muscle region. The fish

larvae were separated into the Tunicamycin treatment group (n=12) and the DMSO control group (n=12). The repeat of both groups were under the same treatment without MS222 imaging sessions to avoid MS222 toxicity (DMSO control group: n=12; Tunicamycin treatment group: n=12). Experimental groups are treated with 0.1 ng/μL tunicamycin. The images were captured at 24 hpt (2dpf) and 48 hpt (3dpf). The experiment was done via the 96-well plate to ascertain that the same fish could be captured at either time point.

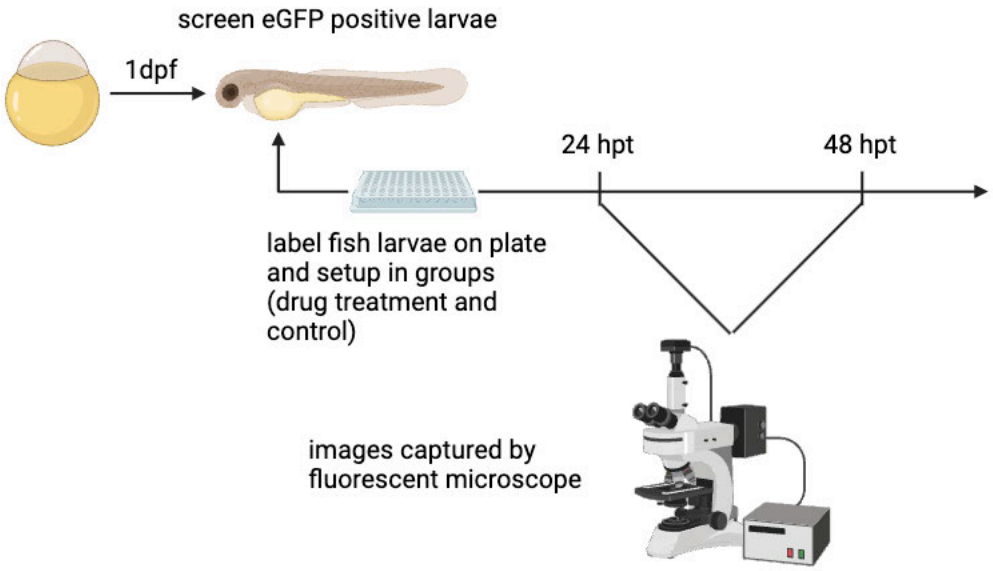


Figure 4.4.1 **Schematic of drug treatment experiment.** The control group larvae (n=12) and experimental group larvae (n=12) were set in a 96-well plate to be treated. The number of each larva was labelled to ensure a one-to-one comparison. Schematics generated in BioRender.

## 4.5 Inducing HRAS$^{G12V}$ expression with 4-hydroxytamoxifen

The K19; UAS: TagBFP-HRAS$^{G12V}$; lysC: NLS-mScarlet adult male and female fish pairs were taken out and set for breeding in 500ml breeding tanks in the afternoon before fertilisation. They were separated by removable dividers and would be taken away at around 9 am to synchronise egg fertilisation time. The collected eggs were kept in petri dishes at 28.5˚ C in fish water, with about 50 eggs per dish. The water was changed every 24 hours till induction. Induction will start from day 2 afternoon

post fertilisation. The induction solution was replaced with fish water and changed every 24 hours. The dishes were kept away from light, promising induction efficiency.

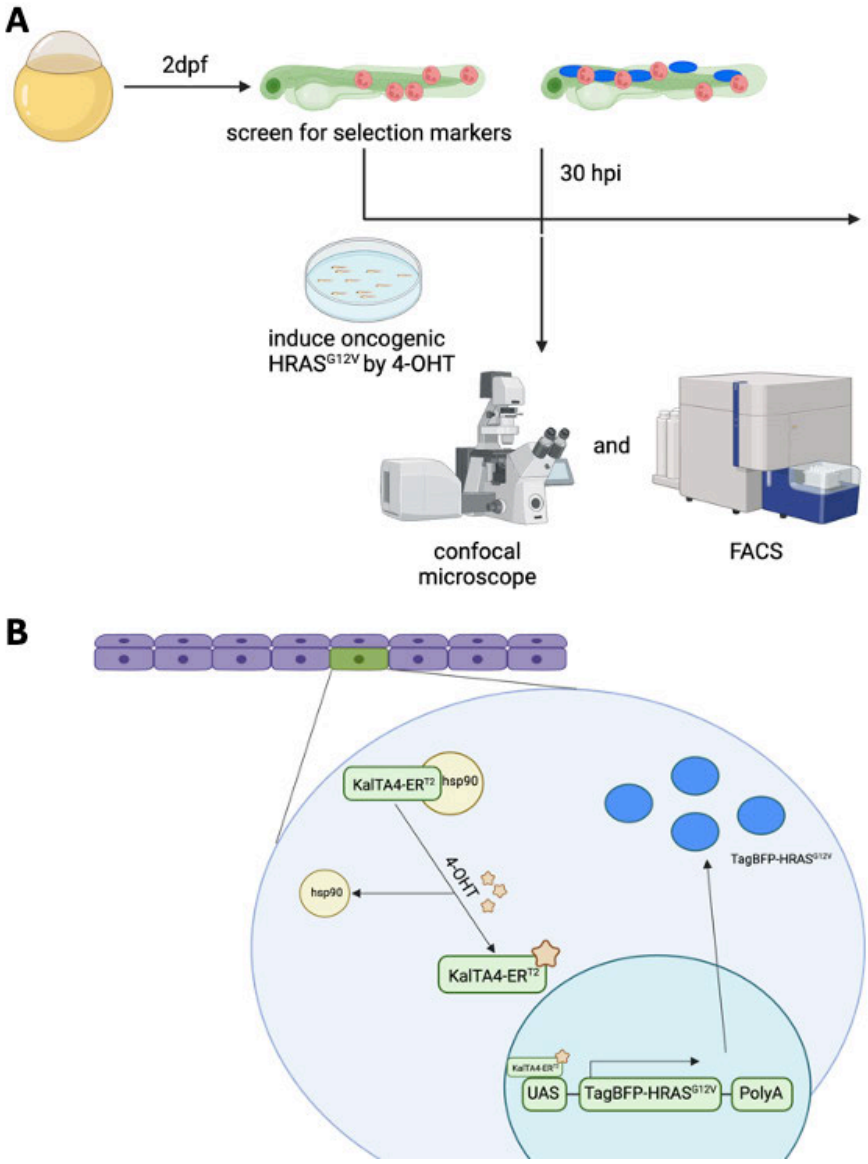


Figure 4.5.1 **Schematics for PNC induction experiment.** A, schematics showing the timeline of the experiment. B, schematics showing the conditionally modulating HRAS$^{G12V}$ expression in basal layer skin cells. Schematics generated in BioRender.

The imaging was captured at 24 hpi.

## 4.6 Imaging ATF6 reporter expression in neutrophils upon PNCs induction and neutrophil recruitment

### 4.6.1 Confocal live imaging

The K19; UAS: TagBFP-HRAS$^{G12V}$; lysC: NLS-mScarlet adult female fish outcrossed with Tg(5XATF6RE:eGFP) male adult fish (F1 generation). The eggs were collected and preselected via transgenic markers. The fish larvae were induced at late 2 dpf and separated into two groups at 24 hpi by the Hras$^{G12V}$ gene expressed in basal layer skin cells and sibling controls. At 24 dpi, both groups of fish larvae (n=8) were mounted into 1% low melting point (LMP) agarose. Live imaging was done at the QMRI CALM facility via a Leica SP8 confocal microscope using the 40X water lens. The basal layer skin cells were imaged above the yolk near the head and caudal haematopoietic(CHT) regions.

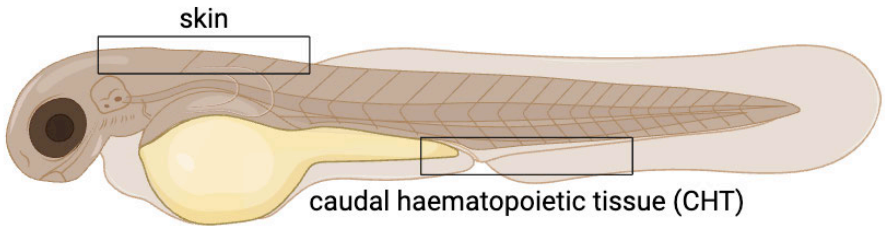


Figure 4.6.1 **Schematic figure showing live imaging region of 3dpf zebrafish larvae.** Schematics generated in BioRender.

### 4.6.2 Time-lapse Imaging

The ATF6 reporter fish were outcrossed with Tg(K19/UAS: TagBFP-HRAS$^{G12V}$; lysC: NLS-mScarlet) with removable dividers to ensure the similar fertilising time. The induction step was started at late 2 pdf and kept away from light for 24 hours in the larvae incubator. Both PNC expressing larvae and the sibling control larvae carrying

atf6 reporter transgene were selected and mounted in 1% LMP agarose (n=7) starting from 24 hpi. Images capturing gap was 35 minutes lasting 4 hours.

## 4.7 FACS analysis of atf6 reporter expression in neutrophils upon PNCs expression

The K19; UAS: TagBFP-HRAS$^{G12V}$; lysC: NLS-mScarlet adult female fish outcrossed with 5XATF6RE:eGFP male adult fish (F1 generation). To promise enough fish larvae number, one male adult fish was paired with two female adult fish (n=12). All fish larvae were pooled together for FACS analysis. The single colour controls were generated from target transgene marker carriers outcrossed with wild-type adult fish. All groups of fish larvae for FACS analysis use a disassociation protocol. The collagenase IV solution is added to media A at a ratio of 1:100 to make a dissociation solution for the sample preparation step.

Zebrafish larvae for the experiment (Table 2) were taken out and sacrificed by using an overdose of MS222. The fish larvae were then separated into 20 larvae in each group and went through the disassociation step. Samples were first added 300µL dissociation solution and pipetted for 30 seconds, then put into 28.5°C incubating for 5 minutes. Then, I took samples out and repeated the previous step to mix them thoroughly. This step was repeated three times before going to 4°C centrifuge. The centrifuge step took 5 minutes at 500 xg then carefully transferred upper layer solution into FACS tubes with cell strainer caps (Falcon #352235) to avoid debris. All prepared cells were kept on ice before FACS analysis. Draq-7 (DRAQ7™ #D15106) was added to all samples.

The FACS was carried out using an Invitrogen Attune CytPix Flow Cytometer at the IRR Flow Cytometry Facility. The data was analysed using Attune Cytometric Software.

| Single colour control | Markers |
|---|---|
| Unstained wildtype | N/A |
| Draq-7 Only | N/A, stained with Draq-7(1:1000) |
| eGFP Only | 5XATF6RE:eGFP |
| mScarlet Only | lysC: NLS-mScarlet |
| TagBFP | BFP beads provided by FCf QMRI |
| | |
| Experimental group 1 | eGFP, NLS-mScarlet, TagBFP, stained with Draq-7(1:1000) |
| Experimental group 2 | eGFP, NLS-mScarlet, TagBFP, stained with Draq-7(1:1000) |
| Control group | eGFP, NLS-mScarlet, stained with Draq-7(1:1000) |

Table 2 Transgenic lines used for FACS analysis. All fish line used in FACS analysis, including single colour control, one control group and two experimental groups repeats. The markers(right) include fluorescent markers and nucleus dye.

## 4.8 Generating neutrophil specific CAS9 expression transgenic zebrafish lines

The *lysC*: cas9[48] plasmid was coinjected with transposase mRNA Tol2 to generate neutrophil specific cas9 expression zebrafish line. The injected embryo was kept in clean fish water in the 28.5°C incubator. The fish water changed daily, and dead eggs were removed. At 3dpf, the eGFP-positive eye marker carriers were selected and

grew up till breeding age. Single adult male or female fish were outcrossed with wildtype fish and kept the eGFP positive eye marker carrying larvae to grow up as F1 generations.

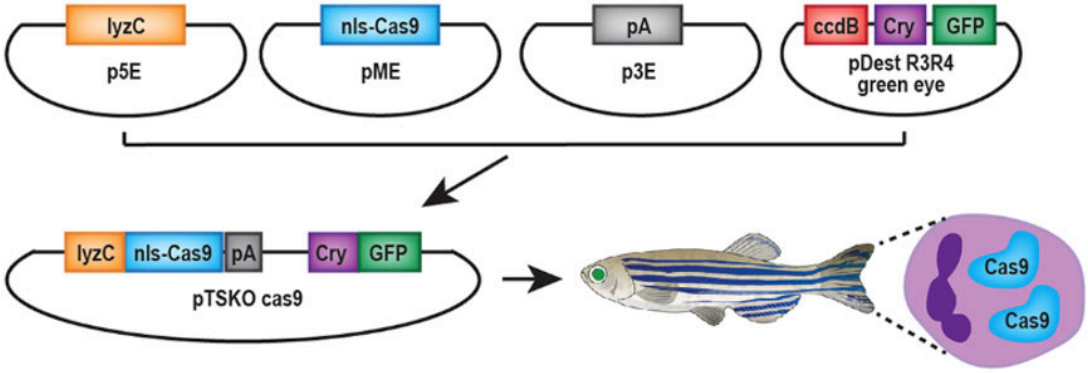


Figure 4.8.1 **Plasmid construct of neutrophil-specific Cas9 expression fish line.** Figure reused from published paper Wang, Yueyang et al. "A robust and flexible CRISPR/Cas9-based system for neutrophil-specific gene inactivation in zebrafish." Journal of cell science vol. 134,8 (2021): jcs258574. doi:10.1242/jcs.258574

## 4.9 Image Data analysis

For the drug treatment experiment, images were captured at the same exposure time(340ms) and gain (3.0) using a Leica M205 modular stereo microscope. The images were analysed using Fiji (ImageJ2) 2.14.0 intensity measurement.

The confocal SP8 live imaging data was visualised by IMARIS 10.1.10.

## 4.10 Statistical Analysis

The statistical analysis, conducted using Prism 9 (GraphPad), mainly involved selecting t-tests (and nonparametric tests) for column analyses to evaluate differences between two sets of data and utilising one-way ANOVA (and nonparametric or mixed methods) to analyse differences among multiple groups in the ER stress induction drug treatment assay. In the analysis, the 95% confidence

interval was calculated from sample data to estimate the range of a population parameter, providing an interval estimate with a confidence level of 95% believed to contain the true population parameter.

The P value, used in statistical hypothesis testing, measures the probability of observing data consistent with the null hypothesis and ranges between 0 and 1. P value $\leqslant$ 0.05 is typically considered statistically significant, and a significance level of 0.05 was employed in this experiment. Conversely, a P value > 0.05 is generally regarded as not statistically significant, indicating insufficient evidence to reject the null hypothesis.

The FACS analysis results were calculated using Microsoft Excel.

### 4.11 ChatGPT

In this thesis, ChatGPT helped with paraphrasing works and grammar checking in the Introduction part of this thesis. No experimental data or discussion was generated using artificial intelligence.

# 5 Results

## 5.1 Establish 5XATF6RE:eGFP line and screen F1 generation and reporter

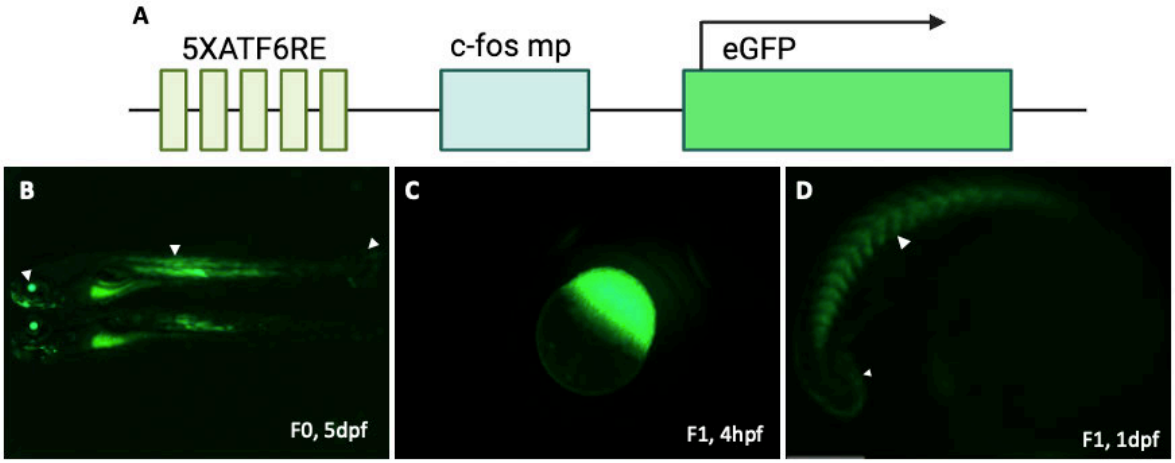


Figure 5.1.1 **Generations of 5XATF6RE:eGFP fish line. Figure A,** a schematic picture of 5XATF6RE:eGFP published by Clark, Eric M et al. (2020). **Figure B,** Transient injection of a Tg(5XATF6RE:eGFP) construct plasmid allows generation of GFP cells on zebrafish larvae (5 dpf). **Figure C & D**, Representative image of 1 dpf (F1 generation) zebrafish embryo with or without maternal 5XATF6RE:eGFP signal fertilisation.

To generate the ATP6 reporter line, I injected the Tg(5XATF6RE:eGFP) construct into one-cell-stage wild-type AB zebrafish fish embryos. I observed transient expression of the eGFP signal in the muscle region and lens (Figure 8, a white arrowhead), coinciding with published data (Clark M et al. 2020). This indicates that the plasmid is working and has been successfully injected into the zebrafish embryo. To generate a stable transgenic zebrafish line, 50 transgenic larvae carrying fluorescent eGFP signals were selected to grow. In the F1 generation, embryos from paternal transgenic adult male and female fish showed different eGFP expression patterns. Embryos from maternal adult fish had a higher baseline of 5XATF6RE:eGFP expression. These suggested that there is a maternal contribution to gene expression. F1 generation zebrafish were outcrossed, and the number of F2

generation embryos was counted as around 50% positive and around 50% embryos negative, meaning the F1 parent carrying a single copy of transgene. The 50:50 ratio proved that endogenous transgene expression can be used for quantitative analysis.

## 5.2 Validating the induction of eGFP expression in Tg(5XATF6RE:eGFP) larvae during drug induced ER stress

To validate the Tg(5XATF6RE:eGFP) zebrafish line reporting ER stress, I induced ER stress in zebrafish larvae using tunicamycin. Tunicamycin inhibits UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT), blocking the initial step of glycoprotein biosynthesis in the ER and causing the accumulation of unfolded glycoproteins, thus inducing ER stress[49]. I

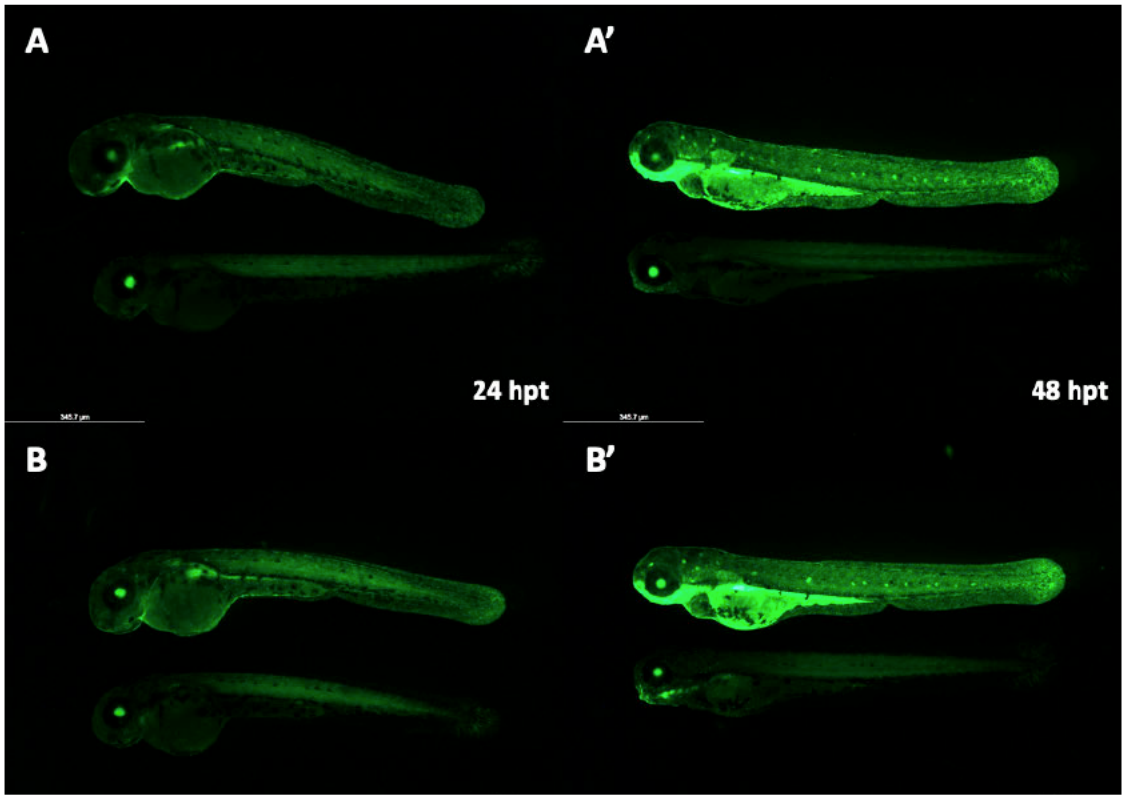


Figure 5.2.1 **Tunicamycin induce eGFP expression in Tg(5XATF6RE:eGFP) larvae at 24 and 48 hours post-treatment.** Figure A and B are 24 hour post treatment groups, and Figure A' and B' 48 hour post treatment groups. The fish larvae on the top of each image were under drug treatment, whereas the bottom ones with low eGFP signal intensity were controls. The scale bar is 345.7µm.

analysed eGFP expression intensity results in this experiment to prove model efficiency.

The results shown in Figure 5.2.1 consistently demonstrated that the eGFP fluorescent signal was stronger in samples treated with the drug for 48 hours compared to the control group. Figures A and B illustrate that the fish larvae treated with the drug exhibited a slightly brighter eGFP signal than the control larvae at 24 hours post-treatment. However, at 48 hours post-treatment (depicted in Figures A' and B'), the difference between the treatment and control groups was more pronounced, with the treatment fish showing a significantly stronger eGFP signal compared to the control larvae. Additionally, comparing the eGFP signal intensity between fish larvae at 24 hours and 48 hours post-treatment revealed that the signal was stronger in the larvae examined at the latter time point. Notably, the control groups showed no detectable eGFP signal, and there were minimal changes observed among the experimental time points.

To quantify the changes in eGFP intensity between 24 hours post-treatment (hpt) and 48 hpt, as well as between the control groups of fish larvae, I employed Fiji (ImageJ2) and Prism 9 (GraphPad) for data analysis.

I measured the average intensity of each fish larva in each group. Figure 5.2.2. This result revealed that limited eGFP signal had been detected in both control groups of fish larvae and that the eGFP intensity had no change over time. This suggested that there is little ER stress, which is predicted as control. In comparison, the drug treatment groups showed a significantly increased GFP intensity from 24hpt to 48hpt with 95% confidence ($P<0.05$). This can also be seen in (Figure 5.2.2), where the mean value increased from 32.00 to 69.05, showing ER stress accumulation over time upon tunicamycin treatment. On the other hand, the drug treatment groups have

significantly higher ($p<0.05$) intensity levels at each time point with respect to the control group. These data demonstrate that I successfully generated a reporter fish line to detect ER stress in vivo.

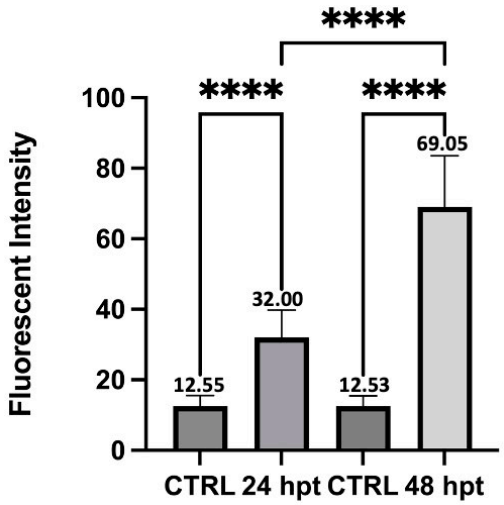


Figure 5.2.2 Drug treatment experiment and data analysis. The figure showed fluorescent intensity changes between two groups at 24 hours and 48 hours treatment time and offered intensity comparison between 24hpt and 48hpt. 24hpt treatment cluster (n=12), 48hpt cluster (n=10). P<0.0001, R2=0.8894.

## 5.3 Confocal live imaging

### 5.3.1 Live image of ATF6 reporter in the PNC model

Zebrafish larvae carrying mScarlet labelled neutrophil nucleus, eGFP labelled heart indicating carrying tissue-specific expression promoter, and cyan labelled lens meaning fish larvae carrying oncogenic HRAS$^{G12V}$ sequence with TagBFP labelled, were screened after 24 hours induction by 4-hydyotomaxifen. From the confocal result, the neutrophils' recruitment to the transformed cell sites was successfully observed, confirming the previous result[50]. However, the 5XATF6RE: eGFP expression had not been found in the neutrophils-surrounded area in this experiment. I could find eGFP fluorescent signals co-localised with HRAS$^{G12V}$ positive cells, meaning the reporter worked.

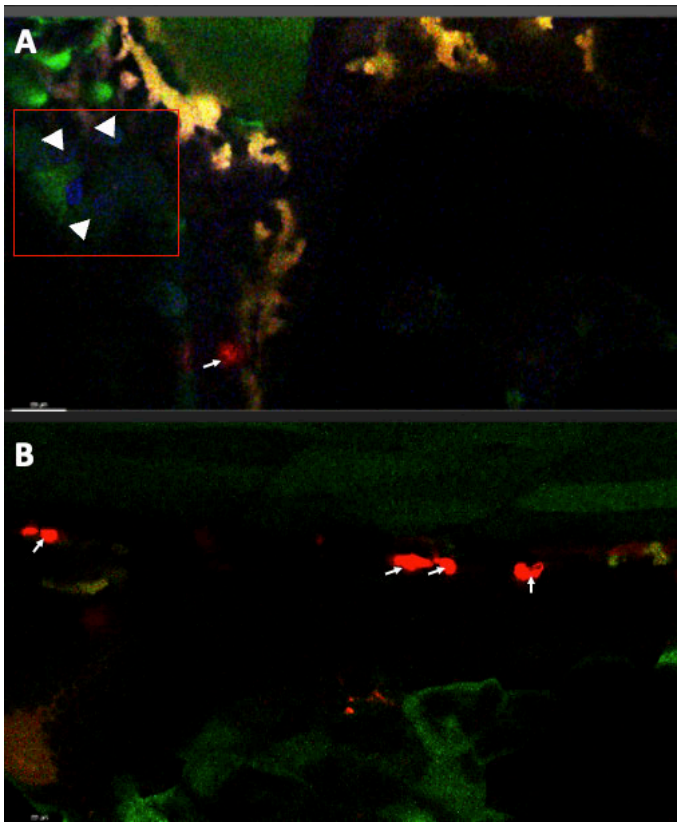


Figure 5.3.1 ATF6 reporter is not detected in PNC associated neutrophils. A. 5XATF6RE:eGFP expression upon HRAS$^{G12V}$ expression at basal skin cell region. B. Neutrophils in zebrafish CHT region. Triangles point eGFP signal colocalised with TagBFP, arrowheads mark neutrophil. Scale bar = 20 µm.

### 5.3.2 Time-lapse capturing eGFP signal in neutrophil upon PNCs expression.

To further investigate and observe 5XATF6RE: eGFP expression in neutrophils, I did a confocal time-lapse assay started at the similar time point of oncogene induction in basal layer skin cells and lasted 4 hours. In the time-lapse video frames, I confirmed the existence of mScarlet-labelled neutrophils, TagBFP-labelled PNCs and eGFP signals. The recruitment of neutrophils was observed to be associated with PNCs. I also visually confirmed the colocalisation of TagBFP and eGFP signals, suggesting the existence of ER stress in PNCs. Yet, the eGFP signal had not been observed co-localised with mScarlet positive neutrophils in the time-lapse frame series.

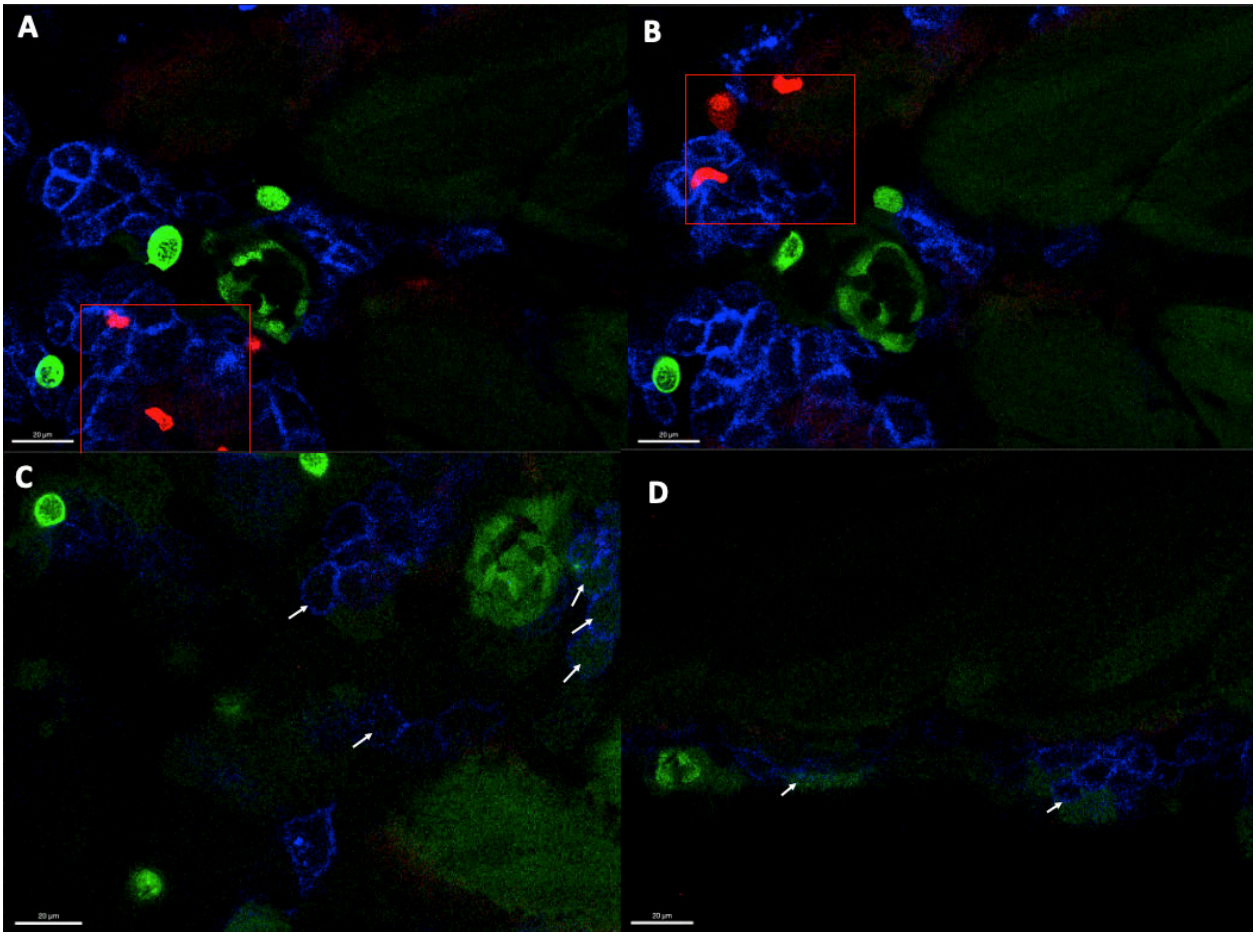


Figure 5.3.2 **Confocal live imaging failed to capture ER stress reporter activation in PNC associated neutrophils. A, B,** Neutrophils (red) were recruited to PNCs (Blue). **C, D,** 5XATF6RE:eGFP expression colocalised with PNCs (Green) (arrowheads). Scale bar = 20 µm.

## 5.4 FACS Analysis indicates a potential subtle increase of ER stress in neutrophils from PNC-bearing larvae

The confocal live imaging only focused on the small region of the larvae, which might miss rare occurring cells. To examine the ATF6 reporter eGFP signal expressed in all neutrophils from PNC bearing larvae, I performed FACS analysis using dissociated larval single-cell suspension.

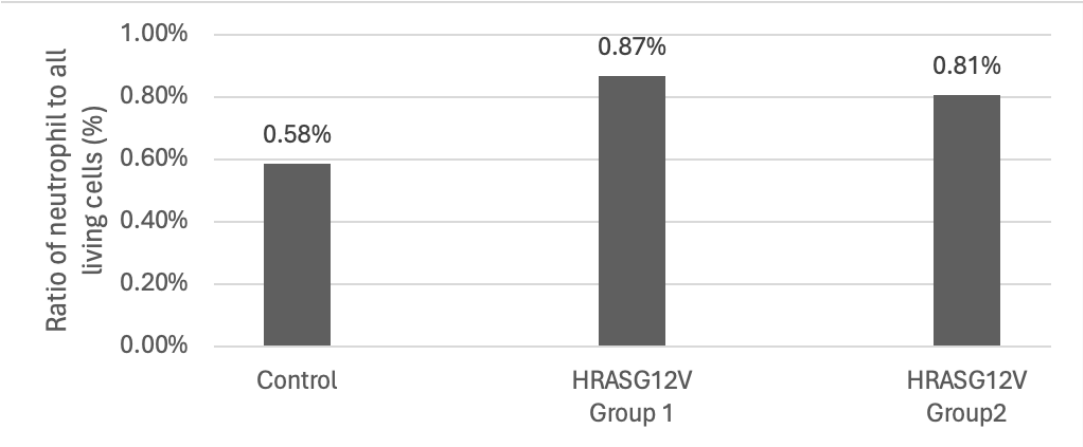


Figure 5.4.1 **The ratio of neutrophils to all living cells is increased in PNC bearing larvae.** One sample from control larvae (n=20) are from Tg(lysC:nls-mScarlet). Two samples from PNC bearing larvae (n=20 each) are shown. No statistical analysis could be carried out.

I found more neutrophils in PNC-bearing larvae than in control larvae (Figure 5.4.1), although due to limitations in experimental repeats, I cannot carry out statistical analysis. Increased neutrophil production would agree with the single-cell RNA sequencing data generated in the lab, which shows enhanced neutrophil proliferation.

I then calculated the number of eGFP-positive cells (reporting ER stress) within mScarlet-positive neutrophils and all living cells. I found that the ratio of eGFP-positive cells within neutrophils was much lower than in all living cells in control larvae, and the ratio of eGFP-positive cells within neutrophils was increased in PNC-

bearing larvae but not within all living cells (Figure 9.4.2). Therefore, this indicates increased eGFP-positive neutrophils in HRAS groups, although no statistical analysis can be carried out due to the limitation of the experimental repeats.

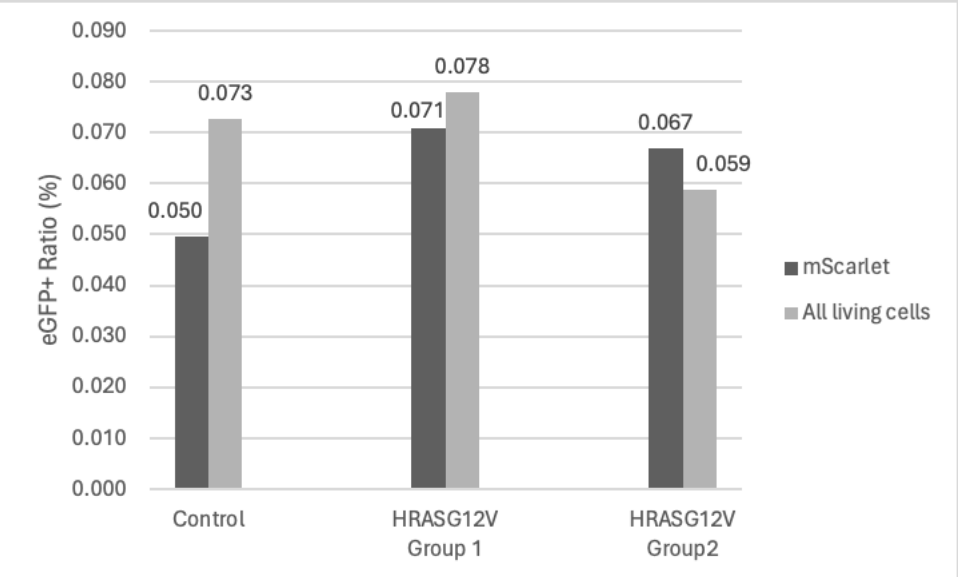


Figure 5.4.2 **The ratio of eGFP+ signal in neutrophils and all living cells.** The control group and two repeat groups of oncogenic $HRAS^{G12V}$ expression fish larvae (n=20) are shown in the figure. The nucleus labelled mScarlet neutrophils are darker bars on the left of each clutch. In comparison, all living cells are shown on the right.

In summary, the FACS analysis suggests a neutrophil response to PNC induction and enhanced neutrophil ER stress in PNC-carrying larvae, although further repeats are needed to confirm this finding.

## 5.5 Generation of neutrophil specific CAS9 expression transgenic fish line

To enable neutrophil specific gene manipulation, which will facilitate the future study of ER stress impact within neutrophils, I used the Tol 2 transposase mediated transgenesis using the *cry*:eGFP-*lyzC*:nlsCAS9 construct, published by Wang, Yueyang et al. (2021) to establish a Tg(lysC:nls-CAS9) line. Similarly to the generation of 5XATF6RE:eGFP fish line, this line was selected by GFP lens marker at 3dpf under the Leica M205 modular stereo microscope. F1 generation was taken

out to cross with wild-type fish and screened by a GFP selection marker. Due to the CAS9 is not fused with any fluorescent marker, I was not able to directly visualise the CAS9 protein expression in neutrophils. I tested a commercially available anti-Cas9 antibody (ab189380) using a wholemount immunostaining protocol; however, no positive signal was detected. Due to the lack of a positive control sample, I could not determine if the anti-Cas9 antibody worked in the wholemount immunostaining protocol I used. Therefore, further validation is still required using F2 larvae to establish a working Tg(lyzC:nlsCAS9) zebrafish strain.

## 5.6 Summaries of key findings

In this study, a transgenic zebrafish line was developed as an ER stress reporter, expressing the 5XATF6RE:eGFP transgene in accordance with the protocols established by Clark et al. in 2020. Screening of the F1 and F2 generations confirmed the presence of the transgene and validated the expression of the reporter for subsequent quantitative analysis. I proceeded to further validate the response of the reporter fish to ER stress inducers and developed a quantification protocol utilising ImageJ.

Subsequently, in the investigation of ER stress dynamics in vivo upon $HRAS^{G12V}$ expression using confocal live imaging, I was unable to detect any activation of the ER stress reporter in neutrophils in PNC-bearing larvae. However, potential ER stress reporter expression enhancement was observed in neutrophils from PNC-bearing larvae. It is important to note that the FACS analysis needs to be repeated to confirm whether the subtle increase observed is indeed "real."

# 6 Discussion

In this project, the transgenic zebrafish line Tg(5XATF6RE:eGFP) was utilised to examine ER stress in neutrophils following the expression of oncogenic $HRAS^{G12V}$ in basal layer skin cells. This investigation involved the use of both confocal live imaging and fluorescence-activated cell sorting (FACS) analysis to assess ER stress dynamics and cellular responses at the molecular level.

The observations presented in Chapter 5.3 and the quantitive data shown in Chapter 9.4 revealed a slight discrepancy in the expression of eGFP signals in neutrophils when comparing the confocal imaging results with those obtained from FACS analysis. Technically, flow cytometry is a versatile technology used in biosensing, cell sorting, and molecular detection by detecting fluorescently labelled cells or microspheres as they flow through a system of fluidics and optics[51]. Confocal microscopy is used to analyse the spatial distribution of proteins in specific areas of tissue cells[52]. The FACS analysis conducted previously exhibited subtle differences, requiring further confirmation. This disparity in results between FACS and confocal imaging may be attributed to the enhanced sensitivity of FACS in detecting fluorescent signals.

ER stress is characterised by an imbalance between the influx of unfolded proteins into the ER and the cellular machinery's capacity to manage this load, initiating three primary responses[53], shown in Figure 1.7.1. Based on previous findings, ER stress has been shown to be conserved across a diverse array of species, encompassing mammals, yeast, plants, and zebrafish[53–56]. Additionally, in zebrafish models, tunicamycin has been demonstrated to activate all branches of the unfolded protein response (UPR), indicating the occurrence of ER stress[54]. The conservation among species ensures the feasibility of the project and lays the groundwork for future

investigations into the functions of ER stress, particularly its mechanisms in neutrophils.

While cell stress reporters monitoring XBP1 splicing and ATF4 translational induction had been documented in various species, only two ER stress reporters have been published for use in the zebrafish model organism: one for monitoring XBP1 activity and the other for dynamically monitoring ATF6 activity[47,57]. In the previously analysed single-cell RNA sequencing data, atf6 showed upregulation in neutrophils associated with PNCs compared to control fish larvae Figure 1.9.1. Given that the ATF6 reporter model reports the released ATF6 protein, I hypothesised that this reporter would provide a readout of ER stress in the PNCs model. The discrepancy between mRNA expression levels and protein abundance may explain the inadequate model for investigating ER stress in neutrophils.

Zebrafish proved to be exceptionally practical for this study, particularly in facilitating in vivo live imaging. Their rapid embryonic development and transparent larvae were invaluable assets to this project. I could visualise cellular mechanisms under the confocal microscope by utilising fluorescent transgenic fish lines, enabling a thorough investigation of "ER stress" through fluorescent signals. Meanwhile, as for immune cells, especially neutrophils, zebrafish neutrophils share morphological and function features among mammals and non-mammalian vertebrates[25]. However, there is a shortage of zebrafish as a model organism. While whole-mount immunofluorescence (IF) is frequently employed to analyse protein expression in zebrafish embryos, this technique can present challenges in obtaining precise colocalisation data[58]. Additionally, certain protocols may be antibody-specific and not universally applicable to all antibody staining, as observed with the anti-Cas9 antibody in 5.5. Indeed, there is a significant discrepancy in the availability of

commercially accessible antibodies, with approximately 112,000 antibodies tailored for use in mice compared to only about 5,300 designed for zebrafish[58]. The significant disparity in product numbers underscores a major technical challenge encountered when utilising zebrafish as a model organism. Furthermore, the absence of specific product manuals for accessing zebrafish immunofluorescence exacerbates the issue, potentially leading to experimental failures.

For future perspectives, there is ample room for improvement in the current experiment. First, optimising the FACS protocol could yield a higher level of singlet neutrophils for re-analysis of eGFP expression in these cells upon induction of PNCs. Secondly, blocking ER stress specifically in neutrophils could be achieved by developing a CRISPR-CAS9 tissue-specific knockout protocol utilising the lyzC:CAS9 transgenic fish that I generated in this project. This would involve measuring downstream ER stress protein markers and assessing neutrophil morphology.

# 7 Conclusion

In the project, I successfully established and validated the Tg(5XATF6RE:eGFP) zebrafish line to monitor the activation of ER stress in vivo in zebrafish larvae, whereas I did not detect ATF6 reporter activation in neutrophils in PNC-bearing larvae using confocal live imaging with my current settings. FACS analysis using my ER stress reporter indicated a possible subtle increase of ER stress in neutrophils in the PNC-bearing larvae. However, further repeats are required to confirm this observation. In the next phase of future experiments, I intend to utilise the lysC:CAS9 transgenic fish generated in this project to manipulate ER stress in

neutrophils specifically. This approach will allow for a deeper analysis of the impact of neutrophil ER stress on PNC growth and associated mechanisms.

This project demonstrates the relationship between neutrophil ER stress and the impact on tumour development, showing the potential of a new therapeutic target and cancer treatment at the early stage. Understanding neutrophil ER stress and its influence on tumour development could pave the way for developing therapies aimed at modulating neutrophil function to inhibit early-stage tumour development, thereby addressing the challenge of weak cancer screening and diagnosis.
Consequently, this research could significantly augment our understanding of the processes involved in early-stage tumour-associated neutrophils and their role in tumour progression. This could further stimulate advancements in the field of oncology and pave the way for the creation of innovative therapeutic strategies.

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