# Transcriptomic analysis of zebrafish prion protein mutants supports conserved cross-species function of the cellular prion protein; and zebrafish as a model for cisplatin induced ototoxicity and transition metals as potential ligands for Tlr4ba and Tlr4bb

By

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## Abstract:

The work presented in this thesis is split between two projects. The first utilises *prp1* and *prp2* knockout zebrafish to investigate physiological functions of the cellular prion protein ($PrP^C$). The second builds upon the use of zebrafish as a model for hearing loss to confirm a role of toll-like receptor 4 (TLR4) in cisplatin induced ototoxicity (CIO) and to investigate group 10 transition metals as potential ligands for zebrafish Tlr4ba and Tlr4bb. Chapter 1 contains a literature review of systemic and central nervous system (CNS) related amyloid disease and how they relate to each other and prion diseases. Functional amyloids are discussed in the context of how control mechanisms for the formation of functional amyloids may help in developing therapeutics for protein misfolding diseases.

In Chapter 2, RNA-sequencing with wild-type and $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ mutant zebrafish at 3 days post fertilisation was used to identify potential roles of prion protein during zebrafish development. Biological process gene ontology analysis showed the process with the largest number of genes showing a significant decrease in transcript abundance was cell adhesion. Of theses, 31 of the 38 genes belonged to the protocadherin family. Protocadherins are involved in the development and maintenance of the CNS. In addition, *ncam1a* and *st8sia2* both showed a significant reduction in transcript abundance after RNA-sequencing and this was confirmed through RT-qPCR. These results closely match those seen in *in vitro* experiments in cells lacking $PrP^C$. Abnormal deposition of neuromasts along the posterior lateral line (PLL) was observed in *prp1*, *prp2* and *prp1/prp2* knockout zebrafish. In *prp1* mutant fish there was a significant decrease in neuromast count along the PLL, in *prp2* mutants there was a significant increase. Combined *prp1/prp2* mutant zebrafish recovered the loss of neuromasts seen in *prp1* mutants but was still higher than wild-type. Together, these results would suggest a cross species conserved role of the cellular prion protein in the early development of organisms.

The second part to this thesis investigates the role of TLR4 in CIO in collaboration with the Amit Bhavsar and Fred West labs at the University of Alberta. Transition metals as potential ligands for zebrafish Tlr4ba and Tlr4bb are also explored. Cisplatin is an effective treatment against cancer but has severe side effects. One of these is permanent, bilateral hearing loss and there is currently no co-treatment to prevent this. This has led to a reduction in usage of cisplatin. Zebrafish PLL neuromasts have become an established model for ototoxicity. Recent work has identified TLR4 as a potential mediator for CIO. TAK-242 is a small compound inhibitor of TLR4. In Chapter 3, morpholino knockdown of *tlr4ba* and *tlr4bb* and inhibition of zebrafish Tlr4ba and Tlr4bb through TAK-242 or synthetic TAK-242 derivatives, termed syntagonists, was used to confirm the role of TLR4 in mediating CIO. Two syntagonists, 134 and 136 significantly reduced the CIO in neuromast cells in 6-7dpf zebrafish. Morpholino knockdown of *tlr4bb* through two separate morpholinos and *tlr4ba* resulted in a significant reduction in CIO. Combined knockout of both *tlr4ba* and *tlr4bb* at the same time reduced CIO though not significantly moreso than either individually. These results confirm TLR4 as a mediator for CIO and established zebrafish as a suitable, high-throughput model for investigating inhibition of CIO going forward.

Finally, utilising the model established in Chapter 3, Chapter 4 contains results investigating whether transition metals are a ligand for zebrafish Tlr4ba and Tlr4bb. In mammalian TLR4 the canonical ligand is lipopolysacharride, though other ligands such as nickel, cobalt and certain viral proteins can also cause TLR4 signalling. Zebrafish PLL neuromasts were exposed to either $NiCl_2$, $PtCl_2$ or $PtCl_4$ and co-treated with syntagonists. Several syntagonists, 138, 150, 166, 168 and 170 all significantly reduced nickel induced ototoxicity. Of these only one had been also tested against CIO, syntagonist 138, in which it had no effect. After co-treatment of syntagonist 134 with $PtCl_2$ or $PtCl_4$ there was no reduction in platinum-induced ototoxicity. This may be due to the concentrations of platinum salts used, or as with nickel

induced ototoxicity, syntagonist 134 is not effective against platinum induced ototoxicity. These results promote optimism that transition metals may activate Tlr4ba or Tlr4bb signalling, though more work is needed to confirm the validity of these results.

## Preface:

This thesis is an original work by Niall M. H. Pollock and the work presented was performed under ethics approval from the University of Alberta Animal Policy and Welfare Committee and in compliance with the Canadian Council on Animal Care (CCAC). The author has completed the mandatory training for animal users as directed by the CCAC on the Care and Use of Animals in Research, Training and Testing.

Chapter 2 has been prepared as a manuscript and submitted to the journal, *Prion*. At the time of writing, the manuscript has been accepted pending minor text revisions. The manuscript was written by NMP with editing contributions from PLA, GN and WTA. Figure contributions: GN contributed RT-qPCR data presented in Figure 2.2 C & D and Supplementary Figure 2.1.

Chapter 3 includes content from the following publication: Babolmorad, Ghazal, Asna Latif, Ivan K Domingo, Niall M Pollock, Cole Delyea, Aja M Rieger, W Ted Allison, and Amit P Bhavsar. 2021. "Toll-like Receptor 4 Is Activated by Platinum and Contributes to Cisplatin-Induced Ototoxicity." *EMBO Reports* n/a (n/a): e51280. https://doi.org/https://doi.org/10.15252/embr.202051280. The manuscript was written through contributions of the authors: GB, AL, NMP, AMR, WTA, and APB. Data contained within the Chapter 3 Figures 3.1 and 3.6 are included in the manuscript and was collected by NMP. The material and methods are as written in the manuscript and were originally provided by NMP and WTA. This thesis chapter was written by NMP with editing contributions from WTA. Contributions to figures: Figure 3.3C contains data collected by Aaron Fox; Figure 3.7 contains chemical structures provided by Ghazal Babolmorad, Ismat Luna and Fred West; Table 3.1 represents *in vitro* cell culture data collected by Asna Latif and Ghazal Babolmorad.

A version of the Chapter 4 material and methods also appears in Chapter 3, and from

Babolmorad, Ghazal, Asna Latif, Ivan K Domingo, Niall M Pollock, Cole Delyea, Aja M Rieger, W Ted Allison, and Amit P Bhavsar. 2021. "Toll-like Receptor 4 Is Activated by Platinum and Contributes to Cisplatin-Induced Ototoxicity." *EMBO Reports* n/a (n/a): e51280. https://doi.org/https://doi.org/10.15252/embr.202051280. Chapter 4 was written by NMP with editing contributions from W. Ted Allison. Contributions to figures: Figures 4.5, 4.6 and 4.7 contain data collected by Aaron Fox.

The work presented in Appendix A contains data collected for the work published in the article: Leighton, Patricia L.A., Richard Kanyo, Gavin J Neil, Niall M Pollock, and W Ted Allison. 2018. "Prion Gene Paralogs Are Dispensable for Early Zebrafish Development and Have Nonadditive Roles in Seizure Susceptibility." Journal of Biological Chemistry 293 (32): 12576–92. https://doi.org/10.1074/jbc.RA117.001171, as well as touch evoked escape response data collected by Michèle DuVal and Natalie Schneider. The appendix material and methods are adapted from Leighton et al. 2018, and were written by PLA and NMP. Figure contributions: Zebrafish images in Figure A.1 were provided by W. Ted Allison and Patricia Leighton. Touch evoked escape response data was provided by Michèle DuVal and Natalie Schneider.

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I am forever grateful to the past and present members of the Allison Lab for creating such a fun, relaxed and stimulating environment in which to carry out my research. In particular, Dr A. Phil Oel, Dr Michèle DuVal, Dr Patricia Leighton and Dr Hadeel Alyenbaawi: Your support and friendship when I first arrived in Canada made settling in to a new country and atmosphere incredibly easy and less stressful than it could have been. Your patience and willingness to help accommodate me to the lab and train me on unfamiliar techniques was utterly invaluable. Thank you to the wider grad school community at the University of Alberta and Department of Biological Sciences: Julia, Logan, Hae-Won, Cody, Reggie, Robin, Fran, Jens, Richard, Katie, Alaina, Kacey and Kacie, Lee, Lindsay, Spencer and Spencer, Sonya and many more. Our weekly (or sometimes more often…) beers and social nights were instrumental in maintaining some semblemce of emotional wellbeing throughout the best and the worst of moments. Thank you to Dave and Piers, who kept me grounded throughout.

Finally a massive thank you to my family. My parents, Kristian and Martin, and my siblings; my twin Lewis, older brother Liam and older sister Joanna. Your support is and always has been immeasurable.

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## List of abbreviations:

| | |
|---|---|
| 4RβS | Four-rung β-solenoid |
| AA | Amyloid-A associated |
| AD | Alzheimer's disease |
| AL | Immunoglobulin light chain |
| ALL | Anterior lateral line |
| ANOVA | Analysis of variance |
| APP | Amyloid precursor protein |
| ATTR | Transthretin related |
| $A\beta_{1-42}$ | Amyloid-β 1-42 |
| Aβso | Soluble amyloid-β oligomers |
| Bap | Biofilm associated protein |
| BBB | Blood brain barrier |
| BSE | Bovine spongiform encephalopathy |
| CIO | Cisplatin-induced ototoxicity |
| Cisplatin | cis-diamminedichloroplatinum(II) |
| CJD | Creutzfeldt-Jakob disease |
| CNS | Central nervous system |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CWD | Chronic wasting disease |
| CWD | Chronic wasting disease |
| DASPEI | 2-[4-(dimethylamino) styryl]-1-ethylpyridinium iodide |
| DAI | DNA-dependent activator of interferon regulatory factors |
| DMF | Dimethylformamide |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dpf | Days post fertilisation |
| E3 | Zebrafish embryo growth medium |
| ECM | Extra-cellular matrix |
| FAD | Familial Alzheimer's disease |

| | |
|---|---|
| ng | Nanogram |
| bp | Base pair |
| PAMPs | Pathogen-associated molecular patterns |
| TEER | Touch evoked escape response |
| TRAM | TRIF-related adaptor molecule |
| RLRs | RIG-I-Like receptor |
| NLRs | NOD-like receptors |
| FAK | Focal adhesion kinase |
| PRR | Pattern recognition receptor |
| FBD | Familial British dementia |
| FDD | Familial Danish dementia |
| FFI | Fatal familial insomnia |
| Gh | Growth hormone |
| Ghrh | Growth hormone releasing hormone |
| GSS | Gerstmann–Sträussler–Scheinker syndrome |
| HDL | High density lipoprotein |
| hpf | Hours post fertilisation |
| IAPP | Islet amyloid polypeptide |
| KCl | Postassium chloride |
| LPS | Lipopolysaccharide |
| MO | Morpholino |
| MRSA | Multi- or Methicillin resistance *Staphylococcus aureus* |
| MS222 | Tricaine methanesulphonate |
| NFT | Neurofibrillary tangles |
| $NiCl_2$ | Nickel chloride |
| $^oC$ | Degrees Celsius |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with 0.1% Tween 20 |
| Pcdh | Protocadherin |
| PD | Parkinson’s disease |

| | |
|---|---|
| PDD | Parkinson's disease dementia |
| PFA | Paraformaldehyde |
| PIRIBS | Parallel in-register β-sheet |
| PLL | Posterior lateral line |
| PNS | Peripheral nervous system |
| PrimI | Primordium |
| $PrP^{C}$ | Cellular prion protein |
| $PrP^{Sc}$ | Prion protein scrapie |
| *PSEN-1* | Presenilin-1 |
| *PSEN-2* | Presenilin-2 |
| $PtCl_2$ | Potassium (II) chloride |
| $PtCl_4$ | Potassium (IV) chloride |
| RHIM | RIP homotypic interaction motifs |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| SAA | Serum amyloid A |
| Sho | Shadoo protein |
| SP-C | Prosurfactant protein C |
| TLR | Toll-like receptor |
| TLR4 | Toll-like receptor 4 |
| TNF | Tumour necrosis factor |
| TRIF | TIR-domain containing adapter inducing interferon-β |
| UK | United Kingdom |
| WT | Wild-type |
| WTTA | Wild-type transthyretin amyloid |
| α-Syn | Alpha-synuclein |
| µl | Micro-litre |
| µM | Micromolar |

# Chapter 1 Literature review: Systemic and central nervous system related amyloid disease, prion disease and functional amyloids

## Chapter 1 Abstract:

Amyloidosis and amyloid-related disease can occur both within the central nervous system and systemically throughout the body. Currently there are over 30 proteins comprising over 70 diseases associated with amyloid formation through the misfolding of physiological protein. Despite affecting different organs and manifesting different symptoms the principal cause of disease, protein misfolding and amyloid fibril formation, remains the same. Amyloid fibrils are polymers of the same protein consisting of repeated units of cross β-sheets and can be identified through their ability to bind to certain dyes such as Congo red and thioflavin-T, as well as resistance to sodium dodecyl sulphate and other ionic detergents. There are many diverse diseases caused by amyloid formation and protein misfolding. Within the CNS, prion diseases are often seen as the prototypical example of template directed misfolding and seeding to adjacent regions of the brain.

Neurodegenerative diseases such as Alzheimer's disease or prion diseases, are amyloid-related diseases as current hypotheses suggest it is the soluble oligomeric pre-cursors to amyloid fibrils which are the pathogenic agent. Prion diseases in particular show an ability for misfolded cellular prion protein ($PrP^{C}$) to 'seed' the further misfolding of additional $PrP^{C}$ into the disease associated conformation, scrapie prion protein ($PrP^{Sc}$) in a template directed manner. There is growing evidence to suggest other neurodegenerative amyloid diseases such as tauopathies, Alzheimer's and Parksinson's disease as well as systemic amyloid diseases may have similar template directed misfolding and seeding properties. The misfolded conformation of $PrP^{Sc}$ is not the same across all prion diseases, leading to different 'strains' of $PrP^{Sc}$ and different, unique disease pathologies. Tauopathies and other amyloid related

diseases such as AD may also show different strains which may account for the variety of disease phenotypes.

Functional amyloids are increasingly being identified across all walks of life including animals, bacteria, and plants. In such cases the amyloid formation process is tightly regulated and controlled, ensuring that the concentration of both soluble oligomeric precursors and the final amyloid fibril do not reach toxic levels. This can involve the presence of a nucleator protein, a rate limiting step in oligomer and amyloid fibril formation. Environmental regulation also occurs such as through bacterial replication producing acidic conditions favourable for amyloid formation. Understanding the control processes behind functional amyloids may aid in therapeutic developments to treat protein misfolding diseases.

A key event in pathology of amyloidosis and amyloid-related diseases is an eventual overwhelming of the proteostatic mechanisms which ordinarily would prevent excessive misfolding of proteins. BRICHOS domains have become increasingly well characterised, their function prevents aggregation of the parent protein, acting as a personal proteostatic mechanism across many proteins which ordinarily would be prone to self-aggregating.

This review chapter will describe some of the more prevalent amyloidosis and amyloid-related diseases, both within the CNS and those which are systemic. Functional amyloids will then be described, including their function and methods of regulation. Finally, BRICHOS domains and their mechanisms behind preventing amyloidosis will be explored in the context of adapting the system to help treat protein misfolding diseases.

## 1.1 Introduction to Amyloids, Amyloidosis and Protein Misfolding Disease:

Amyloids are fibrillar protein aggregates and commonly associated with a variety of diseases both inside and outside of the central nervous system (CNS). They show a characteristic β-sheet secondary structure and while primarily composed of a singular protein can have additional proteins and molecules making up the aggregation (Sipe and Cohen 2000; Benson et al. 2020). Upon this conformational change to the β-sheet structure the subsequent aggregates share several common features including becoming insoluble, non-functional and resistant to degradation. Aggregates are susceptible to staining by certain dyes such as thioflavin T or Congo red (Sunde et al. 1997; Kajava, Baxa, and Steven 2010). Traditionally they were seen as extra-cellular plaques, particularly in the case of disease (Benson et al. 2018). However more recently the definition has been somewhat loosened to include deposits which can occur within the cytoplasm of a cell. 'Amyloid fibril' can refer to any fibril primarily consisting of cross β-sheets (Benson et al. 2020). While largely associated with disease evidence is increasing that there is a growing number of amyloids which serve a distinct biological function, particularly in yeast and bacteria (Pham, Kwan, and Sunde 2014). In humans it has also been proposed that the amyloid plaques and aggregates seen in disease are themselves a defence mechanism of the body rather than the primary pathological aspect of disease. Instead, the smaller soluble oligomeric fibrils which comprise the larger insoluble plaques are increasingly thought to be responsible for disease phenotypes (Reixach et al. 2004; Baglioni et al. 2006; Simoneau et al. 2007). Disease progression typically follows the presence and concentration of these smaller oligomers more-so than the larger plaques. Amyloid formation can be self-perpetuating **(Figure 1)**. once the initial nucleus of an amyloid fibril occurs the addition of further monomers to that fibril can take on an energetically favourable state, causing more monomers to be recruited and extension of the fibril.

There are several different categories of amyloidosis and these can be further categorised as systemic amyloidosis or CNS-related amyloidosis. Systemic amyloidosis disease can affect multiple organs and usually are not associated with prevalent amyloid in the CNS. They include immunoglobulin light chain associated (AL), transthyretin related (ATTR), and amyloid-A associated (AA). AL, ATTR and AA often occur due to complications from other diseases, such as cancer or viral infection.

There are multiple amyloid related diseases which can occur within the CNS (**Table 1)**. Alzheimer's disease (AD) and Parkinson's disease (PD) show proteins (amyloid-β and α-synuclein respectively) which can form β-sheet rich amyloid fibrils following the definition outlined above (Benson et al. 2020). For brevity's sake AD and PD will be the examples focussed on in this review. Whether it is the amyloid-β or α-synuclein (α-Syn) insoluble fibrils, the soluble oligomeric precursors, or both that primarily drive pathology is not yet clear. In prion diseases, where cellular prion protein ($PrP^{C}$) misfolds into scrapie prion protein ($PrP^{Sc}$), the misfolded $PrP^{Sc}$ again forms cross β-sheet fibrils though it is also unclear what the primary pathogenic event is which leads to the characteristic neurodegeneration associated with disease. Smaller, soluble oligomers which act as precursors to amyloid-fibrils can cause cellular toxicity and associated disease symptoms (Baglioni et al. 2006; Simoneau et al. 2007). Oligomer formation is closely linked to amyloid-fibril formation **(Figure 1)**.

In this chapter, non-CNS systemic amyloidosis, CNS-related amyloidosis, and protein misfolding neurodegenerative diseases such as AD, PD and prion disease will be briefly explored alongside common mechanisms of toxicity. In all these diseases the common event is the misfolding of protein leading to oligomer formation and subsequent amyloid fibrils. Therefore, functional amyloids in nature will be described, alongside the control mechanisms which prevent them from resulting in disease, and what lessons may be applied in the treatment and prevention of amyloid and protein misfolding disease.

## 1.2 Amyloid Fibril Formation:

Amyloid formation occurs through polymerisation of peptides or proteins into long fibres consisting of an ever-increasing chain of monomers. There are several different theories as to the process of amyloid formation and they are not necessarily mutually exclusive. They all consist of a nucleation (lag) phase, an exponential growth phase and a saturation phase (Chuang et al. 2018). The amino acid sequence of a peptide can influence amyloid formation. There are hereditary forms of most amyloid diseases, whether they occur in the CNS or systemically. In hereditary disease there are mutations in either the amyloidogenic protein sequence such the prion disease fatal familial insomnia (Alred et al. 2018) or in precursor proteins responsible for production of the amyloidogenic species, such as amyloid precursor protein in AD (Murrell et al. 2000)**.** Other than the propensity to misfold and form amyloid fibrils there does not appear to be any consistency regarding the amino acid sequence or function of the proteins involved in amyloidosis**.**

For amyloid formation to begin there needs to be the presence of a misfolded protein, or a partially or unfolded protein which has the potential to misfold. Once amyloid formation has begun, the misfolded proteins can often be found to act as a template for further protein misfolding, which is particularly prevalent in prion diseases **(Figure 2)**. Over time these misfolded monomers can start to form a nucleus for amyloid formation, becoming short chains of oligomers which can begin to rapidly recruit further monomers, extending the chain (Chuang et al. 2018). One of the more striking and consistent differences in the amyloid of a protein or peptide compared to the normal folding is a reduction in the α-helical content of the final product, and an increase in the β-sheet content. For example compared to $PrP^{C}$ which has about 43% alpha helical content and only 3% beta sheet content, the misfolded $PrP^{Sc}$ isoform is 30% alpha helices and 43% beta sheet (Pan et al. 1993). Typically, the arrangement of this β-sheet core is a parallel arrangement, they have the same N-terminal to

C-terminal orientation, particularly for $A\beta_{1-42}$ and alpha-synuclein fibrils. Amyloids that are smaller in size do form anti-parallel β-sheet cores though these are still the minority of configurations (Sunde et al. 1997). Additional events, such as fibril fragmentation, can then further contribute to amyloid formation, as fibre fragmentation can increase the surface area available to recruit more monomers (Knowles et al. 2009) **(Figure 1)**. This can be particularly problematic as increasing evidence suggests it is the shorter, soluble oligomeric species which are toxic and responsible for disease pathology (P. Huang et al. 2013; Um et al. 2012; Baglioni et al. 2006), and amyloid fragmentation can increase the availability of these oligomers.

There has been rigorous examination as to the need of cofactors in amyloid formation. In certain cases, such as the misfolding of $PrP^{C}$ into $PrP^{Sc}$ cofactors do not appear necessary for misfolding or fibril formation *in vitro*. However the presence of cofactors may provide a more favourable environment for template directed misfolding to occur and increase the infectivity of the subsequent prion strain and will be likely prevalent in *in vivo* systems (Fernández-Borges et al. 2018). In the case of functional amyloids, which do not lead to a disease phenotype, cofactors such as chaperone proteins are involved in the folding of the protein into amyloid structures to carry out their function (Pham et al,. 2014). This discrepancy between a pathological amyloid and a functional amyloid is likely due to evolutionary mechanisms developing over time to restrict the possibility of toxicity to occur in functional amyloid production. Pathological amyloids may be a response from the organism to try and act as a self-defence mechanism against the more toxic, smaller soluble oligomers, sequestering them into insoluble plaques or even a response to microbial insult (Kumar et al. 2016).

## 1.3 Systemic Amyloidosis:

This section will review three of the more prevalent and well characterised systemic amyloidosis diseases: Systemic amyloid-light chain (AL) amyloidosis, transthyretin related (ATTR) amyloidosis, and amyloid-A associated (AA) amyloidosis. These amyloidosis diseases have been well characterised over the last 40-50 years and share similarities with amyloidosis of the CNS. Principle of which the uncontrolled and exponential production of misfolded amyloid fibrils. Treatments exist for systemic amyloidosis and their effectiveness and method may help elucidate possible treatments for those which occur within the CNS. The functions of the amyloidogenic proteins and the pathology of these four diseases will be briefly explored. Later sections will review amyloidosis related disorders of the CNS. Both systemic and CNS disease will then be examined in relation to functional amyloids and how functional amyloid production is controlled and what insights may be learned from this control and how it may help guide the development of therapeutic interventions for amyloidosis and amyloid-related disease.

### 1.3.1 Systemic AL Amyloidosis:

AL is the common form of systemic amyloidosis where amyloid deposition can affect a variety of different organs throughout the body. Early detection is important for treatment. The more advanced the stage of AL the more likely treatments will be ultimately unsuccessful. This is often due to an increase in amyloid deposit occurring in the heart leading to organ failure (Desport et al. 2012). Rapid production of plasma clone cells, or in rare cases clonal B-cells, in the bone marrow leads to a large increase in the production of free, unpaired immunoglobulin kappa and lambda chains (Hasserjian et al. 2007). This can be found in approximately 10% of multiple myeloma patients where there is an overproduction of these light chain units (Gertz 2018). There are still several questions left unanswered regarding the disease mechanisms, such as why only a small subset of the free light chains

169 form amyloid fibrils, and why lambda chains appear more likely to form amyloid fibrils than
170 kappa light chains (Perfetti et al. 2002).

171 AL amyloidosis occurs in multiple myeloma patients, from certain lymphomas, and
172 monoclonal gammopathy of undetermined significance (MGUS) (Kyle et al. 1992; Comenzo
173 et al. 2006). In somewhat similar fashion to prion and protein misfolding diseases of the
174 CNS, AL production of these abnormal light chains can occur before any symptoms become
175 apparent (Wechalekar, Gillmore, and Hawkins 2016; Weiss et al. 2014). As disease
176 progresses patients develop impairments to multiple different organs most critically including
177 the heart, liver and kidney, with cardiac involvement having the worst prognosis (Gertz et al.
178 2005). There are multiple different organ specific biomarkers that can be used to determine
179 the presence of AL, though the reliability and efficacy can be dependent on how they are
180 utilised and they require further validation (Dittrich et al. 2019). Currently the most common
181 biomarker is the presence of the free light chains themselves, the level of which can suggest
182 there is more likely to be cardiac involvement (high light chain serum levels) or renal
183 involvement (low light chain serum levels, Bochtler *et al.*, 2008). The kidney is the second
184 most affected organ behind the heart in AL (Kimmich et al. 2017) and without treatment
185 patients will inevitably develop severe renal failure often within a few years of diagnosis.
186 High protein and particularly albumin concentrations in the urine is one of the most common
187 biomarkers to establish AL renal involvement (Bochtler et al. 2008). Finally, the liver is the
188 next most commonly affected organ, though there are few reliable biomarkers signifying it is
189 affected. Liver pathology is more associated with a rarer form of AL amyloidosis where
190 immunoglobulin M is affected rather than immunoglobulin A or G (Sachchithanantham et al.
191 2016).

192 Similarities can be seen between AL amyloidosis, other systemic amyloidosis diseases and
193 amyloid diseases of the CNS and prion diseases. As mentioned, AL amyloid deposits affect

several different organs. This relies of amyloid being spread from a site of origin around the body. Though this presumably involves the circulatory system specifics are unknown, as is why some organs are more affected over others. This may bear resemblance to the spread of amyloid or prions across the brain in how certain brain regions are more susceptible either in onset or progression of disease depending on the pathology. Of particular interest is the mechanisms of cell toxicity in AL. Broadly speaking in amyloidosis, and even prion diseases, the smaller soluble oligomers are now thought to be more toxic. However, this does not mean the larger insoluble fibrils themselves are not involved. In AL it has been demonstrated that both the smaller soluble oligomers are cytotoxic in addition to larger insoluble fibrils. Depending on whether it was caused by fibrils or oligomers, the mechanism of toxicity was different (Marin-Argany et al. 2016).

### 1.3.2 ATTR Amyloidosis:

ATTR is the second most common form of amyloidosis after AL. Instead of the amyloid deposits consisting of the immunoglobulin free light chains the deposits are primarily made up of the protein transthyretin (Westermark et al. 1990). Transthyretin (TTR) is a transport protein for both thyroxine and retinol binding protein (Van Jaarsveld et al. 1973). It circulates both in cerebral spinal fluid and blood serum and is primarily produced by the liver where it is secreted into the blood but can also be produced by the retinal pigment epithelium and the choroid plexus (Dickson, Howlett, and Schreiber 1985; Dickson et al. 1985).

The hereditary form of ATTR (formerly Familial Amyloid Polyneuropathy, FAP) is the most common hereditary amyloidosis. It most commonly results in polyneuropathy but can also affect the heart, kidney, gastrointestinal system and the eyes (Ando et al. 2013). The non-hereditary form used to be referred to as senile systemic amyloidosis but is now more commonly referred to as wild-type transthyretin amyloid (WTTA). It is similar to AL, most commonly affecting the heart though it generally has a more positive clinical outlook (Ando

et al. 2013). The major risk factor for WTTA is age, and it is thought to affect as many as 80% of the population above the age of 80, particularly males (Connors et al. 2016).

ATTR is progressive and often presents itself first as loss of sensation and neuropathic pain, and as progression occurs leads to motor dysfunction characterised by an altered gait (Planté-Bordeneuve and Said 2011; Çakar, Durmuş-Tekçe, and Parman 2019). Though originally thought to be reasonably rare as diagnosis has become more accurate it is emerging as a more common cause of polyneuropathy and cardiac failure than originally thought. The autonomic nervous system is also eventually affected often presenting as gastrointestinal problems resulting in weight loss and dietary problems. Unlike AL while the kidney can be affected in both WTTA and ATTR, it is not as common and instead the ocular system commonly presents symptoms such as glaucoma (Ando et al. 2013). The similarities between ATTR and AL can lead to misdiagnosis of the two with one being mistaken for the other which can have significant consequences for patients (Naiki et al. 2020).

Pharmaceutical treatment is available, however while there is evidence to show it can be effective in delaying disease progression the options available do not appear to be able to fully treat the disease (Sekijima 2015). Treatment strategies differ on whether the patient has WTTA or ATTR. WTTA can often be managed through a cardiac pacemaker and drugs to manage breathing difficulties and changes associated with blood pressure. These options will improve patient quality of life but will not prevent fatality. For ATTR, tafamidis is the most common pharmaceutical intervention and when given early on can help delay, but not prevent, onset of polyneuropathy (Coelho et al. 2012). The most effective option is liver transplantation which is the only available option to guarantee survival provided there is no cardiac involvement. However this is invasive for the patients and unlikely to be viable in every case due to the constant need and under-supply of available matching organs (Ando et

al. 2013). Moreover, the common problems with organ transplant such as host rejection and the need for immunosuppressants remain.

Liver transplantation can also lead to iatrogenic transmission of ATTR (Holmgren et al. 1991; Gustafsson et al. 2012), reminiscent to that of dura matter grafts or corneal transplants in prion disease (Duffy et al. 1974; Noguchi-Shinohara et al. 2007). This, alongside growing evidence from AA (outlined below), supports that systemic amyloidosis may have transmissible properties similar to prion diseases. ATTR neuropathic symptoms manifested between 6-9 years (Abdelfatah, Hayman, and Gertz 2014). This would suggest the presence of amyloid in the transplanted liver may have been able to seed further amyloidogenesis in the recipient.

### 1.3.3 Systemic AA Amyloidosis:

Formally called secondary amyloidosis, AA is more commonly associated as a secondary event associated with a different disease, such as tuberculosis, and now often with rheumatoid arthritis, irritable bowel syndrome and other autoimmune or autoinflammatory conditions though as many as a quarter of cases have no obvious cause (Westermark *et al*, 2015). These conditions lead to an increase in the release of inflammatory cytokines, causing a signalling cascade resulting in an increase in serum amyloid A (SAA) proteins, primarily by the liver. There are four SAA genes in humans. Proteolytic events can lead to SAA being processed into amyloidogenic AA, particularly SAA1 (Tanaka et al. 2018), though the exact details of how this happens are yet to be elucidated. Ordinary plasma concentrations of SAA ranges between 2-5mg per litre (Hijmans and Sipe 1979) and can rise to as high as 2000mg per litre in a disease state. Inflammatory disorders are the highest risk factor for the development of AA. However it still only occurs in a subset of patients (Kobayashi et al. 1996; El Mansoury et al. 2002), why some are affected and some are not remains unclear.

SAA proteins are apolipoproteins which associate with high-density lipoprotein (HDL) in blood plasma and most commonly are produced by the liver in response to different inflammatory stimuli. They have been shown to be produced by adipocytes and obesity is a contributing risk factor to AA disease (Benditt and Eriksen 1977; Coetzee et al. 1986). They have a variety of functions including cholesterol transport and as part of the immune response they can recruit immune cells to inflammatory sites due to the activity of pro-inflammatory cytokines (Ji et al. 2015; Sano et al. 2015). There is a large increase in the production of SAA as part of the acute-phase response after which levels can fall to pre-response levels very quickly. Of the different SAA proteins, it is SAA1 and SAA2 which are most relevant for forming amyloid deposits (Liepnieks *et al*, 1995). The SAA1 and SAA2 proteins and their isoforms form pre-fibrillar oligomers both of different structural characteristics and at different speeds.

Especially related to prion and prion-like diseases it has also been shown that SAA amyloid formation maybe susceptible to 'seeding', that is to say fibrils can influence their growth through the interaction with normally folded protein (Patke et al. 2013). The effect this has on disease pathology and progression, if any, is uncertain. Typically there is a 76 amino acid sized fragment which constitutes the main unit of SAA amyloid fibrils (Westermark, 1982). Different sized species, both smaller and larger, are also found and may be related to the region of the body affected during disease (Westermark *et al*, 1989). The underlying reasons of how these different sized oligomers are produced are not well determined but may be due to whether there is any proteolytic cleavage before or after fibril formation has occurred, which then further affects the type of fibril produced. Furthermore, for SAA amyloid fibrils to form the SAA protein cannot be in its HDL bound state and first needs to separate, as HDL binding has been associated with an increase in alpha-helical conformation (Elimova *et al*, 2009). While AA derived from SAA is the primary constituent of the amyloid fibrils that

form there is also the presence of other molecules, most often certain glycosaminoglycans and proteoglycans (Pepys et al. 1997; Niewold et al. 1991).

AA is considered the main example of a non-prion transmissible amyloidosis. AA has been transmitted to mice (Hardt 1971; Werdelin and Ranlov 1966), hamsters (Hol et al. 1986), chickens (Murakami et al. 2013), and mink (Sørby et al. 2008). For AA to be transmissible there needs to be a high enough concentration of SAA in the recipient animal, which would likely depend upon an inflammatory response such as that caused from bacterial or viral infection. There is some evidence that transmission of AA can occur between animals in the wild and in captivity which raises parallels to some extent with the chronic wasting disease epidemic in North America. Island foxes and herring gulls both have high incidences of disease which suggests a level of transmission between individuals (Gaffney et al. 2014; Jansson et al. 2018). In captivity, cheetah have been shown to transmission of AA which would also mostly likely suggest oral transmission (Beiru Zhang et al. 2008). In experimental models AA is introduced through injection. Aggregation appears to begin in the spleen, though AA still occurs in animals after splenectomy (Kisilevsky and Benson 1981). Cross-seeding can also be seen using SAA fibrils from other animals in mice. Even the amyloid fibrils from other proteins, such as bacterial curli and spidroin amyloid can accelerate AA amyloidogenesis (Cui et al. 2002; Lundmark et al. 2005). All of this could mean that there is a risk of cross-transmission of AA into humans though there is little evidence to support this happening thus far.

AA could help reveal insights into cross-transmission of prion diseases. As transmission between certain animal populations have already been demonstrated this could help investigate how CWD transmission occurs between deer populations in North America. The influence of different amyloidogenic proteins on the rate of AA amyloidogenesis may also help determine the effects of cross reactivity between fibrils. Curli amyloid may be of

particular interest as there is growing evidence the microbiota of an individual may have an impact on their susceptibility to, or the prognosis of, diseases such as AD. This will be covered later.

### 1.3.4 Summary of systemic amyloids:

To conclude, this section has covered three different common amyloidosis, summarising their pathology and the amyloidogenic proteins involved. Cross reactivity is observed in systemic amyloidosis and may provide an additional model to investigate the potential infectivity of amyloidogenic proteins and prion disease. As there are at least some effective biomarkers present for system amyloidosis progression of amyloidosis may also be determined. It is thought the initial protein misfolding events can occur many years before symptoms of disease present themselves. These biomarkers would likely be easier to follow in systemic amyloidosis than in CNS amyloid disease so may provide powerful complements to elucidating the progression of CNS neurodegenerative diseases such as AD, PD, and prion disease. As is evident in both AL and AA a rise in the concentration of the amyloidogenic proteins is often necessary for disease onset. This could be caused by inflammatory events leading to a rise in the production of amyloidogenic protein. Similar events occur in AD (**section 1.4.4**) and systemic amyloidosis may pose as risk factors for CNS amyloidosis and vice versa. This would suggest the potential for these diseases to be intrinsically linked. Furthering understanding of systemic amyloidosis may therefore result in both furthering understanding of CNS amyloidosis, and prevention of systemic amyloidosis may also reduce the risk of onset of CNS amyloid disease such as AD.

## 1.4 Prion Disease and CNS Amyloid Related Disease:

Here, AD, PD, and prion diseases will be explored. As mentioned, systemic amyloidosis may influence onset or progression of CNS amyloid disease, particularly AD. This includes amyloidosis not covered in this review, such as type II diabetes. Amylin misfolding in type II

342 diabetes possibly contributes to neuronal cell loss alongside amyloid-β misfolding in
343 Alzheimer's disease (Jackson et al. 2013). Patients who have type II diabetes are statistically
344 more at risk of developing cognitive impairment, dementia and AD (Gudala et al. 2013;
345 Roberts et al. 2014) though this is not reciprocal. Amylin has a propensity to spread into the
346 CNS and while Aβ can be detected in the blood serum of patients, and may even become a
347 possible biomarker for disease (Rushworth et al. 2014), it does not appear to have any
348 pathological effects outside of the CNS (Jackson et al. 2013).

349 Particularly for CNS amyloid-related disease it is becoming increasingly accepted that it is
350 not the large, aggregated fibrils which are the main toxic species in disease, though they may
351 still contribute. Instead, oligomeric species ranging from dimers and trimers up to chains of
352 70-80 peptides appear to be the driving force behind pathology and cell death (Baglioni et al.
353 2006; Simoneau et al. 2007; Reixach et al. 2004). Unlike the larger amyloid fibrils these
354 smaller oligomers are soluble and can have potentially a large and diverse set of binding
355 partners which likely contributes to disease. Because of this it is unlikely there is a single
356 primary mechanism behind cell death seen in amyloid related diseases though there are
357 common toxic events that occur. These include an increase in membrane permeability caused
358 by the oligomers forming pores in the cell membrane leading to destabilisation of calcium
359 homeostasis, and an increase in reactive oxygen species (ROS) and associated toxicity
360 (Simoneau et al. 2007). As disease progression occurs there is an ever-growing amount of the
361 misfolded protein. The cellular proteostasis response, the cell's ability to either correct or
362 degrade misfolded proteins, will eventually be overwhelmed (Plate et al. 2016). This means
363 that the cell is not only unable deal with the vast increase in the amyloidogenic protein, but
364 also other proteins which require correction or degradation as they are being produced
365 leading to disruption of cell function and apoptosis.

366 The most common risk factor for all amyloidosis is age, likely due to the correlation with age
367 and general reduction in proteostasis and cellular functions (Chiti and Dobson 2006). Insults
368 caused by ROS increase with age and it is possible these may also contribute to disease
369 (Cadenas and Davies 2000). Despite symptom onset becoming more likely with age the
370 mechanistic events can start occurring significantly before these symptoms manifest (Wesson
371 et al. 2010; Rushworth et al. 2014; Stocker et al. 2020). The majority of amyloidosis is
372 idiopathic with no obvious cause. Though there are genetic cases of diseases, and some are
373 hereditary only **(Table 1)**. In the case of genetic or hereditary forms of amyloidosis the age of
374 onset is typically much younger, presenting themselves during the patient's thirties or forties
375 and are often more aggressive than sporadic cases (Smits et al. 2015; Toniolo et al. 2018;
376 Kim et al. 2020). Reasons for this are unclear but it is likely due to the mutations that result in
377 the hereditary disease leading to a more amyloidogenic form of the protein which will begin
378 to be produced from birth, resulting in an earlier and more rapid build-up of misfold
379 oligomeric species and subsequently larger amyloid fibrils. This would echo what is seen in
380 systemic amyloidosis where increase concentration of the amyloidogenic protein correlates
381 with disease occurrence.

382 Currently the only confirmed risk of infectious transmission of amyloid disease occurs in the
383 case of prion disease (Prusiner 1991; Prusiner 1982). In prion diseases, the normally folded
384 cellular prion protein ($PrP^{C}$) misfolds into the disease-causing isoform, scrapie prion protein
385 ($PrP^{Sc}$). $PrP^{Sc}$ can cause infectious neurodegenerative diseases such as Kuru, passed between
386 humans due to ritualistic practices of cannibalism (Mathews, Glasse, and Lindenbaum 1968).
387 While rare, these can occur across species barriers such as in an outbreak of variant-CJD in
388 the United Kingdom in the late eighties and early nineties due to the consumption of cattle
389 with bovine spongiform encephalopathy (BSE) (Will 2003). There has been speculation
390 whether diseases such as AD may be potentially infectious or have iatrogenic potential,

391 though the evidence for this remains unconvincing (Duyckaerts, Clavaguera, and Potier
392 2019).

393 The subsections below will describe three different protein misfolding disorders of the CNS:
394 Prion disease, Alzheimer's disease, and Parkinson's disease. In each case disease is caused by
395 misfolded protein and the formation of amyloid-like deposits in the brain. Similarities
396 between the onset and progression of the amyloid related diseases in each disease will be
397 explored. Future sections will then discuss functional amyloids and how what is known about
398 non-pathogenic amyloids may help in future efforts to treat amyloidosis and amyloid-related
399 disease.

400 ### 1.4.1 Cellular Prion Protein and Prion Protein Scrapie:

401 Prion diseases are caused by the misfolding of normal $PrP^{C}$ into the misfolding disease $PrP^{Sc}$.
402 This can cause a variety of different neurodegenerative disorders including the eponymous
403 scrapie in sheep, chronic wasting disease in cervids, bovine spongiform encephalopathy
404 (BSE) in cattle and in humans is responsible for: Creutzfeldt Jacob disease (CJD), fatal
405 familial insomnia (FFI), and kuru among others (Mok and Mead 2017). Idiopathic incidences
406 of prion disease, those with no obvious cause, are most common. However, there can also be
407 hereditary as well as acquired causes behind the disease. The protein only hypothesis refers to
408 the model originally proposed by Stanley Prusiner in the 1980s to describe how the
409 pathogenic and infectious agent responsible for disease (in this case scrapie in sheep) was
410 solely a protein and not due to bacterial or viral action (S. Prusiner 1982). It is now widely
411 accepted that $PrP^{Sc}$ is both able to cause disease and infect other organisms without the need
412 of an essential cofactor, though certain molecules or components do seem able to increase
413 infectivity and strain properties of $PrP^{Sc}$ (Fernández-Borges et al. 2018).

414 Hereditary prion diseases include FFI, familial CJD and Gerstmann-Straussler-Scheinker
415 syndrome (GSS). In all cases there are one or two amino acid substitutions which will result

in disease onset producing a consistent series of disease symptoms. FFI is caused by a hereditary D178N mutation where an asparagine residue replaces an aspartic acid residue. It also requires the presence of methionine at position 129, valine can also be present at this position but is not associated with FFI (Alred et al. 2018) and may even be protective against other forms of prion disease (Fernández-Borges et al. 2017).

In prion disease there is evidence there are 'strains' of misfolded protein. This is determined by the secondary structure formed during misfolding leading to different and unique phenotypes and pathology progression. These strains occur despite the amino acid sequence being identical **(Figure 3)** (Moore et al. 2020; Thackray et al. 2007; Solforosi et al. 2013). What determines whether the initial misfolding event will lead to one secondary structure over another is currently unclear although the state of the cellular environment may play a part. Strains do show remarkable fidelity and sustainability. For example, they have been passaged between multiple different generations of mice and retained their strain specific characteristics as seen through onset of encephalopathy and western blot; therefore once a particular misfolded conformation has formed it does not appear likely to change (Thackray et al. 2007). This is perhaps best demonstrated through the passaging and infection of two prion strains from transmissible mink encephalopathy into hamsters, hyper and drowsy (Bessen and Marsh 1992; Bartz et al. 2000). It is possible that these are the characteristics that can lead to eventual crossing of prion diseases between different species, breaking the species barrier (Aguzzi *et al.*, 2007). $PrP^{Sc}$ strains have the potential to make therapeutic intervention particularly difficult, as a treatment which may be effective for one strain may not be effective on another.

A high-resolution structure of $PrP^{C}$ is available (Calzolai and Zahn 2003) but despite significant progress so far a definitive 3D structure of $PrP^{Sc}$ has yet to be identified. Many technical hurdles still exist, primarily due to the insoluble nature of $PrP^{Sc}$ and its propensity to

aggregate. This means that it is difficult to firmly establish its structure using current methods such as Fourier-transform infrared, circular dichroism and nuclear magnetic resonance spectroscopy, electron microscopy and X-ray crystallography (Requena and Wille 2017). Identifying the structure of one strain of $PrP^{Sc}$ does not necessarily mean the information gleaned can then be applied to a different strain of $PrP^{Sc}$, which may have a very different structural conformation (Baskakov et al. 2019). Nevertheless, being able to determine with certainty structures of $PrP^{Sc}$ could be key to preventing its misfolding, or at least understanding the propagation of how it performs template directed misfolding and therefore for identifying different disease properties caused by different misfolding templates resulting in unique strain pathologies. Improvements in cryogenic electron microscopy (cryo-EM) currently describe the structure of $PrP^{Sc}$ as two independent protofilaments with structural units repeating along their axis resulting in a four-rung β-solenoid ((4RβS(Spagnolli et al. 2019)). While this evidence supports a 4RβS $PrP^{Sc}$ structure there is also evidence to suggest an alternate hypotheses where $PrP^{Sc}$ can instead take on a parallel in-register β-sheet (PIRIBS) structure (Spagnolli et al. 2019; Requena and Wille 2017). Functional prions in fungi have been found to form both 4RβS and PIRIBS structures and it may even be that depending on the strain of $PrP^{Sc}$ it could adopt either architecture (Baskakov et al. 2019; Wasmer et al. 2008; Reed B Wickner et al. 2018).

Regardless of the secondary, tertiary or quaternary misfolded structure that results, the mechanisms by which misfolding occurs may be highly similar, and therefore targeting this misfolding mechanism to prevent oligomer formation before actual fibril formation could also be a viable therapeutic strategy. Functional amyloids, which will be explored later in this chapter, can control the process by which oligomer elongation and fibril formation occurs. The use of chaperone and nucleator proteins ensures rapid production of oligomers and subsequent fibrils does not grow out of control causing a toxic outcome **(Figure 2)**. Applying

the knowledge of how these processes are naturally controlled may aid in developing therapeutic interventions for prion and other protein misfolding diseases.

### 1.4.2 Alzheimer's Disease:

Alzheimer's disease is the largest cause of dementia worldwide with cases only expected to rise as life expectancy of global populations continues to increase, while putting considerable economic burdens on national healthcare systems. In the United States it is estimated that in 2020 the cost of AD was $305 billion USD and this is predicted to rise to as high as $1.1 trillion USD by 2050 (Alzheimer's Association 2020). Age remains the biggest risk factor for AD, sporadic onset with no obvious identifiable cause making up approximately 90% cases. Other risk factors include lifestyle risk factors, such as obesity or genetic risk factors such as the *APOE* ε4 allele of apolipoprotein (Farrer et al. 1997). The remaining 10% of cases have a genetic cause and are often referred to as Familial Alzheimer's Disease (FAD). Like other hereditary amyloid diseases, including amyloidosis, they tend to have symptoms which present earlier in the life of an individual, usually in their forties (Mercy et al. 2008) when compared to idiopathic onset. Mutations in amyloid precursor protein (*APP*) and the two presenilin genes presenilin-1 (*PSEN-1*) and presenilin-2 (*PSEN-2*) are the most common causes of FAD (K. Murakami et al. 2003; Tsubuki, Takaki, and Saido 2003; Sherrington et al. 1995).

The main pathogenic species in AD is the amyloid-β peptide (Aβ), along with neurofibrillary tangles primarily comprised of hyperphosphorylated Tau protein (NFTs). The original amyloid cascade hypothesis proposed that neurodegeneration and disease progression was caused by the increase in insoluble Aβ plaques which would deposit in the brain with age (Hardy and Higgins 1992). Over time evidence suggested that this was unlikely to be the complete picture. Insoluble plaque deposition and size does not correlate with either AD severity or progression; instead smaller soluble oligomers of Aβ (Aβo) appear to be the

primary cause of neurotoxicity (Baglioni et al. 2006; Cohen et al. 2013; S. T. Ferreira et al. 2015; Gandy et al. 2011; Walsh et al. 2002). Presence of the plaques may be involved in pathology due to acting as a surface area for oligomer production or wells for soluble oligomers which can be released with plaque fragmentation **(Figure 1)**. Oligomer size can vary with oligomers of length 40 ($A\beta o_{1\text{-}40}$) and 42 ($A\beta o_{1\text{-}42}$) being the most common and concentration correlates much more closely with symptom progression (Baglioni et al. 2006). As it is the smaller soluble oligomers, rather than the amyloid fibrils, which are now thought to be primarily responsible for AD, it is considered as an 'amyloid disease' rather than an amyloidosis (Benson et al. 2020). It has recently been proposed that the characteristic insoluble plaques may be an attempt by the body to sequester away Aβo, to prevent toxicity (Meilandt et al. 2020; Parhizkar et al. 2019). Initial efforts to treat AD, all of which have so far proven unsuccessful, involved immunotherapy using antibodies against Aβ. While some of the antibodies recognised smaller oligomers and amyloid-protofibrils, some also targeted the insoluble amyloid plaques leading to their dissolution. This could lead to an increase in the availability of soluble Aβo and may have acted to increase disease severity rather than decreasing severity and treating the disease **(Figure 1)**(Sengupta, Nilson, and Kayed 2016; Y. H. Liu et al. 2015).

Aβ peptides are produced through either β- or γ- secretase cleavage of the amyloid precursor protein, APP. The β-secretase BACE1 cleaves the APP luminal domain, producing a secreted product (βAPP) before the remaining APP fragment has its transmembrane domain cleaved by γ-secretase producing Aβ (Ehehalt et al. 2003; Sinha et al. 1999). APP can also be first processed by α-secretase rather than β-secretase, which does not result in the production of Aβ as cleavage occurs within the Aβ region of APP and is therefore termed the non-amyloidogenic pathway. A protective mutation against AD has been identified in APP, A673T which appears to increase the α-secretase processing of APP, reducing Aβ production

and likelihood of AD onset (Jonsson et al. 2012). $A\beta_{1-42}$ appears more toxic than $A\beta_{1-40}$ (Klein, Kowall, and Ferrante 1999). A mix of $A\beta_{1-40}$ and $A\beta_{1-42}$ appear to lead to the most toxic outcomes depending on the ratio, producing smaller, stable toxic oligomers (Y. J. Chang and Chen 2014; Johnson et al. 2013; Sengupta, Nilson, and Kayed 2016). Currently there are over 20 mutations identified in APP which can lead to FAD (K. Murakami et al. 2003). Mutations in the two presenilin genes affect the function of the proteins which are involved in the secretase complex responsible for processing APP (Kovacs et al. 1996; De Strooper 2003). Different conformations of Aβo have been described which appear to affect disease pathology, suggesting there may be a similar strain phenomenon as that seen with prion diseases (Petkova et al. 2005; Wei Qiang1 et al. 2017; Condello et al. 2018; Rasmussen et al. 2017).

Two of the most well characterised toxic mechanisms of Aβo are oligomers forming pores within the cell membrane, leading to a disruption of calcium homeostasis and cell death (Sciacca et al. 2012). This mechanism is reminiscent of similar toxicity seen in other amyloid diseases and amyloidosis. The second mechanism involves $PrP^{C}$ acting as a high affinity receptor for $A\beta o_{1-42}$ causing a signal cascade leading to activation of fyn-kinase and cell death (Laurén et al. 2009; Um et al. 2012; Larson et al. 2012). It is important to note that this pathway involves normally folded physiological $PrP^{C}$ and not misfolded $PrP^{Sc}$, and subsequently future treatments for AD may involve targeting of $PrP^{C}$. NFTs are also closely linked with disease pathology and progression though how, if at all, they tie into Aβo toxicity is unclear. It has been proposed that NFTs may be protective against Aβo and NFT formation occurs in response to Aβo mediated cell death and may reduce oxidative stress (Ittner et al. 2016). This is however in direct contrast to previous studies showing that hyperphosphorylated Tau is required for Aβo toxicity to take place (Rapoport et al. 2002). Discrepancies in these studies may once again be due to the smaller, soluble oligomers of

misfolded Tau being responsible for toxicity and the larger tangled assemblies are again a protective response, or simply comparatively less toxic (Penke, Szucs, and Bogár 2020; Kopeikina, Hyman, and Spires-Jones 2012). Aβo toxicity may act as a precursor to NFT toxicity, and Aβo signalling leads to the formation of NFTs (Bloom 2014). This can occur through the Aβo-$PrP^C$ signalling pathway mentioned above (Larson et al. 2012) and there are likely additional pathways as well.

While age is the biggest risk factor for AD there is growing evidence that the onset of disease may be closely linked with the gut microbiota (Dinan and Cryan 2017; Cattaneo et al. 2017). Changes in the bacterial composition of the gut can cause significant proinflammatory responses (Belkaid and Hand 2014; Thevaranjan et al. 2017). Infections leading to an inflammatory response leads to an increase in the production of SAA and is a risk factor for AA. SAA has been found to localise with the amyloid-β senile plaques in AD (J. S. Liang et al. 1997). As cross-seeding is seen in AA, including by bacterial curli or synthetic amyloid (Lundmark et al. 2005; Johan et al. 1998), bacterial infection and impact on the gut could have profound effects of potential amyloidosis both systemically and in the CNS. Endotoxins from *E. coli* have been found to increase amyloid-β fibrillisation *in vitro* (Asti and Gioglio 2014) and in addition to bacterial amyloids may cause inflammatory responses in the CNS resulting in amyloid-β fibril formation and onset of disease. The permeability of the BBB and the gut epithelia increase with age and can be further increased by inflammation and bacterial amyloid and causes a rise in cytokines directly related to AD (Elahy et al. 2015; Bors et al. 2018). This suggests the potential for a significant link between the onset of both systemic and CNS-related amyloidosis. Further investigation of cross-seeding of bacterial and systemic amyloid resulting in CNS-amyloidosis is required to cement the significance, or lack thereof, of the relationship between these different amyloid diseases.

### 1.4.3 Parkinson's Disease:

Parkinson's disease is a neurodegenerative disorder originally classified as the misfolding of alpha-synuclein (α-Syn) causing the formation of Lewy bodies in the substantia nigra region of the basal ganglia (Spillantini et al. 1997; Arima et al. 1999). The most characteristic symptoms of PD involve a progressive loss of control over the motor system, manifesting as a tremor and increasing difficulty in controlling voluntary movement, which occurs due to the loss of dopaminergic neurons in the substantia nigra (Beitz 2014). As symptoms worsen behavioural problems also begin to occur, termed Parkinson's disease dementia (PDD) and are like those seen in AD and other dementias, including mood swings, depression and anxiety (Jankovic 2008). There are additional symptoms which can occur before the motor symptoms manifest though on their own may not be sufficient for diagnosis. The most prevalent and perhaps obvious is a slow decline in olfactory function and loss of smell (Haehner et al. 2009), followed by gastrointestinal symptoms and sleep disruption (Barichella, Cereda, and Pezzoli 2009; Jankovic 2008). The most effective treatment for PD symptoms is the administration of levodopa to try and counteract the loss of dopaminergic neurons, though this does not slow disease progression and so is not an effective long term treatment strategy (Nagatsua and Sawadab 2009).

The biggest risk factor is again age, and other factors include having relatives who develop PD (though the genetic relationship of this is not always clear), pesticide exposure and head trauma (L. M. L. de Lau and Breteler 2006; Semchuk, Love, and Lee 1992). There are genetic risk factors which increase the risk of developing PD, the highest risk gene is *GBA1* which can increase susceptibility to developing PD by up to 7-fold compared to those not carrying the relevant allele (den Heijer et al. 2020). Autosomal dominant mutations in the gene encoding α-Syn (*SNCA*) have also been identified, and are one of the reasons why α-Syn is thought to be the primary pathogenic species in PD (Hernandez, Reed, and Singleton 2016;

590 Konno, Siuda, and Wszolek 2016; Zarranz et al. 2004). Despite these genetic risk factors the
591 majority of PD cases remain idiopathic, with no obvious cause (Beitz 2014).

592 Similar to AD it is not the insoluble, aggregations of α-Syn which are thought to be what
593 leads to toxicity. Instead, evidence suggests it is again the shorter, soluble pre-fibrillar
594 oligomers and so this strictly speaking would classify PD as an 'amyloid disease' rather than
595 a traditional 'amyloidosis' (Mehra, Sahay, and Maji 2019; Benson et al. 2020; Fusco et al.
596 2017). While the α-Syn amyloid fibrils themselves are not be directly responsible for cell
597 toxicity and neurodegeneration they may still play a role in the pathology of PD. The fibrils
598 are important for the spread of PD across the rest of the brain from the disease origin within
599 the substantia nigra (Luk et al. 2009). Fibrils can act as seeds, internalising α-Syn within cells
600 and causing an increase in the formation of oligomers (Volpicelli-Daley, Luk, and Lee 2014;
601 Luk et al. 2012). In this manner the α-Syn fibrils act similarly to $PrP^{Sc}$ in prion diseases, both
602 as a nucleator for the further formation of toxic α-Syn oligomers and because of these also
603 show infectious properties characteristic of prions. Different strains of α-Syn fibrils have also
604 been identified, again similar to prions, which can occur through slight changes in conditions
605 during aggregation incubation (Shahnawaz et al. 2020). These different strains have even
606 been shown to display unique conformation-dependent pathogenesis similar to prion diseases,
607 with specific conformations resulting in distinct disease phenotypes ( Lau et al. 2020). This
608 may suggest a common mechanism of conformation dependent, strain specific, amyloid
609 formations may not be unique to prion diseases, and neurodegenerative diseases such as AD
610 and PD encompass a 'prion-like' method of toxicity with disease characteristics being
611 dependent on the conformation of the pre-fibrillar oligomers and resulting amyloid fibrils.

612 Exact mechanisms behind how α-Syn oligomers may cause toxicity is unknown though may
613 involve ROS generation through the permeabilization of the dopaminergic neurons within the
614 substantia nigra (Danzer et al. 2007; Parihar et al. 2009). Like $PrP^{C}$ knockout mice, α-Syn

knockout mice do not show any obvious serious phenotype(s) and remain healthy and fertile (Abeliovich et al. 2000). This may suggest that PD symptoms are primarily due to a toxic gain of function of α-Syn, rather than loss of function, though there remains the possibility of genetic compensation being more robust in stable knockout-models (El-Brolosy and Stainier 2017). α-Syn overexpression can have conflicting results depending on the amount of overexpression, with high overexpression resulting in cell proliferation and low overexpression resulting in cell toxicity in *in vitro* studies (Rodríguez-Losada et al. 2020).

Mutations in *SNCA*, which cause early-onset PD, affect the fibrillization dynamics of α-Syn, some such as A53T and E46K (Conway, Harper, and Lansbury 1998; Greenbaum et al. 2005) speed up the fibrillization and the A30P and A53E mutations actually slow down fibrillization (Ghosh et al. 2014; J Li, Uversky, and Fink 2001). In these cases, the change in the rate of fibrillization does not show a consistent link with toxicity – as the mutations either speed up or slow down fibrillization but regardless increase the risk of developing PD. It may be beneficial to look at the rate of oligomerization, as mutations which lean towards an earlier onset of PD have an increased rate in oligomerization and those which lean towards a later onset of PD have a comparatively decreased rate in oligomerization (Mehra, Sahay, and Maji 2019). While fibrillization rate is therefore not consistent among the familial mutations of PD, the increase in oligomer concentration or the ability to better sustain a constant oligomer concentration is consistent and is likely the reason why these mutations lead to an increase risk of developing PD, even if the exact mechanisms behind oligomer toxicity are themselves not clear.

### 1.4.4 Conclusion: CNS Amyloid Diseases

Amyloid diseases of the CNS present unique challenges compared to systemic amyloid diseases due to the more closed environment caused by the BBB. A growing body of evidence is beginning to suggest that they may be linked and act as risk factors for each other.

Cross seeding of amyloidosis between systemic proteins such as SAA, bacterial curli and amyloid-β peptides has been demonstrated and contribute to the risk factors associated with ageing. It also opens the possibility of therapeutics for CNS amyloidosis. By maintaining the health of the gut microbiome and reducing inflammatory stimuli which can be exacerbated by age it may help prevent onset of CNS amyloidosis. There are some pressing questions which remain, however. While changes in the gut microbiota and inflammation has been linked with AD, the impact on PD and prion diseases is less clear. $PrP^C$ is abundantly expressed in the gut which may suggest a possible route of transmission from oral consumption of contaminated food. The likelihood of developing PD has also been shown to increase in those that suffer from irritable bowel syndrome (Lai et al. 2014). Cross seeding leading to CNS amyloid disease may also contribute to the unique pathologies seen within the same disease leading to distinct strains of misfolded protein, most evidently seen in prion disease. Lastly, the extent at which systemic or bacterial amyloid has on being the defining event in onset of CNS amyloidosis is unknown. Onset of these neurodegenerative disease may likely occur without inflammatory influence, however it may increase rate of onset. Delaying the occurrence of disease could still prove immensely beneficial to healthcare systems and provide a much needed buffer in which a treatment window is available.

## 1.5 Functional Amyloids:

So far, the topic of review has covered amyloid formation and both systemic or CNS-related amyloid diseases and amyloidosis. In several of these cases, particularly AD and prion diseases, the normal physiological function of the normally folded protein is ambiguous with many different functions being ascribed to $PrP^C$ and amyloid-β. Conversely in systemic amyloidosis the function of the proteins is better understood and misfolding occurs due to a combination of abundance in the availability of the protein leading to proteostatic mechanisms being overrun. Understanding the function of these amyloidogenic proteins may

help guide therapeutic development where treatment is either unavailable, ineffective or with unacceptable side effects.

Functional amyloids are being identified particularly in bacteria and fungi, though also increasingly in higher organisms including mammals (Pham et al., 2014; Jain and Chapman 2019). Exploring their physiological roles, and how the amyloids are constructed in a controlled fashion, may provide insight into how to prevent misfolding in pathogenic amyloidosis. These functional amyloids have various structural similarities to the pathogenic amyloids seen in disease, most notable being the presence of intrinsically disordered domains (IDDs), which is one of the key potential drivers of amyloid formation. IDDs can often be identified as regions made up of amino acid repeat domains (Romero et al. 1997; Dunker et al. 2002). In functional amyloids the actual amyloid formation is tightly regulated and controlled, there are often chaperone or nucleator proteins to aid the process which allows amyloids to form much faster, potentially limiting the number of small soluble oligomeric species which are the main actors in disease (M. L. Evans et al. 2011). Alternatively, functional amyloid production only occurs when the surrounding environment becomes suitable, such as respiration or cell replication lowering pH and promoting amyloid formation under more acidic conditions.

Several of these functional amyloids will be described in the following sections, including those in animals. Then the lessons that can be applied from functional amyloids to pathological amyloids will be explored, and how they may be utilised to give us a better understanding of amyloidosis and potential treatments.

### 1.5.1 Functional amyloids in yeast:

Proteins which can exist in either a soluble, functional state or an amyloid state were first identified in yeast and form the prototypical example of a functional prion (Cox 1965). Often these yeast prions display loss of function phenotypes which can be beneficial depending on

690 the environmental conditions. One of the first yeast prions identified was [*URE3*], the prion
691 form of Ure2 (Aigle and Lacroute 1975; R B Wickner 1994). Ure2 prevents the uptake of
692 ureidosuccinate (USA) which is a component of uracil biosynthesis. Formation of [*URE3*]
693 prions inactivate Ure2 allowing the uptake of USA which would be beneficial to yeast which
694 cannot ordinarily synthesise their own (Lacroute 1971).

695 Prion formation in yeast can also occur due to overproduction of the physiological protein,
696 which can act as a mechanism to prevent protein overactivity by sequestering functional
697 protein into a non-functional prion state (Chernoff, Derkach, and Inge-Vechtomov 1993;
698 Derkatch et al. 1996). This not always sufficient, however, such as with [$PSI^{+}$] prions.
699 Induction of [$PSI^{+}$] does require an increase in concentration of the normal protein, Sup35,
700 however prion formation is increased both by the presence of [$PIN^{+}$] prions or QN-rich
701 prions/protein aggregates in general (Derkatch et al. 2001). [$PIN^{+}$] also increases the
702 formation of both [*URE3*] and [Het-s] prions (Bradley et al. 2002). Yeast proteins capable of
703 forming prions require a prion domain, removal of which inhibits the ability of the protein to
704 form amyloid aggregates (Shewmaker et al. 2007; Masison, Maddelein, and Wickner 1997;
705 J.-J. Liu, Sondheimer, and Lindquist 2002). Structurally the aggregates formed by yeast
706 prions are like what is thought to occur in animals and provide a useful model in which to try
707 and identify mechanisms of $PrP^{C}$ to $PrP^{Sc}$ misfolding. This is seen as yeast prions forming
708 characteristic in-register parallel β-sheet structures (Baxa et al. 2007; Reed B Wickner, Dyda,
709 and Tycko 2008).

710 Unlike in animals, the formation of prions in yeast and fungi are often benign or at least not
711 detrimental to the organism. In certain cases, they may confer an advantage to the yeast, such
712 as with [*URE3*] prions mentioned above. There is some ambiguity as to the beneficial effects
713 of yeast prions, however. [PSI+] was proposed to originally aid yeast react to an increase in
714 temperatures and cellular stress (Eaglestone, Cox, and Tuite 1999). There have been

difficulties in reproducing these effects (True and Lindquist 2000) and [PSI+] response to stress remains inconsistent (Joseph and Kirkpatrick 2008). One of the most clearly beneficial yeast prions is [MOD+], a prion of Mod5p. Fluconazole is an antifungal treatment, the presence of [MOD+] and subsequent reduction in Mod5p function results in an increased resistance to fluconazole (Suzuki, Shimazu, and Tanaka 2012). Reduction in Mod5p function affected its role in the sterol biosynthetic pathway providing resistance to antifungal treatments. Furthermore the presence of antifungal agents caused an increase in [MOD+] prions suggesting that this is an adaptive response to environmental pressures (Suzuki, Shimazu, and Tanaka 2012).

Conversely while under experimental conditions there have been benefits associated with the presence of [PSI+] and [URE3], these prions can often be toxic to yeast. In the case of [PSI+] this is in part because the Sup35 protein is an essential protein, and increase formation of [PSI+] leads to a loss of Sup35 function (McGlinchey, Kryndushkin, and Wickner 2011). While Ure2 is not essential the presence of [URE3] slows growth of yeast in a manner that is not due to loss of Ure2 function which would suggest a toxic effect of [URE3](McGlinchey, Kryndushkin, and Wickner 2011). It may reduction in growth in yeast caused by [URE3] and [PSI+] may be deliberate, to restrict growth and replication until the surrounding environment becomes more favourable.

Functional prions often have some sort of chaperone or self-limiting system to prevent overproduction of the functional prions which may be detrimental to the organism. In yeast, heat shock proteins provide a chaperone system for prion formation. The Hsp104-Hsp70-Hsp40 system aids in seeding and prion propagation. Hsp104 ordinarily is a disaggregase, interacting with substrates to aid in refolding them. Hsp70 targets Hsp140 to a substrate allowing it to aid in its refolding (Winkler et al. 2012). In the case of yeast prions such as [PSI+], activity of Hsp70/Hsp140 can result in the fragmentation of aggregates, increasing

the potential surface area for further prion formation to occur (Chernoff et al. 1995). Inactivation or inhibition Hsp104 prevents [PSI+] formation, demonstrating its importance (P. C. Ferreira et al. 2001; Jung, Jones, and Masison 2002).

Yeast and fungal prions remain the earliest identified example of functional prions. Some of these functions remain ambiguous but are likely a reaction to changes in environmental stresses. They offer an effective means in which to explore template directed seeding of prions and their subsequent structures. Finally, they provide a well characterised model of a chaperone system in the formation of functional amyloids. This chaperone system is vital, as high concentrations of yeast and fungal amyloid are toxic to the organism.

### 1.5.2 Functional amyloids in bacteria:

There are several different bacteria, most commonly gram-negative bacteria, which have been found to produce curli fimbriae, protein fibres with amyloid characteristics (Debenedictiset al., 2017). *Escherichia coli* is the best example of this being studied where the curli act as adhesion molecules to anchor the bacteria to its surroundings as part of the extra-cellular matrix (ECM) biofilms. By acting as an anchor in the ECM it also helps provide resistance to host proteases which may disrupt the bacteria and therefore also contributes to invasion of the target host (Jain and Chapman 2019; White et al. 2006; DePas et al. 2014). While this may be considered the prime example, staining with amyloid dyes has shown amyloid or amyloid-like material in a wide range of different types of bacteria including but not limited to: *Staphylococcus aureus, Mycobacterium tuberculosis, Streptococcus mutans, Klebsiella pneumonia* (Jain and Chapman 2019; Smith et al. 2017). In most of these cases the functional amyloid is related to biofilm formation. However, other functions so far identified also include acting as storage molecules (*K. pneumonia*) and in pili formation (*M. tuberculosis*).

764 Curli amyloids are composed of protein homodimers of CsgA and CsgB, and the larger Csg
765 (curli specific gene) family comprising of CsgC-F aid in localisation and nucleation
766 (Debenedictis et al., 2017; Evans et al. 2011; Robinson et al. 2006). Both CsgA and CsgB
767 have significant IDDs allowing for both flexibility and aggregation. They will spontaneously
768 form amyloid fibrils *in vitro* as CsgA forms the fibrils using CsgB as a nucleator
769 (Debenedictis et al., 2017). CsgA can be considered the 'primary' unit of curli amyloid, and
770 readily forms the cross β-strand structure typical of amyloids. Amyloid formation begins
771 through a nucleation process aided by CsgB (Hammer et al., 2007), producing monomers
772 which can aggregate together to form small oligomers which rapidly continue to mature fibril
773 development (Debenedictiset al., 2017; Wang et al. 2007). Furthermore, similar to pathogenic
774 amyloid formation this process can be sped up through a seeding process by the addition of
775 pre-formed CsgA fibrils. This can even occur through CsgA fibrils from other species of
776 bacteria such as *Salmonella typhimurium* (Zhou et al. 2012). As stated, CsgB is required to
777 nucleate the formation of CsgA amyloid. In cells lacking CsgB, CsgA will still be secreted
778 from the cell membrane but will not undergo amyloidosis (Hammar et al., 1996).

779 Both proteins, CsgA and CsgB, are capable of a level of self-aggregation within the cell
780 cytosol and there are several chaperone proteins involved in preventing this self-aggregation
781 and ensuring transport to the cell membrane (Robinson et al. 2006). In addition, there are
782 proteostatic mechanisms in place to break down any potential aggregates before amyloid
783 production can reach toxic levels. Some of these proteins are considered general chaperone
784 proteins and are not necessarily specific to the prevention of CsgA amyloid formation, such
785 as DnaK and heat shock protein (Hsp) 33, though both have been shown capable of this at
786 least *in vitro* (M. L. Evans et al. 2011). More specifically, CsgC exists as a chaperone protein
787 to prevent premature amyloid formation of both CsgA and CsgB (M. L. Evans et al. 2015).
788 Exactly how CsgC is able to prevent amyloid formation is not entirely clear but it is thought

to prevent the addition of monomers of either CsgA or CsgB to the maturing fibril. Further exploration of the effects of CsgC to prevent amyloid formation may provide insights into disease therapeutics, and this will be discussed later.

There are additional Csg proteins which assist in either the nucleation of CsgA/B or with transport to the membrane. CsgF for example can prime CsgB to begin to act as the nucleator for amyloidosis, though it does not appear required as without CsgF polymerisation of CsgA can still occur, albeit more slowly (Nenninger et al., 2009). CsgG and CsgE are responsible for transport of CsgA/B across the membrane to the cell surface, with CsgG providing a pore for transportation and CsgE effectively acting as a regulator for this pore to function (R. D. Klein et al. 2018). Finally, there is CsgD, which is a transcription factor protein regulating the transcription of all the other curli genes (Arnqvist et al. 1992). The expression of CsgD, and therefore by extension the other curli genes, is regulated by many environmental factors surrounding the bacterium, such as nutrient abundance, oxygen concentration, cell density and temperature (Gerstel and Römling 2001).

While initially identified in gram negative bacteria, gram positive bacteria also contain functional amyloids. *Staphylococcus aureus* is a gram positive bacteria which is arousing concern due to an increase in antibiotic strains appearing (multi-resistant, or methicillin-resistant, *Staphylococcus aureus*, MRSA) (Lakhundi and Zhang 2018). Like *E. coli*, functional amyloids in MRSA are thought to primarily act as a stabilising constituent of the bacterial biofilm. They are referred to as biofilm-associated proteins (Bap) and share similarities with the *csg* family (Lasa and Penadés 2006). Bap mediated amyloidosis is dependent on the environment, with more acidic environments promoting amyloidosis likely due to this correlating with an increase in bacterial replication and glucose metabolism (Taglialegna et al. 2016). Bacterial biofilms are becoming of increasing interest as the larger the structure correlating to an increase in bacterial replication the more resistant the bacterial

infection can be both to the host immune response and antibiotic treatment (Amorena et al. 1999; Monzón et al. 2002). Interestingly, Bap amyloidogenesis does not appear to be as highly controlled by chaperone machinery as the curli proteins or functional amyloids in other bacteria. Instead, a more simplified mechanism appears to take place. At a more neutral pH, Bap is anchored into the cell membrane and processed releasing the N-terminal into the extracellular environment. As pH drops and becomes more acidic, coinciding with bacteria replication, this released N-terminal fragment transitions to a more amyloidogenic state (Taglialegna et al. 2016). This means there is still an element of regulation, as amyloid formation will only take place in a suitably acidic environment caused through bacterial replication and metabolism. As Bap proteins are stable at more neutral pHs this also means oligomer formation will not occur unless this acidic environment is present, and rate of amyloidosis is high meaning there is likely little time for oligomer concentration rising to a point where it could become harmful to the bacteria.

As mentioned above, bacterial amyloid has been found to be able to cross seed SAA amyloid formation (Lundmark et al. 2005; Johan et al. 1998). This can lead to an increased risk of developing AA. Inflammation and AA may also act as a risk factor for developing, or worsening, AD (Elahy et al. 2015; Cattaneo et al. 2017). CsgA can also nucleate amyloid-β fibril formation (Perov et al. 2018). This raises the possibility that adapting the chaperone properties of bacterial amyloid systems may help in developing therapeutic intervention for amyloid diseases in humans. Further studying the interactions between bacterial and animal amyloids and their cross-seeding capability will help determine the specific process in the amyloidogenic pathway that cross-seeding is affecting.

### 1.5.3 Functional Amyloids in Animals:

While the curli genes and proteins in bacteria are currently arguably the best understood of functional amyloids observed in nature, there are increasing observations of functional

amyloid in animals. In non-mammals the functional amyloids appear primarily to be structural components for other products or complexes. *Chrysopa flava* egg stalk silk was one of the first discovered examples of a naturally occurring β-sheet structure (Weisman et al. 2009). In spiders there are spidroin proteins which are the primary constituent of spider dragline silk. There are two spidroin proteins in spider silk, spidroin-1 and spidroin-2 (Kenney et al. 2002). The resulting silk caused through their amyloidosis has a number of remarkable structural properties. The most widely known of these is having the same strength as steel but far more flexibility which has attracted significant interest for potential applications as a biopolymer (Zheng and Ling 2019). While they share some similar properties to proteins involved in amyloidosis, spidroin proteins are significantly larger at the amino acid level with an average side of 3,500 amino acids and the vast majority of the sequences consists of repeat domains (Kenney et al. 2002).

Moving away from the structural amyloids, other amyloids have also been shown to be of functional use within the CNS. *Aplysia californica* is a species of sea slug with a neuronal isoform of cytoplasmic polyadenylation element binding protein (CPEB) involved in memory formation and capable of forming a self-replicating prion like amyloid (Si et al. 2010). In mammals, CPEB3 is closest homolog to that found in sea slugs and contains a similar glutamine rich prion-like domain (Pham et al., 2014; Fioriti et al. 2015; Drisaldi et al. 2015). A *Drosophila* homologue of CPEB3, Orb2, was also found to be involved in learning and memory pathways in fruit flies (Sanguanini and Cattaneo 2018). The non-CNS isoform is an activator or repressor of mRNA depending on its phosphorylation state, these phosphorylation sites are absent in the neuronal isoform (Si et al. 2010). Neuronal CPEB has prion like properties and this prion form appears more active than the non-prion form (Stephan et al. 2015). *A. californica* neuronal CPEB has been found to play roles in learning and memory, When expressed in yeast and mice CPEB3 shows propensity to form both

amyloid fibrils and SDS resistant oligomers and is able to be passed between yeast in a hereditary fashion (Stephan et al. 2015). CPEB3 knockout mice show impaired performance on behavioural studies including in their fear response, novel object recognition tests and reduced performance in the Morris water maze task. CPEB3 formed aggregation of amyloid oligomers in response to both fear response tasks and the Morris water maze and these oligomers were formed due to protein-protein interactions and did not require RNA (Fioriti et al. 2015). The formation of these oligomers and the contribution of CPEB3 to mice learning and memory was dependent on the presence of the glutamine rich N-terminal prion like domain, as re-introduction of this domain rescued long term potentiation (LTP). This was not seen when protein with this N-terminal domain removed was reintroduced instead. Additionally, when this N-terminal domain was deleted this affected CPEB3 activation in mice and its ability to interact with two of its targets, β-actin and GluR2 (Fioriti et al. 2015).

CPEB3 exists in at least two different states, a soluble non-aggregating state, and an insoluble state capable of forming amyloid fibrils. The base state of CPEB3 is SUMOylated causing it to remain soluble and not prone to aggregation (Drisaldi et al. 2015). SUMOylation may be a process designed to prevent aggregation of certain proteins as the aggregation of both α-Syn and the Huntingtin protein associated with Huntington's disease are inhibited when the proteins are SUMOylated (Krumova et al. 2011; Steffan et al. 2004). The way in which CPEB3 carries out these effects is by repressing the translation of mRNA, particularly for the AMPAR subunits GluA1 and GluA2 in its non-aggregated state. Upon de-SUMOYlation and subsequent aggregation it then promotes the translation of AMPAR instead (Fioriti et al. 2015). Activation of CPEB3 is controlled by a non-degrative ubiquitin pathway regulated by the E3 ubiquitin ligase, Neuralized-1, which leads to increased production of CPEB3 and subsequently the GluA1 and GluA2 AMPAR subunits after oligomerisation and amyloid formation (Pavlopoulos et al. 2012; Ford et al. 2019). There does not appear to be a clearly

identified nucleator protein, or evidence to necessarily suggest CPEB3 self nucleates for aggregation. Within the N-terminal prion-like domain of CPEB3 it was identified there were three important domains, two aggregation prone domains and a regulatory domain which interacts with the actin cytoskeleton (Stephan et al. 2015). Upon de-SUMOylation CPEB3 was shown to bind to F-actin in dendritic spines where the local concentration of CPEB3 significantly increases and aggregation into oligomers and larger fibrils begins (Gu et al. 2020).

Removal of the N-terminal region of CPEB3 affects its ability to activate and carry out its biological function (Stephan et al. 2015) which would suggest that CPEB3 is acting as a genuine functional amyloid in a prion-like fashion. After learning events such as the Morris water maze levels of aggregated CPEB3 fall. However when a similar learning event then occurs afterwards subsequent aggregation seems faster than after the first instance (Fioriti et al. 2015). This could be due to levels of aggregated CPEB3 falling to below currently detectable levels but there is still some present to act as a seed for future aggregation events. It is possible this gives credence to the theory that functional amyloids are an evolutionarily ancient form of storing information, which subsequently became largely obsolete due to globular protein structures allowing for a more diverse range of functions and efficiency (Otzen and Riek 2019)**.**

### 1.5.4 Functional Amyloids in Humans:

The final part of this section will cover functional amyloids in humans, in particularly the pre-melanosome protein PMEL and its protein product, often referred to as PMEL17, and the RIP1/3 necrosis pathway.

PMEL17 begins as a protein of 668 amino acids (McGlinchey et al. 2009) and like most amyloidogenic proteins undergoes post translational modifications, particularly glycosylation, and subsequent proteolytic processing to be broken up into smaller fragments

914 (Dean and Lee 2020; Berson et al. 2001). It is these fragments, produced once PMEL17 is
915 localised to a lysosome related organelle called the melanosome, which can form non-
916 pathogenic amyloid fibrils (Hurbain et al. 2008; Dean and Lee 2020). As with the other
917 functional amyloids, proteolytic processing and production of PMEL17 is tightly regulated to
918 ensure overproduction of amyloid fibrils does not take place with amyloids only forming at a
919 more acidic pH, and becoming soluble at neutral pH (McGlinchey et al. 2009) reminiscent of
920 Bap amyloids. Several isoforms of PMEL17 can be produced within the melanosomes.
921 Relevant to amyloid formation are the short repeat domain isoform (SPRT) and long repeat
922 domain isoform (LPRT), distinguished by either having seven repeat domains (short) or ten
923 repeat domains (long) (Dean and Lee 2020). These repeat domains are not exact – they have
924 slightly different amino acid contents. The melanosomes provide an acidic environment in
925 which amyloid fibrils of SPRT and LPRT can form. Interestingly, if fibrils of either isoform
926 are exposed to cytosolic conditions and therefore a more neutral pH they rapidly dissolve and
927 are unable to cause any toxicity (McGlinchey et al. 2009; Dean and Lee 2020; McGlinchey
928 and Lee 2018). Mutations in the PMEL gene which lead to pigmentary glaucoma can disrupt
929 the formation of amyloid fibrils (Lahola-Chomiak et al. 2019) suggesting the amyloid form is
930 less toxic than the alternative non-amyloid conformations. The LRPT is the more abundant
931 isoform, and similar to bacterial curli amyloids recent work has shown that the SRPT may act
932 primarily as a nucleator to kickstart LRPT amyloid formation (Dean and Lee 2020). That this
933 nucleation mechanism appears conserved between bacterial and human functional amyloid
934 suggests this may have developed as a further safeguard in which to control functional
935 amyloid formation to prevent rapid overproduction and subsequent cellular toxicity. Once
936 produced the PMEL amyloid fibrils act to aid melanin storage in the melanocyte and protect
937 the skin and eyes against UV exposure from the sun and consequent damage from ROS. It
938 performs this function by creating a scaffold within the melanosome and binds to both mature

939 melanin and melanin's cytotoxic intermediates involved in melanin production, and also
940 accelerates melanin synthesis (Hurbain et al. 2008).

941 PMEL17 acts as a structural amyloid. Its production occurs across multiple stages linked with
942 melanosome development and the subsequent production of melanin. Melanin precursors are
943 highly toxic to the cell (Hurbain et al. 2008). During and after the production of melanin
944 PMEL17 amyloid fibrils act as a scaffold to sequester both the intermediates and the final
945 product to cause the pigmentary effect and prevent cellular toxicity (Joanne F. Berson et al.
946 2003). Melanosome development is split up into four different stages, PMEL17 fibril
947 formation occurs in the first two stages and melanin production in the final two stages when
948 the melanosome is more mature (Joanne F. Berson et al. 2003; Hurbain et al. 2008). PMEL17
949 undergoes several posttranslational modification events. First both N- and O-linked
950 oligosaccharides are added before transport into intraluminal vesicles and subsequent
951 proteolytic cleavage (Hurbain et al. 2008; Valencia et al. 2007).

952 In stage I melanosomes PMEL17 is cleaved into a membrane subunit, called Mβ, and a large
953 luminal domain peptide called Mα (Dean and Lee 2020; McGlinchey and Lee 2018). These
954 Mα and Mβ are still connected by di-sulphide bonds before further cleavage through BACE2
955 releases the Mα alpha fragment which is then further processed through unknown
956 mechanisms into smaller fibrils which can begin to form amyloid (Rochin et al. 2013;
957 Shimshek et al. 2016; McGlinchey and Lee 2018). The Mα fragment can be further
958 subdivided into different domains: An N-terminal domain consisting of approximately the
959 first 200 amino acids of the fragment, a polycystic kidney disease-like (PKD) domain
960 comprising the next 90 amino acids, and a repeat domain region (RPT) of approximately 130
961 amino acids which consists of imperfect amino acid repeat regions (Hoashi et al. 2006). The
962 RPT and PKD regions are the primary regions required for PMEL17 amyloid fibril formation

963 as identified by their presence in detergent insoluble fractions (Hoashi et al. 2006;
964 McGlinchey and Lee 2017; Watt et al. 2009).

965 The pH of early stage I and stage II melanosomes are approximately pH 4 and as they mature,
966 increases to pH 6 in stage IV melanosomes. As previously mentioned PMEL17 fibrils require
967 an acidic environment to form, and dissolve as pH becomes more neutral (McGlinchey and
968 Lee 2017). In addition to participating in melanin synthesis in melanosomes once the melanin
969 has been produced and binds with the amyloid fibril scaffold this then likely stabilises the
970 amyloid at higher pH, preventing their dissolution and allowing for melanin pigmentation in
971 the relevant cells (McGlinchey and Lee 2018).

972 PMEL17 has become a compelling model for further understanding functional amyloids and
973 how this may aid efforts in treating and elucidating pathogenic amyloidosis and even prion
974 diseases. It is currently the most well studied functional amyloid in humans and shares
975 similarities both with functional amyloids in other organisms and pathogenic amyloids, in
976 particularly amyloid-β in AD. How shorter PMEL fibrils nucleate the amyloid formation of
977 longer fibrils is reminiscent of the functional amyloids mentioned above, particularly the
978 Curli amyloids in bacteria and spidroin amyloids in spider silk. Distinct from Curli and
979 spidroin amyloids however is that PMEL is self-nucleating, with the small nucleator fragment
980 being a result of PMEL post-translational processing (Dean and Lee 2020). This is similar the
981 method of action of BRICHOS domains which are outlined below. Processing of the
982 PMEL17 protein into the Mα and Mβ fragments is similar to that of the processing of
983 amyloid precursor protein (APP) by β- and γ-secretases to produce $A\beta_{1-40}$ and $A\beta_{1-42}$ (Rochin
984 et al. 2013). While the Mβ PMEL17 fragment is not what forms PMEL amyloid fibrils, it is
985 also further processed by γ-secretase reminiscent of what is seen in APP processing, which is
986 first processed by BACE1 and then γ-secretase (Rochin et al. 2013; De Strooper, et al., 2010).

Other functional amyloid proteins so far identified in humans include the RIP1 and RIP3 proteins and are involved in the regulation of programmed necrosis, an alternative cell death pathway to apoptosis which most commonly seems associated with viral infection (Jixi Li et al. 2012; Guo et al. 2015). RIP1 is a regulator of cell fate, able to control a cytokine-directed response resulting in cell death through either apoptosis or necrosis. Alternatively, it can control signals which instead result in cell division and differentiation through NF-κB transcription factor activation (Walczak 2011). Ordinarily RIP related apoptotic signalling occurs due to interactions between RIP1, Fas-associated death domain protein (FADD) and caspase 8. This complex results in activated caspase 8 inactivating RIP1 and RIP3 and apoptosis (He et al. 2009; Wang et al., 2008). If caspases are inhibited, then RIP1 and RIP3 can form the necrosome initiating programmed necrosis. Both RIP proteins contain RIP homotypic interaction motifs (RHIM) which allow for interaction between the two and subsequent amyloid fibril formation and programmed necrosis (S. He et al. 2009; Jixi Li et al. 2012; Mompeán et al. 2018). Once caspase signalling is blocked this can lead to interactions between RIP1, RIP3 and TIR-domain-containing adapter inducing interferon-β (TRIF) and DNA-dependent activator of interferon regulatory factors (DAI) (H. Hu et al. 2020). Alongside RIP1 and RIP3, TRIF and DAI are the only other proteins so far identified to contain RHIM domains (Sun and Wang 2014).

Once events leading to programmed necrosis are in motion RIP1 and RIP3 form a functional amyloid signalling complex through interactions of their RHIM domains. These domains when expressed on their own as fragments have even been shown to readily aggregate with no obvious additional stimulation (Jixi Li et al. 2012). When signalling is started in response to cytokines such as the tumour necrosis factor (TNF), RIP1 acts as the starting actor, binding to RIP3 through their RHIM domains. Upon other responses both TRIF and DAI can also activate RIP1 and RIP3 through the RHIM domains (H. Hu et al. 2020). Unlike through its

pro-apoptotic or pro-cell differentiation pathways to carry out programmed necrosis RIP1, and RIP3, instead act as kinases leading to their autophosphorylation and subsequent aggregation (Cho et al. 2009; S. He et al. 2009), in a manner perhaps similar to that seen in Tau hyperphosphorylation in Alzheimer's disease. This bears similarities with functional amyloids in fungi, where *het* genes are in control of a programmed cell death mechanism, also often as part of a host response to infection (Daskalov et al. 2016).

## 1.6: Proteostasis and Chaperone Proteins

One major difference between pathogenic amyloids in disease and functional amyloids which carry out a normal, physiological function, is the regulation of production of the functional amyloids. Each functional amyloid specified so far except for Bap, whether it is in bacteria or humans, has a highly regulated production pathway to ensure rapid amyloidosis does not occur to overwhelm the cell; but there is much more to be discovered. Often this can involve the use of chaperone proteins to control the rate and location at which amyloidosis occurs. Other safeguards include the proteostatic mechanisms available to the cell to remove misfolded protein and/or creating a suitable environment in which functional amyloidosis can occur. To help control the beginning of amyloid formation the presence of a smaller, nucleator protein is often required to help generate the actual amyloid fibrils. It is likely that when a disease state occurs and pathogenic amyloids begin to accumulate, they will eventually outstrip the ability of a cell's proteostatic capabilities and this, in addition to other toxic properties of amyloids, is a primary perpetrator responsible for eventual cell death in amyloidosis and amyloid related disease. A steady increase in amyloid formation and eventual overwhelming of the cells ability to cope is also a likely factor in the eventual rapid progressive nature of disease. It is thought amyloid formation can occur many years or even decades before symptoms become present (Fagan et al. 2014; Buchhave et al. 2012). Particularly for many prion diseases by the time symptoms are apparent the patient rapidly

deteriorates in a matter of months (Collinge et al. 2006; Garske and Ghani 2010). A combination of build-up of toxic pre-fibrillar oligomers and the deterioration of the body's proteostatic capabilities with age therefore creates a prime environment for disease to take hold. The increase in acceptance of these soluble oligomers as the prime toxic species have led to the suggestion that the amyloid plaques seen in protein misfolding diseases may actually be a defence against these oligomers (Baglioni et al. 2006).

Despite significant efforts to develop treatments for amyloidosis there remain few effective treatment strategies and particularly for CNS-related amyloidosis treatments do not slow disease progression or often ameliorate symptoms. Utilising chaperone proteins may provide a viable addition to developing therapeutics. Proof of concept can be seen by attempts at stabilising Aβ, retaining the α-helical structure and preventing misfolding into a β-sheet rich structure and preventing subsequent oligomerisation (Honcharenko et al. 2019). Here the research surrounding the use of chaperones, both synthetic and those utilised already in functional amyloids, will be discussed in the context of the viability of aiding proteostasis to treat prion and amyloid diseases.

### 1.6.1 Functional Amyloid Chaperone Proteins:

Curli proteins in bacteria remain one of the best understood examples of how functional amyloids are generated and controlled. As previously stated, there are five curli genes including CsgA – the primary constituent of curli amyloid fibrils with CsgB being the nucleator. The chaperone protein is CsgC and once amyloidosis has started CsgC acts to prevents unwanted oligomer formation and fibril aggregation of CsgA which has yet to be secreted from the cell. At the time of writing CsgC has only become established and gathered interest within the last five to six years, gaining prominence after being identified as a highly effective inhibitor of CsgA amyloid formation (Evans et al. 2015). As such, the specific mechanisms behind CsgC regulation of CsgA are still being characterised. Other chaperone

proteins are thought to prevent amyloid formation of proteins through binding to hydrophobic regions on the target protein and thus preventing misfolding. While it is likely it may be a more general issue regarding binding specificity, CsgC may act through a different manner, as it does not recognise the many hydrophobic residues found on Aβ peptides. There does appear to be some cross-reactivity with CsgC however, as it has shown to be highly effective at reducing amyloid formation of α-synuclein, the primary amyloidogenic agent in Parkinson's disease (Evans et al. 2015). This shows that the use of chaperone proteins may help aid in the prevention of oligomer and fibril formation in amyloidosis.

### 1.6.2 The BRICHOS Domain:

Another prevalent example of chaperone proteins is demonstrated by proproteins containing the BRICHOS domain. This was named after the initial proteins it was discovered in: Bri2, chondromodulin-1 and prosurfactant protein C (SP-C) (G. Chen et al. 2017) and has been found in over 300 proteins since . These three BRICHOS proteins, otherwise unrelated, can be responsible for causing several major diseases including dementia and cancer (Sánchez-Pulido et al., 2002) Proproteins with the BRICHOS domain, which is approximately 100 amino acids in size, all have similar regions which can form β-sheet rich amyloid and ordinarily the domain is thought to aid in the proper folding and processing of its parent protein (Willander et al. 2011). There is little similarity between all BRICHOS proteins at the amino acid level. However, they are all predicted to fold into similar secondary structures (Sánchez-Pulido, Devos, and Valencia 2002; Hedlund, Johansson, and Persson 2009). Practically all of the proteins have a β-sheet prone C-terminal region, with the only exception being SP-C, which instead has a transmembrane region with a high valine content and expected to be prone to β-sheet formation (Sáenz et al. 2015).

The first of the BRICHOS proteins, Bri2, is produced in different tissues including the CNS, particularly the hippocampus and cerebellum (Sánchez-Pulido, Devos, and Valencia 2002).

There are two other Bri-proteins, Bri1 and Bri3. Familial British and familial Danish dementia (FBD and FDD respectively) are caused by mutations in Bri2 leading to dementia with clinical symptoms reminiscent of AD (Vidal et al. 1999, 2000). Mutations in Bri2 lead to the release of amyloidogenic peptides referred to as ABri (for FBD) and ADan (for FDD) which will subsequently go on to form soluble oligomers and amyloidogenic fibrils and disease (Marcora et al. 2014). It is still unclear whether it is the amyloidosis of Bri2 or its loss of function in FBD and FDD which results in disease pathogenesis (Tamayev, Giliberto, et al. 2010; Tamayev, Matsuda, et al. 2010). Ordinarily the Bri2 protein is cleaved by furin, a proprotein convertase which is responsible for cleavage of many different proproteins into their active form (Kim et al. 1999, 2002). The products of furin cleavage are the mature Bri2 protein which is integrated into the cell membrane, where its function is still yet to be determined, and a small 23-amino acid peptide referred to as Bri23 (Oskarsson et al. 2018).

In each case, the mutations in the *BRI2* gene lead to an altered stop codon and subsequent extension of the protein (Willander et al. 2011). In FBD the mutation is a substitution in the stop codon. In FDD there is a duplication of 10 amino acids between codons 265 and 266 (Vidal et al. 1999, 2000). In both cases instead of the Bri23 peptide being produced upon furin cleavage, a 34 amino acid fragment is produced (ABri and ADan, respectively) with a high propensity for amyloid formation. The mutations which lead to these extended fragments are not thought to affect the function of the mature Bri23 protein which would suggest that the subsequent dementias may be caused by the oligomerisation and/or the amyloid fibrils formed from the ABri and ADan fragments. Other evidence suggests dementia is caused by loss of Bri2 function (Tamayev, Giliberto, et al. 2010; Tamayev, Matsuda, et al. 2010). As the normal function(s) of Bri23 are not yet fully understood, although a role in neural differentiation has been proposed (Willander et al. 2011), it may be

that ordinarily the Bri23 fragment can further interact with the mature protein and this interaction is lost or altered due to the mutations.

Symptoms of FBD and FDD are very similar to those of AD. There is growing evidence that Bri2 may be involved in the ordinary processing of APP and is able to prevent the accumulation of Aβ in both cell and mice models, possibly due to the influence of the Bri23 peptide (Matsuda et al. 2011; Coomaraswamy et al. 2010; Matsuda et al. 2008; Fotinopoulou et al. 2005; Matsuda et al. 2005). The effects of Bri2 on APP processing also require the furin cleavage of Bri2 to occur first, as only the mature protein and Bri23 fragment appear to interact directly with APP, not the inactivated proprotein (Willander et al. 2011). In contrast, the BRICHOS domain from Bri2 requires cleavage by ADAM10 where it is released into the extracellular space (Martin et al. 2008). How Bri2 and Bri23 affect the processing APP is again unclear but is possibly due to inhibiting the cleavage performed on APP by α-, β- and γ-secretase (Knight et al. 2013). Similarly, the Bri3 protein which is almost solely expressed in the brain and also processed by furin has been shown to inhibit α- and β-, though not γ-, secretase cleavage of APP (Matsuda, Matsuda, and D'Adamio 2009). It also inhibits the oligomerisation of $A\beta o_{1-42}$, though in a less efficient manner than Bri2 (Dolfe et al. 2018). The BRICHOS domain of Bri2 can interact with Bri23, though whether this is needed for its interactions with APP is unclear. Unlike Bri2, the BRICHOS domain of Bri3 is not required for inhibition of APP processing (Matsuda, Matsuda, and D'Adamio 2009).

The BRICHOS domain, once cleaved from its parent proprotein, is thought to aid in proper folding of the mature proprotein by acting as an intramolecular chaperone. By stabilising the C-terminal (or transmembrane region in the case of SP-C) β-sheet prone domain of the parent protein it allows the protein to properly fold and be integrated into the target environment, such as the cell membrane, where it remains stable and no longer prone to amyloidosis (Knight et al. 2013). In this manner it is possible that the BRICHOS domain has evolved as a

natural way to chaperone protein folding so as not to overload more general chaperone proteins such as the heat shock protein family, which also show potential to inhibit amyloid aggregation and seeding (Evans, Wisén, and Gestwicki 2006; Arimon et al. 2008). Alternatively, it may be that the domain is a more of a relic, and with the presence of a developed proteostasis architecture within cells is mostly obsolete. That BRICHOS domains show little if any conservation between their respective proteins may suggest they are more strongly targeted towards their parent protein. However the BRICHOS domain of Bri2 has been shown to interact with other amyloidogenic proteins, including Aβ and islet amyloid polypeptide (IAPP), and to prevent oligomerisation and fibrillation (J. Kim et al. 2008; Oskarsson et al. 2018). Recombinant Bri2 BRICHOS domain was able to reduce both fibril elongation and $A\beta_{1\text{-}42}$ secondary nucleation in a drosophila model of AD (Poska et al. 2016). Recombinant Bri3 can also to an extent prevent $A\beta o_{1\text{-}42}$ fibril formation but appears more efficient at preventing non-fibrillar protein aggregation (Poska et al. 2020).

That the BRICHOS domain of Bri2 shows reactivity with other amyloidogenic peptides is promising regarding the development of potential treatments for related diseases caused by protein misfolding and oligomer toxicity. Furthermore, rather than targeting oligomers or amyloid plaques directly to facilitate their dissolution and removal, BRICHOS domains instead interfere with the nucleation events which could prevent oligomer formation from occurring in the first place. In AD the formation of both the toxic $A\beta_{1\text{-}42}$ soluble oligomers and the larger insoluble amyloid fibrils occurs through nucleation reactions, primary nucleation of monomers into oligomers. Once fibril formation has taken place, secondary nucleation events can occur on the fibril surface to further generate smaller oligomers (G. Chen et al. 2017; Cohen et al. 2013). If the secondary nucleation event is the primary generator of toxic soluble oligomers, molecular interference could prove to be an effective strategy for therapeutic development.

### 1.6.3 Conclusion: Chaperone proteins and the BRICHOS domain

Despite promise there are still challenges that remain. The BRICHOS domains of Bri2, Bri3 and SP-C can reduce amyloid-β fibrillisation, but do not stop it entirely. More work needs to be done to establish whether the BRICHOS domains of other proteins may be beneficial. It is not clear whether BRICHOS domains would be beneficial in relation to other amyloidosis and amyloid diseases. Most current studies have looked at amyloid-β, the effectiveness of BRICHOS for CNS misfolding proteins such as $PrP^{C}$, Tau, α-synuclein and others has not been established. Neither has whether BRICHOS domains can help prevent fibrillisation of system amyloid proteins such as transthyretin, immunoglobulin light chain amyloid or $\beta_2$-microglobulin. In the case of CNS amyloid, there is also the issue of whether the BRICHOS domain could be effectively transported across the blood brain barrier (BBB). Bri2 recombinant BRICHOS domain can pass the BBB but the recombinant domain of SP-C cannot. Any future BRICHOS domains for use in CNS amyloidosis and protein misfolding disease will need to be checked for ability to cross the BBB. Targeting BRICHOS domains to improve efficacy toward the amyloidogenic protein may also provide a challenge. Recombinant constructs comprising of the target protein and the selected BRICHOS domain, such as that from Bri2, may help improve specificity.

## 1.7 Concluding Remarks.

Utilising nucleation inhibition in the treatment of amyloidosis and prion diseases may prove particularly effective if given as prophylactic treatment in those known to be at risk, i.e. those with known genetic mutations which will result in disease onset and progression. Effectiveness in patients who are already symptomatic may not be as dramatic, as damage to the organs will already have occurred though blocking nucleation could slow or prevent further progression of disease. Treating amyloid disorders will require an improvement in

diagnostic capabilities to detect disease as early as possible in addition to the development of therapeutics to treat and prevent symptoms.

Many treatment strategies for CNS-related amyloid disease, such as AD, initially targeted the insoluble plaque aggregates and have so far met with no success for reasons detailed above. The apparent dispensability of the genes responsible for the misfolded proteins in the brain, as seen in animal knockout models of $PrP^C$, APP and α-synuclein would suggest that targeting the proteins directly to affect their initial production, and therefore their availability to act as a template to misfold, may be a viable alternative strategy. However, in such protein misfolding neurodegenerative diseases the normal physiological functions of these proteins remain poorly understood, as their apparent dispensable nature from animal knockout studies also means identifying their functions is a difficult process. The nature of developing stable, chronic knockout animal models is inherently biased towards the selection and breeding of animals which survive, and as research continues subtle but consistent phenotypes are being identified. Acute, transient knockdown of these genes can result in much more severe phenotypes particularly in the development of the organism often causing lethality. Targeting the nucleation and misfolding of protein into soluble oligomers, without blocking protein function, is therefore likely the most desirable treatment strategy for protein misfolding diseases such as amyloid related disease and amyloidosis.

Identifying the normal functions of the proteins is also an important step in understanding the subsequent disease pathology. With disease progression and an increasing pool of misfolded protein resulting in a decreasing pool of available physiological protein there is likely also loss of normal protein function which may require its own targeted therapeutics to aid in treating symptoms. Furthermore, in AD, $PrP^C$ has been identified as a receptor for Aβo resulting in cell toxicity. Understanding $PrP^C$ function will better allow for targeting of this interaction to prevent AD toxicity. Part 1 of this thesis will describe a dispensable role for the

zebrafish *PRNP* homologues, *prp1* and *prp2* in the development of zebrafish and cell adhesion processes in the CNS.

## 1.8 Summary of Thesis Goals

The primary goals of this thesis are split between two distinct parts. First: establishing a function for $PrP^{C}$ in the early development of organisms by taking a transcriptomic approach in zebrafish larvae. Second: adapting an ototoxic model of zebrafish to investigate the role of TLR4 in cisplatin mediated ototoxicity and examining the potential for zebrafish Tlr4 as a mediator for metal ion toxicity. The final chapter of this thesis, Chapter 5, will revisit and summarise the importance of the research outlines below in 1.8.1 and 1.8.2. Further future directions and experiments will be discussed to continue to expand upon the work generated in this thesis.

### 1.8.1 Transcriptomic analysis of prion protein mutant zebrafish

The first goal of this thesis is to further build upon and explore work which suggests a role for $PrP^{C}$ in the early development of organisms. The contents of Chapter 2 describe the results of RNA-sequencing on zebrafish lacking *prp1* and *prp2*, the zebrafish homologues of *PRNP*. Further data supporting the conclusions of Chapter 2 can be found in Appendix I.

$PrP^{C}$ has become a focus for research over several decades due to misfolding into $PrP^{Sc}$ and causing neurodegenerative disease. Significant effort has been made to try and establish what the function(s) of $PrP^{C}$ are in a healthy individual. There are many functions which have been proposed for $PrP^{C}$ and there is still ambiguity about which are the 'primary' functions. Indeed, it may be the case that the promiscuous nature of $PrP^{C}$ is due to it acting as a scaffolding protein to support multiple signalling pathways and cell processes (Linden 2017). Animals lacking $PrP^{C}$ have been found or purposefully generated and do not show distinct phenotypes unless put under stress conditions (Fernández-Borges et al. 2015).

Prior to and after the generation of *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ homozygous mutants were the publication of transcriptomic and proteomic approaches in mice detailing a potential role of $PrP^C$ in cell adhesion processes, particularly those during early development (Mohadeseh Mehrabian et al. 2014; Mohadeseh Mehrabian, Ehsani, and Schmitt-Ulms 2014; Khalifé et al. 2011). In addition, results generated previously by the Allison lab and others in zebrafish morphants suggested roles for Prp1 and Prp2 in zebrafish development (Huc-Brandt et al. 2014; Kaiser et al. 2012; Málaga-Trillo et al. 2009). We hypothesised that $PrP^C$ is involved in development through regulating cell adhesion and differentiation processes.

### 1.8.2 Cisplatin and metal ion toxicity through Toll-like receptor 4

The second goal of this thesis began in collaboration with Amit Bhavsar's lab, utilising zebrafish as an animal model of ototoxicity. Cisplatin is a potent chemotherapeutic used to treat a variety of cancers, particularly solid tumours in children (Dasari and Tchounwou 2014). Like other chemotherapeutics cisplatin has several and severe side effects. One side effect is cisplatin induced ototoxicity (CIO) leading to permanent bilateral hearing loss in patients treated with cisplatin. There is currently no treatment method of cisplatin, or co-treatment, available which prevents ototoxicity.

The Bhavsar Lab had identified Toll-like receptor 4 (TLR4) as a binding partner to cisplatin resulting in CIO. The research contain in Chapter 3 focuses on using zebrafish as a model for ototoxicity, using synthetic compounds derived from the TLR4 antagonist TAK-242 to block Trl4ba and Tlr4bb signalling. Morpholinos were also used to knockdown *tlr4ba*, *tlr4bb* or both. We hypothesised that by blocking, or knocking down, zebrafish Tlr4 we would prevent cisplatin induced ototoxicity in zebrafish neuromasts along the posterior lateral line.

Chapter 4 builds upon the model in chapter 3 to investigate signalling through zebrafish Tlr4ba and Tlr4bb. In mammals, TLR4 primarily recognises lipopolysaccharide (LPS) in the bacterial cell wall of gram-negative bacteria. Upon recognition, TLR4 stimulates the innate

immune response to combat bacterial infection. In contrast it is not known what the primary ligand(s) of zebrafish Tlr4ba and Tlr4bb are. The prevention of CIO through synthetic antagonists and morpholino knockdown of *tlr4ba* and *tlr4bb* led us to hypothesise that Trl4ba and Tlr4bb may bind to transition metal ions.

## 1.9 Chapter 1 Tables and Figures:

### Table 1.1:

List of amyloidogenic proteins associated with human disease and their associated pathology both inside and outside the central nervous system. Table adapted from (Chiti and Dobson 2017).

| Protein | Central Nervous System Amyloidosis and Protein Folding Diseases |
|---|---|
| Amyloid-beta Peptide | Alzheimer's disease, hereditary cerebral haemorrhage with amyloidosis |
| Alpha-Synuclein | Parkinson's disease, Dementia with Lewy Bodies |
| Prion Protein | Transmissable Spongiform Encephalopathies |
| Tau-Protein | Tauopathies, Alzheimer's disease |
| Huntingtin | Huntington's disease |
| Abri | Familial British Dementia |
| Adan | Familial Danish Dementia |
| Cystatin C | Icelandic Type Hereditary Cerebral Haemorrhage with Amyloidosis |
| Protein | Systemic Amyloidosis and Protein Folding Diseases |
| Immunoglobulin Light/Heavy Chain Fragments | AL/AH Amyloidosis |
| Serum Amyloid A Protein | AA Amyloidosis |
| Transthyretin | Transthyretin Related Amyloidosis (ATTR) |
| β2-Microglobulin | Dialysis Related Amyloidosis |
| Apolipoprotein Amyloidosis | Amyloidosis caused by ApoAI, ApoAII, ApoAIV, ApoCII and ApoCIII fragments |
| Gelsolin Fragments | Finnish Hereditary Amyloidosis |
| Lysozyme | Hereditary Non-Neuropathic Systemic Amyloidosis |
| Fibrinogen Alpha Chain Fragments | Fibrinogen Amyloidosis |
| Amylin | Type 2 Diabetes |
| Calcitonin | Thyroid Medullary Carcinoma |
| Atrial Natruiretic Factor | Isolated Atrial Amyloidosis |
| Prolactin | Pituitary Prolactinoma |
| Insulin and Enfuvirtide | Injection Localised Amyloidosis |
| Lactadherin/ Medin | Aortic Medial Amyloidosis |
| Lactotransferrin/ Lactoferrin | Gelatinous Drop-Like Corneal Dystrophy |
| Odontogenic Ameloblast-associated Protein | Calcifying Epithelial Odontogenic Tumours |
| Pulmonary Surfactant-associated Protein C | Pulmonary Alveolar Proteinosis |
| Leukocyte Cell-derived Chemotaxin 2 | Renal LECT2 Amyloidosis |
| Galectin-7 | Lichen and Macular Amyloidosis |
| Corneodesmosin | Hypotrichosis Simplex of the Scalp |
| TGFBI/Keratoepithelin Fragments | Lattice Corneal Dystrophy Type I |
| Semenogelin-1 | Seminal Vesicle Amyloidosis |
| Proteins S100A8/A9 | Prostate Cancer |

1268

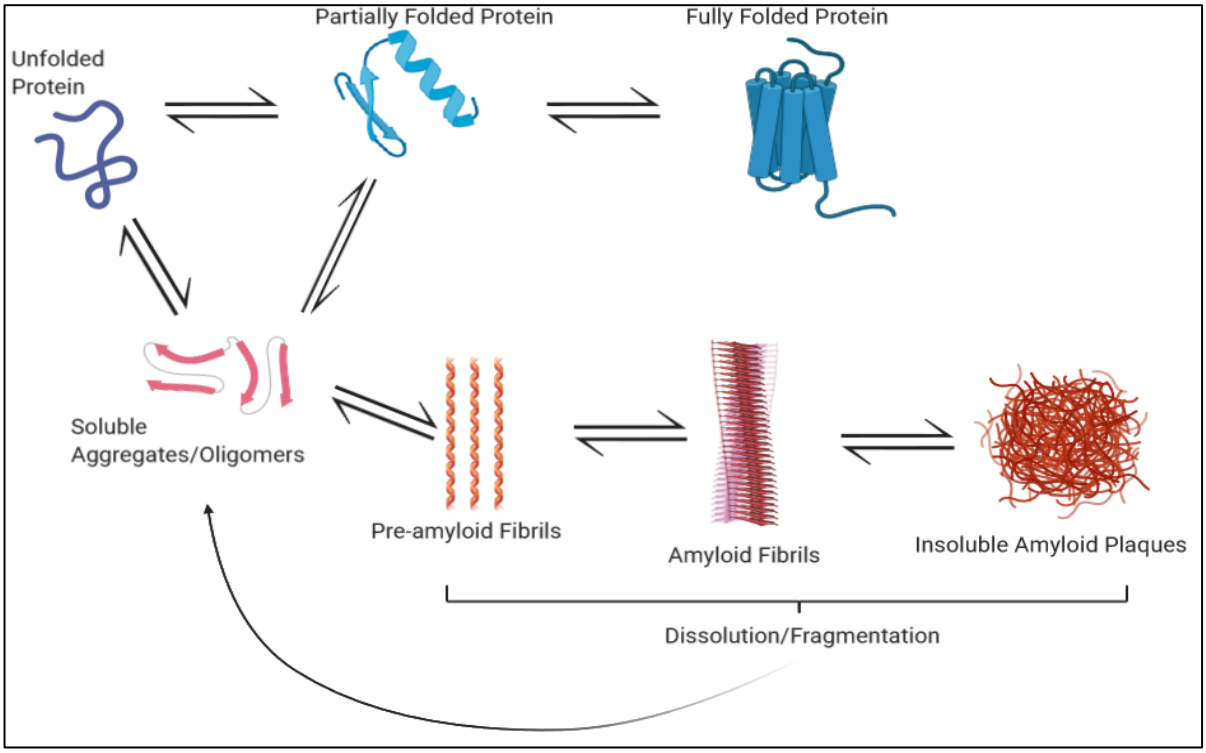


Figure 1.1:
Folding state pathways resulting in either a normally folded protein, or amyloid fibrils and subsequent insoluble amyloid plaques. When normal protein folding goes awry, misfolding can occur leading to the formation of soluble aggregates or oligomers. If the proteostasis system is unable to correct production of misfolded oligomers their concentration can steadily increase forming into pre-amyloid fibrils, which will then form into β-sheet rich amyloid fibrils and finally insoluble amyloid plaques. Longer fibrillar structures can act as a scaffold for further oligomer production, and if fragmented or broken through targeted dissolution can increase the surface area for oligomer production to take place resulting in a steady exponential increase in oligomer concentration. Created with BioRender.com.

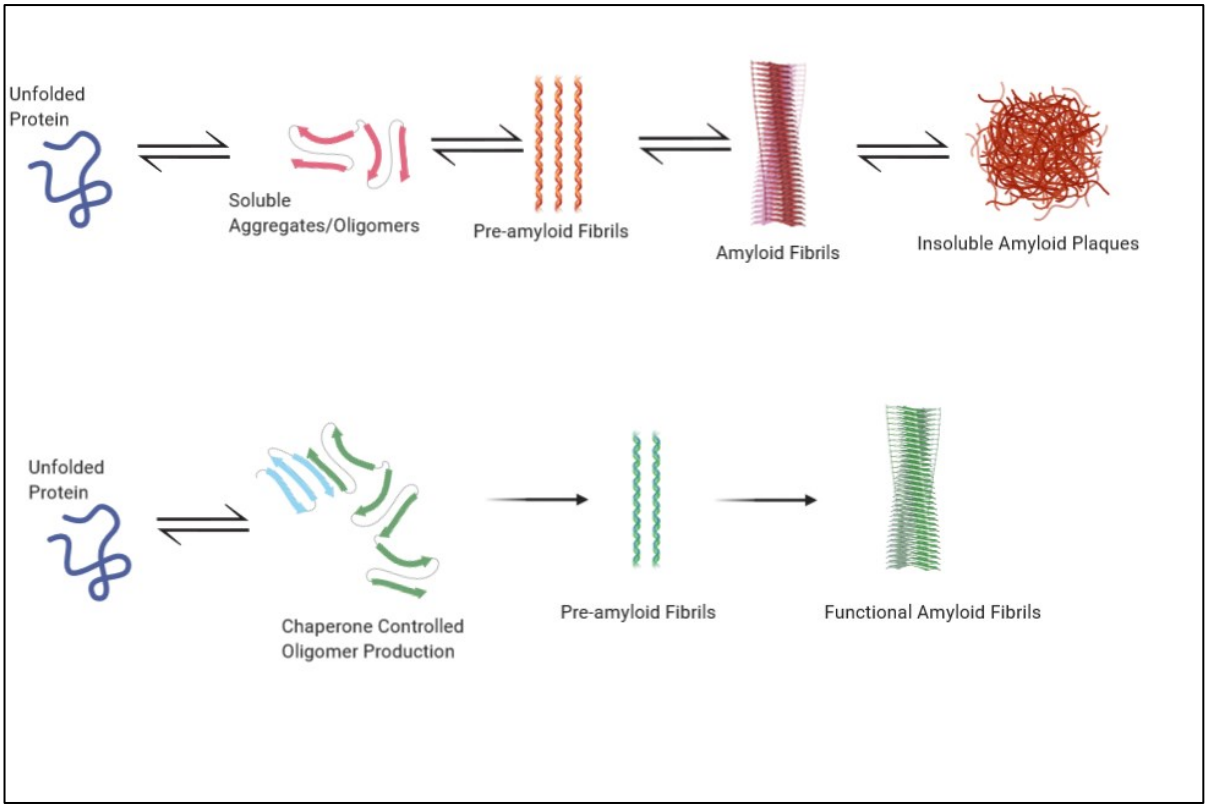


Figure 1.2:
The production of functional amyloids is a heavily controlled process. Pre-fibrillar oligomer production is controlled through a chaperone process, whether a separate nucleator protein or self-chaperoned after post-translational modification of the original sequence, such as with BRICHOS domains. Controlled pre-fibrillar oligomer production regulates oligomer concentration ensuring out-of-control toxic levels are not reached before fibril production resulting in no toxicity in amyloid producing cells. This can be through a variety of mechanisms such as increasing the speed of fibril production or slowing down the construction of pre-fibrillar oligomers. Created with BioRender.com.

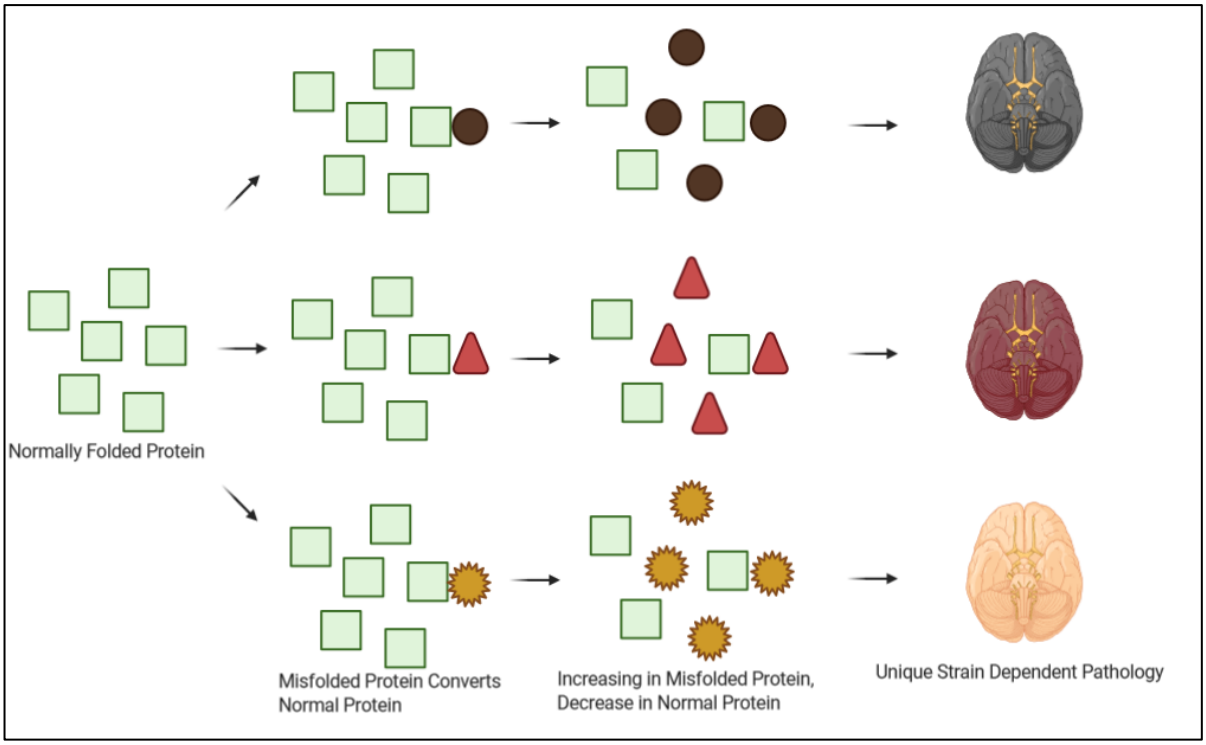


Figure 1.3:

Prion protein scrapie causes misfolding in a template directed manner. The primary amino acid sequence remains the same, however different conformational changes in the misfolded protein (circles, triangles, and suns) can result in different secondary, tertiary, or quaternary structures. These misfolded prions interact with normally folded protein (green squares) causing them to misfold and an ever-increasing pool of misfolded, infectious, and toxic protein and a decreasing pool of normal physiological protein. Created with BioRender.com.

# Chapter 2: Transcriptomic analysis of zebrafish prion protein mutants supports conserved cross-species function of the cellular prion protein:

## Chapter 2 preface:

The following chapter has been prepared as a manuscript and submitted to the journal, *Prion*. At the time of writing, the manuscript has been accepted and is in press. The manuscript was written by NMP with editing contributions from PLA, GN and WTA. Figure contributions: GN contributed RT-qPCR data presented in Figure 2 C & D and Supplementary Figure 1. This chapter is the same as the accepted manuscript, except for minor edits for the clarity of Figure 2.1 and Figure 2.2 for the thesis format.

# Transcriptomic analysis of zebrafish prion protein mutants supports conserved cross-species function of the cellular prion protein

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Key words: Prion knockout, Transcriptome, RNA-Sequencing, Cell adhesion, Scrapie

## Chapter 2 Abstract:

Cellular Prion Protein ($PrP^C$) is a well-studied protein as the substrate for various progressive untreatable neurodegenerative diseases. Normal functions of $PrP^C$ are poorly understood, though recent proteomic and transcriptomic approaches have begun to reveal common themes. We use our compound *prp1* and *prp2* knockout mutant zebrafish at three days post fertilisation to take a transcriptomic approach to investigating potentially conserved $PrP^C$ functions during development. Gene ontology analysis shows the biological processes with the largest changes in gene expression include redox processing, transport and cell adhesion. Within these categories several different gene families were prevalent including the solute carrier proteins, cytochrome p450 enzymes and protocadherins. Continuing from previous studies identifying cell adhesion as an important function of $PrP^C$ we found that in addition to the protocadherins there was a significant reduction in transcript abundance of both *ncam1a* and *st8sia2*. These two genes are involved in early development of vertebrates. The alterations in cell adhesion transcripts were consistent with past findings in zebrafish and mouse prion protein mutants; however E-cadherin processing after prion protein knockdown failed to reveal any differences compared with wild-type in either our double *prp1/prp2* mutant fish or after *prp1* morpholino knockdown. Our data supports a cross species conserved role for $PrP^C$ in the development and maintenance of the central nervous system, particularly by regulating various and important cell adhesion processes.

## 2.1 Introduction:

The cellular prion protein ($PrP^C$) is a well-conserved protein across mammals and to a lesser extent across other vertebrates. It has fascinated researchers since its identification as the cause of a variety of neurodegenerative disorders including: Creutzfeldt Jakob disease (CJD) in humans, scrapie in sheep, chronic wasting disease (CWD) in cervids and bovine spongiform encephalopathy (BSE) in cattle via a conformational change in $PrP^C$ to become scrapie prion protein, or $PrP^{Sc}$ (Kovács et al. 2002; S. Prusiner 1982). Interest is often focussed on the infectious capabilities of the $PrP^{Sc}$ conformation to spread disease, including across species, dubbed 'the protein only hypothesis'(S. Prusiner 1982). In addition to its ability to misfold into $PrP^{Sc}$, normally folded $PrP^C$ has been implicated in the pathology of Alzheimer's disease by acting as a receptor for soluble amyloid-beta oligomers(Um et al. 2012; Laurén et al. 2009; Kostylev et al. 2015; Larson et al. 2012; Özcan et al. 2020a). Despite being subjected to such a large amount of scrutiny, the actual normal physiological functions of $PrP^C$ are not well understood, nor how these functions may be affected under disease conditions(Leighton and Ted Allison 2016). Here we perform transcriptomic analysis on wild-type vs mutant zebrafish, which lack both *prp1* and *prp2* gene products to identify potential functions of $PrP^C$ during early development.

Zebrafish possess two prion protein genes homologous to mammalian *PRNP*, *prp1* and *prp2,* due to a whole genome duplication which occurred in the teleost lineage (John H. Postlethwait et al. 2000; J. H. Postlethwait et al. 1998). While both *prp1* and *prp2* are larger than their mammalian counterpart and therefore share little similarity at the amino acid level, all predicted functional domains of $PrP^C$ are present in both including: an N-terminal signal peptide, a repetitive region, a central hydrophobic domain, a disulphide bridge, two N-linked glycosylation sites and a GPI anchor for attachment to the cell membrane(Cotto et al. 2005)

**(Figure 1)**. This conservation of PrP across evolutionary time indicates that this protein has ancient and important physiological functions.

Transmission of prion diseases to fish by crossing the species barrier has been previously investigated. The difference in size of mammalian to fish PrPs and lower conservation means the chance of transmissibility between species is low but the conserved domains may suggest it is not impossible. Studies have shown that sea bream fed with either scrapie or BSE contaminated brain homogenate results in signs of neurodegeneration and deposits in the brain which reacted to antibodies against sea bream PrP. These deposits developed faster in fish challenged with BSE prions and did not occur in those fed with non-contaminated brain homogenate(Salta et al. 2009). While deposits and histological signs of neurodegeneration were observed there were no clinical symptoms of prion disease and passaging the disease onto additional animals was not reported. Additional *in vitro* studies using mouse cell culture demonstrated that three different fish PrP proteins, including zebrafish Prp1 and Prp2, did not increase the formation of proteinase K resistant prion conversion (Salta et al. 2014). While this supports that it is unlikely fish Prps can misfold into pathogenic species after exposure to mammalian prions the various nature of different $PrP^{Sc}$ strains means it still remains a possibility.

There have been many proposed functions for $PrP^{C}$ including cell adhesion, learning and memory, maintaining circadian rhythm, aspects of the immune response, synaptic function, neuroprotection and more(Castle and Gill 2017; Wulf, Senatore, and Aguzzi 2017). Determining which of these is a direct function of $PrP^{C}$ has proved difficult since animal knockout studies have not shown any obvious overt phenotype in both mice and zebrafish models(Steele, Lindquist, and Aguzzi 2007; Leighton et al. 2018). This is in stark contrast to what can be seen after acute knockdown of $PrP^{C}$, such as morpholino knockdown of *prp1* in zebrafish leading to a lethal phenotype during gastrulation(Málaga-Trillo et al. 2009). This

phenotype is particularly interesting due to a similar phenotype occurring after knockdown of certain ZIP proteins, from which $PrP^C$ may be phylogenetically linked(Schmitt-Ulms et al. 2009). Discrepancies between chronic stable knockout of $PrP^C$ and acute knockdown may suggest robust compensatory mechanisms in mutants allowing for their survival. The lack of overt phenotypes after *Prnp* gene knockout is surprising as $PrP^C$ is evolutionarily well conserved which would suggest an essential function; yet there is little evidence for any particular gene(s) which may be involved in functional redundancy.

Studies in zebrafish, from our own lab and others, support a conserved role of zebrafish prion proteins in cell adhesion(Málaga-Trillo et al. 2009; Huc-Brandt et al. 2014). In addition, proteomic analysis in cell culture has revealed a robust role for mammalian $PrP^C$ during epithelial-mesenchymal transition, a cell adhesion event during development, through affecting NCAM1 polysialylation via ST8SIA2 production(Mohadeseh Mehrabian et al. 2015; M. Mehrabian, Hildebrandt, and Schmitt-Ulms 2016). These studies suggest it plays an important role in the early development of vertebrates and possibly subsequently acts to maintain areas in which it is expressed. Therefore, we have carried out RNA-sequencing analysis in zebrafish larvae to further investigate a role of $PrP^C$ during development.

## 2.2 Results:

### 2.2.1 Compound homozygous $prp1^{ua5003;ua5003}$; $prp2^{ua5001;ua5001}$ knockout mutant exhibited transcriptomic changes:

Wild-type and *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ homozygous compound mutant zebrafish larvae underwent RNA-sequencing analysis. Prion compound mutant fish have engineered small deletion mutations near the beginning of the coding sequence leading to frameshifts in each gene, premature stop codons causing truncated proteins and predicted loss of function(Fleisch et al. 2013; Leighton et al. 2018) **(Figure 1).** Three pools of 50 3dpf wild-type AB fish and *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ compound homozygous mutant fish were

collected and sent to Otogenetics for RNA-sequencing **(Figure 2)**. The age of 3dpf was chosen because it represents a time point, early in development of zebrafish, when the CNS is present, the embryo is available for genetic manipulation and where there is expected to be an overlap in the expression of both *prp1* and *prp2*(Cotto et al. 2005). Using a foldchange cut-off of $\log_2 0.5$ (i.e. there is either 50% more or 50% less transcript abundance) we found a significant change in the transcript abundance of 1249 genes, with 745 showing an increase in transcript abundance and 504 showing a decrease in transcript abundance in compound mutant $prp1^{ua5003/ua5003};prp2^{ua5001/ua5001}$ fish compared to wild-type **(Figure 2A and Table 1)**. We have previously shown a decrease in relative transcript abundance of *prp1* and *prp2* in $prp1^{ua5003/ua5003}; prp2^{ua5001}$ mutants, predicted to be due to nonsense mediated decay of nonsense mRNA(Leighton et al. 2018), and as expected *prp1* and *prp2* were among the top genes showing a decrease in transcript abundance.

RT-qPCR experiments for both *prp1* and *prp2* confirmed a significant reduction in transcript abundance for both genes in our mutants of 79% and 87% respectively **(Figure 2C)**. Initial RT-qPCR of select genes (implicated in eye development) does not strongly support validation of the RNA-sequencing results, this could be due the circadian nature of the expression of those genes, and variability due to the low transcript abundance perhaps being difficult to detect through RT-qPCR, though we have yet to prove either explanation **(Supplementary Figure 1)**. On the other hand, changes in transcript abundance for several other genes were verifiable by RT-qPCR (**Figure *2D*** and described below). For the full results see the published transcriptome **(**GEO accession: GSE164423**)**.

Amongst the ten genes showing the largest increase in transcript abundance in mutants compared to wild-type, five have been linked to proteolytic/hydrolytic processes (*cel.1, ela3l, prss59.1*, *dpp4* and *c6ast4*). While the proteolytic processing of $PrP^C$ itself is becoming increasingly well documented(J. Liang and Kong 2012; Mcdonald et al. 2013; Lewis et al.

2016), its actions in the proteolytic processing of other molecules, whether directly or indirectly is somewhat less appreciated. $PrP^C$ is becoming increasingly associated with cell adhesion(Mohadeseh Mehrabian, Ehsani, and Schmitt-Ulms 2014; Málaga-Trillo et al. 2009; Rousset, Leturque, and Thenet 2016), proliferation(Richardson et al. 2015; Prodromidou et al. 2014) and signalling and it is possible it acts in complexes that process other proteins and molecules as part of these events.

Among the ten genes with the biggest decrease in transcript abundance there does not appear to be a consistent biological process linking them. The gene with the biggest reduction in relative transcript abundance is growth hormone releasing hormone (*ghrh*). Like in mammals Ghrh causes increases in the release of growth hormone during development, particularly in the central nervous system and gut. Secretion of Ghrh is controlled in a circadian manner and has antagonistic effects to somatostatin, with Ghrh promoting short wave sleep while somatostatin promotes deeper REM sleep(Steiger et al. 1992). Growth hormone levels decrease with age and have been suggested to be involved in the ageing process related to a decrease in physiological functions controlled by the hypothalamus(K. Kim and Choe 2019). Interestingly both somatostatin 1 and somatostatin receptor 5 show a significant increase in transcript abundance in prion mutants (60% and 65% respectively). Through recent collaborations we have shown a disruption in the sleep/wake cycle of *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ mutant zebrafish after exposure to amyloid-beta oligomers(Özcan et al. 2020a).

### 2.2.2 Gene ontology analysis of biological processes affected in prp1 and prp2 mutant zebrafish:

The most populous Biological Process categories of genes altered in zebrafish prion mutants are reported in **Figure 3.** Amongst the processes exhibiting a significant increase in transcript abundance, the oxidation/reduction category is represented most often with genes showing a

significant increase in transcript abundance **(Table 2)**. Gene ontology analysis for genes with a significant decrease in transcript abundance again show a similar trend to processes previously linked with $PrP^C$ (Khalifé et al. 2011). **Table 3** shows the most populated biological process categories with a significant decrease in transcript abundance. Cell adhesion is the largest, with the majority of genes belonging to the protocadherin (*pcdh*) family showing a significant reduction in transcript abundance, totalling 31 out of the 38 cell adhesion genes. The *pcdh* genes affected belong to the *pcdh2* alpha and gamma sub clusters. Protocadherins are thought to be particularly involved in the cell adhesion of the early central nervous system(Hayashi and Takeichi 2015), and this reduction in transcript abundance in our mutant fish may in the future help shed light on some of our previous findings suggesting a delay in neural development after prion protein knockdown(Kaiser et al. 2012).

### 2.2.3 Prion protein is involved in cell adhesion processes in early larval development:

Previous work has established a link between $PrP^C$ and cell adhesion. Schmitt-Ulms and colleagues used a proteomic and transcriptomic approach in $PrP^C$ knockout cells, to show a role for $PrP^C$ in the polysialylation of Ncam1(Mohadeseh Mehrabian et al. 2015). In zebrafish, Malaga-Trillo and colleagues found a link between Prp1 and cell adhesion including, though not necessarily limited to, effects on the maturation of E-cadherin during embryogenesis(Málaga-Trillo et al. 2009).

Results from our RNA-sequencing data do not show a significant difference in the transcript abundance of E-cadherin between mutants and wild-type, though this is not surprising if the role of prion protein is in the maturation of the protein (a proteolytic event) and not of the expression of the gene. In zebrafish, *ncam1a* is a homologue of NCAM1 and the Ncam1a protein is also polysialylated by St8sia2(Rieger, Volkmann, and Köster 2008). There is a 30% reduction in the transcript abundance of *ncam1a* and a 33% reduction in the transcript abundance of *st8asia2* in our mutant fish compared to wild-type (**Figure 2D)**. We confirmed

this through RT-qPCR, finding a similar reduction in transcript abundance of *ncam1a*, of approximately 30%, and 50% for *st8asia2*.

After establishing these changes in *ncam1a* and *st8sia2* transcript abundance we next looked at whether there were changes in the processing of E-cadherin in our prion mutant fish compared to wild-type. Previous work has established a role of prp1 in regulating E-cadherin processing in zebrafish(Málaga-Trillo et al. 2009), and our lab has previously shown changes in both E-cadherin and β-catenin localisation after morpholino knockdown of *prp2*(Huc-Brandt et al. 2014). Zebrafish embryos for both wild-type, compound mutant fish and *prp1* morpholino injected fish were stage-selected for those entering the shield stage of embryogenesis, approximately 6hpf. We were not able to identify any changes to the processing or localisation of E-cadherin either in our $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ mutant fish or wild-type fish injected with 5ng *prp1* morpholino (**Figure 4A-C)**. We kept morpholino injected fish and control injected to fish to see if the morpholino was influencing the fish as they developed. We did not see a significant increase in the number of embryos perishing after 1dpf between the morpholino and control injected embryos (data not shown). By 3dpf morpholino injected fish showed clear signs of necrosis and developmental abnormalities compared to the control injected and un-injected control (**Figure 4D-F**). These results would suggest that morpholino knockdown of *prp1* was causing an effect compared to the control injected fish. Why this effect is different compared to what has been previously published is not immediately clear. Morpholinos have come under increased scrutiny due to differences seen in morphants compared to mutants, however this could be due to acute knockdown of genes having more impact than stable, chronic knockout(Rossi et al. 2015; Place and Smith 2017). We previously discussed at length potential explanations for the disparate results during acute knockdown vs. stable mutation of prion proteins(Leighton et al.

2018) and concluded that results from these morpholino reagents should be interpreted with caution.

### 2.2.4 KEGG analysis shows decreased transcript abundance in focal adhesion and actin cytoskeleton regulation pathways:

The most affected pathway in prion mutants is metabolism, exhibiting both an increase and decrease in relative transcript abundance, however due to the sheer size of this KEGG pathway there was little consistency in processes affected, therefore we focussed our attention on the next most populous pathways.

KEGG analysis shows the two most populated pathways with genes having a decrease in transcript abundance are the focal adhesion kinase (FAK) pathway and actin cytoskeleton regulation pathway. There are two FAK homologues in zebrafish, *ptk2ab* (*fak1a*) and *ptk2aa* (*fak1b*). While neither show a significant change in transcript abundance in our zebrafish mutants, the FAK pathway does show several genes with a significant reduction in transcript abundance in close proximity to the FAK genes in the pathway. Genes with direct interactions with FAK showing a significant decrease in transcript abundance include members of the calpain, actinin, talin and integrin families **(Figure 5)**. There is a significant reduction in transcript abundance in *capn2l*, *tln2a*, *actn3b* and *bcar1*. All of these have been heavily linked with regulation of the actin cytoskeleton, affecting cell mobility, division and differentiation(Camacho Leal et al. 2018; Gupta et al. 2012; Thomas-Jinu et al. 2017; Wu et al. 2015; Lepage and Bruce 2008). There is considerable overlap between genes affected in the FAK pathway and the regulation of the actin cytoskeleton pathway:*raf1b, actn3b, bcar1, capn2l, itga9, pak6b, pik3r2, rac1b* show a significant decrease in transcript abundance in both.

Taken alongside the large number of protocadherin family members also showing a reduction in transcript abundance **(Table 3)**, as well as *ncam1a* and *st8sia2* this suggests that *prp1* and

*prp2* help regulate the processes of cell adhesion and differentiation during early development.

## 2.3 Discussion:

### 2.3.1 Conserved roles of $PrP^C$ across species:

Despite numerous animal knockout models there is yet to be a clear and obvious phenotype attributed to the loss of $PrP^C$. This could be due to the age at which the animals were being observed, with evidence both from our lab and others that $PrP^C$ may be important in the early development of organisms. Acute transient knockdown of Shadoo ('shadow of prion protein') in $PrP^C$ knockout mice led to embryonic lethality(Young et al. 2009), though this effect was not seen in a combined knockout model of Shadoo and $PrP^C$ (Daude et al. 2012). Morpholino knockdown of *prp1* in zebrafish led to arrest during gastrulation attributed to deficits in cell adhesion(Málaga-Trillo et al. 2009; Sempou et al. 2016) and our own analysis of *prp1* and *prp2* in zebrafish suggests further, if non-essential, roles in early development(Leighton et al. 2018; Huc-Brandt et al. 2014; Kaiser et al. 2012). This does not account for the relatively diverse and high expression levels of prion protein after development and throughout adulthood suggesting its function may be pleotropic. In the current study we focus on changes to the transcriptome of zebrafish larvae in our $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ mutant fish during early development and identify changes in the transcript abundance of several gene families and related biological processes and further focus on cell adhesion.

Previous transcriptomic and proteomic approaches to investigate changes after the loss of $PrP^C$ in mice or mammalian cells have found changes in a consistent set of biological processes including: cell adhesion, apoptosis, proteolysis, protection against ROS, the immune system and aspects of the cell cycle(Khalifé et al. 2011; Mohadeseh Mehrabian et al. 2014, 2016; Mohadeseh Mehrabian, Ehsani, and Schmitt-Ulms 2014). Here, our own transcriptomic approach and comparison of the biological process gene ontologies finds a

similar group of processes affected. It is worth noting that we did not see great similarity at the individual gene level with that of other studies. This is likely due to the age of the animals in question. Our zebrafish were 3 days post fertilisation (dpf) and would have undergone gastrulation. Similar studies have used either younger zebrafish morphants(Nourizadeh-Lillabadi et al. 2010), or E6.7 and E7.5 mice(Khalifé et al. 2011) which would not have begun or completed gastrulation. This difference in the relative ages and developmental stages, as well as a different species, may account for this lack of gene expression similarity. We also used whole zebrafish larvae, as opposed to specifically the brain or cell culture, as we were interested in a role of $PrP^{C}$ across development of the entire organism. There was still a large overlap in the categories of biological process affected overall.

### 2.3.2 Prp1 and prp2 regulation of cell adhesion genes during development:

One of the more dramatic phenotypes involving prion protein is the gastrulation arrest reported by some scientists in early zebrafish embryos caused by morpholino knockdown of *prp1* leading to disruption of the localisation of E-cadherin(Málaga-Trillo et al. 2009), however we have been unable to replicate this ourselves **(Figure 4).** This may be due to a difference in concentration of morpholino. We have previously shown that higher morpholino doses still cause phenotypes in our *prp1* mutant zebrafish, which suggests that the morpholinos have non-specific effects. As such we elected to use lower morpholino doses which did not result in phenotypes in our mutants (Leighton et al. 2018). As the gastrulation arrest associated with E-cadherin had robust controls demonstrating rescue of the phenotype those results are unlikely to be due to off-target effects at the concentration used (Málaga-Trillo et al. 2009). As previous work has also described the effects of $PrP^{C}$ on cell adhesion, particularly the polysialylation of NCAM1 by ST8SIA2 as a requirement for cells to undergo epithelial to mesenchymal transition(Mohadeseh Mehrabian et al. 2015) and through direct interaction with NCAM1 for neuronal differentiation(Prodromidou et al. 2014), we

investigated whether there were similar changes in expression of the zebrafish *ncam1a* and *st8sia2* and further identified significant decreases in transcript abundance of protocadherins. Transcript abundance of *ncam1a* was significantly reduced in our prion mutants, as was the transcript abundance of *st8sia2*.

Cell adhesion is the largest gene ontology category with a significant decrease in transcript abundance in our mutants. There are 38 genes associated with the cell adhesion process affected at the chosen $\log_2$ fold change cut-off, 31 of which belong to the protocadherin family. Protocadherins are the largest subfamily of cadherin cell adhesion molecules and are primarily expressed within the central nervous system where they are important for its early and continued development(Hayashi and Takeichi 2015). Outside of the chosen fold change cut-off used in the gene ontology analysis are further protocadherins, including members of the *pcdh1* alpha and gamma clusters, and two non-clustered delta protocadherins, *pcdh19* and *pcdh10b*. All of these show a reduction in transcript abundance in our *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ compound mutants compared to wild-type.

The age of the zebrafish used for RNA-sequencing was determined by our previous work on *prp1* morphants and *prp2* mutants while trying to capture a time where both genes are expected to be expressed (Kaiser et al. 2012; Leighton et al. 2018). Combined with the *prp1* morpholino data in **Figure 4**, these results may suggest a role of *prp1* and *prp2* in the expression and regulation of protocadherins and other cell adhesion genes such as *ncam1a* in development of the CNS. Furthermore, genes affected in the FAK pathway would suggest that these processes may be affected through controlling the migration and differentiation of cells which would also support the gastrulation phenotype seen by others (Málaga-Trillo et al. 2009).

### 2.3.3 Prion protein mutant fish show decrease in focal adhesion and actin regulation transcript abundance:

Aside from the metabolism KEGG pathway, KEGG analysis shows that the two most affected pathways with a decrease in gene transcript abundance are the focal adhesion kinase pathway and the regulation of actin cytoskeleton pathway. There are 11 genes affected in the FAK pathway and 13 genes affected in the regulation of actin cytoskeleton pathway; between the two there is an overlap of 7 genes. Combined this suggests the involvement of prion protein in not only cell adhesion processes but also processes which regulate cell motility and differentiation. In addition to cell motility the FAK pathway is heavily involved in angiogenesis(Zhao and Guan 2011) which previous transcriptomic studies have shown to be a biological process affected in developing $PrP^{C}$ knockout mice(Khalifé et al. 2011).

### 2.3.4 Neuroprotection and roles in immune function:

Further, of particular interest is the decreased relative transcript abundance of *pcdh19*, a non-clustered protocadherin which has been shown to be one of the highest genetic risk factors relating to epilepsy(Cooper, Jontes, and Sotomayor 2016). Mice lacking $PrP^{C}$ have been shown to be at an increased risk of seizures(Carulla et al. 2015) and we have also shown this in our *prp1*$^{-/-}$ and *prp2*$^{-/-}$ knockout zebrafish(Kanyo et al. 2020; Leighton et al. 2018). This adds to the increasing amount of data showing $PrP^{C}$ plays a neuroprotective role in vertebrates; this may explain why many phenotypes now becoming apparent occur only after stress is put on the animal.

Finally, Ncam1 has been shown to be expressed in cells involved in the innate immune system including natural killer (NK) cells(Abel et al. 2018), which also express $PrP^{C}$. The expression of $PrP^{C}$ in immune system cells and tissues is an understudied area of research but there is evidence to suggest it is involved in immune quiescence(Bakkebø et al. 2015). This coincides with its higher expression levels in tissues where inflammation could be severely damaging, such as the CNS and testes. Regulation of Ncam1 by $PrP^{C}$ may therefore be a

method in which immune suppression is enacted in these tissues to prevent further damage under stress, however more work is required to properly establish this.

## 2.4 Concluding Remarks:

To conclude, here we present a transcriptome analysis comparing wild-type zebrafish and our *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ mutant zebrafish early in development (3dpf). We find significant changes in transcript abundance of genes in several different biological process gene ontology categories including cell adhesion, proteolysis and oxidation/reduction processes. Importantly, while there is not much overlap at the individual gene level compared to similar studies done in mice our results do overlap considerably at the categorical level. This implies an important, cross-species conserved role of $PrP^C$ in the early development of organisms.

The data support past conclusions that $PrP^C$ participates in cell adhesion pathways. Further, the data implicate a shared role for $PrP^C$ in regulating NCAM1 and its adhesion functions via ST8SIA2; this shared function of $PrP^C$ between mammals and fish is consistent with this being part of an ancient role for $PrP^C$ early in its evolution(Schmitt-Ulms et al. 2009).

## 2.5 Materials and Methods:

### 2.5.1 Animal ethics, zebrafish fish lines and husbandry:

Zebrafish were raised, maintained and bred following Animal Care and Use Committee: Biosciences procedures at the University of Alberta following guidelines set by the Canadian Council of Animal Care. Fish were kept at the University of Alberta fish facility at 28 ℃ under a 14:10 hour light/dark cycle as previously described(Westerfield 2000). The AB strain of zebrafish was used as wild-type (WT) fish as controls for experiments, as well as the background for the *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ compound homozygous mutants which we previously generated in our lab(Fleisch et al. 2013; Leighton et al. 2018).

### 2.5.2 RNA-Sequencing analysis of WT and prp1$^{ua5003/ua5003}$; prp2$^{ua5001/ua5001}$ mutant larvae:

AB WT and *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua500/ua5001}$ fish (ZFIN ID: ZDB-ALT-181113-1 and ZDB-ALT-130724-2) were bred and raised to 3dpf. 50 larvae were taken to form three replicates of each group, WT and mutant, totalling six different samples. Each pool of 50 larvae was homogenised in TRIzol (Invitrogen/ThermoFisher Scientific catalog no. 15596026) with a rotor stator homogeniser (VWR catalog no. 47747-370, Radnor, PA) and shipped to Otogenetics (Atlanta, GA) for Illumina PE100-125 and HiSeq2500 sequencing and DNAnexus Platform standard RNAseq analysis at a depth of greater than 41 million reads. Read alignments and annotation were done using the TopHat and Bowtie pipelines and initial quantification analysis of differential gene expression was done using Cufflinks(Trapnell et al. 2012; Trapnell, Pachter, and Salzberg 2009). Upon receipt of results it was found that two of the three *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001}$samples might have been contaminated with wild-type transcripts. We took a conservative approach and filtered these samples out of the analysis. The integrity of the remaining *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ sample was rigorously screened to ensure it lacked wild-type transcript by assessing SNPs that were consistently present in mutant vs wild-type samples. Further analysis was performed using the R

Programming Language (Version 4.0.0**)** packages CummRbund and ggplot2(R Core Team 2020; Goff, Trapnell, and Kelley 2014; Wickham 2016).

### 2.5.3 RT-qPCR detection of selected genes of interest:

Experiments were performed in compliance with the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments(Bustin et al. 2009)). RNA samples for all genes were extracted from either 3dpf wild-type AB or compound homozygous *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ mutant zebrafish. RNA extraction was done from pools of 15-20 larvae previously stored in RNAlater (Ambion/ThermoFisher Scientific, catalog no. AM7021) and processed using the RNeasy Kit (Qiagen catalogue #74104,Toronto, ON, Canada) following the manufacturers protocol. Homogenisation of larvae was done in RLT buffer with a rotor stator homogeniser as stated above. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was confirmed using an Agilent RNA 6000 NanoChip and Agilent 2100 Bioanalyser for numbers of at least 7/10. cDNA was generated using a qScript Supermix kit (Quanta BioSciences catalogue #95048–100, Beverly, MA, USA) and qPCR carried out as described previously(Leighton et al. 2018). Three technical replicates were used for each biological replicate and transcript abundance was normalised to β-*actin*. Statistical analysis for relative fold change in transcript abundance was done using RQ values. Primers used for the genes were as follows: *prp1* forward: 5'-ATCCGGCACTTATTGAGCAG-3', *prp1* reverse: 5'-CACTTCGGAGATGCTGTGTC-3', *prp2* forward: 5'-CCAACTCTGCAGCTAGTACA-3', *prp2* reverse: 5'-CAGTGTCGCCGTCATTATCA-3', *st8sia2* forward: 5'-GACCAACCATGTCCAGATCAAAC-3', *st8sia2* reverse: 5'-TGGATCTCATCACAAAAGCGAGTA-3', *ncam1a* forward: 5'-GTAGCTGGAAAAAGGCCCCT-3', *ncam1a* reverse: 5'-AACAGTGGCAGCTACCTGTC -3'. All primers were validated before use.

For the RT-qPCR primers used for genes related to eye development see **supplementary Table 1.**

### 2.5.4 Morpholino injections in zebrafish embryos:

An antisense *prp1* morpholino oligonucleotide (MO) was purchased from Gene Tools, LLC (Philomath, OR) and has been previously described by us and others(Kaiser et al. 2012; Málaga-Trillo et al. 2009) (ZFIN ID: ZDB-MRPHLNO-100423-6), a standard negative control morpholino was also acquired and used in experiments (5'-CCTCTTACCTCAGTTACAATTTATA-3'). Injection solutions consisted of 1.0µl KCl, 1.0µl 0.25% dextran red, MO specific volume resulting in a 5ng/µl concentration for *prp1*-MO or 2.5ng/µl for the standard MO and the volume finalised to 10µl with nuclease free water. Embryos identified to be at the 1-2 cell stage were mounted on an agarose plate and injected with 1nl of injection solution with the volume previously calibrated using an ocular micrometer, injecting into mineral oil. Larvae at 3dpf were imaged using a Leica M164 dissecting microscope with a Leica DFC 400 camera.

### 2.5.5 E-Cadherin immunohistochemistry:

Embryos identified at the shield stage of development (approximately 6hpf) were manually dechorionated and fixed in 4% paraformaldehyde and processed for antibody staining. An anti-mouse E-cadherin antibody (BD Biosciences, 610181) at a 1:5000 dilution was used, and embryos were imaged using a Zeiss LSM 700 scanning confocal microscope and Zen 2010 software (Carl Zeiss Imaging). Images were analysed with ImageJ.

### 2.5.6 Gene ontology, KEGG pathway and statistical analysis:

Genes identified to have either a $\log_2$ fold change of 0.5 or greater (increase in transcript abundance) or -0.5 or lower (decrease in transcript abundance) were selected for gene ontology and KEGG pathway analysis using DAVID version 6.8(D. W. Huang, Sherman, and Lempicki 2009b, 2009a). Additional statistical analysis and visualisation was carried out using the tidyverse group of R packages(Wickham et al. 2019) and Microsoft Excel.

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**Declaration of interest statement:** The authors have no competing interests.

## 2.8 Chapter 2 tables and figures

### Table 2.1:

Total number of genes with either a significant increase or decrease in transcript abundance between wild-type and *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ homozygous mutant fish with a $\log_2$ fold change equal to or greater than 0.5.

| | |
|---|---|
| **Total number of genes with a log2 fold change of 0.5 or greater** | **1249** |
| **Increase in transcript abundance** | **745 (60%)** |
| **Top 10** | **cel.1, zp3a.2, zgc:173443, c3a.3, c6ast4, dpp4, paqr3b, prss59.1, pde6h, ela3l** |
| **Decrease in transcript abundance** | **504 (40%)** |
| **Top 10** | **ghrh, zgc:194878, capn2l, mhc1lia, krtcap2, col28a1a, irx4b, prnprs3, si:dkeyp-94g1.1, zgc:112966** |

Table 2.2:

Most populated Biological Process gene ontologies for genes with a $\log_2$ fold change of 0.5 or greater.

| Category | Genes | Number of genes |
|---|---|---|
| Oxidation Reduction Process | hmgcra, haao, hpda, foxred2, nsdhl, aldh1l1, aldh7a1, aox6, cyp1a, cyp2aa1, cyp2aa4, cyp2aa6, cyp2ad2, cyp2ad3, cyp2k18, cyp2k16, cyp2n13, cyp2p8, cyp2p9, cyp2x7, cyp2x9, cyp2k19, cyp24a1, cyp27a1.4, cyp46a1.1, cyp51, cyp7a1, cyp8b1, cyb5r2, dio1, ero1a, fads2, gcdhb, gmpr2, hsd3b7, kmo, ldhbb, msmo1, pipox, pcyox1, pdha1b, rpe65a, rdh8b, sdr42e1, si:dkey-180p18.9, si:dkey-91i10.3, sqlea, srd5a2a, sc5d, sod1, tbxas1, tm7sf2, tdo2a, tyr, uox, zgc:101765, zgc:110783, zgc:136333, zgc:66484, zgc:77938 | 60 |
| Transport | atp5j, abca4a, rhcgb, snf8, ap1m2, apoda.2, apodb, aqp8a.1, aqp8a.2, aqp9b, chmp1a, ero1a, fabp10a, fabp2, fads2, gabra6a, gabrr2a, gabrz, gc, hbbe2, hbz, mb, kcnc1b, kcnf1a, ptgdsb.1, ptgdsb.2, p2rx2, p2rx4a, rlbp1b, rbp2a, rbp2b, rbp4l, snupn, slc1a7b, slc15a1b, slc20a1a, slc25a10, slc25a3a, slc5a1, slc5a11, slc51a, slc52a3, slc6a14, slc6a11a, slc6a13, slc6a19b, slco1d1, spns3, ttpa, tfa, trpv6, zgc:153704, mmp9, metap2a, nln, prep, si:dkey-269i1.4, tll1, tinagl1, usp20, zgc:112285, zgc:174153, zgc:174855 | 52 |
| Metabolic Process | agpat2, hmgcs1, hoga1, aclya, ugt1ab, ugt1a1, ugt1a2, ugt1a4, ugt1a5, ugt1a6, ugt1a7, ugt2a1, ugt2a2, ugt2a3, ugt2a4, ugt2b1, ugt2b3, ugt2b5, ugt5b1, ugt5b3, ugt5b4, ugt5d1, ugt8, acaa1, aldh1l1, aldh7a1, alpi.2, alas1, fah, gla, gcdhb, gstm.3, gstm.1, gsto1, mettl7a, pmt, pfkmb, pdha1b, si:ch211-93g23.2, slc27a1b, scp2a, tyr, uck1, zgc:101040, zgc:101540, zgc:101569, zgc:162780, zgc:66313 | 48 |
| Proteolysis | Ihha, lonrf1l, anpepa, anpepb, ace2, cpa4, cpa5, cpb1, cpb2, caspb, ctsba, ctsl.1, ctsla, ctrl, ctrb1, cfd, ela2l, ela2, ela3l, furinb, enpep, irbp, pcsk1, prss59.1, prss59.2, prss60.2, si:dkey-194e6.1, c6ast4, try, zgc:100868, zgc:112160, zgc:112302, zgc:136872, zgc:85932, zgc:92041, zgc:92480 | 36 |
| Visual Perception | abca4a, grk7a, irbp, opn1lw2, opn1mw1, opn1sw1, opn1sw2, prph2a, prph2b, pde6h, rgra, rom1b, rpe65a, rlbp1b, rho, zgc:73359 | 16 |

Table 2.3:

Most populated Biological Process gene ontologies for genes with a $\log_2$ fold change of -0.5 or less.

| Category | Genes | Number of genes |
|---|---|---|
| **Cell Adhesion** | **cdh5, cntn2, cyr61l1, itga9, tln2a, tinagl1, si:ch211-66e2.3, pcdh2aa1, pcdh2aa15, pcdh2aa3, pcdh2ab1, pcdh2ab10, pcdh2ab11, pcdh2ab12, pcdh2ab3, pcdh2ab5, pcdh2ab6, pcdh2ab7, pcdh2ab8, pcdh2ab9, pcdh2ab2, pcdh2ac, pcdh2g1, pcdh2g10, pcdh2g12, pcdh2g13, pcdh2g16, pcdh2g17, pcdh2g2, pcdh2g28, pcdh2g29, pcdh2g3, pcdh2g4, pcdh2g5, pcdh2g6, pcdh2g7, pcdh2g8, pcdh2g9** | **38** |
| **Proteolysis** | **agtpbp1, cflara, ank2b, atg4c, capn12, capn2l, capn7, capn8, casp2, casp3b, casp6, casp6l1, ctsd, ctslb, f2rl1.2, f9b, f7i, he1b, mmp30, mmp9, metap2a, nln, prep, si:dkey-269i1.4, tll1, tinagl1, usp20, zgc:112285, zgc:174153, zgc:174855** | **30** |
| **Oxidation Reduction Process** | **dhcr7, dao.1, dao.2, sh3pxd2aa, aldh18a1, cyp2aa3, cyp2aa9, gpd1l, hmox1a, loxl1, loxl2b, mdh1ab, ogdha, p4ha2, p4ha1a, p4ha1b, ptgis, ptgr1, pyroxd2, pycr1b, si:dkey-239i20.4, suox, txnl1, ywhae2, cyp2aa2** | **25** |
| **Regulation of Apoptosis** | **bag6l, cflara, dnaja3a, casp2, gdf11, mcl1b, pmaip1, prnprs3, ptgis** | **9** |

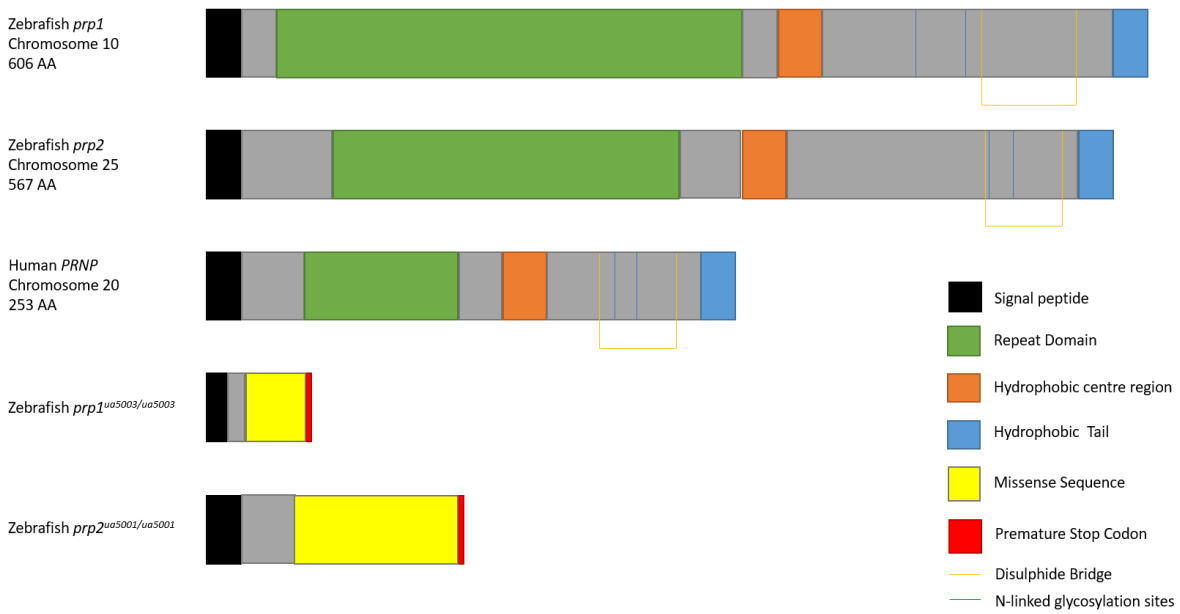
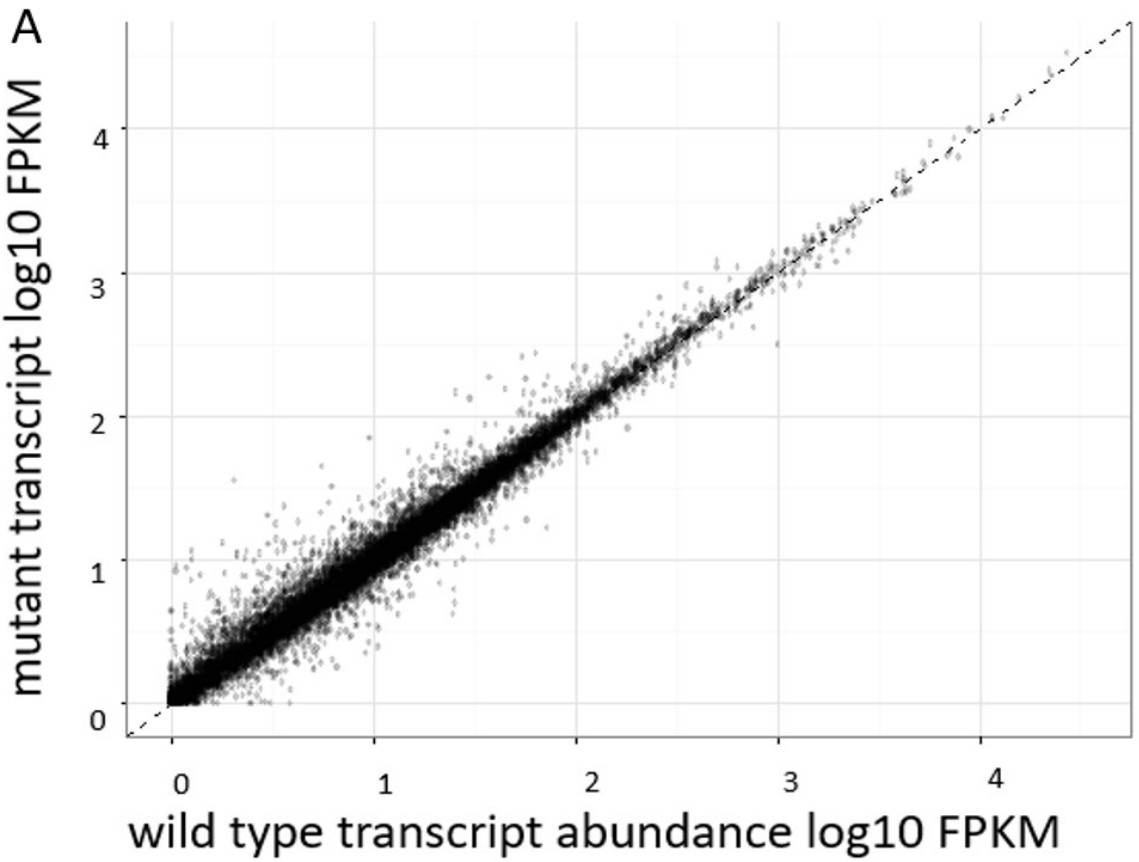
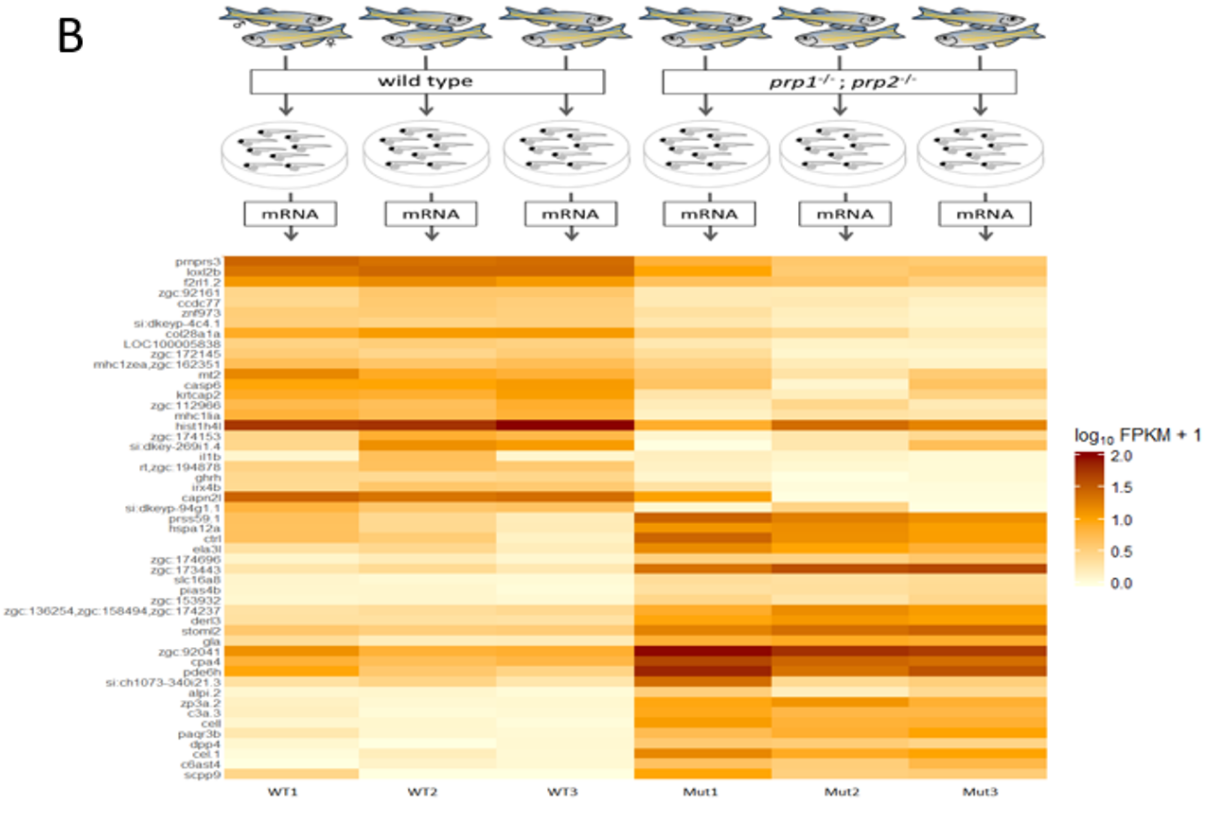



Figure 2.1: Prion proteins of zebrafish Prp1, Prp2, human $PrP^{C}$ and $Prp1^{ua5003/ua5003}$ and $Prp2^{ua5001/ua5001}$ mutant proteins. Zebrafish prion protein genes are larger however have the same conserved domains as human *PRNP*: the signal peptide (black), the repeat domain (green), hydrophobic center region (orange), hydrophobic tail (blue), N-linked glycosylation sites (blue lines) and di-sulphide bridge (orange lines). The $prp1^{ua5003/ua5003}$ and $prp2^{ua5001/ua5001}$ alleles have frameshift deletions near the beginning of the coding exon, leading to a missense sequence of amino acids (yellow), pre-mature stop codons (red) and a shortened, nonsense transcript.


A
mutant transcript log10 FPKM
wild type transcript abundance log10 FPKM
0
1
2
3
4


B
wild type
prp1-/- ; prp2-/-
mRNA
log10 FPKM + 1
2.0
1.5
1.0
0.5
0.0
WT1
WT2
WT3
Mut1
Mut2
Mut3

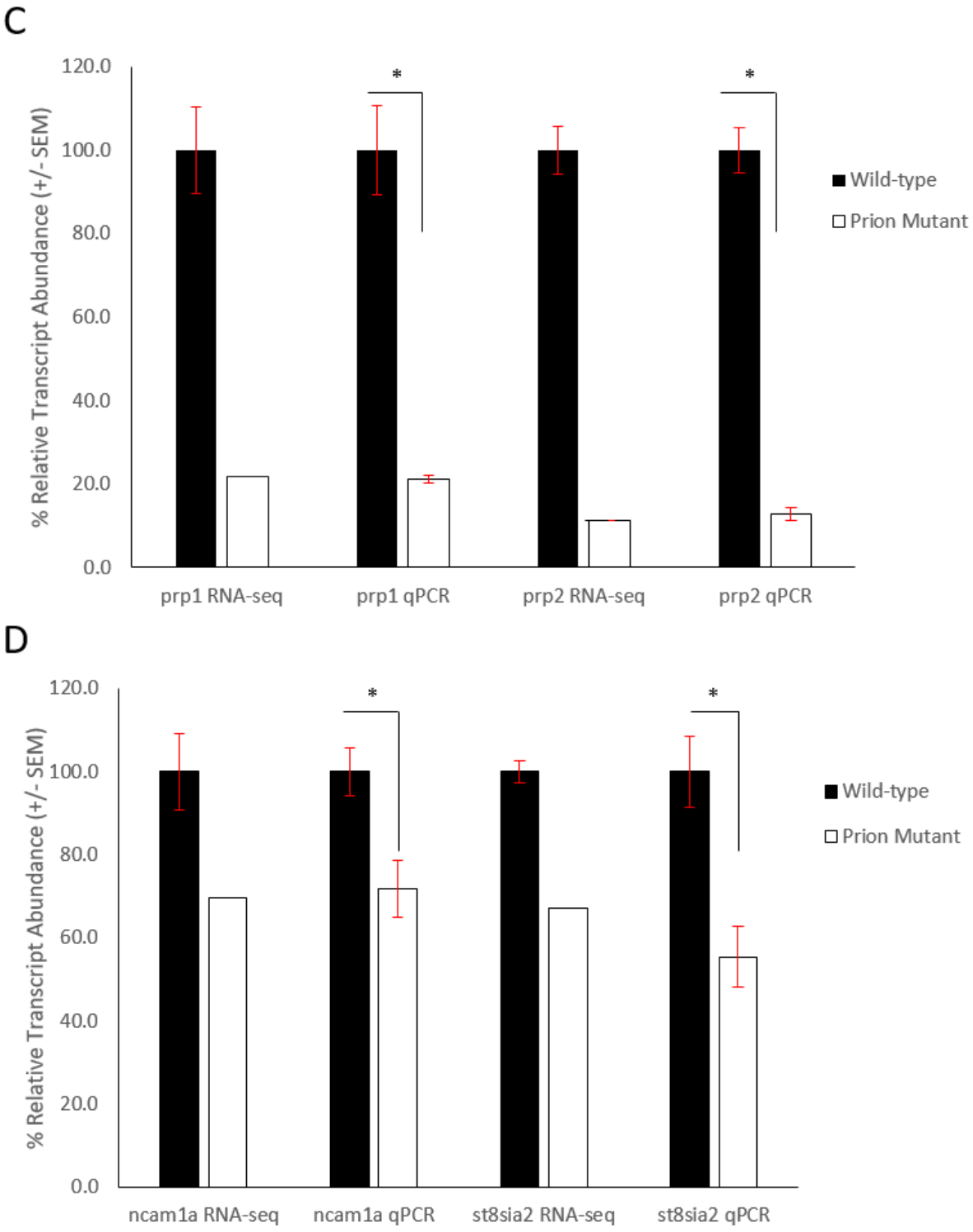



Figure 2.2: RNA-Sequencing show 1249 genes with an increase or decrease of $\log_2$ fold change of 0.5 between wild-type and compound homozygous prion mutant zebrafish larvae. A) Scatter graph showing relative FPKM values for wild-type (X-axis) and mutant (Y-axis) genes after RNA-sequencing. B) Methodology diagram showing RNA-sequencing workflow. Two groups, wild-type and prion mutant (*prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$) homozygous fish,

each with three replicates containing a pool of 50 3dpf larvae were processed and sent for RNA-sequencing. Heatmap displays sample of the 25 genes with a biggest differential abundance in FPKM in wild-types compared to mutants. C) Relative transcript abundance between wild-type and prion mutant fish comparing *prp1* and *prp2* through both RNA-sequencing and RT-qPCR analysis. D) Relative transcript abundance of *ncam1a* and *st8sia2* through both RNA-sequencing and RT-qPCR analysis. * = $P < 0.05$

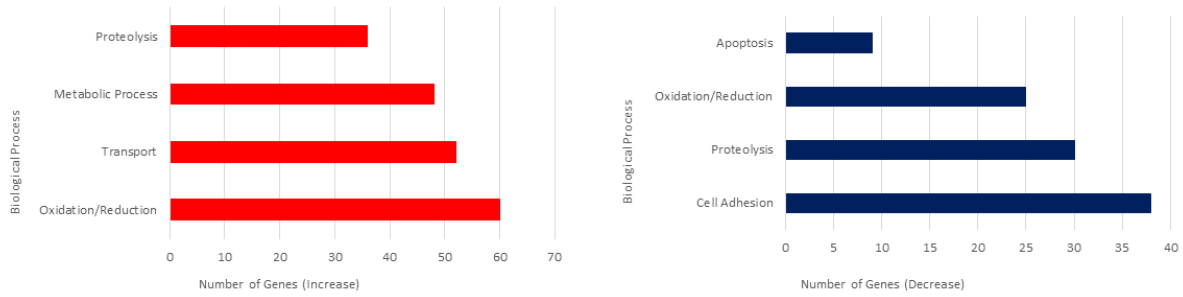



Figure 2.3: Biological Process Gene Ontologies most affected in 3dpf prion mutant (*prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$) zebrafish compared to wild type. Biological processes showing genes with the greatest increase in transcript abundance are shown on the left (red), and genes with the biggest decrease in transcript abundance are shown on the right (blue).

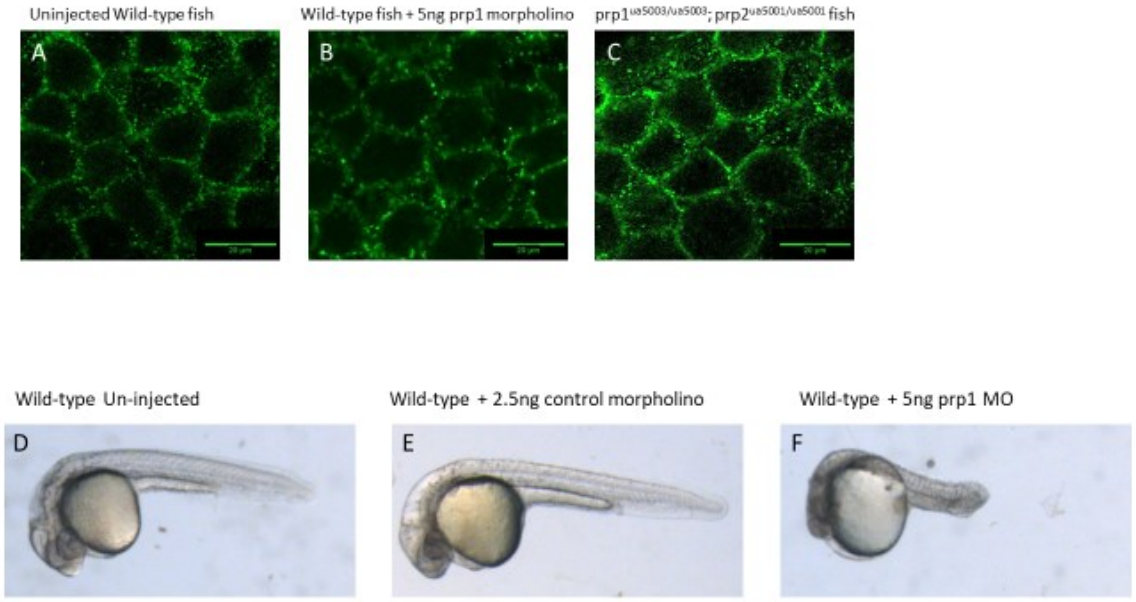



Figure 2.4: A- C) There does not appear to be a difference in the maturation or localisation of e-cadherin in either 5ng *prp1* morpholino injected AB zebrafish or un-injected *prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$ homozygous mutant fish compared to un-injected wild-type controls. F- E) After 3dpf wild-type larvae injected with *prp1* MO (F) show significant signs of necrosis and developmental abnormalities compared to un-injected and control injected larvae (D, E).

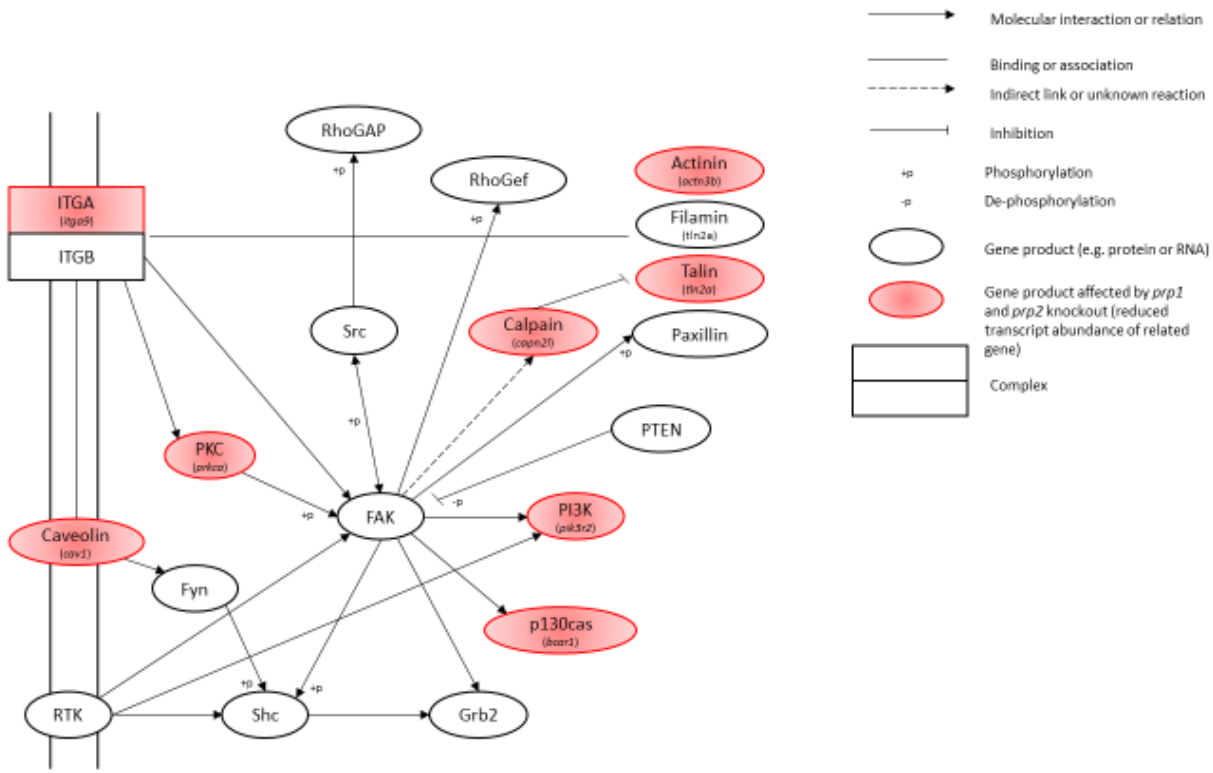
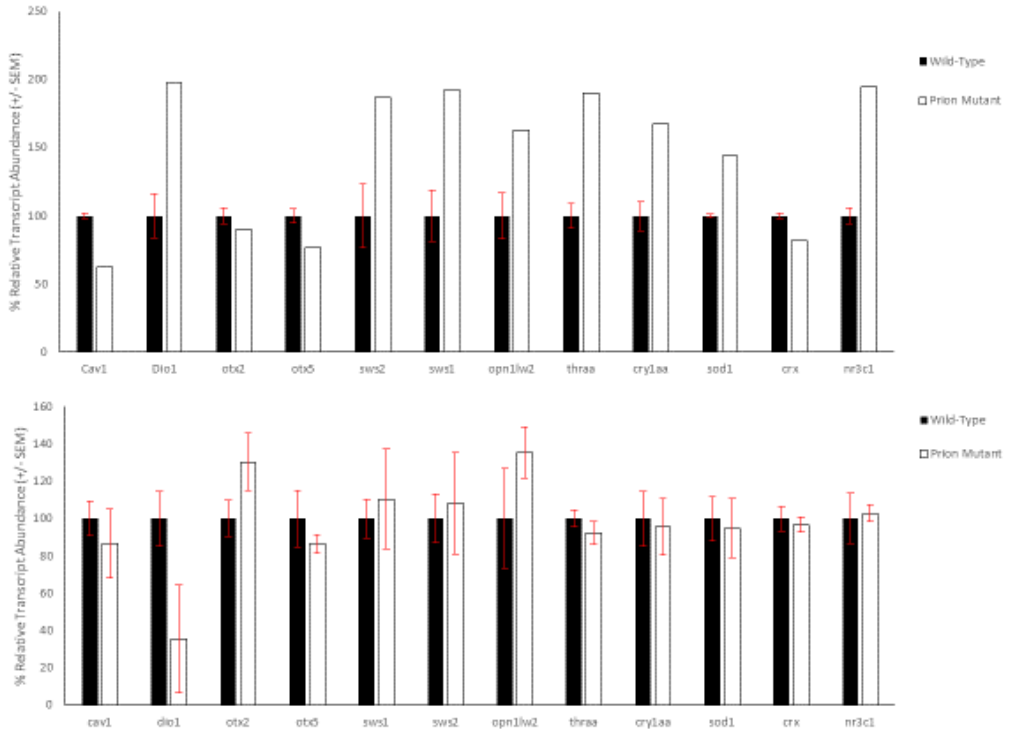



Figure 2.5: Snapshot of the Focal Adhesion Kinase KEGG pathway. Gene products highlighted in red show those with a significant decrease in transcript abundance, with the specific gene italicised underneath.

## Supplementary Table 2.1:

RT-qPCR primer list for genes in supplementary figure 2.1.

| Gene name | Forward Primer 5'-3' | Reverse Primer 5'-3' |
|---|---|---|
| *cav1* | TCA ACC GAG ACC CAA AGC AT | CGA AGC TGT AGG TGC CGG |
| *dio1* | GGA TAT CAG CGT GCA CAA AAA C | CAG GGC ATG GAG GGT CTT |
| *otx2* | TCG AAA CTG TGA TCT GTT GTA ACT GTA | AAT CTA TTA AAA TCA CAG CCG AGT CTT |
| *otx5* | ACA GCG GCG CGA AAG A | GGT ATC GGG TTT TGG AGA ACA G |
| *opn1sw2* | CTA TCT TTG CAA TCT GGG TGG TT | AAA GGC AGG AGG GAA TGG TT |
| *opn1sw1* | TCC TCC CGC AGC ACA TTT AC | AAA GTT ACG GGA TTT GAA CAA TCA G |
| *opn1lw2* | CAA GAG CGC CAC CAT CTA CA | ACC TTC TTT CCA AAG AGC TGC |
| *thraa* | CTG AAA GGC TGC TGT ATG GAG AT | TCT CTC CGC TCA GGG TCA GA |
| *cry1aa* | GGC TGC TTG CTT GCA CTA TGT | GGG ACT GAA TAG GTG TAC GAG ACA |
| *sod1* | ACT CTG TCA GGC CAA CAT TCT | ACT TTC CTC ATT GCC ACC CT |
| *crx* | TCT CCT TTA CTT CAG CGG ATT GG | CGC CTC CAC TTG CTG ACA |
| *nr3c1* | AAG CTA CTG GAC TCC ATG CAC | AAA CTC CAC GCT CAG AGA TT |




Supplementary Figure 2.1: Top: RNA-Sequencing of selected genes between wild-type and prion mutant (*prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$) homozygous fish. Bottom: Initial RT-qPCR of selected genes associated between wild-type and prion mutant (*prp1*$^{ua5003/ua5003}$;*prp2*$^{ua5001/ua5001}$) homozygous fish. RT-qPCR results did not validate that seen in RNA-sequencing, likely due to their labile abundance over circadian cycles.

# Chapter 3 – Toll-like receptor 4 mediates cisplatin induced ototoxicity in a zebrafish model.

## Chapter 3 Preface:

This chapter includes content from the following publication: Babolmorad, Ghazal, Asna Latif, Ivan K Domingo, Niall M Pollock, Cole Delyea, Aja M Rieger, W Ted Allison, and Amit P Bhavsar. 2021. "Toll-like Receptor 4 Is Activated by Platinum and Contributes to Cisplatin-Induced Ototoxicity." *EMBO Reports* n/a (n/a): e51280. https://doi.org/https://doi.org/10.15252/embr.202051280. The manuscript was written through contributions of the authors: GB, AL, NMP, AMR, WTA, and APB. Data contained within the Chapter 3 Figures 1 and 6 are included in the manuscript and was collected by NMP. The material and methods are as written in the manuscript and were originally provided by the author and WTA. This thesis chapter was written by NMP with editing contributions from WTA. Contributions to figures: Figure 3C contains data collected by Aaron Fox; Figure 7 contains chemical structures provided by Ghazal Babolmorad, Ismat Luna and Fred West; Table 1 represents *in vitro* cell culture data collected by Asna Latif and Ghazal Babolmorad.

## Chapter 3 Abstract:

Cisplatin is a highly effective chemotherapeutic used to treat many different types of cancer, particularly in children. Five-year survival rates after treatment with cisplatin are as high as 80%. There are several severe side effects associated with cisplatin treatment. Among them is ototoxicity resulting in permanent bilateral hearing loss. The mechanisms of this cisplatin induced ototoxicity are not clear and there is currently no co-treatment available which can prevent it. TLR4 has been identified as a possible mediator in cisplatin ototoxicity and therefore presents a potential target in which to develop co-treatment. TAK-242 is an inhibitor of TLR4, binding to its intracellular region and preventing further signalling pathways. Here, zebrafish are adapted as a model for ototoxicity by utilising neuromasts in the posterior lateral line. Zebrafish are exposed to cisplatin and co-treated with either TAK-242 or synthetic derivatives and neuromast viability is determined through scoring fluorescent intensity of DASPEI staining. Next, cisplatin toxicity is measured after morpholino knockdown of the zebrafish *TLR4* homologues, *tlr4ba* and *tlr4bb*. Certain TAK-242 synthetic derivatives, but not all, and morpholino knockdown all in ameliorate cisplatin induced ototoxicity in zebrafish posterior lateral line neuromasts. Therefore, using zebrafish as an *in vivo* model, TLR4 is identified as a mediator if cisplatin toxicity. Further work is needed to establish the nature of cisplatin and syntagonist binding to TLR4. This would allow further targeted development of effective co-treatments to prevent cisplatin induced ototoxicity and better overall treatment of childhood cancer.

## 3.1 Introduction

Cisplatin, or cis-diamminedichloroplatinum(II) is a highly effective chemotherapeutic treatment. It is most often used to treat solid tumours in children, and is also effective at treating testicular, ovarian, bladder, cervical, lung, head and neck cancer in adults (Dasari and Tchounwou 2014; Prestayko et al. 1979). The five-year survival rate of cisplatin is as high as 80% when used in child patients (A. C. C. Organisation, 2017). Unfortunately, as with most chemotherapeutics, it also has several severe side effects when used for a prolonged period. These side effects include nephrotoxicity, neurotoxicity, nausea, vomiting and ototoxicity. Nephrotoxicity can be reversed and treated through saline hydration and mannitol diuresis. There is no effective co-treatment or counter-treatment to prevent or reduce ototoxicity, limiting the potential use of one of the most effective chemotherapeutics currently available. (Rybak et al. 2009). Ototoxicity will result in permanent, bilateral hearing loss in patients which can be particularly distressing for child patients. Hearing loss can severely impact their development, particularly social development, at pivotal times in their lives. Cisplatin induced ototoxicity (CIO) in children is associated with an increased risk in learning difficulties (Gurney et al. 2009, 2007). Depending on the age of the patient, treatment duration and concentrations used between 26-90% of children display CIO. Risk of CIO increases in dose dependent manner and almost 100% of patients display CIO when given high doses of cisplatin (K. W. Chang and Chinosornvatana 2010; Kopelman et al. 1988). While cisplatin is a highly effective anti-cancer agent and its use will continue, co-treatment is needed to prevent CIO. Small scale short term trials have identified N-acetylcysteine may be an effective co-treatment, but its effectiveness in larger populations over long periods of time needs to be verified (Sarafraz, Ahmadi, and Daneshi 2018).

Mechanisms of cisplatin toxicity involve the formation of DNA crosslinks which inhibit DNA replication. It forms inter and intra-strand guanine crosslinks preventing DNA

separation as well as alkylating DNA bases resulting in miscoding of the DNA (Pinto and Lippard 1985; Eastman 1987). Once crosslinks or DNA alkylation have taken place multiple signalling pathways can occur to arrest the cell cycle and cause apoptosis (Sarin et al. 2017; Sorenson, Barry, and Eastman 1990). This mechanism makes cisplatin particularly effective against tumour cells as it targets cells which rapidly divide. These mechanisms are unlikely to be related to the primary cause of CIO, because the auditory cells impacted are largely senescent and do not divide. In addition, there are not any efficient mechanisms inside the ear to clear cisplatin as it accumulates. Over time build-up of cisplatin in the cochlea and outer ear hair cells increases and cell toxicity follows (Breglio et al. 2017). This has been linked with an increase in ROS production by the cell due to the increase in cisplatin concentration (Özyurt et al. 2006; Rybak, Mukherjea, and Ramkumar 2019; von Stechow et al. 2013).

Toll-Like receptor 4 (TLR4) has been identified in mediating cisplatin toxicity and as a possible binding partner. An increased risk of CIO was identified in child patients who had the 3A* haplotype of the *TPMT* gene, compared to those which had the wild-type haplotype (Ross et al. 2009; Pussegoda et al. 2013). Studies subsequently showed that cisplatin increased *Tlr4* expression in mouse HEI-OC1 cells in both a time and dose dependent manner. This increase in *Tlr4* occurred in cells expressing the 3A* haplotype but not wild-type *TPMT* (Bhavsar et al. 2017). TLR4 signalling through its primary canonical ligand, lipopolysaccharide (LPS) has been shown to increase ototoxicity in mice (Oh et al. 2011). Outside of the ear TLR4 also mediates cisplatin induced renal toxicity in mice. Separate studies demonstrate mice not expressing *Tlr4* having reduced renal disfunction after cisplatin exposure (Cenedeze et al. 2007; Binzhi Zhang et al. 2008).

TLR4 is a transmembrane protein in the pattern recognition receptor family of toll-like proteins. In mammals its primary ligand is LPS, a component of gram-negative bacteria cell walls. It can also recognise certain viral proteins such as *Mycobacterium tuberculosis* heat

shock proteins (Tatematsu et al. 2016; Bulut et al. 2005). Upon binding of a ligand to the extracellular region of TLR4 an intracellular signalling pathway occurs causing the production of NF-κB or inflammatory cytokines and activation of the innate immune system **(Figure 1A)**. The production of cytokines or NF-κB is dependent on whether there is activation of the MyD88 dependent or independent pathway (Kagan et al. 2008). Cisplatin binds to the intracellular region of TLR4 leading to activation of the MyD88 independent pathway and signalling events distinct to what is seen upon LPS activation (Babolmorad et al. 2021). TAK-242 is a small compound TLR4 antagonist, binding to the intracellular region of TLR4 **(Figure 1B)**(Matsunaga et al. 2011). TAK-242 and synthetic derivatives (hereafter referred to as syntagonists) were supplied by the Fred West lab at the University of Alberta. Inhibition of TLR4 signalling may reduce CIO without affecting cisplatins anti-tumour potential.

This chapter will provide a brief introduction to the use of zebrafish as a model for ototoxicity. Next it will present data on prevention of CIO via inhibition of TLR4 through i) TAK242, ii) syntagonists, and iii) morpholino knockdown of *tlr4ba* and *tlr4bb*. This work suggests that CIO is mediated at least in part by TLR4, and its inhibition through syntagonists is a viable strategy to improve the use of cisplatin as a chemotherapeutic by reducing ototoxicity.

### 3.1.1 Zebrafish as a model organism for CIO:

Accumulation of cisplatin in both the cochlea of the inner ear and the outer hair cells of the Organ of Corti leads to permanent bilateral hearing loss. How cisplatin causes this toxicity is unclear. As previously stated, as a chemotherapeutic cisplatin causes DNA cross links resulting in apoptosis in rapidly dividing cells. Hair cells of the inner and outer ear do not readily divide in mammals. This would suggest there are additional mechanisms to CIO, and selectively targeting these mechanisms should not affect its efficacy as a cancer treatment.

Zebrafish have become an established model of hearing loss, offering many complementary advantages in combination with other *in vivo* models such as mice (Esterberg et al. 2013; Eimon and Rubinstein 2009). They are also a full physiologically relevant system compared to *in vitro* cell culture models. When developing therapeutics, pharmacodynamic interactions are a large hurdle in developing a compound which may seem effective *in vitro* but loses effectiveness when translated to *in vivo* systems (Tuntland et al. 2014). While mice provide an established an effective model organism, relative to zebrafish they are not as well suited for high throughput analysis. They can be costly to for large scale studies, both due to maintenance of animals and the space in which they require. Large numbers of animals required for high throughput analysis can therefore become expensive. Zebrafish provide an excellent addition to help make up for these shortcomings. Breeding pairs of zebrafish can provide 100-150 embryos and can be bred on a weekly basis. Application and uptake of chosen compounds is often a simple case of adding them to the water with the zebrafish. Organ development for all major organs is underway from 24hpf and by 5dpf they are established (Drummond and Davidson 2010; Chu and Sadler 2009). This means that organs involved in pharmacokinetics, often a hurdle for drug development, such as the kidney and the liver are both present and active. This allows for rapid screening and testing of compound libraries to establish *in vivo* effectiveness of potential therapeutics.

Thus, in combination with current *in vivo* and *in vitro* systems they are providing a high throughput method of modelling hearing loss, ototoxicity and drug screening with proven applicability to mammals (Chiu et al. 2008; K. Y. Lee et al. 2017; Rossi et al. 2015; Domarecka et al. 2020; Chapela et al. 2019). Key to this is the availability of neuromasts, bundles of hair cells along the posterior and anterior lateral lines (PLL and ALL respectively, **Figure 2**). Neuromasts of the ALL are deposited along the head of the fish, while PLL neuromasts are along the trunk and tail (Ma and Raible 2009; Iwasaki et al. 2020). Deposition

of neuromasts is a well-defined process during zebrafish larval development. Neuromasts of the PLL in particular are easy to track and follow which makes it simple to identify and observe the same neuromasts between different fish (Sarrazin et al. 2010; Chitnis, Dalle Nogare, and Matsuda 2012).

### 3.1.2 Zebrafish posterior lateral line and neuromast development and screening methods:

PLL development begins after approximately 18hpf. A cranial placode appears close to the otic vesicle and divides into a group of cells which become the PLL ganglion and PLL primordium (primI). Between 22hpf and 40hpf primI migrates along the trunk of the zebrafish towards the tail. As it does so it deposits five groups of cells which become the first PLL neuromasts, L1-L5 (Sarrazin et al. 2010; Colombi, Scianna, and Preziosi 2020). Later in development, from 48hpf onwards, other cells deposited by primI between the L1-L5 neuromasts will mature to form intercalary neuromasts and several terminal neuromasts at the tail will also form. These intercalary neuromasts, and additional lines of neuromasts which occur later in larval development, are produced from two other primordia, primII and primD (Colombi, Scianna, and Preziosi 2020; Chitnis, Dalle Nogare, and Matsuda 2012). This developmental process means identifying the same neuromasts between different fish is a highly reproducible and consistent method.

Neuromasts are bundles of support cells and up to 20 hair cells and are involved in rheotaxis, i.e., sensing changes in water pressure. This aids in predator avoidance, hunting prey and social behaviour such as schooling (Todd et al. 2017; Chiu et al. 2008). While there are certain differences, primary of which being external structures exposed to an aquatic environment, neuromast hair cells are homologous to mammalian inner ear hair cells and the physiological mechanisms of neuromast hair cells are similar to that of inner ear cells (Faucherre et al. 2009). Movement of water causes mechanical stimulation of the neuromast

hairs, and movement of stereocilia towards a kinocilium. This mechanical stimulus is then transferred into an electrochemical response from afferent and efferent fibres connected to the hair cell and recognised as an electrical signal (Van Trump and McHenry 2008). After approximately 3dpf neuromasts begin to mature and are capable of mechano-transduction once the innervating neurons have developed.

Zebrafish are an established model to measure the effects of drugs on neuromast toxicity (Esterberg et al. 2013; Eimon and Rubinstein 2009; Niihori et al. 2015). Compounds can be simply added to the water in a well plate with zebrafish and neuromast viability can be assessed after the chosen exposure time. Neuromast hair cells can be easily stained using fluorescent dyes and fluorescent intensity provides a visual method in which to observe toxicity to both hair cells and their supporting cells. DASPEI (2-(4-(dimethylamino)styryl) -N-ethylpyridinium iodide) is a fluorescent dye which stains active mitochondria of cells (S. K. Lee et al. 2015; Uribe et al. 2018). Simple addition of DASPEI to the water of wells after ototoxin exposure allows selective staining of neuromast cells, as they are the only structure exposed for DASPEI to label. Cisplatin has been shown to cause dose dependent ototoxicity and reduction in fluorescent intensity of neuromasts after exposure (Ou, Raible, and Rubel 2007). This reduction in fluorescent intensity is a visual indicator of the health of cells within the neuromast, as lower fluorescence means a reduction in active mitochondria signalling cell death. Concentration of cisplatin affects how quickly ototoxicity takes place, as even at low concentrations will lead to significant reductions in hair cell loss over time.

### 3.1.3 Hypothesis and chapter summary

Based upon the previous work outlined above identifying TLR4 as a mediator of cisplatin ototoxicity and nephrotoxicity we hypothesise that inhibition of TLR4 signalling will reduce CIO in zebrafish neuromast cells. Here, DASPEI staining of neuromasts is used to measure fluorescent intensity in the presence of cisplatin or co-treatment of cisplatin with TAK-242 or

syntagonists. In addition, fluorescent intensity is also measured after cisplatin exposure in *tlr4ba* and/or *tlr4bb* morpholino knockdown zebrafish larvae. As only cells with active mitochondria are stained by DASPEI, reduced fluorescence would suggest a loss of cell viability. Therefore, a drop in fluorescent intensity would indicate an increase in ototoxicity and death of neuromast hair and support cells. A scoring system developed previously in zebrafish and utilised by groups studying ototoxicity using the zebrafish PLL will be adapted to measure the health of 5 neuromasts along the PLL for each fish (Uribe et al. 2018; Van Trump et al. 2010; Harris et al. 2003). A score of 2 shows no drop in fluorescent intensity signalling healthy and active cells. A score of 0 shows a significant drop in fluorescent intensity signalling apoptosis of neuromast cells caused through CIO.

Syntagonists which reduce CIO in zebrafish neuromasts are identified. Furthermore, morpholino knockdown of *tlr4ba* and *tlr4bb* also reduce neuromast ototoxicity. These results support TLR4 as a viable target to reduce CIO.

## 3.2 Results

### 3.2.1 Cisplatin has a dose response relationship with ototoxicity when modelled in zebrafish neuromasts:

To establish a useful dose and duration of cisplatin treatment in our hands, AB zebrafish larvae at 5dpf were exposed to cisplatin in the wells of a 6-well plate for 20 hours. Each well contained five fish, and the concentrations of cisplatin used were: 0, 5, 10, 25 and 50µM diluted in dimethylformamide (DMF). This was done to establish a dose response curve of cisplatin in relation to its ototoxicity. The concentrations chosen were based upon previously published results in literature (Ou, Raible, and Rubel 2007). There is a loss of fluorescent intensity of DASPEI staining in PLL neuromasts in a dose dependent manner **(Figure 2)**. For subsequent experiments, a concentration of 7.5µM was chosen in 5-6dpf fish as this is expected to cause a significant, but not complete, loss of neuromast hair cells **(Figure 2)**. In

the younger *tlr4ba* and *tlr4bb* morphant fish a concentration of 15µM cisplatin was chosen, as higher concentrations were needed to cause CIO in these younger animals.

### 3.2.2 TAK-242 and synthetic derivatives variously increase and reduce CIO:

TAK-242 is a small compound inhibitor of TLR4 by binding to the intracellular region of TLR4 preventing subsequent interaction with adaptor proteins, preventing continuation of its signalling pathway (Matsunaga et al. 2011). Co-exposure of TAK-242 and syntagonists with 7.5µM cisplatin was performed to establish whether expected inhibition of Tlr4ba and Tlr4bb would reduce neuromast CIO. A concentration of 5µM for TAK-242 and was used based upon preliminary *in vitro* results from the Bhavsar lab. Unexpectedly, zebrafish exposed to both 5µM TAK-242 and 7.5µM cisplatin showed complete loss of fluorescent intensity showing an exacerbation of CIO compared to just cisplatin treatment alone **(Figure 3A)**. At a lower concentration of 2.5µM TAK-242 there was still a significant drop in neuromast score compared to neuromasts only exposed to cisplatin **(Figure 3B)**. Syntagonists 120 and 132 also showed a significant drop in neuromast score at 5µM when compared to zebrafish exposed to just cisplatin. Syntagonist 134 however showed a small but significant increase in neuromast score **(Figure 3B)**. Three other syntagonists were also trialled. Syntagonist 166 had a modest but significant improvement in neuromast score; syntagonist 138 did not cause an increase in neuromast score though it did also not cause an increase in CIO **(Figure 3C)**.

Syntagonists 134 and 136 were tested further for prevention of CIO. After co-exposure of 5µM syntagonist 136 with 7.5µM cisplatin there was a significant increase in neuromast score compared to 7.5µM cisplatin alone. Both 5µM syntagonist 134 and 136 were also tested at 15µM cisplatin but there was no significant recovery of neuromast score **(Figure 4A)**. Increasing the syntagonist concentration to 20µM saw a much larger recover of neuromast score for syntagonist 136, however syntagonist 134 did not see an increase in CIO prevention when compared to 5µM **(Figure 4B)**.

Neither TAK-242 or the syntagonists 120 and 132 show any toxicity on their own with no cisplatin co-exposure **(Figure 5)**. Syntagonists were dissolved in DMF which also did not show any increase in toxicity in vehicle only trials. Therefore, any increase in toxicity is unlikely due to the syntagonist itself or the vehicle causing cisplatin-independent toxicity.

### 3.2.3 Morpholino knockdown of *trlr4ba* and *tlr4bb* cause a reduction in CIO in the zebrafish PLL:

Newly fertilised zebrafish embryos were injected with 5ng of either a *tlr4ba* morpholino, *tlr4bb* morpholino or both. The amount of morpholino injected was based on upon their efficacy in previous studies, which also validated their specificity (Sepulcre et al. 2009; M. Y. Chang et al. 2016). One splice blocking *tlr4ba* morpholino was used and two *tlr4bb* morpholinos were used, one splice blocking and one translation blocking (Sepulcre et al. 2009; M. Y. Chang et al. 2016; Q. He et al. 2015). None of the morpholinos used appeared to affect the overall health of the zebrafish, with all successfully injected morphants surviving with no obvious impact on development or increase in lethality. Fish were exposed to cisplatin at 2dpf for 20 hours and imaged at 3dpf. A higher concentration of 15µM cisplatin was used for the younger fish as this was needed to cause sufficient ototoxicity. This is likely due to the more rapid development of neuromasts at this age. Fish injected with *tlr4ba* and combined *tlr4ba*/*tlr4bb* morpholino showed a significant increase in neuromast score compared to un-injected fish **(Figure 6A)**. Combined *tlr4ba* and *tlr4bb* morpholino injected fish showed a trend towards a further increase in neuromast score compared to single *tlr4ba* or *tlr4bb* morphants but this was not significant.

Zebrafish injected with either the splice blocking or translation blocking *tlr4bb* morpholino showed a significant increase in neuromast score compared to un-injected or control morpholino injected zebrafish **(Figure 6B)**. Both morpholinos had a similar efficacy and neither seemed more effective over the other.

## 3.3 Discussion:

Cisplatin is an effective chemotherapeutic drug; however, its use is complicated by severe side effects including permanent bilateral hearing loss. Zebrafish have become a well-established model for ototoxicity. Here zebrafish larvae are used to investigate the role of TLR4 as a mediator for CIO.

### 3.3.1 Synthetic derivatives of TAK-242 ameliorate CIO:

*In vitro* cell culture assays show TAK-242 can prevent TLR4 signalling caused by cisplatin as shown by a reduction in cytokine production (Babolmorad et al. 2021). In zebrafish, TAK-242 and syntagonists 120 and 132 caused an increase in CIO compared to fish treated with cisplatin alone **(Figure 3A)**. This increase in ototoxicity did not seem to be due to either potential innate toxicity of the syntagonists themselves or the vehicle in which they were dissolved. When exposed to TAK-242 or the syntagonists alone, or the DMF in which they were dissolved, there was no reduction in neuromast score compared to untreated zebrafish **(Figure 5)**. The syntagonists 134, 136 and 166 each caused a significant increase in neuromast score compared to cisplatin alone, reducing CIO **(Figure 3, Figure 4)**. This effect was further increased with higher concentrations of syntagonist 136, with 20µM 136 resulting in an almost complete recovery of neuromast score **(Figure 4B)**. Increased concentrations of syntagonist 134 still resulted in a significant increase of neuromast score compared to cisplatin alone, however there was no increased benefit whether 5µM or 20µM was used. Syntagonist 138 did not recover neuromast score compared to zebrafish exposed to just cisplatin **(Figure 3C)**. Furthermore, higher concentrations of syntagonist 138 appeared toxic to the zebrafish with concentrations of 10µM and above resulting in over 90% lethality in 5-7dpf zebrafish. Collaborators in the Bhavsar lab confirmed cell toxicity of syntagonist 138 in cell culture **(Data not shown)**. Younger 2-3dpf zebrafish larvae were able to tolerate higher concentrations of 138 of at least 10µM (**Data not shown)**. This may suggest that the effectiveness of the syntagonists is linked to their metabolism, which occurs after a certain

stage of zebrafish larval development. This ought to be considered for future experiments designed to establish whether there is added benefit to combined morpholino knockdown of *tlr4ba* and *tlr4bb* with syntagonist treatment.

The reason why TAK-242 and syntagonists 120 and 132 cause an increase in CIO but syntagonists 134, 136 and 166 reduce CIO *in vivo* is not clear **(Table 1)**. The chemical structures of TAK-242 and first generation syntagonists can be seen in **Figure 7**. The benzene ring has different functional groups between all the compounds. In the first carbon ring of TAK-242 and syntagonists 120 and 132 there is a double bond between carbon positions 6-1. In the syntagonists 134, 136, 138, 166 and 170 this double bond has shifted to carbon positions 1-2. The shift of this carbon double bond is predicted to change the Michael reaction properties of the beneficial syntagonists. The syntagonists with the shifted carbon double bond position are expected to form weaker covalent bonds to the intracellular binding position on TLR4 and therefore may dissociate easier compared to TAK-242. In zebrafish, why weaker binding to Tlr4ba or Tlr4bb would result in a reduction in CIO but stronger binding results in increased CIO is again unclear. It may be that by dissociating from Tlr4 there is less disruption to overall function resulting in less toxicity. If this were the case, then one would expect some residual level of toxicity seen when the syntagonists are applied with no cisplatin present but all neuromasts look healthy **(Figure 5A)**. It is possible that antagonism of Tlr4 only results in toxicity if stress is put upon the system, such as the inclusion of cisplatin. These results show the importance of *in vivo* validation of work initially done *in vitro*. Further experiments to establish the binding properties of TAK-242 and syntagonist on zebrafish Tlr4ba and Tlr4bb will help establish the toxic versus beneficial properties and may aid in the development of new syntagonists.

The increase in CIO caused by TAK-242 and syntagonists 120 and 132 compared to syntagonists 134 and 136, which reduce CIO, further demonstrate the importance of *in vivo*

systems such as zebrafish in the drug development pipeline. Compounds which may appear promising *in vitro* do not necessarily translate well when put in a full physiological system. This is due to both the more complex metabolism in a multi-cellular environment as well as the effects different organ systems, particularly the liver, on drug metabolism. An increase in toxicity may also be zebrafish specific as there will be metabolic differences compared to mammals. At a concentration of 10µM syntagonist 138 was lethal to 5-6dpf but not 2-3dpf zebrafish. This syntagonist also appeared toxic *in vitro*. This may suggest the potential for syntagonists to cause toxicity through different pathways to those seen in the increase of CIO by TAK-242. As older zebrafish larvae showed lethal toxicity, after the development of the kidney and liver, this could be due to further organ metabolism of the compounds.

### 3.3.2 Zebrafish *tlr4ba* and *tlr4bb* morpholino knockdown reduces CIO:

Mechanisms of CIO in inner and outer ear cells in patients is not well understood. It is thought to be linked with build-up of cisplatin in the hair cells, and their inability to flush cisplatin out, causing an increase in ROS. The Bhavsar lab has identified TLR4 as a potential mediator of CIO. TLR4 signalling leads to an increase in cytokine production and subsequent inflammatory response which can result in apoptosis **(Figure 1A)**. *In vitro* results show that TAK-242 and different syntagonist derivatives can prevent CIO in cell culture (Babolmorad et al. 2021). Three of these syntagonists, 134, 136 and 166 also prevent CIO in zebrafish **(Figure 3B and C, Figure 4)**. To further establish TLR4 as a mediator for CIO both zebrafish homologues, *tlr4ba* and *tlr4bb* were knocked down using morpholinos previously established in literature (Sepulcre et al. 2009; Q. He et al. 2015; M. Y. Chang et al. 2016). One splice-blocking *tlr4ba* morpholino and two *tlr4bb* morpholinos, one translation blockling (MO1) and one splice blocking (MO2) were used.

Single injection of either the *tlr4ba*, *tlr4bb*-MO1 or *tlr4bb*-MO2 morpholino in all cases led to a significant increase in neuromast score compared to un-injected fish or fish injected with

a control morpholino **(Figure 6)**. Two separate *tlr4bb* morpholinos acting through distinct mechanisms to cause gene knockdown had similar effectiveness. This strongly supports the specificity of *tlr4bb* knockdown resulting in reduced CIO. Combined injection of the *tlr4ba* and *tlr4bb*-MO2 also led to a significant increase in neuromast score compared to un-injected or control morpholino injected fish. Injection of both *tlr4ba* and *tlr4bb* morpholino at the same time did not have a significant increase in neuromast score compared to individual morpholino injections **(Figure 6A)**. Combined efficacy may be more apparent at higher concentrations of cisplatin used in these experiments. Alternatively, an increase in the amount of morpholino injected may further increase the recovery in neuromast score and reduction in CIO. Both *tlr4bb*-MO1 and *tlr4bb*-MO2 resulted in a recovery of neuromast score which would suggest CIO is mediated, at least in part, by signalling through Tlr4bb.

Future experiments will include testing the potential for combined efficacy of *tlr4ba* and *tlr4bb* morpholino knockdown with syntagonist co-treatment. The age of the zebrafish however will be an important consideration when analysing the results. As mentioned above, at 5-6dpf syntagonist 138 was lethal to zebrafish larvae and this was not seen in younger 2-3dpf fish. This suggests that syntagonist metabolism may be required for efficacy. Therefore, syntagonists 134, 136 and 166 which reduced CIO may not show any efficacy in younger zebrafish with or without morpholino injection. Confirming the otoprotective effects of these syntagonists at 2-3dpf will be a priority to establish predictions for combined experiments with morpholino knockdown.

## 3.4 Concluding Remarks:

Here, a zebrafish model for ototoxicity was adapted to investigate whether CIO is mediated by TLR4 signalling. The work detailed in this chapter confirms the hypothesis that TLR4 signalling mediates CIO and that TLR4 antagonists can reduce CIO. Currently it is most likely that the MyD88 independent pathway is responsible for CIO (Babolmorad et al. 2021).

This can be confirmed by inhibiting separate downstream elements of the MyD88 dependent and independent pathways. Three synthetic derivatives of the TLR4 antagonist TAK-242 were able to significantly reduce CIO in 5-6dpf zebrafish larvae. This was observed as an increase in the score of PLL neuromasts correlating with an increase of metabolically active neuromast cells. Morpholino injections to cause a predicted knockdown of *tlr4ba* and *tlr4bb* gene expression also resulted in a significant and reproducible increase in neuromast score and reduction of CIO when measured in 2-3dpf zebrafish. Zebrafish Tlr4ba and Tlr4bb also provide an effective and high throughput model for the further identification of novel synthetic compounds to prevent CIO.

Future experiments will establish whether there is combined efficacy from a combination of morpholino knockdown and syntagonist treatment on PLL neuromasts to prevent CIO. A high throughput method of CIO and syntagonist testing has been established, allowing for identification of additional novel syntagonists in future. Generation of *tlr4ba* and *tlr4bb* knockout zebrafish and transgenic fish expressing mammalian TLR4 will also be generated to further establish the involvement of TLR4 in CIO. The generation of these additional zebrafish models in combination with the experiments outlined in this chapter will continue to provide an invaluable tool in developing new, safe, and effective therapeutics for the treatment of cancer, particularly in children.

## 3.4 Chapter 3 Materials and Methods

### 3.4.1 Animal ethics and zebrafish husbandry:

Zebrafish were kept at the University of Alberta following a 14:10 light/dark cycle at 28°C cycle as previously described (Westerfield 2000). They were raised, bred, and maintained following an institutional Animal Care and Use Committee approved protocol AUP00000077, operating under guidelines set by the Canadian Council of Animal Care.

### 3.4.2 Assessing CIO in larval zebrafish:

Wildtype (AB strain) zebrafish were grown to 5 days post fertilization (dpf) in standard E3 embryo media (Westerfield 2000) and were bath treated with either 0, 5, 10, 25 or 50µM of cisplatin in 6-well plates, with 10-15 zebrafish larvae per well. After a 20-hour incubation with cisplatin at 28$^{o}$C, wells were washed with embryo media before the fish were incubated in media containing 0.01% 2-[4-(dimethylamino) styryl]-1-ethylpyridinium iodide (DASPEI, Sigma-Aldrich) to stain for neuromast mitochondrial activity for 20 minutes. Wells were washed again in embryo media and zebrafish larvae anaesthetized with 4% tricaine. Neuromasts were imaged under a Leica M165 FC dissecting microscope equipped with a fluorescent filter. A standard scoring method for zebrafish hair cell viability was used (Chowdhury et al. 2018): five posterior lateral line (PLL) neuromasts for each fish were assigned a score representing cell viability based on DASPEI fluorescent intensity (2 for no noticeable decline, 1.5 for minor decline, 1 for moderate decline, 0.5 for severe decline and 0 for complete loss of fluorescent intensity). These five scores were summed for each individual (10= all hair cells appear normal and viable; 0=intense ototoxicity).

### 3.4.3 Morpholino knockdown of TLR4 homologs

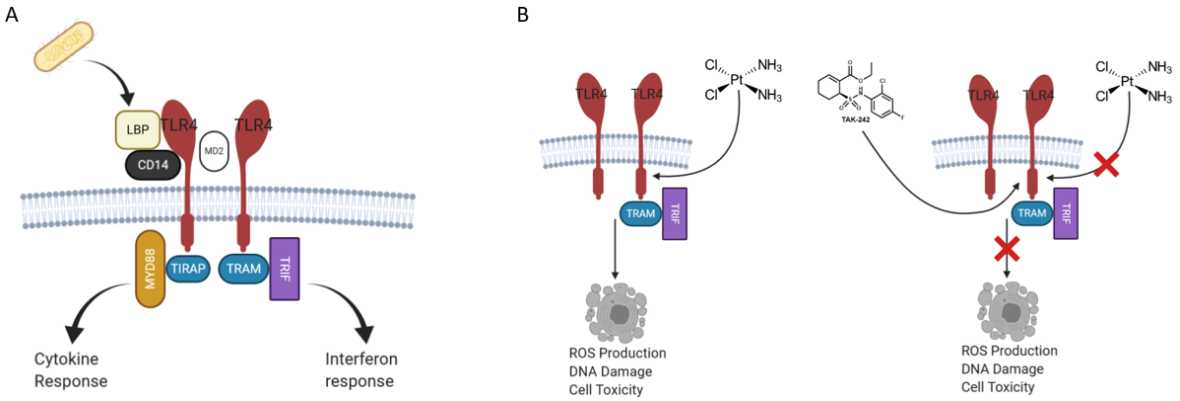
Previously validated anti-sense knockdown reagents (Morpholinos (Sepulcre et al. 2009; M. Y. Chang et al. 2016; Q. He et al. 2015)) against *tlr4ba* and *tlr4bb* (Gene Tools, LLC; Philomath, OR) were delivered to developing zebrafish. Two *tlr4bb* morpholinos were used, the first translation blocking: *trl4bb*-MO1 (5'-AATCATCCGTTCCCCATTTGACATG-3')

the second splice blocking: *tlr4bb*-MO2 (5'-CTATGTAATGTTCTTACCTCGGTAC-3'). A splice blocking *tlr4ba*-MO2 (5'- GTAATGGCATTACTTACCTTGACAG-3') was also used. All gene-specific morpholinos have been previously described and thoroughly vetted for efficacy and specificity to the gene target (Sepulcre *et al.*, 2009; He *et al.*, 2015; Chang *et al.*, 2016). A standard control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a negative control. Injection solution for morpholinos consisted of 0.1M KCl, 0.25% dextran red, either the standard control or gene-specific morpholinos to effective dose and nuclease-free water. One-cell stage newly fertilized embryos were positioned on an agarose plate and injected with 5ng of morpholino. At 2dpf gene-specific morpholino injected fish, control morpholino injected fish and un-injected fish were added to separate wells of a 6-well plate with 10-15 fish per well. Fish were incubated with 15μM cisplatin for 20-hours before being washed, DASPEI stained, imaged, and analysed as described above.

### 3.4.4 Statistical analyses

Neuromast scores were analysed via one-way ANOVA with Tukey's multiple comparisons test. Statistical tests were carried out using R version 4.0 and graphs were constructed using the 'ggplot2' and 'tidyverse' group of R packages (Wickham et al. 2019; Wickham 2016; R Core Team 2020).

## 3.5 Chapter 3 Table and Figures




### Figure 3.1:

A simplified diagram of TLR4 signalling and TAK-242 antagonism. A) Recognised binding partners such as LPS bind to the extracellular region of TLR4 resulting in two potential signalling cascades. The first is the MyD88 pathway resulting primarily in a cytokine response, the second is the MyD88 independent pathway resulting in an interferon response. B) TAK-242 binds to the intracellular region of TLR4. Cisplatin is able to diffuse across the cell membrane and is also thought to bind to the intracellular region of TLR4, resulting in apoptotic pathways. Whether TAK-242 antagonism of cisplatin is a direct or indirect inhibition is not well established. Figure made in Biorender.

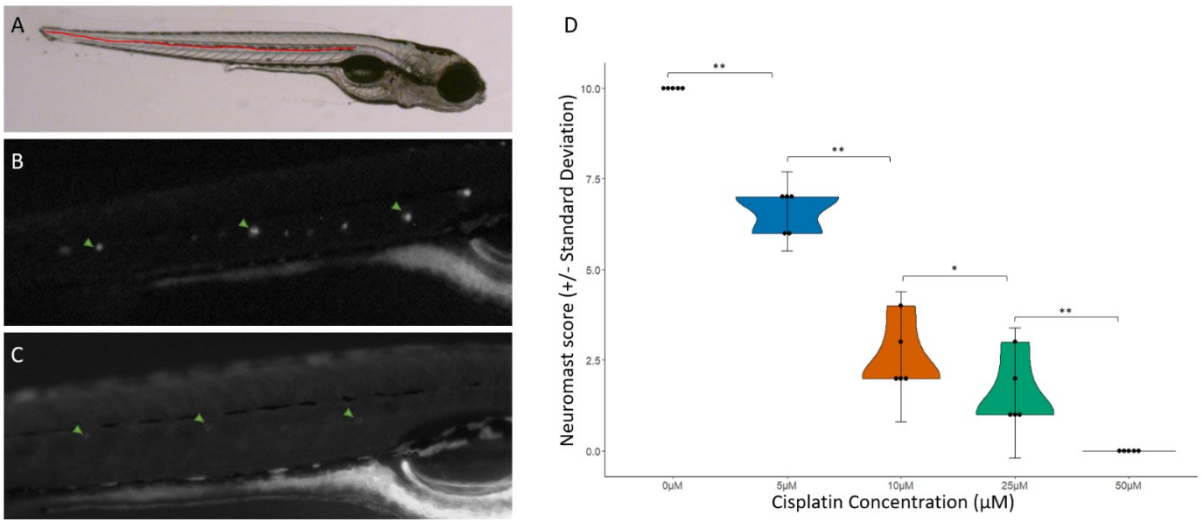



Figure 3.2:
Cisplatin causes cell toxicity in zebrafish neuromasts along the PLL as measured through DASPEI fluorescent intensity. A) The red line in the top panel traces the position of the PLL in a 6dpf zebrafish. B) Green arrows show DAPSEI stained neuromasts in 6dpf zebrafish with no cisplatin exposure. C) Green arrows show DASPEI stained neuromasts in 6dpf zebrafish after 20h of 7.5μM cisplatin exposure. Fluorescent intensity is significantly reduced in cisplatin treated zebrafish compared to untreated zebrafish. Images have been grey scaled for visibility. D) Dose response curve showing a reduction in PLL neuromast score as measured by a decrease in DASPEI fluorescence in 6dpf zebrafish larvae over an increase in cisplatin concentration. Neuromast score represents qualitative analysis of fluorescent intensity correlating with overall neuromast health. A higher score indicates an increase in DASPEI fluorescent intensity, and an increase in active mitochondria and therefore neuromast cell health. Neuromasts were assigned scores of 2, indicating normal fluorescence, 1 indicating a reduction in fluorescent intensity and neuromast health or 0 indicating total loss of fluorescent intensity and significant cell death in the neuromast. Scoring was averaged from 5 neuromasts per zebrafish, giving each a total score out of 10. * = $P < 0.01$, ** = $P < 0.0001$. Significance determined by one way ANOVA with Tukey HSD post-hoc test comparison.

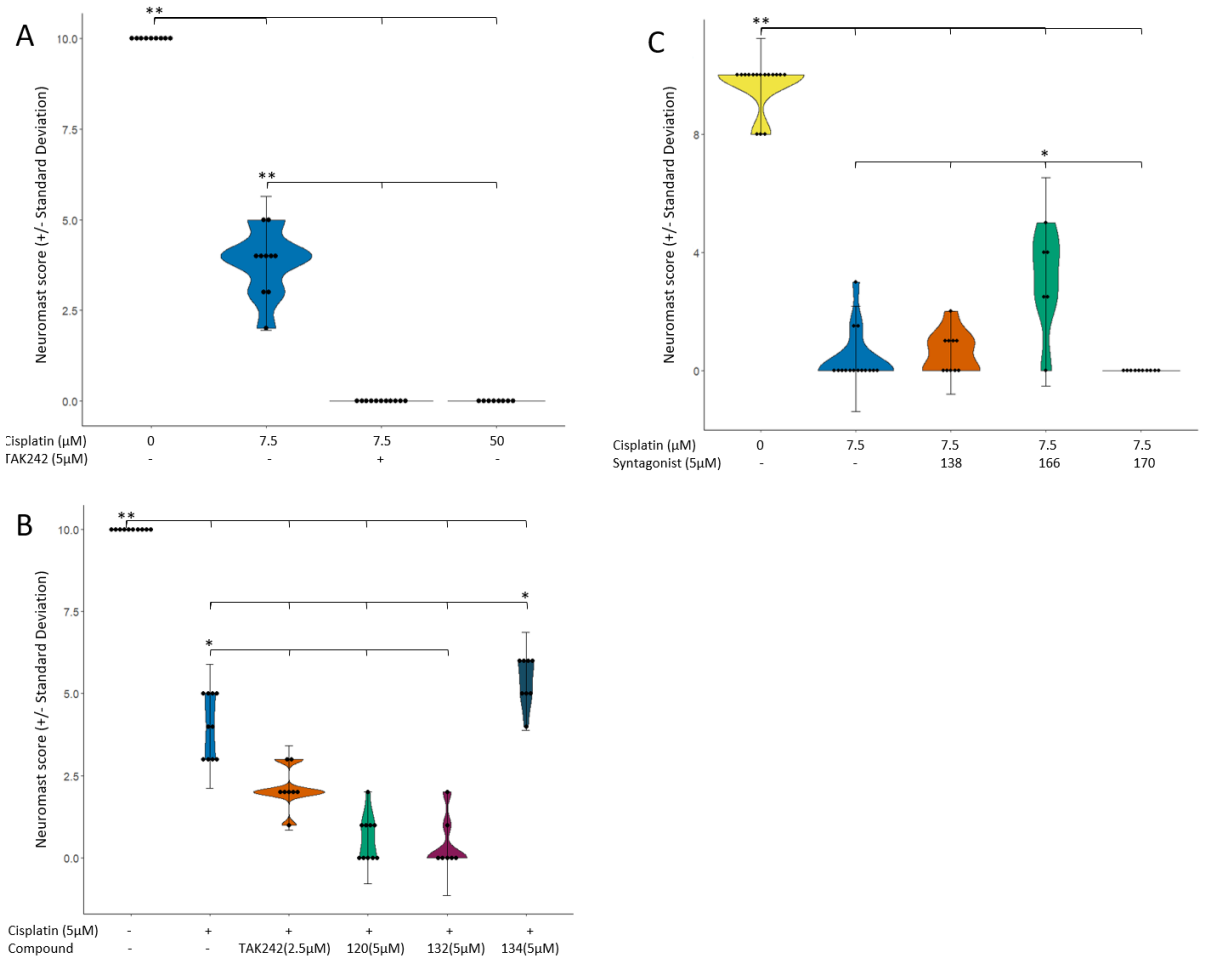



Figure 3.3:

Syntagonists derived from the TLR4 antagonist TAK-242 can exacerbate or ameliorate CIO in 5-6dpf zebrafish. A) 5μM TAK-242 with 7.5μM cisplatin causes an increase in CIO compared to zebrafish exposed to just 7.5μM cisplatin. In zebrafish treated with TAK-242 and cisplatin there was a total loss of fluorescent intensity with each neuromast scoring 0, indicating deterioration of neuromast cell health. B) A lower concentration of 2.5μM TAK-242 (purple) and with 5μM cisplatin continues to cause an increase in CIO. Syntagonists 120 (green) and 132 (cyan) also show an increase in CIO compared to fish treated with just 5μM cisplatin (gold). Syntagonist 134 (blue) causes a significance increase in neuromast score compared to fish treated with TAK-242, syntagonists 120 and 132 or fish treated with just 5μM cisplatin. C) Zebrafish treated with syntagonist 166 (green) show a significant increase in neuromast score compared to just cisplatin or zebrafish co-treated with syntagonist 138

and 170. In A and B zebrafish were scored at 6dpf, in C zebrafish were scored at 7dpf. * = P < 0.01, ** = P < 0.00001. Significance determined by one way ANOVA with Tukey HSD post-hoc test comparison.

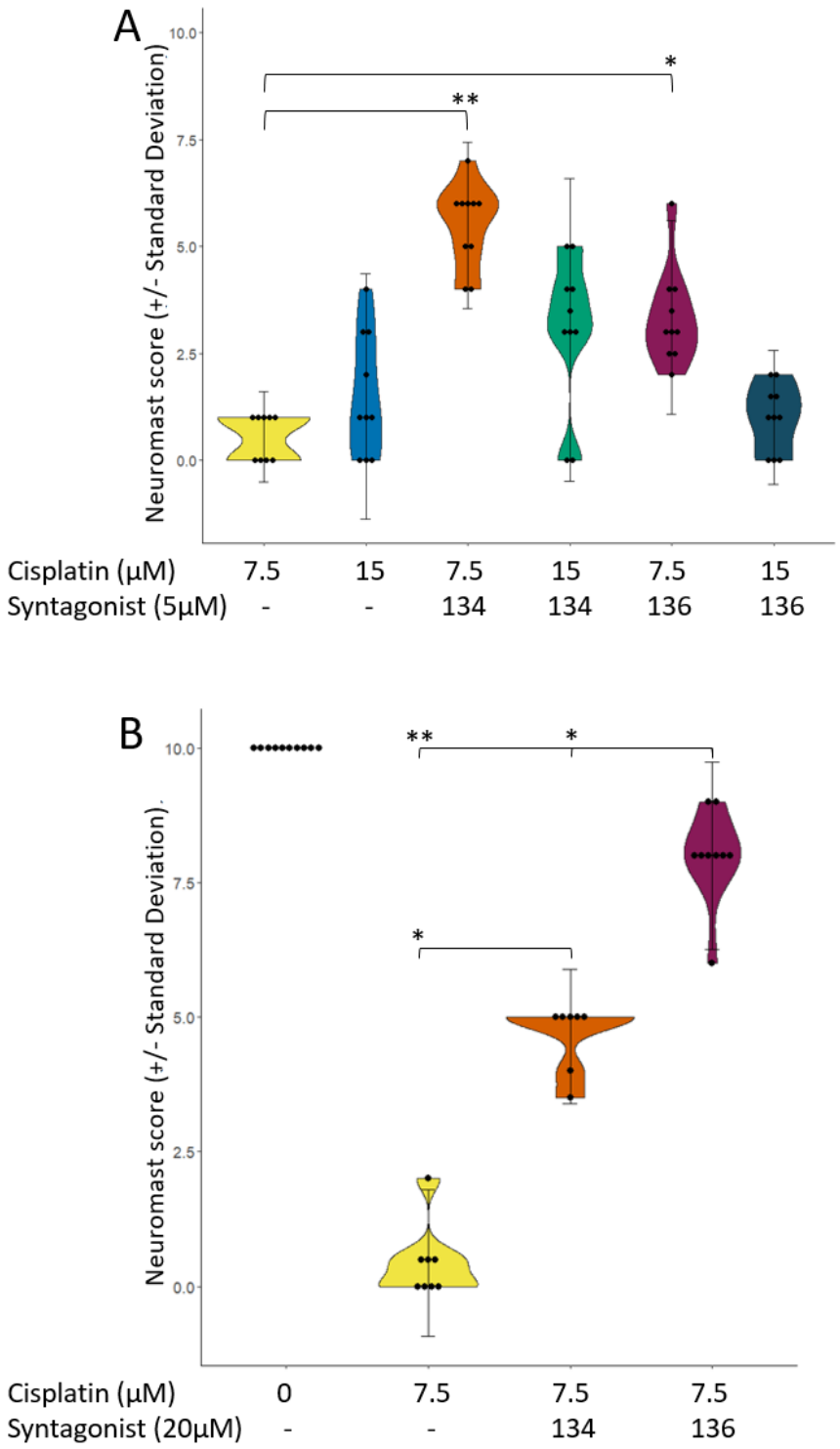



Figure 3.4:

Syntagonists 134 and 136 reduce CIO in 5-6fpd zebrafish exposed to 7.5µM cisplatin. A) Zebrafish co-treated with 5µM of syntagonist 134 (gold) or 136 (cyan) so a significant increase in neuromast score compared to fish exposed to just cisplatin at 7.5µM (pink). This increase in neuromast score does not occur when cisplatin concentration is increased to 15µM at 5µM of either syntagonist 134 or 136. B) Increasing the concentration of syntagonist 136

(cyan) to 20μM further significantly increases neuromast score compared to fish exposed to just 7.5μM cisplatin. Zebrafish were scored at 6dpf. * = $P < 0.001$, ** = $P < 0.00001$. Significance determined by one way ANOVA with Tukey HSD post-hoc test comparison.

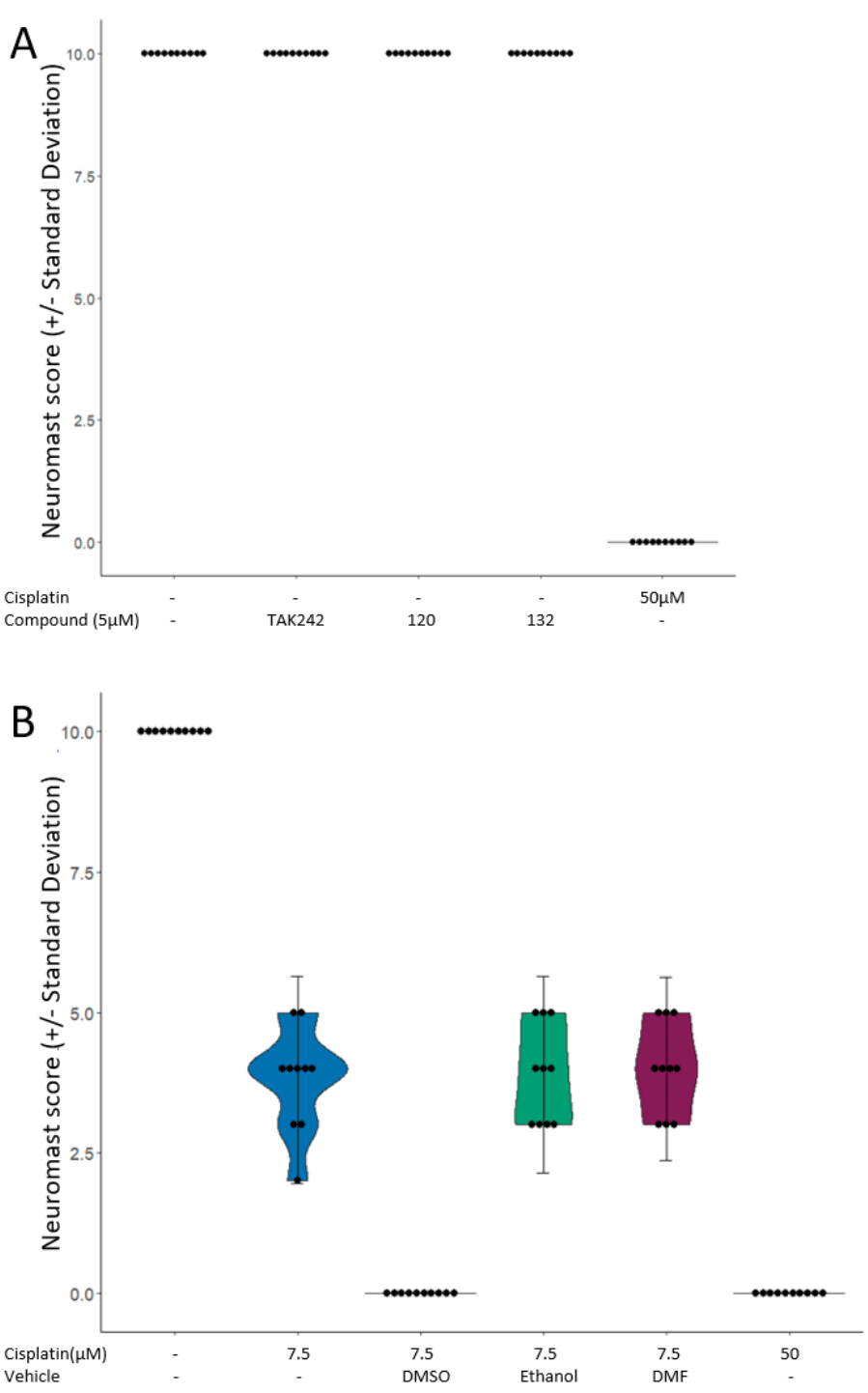



Figure 3.5

TAK-242, syntagonists and compound vehicle are not toxic to zebrafish neuromasts on their own. A) TAK-242 and syntagonists 120 and 132 do not cause any reduction in neuromast score at a concentration of 5µM in 5-6dpf zebrafish. B) Drug delivery vehicle controls show the chosen vehicle for TAK-242 and syntagonists, DMF, does not show any significant change in neuromast score when co-treated with 7.5µM cisplatin compared to fish just treated

2635 with cisplatin. DMSO was included as a control as this has been previously shown to

2636 exacerbate CIO in zebrafish neuromasts. Zebrafish were scored at 6dpf.

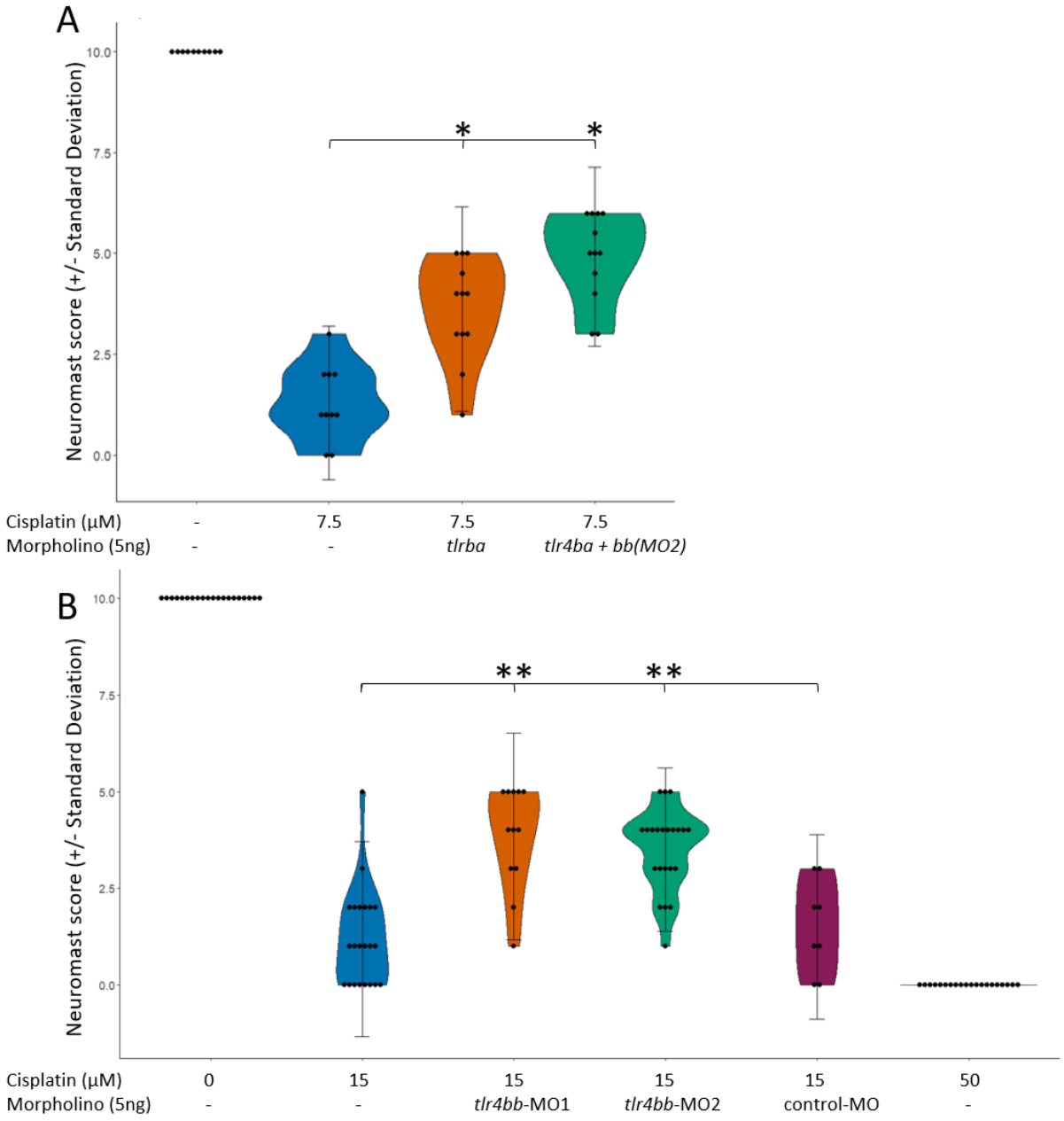



Figure 3.6:

Morpholino knockdown of *tlr4ba* and *tlr4bb* result in recovery of neuromast score in 2-3dpf zebrafish exposed to 7.5μM and 15μM cisplatin. A) Zebrafish embryos injected with 5ng of either *tlrba* morpholino or *tlr4ba* and *tlrba-MO2* morpholinos showed a significant increase in neuromast score compared to un-injected zebrafish exposed to 7.5μM cisplatin. B) Zebrafish injected with either *tlr4bb*-MO1 (blue) or *tlr4bb*-MO2 (pink) both show a significant increase in neuromast score compared to either un-injected fish (gold) or control

morpholino injected fish (cyan) after exposure to 15μM cisplatin. Zebrafish were scored at 3dpf. * = P < 0.001. Significance determined by one way ANOVA with Tukey HSD post-hoc test comparison.

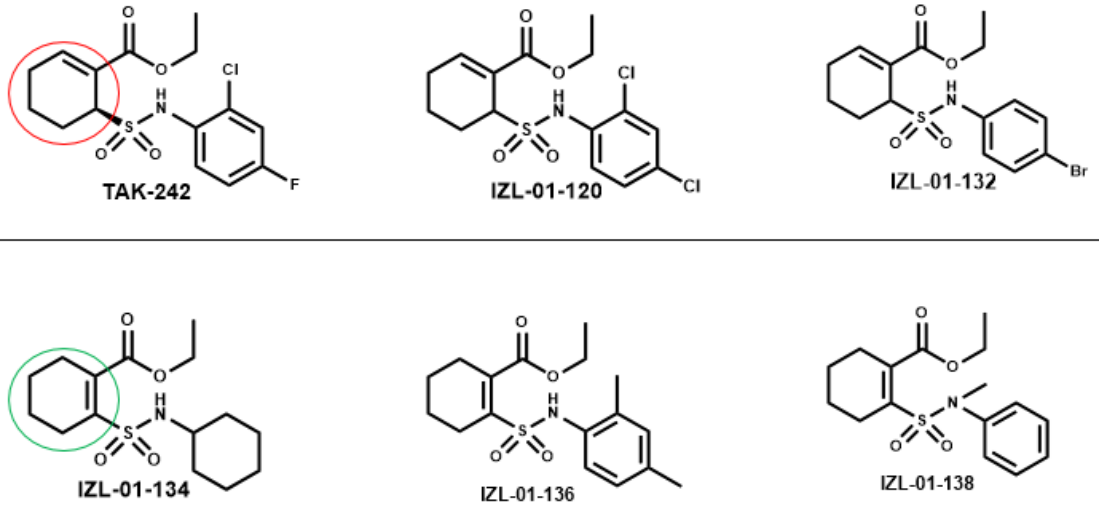
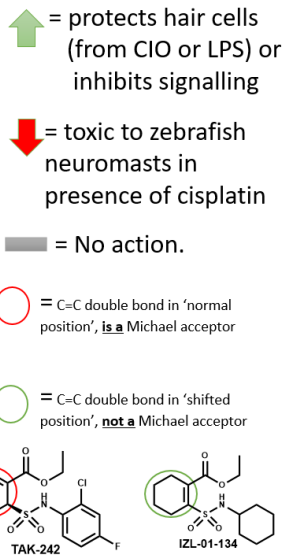



Figure 3.7:
Structures for TAK-242 and first generation syntagonists. TAK-242 and syntagonists 120 and 132 have a carbon double bond in the 6-1 position of the first carbon ring (top row, red circle). Syntagonists 134, 136 and 138 have this carbon double bond shifted to position 1-2 (bottom row, green circle). TAK-242 and syntagonists 120 and 132 cause an increase in CIO, syntagonists 134 and 136 cause amelioration of CIO. Syntagonist 138 does not prevent or increase CIO however appears toxic to zebrafish at higher concentrations.

Table 3.1:

Summary table of the effects of TAK-242 and syntagonists on CIO in *in vitro* experiments and zebrafish PLL neuromasts. LPS is the main canonical ligand to mammalian TLR4 and was used in *in vitro* experiment by collaborators in Amit Bhavsar's lab. Green arrows represent a beneficial effect, reducing CIO or LPS induced toxicity in either mouse HEI-OC1 cell culture or zebrafish PLL neuromasts. Red arrows indicate an increase in toxicity. Grey bands indicate neither a beneficial nor detrimental effect. The position of the carbon double bond is indicated as either a red circle (position 6-1) or green circle (position 1-2). * = Lethal to zebrafish at high concentrations.

| | Cells + cisplatin | Cells + LPS | Zebrafish + cisplatin | Chemistry |
|---|---|---|---|---|
| TAK-242 | ↑ | ↑ | ↓ | ○ |
| 120 | ↑ | ↑ | ↓ | ○ |
| 132 | ↑ | – | ↓ | ○ |
| 134 | ↑ | – | ↑ | ○ |
| 136 | ↑ | – | ↑ | ○ |
| 138 | ↑ | – | – * | ○ |
| 166 | – | ↑ | ↑ | ○ |
| 170 | – | ↑ | – | ○ |



# Chapter 4: Group 10 transition metals as potential ligands for zebrafish Tlr4ba and Tlr4bb

## Chapter 4 preface:

A version of the material and methods also appears in Chapter 3, and from Babolmorad, Ghazal, Asna Latif, Ivan K Domingo, Niall M Pollock, Cole Delyea, Aja M Rieger, W Ted Allison, and Amit P Bhavsar. 2021. “Toll-like Receptor 4 Is Activated by Platinum and Contributes to Cisplatin-Induced Ototoxicity.” *EMBO Reports* n/a (n/a): e51280. https://doi.org/https://doi.org/10.15252/embr.202051280. This chapter was written by NMP with editing contributions from W. Ted Allison. Contributions to figures: Figure 5, 6 and 7 contain data collected by Aaron Fox.

## Chapter 4 Abstract:

Zebrafish Tlr4ba and Tlr4bb are homologues of mammalian TLR4. In mammals the canonical ligand for TLR4 is lipopolysaccharide, however other ligands include viral proteins and metal ions such as nickel. In zebrafish, lipopolysaccharides from gram negative bacteria do not act as a ligand for either Tlr4ba or Tlr4bb and the search for canonical ligands continues. The difference in ligand is likely due to the lack of similarity in the extracellular leucine rich repeat ligand binding domain. Downstream signalling between mammalian and zebrafish Tlr4s remains well conserved. The toll-like receptor (TLR) family has long been implicated in cancer pathology as well as inflammatory signalling due to metal hypersensitivity. Further understanding of the zebrafish Tlr4ba and Tlr4bb signalling pathway through identification of potential ligands may help in developing further *in vivo* cancer and immunological models, particularly for autoimmune diseases and allergens. Platinum is a group 10 transition metal and the principal component of cisplatin. Considering that morpholino knockdown of *tlr4ba* and *tlr4bb* in addition to the application of TLR4 syntagonists can prevent cisplatin induce ototoxicity (CIO) we tested the hypothesis that zebrafish Tlr4s recognise group 10 transition metals. Using the model for CIO outlined in Chapter 3, the toxicity of nickel, platinum (II) or platinum (IV) chloride was established in zebrafish neuromasts. All three group 10 transition models caused significant ototoxicity as measured through a reduction in DASPEI fluorescence correlating with loss of mitochondrial activity and predictive cell death. TLR4 syntagonists were used to block signalling of Tlr4ba and Tlr4bb after exposure to the group 10 transition metals. Neither syntagonist 134 nor 136, which reduced CIO, prevented the metal ion ototoxicity of $NiCl_2$, $PtCl_2$ or $PtCl_4$. Syntagonists 138, 150, 166, 168 and 170 all showed a significant increase in neuromast score after co-treatment with $NiCl_2$ compared to fish treated without syntagonists. While these preliminary results show promise the lack of reproducibility with syntagonist 138 combined with the unreliability of the control group in one experiment demands the need for additional

experiments to confirm syntagonist efficacy against nickel toxicity. Neither $PtCl_2$ nor $PtCl_4$ ototoxicity was reduced after co-treatment with syntagonist 134. In addition to further testing of TAK 242 and its derivative syntagonists, future experiments using morpholino knockdown and CRISPR knockout of *tlr4ba* and *tlr4bb* will also be used to establish whether group 10 transition metals can result in activation of the zebrafish Tlr4 signalling pathway.

## 4.1 Introduction:

In mammals, Toll-like receptor 4 (TLR4) is a pattern recognition receptor and involved in the innate immune response. The primary canonical ligand for mammalian TLR4 is lipopolysaccharide (LPS), a constituent of gram-negative bacterial cell walls. Other ligands such as viral heat shock protein and metal ions including nickel are also recognised (Tatematsu et al. 2016; Bulut et al. 2005; Schmidt et al. 2010). In zebrafish there are two Tlr4 proteins, Tlr4ba and Tlr4bb. Compared to mammals, zebrafish have a reduced response to LPS and it does not activate either Tlr4ba or Tlr4bb (Sepulcre et al. 2009; Sullivan et al. 2009). Currently the ligands for Tlr4ba and Tlr4bb are unknown. In Chapter 3 of this thesis cisplatin induced ototoxicity (CIO) through Tlr4 signalling is described. Morpholino knockdown and pharmacological antagonism of *tlr4ba* and *tlr4bb* significantly reduced CIO. The active element of cisplatin is the platinum molecule at the centre of its structure. This suggests that the zebrafish Tlr4s (zfTlr4) may recognise heavy metals such as the group 10 transition metals. This chapter explores the hypothesis that Tl4ba and Tlr4bb recognise group 10 transition metals by utilising the zebrafish posterior lateral line (PLL) model used in Chapter 3.

The introduction to this chapter will first briefly outline what is known about the zebrafish innate immune response, zfTlr4s in relation to mammalian TLR4 and metal signalling through TLR4.

### 4.1.1 The zebrafish innate immune response:

Zebrafish are coming into their own as an immunological model and for investigating host-pathogen interactions (Lee-Estevez et al. 2018; Beatriz Novoa and Figueras 2012; Mostowy et al. 2013; Hosseini et al. 2014). Most innate immune system components and pathways are well conserved between zebrafish and mammals. These include: the toll-like receptors (TLRs), the complement gene family, interleukins, interferons, signal transducers and

transcription activators (Stein et al. 2007; Seeger, Mayer, and Klein 1996). Conservation of signalling pathways is strongest in the downstream elements, while conservation is not as strong regarding the ligands and ligand-interacting domains of the receptors. This is likely due to the different natural aquatic environment and habitat of zebrafish resulting in their interaction with largely different pathogens. The conservation of the downstream signalling pathways however mean they still provide a relevant and compelling model.

For the first 4-6 weeks of their development zebrafish are strongly reliant upon their innate immune response as their adaptive immune response is not fully developed until around 40 days of age. While immature lymphoblasts which will eventually mature into T and B cells are present as early as 3dpf (days post fertilisation) they do not mature until 4-6 weeks later (Lam et al. 2004). This allows for investigating innate immunity in the absence of adaptive immunity. Understanding innate immunity is important as while it is often thought of as a response to infection from pathogens it is also involved in processes such as wound healing, auto-immunity, cancer and more (Beatriz Novoa and Figueras 2012).

Zebrafish macrophages and neutrophils are well conserved to those in mammals. Both act in a similar manner, circulating in the blood and respond initially to inflammatory signals (Le Guyader et al. 2008; Herbomel, Thisse, and Thisse 1999). Macrophages carry out phagocytosis on pathogens or cellular debris and can stimulate pro-inflammatory gene expression and secretion of cytokines. Throughout embryogenesis there are distinct periods of haematopoiesis which form immune cells. The first wave begins through the tandem effects of two transcription factors, Pu.1 and Gata1 (Rhodes et al. 2005). Like humans Pu.1 and Gata1 negatively regulate each other, when Pu.1 is favourably expressed it leads to the development of myeloid cells. These cells then split into two distinct cell populations. Cells expressing *L-plastin* differentiate into macrophages while those expressing *mpx* differentiate into neutrophils (Tenen et al. 1997; Meijer et al. 2008). Development of the zebrafish innate

immune system begins as soon as 12 hours post fertilisation (hpf) with the development of these myeloid precursors. By 24hpf immature macrophages are present and by 30hpf both cell types are present and active (Lam et al. 2004). While not fully matured at this point the macrophages are still capable of phagocytosing microbes and apoptotic cells. The site and nature of infection can dictate whether the primary immune response is orchestrated by neutrophils or macrophages. Bacterial infection in the blood, or other fluid environments such as the ear, resulted in a phagocytic response after the recruitment of macrophages. When the site of infection was on a predominantly solid tissue surfaces the immune response recruited neutrophils to drive phagocytosis of the pathogen (Colucci-Guyon et al. 2011).

Pattern recognition receptors (PRRs) are responsible for recognising pathogens and are a major component of the innate immune system. TLRs comprise one of the most studied group of PRRs. While there appears to be evolutionary divergence in TLRs between zebrafish and mice or humans many of the downstream components in the signalling pathway remain conserved and this is discussed later in this chapter. Additional PRR groups include: RIG-I-like and NOD-like receptors (RLRs and NLRs, respectively) as well as lectins and scavenger receptors. While there is good conservation of these families between zebrafish and mammals there are notable differences. In mammals the NLR family is relatively small. Zebrafish have orthologues for all 8 mammalian NLR genes, but the family consists of almost 400 genes (Howe et al. 2016). Large numbers of NLR genes are more typically found in organisms which do not have an innate immune system, and the size of this gene family in zebrafish may provide an interesting avenue to explore its evolutionary development. Orthologues for the 3 mammalian RLR genes: RIG-I, MDA5 and LGP2 are all present in zebrafish and provide similar functions (Q.-M. Zhang et al. 2018; Zou et al. 2015, 2014).

The conservation in innate immune system pathways between zebrafish and mammals has led to a powerful translational model in which to investigate the role of the innate immune

response upon pathogen exposure. Methicillin resistant *Staphylococcus aureus* (MRSA) is becoming an increasing bacterial threat to humans due to an increasing prevalence in populations and increasing resistance to anti-biotic treatment (A. S. Lee et al. 2018). The zebrafish innate immune response has been used to show the importance of leukocyte and macrophage for controlling MRSA infection. Morpholino knockout of *pu.1* removed the ability of zebrafish larvae to combat infection (Prajsnar et al. 2008). Further work subsequently established methods of infectivity of MRSA related to its life cycle within cells, where bacteria could form reservoirs in neutrophils and an avenue of sepsis (Prajsnar et al. 2012).

### 4.1.2 Mammalian and zebrafish Tlr4:

The family of toll-like receptor proteins were originally described in *Drosophila* and have since been found to be highly conserved across both vertebrate and invertebrate lineages (Lemaitre et al. 1996; Medzhitov, Preston-Hurlburt, and Janeway 1997). TLRs are transmembrane proteins and constitute an important aspect of the immune response. They produce cytokine or interferon response depending on whether the MyD88-dependent (cytokine) or TRIF-dependent (interferon) pathway is activated upon recognition of pathogen-associated molecular patterns (PAMPs). In mammals there are currently up to 12 identified TLR genes, 10 of which are in humans. Zebrafish in comparison have 20 *tlr* genes with duplicates of *tlr4, tlr5* and *tlr8* (H. Chen et al. 2021; Y. Li et al. 2017). Structurally TLRs have a leucine-rich repeat (LRR) ectodomain responsible for ligand recognition, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain which interacts with adaptor proteins and begins downstream signalling (Kawasaki and Kawai 2014). Ligands for the mammalian TLRs are well established and while some have been identified in zebrafish there are still several to be identified **(Table 1)**.

TLR4 recognition of LPS and subsequent signalling pathway requires multiple adaptor proteins and further signalling molecules. LPS binding requires CD14 to facilitate binding to TLR4 and MD2 to cause homodimerization of TLR4 and subsequent intracellular signalling (Lu, Yeh, and Ohashi 2008). As previously mentioned, there are two signalling pathways through TLR4, the MyD88 dependent pathway and the TRIF dependent pathway (or MyD88 independent) (Premkumar et al. 2010). In the MyD88 dependent pathway the intracellular region of TLR4 recruits MyD88 and the TIRAP protein. This then regulates NF-κβ activation stimulating the production of pro-inflammatory cytokines through further recruitment of IL-1 receptor associated kinases (IRAKs) and TRAF6 (Bagchi et al. 2007; Akira and Hoshino 2003). The TRIF dependent pathway occurs due to viral protein binding to TLR4. Here the intracellular region of TLR4 interacts with TRIF through TRIF-related adaptor molecule (TRAM) resulting in activation of IRF3 and production of anti-viral Type I interferons (W. Hu et al. 2015).

In zebrafish, orthologues for the 10 human TLRs are all present and in total there are 20 TLR variants **(Table 1)**. Due to a genome duplication event in the teleost lineage zebrafish have two *tlr4* genes, *tlr4ba* and *tlr4bb*, a third *tlr4al* gene as well as duplicates of *tlr5* and *tlr8* (Y. Li et al. 2017). Other signalling components and downstream elements are also present in zebrafish, including: MyD88, TRIF, TRAF2, TRAF4 and TIRAP (Y. Li et al. 2017; Fan et al. 2008; Stein et al. 2007)**.** Fish species do react to LPS but appear highly resistant, with much higher concentrations of LPS required to initiate a response in fish compared to mammals. The amount of this discrepancy is the order of requiring micrograms in fish compared to nanograms in mammals (B Novoa et al. 2009). Combined with the lack of TLR4 in some fish species (Palti 2011) this would suggest that the LPS response in fish is not mediated by zfTlr4s and the mammalian response developed after species divergence.

The zfTlr4s do not strongly respond to LPS (Sullivan et al. 2009; Sepulcre et al. 2009). The lack of response to LPS in zfTlr4s can likely be explained to the lack of sequence identity in the extracellular region of the protein **(Figure 2 & 4)**. This suggests a different primary ligand for Tlr4ba and Tlr4bb compared to mammalian Tlr4. The extracellular amino acid sequence similarity between Tlr4ba and Tlr4bb is 61% which may suggest distinct ligands between the two zfTlr4s as well **(Figure 4)**. While sequence identity is low for the extracellular region, it is higher across the intracellular region with Tlr4ba, Tlr4bb and Tlr4al showing 55% identity and 70% similarity **(Figure 3)**. Experiments *in vitro* have shown that by replacing the extracellular region of zebrafish Tlr4ba and Tlr4bb with the region from mouse TLR4 an LPS response can be triggered (Sullivan et al. 2009). Therefore, the signalling pathways downstream of ligand binding in fish and mammalian Tlr4s remain somewhat conserved. This is further supported by the recent identification of a highly diversified zebrafish MD2 homologue which in mammals is required for TLR4 signalling and LPS recognition. The authors demonstrated that *in vitro* experiments could result in an LPS response through Tlr4ba however this required the presence of human CD14. No LPS response was generated through Tlr4bb (Loes et al. 2021). At the time of writing no *CD14* orthologue has been identified in zebrafish. The requirement of human CD14 for LPS response through Tlr4ba would suggest that even with the identification of zebrafish Md2 the search for the natural ligand(s) of Tlr4ba and Tlr4bb continues.

### 4.1.3 Transition metal activation of the TLR4 signalling pathway:

Metal ions have been shown to cause immunological hypersensitivity reactions through TLR4 signalling including nickel, cobalt and palladium (Raghavan et al. 2012; Schmidt et al. 2010). Furthermore, TLR4 has been implicated in iron toxicity. After knockdown of TLR4 there was a significant reduction in cardiac toxicity and progression of heart failure in rats after exposure to iron(Xiaoqing et al. 2019). Nickel is one of the most common contact

allergens used in both day-to-day items such as mobile phones and jewellery as well as medical tools such as stents and dental implants (Moennich, Zirwas, and Jacob 2009; Schmidt et al. 2010). Many mammals do not show nickel contact immunoreactivity, with this most often occurring in humans and other closely related primates such as chimpanzees (Peana et al. 2017). This appears to be due to nickel reactivity occurring due to nickel binding at histidine residues at positions 456 and 458 of the LRR domain which are not conserved across other mammalian species such as mice, and are dispensable for LPS signalling (Schmidt et al. 2010; Peana et al. 2017; Raghavan et al. 2012). These residues are also not conserved in zebrafish Tlr4ba or Tlr4bb. As the LRR region containing the nickel and LPS binding sites are more divergent between zebrafish and humans than between other mammals and humans it may be potential binding sites for nickel and other metals such as platinum reside elsewhere within the zebrafish LRR region. Other mammals which do not have histidine residues at positions 456 and 458, such as rats, can still show nickel induced TLR4 signalling (Gilmour et al. 2004). After sensitisation using LPS, nickel signalling through TLR4 can also occur in mice (Sato et al. 2007). Both nickel and platinum activate TLR4 signalling in a manner independent to that of LPS (Babolmorad et al. 2021; Schmidt et al. 2010; Raghavan et al. 2012). This would suggest that the lack of zebrafish Tlr4ba and Tlr4bb response to LPS may not affect their ability to react to metal ions such as nickel. In addition, as zfTlr4s do not respond to LPS this may also reduce concerns regarding endotoxin contamination causing unreliable results in other *in vitro* and *in vivo* models. MD2 appears required for the activation of TLR4 through nickel signalling (Oblak, Pohar, and Jerala 2015) however homodimerization of TLR4 occurs through independent mechanisms (Raghavan et al. 2012).

Our previous data showed that ototoxicity could occur through a TLR4-dependent response to the platinum-based compound, cisplatin. We chose to use this assay as a proxy for Tlr4

function in zebrafish, to assess a potential role for the zebrafish TLR4 homologues in their response to transition metals.

## 4.2 Chapter 4 Results:

### 4.2.1 Nickel and platinum show significant toxicity in zebrafish neuromasts:

Wild-type AB zebrafish larvae at 6dpf were exposed to different concentrations of either $NiCl_2$, $PtCl_2$ or $PtCl_4$. $PtCl_2$ was used as it is most chemically similar to the platinum in cisplatin. $PtCl_4$ was used as to confirm platinum results in case there was variability in the solubility of the different salts. This was done to establish future concentrations to measure ototoxicity in zebrafish neuromasts as seen through a reduction in the score of neuromasts associated with a drop in DASPEI fluorescent intensity. Nickel showed an increase in ototoxicity at higher concentrations over a range of 0, 2.5, 7.5, 10 and 15µM **(Figure 5A)**. Due to high variability at 5 and 7.5µM a concentration of 10µM was chosen going forwards. Both platinum compounds tested showed significant ototoxicity at the concentrations tested **(Figure 5B)**. $PtCl_2$ appears more toxic than $PtCl_4$ with there being almost total loss of neuromast score at 5, 7.5 and 15µM. DASPEI staining was still observable at 5µM $PtCl_4$ and to some extent at 7.5µM, demonstrating a relatively clear dose-response relationship. These results demonstrate the high potential ototoxicity of group 10 transition metals on the zebrafish PLL. None of the metal compounds appeared to cause any overt toxicity to the zebrafish larvae outside of ototoxicity. All zebrafish larvae appeared to remain healthy with no observable impact on development at the concentrations tested **(Data not shown)**.

### 4.2.2 TLR4 syntagonists reduce nickel toxicity, but not platinum toxicity:

TLR4 syntagonists showed a reduction in cisplatin toxicity as seen in Chapter 3 of this thesis. Syntagonists 134 and 136 showed the most promising protective effects to cisplatin toxicity at concentrations as low as 5µM. Neither syntagonist 134 nor 136 showed protective effects towards nickel toxicity **(Figure 6)**. Interestingly at 5µM, syntagonist 134 appeared to

significantly increase nickel toxicity compared to fish treated with just nickel alone **(Figure 6A, p = 0.003)**. This is reminiscent of the increase in CIO caused by TAK-242 and syntagonists 120 and 132. There was a similar trend with syntagonist 136 increasing nickel toxicity however this was not significant **(Figure 5B)**. Syntagonists 138 and 150 both showed a significant decrease in nickel ototoxicity compared to fish exposed to nickel alone **(Figure 6B)**.

While these results show a statistically significant increase in neuromast score there was a large variability in the results, further experiments to verify the results are needed to confirm the reliability of the TLR4-dependent protective effects against nickel ototoxicity. In another follow up experiment comparing a larger number of syntagonists 138 did not show an increase in neuromast score **(Figure 6)**. There was however a significant increase in neuromast score after co-treatment with syntagonist 166 **(p = 0.02)**, 168 **(p = 0.00004)** and 170 **(p = < 0.000001, Figure 6)**. Unfortunately, the reliability of these results is compromised due to the low neuromast scores seen in untreated wild-type controls. Reasons for this drop in neuromast score will be discussed later.

Platinum ototoxicity was not prevented by syntagonist 134. Both $PtCl_2$ and $PtCl_4$ showed complete loss of neuromast score at 7.5µM, even after co-treatment of 20µM 134 **(Figure 8)**. This result would suggest that while 134 is effective at preventing CIO it is not able to reduce ototoxicity from platinum or nickel. However, while a high concentration of 20µM 134 was used it may be that the concentration of both $PtCl_2$ and $PtCl_4$ was too high and overwhelmed any protective effects there may have been.

## 4.3 Chapter 4 Discussion:

TLR4 is an important component of the innate immune response. In mammals aberrant TLR signalling, and expression has been linked with both cancer pathology (R. Li et al. 2019) and hypersensitivity to metals such as nickel (Schmidt et al. 2010). Nickel can also result in

TLR4 activation and an increase in oxidative stress resulting in increased metastatic potential of lung cancer cells (Xu et al. 2011). Other commonly used animal models such as mice do not naturally show hypersensitivity to nickel or TLR4 activation after nickel exposure (Peana et al. 2017) unless pre-sensitised through the use of TLR4 agonists such as LPS (Sato et al. 2007). Pre-sensitising models using agonists such as LPS do not accurately model nickel induced TLR4 signalling resulting in hypersensitivity in humans. Additional models could help support those currently in use to help establish pathways and treatments against nickel induced hypersensitivity and oxidative stress. Better understanding of the zebrafish Tlr4 signalling pathway through identification of their canonical ligand(s) may help provide an additional *in vivo* model in which to investigate the role of TLRs in cancer progression, auto-immune disease, and functions of the innate immune system. Currently, ligands for zebrafish Tlr4ba and Tlr4bb are yet to be identified. As both morpholino knockdown of *tlr4ba* and *tlr4bb,* and syntagonist inhibition, prevented CIO in zebrafish this led to the hypothesis that group 10 transition metals may be a ligand for zfTlr4 or cause zfTlr4 signalling.

Ototoxicity of $NiCl_2$, $PtCl_2$ and $PtCl_4$ towards zebrafish PLL neuromasts was first confirmed. Both $PtCl_2$ and $PtCl_4$ showed higher ototoxicity compared to $NiCl_2$ **(Figure 5)** and cisplatin **(Chapter 3)**. $NiCl_2$ had a more obvious concentration dose response ototoxicity curve at the concentrations tested **(Figure 5A)**. Synthetic TLR4 antagonists derived from TAK-242 were used to reduce metal ion ototoxicity of either $NiCl_2$, $PtCl_2$ or $PtCl_4$. Several syntagonists were able to significantly reduce ototoxicity caused by $NiCl_2$. Syntagonists 138, 150, 166, 168, 170 all resulted in significantly higher neuromast scores in treated fish compared to $NiCl_2$ alone signalling an increase in active mitochondria and neuromast cell health (**Figure 6B, Figure 7)**. These preliminary results show promise that like human TLR4, nickel causes activation of the Tlr4ba and Tlr4bb signalling pathways.

Significant further work is needed to establish the hypothesis that zfTlr4s do react to nickel. In a follow up experiment this increase in neuromast score through 138 was not replicated **(Figure 7)**. In addition, the reliability of the experiment in **Figure 7** must be called into question due to the surprising reduction in neuromast score in the control group. Zebrafish larvae that were not treated with $NiCl_2$ or syntagonists had reduced DASPEI fluorescence and neuromast scores between 2 and 5 which would ordinarily suggest significant ototoxicity. In previous experiments, including those in Chapter 3, the untreated group neuromasts consistently all scored the maximum of 10. The only exception of this is seen in **Figure 6B** where one fish scored 8 and a second fish scored 9.5. The reasons for the drop in neuromast score in the control group are not apparent. Age of the DASPEI reagent is unlikely to be a factor due to other experiments over a similar timespan not being affected. As DASPEI fluorescence is dependent on active mitochondria and therefore to some extent the physical activity of the zebrafish, it may be by chance reduced movement resulted in reduced uptake of DASPEI and mitochondrial activity resulting in reduced fluorescence. Nevertheless, particularly for syntagonists 168 and 170 the neuromast score is significantly higher than fish treated with 10μM $NiCl_2$ in other experiments where there was no concern surrounding the control group. These results therefore promote optimism that nickel causes activation of Tlr4ba and Tlr4bb signalling pathways.

As platinum is the primary active agent of cisplatin, $PtCl_2$ and $PtCl_4$ were used to investigate whether platinum could stimulate Tlr4ba and Tlr4bb signalling. Syntagonist 134 caused a significant reduction in CIO at concentrations of 5 or 20μM **(Chapter 3)**. Co-exposure of syntagonist 134 with 7.5μM $PtCl_2$ or $PtCl_4$ did not reduce ototoxicity caused by platinum in 6-7dpf zebrafish **(Figure 8)**. This may be due to the concentration of 7.5μM of $PtCl_2$ and $PtCl_4$ being too high, so any potential preventative effects were overwhelmed. While there was a complete loss of neuromast score caused by $PtCl_2$ between 5 and 10μM, $PtCl_4$ did not

show complete loss of score at 5 or 7.5μM **(Figure 5B)**. This would suggest that if syntagonist 134 did have protective effects against $PtCl_4$ ototoxicity it should have had some noticeable effect, especially at a syntagonist concentration of 20μM. As syntagonist 134 had no beneficial effect on $NiCl_2$ ototoxicity, and in fact may have increased ototoxicity **(Figure 6A)** a similar increase in ototoxicity may also be seen after co-treatment with $PtCl_2$ and $PtCl_4$. These results therefore do not show that Tlr4ba and Tlr4bb signalling is initiated by platinum. Lower concentrations of both $PtCl_2$ and $PtCl_4$ in combination with syntagonist 134 will be needed to confirm both either a lack of effectiveness or an increase in platinum mediated toxicity. Further experiments using additional syntagonists or morpholino knockdown of *tlr4ba* and *tlr4bb* will also help demonstrate whether Tlr4ba and Tlr4bb mediate platinum ototoxicity.

## 4.4 Chapter 4 future directions and concluding remarks:

While the ligands for several zebrafish Tlrs have been identified **(Table 1)**, those for Tlr4ba and Tlr4bb are still unknown. The intracellular Tlr pathway in zebrafish is highly conserved between mammals. Based on the identification of TLR4 as a mediator for CIO, the possibility of group 10 transition metals being a ligand for zebrafish Tlr4ba and Tlr4bb was investigated. Syntagonists derived from TAK-242 were able to reduce ototoxicity of $NiCl_2$, but not $PtCl_2$ or $PtCl_4$. Syntagonists 138, 150, 166, 168 and 170, which did not appear to influence CIO or have yet to be tested did show a significant reduction in ototoxicity when co-treated with $NiCl_2$ as observed by an increase in neuromast scoring. The reliability of these results is uncertain due to complications with the control group. Nevertheless, these preliminary results show that Tlr4ba and Tlr4bb may react to group 10 transition metals, particularly nickel. More work will need to be done to establish whether these is a genuine ligand/receptor interaction or due to the inherent reactive nature of metal ions.

Future experiments will continue to test additional syntagonists as they become available and confirm the results of those tested within this chapter. Morpholino knockdown of *tlr4ba* and *tlr4bb* may provide insight into whether one or both zfTlr4s are responsible for mediating metal ion ototoxicity. CRISPR knockout of *tlr4ba* and *tlr4bb* will also allow the observation of how much, if at all, the ZfTlr4s respond to metal ions. The context of how the results presented within this chapter relate to the results for CIO in chapter 3 will be discussed in **Chapter 5.**

## 4.5 Chapter 4 Materials and Methods:

### 4.5.1 Animal ethics and zebrafish husbandry:

Zebrafish were kept at the University of Alberta following a 14:10 light/dark cycle at 28°C cycle as previously described (Westerfield 2000). They were raised, bred, and maintained following an institutional Animal Care and Use Committee approved protocol AUP00000077, operating under guidelines set by the Canadian Council of Animal Care.

### 4.5.2 Assessing nickel and platinum toxicity in larval zebrafish:

Wildtype (AB strain) zebrafish were grown to 6 days post fertilization (dpf) in standard E3 embryo media (Westerfield 2000) and were bath treated with either 0, 5, 7.5. 10, 15µM of nickel chloride hexahydrate (Sigma-Aldrich 654507), platinum(II) chloride (Sigma-Aldrich 520632) or platinum(IV) chloride (Sigma-Aldrich 379840) in 6-well plates, with 10-15 zebrafish larvae per well. Nickel and platinum IV were dissolved in endotoxin-free water (HyClone, SH30529.02), platinum II was dissolved in DMF. After a 20-hour incubation with metal ion salt solutions at 28ºC, wells were washed with embryo media before the fish were incubated in media containing 0.01% 2-[4-(dimethylamino) styryl]-1-ethylpyridinium iodide (DASPEI, Sigma-Aldrich) to stain for neuromast mitochondrial activity for 20 minutes. Wells were washed again in embryo media and zebrafish larvae anaesthetized with 4% tricaine. Experiments utilising TAK-242 derived syntagonists followed the above protocol, with a 1-hour pre-treatment of syntagonist before the addition of metal ion solutions. When metal ion solutions were added, each well was first washed and fresh syntagonist was added alongside the metal ion solutions. Neuromasts were imaged under a Leica M165 FC dissecting microscope equipped with a fluorescent filter. A standard scoring method for zebrafish hair cell viability was used (Chowdhury et al. 2018): five posterior lateral line (PLL) neuromasts for each fish were assigned a score representing cell viability based on DASPEI fluorescent intensity (2 for no noticeable decline, 1.5 for minor decline, 1 for moderate decline, 0.5 for severe decline and 0 for complete loss of fluorescent intensity).

These five scores were summed for each individual (10= all hair cells appear normal and viable; 0=intense ototoxicity).

### 4.5.3 Multiple alignments and statistical analyses

Neuromast scores were analysed via one-way ANOVA with Tukey's multiple comparisons test. Statistical tests were carried out using R version 4.0 and graphs were constructed using the 'ggplot2' and 'tidyverse' group of R packages (Wickham et al. 2019; Wickham 2016; R Core Team 2020). Multiple alignments were done using CLUSTAL Omega, and sequence identity and similarity calculated using EMBOSS needle pairwise sequence alignment (Madeira et al. 2019). Sequence analysis was performed using NCBI Refseq: NP_003257.1 (human TLR4), NP_001124523.1 (Tlr4ba), NP_001315534.1 (Tlr4al) and NP_997978.2 (Tlr4bb).

### 4.5.4 KEGG Pathway Analysis

The TLR KEGG pathway was accessed from the KEGG database (M Kanehisa and Goto 2000; Minoru Kanehisa 2019; Minoru Kanehisa et al. 2021).

## 4.6 Chapter 4 Tables and Figures

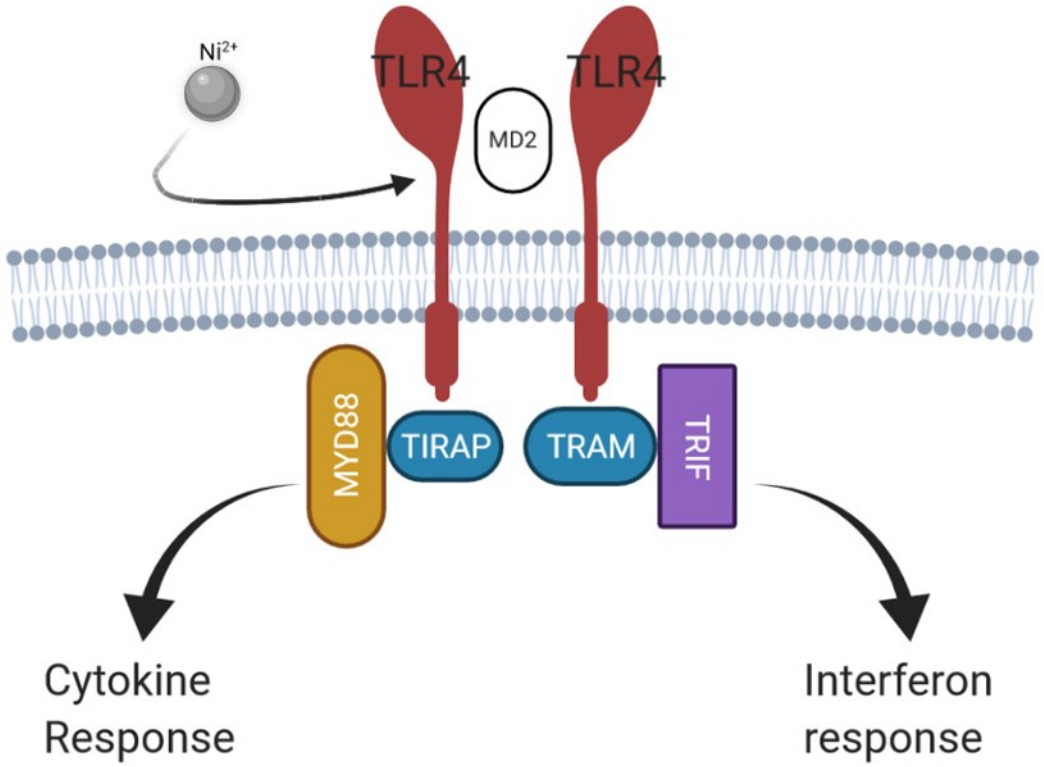



Figure 4.1:

Simplified schematic of nickel interaction with human TLR4. $Ni^{2+}$ binds to histidine residues at positions 456 and 458. Upon $Ni^{2+}$ binding TLR4 homodimerization occurs and TRAM/TRIF signalling leads to an increase in NF-κβ pathways. This signalling pathway requires MD2, though occurs through mechanisms distinct from LPS binding to TLR4.

```
HumanTLR4      -MMSASRLAGTLIPAMAFLSCVRPESWEPCVEVVPNITYQCMELNFYKIPDNLPFSTKNL     59
Tlr4bb         MIMSN---GERMIFLSSIFILVNAGQGQECTELIKNKEYSCSGRNLTCIPGSLPFSVASL     57
zfTlr4ba       ------------------------------------------------------------      0
zfTlr4al       ---------MNFFTISAFIIYFPIGAGQSCTEIIENLHYSCMGRNLSYIPSRIPSSVQTL     51

HumanTLR4      DLSFNPLRHLGSYSFFSFPELQVLDLSRCEIQTIEDGAYQSLSHLSTLILTGNPIQSLAL     119
Tlr4bb         DFSFNFLTSLHKRVFPVMLNLQLLDLTRCYIRQIEKDAFYNVKNLMTLILTGNPITYLAP     117
zfTlr4ba       ------------------MLNVFYLNFRCHIRQIENDAFYNVKNLTTLFLTGNPIIYFAP     42
zfTlr4al       DFSFNDLKWLKKTVFPVFTFLRVLDLSRCHIRQIENDAFYNVKNLTTLFLTGNPIIYFAP     111
                                  .    ** *: **..*: .:.:* **:******  :*

HumanTLR4      GAFSGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEH     179
Tlr4bb         ECLNSLYKLQRLVLVDVRLESLQ-LQINNLTKLQDLKVGTNCIQSMTLPSFMSTFKDFSL     176
zfTlr4ba       GCLNTLYNLQRLVLVDIGLESLQ-LNINNLTKLQELNVGTNYIQSMTLPPFMTTFKDFSL     101
zfTlr4al       GCLNTLYNLQRLVLVDIGLESLQ-LNINNLTKLQELNVGTNYIQSMTLPPFMSTFKDFSL     170
                .:. * .**:** *:  * **: : *.:*..*::*:*. * ***:.** :::.:.::.

HumanTLR4      LDLSSNKIQSIYCTDLRVLHQMPLLNLSLDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDS     239
Tlr4bb         LDLHANNISIIRMDHTAVLRE-IGRNMTLILSRNPLIHIEPGAFKDVILRELHLLAAFIS     235
zfTlr4ba       LDLHANNISIIRTDHTVVLRE-IGRNMTLILTWNPLLHIEPGAFKDVYLRQLDIRSAFVS     160
zfTlr4al       LDLHANNISIIRTNHTVVLRE-IGKNMTLILSRNPLLHIEPGSFKGVHLVELDIRSTFVS     229
               *** :*:*. *   .  **::    *::* *: **: .*:**:** : * :* :   * *

HumanTLR4      LNVMKTCIQGLAGLEVHRLVLGEFRNEGNLEKFDKSALEGLCNLTIEEFRLAYLDYYLDD     299
Tlr4bb         FNAQKECHKALTGLTVDKLFVGRYRMDEKIKVSVPDYLEGLCSINFNEIYL-VQKEWSDS     294
zfTlr4ba       FSAQKAALKALHGLNVKRLIFGKYREDNGFHFVDNDVLDGLCCFNFQEVSY-YVLESAKT     219
zfTlr4al       FASKKQGLNGLHGLNVTRLMFGMYKDDPKLYPSDLDYFDGLCSIHFYEAYY-YMKERLDW     288
               :   *   :.* ** * :*..* :: :  :     . ::*** : : *          .

HumanTLR4      IIDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKL---KSLKRL     356
Tlr4bb         EMHLFRCMVNATKITIKKAYMNSMKHIPFH-RLKELYLSDTGLSVVPF--ISHIPSLEKL     351
zfTlr4ba       TIAIFRCMINATRITVKGGNIFKMETVHFH-KTKELYLINNGLGTLPTKQLSHLHTLEKL     278
zfTlr4al       KMNIFRCMINATVVVVKGGVIRVIGYVPFH-KIKELYLINTQLYTVPGKQLSHIRTLEKF     347
                : :*.*: *.: . :    :  :  . ::   :.* * :  :  .*   :    :*:::

HumanTLR4      TFTSNKGG-NAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMS     415
Tlr4bb         VMKS-PFPITFTGVSDLPLLQYVDLSGNMLILHECCSILFPRTPNIQYLNLSQNSEITFV     410
zfTlr4ba       EITHNSEPIFAEPFTDLPKLQYVDLSDNQLKIKHCCSTLLSGTPQINYLNLSLNSEISVD     338
zfTlr4al       VFTHNSAT-QVEKFLDMPKLQYVDLNSNQITLQSCCIDVLSGTPQIRYLNLSLNPQISLD     406
                :.            *:* *:::**. * : :: **      * .:.**:** *  *:.

HumanTLR4      -SNFLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLE     474
Tlr4bb         NEPFSALDLLEVLDFHHTKLVIVFYFGFFKHLRNLKYLDISYTRVHFN-TLTFQDLHNLT     469
zfTlr4ba       VGGFEGLDSLEILDFSYTRVVRIGYLSVLSNLKNLRYLDVSYSSVTFSNIFCFLGLSSLN     398
zfTlr4al       KGGFEGLESLEILDFHHTKLLGIGSFTLLSNLKNLRYLDISYSSVTFVNVYCFYGLSSLK     466
                  * .*: ** *** ::.:  :  : .:  *:** ***:*:: . .    * .* .*

HumanTLR4      VLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFS     534
Tlr4bb         VLKMAGNSFSGDKLSYFLQNLTSLEVLDISQCGIEKVSMRSFTGTQKLRHLYLSRNKLMV     529
zfTlr4ba       VLKMAGNNFQGDVAKYIFNNLTLLEHLDMSFCHLVELHTSSFKYLQRLRHLNVKGNYLIK     458
zfTlr4al       VLKMAGNSFQGDVANYLFNNLTFLEHLDISYCHVIEIHLTSFKNLQRLRHLNLRGNNLMS     526
               *******.*. :    :: :*  *  **:* * : ::   :*.  . .*: * :  * ::

HumanTLR4      LDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQWI     594
Tlr4bb         LDFLTQPELTHLTSVYIDKNSITTIPLDVLQKLPMNLSEFDLSSNSIDCSCSQTDFILWI     589
zfTlr4ba       IDFLTHPNLKQLTSFYVEKNSITAIPLHVLKNLPMNLSEFDLSFNPIDCSCSQTDFMLWI     518
zfTlr4al       IDFLTDPNLKQLTTFYVNKNSITTIPLDILQKLPMNLSEFDLSFNPIDCSCSQTDFMLWI     586
               :* :    *. *  .  . * * :   . *:::* .*: ::*: * : *:*.: .*: **

HumanTLR4      KDQRQLLVEVERMECATPSDKQGMPVLSLNITCQM--NKTIIGVSVLSVLVVSVVAVLVY     652
Tlr4bb         IQKQNILKQLENIRCKTFSANTDFKAIDFDIDYCVHKKRLTIVLSVICVTFVVVLAILLY     649
zfTlr4ba       INNQKVLKQPENILCKTISPNSDFRVTDFDIDHCVYKKKLIIVLPVFCVVFIVVLSILVY     578
zfTlr4al       INNQRVLKQPENILCKTISPNSDFRVTDFDIDHCVYKKKLIIALLVFCVVFIVVLSILVY     646
               :::.:* : *.: * * * : .: . .::*   :  ::  * : *:.* .: *:::*:*

HumanTLR4      KFYFH----LMLLAGCIKYGRGENIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLH     708
Tlr4bb         KFWFYVQYCFILFSGYRSPGQQECSYDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLH     709
zfTlr4ba       RFQFYLRYCWILLRGYRSPGQQECSYDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLH     638
zfTlr4al       RFQFYLRYCWILLRGYRSPGQQECSYDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLH     706
               :* *:     :*: *  . *: *  ******:** ** ** ***::***:****:*****

HumanTLR4      YRDFIPGVAIAANIIHEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIF     768
Tlr4bb         MRDFQAGKSIASNIIDEGIMGSRKIIVVVSQHFIDSSWCRFEFELAQSRFLMERNANIII     769
zfTlr4ba       MRDFQAGKSIASNIIDEGIMGSRKIIVVVSQHFIASAWCRFEFELAQSRFLMERNANIII     698
zfTlr4al       MRDFQAGKSIASNIIDEGIMGSRKIIVVVSQHFIDSAWCRFEFELAQSRFVVERNANIII     766
                ***  * :**:***.**:  ***:********* * ** **:*:**:  .:. .*.**:

HumanTLR4      IVLQKVEKTLLRQQVELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEGTVG     828
Tlr4bb         IILEDVAERKTKKVFGLHKHLKKNTYLKWSRDPLSNMRFWIRLRKAILQK----------     819
zfTlr4ba       IILEDVAERKTKKILGLHKHLKKNTYLKWSRDPLSNMRFWIRLRKAIVATKQ--------     750
zfTlr4al       IILEDVAERKTKKVLGLHKHLKKNTYLKWSRDPLSNMRFWIRLRKAIVAT----------     816
               *:*:.* :   :: . *:: *.:****:*. . *..  ** ******::

HumanTLR4      TGCNWQEATSI      839
Tlr4bb         -----------      819
zfTlr4ba       -----------      750
zfTlr4al       -----------      816
```

| Identity Sequence | Human TLR4 | Tlr4ba | Tlr4al | Tlr4bb |
|---|---|---|---|---|
| Human TLR4 | 100 | 34 | 37 | 37 |
| Tlr4ba | 35 | 100 | 73 | 60 |
| Tlr4al | 35 | 73 | 100 | 65 |
| Tlr4bb | 35 | 60 | 60 | 100 |

| Gene | AA Length |
|---|---|
| Human TLR4 | 839 |
| Tlr4ba | 750 |
| Tlr4al | 816 |
| Tlr4bb | 819 |

| Similarity Sequence | Human TLR4 |
|---|---|
| Human TLR4 | 100 |
| Tlr4ba | 49 |
| Tlr4al | 54 |
| Tlr4bb | 52 |

Figure 4.2:

Top: Clustal Omega multiple amino acid sequence alignment of human TLR4 with zebrafish Tlr4ba, Tlr4al and Tlr4bb. * denotes amino acid identity, : and . denote varying degrees of similarity. Bottom: Table showing the size of Tlr4 protein in amino acids, the amino acid sequence identity between human TLR4 and zebrafish Tlr4ba, Tlr4al and Tlr4bb and the amino acid sequence similarity between human TLR4, zebrafish Tlr4ba, Tlr4al and Tlr4bb. Sequence identity and similarity was calculated using EMBOSS needle sequence alignment.

```
HumanTLR4TIR      YDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIAANIIHEGFHKSRKV  60
Tlr4baTIR         YDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLHMRDFQAGKSIASNIIDEGIMGSRKI  60
Tlr4alTIR         YDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLHMRDFQAGKSIASNIIDEGIMGSRKI  60
Tlr4bbTIR         YDAFVIFSSYDEAWVMNELMENLENGVPPIQLCLHMRDFQAGKSIASNIIDEGIMGSRKI  60
                  ******:** ** ** ***::***:****:***** ***  * :**:***.**:  ***:

HumanTLR4TIR      IVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKVEKTLLRQQVELYRLLSRNT  120
Tlr4baTIR         IVVVSQHFIASAWCRFEFELAQSRFLMERNANIIIIILEDVAERKTKKILGLHKHLKKNT  120
Tlr4alTIR         IVVVSQHFIDSAWCRFEFELAQSRFVVERNANIIIIILEDVAERKTKKVLGLHKHLKKNT  120
Tlr4bbTIR         IVVVSQHFIDSSWCRFEFELAQSRFLMERNANIIIIILEDVAERKTKKVFGLHKHLKKNT  120
                  ********* * ** **:*:**:  .:. .*.**:*:*:.* :   :: . *:: *.:**

HumanTLR4TIR      YLEWEDSVLGRHIFWRRLRKAL        142
Tlr4baTIR         YLKWSRDPLSNMRFWIRLRKAI        142
Tlr4alTIR         YLKWSRDPLSNMRFWIRLRKAI        142
Tlr4bbTIR         YLKWSRDPLSNMRFWIRLRKAI        142
                  **:*. . *..  ** *****:
```

| Identity Sequence | Human TLR4 | Tlr4ba | Tlr4al | Tlr4bb |
|---|---|---|---|---|
| Human TLR4 | 100 | 55 | 55 | 55 |
| Tlr4ba | 55 | 100 | 97 | 97 |
| Tlr4al | 55 | 97 | 100 | 97 |
| Tlr4bb | 55 | 97 | 97 | 100 |

| Gene | AA Length |
|---|---|
| Human TLR4 | 142 |
| Tlr4ba | 142 |
| Tlr4al | 142 |
| Tlr4bb | 142 |

| Similarity Sequence | Human TLR4 |
|---|---|
| Human TLR4 | 100 |
| Tlr4ba | 70 |
| Tlr4al | 70 |
| Tlr4bb | 70 |

Figure 4.3:

Top: Clustal Omega multiple amino acid sequence alignment of the intracellular TIR domain of human TLR4 with zebrafish Tlr4ba, Tlr4al and Tlr4bb. * denotes amino acid identity, : and . denote varying degrees of similarity. Bottom: Table showing the size of the Tlr4 TIR domain in amino acids, the amino acid sequence identity between human TLR4 and zebrafish Tlr4ba, Tlr4al and Tlr4bb and the amino acid sequence similarity between human TLR4, zebrafish Tlr4ba, Tlr4al and Tlr4bb TIR domains. Sequence identity and similarity was calculated using EMBOSS needle sequence alignment.

```
HumanTLR4Extra      ----------------------------------------------------STKNLDLS        8
Tlr4bbExtra         MIMSNGERMIFLSSIFILVNAGQGQECTELIKNKEYSCSGRNLTCIPGSLPFSVASLDFS        60
Tlr4baExtra         ------------------------------------------------------------        0
Tlr4alExtra         ------MNFFTISAFIIYFPIGAGQSCTEIIENLHYSCMGRNLSYIPSRIPSSVQTLDFS        54

HumanTLR4Extra      FNPLRHLGSYSFFSFPELQVLDLSRCEIQTIEDGAYQSLSHLSTLILTGNPIQSLALGAF        68
Tlr4bbExtra         FNFLTSLHKRVFPVMLNLQLLDLTRCYIRQIEKDAFYNVKNLMTLILTGNPITYLAPECL        120
Tlr4baExtra         --------------MLNVFYLNFRCHIRQIENDAFYNVKNLTTLFLTGNPIIYFAPGCL        45
Tlr4alExtra         FNDLKWLKKTVFPVFTFLRVLDLSRCHIRQIENDAFYNVKNLTTLFLTGNPIIYFAPGCL        114
                                    .    ** *: **..*: .:.:* **:******  :*  .:

HumanTLR4Extra      SGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEHLDL        128
Tlr4bbExtra         NSLYKLQRLVLVDVRLESLQ-LQINNLTKLQDLKVGTNCIQSMTLPSFMSTFKDFSLLDL        179
Tlr4baExtra         NTLYNLQRLVLVDIGLESLQ-LNINNLTKLQELNVGTNYIQSMTLPPFMTTFKDFSLLDL        104
Tlr4alExtra         NTLYNLQRLVLVDIGLESLQ-LNINNLTKLQELNVGTNYIQSMTLPPFMSTFKDFSLLDL        173
                    . * .**:** *:  * **: : *.:*..*::*:*. * ***:.** :::.:.::. ***

HumanTLR4Extra      SSNKIQSIYCTDLRVLHQMPLLNLSLDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDSLNV        188
Tlr4bbExtra         HANNISIIRMDHTAVLRE-IGRNMTLILSRNPLIHIEPGAFKDVILRELHLLAAFISFNA        238
Tlr4baExtra         HANNISIIRTDHTVVLRE-IGRNMTLILTWNPLLHIEPGAFKDVYLRQLDIRSAFVSFSA        163
Tlr4alExtra         HANNISIIRTNHTVVLRE-IGKNMTLILSRNPLLHIEPGSFKGVHLVELDIRSTFVSFAS        232
                     :*:*. *   .  **::    *::* *: **: .*:**:** : * :* :   * *:

HumanTLR4Extra      MKTCIQGLAGLEVHRLVLGEFRNEGNLEKFDKSALEGLCNLTIEEFRLAYLDYYLDDIID        248
Tlr4bbExtra         QKECHKALTGLTVDKLFVGRYRMDEKIKVSVPDYLEGLCSINFNEIYL-VQKEWSDSEMH        297
Tlr4baExtra         QKAALKALHGLNVKRLIFGKYREDNGFHFVDNDVLDGLCCFNFQEVSY-YVLESAKTTIA        222
Tlr4alExtra         KKQGLNGLHGLNVTRLMFGMYKDDPKLYPSDLDYFDGLCSIHFYEAYY-YMKERLDWKMN        291
                     *   :.* ** * :*..* :: :  :     . ::*** : : *          .  :

HumanTLR4Extra      LFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKL---KSLKRLTFT        305
Tlr4bbExtra         LFRCMVNATKITIKKAYMNSMKHIPFH-RLKELYLSDTGLSVVPF--ISHIPSLEKLVMK        354
Tlr4baExtra         IFRCMINATRITVKGGNIFKMETVHFH-KTKELYLINNGLGTLPTKQLSHLHTLEKLEIT        281
Tlr4alExtra         IFRCMINATVVVVKGGVIRVIGYVPFH-KIKELYLINTQLYTVPGKQLSHIRTLEKFVFT        350
                    :*.*: *.: . :    :  :  . ::   :.* * :  :  .*   :    :*::: :.

HumanTLR4Extra      SNKGG-NAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMS-SN        363
Tlr4bbExtra         S-PFPITFTGVSDLPLLQYVDLSGNMLILHECCSILFPRTPNIQYLNLSQNSEITFVNEP        413
Tlr4baExtra         HNSEPIFAEPFTDLPKLQYVDLSDNQLKIKHCCSTLLSGTPQINYLNLSLNSEISVDVGG        341
Tlr4alExtra         HNSAT-QVEKFLDMPKLQYVDLNSNQITLQSCCIDVLSGTPQIRYLNLSLNPQISLDKGG        409
                              *:* *:::**. * : :: **      * .:.**:** *  *:.

HumanTLR4Extra      FLGLEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLK        423
Tlr4bbExtra         FSALDLLEVLDFHHTKLVIVFYFGFFKHLRNLKYLDISYTRVHFN-TLTFQDLHNLTVLK        472
Tlr4baExtra         FEGLDSLEILDFSYTRVVRIGYLSVLSNLKNLRYLDVSYSSVTFSNIFCFLGLSSLNVLK        401
Tlr4alExtra         FEGLESLEILDFHHTKLLGIGSFTLLSNLKNLRYLDISYSSVTFVNVYCFYGLSSLKVLK        469
                    * .*: ** *** ::.:  :  : .:  *:** ***:*:: . .     * .* .* ***

HumanTLR4Extra      MAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDT        483
Tlr4bbExtra         MAGNSFSGDKLSYFLQNLTSLEVLDISQCGIEKVSMRSFTGTQKLRHLYLSRNKLMVLDF        532
Tlr4baExtra         MAGNNFQGDVAKYIFNNLTLLEHLDMSFCHLVELHTSSFKYLQRLRHLNVKGNYLIKIDF        461
Tlr4alExtra         MAGNSFQGDVANYLFNNLTFLEHLDISYCHVIEIHLTSFKNLQRLRHLNLRGNNLMSIDF        529
                    ****.*. :    :: :*  *  **:* * : ::   :*.  . *: * :  * :: :*

HumanTLR4Extra      FPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQWIKDQ        543
Tlr4bbExtra         LTQPELTHLTSVYIDKNSITTIPLDVLQKLPMNLSEFDLSSNSI----------------        576
Tlr4baExtra         LTHPNLKQLTSFYVEKNSITAIPLHVLKNLPMNLSEFDLSFNPI----------------        505
Tlr4alExtra         LTDPNLKQLTTFYVNKNSITTIPLDILQKLPMNLSEFDLSFNPI----------------        573
                    :    *. *  .  . * * :   . *:::* .*: ::*: * :

HumanTLR4Extra      RQLLVEVERMECATPSDKQGMPVLSLNI        571
Tlr4bbExtra         ----------------------------        576
Tlr4baExtra         ----------------------------        505
Tlr4alExtra         ----------------------------        573
```

| Identity Sequence | Human TLR4 | Tlr4ba | Tlr4al | Tlr4bb |
|---|---|---|---|---|
| Human TLR4 | 100 | 32 | 31 | 31 |
| Tlr4ba | 32 | 100 | 63 | 51 |
| Tlr4al | 31 | 72 | 100 | 57 |
| Tlr4bb | 31 | 58 | 57 | 100 |

| Gene | AA Length |
|---|---|
| Human TLR4 | 571 |
| Tlr4ba | 505 |
| Tlr4al | 573 |
| Tlr4bb | 576 |

| Similarity Sequence | Human TLR4 | Tlr4ba | Tlr4al | Tlr4bb |
|---|---|---|---|---|
| Human TLR4 | 100 | 47 | 47 | 43 |
| Tlr4ba | 47 | 100 | 73 | 61 |
| Tlr4al | 47 | 73 | 100 | 69 |
| Tlr4bb | 43 | 61 | 69 | 100 |

Figure 4.4:

Top: Clustal Omega multiple amino acid sequence alignment of the extracellular leucine rich repeat (LRR) domain of human TLR4 with zebrafish Tlr4ba, Tlr4al and Tlr4bb. * denotes

amino acid identity, : and . denote varying degrees of similarity. Bottom: Table showing the size of the Tlr4 LRR domain in amino acids, the amino acid sequence identity between human TLR4 and zebrafish Tlr4ba, Tlr4al and Tlr4bb and the amino acid sequence similarity between human TLR4, zebrafish Tlr4ba, Tlr4al and Tlr4bb LRR domains. Sequence identity and similarity was calculated using EMBOSS needle sequence alignment.

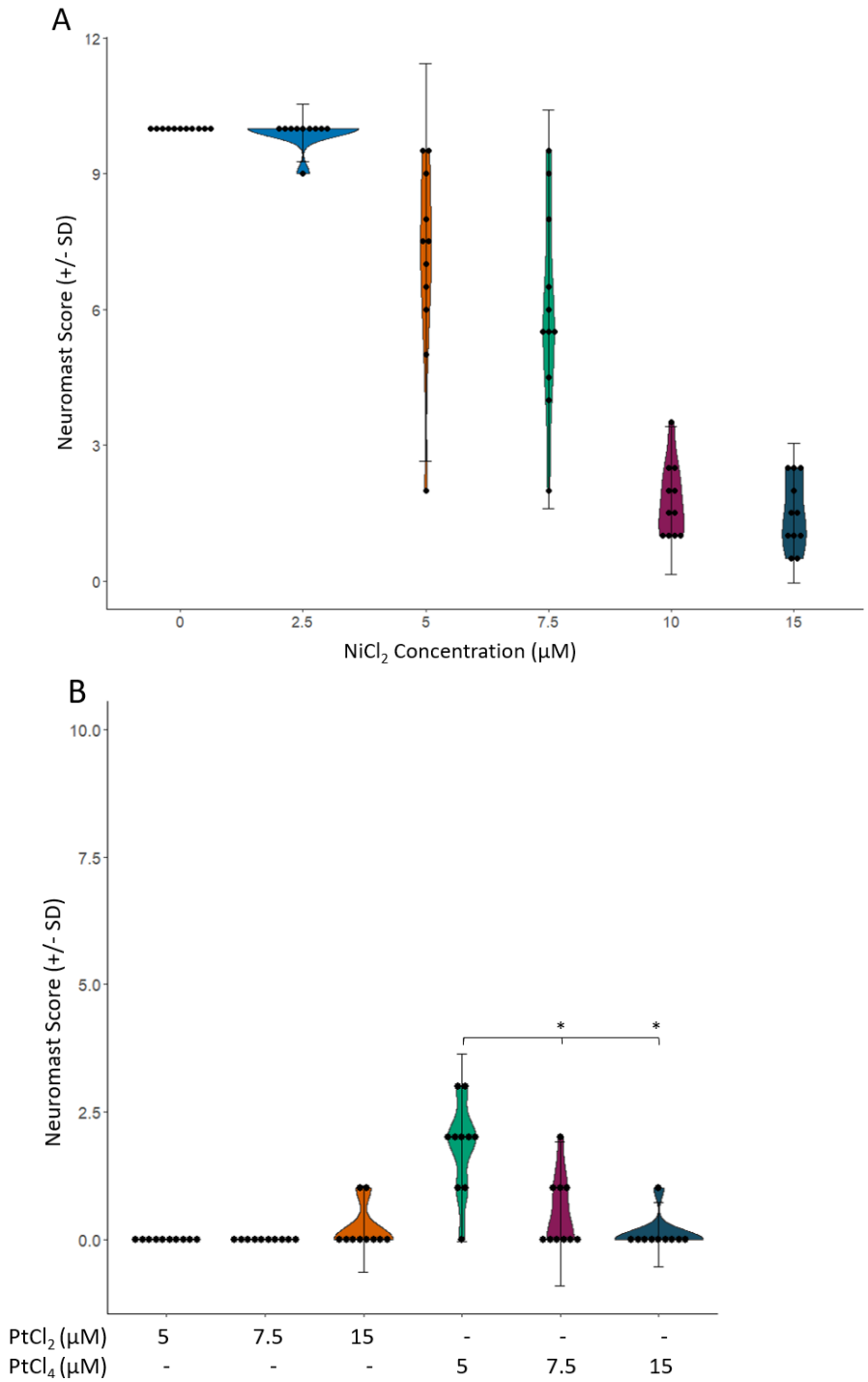



Figure 4.5:

Dose response of nickel, $PtCl_2$ and $PtCl_4$ chloride toxicity in 6-7dpf zebrafish PLL neuromasts. A) There is a significant reduction in PLL neuromast score over increasing concentrations of $NiCl_2$ at 2.5, 5, 7.5, 10 and 15µM. B) PII shows almost a complete loss of neuromast score at 15µM and a total loss of neuromast score at 5 and 7.5µM. PIV shows significant dose dependent toxicity at 5, 7.5 and 15µM though slightly less compared to PII.

Both platinum salts show significantly more toxicity compared to $NiCl_2$. * = $p < 0.0001$ through one-way ANOVA with Tukey HSD. Abbreviations: $PtCl_2$ = platinum (II) chloride, $PtCl_4$ = platinum (IV) chloride, $NiCl_2$ = nickel chloride. SD = standard deviation, dpf = days post fertilisation.

Figure 6:

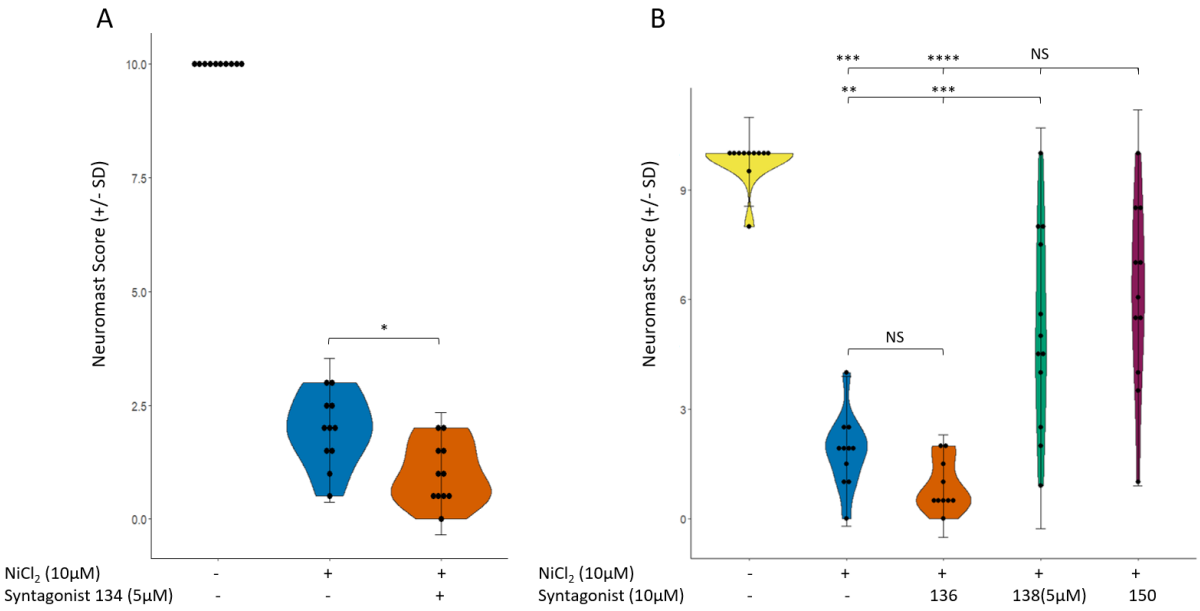



Figure 4.6:

Syntagonist derivatives of TAK-242 reduce or exacerbate nickel chloride toxicity in 6-7dpf zebrafish PLL neuromasts after 20h exposure. A) Syntagonist 134 (orange) shows a significant reduction in neuromast score after 10µM $NiCl_2$ exposure compared to fish only exposed to 10µM $NiCl_2$ alone (blue) or untreated fish. This contrasts with the effects of syntagonist 134 on CIO, where toxicity was reduced. B) After exposure to 10µM $NiCl_2$ syntagonists 138 (green) and 150 (magenta) cause a significant recovery in neuromast score compared to fish treated with just $NiCl_2$ (blue). * = $p < 0.003$, ** = $p < 0.001$, *** = $p < 0.0001$, **** = $p < 0.00001$ through one way ANOVA with Tukey HSD. Abbreviations: $NiCl_2$ = nickel chloride. SD = standard deviation, dpf = days post fertilisation.

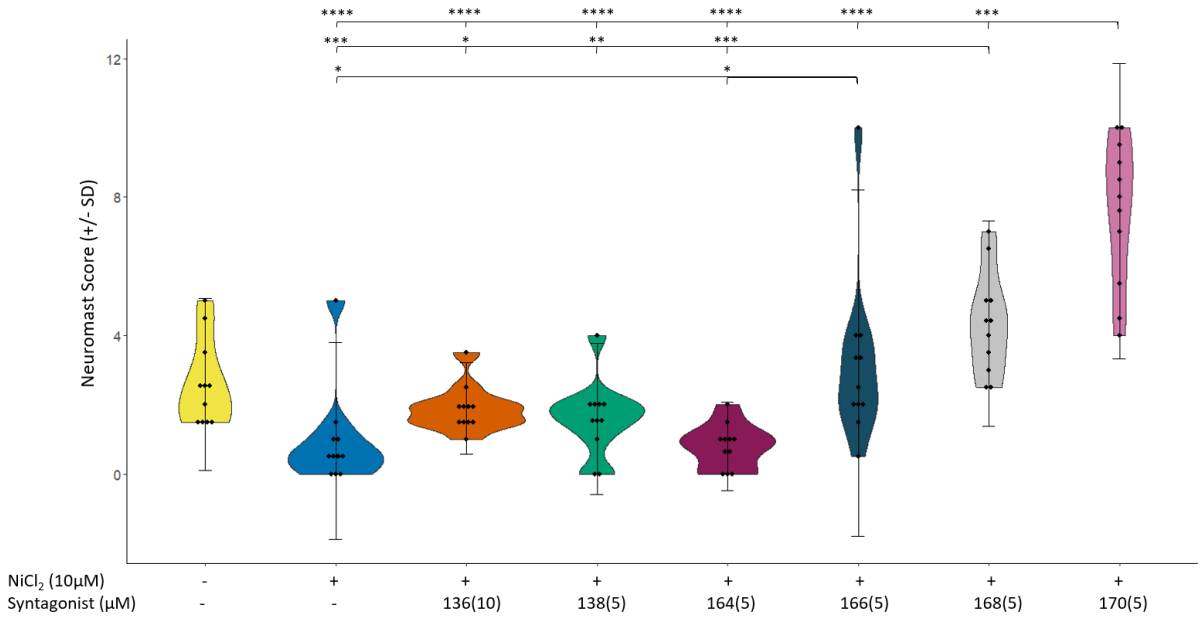



Figure 4.7:

Syntagonist derivatives of TAK-242 reduce nickel chloride toxicity in 6-7dpf zebrafish PLL neuromasts after 20h exposure. A) Syntagonists 166 (dark blue), 168 (grey) and 170 (pink) all show a significant increase in neuromast score after 10µM $NiCl_2$ exposure compared to fish only exposed to 10µM $NiCl_2$ alone (blue) or fish treated with syntagonists 136 (orange), 138 (green) and 164 (magenta). Control fish (yellow) not treated with either $NiCl_2$ or $NiCl_2$ with a syntagonist showed a surprising reduction in neuromast score in the absence of any toxic agent. This may be due to the basal activity and movement of the fish (see 4.2 Results). Repeat experiments are needed to confirm the efficacy of syntagonists in preventing $NiCl_2$ toxicity. * = $p < 0.02$, ** = $p < 0.002$, *** = $p < 0.0002$, **** = $p < 0.000002$ through one way ANOVA with Tukey HSD. Abbreviations: $NiCl_2$ = nickel chloride. SD = standard deviation, dpf = days post fertilisation.

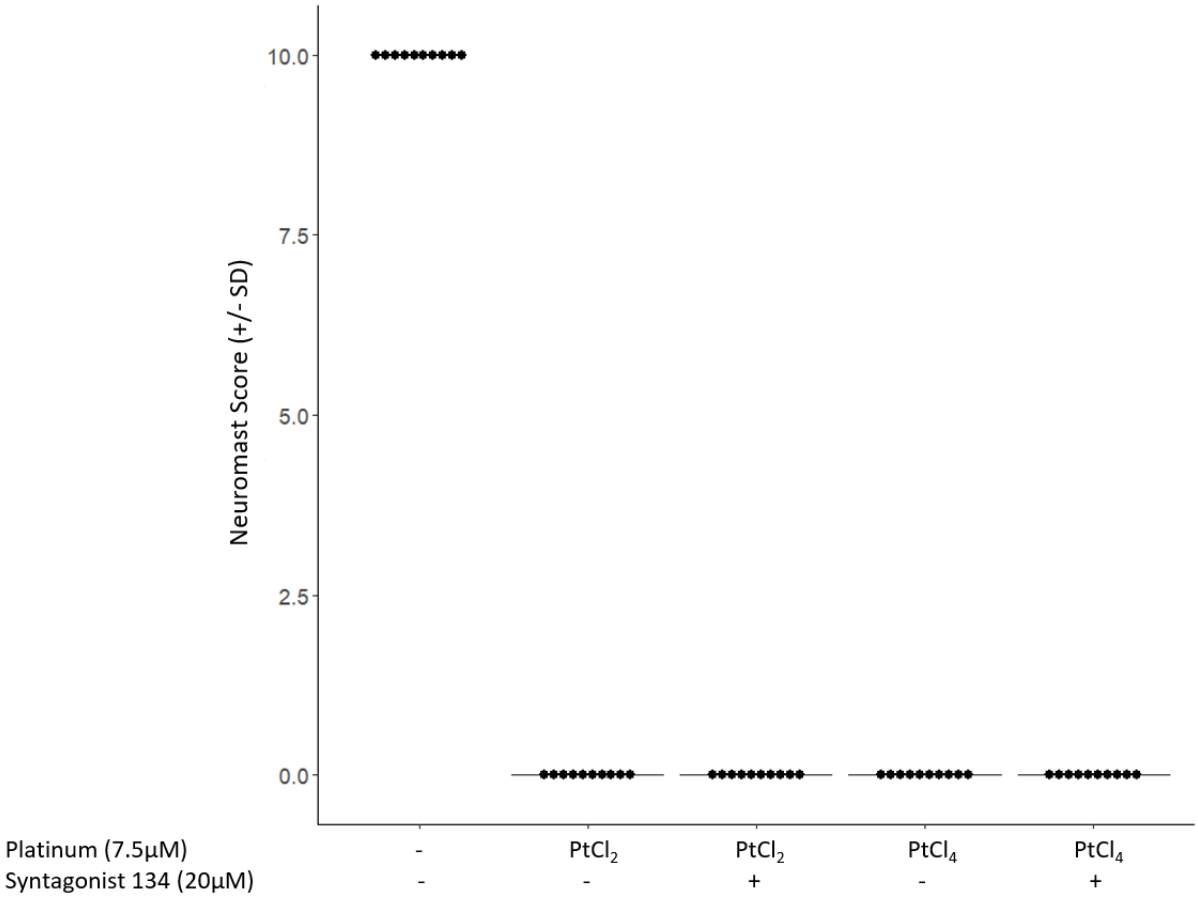



Figure 4.8:

Syntagonist 134 had to effect on either PII or PIV toxicity in 6-7dpf zebrafish neuromasts after 20h. At a concentration of 20µM which caused a significant increase in neuromast score in CIO experiments there was still a complete loss of neuromast score after exposure to 7.5µM of either PII or PIV chloride. Syntagonist 134 also failed to protect against neuromast cell toxicity after exposure to $NiCl_2$ **(Figure 6)**. Other syntagonists which were beneficial against $NiCl_2$ toxicity will need to be tested to see if their protective effects extend to platinum. Abbreviations: $PtCl_2$ = platinum(II) chloride, $PtCl_4$ = platinum (IV) chloride, $NiCl_2$ = nickel chloride. SD = standard deviation, dpf = days post fertilisation.

Table 4.1:

List of zebrafish toll-like receptors and their respective ligands, if known. Table is adapted from information within (Y. Li et al. 2017; H. Chen et al. 2021)

| Zebrafish Tlr | Ligand |
|---|---|
| Tlr1 | Tlr1-Tlr2 heterodimer |
| Tlr2 | Lipopeptides, Pam3CSk4 |
| Tlr3 | Double stranded RNA, polyI:C |
| Tlr4al | Unknown |
| Tlr4ba | Unknown |
| Tlr4bb | Unknown |
| Tlr5a | Flagellin |
| Tlr5b | Flagellin |
| Tlr7 | Unknown |
| Tlr8 | Unknown |
| Tlr8a | Unknown |
| Tlr8b | Unknown |
| Tlr9 | CpG-ODNs |
| Tlr18 | Unknown |
| Tlr19 | Unknown |
| Tlr20a | Unknown |
| Tlr20d | Unknown |
| Tlr21.3 | Unknown |
| Tlr21.1 (Tlr21) | CpG-ODNs |
| Tlr21.2 (Tlr22) | Double stranded RNA, polyI:C |

# Chapter 5: Summary of presented work and future directions:

## Chapter 5 Abstract:

The work presented within this thesis can be split into two distinct parts. First, the physiological functions of prion protein during early development were investigated by taking a transcriptomic approach in zebrafish lacking *prp1* and *prp2*. Ontological analysis showed a considerable overlap in biological processes affected between zebrafish and embryonic mice. Cell adhesion was the most populated process with genes showing a significant reduction in transcript abundance, with 31 of 38 of the genes affected belonging to the protocadherin family. Furthermore, reductions in the transcript abundance of *ncam1a* and *st8sia2* closely match the results found in *in vitro* studies in cells lacking cellular prion protein. KEGG pathway analysis showed the focal adhesion kinase pathway had a significant decrease in transcript abundance of genes which directly interact with the focal adhesion kinase genes. Taken together these results suggest a cross species conserved function of prion protein in the development of organisms.

The second part of this thesis investigates the role of TLR4 in cisplatin induced ototoxicity. Zebrafish have two homologues of mammalian TLR4, Tlr4ba and Tlr4bb. Chemical inhibition and morpholino knockdown of *tlr4ba* and *tlr4bb* were used alongside an established neuromast scoring method to investigate cisplatin induced ototoxicity in a zebrafish model. Inhibition of Tlr4ba and Tlr4bb was done using the TLR4 antagonist, TAK-242 and synthetical derived antagonists, termed syntagonists. Morpholino knockdown was done using morpholinos previously established in literature. Both Tlr4ba and Tlr4bb antagonism or morpholino knockdown led to a significant reduction in cisplatin induced ototoxicity. These results strongly support a role for TLR4 as a mediator for cisplatin induced ototoxicity and provide a potential target for the development of co-treatments to improve cancer treatment.

The ligands for Tlr4ba and Tlr4bb have not yet been identified. The prevention of cisplatin ototoxicity through knockdown or inhibition of Tlr4ba and Tlr4bb led us to hypothesise that they may recognise transition metals. The same model was utilised after zebrafish neuromasts were exposed to nickel or platinum. Syntagonists were able to significantly reduce nickel induced ototoxicity, but not platinum induced ototoxicity. Further experiments with syntagonists will help confirm these results. In addition, morpholino knockdown of *tlr4ba* and *tlr4bb* will help establish if one or the other is more reactive to transition metal signalling. Finally, CRISPR knockout of Tlr4ba and Tlr4bb will be used to create a stable knockout model for the further investigation of zebrafish Tlr4 function and ligand identification.

## 5.1 Part 1: Cellular prion protein plays a conserved cross-species but ultimately dispensable role in the development of organisms:

The cellular prion protein ($PrP^{C}$) has fascinated researchers since the discovery that it can misfold into prion protein scrapie ($PrP^{Sc}$) (S. Prusiner 1982). This misfolding event can lead to a variety of phenotypically distinct, progressive, and incurable neurodegenerative diseases. In humans these include Creutzfeldt–Jakob disease (CJD), Kuru and fatal familial insomnia (FFI) among others. Prion disease in other animals include the eponymous scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD). Importantly, prion diseases are infectious despite being caused by just $PrP^{Sc}$ protein, dubbed 'the protein only' hypothesis. This infectivity can even cross species barriers. While this is rare, and many factors are likely involved, transmission from other animals into humans has occurred. In the late 1980s and early 1990s there was an outbreak of variant-CJD (vCJD) in the United Kingdom linked with eating contaminated beef (Will 2003). Currently there is an increasing prevalence in CWD in North America, with incidences also occurring in Scandinavia and South Korea (Osterholm et al. 2019).

$PrP^{C}$ misfolding into $PrP^{Sc}$ has largely been the focus of prion research since its identification as the cause of scrapie. Currently the normal physiological functions of $PrP^{C}$ are ambiguous. It has been implicated in many diverse functions including circadian rhythm, neuronal differentiation, cell adhesion, metal ion homeostasis and more (Ochs and Málaga-Trillo 2014; Linsenmeier et al. 2017). Increasing focus on identifying $PrP^{C}$ function was renewed after the observation that $PrP^{C}$ can act as a high affinity receptor for soluble amyloid-β oligomers (Aβso) (Um et al. 2012; Larson et al. 2012; Laurén et al. 2009). Aβso are currently thought to be the main toxic species in Alzheimer's disease (AD). The discovery that $PrP^{C}$ may facilitate one of the toxic pathways of AD has raised the possibility that it would be a viable

therapeutic target in AD treatment. Understanding the function of $PrP^C$ would allow better allow for preventing Aβso-$PrP^C$ interaction without preventing $PrP^C$ function.

Definitively attributing function(s) to $PrP^C$ has proved difficult as animal models generated do not appear to show any obvious overt phenotypes after knockout of $PrP^C$ (Fernández-Borges et al. 2015). This is in stark contrast in certain animal models when acute $PrP^C$ knockdown is performed. In zebrafish this has led to developmental arrest and death during gastrulation (Málaga-Trillo et al. 2009). In mice Shadoo protein (Sho), or 'shadow of prion protein' has been proposed to compensate for loss of $PrP^C$. In stable $PrP^C$ knockout mice acute knockdown of Sho leads to embryonic lethality (Young et al. 2009). This is again in contrast to stable, chronic knockout of both $PrP^C$ and Sho which do not show embryonic lethality and again develop seemingly normally with no obvious phenotype (Daude et al. 2012). This would suggest that removal or antagonism of $PrP^C$ is a viable treatment strategy for AD and prion disease.

In zebrafish our lab has identified an increase in seizure susceptibility in *prp1* and *prp2* knockout zebrafish (Leighton et al. 2018). In mice a peripheral myelopathy was generated in a new mouse model in a genetically pure background strain (Küffer et al. 2016). At the time of the start of the work presented in **Chapter 2** *in vitro* transcriptomic and proteomic experiments had identified changes in transcript abundance of genes related to cell adhesion during early development (Mohadeseh Mehrabian et al. 2015, 2014). Prior to that still, in 2011 transcriptomic experiments in developing mouse embryos showed changes in transcript abundance of genes important for embryonic development (Khalifé et al. 2011). With these findings we set out to use zebrafish to investigate the role of $PrP^C$ in early development. Generation of single mutant or compound homozygous $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ mutant zebrafish appeared normal however we did identify small yet consistent phenotypes (Leighton et al. 2018). Both $prp1^{ua5003/ua5003}$ and $prp1^{ua5004/ua5004}$ zebrafish were smaller than

wild-type zebrafish. In contrast, compound homozygous *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ fish were consistently larger **(Appendix A** and (Leighton et al. 2018)). Furthermore, it appeared that *prp1* and *prp2* may play antagonistic roles in development. There was a significant reduction in the deposition of neuromasts along the posterior lateral line (PLL) of both *prp1*$^{ua5003/ua5003}$ or *prp1*$^{ua5004/ua5004}$. In contrast, *prp2*$^{ua5001/ua5001}$ zebrafish showed a significant increase in neuromast number. In compound homozygous mutant fish, the increase or decrease of neuromasts was recovered, though remained still slightly and significantly higher than wild type.

These phenotypes suggested roles for *prp1* and *prp2* in cell adhesion and differentiation during zebrafish larval development. In **Chapter 2,** transcriptomic analysis via RNA-sequencing was carried out in *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua500/ua5001}$ zebrafish at 3 days post fertilisation (dpf). Gene ontology analysis revealed that the biological processes with the biggest increases or decreases in transcript abundance were similar to embryonic mice (Khalifé et al. 2011). There was little overlap at the individual gene level however this is not necessarily surprising due to the different relative stages of development. In the mouse study, the animals would have been earlier in development, before gastrulation. Zebrafish by comparison develop much more rapidly and at 3dpf would already have completed gastrulation. The biological process with the most genes showing a reduction in transcript abundance was cell adhesion. Within this process, protocadherin genes accounted for 31 of the 38 genes affected. The protocadherin family is thought to play an important role in the development and maintenance of the central nervous system (Cooper et al. 2015).

This overlap in gene ontology would suggest that the role of $PrP^C$ in development is evolutionarily conserved across species. Furthermore *in vitro* studies showed that $PrP^C$ may play a role in epithelial to mesenchymal transition (EMT), and important cell adhesion developmental process during gastrulation (M. Mehrabian, Hildebrandt, and Schmitt-Ulms

2016). This was due to knockout of $PrP^C$ resulting in a decrease in *ST8SIA2* transcription and subsequent polysialylation of NCAM1. In our *prp1* and *prp2* knockout zebrafish there was both a significant reduction in the transcript abundance of both *St8sia2* and *Ncam1a*, the zebrafish homologue of *NCAM1*. This reduction in transcript abundance was confirmed through RT-qPCR. Additionally, KEGG pathway analysis showed many genes had a significant reduction in transcript abundance surrounding the focal adhesion kinase (FAK) pathway. While neither *ptk2aa* or *ptk2ab*, the zebrafish FAK homologues, showed a significant reduction in transcript abundance themselves many genes which directly interact with them and are involved in other cell adhesion processes, such as calpain, had significantly reduced transcript abundance.

Taken together, the results presented in **Chapter 2** and **Appendix A** support an evolutionarily conserved, cross species role for $PrP^C$ in the early development of organisms. Stable knockout models would suggest that this role may ultimately be dispensable however combined with the severe phenotypes associated with acute loss of $PrP^C$ this may promote caution through the development of treatment for neurodegenerative diseases including prion diseases and AD that target $PrP^C$ function. Other evidence from our lab suggests neuroprotective properties of prion protein (Leighton et al. 2018), and more work needs to be done to establish the function of $PrP^C$ past development into adults. Our zebrafish mutants may act as a valuable addition to current animal models for determining $PrP^C$ function. Camera tracking technologies such as Ethovision (Noldus, Spink, and Tegelenbosch 2001) and Zantiks can allow for high throughput behavioural experiments before and after appropriate stimulus. Collaborations between others in the Allison lab with the Rihel lab have utilised such models to link prion protein and Aβso with changes in zebrafish sleep/wake cycles (Özcan et al. 2020b). Further building upon this work will help identify how prion protein function is affected in disease.

## 5.2 Part 2: Cisplatin and transition metal activation of the toll-like receptor 4 pathway:

### 5.2.1 Antagonism or knockdown of TLR4 ameliorates cisplatin induced ototoxicity in a zebrafish model:

Toll-like receptors (TLRs) are an evolutionary well conserved family of proteins involved in the innate immune response of organisms. TLR4 has been implicated as a mediator of cisplatin induced ototoxicity (CIO) after treatment of the potent chemotherapeutic, cisplatin (Oh et al. 2011; Bhavsar et al. 2017). This leads to severe and permanent bilateral hearing loss in patients. Cisplatin is particularly used to treat cancers in children and the ototoxic side effects can severely impact their social development. This means while being a highly effective treatment for cancer its use is becoming less common due in large part to these ototoxic side effects. Developing co-treatments to prevent ototoxicity after cisplatin treatment without affecting its ability to kill tumour cells would therefore be highly beneficial for the treatment of cancer. As CIO is thought to occur due to a build-up of cisplatin in largely senescent populations of inner and outer ear cells the toxic pathways are likely distinct from its toxicity to rapidly dividing tumour cells (Breglio et al. 2017).

**Chapter** 3 presents work done in collaboration with the Amit Bhavsar and Fred West labs at the University of Alberta and builds upon the previous identification of TLR4 as a potential mediator of cisplatin toxicity (Bhavsar et al. 2017; Binzhi Zhang et al. 2008). We use neuromasts in the PLL of zebrafish as a model for TLR4 mediated CIO. The zebrafish PLL has become an established and widely accepted model of ototoxicity (Domarecka et al. 2020). By adapting a scoring method used to assess neuromast cell health through DASPEI fluorescent intensity (Uribe et al. 2018; Van Trump et al. 2010; Harris et al. 2003) the effects of either morpholino knockdown of *tlr4ba* and *tlr4bb* or chemical inhibition of the zebrafish Tlr4s on CIO were observed. Chemical inhibition of Tlr4ba or Tlr4bb was performed using the TLR4 antagonist, TAK-242, or synthetic derivatives termed syntagonists. Surprisingly,

TAK-242 and two syntagonists, 120 and 132 increased CIO. Syntagonists 134 and 136 both prevented CIO, and at 20μM there was almost complete recovery of neuromast score after co-treatment of cisplatin and syntagonist 136.

This difference in outcome, with TAK-242 and syntagonists 120 and 132 exacerbating CIO while syntagonists 134 and 136 reducing CIO may be due to the change in the position of a carbon-carbon double bond caused during the synthesis of syntagonists 134 and 136. This change in position of the double bond means syntagonists 134 and 136 are predicted to no longer form a covalent bond with Tlr4ba or Tlr4bb. Any interaction between the syntagonists and zebrafish Tlr4s will therefore be due to electrostatic interactions, Van der Waals forces or hydrogen bonding resulting in a comparatively weaker interaction than the covalent bonds formed with TAK-242 and syntagonists 120 and 132. Why this difference would result in an increase in CIO is not clear. It may be that stronger inhibition of Tlr4ba and Tlr4bb itself leads to apoptosis of the cell. These results confirm TLR4 is a viable target to reduce CIO and zebrafish provide a suitable high throughput model for investigating TLR4 mediated CIO. Further work to establish the binding properties of TAK-242 and syntagonists to TLR4 may help shed light on how the interaction between the syntagonists and zebrafish Tlr4ba and Tlr4bb results in either protective or damaging outcomes.

### 5.2.2 Group 10 transition metals as a possible ligand for zebrafish Tlr4ba and Tlr4bb:

In mammals, lipopolysaccharide (LPS) is the canonical ligand of TLR4. Other 'non-canonical' ligands include viral proteins and transition metals such as nickel, cobalt, palladium (Tatematsu et al. 2016; Bulut et al. 2005; Schmidt et al. 2010). Recently platinum may be considered added to the list of metals able to activate TLR4 signalling (Babolmorad et al. 2021). Zebrafish have two homologues of TLR4, Tlr4ba and Tlr4bb. Neither zebrafish Tlr4 responds to LPS, and their current ligands have not yet been identified (Sullivan et al. 2009). The difference in ligand(s) between mammalian TLR4 and zebrafish Tlr4 is likely due

to low conservation in the extracellular leucine rich repeat (LRR) domain of the protein. Conservation is higher across the intracellular TIR domain and TLR downstream signalling is well conserved across vertebrate species.

Human TLR4 signalling can be activated by transition metals. In **Chapter 3** inhibition or knockdown of Tlr4ba and Tlr4bb ameliorates CIO in zebrafish. This led us to propose the hypothesis that zebrafish Tlr4ba and Tlr4bb may respond to transition metals, which can activate their downstream signalling. To do this we utilised the same zebrafish ototoxicity model established in literature and used in Chapter 3 (Uribe et al. 2018; Van Trump et al. 2010; Harris et al. 2003). Zebrafish were bath exposed to nickel or platinum in the forms of nickel chloride ($NiCl_2$), platinum (II) chloride ($PtCl_2$) or platinum (IV) chloride ($PtCl_4$). Regardless of which metal salt was used, zebrafish showed significant ototoxicity after exposure. $PtCl_2$ and $PtCl_4$ were more toxic than $NiCl_2$, which showed a more obvious dose response toxicity curve.

Syntagonists 134 and 136 both significantly reduced CIO. After exposure to $NiCl_2$, neither syntagonist 134 nor 136 had any protective effects against nickel induced ototoxicity. Syntagonist 134 may even have increased ototoxicity in a similar fashion to TAK-242 and syntagonists 120 and 132 after cisplatin exposure. However, syntagonists 138, 150, 166, 168 and 170 all significantly reduced ototoxicity caused by $NiCl_2$. At the time of writing of these syntagonists only 138 has also been tested against CIO in which it had no effect. That five different syntagonists were able to prevent $NiCl_2$ strongly suggests that nickel can activate Tlr4ba and or Tlr4bb signalling. Caution is needed when interpreting the results, however. The otoprotective effects of syntagonist 138 were not repeated. This follow up experiment which showed the efficacy of syntagonists 150, 166, 168 and 170 had a surprising reduction in neuromast score in the control group. This group was not treated with either syntagonists or $NiCl_2$ yet appeared to show significant ototoxicity. The reasons for this are unclear but

may be due to DASPEI fluorescence requiring fish to remain active during incubation with the dye. Repeat experiments will be needed to properly validate the otoprotective effects of these syntagonists.

Finally, zebrafish were co-treated with syntagonist 134 and either $PtCl_2$ or $PtCl_4$. In both cases syntagonist 134 was unable to prevent platinum ototoxicity. At a concentration of 7.5μM there was complete loss of DASPEI fluorescence demonstrating severe ototoxicity. Lower concentrations of platinum will be tested to ensure this was not due to any otoprotective effects being overwhelmed. Interestingly after exposure to $NiCl_2$ 134 appeared to increase ototoxicity rather than reduce it. A similar outcome may be occurring after exposure to $PtCl_2$ or $PtCl_4$, lower concentrations will help to confirm whether this is or is not the case. That certain syntagonists may prevent CIO but increase metal ion ototoxicity is surprising. Cisplatin interacts with the intracellular region of TLR4 and depending on the site of syntagonist binding this may affect the outcome of inhibition of Tlr4ba and Tlr4bb signalling.

Overall, these results provide optimism that in zebrafish, transition metals may act as ligands for Tlr4ba and Tlr4bb. While more work is needed, if these results are confirmed then this will help provide an additional *in vivo* model for applications in autoimmunity, cancer and modelling the innate immune response.

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# Appendix A: Zebrafish Prp1 and Prp2 are involved in early developmental processes.

## Appendix A preface:

The work presented in this appendix contains data collected for the work published in the article: Leighton, Patricia L.A., Richard Kanyo, Gavin J Neil, Niall M Pollock, and W Ted Allison. 2018. "Prion Gene Paralogs Are Dispensable for Early Zebrafish Development and Have Nonadditive Roles in Seizure Susceptibility." Journal of Biological Chemistry 293 (32): 12576–92. https://doi.org/10.1074/jbc.RA117.001171, as well as touch evoked escape response data collected by Michèle DuVal and Natalie Schneider. The appendix material and methods are adapted from Leighton et al. 2018, and were written by PLA and NMP. Figure contributions: Zebrafish images in **Figure 1** were provided by W. Ted Allison and Patricia Leighton. Touch evoked escape response data was provided by Michèle DuVal and Natalie Schneider.

## A1.1 Introduction

Functions of $PrP^C$ have been linked to: circadian rhythm, cell adhesion, cell differentiation and development, neuroprotection, metal ion homeostatsis, intercellular signalling, apoptosis, metabolism and more (Zomosa-Signoret et al. 2008; Castle and Gill 2017). The numerous different, and often unrelated, processes $PrP^C$ has been linked with makes its apparent dispensability even more surprising; with a variety of animals, including mice, goats and cattle, showing few overt serious phenotypes when lacking $PrP^C$ expression (Fernández-Borges et al. 2015; Büeler et al. 1993; Weissmann et al. 1994; Richt et al. 2007; Benestad et al. 2012). $PrP^C$ is highly conserved between mammals, and relatively well conserved across all vertebrates which would suggest some sort of essential, or at least important, function (Premzl and Gamulin 2007). The apparent dispensability may be due to robust genetic compensation occurring after stable knockout of the prion gene in animal models. As yet what gene(s) is responsible for this compensation, if any, has not yet been identified.

Sporadic onset prion diseases, and protein misfolding diseases in general, are often associated with age due to symptoms manifesting the latter half of the age of the patient, most frequently occurring after 60 years of age. As such animal model efforts to characterise the function of $PrP^C$ initially looked at possible roles in older animals; there is however evidence suggesting that $PrP^C$ may be important during the early development of an organism, particularly related to the CNS, and subsequent continued maintenance of neurons (Halliez et al. 2014). Early on in $PrP^C$ research, dynamic expression of $PrP^C$ was demonstrated in the developing mouse embryo (Manson et al. 1992), expression begins in the CNS and is followed by further expression in the heart before eventually the entire embryo (Tremblay et al. 2007). In zebrafish expression of prion protein begins in the midblastula stage (approximately 2.5-5.5hpf (CB et al. 1995)) though there are conflicting results as to whether this is expression of *prp1* or *prp2* and by 2dpf there is clear expression of *prp2* and our own work shows

detectable RT-qPCR transcript abundance of *prp1* by 50hpf (Cotto et al. 2005; Málaga-Trillo et al. 2009; Leighton et al. 2018).

Here, experiments suggesting roles of *prp1* and *prp2* in early and neural development of zebrafish using *prp1*$^{ua5003/ua5003}$ and *prp2*$^{ua5001/ua5001}$ mutant zebrafish are presented. Zebrafish neuromasts consist of bundles of hair cells and support cells along the posterior lateral line (PLL) of the trunk and tail or the anterior lateral line (ALL) distributed on the head. Neuromasts provide a powerful and easily accessible tool to model development. Changes in neuromast development and deposition are described. In addition, phenotypes related to the general development of zebrafish in *prp1*$^{ua5003/ua5003}$ and compound homozygous *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ mutant zebrafish are also characterised. Together this data suggests role of prion protein in early organism development, though this role appears dispensable.

## A 1.2 Results:

### A 1.2.1 *Prp1* and double *prp1* and *prp2* mutants show mild, non-severe developmental phenotypes.

Single *prp1*$^{ua5003/ua5003}$ or *prp1*$^{ua5004/ua5004}$ and Compound homozygous *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ zebrafish were grown to 50hpf/2dpf and size measured to look for any sign of developmental delay or abnormality. Visually there was no discernible phenotype in any mutant group compared to wild type at 50hpf **(Figure 1A)**. Homozygous *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua500/ua5001}$ mutant zebrafish grow normally into adulthood without showing any further visual phenotypes **(Figure 1B)**. Fish appear fertile and behaviourally normal under non-stressful conditions. Both of *prp1*$^{ua5003/ua5003}$ and *prp1*$^{ua5004/ua5004}$ mutant zebrafish showed a significant decrease in size compared to age match wild type controls at 50hpf **(Figure 1C)**. On average fish with either mutant allele were 0.17mm smaller. In contrast, *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ zebrafish showed a small increase in size when compared to wild type of

0.26mm at 50hpf **(Figure 1C)**. The size differences may suggest abnormal development of some capacity in larval zebrafish, though any biological significance does not appear severe.

To test physical responsiveness of prion knockout zebrafish, touch evoked escape response (TEER) experiments were performed to measure the reaction of *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ fish towards physical stimuli. The prion protein mutants showed a significant decrease in both the total distance moved and velocity of movement compared to age-matched wild type controls at 2dpf **(Figure 1D, E).** This was characterised as a reduced average distance moved of 2.3cm and a slower average velocity of 0.5cm/s. This can also be observed as a reduced range of distance and velocity. Wild type zebrafish have a higher average max distance travelled and velocity of 3.6cm and 1.33cm/s compared to 1.3cm and 0.78cm/s respectively.

### A 1.2.2 Knockout of *prp1* and *prp2* leads to developmental abnormality of neuromast deposition along the PLL:

By 2dpf cldnb:gfp imaging of zebrafish shows 6 neuromasts deposited along the PLL of wild type zebrafish, compared to only 4 neuromasts in *prp1*$^{ua5004/ua5004}$ mutant zebrafish **(Figure 2, top)**. Further quantification of PLL neuromast deposition was performed on either *prp1*, *prp2* or *prp1/prp2* knockout mutant lines using alkaline phosphatase staining at 3dpf to account for developmental delay. Discrepancies in the number of neuromasts in *cldnb:gfp* fish compared to alkaline phosphatase stained fish is due to alkaline phosphatase only staining mature neuromasts, while *gfp* expression occurs in both mature and immature neuromasts under the *cldnb* promoter. Regardless of whether *cldnb:gfp* fish or alkaline phosphatase staining was used the correlation of neuromast count between different *prp1* and *prp2* mutants remained the same.

In *prp1*$^{ua5004/ua5004}$ fish there is a significant reduction in the number of PLL neuromasts, though this reduction is not significant in *prp1*$^{ua5003/ua5003}$ fish. This may be due to the alleles

having different effects on the rate of developmental delay in the zebrafish larvae. The opposite is seen in *prp2*$^{ua5001/ua5001}$ mutants where there is a significant increase in PLL neuromasts compared to age matched wild type AB fish **(Figure 2, bottom)**. In *prp1*$^{ua5003/ua5003}$; *prp*$^{ua5001/ua5001}$ fish there was a significant increase in neuromast count compared to wild type though not as high as single *prp2*$^{ua5001/ua5001}$ fish **(Figure 2, bottom)**. This would suggest that *prp1* and *prp2* may have antagonistic, rather than complimentary, effects on zebrafish PLL development.

## A 1.3 Discussion:

Roles for $PrP^{C}$ and the closely related member of the prion-like superfamily Shadoo (Sho), have been described in the early embryonic development of organisms (Young et al. 2009, 2011). Despite this, $PrP^{C}$ and Sho knockout animals, including double knockouts of both proteins at once, appear to develop normally into healthy adults (Daude et al. 2012). Lethal phenotypes are seen in acute morpholino knockdown of *prp1* in zebrafish and acute knockdown of the gene encoding Sho, *SPRN*, in stable *PRNP* knockout mice (Young et al. 2009; Málaga-Trillo et al. 2009). Discrepancies in the results between acute knockdown or stable knockout of *PRNP* and *SPRN* may be due to compensatory genetic mechanisms though the search for what the gene(s) may be continues.

Previously the effects of *prp1* and *prp2* knockdown in zebrafish development was described (Kaiser et al. 2012; Huc-Brandt et al. 2014; Fleisch et al. 2013). Morpholino knockdown of *prp1* suggested synergistic roles in cell adhesion and neuroprotection with *appa* which was rescuable by the re-introduction of zebrafish *prp1* or mammalian *Prnp* mRNA(Kaiser et al. 2012). After *prp2* morpholino injection various developmental phenotypes were described however as these were not rescuable through ectopic delivery of *prp2* mRNA the specificity of these phenotypes cannot be confirmed (Málaga-Trillo et al. 2009; Kaiser et al. 2012; Huc-Brandt et al. 2014).

### 1.3.1 Zebrafish prion protein mutants are show only mild developmental phenotypes:

Here the effects of knockout of *prp1* and combined knockout of *prp1* and *prp2* on early zebrafish larval development are explored. While zebrafish prion protein mutants grow to healthy, fertile adults **(Figure 1A & B)** the developmental phenotypes seen here support the hypothesis that *prp1* and *prp2* may play antagonistic roles to each other (Leighton et al. 2018). Both $prp1^{ua5003/ua5003}$ and $prp1^{ua5004/ua5004}$ alleles show a significant reduction in size when compared to wild type fish **(Figure 1C)**. This reduction in size was reversed in $prp1^{ua5003/ua5003}$; $prp2^{5001/ua5001}$ fish. Previous work has established contrasting and non-additive neuronal hyperexcitability defects in *prp1* and *prp2* knockout zebrafish (Leighton et al. 2018; Kaiser et al. 2012; Fleisch et al. 2013). Single $prp2^{ua5001/ua5001}$ zebrafish showed an increase susceptibility to seizures compared to wild type and $prp1^{ua5003/ua5003}$ mutants. This susceptibility was slightly blunted in compound homozygous $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ zebrafish and not additive (Leighton et al. 2018). As baseline activity was seen to be decreased, touch evoked escape response (TEER) experiments were done to measure $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ fish response to physical stimuli. Prion protein mutant zebrafish moved a shorter distance at a slower velocity compared to wild type fish **(Figure 1 D-E)**.

Abnormal neuromast patterning is seen *prp1* and *prp2* knockout zebrafish. Compared to wild-type fish, $prp1^{ua5004/ua5004}$ knockout fish show a significant reduction in neuromast count. A similar trend is seen in $prp1^{ua5003/ua5003}$ fish, but this was not significant. In comparison, $prp2^{ua5001/ua5001}$ show a significant increase in neuromast number along the PLL. These results suggest that *prp1* and *prp2* have opposing functions on neuromast development. Double knockout of *prp1* and *prp2* appeared to revert the opposing phenotypes. While there was still a significantly higher neuromast count compared to wild-type in $prp1^{ua5003/ua5003}$; $prp2^{ua5001/ua5001}$ fish this was reduced compared to $prp2^{ua5003/ua5003}$ mutant fish.

These results demonstrate mild and consistent developmental phenotypes after *prp1* or double *prp1* and *prp2* knockout in zebrafish.

## A 1.4 Materials and methods

### A 1.4.1 Animal ethics and zebrafish husbandry:

Zebrafish were kept at the University of Alberta following a 14:10 light/dark cycle at 28°C cycle as previously described (Westerfield 2000). They were raised, bred, and maintained following an institutional Animal Care and Use Committee approved protocol AUP00000077, operating under guidelines set by the Canadian Council of Animal Care.

### A 1.4.2 Fish lines/strains:

Zebrafish of the AB strain were used as the WT fish in this study and were the background strain for the targeted mutagenesis. Our previously published prp2ua5001 allele (ZFin ID: ZDB-ALT-130724-2), which has a 4-bp deletion and is predicted to produce a truncated protein lacking all recognizable protein domains (16), was maintained on an AB background. Tg(cldnb:gfp) larvae (Tg (-8.0cldnb:Ly-EGFP, ZFin ID: ZDB- ALT-060919-2); referred to herein as cldnb:gfp ((Haas and Gilmour 2006))) were kindly provided by Pierre Drapeau and were bred into fish with the newly generated prp1 ua5004 allele upon reaching adulthood.

### A 1.4.3 Measuring the length of larval zebrafish:

2 dpf larvae were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4, with 5% sucrose at 4 °C. Larvae were then rinsed several times with 1x PBS and imaged and photographed with a Leica M165FC dissecting microscope and a Leica DFC 400 camera. The scale bar feature in the Leica software was then used to measure the length of each fish from the forebrain to the tip of the caudal fin.

### A 1.4.4 Analysis of neuromast number and position:

Trunk neuromasts of the PLL were visualized by detection of GFP fluorescence in Tg(cldnb:gfp) fish using a Leica M165FC dissecting microscope. An observer, who was blinded to the genotype of the fish, counted the number of neuromasts.

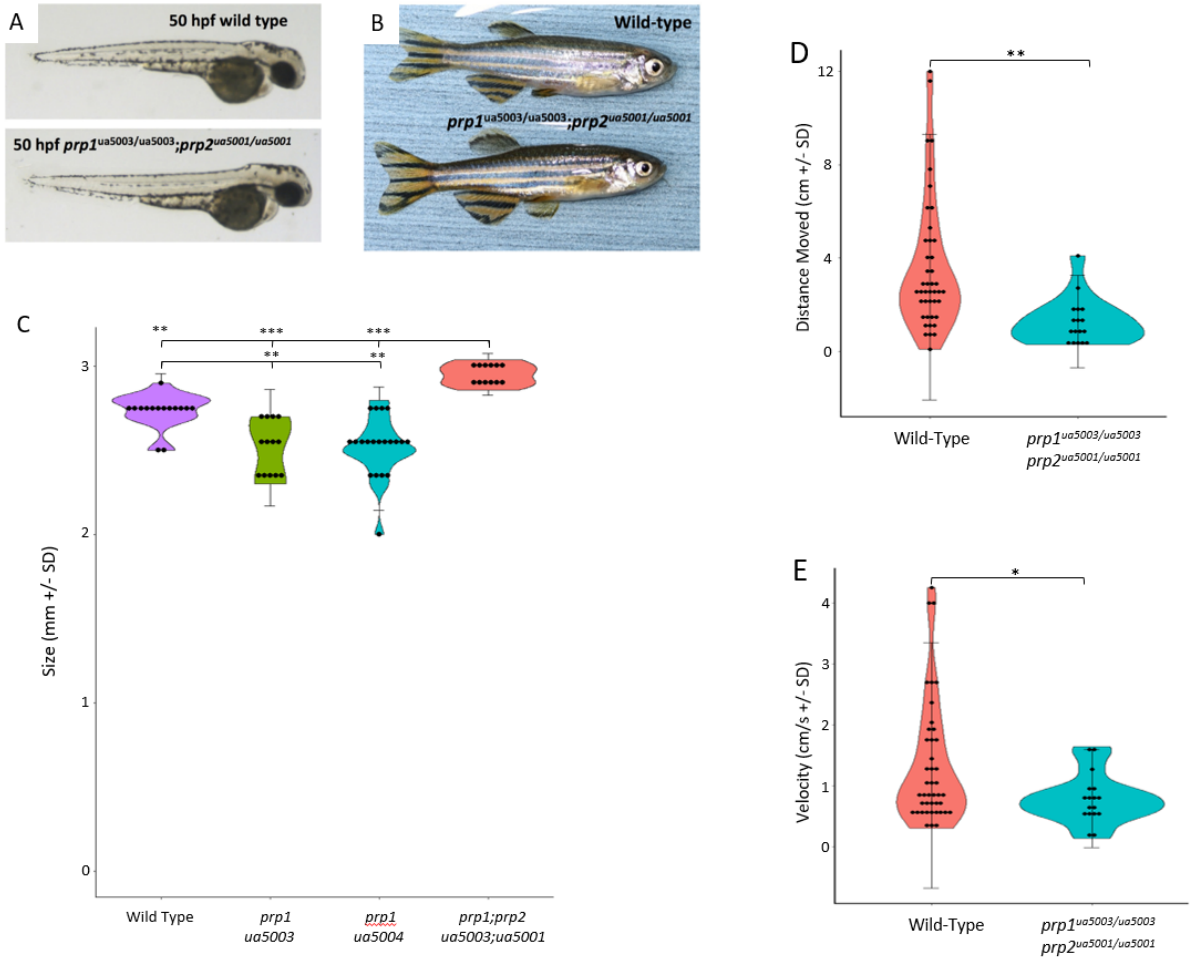
### A 1.4.5 Touch evoked escape response test:

Wild-type or *prp1*$^{ua5003/ua5003}$*; prp2*$^{ua5001/ua5001}$ zebrafish were grown to 3dpf. Individual zebrafish were placed within a petri dish containing 25ml E3 medium. Ethovision (Noldus, Spink, and Tegelenbosch 2001) was calibrated to recognise zebrafish larvae. Larvae were exposed to a physical stimulus by contact with a fish wire to the tail. Ethovision software tracked the velocity and distance travelled of zebrafish after physical stimulus and data was then plotted.

### A 1.4.6 Statistical analyses

One-way ANOVA with Tukey's multiple comparisons test or unpaired student t-tests were carried out using R version 4.0 and graphs were constructed using Microsoft Excel or the 'ggplot2' and 'tidyverse' group of R packages (Wickham et al. 2019; Wickham 2016; R Core Team 2020).

## A 1.4 Appendix 1 Figures:




### Figure A.1:

Zebrafish lacking *prp1* and *prp2* develop normally with only mild phenotypes. A and B) There is no visually obvious distinguishable difference between wild-type and *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$. C) Zebrafish lacking *prp1* are slightly but consistently significantly smaller than wild-type zebrafish, while *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ fish are larger. D and E) After TEER stimulation, 3dpf *prp1*$^{ua5003/ua5003}$; *prp2*$^{ua5001/ua5001}$ move a significantly smaller distance at a significantly smaller velocity than wild-type fish. *** = $p < 0.00001$, ** = $p < 0.001$, * = $p < 0.01$. Significance in C determined through one way ANOVA with Tukey HSD. Significance in D and E determined through unpaired t-test.

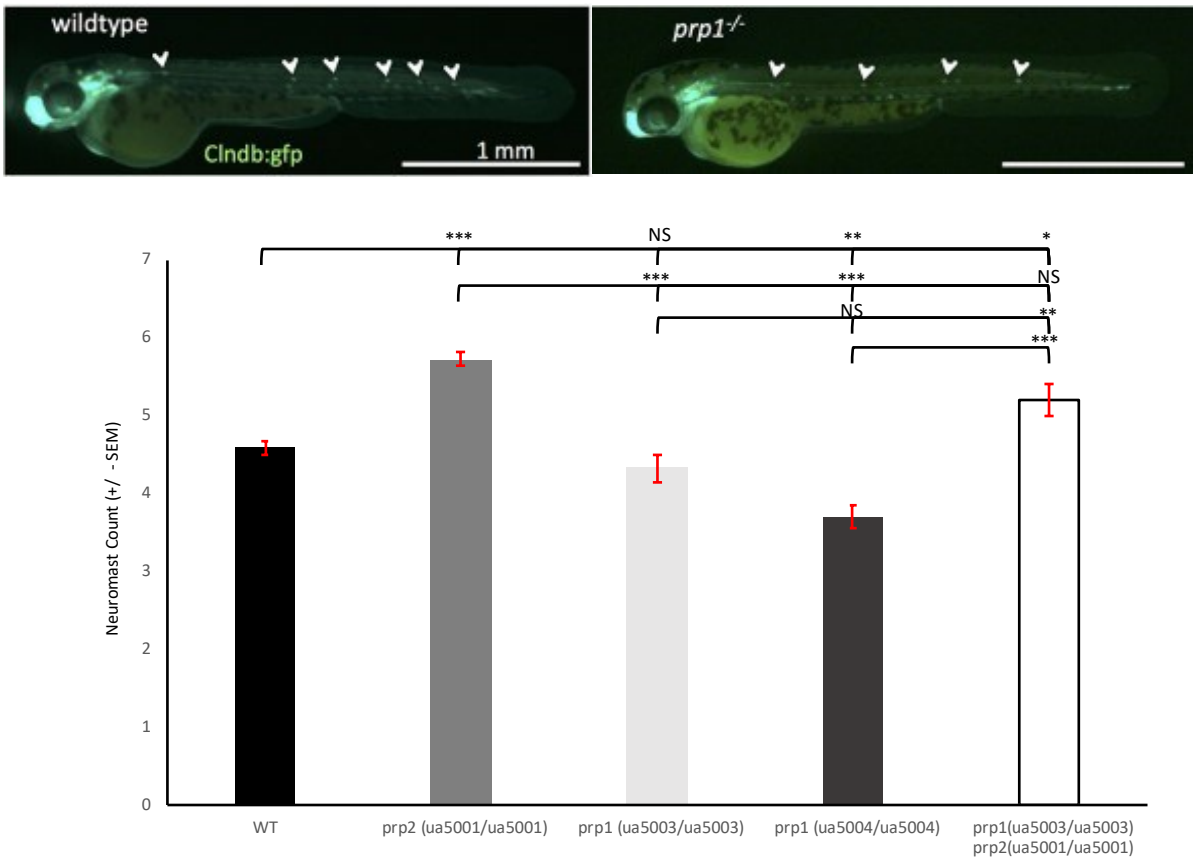



Figure A.2:

Prion mutant zebrafish show abnormal neuromast deposition along the posterior lateral line. Top) At 2dpf cldnb:gfp wild-type zebrafish (left) have 6 trunk neuromasts while 2dpf cldnb:gfp *prp1*$^{ua5004/ua5004}$ mutant fish (right) have 4. Bottom) Loss of *prp2* increased neuromast count while loss of *prp1* reduced neuromast count. Loss of *prp2* rescued the effects of loss of *prp1* however there was still a slight increase in neuromast count compared to wild-type. *** = $p < 0.00001$, ** = $p < 0.0001$, * = $p < 0.01$. Significance determined by one way ANOVA with Tukey HSD.