CO-PURIFICATION OF NUCLEAR RECEPTOR LIGAND(S) AND INTERACTING PROTEINS FROM ZEBRAFISH EMBRYOS

By

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Co-purification of Nuclear receptor ligand(s) and interacting proteins from zebrafish embryos

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Abstract

The main focus of this project was to optimize a protocol for small molecule ligand co-purification from an in-vivo tissue source. For this purpose, I employed a transgenic zebrafish line called the pLT-gypsy, which expresses a fusion protein containing a tagged-NR LBD (Tiefenbach et al., 2010). The particular line I used to optimize the ligand identification protocol is the pLT-PPARγ zebrafish line, which expresses the tagged-PPARγ receptor's LBD (also called PPARγ-fusion protein). By using rosiglitazone (a known PPARγ ligand) as a positive control, I managed to optimize a protocol to purify the PPARγ-fusion protein and identify the co-purified ligand by mass spectrometry. This protocol can be used to identify the physiological/endogenous ligand for the PPARγ receptor as well as other orphan NRs. Compared to previous methods of ligand identification, this method allows for the identification of the ligand from the tissues where it is functional.

(See supplemental file for full abstract)

Poem of Acknowledgments:

So here I am writing my thesis, failed my reclass and still kind of pissed.

But would things be better if I had passed? How long would I stand? How long would I last?

But what's done is done, no point looking back. Must keep looking ahead. Staying focused, staying on track.

A huge thanks to Mum and huge thanks to Dad, for the great education that so far I had.

You sent me away to a land overseas, and hope I return with a great PHD.

In this respect, I did not make it. But one day I may be back, to see if I can take it.

To James and Ronald, Vivek, Khan and Mustafa. We definitely had great times with plenty of laughter.

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To all my friends, both new and old. Our friendship is worth way more than gold.

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To my committee members, Aled Edwards and Ian Scott. For all your advice, I thank you a lot.

Though I failed the reclass, it opened the door. To a world of possibilities and much, much more

And last but not least before I go off, I thank Henry Krause the most awesome Prof.

Through bad times and worse you gave me consolation. And despite my annoyances you still have patience.

Unlike others, you saw potential in me. To most others, an idiot they see.

But soon I will leave Toronto behind. Another prof like you will be hard to find.

Soon I'll be free, released from enslavement. Free to find happiness, free to find enjoyment.

I have fallen over, but I will keep going. I may fall again, but I will keep trying.

There are many roads to fame, many roads to success. I will keep trying, and do nothing less.

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1 Introduction

1.1 Nuclear receptors - overview

Nuclear receptors (NRs) are a class of transcription factors that can be regulated by small hydrophobic molecules, including but not limited to the steroid hormones, most of which are thought to pass freely between cells and tissues (Gronemeyer et al. 2004; Huang et al. 2010). This ability makes the NRs different from other cell surface receptors that rely on transduction of a secondary intracellular messenger to relay the message to the nucleus (Pardee et al. 2011).

Apart from their ability to be targeted by small hydrophobic molecules, they are able to function as transcription factors and bind onto DNA sequences. The particular DNA sequences on which they bind are termed hormone response elements (HREs). These HREs are located in the enhancer sites of target genes. By binding to the HREs, the NRs are directed to specific target genes where they can then promote transcriptional activation or repression of that gene (Pardee et al. 2011; Bain et al. 2007).

Without ligand binding, some NRs can bind to their HREs, but are associated with co-repressors. This causes transcriptional repression of the target genes. Upon binding to their cognate ligand, these receptors undergo conformational change that allows them to bind co-activators. These co-activators recruit the RNA Polymerase II complex and the basal transcription machinery, resulting in the transcriptional activation of the target gene (Gronemeyer et al. 2004). Other receptors that are transcriptional repressors bind to their cognate ligand, leading to even stronger repression of the target gene. Steroid receptors generally require ligand binding to enter the nucleus and/or bind to their HREs.

NRs have essential roles in the control of many biological processes such as lipid metabolism, embryogenesis and reproduction. Furthermore, they have been implicated in the pathology of various human diseases – for example obesity, inflammation, cancer and autoimmune disorders. As a result, there is a huge interest to identify both synthetic and endogenous ligands that can target these molecules (Doshi et al., 2010). However, only half of the NRs have known, endogenous ligands (Pardee et al. 2011).

Steroid hormones were the first NR ligands discovered, and were known long before the discovery and cloning of their target receptors. These hormones bind with high-affinity to their cognate receptors and thus, were employed as a tool for tracking the receptors during purification and further biochemical analyses (Willson and Moore, 2004; Jensen et al. 1982). The use of radiolabeled-estrogen showed that after binding to its cognate receptor, the complex translocated from the cytoplasm into the nucleus (Sarff and Gorski, 1971; Juliano and Stancel, 1976). This led to the idea that NRs are also involved in gene regulation. Despite this finding, it was later discovered that the majority of un-liganded estrogen receptors are residing inside the nucleus even before ligand stimulation (Leclercq et al., 2006; Kawata et al., 2001).

The advancement of molecular biology in the 1980s allowed for the isolation of the genes that encode these receptors. This led to the cloning of the steroid hormone receptors (Govindan et al., 1985; Miesfeld et al., 1984; Walter et al., 1985). Following the cloning of the steroid hormone receptors, the discovery of conserved domains allowed the identification and cloning of additional family members without knowledge of their endogenous ligands (Robinson-Rechavi et al., 2001). Upon completion of the human genome sequence, this number rose to 48 family members, approximately half of which have no known endogenous ligand, and are termed 'orphan' receptors (Table 1-1; Pardee et al., 2011).

1.1.1 Nuclear receptor structure

Generally, the structure of an NR protein consists of an N-terminal domain (also called A/B domain), a central DNA-binding domain (DBD; also called C domain), a Hinge region (D domain) and a C-terminal ligand-binding domain (LBD; E domain) (Pardee et al., 2011; Gronemeyer et al., 2004). Some NRs have an extra C-terminal region called the F-domain (Figure 1-1).

The poorly conserved A/B domains (N-terminal domain) act as regulators of transcription and also as sites of kinase-dependent phosphorylation (Burns and Heuvel, 2007; Hammer et al., 1999). The DBD, which is highly conserved, contains two zinc-finger DNA binding motifs that stabilize the DBD, allowing it to bind to response elements in the enhancer sites of target genes. The LBD generally consists of 12 helices that form the ligand-binding pocket, which contains mostly hydrophobic amino acids on its internal surface for interaction with the ligand (Olefsky,

2001). Apart from ligand interaction, the LBD is also used for dimerization and interactions with cofactor/coregulator proteins.

Figure 1-1. Nuclear receptor structure. The NR protein can be divided into several domains. From the N-terminus, there is the A/B domain, the DNA-binding domain (DBD; also called the C-domain), the Hinge region (D-domain), Ligand binding domain (LBD; also called E-domain) and a C-terminal F-domain (Pardee et al., 2011). (With kind permission from Springer Science + Business Media: Subcellular Biochemistry, Nuclear receptors: Small molecule sensors that coordinate growth, metabolism and reproduction (Chapter 6 in A handbook of transcription factors), vol. 52, 2011, p124, Pardee K., Necakov A.S., Krause H., (ed: Hughes, T.R.), Fig. 6.1, License number: 2786080560618)

1.1.1.1 Ligand-binding domain (LBD)

The LBD is the region of the NR protein used for binding to the receptor's cognate ligand. Generally, the structure of the LBD consists of 12 α-helices and 3 β-strands, folded into a globular "α-helical sandwich" surrounding a non-polar cavity (the ligand-binding pocket) (Weatherman et al., 1999). Helices 3, 7 and 10 form the shape of the ligand-binding pocket, which contains mostly of hydrophobic amino acids on its internal surface for interaction with the ligand (Figure 1-2; Li et al., 2003; Huang et al., 2010). Although the sequence similarity between different NR LBDs can be relatively low, the overall structure is conserved (Pardee et al., 2011).

The ligand-binding pocket can vary in size from almost nothing (filled with large hydrophobic side-chains) to approximately 1500 Angstroms cubed (Huang et al., 2010). However, upon ligand binding, the LBD pocket can enlarge or change shape to accommodate the ligand. For example, the crystal structure of apo-Rev-erbβ did not show any space for ligand binding, as the pocket is filled with large hydrophobic side chains. However, upon heme binding, the Rev-erbβ LBD adopts a larger pocket to accommodate the ligand (Pardee et al., 2009).

For most non-steroid binding NRs, the LBD in the apo-form (not bound by a ligand) will be associated with co-repressors such as NCoR (Nuclear receptor Co-repressor) or SMRT (Silencing mediator for retinoid and thyroid hormone receptor), which recruit histone deacetylases, leading to the repression of target genes. In this state, helix 12 of the LBD is in a loose and outward position (Figure 1-2A; Nagy et al., 1997; Pardee et al., 2011). In this state, co-repressors containing a CoRNR box (Co-repressor Nuclear receptor box) motif, LCCI/HIXXXI/L, can bind to a hydrophobic groove between H3 and H4 (Gronemeyer et al., 2004).

Upon agonist binding, helix 12 folds over to interact with the ligand forming a lid/cover over the pocket (Figure 1-2B; Zoete et al, 2007). This causes a structural change in the LBD, leading to dissociation of co-repressors and association with co-activators (Figure 1-2C). Co-activators containing LXXLL motifs are able to bind to the hydrophobic cleft formed between H12, H3 and H4 (Gronemeyer et al., 2004). Co-activators recruit histone acetyl transferases and the RNA polymerase II complex, leading to transcription of the target gene.

Traditionally, it is believed that the degree of ligand-induced agonism is related to the degree of stabilization of helix 12 (Pardee et al., 2011). Whether directly or indirectly, agonists will generally stabilize the helix 12. On the other hand, antagonists, which are usually larger molecules, will prevent stabilization of the helix 12, and thus coactivator binding (Huang et al. 2010). However, many ligands do not simply agonize or antagonize an NR, but are capable of limited agonism (partial agonists) or tissue-selective action (selective nuclear receptor modulators (SNuRMs)). Previously, it was believed that partial agonists stabilized the helix 12 in a halfway position between the full agonist- and antagonist-bound position (Pochetti et al., 2007). However, more recent data has shown that some partial agonists, like MRL-24, are able to bind inside the ligand-binding pocket of the PPARγ receptor without stabilizing helix 12 (Choi et al., 2010b). Instead this ligand stabilizes a portion of helix 3 (Bruning et al., 2007). As a result, these partial agonists only switch-on the anti-diabetic effect of the PPARγ receptor, without activating the full transcriptional program.

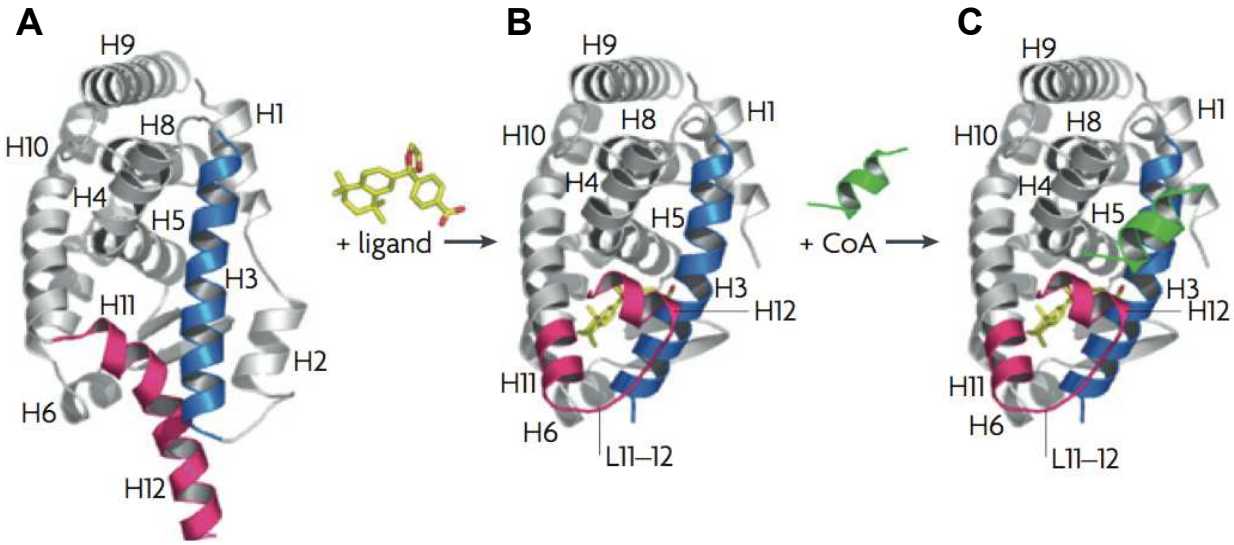

Figure 1-2. Structural basis for ligand-response. The structures shown demonstrate the canonical agonist-induced conformational change of NR LBDs. (A) The un-liganded or apo-structure of RXRα shows H12 pointing away from the LBD. (B) Upon agonist (SR11237) binding, H12 folds over to interact with the ligand forming a lid/cover over the pocket. (C) This structural change allows for LXXLL motif-containing co-activators (CoA) to bind the LBD (de Lera et al., 2007). (Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Drug Discov.] de Lera, A.R., Bourguet, W., Altucci, L., and Gronemeyer, H. Design of selective nuclear receptor modulators: RAR and RXR as a case study, vol. 6, pp811-820, copyright 2007. License number: 2790361508430)

1.1.1.2 DNA-binding domain

The DNA-binding domain (DBD), which has the highest sequence conservation compared to other NR domains, is able to bind hexa-nucleotide repeats called hormone response elements (HREs) in the enhancer sites of target genes (Bain et al., 2007). This allows the localization of NRs to enhancers, where they can activate or repress target genes (Schwabe and Teichmann, 2004). The structure of the DBD consists of two zinc-finger motifs, each containing 4 cysteine residues coordinated around one zinc atom. A hinge domain connects the DBD to the LBD, allowing for flexibility and interactions between the two domains (Warnmark et al., 2003; Chandra et al., 2008).

The various HREs are derivatives of the sequence A/G-GGTCA (Gronemeyer et al., 2004). The selectivity of binding for the specific NRs to their response element is dictated by the sequence itself, extensions, repeat orientation and spacing (Pardee et al., 2011). Several studies have

suggested that the NR response elements may also affect NR structure and conformation (Thompson and Kumar, 2003).

1.1.1.3 N-term domain

Compared to the other NR domains, the N-terminal domain or A/B domain is least understood. Although its composition and structure is variable, it plays a generally important role in the regulation of NR transcriptional activity (Warnmark et al., 2003; He et al., 2002). Interestingly, fusing the N-terminal domain of most NRs to a heterologous DNA-binding domain will lead to the transcriptional activation of the target gene (Tontonoz et al., 1994). Paradoxically, deleting the N-terminal region of some receptors results in a higher transcriptional activity of the receptor (Tontonoz and Spiegelman, 2008).

This domain also acts as a site for kinase-dependent phosphorylation. An example is the phosphorylation of Serine 112 of the PPARγ receptor by Erk 1 and 2, members of the MAP kinase family, leading to decreased transcriptional activity of the receptor (Adams et al., 1997; Hu et al., 1996). Furthermore, a Serine to Aspartic acid substitution of the Serine 112 residue has been shown to inhibit ligand binding to the PPARγ receptor (Shao et al., 1998).

1.1.2 Transcriptional regulation by NRs

Based on their ability to hetero-dimerize to other NRs, and their binding to respective response elements, the NRs can be classified into four classes (Figure 1-3; Mangelsdorf et al., 1995; Pardee et al., 2011). Class I NRs are steroid hormone receptors that bind to inverted repeats as homo-dimers. As apo-receptors, the Class I NRs are usually located in the cytoplasm, bound and repressed in a complex containing heat-shock proteins (Figure 1-3; Smith and Toft, 2008; DeFranco et al., 2000). Generally, ligand binding to the Steroid hormone receptors causes the dissociation of the repression complex, allowing the translocation of the receptor-ligand complex into the nucleus. Once inside the nucleus, the Steroid hormone receptors will homo-dimerize with another receptor and bind onto inverted hexa-nucleotide repeats. Despite this classical view of the steroid receptors, a fraction of the un-liganded receptors are already present in the nucleus. Even in the absence of ligand binding, the majority of un-liganded Estrogen

receptor is already residing in the nucleus (Leclercq et al., 2006; Kawata et al., 2001). Class I receptors bind their cognate steroid ligands with high affinity.

Class II NRs form hetero-dimers with RXRs and bind onto direct hexa-nucleotide repeats (Figure 1-3). As apo-receptors, the Class II NRs are usually retained inside the nucleus in complex with the RXRs and bound onto the hexa-nucleotide direct repeats. In this apo state, the Class II NRs associate with co-repressor proteins, leading to repression of the target gene (Mangelsdorf et al., 1995). Upon ligand binding, the Class II NRs undergo conformational changes that allow for dissociation from co-repressor proteins, and the recruitment of co-activators, resulting in target gene transcription. Class II NRs usually bind a variety of ligands with relatively low affinity (Pardee et al., 2011).

Class III NRs do not bind steroid hormones but bind onto direct repeats as homo-dimers. On the other hand, Class IV NRs bind as monomers onto single response elements (Figure 1-3). Most of the receptors in Class III and IV are still orphan receptors (Bain et al., 2007).

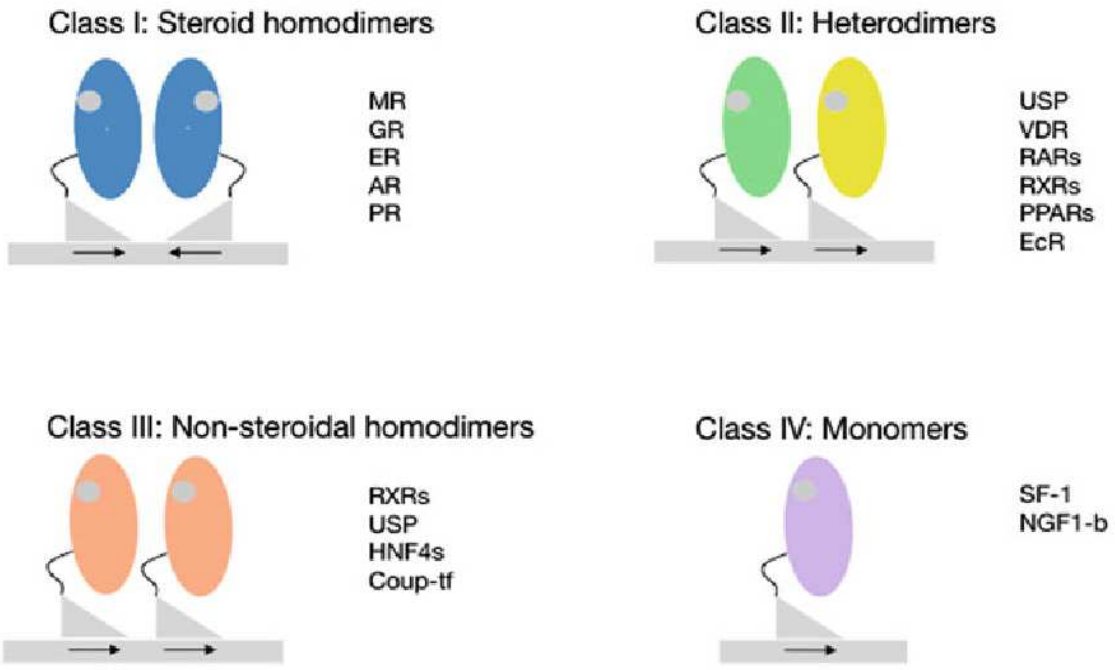

Figure 1-3. Classification of nuclear receptors based on dimerization and binding to Hormone response elements (HREs). NRs can be grouped into four classes: Steroid receptors (Class I), RXR hetero-dimers (Class II), Non-steroidal homo-dimers (Class III) and Monomeric receptors (Class IV). (Pardee et al., 2011; Mangelsdorf et al., 1995). (With kind permission from Springer Science + Business Media: Subcellular Biochemistry, Nuclear receptors: Small molecule sensors that coordinate growth, metabolism and reproduction (Chapter 6 in A handbook of transcription factors), vol. 52, 2011, p124, Pardee K., Necakov A.S., Krause H., (ed: Hughes, T.R.), Fig. 6.2, License number: 2786080560618). (Original copyright notice: Reprinted from Cell, vol. 83, Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M., The nuclear receptor superfamily: the second decade, pp835-839, Copyright 1995, with permission from Elsevier, License number: 2786081039243).

1.1.3 NR ligands and binding chemistry

NR ligands tend to be small hydrophobic molecules that are able to pass relatively freely from cell to cell (Huang et al., 2010; Sladek, 2010). Ligand binding to their cognate NRs induces structural change that regulates the receptor's affinity for different co-activator or co-repressor proteins. It is through these changes that NR ligands are able to control the transcription of the target genes. Generally, NR ligands are classified as agonists or antagonists based on their effects on the expression of the target gene (Gronemeyer et al., 2004). For receptors that are transcriptional activators, an agonist ligand, when bound to its cognate receptor, will lead to an up-regulation of the target gene expression. For other receptors that are transcriptional

repressors, agonist binding will lead to increased repression of the target gene. On the other hand, antagonists (which are usually synthetic ligands), will bind to the receptor and block endogenous agonists from binding, thereby leading to the repression of the target gene (Sladek, 2010; Noy, 2007). However, there are many cases where the ligand does not simply act as agonist or antagonist (see below).

Ligand binding to their cognate NRs stabilizes the receptor-ligand complex by filling the hydrophobic core, forming new inter-molecular and intra-molecular interactions. Although NR ligands are largely hydrophobic molecules, they usually contain at least one small polar group. It is these small polar groups that form inter-molecular interactions with the receptor and thus, help to position the ligand inside the ligand-binding pocket (Nagy and Schwabe, 2004; Shiau et al., 1998; Williams et al., 2003).

The steroid hormone receptors are able to bind a single natural ligand at high affinity (Pardee et al., 2011). Therefore it was originally assumed that NR ligands for the orphan receptors would bind in the same fashion. This formed the basis for the traditional in-vitro screening approach for ligand identification of orphan NRs (Kliewer et al., 1999). However, it was later found that many newer receptors (discovered more recently) are able to bind a variety of natural ligands at relatively low affinity (Table 1-1).

1.1.3.1 Unconventional ligand regulation

The traditional concept of ligand-dependent regulation is based on studies of the few steroid hormone receptors that were initially cloned from humans (Sladek, 2010). Despite the traditional model of ligand-dependent transcriptional regulation, there exist examples of regulation that do not fit into this model (Pardee et al., 2011).

The RORβ receptor is active even in the absence of a ligand. However, upon all-trans retinoic acid binding to the receptor, transcription is repressed (Stehlin-Gaon et al., 2003). Furthermore, some receptors such as Nurr1, which are active in the absence of a ligand, contain large hydrophobic side-chains filling the ligand-binding pocket. This has led to the assumption that these receptors may not have an endogenous ligand (Wang et al., 2003b). However, from examples such as Rev-erbβ, it is known that NRs are able to alter their conformation to allow binding of a ligand, even though the apo-structure does not reveal a spacious ligand-binding pocket (Pardee et al., 2009).

Some ligands bind to their cognate receptors as permanent cofactors rather than exchangeable ligands. For example the *Drosophila* receptor, E75, requires heme to be permanently homebound in order to fold properly and accumulate (Reinking et al., 2005). On the other hand, its human homologues, the Rev-erbs, are able to bind and release heme with little effect on stability (Raghuram et al., 2007; Yin et al., 2007; Pardee et al., 2009).

Furthermore, many post-translational modifications of the NR proteins, such as acetylation, phosphorylation, sumoylation and ubiquitylation are involved in transcriptional regulation of their target genes (Hwang et al., 2009; Hammer et al., 1999). Although largely focused on the N-terminal domain, post-translational modification can also occur at other sites on NR proteins (Lee et al., 2006). Cdk5, for example, is able to phosphorylate Ser273 of the PPARγ receptor's LBD, resulting in reduction of adiponectin and increased insulin-resistance (Choi et al., 2010), possibly through the differential recruitment of co-regulator proteins.

Some ligands can also act as partial agonists or SNuRMs (Selective nuclear receptor modulators; Gronemeyer et al., 2004; Pardee et al., 2011). These partial agonists may confer the up-regulation of only a subset of genes, or have tissue-selective activation. A famous example of this is tamoxifen, which acts as an estrogen receptor antagonist in breast tissue, while acting as an agonist in the bone (Brzozowski et a., 1997; Shiau et al., 1998). The helix 12 of tamoxifen-bound estrogen receptor partially blocks the coactivator-binding site, allowing only or a subset of coactivators to bind. As a result, tamoxifen acts as an agonist in the endometrial cells that is expressing a high level of SRC-1, while acts as an antagonist in the lower SRC-1-expressing breast cells (Shang and Brown, 2002).

1.1.4 Current and previous methods of ligand identification

Considering that NRs are involved in the regulation of a variety of biological processes and diseases, many researchers have put significant effort into the identification of endogenous ligand(s) for the orphan NRs (Schupp and Lazar, 2010). Previously, the main approach for endogenous ligand identification for orphan receptors has involved the screening of natural small molecule libraries in a cell-culture based assay. This approach has yielded potential ligands for receptors such as the RXRs and LXRs (Levin et al., 1992; Makishimi et al., 1999). However, this method is problematic, due in part to the ability of some orphan receptors to bind

many different small molecules at low-affinity (micromolar range) (Noy N, 2007). Through this method, the PPARs were found to bind to a variety of fatty acids and it was unclear as to which one is the true endogenous ligand (Forman et al., 1995; Forman et al., 1997). Some ligands like 15d-PGJ2, identified by this method as the PPARγ ligand, have not been shown to be relevant in-vivo (Bell-Parikh et al., 2003). Other problems with the in-vitro screening approach are that one may not have the actual ligand available within small molecule collections, and that cellular environments may not be well or fully represented in the assay.

Some groups have managed to identify NR ligands through expression and purification of the NR's LBD for protein crystallography, and fortuitously finding ligands bound inside the solved structure of the LBD (Dhe-Paganon et al., 2002; Kallen et al., 2002). For example, in-vitro screens did not yield any ligand for RORα, however when expressed in Sf9 insect cells and then crystallized, cholesterol was found in its ligand-binding pocket. Follow-up experiments with mass spectrometry also showed the ability of cholesterol to bind to RORα. However, due to the difficulties of crystallography, and the heterologous nature of over-expression systems, this method has shown limited success.

Some ligands for orphan receptors have been identified by expressing and purifying a tagged-LBD from bacteria or Sf9 insect cells and identifying the ligands by mass spectrometry (Bitsch et al., 2003; Potier et al., 2003). For example, with the identification of heme as the endogenous ligand for the *Drosophila* nuclear receptor E75 (orthologue of the mammalian Rev-erbs), heme was co-purified with a tagged-E75 LBD expressed in bacteria and identified by non-denaturing ESI-MS (Reinking et al., 2005). Following this identification, its in vivo relevance was confirmed in flies. Although useful for facilitating increased protein solubility and high levels of expression, purification from bacterial systems have not yielded all that many true ligands (Pardee, 2010).

More recently, several groups have attempted to express the tagged-LBD in tissues or cells relevant to the function of the NR (Schupp and Lazar, 2010; Yuan et al., 2009; Chakravarthy et al., 2009). For example, Chakravarthy et al. (2009) expressed a tagged-LBD of the PPARα NR in mice using the adenovirus expression system, followed by affinity purification of the tagged-LBD from mouse livers, and identified a suspected ligand, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine, by mass spectrometry. This approach of affinity-purification followed by mass spectrometry should allow for capture of the endogenous ligand in its native environment and

overcomes many problems and biases of the previously mentioned in-vitro screening approach. This is a similar approach to the ligand identification protocol that I will be optimizing. It is worth noting in the case above, however, that the NR used was over-expressed, and the researchers were looking for a pre-suspected ligand type. Dissecting out the mouse liver (or other specific organ) for affinity purification also reduced the complexity of the tissue source. Thus, without prior knowledge of the ligand's identity and tissue location, this approach requires further improvements.

My approach adds features such that ligand identification through affinity purification of a tagged-NR protein can be used from the whole organism. This overcomes the bias of using a specific organ or a specific cell type (cell culture) for ligand identification. Furthermore, this protocol allows for the identification of previously unknown ligands.

1.1.5 Zebrafish as a model system

Propelled by the work of George Streisinger, the zebrafish has become a popular model organism (Grunwald and Streisinger, 1992b). Due to its fast developmental timing, ex-vivo development and transparent embryos, it has been widely used by developmental biologists (Kimmel et al., 1995). Most zebrafish organs are fully developed by 4dpf (days-post-fertilization), meaning that embryogenesis and organogenesis can be studied in a short period of time. Optical clarity of the embryos also means that fluorescent protein markers along with live-imaging techniques can be employed to view the mechanisms of development in real-time (Dooley et al., 2003). Furthermore, there are a variety of molecular biology tools that can be used for genetic manipulation, such as morpholinos which are synthetic nucleic acid analogs that are used to ‘knock-down’ gene expression via interference with mRNA splicing or translation. Also extremely helpful are transposon-mediated transgenesis approaches.

Although traditionally used as a model system for developmental biology, there is a growing trend in using zebrafish for drug discovery (Tan and Zon, 2011). Zebrafish embryos are DMSO-tolerant and are able to absorb drugs from the surrounding medium. This has allowed for ease of drug treatment to the embryos. The maintenance cost for housing zebrafish is much less compared to mice. Furthermore, the small size of the zebrafish embryos and hatchlings allow them to be arrayed into multi-well plates and can survive in a very small volume of water. This

makes the zebrafish model amenable for large-scale drug screens. Also, the clarity of the embryos means that certain effects of the drugs can be seen in the internal organs. Drug discovery using zebrafish has yielded several drugs, for example, rosuvastatin, used for the treatment of prostate cancer (Wang et al., 2010).

1.1.5.1 Zebrafish for NR research

Even though there is an estimated 450 million years of divergence between humans and zebrafish, there is still a relatively high sequence similarity in the genes from the two species (Liu et al., 2002; Delvecchio et al., 2011). In terms of NR research, for nearly every human NR, there is at least one homologue representing it in the zebrafish (Bertrand et al., 2007). There is also evidence that the zebrafish homologues of the human NRs are functioning very similarly to the human receptors and can bind the same ligands. Zebrafish steroid hormone receptors, for example, are responsive to human steroid hormones (Alsop and Vijayan, 2008; Archer et al. 2008; Linville et al. 2009; Costache et al. 2005). While drugs targeting human NRs are known to also bind and activate their homologous zebrafish NRs. Therefore, a ligand discovered in the zebrafish would likely be functioning in humans as well.

1.1.5.2 pLT-gypsy transgenic zebrafish lines

Currently, our lab has zebrafish lines that express "ligand trap" fusion protein constructs with a number of human LBD inserts. These ligand trap vectors, (pLT-gypsy) contain two functional components: the left half encoding an NR fusion protein, and the right half containing a GFP reporter system (Figure 1-4). The fusion protein construct (left side) consists of an NR-LBD, fused to the Gal4 DBD, as well as an “FSH” (3xFLAG, StrepII, 6xHis) affinity tag. This fusion protein is expressed under the control of a zebrafish heat-shock promoter (hsp70). Upon heat induction, the expressed fusion protein can be purified, and the bound ligand extracted and identified by mass spectrometry. The use of this fusion protein approach, consisting of the nuclear receptor LBD fused to a Gal4 DBD and N-terminal tags, instead of using a full length NR protein, will avoids potentially complicating and disruptive interference with endogenous NR proteins, and vice versa.

The other half of the LT construct encodes a Green fluorescent reporter protein (eGFP), under the control of a 14xUAS_{GAL4} enhancer sequence (Figure 1-4). The NR fusion protein is able to bind the UAS element using its Gal4 DBD, and upon ligand-induced activation of the LBD,

leads to the expression of GFP. Therefore, GFP can be seen in the tissues of the zebrafish embryo where the nuclear receptor ligand is present (if an agonist). Hence, these transgenic zebrafish lines can be used to detect, purify and identify endogenous as well as exogenously provided ligands.

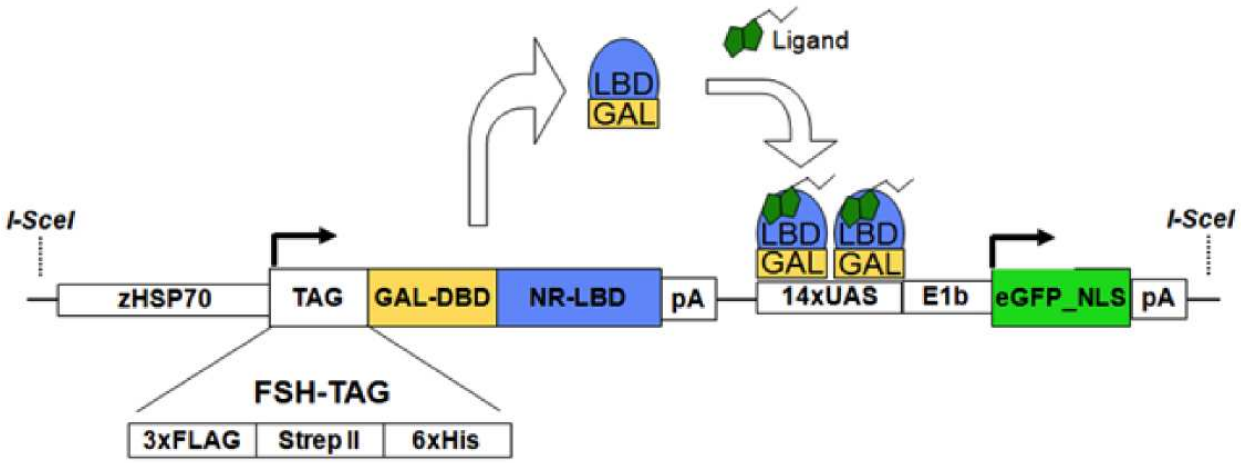

Figure 1-4. Schematic of transgenic construct harbored by pLT-gypsy transgenic zebrafish lines. (Tiefenbach et al., 2010).

1.1.5.3 Zebrafish heat-shock promoter

Since the transgenic pLT-gypsy vector employs the zebrafish *hsp70* promoter for expression of NR fusion proteins, and heat shock proteins are also known to play roles in NR function, an introduction to heat-shock proteins is in order.

Heat-shock proteins of various molecular weights are produced by all eukaryotes under heat-shock or other environmental stresses. The heat-shock proteins, such as the Hsp70 family, have been shown to act as chaperones for mediating the correct assembly and folding of proteins under stress conditions (Wu, 1995). But even under normal growth conditions, these proteins are expressed at relatively high levels to aid in protein folding. With respect to NRs, some of the heat-shock proteins, such as Hsp70, are known to bind to un-liganded steroid hormone receptors in the cytosol to prevent them from homo-dimerizing and translocating to the nucleus (Smith and Toft, 2008).

The *hsp70* promoter has become popular for transgenic over-expression of proteins in the zebrafish model system (Shoji and Sato-Maeda, 2008). This promoter has allowed experimental control over the timing and duration of the expressing protein. Regarding the mechanism of *hsp70* gene expression, the *hsp70* gene is under the control of a transcription factor called the heat-shock factor (HSF). Under normal conditions, the HSF protein is bound within a protein complex consisting of the hsp90 protein (Zou et al., 1998). Upon heat-induced stress, the unfolding of proteins in the cell causes the Hsp90 protein to be recruited away from the HSF complex, allowing HSF protein to bind heat-shock elements on the *hsp70* gene promoter and activate transcription.

1.1.6 PPARγ

In order to optimize the ligand identification protocol, I employed the use a transgenic pLT-gypsy zebrafish line expressing a tagged fusion protein containing the PPARγ receptor LBD (pLT-PPARγ fish line; Tiefenbach et al., 2010). This particular line was chosen because the receptor is a known drug target for the Type II diabetes drug, rosiglitazone (Avandia) (Lehmann et al. 1995), and J. Tiefenbach in our lab had shown that pLT-PPARγ embryos respond well to rosiglitizone. My objective was to treat PPARγ transgenic embryos, following heat-induced expression and rosiglitazone treatment, to see if rosiglitazone could be co-purified with the activated fusion protein. I will give a brief introduction to the background of the PPARγ receptor.

The PPARγ receptor is abbreviated from the name Peroxisome Proliferator-Activated Receptor gamma. This name is derived from a related receptor, the PPARα, which when activated by fibrate drugs, is found to induce proliferation of peroxisomes, which are organelles that function to breakdown long-chain fatty acids. The resulting shorter-chain fatty acids can then be transported into the mitochondria and broken-down for energy production. The PPARγ receptor was discovered by sequence homology to the PPARα receptor (Tontonoz and Spiegelman, 2008).

In terms of its structure, the PPARγ receptor contains the same domains as other NRs. Like the other PPARs, they also bind to RXRs as obligate hetero-dimers in order to bind to HREs on the enhancers of target genes (Viswakarma et al., 2010).

The PPARγ receptor has been named the "Master regulator" of adipogenesis because it plays a crucial role in the terminal differentiation of pre-adipocytes into mature adipocytes. An adipose-specific knockout of PPARγ in mice results in malformation and distribution of white and brown adipose tissue (Jones et al., 2005). Furthermore, ectopic expression of PPARγ in fibroblast cell lines results in the accumulation of lipid droplets and up-regulation of adipocyte-specific genes (Tontonoz et al., 1994). Apart from adipose tissue, the PPARγ receptor also plays important roles in other tissues and cells, for example in macrophages and the brain (Weisberg et al., 2003; Lu et al., 2011).

Although the biological processes and functions governed by PPARγ has been heavily studied, the identity of its endogenous ligand is still relatively unclear. From screens of natural fatty acids, PPARγ was found to bind prostanoids such as 15-deoxy-12,14-prostaglandin J2 (Forman et al., 1995; Kliewer et al., 1995). However, based on its affinity for the receptor, this ligand was found to be present in-vivo at levels too low to be the biologically significant (Bell-Parikh et al., 2003). Other lipids such as 9-HODE and nitrated fatty acids have also been reported to activate the PPARγ receptor (Nagy et al., 1998; Schopfer et al., 2005), but are also not widely regarded as significant in vivo ligands.

1.1.7 Mass spectrometry

Mass spectrometry is an analytical technique often used to identify unknown compounds in a sample. The identification of the compounds or molecules by mass spectrometry is based upon the movement of charged particles in an electromagnetic field (Ramanathan, 2008).

The mass spectrometer can be separated into three main parts: an Ion source, a mass analyzer and a detector (Figure 1-5). The ion source induces the neutral compounds to form charged particles that can be accelerated into the mass analyzer by an electric or magnetic field. Using this electric or magnetic field, the mass analyzer acts as a filter to separate the ion particles by its

mass-to-charge ratio (m/z). A detector then measures the abundance of ions with a specific mass-to-charge ratio at a particular point in time (Ramanathan, 2008).

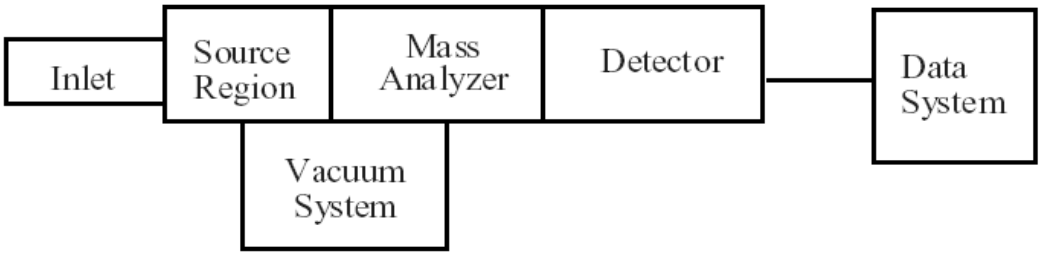

Figure 1-5. Basic components of a mass spectrometer (Van Bramer, 1997). (Reprinted from Van Bramer S.E., An introduction to Mass Spectrometry, Widener University: Department of Chemistry, pp1-38, Revised: 2nd September 1998, Copyright 1997, with permission from Dr. Scott E. Van Bramer)

1.1.7.1 ESI-MS

Various methods can be used to ionize the compounds for analysis by mass spectrometry. For optimization of the ligand identification protocol Electrospray ionization MS (ESI-MS) was used. ESI is typically used to ionize large or thermally sensitive molecules (Ramanathan, 2008). This technique is considered a "soft" method of ionization, allowing for the formation of relatively large molecular or parent ions, as opposed to other more energetic techniques (e.g. electron bombardment) that may cause extensive fragmentation of the analyte into smaller ions. ESI is used to ionize compounds with a mass range between 100 to 1,000,000 Da, such as proteins, peptides polymers and small molecule ligands (Fenn et al., 1989).

The samples or analytes are mixed with a volatile solvent and pumped through a capillary tube. At the end of the tube an electric field is applied. As the liquid leaves the tube as an aerosol, the electric field induces the particles in the droplets to form ions, which are then accelerated into the mass analyzer (Kebarle and Verkerk, 2009). For most applications, an HPLC column is coupled with the ESI-MS. Liquid chromatography can be used to separate a mixture of compounds before injection into the mass spectrometer (Kim et al., 2009).

1.1.7.1.1 Denaturing ESI-MS

To identify the co-purified ligand, denaturing ESI-MS was performed following affinity purification of the PPARγ-fusion protein. For denaturing ESI-MS, organic solvents are used to

denature the purified fusion protein and thus releasing the bound ligand. Solvents such as chloroform can be used to help dissolve and separate hydrophobic ligands from the protein portion (Chakravarthy et al., 2009). For ionization of proteins, acetonitrile or methanol can be used to increase the solubility of the protein samples. The ligand or protein samples can then be put into the LC-MS for mass spectrometry analysis.

1.1.7.1.2 Mass analyzer: Triple Quadrupole

Following ionization, the ions are accelerated into the mass analyzer by an electric field, where they are separated according to their mass-to-charge (m/z) ratio. At a certain point in time, a single ion of a specific m/z will be transmitted to the detector. Although several types of mass analyzers are available, for this project a Triple Quadrupole mass spectrometer was used. I will first explain the workings of a quadrupole mass spectrometer before discussing the triple quadrupole.

A quadrupole mass spectrometer consists of 4 circular rods or electrodes set parallel to each other (Figure 1-6B). A radio frequency (RF) voltage or alternating current (AC) is supplied between the two pairs of opposing rods in an alternating or oscillating fashion. At a particular RF frequency and voltage, the quadrupole acts to filter out ions that have m/z values lower than the cut-off, while allowing the transmission of ions with higher m/z values (de Hoffmann, 1996). The transmitted ions will travel in the center of the quadrupole, between the four electrodes (Figure 1-6B). Ions with lower m/z values will have a higher acceleration rate and therefore will be attracted to and collide with the electrodes, followed by removal by the vacuum system.

A direct current (DC) is also superimposed on the RF. In combination with the RF, the DC voltage acts to filter out the ions with m/z values higher than the cut off, while allowing the transmission of ions with lower m/z values. Since ions with a higher m/z value cannot refocus quickly enough during the RF cycle, the DC voltage has a greater influence on their movement. The resulting effect is that the higher m/z ions slowly drift from the center of the quadruprole and eventually collide with the electrodes (de Hoffmann, 1996).

As the ions travel through the quadrupole between the 4 electrodes, they are filtered according to their m/z ratio. The resulting effect is that at a specific DC and RF voltage, only ions of a single m/z value can be transmitted to the detector. Adjustment of the RF and DC voltages allows the selection of different m/z values (de Hoffmann, 1996).

Building on this, a triple quadrupole mass spectrometer consists of 3 quadrupoles connected in succession (Figure 1-6A). The first quadrupole acts as a filter to separate ions by m/z value, while the second (middle) quadrupole acts as a collision cell for performing collision-induced dissociation to fragment molecular/parent ions that passed through the first quadrupole, into smaller product ions. These fragmented product ions are then transferred into the third quadrupole for analysis.

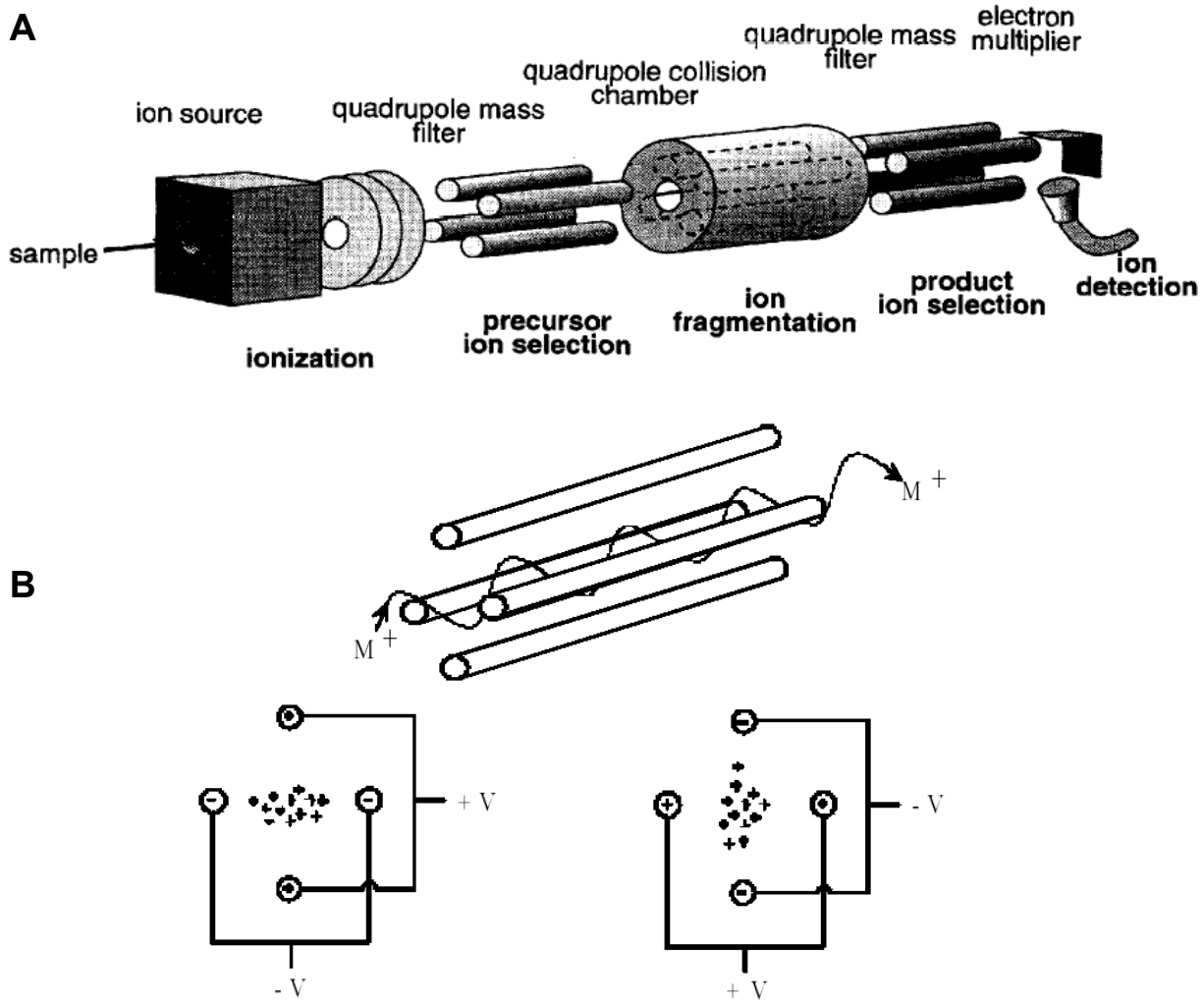

Figure 1-6. Triple quadrupole mass spectrometer. (A) Components of a Triple quadrupole mass spectrometer. (B) Movement of an ion through a quadrupole is dictated by the RF and DC voltages applied to the four electrodes. Adapted from de Hoffmann, 1996 and Van Bramer, 1997. (Reprinted from Journal of Mass Spectrometry, vol. 31, de Hoffmann E., Tandem mass spectrometry: a Primer, pp129-137, Copyright 1996, with permission from John Wiley and Sons, License number: 2790380477667). (Reprinted from Van Bramer S.E., An introduction to Mass Spectrometry, Widener University: Department of Chemistry, pp1-38, Revised: 2nd September 1998, Copyright 1997, with permission from Dr. Scott E. Van Bramer).

1.1.7.1.3 LC-MRM

The identification of ligands in this project employed two modes of mass spectrometry analysis, LC-MRM and LC-MS scanning. Multiple reaction monitoring (LC-MRM) is an analysis mode that allows for the identification, at high sensitivity, of a particular analyte within a mixture of many compounds (Anderson and Hunter, 2006). This is done by setting the Triple Quadrupole

mass spectrometer to a fixed DC and RF voltage to only allow ions of a specific m/z ratio to be transmitted to the detector. Prior knowledge of the compound's identity and its fragmentation pattern must be known. From this knowledge, the parameters can be set in the first and third quadrupoles to select for the desired parent and product ions, respectively. Since the parameters are fixed for the duration of the experiment, many ions of the specific m/z value are transmitted to the detector, leading to higher sensitivity (de Hoffmann, 1996; Ramanathan, 2008).

1.1.7.1.4 LC-MS scanning

LC-MS scanning mode allows for the potential identification of all components in a mixture of compounds. The parameters (RF and DC voltage) are varied over time to "scan" through all the possible m/z values and identify all the compounds in the mixture. This will produce a mass spectrum of the compounds. Unlike LC-MRM, prior knowledge of the molecules' identity and fragmentation pattern need not be known. However, this method is much less sensitive compared to LC-MRM in identifying the presence of individual molecules. This is because the parameters (RF and DC voltage) are not fixed, and therefore selective for an individual molecule for only a shorter period of time compared to LC-MRM (de Hoffmann, 1996; Ramanathan, 2008).

To find the identity of these unknown molecules, the respective molecular ions of a specific m/z value can be selected in the first quadrupole, transferred into the second quadrupole to be fragmented and then 'scanned' in the third quadrupole to find the fragmentation pattern. From the molecules' fragmentation pattern, the identity of the molecule may be figured out. If sufficiently pure, abundant and cooperative, unknown co-purified ligands can be identified.

1.1.7.1.5 Non-denaturing ESI-MS

Non-denaturing ESI-MS allows for the study of intact protein-ligand complexes (Loo, 1997). Following affinity purification of the protein-ligand complex, the analytes can be transferred into a non-denaturing aqueous buffer such as ammonium acetate. This will keep the protein-ligand complex intact. The whole protein-ligand complex can then be ionized ‘gently’ using ESI and identified by mass spectrometry. Subsequent collision-induced dissociation (CID) can then be performed to break the protein-ligand complex, thus releasing the ligand. The mass difference between the bound and unbound proteins should reflect the mass of the interacting ligand. Since

the mass of the bound ligand can also be detected, this method gives a considerable advantage over denaturing MS to identify unknown NR ligands.

1.2 Objective

The aim of my project is to optimize a ligand identification protocol that can be used for the co-purification of endogenous ligand(s) from a native in vivo tissue source. Traditional approaches for ligand identification have mostly come about through in vitro screening of natural small molecules to test for binding in cell culture (Kliewer et al., 1999; Levin et al., 1992). However, this approach has proven ineffective in elucidating the endogenous ligands for many orphan NRs.

To standardize the protocol for ligand identification, I treat 1dpf pLT-PPARγ zebrafish embryos with 1μM rosiglitazone (Tiefenbach et al., 2010). The particular line of zebrafish we are using is expressing a tagged-fusion protein containing the PPARγ LBD (Figure 1-4). Since rosiglitazone binds inside the hydrophobic PPARγ ligand-binding pocket in much the same way as other NR ligands, affinity purification conditions should be useful for all NR ligand co-purifications (Lehmann et al. 1995; Nolte et al., 1998; Huang et al., 2010). Identification of the bound rosiglitazone by mass spectrometry following affinity purification of the PPARγ-fusion protein from the rosiglitazone-treated zebrafish embryos would serve as a proof of concept for a functional and optimized protocol. This protocol could then be used for the identification of endogenous ligands for other orphan receptors. Some variations may need to be introduced for extraction and mass spectrometry identification steps, depending on the hydrophobicity, charge, size and ionization properties of various ligands.

Upon optimization of the ligand-identification protocol, I will be able to use this protocol to identify endogenous ligands for ‘orphan’ NRs (Table 1-1). The identification of these ligands will open doors into understanding how these receptors are regulated and which biological processes they control. This may also eventually lead to the discovery of new drugs that target these receptors (Shi, 2007).

A drug screen was performed in the lab to identify molecules that activate the PPARγ receptor using the pLT-PPARγ zebrafish line (JT, in preparation). The ligand identification protocol can

be used to identify direct binding between any positively acting compounds and the PPARγ LBD. In addition, the pLT-PPARγ fish can also be used to co-purify novel proteins that interact with PPARγ in a ligand-dependent fashion.

Table 1-1. Human nuclear receptors and identified endogenous ligand(s)

Human Nuclear Receptors	**Isoforms**	**Potential endogenous ligand(s)**
Thyroid hormone receptor	TRα	Thyroid hormone
	TRβ	Thyroid hormone
Retinoic acid receptor	RARα	Retinoic acid
	RARβ	Retinoic acid
	RARγ	Retinoic acid
Peroxisome proliferator-activated receptor	PPARα	16:0/18:1-GPC
	PPARβ	Fatty acids
	PPARγ	Fatty acids
Reverse-erbA	Rev-erbα	Heme
	Rev-erbβ	Heme
RAR-related Orphan receptor	RORα	Cholesterol
	RORβ	All-trans retinoic acid
	RORγ	Retinoic acid
Liver X receptor	LXRβ	Oxysterols
	LXR	Oxysterols
Farnesoid X receptor	FXR	Bile acids, Lanosterol
Vitamin D receptor	VDR	Vitamin D_3, Bile acids
Pregnane X receptor	PXR	Xenobiotics
Constitutive androstane receptor	CAR	Xenobiotics, Androstane
Hepatocyte nuclear factor 4	HNF4α	Linoleic acid
	HNF4γ	Fatty acids
Retinoid X receptor	RXRα	9-cis-Retinoic acid
	RXRβ	9-cis-Retinoic acid
	RXRγ	9-cis-Retinoic acid
Testicular receptor	TR2	Orphan
	TR4	Orphan
Tailess	TLL	Orphan
Photoreceptor cell-specific nuclear receptor	PNR	Orphan
Chicken ovalbumin upstream-promoter transcription factor	COUP-TFI	Orphan
	COUP-TFII	Orphan
ErbA2-related gene-2	EAR2	Orphan
Estrogen receptor	ERα	17b-Estradiol
	ERβ	17b-Estradiol
Estrogen-related receptor	ERRα	Orphan
	ERRβ	Orphan

	ERRγ	Orphan
Glucocorticoid receptor	GR	Cortisol
Mineralocorticoid receptor	MR	Aldosterone
Progesterone receptor	PR	Progesterone
Androgen receptor	AR	Testosterone
Nerve growth factor 1B	NGF-1B	Orphan
Nuclear receptor-related 1	Nurr1	Orphan
Neuron-derived orphan receptor 1	NOR1	Orphan
Steroidogenic factor 1	SF1	Orphan
Liver receptor homolog 1	LRH-1	Orphan
Germ cell nuclear factor	GCNF	Orphan
Dosage-sensitive sex reversal, adrenal hypoplasia critical region, chromosome X gene 1	DAX1	Orphan
Short Heterodimeric Partner	SHP	Orphan

Compiled from Noy, 2007; Pardee et al., 2011; Mangelsdorf et al., 1995. (With kind permission from Springer Science + Business Media: Subcellular Biochemistry, Nuclear receptors: Small molecule sensors that coordinate growth, metabolism and reproduction (Chapter 6 in A handbook of transcription factors), vol. 52, 2011, p124, Pardee K., Necakov A.S., Krause H., (ed: Hughes, T.R.), Table 6.1, License number: 2786080560618). (Reprinted from Cell, vol. 83, Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M., The nuclear receptor superfamily: the second decade, pp835-839, Copyright 1995, with permission from Elsevier, License number: 2786081039243). (Adapted with permission from: Noy, N. (2007). Ligand Specificity of Nuclear Hormone Receptors: Sifting through Promiscuity. Biochemistry 46, 13461-13467. Copyright 2007 American Chemical Society).

1.3 Summary

Nuclear receptors play a crucial role in many biological processes and its dysregulation has been implicated in many diseases. Although some receptors such as the ERs have been heavily studied, research on a large number of orphan receptors have been impeded by the lack of molecular biology tools available (Edwards et al., 2011). NR ligands are one such tool that can be used to study a particular receptor. Therefore, optimization of the ligand identification protocol will allow for the identification of endogenous ligand(s) for the orphan receptors, and thus promote research into the biology of these receptors. The identification of these ligands will open doors into understanding how these receptors are regulated and also the biological/physiological processes that these receptors control. Furthermore, by understanding the biochemical interaction between the ligand and its cognate receptor, we will be able to relate

the structural features of the ligand to the downstream activity of the receptor. This may eventually lead to the discovery of drugs to target these receptors.

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Chapter 2
Methods and Results

2 Methods and Results

2.1 Method

2.1.1 Drugs Used

Rosiglitazone (Cayman, #71740) was stored in DMSO at stock concentration of 1mM and treated to zebrafish embryos at a final concentration of 1μM. GW9662 (Cayman, #70785) was stored in DMSO at a stock concentration of 1mM and treated to zebrafish embryos at a final concentration of 2μM. Ibuprofen (Cayman, #70280), capsaicin (Cayman, #92350) and idebenone (Sigma-Aldrich, #I5659) were stored in DMSO at a stock concentration of 100mM and treated to zebrafish embryos at a final concentration of 4μM.

2.1.2 Zebrafish heat-shock

Zebrafish were maintained on a 14/10 hr light/dark cycle and crossed once a week. For heat-shock, 1dpf zebrafish embryos were dropped into a bottle of pre-heated fish water (0.075g/L $NaHCO_3$, 0.018g/L Sea salt, 0.0084g/L $CaSO_4.2H_2O$) at 37°C (+/- drugs) and heat-induced for a duration of 1hr. Following a 1hr 30min recovery at 28.5°C, embryos were frozen in Liquid nitrogen.

2.1.3 Affinity purification (ligand co-purification)

Purification steps were performed at 4°C. Frozen embryos were homogenized in lysis buffer (50mM Hepes ph7.5, 1M NaCl, 0.1% Triton X-100, 1mM DTT, 1mM EDTA, 1ppm BHT, Complete protease inhibitor cocktail tablet (Roche, 1 tablet/50ml buffer)) at a ratio of 4ml lysis buffer/gram embryos. After 20min incubation on ice, the lysate was centrifuged at 14000 x g for

15mins. The supernatant was passed through a column of pre-washed (lysis buffer) avidin-conjugated agarose slurry (Pierce, #20219; 1:30 slurry:lysate) and the resulting flow-through incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich, #A2220; 1:150 slurry:lysate) for 2hrs. After incubation the beads were transferred to a 2ml-gravity disposable column (Biorad, #732–6008), washed 3 times with 6 column volumes (bead volume) of lysis buffer and eluted three times with 4, 3 and 3 column volumes of 300ug/ml 3xFLAG peptide dissolved in lysis buffer (each elution requires 30mins incubation). The Flag elutions were combined and incubated with StrepTactin slurry (IBA, #2-1201-0251; 1:5 slurry:flag elution) for 1.5hrs. The beads were then transferred to a 2ml-gravity disposable column and washed 3 times with 6 column of Last Wash Buffer (800mM ammonium acetate pH7.5, 1ppm BHT). The beads were then transferred to a glass vial and the samples prepared for mass spectrometry.

2.1.4 Affinity purification (cofactor purification)

Cofactor purification was also performed at 4°C. Frozen embryos were homogenized in lysis buffer (50mM Tris-Cl pH7.4, 150mM NaCl, 0.1% Triton X-100, 1mM DTT, 1mM EDTA, protease inhibitor (Roche, 1 Tablet/50ml buffer) at a ratio of 4ml lysis buffer/gram embryos. After a 20min incubation on ice, the lysate was centrifuged at 12000 x g for 15mins. The supernatant was then passed through a column of pre-washed (lysis buffer) avidin-conjugated agarose (1:30, slurry:lysate) and the resulting flow-through incubated with StrepTactin slurry (1:125, slurry:lysate) for 2hrs. After incubation the beads were transferred to a 10ml-gravity disposable column (Biorad; #731–1550), washed 3 times with 6 column volumes (bead volume) of lysis buffer and eluted three times with 3, 2 and 2 column volumes of 5mM d-desthiobiotin dissolved in lysis buffer (each elution requires 30mins incubation). Strep elution fractions were combined and incubated with anti-FLAG M2 affinity gel (1:20, slurry:lysate) for 1.5hrs. After incubation the beads were transferred to a 2ml-gravity disposable column, washed 3 times with 6 column volumes (bead volume) of lysis buffer and eluted three times with 4, 3 and 3 column volumes of 300ug/ml 3xFLAG peptide dissolved in lysis buffer (each elution requires 30mins incubation).

2.1.5 Mass spectrometry analysis of drugs bound to the NRs

For analysis of drugs bound to the NR, two sample preparation procedures were used. For rosiglitazone, the beads from affinity purification were treated with 2:1 chloroform/methanol (in a ratio of 1:4, beads/solution : chloroform/methanol) and then centrifuged. The chloroform phase was collected and dried under a stream of nitrogen gas. The dried residue was reconstituted in 10% acetonitrile prior to LC-MRM. For ibuprofen, idebenone and capsaicin, 1ml of 50% acetonitrile/5% formic acid was added to the beads, incubated for 5min and filtered. The filtrates were dried and resuspended in 60ul of 50% acetonitrile/0.2% formic acid prior to LC-MRM.

LC-MS analysis was performed on a triple quadrupole mass spectrometer (Quattro micro^{TM}, Micromass, Manchester, UK) coupled with an Agilent 1100 HPLC. A reverse phase column (Luna C-18(2); 3 μM, 1.00 mm X 15 cm, Phenomenex, Torrance, CA) was used for LC separation. For analysis of rosiglitazone, an elution gradient was used, starting with 0.005% formic acid, 5% acetonitrile in water (buffer A) at time zero, with a flow rate of 70μl/min, increasing to 0.05% formic acid, 50% acetonitrile in water (buffer B) by 10 min, kept stable for 5 min, and then increasing to 0.095% formic acid, 95% acetonitrile in water (buffer C) over the next 5 min and then returning to Buffer A over the next 5min. For analysis of ibuprofen, idebenone and capsaicin, a gradient starting with Buffer A, with a flow rate of 100 μl/min was used, changing to Buffer C by 15 min, kept stable for 5 min, and then returning to Buffer A over the next 5 min.

General MS conditions were as follows: Capillary voltage was 3.5 kV, cone voltage was 30 V, and LM/HM resolution for both MS and MS2 was set to 15/15 so that a resolution of 1100 at FWHM was achieved for peak at 370 m/z. For LC-MRM, argon was used as a collision gas at a pressure of $3.1X10^{-3}$ mbar.

MRM conditions for each drug were as follows:

Ibuprofen, ES-, transition 205----101. Collision Cell Voltage 5 V,

Rosiglitazone, ES+, transition 358----135. Collision Cell Voltage 27 V,

Idebenone, ES+, transition 339----197. Collision Cell Voltage 18 V,

Capsaicin, ES+, transition 306----137. Collision Cell Voltage 18 V

2.1.6 MALDI-TOF Mass spectrometry analysis of co-purified proteins

Affinity purification elution samples were mixed with 4xSDS loading buffer in a ratio of 3:1 (elution sample:loading buffer). The samples were then run on SDS-PAGE and subjected to silver staining. Bands of interests were sliced out and stored in 1% acetic acid at 4°C, overnight. After a brief wash with HPLC grade water, the excised gel bands were de-stained in a 1:1 solution of 100mM sodium thiosulfate [$Na_2S_2O_3$]:30mM potassium ferricyanide [$K_3Fe(CN)_6$]. The gel pieces were then incubated with a solution of 50mM NH_4HCO_3 (pH 8.0) for 10min followed by 50mM NH_4HCO_3/50% acetonitrile (pH8.0) for another 10min. Then the gel pieces were incubated with acetonitrile for 15min. Following this, the gel pieces were reduced with 10mM dithiothreitol (in 100mM NH_4HCO_3) pH8.5 at 50°C for 60 min. After another brief acetonitrile wash, gel pieces were submerged in 100mM NH_4HCO_3 55mM iodoacetamide for 20 minutes at room temperature in the dark. The gel particles were then washed with 100mM NH_4HCO_3 followed by acetonitrile, and then dried.

Dried gel particles were then digested in trypsin solution (6.25ng/ml trypsin, 50mM NH_4HCO_3, 0.05% CaCl2, 0.1% N-octyl Glucoside, 0.06mM HCl) at 37°C overnight. The digestion reaction was stopped with addition of acetic acid. The digested peptides were extracted from gel particles by shaking in 100mM NH_4HCO_3 at 37°C for 1hour.

The digested peptides are purified using C-18 beads (Sigma). The extracted peptides were incubated with C-18 beads at 37°C for 30min and washed with a solution of 2% acetonitrile, 1% acetic acid. The peptides were eluted with 75 % acetonitrile, 1% acetic acid at room temperature for 10min. The purified peptides were then analyzed using MALDI-TOF-MS.

2.1.7 ORBITRAP Mass spectrometry analysis of co-purified proteins

Elution samples were mixed with TCA (100%, w/v, 6.1N) in a ratio of 4:1 protein sample:TCA and incubated at 4^oC overnight. After centrifugation of the sample, pellets (precipitated protein samples) were washed twice with acetone at -20°C for 30min per wash, dried at room temperature, dissolved in 2mM TCEP-HCl/50mM ammonium bicarbonate and incubated at room temperature for 45min. Iodoacetamide was added (final concentration 10mM) and the

samples incubated at room temperature in darkness for another 40 mins. Following addition of $CaCl_2$ (final concentration of 1mM), the samples were subjected to overnight incubation with Trypsin (10ng/μl) at 37°C. The reaction digest was then stopped with the addition of Formic acid (Fluka) to 1% final concentration.

Micro-chromatography columns for reverse phase HPLC were prepared by packing a 100mm x 75mm column with Luna C-18 (2) beads (Phenomenex, Torrance, CA). Trypsin digested samples were loaded onto the column and driven by the EASY-nLC system (Proxeon, Odense, Denmark) using a buffer gradient of 98% buffer A (5% acetonitrile/0.1% formic acid) to 90% buffer B (95% acetonitrile/0.1% formic) over a duration of 105mins at a flow rate of 300nL/min. Eluted peptides were directly sprayed into an LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA) using a nanospray ion source (Proxeon). A spray voltage of 2.5 kV was applied.

2.2 Results

2.2.1 Introduction: Optimization of ligand identification protocol

In order to optimize the ligand identification protocol, I employed the use of a transgenic pLT-PPARγ zebrafish line, which expresses an affinity tagged (Flag-His-Strep II) fusion protein containing the PPARγ receptor LBD fused to a GAL4 DBD (Figure 1-4; Tiefenbach et al., 2010). This line was previously shown to respond to the PPARγ -specific drug rosiglitazone (Avandia), which has been used for the treatment of Type II diabetes (Lehmann et al. 1995). In untreated 1dpf pLT-PPARγ embryos, GFP signal corresponding to endogenous ligand response is limited to the tail region as well as a few dots elsewhere in the body. When rosiglitazone is added, an increase in GFP signal, produced under control of the UAS_{GAL4}-dependent promoter is seen (Figure 2-2). This shows that rosiglitazone is absorbed by the zebrafish embryos, and that the expressed PPARγ-fusion protein is able to fold properly into a functional LBD and bind to the drug. This acts as a proof of concept, showing that the pLT-gypsy transgenic zebrafish lines can serve as a tool for ligand identification. It also indicates that this PPARγ fusion protein fish line, after treatment with rosiglitazone, should serve as a suitable platform for optimization of my ligand co-purification and identification protocol (Figure 2-5).

The first step to optimizing the ligand identification protocol is to maximize the amount of heat-shock-induced expression of the PPARγ-fusion protein. The following steps would then be to optimize lysate production and affinity purification such that PPARγ yield is optimized, and then optimizing ligand extraction and mass spectrometry approaches. Thus, the overall goal is to optimize the ligand identification protocol so that maximal amounts of ligand are co-purified, extracted and detected by mass spectrometry.

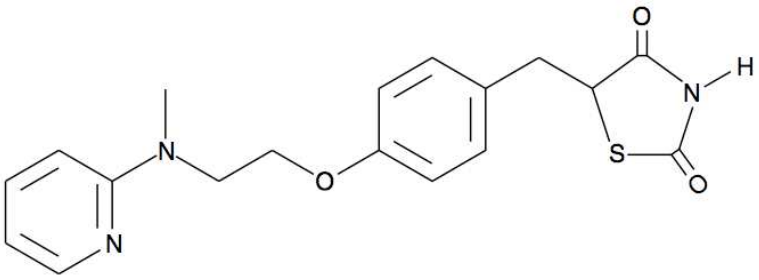

Figure 2-1. Chemical structure of rosiglitazone.

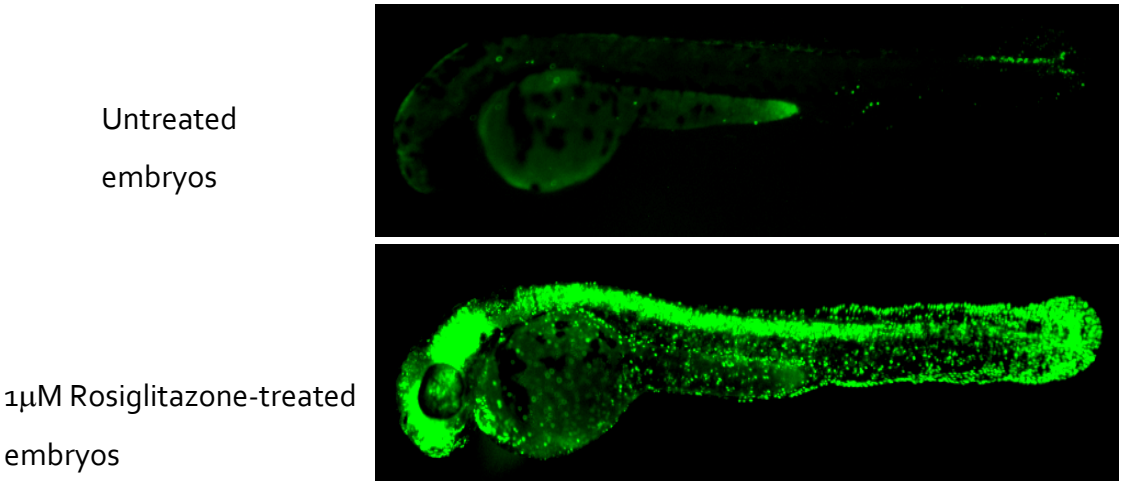

Figure 2-2. Transgenic "Ligand-trap" zebrafish embryos expressing a fusion protein containing the PPARγ ligand-binding domain. 1dpf (days post fertilization) embryos were untreated (top) or treated with 1μM rosiglitazone (bottom) and imaged at 2dpf.

2.2.1.1 Optimization of heat-shock induced fusion protein expression

Since the PPARγ-fusion protein construct is under the control of a zebrafish hsp70 promoter, I decided to vary the heat-shock conditions in order to maximize the amount of expressed PPARγ-fusion protein.

Firstly, I needed to know which stage of development would be best for heat shock induction and protein purification. I started by comparing the levels of heat-shock-induced fusion protein expression at different stages of zebrafish development (Figure 2-3). In each case, the pLT-PPARγ fish were heat shocked at 37°C for 1hr. The level of heat shock-induced PPARγ-fusion protein was much higher in earlier stages of development (1 to 3 days-post-fertilization; dpf) compared to later stages of development, at larval stages or even at adulthood. 3dpf zebrafish embryos gave the highest heat-shock-induced level of PPARγ-fusion protein.

Despite this, I am currently collecting 1dpf zebrafish embryos for my protein purification due to the ease of manipulation and collection. 1dpf zebrafish embryos, which are still protected by a chorion, can be easily collected and pooled from many 15cm cell-culture plates into a tea strainer. On the other hand, older embryos that are outside of the chorion will pass through the tea strainer and cannot be pooled easily. Using smaller meshes to capture the 3dpf was found to kill them. Furthermore, older embryos start swimming, which makes them hard to pipette and pool into a smaller volumes of liquid. For future experiments that require the use of older embryos or larvae, new approaches will need to be adapted to pool the larvae together for heat-shock.

For the 1dpf embryos, I varied the length of time that the embryos were heat shocked at 37°C to find the condition that gave the highest level of PPARγ-fusion protein (Figure 2-4). My results showed that heat shocking 1dpf embryos at 37°C for 1hr gave the highest level of fusion protein expression. I did not increase the heat-shock temperature because 37°C heat-shock for zebrafish embryos is standard. Furthermore, in agreement with previous results, I found that heat-shocking the zebrafish 1dpf embryos at 39/40°C led to embryonic lethality (Krone et al., 1997b).

Currently, the zebrafish 1dpf embryos are heat-shocked at 37°C for 1hr, followed by a recovery period at 28°C for 1hr, and then flash-frozen in liquid nitrogen. I also quantified the level of expressed PPARγ-fusion protein at different time-points after heat-shock, and found that, while

the fusion protein lasted for as long as 7 hours after heat-shock, optimal levels peaked at approximately 1 hr after induction (data not shown).

The current heat-shock protocol is as follows: 1) Preheat embryo medium (0.00003% Methylene blue in fish water) in a 37°C water bath (rosiglitazone or other drugs may be added to the medium at this time; 150ml medium per 250ml Glass bottle). 2) 1dpf zebrafish embryos are dropped directly into preheated medium (4ml embryos/liquid per 150ml medium) and heated for 1hr. 3) Embryos are then poured into a 150mm x 20mm Petri-dish and left to recover (open lid) at 28°C for 1hr 15mins. 4) Finally, embryos are aliquot into tubes and frozen in liquid nitrogen.

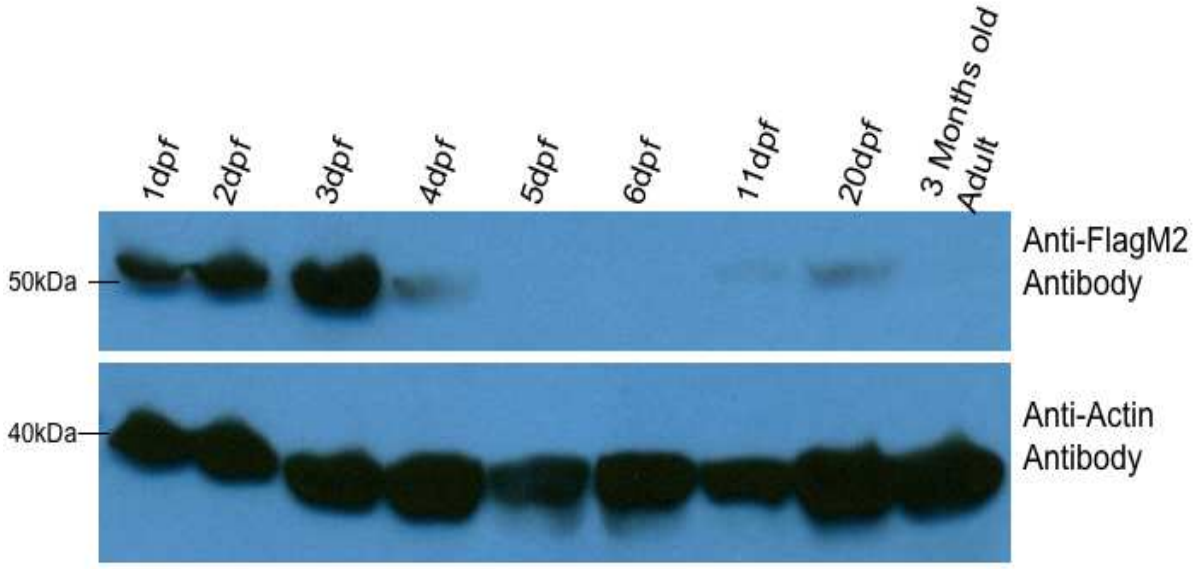

Figure 2-3. Heat-induced PPARγ-fusion protein expression at different stages of development. Transgenic PPARγ zebrafish embryos/larvae were heat-shocked at varying stages in a 37°C water-bath for 1hr. Following heat-shock, the embryos were recovered for 1hr before freezing in liquid N2. PPARγ-fusion protein (53kDa) was detected using Anti-Flag Antibody.

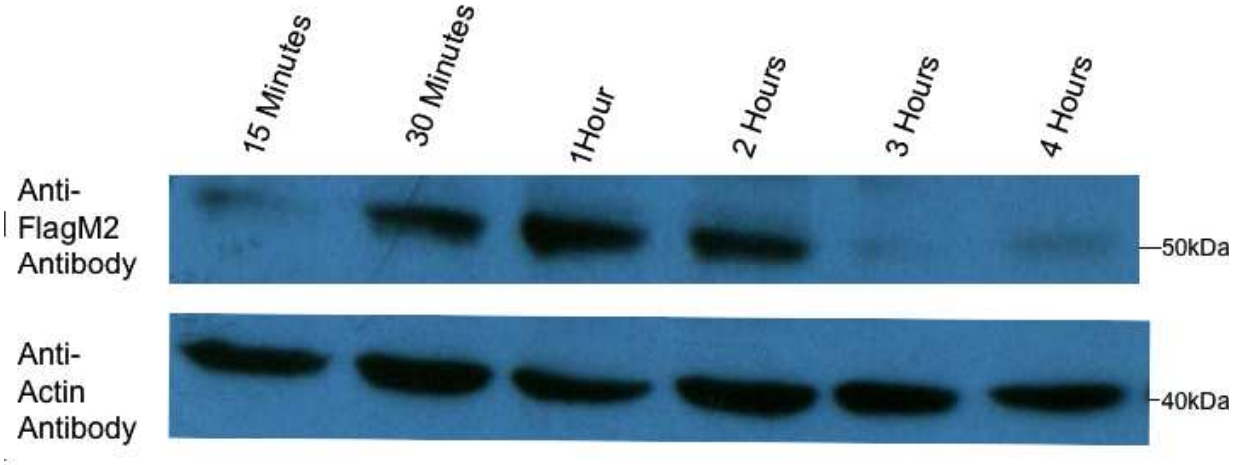

Figure 2-4. Level of PPARγ-fusion protein expressed with respect to duration of heat-induction. PPARγ 1dpf embryos were heat-shocked in a 37°C water-bath for varying amount of time. Following heat-induction, the embryos were recovered for 1hr and then frozen in liquid N2. PPARγ-fusion protein (53kDa) was detected using Anti-Flag Antibody.

2.2.1.2 Optimization of protein extraction from tissues and cells

In order to isolate and purify the receptor-ligand complex, tissues need to be homogenized and the cell membranes broken to release the intracellular material. At the same time, the buffer used needs to allow for efficient cell lysis, while preventing protein denaturation, thereby keeping the ligand inside the ligand-binding pocket.

For protein extraction, I am currently homogenizing the frozen embryos in a high-salt lysis buffer (50mM Hepes ph7.5, 1M NaCl, 0.1% Triton X-100, 1mM DTT, 1mM EDTA, 1ppm BHT, protease inhibitors), followed by centrifugation at 12000xg for 15mins at 4°C. This is expected to help lyse cells, extract protein from nuclei and prevent ligand dissociation from the hydrophobic ligand binding pockets of NR LBDs. The supernatant is then taken for affinity purification. Using this approach, nearly all (>90%) of the PPARγ-fusion protein is present in the supernatant fraction (Figure 2-5; Table 2-1). Some detergent was also used to help with cell lysis and protein solubility. The relatively high-salt concentration used prevents the detergent from facilitating ligand release from the hydrophobic ligand-binding pocket. DTT is also added to prevent formation of disulfide bonds that are not normally present. The EDTA is added to inhibit Ca and Mg-dependent proteases. BHT (Butylated hydroxy toluene) is an anti-oxidant that is used to prevent possible oxidation of the ligand during lysis and purification.

Initially, fearing the possibility that detergents would release ligands from the LBD, I originally used a buffer with only high salt and no detergents. With this lysis buffer very little of the fusion protein was extracted from the cells, with most of it remaining in the pellet after centrifugation. The use of sonication along with this buffer helped release more fusion protein from the cells. However, sonication has a tendency to heat up the lysate if used with long pulses or high frequency. Furthermore, this increased the lysis procedure by approximately 1.5hrs. As similar yields were obtained with detergent alone, the sonication step was removed. Furthermore, most ligand-NR interactions are extremely tight, and cannot be disrupted by detergent alone.

2.2.1.3 Optimization of Affinity purification for small-molecule co-purification

With the current affinity purification protocol, the supernatant from the lysate is incubated with Flag beads for 2hrs, washed with lysis buffer (50mM Hepes ph7.5, 1M NaCl, 0.1% Triton X-100, 1mM DTT, 1mM EDTA, 1ppm BHT, protease inhibitors), and then eluted by competition with 3xFLAG peptide. The Flag elutions are then incubated with Streptactin sepharose beads for 1.5hrs, washed with Last wash buffer (800mM ammonium acetate pH7.5, 1ppm BHT), and the beads then mixed with a solution of 2:1 chloroform : methanol to denature the LBD and solubilize the ligand. The hydrophobic ligand should enter the bottom (chloroform) phase, which is taken for mass spectrometry analysis (Figure 2-5).

Initially, for affinity purification I used a buffer containing 50mM Hepes pH7.5, 1M ammonium sulfate, 0.1% Triton X-100, 1mM DTT, 1mM EDTA and protease inhibitor. However, such a high salt concentration also caused sticking of non-specific proteins to the fusion protein and affinity beads. Dropping the salt concentration to 1M NaCl provided a much cleaner purification.

Originally, the fusion protein was eluted from the second column Streptactin beads using 5mM d-desthiobiotin dissolved in lysis buffer. Despite producing a clean purification and being able to identify rosiglitazone by LC-MRM*, this elution buffer produced a lot of background peaks in LC-MS scanning mode*. This is the mode that would be used to identify unknown endogenous ligands. The component of the elution buffer that was found to cause the majority of background peaks in LC-MS was the Triton X-100 detergent. Protease inhibitors and d-desthiobiotin also caused some background peaks. The protease inhibitors could be removed from these last steps without affecting the purification process. However, without Triton X-100 or d-desthiobiotin the

protein could not be eluted from the Streptactin beads. Removal of Triton X-100 also affected elution from Flag beads.

* LC-MRM (Multiple Reaction Monitoring) is a mode of mass spectrometry used to specifically identify a particular ion species at a very high sensitivity within a mixture of compounds

* LC-MS scanning mode is used to identify all the ion species within a mixture of compounds

Different detergents, namely Tween-20, deoxycholate and N-lauroyl sarcosine, were tried in the hope that they would not give background peaks in LC-MS. Since the elutions were mixed with a solution of chloroform: methanol in preparation for mass spectrometry, it was hoped that the ionic detergents (deoxycholate and N-lauroyl sarcosine) would stay in the methanol phase and not enter the chloroform phase, which is taken for MS analysis. Nevertheless, all the detergents that I tried gave background peaks in the LC-MS.

Therefore, a new variation was adopted. Following Strep binding, the beads were washed with a solution of ammonium acetate to remove as much of the detergent as possible, then mixed directly with chloroform:methanol so that the ligand is directly extracted off from the bead-bound fusion protein. This protocol allows for majority of detergent to be removed and gave very little background in the LC-MS scanning mode.

With the currently optimized affinity purification protocol, I managed to acquire an overall bait protein yield of approximately 72%. Using 1.7g of 1dpf pLT-PPARγ zebrafish embryos, I was able to purify approximately 1.4 μg of PPARγ-fusion protein with a purity of approximately 56% (Figure 2-6; Table 2-1).

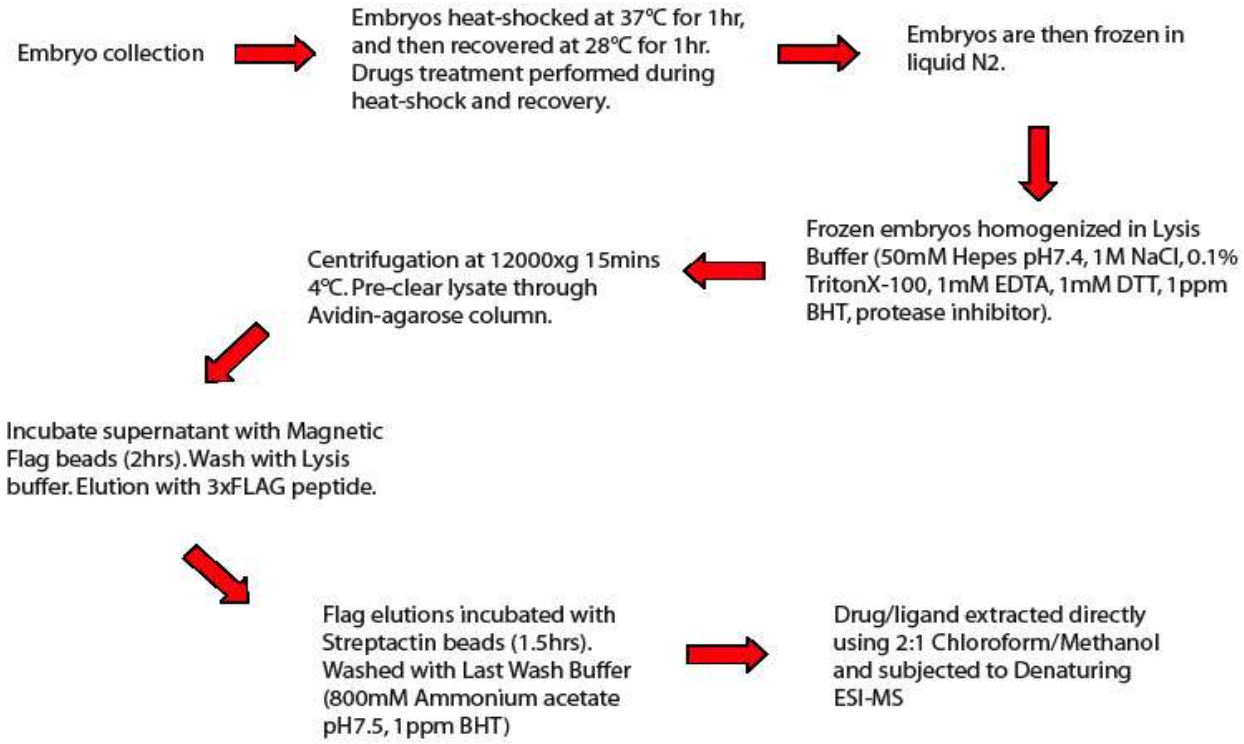

Figure 2-5 Outline of the ligand identification protocol

	Total Protein (µg) *	Fusion protein (µg) ¶	Yield of Fusion protein (%) Δ	Purity (% Fusion protein in total protein) Ω	Purification Factor ø
Lysate	49300.0	1.9	100	-	-
Soluble Supernatant	48900.0	-	94.4	-	-
Resuspended Pellet	1430.0	-	5.6	-	-
Input (Purification)	44300.0	1.8	94.4	-	-
Flag Flowthrough	42900.0	-	8.5	-	-
Flag Wash	21.0	-	0	-	-
1st Flag Elution	5.4	-	42.3	-	3781.3
2nd Flag Elution	2.9	-	25.4	-	
3rd Flag Elution	2.0	-	11.3	-	
Flag Beads	6.3	-	6.9	-	-
Strep Input	9.2	-	79.0	-	-
Strep Flowthrough	6.1	-	6.8	-	-
Strep Wash	0	-	0	-	-
Strep Beads	2.5	1.4	72.2	56	14237.8

Table 2-1. Purification summary of PPARγ-FSH fusion protein (from 1.7g 1dpf zebrafish embryos). Protein concentrations, yield and purification values corresponding to the fractions represented in Figure 2-6.

***** Amount of total protein in each fraction measured by Bradford assay

¶ Amount of purified PPARγ-FSH fusion protein (Strep Beads fraction) was measured by comparison to a BSA standard. Amount of PPARγ-FSH fusion protein in the lysate and input (purification) fraction was then estimated with respect to the percentage yield

Δ Yield of fusion protein for each fraction was based upon the relative western blot band signal intensity in comparison to that of the lysate

Ω Purity of the purified fraction (Strep beads) was the amount of purified PPARγ-FSH fusion protein as a percentage of the total protein in the fraction

Ø Purification factor was calculated as follows: (Total protein in fraction / Total protein in lysate) x (Purification yield for that fraction / 100). Combined purification factor for all three Flag elution fractions (1^{st}, 2^{nd} and 3^{rd} Flag elution) were calculated.

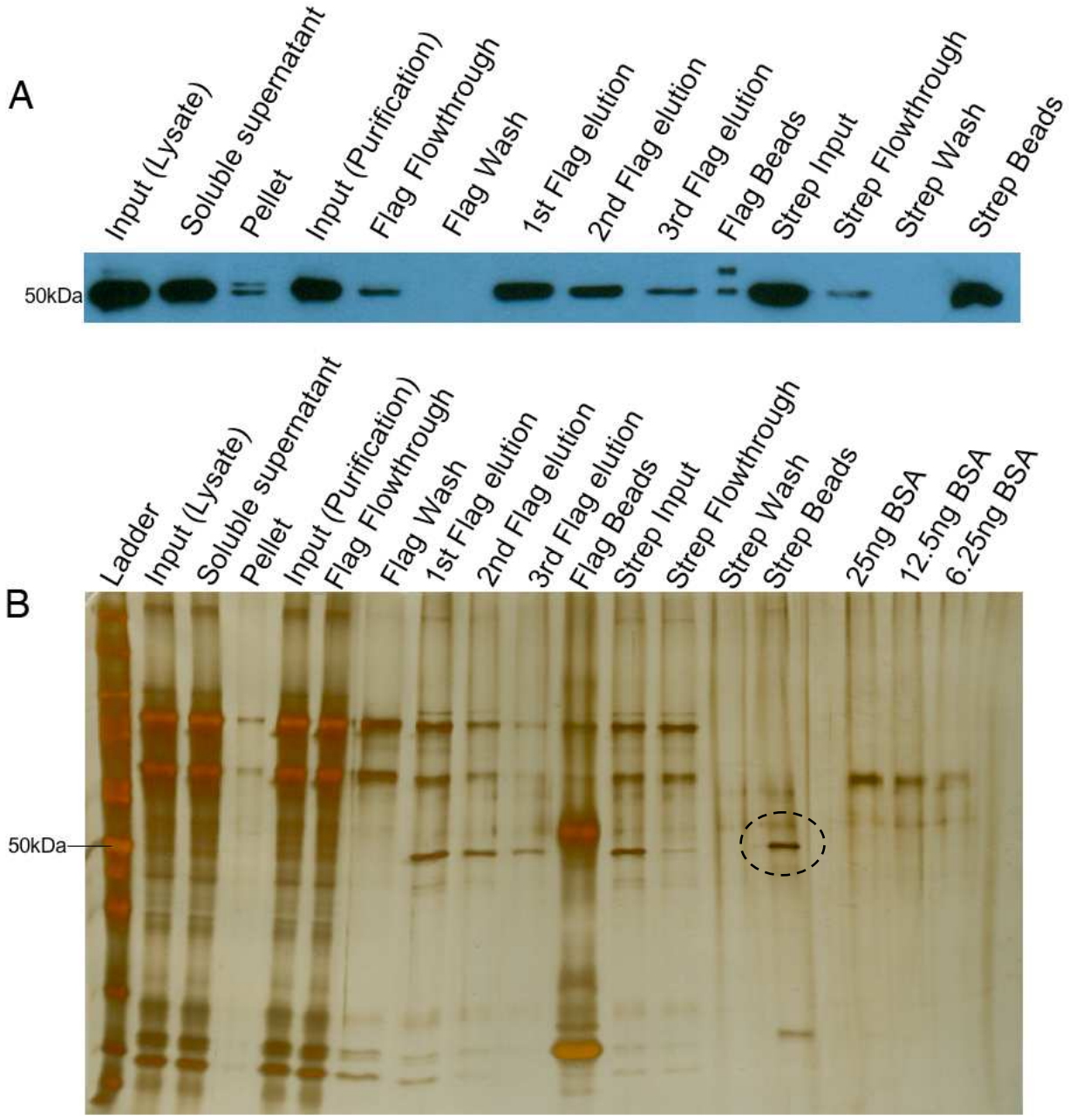

Figure 2-6. Purification of PPARγ-FSH fusion protein from 1dpf zebrafish embryos. (A) Detection of PPARγ-FSH fusion protein in each purification fraction by Western blot analysis using anti-Flag M2 Antibody (Sigma). (B) Analysis of protein purification by SDS-PAGE followed by silver staining; circled band (dashed line) represents the purified PPARγ-FSH fusion protein bound to the Streptactin beads. (PPARγ-FSH fusion protein = 53kDa)

2.2.1.4 Ligand extraction and mass spectrometry for small molecule ligand identification

Using the current ligand co-purification protocol, rosiglitazone was identified by both LC-MRM and LC-MS scanning mode (Figure 2-7; Figure 2-8). Originally, rosiglitazone could only be identified by LC-MRM and not LC-MS scanning, due to the high level of detergents in the elution buffer. However, the current protocol bypasses the need to use detergents in the elution buffer.

From 1.7g of 1dpf pLT-PPARγ zebrafish embryos, 1.4 μg of PPARγ-fusion protein was purified (Table 2-1). From the 1.4 μg of PPARγ-fusion protein, 372fmol of rosiglitazone was quantified by LC-MRM. However, comparison of the molar ratio of rosiglitazone to the amount of purified PPARγ-fusion protein (372fmol : 26415fmol), suggests that only ~1.4% of the fusion protein was bound by rosiglitazone. Alternatively, a significant portion of the ligand may have been lost or modified during various stages of the purification, extraction and MS procedures.

To give an idea of the amount of material used for the purification, one 1dpf embryo weighs approximately 1mg (embryos are still inside the chorion). Therefore 1.7g of 1dpf embryos amounts to 1700 embryos. Considering that 1 female is able to produce 300 embryos per crossing, 6 females are needed to produce this amount of embryos.

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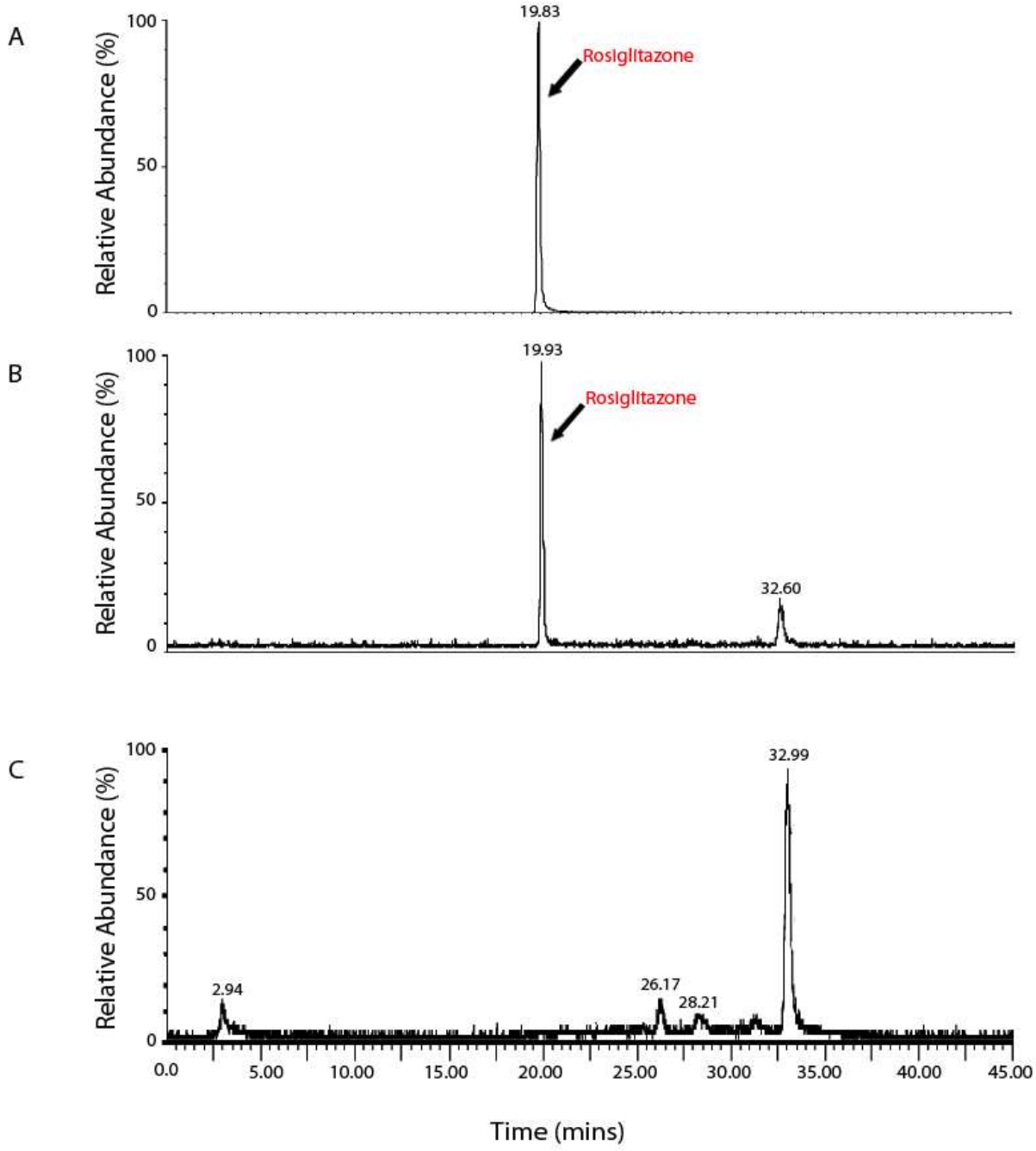

Figure 2-7. Detection of rosiglitazone by LC-MRM (Liquid Chromatography – Multiple Reaction Monitoring) on ESI-MS with respect to its retention time in the HPLC column. (A) Pure rosiglitazone (1picomole) is detected by LC-MRM. (B) pLT-PPARγ 1dpf zebrafish embryos were heat-shocked and treated with 1μM rosiglitazone. The embryos were then homogenized and the PPARγ-FSH fusion protein purified by a 2-step affinity tag purification, followed by identification of rosiglitazone by ESI-MS. Amount of rosiglitazone recovered from PPARγ-FSH fusion protein binding is 372fmole. (C) Wild type 1dpf zebrafish embryos (Control) were heat-shocked and treated with 1μM rosiglitazone, then processed in the same manner as the pLT-PPARγ zebrafish embryos (figure 2B).

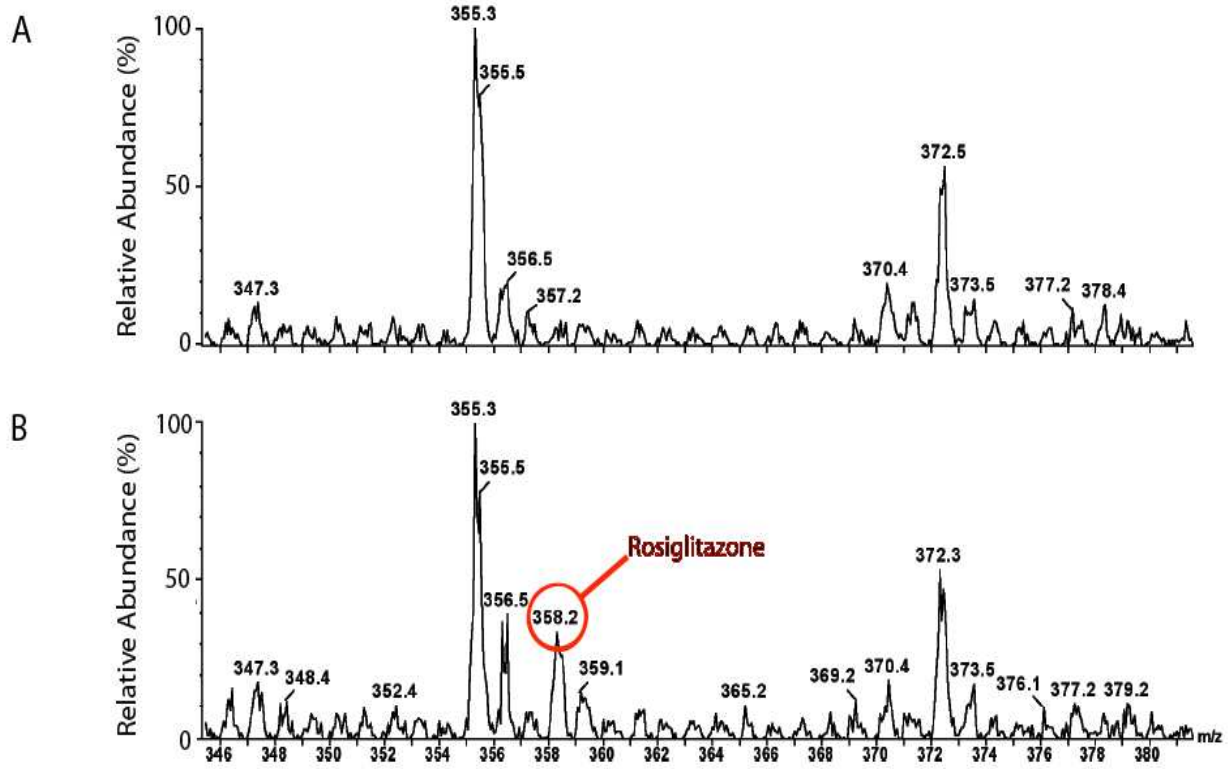

Figure 2-8. Detection of rosiglitazone by LC-MS scanning. (A) Wild type 1dpf zebrafish embryos (Control) were heat-shocked and treated with 1μM rosiglitazone, and then the PPARγ-FSH fusion protein purified by a 2-step affinity tag purification. No rosiglitazone is detected in wild type samples. (B) pLT-PPARγ 1dpf zebrafish embryos were heat-shocked and treated with 1μM rosiglitazone. The PPARγ-FSH fusion protein was then purified by a 2-step affinity tag purification. Rosiglitazone (m/z = 358.2) identified in pLT-PPARγ samples. Red circle represents peak for rosiglitazone.

2.2.2 New PPAR binding compounds

Considering that NRs have been implicated in many human diseases and that they have the ability to bind small molecules, the NRs have become promising targets for drug discovery (Schupp and Lazar, 2010). Not surprisingly, there are many compounds used to target these receptors. However, some of the drugs that target NRs present harmful side effects. Rosiglitazone (Avandia), which is used for the treatment of Type II Diabetes, has been associated with heart failure (Nissen and Wolski, 2007). The current focus of NR-based drug discovery is to find Selective Nuclear receptor modulators (SNuRMs) that perform the desired function with minimal side effects (Gronemeyer et al., 2004).

With this goal in mind, a relatively small compound library screen was performed by Dr. Jens Tiefenbach of InDanio Biosciences to identify drugs that can activate the PPARγ receptor. This screen was performed by treatment of heat-induced 1dpf pLT-PPARγ zebrafish embryos with various drugs, followed by the quantification of GFP signals using an Isocyte plate-reader. Upon drug treatment of the zebrafish embryos, an increase in the GFP signal relative to the untreated embryos would suggest that the compound is activating the PPARγ LBD.

By using the pLT-gypsy transgenic zebrafish lines for drug screening, the compounds can be tested not only for their ability to bind and activate the receptor, but also for tissue specific activation and associated phenotypes. Considering that most compounds that reach the clinical trial phase fail due inappropriate uptake, metabolism or off target effects (ADME-Tox), the zebrafish can be used as a high-throughput predictive model that reveals ADME-Tox issues early in the drug development process (Delvecchio et al., 2011; Tan and Zon, 2011).

From the BioMol library (~650 FDA-approved drugs) screen performed by Dr. Jens Tiefenbach, the compounds idebenone, capsaicin and ibuprofen were found to activate the PPARγ-fusion protein (Figure 2-9; Figure 2-10). When the heat-induced pLT- PPARγ embryos were treated with these compounds in excess, the GFP signal was increased more modestly than with rosiglitazone, and in fewer tissues. This may be explained by differential cofactor recruitment in various tissues, less effective co-activator recruitment (partial agonism) or differential/reduced bioavailability. Of the drugs tested and found to cause an increase in GFP signal, I was able to use the ligand identification protocol to assess whether the activation of PPARγ was a result of a direct binding of the drug to the LBD. The fish were treated with optimal levels of each drug, affinity purification performed and the drugs identified by mass spectrometry (Figure 2-5).

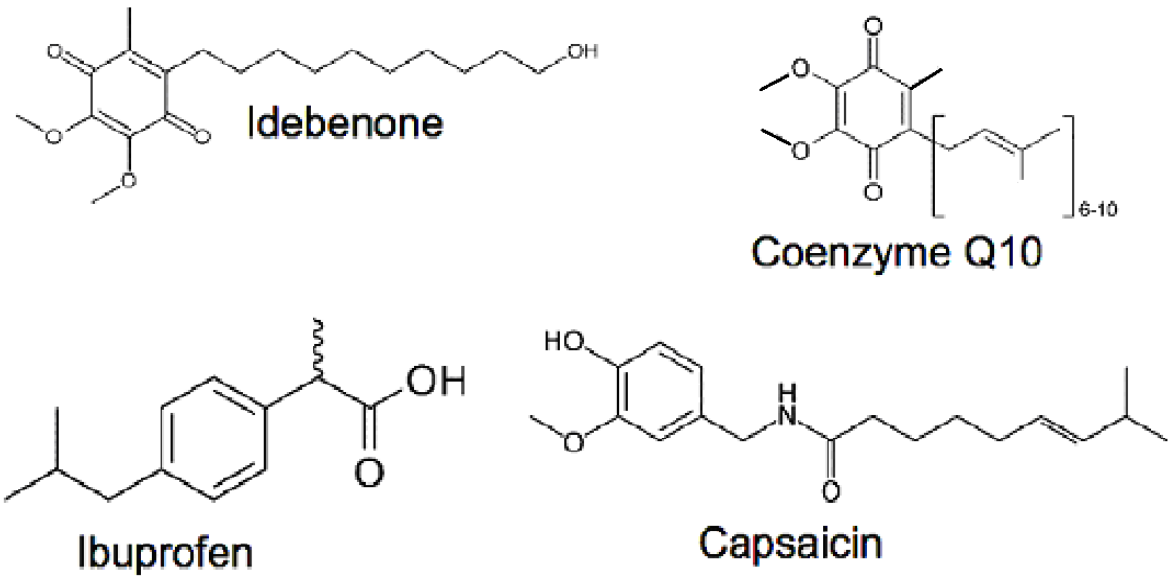

Figure 2-9. Chemical structures of idebenone, coenzyme Q10, ibuprofen and capsaicin.

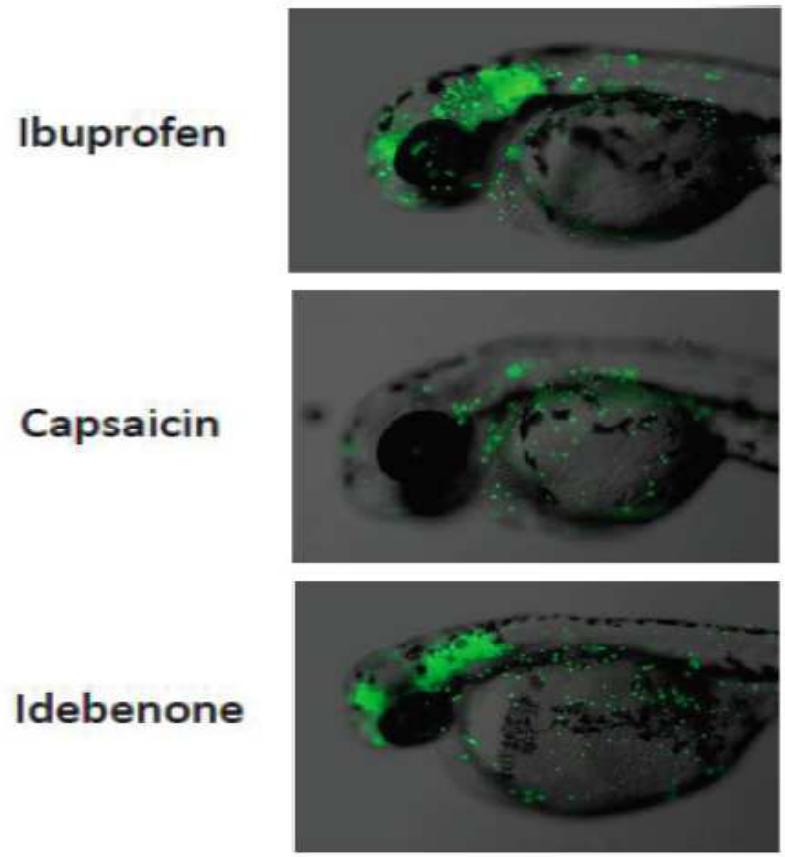

Figure 2-10. Drug-induced GFP response in pLT-PPARγ zebrafish lines. 1dpf pLT-PPARγ zebrafish embryos were heat-induced and treated with 2μM of idebenone, ibuprofen or capsaicin. (Tiefenbach, unpublished data)

2.2.2.1 Results

Idebenone, capsaicin and ibuprofen were found to cause activation of the PPARγ-fusion protein receptor (Figure 2-10). Following treatment of pLT-PPARγ zebrafish embryos with each of these drugs, affinity purification and mass spectrometry were performed. A control zebrafish line was also treated with the same drugs, and the expressed fusion protein purified using the same protocol. This control zebrafish line expressed a tagged-NR fusion protein with containing the RORβ LBD.

Idebenone and capsaicin were identified by LC-MRM only in the purified fraction from pLT-PPARγ embryos treated with these drugs (Figure 2-12). Idebenone and capsaicin were observed in the purified fraction from pLT-RORβ embryos treated with the same drugs. This suggests that Idebenone and capsaicin are able to bind specifically and directly to the PPARγ-LBD. From the 500ng of PPARγ-fusion protein, 22fmol of idebenone was quantified by LC-MRM. Comparison of the molar ratio of idebenone to the amount of purified PPARγ-fusion protein (22fmol : 9434fmol), suggests that only ~0.23% of the fusion protein was bound by idebenone. On the other hand, ibuprofen was not identified by mass spectrometry in the purified fraction from either pLT-PPARγ or pLT-RORβ embryos treated with the drug (Figure 2-11).

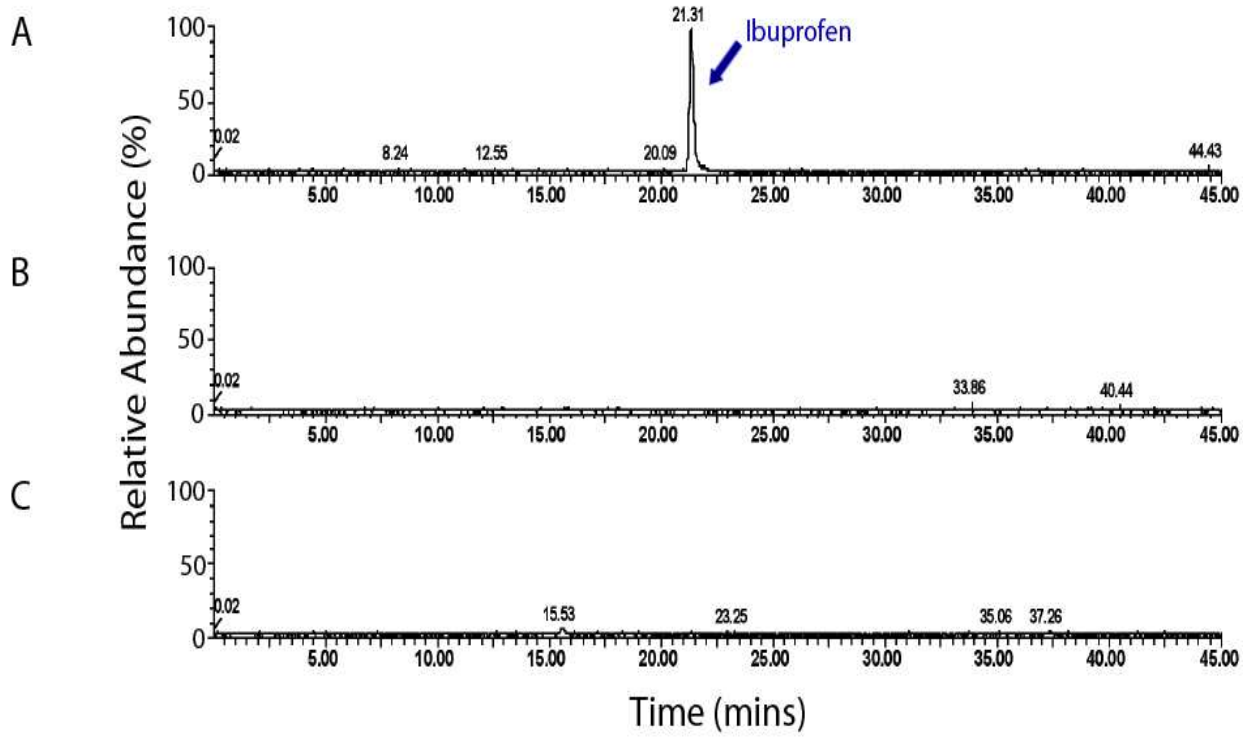

Figure 2-11. Ibuprofen does not bind to PPARγ ligand binding domain. (A) Pure ibuprofen is detected by LC-MRM (B) 1dpf pLT-PPARγ zebrafish embryos were heat-shocked and treated with 4μM ibuprofen. The fusion protein was purified. No ibuprofen was identified by ESI-MS. (C) 1dpf pLT-RORβ (control) zebrafish embryos were heat-shocked and treated with 4μM ibuprofen. The fusion protein was purified. No ibuprofen was identified by mass spectrometry from pLT-RORβ zebrafish embryos.

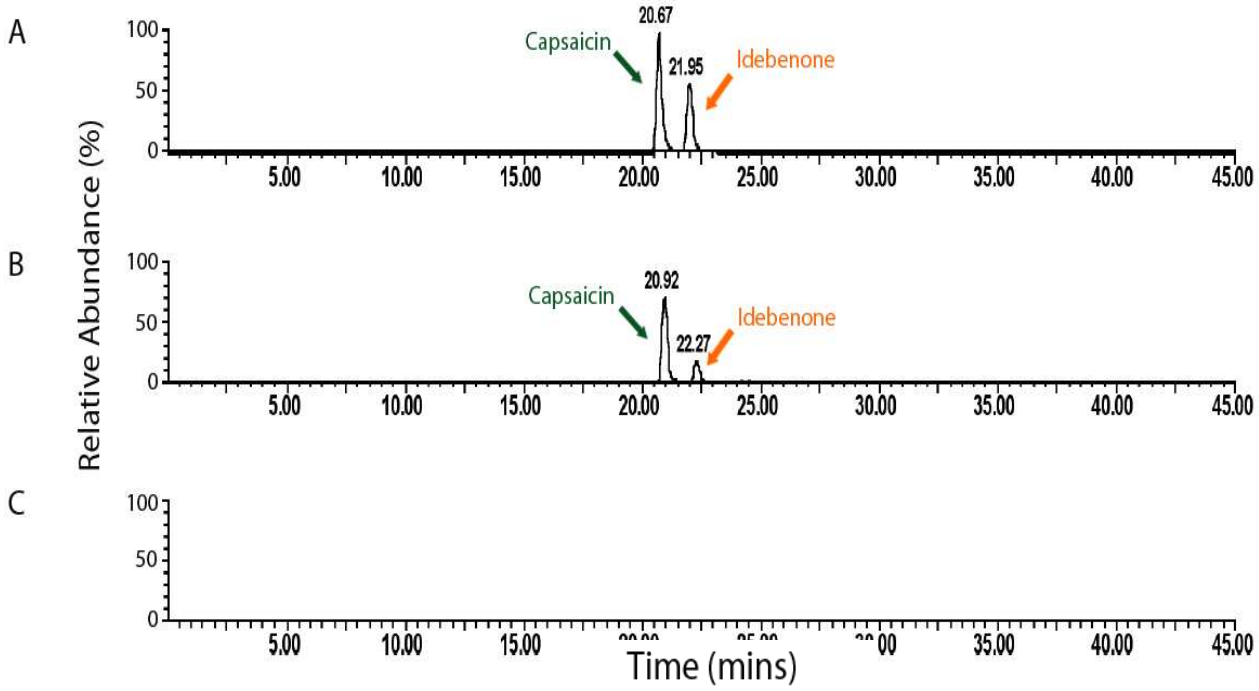

Figure 2-12. Idebenone and capsaicin bind to the PPARγ ligand-binding domain. (A) Pure idebenone and capsaicin (pooled) detected by LC-MRM (B) Pooled eluates from pLT-PPARγ zebrafish embryos treated with 4μM Idebenone or 4μM capsaicin. (C) 1dpf pLT-RORβ (control) zebrafish embryos treated with 4uM idebenone or 4μM capsaicin. Idebenone and capsaicin were not detected by mass spectrometry.

2.2.3 Co-purification of interacting proteins

Apart from its use for ligand co-purification, the pLT-gypsy transgenic zebrafish lines are also useful in identifying novel protein-protein interactions. In this part of the project, the pLT-PPARγ zebrafish line was used to identify novel interacting proteins for the PPARγ receptor.

The co-purification protocol to identify protein-protein interactions is adapted from Tiefenbach et al. 2010. The expressed PPARγ-fusion protein was extracted and purified under relatively low-salt conditions to maintain protein interactions. The co-purified proteins were identified either by MALDI-TOF or Orbitrap-mass spcctrometry.

In this particular purification, two groups of 1dpf pLT-PPARγ zebrafish embryos were treated with either 1μM rosiglitazone (a PPARγ agonist) or 2μM GW9662 (a PPARγ antagonist). Considering that the activated and inactivated NR LBD should assume different conformations, the agonist-bound PPARγ LBD should be associating with a different set of interacting proteins

compared to the antagonist-bound LBD. Ideally, by using optimal drug levels, all of the PPARγ-fusion protein should be bound by drug and adopt an active structural conformation. Therefore, different subsets of interacting proteins should bind to the PPARγ LBD under agonist and antagonist treated conditions.

2.2.3.1 Results

My affinity purification of the PPARγ-fusion protein yielded a number of specifically co-purified proteins identified by either MALDI-TOF or Orbitrap mass spectrometry. As expected, the purified PPARγ bait protein gave the highest spectral count (number of identified peptides corresponding to the protein of interest) from both the rosiglitazone-treated and GW9662-treated purification conditions. Notably, RXR alpha/beta/gamma, a group of proteins known to interact with the PPARγ receptor, were also identified with high confidence by mass spectrometry. Importantly, the RXRs were co-purified with only the rosiglitazone-bound PPARγ LBD (Figure 2-13). Previously, the interaction between the PPARγ receptor and RXRs is shown to be ligand-independent. This finding that RXRs were only co-purified with the agonist-bound PPARγ LBD suggests a requirement for the PPARγ DBD for the interaction with the RXRs in the absence of an agonist (Chandra et al., 2008).

Apart from this, both the rosiglitazone-treated and GW9662-treated purification conditions yielded a number of potential interacting proteins. However, most of these identified proteins gave a very low spectral count and several are novel proteins (e.g.: Zgc) that have not been previously studied. One of the potential interacting proteins, Major Vault Protein, was identified only from GW9662-treated embryos. In addition, several heat-shock proteins were co-purified only from the rosiglitazone-treated embryos (Figure 2-13).

Despite this limited success, a large number of the identified proteins are likely to be non-specifically interacting. These include abundant proteins such as kerratin, actin, myosin and vitellogenin.

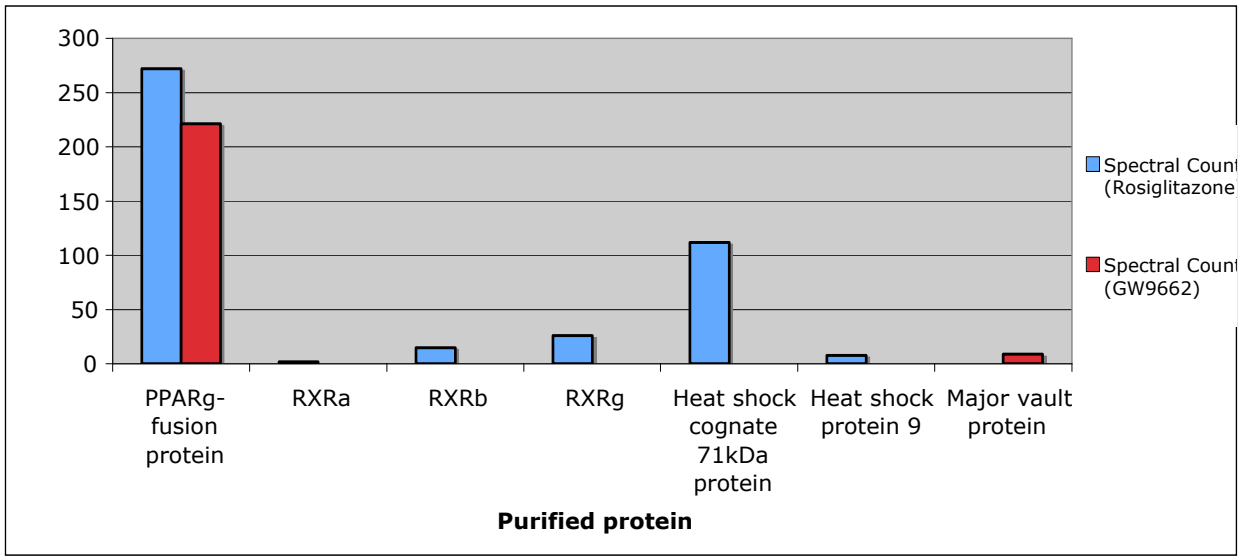

Figure 2-13. Spectral counts of co-purified proteins. pLT-PPARγ embryos were treated with either 1μM rosiglitazone or 2μM GW9662. The PPARγ-fusion protein was purified and the interacting proteins were identified by LC-MS (Orbitrap). The spectral counts of several co-purified proteins are shown.

Chapter 3

Discussion and Future directions

3 Discussion and Future directions

3.1 Discussion and future directions

The results presented in this thesis describe the development of a ligand identification protocol for orphan nuclear receptors, and its use to identify directly bound small molecules and cofactors. First, optimization of the ligand identification protocol will be discussed. Following this, I will discuss the significance of the drugs and interacting proteins that were found to bind to the PPARγ LBD.

3.1.1 Heat-shock optimization

The original goal to optimize the heat-shock protocol was to maximize the amount of expressed PPARγ-fusion protein, which should maximize the amount of co-purified ligand. Initially, the embryos and larvae at different stages of development were heat-induced and the relative level of the PPARγ-fusion protein expression compared. A comparison between early stage embryos showed that 3dpf embryos have a higher level of PPARγ-fusion protein expression relative to the 1dpf (Figure 2-3). This is consistent with previous studies on hsp70 protein induction (Krone et al., 1997). From 4dpf to the adult stage there was a huge decrease in heat-induced PPARγ-fusion protein expression relative to the earlier embryos. Preliminary experiments on pLT-RORβ zebrafish lines also suggest that earlier embryos produce more of the fusion protein compared to adult fish. Most of the previous studies on zebrafish Hsp70 protein expression have focused on earlier embryonic stages. Although there are studies that use adult zebrafish for heat-shock, there has been no direct comparison between hsp70 promoter-controlled expression of adult zebrafish and zebrafish embryos (Murtha and Keller, 2003). One possible explanation is that inducibility is a function of the site of ISceI meganuclease-directed insertion, with the lines characterized inserted in sites that are silenced after 3dpf (Grabher et al., 2004). It remains to be

seen whether the same pattern of heat shock-induced fusion protein expression occurs with other pLT-gypsy transgenic lines. Another possibility is that the heat shock response, in general, is dampened as development progresses. If so, this would likely be vertebrate or zebrafish specific, as a reduction in HS inducibility has not been observed in other model systems such as *Drosophila* (Niedzwiecki et al., 1991).

For optimization of the ligand identification protocol I am using 1dpf embryos, despite the fact that they produced less PPARγ-fusion protein compared to 3dpf embryos. This choice was due to their ease of manipulation. While the 1dpf are still inside the protective chorion they are easier to collect using a tea strainer or a mesh. Collection of 3dpf embryos using a mesh results in death, mostly likely due to abrasion against the mesh material. On the other hand, transfer of the 3dpf embryos using a pipette is time consuming. For future purposes, when 3dpf embryos or older larvae are to be used for heat-shock, better methods needs to be optimized. Tricaine (Ethyl 3-aminobenzoate methanesulfonic acid) may be used to anesthetize the 3dpf embryos before collecting in a mesh to avoid abrasion from the embryos moving against the mesh surface.

For the 1dpf embryos, I optimized the heat-shock procedure by varying the duration that embryos were exposed to heat-shock at 37°C. Consistent with previous studies, heat shock for 1hr produced the highest level of protein expression, which gradually decreased following longer heat shock durations (Figure 2-4; Lele et al., 1997). The heat-shock temperature was not increased due to the well-established use of 37°C in other studies. Indeed, going to higher temperatures (39/40°C) led to a high proportion of the embryos dying during or after heat-shock (Krone et al., 1997b).

In terms of the recovery time, the PPARγ-fusion protein level was also measured by Western blotting of samples taken at different time points after heat-shock. A significant level of the PPARγ-fusion protein lasts up to 7hrs. However, we don't know how much time it takes the protein to properly fold into a functional LBD and capture the ligand. Currently I am heat-shocking the 1dpf embryos at 37°C for 1hr and recovering the embryos at 28°C for 1hr (Figure 2-5). I decided to use this time of recovery partly due to previous data showing that following heat pulse, approximately 20 minutes was required to fully induce expression of *hsp70* promoter-regulated genes, with another 40 min required for maximal folding and accumulation of the encoded fusion proteins (Nasiadka et al., 1999). Longer recovery times did not lead to an increase in protein expression levels, and ligand binding at effective doses is extremely rapid.

Using this recovery time, I was able to identify bound rosiglitazone by mass spectrometry, and was also able to identify RXR alpha, beta, and gamma, proteins known to interact with functional PPARγ protein (Figure 2-7; Figure 2-8; Figure 2-13). This data suggests that the current recovery time allows proper protein folding. However, given the low stoichiometry of ligand and cofactor co-purification, additional recovery times should be tested to see if there is an increase in the percentage of fusion protein bound to RXRs and rosiglitazone.

3.1.2 Protein lysis and affinity purification

With the current ligand identification protocol, I was able to purify approximately 1400ng of PPARγ fusion protein from 1.7g of embryos. The purification level was approximately **20,000**-fold, with a yield of approximately 72.2% and final purity of ~56% (Table 2-1; Figure 2-6). In comparison to previous protein purification approaches, this yield is significantly higher. For example, in Yang et al. 2006, a two-tag purification, using Flag and His tags to purify either the E75- or dHNF4-fusion protein, had a yield of 13.94% and 10.6%, respectively, while use of the more conventional TAP-tag (Protein A and CBP) gave yields of 1.33% and 0.28%, respectively. In my protocol, a high-salt concentration buffer was used with the intention of keeping the hydrophobic ligand inside the ligand-binding pocket of the PPARγ-fusion protein. The high-salt concentration should also limit cofactor, protease and other modifying protein interactions with the bait protein. Other studies that have identified ligands from an in-vivo tissue source, namely mice liver, have used a relatively low salt concentration or even a higher detergent concentration in the lysis buffer. However, these studies already had prior knowledge to the ligand's identity before co-purification. This allowed for the identification of very small amounts of co-purified ligand (less than 10fmol), using LC-MRM. Despite having no firm proof, I hypothesize that using high salt concentrations in both the lysis and purification buffer would be a safer method to keep the hydrophobic ligand inside the ligand binding pocket. Ideally, this would maximize the amount of ligand being co-purified, allowing easier identification of a previously unknown ligand by LC-MS scanning mass spectrometry.

The study that co-purified ligands from mouse liver did not use detergents in the lysis and purification buffers (Chakravarthy et al., 2009). A nuclear extract was made instead by first using a hypotonic low salt buffer to lyse the cell and pellet the nuclei, followed by using a

hypertonic high salt buffer to break open the nuclei. A problem with this method is that some of the ligand and fusion protein may leak out of the nuclei during the extract preparation. With the optimized ligand identification protocol, a whole-cell extract is made by detergent-based homogenization of the zebrafish embryos. On the other hand, the latter may lead to some ligand loss due to high detergent levels.

3.1.3 Identification of an endogenous ligand for an orphan NR

3.1.3.1 Identification of Rosiglitazone by ESI-MS

From the 1.7g of 1dpf embryos treated with 1μM rosiglitazone, the drug could be identified by mass spectrometry following affinity purification (Figure 2-7; Figure 2-8). However, only 372fmol of the drug was identified by mass spectrometry from the 26415fmol (1400ng) of purified PPARγ-fusion protein, which suggest that only 1.4% of the fusion protein is bound by rosiglitazone. This is a relatively small yield of bound ligand compared to previous experiments of ligand co-purification. For example, the co-purification of heme with tagged-E75 fusion protein yielded essentially 100% of the fusion protein bound by heme (Reinking et al., 2005).

I assumed that 1μM rosiglitazone is enough to saturate all the expressed fusion protein based on previous data produced by Dr. Chris Delvecchio. The GFP signal from the PPARγ 1dpf embryos was quantified upon increasing the concentrations of different drugs, including rosiglitazone. At approximately 100nM of rosiglitazone, the GFP response reached its maximum level (data not shown). Therefore I assumed that using the higher concentration of 1μM rosiglitazone would saturate all the fusion protein receptors. However, the heat-shock condition used by Dr. Delvecchio may have induced less fusion protein expression compared to my optimized protocol. This means that more rosiglitazone would be required to saturate all the fusion protein than previously expected.

There could be several possibilities to explain the low amount of rosiglitazone identified by mass spectrometry. Firstly, rosiglitazone is very hydrophobic and was found to stick to HPLC and mass spectrometry machine components. Thus, a portion of the co-purified rosiglitazone may not be quantified. This effect could be exaggerated with lower levels of the drug. One way to test this would be to use non-denaturing MS to see what proportion of the purified bait protein

is bound by rosiglitazone prior to ligand extraction. If the drug was lost later due to incomplete extraction, or to stickiness within the vessels and apparatus used for MS, then a higher ratio of bound versus apo receptor would be observed using the non-denaturing method. If this is observed, then one way to improve things would be to use different solvents and buffers for ligand extraction and LC-MS

As previously mentioned, a low percentage of rosiglitazone binding to fusion-protein may also suggest that not enough time was given for the fusion protein to properly fold and become functional for capturing the drug. It is worth noting that the GFP response experiments required many more hours for optimal GFP production. While this was expected to be due to the time required for GFP folding, accumulation and activation, some of this time may also be required for full PPAR folding and ligand binding. In this case I will vary the recovery time after heat shock to see if more rosiglitazone is able to bind to the fusion protein.

Another possibility is that rosiglitazone may be falling out of the pocket during the purification process. However, this seems unlikely, due to the high salt concentrations used in the purification buffer. One way to test this would be to quantify (by ESI-MS) the amount of rosiglitazone present in each fraction of the purification (i.e. washes and flow-through) and identify any steps where rosiglitazone titers are decreasing. Likewise, I could employ the use a fluorescent PPARγ ligand (e.g. Fluormone PPAR green, Invitrogen; DeGrazia et al., 2003) or a radioactive rosiglitazone. One complication is that I may not be able to distinguish whether bound rosiglitazone is falling off during the purification process or if unbound rosiglitazone (due to excess concentration of rosiglitazone given to saturate the PPARγ-fusion protein) is being washed away. Another alternative would be to use the non-denaturing MS approach described above. If loss of rosiglitizone during affinity purification does turn out to be a (the) problem, then varying buffer conditions at key steps during the purification may improve the quantity of rosiglitazone capture.

Alternatively, there is also the possibility that an endogenous ligand is competing with rosiglitazone for binding to the PPARγ-fusion protein, which would explain the low amount of identified rosiglitazone. If increasing the recovery time after heat-shock does not recover anymore rosiglitazone then this possibility may be true.

3.1.3.2 Non-denaturing MS

The current ligand identification protocol that has been optimized relies on ESI-MS with denaturing mass spectrometry, where the purified PPARγ-fusion protein is denatured to release the ligand by organic extraction using chloroform/methanol solution. The disadvantage of this approach is that many potential peaks will be identified by mass spectrometry, and thus it will take effort and time to find the molecular identity of the peaks and screen through them to find the ligand.

By using the current affinity purification protocol coupled with non-denaturing MS, this problem may be overcome. With non-denaturing MS, the intact fusion protein-ligand complex must be eluted from the beads, and then the whole complex can be ionized and detected by mass spectrometry. The ligand can then be released from the complex by collision-induced dissociation (CID). The mass difference between the ligand-bound NR fusion protein and the unbound fusion protein should ideally reflect the molecular weight of the bound ligand. This method would allow easier identification of the endogenous ligand compared to the use of denaturing MS. Furthermore, by being able to detect the protein-ligand complex, the binding stoichiometry can also be measured.

The disadvantage of this method is that a larger amount of fusion protein is expected to be required for detection by mass spectrometry. The previous protocol developed by Dr Keith Pardee, for ligand identification using non-denaturing mass spectrometry, used either *E. coli* or Sf9 insect cells for over-expression of the fusion protein (Pardee, 2010). These expression systems are able to produce a larger amount of fusion protein compared to zebrafish embryos. However, we do not know the minimal amount of fusion protein required to be detected.

Furthermore, I have made several failed attempts to couple the optimized affinity purification protocol with non-denaturing mass spectrometry. Previous trials have shown that reagents namely, detergents and d-desthiobiotin, are needed for the successful elution of the intact protein-ligand complex from the Streptactin beads. However, both of these reagents create a lot of background noise in the mass spectrometer, preventing the protein-ligand complex from being identified. For the successful identification of the protein-ligand complex by non-denaturing MS, the elution samples will need to be cleared of these components before injecting the samples for mass spectrometry. This can be achieved with the use of a detergent-removal spin column (Pierce, #87780).

3.1.3.3 Identification of an endogenous ligand for an orphan NR

The following step would be to upscale the purification process to identify an endogenous ligand for the PPARγ receptor and other orphan NRs. The affinity purification should be performed on the particular zebrafish line that the ligand is to be identified, and also a negative control zebrafish line. The control zebrafish should ideally be another transgenic line expressing the LBD of a different NR. After affinity purification the purified material can be identified by an LC-MS scan. In the case of the PPARγ receptor, the ligand identification can also be performed on pLT-PPARγ embryos treated with rosiglitazone, as a positive control. The peaks that appear in the purified fraction from untreated pLT-PPARγ embryos, but not in the rosiglitazone-treated pLT-PPARγ positive control or the negative control zebrafish embryos, may potentially represent the endogenous PPARγ ligand. The identity of the molecules represented by these peaks can be identified.

Considering that idebenone, which is an analog of coenzyme Q10, is able to bind and activate the PPARγ receptor LBD, it should be investigated whether or not coenzyme Q10 is a/the endogenous ligand for PPARγ in-vivo. To this end, LC-MRM can be performed on purified fractions from the pLT-PPARγ zebrafish embryos, to specifically detect the presence of coenzyme Q10 in the mixture.

Dr. Jens Tiefenbach, in our lab, had shown that pLT-PPARγ embryos respond similarly to both idebenone and coenzyme Q10. Interestingly, JT had also shown that coenzyme Q10 is able to activate the PPARα receptor, and is a much stronger agonist for PPARα.

3.1.3.4 Confirmation of NR ligand

After identifying the ligand, confirmation is needed to prove that it is a genuine NR ligand, as opposed to a non-specifically associating molecule. The set of identified molecules can be screened in an assay to confirm binding to the receptor. The ligand can be added to the pLT-gypsy transgenic zebrafish lines to assay for an increase or decrease in the GFP signal relative to the untreated/endogenous signal. An increase in GFP will suggest that the ligand is an agonist, while a decrease will suggest antagonistic activity. Furthermore, by doing this experiment, the tissue specific pattern of ligand activation can be seen. By treating the zebrafish embryos with this ligand, the EC50 value can be found with respect to the GFP response. Apart from this,

specific phenotypes/defects, such as heart edema or lipid accumulation can be assessed for their ED50 and LD50 values.

These results can be confirmed using in vitro approaches. For example, a stargazer assay could be performed. The protein aggregation curve can be made for the full-length nuclear receptor protein without the ligand. Upon ligand addition, if there is an increase in the temperature required to cause receptor protein aggregation, this would suggest that the ligand binding is specific and acts to stabilize the receptor protein.

ITC (Isothermal Titration of Calorimetry) could also be performed, where a known amount of the ligand can be titrated into a known amount of purified full-length nuclear receptor protein, and the change in temperature corresponding to the ligand binding can be measured. From here the K_D values can be obtained, which will tell us the affinity of the ligand for the receptor.

Assuming that a genuine NR ligand would bind inside the ligand-binding pocket, many small-scale purifications could be performed from embryos treated with a varying concentration of a known drug that targets this receptor. This should result in displacement of the endogenous ligand. Using LC-MRM mode on ESI-MS, it can be seen if increasing the concentration of drug will displace the endogenous ligand in a concentration-dependent manner. For the case of the PPARγ receptor, we know that rosiglitazone binds inside the ligand-binding pocket. A displacement of the ligand by rosiglitazone would suggest that the ligand is binding specifically inside the ligand-binding pocket. However, this test will only be feasible if there are known drugs targeting the NR.

3.1.3.5 Biological role of the endogenous ligand

After the ligand is confirmed to bind specifically to the NR-LBD, the question of how the ligand is binding inside the pocket can be asked. To solve this problem, co-crystallization of the ligand and the NR-LBD can be attempted, and the crucial amino acids involved in the interaction can be identified. The importance of these amino acid residues could also be assessed by expressing substitution mutant forms of the NR-LBD fusion protein in a cell culture assay (NR-LBD-Gal4DBD/UAS-Luc system). A decrease in luciferase activity upon ligand binding compared to native NR-LBD sequence will suggest importance of those particular amino acids in binding the ligand.

We can also ask the question of what cofactors this ligand recruits. Affinity purification of tagged NR-fusion protein can be performed from zebrafish embryos treated with and without ligand to identify any novel interacting proteins. A stargazer screen using liganded LBD could also be done, while adding various known cofactor peptides/proteins and assaying for increased or decreased stabilization. The NR-fusion protein can be co-expressed in cell culture with known cofactors, in the presence of the ligand. Co-IP experiments can then be performed to test whether some or all of the previously identified cofactors are interacting in the presence of this ligand.

Also, microarray analysis using whole zebrafish embryos can be performed to find genes that are up/down-regulated by this ligand. Zebrafish wildtype embryos can be either be treated with excess of the ligand, injected with morpholinos against the synthesizing enzyme(s) for the ligand, or untreated. One problem with this approach is that target genes may only respond in a small subset of tissues, or may be up-regulated in one tissue but down-regulated in another. However, these issues should be avoidable by selecting developmental stages where GFP responses in the corresponding ligand trap lines show widespread responsiveness.

Some of the genes that show responsiveness in the microarray experiment may be indirect targets. To identify direct targets, ChIP-chip or ChIP-seq could be used to find directly bound target sites, and cell based reporter assays to confirm functional responsiveness.

These experiments will link the structural information of ligand binding to the NRs, with the activity of the particular NR upon binding its cognate ligand. This will allow us to understand the physiological processes that these ligands are regulating. Furthermore, the identification and functional characterization of the ligand may eventually lead to developing drugs to target their cognate NR and associated diseases.

3.1.4 Drug binding to the PPARγ LBD

From the drug screen used to find novel activators of the PPARγ receptor, ibuprofen, idebenone and capsaicin were found to activate the PPARγ LBD in the pLT-PPARγ transgenic zebrafish embryos (Figure 2-9; Figure 2-10). By using my optimized ligand identification protocol I was able to answer the question of whether these drugs are able to bind the PPARγ LBD directly.

The drugs were added to heat-induced 1dpf pLT-PPARγ zebrafish embryos, and affinity purification was performed to isolate the PPARγ-fusion protein. LC-MRM was then used to identify the drugs by mass spectrometry (Figure 2-5). [Before discussing the data and results, I will give a brief review of the three drugs that were identified as positive hits from the zebrafish fluorescent screen.]

3.1.4.1 Ibuprofen

Frequently used for the treatment of pain, fever and inflammatory diseases, ibuprofen has become a popular over-the-counter drug available worldwide. Ibuprofen was discovered in the 1960s and was used for the treatment of rheumatoid arthritis. Like most other Non-Steroidal Anti-inflammatory drugs (NSAIDs), ibuprofen is an inhibitor of the cycloxygenase enzyme (COX), an enzyme that is involved in the production of prostaglandins from arachidonic acid (Rao and Knaus, 2008). By reducing the levels of prostaglandins, which normally act as chemical messengers for pain and inflammation, ibuprofen has been used as an analgesic drug.

Previous studies have shown that ibuprofen and several other NSAIDs can cause the activation of the PPARγ receptor in a cell culture luciferase assay (Lehmann et al., 1997). Furthermore, ibuprofen had the ability to compete with radioactive rosiglitazone for binding to the PPARγ LBD. Ibuprofen has also been shown to reduce the risk of Alzheimer's disease. By binding to the PPARγ receptor, ibuprofen causes transcriptional repression of the gene encoding the β-secretase enzyme. This leads to a reduction in the level of Alzheimer's disease-causing β-amyloid peptides (Sastre et al., 2006). Furthermore, ibuprofen has also been shown to inhibit RhoA signaling, through its binding to the PPARγ receptor. This leads to neurite outgrowths in cultured Dorsal Root Ganglion cells (Dill et al., 2010).

3.1.4.2 Capsaicin

Capsaicin, a compound present in chili peppers, acts as an irritant. Capsaicin is believed to be produced from the addition of coenzyme A to a vanillylamine group. As a member of the vanilloid group of molecules, capsaicin is able to bind to the vanilloid receptor 1 (VR1), also known as the TRPV1 (Everaerts et al., 2011). TRPV1 is an ion channel of the TRP family that is highly expressed in the neurons of the Dorsal Root Ganglion (DRG). When activated by capsaicin, TRPV1 allows non-selective passage of cations through the plasma membrane into

the cell. Apart from capsaicin, TRPV1 can also be activated by high temperatures (Huang et al., 2002).

Capsaicin has also previously been found to bind to the PPARγ receptor (Kang et al., 2010). The effect of capsaicin binding to the PPARγ receptor includes inhibition of the pro-inflammatory factor, TNFα, through inactivation of the transcription factor NF-κB by the PPARγ receptor (Park et al., 2004). The PPARγ receptor can bind directly to the NF-κB transcription factor, preventing interaction with its transcriptional co-activators, and thus leading to its inactivation.

3.1.4.3 Idebenone

Idebenone is a drug originally developed by Takeda Pharmaceuticals for the treatment of Alzheimer's disease. Currently it is in clinical trials for the treatment of neurodegenerative and mitochondrial related diseases, such as Duchenne Muscular Dystrophy, Mitochondrial encephalopathy and Friedreich's Ataxia by promoting mitochondrial function and proliferation in muscle (Becker et al., 2010; Rustin et al., 1999). As an anti-oxidant and an analog of coenzyme Q10, it is also believed to act via the prevention of lipid peroxidation through the binding of free radical molecules (Figure 2-9). This prevents damage to cell and organelle membranes from the free radicals (Villalba et al., 2010).

Coenzyme Q10 is an abundant, fat-soluble substance that is a crucial component of the electron transport chain, whereby it transfers electrons from complex I and II to complex III, resulting in the movement of electrons and the pumping of protons across the inner mitochondrial membrane for ATP production. Idebenone can be used as a replacement of coenzyme Q10 in the treatment of coenzyme Q10 deficiency (Villalba et al., 2010). No previous studies have shown idebenone binding to the PPARγ receptor.

3.1.4.4 Drug binding to the PPARγ LBD

Capsaicin and idebenone were found to be bound to the PPARγ LBD, while ibuprofen could not be identified (Figure 2-11; Figure 2-12). Considering that previous studies have shown capsaicin binding to the PPARγ receptor, it is not surprising that capsaicin was identified by mass spectrometry following the affinity purification (Park et al., 2004). Capsaicin was found only in the purified fraction from pLT-PPARγ embryos and not from pLT-RORβ control embryos,

consistent with specific binding to the PPARγ LBD. This data acts as another proof-of-concept, showing the usability of the pLT-gypsy transgenic zebrafish lines for ligand identification.

On the other hand, ibuprofen was not identified by mass spectrometry following affinity purification, despite previous studies showing its interaction with the PPARγ receptor (Figure 2-11; Lehmann et al., 1997). This may be due to insufficient time given for ibuprofen to be absorbed by the zebrafish embryos and bind to the PPARγ-fusion protein. Another possibility is that the drug is less tightly associated with the ligand-binding pocket and is coming out during the purification procedure.

The finding that idebenone is able to bind to the PPARγ LBD is a novel one (Figure 2-12). From the 500ng of PPARγ-fusion protein, 22fmol of idebenone was quantified by LC-MRM. Comparison of the molar ratio of idebenone to the amount of purified PPARγ-fusion protein (22fmol : 9434fmol), suggests that only ~0.23% of the fusion protein was bound by idebenone. Activation of the PPARγ receptor has previously been shown to cause a reduction in the level of β-secretase enzyme, thus lowering the amount of Alzheimer's disease-causing β-amyloid peptides (Mandrekar-Colucci et al., 2011; Sastre et al., 2006). The ability of idebenone to bind and activate the PPARγ receptor may explain this compound's ability to ameliorate several neurodegenerative diseases including Alzheimer's disease (Becker et al., 2010; Rustin et al., 1999). Furthermore, it may also be worthwhile to investigate whether idebenone binding to PPARγ has any insulin-sensitizing effects. Since idebenone is an analog of coenzyme Q10, an abundant molecule in every cell of the body, there is a possibility that coenzyme Q10 could serve as an endogenous ligand for the PPARγ receptor (Bentinger et al., 2010).

3.1.4.5 Future directions

Idebenone (marketed as Catena) is an FDA-approved drug, used for the treatment of neurodegenerative diseases such as Friedreich's Ataxia. The binding of idebenone to the PPARγ receptor's LBD is a novel finding that may lead to the use of idebenone for the treatment of diseases, other than neuromuscular disorders. Considering that the PPARγ receptor has been implicated in the pathology of diseases such as Type II Diabetes, idebenone may be used as a treatment for these same diseases. With this in mind, clinical trials can be performed to test idebenone's effect on blood sugar levels in patients with Type II Diabetes.

3.1.5 Technical challenges to ligand discovery

At present, my optimized ligand identification protocol has yet to be proven successful with the identification of a ligand for an orphan NR. Currently, rosiglitazone can be identified by an LC-MS scan following affinity co-purification from zebrafish embryos treated with 1μM of the drug (Figure 2-8). This acts as a proof-of-concept to show that the optimized ligand identification protocol is usable for ligand identification for an orphan NR. However, there are some technical challenges to the discovery of the endogenous PPARγ ligand.

First, the expression of the PPARγ-fusion protein in the pLT-PPARγ zebrafish is only maximally responsive to heat-induction at 1-3dpf (Figure 2-3). Preliminary studies on the pLT-RORβ lines have also shown similar results. Therefore, the stage of development at which the ligand identification can be performed is limited for these lines. However, it remains to be seen whether other pLT-gypsy transgenic zebrafish lines will have the same pattern of heat-induction. Another potential problem lies in the possibility that the endogenous ligand may not be produced at that particular stage. For example, the PPARγ receptor is heavily studied for its role in adipogenesis and the endogenous ligand related to this process is highly sought after (Tzameli et al., 2004). However, adipose tissue is first seen in the zebrafish at 12dpf, and activation of the zebrafish PPARγ receptor is thought to occur normally upon ingestion of a high-fat diet (Flynn et al., 2009; Imrie and Sadler, 2010). However, at these later stages of development, there is very low heat-induced expression of the PPARγ-fusion protein. Therefore it is unlikely that a PPARγ ligand can be co-purified from this developmental stage.

Second, there is the possibility that the endogenous ligand is produced at a quantity too low to be detected and identified by mass spectrometry or is present only transiently. Since some of the previously identified ligands, such as heme, are found at very abundant levels, it is likely that the ligands for some orphan receptors will also be at an abundant level (Reinking et al., 2005). Nevertheless, the possibility of low ligand abundance for PPARγ still holds. The hope is that the current ligand identification protocol will be able to capture the more abundant ligands, leaving the less abundant ones to be identified as the technology improves.

3.1.6 Significance of the ligand identification protocol

The traditional approach to ligand identification for orphan receptors has been to screen libraries of natural and endogenous small molecules. This approach was successful in the discovery of endogenous ligands for several receptors, but did not yield any ligand for many other orphan receptors (Schupp and Lazar, 2010). Other studies managed to identify NR ligands serendipitously, through protein crystallography of the NR protein (Pardee et al., 2011).

The approach taken in this project is aimed at the co-purification of an endogenous NR ligand from an in-vivo tissue source (Figure 2-5). Compared to the approach of testing compounds in-vitro, the optimized ligand identification protocol allows for the co-purification of the endogenous ligand directly from the tissue and cells that it normally has an effect. The traditional ligand screening approach has previously yielded many ligands, but for some of these ligands there is no definitive proof of its relevance in-vivo.

The expression and purification of NR proteins from bacteria have yielded some bound ligands. Heme was co-purified from bacterially expressed tagged-E75 protein, and then its in-vivo relevance was later confirmed in flies (Reinking et al., 2005). However, for some NRs, this approach has proven ineffective in identifying an endogenous ligand. This may be because of the absence of the correct ligand in bacteria. The optimized ligand identification protocol may overcome this problem by purifying from an in-vivo tissue source of an organism closely related to humans.

Considering that zebrafish have a relatively similar physiology to humans, and that there is a relatively high sequence similarity between zebrafish and human NRs, zebrafish can be used as a system for human NR ligand identification (Delvecchio et al., 2011; Bertrand et al., 2007). For every human NR, there is at least one homologue representing it in the zebrafish, and it is known that zebrafish NRs are able to respond to human ligands (Alsop and Vijayan, 2008). Furthermore, ligands discovered for *Drosophila* NRs have been found to bind the human homologues of these NRs (Pardee et al., 2009; Horner et al., 2010). Therefore, ligands discovered using the zebrafish, which is evolutionarily closer to humans than flies, would also likely function as human NR ligands.

More recently, several studies have identified endogenous NR ligands from mouse liver (Chakravarthy et al., 2009; Yuan et al., 2009). Although these studies identified the ligand from

an in-vivo tissue source, prior knowledge of the ligand's identity allowed the identification of the ligand by LC-MRM from only 100mg of mouse liver. In one particular study, which identified linoleic acid as a ligand for the HNF4α receptor, the ligand had been co-purified from COS-1 cells and identified by an LC-MS scan before confirming its presence by co-purification from mice liver. The optimized ligand identification protocol allows for the identification of the NR ligand from whole zebrafish embryos without prior knowledge of the ligand's identity (Figure 2-8).

3.1.7 Identification of interacting proteins for the PPARγ receptor

Affinity purification experiments from both the rosiglitazone-treated and GW9662-treated pLT-PPARγ embryos yielded a large list of co-purified proteins that were identified by either a MALDI-TOF or Orbitrap mass spectrometry. RXR alpha/beta/gamma, a group of proteins known to interact with the PPARγ receptor, were also identified by mass spectrometry (Figure 2-13). This suggests that the PPARγ-fusion protein is able to fold into a functional LBD and is also proof that our ligand trap fusion protein is able to co-purify relevant interacting co-factor proteins. Although it has been established that the PPARs are able to hetero-dimerize with the RXRs, even when in the inactive state, the RXRs were co-purified only with the rosiglitazone-bound PPARγ LBD. This maybe due to requirements for the PPARγ DBD for hetero-dimerization in the absence of agonist (Chandra et al., 2008).

Apart from this, several potential interacting proteins were identified such as the Major Vault Protein (MVP; Figure 2-13). This ubiquitously expressed protein is the major component of the Vault complex, a large ribonucleo-protein complex, implicated in many cellular processes such as signal transduction, immune response and nucleo-cytoplasmic transport (Berger et al., 2009). MVPs can associate with the Nuclear Pore Complex, and have been implicated in transport of proteins between the nucleus and cytoplasm. Interestingly, agonist-bound Estrogen receptor has also been shown to interact with MVP (Abbondanza et al., 1998). It is thought that MVP regulates the transport of Estrogen receptor into the nucleus upon binding to its cognate ligand. Considering this evidence, it is possible that there is a genuine interaction between MVP and the PPARγ receptor. Since MVP was co-purified exclusively with the GW9662-bound

PPARγ receptor, this interaction may be implicated in the transport of apo or antagonist bound PPARγ receptor into or out of the nucleus.

A large number of the co-purified proteins are assumed to be non-specifically binding, such as the abundant cytoskeletal proteins, actin and myosin. Since 1dpf embryos were used for the purification, a large amount of vitellogenin (yolk protein) was also identified.

Furthermore, due to the use of heat-shock for expression of the PPARγ-fusion protein, it may not be surprising that heat-shock proteins would also be co-purified. Interestingly though, heat-shock protein 70kDa and heat-shock cognate 71kDa protein were identified only with the rosiglitazone-treated purification (Figure 2-13). Considering that heat-shock proteins have been known to associate with several other NRs, these heat-shock proteins may play functional roles in PPARγ LBD function (Smith and Toft, 2008; DeFranco et al., 2000).

3.1.7.1 Future directions

The affinity purification experiments from both the rosiglitazone-treated and GW9662-treated pLT-PPARγ embryos yielded a large list of co-purified proteins that were identified by either the MALDI-TOF or Orbitrap mass spectrometer. Given that a large number of the potential interacting proteins were identified at a very low spectral count, a repeat of the same purification conditions should be performed. Wild type zebrafish embryos treated with either rosiglitazone or GW9662 could be used as a control to eliminate non-specifically binding proteins.

Following the purification repeat, the identified proteins could be screened by Co-IP experiments to confirm interaction to the PPARγ-LBD. Antibodies targeting the identified protein could be used for pull-downs from the pLT-PPARγ zebrafish embryos, followed by probing the purified fractions with either an anti-FLAG M2 or anti-PPARγ antibody.

3.2 Conclusion

Overall, the ability to co-purify and identify a NR ligand from an in-vivo tissue source is a potentially powerful one. This protocol for ligand identification overcomes many problems and limitations of previous methods of ligand identification for orphan NRs, allowing for the identification of the true endogenous ligand from the tissues in which it is functioning. The

identification of rosiglitazone by LC-MS scanning from a relatively small number of zebrafish embryos suggests that the protocol can easily be scaled up and used to identify the endogenously relevant ligand for PPARγ and other orphan NRs (Figure 2-8). Despite this success, there is still room for improvement in terms of the quantity of co-purified rosiglitazone.

This approach confirmed the direct binding of idebenone to the PPARγ LBD (Figure 2-12). This novel finding may explain previous studies showing that both idebenone and previously established PPARγ agonists caused the alleviation of certain neurodegenerative diseases (Sastre et al., 2006; Becker et al., 2010). Apart from this, it could also be investigated whether idebenone leads to increased insulin sensitivity.

Lastly, the co-purification of the RXRs with the PPARγ-fusion protein serves as a proof that the expressed fusion protein is able to fold into a functional LBD (Figure 2-13). Despite the large amount of co-purifying non-specific proteins, such as kerratin and actin, new potential interacting proteins have also been identified. An example is Major vault protein, which was only associated with the antagonist-bound PPARγ LBD.

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Figure 1-1. Nuclear receptor structure.

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Figure 1-2. Structural basis for ligand-response.

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Figure 1-3. Classification of nuclear receptors based on dimerization and binding to Hormone response elements (HREs).

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Figure 1-5. Basic components of a mass spectrometer.

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Figure 1-6. Triple quadrupole mass spectrometer.

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Table 1-1. Human nuclear receptors and identified endogenous ligand(s).

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