Title of Thesis

Characterisation of a Novel Nuclear Receptor-like Protein

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Faculty of Medicine
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Abstract

A genome threading algorithm was employed by Inpharmatica to identify a number of proteins with a predicted structure similar to that of the ligand binding domain (LBD) of nuclear receptors (NRs), an approach that has been successfully used to annotate the yeast transcription factor Oaf1 (Phelps et al., 2006). This work focuses on one such protein termed NR3, which is identical to TRPC4AP or TRUSS, a protein proposed to function as a scaffold protein in cell signalling processes and as a cell cycle regulator. It is conceivable that NR3 does not function as a transcription factor in contrast to bona fide NRs and that the putative LBD may function as an allosteric switch to control functional activity.

To investigate the idea that NR3 may possess a fold similar to the LBD of NRs preliminary structural work has been undertaken, which has suggested the putative LBD folds into an autonomous domain as it is region resistant to proteolysis. In addition, the potential role of the putative LBD fold as a molecular switch was examined by using constitutively active fusion proteins in reporter gene assays. It was determined that the putative NR3 LBD acts in a repressive manner, potentially due to the alteration in subcellular localisation exerted by the putative NR3 LBD on the fusion protein. To further assess the role of the putative LBD a ligand screen was undertaken to identify compounds that may reduce its repressive activity, however no ligand was identified and it is conceivable NR3 may act in a ligand independent manner similar to some orphan receptors.

Initial analysis of NR3 function indicates that its expression may have a positive effect on cell proliferation. To further assess the role of NR3 protein interaction assays were established to screen for binding partners. This identified the E3 ubiquitin ligase component DNA damage-binding protein 1 (DDB1) as an interacting protein involved in the regulation of cell cycle progression and DNA repair. Mapping studies suggest NR3 binds to the substrate docking site of DDB1 and further analysis showed NR3 to be ubiquitinated, affecting the stability of the protein. It is reported that the arylhydrocarbon receptor binds to a DDB1 complex, which then acts as a ligand regulated E3 ubiquitin ligase complex (Ohtake et al., 2007). This raises the possibility that NR3 may act in functionally analogous manner. To address NR3 function within the whole organism a targeting vector designed to inactivate the NR3 gene has been generated and currently a conditional knockout mouse line are being bred.
Statement of Originality

All work presented in this thesis was performed by myself unless otherwise stated in the text.
Acknowledgments

Firstly I would like to thank Professor Malcolm Parker for providing me with the opportunity to undertake my Ph.D. in his laboratory. His guidance and supervision have been instrumental in the success of this project. I would also like to thank Roger White for his advice and ideas, which has been a huge help throughout. My thanks also go to Mark Christian, who has always had the answers to the never-ending questions, and to Magnus Hallberg, the knowledge he provided has been invaluable. I’d like to thank Evangelos Kiskinis for his advice and support, and also the other members of the Molecular Endocrinology laboratory, both past and present, for their help and for making my time in the laboratory an enjoyable one. My thanks also go to Janet Allen, Rick Fagan and Adrian Kinkaid at Inpharmatica who have provided help and advice during the course of this project.

I would like to thank my Mum, Dad and sisters for their support outside the laboratory. Finally, I would like to thank my girlfriend Ana who has offered her support and understanding throughout my project, helping me endure the low points and celebrate the high points.
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<tbody>
<tr>
<td>AEBSF</td>
<td>4-(2-Aminooethyl) Benzenesulfonyl Fluoride HCl</td>
</tr>
<tr>
<td>AF</td>
<td>Activation Function</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl Hydrocarbon Nuclear Translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BPA</td>
<td>β-propeller Domain A</td>
</tr>
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<td>BPB</td>
<td>β-propeller Domain B</td>
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<tr>
<td>BPC</td>
<td>β-propeller Domain C</td>
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<td>BSA</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CDT</td>
<td>Chromatin Licensing and DNA Replication Factor</td>
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<td>Colony Forming Units</td>
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<td>CTD</td>
<td>C-terminal domain</td>
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<td>CUL</td>
<td>Cullin</td>
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<td>DAX-1</td>
<td>Dosage sensitive sex reversal - Adrenal hypoplasia congenita gene on the X chromosome, gene 1</td>
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<tr>
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<tr>
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<td>Mouse Double Minute 2</td>
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<td>Nucleotide Excision Repair</td>
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<td>PMSF</td>
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<tr>
<td>SRS</td>
<td>Son-of-Sevenless Recruitment System</td>
</tr>
<tr>
<td>ssssDNA</td>
<td>Sonicated Salmon Sperm DNA</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumour necrosis factor receptor 1</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>TRPC4AP</td>
<td>Transient receptor potential cation channel, subfamily C, member 4 associated protein</td>
</tr>
<tr>
<td>TRUSS</td>
<td>TNF receptor-associated ubiquitous scaffolding and signalling protein</td>
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Introduction
1.1 Overview

Nuclear receptors (NRs) can be defined as ligand activated transcription factors that regulate the expression of genes involved in diverse processes (Rosenfeld et al., 2003). The nuclear receptor superfamily currently consists of 48 members within the human genome, a number that has been confirmed by bioinformatic DNA sequence homology searches (Robinson-Rechavi et al., 2001). Although the protein sequence of NRs may be divergent throughout the superfamily, members possess a conserved canonical modular domain structure. The ligand binding domain (LBD) region is moderately homologous at the primary sequence level, yet 3-dimensional structures gained from X-ray crystallography show that the overall fold for the LBD is conserved between NRs (Renaud et al., 1995; Wagner et al., 1995). This domain is proposed to act as a multifunctional switch controlled by the binding of ligands.

A bioinformatic program named Genome Threder has been employed to produce protein alignments based on the 3-dimensional structure of NR LBDs rather than primary sequence homology. This method has identified several novel nuclear receptor-like proteins, predicted to contain a fold similar to that of NR LBDs. A number of the identified proteins were initially characterised in the literature, and although none are reported to act as direct regulators of transcription, the presence of a ligand binding domain within such proteins may indicate the ability of a small molecule to modulate a number of biological pathways within which the putative receptor protein functions. This project will focus on novel receptor 3 (NR3), which is strongly predicted to possess a NR LBD-like fold.

1.2 Identification and Classification of Nuclear Receptors

Initially the concept of nuclear receptors developed from observations that steroid hormones bound to specific tissues, providing evidence that hormone binding factors were present (Jensen, 1962). The oestrogen receptor (ER) was the first NR to be isolated over forty years ago from rat uterus homogenate (Toft and Gorski, 1966), and initial functional characterisation showed that upon activation the receptor produced an increase in mRNA and protein synthesis, and thus was thought to be involved in the regulation of gene transcription (O'Malley and McGuire, 1968). The purification and characterisation of the glucocorticoid receptor (GR) showed that the
receptor could bind to mammary tumour virus DNA upstream of the transcriptional start site in a sequence specific manner, suggesting that the mechanism by which NRs regulate transcription involved receptors recognising and binding to distinct DNA sequences at target genes (Payvar et al., 1981). Additional work from the same laboratory later showed that the sequences to which GR binds acted as enhancer elements, termed response elements (REs), that were separate from elements within the core promoter region already known to be essential for transcription (Chandler et al., 1983).

Further identification and characterisation of REs showed they were inverted repeat motifs (Evans, 1988), lending support to the postulation that nuclear receptors bound to REs in dimeric complexes, which arose from the observations that receptors could be purified as dimers (Schrader et al., 1975). From these initial descriptive experiments a simple model emerged for the steroid hormone activation of their respective nuclear receptors in which ligand binding can induce receptor dimerisation, binding to specific response elements and the modulation of target gene transcription (figure 1.1).

During the 1980s and 1990s much emphasis was placed on the cloning of nuclear receptors. Comparison of the complementary DNA (cDNA) and deduced amino acid sequences showed the receptors shared a high level of homology, and thus are thought to form a superfamily (Evans, 1988). Low stringency hybridisation techniques using probes corresponding to the highly homologous DNA binding domain (DBD) region of NRs were initially used to identify members of the superfamily. Completion of the human genome project aided the identification of additional NR superfamily members by allowing homology searches based on DNA sequence. At present, such searches show that there are 21 nuclear receptors in the Drosophila genome, 48 in the human, 49 in the mouse, and over 270 receptors currently identified in the Caenorhabditis elegans genome (Robinson-Rechavi et al., 2001; Robinson-Rechavi et al., 2003). The superfamily may be subdivided and classified in different ways. Historically, nuclear receptors have been divided into 4 different classes based upon the dimerisation and DNA binding properties of individual receptors (Mangelsdorf et al., 1995). The type I receptors, also known as steroid receptors, form homodimers and bind to inverted repeat REs, whereas type II receptors form heterodimers with retinoid X receptor (RXR) and bind direct repeat
Figure 1.1 Simplified Model of NR Action
Cartoon showing a basic model of nuclear receptor function. Upon ligand binding receptors dimerise and bind to hormone specific response elements within the promoters of target genes, producing an upregulation of gene transcription.

A) Classical receptors homodimerise and bind to inverted repeats. ER binds to half sites.

B) Non-classical receptors heterodimerise with RXR and bind to direct repeats.
REs. Type III receptors, which include RXR, bind to direct repeat REs as homodimers, although RXR may also heterodimerise with type II receptors. Type IV receptors bind to extended half site REs as monomers.

Alternatively, the nuclear receptor superfamily may be divided based on the type of ligand they bind (figure 1.2A) (Sonoda et al., 2008). Such a classification possesses three groups of receptors, endocrine receptors that bind a defined hormone, adopted orphan receptors for which a ligand has been identified, and orphan receptors for which a ligand has yet to be identified or may not bind a ligand. However, receptor classification may be problematic due to the complexity and redundancy in the nomenclature. As such a unified nomenclature was devised to offer simplicity and scope for the addition of newly discovered receptors to the superfamily. This system is based on the phylogenetic tree derived from the receptors from vertebrates, arthropods and nemotodes (Laudet, 1997). This classification produces 6 subfamilies of nuclear receptor, named subfamilies 1-6, and a separate subfamily, named subfamily 0, was used to group receptors not conforming to the standard structural architecture of the superfamily (figure 1.2B) (Nuclear Receptors Nomenclature Committee, 1999). Each subfamily is grouped by receptor family and each member of the receptor family is numbered. This method of classification also provides a form of nomenclature that names receptors based on subfamily and grouping.

Recent work investigating the receptors present within the *Schistosoma mansoni* genome has led to the postulation of creating a new subfamily, named subfamily 7, which consists of 3 NRs from the *S. mansoni* genome, termed 2DBDα,β,γ (Wu et al., 2006; Wu et al., 2007). The receptors possess two DBDs in tandem repeat, a feature that appears to be restricted to the flatworms. The first DBD is most homologous with the DBD of *Drosophila* proteins Knirps, KNRL and Eagle, and the second DBD shares similarities with the DBD of the receptors grouped in subfamily 1, indicating the ancestral origins of the DBDs in the receptors are not monophyletic (Wu et al., 2006). However, it remains unclear as to the timings of the DBD duplication, and thus whether the event occurred before the divergence of the flatworm phylum and one DBD was subsequently lost in receptors belonging to other phyla, or whether the duplication event occurred after phylum divergence suggesting the appearance of two DBDs within a receptor is unique to flatworms.
Figure 1.2 Classification of Human Nuclear Receptors
Human NRs classified by A) the type of ligand they bind and B) subfamily based on phylogenetic analysis.
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Such phylogenetic studies have made it clear that the members of the NR superfamily are evolutionarily related. In addition, structural studies have shown that NRs also share a similar architecture consisting of distinct regions, some of which are modular autonomous domains responsible for different aspects of NR function.

1.3 Structure of Nuclear Receptors

1.3.1 Structural Paradigm for the Nuclear Receptor Superfamily

Receptor homology across the superfamily is variable, for example aligning ERα (accession NP_000116) with peroxisome proliferator activated receptor (PPAR) α (accession AAB32649) shows there is 16.5% identity between the proteins. Despite the variability between protein sequences, NRs share a canonical protein architecture within which there are several identifiable domains (figure 1.3). The idea of a domain-like structure emerged from the observations that limited proteolysis of the dexamethasone bound GR could separate the ligand binding and DNA binding regions of the receptor (Wrang and Gustafsson, 1978). Currently up to six regions have been identified within NRs on the basis of sequence homology, denoted regions A-F (Krust et al., 1986). Of these six regions, regions A-E are relatively well characterised, however the biological function of region F remains unclear.

The A/B region, also known as the modulator domain, is the most variable region between nuclear receptors. This variation may be generated through alternative splicing, gene transcription occurring from different promoters, and translation beginning from different start sites (reviewed in Giguere, 1999). The region is thought to be a largely disordered domain and contains activation function (AF) -1, which provides constitutive activity and may provide a mechanism for receptor activation in the absence of ligand. It is likely that the transcriptional activation conferred by the A/B region is due to an interaction between the A/B domain and coactivators (Giguere, 1999). The level of A/B domain activity may be dependent on phosphorylation status of the receptor as exemplified by ERα, which is activated by direct phosphorylation at serine-118 by mitogen-activated protein kinase pathways (Bunone et al., 1996; Kato et al., 1995). In addition to providing constitutive and ligand-independent activity, the A/B region can also confer receptor dependent ligand-responsiveness and cell type specific activity (Ali et al., 1993).
Figure 1.3 Schematic of the Canonical Structure of the Nuclear Receptor Superfamily
The schematic show the 6 identifiable regions of nuclear receptors, termed A-F. The A/B region (modulator) contains the constitutively active activation function-1 (AF-1) and region E (LBD) contains the ligand dependent activation function-2 (AF-2).
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The DBD (region C) is the most highly conserved domain and is responsible for recognising the appropriate response element within the promoters of target genes. There are several identifiable regions within the DBD. The DBD possesses two Cys2/Cys2 zinc finger motifs, which are the core of the DBD and are responsible for binding DNA, and a region of approximately 25 amino acids termed the carboxyterminal-extension (CTE). Within the zinc finger motifs are the P-box and D-box, named according to their relative position within the DBD, which have been shown to be involved in the recognition of different nuclear receptor REs (Umesono and Evans, 1989). Crystallographic studies have provided insightful structural models of the way NRs bind and recognise the appropriate response elements. The domain includes two main α-helices, of which the N-terminal helix possesses the P-box and the C-terminal helix resides between the D-box and the CTE. Such studies on the DBD have indicated that the N-terminal helix binds to DNA in the major groove making direct contacts to the nucleotides of the RE, and the C-terminal helix overlaying the structure (Luìsi et al., 1991; Rastinejad et al., 1995; Zhao et al., 1998). In addition, the D-box has also been shown to be important in the determination of partner when receptors dimerise (Zechel et al., 1994). The CTE is also reported to be involved in dimerisation and may make additional contacts with DNA (Rastinejad et al., 1995; Zhao et al., 1998).

The hinge region (region D) lacks sequence conservation across the superfamily and acts as a linker between the DBD and LBD. It confers flexibility between the two domains and plays a role in receptor dimerisation and RE recognition. Although relatively little is known about the role of this region, it appears it may be involved in transcriptional repression both by a corepressor independent mechanism (Liao et al., 2003), and also by potentially stabilising corepressor binding (Wang et al., 2001). The LBD (region E) is moderately conserved throughout the superfamily, and is a multifunctional domain. Ligand binding can induce nuclear localisation, receptor dimerisation (Fawell et al., 1990), and the binding of ligand dependent cofactors (Cavailles et al., 1994; Onate et al., 1995). The LBD forms a second independent activation domain, termed AF-2 that contrasts the function of AF-1 due to its ligand dependence (Danielian et al., 1992). Although these actions are receptor specific and thus do not apply to all NRs, the LBD can be thought of as a molecular switch that,
upon ligand binding, is responsible for a conformational change resulting in activation of the receptor.

The structural paradigm of the nuclear receptor superfamily clearly indicates NRs possess distinct modular regions that underpin the biological function of the proteins. The LBD is of particular interest to both academia and the pharmaceutical industry as it is responsible for activating NRs in response to ligand, and as such is an attractive region to target pharmacologically. Structural studies and molecular dissection of the LBD have proved fruitful in aiding the understanding of the mechanisms by which the domain functions.

1.3.2 Structure of the Ligand Binding Domain

Solution of the 3-dimensional structures of several nuclear receptor LBDs in the apo and holo configurations through X-ray crystallography has shown a strong conservation in overall structure. The first ligated LBDs structures to be solved were the thyroid hormone receptor (TR) α and retinoic acid receptor (RAR) γ (Renaud et al., 1995; Wagner et al., 1995), and such structural studies have shown that the secondary structure of LBDs generally consists of 12 α-helices separated by 3 antiparallel β-sheets producing a box-like structure. The structural conservation of the LBD is observed across the superfamily, even in receptors that possess a relatively low homology. Comparison of the PPARα and TRα LBD crystal structures show conservation in 3-dimensional structure, yet they only share ~22% homology (figure 1.4) (Cronet et al., 2001; Ye et al., 2003).

Within the 3-dimensional structure of the LBD a region has been identified termed the ligand binding pocket, which contains residues responsible for physical interaction with the ligands. However, the crystal structures of several NR LBDs has shown the exact positioning of the ligand binding pocket within the LBD is variable (Love et al., 2002; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995; Watkins et al., 2001). Furthermore, the size of the ligand binding pocket varies greatly across the superfamily ranging from 30Å³ within the Drosophila DHR38 receptor to 1400Å³ in the PPAR receptors (reviewed in Li et al., 2003). It appears that those receptors with larger binding pockets, such as the PPARs and the pregnane X receptor (PXR), function to sense metabolites and may be regarded as promiscuous as
Figure 1.4 The NR LBD Architecture is Conserved Across the Superfamily
Schematic of the LBD crystal structures of A) PPARα bound to AZ242 (protein data bank ID: 1I7G; Cronet et al, 2001) and B) TRα bound to IH5 (protein data bank ID: 1NAV; Ye et al, 2003). The 3-dimensional architecture of the LBDs is well conserved despite the divergence of the amino acid sequence. In both structures helix 12 (H12) is positioned over the ligand binding pocket and provides a surface upon which cofactors may bind through LXXLL domains to modulate transcription. Image was generated using 3D Molecule Viewer which forms part of the Vector NTI software suite.
they bind a wide range of ligands, which contrasts with the steroid receptors that bind a narrower range of ligands and possess smaller cavities (Li et al., 2003). It is conceivable that a larger ligand binding pocket enables a receptor to bind a number of different ligands with potentially different amino acids within the cavity forming molecular interactions to mediate such binding. This has been found to be the case for PXR, which is not only promiscuous in its ligand binding, but also may bind a single ligand in a number of orientations (Watkins et al., 2001). Interestingly, a recent study has shown that the large cavity within PPARγ can accommodate two copies of oxidised fatty acids at the same time, which is the first example of such ligand binding (Itoh et al., 2008). Furthermore, a number of ligand binding determinants were observed for the different ligands bound within the PPARγ binding pocket.

Structural studies have also provided mechanistic insights to the effects of ligand binding. Potentially the most important mechanistic observation made from structural studies is that upon the binding of ligand, receptor LBDs undergo a conformational change that allows helix 12 to be repositioned above the ligand binding pocket and held tight towards the body of the LBD (figure 1.4) (Bourguet et al., 1995; Cronet et al., 2001; Love et al., 2002; Renaud et al., 1995; Wagner et al., 1995; Ye et al., 2003). Such repositioning is thought to promote the release of corepressor molecules that bind to unligated receptors, and also provides a surface to which coactivators can bind and positively modulate the transcription of target genes.

Although there is a strong conservation of 3-dimensional structure across the superfamily, the amino acid sequences of NR LBDs can be rather divergent when aligned. However, such alignments show that certain key residues are conserved within most NR LBDs. Receptor alignments show the (F/Y/W)(A/S/I)(K/R/E/G)XXXX(F/L)XX(L/V/I)XXX(D/S)(Q/K)XX(L/V)(L/I/F) motif is present in all receptors, however a much simpler form of the motif is present in most receptors consisting of the consensus (F/W)AKXXXXFXXLXXXDQXXLL (figure 1.5A) (Wurtz et al., 1996). These residues form parts of helix 3 to helix 5 and are thought to be in close proximity to the core of the ligand binding pocket, and thus are potentially involved in the downstream effects following ligand binding. In addition, the AF-2 region of helix 12 is well conserved across the super family. The consensus sequence of AF-2 is represented by a glutamic acid residue flanked either side by 2
1.5 Consensus Sequences of the Nuclear Receptor Ligand Binding Domain

A) The conserved motif present within helices 3 and 5 of all nuclear receptor LBDs, and the simplified consensus sequence present in most nuclear receptors (Wurtz et al., 1996).

B) The conserved AF-2 motif present in helix 12 of nuclear receptor LBDs (Danielian et al., 1992; Wurtz et al., 1996). The glutamic acid required for full AF-2 activity is shown in red.
hydrophobic residues (figure 1.5B). The glutamic acid present at the centre of the amphipathic helix is required for AF-2 mediated receptor activity (Danielian et al., 1992).

In the structural paradigm described, the ligand binding domain of nuclear receptors clearly plays an important role in the function of the receptor by acting as a molecular switch to activate the receptor to mediate the recruitment and interaction with other signalling proteins. However the orphan NR, Nur77-related factor 1 (Nurr1) is considered to be a constitutively active receptor important in developmental processes. Initially studies focused on the identification of the endogenous ligand for Nurr1, however the solution of the Nurr1 LBD crystal structure showed that it was unlikely to bind ligand (Wang et al., 2003). The LBD exists in a conformation similar to that of an activated NR, however no ligand binding pocket appears to be present due to the tight packing of bulky side chains from amino acid residues within the surrounding helices. Similarly, the adopted orphan receptor hepatocyte nuclear factor (HNF) 4α binds to fatty acids constitutively, which retains the LBD in an active conformation (Wisely et al., 2002). This allows the receptor to possess constitutive activity, which is thought may be regulated by post translational modifications to the receptor (Viollet et al., 1997; Wisely et al., 2002).

1.4 Function of Nuclear Receptors

1.4.1 Regulation of NR Mediated Transcription

The actions of nuclear receptors with regards the transcriptional control of genes is well characterised. However, observations in the 1990s showed that NR function was regulated by a class of proteins termed coregulators, as had been predicted by initial squelching experiments (Tasset et al., 1990). Later reports showed they could be recruited in a ligand dependent fashion, and provided another layer of control to the transcriptional activation of NR target genes (Cavailles et al., 1994; Halachmi et al., 1994).

The NR coregulators consist of coactivator and corepressor molecules, which function as platforms to recruit other activating or repressive proteins capable of remodelling the chromatin to which they are bound. Coactivators include proteins such as p300, CREB binding protein, thyroid hormone receptor-associated protein,
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and the p160 family of proteins, which consist of steroid receptor coactivator-1, glucocorticoid receptor interacting protein-1 and receptor-associated coactivator-3 (Chakravarti et al., 1996; Fondell et al., 1996; Hong et al., 1996; Li et al., 1997; Onate et al., 1995). Their recruitment occurs through the NR box, an amphipathic α-helical LXXLL that is critical for NR interaction (Heery et al., 1997). Some coactivators possess intrinsic histone acetyltransferase activity, which produces an open chromatin structure associated with transcriptionally active genes acetylating histones H3 and H4 within the chromatin (Spencer et al., 1997). The open configuration of transcriptionally active chromatin then allows the recruitment of general transcription factors and the basal transcription machinery to the promoter.

Conversely corepressors, such as SMRT and N-CoR, bind to NRs in the absence of ligand (Chen and Evans, 1995; Horlein et al., 1995). This interaction occurs through a motif that is analogous to the NR box, termed the CoRNR box. The consensus sequence for the this motif is (L/I)XX(I/V)I, of which the corepressors SMRT and N-CoR possess two each (Hu and Lazar, 1999). This consensus duplication is thought to have a functional role in the mechanism by which corepressors interact with NRs, and may provide a receptor-dimer specificity allowing the differentiation between receptor complexes. In contrast to other corepressors RIP140 is binds to NRs in the presence of agonist, which initially led to the assumption that the protein acted as a coactivator (Cavailles et al., 1995). In addition, RIP140 bound to NRs via the coactivator NR box motifs, 10 of which are present within the structure of RIP140 (Heery et al., 1997). However, further functional analysis identified RIP140 as a corepressor of nuclear receptors (Joyeux et al., 1997; Lee et al., 1998; Lee and Wei, 1999; Subramaniam et al., 1999; Treuter et al., 1998). The binding of corepressors results in the recruitment of histone deacetylases that possess enzymatic activity capable of removing the activating acetyl groups from the histones within the chromatin (Nagy et al., 1997). This epigenetic regulation of target gene promoters allows chromatin to condense preventing the recruitment of transcription factors.

1.4.2 Elucidation of NR Function Using Null Animals

The use of knockout mice in which the target gene of interest has been disrupted has led to many insights into nuclear receptor physiology. One of the most well
studied models is that of the ER knockout (ERKO) mice. ERα (αERKO), ERβ (βERKO) or both receptors (αβERKO) have been deleted in mice (Couse et al., 1999; Dupont et al., 2000; Krege et al., 1998; Lubahn et al., 1993), producing different phenotypes dependent on the physiological signalling of the respective receptor. Deletion of ERα in mice resulted in infertility in both sexes (Lubahn et al., 1993), which was later shown to be independent of oestrogen synthesis for male mice, suggesting the ERα signalling required for fertility in males may be ligand independent (Fisher et al., 1998). In βERKO mice, females displayed a compromised reproductive phenotype although they were fertile, indicating that both ERα and ERβ are required for normal female fertility and only ERα is required for normal male fertility (Krege et al., 1998). Unsurprisingly, αβERKO mice are infertile, similar to the phenotype of αERKO (Couse et al., 1999; Dupont et al., 2000). Examination of the respective phenotypes of ERKO mice has demonstrated a role for both ERα and ERβ in regulating bone density. αERKO mice possess bones with decreased density in both sexes, whereas βERKO females possess denser bones but males appear normal (Vidal et al., 2000). This suggests ERα plays a positive role in the mineralisation of bone in both sexes but ERβ is a negative regulator of mineralisation in females.

The use of knockout mouse technology has clearly proven to be useful in understanding the physiological role of proteins within the context of the whole organism. However, disruption of a gene that is required for development may lead to embryonic lethality or, as exemplified by GR null animals, can result in gross phenotypic abnormalities that can be lethal at birth (Cole et al., 1995; Schmid et al., 1995). In the case of GR a conditional knockout mouse system was used that allows the disruption of a targeted gene in specific tissues. Conditionally disrupting GR by crossing floxed GR mice with mice expressing Cre recombinase from the nestin promoter showed that GR expression was not required in the nervous system for development (Tronche et al., 1999). Furthermore, such animals were shown to possess reduced stress levels, implicating GR signalling in such processes. The use of knockin mice has also proved fruitful when studying the physiological roles of GR. Mice carrying the point mutation A458T were generated by targeting exon 4 of the gene (Reichardt et al., 1998). The resultant mice, named GR$^{\text{dim/dim}}$ mice, possessed a mutant GR receptor that failed to dimerise, bind to glucocorticoid REs and
transactivate target genes, although the expressed mutant GRs were still capable of transrepression (Reichardt et al., 1998). The use of such mice has allowed the dissection of the pathways involved in physiological functions that GR regulates. The growth, development, and spatial learning of an animal is dependent on GR expression but not its ability to directly activate target genes (Bayo et al., 2008; Cole et al., 1995; Oitzl et al., 2001; Schmid et al., 1995; Tronche et al., 2004). This is in contrast with the ability of GR to regulate T-lymphocyte development, which is dependent on direct gene regulation by GR (Reichardt et al., 1998). However, the involvement of GR in the regulation of stress responses appears to require both the direct and indirect regulation of target genes (Reichardt et al., 1998).

The use of animal studies to examine the effects of gene disruption has proved to be a powerful research tool and provided an insight into the physiological functions of nuclear receptors. However, studies using explanted tissue and cell based assay systems have also been useful in understanding the function of nuclear receptors. Such studies have shown that the effects mediated by nuclear receptor signalling may not necessarily result in transcription of target genes directly, but may result in stimulation of intracellular signalling pathways. These are regarded as non-genomic effects exerted by nuclear receptors.

1.4.3 Non-genomic Actions of NRs

Although much is clearly known about NR function within the context of direct target gene regulation, evidence is emerging to suggest NRs may be involved in non-genomic functions. The most well characterised receptor in terms of actions outside those of transcriptional regulation is the ER. Preliminary observations almost three decades ago first showed that oestrogen could bind to the outer surfaces of endometrial and liver cells (Pietras and Szego, 1977). It was later shown that macromolecules capable of oestrogen binding could be isolated from purified membranes of hepatocytes derived from ovariectomised rat liver (Pietras and Szego, 1980), giving rise to the idea of functional membrane steroid receptors. In the proceeding years there were many observations that steroid hormone administration could produce rapid effects on intracellular signalling pathways associated with membrane receptors (Castoria et al., 2001; Migliaccio et al., 2000; Migliaccio et al., 1996; Migliaccio et al., 1998). However, confusion arose as to whether the receptors
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responsible for mediating the effects of the steroid hormones acting at the plasma membrane were identical to those responsible for transcriptional regulation. To address this confocal laser microscopy was used to confirm the presence of the ER nuclear receptor at the cell membrane (Pappas et al., 1995). Furthermore, it was confirmed through transfection of Chinese hamster ovary cells, which do not express endogenous ER, that both exogenous ERα and ERβ isoforms are present at the cell membrane (Razandi et al., 1999).

The mechanism by which the ER is localised to the membrane remains unclear as the receptor itself is lacking in any hydrophobic region thought to be typically necessary for integration into the plasma membrane. It is known that the cysteine residue at position 447 is palmitoylated, a post-translational modification that is important in localising ERα to the cell membrane and for interactions with other proteins (Acconcia et al., 2005). Such interactions may also be a potential mechanism by which the ER is localised to the membrane, as an interaction with a membrane integrating protein would tether the receptor to the plasma membrane. ERα interacts with caveolin-1 (Razandi et al., 2003), a typical marker for caveolae (Rothberg et al., 1992), suggesting ERα may be recruited to lipid microdomains at the cell surface. Mutation of serine 522 in ERα prevented this interaction, although this effect did not ablate ERα recruitment to the plasma membrane (Razandi et al., 2003), thus other mechanisms must be involved in ERα membrane recruitment. There is evidence to suggest ER interacts with other cell surface molecules, supporting the idea that cross-talk between ER and other receptors occurs to activate intracellular signalling pathways. It has been observed that ER can form ligand dependent ternary complexes with Shc and IGFR, and with c-Src and PELP1 to modulate mitogen activated protein kinase signalling pathways (Song et al., 2004; Wong et al., 2002). However, the relevance of such interactions in determining the localisation of the ER to the plasma membrane is unclear, as the interactions have been described to be ligand dependent, yet the ER can be observed at the cell membrane in the absence of ligand (Pappas et al., 1995).
1.4.4 Non-Nuclear Receptor Mediated Steroid Signalling

In addition to the observations that oestrogen could signal through ER mediated non-genomic pathways, further complexity arose from reports that oestrogen could signal through receptors at the membrane that were not related to the ER. GPR30 is an orphan G-protein coupled receptor, for which evidence is building to suggest its involvement in non-genomic oestrogen signalling. SKBR3 cells that lack both ERα and ERβ are responsive to oestrogen, resulting in activation of extracellular signal-regulated kinase (ERK) through cross-talk with the epidermal growth factor receptor in a heparin-bound epidermal growth factor dependent manner (Filardo et al., 2000). In addition the same study showed that overexpression of GPR30 in MDA-MB-231 cells, which lack ERα and GPR30, but express ERβ, also resulted in ERK activation in response to oestrogen. Later work from the same group in a similar set of experiments showed that GPR30 also activates adenylyl cyclase in response to oestrogen (Filardo et al., 2002), suggesting the receptor couples with Gs proteins. Further evidence that the rapid activation of ERK and adenylyl cyclase in response to oestrogen is mediated through GPR30 is gained from the observation that antioestrogens act as agonists in the above adenylate cyclase and ERK activity assays (Filardo et al., 2000; Filardo et al., 2002). Therefore questions are raised as to exactly what effects antioestrogenic drugs have when taken therapeutically and also whether it is possible to produce selective drugs that can target either nuclear receptor or GPR30 activated pathways.

It is evident that the LBD region of NRs plays a role in the different NR modes of action when transducing endocrine signalling in the nucleus, cytoplasm and at the cell surface within the context of both gene regulation and effects on non-genomic signalling pathways. Thus it is conceivable that a ligand binding domain-like region may be present within proteins not directly related to NRs, functioning as a regulatory domain or molecular switch within cell signalling pathways.

1.5 Evolution of the Nuclear Receptor Superfamily

It has been known for some time that the nuclear receptor superfamily existed prior to the evolutionary divergence of deuterostomes and protostomes (Laudet et al., 1992), which raises questions regarding the evolutionary origin of the superfamily. It
has been postulated that the superfamily is likely to have arisen from a single ancestral gene and that gene duplication events played an important role in the expansion and divergence of the superfamily (Laudet, 1997). Two waves of such duplication are thought to have occurred, with the first wave happening prior to the divergence of arthropods and vertebrates resulting in the emergence of the six subfamilies receptors within the superfamily (Escriva et al., 1997; Laudet, 1997). The second wave of gene duplication is thought to be responsible for the diversification of the superfamily, giving rise to subfamilies of receptor type, such as the α and β forms of ER and the α, β and γ forms of the oestrogen related receptors (Laudet, 1997). Other analyses support the idea that gene duplication played a central role in the expansion and diversification of the superfamily, as it is thought that such duplication events account for the presence of over 270 receptors within the genome of C. elegans (Sluder et al., 1999), many of which are related to HNF4 (Robinson-Rechavi et al., 2005). The members of the nuclear receptor superfamily possess divergent ligand binding properties as there are orphan receptors that are predicted not to bind ligand, adopted orphans that are promiscuous in their ligand binding characteristics, and some receptors, such as the steroid receptors, that are more specific in their ligand binding. Study of the evolution of the superfamily may provide some clues as to how such divergence in ligand binding properties arose from a putative single ancestral gene. Phylogenetic analysis has suggested that orphan receptors with no known ligand may be more ancient than receptors possessing ligand, leading to the suggestion that the putative ancestral gene was an orphan receptor and ligand binding was an evolutional gain of function (Escriva et al., 1997). Recent work has reported that heme binds within the ligand binding pocket of the Drosophila E75 receptor and appears to be required for stability of the LBD (Reinking et al., 2005). The oxidative state of the heme molecule may be altered when bound to the LBD of E75, suggesting the receptor acts as a redox sensor (Reinking et al., 2005). Further studies have shown that the mammalian orthologues of E75, Rev-erbα and Rev-erbβ, also bind heme as a ligand, although the receptors were capable of forming stable structures in the apo state (Raghuram et al., 2007). In addition, the ecdysone receptor, which is the Drosophila orthologue of the farnesoid X receptor in mammals, is known to bind ecdysteroids to regulate target genes (Koelle et al., 1991). It is therefore clear that
gain of function for receptors to bind ligands emerged prior to evolutionary divergence of deuterostomes and protostomes, or that the ability to bind ligand simultaneously emerged in both taxa.

Analysis of the steroid receptor evolution suggests that the ability to bind ligand predates the evolutionary divergence of deuterostomes and protostomes. The cloning of a mollusc ER orthologue from *Aplysia californica* suggested that the steroid subfamily of NRs was present in invertebrates (Thornton et al., 2003). Protein sequence alignments of the mollusc ER to the human ERα showed the mollusc receptor was 88% similar to the human ERα DBD, and 35% similar to the LBD. Functional characterisation of the mollusc receptor in reporter gene assays showed that the DBD was capable of binding to an oestrogen RE, and that the LBD possessed an AF-2 dependent constitutive activity that was unaltered by ligand administration. Furthermore, the LBD did not bind oestradiol (Thornton et al., 2003). To determine whether oestrogen binding is a gain of function in mammalian ERs, or a loss of function in mollusc ERs an ancestral LBD was generated by Thornton et al (2003) using maximum likelihood reconstruction techniques to infer the amino acid sequence. The ancestral LBD was observed to activate transcription in reporter gene assays in response to oestradiol, and also bind oestradiol with a Kd of 198nM, which is a relatively low affinity compared to the dissociation constant of 0.8nM associated with the human ERα LBD (Thornton et al., 2003). The evidence presented by Thornton et al (2003) suggests that the steroid subfamily of nuclear receptors have emerged prior to the evolutionary divergence of deuterostomes and protostomes, and that such ancestral steroid receptors may have bound a ligand to regulate their activity.

The view that mollusc receptors may bind ligand has also been strengthened by the cloning of the *Biomphalaria glabrata* orthologue of RXR (Bouton et al., 2005). Electrophoretic mobility shift assays with a number of direct repeat RE binding sites showed that the mollusc RXR could weakly bind to the REs as homodimers, and much more strongly as heterodimers with human FXR and PPARα. In addition, it was observed that the mollusc RXR binds 9-cis retinoic acid, which could result in transactivation in reporter gene assays (Bouton et al., 2005). These data indicate that the RXR orthologue potentially possesses a ligand similar to 9-cis retinoic acid in the mollusc, which may regulate its activity. As the ligand for the mollusc RXR is likely to be related to that from mammals, it is possible that the emergence of an ancestral
nuclear receptor that could bind ligand occurred prior to evolutionary divergence of deuterostomes and protostomes.

The multiple domain architecture of nuclear receptors has also led to the idea that the ancestral NR gene may be the product of genomic rearrangements that brought together DNA coding for pre-existing protein modules (Barnett et al., 2000; Laudet et al., 1992). The zinc finger-possessing DBD of the NR superfamily shares homology to those of the LIM and GATA transcription factors, orthologues of which are found in yeast, suggesting a common evolutionary ancestor for the module (Clarke and Berg, 1998). Furthermore, primary sequence alignments have provided evidence that the LBD region of NRs is related to Pex11p (Barnett et al., 2000). Pex11p is present in eukaryotes, and is implicated in peroxisome proliferation (Li et al., 2002a; Li et al., 2002b). Reported alignments show that Pex11p is most similar to the LBDs of the PPAR family of NRs, and possesses moderately conserved key residues pertaining to NR LBDs (Barnett et al., 2000). Analysis of the predicted secondary structure of Pex11p in conjunction with the crystal structures of the PPARs allowed the authors to conclude that Pex11p is likely to possess a similar folded structure as the LBD of NRs (Barnett et al., 2000). The suggestion may therefore be raised that the ancestral nuclear receptor gene may have arisen from a fusion of the zinc finger DBD, similar to that found in LIM and GATA transcription factors, and an LBD-like module related to Pex11p. Both modules are present within eukaryotes outside the animal kingdom. However the putative single ancestral gene that the NR superfamily arose from is generally thought to be metazoan in origin, as NRs appear to be restricted to the animal kingdom (Escriva et al., 1997). However a recent study in yeast has indicated NR superfamily members may be present in unicellular eukaryotes (Phelps et al., 2006), adding support to the idea that the ancestral gene or genes related to the nuclear receptor superfamily may date further back in evolution than initially anticipated.

1.6 NRs that do not Conform to the Structural Paradigm

Although the nuclear receptor superfamily shares a common domain and 3-dimensional architecture, there are examples of receptors that do not fit in the generalised model above. The orphan nuclear receptors dosage sensitive sex reversal - adrenal hypoplasia congenita gene on the X chromosome, gene 1 (DAX-1) and short
heterodimers partner (SHP) also do not possess the canonical zinc finger DBD associated with the nuclear receptor superfamily. DAX-1 was originally identified as the gene responsible for the developmental disorder X-linked adrenal hyperplasia congenital, and SHP was identified through a yeast two-hybrid screen for proteins that interact with nuclear receptors (Seol et al., 1996; Zanaria et al., 1994). Both receptors have been shown to interact with other nuclear receptors, and there is evidence to suggest they function as repressors. However, the mechanisms by which the receptors exert their respective repressive may differ. DAX-1 is thought to recruit corepressors, such as N-CoR, and function as a trans-repressive molecule (Crawford et al., 1998). SHP has also been reported to inhibit the transcriptional effects of several nuclear receptors, although the mechanism by which this occurs has yet to be elucidated (Ourlin et al., 2003). Furthermore, more recently SHP has also been shown to interact with non-nuclear receptor transcription factor HNF3, exerting a repressive effect on its transcriptional activity through an interaction with the forkhead domain of HNF3, resulting in inhibition of DNA binding (Kim et al., 2004). As neither receptor possesses a DBD, they differ from the rest of the superfamily members, however it is unclear as to whether their evolution has resulted from the loss of the DBD from an ancestral NR, or if their existence supports the idea that NRs originated from two distinct modules that were fused as a result of DNA rearrangements.

It is clear that in general the members of the nuclear receptor superfamily are subject to the same structural paradigm. However nuclear receptors such as DAX-1 and SHP represent a class of nuclear receptor superfamily members that do not conform precisely to the suggested structural model. It is therefore conceivable that there may be a number of other proteins that share some similarities in structure to NRs, yet are not homologous nor possess all the domains associated with the typical NR architecture.

1.7 Bioinformatic Predictions for Novel Nuclear Receptors

Clearly nuclear receptors are important regulators within the cell, and as such are implicated in many disease processes, including cancer, obesity and osteoarthritis. Furthermore, as NRs are known to be responsive to endogenous ligands they represent a prime target for the development of therapeutics. Together this has led to much interest from both academia and the pharmaceutical industry in characterising
NR biology. Currently, bioinformatic analysis using sequence homology searches with the databases available online suggests that there are a total of 48 nuclear receptors within the human genome (Robinson-Rechavi et al., 2001). Inpharmacis are a biotechnology company specialising in the prediction of protein folds using bioinformatic annotations. By employing sequence-structure compatibility or “threading” techniques using their protein fold prediction algorithm, Inpharmacis have identified several proteins thought to possess regions within their structure that fold in a similar way to the ligand binding domain of nuclear receptors.

As discussed above, the NR LBD is moderately conserved at the primary sequence across the superfamily, however possesses an overall conserved 3-dimensional structure. Detecting structural relationships between sequences of ~20% homology, as in the case for TRα and PPARα, may not be possible through primary sequences alignments. To overcome this issue, threading techniques were pioneered to enable protein fold recognition, and thus detect groups of proteins that may be analogous in function yet lack primary sequence homology. Such techniques involve the threading of the target sequence through a representation of a fold family, whilst estimations are made as to whether the target sequence and the fold representation are compatible (Shortle, 1997). This has been shown to provide an accurate method of fold recognition, and in the guise of GenTHREADER has been developed such that the technique may be automated, vastly improving sequence throughput (Jones, 1999; Shortle, 1997).

Building on the GenTHREADER technology, Inpharmacis have developed a powerful algorithm termed Genome Threader, which compares all the sequences available in the proteome to all the crystal structures available in the protein data bank to determine compatibility. When compatible sequences are identified their predicted secondary structures are aligned to those of target proteins, and conserved residues are searched for. This technology has been used to successfully identify the yeast transcription factor Oaf1 as possessing a LBD fold (Phelps et al., 2006). Oaf1 is known to upregulate target genes in the absence of glucose and presence of oleate, and was shown to be critical for oleate signal transduction (Baumgartner et al., 1999). The structural predictions assigned the LBD of Oaf1 to the region of the protein between amino acids 254 and 563, with the highest compatibility observed with the structure of human ERβ, despite only sharing 12% sequence identity. Alignment of
the predicted secondary structure of Oaf1 to that of human nuclear receptors showed an overall conservation (Phelps et al., 2006). The motif present within helices 3 and 5 of the LBD as described by Wurtz et al (1996) is not conserved in Oaf1, and the hydrophobic motif pertaining to AF-2 is semi-conserved, however lacks the glutamic acid residue reported to be important for AF-2 mediated receptor activation (Danielian et al., 1992). Functional analysis showed that oleate was able to activate Oaf1 as observed in reporter gene assays, which was abrogated following mutation of residues thought to line the putative ligand binding pocket (Phelps et al., 2006). Furthermore, binding studies also showed that the region predicted to fold as a ligand binding domain within Oaf1 is capable of binding oleate with a $K_d$ of 17nM. The functional characterisation of Oaf1 as a protein with a region capable of binding oleate as a ligand, lends support to the structural predictions made by Inpharmatica, and begins to prove the principle that it is possible to detect similar structures between proteins that do not share sequence homology.

Inpharmatica have used the threading approach offered by Genome Thresher to identify novel proteins within the human proteome that possess putative folds within their structure, particularly those that possess kinase-like domains and nuclear receptor-like ligand binding domains. In the case of the NR LBDs, a number of novel proteins predicted to possess a similar fold were identified, which were named novel receptors. To further investigate the likelihood that the identified novel receptors possessed a similar structure to NR LDBs, the identified sequences were individually aligned to the LBD regions pertaining to bone fide NRs and conservation of key residues forming part of the conserved NR LBD motifs were examined. Of these annotated proteins, the candidate with the strongest prediction was NR3, which possessed a prediction that was at least comparable to the strength of prediction observed for Oaf1 (Phelps et al., 2006). NR3 is identical to the protein transient receptor potential cation channel, subfamily C, member 4 associated protein (TRPC4AP), which is also known as TNF receptor-associated ubiquitous scaffolding and signalling protein (TRUSS), which has been proposed to modulate TNFα signalling (Soond et al., 2003; Soond et al., 2006).
1.8 NR3 Bioinformatic Predictions

Genome Threader identified NR3, which comprises 797 amino acids, as possessing a NR LBD structure between amino acids 477-722. An armadillo repeat structure was also predicted to be present between amino acids 53-525, a motif which is known to be important in protein-protein interactions in many processes such as intracellular signalling, cell-cell interactions and nuclear import (Herold et al., 1998; Kaufmann et al., 2000; Song et al., 2003). A summary of the bioinformatic predictions for NR3 is presented in figure 1.6. The putative LBD structure within human NR3 is predicted to be compatible with the structures of 18 bona fide nuclear receptors from the PDB, yet the strongest match is with RARγ, which shares only 16% identity. Examining other species, the NR3 protein sequences from the mouse, rat, pufferfish, zebrafish and a simple chordate named Oikopleura dioica were also predicted by the Genome Threader algorithm to possess a NR LBD fold, supporting the bioinformatic predictions for the human protein. Alignment of these sequences shows the region is very well conserved, with absolute conservation between the human, mouse and rat (figure 1.7). Alignment of both the full length and putative LBD sequences of human NR3 to the other species on an individual basis allows the level of identity and similarity between species to be determined (table 1.1). This analysis shows the homology between mouse and rat sequences to the human sequence is almost 100% in terms of the full length protein. Homology between pufferfish and zebrafish sequences is also high when compared to the human sequence, ranging from 70-80% identity. Homology between Oikopleura and human sequences is less conserved in terms of amino acid identity and similarity, which is not unexpected due to the evolutionary distance between the species. This analysis therefore further highlights the level of conservation of the NR3 across different species, which may suggest NR3 plays an important biological role.

The predicted secondary structure of NR3 is mainly alpha helical overall, and predicted secondary structure alignment of the NR3 putative LBD and the LBD of bona fide nuclear receptors shows the proteins have a reasonable degree of similarity (figure 1.8). Furthermore, the (F/W)AKXXXXFXXLXXXDQXXLL motif thought to form part of the ligand binding pocket core is semi-conserved in NR3 (figure 1.8) (Wurtz et al., 1996). There is also semi-conservation of the hydrophobic residues present within the putative helix 12 of NR3, although it does not possess the glutamic
Figure 1.6 Summary of the Bioinformatic Predictions for NR3
The Armadillo Repeat-like structure is predicted to reside between amino acids 53 and 525. The NR LBD-like structure is predicted to span amino acids 477-722.
Figure 1.7 Alignment of the Putative NR3 LBD Protein Sequence from Different Species

Residues identical to those in human NR3 are shown in yellow and residues with similar properties are shown in green. Semi-conservation of key residues within NR LBD motifs are highlighted in boxes (Danielian et al., 1992; Wurtz et al., 1996). Zebrafish 1 and 2 refer to the 2 protein sequences available from the NCBI database.
Table 1.1 Homology Between the Human NR3 Sequence and Different Species
Comparison by sequence alignment of the percentage identity (ID) and similarity (Sim) of residues between the human full length and putative LBD sequences to those of different species.

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<tr>
<td>Oikapleura</td>
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</table>
Figure 1.8 Alignment of the Putative NR3 LBD with Bona Fide Nuclear Receptor LBDs

Predicted secondary structure and sequence alignments of ultraspiracle from *Drosophila*, human RARγ, mouse ERα and human NR3. Red denotes predicted α-helical structure. Blue denotes predicted β-strands. Non-coloured regions are predicted coil regions. Boxes represent the semi-conservation of the (F/W)AKXXXXFXXLXXXDQXX LL motif (Wurtz et al., 1996), and AF-2 (Danielian et al., 1992).
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acid residues present in all NRs (figure 1.8) (Danielian et al., 1992). In addition, sequence homology searches performed by Inpharmatica using PSI-BLAST to search for proteins related to NR LBDs aligns the Danio Rerio orthologue of NR3 with 4 different nematode NR LBDs at the third iteration, suggesting NR3 is related at the sequence level.

Examination of the NR3 protein sequence reveals there are no obvious DNA binding motifs present. Furthermore, NR3 has been initially characterised within the literature, and functional studies to date suggest NR3 is involved in TNFα signalling through the TNFR1 pathway (Soond et al., 2003; Soond et al., 2006), and potentially involved in cell cycle regulation (Nicassio et al., 2005), thus it is unlikely that NR3 acts in the same manner as bone fide nuclear receptors. The LBD of NRs may be considered to be a molecular switch that undergoes a conformational change upon ligand binding (Nagy and Schwabe, 2004), and as such it is conceivable that any ligand binding domain present in the NR3 structure may act as a molecular switch to modulate the signalling pathways with which it is involved.

1.9 Functional Characterisation of NR3

1.9.1 Role in TNF Signalling

In parallel with the bioinformatic predictions made regarding the structure of NR3, the protein was initially described as TRUSS in the literature when it was identified as a binding partner for the membrane proximal region of TNFR1 in a transcription-based yeast two-hybrid screen (Soond et al., 2003). Further characterisation of the putative protein-protein interaction between TRUSS and TNFR1 by GST pulldown assays showed TRUSS could directly interact with TNFR1, an interaction that was mapped to amino acids 207-300 of TNFR1 by using TNFR1 deletion mutants fused to GST (Soond et al., 2003). Co-immunoprecipitation of full length HA-tagged TRUSS and a series of deletion mutants co-overexpressed with FLAG-tagged TNFR1 mapped the region within TRUSS responsible for the interaction to amino acids 1-440 (Soond et al., 2003). The initial characterisation of NR3 examined the proteins within the TNFR1 signalling pathway that interact both directly and indirectly with TRUSS. Upon activation, TNFR1 recruits a signalling complex formed by TRADD, TRAF2, RIP, and the IKK complex consisting of IKKα,
IKKβ and IKKγ. It was shown that TRUSS coprecipitates with all members of this signalling complex in immunoprecipitation assays where TRUSS and the protein of interest were overexpressed (Soond et al., 2003; Soond et al., 2006). In addition, it was shown that TRUSS can interact directly with TRADD, TRAF2, IKKα, IKKβ and IKKγ, but not RIP, in GST and HIS in vitro pulldown assays (Soond et al., 2003). To examine the function of the protein, Soond et al (2003) overexpressed TRUSS in NIH/3T3 cells in NF-κB reporter gene assays. TRUSS expression increased NF-κB activation in a biphasic dose dependent manner, which was potentiated by treatment with TNFα (Soond et al., 2003). Additionally, truncating TRUSS to amino acid 723 abolished the response, suggesting the putative NF-κB activation domain resides between amino acids 723-797. In a later paper the same authors showed that TRUSS overexpression resulted in c-Jun NH2-terminal kinase and AP-1 activation, which was thought to be due to an interaction with TRAF2 (Soond et al., 2006).

1.9.2 Role in Cell Cycle and Implication in Alzheimer’s Disease

In addition to the reported role of NR3 in TNF signalling (Soond et al., 2003; Soond et al., 2006), NR3 has been proposed to be involved in regulation of the cell cycle (Nicassio et al., 2005). Deletion of the cell cycle inhibitor retinoblastoma protein or overexpression of the E2F family of transcription factors, which are positive regulators of the cell cycle, is insufficient to induce re-entry into the cell cycle in terminally differentiated cells (Camarda et al., 2004; Pajalunga et al., 1999). However, expression of E1A can induce terminally differentiated cells to re-enter the cell cycle, thus Nicassio et al (2005) performed a biased screen to identify genes in terminally differentiated cells that upon E1A transformation are upregulated, but are not so following E2F1 overexpression or retinoblastoma protein deletion. The authors identified 5 such genes (Nicassio et al., 2005). These were SF3B1, Ch-TOG, SKIN, SMU-1 and TRPC4AP (NR3). The expression of all five genes was unaltered during the cell cycle progression of proliferating cells, and as such it was concluded that their over expression following re-entry into the cell cycle was not a downstream effect of cell cycle progression (Nicassio et al., 2005). The expression of the identified genes were also semi-quantitatively analysed in cancerous samples as compared to normal surrounding tissue from the same donor using tissue microarray techniques (Nicassio
et al., 2005). Although the results are only semi-quantitative the authors report that NR3 was found to be significantly overexpressed in 41-60% of colon cancers, and 21-40% of lung and stomach cancers tested in the microarray. Nicassio et al (2005) focused on the identified gene SKIN to determine that knockdown with siRNA resulted in a negative effect of cell proliferation, however similar studies were not reported for TRPC4AP (Nicassio et al., 2005). The work performed by Nicassio et al (2005) does implicate NR3 in the regulation of the cell cycle, and as such the expression of NR3 may have an effect on the proliferation of cells.

NR3 has also been identified in a genome-wide analysis of the single nucleotide polymorphisms (SNPs) associated with late-onset Alzheimer’s disease by microarray analysis (Poduslo et al., 2008). The screen was performed on two families in which there are multiple members with the disease, representing rare large groups of related sufferers. Of the SNPs identified in the microarray analysis, four were found in the NR3 gene, however all were found in intronic regions (Poduslo et al., 2008). It is possible that the regulatory elements may be present within introns (Fedorova and Fedorov, 2003), and that their disruption may result in deleterious effects. However as the SNPs identified by Podulso et al (2008) do not code for amino acids mutations it is unclear as to the significance of the findings with regards a physiological role for NR3 in the development of late-onset Alzheimer’s disease.

1.10 Aims and Hypothesis

The aim of this project is to characterise the biological role of NR3, and examine the role of the LBD region within that function. It is hypothesised that the LBD region possesses a structure similar to that of a nuclear receptor LBD, and may bind a ligand, allowing the region to act as a molecular switch to modulate the activity of NR3 within its biological function.
Chapter 2

Materials and Methods
2.1 Materials

All chemicals and solvents used were of analytical grade and obtained, as indicated, from Abcam, Cambridge, UK; Ambion, Abingdon, UK; Amersham Biosciences/GE Healthcare, Amersham, UK; Applied Biosystems, Cheshire, UK; BD, Oxford, UK; BDH/VWR Chemicals, Lutterworth, UK; Bioline, London, UK; Biomol, Exeter, UK; Bio-Rad, Hemel Hempstead, UK; Calbiochem/Novagen, Nottingham, UK; Cambrex, Iowa, USA; Chemicon, Harrow, UK; Corning, New York, USA; DAKO Cytomation, Glostrup, Denmark; Dharmacon, Louisiana, UK; Finnzymes, Espoo, Finland; Fisher Scientific, Loughborough, UK; Genome Research Ltd, Cambridge, UK; Invitrogen/Gibco BRL/Zymed, Paisley, UK; LUX Biotechnology, Edinburgh, UK; MRC Geneservice, Cambridge, UK; National Diagnostics, Georgia, USA; New England Biolabs, Hitchin, UK; Perkin Elmer, Massachusetts, USA; Pierce, Illinois, USA; Premier Foods, St. Albans, UK; Promega, Southampton, UK; Q-Biogene, Cambridge, UK; Qiagen, Crawley, UK; Roche, Lewes, UK; Santa Cruz Biotechnology, Heidelberg, Germany; Sigma-Aldrich Company Ltd, Poole, UK; Stratagene, Texas, USA; Thermo, Fife, UK; Web Scientific, Crewe, UK; Zymo Research, California, USA.

2.1.1 Chemicals, Reagents and Solvents

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<td>Materials and Methods</td>
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<td>Nonylphenyl Polyethylene Glycol (NP40) Alternative</td>
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<td>Oligo (dT)₁₂-₁₈ Primer</td>
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<td>Protease Inhibitor Cocktail</td>
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<td>Stratagene</td>
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### Chapter 2  
#### Materials and Methods

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<td>Tween-20</td>
<td>Sigma</td>
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<td>West Pico Detection System</td>
<td>Pierce</td>
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#### 2.1.2 Radiochemicals

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<td>L-[^35]S Methionine</td>
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<td>[α-[^32]P] dATP</td>
<td>&gt;1000Ci/mmol, Amersham Biosciences</td>
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#### 2.1.3 Enzymes

<table>
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<tr>
<td>Accuprime Taq Polymerase</td>
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<tr>
<td>Bacterial Alkaline Phosphotase</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DNase I (RNase-free)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>EcoICRI Restriction Endonuclease</td>
<td>Promega</td>
</tr>
<tr>
<td>Endoproteinase Glu-C from Staphylococcus aureus V8</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>KOD DNA Polymerase</td>
<td>Novagen</td>
</tr>
<tr>
<td>MMLV Reverse Transcriptase</td>
<td>Sigma</td>
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<td>Pfu Turbo DNA Polymerase</td>
<td>Stratagene</td>
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<tr>
<td>Phusion DNA Polymerase</td>
<td>Finnzymes</td>
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<tr>
<td>Restriction Endonucleases (except EcoICRI)</td>
<td>New England Biolabs</td>
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<tr>
<td>RNASin Ribonuclease Inhibitor</td>
<td>Promega</td>
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<td>T4 DNA Ligase</td>
<td>Invitrogen</td>
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<td>T4 PNK</td>
<td>New England Biolabs</td>
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<td>Taq Polymerase</td>
<td>Invitrogen</td>
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</table>
Chapter 2  Materials and Methods

2.1.4 Miscellaneous

3mm non-acid washed glass beads    Sigma-Aldrich
1mL Disposable Pipette     BD Biosciences
Cell Lifter               Corning
Dynabeads Oligo (dT)$_{25}$      Dynal
ECL Hyperfilm             Amersham Biosciences
Electroporation Cuvette (1mm gap) Web Scientific
Glass Plates for Setting Polyacrylamide Gels Web Scientific
Hybond PVD-F Membrane         GE Healthcare
Optical 96-well Plates for QRT-PCR Abgene

2.1.5 Kits

Cytrotrap XR Library Construction Kit   Stratagene
GeneClean Spin Kit         Q-Biogene
HiSpeed Plasmid Maxi Kit    Qiagen
Large Construct Kit         Qiagen
LucLite Reporter Assay Kit  Perkin Elmer
pcDNA3.1 Directional TOPO Expression Kit Invitrogen
QIAprep Spin Miniprep Kit   Qiagen
QIAquick Gel Extraction Kit Qiagen
Power SYBR Green Master Mix Applied Biosystems
TNT Coupled Reticulocyte Lysate System Promega
TNT Quick Coupled Transcription/Translation System Promega
Zymoprep II Yeast Plasmid Minipreparation Kit Zymo Research

2.1.6 Plasmids

Working stocks of plasmid DNA were stored at 4°C at a concentration of 0.25-1mg/mL. Plasmid DNA was stored long term at -20°C.

G112     Genoway
G139     Genoway
pBACe3.6-NR3 Genome Research Limited, bMQ-418A20
### Chapter 2  Materials and Methods

- pCMV-SPORT6-14-3-3\(\eta\)  
  MRC Geneservice, IMAGE 4949445

- pCMV-SPORT6-DDB1  
  MRC Geneservice, IMAGE 3487617

- pCMV-SPORT6-NR3  
  MRC Geneservice, IMAGE 3673725

- pCR2.1  
  Invitrogen

- pCR4  
  Invitrogen

- pcDNA3.1-V5-6H  
  Invitrogen

- pcDNA3.1-DDB1_{1-353}-V5-6H
- pcDNA3.1-DDB1_{1-704}-V5-6H
- pcDNA3.1-DDB1_{1-1043}-V5-6H
- pcDNA3.1-DDB1_{1-1140}-V5-6H
- pcDNA3.1-DDB1_{361-1140}-V5-6H
- pcDNA3.1-DDB1_{396-704}-V5-6H
- pcDNA3.1-DDB1_{\Delta BPB}-V5-6H
- pcDNA3.1-NR3-V5-6H
- pcDNA3.1-NR3_{477-797}-V5-6H
- pcDNA3.1-NR3_{1-722}-V5-6H

- pGEX-6P2  
  Amersham

- pGEX-6P2-NR3
- pGEX-6P2-NR3_{1-722}
- pGEX-6P2-NR3_{1-120}
- pGEX-6P2-NR3_{1-239}
- pGEX-6P2-NR3_{61-120}
- pGEX-6P2-NR3_{61-239}
- pGEX-6P2-NR3_{120-722}
- pGEX-6P2-NR3_{239-722}
- pGEX-6P2-NR3_{358-722}
- pGEX-6P2-NR3_{419-722}
- pGEX-6P2-NR3_{477-722}

- pGL3-NF\(\kappa\)B
- pGL3-GAL4

- pMyr XR  
  Stratagene

- pSG5  
  Stratagene
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pSG5-GAL4-ER
pSG5-VP16
pSG5-VP16-GAL4-CTRL
pSG5-VP16-GAL4-ER
pSG5-VP16-GAL4-NR3

pSOS
pSOS-NR3
pSOS-NR3 LBD
pRL-EF1α
pRL-TK
RWH1-GA1-linker
RWH1-HR1
RWH1-LA-LoxP
RWH1-LA-bac
RWH1-LSA-Neo
RWH1-Neo/SA
RWH1-SA-C+
RWH1-SAmod

2.1.7 siRNA

siRNA was stored at -80°C at a concentration of 20µM or 50µM depending on the manufacturer’s guidelines.

Custom synthesised control siRNA  Dhharmacon
Smartpool siRNA to TRPC4AP  Dhharmacon
Silencer Negative Control #2  Ambion
siTOX  Dhharmacon
2.1.8 Antibodies

Primary Antibodies

14-3-3 (SA-483)  Biomol
CDT1 (ab22716)  Abcam
DDB1 (ab13562)  Abcam
GAL4 (sc-510) (used for western blotting)  Santa Cruz
GAL4 (sc-577) (used for immunostaining)  Santa Cruz
GAPDH (MAB374)  Chemicon International
TNFR1 (ab19139)  Abcam
Ubiquitin (P4D1)  Santa Cruz
V5 (46-0705)  Invitrogen

Secondary Antibodies

Anti-Mouse FITC  DAKO Cytomation
Anti-Mouse HRP  DAKO Cytomation
Anti-Rabbit FITC  DAKO Cytomation
Anti-Rabbit HRP  DAKO Cytomation

2.1.9 Bacterial Strains

BL21 [F, ompT, hsdSB (rB-rB), dcm, gal, (DE3), pLysS, Cmr] are chemically competent E. coli for use with T7 RNA polymerase-based protein expression systems.

DH5α [F mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15] are chemically competent or electrocompetent Escherichia coli (E. coli) used when cloning DNA fragments into vectors and for amplification of plasmids. They replicate DNA with high efficiency, making them suitable for preparing DNA in small and large quantities.

TOP10 (Invitrogen) [F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG] are chemically competent E. coli used for transformation with the resulting plasmid from a TOPO reaction.
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XL10 Gold (Stratagene) Tetr Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F’ proAB lacIqZΔM15 Tn10 (Tetr) Tn5 (Kanr) Amy] are chemically competent cells used for transformation of plasmids when constructing and amplifying a cDNA library to be used with the CytoTrap yeast two-hybrid system (Stratagene).

2.1.10 Yeast Strains

cdc25H (Stratagene) Mata ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal+ are chemically competent yeast cells used in the CytoTrap (Stratagene) method for yeast two-hybrid screens. Cell division in this strain is temperature dependent, with 37°C being non-permissive for growth in the absence of Ras activation.

2.1.11 Cell Lines

COS-1  African green monkey kidney cell line immortalised with SV40 (Gluzman, 1981)
HEK-293  Human embryonic kidney cell line immortalised with adenovirus type 5 DNA (Graham et al., 1977)
NIH/3T3  Mouse fibrosblast cell line (Todaro and Green, 1963)

2.1.12 Bacterial Media

Bactotryptone, yeast extract and Bactoagar were supplied by BD.

LB-Agar

1% (w/v) Bactotryptone
0.5% (w/v) Yeast Extract
0.5% (w/v) NaCl
0.1% (w/v) Glucose
1.5% (w/v) Bactoagar
Chapter 2  Materials and Methods

2x LB-Agarose  2% (w/v) Bactotryptone  1% (w/v) Yeast Extract  1% (w/v) NaCl  0.2% (w/v) Glucose  0.3% (w/v) SeaPrep Ultralow Temperature Agarose

LB-Broth  1% (w/v) Bactotryptone  0.5% (w/v) Yeast Extract  0.5% (w/v) NaCl  0.1% (w/v) Glucose

SOC Media (Invitrogen)  2% (w/v) Bactotryptone  0.5% (w/v) Yeast Extract  10mM NaCl  2.5mM KCl  10mM MgCl₂  10mM MgSO₄  20mM Glucose

2.1.13 Yeast Media

Yeast extract, Bactopeptone and Bactoagar supplied by BD. All amino acids and yeast extract without amino acids were supplied by Sigma-Aldrich.

YPAD Broth  1% (w/v) Yeast Extract  2% (w/v) Bactopeptone  2% (w/v) Dextrose  40mg/L Adenine Hemisulfate

YPAD Agar  1% (w/v) yeast extract  2% (w/v) Bactopeptone  2% (w/v) Dextrose  40mg/L Adenine Hemisulfate  2% (w/v) Bactoagar
### Materials and Methods

**Chapter 2**

| 10x Dropout Solution | 300mg/L L-Isoleucine  
| 1500mg/L L-Valine  
| 200mg/L L-Adenine Hemisulfate Salt  
| 500mg/L L-Arginine HCl  
| 200mg/L L-Histidine HCl Monohydrate  
| 500mg/L L-Lysine HCl  
| 200mg/L L-Methionine  
| 500mg/L L-Phenylalanine  
| 2000mg/L L-Threonine  
| 500mg/L L-Tryptophan  
| 500mg/L L-Tyrosine  
| 1000mg/L L-Bradipene  
| 1000mg/L L-Aspartate  
| 400mg/L L-Serine |

**SD Glucose Medium (-UL)**

| 0.17% (w/v) Yeast Nitrogen Base without amino acids  
| 0.5% (w/v) Ammonium Sulfate  
| 2% (w/v) Dextrose  
| 1x Dropout Solution |

**SD Glucose Plates (-UL)**

| 0.17% (w/v) Yeast Nitrogen Base without amino acids  
| 0.5% (w/v) Ammonium Sulfate  
| 2% (w/v) Dextrose  
| 1.9% (w/v) Bactoagar  
| 1x Dropout Solution |

**SD Galactose Medium (-UL)**

| 0.17% (w/v) Yeast Nitrogen Base without amino acids  
| 0.5% (w/v) Ammonium Sulfate  
| 2% (w/v) Galactose  
| 1% (w/v) Raffinose  
| 1x Dropout Solution |
**2.1.14 Cell Culture Materials**

Dextran charcoal stripped foetal bovine serum (FBS) was routinely prepared by colleagues in the laboratory. Where appropriate some cell culture plates were coated with 0.01% (w/v) gelatine for 20 minutes.

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<td>Gibco</td>
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<td>G418</td>
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<td>L-Glutamine</td>
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<td>Optimem</td>
<td>Gibco</td>
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<td>Penicillin (100U/mL)</td>
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<td>Streptomycin (50µg/mL)</td>
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<td>Tissue Culture Grade Plasticware</td>
<td>Corning</td>
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<td>Trypsin</td>
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**2.1.15 Buffers, Solutions and Gels**

**DNA Techniques**

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<th>Buffer</th>
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<td>Annealing Buffer</td>
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<tr>
<td></td>
<td>20mM Tris pH7.5</td>
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<tr>
<td></td>
<td>2mM EDTA pH8.0</td>
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</table>
10x DNA Loading buffer  
0.2% (w/v) Bromophenol Blue  
40% (v/v) Glycerol  
100mM EDTA pH8.0  

10x TBE  
900mM Tris Borate  
20mM EDTA pH8.0  

TE Buffer  
10mM Tris HCl pH8.0  
1mM EDTA pH8.0  

**Protein Analysis**

*Immunostaining Buffers*

Blocking Buffer  
140mM NaCl  
2.5mM KCl  
1.5mM KH$_2$PO$_4$ pH7.2  
10mM Na$_2$HPO$_4$ pH7.2  
1% (w/v) BSA  

Permeabilising Buffer  
140mM NaCl  
2.5mM KCl  
1.5mM KH$_2$PO$_4$ pH7.2  
10mM Na$_2$HPO$_4$ pH7.2  
0.1% (v/v) Triton X-100  

*Lysis Buffers*

HEPES Lysis Buffer  
150mM NaCl  
50mM HEPES pH7.6  
5mM EDTA pH8.0  
1% (v/v) NP40 Alternative  
Protease Inhibitor Cocktail (50µL/10mL)  
2mM PMSF
### Chapter 2  Materials and Methods

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
<th>Notes</th>
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<td>20mM Tris HCl pH8.0</td>
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<td>1mM EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5% (v/v) NP40 Alternative</td>
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<td></td>
<td></td>
<td>Complete Protease Inhibitors (1 tablet/50mL)</td>
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<td></td>
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<td>1mM DTT</td>
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<td></td>
<td></td>
<td>2mM PMSF</td>
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<td>Nuclear Extract Buffer A</td>
<td>10mM HEPES</td>
<td>10mM KCl</td>
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<td>0.1mM EDTA</td>
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<td></td>
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<td>0.1mM EGTA</td>
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<td></td>
<td>2mM DTT</td>
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<td></td>
<td>Protease Inhibitor Cocktail (50µL/10mL)</td>
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<td>10mM KCl</td>
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<td>0.1mM EDTA</td>
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<td></td>
<td></td>
<td>0.1mM EGTA</td>
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<tr>
<td></td>
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<td></td>
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<td>400mM NaCl</td>
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<td>1% (v/v) NP-40</td>
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<td>Protease Inhibitor Cocktail (50µL/10mL)</td>
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<td>SDS-PAGE</td>
<td>4% (w/v) SDS</td>
<td>20% (v/v) Glycerol</td>
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<td>2x Laemmli Sample Buffer</td>
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<td>10% (v/v) β-mercaptoethanol</td>
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<tr>
<td></td>
<td></td>
<td>0.004% (w/v) Bromophenol Blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125mM Tris HCl pH6.8</td>
</tr>
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</table>
### Polyacrylamide Gels (resolving)
- 375mM Tris HCl pH8.8
- 10-12% (w/v) Acrylamide/Bis
- 0.1% (w/v) SDS
- 0.1% (w/v) Ammonium Persulfate
- 0.04% (v/v) TEMED

### Polyacrylamide Gels (stacking)
- 125mM Tris HCl pH6.8
- 5% (w/v) Acrylamide/Bis
- 1% (w/v) SDS
- 1% (w/v) Ammonium Persulfate
- 0.1% (v/v) TEMED

### SDS-PAGE Fixing Buffer
- 30% (v/v) Methanol
- 10% (v/v) Acetic Acid

### SDS Running buffer
- 25mM Tris base
- 190mM Glycine
- 0.1% (w/v) SDS

### Silver Fixing Buffer
- 50% (v/v) Ethanol
- 10% (v/v) Acetic Acid

### Western Blotting Buffers
#### Milk Solution
- 5% (w/v) skimmed milk powder
- 130mM NaCl
- 20mM Tris pH7.6
- 0.1% (v/v) Tween-20

#### TBS-T
- 130mM NaCl
- 20mM Tris pH7.6
- 0.1% (v/v) Tween-20
Chapter 2  Materials and Methods

Transfer Buffer
- 25mM Tris Base
- 190mM Glycine
- 20% (v/v) Methanol

Yeast Two-Hybrid Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
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<tbody>
<tr>
<td>LiSORB</td>
<td>100mM LiOAc&lt;br&gt;10mM Tris-HCl p8.0&lt;br&gt;1mM EDTA pH8.0&lt;br&gt;1M Sorbitol</td>
</tr>
<tr>
<td>PEG/LiOAc</td>
<td>10mM Tris HCl pH8.0&lt;br&gt;1mM EDTA pH8.0&lt;br&gt;100mM LiOAc pH7.5&lt;br&gt;40% (w/v) Polyethylene Glycol 3350</td>
</tr>
<tr>
<td>STE Buffer</td>
<td>100mM NaCl&lt;br&gt;20mM Tris HCl pH7.5&lt;br&gt;10mM EDTA</td>
</tr>
</tbody>
</table>

Miscellaneous Buffers and Solutions

<table>
<thead>
<tr>
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<th>Components</th>
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<tbody>
<tr>
<td>PBS</td>
<td>140mM NaCl&lt;br&gt;2.5mM KCl&lt;br&gt;1.5mM KH₂PO₄ pH7.2&lt;br&gt;10mM Na₂HPO₄ pH7.2</td>
</tr>
<tr>
<td>Polyacrylamide Gel Fixing Solution</td>
<td>30% (v/v) Methanol&lt;br&gt;10% (v/v) Acetic Acid</td>
</tr>
<tr>
<td>Renilla Buffer</td>
<td>500mM HEPES pH7.8&lt;br&gt;40mM EDTA&lt;br&gt;10µg/mL Coelenterazine</td>
</tr>
</tbody>
</table>
Defined Qiagen Supplied Buffers and Solutions

Qiagen Buffer EB  
10mM Tris HCl pH8.0

Qiagen Buffer P1  
50mM Tris HCl pH8.0
10mM EDTA
100µg/ml RNase A

Qiagen Buffer P2  
200mM NaOH
1% (v/v) SDS

Qiagen Buffer P3  
3M KAc pH5.5

Qiagen Buffer QBT  
750mM NaCl
50mM MOPS pH7.0
15% (v/v) Isopropanol
0.15% (v/v) Triton X-100

Qiagen Buffer QC  
1M NaCl
50mM MOPS pH7.0
15% (v/v) Isopropanol

Qiagen Buffer QF  
1.25M NaCl
50 mM Tris HCl pH8.5
15% (v/v) Isopropanol

Qiagen TE Buffer  
10mM Tris HCl pH8.0
1mM EDTA

Dharmacon Supplied Buffers

5x siRNA Buffer  
300mM KCl
30mM HEPES
1mM MgCl₂
40mM KOH
Dynal Supplied Buffers

Binding Buffer  
20mM Tris-HCl pH7.5  
1M LiCl  
2mM EDTA

Wash Buffer B  
10mM Tris-HCl pH7.5  
150mM LiCl  
1mM EDTA

Invitrogen Supplied Buffers

MOPS Running Buffer  
50mM MOPS  
50mM Tris Base  
0.1% (w/v) SDS  
1mM EDTA

5x T4 Ligase Buffer  
250mM Tris HCl pH7.6  
50mM MgCl₂  
5mM ATP  
5mM DTT  
25% (w/v) Polyethylene Glycol-8000

10x DNase I Reaction Buffer  
200mM Tris-HCl pH8.4  
20mM MgCl₂  
500mM KCl

New England Biolabs Supplied Buffers

NEBuffer 1  
10mM Tris Propane HCl pH7.0  
10mM MgCl₂  
1mM DTT
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NEBuffer 2  
10mM Tris HCl pH7.9  
10mM MgCl$_2$  
50mM NaCl  
1mM DTT

NEBuffer 3  
50mM Tris HCl pH7.9  
10mM MgCl$_2$  
100mM NaCl  
1mM DTT

NEBuffer 4  
20mM Tris Acetate pH7.9  
10mM MgCl$_2$  
50mM KAc  
1mM DTT

Perkin Elmer Supplied Buffers

2x LucLite Reagent  
10mL LucLite buffer added to 1 bottle of lyophilised LucLite solution  
1mM CaCl$_2$  
1mM MgCl$_2$

Sigma Supplied Buffers

10x Reverse Transcriptase Buffer  
500mM Tris-HCl pH8.3  
500mM KCl  
30mM MgCl$_2$  
50mM DTT

2.1.16 In Silico Materials

Software Packages

Vector NTI Software Suite  Invitrogen
Office Software Suite  Microsoft
**Chapter 2  Materials and Methods**

**Websites and Databases**

http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/
http://genome.ucsc.edu/
http://www.ensembl.org/
http://www.strubi.ox.ac.uk/RONN

**2.2 Methods**

**2.2.1 Bacterial Techniques**

All bacterial techniques were performed aseptically.

**Storage of Bacteria**

Amplification of DNA plasmids was performed in Escherichia coli strain DH5α. All the plasmids described possessed resistance genes to ampicillin, kanamycin, or chloramphenicol. Transformed bacteria were grown in LB-broth or LB-agar containing either 100µg/mL ampicillin, 50µg/mL kanamycin or 30µg/mL chloramphenicol. Bacterial stocks were stored in LB-broth containing 40% (v/v) glycerol at -80°C.

**Preparation of Competent Bacteria for Transformation by Heatshock**

DH5α were streaked onto an LB-agar plate without antibiotic and incubated at 37°C overnight. A single colony was used to inoculate 100mL LB-broth in a 1L flask and cultured at 37°C until the bacteria were in log-phase growth, as judged when the OD$_{600}$ measured 0.5. The culture was then transferred to two pre-chilled centrifuge tubes and incubated on ice for 10 minutes. The cells were then recovered by centrifugation at 4500xg and 4°C for 10 minutes, and the media discarded. The pellets were resuspended in 10mL pre-chilled 0.1M CaCl$_2$, the bacteria recovered by centrifugation at 4500xg and 4°C for 10 minutes, and the supernatants discarded.
Each pellet was then resuspended in 2mL pre-chilled 0.1M CaCl$_2$ containing 10% (v/v) glycerol and aliquoted for storage at -80°C.

**Transformation of Bacteria by Heatshock**

Chemically competent DH5α cells were thawed on ice and 50µL transferred to a pre-chilled microfuge tube. Between 250-500ng plasmid DNA or 1µL of a TOPO cloning reaction was added to the competent cells and mixed. The bacteria/DNA mixture was incubated on ice for 30 minutes before heatshock at 42°C for 30 seconds. Cells were placed back on ice for 2 minutes and 150µL SOC media added to each transformation. Transformation reaction were then incubated at 37°C and 220rpm for 1 hour, plated on selective plates and incubated at 37°C overnight.

**Preparation of Competent Bacteria for Transformation by Electroporation**

DH5α were streaked onto an LB-agar plate without antibiotic and incubated at 37°C overnight. A single colony was used to inoculate 10mL LB-broth and cultured overnight. The overnight culture was used to inoculate 1L LB-broth, which was split into four 250mL cultures and incubated at 37°C until the bacteria were in log-phase growth, as judged when the OD$_{600}$ measured 0.5-0.8. The 250mL cultures were then transferred to pre-chilled centrifuge tubes and centrifuged at 4500xg and 4°C for 10 minutes. The media was discarded and the pellets washed by resuspending in 250mL of pre-chilled sterile H$_2$O and the bacteria recovered by centrifugation at 4500xg and 4°C for 10 minutes. Each pellet was then washed again with 125mL H$_2$O, and then resuspended in 5mL H$_2$O containing 10% (v/v) glycerol and pooled together. The bacteria were centrifuged at 4500xg and 4°C for 10 minutes, and the pellets resuspended in 2mL pre-chilled H$_2$O containing 10% (v/v) glycerol and aliquoted for storage at -80°C.

**Transformation of Bacteria by Electroporation**

Electrocompetent DH5α cells were thawed on ice and 50µL transferred to a pre-chilled electroporation cuvette. 0.5µL of a ligation reaction was added to the competent cells and mixed. The bacteria/DNA mixture was electoporated using a Bio-
Rad gene pulser, at 1.67kV, 25µF and 200Ω, providing a time constant of between 4-5 milliseconds. Cells were placed back on ice for 2 minutes and 150µL SOC media added to each transformation. Transformation reaction were then incubated at 37°C for 1 hour, plated on selective plates and incubated at 37°C overnight.

2.2.2 Nucleic Acid Manipulation

Restriction Digest

Restriction digests were performed in accordance with New England Biolabs guidelines.

Analytical Restriction Digests

200-500ng of miniprep DNA was digested with 0.2µL of appropriate restriction endonucleases in a 10µL reaction containing the recommended supplied buffer and 10ng/µL BSA if required, and incubated at the appropriate temperature, typically 25°C, 37°C or 65°C, for 1 hour. Restriction digest products were analysed by agarose gel electrophoresis.

Restriction Digests for Cloning

To generate vector backbone DNA, 1µg of plasmid DNA was digested with 1µL of the appropriate restriction endonucleases in a 50µL reaction containing the recommended buffer and 10ng/µL BSA if required, and incubated at the appropriate temperature for 3 hours. 1µL bacterial alkaline phosphotase was added to each digest and the reaction incubated at 65°C for 30 minutes. The restriction products were then analysed by agarose gel electrophoresis, the vector backbone DNA excised from the gel and purified by Geneclean Spin Kit or QIAquick Gel Extraction Kit.

Insert DNA was derived from digesting 1µg of the appropriate plasmid DNA or the purified product from a PCR reaction. 1µL of the appropriate restriction enzymes were used in a 50µL reaction and incubated for 3 hours at the appropriate temperature. Restriction products were then analysed by agarose gel electrophoresis, the insert DNA excised and purified using Geneclean Spin Kit or QIAquick Gel Extraction Kit.
Oligonucleotide Phosphorylation and Annealing

Lists of oligonucleotides used for cloning applications can be found in appendix A. Oligonucleotides were phosphorylated by combining T4 PNK and buffer with 1.4µL of the appropriate forward and reverse 100µM oligonucleotides separate 25µL reactions, and incubated for 30 minutes at 37°C. The phosphorylated oligonucleotides were then combined and 50µL annealing buffer added. The oligonucleotides were then incubated at 100°C for 15 minutes and allowed to cool slowly to room temperature before being placed on ice.

Polymerase Chain Reaction

Generating DNA Fragments for Cloning

List of oligonucleotides used for PCR can be found in appendix A. Polymerase chain reactions (PCRs) were performed with Accumprime Taq polymerase, KOD polymerase, Pfu Turbo polymerase or Phusion polymerase. Reactions were typically 50µL and were performed in accordance with polymerase manufacturer’s guidelines and with the supplied buffers. Briefly, the reactions contained 200-400µM dNTPs, 0.2-1µM forward and reverse oligonucleotide primers, 1.5-2mM MgCl₂, either 2% (v/v) DMSO or 1M betaine, and 50-100ng of DNA template for Pfu Turbo PCRs and Accuprime Taq PCRs, or 5-10ng of DNA template for KOD PCRs and Phusion PCRs. The amount of enzyme added to each reaction was as directed by the manufacturers. Cycling conditions were determined empirically for each reaction, and were within the polymerase manufacturer’s guidelines, however typical cycling conditions for each polymerase are shown in table 2.1. PCR products were analysed by agarose gel electrophoresis, excised from the gel and purified using Geneclean Spin Kit or QIAquick Gel Extraction Kit. Purified products could then be used for TOPO cloning or for restriction digest and ligation into a vector.

Screening Bacterial Clones by PCR

PCR reactions to screen bacterial clones were performed with Taq polymerase in accordance with manufacturer’s guidelines. Briefly, reactions were 50µL and
Table 2.1 Summary of PCR Cycling Conditions for Different Polymerases

The different polymerases used for PCR reactions required different cycling conditions. A summary is shown above, although some reactions required conditions to be determined empirically. Tm refers to the lowest melting temperature of the primers in the reactions. Product extension times are given per kilobase of amplicon.

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Accuprime Taq</th>
<th>KOD</th>
<th>Pfu Turbo</th>
<th>Phusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Template Denaturation</td>
<td>94 2 mins</td>
<td>95 2 mins</td>
<td>95 4 mins</td>
<td>98 30 secs</td>
</tr>
<tr>
<td>Template Denaturation</td>
<td>94 30 secs</td>
<td>95 20 secs</td>
<td>95 45 secs</td>
<td>98 10 secs</td>
</tr>
<tr>
<td>Primer Annealing</td>
<td>Tm-5 30 secs</td>
<td>Tm 10 secs</td>
<td>Tm-5 45 secs</td>
<td>Tm+3 30 secs</td>
</tr>
<tr>
<td>Product Extension</td>
<td>68 1 min/kb</td>
<td>70 10 secs/kb</td>
<td>68 1 min/kb</td>
<td>72 15 secs/kb</td>
</tr>
<tr>
<td>Final Product Extension</td>
<td>68 10 mins</td>
<td>70 10 mins</td>
<td>68 10 mins</td>
<td>72 10 mins</td>
</tr>
</tbody>
</table>
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contained 200µM dNTPs, 1.5mM MgCl₂, 1µM forward and reverse primers, and 0.2µL Taq polymerase. Bacterial colonies were harvested by transferring from a selective agar plate and adding directly to the PCR mix using a 200µL pipette tip, which were saved and used to inoculate selective LB-broth to grow positive colonies up for miniprep. Reactions were subjected to an initial denaturing step of 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, primer Tm-5°C for 30 seconds, and 72°C for 1 minute per kilobase of amplicon. A final product extension step of 72°C for 10 minutes was included. Reactions were analysed by agarose gel electrophoresis to determine positive clones.

Agarose Gel Electrophoresis

0.8-1% (w/v) agarose gels were cast by dissolving agarose into TBE buffer by heating in a microwave, allowing the solution to cool, adding 1µL 0.2µg/mL ethidium bromide per 50mL gel, and pouring into an appropriate size mould. Gels were then submerged in TBE buffer and DNA samples loaded into wells following the addition of 10x DNA loading buffer to the samples. Electrophoresis was performed at 110V until the DNA fragments were resolved. Ethidium bromide stained DNA fragments were then visualised in a transilluminator (UVP). The size of fragments was estimated by comparing to HyperLadder DNA markers. DNA fragments required for cloning were then excised from the gel using a scalpel and purified using Geneclean Spin Kit or QIAquick Gel Extraction Kit.

DNA Purification by Geneclean Spin Kit

DNA fragments were purified in accordance with manufacturer’s instructions. 400µL of suspended glassmilk was added to each gel slice in a microfuge tube. The gel slice was heated to 55°C with occasional agitation to for 5 minutes or until the gel had melted, and the sample transferred to a spin filter. Samples were centrifuged 17530xg for 1 minute to pass the solution though the spin filter into the catch tube to be discarded. The glassmilk retained on the spin filter was then resuspended with 500µL wash buffer and centrifuged as before to pass the wash buffer into the catch tube to be discarded. The glassmilk was dried by centrifuging the filter and catch tube at 17530xg for 2 minutes to allow any traces of wash buffer to pass into the catch tube.
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The filter was then transferred to a fresh microfuge tube and the purified DNA eluted by resuspending the glassmilk in 15mL elution solution and centrifuging at 17530xg for 2 minutes. 1µL of purified DNA was then analysed by agarose gel electrophoresis to determine the size and concentration of the DNA fragment.

**DNA Purification by QIAquick Gel Extraction Kit**

DNA fragments were purified in accordance with the manufacturer’s guidelines. Briefly, excised gel slices containing the required DNA fragments were placed in a fresh microfuge tube and weighed. 3 gel volumes of buffer QG was added to 1 volume of gel slice and incubated at 50ºC with occasional agitation for 10 minutes or until the gel melted. 1 gel volume of isopropanol was then added to samples, the samples transferred to a spin column, and centrifuged at 17530xg for 1 minute. The flowthrough was discarded and the DNA washed by applying 500µL buffer QG to the spin column and centrifuged as before. The flowthrough was then discarded and the DNA washed with 750µL buffer PE. The flowthrough was discarded and the spin column dried by centrifugation at 17530xg for 2 minutes. 20µL EB was then applied to the spin column in a fresh microfuge tube and incubated for 1 minute at room temperature before centrifugation at 17530xg for 2 minutes to recover the DNA. 1µL of the eluted product was then subjected to agarose gel electrophoresis to determine the size and concentration of the recovered DNA.

**Purification of DNA by Phenol-Chloroform Extraction**

Phenol-chloroform methods were used to purify DNA from protein in samples. An equal volume of phenol-chloroform was added to the DNA samples and vortexed. Samples were centrifuged at 17530xg for 2 minutes, and the upper aqueous layer was transferred to a fresh microfuge tube. An equal volume of chloroform was added and the samples vortexed, before centrifugation at 17530xg for 2 minutes. The upper aqueous layer was transferred to a fresh microfuge tube and the DNA was ethanol precipitated from the sample.
DNA Precipitation

DNA was precipitated by addition of salt and either ethanol or isopropanol and resuspended in an appropriate volume to increase the concentration of the DNA and purify DNA from samples. 0.1 sample volumes of 3M NaAc was added to the DNA and mixed, followed by either 2.5 volumes of ethanol or 0.7 volumes of isopropanol, and incubated on ice for 1 hour. Samples were centrifuged at 17530xg and 4°C for 30 minutes to pellet the DNA. The pellets were washed with 1 volume of 70% (v/v) ethanol and centrifuged as before for 15 minutes. The supernatant was discarded and the pellets allowed to air-dry. The DNA was resuspended in an appropriate amount of TE buffer or H₂O and quantified by measuring absorbance at 260nm.

DNA Ligation

Ligation reactions were performed in accordance with the enzyme manufacturer’s instructions. Backbone vectors and PCR product inserts were prepared by restriction digest, purified, and quantified by agarose gel electrophoresis. Oligonucleotide inserts with the appropriate 5’ and 3’ overhangs were prepared by annealing and phosphorylating. Ligation reactions were 10µL in total volume and consisted of 1x T4 ligase buffer, 1µL T4 ligase and 30ng DNA. The DNA used in ligation reactions consisted of vector and insert combined in a 1:1 or 3:1 ratio, or of vector alone for control reactions. Ligation reactions were incubated at 16°C overnight and 1µL was used to transform electrocompetent bacteria. The presence of the insert in the vector backbone was determined by PCR or by preparing minprep DNA and performing an analytical restriction digest.

TOPO Cloning

TOPO reactions were performed in accordance with Invitrogen’s guidelines. Inserts possessed the 5’-CACC strand invasion sequence required to provide directional ligation into pcDNA3.1-V5-6H, and were either oligonucleotides or generated by PCR using KOD, Phusion or Pfu Turbo polymerases. 0.5µL Insert was added to 0.5µL TOPO vector and 2µL H₂O in a fresh microfuge tube and incubated at room temperature for 5 minutes. The reaction was then transformed into 50µL TOP10 bacteria and plated on ampicillin selective plates. The presence of the insert in the
vector backbone was determined by preparing minprep DNA and performing an analytical restriction digest.

**DNA Preparation**

All DNA preparation techniques were performed in accordance with Qiagen or Zymo Research guidelines.

*Small Scale DNA Preparation by Qiagen QIAprep Spin Miniprep Kit*

Qiagen QIAprep Spin Miniprep Kits were routinely used to prepare plasmid DNA on a small scale, typically generating 15-25µg of DNA. Briefly, individual transformed colonies were selected from agar plates to inoculate 5mL selective LB-broth and incubated overnight at 37°C. The cultures were then centrifuged at 4500xg and 4°C for 10 minutes to pellet the bacteria and the media removed. Pellets were resuspended in 250µL buffer P1 and the cells lysed by addition of 250µL buffer P2. The lysis reaction was neutralised and bacterial genomic DNA and proteins precipitated by addition of 350µL buffer N3. Samples were then centrifuged at 17530xg for 10 minutes and the supernatant applied to the provided spin column, which binds DNA to a silica resin under high salt concentrations. Plasmid DNA was bound to the resin by passing the supernatant through the column by centrifugation or by using a vacuum manifold. The DNA was then washed with 750µL buffer PE and dried by centrifugation. The purified plasmid DNA was eluted with 50µL buffer EB by incubating at room temperature for 1 minute and centrifugation at 17530xg for 2 minutes. The concentration of the eluted DNA was determined by measuring absorbance at 260nm. Miniprep DNA was then used for analytical restriction digests, DNA sequencing, *in vitro* protein expression or downstream cloning procedures.

*Large Scale DNA Preparation by Qiagen HiSpeed Plasmid Maxi Kit*

For larger scale DNA preparations, Qiagen HiSpeed Plasmid Maxi Kits were routinely used to prepare 0.5-1mg of plasmid DNA. The Maxi Kits work by the same principle as the miniprep procedure. Briefly, an individual colony from a selective plate was used to inoculate 5mL selective LB-broth and incubated for 8 hours at 37°C.
200µL of this starter culture was then used to inoculate 200mL of fresh selective LB-broth and incubated overnight at 37°C. Cultures were centrifuged at 4500xg and 4°C for 10 minutes to pellet the bacteria and the media removed. Pellets were resuspended with 10mL buffer P1, lysed with 10mL buffer P2, and the reaction neutralised by 10mL buffer P3. Bacterial lysates were then filtered with the provided QIAfilter Cartridge and applied to a HiSpeed Maxi Tip previously equilibrated with 10mL buffer QBT. The lysate was passed through the tip by gravity flow, allowing the plasmid DNA to bind to the silica resin. The DNA was washed with 60mL buffer QC and eluted with 10mL buffer. 10.5mL isopropanol was added to the eluate and incubated at room temperature for 5 minutes to precipitate the DNA. Precipitated DNA was then applied to the provided QIAPrecipitator, washed with 2mL 70% (v/v) ethanol, and air-dried. The plasmid DNA was eluted in 1mL TE buffer, and the concentration measured by absorbance at 260nm. Maxiprep DNA was then used for transfection, *in vitro* protein synthesis or downstream cloning procedures.

**BAC Clone DNA Preparation by Qiagen Large Construct Kit**

To prepare BAC clone DNA free from bacterial genomic DNA contamination, Qiagen Large Construct Kits were used. Typical yields were up to 50µg. The kit is similar in principle to the Maxiprep procedure, but also incorporates an additional exonuclease digestion step to remove non-circular DNA such as contaminating genomic DNA or sheared BAC clone DNA. Briefly, a single colony transformed with pBACe3.6-NR3 was transferred to 5mL LB-broth with ampicillin and incubated at 37°C for 8 hours. 500µL of this starter culture was then used to inoculate 500mL LB-broth with ampicillin and incubated overnight at 37°C. The bacteria were recovered by centrifugation at 4500xg and 4°C for 10 minutes and the resultant pellets resuspended in 20mL buffer P1, lysed in 20mL buffer P2 and the reaction neutralised by 20mL buffer P3. The lysate was then incubated on ice for 10 minutes before clearing by centrifugation at 15000xg for 30 minutes and filtration through pre-wetted filter paper. BAC DNA was precipitated from the cleared lysate by addition of 36mL isopropanol and centrifugation at 15000xg for 30 minutes. The DNA pellet was washed with 5mL 70% (v/v) ethanol, allowed to air-dry, and resuspended in 9.5mL buffer EX. 200µL of the provided ATP-dependent exonuclease and 300µL 100mM ATP were added to the DNA and incubated for 1 hour at 37°C. 10mL buffer QS was
added and the sample applied to a Qiagen-Tip 500 pre-equilibrated with 10mL buffer QBT, and the DNA allowed to bind the silica resin by gravity flow. The DNA was washed with 60mL buffer QC and eluted with 15mL buffer QF heated to 65°C. The BAC DNA was then precipitated by adding 10.5mL isopropanol and centrifugation at 15000xg. The DNA pellet was washed with 5mL 70% (v/v) ethanol, air-dried, and resuspended in 100μL TE buffer. The concentration of the eluted DNA was measured by absorbance at 260nm. The BAC clone DNA was then used for downstream cloning applications.

*Plasmid Preparation from Yeast using Zymoprep II Kit*

Plasmid preparation from yeast cells were performed on patches grown on selective plates. A ~10μL volume of yeast was transferred from the plate to 200μL Zymoprep solution 1 and cells dispersed. 3μL Zymolase added to each preparation and incubated at 37°C for 1 hour. 200μL Zymoprep solution 2 was added and mixed before addition of 400μL Zymoprep solution 3. Samples were centrifuged at 17530xg for 3 minutes and the supernatant solution applied to a Zymo-Spin-I column. Samples were centrifuged 17530xg for 30 seconds to bind plasmid DNA to the column. After discarding the flow-through solution, column-bound DNA was washed by applying 550μL Zymoprep wash buffer to the columns and centrifugation at 17530xg for 2 minutes. The column was then placed in a fresh 1.5mL microfuge tube and DNA eluted by applying 10μL TE buffer to the column and centrifuging at 17530xg for 2 minutes. Zymoprep DNA was then used for PCR or to transform into DH5α cells to generate miniprep DNA.

**DNA Sequencing**

Lists of oligonucleotides used for sequencing can be found in appendix A. DNA sequencing was performed on an Applied Biosystems 3100 Genetic Analyser and typically 0.5-1μg miniprep DNA or maxiprep DNA that had been precipitated and resuspended in H₂O was used. The DNA was combined with 8μL Big-Dye Terminator 3.0, 3.2pmol of the appropriate oligonucleotide sequencing primer, and 5% (v/v) DMSO in a final reaction volume of 20μL. Although annealing temperatures
for primers were determined empirically, cycling conditions typically consisted of an initial denaturating step of 96°C for 3 minutes, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Ramping rates were set to 1°C per second. Following the PCR reaction, 80µL 75% (v/v) isopropanol was added, and the samples centrifuged at 2600xg for 50 minutes. The supernatant was decanted and 150µL 70% (v/v) isopropanol was added and the samples centrifuged as before for 20 minutes. The supernatant was decanted and the pellets were dried by centrifuging inverted at 522xg for 1 minute. Samples were then processed by the sequencing services at the Institute of Reproductive and Developmental Biology or the MRC Genomics department, Hammersmith Hospital. DNA sequence files were analysed using Contig Express software package, which is part of the Vector NTI software suite.

RNA Extraction

RNA extraction was performed using Trizol reagent in accordance with the manufacturer’s guidelines. 1mL Trizol was added per 50mg of tissue, which was then homogenised in a power homogeniser (Polytron), for extraction of RNA from tissue. To extract RNA from cells cultured as a monolayer 1mL Trizol was added to 1 well of a 6-well plate to lyse the cells directly and the lysate was transferred to a 1.5mL microfuge tube free from ribonucleases. For extractions of RNA from cells cultured in smaller formats the volume of Trizol was adjusted accordingly. Samples were incubated at room temperature for 5 minutes to allow dissociation of protein complexes with nucleic acid. 0.2mL chloroform per 1mL Trizol was added to each sample, which were then shaken vigorously for 15 seconds, before incubating at room temperature for 3 minutes. Samples were then centrifuged at 12000xg and 4°C for 15 minutes to separate the phases of the samples. The upper aqueous phase was then transferred to a fresh microfuge tube free from ribonucleases. 0.5mL isopropanol per 1mL Trizol was added to each sample, incubated at room temperature for 10 minutes and centrifuged at 12000xg and 4°C for 15 minutes to precipitate RNA. The supernatant solution was discarded and precipitated RNA pellets were washed by adding 1mL 75% (v/v) ethanol and centrifuging at 7500xg and 4°C for 5 minutes. The supernatant solution was discarded and pellets allowed to air-dry, before resuspension in an appropriate amount of DEPC-treated H$_2$O. RNA was then quantified by
measuring absorbance at 260nm. Extracted RNA was then used for purifying mRNA or for cDNA synthesis.

**Purification of mRNA**

Messenger RNA was purified from total RNA using Dynabeads in accordance with the manufacturer’s guidelines. 150µg total RNA was placed in a 1.5mL microfuge tube free from ribonuclease and the volume adjusted to 200µL with DEPC-treated H₂O. Samples were then heated to 65°C for 2 minutes and placed on ice to disrupt secondary structure. 400µL Dynabeads were placed in a microfuge tube free from ribonucleases and washed by placing on a magnet (Dynal) for 2 minutes to collect the beads, the supernatant solution was removed and 200µL binding buffer added and mixed. The beads were then collected again using the magnet and the supernatant solution removed. The beads were resuspended in 200µL binding buffer and 200µL total RNA added. Samples were then rotated at room temperature for 5 minutes to allow the oligo (dT) to anneal to the poly-A tail of mRNA molecules, and the beads collected using a magnet as before. The supernatant solution was discarded and the beads were washed twice with wash buffer B by placing tubes on a magnet and discarding the supernatant solution. The mRNA was eluted from the beads by addition of 10mM Tris-HCl pH7.5 and incubating at 80°C for 2 minutes to disrupt the interaction between mRNA molecules and oligo (dT), before placing the samples on the magnet to collect the beads. The mRNA present in the supernatant solution was transferred to a fresh microfuge tube free from ribonuclease. Purified mRNA was then used for cDNA library construction.

**cDNA Synthesis**

Synthesis of cDNA was performed using MMLV reverse transcriptase in accordance with manufacturer’s instructions. Briefly, 1µg total RNA, 0.5U DNase I and 0.5µL 10x DNase I reaction buffer were combined in a microfuge tube free from ribonuclease, the volume was adjusted to 5µL with DEPC-treated H₂O, and samples were incubated at room temperature for 15 minutes to digest contaminating DNA in RNA samples. The reaction was inactivated by addition of 0.5µL 25mM EDTA.
pH 8.0 and incubation at 65°C for 10 minutes. 1 µg oligo (dT)\textsubscript{12-18} primer and 1 µL 10 mM dNTP mix were added to samples, and the volume adjusted to 10 µL with DEPC-treated H\textsubscript{2}O. Samples were incubated at 70°C for 10 minutes then placed on ice. 2 µL 10x reverse transcriptase buffer, 20 U RNase inhibitor, and 200 U MMLV reverse transcriptase were added to samples and the volume adjusted to 20 µL with DEPC-treated H\textsubscript{2}O. Tubes were incubated at room temperature for 10 minutes, followed by incubation at 37°C for 50 minutes. The reaction was inactivated by heating at 94°C for 10 minutes. Synthesised cDNA was then used for quantitative real-time PCR (QRT-PCR).

**Quantitative Real-Time PCR**

Gene expression was analysed using QRT-PCR, the principle of which requires that cDNA is PCR amplified in a manner that produces a fluorescent signal proportional to the amount of target cDNA present. This may be done with either SYBR Green, which fluoresces when intercalated in DNA, or with a fluorescent probe that anneals to the target DNA and is degraded during the PCR reaction, which allows emission of the fluorescent dye. The fluorescent signal is detected in each sample following each cycle of the PCR reaction. The data is analysed by the setting of a threshold within the linear phase of the reaction, termed Ct value, and determining the number of cycles required for the fluorescent signal to reach that threshold for each PCR reaction amplifying target cDNA. The levels of cDNAs of interest are then calculated relative to that of a housekeeping gene, and fold differences are determined across samples.

QRT-PCR reactions were performed in duplicate using Power SYBR Green Master Mix or SYBR Green Master Mix according to manufacturer’s instructions. Primers specific to the mouse ribosomal gene L19 were designed previously by others in laboratory and were used routinely, and primers specific to mouse NR3 were identical to those used elsewhere (Nicassio et al., 2005). Sequences of primers used for QRT-PCR are listed in appendix A. For QRT reactions 1 µL of cDNA was added to 12.5 µL master mix solution in each well of an optical 96-well plate. 2 pmol of mouse NR3 primers or 7.5 pmol of mouse L19 primers were added to reactions and volumes adjusted to 25 µL with DEPC-treated H\textsubscript{2}O. Reactions were then mixed by
inversion and centrifuged at 522xg for 1 minute to collect the reactions at the bottom of the plate. Thermocycling was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with 40 cycles of 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, for a total of 40 cycles.

NR3 levels in cDNA samples were determined by normalising to L19 expression. This is performed by taking the mean Ct value for duplicate QRT-PCR reactions and calculating the $\Delta$Ct value for NR3 in a cDNA sample by subtracting the mean Ct for L19 from the mean Ct of NR3. The levels of NR3 in cDNA samples were then compared by selecting a cDNA sample to relate other samples to and calculating the $\Delta\Delta$Ct value by subtracting the $\Delta$Ct of the selected sample to relate to from the $\Delta$Ct values of other samples. $\Delta\Delta$Ct values for samples were then converted to fold differences using the expression $2^{-\Delta\Delta C_t}$, which assumes the efficiency of each PCR cycle produces a 2-fold increase in amplicon levels. Fold differences were then multiplied by a factor of 100 to express the data as percentage mRNA expression levels.

2.2.2 Cell Culture Methods

Maintenance of Cell Stocks and Storage of Cells

HEK-293, NIH/3T3 and COS-1 cells were maintained in standard medium consisting of DMEM supplemented with 10% (v/v) FBS, 100U/mL penicillin, 100ng/mL streptomycin and L-glutamine at 37°C and 10% CO$_2$. Cells were grown as a monolayer on tissue culture grade plastics. To maintain cell stocks NIH/3T3 cells were typically split 1:10 twice each week, and HEK-293 and COS-1 cells were split 1:20 twice per week. Cells were stored in DMEM containing 10% (v/v) DMSO under liquid nitrogen.

Transfection Techniques

DNA Transfection

For transfection using Fugene 6 transfection reagent (Roche) cells were seeded into either a 96-well plate, a 10cm$^2$ dish, an 8-well chamber slide or a T150 cell culture flask at ~30% confluence. For transfection using Lipofectamine 2000
transfection reagent (Invitrogen) cells were seeded into either a 96-well plate at ~50% confluence. Cells were seeded in phenol red free DMEM supplemented with 5% dextran-coated-charcoal stripped FBS. The following day cells were transfected according to manufacturer’s guidelines at 50-80% confluence using Fugene 6, or at ~90% confluence using Lipofectamine 2000. The appropriate amounts of transfection reagent and Optimem were combined in accordance with the instructions of the transfection reagent manufacturer for each cell culture format, and incubated for 5 minutes. DNA mixes were prepared with the manufacturer’s recommended amount of DNA for each cell culture format and empty pcDNA3.1 or pSG5 was used to keep transfection amounts constant within each assay and to perform mock transfections. The transfection reagent/Optimem solution was then added the DNA, mixed, and incubated for 20 minutes at room temperature to allow the DNA to complex with the transfection reagent. Complexes were added to the cells and incubated at 37°C. After 18-24hrs cells were subjected to appropriate treatments and harvested 24hrs later.

siRNA Transfection

NIH/3T3 cells were seeded in phenol red free media containing 5% (v/v) dextran charcoal stripped FBS into gelatine coated 12-well plates at a density of 6x10^4 per well or 6-well plates at a density of 1.2x10^5 per well, and cells transfected 4 hours later with Lipofectamine 2000 transfection reagent in accordance with manufacturers instructions. Briefly, 40pmol siRNA was diluted with Optimem to a final volume of 100µL. 2µL Lipofectamine 2000 was added to 98µL Optimem and incubated at room temperature for 5 minutes. The Lipofectamine 2000 and siRNA mixes were then combined and incubated at room temperature for 20 minutes. 200µL cell culture media was then replaced with the transfection mix, the cells incubated at 37°C overnight, and the transfection procedure repeated the following morning.

Generation of Cell Lines Stably Expressing NR3-V5

Cells were transfected with DNA plasmids coding for NR3-V5 or empty vector possessing a neomycin resistance gene and cultured for 48 hours. Cells were then maintained in standard medium containing 300mg/mL G418 to select for transfectants, and as a positive control untransfected cells were maintained under the same
conditions. Cells were cultured in these conditions and cell numbers observed to diminish until all cells in the untransfected cultures had undergone cell death. Transfectants were maintained in 300mg/mL for a further 48 hours and then cultured in standard medium containing 150mg/mL G418.

### 2.2.3 Protein Analysis

**Protein Expression in Bacteria**

DNA fragments were cloned into pGEX-6P2 in frame with GST to produce fusion proteins, and the resultant plasmids transformed into BL21 bacteria. A single colony was used to inoculate 5mL LB-broth with 100µg/mL ampicillin and incubated at 37°C overnight. Overnight cultures were then added to 45mL LB-broth and incubated at 37°C for 1 hour. Protein expression was induced by the addition of 25µL 1M IPTG to give a final concentration of 500nM IPTG, and incubation at 24°C for 3 hours. Cells were then collected by centrifugation at 4500xg and 4°C for 10 minutes and pellets lysed in 5mL NETN. Lysed cells were then sonicated twice for 10 seconds to break up the bacterial DNA. GST fusion proteins were purified by adding 125µL Glutathione Sepherose 4B beads previously washed with NETN and incubating with agitation for 2 hours at 4°C. For negative control samples containing GST alone, 250µL lysate was adjusted to a volume of 5mL with NETN and used for purification to account for differences in protein expression levels. The beads were washed five times by centrifuging at 5720xg and resuspension in 5mL NETN, collected by centrifugation at 5720xg, and resuspended in an appropriate buffer for further processing. Typically, bacterially expressed proteins were used for GST pulldown assays, or a purification scaled up 3-fold was used for proteolytic cleavage.

**In Vitro Protein Synthesis**

In vitro protein synthesis was performed using the TNT Coupled Reticulocyte Lysate System or the TNT Quick Coupled Transcription/Translation System. These systems couple RNA transcription from a DNA template possessing a T3, T7 or SP6 promoter with translation of the transcribed RNA in a single reaction that utilises rabbit reticulocyte lysate. Reactions were assembled on ice according to the
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manufacturer’s guidelines and performed in a total reaction volume of 50µL using 1µg of plasmid DNA and 20µCi L-[35S] methionine. Reactions were incubated at 37°C for 90 minutes and the product used in GST pulldown experiments.

**GST Pulldown**

GST fusion protein bound beads were resuspended in 6mL NETN and radiolabelled *in vitro* synthesised proteins were diluted 1:5 by adding 200µL NETN containing 10% (v/v) glycerol. 600µL of beads and 50µL diluted radiolabelled protein were then combined and rotated overnight at 4°C. Beads were washed five times by centrifugation at 5720xg and resuspension in 1mL NETN. The beads were then collected by centrifugation at 5720xg and dried in a Savant DNA120 Speed Vac, before resuspension in 40µL Laemmli sample buffer. GST fusion protein loading controls were prepared by centrifuging 600µL of the GST fusion protein-bound beads by centrifuging at 5720xg, drying in a Speed Vac, and resuspension in 40µL Laemmli sample buffer. Loading Controls or samples and 10% inputs for the radiolabelled protein were loaded onto polyacrylamide gels and subjected to SDS-PAGE. Loading control gels were then subjected to Coomassie staining, and gels containing samples and 10% inputs were fixed in SDS-PAGE fixing buffer for 10 minutes and incubated in Amplify for 30 minutes. Gels were then dried on to filter paper using a Savant SGD2000 Slab Gel Dryer and sample gels exposed to a Molecular Dynamics Low Energy Phosphor Screen overnight, which was scanned used a Typhoon 8600 Variable Mode Imager.

**Expression and Extraction of Proteins in Mammalian Cells**

NIH-3T3 or HEK-293 cells seeded were either transiently or stably transfected with plasmid DNA constructed to express the gene of interest in mammalian cells. Cells transiently or stably transfected with empty vector were used as negative controls. For TNFα treatments, cells were treated 18 hours post transfection with 10ng/mL TNFα for 6 hours. For treatments with MG132, cells were treated with 20µM MG132 at the appropriate timepoint prior to lysis. Whole cell extracts were taken by lysing cells directly in Laemmli sample buffer heated to 100°C, subjected to
a freeze thaw cycle, and heated at 100°C for 5 minutes to denature the proteins. Denatured whole cell extracts were then centrifuged at 17530xg for 5 minutes before loading into a polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein extracts were taken by lysing cells with HEPES lysis buffer and rotating at 4°C for 20 minutes. Lysates were then subjected to a freeze-thaw cycle and were centrifuged at 17530xg and 4°C for 20 minutes to clear the lysates. The concentration of protein in cleared lysates was then measured by either BCA Protein Assay or Bio-Rad Protein Assay.

**Pulse Chase Experiments**

HEK-293 cells stably expressing NR3-V5 were seeded into gelatine-coated 24-well plates at ~60% confluence and allowed to acclimatise overnight. Cells were then pre-treated with 20µM MG132 or DMSO for 3 hours, treated with 20ng/mL cycloheximide and cells harvested at the appropriate time point using Laemmlli sample buffer. Samples were then subjected to Western blot.

**Measurement of Protein Concentration**

Protein concentration was determined by using BCA Protein Assay kit or Bio-Rad Protein Assay. Reactions were performed in accordance with the manufacturer’s guidelines.

**BCA Protein Assay**

The BCA Protein Assay procedure is based on the ability of peptide bonds to reduce Cu$^{2+}$ from cupric sulphate to Cu$^{1+}$, which then chelates with two molecules of bicinchoninic acid forming a purple solution that absorbs light at 592nm. Protein extracts were diluted as necessary in HEPES lysis buffer, typically 1:10 or 1:100, and 25µL added to one well a 96-well plate. Protein standards ranging from 25µg/mL to 2mg/mL were prepared from BSA, and 25µL of each standard added to the 96-well plate. BCA working reagent was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B and 200µL was added to each standard and sample. The reaction was incubated for 30 minutes at 37°C and absorbance was measured at 592nm.
Bio-Rad Protein Assay

The Bio-Rad Protein Assay is based on the Bradford assay, in which acidic solutions of Coomassie Brilliant Blue G-250 absorb light at 595 nm when bound to protein. The concentrated reagent was diluted 1:5 to generate a working solution. Protein standards were prepared from BSA ranging from 20 mg/mL to 625 mg/mL, and samples were prepared by diluting appropriately, typically 1:10. 10 µL of standard or sample was added to 500 µL of diluted reagent, vortex mixed, incubated at room temperature for 5 minutes and absorbance at 595 nm was measured.

Immunoprecipitation from Protein Extracts

Pre-clearing and Denaturing Samples

Immunoprecipitations were performed on protein extracts possessing the same amount of total protein as determined by BCA protein assay or Bio-Rad Protein Assay under native or denatured conditions. Lysates were precleared with 25 µL protein-A agarose for 1-16 hours rotating at 4°C, and centrifuged at 5720 xg and 4°C for 1 minute. Samples requiring immunoprecipitation under native conditions were then used in the immunoprecipitation procedure outlines below. For samples requiring denaturing prior to immunoprecipitation, SDS and DTT were added to give final concentrations of 1% (w/v) SDS and 5 mM DTT, and samples were heated at 100°C for 10 minutes. 9 sample volumes of HEPES lysis buffer was then added to dilute the SDS and DTT such that their effect on the efficiency of the immunoprecipitation would be minimised.

Immunoprecipitation Procedure

Denatured or native samples were incubated with 0.5 µg anti-V5 antibody and 25 µL protein-A agarose for 16 hours rotating at 4°C. Samples were then centrifuged at 5720 xg and 4°C for 1 minute. Beads were washed four times with 1 mL HEPES lysis buffer by resuspension and centrifugation. The beads were then either resuspended in 1 mL 10 mM Tris pH 8.0 and used for proteolytic cleavage experiments or resuspended in 100 µL Laemmli sample buffer and subjected to SDS-PAGE followed by silver staining or Western blotting.
Limited Proteolysis

Mammalian expressed immunoprecipitated or bacterially expressed GST purified protein bound to the appropriate beads for purification was washed three times by centrifugation at 5720xg and resuspension with 1mL 10mM Tris pH8.0, and immunoprecipitated protein beads resuspended in 500µL 10mM Tris pH8.0 and GST purified protein beads resuspended in 100µL 10mM Tris pH8.0. V8 protease was then added to the samples to give final concentrations of 0, 0.2, 0.6 and 2.0µg/mL for immunoprecipitated protein, or 0, 0.2 and 2.0µg/mL for GST purified protein, and the samples rotated at room temperature for 90 minutes before stopping the reaction with 1mM AEBSF. Beads were then collected by centrifugation at 5720xg for 2 minutes and the supernatant retained from GST purified samples, and the beads retained from immunoprecipitated samples. Supernatants were then mixed 1:1 with 2x Laemmli sample buffer and beads were resuspended in 100µL Laemmli sample buffer. Samples were then subjected to SDS-PAGE and further analysis by Coomassie staining or by Western blot.

Nuclear Extract Method

10cm plates were seeded with HEK-293 cells and transfected with 1µg test construct. At 36-48hrs post transfection cells were washed with chilled PBS and harvested by scraping with a cell lifter in 200µL nuclear extract buffer A. Cells were transferred to a 1.5mL centrifuge tube and incubated on ice for 20 minutes. 15µL 20% (v/v) NP-40 diluted in nuclear extract buffer A was added and the cells vortexed for 10 seconds. The lysates were centrifuged at 17530xg for 30 seconds at 4°C, the cytosolic supernatant removed and snap frozen on dry ice. 100µL Nuclear extract buffer B was added to the cell pellet, and pipetted up and down. The pellet was then incubated with the solution in a rotator at 4°C for 15 minutes to resuspend nuclear proteins. The lysates were then centrifuged at 17530xg at 4°C for 5 minutes, the nuclear supernatant removed and snap frozen on dry ice. Lysate fractions were stored at -80°C.
TCA Precipitation

Protein samples were concentrated by precipitation with TCA and resuspension in Laemmili sample buffer. 0.1 sample volumes of TCA was added to the sample and incubated on ice for 45 minutes. Samples were centrifuged at 17530xg for 20 minutes and the pellets washed with 0.5mL chilled acetone. Samples were centrifuged at 17530xg for 5 minutes and the residual acetone removed. The pellet was air-dried and resuspended in an appropriate amount of Laemmli sample buffer. TCA precipitated proteins were then used for SDS-PAGE.

SDS-PAGE

Polyacrylamide gels consisted of NuPage 4-12% (w/v) polyacrylamide gels, 10% (w/v) polyacrylamide gels, or 12% (w/v) polyacrylamide gels. 10% and 12% resolving gels were poured by combining the components listed in the section 2.1.12, pipetting the gel solution between glass plates, and overlaying with H₂O to ensure the gel is level. 5% stacking gels were then poured over set resolving gels and a comb inserted. Protein samples were whole cell extracts, protein extracts mixed 1:1 with Laemmli sample buffer, or protein extracts that had been subjected to TCA precipitation and resuspended in Laemmli sample buffer. Samples were heated at 100°C for 5 minutes prior to loading onto gels. Typically 50µg of protein was loaded into one well of a polyacrylamide gel, alongside either Full-Range Rainbow Molecular Weight Marker or Novex Sharp Pre-Stained Protein Standards, and subjected to electrophoresis at 150V until the marker had sufficiently separated.

Coomassie and Silver Staining

Coomassie and silver staining procedures were performed on polyacrylamide gels in accordance with the stain manufacturer’s guidelines.

Coomassie Staining

Following SDS-PAGE gels were rinsed three times for 5 minutes with an excess of distilled H₂O, fixed for 15 minutes in SDS-PAGE fixing buffer, and rinsed again with distilled H₂O. Gels were incubated with EZBlue Gel Staining Reagent for 1 hour
or overnight, and washed with distilled H\textsubscript{2}O until background staining was reduced. Gels were then dried on to filter paper using a Savant SGD2000 Slab Gel Dryer.

**Silver Staining**

SDS-PAGE gels were fixed in silver fixing buffer for 1 hour or overnight, followed by washing in 30\% (v/v) ethanol for 10 minutes. Gels were then washed with distilled H\textsubscript{2}O for 10 minutes, and incubated with Sensitiser solution for 10 minutes, followed by a further wash with distilled H\textsubscript{2}O for 10 minutes and incubation with Silver solution for 10 minutes. Gels were then washed for 90 seconds with distilled H\textsubscript{2}O and developed in Developer solution until the appropriate staining intensity was achieved. Gels were either dried on to filter paper using a Savant SGD2000 Slab Gel Dryer, or stained protein bands cut out and subjected to mass spectrometry. Mass spectrometry was carried out by Dinah Rahman, Proteomics Facility, MRC Clinical Sciences Centre, Hammersmith Hospital.

**Western Blotting**

Following SDS-PAGE, proteins were transferred to Hybond PVD-F membrane by electroblotting using the Mini-Trans Blot system (Bio-Rad). The membrane was activated by briefly placing in methanol, and then rinsing in transfer buffer. It was then placed directly onto the gel, ensuring no air bubbles were present, and the gel and membrane placed between two pieces of 3mm filter paper and two sponge pads. This was assembled in transfer buffer and held together in a transfer cassette. Electroblotting was performed at either 50V at 4\(^\circ\)C overnight or 120V at 4\(^\circ\)C for 1 hour. Membranes were dried and reactivated by brief immersion in methanol and rinsing in TBS-T, and additional protein binding sites on the membrane were blocked with milk solution for 1 hour at room temperature or overnight at 4\(^\circ\)C. Primary antibodies were diluted in milk solution, typically 1:1000, and supplemented with sodium azide before incubation with the membrane at either room temperature for 1 hour or at 4\(^\circ\)C overnight. Membranes were washed three times for 5 minutes with TBS-T, before incubation with the appropriate HRP-conjugated secondary antibody diluted 1:5000 in milk solution for 1 hour at room temperature. Membranes were
again washed three times for 5 minutes in TBS-T, and bound secondary antibody was then detected by incubation with ECL, ECL plus, or West Pico reagents.

**Immunostaining**

COS-1 cells were seeded into 8-well chamber slides and transfected with 10ng test construct. Transfection amounts were adjusted to 55ng using empty vector. Negative controls consisted of cells transfected with empty vector only. 36-48hrs post transfection cells were fixed in chilled methanol for 15 minutes, washed with sterile filtered PBS and permeabilised for 5 minutes with permeabilising buffer. Cells were washed as before, blocked with sterile filtered blocking buffer 1 for 30 minutes, and incubated for 30 minutes with blocking buffer 2 using normal serum from the species the secondary antibody was produced in. Cells were then washed in blocking buffer and incubated with primary antibody diluted 1:200 in blocking buffer overnight at 4°C. Cells were then washed with blocking buffer and incubated with FITC-conjugated secondary antibody for 1 hour at 25°C. Cells were washed with blocking buffer, mounted, and DAPI stained using Vectashield Mounting Medium with DAPI or ProLong Gold. Cells were then subjected to fluorescence microscopy and images taken at 250ms exposure.

**2.2.4 Yeast Two-Hybrid Screen**

Yeast two-hybrid screening was performed using the CytoTrap system, which is based on the Son-of-Sevenless (SOS) Recruitment System (SRS) (Aronheim et al., 1997). Screening for protein-protein interactions was performed in accordance with the manufacturer’s instructions.

**CytoTrap Library Construction**

All enzymes and buffers were provided by Stratagene. The method used to construct of the cDNA library for screening protein-protein interactions provides an enrichment of full length cDNA. The cDNA fragments are size fractionated and directionally cloned into the yeast expression vector pMyr.
First-Strand cDNA Synthesis

5µg mRNA purified from RNA samples extracted from embryonic/placental and brain tissue and first strand cDNA synthesis was performed by combining the mRNA with the supplied first-strand buffer, 2.8µg oligo-dT primer containing a XhoI restriction site, 40U RNase Block Ribonuclease Inhibitor and 600nM dNTPs containing 300nM methyl-dCTP to provide hemimethylated cDNA in an RNase-free microfuge tube and the volume adjusted to 48.5µL with DEPC-treated H₂O. The primer was annealed to the template by incubating at room temperature for 10 minutes, and 1.5µL of the supplied 75U Stratascript reverse transcriptase enzyme added and the reactions incubated at 42°C for 1 hour.

Second-Strand cDNA Synthesis

Second strand synthesis was performed by adding 10x second-strand buffer, 300nM dNTPs containing 780nM dCTP, 300nM [α-³²P] dATP, 3U RNase H, and 99U DNA polymerase I, and the volume adjusted to 200µL with DEPC-treated H₂O. Reactions were incubated at 16°C for 2.5 hours and the resultant double-stranded cDNA fragments were blunted by adding 5U Pfu polymerase and 255nM dNTPs. The blunted cDNA was purified by phenol-chloroform extraction.

Ligation of EcoRI Adaptors, Phosphorylation of cDNA and Digestion with XhoI

The purified cDNA pellet was resuspended and combined with 3.2µg EcoRI adapters, ligase buffer, 0.9mM rATP and 4U T4 DNA Ligase in final reaction volume of 11µL. Ligation reactions were incubated at 8°C overnight and the ligase inactivated by incubation at 70°C for 30 minutes. cDNA fragments were phosphorylated by addition of 10U T4 PNK, 1µL 10mM rATP, and 1µL 10x ligase buffer, and adjusting the reaction volume to 21µL. Reactions were incubated at 37°C for 30 minutes before heat inactivation at 70°C for 30 minutes. 28µL XhoI buffer supplement and 120U of XhoI were added in a final reaction of 52µL, and the reactions incubated at 37°C for 1.5 hours. The cDNA digested with hemimethylated DNA sensitive XhoI was then purified by ethanol precipitation and the resulting cDNA pellet resuspended in 14µL.
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STE and 3.5μL column loading dye was added. The processed hemimethylated cDNA fragments now possess EcoRI and XhoI sticky ends for directional cloning into the pMyr vector backbone (figure 2.1).

Size Fractionation of cDNA Fragments

The gel filtration column was prepared by packing Sepharose CL-2B gel filtration medium into a 1mL disposable pipette as indicated by Stratagene’s guidelines. The column was washed with 20mL of STE, and the cDNA loaded onto the column. 12 100μL fractions were collected possessing 2 peaks of radioactivity, the first of which represents the cDNA containing fractions, and the second contains unincorporated radiolabelled nucleotides. The cDNA was then purified from the appropriate fractions by phenol-chloroform extraction, resuspended in 3.5μL DEPC-treated H₂O, and assessed for nucleotide concentration by spotting onto an agarose plate containing ethidium bromide.

Ligation of cDNA Fragments into pMyr Vector

Fractions containing cDNA were ligated into the supplied pMyr vector pre-digested with EcoRI and XhoI by combining 60ng cDNA insert, ligase buffer, 1mM rATP, 100ng of pMyr and 2U T4 DNA ligase in a final reaction volume of 5μL. Reactions were incubated at 12°C overnight and pilot transformations into XL10-Gold Kan cells performed to examine transformation efficiency of each ligated fraction.

Determining Transformation Efficiency

To ensure that there is no over representation of a specific range of cDNA sizes, the transformation efficiency of each ligation reaction is determined. Transformations were made up to 1mL with SOC medium and 1μL and 10μL were plated onto LB-chloramphenicol plates, and the number of colonies assessed. The number of colonies on the 1μL and 10μL plates were then multiplied by 1000 and 100 respectively to determine the number of colony forming units (CFU)/μL for each transformation.
Figure 2.1 Schematic of cDNA Fragments Generated for CytoTrap Library Construction
cDNA fragments synthesised to possess EcoRI and XhoI sticky ends for directional cloning into the pMyc XR vector. XhoI is methylation sensitive and will not cut within the cDNA fragments.
Transformations were repeated but scaled-up 5-fold and the amount of cDNA insert normalised based on the number of CFU determined in each transformation.

**Preparing the cDNA Library**

Transformation reactions were pooled and 1µL and 10µL plated to determine the CFU/µL as before. The rest of the transformants were then plated on 15cm LB-chloramphenicol plates, plating 20,000-30,000 CFU per plate, and incubated overnight at 37°C. The resulting colonies were then scraped in 6mL LB-broth, the plates washed 2mL LB-broth to recover the residual bacteria, and bacteria pooled. Half the bacteria were used to prepare library DNA by Maxiprep and the average size of insert was determined by PCR. 0.2 volumes of 80% (v/v) glycerol was added to the remaining bacteria, which were then stored at -80°C. Prepared library DNA was then used for transformation into cdc25H yeast cells to screen for protein-protein interactions.

**CytoTrap Yeast Two-Hybrid Screening**

**Generation of Competent Yeast Cells**

Competent cdc25H yeast cells were prepared by streaking cdc25H cells onto a YPAD agar plate and incubating at room temperature for 4 days. A single colony was selected and dispersed into 1mL YPAD broth, which was then added to 50mL YPAD broth and incubated at 22°C and 220rpm for 14-19 hours until the OD$_{600}$ measured greater then 1. The 50mL cultures were then added to 250mL YPAD broth, and incubated at 22°C and 220rpm for 3 hours. The OD$_{600}$ was measured to ensure it was greater than 0.7. Yeast cells were then pelleted by centrifugation at 4500xg for 10 minutes, and cells were washed with 50mL distilled H$_2$O resuspension and centrifugation before resuspension in 50mL LiSORB. Cells were then incubated for 30 minutes at room temperature, collected by centrifugation at 4500xg for 10 minutes, and resuspended in 300µL LiSORB. 400µL 20mg/mL sssDNA was denatured by heating to 100°C for 10 minutes, allowed to cool to room temperature and added to 600µL LiSORB. 400µL of the sssDNA/LiSORB mix, 5.4mL PEG/LiOAc and 530µL DMSO was added to the cells, which were then used immediately for transformation.
Co-transformation of Competent Yeast Cells

Competent yeast cells were co-transformed by adding 20µg library DNA derived from brain, 20µg embryonic/placental library DNA, 40µg of either pSOS-NR3 or pSOS-NR3 LBD, and 200µL 1.4M β-mercaptoethanol to 10mL of competent yeast cells, aliquoting into 20 microfuge tubes and incubating at room temperature for 30 minutes with occasional mixing. Control transformations were set up by combining 2µg pSOS DNA, 2µg pMyr DNA, 500µL competent yeast cells and 10µL 1.4M β-mercaptoethanol. The transformations were heat-shocked at 42°C for 20 minutes, incubated on ice for 3 minutes, and collected by centrifugation at 3220xg for 10 minutes. The pellets were washed by resuspension in 500µL 1M sorbitol and centrifuged at 3220xg for 10 minutes and resuspended in 250µL 1M sorbitol. Each transformation was then spread onto a 15cm SD glucose agar plate using sterile 3mm non-acid washed glass beads. Plates were incubated at room temperature for 48 hours, before replica plating onto 15cm SD galactose agar plates to induce library protein expression and incubated at the selective temperature of 37°C to identify interactor candidates.

Primary Test for Interaction

After 6 days and 10 days, colonies growing on SD galactose agar plates at 37°C were transferred with a sterile loop to SD glucose agar plates to prevent library protein expression, and incubated at room temperature for 48 hours. As a primary test for an interaction between interactor candidates and the bait protein, colonies were patched back onto SD galactose agar plates and onto 2 sets of SD glucose plates by picking colonies and suspending in sterile distilled water. 0.5µL was then spotted onto the plates. SD galactose agar plates were incubated at 37°C. As a negative control one set of SD glucose agar plates was also incubated at 37°C to ensure that colony growth was dependent on library protein expression. The second set of SD glucose agar plates was incubated at room temperature.
Secondary Test for Interaction and Isolation of Putative Interacting Constructs

As a secondary test for an interaction between candidates and bait protein, patching was performed as in the primary test onto one set of SD galactose agar plates and one set of SD glucose agar plates, using the SD glucose agar plate from the primary test grown at room temperature as a source. After 48 hours, patches growing on SD galactose agar plates, but not SD glucose agar plates at 37°C, were picked and used to inoculate 5mL SD glucose broth. The cultures were incubated at room temperature and 220rpm for 3 days, and the cells pelleted by centrifugation at 4500xg. The pellets were resuspended in yeast lysis buffer and transferred to a fresh microfuge tube. 50µL 0.5mm acid-washed glass beads and 300µL phenol-chloroform were added and the cells vortexed for 1 minute. The samples were centrifuged at 17530xg for 2 minutes and the upper aqueous phase transferred to a fresh microfuge tube. The DNA was then isolated by ethanol precipitation, resuspended in 40µL H₂O, and transformed by electroporation into DH5α bacteria. The DNA was then isolated by miniprep.

Verification of Interaction

2µg of the isolated putative interacting constructs were cotransformed with 2µg pSOS-NR3 or pSOS-NR3 LBD into 500µL of freshly prepared competent cdc25H cells, and spread onto SD glucose agar plates using glass beads as before, and incubated at room temperature for 6 days. Colonies were then patched as before onto SD galactose agar plates and SD glucose agar plates and incubated at 37°C. After 4 days patched colonies were assessed for growth on SD galactose agar plates and lack of growth on the SD glucose agar plates. Colonies were then selected and prey plasmid DNA prepared by Zymoprep.
2.2.5 Functional Assays

**Reporter Gene Assays**

*TNFα Assays*

For TNFα assays NIH-3T3 or HEK-293 cells were seeded into a 96-well plate and transfected either using Lipofectamine 2000 with 200ng of DNA consisting of 30ng pGL3-NFκB firefly luciferase reporter, 5ng pRL-EF1α renilla luciferase reporter and varying amounts of test construct (NIH-3T3), or using Fugene 6 with 55ng of DNA consisting of 15ng pGL3-NFκB luciferase reporter, 10ng pRL-TK renilla luciferase reporter and varying amounts of test construct (HEK-293). 125ng p65 was used as a positive control in NIH-3T3 transfections, and 30ng p65 was used as a positive control in HEK-293 transfections. For transfections with TNFR1, an IMAGE clone coding for the full length mouse TNFR1 (IMAGE 30061894, MGC 60564, accession BC052675) was obtained (MRC Geneservice, Cambridge) and 10ng cotransfected into HEK-293 cells with Fugene 6. Cells were treated with 10ng/mL TNFα or the appropriate amount of vehicle 18hrs later, and harvested 6hrs post-treatment. Experiments were performed in replicates of at least two.

*Ligand Screening Optimisation*

For optimisation of ligand screening assays HEK-293 cells were seeded into a 96-well plate and transfected with 55ng of DNA consisting of 15ng pGL3-GAL4 firefly luciferase reporter, 10ng pRL-TK renilla luciferase reporter and varying amounts of test construct. Cells were treated with 10nM oestradiol or the appropriate amount of vehicle 36-48hrs later, and harvested 24hrs post-treatment.

*Ligand Screening*

For Ligand screening assays HEK-293 cells were seeded into T150 flasks and transfected with 27.5μg of DNA consisting of 7.5μg pGL3-GAL4 firefly luciferase reporter, 5μg pRL-TK renilla luciferase reporter, 10μg empty pSG5, and either 5μg pSG5-VP16-GAL4-NR3 construct for test and low signal control samples, or 5μg pSG5-VP16-GAL4-CTRL construct for high signal control samples. 24hrs post...
transfection cells were split into 96-well plates and treated with the appropriate compound at a concentration of 10μM, and control cells left untreated. 24hrs post treatment cells were harvested.

**Cell Harvesting**

To harvest from 96-well plates, 50μL of the cell culture medium was removed from the cells, 50μL of 2x LucLite reagent added, and the cells incubated in the dark for 15 minutes. After ensuring cell lysis was complete, reactions were transferred to a white plate to measure luminescence on a Wallac Victor2 1420. 25μL Renilla buffer was then added to each reaction, allowed to equilibrate for 30 minutes at room temperature, and luminescence reading was repeated. Firefly luciferase values were normalised using renilla luciferase values to account for experimental variation.

**Cell Proliferation Assay**

For cell proliferation assays cell number was determined by MTS assay using CellTiter 96 AQueous MTS reagent in accordance with manufacturer’s guidelines. The system is based on the conversion of MTS tetrazolium salt to formazan by dehydrogenase enzymes in metabolically active cells, and is routinely used to determine cell number. NIH/3T3 cells transfected with siRNA or HEK-293 cells stably transfected with NR3-V5 or empty vector were seeded into 96-well plates at a density of 0.5x10^4 cells per well in a culture volume of 100μL. Time points were taken at 0, 24, 48 and 72 hours post seeding by addition of 20μL MTS reagent, and incubation at 37°C for 1 hour. Absorbance at 490nm was read on a Wallac Victor2 1420. RNA extraction for siRNA controls were performed in cells harvested at the 72 hour time point.

**2.2.6 In Silico Techniques**

Sequence alignments were performed using the Align X program, which forms part of the Vector NTI software suite. The software package calculates the percentage identity and similarity between protein, DNA or RNA sequences. 3D molecule
images were generated using the 3D Molecule Viewer, which also forms part of the Vector NTI software suite.

Predictions of protein disorder as an indication of structured regions were generated using the Regional Order Neural Network (RONN) prediction website. Predictions of transcription factor binding sites were generated with the Consite website with genomic sequences taken from the Ensembl and UCSC websites. Blast and Blat searches were performed using the NCBI and UCSC websites respectively.

2.2.7 Statistics

Probability values for one-tailed two-sampled t-tests were performed using the Excel software, which forms part of the Office software suite. T-tests were performed between measurements taken on samples from different cell transfections harvested at the same time point. Z-prime statistical tests were performed using the calculation below, where the terms high and low refer to the values for high and low controls for the assay.

\[
Z' = 1 - \frac{(3 \times SD_{\text{high}} + 3 \times SD_{\text{low}})}{(\bar{x}_{\text{high}} - \bar{x}_{\text{low}})}
\]
Chapter 3

Characterisation of the Putative Nuclear Receptor-like Ligand Binding Domain of NR3
3.1 Introduction

The putative LBD region of NR3 is predicted by the Genome Threader algorithm to reside between amino acids 477 and 722 within the NR3 protein. To determine whether this region may be regarded as a bona fide LBD, two different approaches were employed.

Firstly, the question of whether the putative LBD region of NR3 possesses a similar structure to that of bona fide NRs was addressed. A comprehensive method for determining protein structure is to employ X-ray crystallography. However, prior to such work preliminary structural experiments can be performed to determine the candidate protein regions for crystallography, and to ensure that the protein region of interest possesses a structured fold. In the absence of any data regarding the structure of NR3, limited proteolysis was carried out to determine the protein regions possessing a structured fold, which renders them resistant to proteolytic cleavage (Hubbard, 1998).

Secondly, the question of whether the NR3 LBD binds a ligand was addressed by screening for a synthetic ligand that may bind and modulate the activity of NR3 in a luciferase reporter gene assay. Inpharmatica provided a library of ligands that had been synthesised for screening against the PPAR nuclear receptors, and as such represented a library of small hydrophobic molecules generated to screen against NR LBDs. This library was used to screen for compounds that could activate NR3 in the reporter gene assay system.

3.2 Structural Analysis of NR3

The folded 3-dimensional structure of NRs has been shown in many crystallographic studies to be well conserved across the superfamily. Such structured domains are known to be resistant to proteolytic digestion as the folding of the peptide renders the substrate residues inaccessible to the protease (Hubbard, 1998). As the putative LBD of NR3 is predicted to possess the same folded structure as the LBD of the NR superfamily, and due to the lack of reports discussing the 3-dimensional structure of NR3, limited proteolysis was performed to determine whether the region in which the LBD is predicted to reside is resistant to proteolytic degradation.
The protein sequence of NR3 was firstly assessed *in silico* for the presence of regions thought to be disordered using the RONN prediction methods (Yang *et al.*, 2005) (figure 3.1). If a region is disordered it is unlikely to fold into a structured domain, and as such would be susceptible to digestion with proteases. NR3 is predicted to be structured overall, with regions of disorder between amino acids 1-34, 81-87, 226-231, 260-285, 343-371, 434-445 and 713-717. Therefore these predictions support the idea that the putative LBD region between amino acids 477-722 is a structured domain that should be resistant to proteolysis. Sequence analysis shows there are numerous substrate sites for V8 within NR3, however it would be expected that those in the disordered regions of NR3 would be cleaved more readily. As such, a schematic mapping cleavage sites that would be preferentially digested during limited proteolysis due to protein disordered can be produced (figure 3.1). These data show that if V8 digests NR3 preferentially at the regions predicted to be disordered the LBD region should reside in a peptide fragment 41.0kDa in size.

### 3.2.1 Limited Proteolysis of Bacterially Expressed NR3

To determine whether there are structured, proteolytic resistant domains present within NR3 limited proteolysis was carried out on bacterially expressed protein. Glutathione S-transferase (GST) tagged NR3 and, as a control, GST only were expressed in BL21 cells and purified by binding to glutathione conjugated sepharose 4B beads. Purified bound GST proteins were then digested with V8 protease and the size of the peptide fragments present in the supernatant solution following the reaction were analysed by SDS-PAGE and Coomassie staining (figure 3.2). A band corresponding to the size of GST (25.7kDa) was present in undigested GST control sample and GST control sample digested with 2.0µg/mL V8 protease (figure 3.2, lanes 1 and 3). However surprisingly the band is absent from the GST control sample digested with 0.2µg/mL V8 protease (figure 3.2, lane 2), however a band was observed on other occasions (data not shown). A further band is also present at ~45kDa in both undigested GST and GST-NR3 (figure 3.2, asterisk in lanes 1 and 4), which is likely to be a non-specific protein co-purifying from the bacterial lysate as its migration suggests it is too large to be GST. As this band is absent from digested samples, it is appears to be readily digested by V8 protease (figure 3.2, lanes 2, 3, 5 and 6).
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Figure 3.1 Prediction of Protein Disorder and Protease Site Mapping for NR3
The mouse NR3 protein sequence was used to predict the regions of disorder within the protein using the RONN server (http://www.strubi.ox.ac.uk/RONN). Peaks above 0.5 on the plot show regions predicted to be disordered. A schematic of the NR3 protein is overlaid highlighting where V8 protease substrate sites are present within regions of disorder (vertical black lines). The expected sizes of fragments following digestion are also shown.
Figure 3.2 Limited Proteolysis of GST-NR3
Immobilised GST proteins were digested with the indicated concentrations of V8 protease following expression in BL21 cells and purification by binding to glutathione conjugated sephrose 4B beads. The digested fragments present in the supernatant of GST (lanes 1-3) and GST-NR3 (lanes 4-6) samples were subjected to SDS-PAGE and Coomassie staining to analyse the size of fragments resistant to proteolysis. GST sample from lane 2 was not present in the experiment shown and was observed when the experiment was performed on other occasions. Asterisks denote non-specific bands from bacterial protein contamination.
Several bands can be observed in both undigested and digested GST-NR3 samples (figure 3.2, lanes 4, 5, and 6). In the undigested GST-NR3 sample distinct bands can clearly be seen at ~120kDa, which is likely to correspond to the full length GST-NR3 protein (118.3kDa), ~90kDa, which is likely to correspond to the full length NR3 with the GST tag cleaved (92.3kDa), ~75kDa, ~60kDa, the non-specific band at ~45kDa, ~40kDa and a band corresponding to the size of GST at ~25kDa (figure 3.2, lane 4). Following digestion with 0.2µg/mL V8 protease a decrease in the levels of the ~120kDa and ~90kDa bands, and no change in levels of the bands at ~75kDa and ~60kDa can be observed (figure 3.2, lane 4). In addition, an increase in the levels of the band at ~40kDa, a stabilisation of a band at ~55kDa and a band that appears to migrate at a size above that of GST at ~25kDa, giving the appearance of a smeared band (figure 3.2, lane 5). Following proteolysis with 2.0µg/mL V8 protease most bands present within the sample are digested with the exception of the bands at ~75kDa and ~60kDa, and the smeared band present between 25-30kDa (figure 3.2, lane 6). As there is no change in the levels of the ~75kDa and ~60kDa bands following digestion these are unlikely to represent NR3 fragments, and as such may be proteolytic resistant bacterial that are either contaminants or proteins that interact with NR3.

*In silico* analysis of the cleavage sites showed that if digestion occurred preferentially at substrate sites with regions predicted to be disordered, the LBD would reside in a 41.0kDa fragment (figure 3.1), which may correspond to the peptide fragment of ~40kDa observed following digestion. Furthermore, the predicted size of the putative LBD is 28.5kDa, thus it is possible that the smeared band observed between 25-30kDa in digested GST-NR3 samples (figure 3.2, lanes 5 and 6) may correspond to a fragment resulting from digestion at sites in close proximity to the predicted LBD boundaries. These data show there are a number of NR3 specific peptide fragments that are resistant to digestion with V8 protease, which suggests there are structured regions with NR3, including a region which may correspond to the size of the putative NR3 LBD.

### 3.2.2 Limited Proteolysis of NR3 Expressed in Mammalian Cells

The data generated from limited proteolysis in bacterially expressed protein provides evidence that there are structured domains resistant to proteolysis within NR3.
However, it is unclear as to where the boundaries of these domains reside and if the putative LBD region is one such domain. To map the regions of protease resistance V5 tagged NR3 was stably expressed in mammalian cells by transfection of pcDNA3.1-NR3-V5-6H, or empty vector as a negative control, into HEK-293 cells and selecting for transfected cells with G418. A protein extract was then taken and tagged NR3 purified by immunoprecipitation against the V5 tag. Immobilised tagged NR3 was then digested with V8 protease, and the fragments that remained bound to the protein-A agarose beads subjected to western blot against the V5 tag (figure 3.3). Comparison of mock samples to NR3-V5 samples show that the heavy and light chains of the antibody used for immunoprecipitation migrate at ~55kDa and ~23kDa respectively, and are resistant to digestion with V8 protease (figure 3.3, asterisk in all lanes). In addition a band at ~95kDa can also be observed, which is likely to be cross-well contamination from NR3 samples (figure 3.3, lane 4). Distinct bands specific to NR3 samples over mock samples can clearly be observed at ~95kDa, ~70kDa, and ~45kDa in undigested samples (figure 3.3, lane 5). Following digestion with increasing concentrations of V8 protease bands are also present at ~40kDa, ~18kDa and ~16kDa (figure 3.3, lanes 6-8).

As the V5 tag is present at the C-terminus of NR3, it can be used to map the protease resistant regions of NR3. If NR3 is digested either side of the putative LBD boundaries, fragments of 42.6kDa and 14.1kDa should be observed (figure 3.3). These are similar in size to the fragments observed at ~40kDa, ~18kDa and ~16kDa, which may mark the boundaries of a structured region in NR3 that corresponds to the putative LBD. The N-terminal boundary of the 41.0kDa fragment that is predicted to possess the putative LBD region following digestion with V8 protease as indicated from the NR3 disorder prediction (figure 3.1), would be expected to give a fragment of 55.6kDa in this limited proteolysis assay. This fragment is not observed here, which may be due to it being masked by the heavy chain of the antibody used for immunoprecipitation.

Taken together, the data from the limited proteolysis of NR3 expressed in bacterial and mammalian cells, and the in silico predictions of NR3 disorder, suggest the region that is predicted to possess a fold similar to that of a bona fide NR LBD is a structured and protease resistant domain. Therefore these data provide support for the
Figure 3.3 Limited Proteolysis of NR3-V5
Protein extracts from V5-tagged NR3 and mock stably transfected cells were subjected to Immunprecipitation, and immobilised samples were digested with the indicated concentrations of V8 protease. The digested fragments remaining on the beads in mock (lanes 1-4) and NR3-V5 (lanes 5-8) samples were then subjected to western blot, probing for V5 to determine their size. Asterisks denote bands produced from the antibody used in the immunoprecipitation step. Cartoon depicts the predicted sizes of V5 containing fragments following V8 digestion either side of the LBD and in the regions of disorder closest to the LBD boundaries (vertical lines).
idea that the NR3 LBD region is a candidate for crystallography to determine its structure, which would provide strong evidence either supporting or against the idea that NR3 possess a NR-like LBD.

3.3 Screening for NR3 Ligands

The preliminary structural work presented above suggests there is an ordered protease resistant region spanning the amino acids predicted to be an NR-like LBD within NR3. It is possible therefore that this region does possess a fold similar to that of an NR LBD and as such may bind ligand. To try to identify a ligand an assay was optimised and used to screen a library of synthetic compounds at Inpharmatica.

3.3.1 Construction of Expression Vectors for Ligand Screening

To screen the compound library of Inpharmatica, a heterologous reporter gene assay system was used that utilised fusion proteins possessing a GAL4 DBD fused to VP16 activation domain. The VP16 activation domain provides constitutive transcriptional activation of the reporter gene (Sadowski et al., 1988), allowing the screening of compounds to identify both agonists and inverse agonists and not relying on the supposition that the putative NR3 LBD is able to recruit coactivators. Three fusion proteins were used named VP16-GAL4-NR3, VP16-GAL4-ER and VP16-GAL4-CTRL. These were generated by PCR amplifying DNA fragments coding for the GAL4 DBD, the mouse ERα LBD and a multiple cloning site, and ligating in frame with VP16 in a pSG5 vector backbone (figure 3.4A). The putative NR3 LBD was also amplified by PCR and ligated in frame with VP16-GAL4 in both the correct and incorrect orientations replacing the ERα LBD (figure 3.4B). Ligating DNA coding for the NR3 LBD region in the incorrect orientation to that coding for VP16-GAL4 produces the VP16-GAL4-CTRL fusion protein, which codes for 11 amino acids, LGVFLAVLHSL, downstream of the GAL4 DBD as a stop codon is present in the position corresponding to the 12th amino acid (figure 3.4B). VP16-GAL4-ER serves as a positive control for the system able to be activated or repressed by ligand, and VP16-GAL4-CTRL serves as a negative control as it represents basal activation of the system by VP16.


**Figure 3.4 Schematics of VP16-GAL4 Fusion Proteins used for Screening**

A) A fragment generated by PCR from pSG5-GAL4-ER containing the GAL4 DBD fused to the mouse ERα LBD was cloned into pSG5-VP16 to create pSG5-VP16-GAL4-ER. B) pSG5-VP16-GAL4-NR3 and pSG5-VP16-GAL4-CTRL were produced by subcloning the appropriate LBDs into pSG5-VP16-GAL4-ER digested with the appropriate enzymes. Underlined text denotes the enzymes used for subcloning.
3.3.2 Transcriptional Activity of VP16-GAL4 Fusion Proteins

Transfection of VP16-GAL4-ER and VP16-GAL4-CTRL into HEK-293 cells produced a 9.5-fold and 6-fold increase in transcriptional activity of the reporter gene over mock transfected cells in the absence of ligand (figure 3.5), a response that was dependent on the amount of construct transfected (data not shown). The level of transcriptional activation remained unchanged upon treating cells expressing VP16-GAL4-CTRL with 10nM 17β-oestradiol, however as expected such treatment enhanced the activity of VP16-GAL4-ER a further 29-fold (figure 3.5).

Surprisingly, cells transfected with VP16-GAL4-NR3 did not produce a constitutive level of transcriptional activation, and as expected remained unresponsive to 10nM 17β-oestradiol administration (figure 3.5). A level of activity similar to that seen with untreated VP16-GAL4-ER and VP16-GAL4-CTRL expressing cells would have been expected, yet the level of activity was similar to that of mock transfected cells. Thus it is clear the putative LBD of NR3 suppresses transcription when fused to a GAL4 DBD and VP16 activation domain, and expressed in a heterologous reporter gene assay system. In order to activate the system, the fusion proteins must be translocated to the nucleus. It is possible that the NR3 LBD prevents the nuclear localisation of the fusion protein, or that VP16-GAL4-NR3 is not expressed to high levels when transfected into cells.

3.3.3 Subcellular Localisation of VP16-GAL4 Fusion Proteins

To determine the localisation of the fusion proteins, HEK-293 cells were transfected with the appropriate VP16-GAL4 construct, treated with 17β-oestradiol, subjected to immunostaining with anti-GAL4 DBD antibody and co-stained with DAPI. However, no staining could be observed with the anti-GAL4 DBD antibody (data not shown), which may be due to the fusion proteins being expressed at a level below the detection limit of the antibody. To address this, the assay was repeated in COS-1 cells (figure 3.6). COS-1 cells have been immortalised with SV40 virus and express large T antigen. Due to the presence of the SV40 origin of replication in the pSG5 vector backbone, large T antigen expressing cells will replicate the plasmid
Figure 3.5 Luciferase Response to Expression of VP16-GAL4 Fusion Proteins in a Heterologous Reporter Gene Assay System
HEK293 cells were cotransfected with pGL3-GAL4 reporter, pRL-TK internal control reporter, and the appropriate VP16-GAL4 construct and incubated in the absence (open bars) or presence (closed bars) of 10nM 17β-oestradiol. Responses are fold induction of unstimulated mock transfected cells. Error bars represent standard deviation between duplicate samples.
Figure 3.6 Immunostaining of VP16-GAL4 Fusion Proteins
COS-1 cells were transfected with the appropriate VP16-GAL4 fusion protein, treated for 24 hours with 10nM 17β-oestradiol, and subjected to immunostaining (first and third panels). Nuclei were counterstained with DAPI (second and fourth panels). Arrows denote nuclei of stained cells.
(Gluzman, 1981), inducing higher expression of the gene of interest. Mock transfected cells showed no staining, as did cells transfected with VP16-GAL4-CTRL (data not shown). Cells transfected with pSG5-VP16-GAL4-ER possess a diffuse staining throughout the whole cell, whereas cells transfected with VP16-GAL4-NR3 show the staining is mainly excluded from the nucleus (figure 3.6). The staining patterns for both fusion proteins were unaltered following treatment with 10nM 17β-oestradiol, a result that is concordant with previous studies investigating the cytosolic and nuclear distribution of ERα (Picard et al., 1990).

To confirm these results, expression and localisation was examined by determining the distribution of the fusion proteins in cytosolic and nuclear fractions from a nuclear extract. HEK-293 cells were transfected with the appropriate construct and a nuclear extract generated. Nuclear and cytosolic fractions were then subjected to western blot with anti-GAL4 DBD antibody. Anti-RNA polymerase II and anti-GAPDH antibodies were also used to assess cross fraction contamination (figure 3.7). The anti-GAL4 DBD antibody gave a specific band at the correct sizes for VP16-GAL4-ER (58.4kDa) and VP16-GAL4-NR3 (54.5kDa). A non-specific band was also seen in the nuclear fractions, as indicated by its presence in samples derived from mock transfected cells (figure 3.7, asterisk). VP16-GAL4-CTRL could not be detected (27.1kDa), which is concordant with immunostaining experiments.

Examination of VP16-GAL4-NR3 protein levels show that the construct is expressed, however it is enriched in the cytosolic fraction over the nuclear fraction. Contrastingly VP16-GAL4-ER appears to be evenly distributed between the cytosolic and nuclear fractions, which was as expected (Picard et al., 1990). Examination of the controls shows there is low level contamination between the fractions (figure 3.7). These data highlight that NR3 appears to be largely absent from the nucleus and as such are in accordance with the data generated by immunostaining of transfected cells. This absence of high protein levels within the nucleus could account for the lack of activity observed in reporter assays.

### 3.3.4 Screening Ligands for NR3

The difference in transcriptional activation of the GAL4 reporter gene observed between cells expressing the control fusion proteins, and the cells expressing VP16-
Figure 3.7 Subcellular Localisation of VP16-GAL4 Fusion Proteins
HEK-293 cells were transfected with the appropriate VP16-GAL4 fusion protein or empty vector (mock) and subjected to nuclear extract. The distribution between the cytosolic (C) and nuclear (N) fractions of each fusion protein was determined by western blotting. PolII and GAPDH were also blotted for as controls for cross contamination. Arrows denote bands corresponding to fusion proteins, or for VP16-GAL4-CTRL, the expected size of the protein. Asterisks denote non-specific bands in the nuclear fraction.
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GAL4-NR3 provided the opportunity to use the system to screen for potential ligands. This is based on the assumption that a ligand binding to the putative NR3 LBD could induce a conformational change that prevents the retention of the fusion protein within the cytosol, allowing translocation to the nucleus and transcription of the reporter gene. Inpharmatica possess a compound library that was screened using the developed system to determine whether a synthetic compound could induce an alteration in NR3 LBD associated transcription.

In total 2320 compounds were screened individually at a concentration of 10µM over 29 assay plates. Test wells contained cells transfected with VP16-GAL4-NR3 that were then treated with test compound, and control wells consisted of either low signal controls provided by cells transfected with VP16-GAL4-NR3 and left untreated, or high signal controls that were cells transfected with VP16-GAL4-CTRL, and again left untreated. Each assay plate consisted of 8 low signal control wells, 8 high signal control wells and 80 test wells. Assay plates were analysed with z-prime statistic, which is a standard method of assessing the quality of high throughput screening data (Zhang et al., 1999). It compares the mean values of the low signal controls to the mean value of the high signal controls taking into account the variation of each. A high ratio and low variation between the sets of controls produces a high z-prime statistic. A perfect assay returns a z-prime statistic of 1, and a poor assay produces a z-prime statistic of below 0.5. Of the 29 plates screened 24 plates returned a z-prime statistic of greater than 0.5.

Of the 2320 compounds screened, 89 compounds produced hits. That is they gave a greater value than a threshold value of 3 standard deviations above the mean of the low signal controls (data not shown). Of the 89 hits generated, the 15 greatest hits, which all produced responses more than 6 standard deviations above the mean of the low controls, were taken forward for further screening. 12 of these compounds were assayed on plates scoring a z-prime statistic of above 0.5 in the primary screen, and 3 compounds were assayed on a plate scoring 0.39. However, these compounds were still further investigated due to their responses being over 6 standard deviations above the mean of the low signal controls.

For a secondary screen, compounds were screened at increasing concentrations in either mock transfected cells or cells transfected with VP16-GAL4-NR3 (figure 3.8). Compounds 1C1, 1G1, 2H2, 2H8, 1A4, 2B9, 1H1, 2D9, 2C2, 1F2 and 1C2
Figure 3.8 Luciferase Response in a Secondary Screen for Hit Compounds
Cells were cotransfected with pGL3-GAL4 firefly luciferase reporter, pRL-TK renilla luciferase reporter internal control, and either empty pSG5 vector (open bars) or pSG5-VP16-GAL4-NR3 (closed bars), and subsequently treated with increasing concentrations of compound. Each experiment was performed three times, except for compound 1C1 which was performed twice. Error bars show the standard deviation between the experiments.
produced a dose dependent increase in reporter gene response in both mock and VP16-GAL4-NR3 transfected cells. Compound 2C8 produced a biphasic response in both mock transfected cells and cells expressing VP16-GAL4-NR3. The reduced response when cells were treated with 30mM 2C8 is possibly due to cell death, as the firefly and renilla luciferase values are greatly reduced (data not shown). Compounds 2F2, 2C4 and 2B8 had little effect on the luciferase response observed in both mock and VP16-GAL4-NR3 transfected cells, producing a flat response in both. As the responses observed for all compounds were not significantly increased in VP16-GAL4-NR3 transfected cells over mock transfected cells, it may only be concluded that any increase seen in a dose dependent manner is independent of VP16-GAL4-NR3 expression and thus an artefact of the assay system.

3.4 Summary and Conclusions

Two approaches were taken to investigate whether the region of the NR3 protein was indeed a LBD. Firstly, preliminary structural work was performed to begin to determine whether the putative NR3 LBD possesses a fold similar to that of bona fide NRs. In silico predictions suggest that NR3 is an overall structured protein with little disorder, which would indicate NR3 may possess domains that are resistant to proteolytic digestion. By performing limited proteolysis on the NR3 protein expressed in both bacterial and mammalian system it was shown that there are clearly a number of domains that were resistant to proteolysis, and mapping studies showed that the LBD region may be one such domain. Therefore, the LBD region of NR3 represents a good candidate for further structural work by X-ray crystallography.

In addition, a screen was performed to identify a synthetic ligand that may activate NR3 in a luciferase reporter gene system. It was shown that the NR3 LBD suppresses activity of a fusion protein in such a system, which may be explained by the exclusion of the VP16-GAL4-NR3 fusion protein from the nucleus. This system was used to screen a compound library provided by Inpharmatica based on the principle that a ligand binding to the NR3 LBD may induce a conformational change such that the inhibitory effect of the LBD is abrogated and the fusion protein may translocate to the nucleus to drive transcription of the reporter gene. Following the screening of 2320 compounds no ligand specific to NR3 activated the reporter system, and as yet a ligand remains to be found.
Chapter 4

Screening for Binding Partners and Assessing the Function of NR3
4.1 Introduction

NR3 is reported to function within the signalling pathways stimulated by TNFR1 (Soond et al., 2003; Soond et al., 2006). To address the function of NR3, experiments investigating the involvement of NR3 in TNF signalling were repeated here. In addition, an unbiased approach was taken to further characterise the biological function of NR3 by screening for interacting proteins. Identification of interacting proteins may place NR3 in another characterised signalling pathway, and as such the functional significance of the protein interactions was investigated.

4.2 Effect of Overexpression of NR3 on TNF Signalling

NR3 has been described as a scaffold protein that interacts with TNFR1 and members of the TNFR1 signalosome independent of receptor activation. Upon stimulation with TNFα, overexpression of NR3 results in a potentiation of NF-κB signalling in reporter gene assays (Soond et al., 2003). The authors showed that responses elicited by NR3 were dose dependent in transiently transfected cells. In preliminary experiments performed here, HEK-293 cells were transiently transfected with an increasing amount of NR3 expression vector and treated with TNFα in NF-κB reporter gene assays. No evidence of a dose dependent increase in NF-κB activation could be demonstrated in the absence or presence of TNFα (figure 4.1A). The reporter gene assay was then repeated in NIH/3T3 cells, as this cell type was used for such assays by others (Soond et al., 2003). These results were concordant with the data obtained in HEK-293 cells, although the overall magnitude of response produced was much lower (figure 4.1B). In addition, the interaction between NR3 and TNFR1 was investigated by immunoprecipitation of from HEK-293 cells stably expressing V5-tagged NR3. However, TNFR1 could not be detected in immunoprecipitates (figure 4.1C). In view of the failure of the preliminary data presented here to recapitulate those reported by Soond et al (2003), an approach was employed that presumes no prior function for NR3 by screening for binding partners. Yeast two-hybrid and immunoprecipitation based assays were used to identify NR3-interacting proteins.
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Figure 4.1 Effect of NR3 Expression on NF-κB
A and B) HEK-293 cells (A) and NIH/3T3 cells (B) were co-transfected with NF-κB firefly luciferase reporter, renilla luciferase reporter as an internal control and increasing amounts of NR3 expression construct using Fugene 6 (HEK-293) or Lipfectamine 2000 (NIH/3T3), and incubated in the absence (open bars) and presence (closed bars) of 10ng/mL TNFα. As a positive control NIH/3T3 cells were co-transfected with 125ng p65 in place of NR3. Responses represent fold induction over unstimulated mock transfected cells (0ng). Error bars show the standard deviation between four replicates.

C) NR3 tagged with V5 was immunoprecipitated from HEK-293 cells stably transfected with NR3-V5 or empty vector as a negative control, and probed in a western blot for V5 (upper blot) and TNFR1 (lower blot). Arrowheads denote band specific to TNFR1.
4.3 Screening for Binding Partners by Yeast Two-Hybrid

The CytoTrap yeast two-hybrid system is based on the SRS system (Aronheim et al., 1997). This system fuses the bait protein to human SOS and the prey protein to a myristylation signal that targets the protein to the yeast cell membrane. The host strain is the temperature sensitive mutant *S. cerevisiae* strain cdc25H. This strain possesses a point mutation at amino acid 1328 of cdc25 protein, which inhibits growth at 37°C due to a deficiency in the Ras signalling pathway (Petitjean et al., 1990). An interaction between the bait and prey proteins results in recruitment of the bait SOS fusion protein to the yeast cell membrane where SOS can activate the Ras pathway and stimulate growth at the non-permissive temperature of 37°C (figure 4.2). Screening for binding partners can be performed by expressing both a prey protein from a cDNA library and a bait protein in cdc25H cells. Selection for protein-protein interactions is performed by incubating transformed yeast cells at 37°C and screening for growth of colonies.

4.3.1 Construction of the cDNA Library

When performing a yeast two-hybrid screen for protein-protein interactions it is important to use a cDNA library that incorporates a large number of genes that encode full length or long length cDNAs in order to express prey fusion proteins capable of interacting with the bait protein. It is also important the use a cDNA library in which there is as little over-representation or under-representation of genes as possible to allow genes that are not as highly expressed as others to be included in the screen.

To generate prey cDNA libraries that represented a large number of genes double stranded 32P-labelled cDNA was synthesised from mRNA extracted from tissue derived from a number of murine embryos and placentas combined together to generate one library, and also from murine brain to generate a second library. The cDNA was size fractionated in to 12 fractions to prevent an enrichment of very small fragments in the library, and thus ensuring full length and long length cDNAs were used for library construction. A peak of radioactivity corresponding to the 32P-labelled cDNA was present in fractions 3-7 for the brain sample and 4-8 for the
Figure 4.2 Principle of Screening for Protein Interactions in the SRS system
Prey proteins are tethered to the cell membrane via a myristylation signal to which they are fused. Interacting bait proteins may rescue phenotype of the temperature cdc25H yeast strain through their fusion to human SOS (hSOS), which activates the Ras pathway, permitting growth at the selective temperature of 37°C.
embryonic/placental sample. Unincorporated $^{32}$P-dATP used for cDNA synthesis was present in fractions 11-12 for both samples, suggesting these fractions possessed little cDNA or very small fragments of cDNA.

Pilot reactions to ligate the cDNA into the pMyr XR vector were performed for each fraction and transformed into bacteria to determine the transformation efficiency. Brain fractions 3-10 and embryonic/placental fractions 4-10 were used to ensure no cDNA was excluded. The number of CFU per fraction was calculated, which estimates the number of clones in each fraction and as such provides a guide as to the number of cDNA fragments ligated into the pMyr XR vector in each fraction (table 4.1). These data allow the downstream library generation and amplification to be normalised, preventing the overrepresentation of genes in fractions with higher CFU within the library. As a negative control for the reactions a ligation was performed without a cDNA insert, which showed the background to the ligation reactions was $1\times10^2$ CFU. Fractions 3-7 in brain samples and 5-8 in embryonic/placental samples possessed between $1.5\times10^3$-$1\times10^4$ CFU (table 4.1, columns 1 and 2), and were used for downstream processing. Fraction 10 for the brain samples was not used because although it possessed $1.3\times10^3$ CFU it is likely to contain very small fragments.

Selected fractions were processed further by scaling-up ligations and transformations normalising the amount of DNA used for ligation based on the observed CFU for each fraction. The number of CFU resulting from each scaled-up reaction was then determined to assess the success of the normalisation process. This showed that brain fractions were reasonably consistent and were between $1\times10^4$ and $1.6\times10^4$ CFU (table 4.1, column 3). The embryonic/placental samples showed more variation, fractions 5-8 were between $7.5\times10^3$ and $1.2\times10^4$ and the ligation from fraction 4 possessed a CFU of only $5\times10^3$, however as all the cDNA was used in the ligation reaction it is likely that this is representative of the fraction not being enriched with cDNA (table 4.1, column 4).

The scaled-up transformations were then pooled and plated to allow transformed bacterial colonies to grow. The number of CFU in the pooled bacteria was again determined to assess the number of clones each library is generated from, which indicates the number of cDNA fragments present in each library. Pooling and plating transformation reactions also acts as an amplification step as the colonies are then harvested and the library DNA prepared. The brain library possessed $8.4\times10^4$ CFU
Table 4.1 CFU of Transformation Reactions for Constructing cDNA Libraries

Size fractionated cDNA inserts were generated from mRNA derived from brain or a combination of embryonic and placental tissue, ligated into the pMyr XR vector, and transformed into bacteria. Small scale pilot reactions were performed to estimate the number of colony forming units (CFU) in each fraction (columns 1 and 2), and reactions were normalised and scaled-up to prevent over representation of genes in fractions with a higher CFU value. The CFU of normalised larger-scale reactions were again estimated to assess the level of variation in CFU among the fractions (columns 3 and 4).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CFU for Pilot Reactions</th>
<th>CFU for Normalised Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Embryonic/Placental</td>
</tr>
<tr>
<td>3</td>
<td>6.2x10^3</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>5x10^3</td>
<td>2x10^2</td>
</tr>
<tr>
<td>5</td>
<td>4x10^3</td>
<td>3.2x10^3</td>
</tr>
<tr>
<td>6</td>
<td>1x10^4</td>
<td>8.2x10^3</td>
</tr>
<tr>
<td>7</td>
<td>6.2x10^3</td>
<td>4.6x10^3</td>
</tr>
<tr>
<td>8</td>
<td>7x10^2</td>
<td>1.5x10^3</td>
</tr>
<tr>
<td>9</td>
<td>6x10^2</td>
<td>7x10^2</td>
</tr>
<tr>
<td>10</td>
<td>1.3x10^3</td>
<td>6x10^2</td>
</tr>
</tbody>
</table>
and the embryonic/placental \(3.5 \times 10^4\) CFU, indicating the libraries possess \(8.4 \times 10^4\) and \(3.5 \times 10^4\) cDNA fragments as inserts respectively. However, the fragments were cloned into the pMyr XR vector directionally as outlined in chapter 2.2.4. Therefore there is a 1 in 3 probability of an insert being in the correct reading frame with the myristylation signal. This indicates that there are \(2.8 \times 10^4\) and \(1.2 \times 10^4\) functional prey clones within the brain and embryonic/placental libraries respectively.

To examine the average insert size of each library colony PCR reactions were performed (figure 4.3). Both libraries possessed an average insert size of ~1kb, which ranged from 200bp to 4kb for the brain library, and 200bp to 2.5kb for the embryonic/placental library. cDNA libraries generated from mouse brain or embryonic tissue that are compatible for protein-protein interaction screening using the CytoTrap yeast two-hybrid method were not available from Stratagene at the time of library construction, however such products are now available and may be compared to the libraries generated here. Stratagene’s brain library possesses an average insert of 1.9kb and a CFU of \(5.9 \times 10^6\), and the embryonic library possesses an average insert of 1.1kb and a CFU of \(3.95 \times 10^6\). These data indicate that the libraries generated here possess around 100-fold less cDNA inserts than the Stratagene libraries, however the average insert length is comparable between to those present in the libraries offered by Stratagene.

### 4.3.2 The NR3 LBD Interacts with 14-3-3η in a Yeast Two-Hybrid Assay

To identify proteins that potentially interact with NR3, SOS-NR3 and SOS-NR3 LBD were used as bait proteins in the yeast two-hybrid system to screen against the constructed libraries. Yeast cells were transformed in a 2:1:1 ratio of SOS fusion protein DNA, brain library DNA and embryonic/placental library DNA and cultured as outlined in chapter 2.2.4. Cells transformed with SOS-NR3 provided 124 colonies and SOS-NR3 LBD provided 82 colonies that grew in non-permissive conditions, which were patched on to plates for primary and secondary tests of interaction as outlined in chapter 2.2.4. 11 full length NR3 colonies and 31 NR3 LBD colonies passed these tests. cDNA library plasmid DNA from the positive candidates was then isolated and transformed into chemically competent DH5α bacteria to enable
Figure 4.3 Average Insert of cDNA Libraries used for Two-Hybrid Screening
Transformed bacterial colonies generated from normalised large-scale cDNA ligation reactions were subject to colony PCR to determine the average and range of insert size in both embryonic/placental and brain libraries. The average insert can be estimated at around 1kb for both libraries, and the range of insert sizes appears to be between 0.2-2.5kb and 0.2-4.0kb for the embryonic/placental and brain libraries respectively.
miniprep DNA to be generated. Miniprep DNA was then sequenced to identify the cDNA library insert, the results of which are outlined in table 4.2.

In the screen against SOS-NR3, surprisingly only 3 of the 11 candidates possessed a cDNA insert in frame with the myristylation signal within the pMyr XR vector and are fragments that correspond to coding regions of the mRNAs from which they are derived. These candidates were then co-transformed into yeast cells in a 1:1 ratio with SOS-NR3 to verify the interaction within the two-hybrid system. No growth was observed at non-permissive temperatures, suggesting there was no interaction between the candidates and SOS-NR3 (table 4.2). Stratagene state that the phenotype of the cdc25H yeast cells may revert and allow growth at 37°C. It is possible that the positive results for the 11 identified colonies observed in the screen against SOS-NR3 were due to such a reversion. However, in the time intervening between the screen of full length NR3 and the screen of the NR3 LBD, it became apparent that due to the nature of the co-transformation procedure more than one prey plasmid may be taken up by yeast cells. When screening the NR3 LBD for interacting proteins, plasmids prepared from yeast cells were subject to PCR to identify those colonies with more than one prey plasmid. Most colonies appeared to possess a single construct, however some colonies possess up to four (figure 4.4). It is therefore plausible that in the 11 colonies selected from the full length NR3 screen there were more than one prey plasmid, and thus it may have been another unidentified plasmid that stimulated growth. These data allow the conclusion to be drawn that the 11 prey proteins identified did not interact with NR3, however it is not clear whether the reason for growth of colonies at the non-permissive temperature is due to a phenotypic reversion or due to the presence of a non-identified prey plasmid.

For the NR3 LBD screen, prey plasmids isolated from candidates were sequenced, identifying inserts from candidates possessing a single plasmid or multiple plasmids. Of the 31 clones tested 29 were identified as containing a SOS prey plasmid. These were regarded as false positives as direct recruitment of SOS to the cell membrane would bypass the two-hybrid system and activate the Ras pathway, allowing growth at the non-permissive temperature. Two clones were not regarded as false positives and possessed inserts of Flt 3 interacting zinc finger protein 1 (BC106113) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, η polypeptide (BC061497), the latter of which is identical to 14-3-3η.
Table 4.2 The Putative LBD of NR3 Interacts with 14-3-3ζ in a Yeast Two-Hybrid Screen

Full length NR3 (upper rows) and the LBD of NR3 (lower rows) were fused to human SOS and used for screening against the constructed cDNA libraries in the CytoTrap yeast two-hybrid system to search for interacting proteins. The library plasmid DNA was recovered and sequenced for each potential positive result to determine the identity of the candidate (column 1), the reading frame of the insert, and whether it corresponded to the coding region of the cDNA (column 3, Y=Correct Reading Frame, N=Incorrect Reading Frame, 3’UTR=insert corresponds to the 3’ untranslated region of the cDNA). Candidates in frame with the myristylation signal and representing the coding region of the cDNA were re-screened in the system to assess the validity of the interaction (column 4, Y=Positive interaction, N=Negative interaction). No candidate was shown to robustly interact with full length NR3. Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase Activating Protein, which is also known as 14-3-3ζ, was found to interact with the NR3 LBD in this system.

<table>
<thead>
<tr>
<th>Identity of Candidate</th>
<th>Accession</th>
<th>Reading Frame</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Follistatin-like 1</td>
<td>NM_007085</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>2. Alpha Tubulin 2</td>
<td>NM_011654</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>3. Hypothetical Domain of Unknown Function DJF143 Containing Protein</td>
<td>AK010366</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4. Secreted Phosphoprotein 1</td>
<td>NM_009263</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>5. Scavenger Receptor Class B, Member 1 (Scarb1)</td>
<td>NM_016741</td>
<td>3’UTR</td>
<td></td>
</tr>
<tr>
<td>6. Niemann Pick Type C2 (Npc2)</td>
<td>NM_023409</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>7. Similar to Ribosomal Protein S2</td>
<td>XM_001477049</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>8. Cyclin D1</td>
<td>NM_007631</td>
<td>3’UTR</td>
<td></td>
</tr>
<tr>
<td>9. Similar to Rpt136 Protein</td>
<td>XM_001480807</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>10. NADH Dehydrogenase (Ubiquinone) 1 alpha Subcomplex</td>
<td>NM_010886</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>11. Cytotoxic T Lymphocyte-associated Protein 2 alpha</td>
<td>NM_007796</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>14. FLT3 Interacting Zinc Finger Protein 1</td>
<td>BC106113</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>20. Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase Activating Protein</td>
<td>BC061497</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Figure 4.4 PCR of Prey Plasmid Inserts from Zymoprep DNA

Prey plasmid DNA was prepared from yeast colony candidates from the yeast two-hybrid screen against the NR3 LBD and the plasmid inserts were amplified by PCR to identify the number of prey plasmids within each candidate. Arrow denotes 1kb.
frame with the myristylation signal (table 4.2). Upon co-transformation of the positive candidates into cdc25H yeast cells and testing for interaction at the non-permissive temperature of 37°C, 14-3-3\(\eta\) provided a positive result and thus appears to interact with NR3 in the CytoTrap yeast two-hybrid system, however an interaction between NR3 and Flt 3 interacting zinc finger protein 1 could not be confirmed (table 4.2).

### 4.3.3 Verification of the Interaction between NR3 and 14-3-3\(\eta\)

An interaction between proteins in a yeast two-hybrid system suggests the proteins are likely to directly interact. To verify this between NR3 and 14-3-3\(\eta\), a GST pulldown assay was used to examine whether NR3 or the NR3 LBD and 14-3-3\(\eta\) may directly interact in vitro. GST-NR3, GST-NR3 LBD or GST alone as a negative control were used in conjunction with in vitro synthesised radiolabelled 14-3-3\(\eta\) in a GST pulldown assay as outlined in chapter 2.2.3. 14-3-3\(\eta\) did not co-purify with GST, GST-NR3 or GST-NR3 LBD in the pulldown (figure 4.5A, upper panel), suggesting NR3 and 14-3-3\(\eta\) do not interact directly. It is possible that the lack of interaction could be due to a low level of GST fusion protein in the assay. As a control, purified GST proteins were subjected to SDS-PAGE and Coomassie staining to assess the levels of expression and purification (figure 4.5A, lower panel), which shows relatively even loading and that the proteins are expressed to a level detectable by Coomassie staining, thus it is unlikely that the lack of interaction is due to low levels of GST fusion protein.

It is possible that an interaction in a yeast two-hybrid screen is indirect, as the interaction may be mediated by proteins expressed endogenously in the host cell. To further examine the potential interaction between NR3 and 14-3-3\(\eta\) in vivo protein extracts from HEK-293 cells stably expressing NR3 tagged with V5 were used in immunoprecipitation experiments, followed by western blotting to examine whether 14-3-3 proteins co-precipitated with NR3. HEK-293 cells stably transfected with empty vector were used as a negative control. Immunoprecipitation with anti-V5 antibody clearly purified NR3-V5 (figure 4.5B, upper panel), however there is no evidence that members of the 14-3-3 family of proteins co-precipitate with NR3-V5, as probing the blot with an anti-14-3-3 antibody that detects all family members provides no signal in immunoprecipitated samples (figure 4.5B, lower panel). This
Figure 4.5 NR3 Does Not Interact with 14-3-3η in GST Pulldown and Immunoprecipitation Assays

A) GST, GST-NR3 and GST-NR3 LBD were incubated with radiolabelled 14-3-3η, interacting complexes purified, and subject to SDS-PAGE. 14-3-3η did not co-purify with the GST fusion proteins to a detectable level (upper panel). GST fusion protein loading was assessed by Coomassie staining (lower panel). Arrow heads denote bands that correspond to the expected sizes of GST fusion proteins.

B) Immunoprecipitations against V5 were performed in HEK-293 cells stably transfected with empty vector (mock) or NR3-V5. Precipitates were subject to western blotting with anti-V5 antibody and an antibody that recognises all members of the 14-3-3 protein family. Co-purification of 14-3-3 proteins with NR3-V5 was not detected.
suggests an interaction between NR3 and 14-3-3 in vivo, direct or indirect, may not be detectable. Coupled with the data provided by the GST pulldown experiments, these data suggest that NR3 does not interact with 14-3-3 in vitro or in vivo, which in turn indicates the interaction observed in the yeast two-hybrid screen may be an artefact of the system.

4.4 Screening for Binding Partners for NR3 by Immunoprecipitation

Screening for NR3-interacting proteins was also approached by utilising V5 tagged NR3 in immunoprecipitation experiments. Co-precipitating proteins can be isolated from samples and identified by mass spectrometry.

4.4.1 NR3 Interacts with DDB1

NIH/3T3 cells were transfected with empty vector as a negative control, or DNA coding for NR3-V5, NR3_{477-797}-V5 or NR3_{1-722}-V5. 42 hours later the cells were treated with 10ng/mL TNFα for 6 hours as NR3 has been implicated in TNF signalling (Soond et al., 2003; Soond et al., 2006), and stimulation of these pathways may effect the recruitment of proteins to NR3. Protein extracts were then generated and immunoprecipitations performed using an antibody recognising the V5 tag. Precipitates were subject to SDS-PAGE and silver staining. Numerous bands were present in the silver-stained gel, however the majority of these correspond to proteins that co-purified non-specifically in the precipitation as they are present in mock samples transfected with empty vector (figure 4.6A, lanes 1 and 5). In addition, the pattern of the bands observed was unaffected by treatment with TNFα (figure 4.6A, lanes 1-4 compared to lanes 5-8). Specific bands corresponding to the expected sizes of the different tagged NR3 constructs expressed in the cells were present at ~95kDa, ~40kDa and ~80kDa for NR3-V5, NR3_{477-797}-V5 and NR3_{1-722}-V5 respectively (figure 4.6A, lanes 2-4 and 6-8). In addition, a band was observed at ~130kDa in NR3-V5 and NR3_{1-722}-V5 precipitates that appeared to co-purify at stoichiometric levels. This band was absent from mock and NR3_{477-797}-V5 precipitates (figure 4.6A, all lanes). These data therefore suggest the ~130kDa protein interacts in stoichiometric amounts with the N-terminus of NR3, and is not effected by treatment with TNFα.
Figure 4.6 NR3 Interacts with DDB1

A) NIH/3T3 cells were transfected with empty vector or V5 tagged NR3 constructs and treated with TNFα for 6 hours. Fusion proteins were immunoprecipitated using the V5 tag and subject to SDS-PAGE and silver staining. Arrowheads denote immunoprecipitated NR3 constructs and asterisks denote bands corresponding to antibody heavy chains (~50kDa) and light chains (~25kDa). Red ellipses denote a co-precipitated protein that was identified as DDB1 by mass spectrometry.

B) Immunoprecipitation with anti-V5 antibody was performed using protein extracts from HEK-293 cells stably expressing NR3-V5 or stably transfected with empty vector (mock). Precipitates were then subject the western blot with anti-V5 and anti-DDB1 antibodies.
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The silver-stained protein bands at ~130kDa were excised from the gel and subject to quadruple time-of-flight mass spectrometry. Numerous peptide fragments were identified, however only one fragment was regarded as a significant hit. This peptide fragment had the sequence VTLGTQPTVLR, which corresponds to a 127kDa protein named damage-specific DNA-binding protein (DDB) 1, as identified by protein BLAST searches. DDB1 is a protein that possesses multiple domains and acts as a substrate recognition subunit of an E3 ubiquitin ligase complex that is reported to be involved in regulation of the cell cycle and nucleotide excision repair (NER) of DNA damaged by UV light (Bondar et al., 2006; Hayes et al., 1998; Li et al., 2006a). These data therefore implicate NR3 in one or both of those processes.

To confirm that DDB1 co-precipitates with NR3, immunoprecipitation experiments were also performed in HEK-293 cells stably transfected with NR3-V5 or empty vector as a negative control. Following immunoprecipitation, western blotting was performed. Control blots with anti-V5 show the presence of NR3-V5 in precipitated samples (figure 4.6B, upper panel). Probing with anti-DDB1 antibody detects a band at ~130kDa in NR3-V5 precipitates, which is absent from mock precipitates (figure 4.6B). Coupled with the data generated by the silver-staining experiments, this supports the notion that NR3 interacts with DDB1 as NR3-V5 and DDB1 co-purify in immunoprecipitates from both HEK-293 cells and NIH/3T3 cells.

4.5 Mapping the Interaction between NR3 and DDB1

DDB1 is a member of an E3 ubiquitin ligase complex, and is responsible for the recruitment of substrates and substrate adaptors to the complex. The interaction between NR3 and DDB1 leads to the hypothesis that NR3 may be either a substrate for ubiquitination, an adaptor molecule involved in the recruitment of substrates, or a regulator of the process. Such functions may not necessarily be mutually exclusive and therefore NR3 may fulfil more than one of these roles. Characterisation of the interaction between DDB1 and NR3 will provide a better understanding of the nature of the interaction and may indicate the likely consequence of such an interaction.

X-ray crystallographic studies of DDB1 when sequestered by the V protein of the simian virus 5 showed that DDB1 possesses four domains, there are 3 β-propeller domains named A-C (BPA, BPB, BPC), and an α-helical C-terminal domain (CTD) (figure 4.7) (Angers et al., 2006; Li et al., 2006b). The BPA and BPC domains form a
Figure 4.7 Crystal Structure of DDB1 and Interacting Proteins

A) Crystal structure of DDB1 showing the three $\beta$-propeller domains (BPA, BPB and BPC) and $\alpha$-helical C-terminal domain (CTD) (protein data bank ID: 2B5M; Li et al., 2006).

B) Crystal structure of DDB1 in the reverse orientation to A).

C) Crystal structure of the archetypical DDB1 complex bound to the V protein of the SV-5 virus (cyan) (protein data bank ID 2HYE; Angers et al., 2006). DDB1 (blue) binds substrates in the cleft between its BPA and BPC domains. The CTD domain resides underneath this cleft. The BPB domain interacts with CUL4 (green), which acts as the enzymatic core of the ligase with ROC1 (yellow).

Image was generated using 3D Molecule Viewer which forms part of the Vector NTI software suite.
clam-shell like structure that provides a cleft into which substrates or adaptor molecules dock. Furthermore, such studies have solved the structure of the archetypical DDB1 containing complex, which also comprises cullin (CUL) 4 and RING of cullin (ROC1) (figure 4.7C) (Angers et al., 2006). CUL4 and ROC1 form the catalytic core of the complex, and are responsible for the recruitment of the E2 ubiquitin ligase. The N-terminus of the CUL4 interacts with the BPB region of DDB1 (Angers et al., 2006), and therefore mapping the NR3 interaction to a domain of DDB1 may provide information as to the role NR3 plays when complexed with DDB1. To address this, GST pulldown assays were employed to screen deletion mutants of both NR3 and DDB1, and also to determine whether NR3 and DDB1 can interact directly.

4.5.1 BPC of DDB1 is Required for Interaction with NR3

The crystallisation of DDB1 has shown it possesses a complex structure (Angers et al., 2006). Although BPA, BPB and CTD are continuous polypeptide chains forming their respective 3-dimensionally structured domains, BPC is not continuous as the first blade of the β-propeller and the rest of BPC are interspersed by the BPA and BPB domains (figure 4.8). The structural characteristics of DDB1 were taken into account or the mapping studies, and mutant constructs were generated that delete portions of the protein according to the position of the individual domains. The radiolabelled mutant proteins were then tested for interaction with GST or GST-NR3 as outlined in chapter 2.2.3.

Full length DDB1 interacts with GST-NR3, producing a band of at least equal intensity to that of the input control. This supports the idea that NR3 and DDB1 interact, and indicates the interaction is direct in nature (figure 4.8, DDB1). Deletion of the CTD allows DDB1 to retain the interaction with GST-NR3 although the level of signal detected is reduced compared to that of full-length DDB1, suggesting the interaction is not as high affinity (figure 4.8, DDB11-1043). However, deletion of the CTD and the C-terminal portion of BPC (figure 4.8A, DDB11-706), or deletion of the N-terminal portion of BPC resulted in an ablation of the interaction (figure 4.8, DDB117-1140), indicating BPC is required for NR3 binding. Deletion of the BPB domain retained the interaction with GST-NR3, which suggests BPB is not required...
Figure 4.8 NR3 Interacts with the BPC region of DDB1
Mutant DDB1 constructs were generated according to the domain structure of DDB1, and the indicated radiolabelled proteins were subject to GST pulldown assays with GST and GST-NR3. Full length DDB1 (DDB1-1140) interacts directly with GST-NR3. The interaction is retained following deletion of CTD (DDB1-1043) and BPB (DDBΔBPB), and abolished following deletion of the N-terminal (DDB1ΔN) and C-terminal (DDB1ΔC) portions of BPC.
for DDB1 binding NR3 (figure 4.8, DDB1\(_{\Delta\text{BPB}}\)). These data indicate that NR3 binds to the BPC domain of DDB1, which is the region implicated in substrate recognition. Therefore it is possible that NR3 binds to DDB1 as a substrate or adaptor protein.

4.5.2 The N-terminus of NR3 interacts with DDB1

Immunoprecipitation experiments have implicated the N-terminus of NR3 as being required for the interaction with DDB1 (figure 4.6A). To further investigate the region of NR3 that binds to DDB1, deletion mutants of NR3 were fused to GST and used in conjunction with radiolabelled DDB1 in a GST pulldown assay. The positive control of GST-NR3 showed that DDB1 co-purified in the assay, which was not affected by C-terminal deletion (figure 4.9A, NR3 and NR3\(_{1-722}\)). However, deletion of the first 120 amino acids of NR3 resulted in loss of interaction with DDB1 (figure 4.9A, NR3\(_{120-722}\)). Furthermore, it was observed that amino acids 1-120 of NR3 fused to GST were sufficient to interact with DDB1 (figure 4.9A, NR3\(_{1-120}\)). No binding was observed with GST-NR3 LBD, which served as a negative control (figure 4.9A, NR3 LBD). Coomassie staining of purified GST-fusion proteins shows all the fragments were expressed at the expected sizes (figure 4.9B). Interestingly bands could be observed in a number of samples at \~75kDa and \~60kDa, as observed in the limited proteolysis experiments outlined in section 3.2.1. The polypeptides were present in GST-NR3, GST-NR3\(_{1-722}\), GST-NR3\(_{120-722}\) and GST-NR3 LBD samples, supporting the idea that they correspond to bacterial proteins that co-purify with GST-NR3 and indicates that putative LBD of NR3 may be important for their co-purification. fusion proteins possessing the putative LBD region, This supports the idea that these polypeptides coThe data indicate the N-terminus of NR3 is responsible for the interaction with DDB1 and the site of interaction resides between amino acids 1 and 120. The bioinformatic predictions for NR3 suggest the N-terminus possesses an armadillo repeat-like structure, which is a motif known to be involved in mediating protein-protein interactions (Herold et al., 1998; Kaufmann et al., 2000; Song et al., 2003), and as such the results presented here support the notion that the N-terminus of NR3 possesses a structure that allows interaction with other proteins.
Figure 4.9 DDB1 Interacts with the N-terminus of NR3  
Radiolabelled full length DDB1 was incubated with GST-fused NR3 deletion mutants in a GST pulldown assay.

A) Deletion of the NR3 C-terminus retains the interaction between GST-NR3 and DDB1 (NR3<sub>1-722</sub>). Deletion of the N-terminus of NR3 abolishes binding (NR3<sub>120-722</sub>). The N-terminal 120 amino acids of NR3 are sufficient to bind to DDB1 (NR3<sub>1-120</sub>). GST-NR3 LBD serves as a negative control.

B) Coomassie stained gel of GST-fused NR3 deletion mutants. Arrowheads denote the band corresponding to the correct fusion protein.
4.6 NR3 is Ubiquitinated and Degraded by the Proteasome

DDB1 is the substrate recognition subunit of an E3 ubiquitin ligase complex involving CUL4 and ROC1. NR3 binds to the region that is important for substrate and adaptor docking (Angers et al., 2006), which are biological roles that are not necessarily mutually exclusive (Sugasawa et al., 2005). It may be hypothesised that NR3 is ubiquitinated, and as polyubiquitination is likely to lead to protein degradation at the proteasome (reviewed in Varshavsky, 2005), it is possible this is important for regulating NR3 levels.

To determine whether NR3 levels are regulated by the proteasome, it is necessary to block its action, which may be done by administering MG132. The chemical has previously been used at concentrations of 10-50µM for between 0.5 and 6 hours (Guo and Wang, 2007; Wassler et al., 2008). To optimise the MG132 treatment for experiments examining the ubiquitination status of NR3, a time course treatment with 20µM MG132 was performed. The protein levels of the DDB1 target chromatin licensing and DNA replication factor (CDT) 1 were determined as an indicator of proteasomal blockade. Levels and NR3-V5 were also examined in response to MG132 administration. DDB1 and GAPDH served as negative controls for the optimisation as proteasomal blockade was unlikely to affect their protein levels. Treatment with MG132 produced an accumulation of CDT1 after 1-2 hours, which peaked at ~4 hours (Figure 4.10A). However, no accumulation of protein was observed for DDB1, NR3-V5, or the loading control GAPDH. These data show that proteasomal blockade was achieved effectively between 2-4 hours, and as such following MG132 pre-treatments were performed for 3 hours at a concentration of 20µM.

To investigate the ubiquitination status of NR3, a V5 tagged version of the protein was immunoprecipitated under native and denatured conditions from stable NR3-V5 expressing HEK-293 cells following treatment with MG132, as outlined in chapter 2.2.3. Immunoprecipitations were also performed in cells stably transfected with empty vector as a negative control. Samples were then probed for NR3-V5 and ubiquitin in a western blot to determine whether ubiquitinated material co-precipitated with NR3. For both native and denatured immunoprecipitations, probing for V5
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**Results**

**Figure 4.10 NR3 is Ubiquitinated and Degraded by the Proteasome**

**A)** HEK-293 cells stably expressing V5-tagged NR3 were subject to treatment with 20μM MG132 for increasing lengths of time. Western blots were performed with antibodies to recognise the indicated proteins.

**B and C)** HEK-293 cells stably transfected with NR3-V5 or empty vector (mock) were left untreated or treated with 20μM MG132 for 3 hours and immunoprecipitations with anti-V5 antibody under **B)** native and C) denatured conditions were performed. Immunoprecipitates were probed for V5 or ubiquitin (Ub) in a western blot. Arrowheads denote where NR3-V5 is expected to migrate. Asterisks denote bands corresponding to immunoprecipitation antibody heavy and light chains.

**D)** Pulse chase experiments with HEK-293 cells stably expressing NR3-V5. Cells were left untreated or pre-treated with 20μM MG132 for 3 hours, and treated with 20ng/mL cyclohexamide (Chx) for the length of time indicated. Levels of NR3-V5 were assessed by western blot against the V5 tag. GAPDH was blotted for as a loading control.
detects numerous bands of differing molecular masses in NR3-V5 samples, and no bands in mock samples, indicating the bands are specific to NR3-V5 (figure 4.10B, lanes 1-4; figure 4.10C, lanes 1-4). The bands at lower molecular masses are likely to correspond to degraded fragments of NR3-V5, and the smear observed above the expected migratory size for NR3-V5 is likely to be modified NR3-V5. The lower molecular mass bands are enriched following administration of MG132 (figure 4.10B compare lane 3 to 4), suggesting some of the bands are partially proteasomally degraded NR3-V5. The upper molecular mass smear is also slightly enriched in native samples and enriched in denatured samples upon treatment with MG132 (figure 4.10B and C, compare lanes 3 and 4), which may indicate a portion of the smear corresponds to polyubiquitination.

Probing the samples with an anti-ubiquitin antibody shows that ubiquitin does indeed co-precipitate with the samples immunoprecipitated under native conditions (figure 4.10B, lanes 5-8), indicating NR3 is associated with ubiquitinated material. This was recapitulated in immunoprecipitations performed following denaturation of samples (figure 4.10C, lanes 5-8). Denaturing samples prior to immunoprecipitation disrupts the protein structure and breaks protein-protein interactions, ensuring no associated proteins are co-precipitated. However, covalent modifications are retained. Therefore these data show that NR3 is ubiquitinated, and due to the smear pattern in the western blot, this is likely to be polyubiquitination, supporting the results observed when western blotting for V5 (figure 4.10B and C, lanes 1-4).

Ubiquitinated material can also be observed in mock native immunoprecipitated samples treated with MG132, but not those left untreated (figure 4.10B, lane 5 and 6), indicating that some of the precipitated material in MG132 treated samples is non-specific. However, the co-precipitated ubiquitinated material in native NR3-V5 samples treated with MG132 is highly enriched implying most of the material present is specific to NR3-V5 (figure 4.10B, lanes 6 and 8). Some ubiquitinated material is present in native precipitates from NR3-V5 cells cultured in the absence of MG132, which contrasts the samples from mock cells cultured under the same conditions (figure 4.10B, lanes 5 and 7). This suggests NR3 is associated with ubiquitinated material in the absence of proteasomal blockade, although the quantity of material is lower than precipitates from NR3-V5 cells treated with MG132 (figure 4.10B, lanes 7 and 8). No ubiquitinated material is present in denatured mock samples, or denatured
NR3-V5 samples derived from cells cultured in the absence of MG132 (figure 4.10C, lanes 5-7).

Comparing the relative quantities of ubiquitinated material precipitated under native and denatured conditions shows that a greater amount of material is present in precipitates derived from native samples (compare figure 4.10A, lanes 5-8 and figure 4.10C, lanes 5-8). Although a portion of the ubiquitinated material in native precipitates is non-specific, it is possible that NR3 is also associated with other ubiquitinated proteins. Taking the results from the immunoprecipitations performed on native and denatured protein samples together, these data show that NR3 is associated with ubiquitinated material and is polyubiquitinated itself.

Polyubiquitination generally leads to degradation at the proteasome (Varshavsky, 2005), which coupled with the data presented here showing NR3 polyubiquitination is detectable following treatment with the proteasome blocking agent MG132, strongly suggests the ubiquitin-proteasome may be important in regulating the protein levels of NR3. To address this idea, pulse chase experiments were performed in HEK-293 cells stably expressing NR3-V5. Cells were pre-treated for 3 hours with 20µM MG132 or left untreated, and treated with cyclohexamide to block protein translation. Cells were harvested at different time points and protein samples subject to western blot to determine the half life of NR3-V5. Following cyclohexamide administration, NR3-V5 degrades rapidly with a half life of ~1 hour and is stabilised by pre-treatment with MG132 prior to cyclohexamide administration (figure 4.10D). This indicates that NR3 is degraded at the proteasome, and that the polyubiquitination observed in previous experiments is likely to be responsible for this. Interestingly administration of MG132 provided an accumulation of NR3-V5 protein (figure 4.10D), which contrasts the results observed for the MG132 timecourse experiments (figure 4.10A). However, the reasons for this are unclear as the level of cell confluence does not appear to affect NR3-V5 accumulation (data not shown), and the conditions between experiments were kept constant.

Drawing together the data from this set of experiments with the data from the interaction and mapping studies it can be concluded that the NR3 binds to the substrate docking region of DDB1 and is polyubiquitinated, which results in degradation at the proteasome.
4.7 Effect of NR3 Expression on Cell Proliferation

A potential role for NR3 in the regulation of cell cycle progression has been identified previously by screening for novel genes upregulated in cancer, suggesting it may play a role in cell cycle regulation (Nicassio et al., 2005). However, the authors did not characterise the effect of NR3 expression on cell proliferation. DDB1 is known to participate in cell cycle regulation by polyubiquitinating important regulatory proteins and marking them for destruction at the proteasome (Banks et al., 2006; Bondar et al., 2006; Cang et al., 2006; Higa et al., 2006). NR3 is a potential substrate for DDB1, and thus lends support to Nicassio and colleagues’ initial hypothesis that NR3 may be implicated in regulation of the cell cycle.

To test the effect of NR3 expression on cell proliferation, ectopic expression of NR3 was examined by determining the proliferative rate of HEK-293 cells stably expressing murine NR3 in comparison to HEK-293 stably transfected with empty vector. Transient transfection is inappropriate for such experiments as only a proportion of cells will be transfected, which may mask any effects produced by overexpression. Although genomic integration results in lower levels of expression in stable cell lines, 100% of the cells used in the assay ectopically express NR3, avoiding the problems associated with non-transfected cells in transient transfection assays. Cell number was monitored over a period of 72 hours by MTS assay. After 72 hours there was a statistically significant increased rate of proliferation in the cells ectopically expressing NR3, although this was not apparent after 24 and 48 hours (figure 4.11A). This suggests that the HEK-293 cells ectopically expressing murine NR3 possess a proliferative advantage over cells stably transfected with empty vector.

To further examine whether NR3 plays a role in cell proliferation, NR3 was knocked down using siRNA in NIH/3T3 cells. Although the cells have undergone transformation during the generation of the cell line (Todaro and Green, 1963), they are a murine fibroblasts that have not been virally immortalised or derived from a tumour. As such, they are a cell type that possess a relatively unperturbed cell cycle and represent a more physiologically relevant model of cell cycle regulation. Knockdown was performed by transfecting cells with either a pool of siRNA targeting NR3 or a non-specific siRNA as a negative control, and cell number over a 72 hour period was measured by MTS assay. Transfection of siRNA targeting NR3 achieved
Figure 4.11 Effect of NR3 Expression on Cell Proliferation

Cell number was determined over a time course by MTS assay at the given time point to examine the effect of NR3 levels on cell proliferation. Data points represent the mean of 4 independent experiments performed in at least duplicate and error bars represent standard error between experiments. Statistical significance (denoted by asterisk) was tested by the Mann-Whitney U test between control and test samples at the given time point.

A) HEK-293 cells were stably transfected with NR3-V5 (closed circles) or empty vector (open circles) and proliferation examined over 72 hours.

B) NIH/3T3 cells were transfected with a non-targeting control siRNA (siControl, open circles) or siRNA targeting NR3 (siNR3, closed circles) and proliferation examined over 72 hours. Inset shows representative NR3 mRNA levels in cells harvested at the 72 hour time point.
Results

~95% knockdown at the mRNA level over non-targeting siRNA (figure 4.11B, inset), however no significant difference in cell proliferation was observed after 72 hours between non-targeting siRNA and siRNA targeting NR3 as the gradients pertaining to the growth curves for each transfection are similar (figure 4.11B). This data appears to indicate that NR3 is not required for cell proliferation in NIH/3T3 cells. However, it is also possible that 95% knockdown of NR3 at the mRNA level is not sufficient to abrogate the physiological function of NR3.

From these experiments it can be concluded that HEK-293 cells expressing murine NR3 proliferate at an increased rate than cells transfected with empty vector, which may be due to the increased NR3 levels within the cells. However, it appears NR3 is not to be required for cell proliferation, as reduction of the NR3 transcript by ~95% had no effect on the rate of cell proliferation.

4.8 Summary and Conclusions

Screening for proteins that interact with NR3 was approached in two ways. Immunoprecipitation experiments with V5-tagged NR3 proteins were performed and precipitating proteins were detected by SDS-PAGE followed by silver staining. This technique provided a detectable interaction with DDB1, which was confirmed in a different cell system by immunoprecipitation of V5-tagged NR3 and co-precipitation of endogenous DDB1. GST pulldown experiments showed the interaction was direct and mapping studies showed the BPC domain of DDB1 was required for interaction, which is involved in ubiquitination substrate recognition. The interaction was also mapped to the N-terminus of NR3, which is predicted to possess an armadillo repeat-like structure, a motif important in protein-protein interactions (Herold et al., 1998; Kaufmann et al., 2000; Song et al., 2003). Therefore, it may be concluded that the N-terminus of NR3 binds to the region of DDB1 that is involved in the recognition of substrates for ubiquitination. In addition, a yeast two-hybrid based approach was also employed. This system implicated 14-3-3η as a protein that interacts with the LBD of NR3. However these results could not be confirmed using other assays, indicating the interaction may not be physically relevant.

DDB1 is part of an E3 ubiquitin ligase complex also including CUL4 and ROC1. The role of DDB1 is to directly recruit substrates for ubiquitination or adaptor molecules that in turn recruit substrates (Angers et al., 2006). The interaction between
NR3 and DDB1 gives rise to the idea that NR3 may be ubiquitinated, which often leads to degradation at the proteasome (Varshavsky, 2005). Immunoprecipitation experiments show that NR3 ubiquitination can be detected upon proteasome blockade. Furthermore, pulse chase experiments show NR3 protein levels are stabilised in the presence of MG132. From these data it can be concluded that NR3 is polyubiquitinated and as a result is degraded at the proteasome.

DDB1 is known to possess a role in the ubiquitination of cell cycle regulators (Banks et al., 2006; Bondar et al., 2006; Cang et al., 2006; Higa et al., 2006). In addition, NR3 has been implicated as possessing a potential role in cell cycle regulation (Nicassio et al., 2005). As such the effect of NR3 expression levels on cell proliferation was addressed. HEK-293 cells that stably expressed mouse NR3 possess a significant proliferative advantage over cells stably transfected with empty vector. However, when depleted of NR3, NIH/3T3 cells show no difference in cell proliferation over control cells. These data suggest that NR3 expression may have a positive effect on the cell cycle, however further experiments must be performed to confirm this. It may also be concluded that NR3 expression is not required for cell proliferation in NIH/3T3 cells.
Chapter 5

Generation of NR3 Null Mice
5.1 Introduction

Due to the interaction between NR3 and DDB1 previous experiments have suggested NR3 may play a role in regulating the functional pathways in which DDB1 is involved. To assess the biological function of NR3 *in vivo* a conditional knockout mouse model is currently being generated in conjunction with the services offered by Genoway, Lyon. The initial cloning steps and targeting vector construction were undertaken within the laboratory here and the completed targeting vector was then passed to Genoway, who were responsible for the generation of heterozygous floxed mice.

5.2 Gene Structure of NR3 and Strategy for Deletion

Blat searches with the NR3 cDNA sequence (accession BC033274) on the UCSC genome bioinformatics server (http://genome.ucsc.edu/) reveals the NR3 gene consists of 19 exons spanning a 58kb region within the qH1 region of chromosome 2 (figure 5.1). The start ATG of the gene is present in exon 1, and the termination codon is present within exon 19. The NR3 gene is flanked downstream by myosin heavy chain 7B cardiac muscle β gene in the reverse orientation and upstream by the α-mannosidase gene in the same orientation, separated by 0.3kb and 9.3kb of genomic DNA respectively. The targeting strategy selected involves flanking exon 1 with *loxP* sites allowing a tissue specific deletion of the start ATG codon within exon 1 upon crossing floxed mice with mice expressing Cre recombinase in the tissue of interest. With such a strategy it is important to avoid disrupting important regulatory elements, thus allowing endogenous expression of the targeted gene in the absence of Cre recombinase.

To identify appropriate sites for *loxP* insertion, the mouse genomic DNA sequences either side of exon 1 of the NR3 gene were analysed in conjunction with the equivalent human sequences using Consite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite) to identify conserved potential transcription factor binding sites. This revealed there are three conserved clusters of transcription factor binding
Figure 5.1 Gene Structure of NR3
Schematic of the gene structure of NR3. The NR3 gene resides on region qH1 of chromosome 2 in mice, and is flanked either side by the myosin heavy chain 7B cardiac muscle β and α-mannosidase genes. The NR3 gene consists of 19 exons, with the translational start site in exon 1 and the termination codon in exon 19.
sites upstream of the NR3 core promoter (figure 5.2A). Cluster 1 resides furthest from
the transcriptional start site between -5783bp and -4093bp and contains the greatest
number of conserved transcription factor binding sites. Cluster 2 is the smallest
cluster and is situated between -2167bp and -2152bp. Cluster 3 is the shortest distance
from the transcriptional start site, residing between -712bp and -18bp. In addition to
regulatory elements that are present within the promoter, it is possible elements may
reside in intron 1, including binding sites for transcription factors (Fedorova and
Fedorov, 2003). Intron 1 spans 19kb in the mouse and 14kb in the human, therefore a
similar transcription factor binding site analysis was performed on the 10kb region of
intron 1 immediately downstream of exon 1 (figure 5.2B). The conserved
transcription factor binding sites could again be grouped into 3 clusters. Intronic
cluster 1 is the smallest cluster and closest in proximity to exon 1, situated between
16bp and 603bp downstream of exon 1. Cluster 2 is the largest group and resides
between 1319bp and 3260bp downstream of exon 1. Cluster 3 is the furthest group
from exon 1 being present between 4245bp and 5364bp downstream of exon 1.

Based on this analysis, it was decided that the distal \textit{loxP} site should be placed
within the BstBI restriction enzyme recognition site positioned at 553 base pairs
upstream of the translational start site, and the proximal \textit{loxP} site be placed within
intron 1, 1799 base pairs downstream of the translational start site within exon 1,
representing a distance of 2.35kb between the two sites. Placing the \textit{loxP} sites in these
positions avoids the disruption of any of the potential transcription factor binding sites
identified by \textit{in silico} analysis of the promoter and intron 1. A schematic of the final
targeting vector is shown in figure 5.3. The targeting vector possesses short and long
homology arms that allow the \textit{loxP} sites to be homologously recombined at the
correct genomic locus. The long homology arm contains distal \textit{loxP} site, and the
proximal \textit{loxP} site resides between the long and short homology arms. Insertion of the
neomycin resistance cassette in conjunction with the proximal \textit{loxP} site allows the
initial selection of clones in which homologous recombination has successfully
occurred within the short homology arm. The diphtheria toxin A gene located
upstream of the long homology arm allows for selection against clones that have not
successfully undergone homologous recombination within the long homology arm
region (figure 5.3). However, it is possible that recombination may occur downstream
of the distal \textit{loxP} site. Thus clones are then screened for presence of the distal \textit{loxP}
The genomic DNA sequences surrounding exon 1 of the NR3 gene in the mouse and human genomes were aligned and analysed in silico using Consite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite) to identify conserved transcription factor binding sites. Analysis is of the 10kb region A) upstream of the translation start codon and B) downstream of exon 1. For each set of data three clusters of conserved transcription factor binding sites could be identified. The proposed sites for loxP insertion are denoted by the arrow in each figure.
Figure 5.3 Schematic of the Floxed NR3 Gene
Schematic of the targeting vector being constructed. The long homology arm consists of a 5.9kb region of DNA that includes a BstBI restriction site 0.5kb upstream of exon 1, where the distal \textit{loxP} site is inserted. The short homology arm consists of a 1.75kb region. The proximal \textit{loxP} site and neomycin resistance cassette (Neo) are inserted into the EcoRI restriction site between the long and short homology arms. The neomycin resistance cassette is flanked by FRT sites, allowing excision with \textit{Flp} recombinase. The diphtheria toxin A (DTA) gene represents a method of selecting for clones that have undergone homologous recombination within the long arm.
site by PCR and Southern blot to determine whether recombination has occurred at an appropriate upstream position or downstream of the distal \textit{loxP} site. To minimise the disruption of any regulatory elements within intron 1 the neomycin selection cassette will be removed in floxed mice. To enable this, the neomycin cassette is flanked by FRT sites that will recombine in the presence of \textit{Flp} recombinase (figure 5.3), thus deleting the neomycin selection cassette while retaining the inserted proximal \textit{loxP} site.

### 5.3 Targeting Vector Construction

The cloning strategy employed to generate the homology arms and for targeting vector construction involves the isolation of a 1.75kb short homology arm downstream, and a 5.9kb long homology arm upstream of the proximal \textit{loxP} site and neomycin cassette. It is within the long homology arm that the BstBI site proposed for distal \textit{loxP} site insertion is present (figure 5.3).

#### 5.3.1 Generation of the Short Homology Arm

Isolation of the short homology arm was performed in triplicate by PCR to generate a 3.5kb amplicon containing the region spanning the EcoRI and BstZ17I restriction sites from 129S genomic DNA, which was then TOPO cloned into the pCR4 vector. Clones containing the separate fragments were then sequenced to determine polymorphisms between the 129S and C57Bl sequences by alignment of the individual fragments and the C57Bl sequence, which showed the short homology arm region is 99.32% homologous between the two mouse strains. PCR induced mutations could also be identified in this manner by searching for differences between sequences of the three separate clones. Clone 5 possessed a number of mutations and was discarded from further cloning steps. Clone 19 possessed five PCR induced mutations in total (figure 5.4). Three transversion mutations were present, two C-T mutations 1533bp and 1952bp downstream of the 5’ terminus of the PCR fragment, and a T-C mutation at 3350bp. A transition mutation in the form of A-T was present at 3032bp, and an insertion of cytosine base was observed at 2232bp. Clone 20 possessed two transversion mutations, T-C at 678bp and A-G at 787bp. There were
Figure 5.4 Correction of Mutations Generated by PCR During the Cloning of the Short Homology Arm

Schematic representing the PCR fragment generated to isolate the short homology arm. Three independent fragments were produced and cloned, of which clones 19 and 20 were used in subsequent cloning steps. Blue asterisks shows PCR induced mutations in clone 19, and red asterisks denote mutations within clone 20. The AatII-NsiI fragment from clone 19 was subcloned into clone 20 to produce a clone free from mutation within the short homology arm region.
also instances of a deletion and an insertion of a thymine base at 2629bp and 3258bp respectively (figure 5.4). Due to the positioning of an NsiI restriction site within the short homology arm region, subcloning of a fragment digested with AatII and NsiI from clone 19 and ligation into clone 20 digested with the same enzymes, resulted in a modified clone named RWH1-SAmod free from PCR induced mutations within the 1.75kb short homology arm region (figure 5.4). The modified clone was therefore used for the further cloning steps outlined in section 5.3.3. The 2.9kb control short homology arm region present within RWH1-SAmod was used by Genoway to optimise PCR conditions to screen for homologous recombination within the short homology arm region as outlined in appendix B.

5.3.2 Generation of the Long Homology Arm

The long homology arm was isolated by digestion of a BAC clone from a 129S genetic background containing the NR3 genomic DNA. The BAC clone was purified and digested with XhoI and NsiI, which should produce 46 fragments ranging in size between 22bp and 12299bp. The fragments were ligated into empty pCR2.1 vector digested with XhoI and NsiI. A total of 46 colonies were screened by PCR for the presence of the required 6.7kb insert. Clones 16 and 33 produced the expected amplicon of 0.5kb as indicated by the positive controls of the purified BAC clone DNA and also a colony PCR performed using a small amount of BAC clone glycerol stock (figure 5.5A). As such, clones 16 and 33 were further analysed by restriction analysis for presence of the 6.7kb XhoI-NsiI fragment. Upon digestion of clones 16 and 33 with XhoI and NsiI, several bands were observed for each clone (figure 5.5B), including a band at the expected size of 6.7kb. The 6.7kb fragment generated from the digestion of clone 33 was isolated and subcloned into empty pCR2.1 digested with XhoI and NsiI to generate vector RWH1-LAbac. This was then sequenced to determine polymorphisms between 129S and C57Bl mice, which showed the two strains were 98.62% homologous. RWH1-LAbac was then used for the subsequent subcloning steps outlined in section 5.3.3.
**Figure 5.5 Cloning the Long Homology Arm for Targeting Vector Construction**

A) Agarose gel showing clones possessing an insert corresponding to the long homology arm region. PCRs were performed using colonies from transformed cells as a template and identified two positive clones (clones 16 and 33). As positive controls PCRs were performed using the purified BAC construct (BAC) and also a glycerol stock of the BAC construct (Glyc) as template.

B) Agarose gel showing restriction patterns of clones 16 and 33 following digestion with XhoI and NsiI. Neither clone produced a single band upon digestion. However, both clones possess a band at 6.7kb, which corresponds to the size of required the long homology arm fragment.
5.3.3 Generation of the Final Targeting Vector

RWH1-SAmo and RWH1-LAbac were used in a series of subcloning steps to generate the final vector designed to target the NR3 locus. Genoway provided three of the vectors they use routinely for this procedure. G139 possesses the neomycin selection cassette, pGA1 was used the backbone for the targeting vector, and G112 possessed the diphtheria toxin A negative selection gene. Details of vector maps can be found in appendix B.

Subcloning the Short Homology Arm into Neomycin Cassette Vector G139

To subclone the short homology arm into the G139 vector, which possesses the neomycin selection cassette and proximal $loxp$ site, the control short homology arm region spanning 2.9kb was inserted (figure 5.6A). The resultant vector can then be used for optimisation of the PCR screen to determine presence of homologous recombination in embryonic stem (ES) cells, and also used for subsequent cloning steps. RWH1-SAmo was digested with the restriction endonucleases EcoRI and BstZ17I and ligated into vector G139 digested with EcoRI and EcoICRI, and transformed into DH5α. Both BstZ17I and EcoICRI leave the digested DNA blunt and are therefore compatible for cloning applications. Miniprep DNA was isolated from a total of 8 bacterial colonies and diagnostic restriction digest analysis with SpeI of the resultant vector named RWH1-SA-C+ performed. SpeI cuts within the neomycin cassette and the short homology arm region and gave the expected fragments of 6.3kb and 1.3kb in clone 2 (figure 5.6B). RWH1-SA-C+ from clone 2 was sequenced and an aliquot sent to Genoway for optimisation of the screening strategy. RWH1-SA-C+ was also used for downstream subcloning applications.

Insertion of the Distal $loxp$ Site into the Long Homology Arm

In the strategy agreed with Genoway, the distal $loxp$ site is inserted into the BstBI restriction site in the long homology arm (figure 5.3). The $loxp$ site was inserted by linearising vector RWH1-LAbac with BstBI and dephosphorylating the resultant fragment. A pair of complementary oligonucleotides possessing
Figure 5.6 Subcloning the Short Homology Arm into Vector G139
A) Schematic detailing the strategy for subcloning the short homology arm region into vector G139 downstream of the neomycin selection cassette (Neo) and proximal loxP site. Restriction enzyme sites depicted in red show the sites used for subcloning. Underlined restriction enzyme sites denote enzymes used for diagnostic digest.

B) Agarose gel of the diagnostic digests of RWH1-SA-C+ with SpeI following ligation of the short homology arm region into vector G139. The lanes of positive samples possessing the correct diagnostic restriction pattern are identified above the gel. Expected fragment sizes are 6.2kb and 1.3kb.
EcoNI-\textit{loxP}-BamHI-NsiI sites and with compatible ends to BstBI were phosphorylated, annealed and ligated into the linearised RWH1-LAbac vector to give RWH1-LA-\textit{loxP}, as outlined in figure 5.7A. Miniprep DNA was purified from 24 colonies and diagnostic digestion with BamHI carried out, which cuts in the inserted oligonucleotides and within the pCR2.1 vector backbone. Diagnostic digests generated the expected fragments of 7.1kb and 3.6kb in all but 4 of the selected colonies (figure 5.7B). Positive miniprep DNA samples were then sequenced to determine which possessed the \textit{loxP} site inserted in the correct orientation. This confirmed clones 2, 3, 4, 5, 17, 20 and 21 were correct, and clone 2 was selected for downstream cloning steps involving RWH1-LA-\textit{loxP}.

**Subcloning the Neomycin Cassette and Homology Arms into pGA1**

The backbone for the targeting vector is the Genoway plasmid pGA1. RWH1-SA-C+ was used to subclone the neomycin resistance cassette, proximal \textit{loxP} site and the short homology arm into pGA1, and RWH1-LA-\textit{loxP} was used to subclone in the long homology arm with the distal \textit{loxP} site inserted. An overview of these subcloning steps is given in figure 5.8. The selected cloning strategy involved the synthesis of complementary oligonucleotides spanning 890bp in length that possessed 92bp from the 3’ end of the long homology arm connected via a 21bp linker to 733bp of the 3’ terminus of the short homology arm (figure 5.8). The sequence of the complementary oligonucleotides is detailed in appendix B. The oligonucleotides were synthesised, cloned into the pGA1 vector, and sequence verified by Top Gene Technologies, Quebec to generate RWH1-GA1-linker. The neomycin cassette, proximal \textit{loxP} site and 5’ region of the short homology arm were then subcloned into this vector by digesting RWH1-SA-C+ with HpaI and Bsu36I and ligating into RWH1-GA1-linker digested with the same restriction endonucleases to produce RWH1-Neo/SA. The ligation reaction was transformed into DH5α and 8 colonies were selected for miniprep. Miniprep DNA was then analysed by diagnostic restriction digest with AvrII, which cuts within the neomycin cassette and short homology arm. All 8 colonies possessed the correct insert as reactions produced fragments of 5.3kb and 1.2kb, although digestion was incomplete (figure 5.9A). Miniprep DNA from colony 1 was selected and the sequence of RWH1-SA/Neo was verified by DNA sequencing.
Figure 5.7 Subcloning the Distal loxP Site into the Long Homology Arm

A) Schematic showing the strategy for subcloning the distal loxP site into the long homology arm region. Restriction enzyme sites depicted in red show the sites used for subcloning. Underlined restriction enzyme sites denote enzymes used for diagnostic digest.

B) Agarose gel of the diagnostic digests of RWH1-LA-loxP with BamHI. The lanes of positive samples possessing the correct diagnostic restriction pattern are identified above the gel. Expected fragment sizes are 7.1kb and 3.6kb.
Figure 5.8 Subcloning the Short and Long Homology Arms into pGA1
Schematic detailing the strategy for subcloning the short homology arm, neomycin selection cassette (Neo) and proximal \textit{loxP} site into the vector pGA1, followed by the long homology bearing the distal \textit{loxP} site. Restriction enzyme sites depicted in red show the sites used for subcloning. Underlined restriction enzyme sites denote enzymes used for diagnostic digest.
Figure 5.9 Restriction Analysis of the Subcloned Short and Long Homology Arms in pGA1

Agarose gels of the diagnostic digests of A) RWH1-Neo/SA and B) RWH1-LSA/Neo. The lanes of positive samples possessing the correct diagnostic restriction pattern are identified above the gel. Expected fragment sizes are given below the gels.
A fragment possessing the 5’ terminus of the long homology arm with the distal $loxp$ site inserted within the proposed BstBI restriction site was generated by digesting RWH1-LA-$loxp$ with XhoI and AatII (figure 5.8). This was then ligated into RWH1-SA/Neo digested with the same enzymes to produce RWH1-LSA/Neo, and transformed into DH5$\alpha$ bacteria. Colonies were selected for miniprep and analysed by diagnostic digest with BamHI (figure 5.9B). Samples 2, 4, 7, 14 and 16 possessed the correct size fragments of 9.6kb and 2.7kb following digestion. The samples were sequenced and sample 4 was used for further subcloning applications.

**Subcloning the Diphtheria Toxin Selection Cassette the Targeting Vector**

To generate the final targeting vector, the negative selection cassette containing the diphtheria toxin A gene must be inserted upstream of the long homology arm. DNA coding for the diphtheria toxin A negative selection cassette was provided by Genoway in the G112 plasmid. This was digested with Ascl and NotI which cut either side of the cassette (figure 5.10A), and also with Tth111I, which cuts the DNA within the vector backbone to aid the isolation of the selection cassette. The Ascl and NotI digested selection cassette fragment was ligated into RWH1-LSA/Neo digested with the same restriction endonucleases to generate the final targeting vector named RWH1-HR. The ligation reaction was transformed into DH5$\alpha$ cells, and 12 colonies selected for miniprep isolation of plasmid DNA. Diagnostic restriction analysis of the purified miniprep samples with ApaI shows that samples 1-9 and sample 12 possessed the correct restriction pattern of fragments at 9.8kb and 5.9kb (figure 5.10B). The presence of the correct insert in samples 10 and 11 could not be confirmed due to a low yield following miniprep purification of plasmid DNA. Samples 6, 7 and 8 were DNA sequenced to ensure they were correct. An aliquot of the final targeting vector RWH1-HR from sample 8 was then passed to Genoway for the downstream processes involved in the generation mice floxed at the NR3 gene locus. A summary of this work is provided in appendix B.
Figure 5.10 Construction of the Final Targeting Vector

A) Schematic showing the strategy for subcloning the diphtheria toxin A (DTA) selection cassette into RWH1-LSA/Neo to yield the final targeting vector RWH1-HR. Restriction enzyme sites depicted in red show the sites used for subcloning. Underlined restriction enzyme sites denote enzymes used for diagnostic digest.

B) Agarose gel of the diagnostic digest of the final targeting vector RWH1-HR with ApaI. Numbers above lanes represent miniprep samples. Expected fragment sizes are 9.8kb and 5.9kb.
5.4 Summary of the Generation of NR3 Knockout Mice

This chapter describes the cloning and subcloning procedures undertaken to generate a vector designed to target the NR3 locus. Correct homologous recombination between the targeting vector and genomic DNA in ES cells results in exon 1 of the NR3 gene being flanked with $\textit{loxP}$ sites, which are placed in positions that are predicted to be of minimal disruption to NR3 expression prior to deletion of the exon. Gene deletion may be performed in either a global or a tissue specific manner in mice homozygous for the floxed gene by crossing them with mice expressing Cre recombinase in the appropriate tissues. Such breeding work is currently being undertaken by Genoway.
Chapter 6

Discussion
6.1 Structure of the Putative LBD Region of NR3

Inpharmatica have used a threading algorithm to predict a number of proteins encoded by the human genome that may possess a structure similar to that of a ligand binding domain found in bona fide NRs. NR3 is one such protein that is predicted to fold into a ligand binding domain-like structure between amino acids 477 and 722. This region was shown to be resistant to limited proteolysis which indicates that it may form a structured domain. This is concordant with disorder predictions for NR3. However, the protease resistant region corresponding to the putative LBD was mapped using a C-terminal tag and mapping studies performed with an N-terminal tag may have also provided structural information to support this conclusion. Immobilised bacterially expressed GST-fusion proteins may have been useful for such studies by examining the size of fragments present on beads following digestion.

Protease mapping experiments performed on bona fide nuclear receptors have shown that the size of the LBD following digestion of full length receptor is ~30kDa (Couette et al., 1996; Keidel et al., 1994; Modarress et al., 1997; van den Bemd et al., 1996). The size of the protease resistant putative LBD region within NR3 is estimated to be ~27kDa, supporting the idea that this region of NR3 possesses a folded domain of a similar size to that of a NR LBD. The binding of ligand is thought to stabilise the structure of the LBD, which may account for the dearth of reported apo-LBD crystal structures relative to those in the holo-conformation. In keeping with this notion, others have found that the apo-LBD is more susceptible to digestion than the holo-LBD (Couette et al., 1996; Keidel et al., 1994; Modarress et al., 1997; van den Bemd et al., 1996), however such experiments were performed using up to 100-fold higher concentrations of protease. At similar concentrations of protease to those used here, it is observed that the NR apo-LBDs are resistant to digestion. If the putative NR3 LBD does require a stimulus to induce a more structured conformation in a manner similar to that of nuclear receptors, the peptide fragments observed here following digestion may reflect either a less structured inactive conformation or a more structured activated conformation.

The results from limited proteolysis presented here provide evidence that the putative LBD region of NR3 is a structured domain, and as such represents a candidate for further structural work. X-ray crystallography is a technique that allows the modelling the 3-dimensional structure of a protein or protein domain in vitro,
providing a good representation of the conformation a protein is likely to reside in under physiological conditions. Therefore crystallographic studies offer the opportunity of producing an unequivocal answer to the question of whether NR3 possesses a NR LBD-like fold within its structure.

### 6.2 Ligand Binding of NR3

Within the superfamily of nuclear receptors, two thirds of receptors are regulated by a ligand (figure 1.1A). In addition, the oestrogen-related receptors are orphan receptors that are known to fold into an active conformation, but may bind synthetic molecules within the LBD (Chao et al., 2006; Greschik et al., 2004; Kallen et al., 2004). As the preliminary structural work reported here suggests the putative LBD of NR3 forms a protease resistant fold and the bioinformatic predictions of Inpharmatica suggest that fold is similar to that of a NR LBD, a ligand binding screen was performed. 2320 compounds were screened in a reporter gene based assay but no ligand was identified. One may speculate as to the reason for this.

Firstly, the compound library used for ligand screening was provided by Inpharmatica and was focussed towards screening against the PPARs, which may bias the library towards structures that are more readily accommodated within the PPAR ligand binding pocket. The ligand binding pocket of the PPARs is large in comparison to the rest of the superfamily, possessing a cavity of around 1400Å$^3$, which has been shown to be large enough to accommodate two fatty acids bound simultaneously (Itoh et al., 2008). It may be that the compounds present within the library are of appropriate structures to bind to a large cavity but may not bind effectively to smaller cavities. As it is impossible to estimate the size of a putative ligand binding pocket within NR3, it is conceivable that the compound library may not have been appropriate to identify a ligand for NR3. However, it has been determined that ligand binding within the LBD of nuclear receptors with large binding pockets such as PXR may be mediated by a number of different amino acids, which may account for their promiscuous binding properties (Watkins et al., 2001). In addition a recent report has shown that residues within the large binding pocket of PPAR$\gamma$ make different contacts with different fatty acid ligands (Itoh et al., 2008). As such, it is conceivable that a library focussed toward the screening of PPAR ligands may contain compounds with wide ranging structures. Furthermore, although details of the compound structures
within the library were not made available, Inpharamatica have indicated that the library possesses compounds that are structurally similar and dissimilar to known PPAR ligands (Adrian Kinkaid, personal communication). This suggests that a variety of different chemical structures were available within the library for screening, and not just those that may bind preferentially to the PPAR receptor. In addition, as the library is designed to be used for screening against members of the nuclear receptor superfamily the compounds within the library are likely to be a selection of small hydrophobic molecules, which is a description that fits most synthetic ligands for NRs. This suggests the library available was more appropriate for ligand screening against a putative NR-like LBD than a more general library that possesses a much larger range of chemical structures.

The limitations of the reporter gene assay used for the NR3 ligand screen are another consideration when interpreting the results. The screen was based on the idea that the VP16-GAL4-NR3 fusion protein used was enriched in the cytoplasm, and thus may not drive transcription of the reporter gene in the nucleus. It is possible that the putative NR3 LBD binds to transcriptional repressors to inhibit the activity of the fusion protein, however from the interactions studies performed here there is no evidence to suggest this. The ligand screen was performed based on the hypothesis that a ligand binding to the putative LBD of NR3 may induce a conformational change such that the fusion protein is translocated to the nucleus and the reporter gene transcribed. As the system employed a VP16 activation domain, which is known to be active in such assays (Sadowski et al., 1988), it does not rely on the supposition that the putative LBD of NR3 is able to recruit coactivators to stimulate transcription of the reporter gene. It is conceivable that one of the ligands screened may have bound to the putative NR3 LBD and, regardless of any conformational change that may have occurred, did not induce translocation of the fusion protein to the nucleus. Under such circumstances the ligand would not have been identified as a positive hit. However, in the absence of another screening assay such an idea cannot be confirmed.

It must also be noted that the number of ligands screened against the putative NR3 LBD is up to 100-fold smaller than screens performed elsewhere (Willy et al., 2004). Therefore, it may be suggested that one reason a ligand was not identified may be related to the scale of the screen performed. However, there are also examples of screens against nuclear receptors using much smaller numbers of compounds than that of Willy et al (2004) that have proved fruitful in their synthetic ligand identification.
(Mao et al., 2008; Wu et al., 2005). In addition, there are also a number of orphan receptors yet to have either an endogenous or synthetic ligand to which they bind identified (figure 1.1A). It is also known that although some receptors are promiscuous in their ligand binding (Li et al., 2003), crystallography studies have shown that some receptors such as Nurr1, which possesses bulky amino acid residue side chains in the cavity that represents the ligand binding pocket, and DHR38, which possesses a small ligand binding pocket of 30Å³, are not thought to bind ligand at all (Baker et al., 2003; Wang et al., 2003). It is possible that should NR3 possess an NR-like LBD, that it may be more closely related to that of a receptor that is incapable of binding a ligand. It is also postulated that the ancestral LBD of NRs is likely to be an orphan receptor and that ligand binding was a gain of function during evolution (Escriva et al., 1997). Furthermore, it is suggested that the LBD region of NRs emerged initially as a separate module, and that DNA rearrangements may have fused it to the DBD of a transcription factor (Barnett et al., 2000; Laudet et al., 1992). NR3 does not conform to the domain organisation associated with nuclear receptors as outlined in chapter 1.3, and also lacks homology to an NR LBD at the amino acid level. It is therefore conceivable that NR3 may be more related to the putative ancestral LBD, and as such may be similar to an orphan receptor with regards its ability to bind a ligand.

In the instance that the putative LBD of NR3 is similar in structure to the LBD of bona fide nuclear receptors yet does not bind ligand it is possible that post translational modifications are responsible for inducing an active conformation. The study of post translational modifications of NRs has shown that phosphorylation status of AF-1 may regulate transcriptional activity for some receptors, which in turn may be influenced by ligand binding (Chen et al., 2000; Wang et al., 2002b). It has been reported that phosphorylation may also regulate the subcellular localisation (Qiu et al., 2003). Studies on the LBDs of receptors have shown that this region may also be post translationally modified. Y537 within the human ERα LBD, and its murine equivalent Y541, is positioned very close to AF-2 and known to influence the activity of the receptor (Weis et al., 1996; White et al., 1997). Mutation to a small non-aromatic residue resulted in constitutive activation of the receptor (Weis et al., 1996; White et al., 1997; Yudt et al., 1999). Recent work has also suggested the phosphorylation of the residue may be important for the interaction ERα and c-Src,
suggesting such modification may have a role in the non-genomic actions of the receptor (Barletta et al., 2004). Similarly phosphorylation and sumoylation have also been studied in the androgen receptor, glucocorticoid receptor and mineralocorticoid receptor LBDs, however the functional significance of these modification is also unclear (Lin et al., 2001; Tallec et al., 2003; Tian et al., 2002; Wen et al., 2000). Studies of LBD phosphorylation in RXR and RAR have shown that such modifications can regulate their activity (Rochette-Egly et al., 1995; Solomon et al., 1999), and that phosphorylation of ser-260 in RXR inhibits recruitment of coactivators to the receptor, which may be due to a disruption of the AF-2 positioning following ligand binding (Macoritto et al., 2008). This contrasts the mechanism of regulation conferred by phosphorylation of ser-369 within the RAR LBD, which increases phosphorylation of ser-77 in the AF-1 region resulting in increased transcriptional activity (Gaillard et al., 2006). Should NR3 possess a fold that is structurally similar to that of the LBD region of a nuclear receptor, it is possible that it may be regulated by post translational modifications that may modulate the activity of the protein.

6.3 Proteins that Interact with NR3

6.3.1 Screening for Interactions Using a Two-Hybrid Assay

In this study proteins with which NR3 interacts were screened for by using a yeast two-hybrid assay that is based on the SRS system, originally described by Aronheim et al (1997). The results of the screen indicated that putative NR3 LBD interacts with 14-3-3η, which is one of the 7 members of the 14-3-3 family of proteins. This family is involved in the regulation of numerous cellular processes, such as transcription, cell cycle progression, signal transduction, metabolism, stress responses, and apoptosis (reviewed in Tzivion et al., 2006). The family is known to interact with over 200 proteins, modulating their function by altering activity, localisation, recruitment to complexes, post translational modifications, and protein stability (Tzivion et al., 2006). However, the interaction observed between 14-3-3η and NR3 in the yeast two-hybrid system could not be confirmed using other assay systems, suggesting the result is specific to the two-hybrid system and is therefore of questionable physiological significance.
Aronheim et al (1997) proposed the SRS system as an alternative to yeast two-hybrid methods that are based on a transcriptional readout. It is suggested that the SRS system does not possess the limitations associated with transcription-based methods, including those that may arise from the screening of transcriptional regulators that may constitutively activate or repress the system and the screening of proteins that do not translocate to nucleus (Allen et al., 1995; Aronheim et al., 1997). Based on the results presented here regarding the localisation of a VP16-GAL4-NR3 LBD fusion protein the SRS system is a more appropriate yeast two-hybrid assay to use over one based on a transcriptional readout, as there is the likelihood that the NR3 bait protein may not translocate to the yeast nucleus and fail to allow the transcription of the reporter gene.

Although the SRS system was developed as an alternative to conventional yeast two-hybrid assays (Aronheim et al., 1997), it is not without limitations itself. The assay utilises a yeast strain that carries a mutation within the cdc25 gene, which inhibits growth at the selective temperature of 37°C. The prey protein is tethered to the membrane via a myristylation signal and recruitment of the bait protein fused to SOS allows activation of the Ras signalling pathway, enabling growth at 37°C. However, as indicated by the results presented here, the presence of SOS within the cDNA library may bypass the necessity for an interaction between bait and prey proteins. Furthermore, the phenotype of the yeast strain used is susceptible to spontaneous reversion allowing growth at 37°C. Although there are such problems associated with the method, there are numerous reports outlining the use of the system to identify interacting proteins (Aronheim et al., 1997; Chang, 2002; Gil et al., 2002; Hendron et al., 2002; Su et al., 2002; Wang et al., 2002a).

6.3.2 Screening for Interactions Using Immunoprecipitation

Proteins that interact with NR3 were also screened for using immunoprecipitation. This showed that NR3 interacts with DDB1, which was confirmed by both GST pulldown and further immunoprecipitation experiments. The mapping studies presented here show that the N-terminus of NR3 interacts with the substrate docking region of DDB1 (Angers et al., 2006; Li et al., 2006b). The N-terminus of NR3 is predicted to possess a structure similar to that of the armadillo repeat motif, which is involved in mediating protein-protein interactions and therefore
such a structure may be responsible for mediating the interaction with DDB1 (Herold et al., 1998; Kaufmann et al., 2000; Song et al., 2003). During the course of these studies, NR3 has been reported to interact with both DDB1 and its binding partner CUL4, although this did not represent the focus of the report (Angers et al., 2006). However, such work serves to confirm the interaction between NR3 and DDB1.

DDB1 is a substrate recognition subunit of an E3 ubiquitin ligase complex also involving CUL4 and ROC1 (Angers et al., 2006). It is known that DDB1 can recruit ubiquitination substrates to the complex both directly and indirectly via adaptor proteins (Angers et al., 2006; He et al., 2006; Sansam et al., 2006; Sugasawa et al., 2005). It may therefore be hypothesised that NR3 binds to DDB1 either as a substrate, an adaptor, or a regulatory molecule. To address this, the ubiquitination status of NR3 was examined. NR3 was shown to be polyubiquitinated in the presence of the proteasome blocker MG132, indicating that the modification leads to degradation at the proteasome. These data were supported by pulse chase experiments showing that NR3 could be stabilised by blockade of the proteasome. Therefore NR3 may be a substrate for ubiquitination by an E3 ubiquitin ligase that contains DDB1.

The use of tandem affinity purification performed separately on DDB1 and CUL4 has shown that a group of 16 proteins termed the DDB1-CUL4 associated factors (DCAFs) that interact with both DDB1 and CUL4 possess WD40 repeat motifs (Angers et al., 2006). These motifs are thought to mediate the interaction with DDB1 (He et al., 2006), and of the DCAFs identified some are known to be adaptors and substrates for DDB1 ligase complexes (Angers et al., 2006). Sequence analysis of NR3 shows that it is not predicted to possess a WD40 repeat motif, and therefore is unlikely to bind to DDB1 in the same manner as the DCAFs. However, the adaptor molecule de-etiolated 1 (DET1) was also identified as an interacting protein with both DDB1 and CUL4, yet does not possess a WD40 repeat motif (Angers et al., 2006). Therefore the role of NR3 as an adaptor molecule for DDB1 complexes may not be ruled out on the basis that it does not possess a WD40 repeat motif. It should also be noted that adaptor molecules recruited to DDB1 may also be substrates, as exemplified by DDB2 (Sugasawa et al., 2005). This protein mediates the recruitment of DDB1 substrate XPC to an E3 ubiquitin ligase complex consisting of DDB2-DDB1-CUL4 in response to UV induced damage of DNA. Following polyubiquitination of XPC, DDB2 is polyubiquitinated itself to mark it for
Chapter 6

Discussion

degradation at the proteasome (Sugasawa et al., 2005). Thus it is plausible that NR3 may be an adaptor and a substrate molecule for DDB1.

Previous studies have reported that the activity of E3 ubiquitin ligase complexes that possess CUL4 may be modulated by binding regulatory proteins and complexes. The protein CAND1 binds to cullin family of proteins and prevents interaction with substrate recruitment subunits such as DDB1 (Goldenberg et al., 2004). This interaction is prevented by neddylation of the cullin protein, which is thought to sterically hinder the binding of CAND1 (Goldenberg et al., 2004). The COP9 signalosome is responsible for deneddylation of cullins, and thus allow CAND1 access to its binding site (Cope and Deshaies, 2003; Goldenberg et al., 2004). Studies examining the association of DDB1 and CAND1 with CUL4 suggest this mechanism of regulation appears to apply to the CUL4-DDB1 interaction (Hu et al., 2004). In addition, more recent studies have found that a complex containing DDB1 may also regulate the activity of CUL4 ligases. The DDD complex comprises of DDB1, a protein called DET1 and DDB1 associated (DDA1), and substrate adaptor molecule DET1. It regulates the activity of CUL4 E3 ligase complexes by allowing the recruitment of E2 ubiquitin ligases, but preventing the maintenance of the ubiquitin thioester bond that is necessary for the transfer of the ubiquitin molecule to the substrate (Pick et al., 2007). DDA1, the primary role of which seems to be regulatory, has been shown to bind to the BPA domain of DDB1 (Pick et al., 2007). As this region forms part of the substrate docking cleft of DDB1 (Angers et al., 2006), it is possible that NR3 may play a role similar to DDA1, and thus regulate the activity of the complex.

6.4 Assessing the Function of NR3

6.4.1 Potential Role of NR3 in Cell Cycle Regulation

The interaction between NR3 and DDB1 implicates NR3 in the processes with which DDB1 is involved. E3 ubiquitin ligase complexes containing DDB1 control the levels of proteins known to be involved cell cycle progression and NER (Bondar et al., 2006; Li et al., 2006a). It is also speculated by others that NR3 may be involved in the regulation of the cell cycle (Nicassio et al., 2005). To provide evidence for such a role, the effect of NR3 expression on cell proliferation was examined. The results
presented here indicate that a stable human cell line overexpressing mouse NR3 possesses a proliferative advantage over control cells, although it is unclear as to whether the observed effect is specific to NR3 overexpression. It is possible that stable integration of the cDNAs coding for NR3 and the selective marker into the genome of the transfected cells may have disrupted regulatory mechanisms inducing an increased proliferative rate. However, this is unlikely as the cell line is a polyclonal line that is derived from a mixed population of transfected cells. Therefore genomic integration of the cDNAs will have occurred at different loci in the different cells that survived the selection process, indicating the response may be specific to NR3 overexpression. To confirm this, further experiments are required, such as the use of an inducible overexpression system to determine whether the same effect is observed in the same cell line in the absence and presence of NR3 overexpression.

In addition to NR3 overexpression, the effect of NR3 depletion using siRNA was examined in NIH/3T3 cells. No effect was observed, indicating that NR3 is not required for proliferation in that cell type. However, knockdown was typically observed to be ~95% at the mRNA level. In the absence of an antibody that detects endogenous levels of NR3 in a western blot, it is impossible to determine the effect of such knockdown on protein levels and it cannot be assumed that it will be of a similar magnitude. Furthermore, it is plausible that ~95% depletion of NR3 is not sufficient to abrogate the function of NR3 to a detectable level. However, it must also be noted that NR3 was observed to be upregulated following viral transformation of cells and in some tumours (Nicassio et al., 2005), and NIH/3T3 cells do not represent such a cell type (Todaro and Green, 1963). Therefore it is possible that NR3 expression may be more important in the regulation of the cell cycle following viral infection or tumourogenesis.

The regulation of cell cycle control proteins mediated by DDB1 appears to involve their polyubiquitination to mark them for degradation. Negative cell cycle regulator p27^Kip1^ has been described as a target for ubiquitination and degradation following association with a DDB1-CUL4 complex that utilises S-phase kinase associated protein 2 (Skp2) as an adaptor molecule (Bondar et al., 2006). This degradation is thought to occur in S-phase and allow the progression of the cell cycle. CDT1 is a DNA replication licensing factor, the activity of which must be controlled to prevent re-replication of DNA during S-phase. Such regulation may occur by inhibition of CDT1 by geminin (Tada et al., 2001; Wohlschlegel et al., 2000), or by
polyubiquitination and degradation of CDT1 (Arias and Walter, 2006; Nishitani et al., 2006; Sansam et al., 2006). The proteolytic regulation of CDT1 is mediated by two distinct pathways, one involving DDB1-CUL4 complexes and one involving an analogous complex possessing another cullin family member comprised of CUL1 and Skp2 (Nishitani et al., 2006). DDB1-CUL4 induced polyubiquitination has been reported to require proliferating cell nuclear antigen (PCNA), which may act as a clamp to target the DDB1 complex to chromatin (Arias and Walter, 2006), and is thought to be mediated by adaptor protein CDT2 (Sansam et al., 2006). Furthermore, the study of a DDB1 complex containing CUL4, CDT2, PCNA and mouse double minute 2 (MDM2) has shown that it regulates the levels of tumour suppressor gene p53 in unstressed cells, and lack of such regulation leads to cell cycle arrest in G1-phase (Banks et al., 2006).

In addition to their role in cell cycle regulation in unstressed cells, DDB1-CUL4 complexes also regulate the cell cycle in response to DNA damage. Following treatment with UV light to induce DNA damage, MDM2 is degraded by a DDB1-CUL4-PCNA complex, which leads to stabilisation of p53 (Banks et al., 2006). Cellular stresses such as DNA damage lead to activation of p53, resulting in cell cycle arrest and activation of DNA repair pathways (reviewed in Jin and Levine, 2001). Similarly, DDB1 target CDT1 is also degraded in response to UV light-induced DNA damage, preventing the licensing of replication origins and protecting the cell from replicating damaged DNA (Higa et al., 2006; Hu et al., 2004; Sansam et al., 2006).

In the results presented here, NR3 has been shown to be polyubiquitinated which marks it for degradation at the proteasome. Given the interaction observed with the substrate docking region of DDB1, it is possible that such ubiquitination is mediated by DDB1, and therefore one may speculate that NR3 may indeed be involved in the regulation of the cell cycle similar to other DDB1 substrates.

### 6.4.2 Potential Role of NR3 in Nucleotide Excision Repair

The DDB1-CUL4-ROC1 complex is also thought to be directly involved in the NER process to remove DNA lesions following UV light exposure (El-Mahdy et al., 2006; Li et al., 2006a; Moser et al., 2005; Sugasawa et al., 2005). Following DNA damage the DDB1 complex bound to adaptor molecule DDB2 recognises and binds to the UV-induced lesions. NER protein XPC is recruited to lesions and binds to DDB2,
resulting in the polyubiquitination of XPC. Such modification allows XPC to more tightly associate with damaged DNA and enhance repair (Sugasawa et al., 2005). DDB2 is then polyubiquitinated by DDB1-CUL4-ROC1, marking it for degradation (El-Mahdy et al., 2006; Sugasawa et al., 2005). Furthermore, the DDB2-DDB1-CUL4-ROC1 complex has been shown to remodel chromatin by the polyubiquitination of histones H2A, H3 and H4 following DNA damage with UV light, a response which is thought to enable the recruitment of repair complexes to sites of damage (Kapetanaki et al., 2006; Wang et al., 2006). It is conceivable that NR3 may play a role in the direct regulation of NER performed by DDB1. DDB2 is thought to bind directly to DDB1, as they co-purify from damaged DNA as a heterodimer (Keeney et al., 1994). As DDB2 is both a substrate and adaptor for DDB1-CUL4-ROC1 mediated ubiquitination, it would be predicted that the binding site within DDB1 for DDB2 would overlap that shown here to be important for interaction with NR3. Therefore it would also be predicted that any role NR3 possessed in the direct regulation of NER would not involve it complexing with both DDB1 and DDB2 simultaneously.

6.4.3 DDB1 Studies in the Whole Organism and Potential Other Roles for DDB1 Complexes

Studies involving the effects of loss of DDB1 in flies by gene disruption and depletion with siRNA have shown that DDB1 is involved in the development of the organism, and is required for cell proliferation genomic stability (Shimanouchi et al., 2006; Takata et al., 2004). Global knockout of the DDB1 gene in mice unsurprisingly resulted in embryonic lethality at day E12.5 (Cang et al., 2006). Studies were therefore performed on conditional knockout mice which showed similar results to those observed in Drosophila. Deletion of DDB1 in the brain and lens, and in the epidermis of mice leads to p53 induced apoptosis of proliferating cells and genomic instability (Cang et al., 2006; Cang et al., 2007). These studies serve to underline the necessity for DDB1 in the regulation of the cell cycle and DNA repair.

However, it is noteworthy that most of the DCAF proteins identified by Angers et al (2006) remain functionally uncharacterised. It is therefore possible that they may not be involved in regulation of the cell cycle and NER, implicating DDB1 in other biological processes. This leads to the speculation that NR3 may be involved in a
process unrelated to the regulation of the cell cycle and NER, and as such its interaction with DDB1 may represent a novel process in which DDB1 is involved.

6.4.4 DDB1 Forms Part of a Ligand Regulated E3 Ubiquitin Ligase Complex

The aryl hydrocarbon receptor (AhR) is a protein that is functionally analogous to NRs. The AhR is generally regarded as a xenobiotic receptor that heterodimerises with the aryl hydrocarbon nuclear translocator (ARNT) and transcribes target genes, such as the cytochrome P450 enzymes, in response to environmental toxins (reviewed in Barouki et al., 2007). However, a role for the AhR outside detoxification has also been implied (Barouki et al., 2007), which has led to the suggestion of eicosanoids and their metabolites as endogenous ligands (Chiaro et al., 2008; Schaldach et al., 1999; Seidel et al., 2001). Recent work has shown that the AhR-ARNT heterodimer can form a complex with DDB1-CUL4-ROC1 and transducin-β-like 3 in a ligand dependent manner, thus forming a ligand regulated E3 ubiquitin ligase (Ohtake et al., 2007). The assembled E3 ligase polyubiquitinates ERα to target it for degradation at the proteasome, a process that appears to be independent of ERα ligand and ser-118 phosphorylation induced degradation (Ohtake et al., 2007). The existence of a ligand regulated E3 ubiquitin complex involving DDB1 is an intriguing prospect. If NR3 does possess an LBD that may bind ligand, it is possible that when part of a complex with DDB1 it may function analogously to the AhR E3 ligase. However, it is worth noting that any such analogous function is likely to be mechanistically different as the interaction between the AhR-ARNT heterodimer and the E3 ligase is mediated by CUL4, and not DDB1 as in the case for NR3 (Ohtake et al, 2007).

6.4.5 Elucidation of NR3 Function by the Generation of Null Mice

To understand the biological role of NR3 in vivo mice that do not express NR3 are being generated. This has involved the construction of a targeting vector that allows the employment of a strategy to insert loxP sites flanking exon 1 of the NR3 gene, in which the translational start codon is located. The construction of the targeting vector was performed here by cloning the long and short homology arms, and inserting them with the appropriate loxP sites and selection cassettes into the vector backbone. This was then passed to Genoway to generate heterozygous floxed
mice as outlined in appendix B. Currently heterozygous floxed mice have been
generated by Genoway, in which no severe defect or phenotype has been observed,
indicating that the insertion of \textit{loxP} sites at the chosen loci has not disrupted gene
expression. These mice possess a neomycin selection cassette that has been excised in
some but not all cells by the backcrossing of floxed mice with \textit{Flp} expressing mice.
Currently a breeding program to completely excise the neomycin selection is being
undertaken. Following the generation of heterozygous floxed mice in which the
neomycin selection cassette has been excised in all cells, homozygous floxed mice
will be bred. Such animals will be crossed with mice globally expressing Cre
recombinase to delete NR3 in the whole animal, and initial phenotypic analysis will
be performed on homozygous NR3 knockout mice.

One may only speculate as to what the phenotype of NR3 null mice may be. The
data of others suggest NR3 possesses a role in TNF signalling (Soond \textit{et al.}, 2003;
Soond \textit{et al.}, 2006), and therefore null mice may possess defects in such signalling
pathways. Given the link with NR3 and the regulation of the cell cycle based on the
data of Nicassio et al (2005) and the interaction with cell cycle regulator DDB1
presented here, it is possible null animals may possess a defect in cell proliferation.
This may provide a similar phenotype to that of DDB1, which were shown to possess
developmental defects lethal to embryos (Cang \textit{et al.}, 2006). If NR3 were to be
involved in DNA repair, it may be predicted that null mice may possess genomic
instability as observed in DDB1 knockout mice (Cang \textit{et al.}, 2006; Cang \textit{et al.}, 2007),
or be prone to tumours similar to mice lacking DDB2 (Itoh \textit{et al.}, 2004; Yoon \textit{et al.},
2005). It is also possible that mice lacking NR3 may not show an overt phenotype.
This seems unlikely as one may predict the role of NR3 to be important. The
conservation of the protein extends to the genome of the simple chordate
\textit{Oikopleura dioica}, which possesses one of the smallest genomes in chordates (Seo \textit{et al.},
2001). NR3 also does not appear to be a member a family of homologous proteins as
indicated by BLAST searches, and as such one might predict that NR3 deletion would
not produce a phenotype that is masked by a redundancy effect from proteins
possessing overlapping function.
6.5 Overall Conclusion

NR3 is predicted by bioinformatic methods to possess a NR-like LBD structure. Although it is unlikely NR3 acts as a transcription factor due to the lack of a characterised DBD, it is possible that the LBD may modulate the activity of NR3. The aim of this project was to characterise the biological function of NR3 and to examine the role of the putative LBD region within that function. Others have suggested NR3 may possess a role in TNF signalling and the regulation of the cell cycle (Nicassio et al., 2005; Soond et al., 2003; Soond et al., 2006). The work presented here has shown that NR3 associates with DDB1, a member of an E3 ubiquitin ligase complex involved in regulation of the cell cycle and NER, indicating NR3 may be involved in those processes. The generation of NR3 null animals may provide evidence to support this implication.

Preliminary structural work performed on the putative LBD suggests the region is ordered and structured, lending support to the idea it may be structurally similar to the LBD of a bona fide nuclear receptor. It is hypothesised that the putative LBD region regulates the activity of NR3 in a functionally analogous manner to which the LBD of bona fide nuclear receptors regulates their activity. In the absence of a confirmed biological function for NR3 it is not possible to test this hypothesis. However, given the characterisation of a DDB1 E3 ubiquitin ligase complex involving the AhR that may be regulated by ligand (Ohtake et al., 2007), it is possible NR3 may possess an analogous role to the AhR within the DDB1 complex.
Appendix A

List of Primers
### List of Primers

#### General Sequencing Primers

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<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' - 3')</th>
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<td>BGH Rev D-TOPO</td>
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<tr>
<td>M13 Fwd TOPO-FA</td>
<td>CTAAAACAGGCCG</td>
</tr>
<tr>
<td>M13 Rev TOPO-FA</td>
<td>CAGGAAAGATCTTAC</td>
</tr>
<tr>
<td>SP6 Promoter</td>
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<tr>
<td>T7 D-TOPO</td>
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#### Primers used for CytoTrap Constructs

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<th>Primer Name</th>
<th>Primer Sequence (5' - 3')</th>
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Appendix A
### Appendix A

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### QRT-PCR Primers

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Appendix B

Summary of Vectors and the Work of Genoway During the Generation of NR3 Knockout Mice
B.1 Summary of the Plasmids used in the Generation of the NR3 Targeting Vectors

Schematic diagrams of the plasmids used for generation of the final targeting vector as outlined in chapter 6 are shown in figures B.1-B.10. All schematics were generated by Genoway, Lyon. The sequence of the sense oligonucleotide synthesised by Top Gene Technologies is shown in figure B.11.
**Figure B.1 Schematic of the Vector G139**
G139 possesses the proximal *loxP* site and neomycin positive selection cassette consisting of the neomycin resistance gene flanked by FRT sites.

**Figure B.2 Schematic of the Vector G112**
G112 possesses the diphtheria toxin A negative selection cassette (DTA).
Appendix B

Figure B.3 Schematic of the Vector RWH1-SA
RWH1-SA mod possesses the corrected short homology arm (SA) and the control homology arm (SA max) regions.

Figure B.4 Schematic of the Vector RWH1-LA
RWH1-LA bac possesses the long homology arm (LA) in a pCR2.1 backbone.
Figure B.5 Schematic of the Vector RWH1-SA-C+
RWH1-SA-C+ possesses the short homology arm (SA) and the control homology arm (SAmax) regions, the proximal loxP site, and the neomycin positive selection cassette.

Figure B.6 Schematic of the Vector RWH1-LA-LoxP
RWH1-LA-LoxP possesses the long homology arm (LA), the proximal loxP site in a pCR2.1 backbone.
Figure B.7 Schematic of the Vector RWH1-GA1-linker
RWH1-GA1-linker possesses a 3’ region of the long homology arm and a 3’ region of the short homology arm interspersed by a polylinker sequence.

Figure B.8 Schematic of the Vector RWH1-Neo/SA
RWH1-Neo/SA possesses the short homology arms (SA), the proximal loxP site, and the neomycin positive selection cassette.
Figure B.9 Schematic of the Vector RWH1-LSA-Neo
RWH1-LSA-Neo possesses the long (LA) and short homology arms (SA), the distal and proximal loxP sites, and the neomycin positive selection cassette.

Figure B.10 Schematic of the Final Targeting Vector RWH1-HR
The final targeting vector possessing the long (LA) and short homology arms (SA), the diphtheria toxin A (DTA) negative selection cassette, the distal and proximal loxP sites, and the neomycin positive selection cassette.
Figure B.11 Sequence of the Sense Oligonucleotide Synthesised by Top Gene Technologies

Complementary oligonucleotides of the sequence above were synthesised and cloned into the Genoway vector pGA1 by Top Gene Technologies. Blue text denotes the 3’ region of the long homology arm and red text denotes the 3’ region of the short homology arm.
B.2 Summary of the Work Carried Out at Genoway

Genoway are responsible for carrying out the work in the generation of NR3 floxed mice following the completion of targeting vector construction. All work described in this section was performed by Genoway.

B.2.1 Electroporation of ES Cells and Detection of Homologous Recombination

To prepare the targeting vector for electroporation into ES cells, Genoway linearised it using NruI restriction endonuclease, which digests the vector downstream of the short homology arm. In four separate transfections, the linearised vector was electroporated into ES cells, where recombination may occur between genomic DNA and the targeting vector. ES cells that had undergone recombination were selected by treatment with 200µg/mL G418 48 hours post transfection. A total of 565 clones resistant to G418 were isolated and amplified in 96-well plates. Duplicates of the clones were made, using one copy to store at -80°C and the other to purify genomic DNA. Genomic DNA was then screened for homologous recombination to ensure the NR3 locus was correctly targeted.

The initial screening strategy Genoway employed was one based on PCR. To screen for homologous recombination of the short homology arm, the PCR reaction was optimised using RWH1-SA-C+ as this possesses an extended 3’ region of the short homology arm (figure B.12A). The forward primer is designed to anneal within the neomycin selection cassette and the reverse primer downstream of the short homology arm, producing a 3kb amplicon. This screening method provides a convenient and accurate way of determining whether recombination downstream of the neomycin resistance cassette has occurred at the NR3 locus. Genoway used RWH1-SA-C+ diluted in 10ng of genomic DNA. Dilutions represented the either 0.1, 1 or 10 copies of plasmid per copy of genome to determine the sensitivity of the PCR. The positive controls in the absence of genomic DNA give an increase in PCR product with increasing template. PCR products of the correct size are observed in the presence of genomic DNA, although with a lowered yield (figure B.12B). The negative control reactions of genomic DNA alone and no template do not provide a PCR product, indicating the primers are specific to the targeting vector. A dilution of
Figure B.12 PCR Screening Strategy for 3’ Homologous Recombination

**A)** Schematic depicting the position of primer hybridisation during the PCR reaction. The forward primer anneals to the neomycin cassette (Neo) and the reverse primer anneal downstream of the short homology arm.

**B)** Agarose gel of PCR reaction optimisation performed by Genoway. RWH1-SA-C+ was diluted to give the equivalent of 0.1, 1 or 10 copies of the vector per copy of the genome within 10ng of wild-type (WT) genomic DNA. This was then used as a template alone for PCR as a positive control, or mixed with 10ng WT genomic DNA to determine the sensitivity and specificity of the PCR reaction. Negative control reactions used 10ng WT genomic DNA alone or no template (H2O).
1 copy of RWH1-SA-C+ per copy of genome emulates the conditions that would be expected following homologous recombination. These results indicate the PCR system optimised by Genoway is sensitive and specific enough to detect homologous recombination of the short homology arm. Using this method Genoway identified a total of 40 clones that provide PCR products of the expected size (data not shown).

Similarly a PCR based method was employed by Genoway to determine correct recombination at the 5’ end of the targeting vector, thus integrating the distal loxP site at the NR3 locus. However, both primers were designed by Genoway to hybridise to genomic sequences and as such the PCR fragments require DNA sequencing to determine the presence of the distal loxP site. As the loxP site integration occurs at one allele, the sequencing data displays a double signal at the appropriate locus. Using this method Genoway screened the 40 positive clones identified as having undergone homologous recombination within the short homology arm region, which indicated 14 clones to have also undergone homologous recombination at within the 5’ region of long homology arm, thus allowing the integration of the distal loxP site within the NR3 locus (data not shown).

A secondary screen was also employed by Genoway to confirm the recombination event in positive clones was homologous using the method of Southern blot. To detect homologous recombination within the 5’ region of the long homology arm, genomic DNA was digested with NsiI and subject to Southern blot using a probe designed to hybridise upstream of the long homology arm (figure B.13A). Non-targeted alleles should possess a fragment of 10.9kb, whereas targeted alleles should produce a fragment 7.7kb in size. For detecting homologous recombination within the short homology arm, genomic DNA was digested with EcoRV and a probe used that hybridises within the short homology arm (figure B.13B). This should detect a 5.9kb fragment for targeted alleles and a 11.1kb fragment for non-targeted alleles. In both cases clones in which homologous recombination has occurred should produce bands for both targeted and non-targeted alleles, as only one allele is floxed. Figure B.13 shows data representative of Genoway’s Southern blot screening method. Clone 9-1C was indicated by PCR to have undergone homologous recombination within the short homology arm and the 3’ region of the long homology arm, thus is negative in the Southern blot for distal loxP site integration (figure B.13A), but positive for recombination within the short homology arm (figure B.13B). Clone 10-1A was
Figure B.13 Southern Blot Screening for Homologous Recombination
Southern blot analysis of homologous recombination occurring within A) the 5’ region of the long homology arm and B) the short homology arm. Schematics depict the position of probe hybridisation and expected fragment size based on recombination status. Restriction enzymes in red denote those used to digest DNA for Southern blot analysis. Gels are representative of the data generated by Genoway.
determined to be positive for both homologous recombination within the short homology arm and 5’ region of the long homology arm, which is confirmed by Southern blot (figure B.13A and B). In total 5 of the 14 clones suggested to be positive by PCR were confirmed to have undergone homologous recombination at the appropriate locus by Southern blot performed at Genoway. These clones are referred to by Genoway as 10-1A, 28-2H, 30-4F, 31-3B and 31-1C.

**B.2.2 Generation of Floxed Mice and Current Status of the Project**

The ES cells used by Genoway are removed from 3.5 day old embryos at the blastocyst stage, which allows their reintroduction by microinjection to blastocysts having following genetic manipulation. Clones 10-1A, 28-2H, 31-3B and 31-1C were injected into harvested blastocysts, which were then implanted into foster mothers. Pregnant foster mothers give birth to chimeric pups that possess cells derived from the injected floxed ES cells or from the ES cells pertaining to the blastocyst. The ES cells used by Genoway for transfection and subsequent blastocyst injection are derived from 129S strain of mouse, which possess an agouti coloured coat. The blastocysts are harvested from C57Bl mice, which have a black coloured coat. The dependency of the coat colour on the genetic background of the mice allows the estimation of the proportion of cells derived from the floxed ES cells as chimeric offspring possess a mixed coat colour. The work undertaken by Genoway to generate chimeras is summarised in table B.1.

Following the generation of chimeric offspring, chimeras must be backcrossed with wild-type mice to enable germline transmission of the floxed allele. Genoway have carried out breeding programs for the male chimeric mice generated by ES cell microinjection and blastocyst re-implantation that are estimated to display a level of chimerism of ~80%. Such breeding has been performed with male mice derived from ES cell clones 10-1A, 28-2H, 31-3C and with female mice globally expressing Flp recombinase that will remove the neomycin selection cassette from the floxed allele. Breeding from mice derived from clones 10-1A and 31-3C did not yield germline transmission of the floxed allele. However, germline transmission was observed in chimeric mice derived from clone 28-2H, and a total of 11 agouti pups were generated by the breeding program. As the ES cells from which these animals were derived
### Table B.1 Summary of the Work Carried out by Genoway to Generate Chimeric Mice

Transfected ES cells clones identified by Genoway using PCR and Southern blot techniques as having undergone homologous recombination were microinjected into blastocysts harvested from pregnant mice. Injected blastocysts were then implanted into foster mothers to generate chimeric offspring. The level of chimerism is an estimation of the percentage contribution of cell genotype from injected ES cells based on coat colour.

<table>
<thead>
<tr>
<th>Floxed Clone</th>
<th>No. of Injected Blastocysts</th>
<th>No. of Reimplantations</th>
<th>No. of Pregnancies</th>
<th>Total No. of Pups Born</th>
<th>No. of Pups Still Born</th>
<th>Total Number</th>
<th>Level of Chimerism</th>
<th>Total Number</th>
<th>Level of Chimerism</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-1A</td>
<td>46</td>
<td>3</td>
<td>3</td>
<td>17</td>
<td>0</td>
<td>8</td>
<td>20%</td>
<td>5</td>
<td>20%</td>
</tr>
<tr>
<td>28-2H</td>
<td>71</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>80%</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>31-3B</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>80%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31-3C</td>
<td>28</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>3x80%, 2x50%</td>
<td>1</td>
<td>80%</td>
</tr>
</tbody>
</table>
possess both a targeted allele and a non-targeted allele, 50\% of the animals will be heterozygous mice floxed at the NR3 locus. Genoway performed PCR genotyping to determine which offspring were heterozygous for the floxed NR3 locus, and to ensure the neomycin selection cassette was excised in those mice. Although \sim50\% of the offspring were found to be heterozygous floxed mice, such screening using primers that are designed to amplify the selection cassette showed only partial excision was achieved (data not shown). This indicates that the excision event has occurred in some but not all cells within the heterozygous floxed mice. Therefore the mice offspring must be backcrossed with the Flp expressing line again to ensure excision of the neomycin selection cassette in all cells. This backcross is currently being undertaken by Genoway.
References


References


References


References


References


References


