An investigation into the relationship between Notch and Ero1

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Declaration

I declare that the composition of this thesis, and all data presented herein, is the result of my own work. No part of the material offered has previously been submitted for a higher degree. The body of work has been achieved under the supervision of Dr. Adam M. Benham.

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Finally to my friends and family for keeping my happy and sane through the whole process.

Abstract

Ero1 is an endoplasmic reticulum (ER) resident protein responsible for PDI oxidation in disulphide bond formation. Recent work has shown a link between Ero1 and Notch pathway proteins. We wished to see if the oxidative folding of notch was directly catalysed by the Ero-PDI pathway or if Ero1 was involved in transcriptional modulation of Notch signalling. Using western blot analysis, immunoprecipitation and immunofluorescence analysis we were able to characterise and localise relevant proteins within cells. The range of cell lines and conditions under which the Ero1 antibody 2G4 were confirmed and extended.

A novel link between Ero1 and Jagged1, a notch ligand was found. In immunoprecipitation studies Ero1α was able to retrieve Jagged 1 and the opposite was also true, with Jagged 1 pulling down Ero1α as well. This suggests a novel and important link between the Ero1 and the function of the notch signalling pathway.

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1. Introduction

In order for a living cell to function viably it must be able to successfully synthesise and secrete functional proteins. A critical part of a cell secreting a working protein is ensuring that correct protein folding takes place. Protein folding has attracted a large amount of research due to the association of protein misfolding in several types of diseases including ageing disorders, diabetes, cancer and cystic fibrosis (Dobson, 2003). For example, nearly 90% of all patients of cystic fibrosis have mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) at position 508. The CFTR is an ion channel responsible for transport of chlorine and its loss of function leads to cystic fibrosis. CFTR undergoes a series of strict post translational modifications in the ER including, glycosylation and oligosaccharide chain modification, with only a 33% of all proteins produced being released from the ER in their native states. Mutation of the phenylalanine at position 508 completely blocks the maturation and export of the CFTR from the ER. This is believed to occur due to an altering of the binding site of the vesicle coat protein sec24 to the CFTR which prevents the CFTR from being exported from the ER by COP II vesicles (Riordan, 2008). As well as cystic fibrosis, aggregation of proteins due to misfolding or mistranslation can cause diseases such as Huntington's, Parkinson's and Alzheimer's (Vembar and Brodsky, 2008).

The endoplasmic reticulum (ER) is a highly specialised compartment within the cell which deals with a huge amount of protein maturation, including ensuring correct protein folding takes place (Helenius et al, 1992). To achieve this role the ER has evolved a large variety of molecular chaperones optimised to the role of protein folding. There are a huge variety of these chaperones in the ER, each with its own role

and function in ensuring proteins achieve their native state. Protein folding begins during synthesis as a vectorial process (Braakman & Bulleid, 2011) and is limited by the rate of protein transcription. Folding complexes compete with the protein itself to delay interactions within the protein, allowing the protein to be fully translated before it begins folding and prolonging the duration that the protein is in its less folded state. This helps prevent the protein from becoming misfolded before it has even been fully translated.

Posttranslational folding makes up the bulk of the work which is carried out by the ER. Research has provided a large list of proteins which have been shown to affect protein folding directly or that are assumed to have a folding role based on their structural similarities to other ER proteins. Examples of these proteins and their roles are wide ranging, and include Binding immunoglobulin protein (BiP) which has a very broad specificity and BiP helps maintain proteins in a optimal state for folding and the recruitment of co-chaperones. Other roles provided by ER proteins include glycan-binding proteins like the Calnexin family of proteins, protein disulphide isomerases such as PDI and peptidyl prolyl *cis/trans* isomerases. Acting as folding catalysts, these chaperones ensure that unfolded polypeptides first achieve their correct tertiary structure and then later act as a quality control mechanism, ensuring only native folded proteins are produced by the cell (Hurtley and Helenius, 1989). Enzymatic modification of proteins via carbohydrate addition and the formation or translocation of disulphide bonds occurs in the ER as a level of protein quality control (Sevier and Kaiser, 2008).

If the folding of proteins is disrupted within a cell or the folding demand passes a cells capacity to fold proteins and misfolded proteins begin to build up in the ER the cell activates the unfolded protein response (UPR) (Ron and Walter, 2007) which causes

the cell to begin to produce more ER associated chaperone molecules, expand the ER lumen and increase the rate of removal of terminally misfolded proteins from the cell via ER-associated degradation (ERAD) (Cox et al, 1993). ERAD acts in three main capacities to ensure that any misfolded protein is removed from the cell. Molecular chaperones, such as the Hsp-70 family (Vembar and Brodsky, 2008), are able to bind to proteins which are to be targeted for ERAD. Detection of ERAD is dependent on factors such as exposed hydrophobic regions, the presence of immature N-linked glycans as recognised by UDP-glucose:glycoprotein glucosyltransferase (Caramelo et al, 2003) or failures in the protein folding, such as unpaired cysteine residues which can be recognised by complexes of PDI (see later) and BiP (Molinari et al, 2002). After being targeted for ERAD proteins are exported to the cytoplasm by the Sec61 translocation channel and transported to the ubiquitin-proteasome system (UPS). The proteins are tagged for degradation by the 26S proteasome (Vembar and Brodsky, 2008) via ubiquitination which is carried out by three types of enzyme, E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligases. These processes of ERAD act together in tandem to ensure that no misfolded or otherwise incorrect protein is able to leave the ER.

One integral part of the protein folding process is the formation of disulphide bonds. Disulphide bonds are estimated to be involved in the formation of around one third of all human proteins (Hatahet and Ruddock, 2009) and are required for protein stability, regulation of protein activity and redox enzymatic function (Ellgaard et al, 1999). A disulphide bond is a covalent link between two thiol (-SH) side chains on cysteine residues within polypeptides. Reduction-oxidation (redox) reactions are reactions in which one chemical species attains a higher oxidation state via the loss of electrons or gain of oxygen, whilst conversely another species gains a lower oxidation state via the

gaining of an electron or loss of oxygen (Hatahet and Ruddock, 2009). Within thiol groups the sulphur has the oxidation state of -2 and when two groups are oxidised together to form a disulphide bond they are both lowered in oxidation state to -1. For this to occur, the reaction needs to lose two electrons and two protons to an oxidant, such as molecular oxygen. Any cysteine residue in a protein has the potential to form a disulphide bond with another, giving huge numbers of potential redox states and potential tertiary configurations for a protein, and yet most proteins have only a very select number of disulphide bond configurations in their native state.

The classic experiments by Anfinsen et al (Anfinsen et al, 1963) showed that formation of disulphide bonds is a spontaneous process *in vitro* with only the polypeptide required in order to achieve its native state. However due to disulphide bond formation relying on a redox reaction, it is naturally a slow process and would also require an electron acceptor, such as oxygen, *in vivo*. These factors would suggest that disulphide linked folding must be an assisted process within the ER (Tu and Weissman, 2002).

The first work identifying the proteins that assisted disulphide bond formation in the ER was undertaken by two groups during the 1960's. Venetianer and Straub (Venetianer and Straub, 1963) found that they were able to reoxidise reduced ribonuclease with extracts from the pancreas of either chickens or pigeons. At the same time work by Anfinsen et al (Anfinsen et al, 1963) showed that microsomal systems from rat liver sped up the reactivation of ribonuclease. Later purification of the enzyme responsible showed it was able to catalyse thiol-disulfide exchange reactions and required a cofactor for its activity, which could be replaced by the addition of flavo adenine dinucleotide (FAD). The enzyme discovered was named protein disulphide isomerase (PDI) and was the first folding catalyst reported. It is

interesting to note that this work commented on a cofactor containing FAD, which was later named Ero1, nearly 30 years before it was fully characterised and found to be required for PDI activity.

PDI is a 491 amino acid protein present in the ER in high concentrations and has a KDEL-ER retrieval motif (Pellham, 1990), marking it as an ER located protein. PDI has a multidomain structure, with 4 distinct domains **a, a', b** and **b'** as well as a C-terminal extension **c** (Hatahet and Ruddock, 2009). The **a** and **a'** domains of PDI contain the catalytic domains of PDI, found at the N-terminus of the α2 helix whilst the **b** and **b'** domains have been shown to be the principal binding sites of PDI (Klappa et al, 1998).

PDI has been shown to have other roles within the cell, including acting as subunits of other protein complexes (Hatahet and Ruddock, 2009). For example, the P4HB gene, which codes for PDI has been linked with collagen formation. Here PDI acts as a subunit of a complex known as Prolyl 4-hydroxylase, which catalyses the formation of 4-hydroxyproline residues that act to stabilise the triple helices seen in collagen structure (Pihlajaniemi et al, 2005). However, for my work I will focus on its roles in oxidative protein folding.

PDI acts as an oxidant, containing a disulphide bond which is transferred over to a folding protein and thus becomes reduced to a dithiol state (Hatahet and Ruddock, 2009). As mentioned above proteins have a huge number of potential configurations of disulphide bonds and free thiol groups, of which one will be its native state. Due to the random nature of protein folding, which could potentially bring any combination of cysteine residues together to form disulphide bonds, both native and non native state proteins can be found in the ER. Proteins fold rapidly with random thermal changes

leading the folding to move energetically towards the native structure, however the process is not perfect and non-native structures do occur (Dill and MacCallum, 2012). Thus the cell must be able to convert non native proteins into their native state.

There are two potential pathways suggested to allow for these changes. The protein can either undergo a series of redox reactions, where the disulphide bonds are first reduced to their thiol state before being reoxidised in a new configuration, or undergo direct isomerisation. PDI can catalyse either of these reactions. PDI reacts with molecular oxygen slowly, meaning that all reactions catalysed by PDI must be thiol-disulphide exchange reactions. This gives PDI the potential to catalyse three types of reactions.

The first type of reaction which PDI can support is an oxidation reaction. This is done by losing one of the disulphide bonds from one of the active sites to a protein, causing a dithiol group on the protein to be oxidised to a disulphide state. To then finish this cycle PDI must be reoxidised. The reverse of this reaction is also catalysed by PDI, in which it reduces a protein disulphide bond to its dithiol state and in turn gains an active site disulphide bond itself. The PDI is then reduced to complete this cycle. Finally, in direct isomerisation PDI assists in the rearrangement of disulphide and thiol groups within a protein. This involves no change in the redox potential of the system and thus no extra reagents are needed.

Before 1998 general opinion was that PDI was oxidised *in vivo* by reaction with oxidised glutathione (GSSG). However the discovery of the Endoplasmic Reticulum Oxidoreductin (Ero) family of proteins changed this, showing that GSSG is in fact a non functional by-product of the disulphide bond formation pathway. More recent work has shown that there is a complex interplay between reduced glutathione (GSH)

and Ero1 family members. GSH is responsible for the transfer of reducing equivalents from the cytosol into the ER (Molteni et al, 2004). Then GSH is oxidised to GSSG, forming a redox buffer to help maintain ER homeostasis.

As mentioned above when Anfinsen et al (Anfinsen et al, 1963) first purified PDI, they found that a small low molecular weight molecule was also needed for PDI activity. The molecule was lost during dialysis but the function of PDI could be restored by adding FAD to the PDI, and was the first retrospective evidence of Ero activity. Frand *et al* (Frand *et al*, 1998) and Pollard *et al* (Pollard *et al*, 1998) were the first to report a homologue of this gene product, Ero1p, in *S.cerevisiae* as a molecule required for the formation of disulphide bonds in the ER. Homologues in many other eukaryotes were soon found.

The Ero1 family are flavoproteins with two distinct di-cysteine active sites that act in the transfer of electrons from the substrate to the FAD molecule (Appenzeller-Herzog et al, 2008). One active site, the outer site, interacts and binds with the substrate whilst the second, inner site is next to the FAD molecule. The cysteine residues located on the outer site are able to move via a flexible polypeptide segment and shuttle electrons to the inner active site (Gross et al, 2004).

Ero1p was shown to be a cysteine rich and highly glycosylated molecule associated with the ER membrane. Studies by Frand and Kaiser (Frand and Kaiser, 1999) showed that Ero1p was required for the oxidation of the yeast PDI analogue Pdi1p and that the N-terminal active site of Pdi1p formed mixed disulphide bonds with Ero1p *in vivo* showing that Ero1p directly oxidises Pdi1p. Ero1 family members have been shown to have a large amount of cysteine residues in their structure which are essential for their

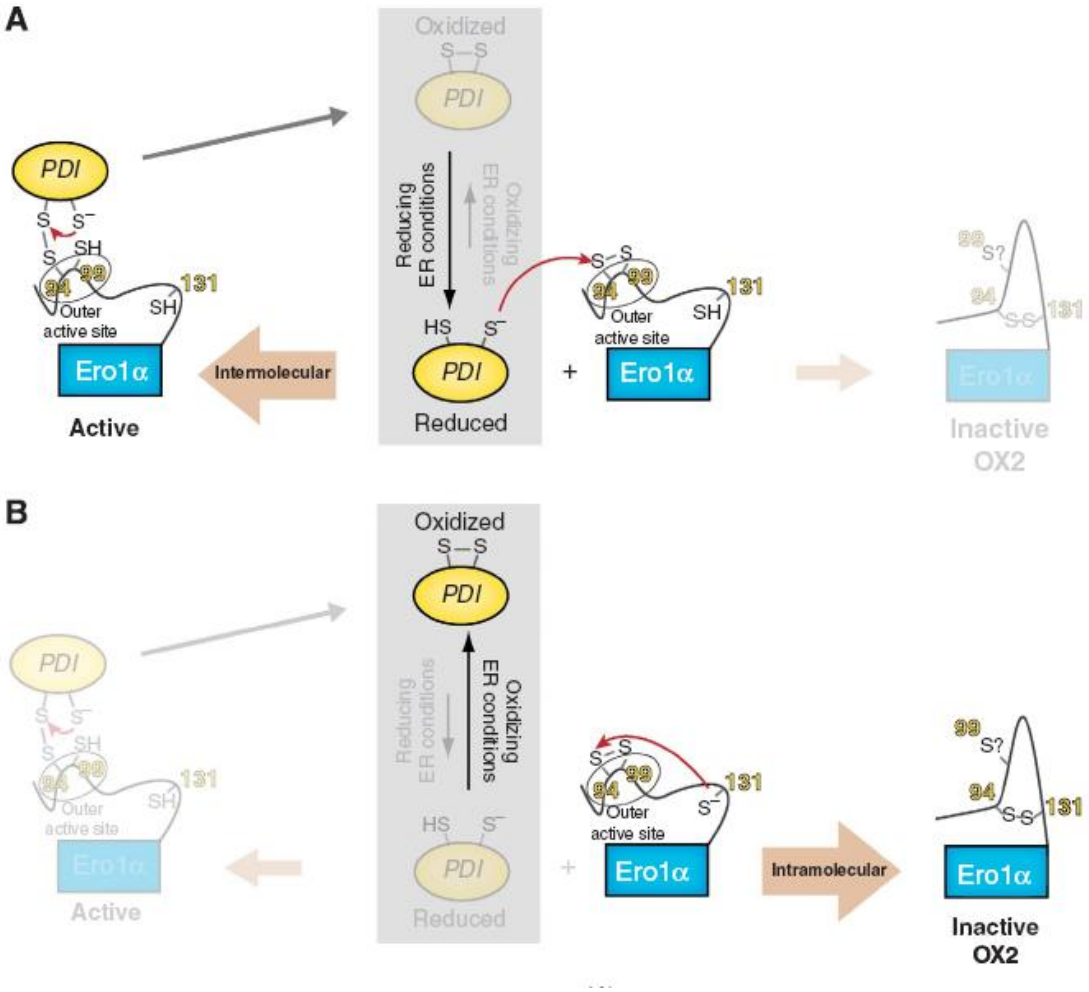

Figure 1. **Model for control of the PDI redox state by Cys131 dependant feedback regulation of Ero1α** a, Under reducing ER conditions, PDI accumulates in the reduced form. Reduced PDI is a substrate for oxidation by Ero1a. A thiol–disulphide exchange reaction between Ero1a (in the active form) and PDI then results in the formation of oxidized PDI. By this mechanism, the activity of Ero1a counterbalances reducing ER conditions. b, Under oxidizing ER conditions, PDI is predominantly oxidized and the outer active-site disulphide of Ero1a will, therefore, preferentially react with Cys131. The resulting OX2 configuration of Ero1a is inactive because the outer active Cys94 is covalently blocked by Cys131. (Diagram and description adapted from Appenzeller-Herzog et al, 2008).

folding and function (Tu et al, 2000). The number of different disulphide bond states was later found to be part of a regulatory system of Ero1, with at least one of them most likely being a quiescent state for the protein. Benham et al (2000) showed that Ero1α exists in two oxidised redox forms *in vivo*, OX1 and OX2, and the switching between the two directly regulates enzymatic activity (Appenzeller-Herzog et al, 2008). Also, the activation of Ero1α is directly linked to availability of reduced PDI, giving evidence of a feedback loop control of ER redox (see Figure 1). Work by Sevier et al (Sevier et al, 2007) has identified two disulphide bonds in Ero1p that must be reduced in order for the protein to have catalytic activity. The cysteine pairs located at Cys90-Cys349 and Cys150-Cys295 (see Figure 2) affect the activity of the protein in a redox dependent manner. When these cysteines are in their reduced form the Ero1p is catalytically active and it is not when they are found in the oxidised disulphide form. Sevier et al (Servier et al, 2007) suggest that the regulation of the activity of Ero1p in this manner aids the cell in governing the flow of oxidising equivalents within the ER which allows the cell to maintain the optimum conditions for native disulphide formation. The method by which Ero1α in mammalian cells is regulated is based on the same principle of disulphide bond formation but the process is different. Whereas the yeast protein is activated by the reduction of two cysteine pairs, Ero1α is controlled by the formation of regulatory disulphide bonds between non-catalytic cysteines and cysteines located within the first active site of the protein (Baker et al, 2008). The formation of a disulphide bond between Cys94 and Cys131 covalently blocks Cys94 which is the first shuttle cysteine and catalytically essential to the Ero1α function (Appenzeller-Herzog et al, 2008).

The identification of these regulatory bonds in both yeast Ero1p and mammalian Ero1α showed that whilst both used the formation of these disulphide bonds as a

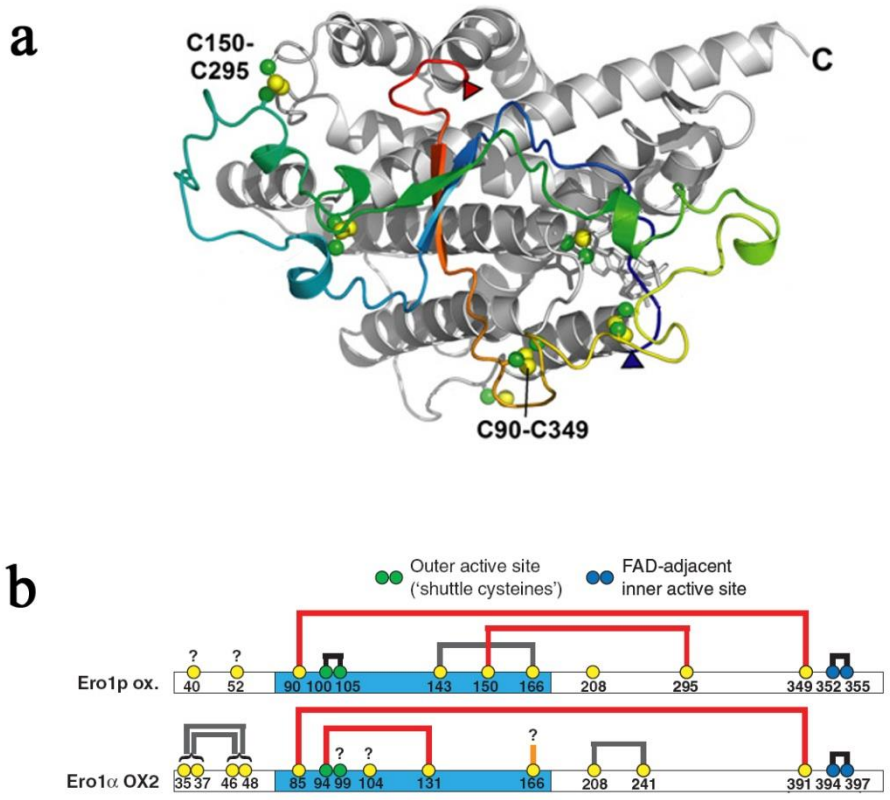

Figure 2. **Comparison of the regulatory cysteine residues for Ero1p and Ero1α**. a, Structure of Ero1p showing the locations of regulatory cysteine residues. Cystein side chains are depicted by green and yellow spheres. b, Schematic representations of the locations of the cysteine residue connectivity in Ero1p and Ero1α. The cysteines are shown as yellow, green (outer active site) or blue (inner active site) circles with amino-acid numbering, and disulphides as thick grey (likely structural), black (active site) or red (reported or inferred regulatory function) lines. (2a was adapted from Gross et al, 2004 and 2b was adapted from Appenzeller-Herzog et al, 2008)

control mechanism, the relative positions of the bonds and the method of activation were different. This difference is as yet unaccounted for and it is unknown why this change has occurred or what its implications are to the functions of the otherwise similar molecules.

Two individual Ero1 proteins have been found in mammals and humans, Ero1α and Ero1β, both of which are able to rescue *S.cerevisiae* from several phenotypic changes seen by the loss of Ero1p activity (Cabibbo et al, 2000). The CKY559 is a thermosensitive Ero1-1 yeast mutant strain. The cells are unable to grow at 37°C, show inefficient ER-Golgi transport of disulphide rich proteins and have an increased sensitivity to DTT. A series of experiments showed that transformation with the human version of Ero1α was able to rescue the yeast from each of these defects with the transformed cells able to grow viably at 37°C, having a reduced sensitivity to DTT and increased transport of a reporter protein CPY along the ER-Golgi pathway.

Ero1α is up regulated by hypoxia (Gess et al, 2003) and ER stress (Marciniak et al, 2004) whilst Ero1β expression is induced via the UPR (Pagani et al, 2000). Some tissue specificity has also been noted between the two sub families, with Ero1α being more universally expressed whilst Ero1β is more tissue specific, being highly expressed in the pancreas, stomach and testes (Dias-Gunasekara et al, 2005).

Benham *et al* (2000) looked into the complexes formed between Ero1 and PDI using pulse chase experiments to determine if Ero1-PDI complexes were the intermediates in the oxidation of PDI or if Ero1 was acting as a folding substrate for PDI instead. As the group was only able to see the complexes one hour after the start of the experiment it was determined that the complexes were intermediates in PDI oxidation. Work by Tu et al (Tu et al, 2000) followed up on the previous observations by Anfinsen et al

(Anfinsen et al, 1963) into the role of FAD in the pathway. It was found that Ero1p was a FAD-binding molecule and that they could reconstitute an oxidative folding pathway using RNaseA as a substrate, using only Ero1p, PDI and FAD. Next, mutagenesis assays on the structure of both Ero1α and Ero1p (Bertoli et al, 2004) showed that there is a complex system of inter- and intra-molecular disulphide bond transfers between FAD and PDI. Using a panel of cysteine replacement Ero1 mutants to assess the underlying molecular mechanisms of the proteins action the group showed that two cysteine triads, Cys85-Cys94-Cys99 and Cys391-Cys394-Cys397, aid in electron transfer, with PDI forming mixed disulphides with Cys94 of Ero1. Deletion of these triads resulted in reduced disulphide bond formation and ability to rescue Ero1 knock out mutant yeast.

As for the final electron acceptor in the chain, it is believed that Ero1 family members will pass their electrons onto molecular oxygen *in vivo* although this has only been demonstrated by *in vitro* experiments. The electrochemistry of Ero1 suggests that oxygen will indeed be at least one of the electron acceptors *in vivo*, but this will lead to the unwanted generation of reactive oxygen species (ROS) by the bond formation. Each disulphide bond formed by Ero1 in this way would generate one molecule of hydrogen peroxide (Hoober et al, 1996). This generation of ROS species, as well as the need of the redox potential of the ER to be most favourable for disulphide bond formation, suggests that there must be tight regulation of Ero1 proteins, an observation corroborated by the presence of regulatory disulphide bonds in the Ero structure. However recent work by the likes of Tavender and Bulleid (Tavender and Bulleid, 2010) has shown the presence of ER peroxidases which help protect cells from such oxidative stress from disulphide formation. Peroxiredoxin IV (PrxIV) was shown to

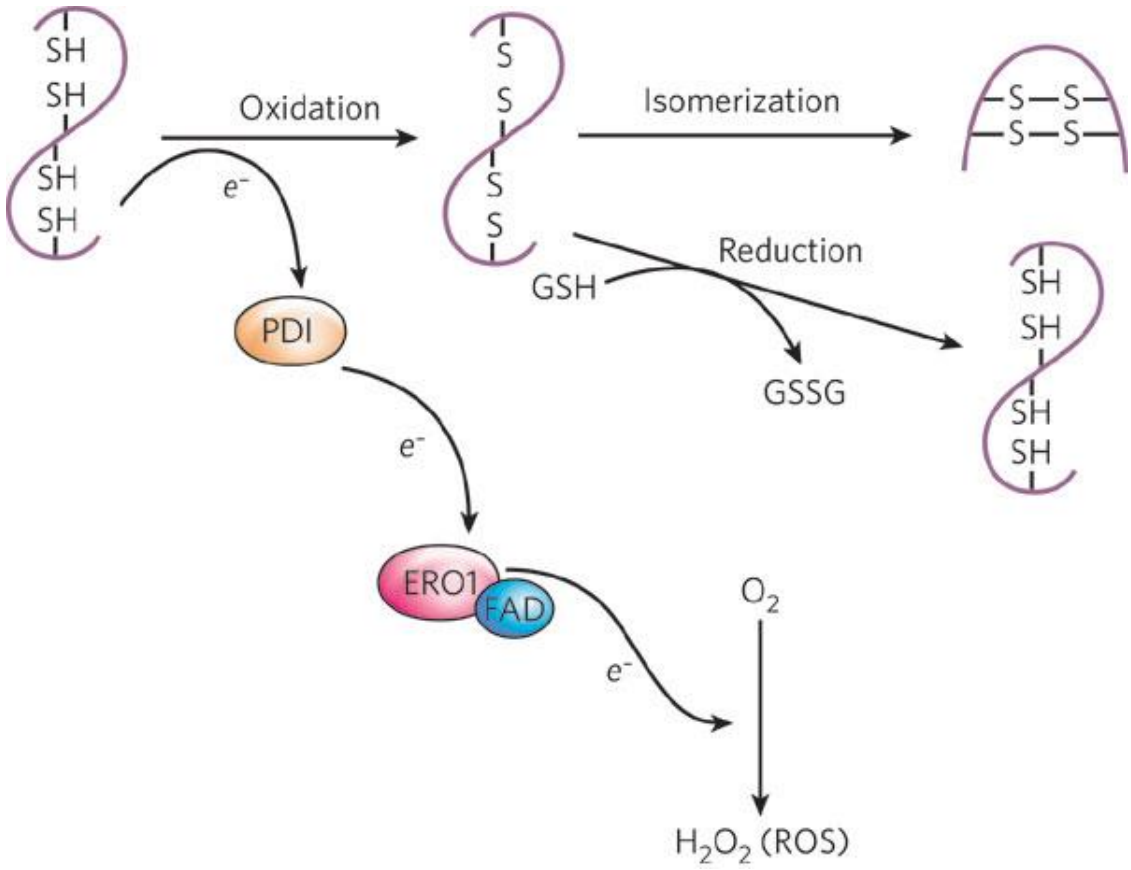

Figure 3. **Summary of oxidative protein folding.** PDI accepts electrons from protein-folding substrates, thereby oxidizing the thiol (SH) groups in the protein's cysteine residues and resulting in the formation of disulphide bonds. Ero1 uses an FAD-dependent reaction to transfer electrons from PDI to molecular oxygen, resulting in the production of ROS in the form of hydrogen peroxide (H_2O_2). Reduced glutathione (GSH) can assist in disulphide-bond reduction, which occurs when there is a overload of proteins to fold or an accumulation of misfolded proteins, and results in the production of oxidized glutathione (GSSG). (Diagram and description adapted from Zhang and Kaufman, 2009)

metabolise the hydrogen peroxide produced by Ero1 and PrxIV was preferentially hyperoxidised over other peroxiredoxins due to the increased levels of H_2O_2 from Ero1 disuphide bond formation.

Overall the PDI and Ero1 families work together to produce the main source of disulphide bonds in eukaryotic cells. Ero1 couples with FAD to use the oxidising power of oxygen to generate disulphide bonds which are then passed over to PDI, which in turn transfers them over to folding secretory proteins, all via a series of thiol-disulphide exchanges (see Figure 3).

As mentioned previously, experiments with human Ero1α and Ero1β have shown that the proteins are able to rescue the lethal effects of Ero1p knock out in yeast (Cabibbo et al, 2000). This suggests that the function of the Ero family is most likely evolutionarily conserved from yeast in multicellular organisms. However there are some indications that Ero proteins in mammals are required for specific proteins in vivo in different physiological contexts.

A 2008 paper by Tien *et al* (Tien et al, 2008) has suggested a novel function for Ero1 in *Drosophila melanogaster* which raises some interesting new ideas with regard to Ero1 function. In their study, using a mosaic screen in *Drosophila* flies, isolated mutations in Ero1L were found to cause Notch-related phenotypes. Using the eyeless-flipase system the group produced mutants of the 3L chromosome, which displayed a bristle-tufting phenotype of the head and thorax epidermis in homologous mutants. Also seen in this mutant group was notching of the wing margin and thickening of wing veins. These phenotypes are indicative of a problem with lateral inhibition in the flies, which is normally then linked back to an issue with the Notch signalling pathway. Genetic analysis and loss of function rescue experiments then confirmed that

the mutations were in the *Drosophila* Ero1L gene and that the loss of Ero1L was causing a build up of misfolded Notch receptor proteins in the ER causing induction of the UPR.

The Notch signalling pathway is an evolutionarily conserved pathway used by metazoans in a large variety of developmental processes across different organisms (Tien et al, 2008) as a means of controlling cell fates via short range cell-cell interactions. Notch is highly conserved between organisms, with Notch like proteins being characterised in many organisms including *Caenorhabditis elegans*, sea urchins and many vertebrate species, including humans (Artavanis-Tsakonas et al, 1999). In order for proper development to take place, multicellular organisms need to spatially and temporally regulate and coordinate cell behaviour and growth. Notch signalling is one way in which organisms are able to do this.

Moohr first noted a phenotype related to the Notch receptor in 1919 (Moohr, 1919) during studies into *Drosophila melenogaster*. He noted that a partial loss of function resulted in notches forming along the wing margins. Later studies by Poulson (Poulson, 1940) showed that lethal loss of function mutations caused tissue that was originally destined to become epidermis was instead forming neural tissue.

Interaction studies, both molecular and genetic, showed several other proteins which interacted with Notch to either regulate or transmit the Notch signal. Studies in *Drosophila* showed two partially redundant Notch ligands, Delta and Serrate. Mammalian studies showed similar results with Delta-like (Dll) and Jagged, a Serrate homologue (Li et al, 1997). Downstream of Notch activation was shown to be the transcription factor Suppressor of Hairless, Su(H), or in mammals RBP-J_k, activation. These proteins are the main downstream effectors of the Notch pathway and activate

expression of the Notch pathways primary targets, the genes of the *Enhancer of split* locus. These genes express nuclear basic helix-loop-helix proteins and these factors in turn affect the regulation of other genes such as the *Achaete-Scute* complex, which contains proneural genes which express proteins that are involved in the isolation of epidermal and neuronal cell fates (Bailey and Posakony, 1995).

Over the years further studies have elaborated on this initial work to give our current more detailed understanding of the Notch pathway and its constituent parts as seen in Figure 4.

Notch signalling is a short range cell to cell signalling pathway. The short range of the pathway is due to the transmembrane proteins that make up its components. Both the Notch receptor and ligands are cell surface type I transmembrane proteins (Schweisguth, 2004). Vertebrates have four different Notch receptors, Notch 1, 2, 3 and 4 and two ligand families, Delta-like and Serrate/Jagged. Notch 1 is seen in most fetal tissues and is abundant in the spleen, lungs and brain stem and is in most adult tissues, primarily in lymphoid tissue. Notch 2 is universally expressed in the embryo and is found in the brain, heart, kidney, lung, skeletal muscle and liver of adults (Simpson et al 2011). Notch3 is ubiquitously expressed in the both the embryo and adult vertebrates. Notch 4 has not been seen widely in adult tissue but is highly expressed in the fetal heart, moderately in the lung and placenta and at low levels in the liver, skeletal muscle, kidney, pancreas, spleen, lymph node, thymus, bone marrow and fetal liver.

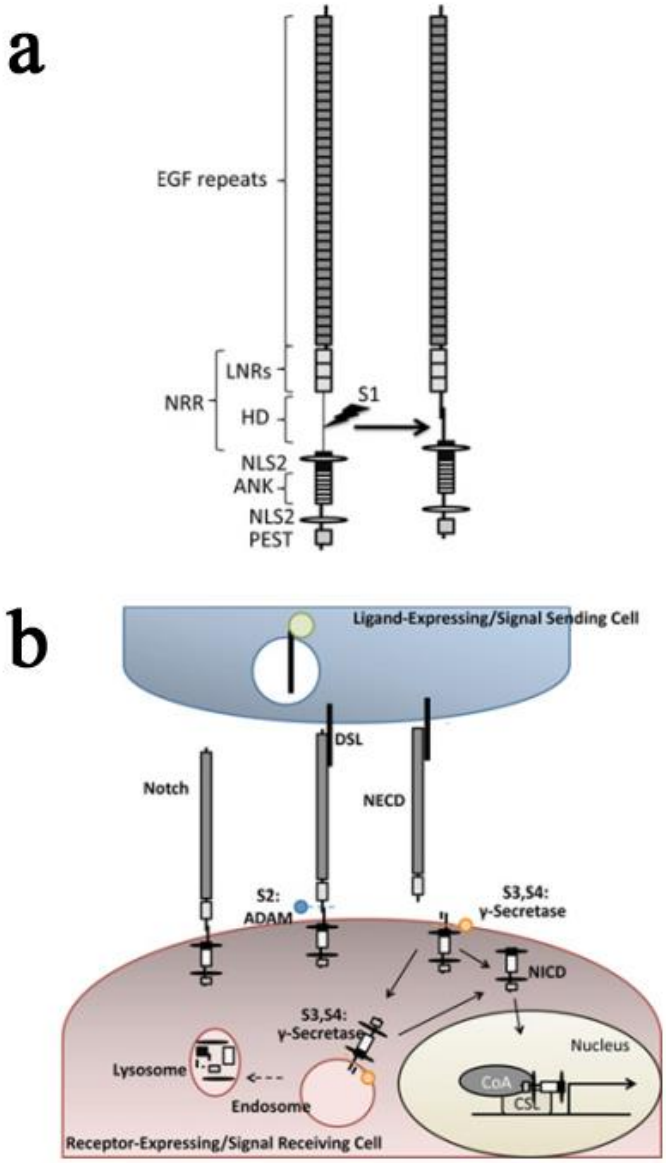

Figure 4. **Schematic diagram of Notch structure and pathway.** a, Schematic diagram of Notch structure, showing EGF-repeats, LNR and HD domain. Vertebrate Notch undergoes S1 cleavage within the secretory pathway to generate the heterodimeric receptor that is found on the cell surface. b, Notch is activated by binding to ligands of the Delta/Serrate/Lag-2(DSL) family. Activated Notch undergoes sequential cleavages, first by an ADAM family metalloproteinase at the S2 site, then at the S3 and S4 sites by a γ-secretase. These cleavages release the Notch intracellular domain (NICD), which translocates to the nucleus where it interacts with members of the CSL family of transcription factors, and recruits co-activators to activate transcription of Notch target genes. (Diagram and description adapted from Pratt et al, 2011)

Serrate/Jagged is universally expressed in humans whilst Delta-like is expressed mainly in the heart and pancreas, with lower expression levels in the brain and muscles. The Serrate/Jagged family differs from the Delta-like family due to the presence of a cysteine-rich domain which is lacking in Delta-like.

After its synthesis and processing Notch is presented at the plasma membrane as a heterodimer, made up of its membrane tethered intracellular domain, known as the NTM, and the ectodomain called the Notch extracellular domain (NECD). The two domains have been shown to interact in a non-covalent Ca^{2+} dependant manner (Rand et al, 2000).

After a ligand binds to the Notch receptor, Notch is then subject to two separate proteolytic cleavages. Ligand binding makes the S2 domain of the NTM sensitive to cleavage (Brou et al, 2000). The primary cleavage is mediated by an ADAM family metalloproteinase, such as ADAM10 or 17 (Watt et al, 2005). This S2 cleavage causes the formation of a membrane bound active form of the receptor known as the Notch extracellular truncation (NEXT) and the release of the ectodomain. NEXT is then subject to two cleavage events at the S3 and S4 sites, catalysed by a γ-secretase complex, Presenilin-Nicastrin-Aph1-Pen2 protein complex (Fortini, 2001). These cleavages cause the release from the plasma membrane of the Notch intracellular domain (NICD) into the cell and the expulsion of a small peptide Nβ to the exterior of the cell. The NICD is the active form of the Notch receptor and the function of Nβ, if any, is currently unknown.

The NIDC then passes into the nucleus where it complexes with a member of the RBP-J_k (also known as the CSL) family of DNA binding proteins. The complex then recruits a Mastermind-like (MAML) transcriptional co-activator protein and the

transcription of the target genes, such as the Hes and Hey family, begins. In the absence of Notch, RBP-J_k recruits repressor complexes which bind the *cis*-regulatory region of the target genes (Schweisguth, 2004). Thus Notch activation is a switch from gene repression to activation. Of note is that the NICD has not been detected in the nuclei of signal receiving cells, leading some to suggest that the NICD works at very low concentrations below that detectable by normal immune-detection techniques (Schroeter et al 1998).

The Notch1 gene encodes for a 300kD single pass transmembrane receptor. The extracellular domain of Notch includes 36 tandem EGF-like repeats and three cysteine rich LIN-12/Notch repeats (LNRs) known as the negative regulatory region (NNR), as well as a heterodimerisation domain (HD). The NNR acts to prevent ligand independent activation of Notch, seen in knock out experiments of this domain which result in a constitutively active form of Notch (Weng et al, 2004). Conversely the intracellular domain includes six tandem ankyrin repeats, a PEST sequence and a glutamine rich domain (Artavanis-Tsakonas et al, 1999).

Genetic mosaic studies (Heitzler and Simpson, 1991) have shown Delta and Serrate to be short range and non-autonomous, which correlates with the ligands being transmembrane proteins can only affect adjacent cells. EGF repeats of Notch and the conserved extracellular DSL region of the ligands mediate the interaction between Notch and its ligands. Figure 5 shows a schematic representations and comparisons of the full structures of Notch and DSL ligands from humans, *Drosophila* and *C. elegans.*

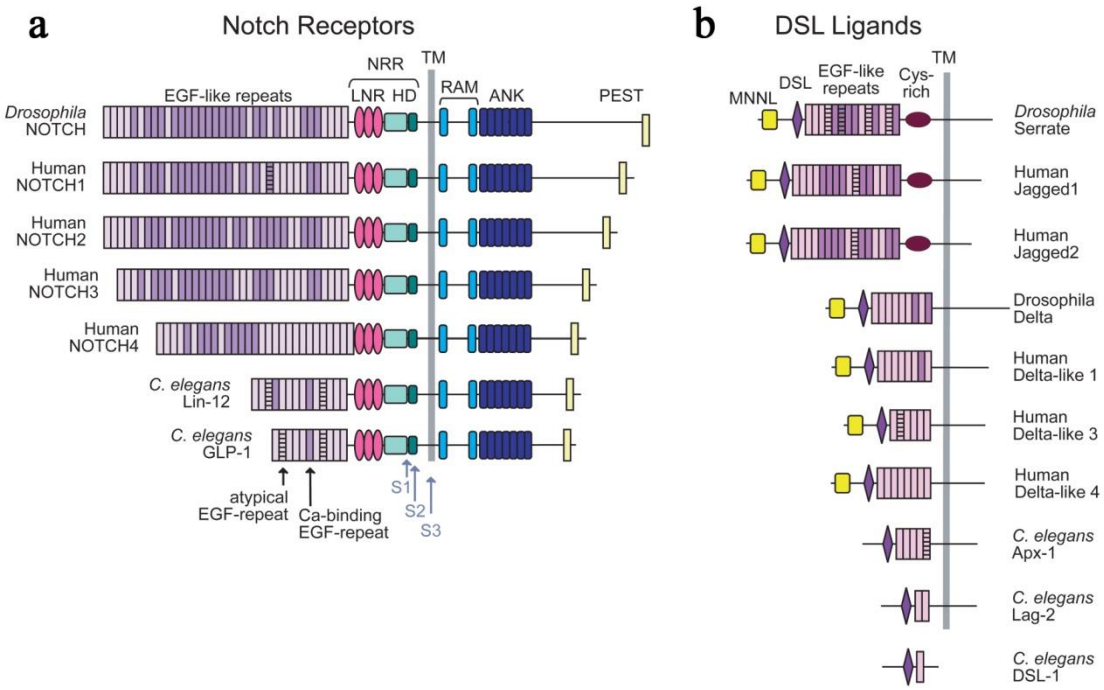

Figure 5. **Schematic diagrams of the structure and domain organizations of Notch receptors (a) and DSL ligands (b) in *Homo sapiens, Drosophila melanogaster and Caenorhabditis elegans.*** (Diagram taken from Gordon et al, 2008)

The Notch pathway is an example of signalling that involves regulated intramembrane proteolysis (RIP) (Brown et al, 2000). This has several implications on how Notch functions and thus how it is able to be regulated by the cell. As the cleavage of Notch is an irreversible event, coupled with the fact that the cleavage releases the ligand which has bound to Notch, each molecule is able to act only once. This means that the regulation of the signal can't be dependent on receptor desensitization (Schweisguth, 2004). Also the signaling is direct, with no involvement of any secondary molecules. These factors limit any potential to amplify the signal.

Evidence from *Drosophila* has shown that normal development is highly dependent on Notch and Delta gene dosage. This would seem to suggest that the amount of either the receptor or ligand is an important and highly regulated factor in development. The timing and activation of Notch is clearly dependant on the presence of both the receptor and ligand. However the pattern of Notch activation does not coincide with the extensive expression of its ligands, suggesting that there must be regulatory mechanisms which restrict Notch activation.

One such mechanism for regulation involves the Dll ligand. Work on *Drosophila* lead to the study of the *neutralised* (*neur)* gene, which was shown by (Lai et al, 2001) to cause Notch-like mutant phenotypes. This gene was found to act in the regulation of the ubiquitylation of Dll and that when tagged with ubiquitin, Dll is marked for endocytosis by the cell. Clonal analysis showed that *neur* acted to upregulate the signalling activity of Dll, after which (Parks et al, 2000) hypothesised that the endocytosis of Dll in the signal sending cell in some way helps promote Notch activation in the receiving cell.

Endocytosis is involved in many signalling pathways and has also been hypothesised to be involved with Notch, as mentioned above. In addition to this example, other studies have shown other functions of endocytosis in Notch signalling. Parks et al (Parks et al, 2000) suggested that endocytosis of a ligand bound to Notch may be needed to provide a pulling force that leads to the exposure of the S2 domain, either by a conformational change or by dislocating the Notch heterodimer. Other potential functions of endocytosis in the pathway may be to allow γ-secretase activity, which is mostly seen in an intracellular compartment (Gupta-Rossi et al, 2004), or that receptor endocytosis and lysosomal targeting may be needed in order to help prevent any ligand independent activation of the pathway (Childress et al, 2005).

Another example of Notch regulation is the addition of carbohydrate chains to Notch, which can alter the ability of the ligands to activate the receptor. This addition takes place in the Golgi by O-fucosytransferase 1 (Ofut1), potentially an important point to consider when looking at Notch-Ero interactions (Artavanis-Tsakonas et al, 1999). Fructose molecules are added to specific Serine and Threonine residues in the Notch EGF-like repeats (Schweisguth, 2004) and loss of Ofut1 function mimics loss of Notch function in *Drosophila* and mice (Shi and Stanley, 2003). Transfection studies have shown that when Ofut1 acts on Notch, interaction between the ligands and receptors is increased; however its overexpression leads to loss of correct lateral inhibition in development in *Drosophila* and several phenotypical mutations also seen in lack of Notch function. Okajima and Irvine (Okajima and Irvine, 2002) has suggested that since the activity of Ofut1 is controlled developmentally and it's over expression stops receptor-ligand binding, that one of its many apparent functions may be one method of Notch regulation. These opposing influences of Ofut1 on notch signalling could

involve positive and negative roles for distinct EGF domains on Notch and their differing affinities for Ofut1

Fringe, a glycosaminyl transferase involved in boundary formation in *Drosophila* and mammals, also interacts with Notch and modifies O-linked fructose on specific EGF-like repeats. One such repeat is EGF12 which has been shown to act in ligand binding. Binding assays in cultured cells by Lei et al (Lei et al, 2003) have shown that Notch modified by Fringe has a greater binding affinity for Delta and a reduced affinity for Serrate. Together with other studies in this area, it has been suggested that the Notch ligands have lectin-like properties, as they are able to bind to Notch with differing affinities depending on the type and extent of sugar modifications of Notch.

Evidence of a feedback loop of regulation in Notch signalling was first seen in genetic mosaic experiments in *Drosophila* and later direct evidence came from the studies of Heitzler and Simpson (Heitzler and Simpson, 1991) with *C.elegans.* Dynamic changes in the expression of LIN-12 and LAG-2 (homologues of Notch and Jagged) in developing cells in the worms were observed to be consistent with those seen in other transcriptional feedback loops in other biological systems. This feedback loop provides a mechanism by which Notch signalling can amplify small initial differences between cells in order to consolidate those differences. Thus intrinsic or extrinsic factors can bias developing cells so that the critical level of Notch signalling can be obtained to alter the cells developmental path (Seugnet et al, 1997).

Posttranscriptional proteolytic cleavage events help to regulate Notch activity. Notch is cleaved at the S1 site in the trans-Golgi network by a furin-like convertase before its presentation to the cell surface as a heterodimer (see Figure 4a). This generates the extracellular and transmembrane subunits of Notch, held together by the HD domain.

This processing is required for active signalling in mammals but seems to be a dispensable process in *Drosophila* (Kidd and Leiber, 2002).

Notch is used widely developmentally, resulting in different outputs by the cell depending on the situation in which it finds itself. Thus different NICD- RBP-J_k targets are expressed in different cells by the same ligand activation (Cooper et al, 2000). Therefore the selective genome response to Notch signalling must also be highly regulated. This area is still being investigated with the nature and co-factors of the NICD- RBP-J_k complex the most likely area of control, but chromatin structure and NICD competition with repressor complexes have also been suggested as possible regulatory mechanisms (Schweisguth, 2004).

Over time extensive loss and gain of function mutation studies using Notch have been carried out across the animal kingdoms, with studies into *Drosophila*, fish, birds, mice and humans to name but a few. These studies have all shown a remarkable conservation of function for the Notch pathway, with mutations leading to disruption of somite formation, lymphoid development, angiogenesis and many neural defects in vertebrates (Egan et al, 1998). In *Drosophila* where data is most abundant it seems that practically all tissues, across all three germ layers, are affected by Notch.

The Notch pathway has been shown phenotypically in both vertebrates and non-vertebrates to have a deep-seated role in controlling cell fate choices between adjacent cells regardless of their primary developmental similarity (Artavanis-Tsakonas et al, 1999). Notch signalling is therefore vital to lateral inhibition events, which lead to segregation of groups of cells, definitions of borders between areas and causing lineage differentiation from within groups of cells. Data from Heitzler and Simpson (Heitzler and Simpson, 1991) has shown, by placing cells expressing differing levels

of either Notch or one of its ligands next to one another that only a small difference in the amounts of one ligand ratio between cells can cause one cell to take on a lateral inhibition signalling role. Then as development proceeds, these initial differences, caused by random events or any number of factors, are stabilised or augmented by Notch signalling leading to the dictation of a cells final fate. Examples of Notch in lateral inhibition processes are evident in several areas of *Drosophila* development such as in the developing eye disk (Fanto and Mlodzik (1999). Adjacent photoreceptor precursor cells R3 and R4 experience a Wingless signal gradient emanating from the imaginal disk, which upregulates the expression of Delta. R3 which is closer to the source of this signal expresses higher levels of Delta giving rise to R3 fate. At the same time this activates Notch in the adjacent R4 precursor cell, pushing it into its R4 fate. This is a prime example of the non-autonomous effects of Notch, and how it can act to push cells into differing cell fates over a range of only two cells.

However, it must be noted that for Notch to be able to influence so many developmental processes it must also interact with many other factors and also must be highly dependent on the developmental context in which it is found. Whilst the activation of Notch can be seen in many cells to lock them into a certain developmental fate, preventing other paths being taken, cells are not terminally locked into this fate by Notch and can react to other factors. Ahmad et al (Ahmad et al, 1995) note that Notch acts in terminally differentiated cells such as post-mitotic neurones, and suggests that Notch may confer on them some level of developmental plasticity.

Related to the Notch pathways role in lateral inhibition and other such developmental processes is the function of the Notch ligands in cell adhesion. Lindner et al (Lindner et al, 2001) have shown that secreted forms of the Jagged1 extracellular domain

reduce adhesion of cells to the extracellular matrix, lose focal adhesion formation, and increase the amount of cadherin based intercellular junctions. Conversely, Dll1 promotes cell adhesiveness. Cell mixing experiments (Lowell and Watt, 2001) have shown that keratinocytes expressing a higher level of Dll1 were more cohesive in culture, but the effect did require the cytoplasmic domain of Dll1.

Notch and Jagged have also been studied into widely in regards to their role in skin and hair follicle development in mammals, with expression profiles for both well mapped out (Watt et al, 2008). These studies have noted that Notch expression differs between embryonic and adult epidermis with the expression of the different pathway components being highly dynamic as the epidermis develops and is maintained. This change in function over time is shown well by Pan et al, who showed by deletion of γ-secretase that Notch function is not required to initiate the formation of hair follicles in the embryo (Pan et al, 2004). However, in postnatal skin the lack of Notch function causes the interfollicular regions to hyperproliferate, and the hair follicles to convert into cysts which undergo proliferation akin to that seen in the interfollicular epidermis.

Notch signalling also displays a similarly wide range of functions in cancers and tumour formation. Whilst Notch can act as a tumour suppressor in the skin of mice (Nicolas et al, 2003), where loss of Notch or its suppression by inhibition of the function of MAML1 caused spontaneous squamous cell carcinomas, it can also have the opposite effect. In human T cell acute lymphoblastic leukaemia, studies have shown that a gain of Notch function is one of the causes of the condition. As well as this, mutations in the ADAM metalloprotease Kuzbanian (Kuz), involved in the primary cleavage of Notch, have been linked to Alzheimer's disease development via β-amyloid precursor processing (Hardy and Israel, 1999).

Tien et al (Tien et al, 2008) produced data indicating that Ero1L in *drosophila* specifically affects Notch signalling during fly development. Lateral inhibition, follicle cell differentiation and inductive signalling were all shown to be defective in the Ero1L mutants, phenotypes usually seen when there are problems in Notch signalling. However, several other pathways such as decapentaplegic and hedgehog, involved in anterior-posterior boundary formation in wing development, were unaffected in the mutants. This coupled with the fact that several other membrane proteins were expressed and localised normally in the Ero1L mutants showed that Ero1L was specifically affecting Notch activity and not that of other similar pathways. Finally the cells would normally induce the UPR in the mutants to deal with the excess of unfolded Notch that was building up inside the ER lumen. In Ero1L/Notch double knock outs, this effect was reduced. Taking all this data into account, the group suggested that Notch is either a very sensitive target when Ero1L is disrupted or that Notch is in fact a major substrate for Ero1L.

Analysis of the NECD showed the group that Ero1L was involved in some form in the folding of this protein region. This gave two possibilities for the domains that the Ero1L was aiding the folding of, either the EGF repeats or the LNR region. In the *kiga* Ero1L mutants, only Notch was seen to exhibit high levels of ER retention and not any other EGF containing membrane proteins (Tien et al, 2008). The LNR was then investigated to see if its cysteine bridges were affected by the Ero1L loss. LRN are found in all known Notch homologues including Lin12 and human Notch 1-4 and are only seen on two other unrelated groups of mammalian proteins, which have no homologues in *Drosophila*. The biochemical data generated by the group indicated that it was the disulphide bonds in the LNR regions that were affected when Ero1L function was lost. This was done by examining the secretion efficiency of LNR and

EGF domains tagged with a signal peptide for secretion when they were properly folded, in S2 cells. When Ero1 function was reduced through iRNA the EGF secretion was unaffected, however a significant decrease was seen in LNR domain levels in the cell media, suggesting Ero1 is needed for correct formation of the LNR region. Western blot analysis of also confirmed this by showing that when Ero1 was knocked down there was an increase in free thiol groups on the LNR regions, shown via an increase in AMS binding to the LNR. AMS is able to bind to free thiol groups and increase the molecular mass of the protein by 490D per cysteine residue.

The loss of function phenotypes seen in the *drosophila* Ero1 mutants were rescued by over expression of human Ero1α, and this coupled with the knowledge that human Notch has the same unique LNR regions as the *Drosophila* suggests that the role of Ero1 in the Notch pathway might be conserved in mammals.

My project aims to gather more information on this potential link between Ero1α and the Notch pathway proteins in mammalian cells. Specifically, the two main hypotheses we shall be investigating are:

- The oxidative folding of the Notch protein is directly catalysed by the Ero-PDI pathway in the ER.
- Ero1 and the Notch pathway interact with each other causing changes to the actions of one or the other pathway, for example via transcriptional modulation of the Ero1 protein by Notch signalling.

As has been shown by the data discussed above these links have been previously shown to exist in insect systems and we wish to investigate if these links have been conserved in mammalian development. By using a combination of western blotting, immunoprecipitation and immunocytochemistry we shall investigate if mammalian

Ero1 and Notch interact in a manner as has been suggested by previous work or if this has been evolutionarily lost in favour of a different system

2. Materials and Methods

2.1 Antibodies

The polyclonal rabbit anti-serum against PDI was raised against purified rat PDI as described (Benham et al., 2000). αEro1α (D5) was raised in rabbit against reduced, non-reduced and denatured forms of an *E. coli* expressed and purified MBP fusion protein (Mezghrani et al., 2001). The monoclonal mouse antibody, raised against recombinant Ero1α (2G4) was a gift from Prof. Roberto Sitia, Milan. The polyclonal goat anti-serum, against hJagged1 (C20) was a gift from Dr. Carrie Ambler and is available commercially (Santa Cruz Biotechnology Inc.). The polyclonal mouse anti-serum, against the V5 (antigen GKPIPNPLLGLDST, Sigma), the monoclonal anti-Myc antibody clone 9B11 and the polyclonal antibody anti-Myc (antigen EQKLISEEDL, both Cell Signalling) were commercially available.

2.2 Molecular Biology

2.2.1 cDNA constructs

The Ero1α-wt cDNA was a gift from Prof. Roberto Sitia, Milan. The Ero1α mutants, C394A and C397A were made by Dr. Adam Benham by site directed mutagenesis using the Stratagene Quikchange kit according to the manufactures instructions (Benham et al., 2000). The hJagged1, hNotch1, NICD-FL and NICD-ER neo cDNAs were gifts from Dr. Carrie Ambler, Durham. The Ero1β-HA was a gift from Prof. Roberto Sitia, Milan.

2.2.2 *Escherichia coli* transformation

Plasmid cDNA was used to transform DH5α and XL10-Blue supercompetent cells (a gift from Max Brown, commercially available). One μl plasmid DNA was mixed with 50 μl bacteria cells and incubated on ice for 30 min. Bacteria were heat shocked at 42^{o}C for 20 sec, incubated on ice for a further 2 min prior to mixing with 950 μl of pre-heated (42^{o}C) LB-broth (Tryptone 10g/l, yeast extract 5g/l, NaCl 5g/l) supplemented with 0.1mg/ml ampicillin at 37^{o}C for 1 hr with constant agitation. Bacteria were centrifuged at 6,200 rpm for 2 min at room temperature and the resuspended bacterial pellets were spread on LB-agar plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar) supplemented with 0.1 mg/ml ampicillin and grown overnight at 37^{o}C with constant agitation. The following day a single colony was inoculated in 5 ml of LB-broth supplemented with ampicillin (0.1 mg/ml), and grown overnight at 37^{o}C with constant agitation. The overnight culture was used to extract plasmid DNA (using the Qiagen MiniPrep according to the manufacturers' protocol)

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse DNA restriction digest products. The appropriate amount of agarose (Bioline) to make a 1% solution was disolved in a suitable volume of TAE (20 mM Tris, 0.002% glacial acetic acid, 0.5 mM EDTA, pH 8.0) buffer by heating in a microwave. Ethidium bromide was added to a final concentration of 0.6 μg/ml prior to pouring into a tray to set. The gel was submerged in TAE buffer and DNA samples were loaded into the wells after mixing 6:1 with 6x loading buffer (15% Ficoll; type 400, (Pharmacia), 5 mM EDTA, pH 8.0, 0.25%

bromophenol blue). Following electrophoresis, gels were visualised by UV illumination and photographed using a BioRad Gel Doc.

2.3 Cell Culture

Human cervical carcinoma HeLa cells were maintained in minimum essential medium (MEM, Invitrogen) and human embryonic kidney carcinoma HEK293 cells were maintained in Dulbecco's modified Eagles's medium (DMEM, Invitrogen), both supplemented with 8% fetal bovine serum (FBS, Sigma), 2 mM glutamax (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 5% CO_2. Both cell lines were serially passaged at 70-80% confluency using 0.05% trypsin (Trypsin-EDTA, Invitrogen) every three to four days.

2.4 Cell transfection

2.4.1 Lipofectamine transfection

Transfections with Lipofectamine 2000 (Invitrogen) were performed according to manufacturer's instructions. Sub-confluent cells in 4cm dishes were washed either singly (HEK293) or twice (HeLa) with PBS (Invitrogen), and transfected with 1 μg DNA and 3μl Lipofectamine for 5 hours in Optimem serum free medium (Invitrogen). The medium was replaced after 5 hours with the relevant complete medium for the cell line and the cells analysed 24 hours post-transfection.

2.4.2 Roche X-tremeGENE transfection

Transfections with X-tremeGENE (Roche) were performed according to manufacturer's instructions. Sub-confluent cells in 4cm dishes were washed either singly (HEK293) or twice (HeLa) with PBS (Invitrogen), and the relevant complete

medium for the cell line was replaced. The cells were then transfected with 1 µg of DNA in 3µl of reagent and the cells analysed 24 hours post-transfection.

2.5 Cell lysis

Lysates were prepared from cell lines 24 hours after transfection. Cells were washed either singly (HEK293) or twice (HeLa) with PBS (Invitrogen) and placed onto an ice cold metal tray. 400 µl of lysis buffer (20 mM MES, 30 mM Tris-HC, 100 mM NaCl, 1% TX100, 1µg/ml CLAP, pH 7.1), supplemented with 20 mM N-ethylmaleimide (NEM) where required, was added to each dish and the cells removed from the dish with a plastic scraper. The lysate was transferred to a 1ml eppendorf tube and centrifuged for 10 minutes at 14,200g to remove the nuclei. The resulting lysates were either used immediately or flash frozen in liquid nitrogen and stored at -20°C for later use.

2.6 Immunoprecipitation

Immunoprecipiations (IP) were carried out using the required lysate and 1 µl of monoclonal antibodies or 8 µl of polyclonal antibodies immobilised on 50 µl of a 20 % suspension of Protein A or G sepharose beads (Sigma) for 2 hours at 4^{o}C. Beads were spun down at 4000g for 30 seconds and the lysis buffer was replaced. Beads were agitated and then washed again in the same manner. Fifty or one hundred µl of the relevant beads were added to 100µl of lysate and rotated together for 1 hour at 4°C. The beads were then washed three times as described previously. The resulting beads were prepared for SDS-PAGE and Western blotting as described below.

2.7 SDS-PAGE

Cell lysates or immunoprecipitates were mixed 1:1 with sample buffer ((Laemmli, 1970) comprising 0.125M Tris HCl, 2% SDS, 10% glycerol, 0.01% bromophemol blue, pH 6.8) and supplemented with 50mM dithiothreitol (DTT), heated for 5 minutes at 95˚C and then analysed by SDS-PAGE (resolving gel consists 10% acrylamide, 0.375 M Tris (pH 8.8) and 0.1% SDS, 0.1% APS, 0.04% TEMED; stacking gel consists of 5% acrylamide, 0.125M Tris (pH 6.8), 0.1% SDS, 0.075% APS and 0.075% TEMED). Samples were loaded and run at 50mA for 30 minutes using a Bio-rad power pack 200.

2.8 Western Blotting

Following SDS-PAGE proteins were transferred to PVDF membranes (Millipore), (primed in methanol), in transfer buffer (190 mM glycine, 25mM Tris in 20% Methanol) at 150mA for 2 hrs or 30V overnight using a Bio-rad powerpack 200. The membranes were then blocked in 8% milk/TBST (TBST- 150mM NaCl, 10mM Tris base (Sigma), pH 8.0 plus 0.8% Tween) for 1 hr or overnight. The primary antibody was used at its required concentration (see individual experiements for concentration used) for immunoblotting for 1 hour on a roller. Membranes were then washed 4 times with TBST, each wash lasting five minutes. Membranes were then incubated with corresponding secondary antibodies (DAKO) at 1:3000 for 1 hour and again washed 4 times with TBST, each wash lasting five minutes. Membranes were visualised by ECL+ (GE Healthcare) and exposure to film (Kodak).

2.9 Immunofluorescence

HEK293 cells were grown on 16mm coverslips treated with a 1:10 poly-1-lysine solution to aid cell binding. When the cells were 70-80% confluent they were transfected with the appropriate cDNA construct (See section 2.4). Coverslips were washed 3 times in PBS++ (PBS (Invitrogen), 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$) the fixed in 4% paraformaldehyde (in PBS++) for 10 minutes. Coverslips were then placed in 50mM NH_4Cl for 15 minutes to quench free aldehyde groups before washing three time in PBS++. Where needed cells were then permeabilised by placing in 0.1% TX100 (in PBS++) solution for ten minutes at room temperature. Coverslips were washed three times in PBS ++ with 0.2% BSA (Sigma). Primary antibodies (αPDI, 1:500 or αMyc, 1:1000 or αV5, 1:200) were applied in the presence of 0.2% BSA for one hour at room temperature before washing three times in PBS++ for five minutes. Secondary fluorescent antibodies (ALEXA, Invitrogen) or rhodamine labelled swine anti-rabbit Ig (TRITC) were incubated on the coverslips at 1:1000 concentrations for one hour. Next coverslips were washed twice in PBS++ supplemented with 0.2% BSA (Sigma) then five times in PBS++. Cell nuclei were stained with 1:1000 DAPI and washed a final time in PBS++. Mounting of the coverslips onto microscope slides was done using vectashield (VECTOR) and analysis was under a Zeiss UV inverted microscope system.

3. Results

3.1. Characterisation of plasmids

3.1.1. Establishing cDNA stocks for cell transfection

In order to study the interactions between Ero1α and the Notch pathway proteins we needed to generate stocks of cDNA plasmids containing the genes required to cause ectopic protein expression in mammalian cell lines. The constructs made available are noted in Table 1.

Table 1 – Plasmid constructs used in this project

Construct	Tag	Plasmid	Restriction Enzyme used
Human Jagged 1	--	pcDNA 3.1	Xho1/BamH1
Full Length Jagged 1	V5	pcDNA 3.1	Xho1/BamH1
Human Notch 1	--	pcDNA 3.1	Xho1
NICD-Full length (Inducible)	--	pcDNA 3.1	Xho1
NICD-Full length (Non-inducible)	Myc	pcDNA 3.1	Xho1
Ero1-α (Wild Type)	--	pcDNA 3.1	Xho1
Ero1-α C394A	Myc	pcDNA 3.1	Xho1
Ero1-α C397A	Myc	pcDNA 3.1	Xho1

The plasmids were used to transform XL10-Blue supercompetent cells as described in 2.2.2. After using the Qiagen MiniPrep kit according to manufacturer protocols to extract the cDNA from the *E. coli*, the concentration of the cDNA collected was measured using a nanodrop protocol. An example of the results obtained are shown in Table 2; **λ230** is the absorbance measured at a wavelength of 230nm, **A260** is the absorbance of the sample at 260nm, **A280** is the absorbance of the sample at 280nm,

260/280 is the ratio of the sample absorbance at 260nm and 280nm, used to assess the purity of the sample with a value of around 1.8 generally accepted as 'pure' DNA, **260/230** is the ratio of sample absorbance at 260nm and 230nm used as a secondary measure of purity with values in the range of 1.8-2.2 accepted as pure, **ng/µl** is the sample concentration in ng/µl based on the absorbance at 260nm electronically calculated using Beer's Law). Yields in the range of hundreds of ng/µl are common for MiniPrep procedures.

Table 2 – Absorbance measurements of plasmid constructs.

Construct	λ 230	A260	A280	A260/280	A280/ λ230	ng/ µ l
Human Jagged 1	7.049	15.893	8.339	1.91	2.25	**794.7**
Full Length Jagged 1	6.507	13.825	7.564	1.83	2.12	**691.3**
Human Notch 1	5.430	11.467	6.116	1.87	2.11	**573.3**
NICD-Full length (Inducible)	18.423	41.071	21.721	1.89	2.23	**2053.5**
NICD-Full length (N-I)	9.660	21.717	11.304	1.92	2.25	**1085.8**
Ero1-α (Wild Type)	13.726	30.814	16.145	1.91	2.25	**1540.7**
Ero1-α C394A	18.654	41.487	21.962	1.89	2.22	**2074.4**
Ero1-α C397A	13.418	30.048	15.597	1.93	2.24	**1502.4**

The three Ero1 constructs have been previously used and characterised by the group. The Ero1-α mutants C394A and C397A cannot form the CxxC active site, do not compliment Ero1α and act as strong dominant negatives. Since these mutants are catalytically inactive, it is of interest to determine whether or not they will functionally/physically interact with Notch compared with wild type Ero1a.

The cDNA for hNotch1, hJag1, FLJag1, NICD-FL*i* and NICD-FL*ni* were gifts from Dr. Carrie Ambler and not before used by the group. As such samples of these constructs were linearised by restriction digest using the enzyme listed in Table 1. 5µl of each construct was digested with 1µl of the relevant restriction enzyme,) and 13µl of deionised H_2O and placed in a water bath at 37°C for one hour. Samples were then prepared as described in Section 2.2.3 and run on a 1% agrose gel in order to check the molecular mass of the constructs, to confirm identity and to check for contamination of the samples.

Constructs 1-5 all ran between approximately 10kb and 13kb as expected (personal communication, Dr. Carrie Ambler). As seen in Figure 1 all constructs were found to have the correct approximate molecular mass. No contamination or artefacts were found to be present in any of the samples.

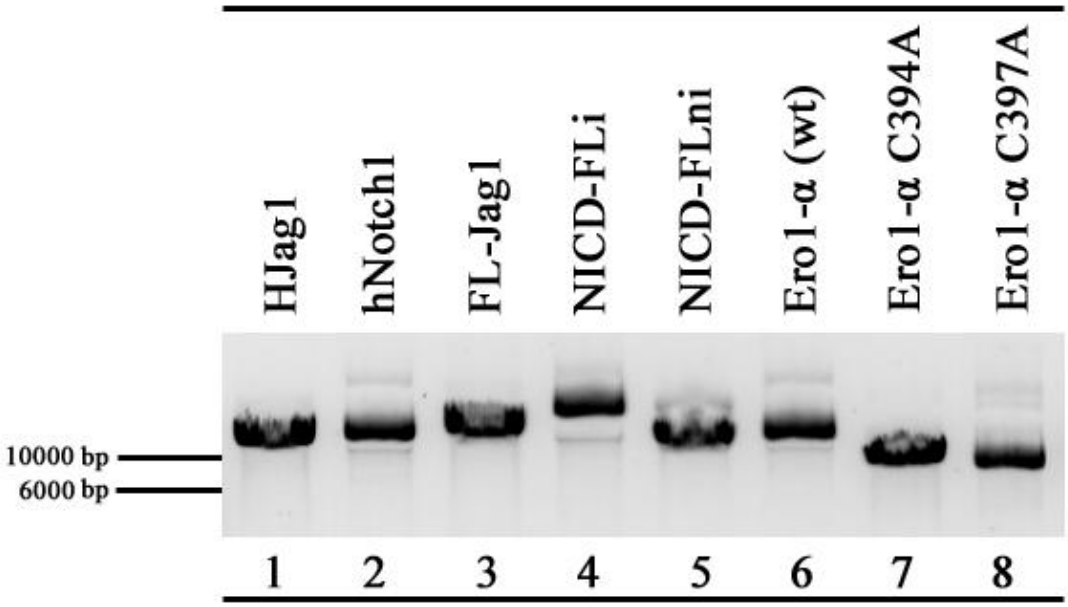

Figure 1. **Restriction digest of plasmid constructs.** Plasmid constructs, Human Jagged 1 (lane 1), human Notch 1 (lane 2), full length Jagged 1 (lane 3), full length NICD inducible (lane 4), full length NICD non-inducible (lane 5), Ero1α wild type (lane 6), Ero1α C394A mutant (lane 7) and Ero1α C397A mutant (lane 8) were linearised for 1h using relevant restriction enzyme (see Table 1) and run on a 1% agrose gel.

3.1.2. Characterisation of Ero1α antibody 2G4

Antibodies are serum immunoglobulins able to bind to specific antigens with high affinity. Due to this property they are often used in biology as useful tools in Western blots and immunoprecipitation to identify and isolate proteins, and in immunofluorescence for localisation studies. When trying to identify and stain proteins from cell lysates the specificity of antibodies is vital to ensure that the correct protein is detected and analysed.

The 2G4 antibody is a monoclonal mouse antibody raised against recombinant Ero1α. An analysis of the batch used was needed to confirm its binding specificity and to confirm that it identified Ero1α in cell lysates from different cell types.

To investigate 2G4, HeLa lysates were separated by SDS-PAGE and transferred onto a PVDF membrane for Western blotting as described in Chapter 2. Two lanes were then immunoblotted with 2G4 and the other two lanes with αPDI (see Figure 1A). The control lanes 3 and 4 showed PDI staining as a control for correct protein loading. The PDI antibody detects a doublet. Previous unpublished data from our group has found that the lower band is PDI and the top band is albumin which has cross reacted with the αPDI antibody. This doublet usually occurs in lysate samples in cell washing has not removed all the albumin from the cell preparation (Personal communication with Dr. Adam Benham).

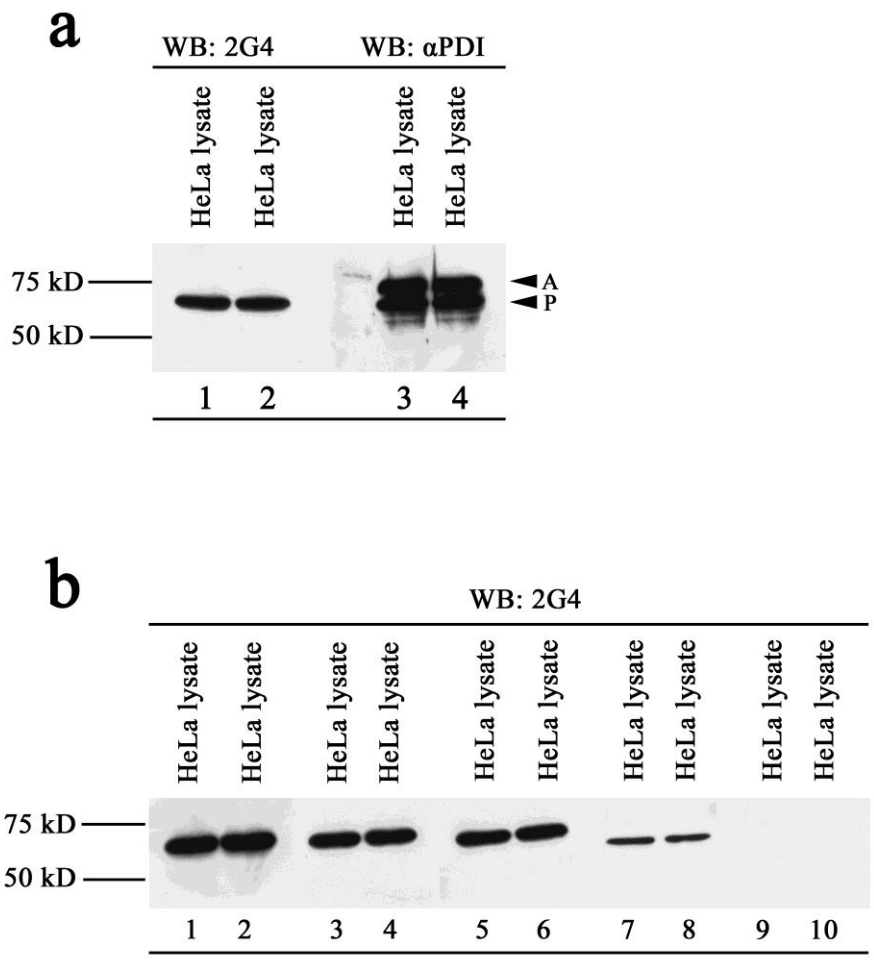

Figure 1. **2G4 binds to Ero1α at varying concentrations.** A, HeLa lysates were subjected to Western blotting after SDS-PAGE using 2G4 at 1:2 concentration (lanes 1 and 2) and αPDI at 1:1000 (lanes 3 and 4). A doublet is seen in lanes 3 and 4, with the lower band (P) indicating PDI and the higher band (A) albumin which has cross reacted with the αPDI. B, the same HeLa lysates were separated by SDS-PAGE and immunoblotted with varying concentrations of 2G4; Lanes 1 and 2 at 1:2, lanes 3 and 4 at 1:5, lanes 5 and 6 at 1:10, lanes 7 and 8 at 1:50 and lanes 9 and 10 at 1:100.

The 2G4 staining was very strong in lanes 1 and 2, with a distinct band seen in each and with no background staining. The molecular mass between the 50kD and 75kD markers is consistent with previous data, and is consistent with the molecular mass of Ero1α which is 55kDa (without glycans). It was concluded that 2G4 does bind to Ero1α and that Ero1α was expressed at a detectable level in the HeLa cells.

The antibody is however in limited supply and is not a commercially available product. In order to discover the lowest concentration at which 2G4 is able to provide a clear signal for Ero1α, a titration experiment was performed (see Figure 1B). Twenty µl of HeLa lysate was loaded into each lane and separated by SDS-PAGE as before and immunoblotted using different dilutions of 2G4 from the hybridoma supernatant, to investigate the optimum concentration. Lanes 1 and 2 were at a 1:2 dilution, lanes 3 and 4 at 1:5, lanes 5 and 6 at 1:10, lanes 7 and 8 at 1:50 and finally lanes 9 and 10 at 1:100. The film was exposed to the samples for 30 seconds.

As can be seen in Figure 1B lanes 1-8 all show Ero1α staining, whilst lanes 9 and 10 show no protein detection at all. Lanes 7/8 show the clearest staining, with a small specific band as opposed to the somewhat overexposed bands seen on lanes 1-6. At a 1:2 dilution there is also some level of staining not seen in Figure 1A, suggesting either insufficient blocking of the membrane or non-specific binding by the 2G4 antibody. As the samples were all run and blocked together on the same gel, it is more likely that the additional staining is mainly from strong exposure. The lowest concentration of 1:100, seen in lanes 9 and 10, showed no detection at all. This would suggest that the 2G4 has fallen to a concentration at which is it unable to bind to Ero1α in high enough amounts to be detected.

Thus from this data, the optimum dilution at which to use the 2G4 antibody for detecting Ero1α is at 1:50 as this gives a sharply defined band of marked protein, with no background staining.

As mentioned previously the specificity of the binding of an antibody is important. If it binds to more than one protein this must be known if the results are to be correctly interpreted. In the case of the 2G4 antibody we need to be sure that it only binds to Ero1α and not to any other related proteins such as Ero1β which is also found in some cells and tissues.

To test this, HeLa cells were transfected with either Ero1α mutant C397A-myc (see 3.1.1 Table 1), Ero1β-HA or a combination of MHCII plasmids (HLA-D, HLA-DM and invariant chain) as negative controls. Lysates were prepared from these transfectants as described in Chapter **2.5**. The lysates were loaded onto a 10% resolving gel, separated by SDS-PAGE and immunoblotted with either 2G4 or 9B11, an anti-Myc antibody (two separate membranes).

Figure 2A shows the 2G4 western blot, using the same 1:50 dilution as optimised previously in Figure 1. In lanes 1 and 2 where the C397A-myc lysate was loaded a single band can be seen between the 75kDa and 50kDa markers, corresponding to Ero1α. Lanes 3 and 4 were blank, whilst 5 and 6 showed a very faint band running just above the 50kDa marker.

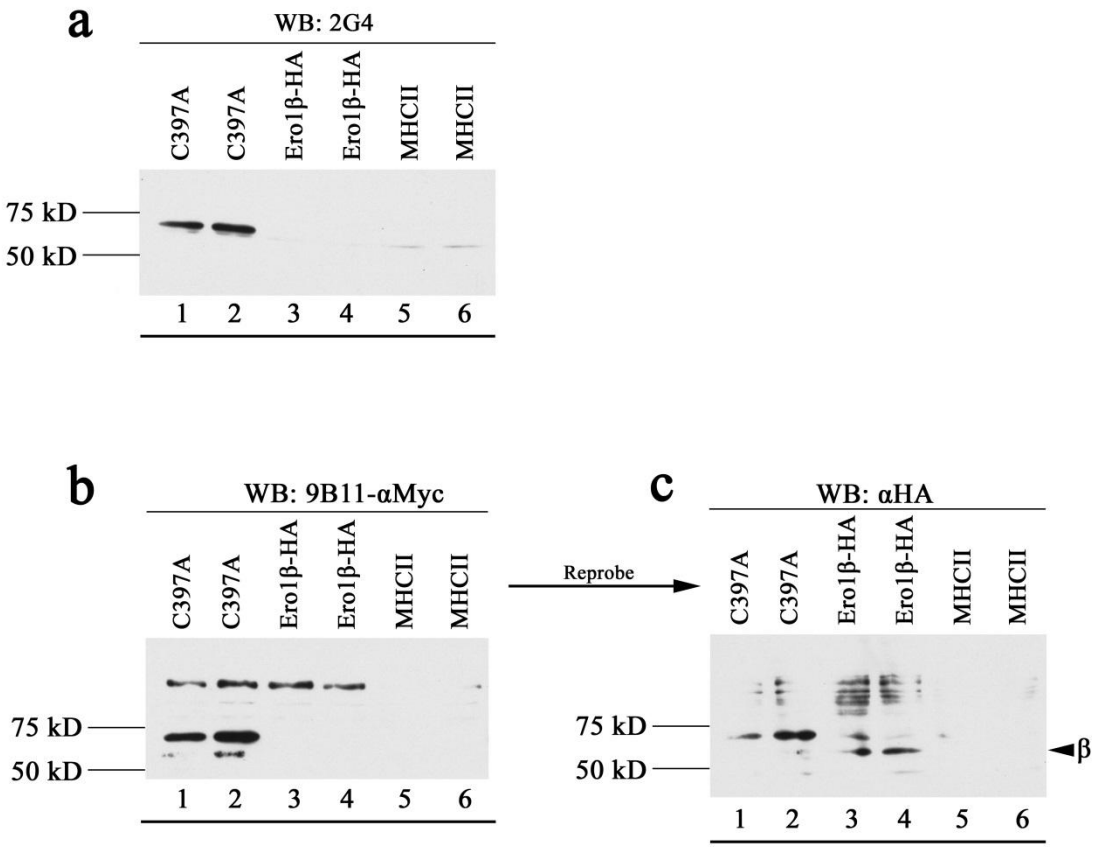

Figure 2. **2G4 binds specifically to Ero1α.** A, HeLa cell lysates from transfection with Ero1α C397A-myc (lanes 1 and 2), Ero1β-HA (lanes 3 and 4) and MHCII (lanes 5 and 6) were immunoblotted with 2G4 at 1:50 concentration after separation via SDS-PAGE. B, HeLa lysates were run in the same manner as A then immunoblotted with 1:2000 αMyc 9B11. C, The membrane from B was reprobed with αHA. The arrow β indicates the running level of Ero1β in lanes 3 and 4.

In Figure 2B the samples were run in the same order and manner as in Figure 2A but were instead immunoblotted with 9B11 which binds specifically to the tag sequence Myc. As can be seen in Table 1 in section 3.1.1 Ero1α C397A has been tagged with Myc whilst the other constructs used in this experiment are not. As such we would expect to see bands for the myc-tagged Ero1α mutant running at a similar level to wild type Ero1α, and no bands apparent in the other lanes.

In figure 2B, lanes 1 and 2 both had distinct strong bands corresponding to Ero1α, showing that the transfection with the Ero1α C397A construct has been successful and the cells in the lysate were producing the mutant variant of Ero1α. This also corroborates the previous evidence that 2G4 binds specifically to Ero1α proteins, as when the data from Figure 2A and 2B were compared the bands in lane 1 and ran at appropriate molecular weights. From this can be concluded that these bands represent Ero1α C397A-myc protein and that 2G4 was able to detect the mutant Ero1α as well as the wild type, as seen in Figure1.

As well as the Ero1α bands, lanes 1-4 all showed a band of higher molecular weight, running above the level of the C397A signal at approximately 100kDa. This band is most probably corresponds to a cross reactive protein recognised by the 9B11 antibody leading to these background signals. These bands are however useful as a loading control for the lanes 3 and 4. From them we can see that there was indeed protein present on the membrane. When combined with the fact that there is no equivalent Ero1α band as seen in lanes 1 and 2, we can conclude that the 9B11 antibody specifically only binds to the myc-tagged proteins.

The membrane used to produce Figure 2B was rehydrated in TBST solution for 1 hour before being reprobed with an anti-HA antibody, without prior stripping of the previous antibody. The results of this reprobe can be seen in Figure 2C.

In lanes 1 and 2, the signals seen can be attributed to the 9B11 antibodies still on the membrane from the previous immunoblot. Lanes 3 and 4 also showed a similar pattern of additional bands but to a stronger degree than that of lane 1 or 2. Of greater note however is the band seen at the level marked by the arrow β. This band ran at a lower height than any of the previous experiments. As noted by Dias-Gunasekara et al (2005) this lower weight band represents the HA tagged Ero1β protein. The signal was more clearly visible in lane 4 than lane 3.

The data in Figure 2C demonstrates that 2G4 only binds to Ero1α and not the Ero1β. The HA blot of Ero1β showed that Ero1β was produced by the transfected cells and so would also have been in the lysate samples run in Figure 2A. As there is no band in lanes 3 and 4 of Figure 2A we can be sure that 2G4 did not bind to Ero1β or to the MHCII proteins, which are involved in antigen presentation and are not related to the Ero or PDI families.

3.1.3. Detection of Ero1 and Notch proteins by antibody staining

Western blots were carried out in order to assess the protein expression following transfection with various cDNA, as well as the endogenous levels of protein expression in non transfected cells. This also served the secondary purpose of confirming that the cDNAs expressed the protein expected other second cell lines.

HEK293 cells were transfected individually with each of the eight constructs detailed in Table 1 of 3.1.1 as described in Chapter 2.4.2 and lysed in the presence of lysis buffer containing 20mMol NEM as described in Chapter 2. In addition a control dish of cells was prepared by treating the dish with the X-tremeGENE (Roche) kit according to manufacturer's instructions without the addition of any cDNA. The lysates were loaded onto a 10% resolving gel and transferred to PVDF membranes. In all cases the samples were run in the order shown in Table 3 below.

Table 3 – Constructs used for Western blotting and their respective tags

Construct	Tag
Human Jagged 1	--
Human Notch 1	--
Full Length Jagged 1	V5
NICD-Full length (Inducible)	--
NICD-Full length (Non-inducible)	Myc
Ero1-α C394A	Myc
Ero1-α C397A	Myc
Ero1-α (Wild Type)	--
Mock transformation	--

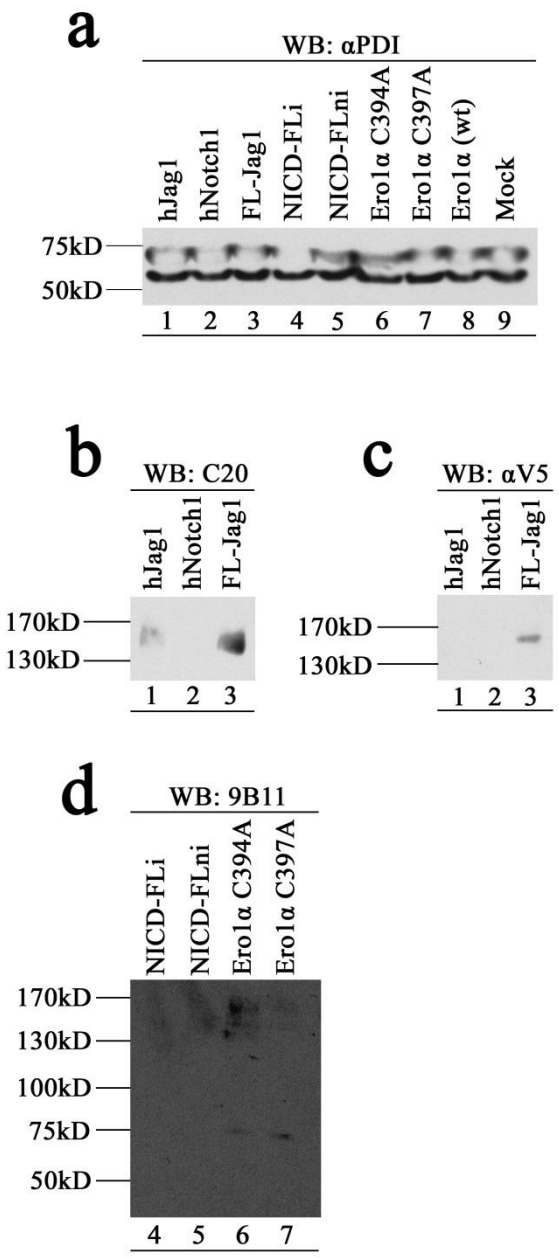

Figure 3. **Antibody detection of proteins in HEK293 cells.** A, HEK293 cell lysates from transfection with hJag1 (lane 1), hNotch1 (lane 2), FL-Jag1 (lane 3), NICD-FL1 (lane 4), NICD-FLni (lane 5), C394A (lane 6), C397A (lane 7), Ero1α-wt (lane 8) and a HEK293 mock transfection lysate (lane 9) were separated by SDS-PAGE and immunoblotted with αPDI antibody. B, HEK293 cell lysates from transfection with hJag1 (lane 1), hNotch1 (lane 2), FL-Jag1 (lane 3) were immunoblotted with C20 antibody or C, with αV5 antibody. D, lysates from HEK293 cells transfected with NICD-FL1 (lane 4), NICD-FLni (lane 5), C394A (lane 6) and C397A (lane 7), were immunoblotted with the αmyc-tag antibody 9B11.

Figure 3A shows an immunoblot control probing for PDI. In this experiment universal expression of PDI in all lysates was seen as expected. This acted as a control and showed that equal amounts of protein had been loaded in the samples and transferred from the gel. As seen in Figure 1A of 3.1.1. a doublet was present in all cases with PDI being the lower more strongly stained band. This would suggest that the cells retained bound albumin. However the transfected HEK293 cells tended to detach during additional washing leading to an unacceptable level of cell loss.

Figure 1B shows staining with C20, a commercially available antibody to Jagged 1. Consistent with the manufacturer's description of this antibody, hJag1 can be seen at a running height of 150kD, with a smear seen instead of a defined band. In this blot Jagged 1 was detected in lanes 1 and 3, with lane 3 staining more strongly than lane 1 at a height corresponding to approximately 150kD. These two lanes correspond to the cell lysates which had been transfected with the two versions of hJagged1. Lane 2 showed no detection of any Jagged1 protein as expected since this was a negative control for the antibody.

This experiment suggests that the cDNA constructs for Jag1 were taken up and expressed in the cells causing the high level of expression shown in lanes 1 and 3. The higher level in lane 3 would imply that either there was a greater transfection percentage in cells transfected with FL-hJag1 cDNA or that the construct caused the cells to produce a larger amount of Jag1 than the hJag1 construct.

Figure 1C shows staining with an antibody against the V5 tag. As only the full length Jagged 1 construct has been tagged with a V5 tag the expected result was to see expression in lane 3 at 150kDa, the molecular mass of Jagged 1. As can be seen this was the case. No other lanes showed any V5 staining and there was no other

significant background staining. Lack of detection of the hJag1 in lane 1 shows the specificity of the antibody to the V5 tag on the Jagged 1 protein in lane 3, not the protein itself. Lane 2 is shown as a negative control, confirming that the V5 antibody does not bind to Notch.

Figure 3D shows staining with 9B11, an antibody against the myc tag. Lanes 6 and 7 showed detection of protein with a molecular mass of approximately 75kDa corresponding to the two myc tagged Ero1α mutants, C394A and C397A. It was expected to see some detection in lane 5 at approximately 80kDa, the molecular weight of the NICD. However no protein was detected, suggesting either a lack of expression of the NICD due to low transfection efficiency or levels of NICD in the lysates not detectable by the 9B11 antibody. Lane 4 contained NICD-FLi, which was not myc-tagged and thus no protein was detected. All lanes showed some non-specific binding between the 130kDa and 170kDa markers, most likely corresponding to cross reaction of the 9B11 to endogenous proteins. As the myc-tag is derived from the c-Myc transcription factor this level of background staining can sometimes be expected.

The results shown in Figure 3 allow two main conclusions to be drawn from the data. Firstly, it helps to confirm that the cDNA constructs express the expected proteins, with all the lysates from each transfection behaving as expected for each antibody, with the exception of the NICD-FL*ni* in Figure 1D. Also the experiments confirmed that the transfection worked correctly, with the plasmids being taken up by the cells and the proteins of interest expressed.

3.1.4. Location of transfected proteins by immunofluorescence

Immunofluorescence experiments were carried out in order to localise the expression of transfected proteins. Immunofluorescence allows the subcellular location of a protein to be visualised within the cell and can be used to assess co-localisation with potential partner proteins. Cells were stained with both DAPI, to view the nucleus of the cell, and antibodies specific to the protein. The primary antibodies are then reacted with fluorescent secondary antibodies which allow the proteins to be viewed under either a green or red filter.

HEK293 cells were grown on coverslips and then mock transfected using the lipofectamine protocol before being prepared for immunofluorescence according to protocol outlined in 2.9. In this instance the cells were permeabilised with 0.1% TX100.

Under the DAPI filter, the staining of the cell nuclei can be seen. The images on the left of Figure 4; a, c, d and e all show the DAPI staining of the cell nuclei. The equivalent image to their left; b, d, f and h respectively, show the same coverslip under a different relevant filter.

The coverslip seen in Figure 4a and 1b was stained for PDI with Alexa fluor-488 secondary anitbody which was then viewed under the relevant filter. The PDI protein was resident in the ER of the cell as expected, evidenced by perinuclear staining around the nuclei (see labelled arrows x and y for example) corresponding to the location of the ER within the cell. 4x and 4y show expanded images of the cells visualised to better show the typical ER staining seen. Closer inspection of cell x showed the cell to be in the process of mitosis, due to its distinct telophase structure and evidence of cleavage furrows, showing the cells were still actively growing and

dividing. These strong staining patterns seen in our cells correspond to previous data from our group (van Lith et al, 2005) and other comparative sources, so we are confident that the staining seen is of PDI in the ER of the cell.

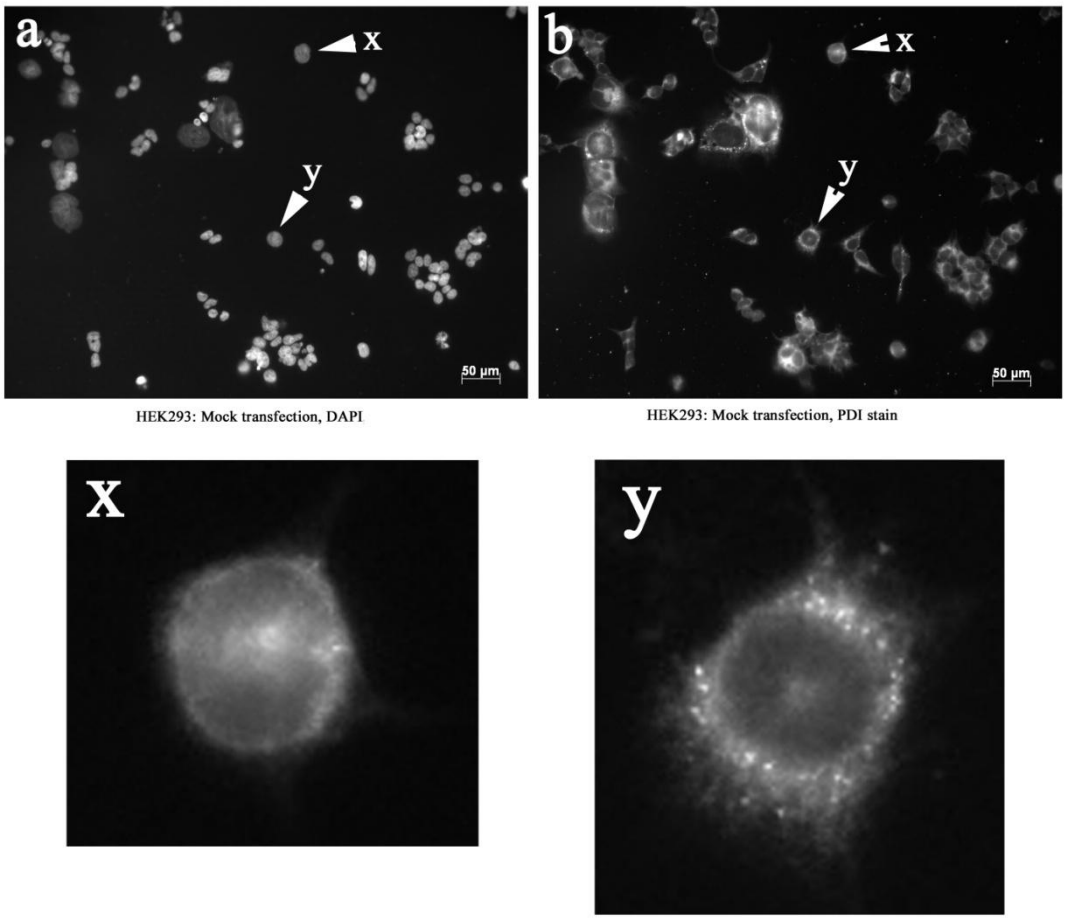

Figure 4. **Immunofluorescence detection of proteins in HEK293 cells.** HEK293 cells were probed with DAPI (a) and αPDI (b) and photographed at 20x magnification under the according filters. x and y show individual cells stained with αPDI.

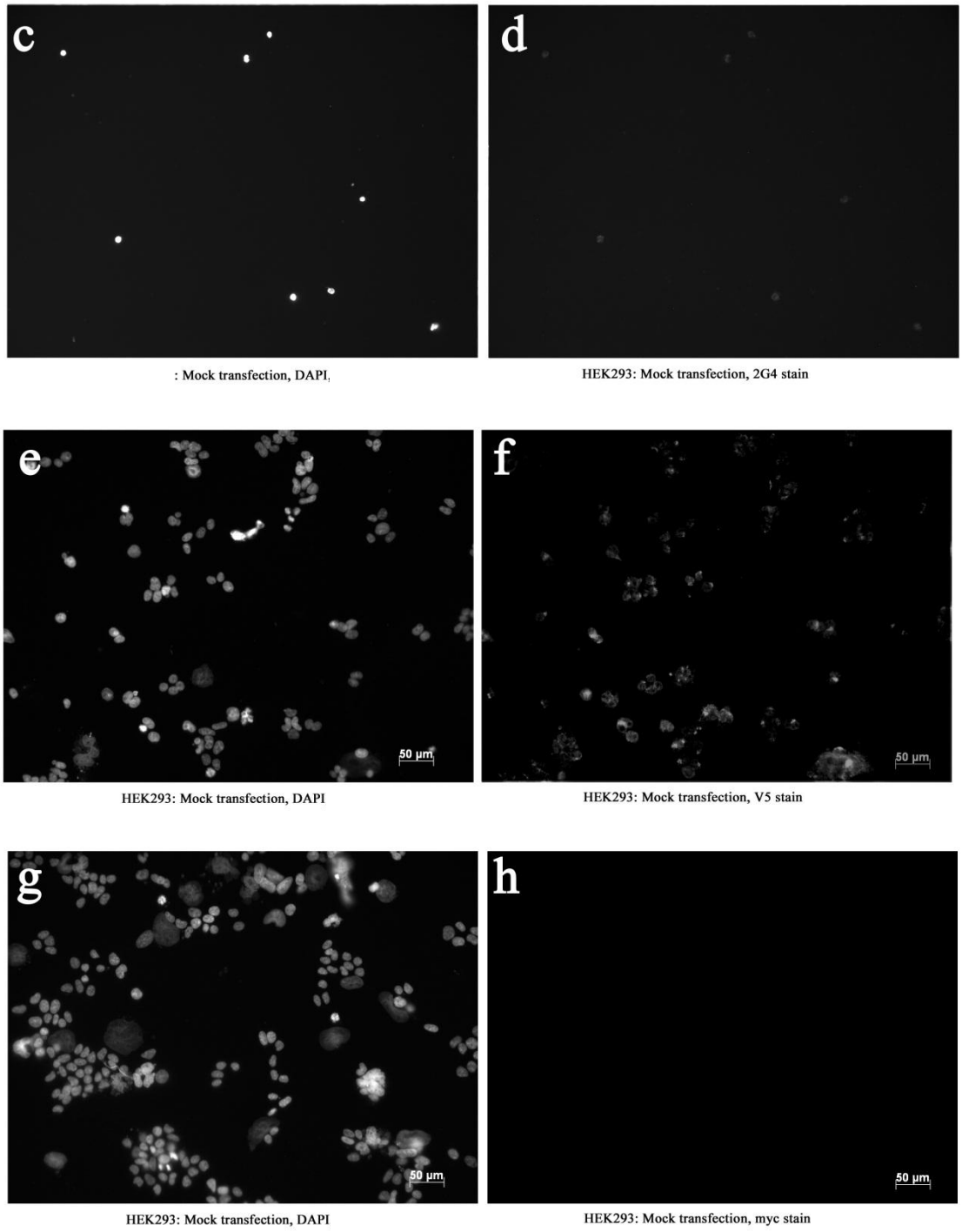

Figure 4 (continued). **Immunofluorescence detection of proteins in HEK293 cells.** HEK293 cells were probed with DAPI (c, e and g) and either 2G4 (d), αV5 (f) or αMyc (h) and photographed under the according filters.

In figure 4c/d, the coverslip was probed using 2G4 to localise Ero1α expression within the cell. Previous work has shown Ero1α to be resident to the ER, and to co-localise with PDI and so expression of Ero1α was expected to be very similar to the PDI staining seen in 4b. Figure 4c showed DAPI staining the nuclei as expected. Figure 4d showed protein detection in the ER like that seen in 4b, however the level of detection was much lower than for PDI and not as widely expressed through the ER. We can see by comparing 4c to 4d that not all the cells present have been successfully stained for Ero1α. This would suggest that the dilution of the 2G4 used was too low to detect Ero1α in all the cells present. Nevertheless the cells which have been successfully probed with the antibody show the protein detection pattern that was expected for Ero1α.

The coverslip shown in 4e/f was probed with αV5 antibody in addition to the DAPI staining. Figure 4e shows DAPI staining the nuclei as expected. As the V5 antibody detects the V5 tag sequence which is not found on endogenous proteins, no protein detection was expected. Figure 4f did show some faint staining in the cells which seems to correspond to the ER. This was therefore most likely to be non specific cross reactions between the αV5 antibody and one or more endogenous proteins located in the ER of the cell. This data would suggest that the antibody is either unsuitable for use in immunofluoressence or is needed at much higher concentrations to give detection of the protein.

Cells in Figure 4g and 4h were stained with an anti-myc tag antibody at 1:1000 dilution. The myc tag is a 1202kDa polypeptide protein tag normally fused to either the C or N-terminus of a protein. Whilst the cells do express the c-Myc transcription factor for which the myc-tag is derived, this should not be detected by the antibody used as it is a nuclear protein. Therefore, as the sequence is not normally found in

naturally occurring proteins outside of the nucleus, no myc signal was expected as seen in Figure 4h. The presence of cells was confirmed by the DAPI nuclear staining in 4g, confirming that there were cells present on the coverslip and that no staining has occurred on any proteins from the anti-myc antibody as expected.

3.2 Investigations into Ero1α/Notch pathway interactions

3.2.1 Immunofluorescence of transfected HEK293 cells

The results from 3.1.4 have shown the base endogenous protein expression in HEK293 cells. To expand on this, cells were next transfected with cDNA constructs to investigate the localisation of the ectopic proteins as well as the transfection efficiency. HEK293 cells were grown and then transfected with 1ng of the relevant cDNA using the lipofectamine protocol before being prepared for immunofluorescence according to protocol outlined in section 2.9. All cells were permeabilised with 0.1% TX100.

Figure 5 shows data from HEK293 cells transfected with wild type Ero1α. In all cases the DAPI staining is shown on the left of the row, the antibody staining is central and the right hand image is a false colour combination of the other two images to allow for comparison. Figure 5 a, b and c shows data from an αPDI detection with Alexa fluor 488 fluorescent secondary antibody, whereas Figure 5d, e and f used Alexa fluor 594 secondary antibody. PDI is known to be a marker of the ER, and consistent with earlier experiments, PDI is located at the nuclear periphery of the cell. Both false colour combinations allowed this to be seen more clearly as the overlay clearly shows perinuclear staining against the nuclear localised DAPI stain. The V5 staining shown in 5h was again subject to very high background levels. The staining seemed to be universal throughtout the cell, not confined to any paticular region. This was in contrast to the previous example in Figure 4, where the V5 background was limited to the ER. Several atempts were made to visualise Ero1α using 2G4. However the high levels of background prevented reliable localisation and co-localisation of

endogeneous Ero1α. Also due to the wild type Ero1α used it is impossible to differentiate which cells

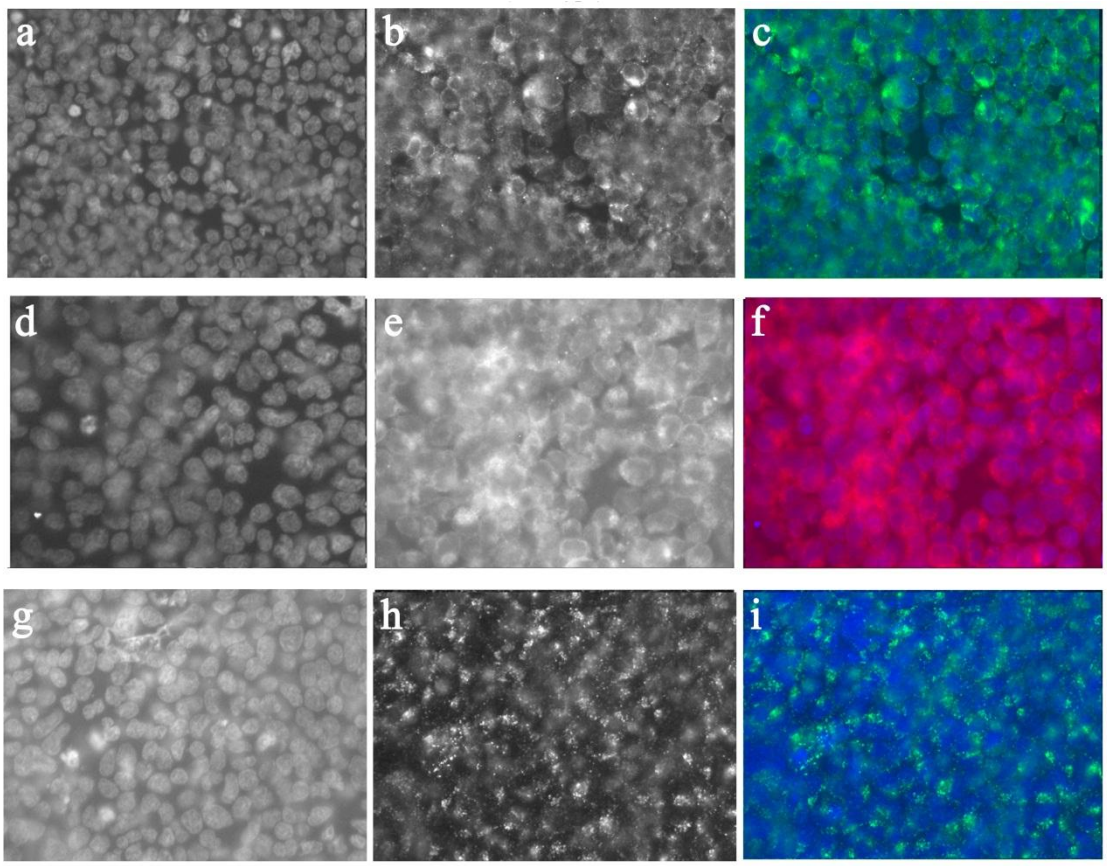

Figure 5. **Immunofluorescence detection of proteins in HEK293 cells transfected with Ero1α-wt.** HEK293 cells were stained with DAPI (a, d and g) and either PDI-G (b), PDI-R (e) or αV5 (h) and photographed at 20x magnification under the relevant filters. c, false colour combination of a (blue) and b (green). f, false colour combination of d (blue) and e (red). i, false colour combination of g (blue) and h (green).

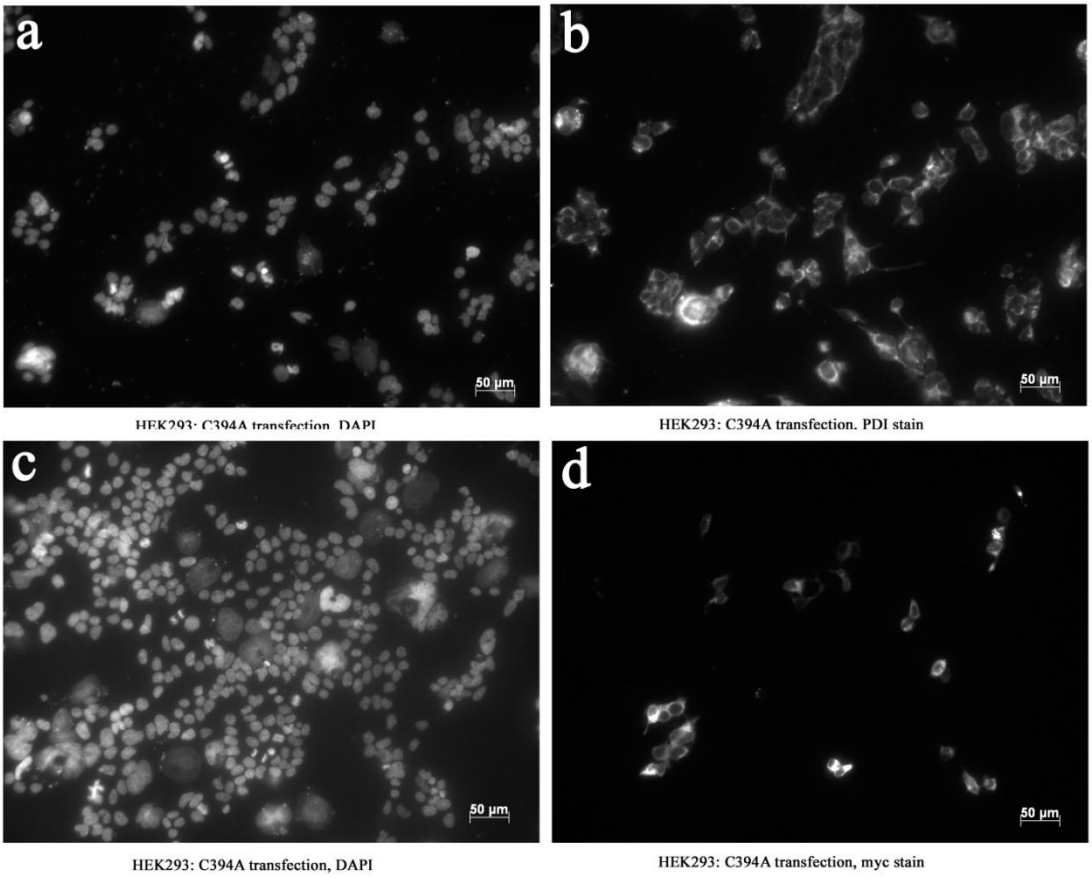

Figure 6. **Immunofluorescence detection of proteins in HEK293 cells transfected with C394A Ero1α mutant.** HEK293 cells were probed with DAPI (a and b) and either αPDI (b) or αMyc (d) and photographed under the according filters.

were sucessfully transfected and expressing the ectopic version of the protein using the 2G4 antibody which will bind to both the ectopic and endogenous version of the protein.

Given that endogenous Ero1α was hard to detect in HEK293 cells, we continued the analysis with transfected Ero1α. Figure 6 shows an example of data from transfection with the C394A Ero1α mutant. The coverslip for Figure 6a/b was probed with αPDI antibody and the coverslip in Figure 6c/d was probed with αMyc antibody. From this experiment it was hoped to see the localisation of the myc tagged Ero1α mutant, which should be expressed in the ER as with the wild type.

Figure 6b shows PDI detection in the ER as expected. Figure 6d shows the staining with the αMyc antibody, which has detected protein expression in approximately 11% of the cells. The active site mutant of Ero1α shows very similar ER localisation to previous data from both wild-type Ero1α and PDI. Thus it can be concluded that the detected protein is the C394A mutant variant of Ero1α. Whilst the transfection efficiency is lower than would be expected for this cell line and protocol, this demonstrates that HEK293 cells can express detectable mutant Ero1 proteins.

Figure 7 shows data from transfection with the hNotch1 cDNA. The coverslip for Figure 7a/b was probed with αPDI antibody and the coverslip in Figure 7c/d was probed with αMyc antibody as a negative control. From this experiment we were able to demonstate the specificity of the myc antibody, which was unable to detect the Notch protein. Despite several attempts, we were unable to detect Notch using the αNotch antibody which was unsuitable for use by immunofluorescence (data not shown). The issues with this antibody in immunofluorescence have also been seen by Dr. Ambler's group.

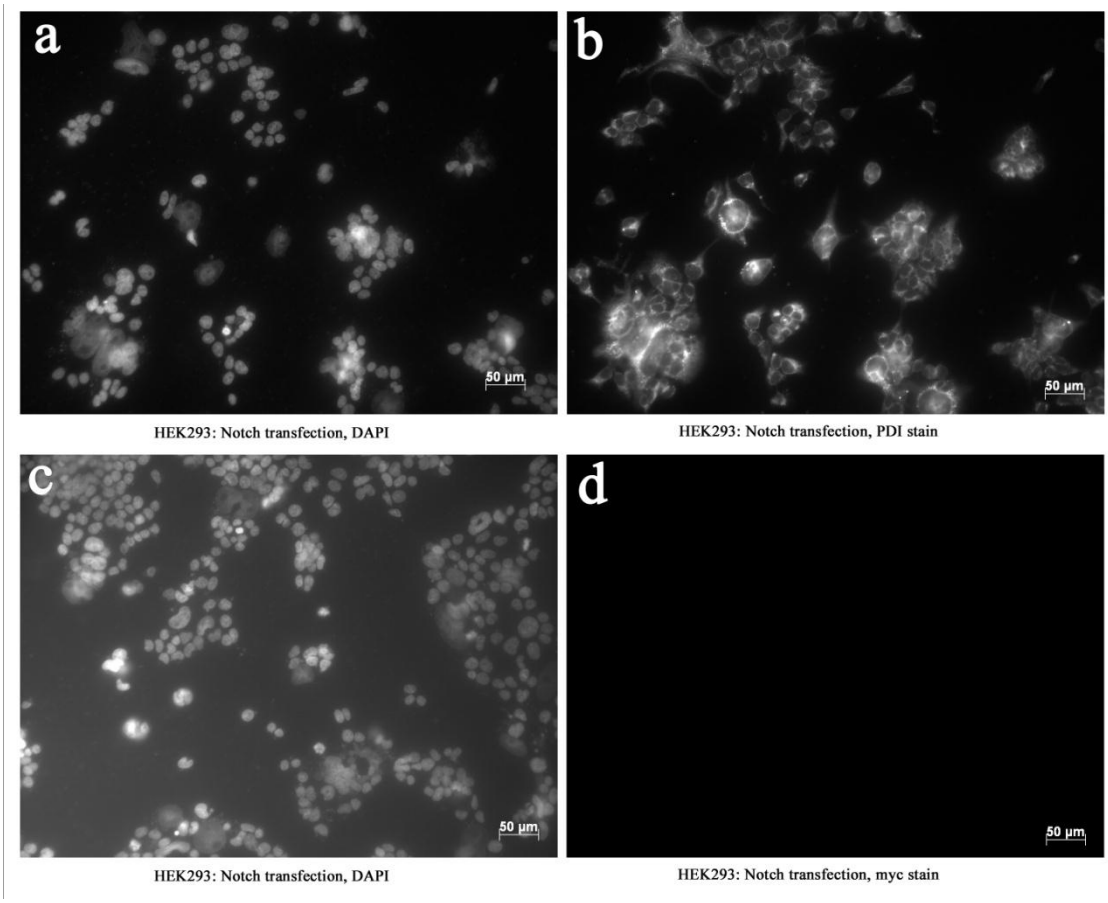

Figure 7. **Immunofluorescence detection of proteins in HEK293 cells transfected with hNotch1 cDNA.** HEK293 cells were probed with DAPI (a and b) and either αPDI (b) or αMyc (d) and photographed at 20x magnification.

Figure 7a showed PDI localisation that was consistent with earlier experiments, with PDI being located in the ER around the nuclei of the cell. Figure 7b showed strong PDI staining with clear definition on the ER, which did not overlap with the nuclear DAPI staining of the same coverslip in Figure 7a. Figure 7c showed DAPI nuclear staining, confirming cells present on the coverslip but Figure 7d was blank, confirming that the αMyc antibody is unable to bind to the Notch protein. However, due to a lack of any comparative Notch staining, we cannot confirm that the Notch plasmid has indeed been taken up and expressed by the cell. Western blot analysis of cell lystates would hopefully be able to confirm if the Notch ligand has been taken up.

Figure 8 shows data from transfection with the NICD-FLni cDNA. The coverslip for Figure 8a/b was probed with αPDI antibody and the coverslip in Figure 8c/d was probed with αMyc antibody**.** From this we wished to localise the myc-tagged NICD protein and confirm its expression after transfection.

The staining of the PDI control in Figure 8a/b was consistent with previous data. This NICD was expected to move to the ER/nuclear membrane in order to enter the nucleus, where it is functional active in the cells. Staining in the nucleus cytoplasm or around the nuclear membrane was therefore expected. Whilst the NICD should be able to enter the nucleus, the excess amount of protein the cell would hopefully produce from the transfection should lead to a build up around the nuclear membrane for detection. As can be seen in 8d this localisation is what appeared to occur in the cells. The NICD was detected around the very edge of the nuclear membrane and central ER. The examples expanded in x and y shows this pattern more clearly. The signal localised to a much smaller and more concentrated area around the nucleus than in the equivalent PDI staining of the ER.

Having demonstrated that the NICD domain of Notch could be transfected into HEK293 cells and detected by the myc antibody, we moved on to examine the Notch ligand Jagged1. Figure 9 showed the coverslips with cells which have undergone transfection with hJagged1. Figure 9a/b shows the PDI control, which shows strong nuclear staining in 9a and equivalent PDI staining in the ER in 9b, despite the slightly lower cell retention on the slide than would be ideal. Figure 9c shows the DAPI staining of the second coverslip which gave strong nuclear detection. Figure 9d shows the αV5 image, in which can be seen staining of the ER of the cell. When compared to the mock transfection the strong specific staining with V5 compared to the mock transfection suggests that the Jagged1 protein is being expressed by these cells and is present in the ER. The transfection efficiency was still lower than the 30% usually expected for this transfection reagent in HEK293 cells, at only approximately 9%, but nevertheless Jagged expression was clearly seen in the transfected cells.

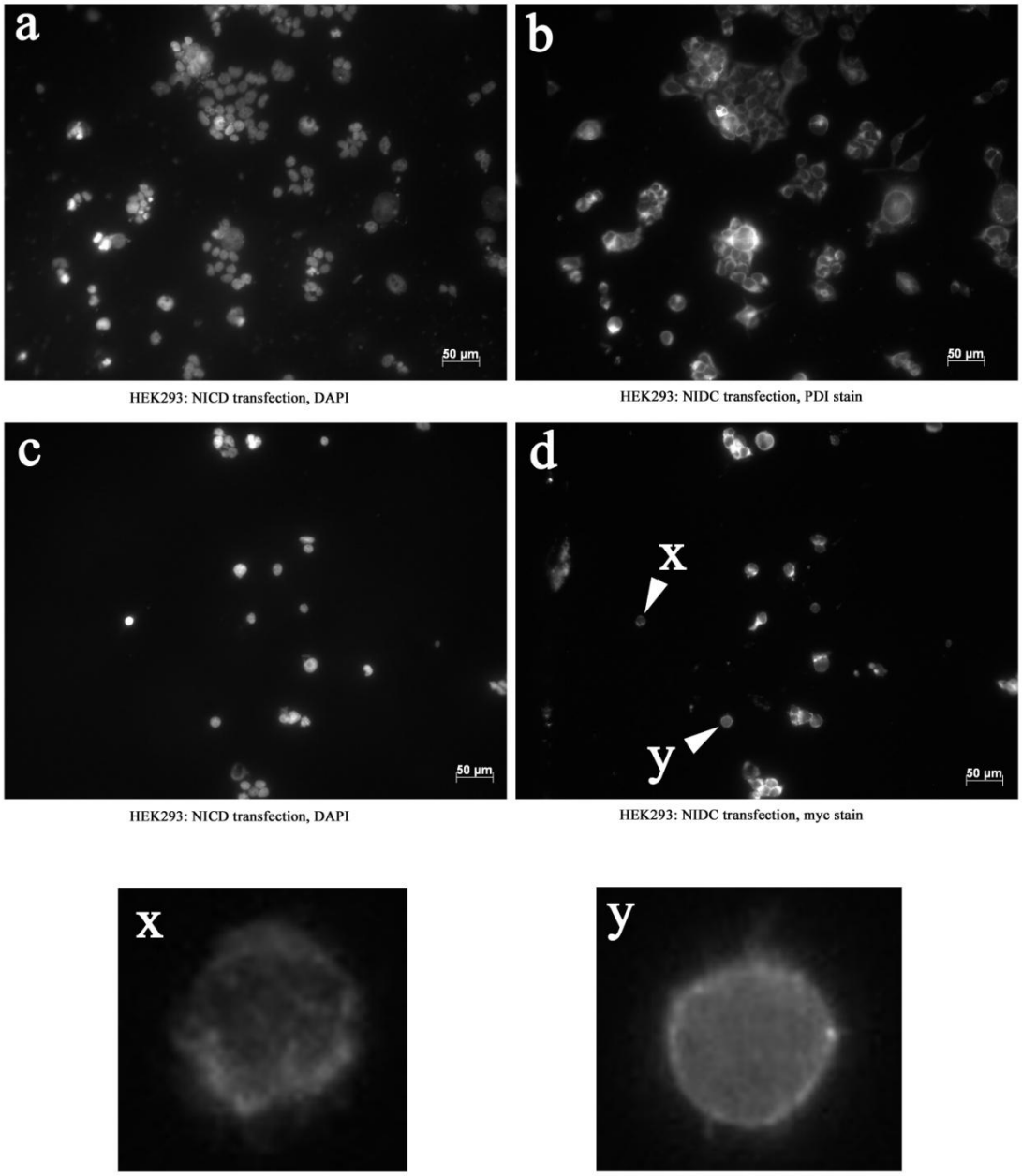

Figure 8. **Immunofluorescence detection of proteins in HEK293 cells transfected with NICD-FL*ni* cDNA.** HEK293 cells were probed with DAPI (a and b) and either αPDI (b) or αMyc (d) and photographed at 20x magnification under the relevant filters. x and y shows expansions of the cells accordingly marked in 4b.

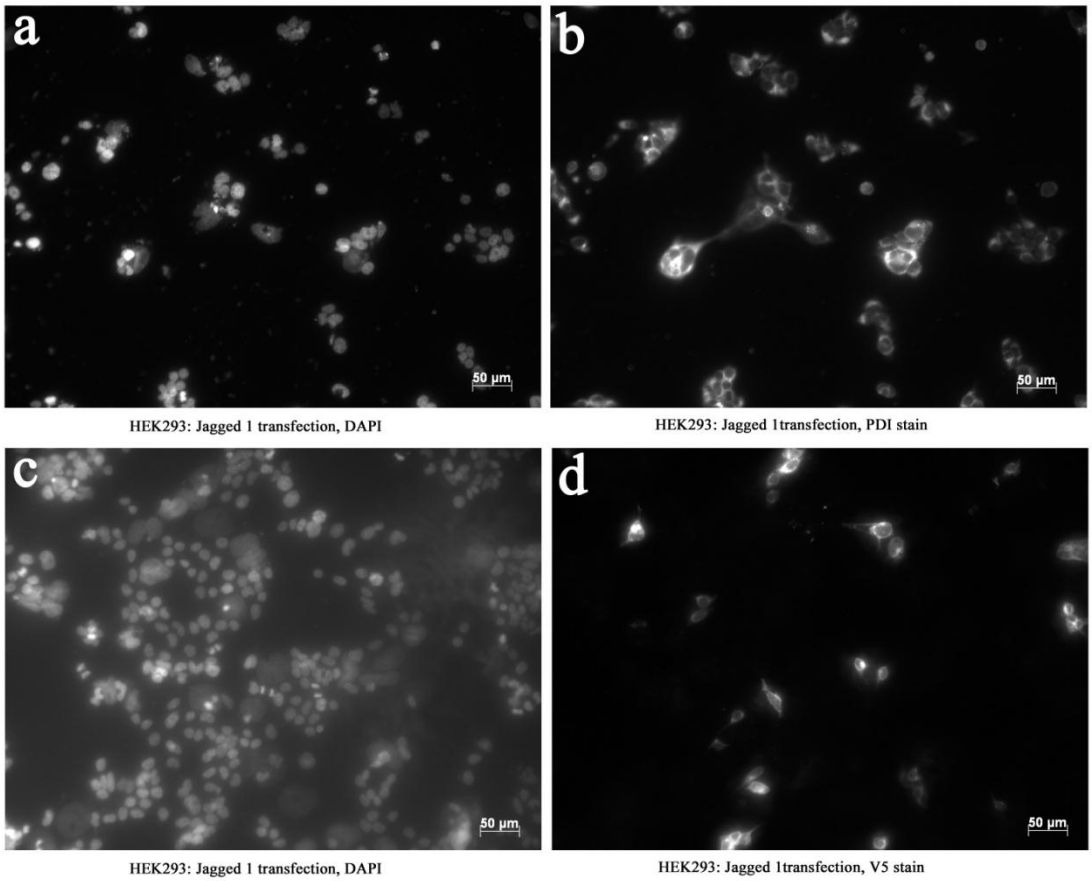

Figure 9. **Immunofluorescence detection of proteins in HEK293 cells transfected with FL-Jagged1 cDNA.** HEK293 cells were probed with DAPI (a and b) and either αPDI (b) or αV5 (d) and photographed under the according filters.

3.2.2. Jagged 1 perforated verses non-perforated cell immunofluorescence

The presence of Jagged1 in the ER of transfected cells in 3.2.1 caused us to question whether there is a potential interaction between Jagged 1 and Ero1α in addition to the Notch1-Ero1α interaction noted in the Tien paper (Tien et al, 2008).

In order to study this potential interaction we needed to study further the behaviour of Jagged 1 in our cell lines. Using a combination of immunofluorescence and Western blots we should be able to distinguish where in the secretory pathway Jagged is expressed and whether it interacts with Ero1α or other components of the oxidative folding machinery in the ER.

In order to probe the expression of Jagged1 in the transfected HEK293 cells we devised a new experiment. Jagged 1 is a transmembrane protein normally found on the plasma membrane of a cell. Thus cells were prepared for immunofluorescence with and without permeabilisation by TX100. Thus if the Jagged 1 was being expressed by the cells and trafficked properly to the cell surface it would be detected there by the αV5 antibody in non-permeabilised cells. Control tests were also performed using αPDI and 2G4 as controls, in which the permeabilised cells should show ER localisation and the non-permeabilised cells would show no staining, as the antibodies were unable to enter the cell to bind to the ER resident proteins which they were targeting.

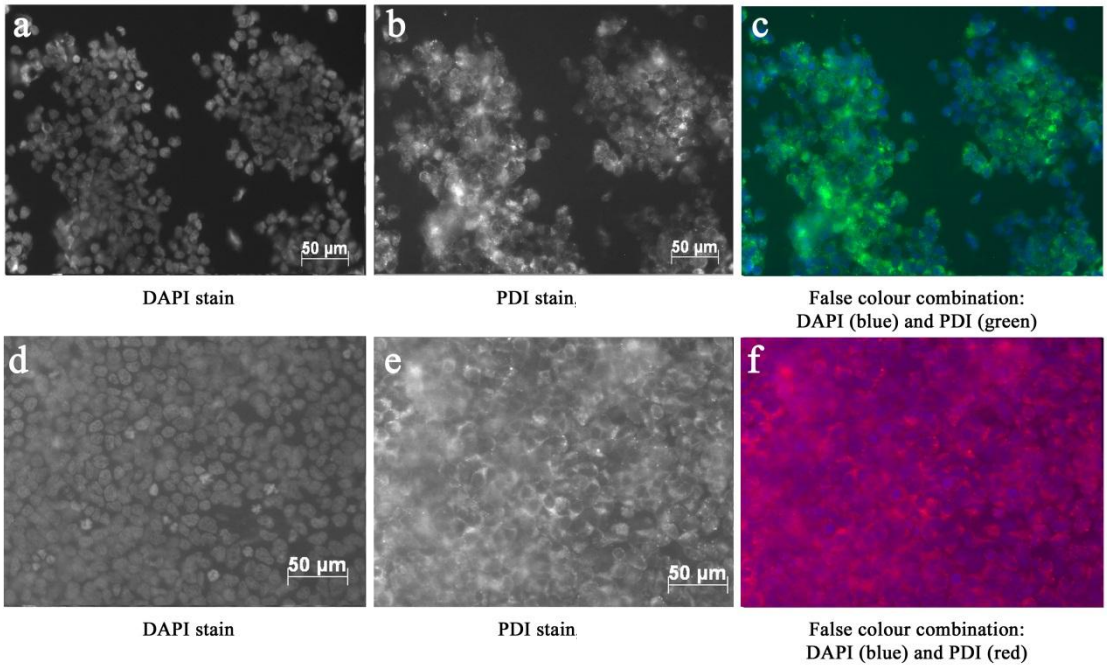

Figure 10. **Immunofluorescence detection of PDI proteins in HEK293 cells transfected with FL-Jag1.** HEK293 cells were probed with DAPI (a and d) and either αPDI with Alexa-fluor 488 [PDI-G] (b) or αPDI with Alexa fluor 610 [PDI-R] (e) and photographed at 20x magnification under the appropriate filters. c, false colour combination of a (blue) and b (green). f, false colour combination of d (blue) and e (red).

HEK293 cells were transfected with the FL-Jag1 construct or the Ero1α construct using the lipofectamine transfection method and prepared for immunofluorescence according to the protocol outlined in 2.9. Depending on the sample in question, the cells were either permeabilised with 1% TX100 solution or the permeabilisation step was omitted. Non-permeabilised samples were kept in PBS++ buffer whilst the other samples were undergoing permeabilisation treatment before continuing with the protocol as detailed in section 2.9.

Figure 10a, b, c, d, e and f show the cells probed with DAPI and αPDI with either Alexa fluor 488 or 610. As seen previously, the PDI staining (Figure 10b and e) corresponds to the ER, which could be seen to lay around the nuclei (Figure 10a and c) of the cells in the false colour images overlays in 10c and 10f. Non permeabilised cells were also exposed to the αPDI antibody to act as a control for the cell membrane staining, and did not give a specific ER staining pattern, as expected (data not shown).

The result from the Ero1α localisation in both permeabilised and non-permeabilised HEK293 cells are shown in Figure 11. Figure 11a shows the DAPI staining of the nuclei of the cells which were permeabilised, and corresponds to the 2G4 staining in Figure 11b. Figure 11c is a false colour overlay of both Figure 11a and Figure 11b in order to co-localise nucleus and ER. As was previously noted the 2G4 antibody binds to Ero1α, and this has occurred in approximately 20% of the cells shown. Figure 11d and 11e show the 2G4 probe for the non permeabilised HEK293 cells. The DAPI staining in Figure 11d has occurred as expected showing the cell nuclei, as the DAPI stain is able to pass freely into the cell through the cell membrane without permeabilisation. No 2G4 detection of Ero1α was seen in Figure 11e as was expected. Ero1α is a resident protein to the ER and is not seen at the cell surface. Thus with no means

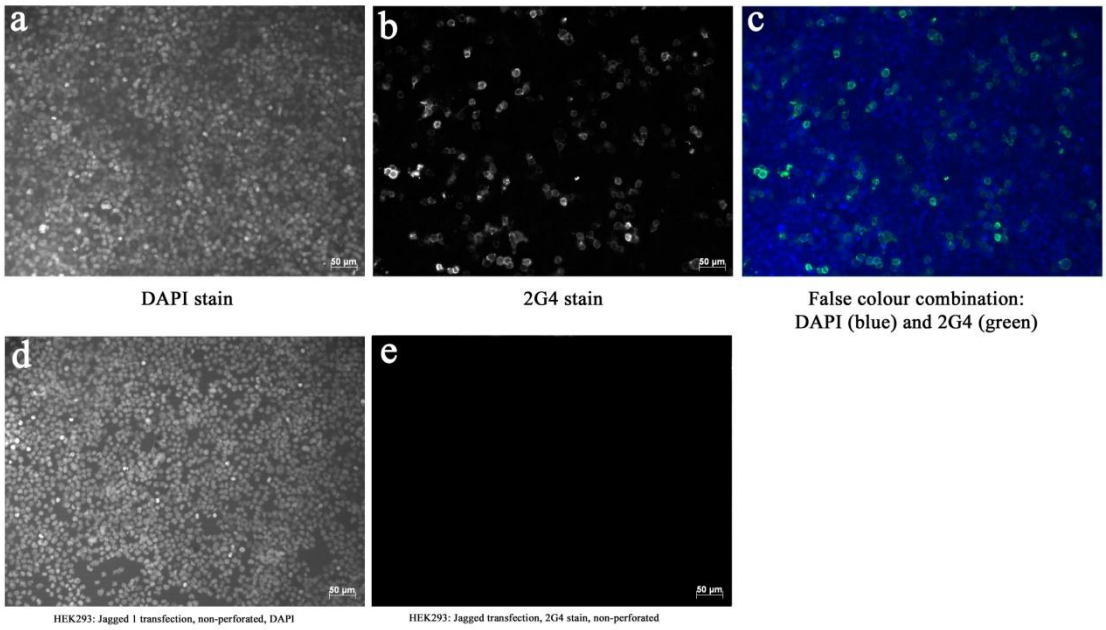

Figure 11. **Immunofluorescence detection of Ero1α proteins in HEK293 cells transfected with FL-Jag1.** HEK293 cells were probed with DAPI (a and d) and 2G4 (b and e) and photographed at 20x magnification. c, false colour combination of a (blue) and b (green).

of entering the cell, the 2G4 antibody (see earlier sections) was unable to bind to the Ero1α and thus is simply removed from the cells during the wash steps.

Figure 12 shows the HEK293 cells which were permeabilised and then probed with αV5 in order to detect Jagged 1 expression within the cell. As seen before, the V5 tagged Jag is not apparent in all cells showing approximately 10% transfection efficiency. As was seen in 3.2.1 the V5 tagged detection of the Jagged 1 is higher than the background levels seen in the untransfected HEK293 cells. The V5 detection was centred on the ER of the cell just around the area of DAPI nuclear staining, seen well in the merged images. Images were also taken of this at 40x magnification (see Figure 12 c, d and e) which allowed clear localisation of the Jagged 1 to the ER of the cell. The region marked x in 3f is expanded 3x to allow the ER localisation to be seen clearly.

Figure 13 shows the data collected from the HEK293 cells which were not permeabilised and then probed for expression of Jagged1 at the cell surface. Figure 13a, b and c were taken at 20x magnification and the data showed the V5 tagged protein expressed at the cell surface, as can most clearly seen in Figure 13c. Here the Jagged1 is seen surrounding but not in contact with the area around the cell nuclei suggesting that it is being presented at the cell surface. This can be more clearly seen in Figure 13d-i at 40x magnification. This data showed the Jagged1 presented at the cell membrane, and several good examples can be seen expanded in Figure 13x,y and z. Co-localisation with another cell membrane protein to confirm localisation was not possible due to time and resource constraints

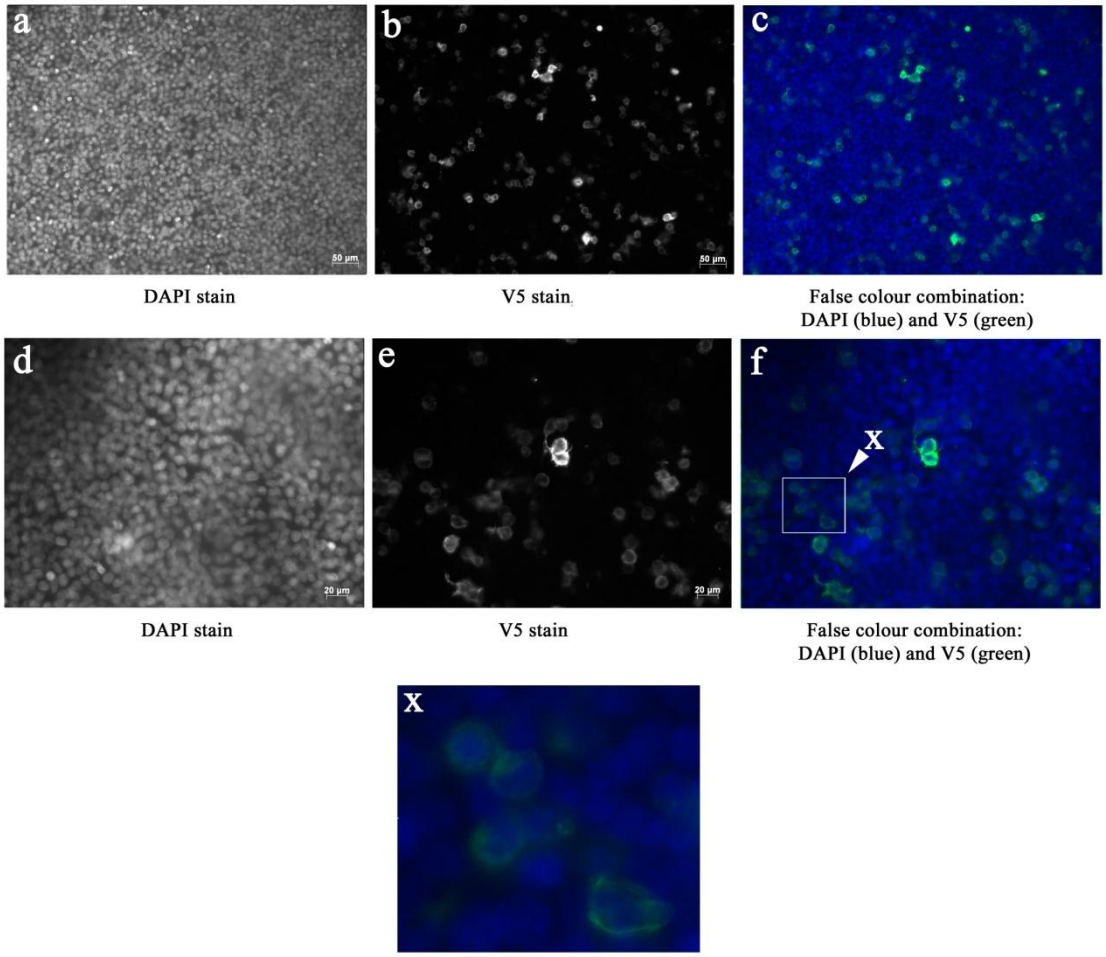

Figure 12. **Immunofluorescence detection of Jag1 proteins in HEK293 cells transfected with FL-Jag1.** HEK293 cells were probed with DAPI (a and d) and αV5 (b and e) and photographed at 20x magnification under the according filters. c, false colour combination of a (blue) and b (green). f, false colour combination of d (blue) and e (green). x, expansion of the area marked in 3f.

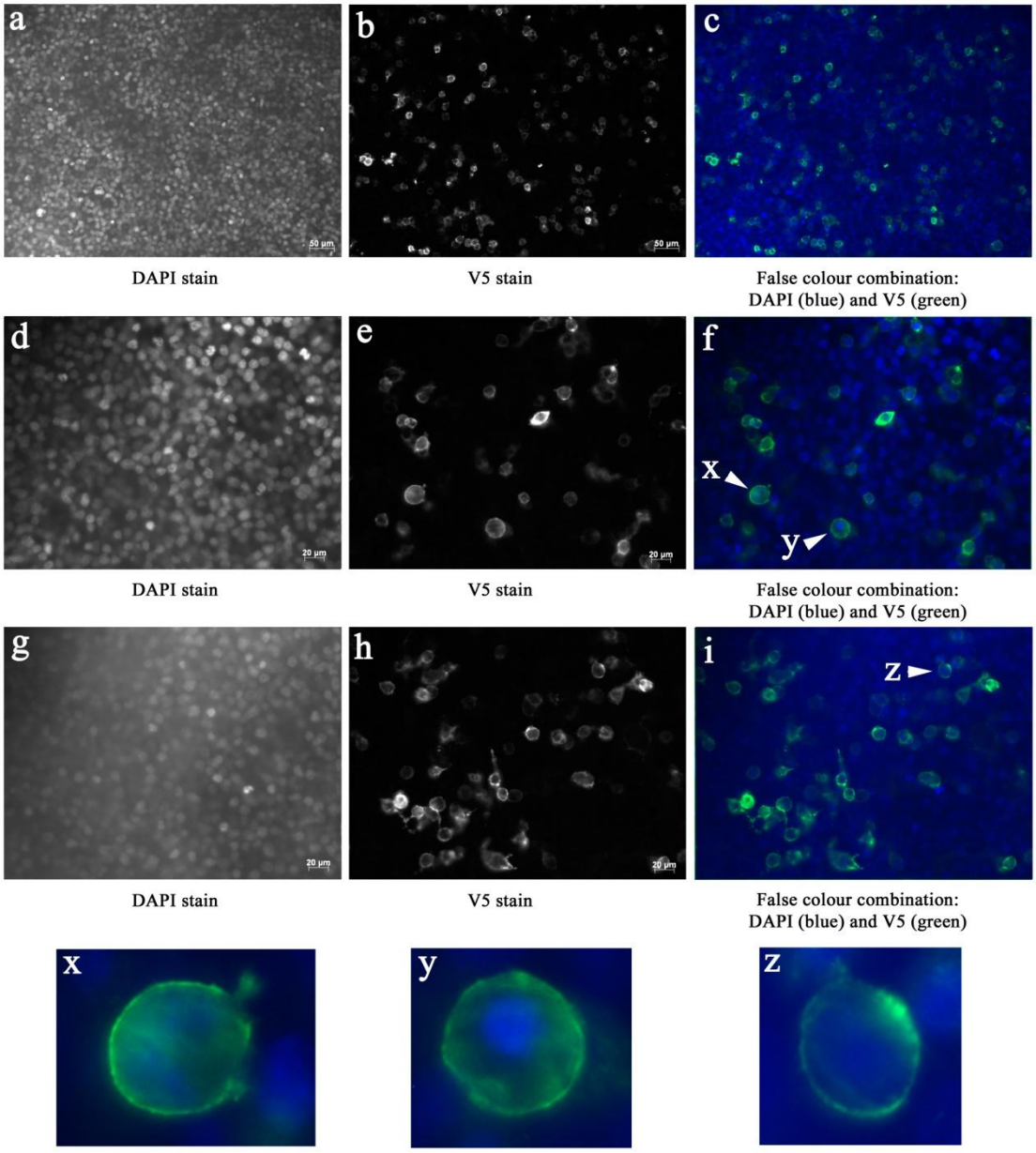

Figure 13. **Immunofluorescence detection of Jag1 proteins in non permeabilised HEK293 cells transfected with FL-Jag1.** HEK293 cells were probed with DAPI (a, d and g) and αV5 (b, e and h) and photographed at 20x magnification under the according filters. c, false colour combination of a (blue) and b (green). f, false colour combination of d (blue) and e (green). i, false colour combination of g (blue) and h (green). Cells marked in 4f and i are expanded in x, y and z.

In conclusion, the data suggests that not only is Jagged1 being expressed in some of the cells, but that it is being fully processed as it would be in vivo and then transported to the cell surface where it is being presented on the plasma membrane, as it normally would be to undertake its normal function in Notch signalling. The data shown in both Figure 12 and 13 shows us that Jagged1 is being expressed in some of the cells, but not all in both cases. The fact that the staining is better on the non permeabilised cells may be due to the antibody being able to bind to the jagged better when it is properly presented at the cell membrane, possibly due the difference in the shape or presentation of the binding region to the antibody or it is easier for the antibody to bind at the surface than to infiltrate the cell and bind to the Jagged1 in the ER.

3.2.3 Co-immunoprecipitation studies with Ero1α and Notch pathway proteins

Immunoprecipitation is a technique used to isolate individual proteins from cell lysates by the use of antibodies which specifically bind to the protein of interest. Antibodies are bound to a solid substrate such as sepharose beads preloaded with protein A or G which bind to the heavy chains of the antibody.

Co-immunoprecipitation was used on lysates in order to pull down a known protein and investigate other proteins to which it might be bound within the cell. Co-immunoprecipitation of protein complexes can help to elucidate novel members of protein networks and reveal new protein interactions. Initially we wanted to look into the potential interaction between Ero1α and Notch1 in mammalian cells. However, the lack of a suitable Notch antibody limited this approach. Instead we investigated if there was an interaction between Ero1α and Jagged1, as the previous immunofluorescence data had shown that both could be expressed in the HEK293 cell line. Jagged also has the most similar structure to Notch of the proteins available to test, with both EGF repeats and an area of cysteine residues similar to the LNR region of Notch which is where Ero1α may bind to Notch. As such it is also possible that Ero1 may bind to this cysteine rich region of Jagged to aid in disulphide bond formation. If shown to be true this would elucidate a potentially new folding client for the Ero1α-PDI pathway as well as further expanding our knowledge of Notch biology.

The first experiment undertook was to see if 2G4 was able to detect Ero1α pull down by PDI. Protein A beads were incubated with αPDI antibody as described in 2.6 and then 50µl of beads were added to either HeLa or HEK293 lysates which had been transfected using Ero1α C394A, Ero1α C397A, Jagged1 or Jagged 1 with Ero1α

C974A as well as a mock for control. The original lysates as well as the IPs were then separated by SDS-PAGE and visualised.

Figure 14a shows the results of the HeLa lysates and PDI-IPs, blotted for with 2G4. The lysates all showed detection of Ero1α concordant with previous results. The mock transfected lysate in lane 1 shows lower detection of Ero1α, whereas the lysates in lanes 2 and 3 show stronger Ero1α detection. The higher protein level showed that the transfection had been successful and the cells were expressing the mutant version of Ero1α. Interestingly, the lysate transfected with Jagged 1 in lane 4 also showed higher levels of Ero1α detection. This would imply that the increased levels of Jagged 1 in the cell were causing an increased level of Ero1α production in the cell. Lane 5 contained the lysate transfected with both Ero1α C974A and Jagged 1. As such the increased detection of Ero1α seen, when compared to the mock transfectant in lane 1, either means that the cells were producing the mutant version of Ero1α or that the Jagged 1 production was affecting the cells Ero1α levels as in lane 4. Another possibility is that the different detection levels are not cause by differences in protein levels but is from a transfer or detection issue from the gel. If this is the case a re-run of the gel should be able to prove which of the two ideas is correct.

The IPs in figure 14a did not show any detection of Ero1α. This either means the IP was unsuccessful in pulling down any of the protein or that the levels of protein pulled down were unable to be detected. Following up this experiment by either re-blotting the gel with anti-myc antibody to see if the myc tagged versions of Ero1α are detectable or by using a higher 2G4 concentration to attempt to get a greater sensitivity of detection would possibly provide more data as to what has occurred. The low weight bands seen in each lane are most likely to be from cross reactivity of the

antibody light chains, but this signal is normally much weaker. Also the antibody heavy chains normally seen at 50kDa are not seen

Figure 14b shows the data from the HEK293 cells. The lysates in lanes 1-5, showed detection levels of Ero1α in line with what was seen in 14a, albeit at slightly lower overall levels than seen in the HeLa cells. The HEK293 IPs again showed no Ero1 detection but did show detection of the antibody heavy chains at around 50kDa which were not seen in Figure 14a. Lane 11 showed a very faint detection of protein at the level at which Ero1α is normally seen. This suggested that the IPs were in fact working but that they were just pulling down very low levels of Ero1α or that Jagged is inducing a slight increase in the levels of Ero1 produced in the cell. Again, a re-blot using a higher concentration of 2G4 may allow better detection of any Ero1α proteins that are present.

Since none of the previous immunoprecipitations had been able to detect Ero1α a second experiment was performed. IPs were carried out on lysates of HeLa and HEK293 cells, both transfected with Ero1α C974A, and this time using 100µl of Protein A beads which had been incubated with αPDI antibody, in an attempt to pull down more proteins for detection. As can be seen in Figure 14c, both lysates IPs now showed detection of Ero1α when using the higher levels of beads. This would suggest that the previous conclusions that the levels of protein pulled down in Figures 14a and b were too low to be detected.

Together this data confirms that the immunoprecipitations were successful and that PDI and Ero1α complex together in the cell as shown in previous published work.

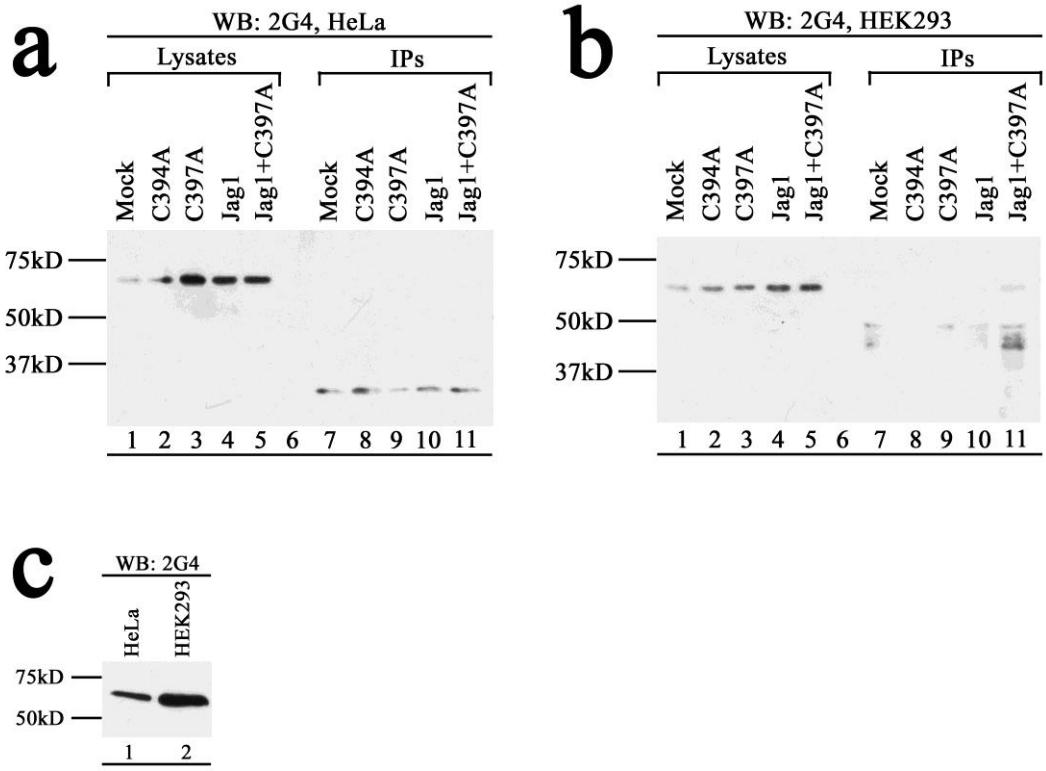

Figure 14. **Detection of Ero1α from immunoprecipitations of cell lysates using αPDI antibodies bound to protein A beads.** A, HeLa cell lysates from transfection with Ero1α C394A (lane 2), Ero1α C397A (lane 3), Jagged1 (lane 4), Jagged 1 with Ero1α C974A (lane 5) or mock transfected (lane 1) as well as immunoprecipitations using αPDI bound to Protein A beads with the lysates, Ero1α C394A (lane 7), Ero1α C397A (lane 8), Jagged1 (lane 9), Jagged 1 with Ero1α C974A (lane 10) or mock transfected (lane 11) were immunoblotted with 2G4 at 1:50 concentration after separation via SDS-PAGE. Lane 6 is blank. B, As described in A instead using HEK293 cells. C, Lysates from HeLa cells (lane 1) and HEK293 cells (lane 2) were transfected with Ero1α C974A and immunoprecipitated using 100µl of αPDI bound to Protein A beads before immunoblotting with 2G4 at 1:50 concentration after separation by SDS-PAGE.

Figure 15 shows the results for the immunoprecipitation experiments investigating whether Ero1α can successfully pull down the V5 tagged Jagged1 and *vice versa*. Due to the higher levels of detection seen in 14c HEK293 cells were used. Figure 15a shows the results from an IPs with either V5 or 2G4 bound to 100µl of protein A sepharose beads which was then blotted back with 2G4 and 15b shows the equivalent samples blotted back with αV5 antibody.

Figure 15a shows the immunoprecipitation of Jagged 1 transfected HEK293 cells with either 100µl of protein A beads incubated with αV5 antibody (lane 1) or 2G4 (lane 2). Lane 2 showed Ero1α detection from the lysate which is what was expected from the previous data gathered. Lane 1 showed a detection of Ero1α from a pull down with the αV5 antibody. This suggests a potentially novel interaction, that Jagged 1 forms a complex with Ero1α within the cell.

Figure 15b shows the reciprocal interaction to what was seen in Figure 15a. Lane 2 shows the result of Ero1α pull down via the 2G4 antibody, with the V5 antibody detecting Jagged 1 as seen in previous experiments. This suggests that Ero1α is also able to pull down Jagged 1, strengthening the conclusion from Figure 15a that the two proteins form a complex with each other. The result from lane 1 was unexpected as a similar Jagged 1 signal to that seen in lane 6 was anticipated. The lower weight protein band detected between 75kDa and 50kDa is likely to be cross-reactive antibody heavy chains.

This data shows that in immunoprecipitation Ero1α was able to retrieve Jagged 1 and that the opposite was also true, with Jagged 1 pulling down Ero1α as well. This suggests a novel interaction between these two proteins, suggesting that Ero1α may have a role in the folding of Jagged1.

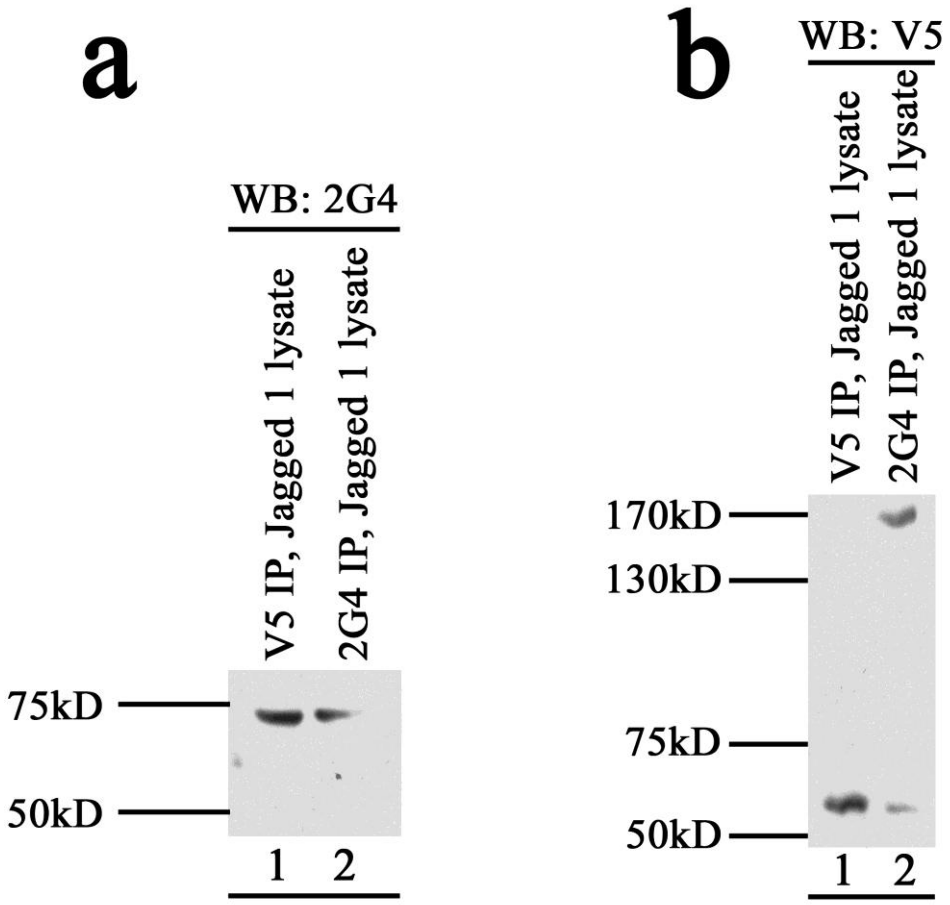

Figure 15. **Jagged 1 and Ero1α form a complex within the cell.** A, HEK293 lysates were transfected with Jagged 1 and immunoprecipitated with either αV5 (lane 8) or 2G4 (lane 9) antibodies bound to protein sepharose A beads, then immunoblotted with 2G4 at 1:50 concentration after separation by SDS-PAGE. B, HEK293 lysates were transfected with Jagged 1 and immunoprecipitated with either αV5 (lane 8) or 2G4 (lane 9) antibodies bound to protein sepharose A beads, then immunoblotted with αV5 after separation by SDS-PAGE

4. Discussion

4.1 Characterisation of cDNA and ectopic proteins

Ectopic expression of proteins via cDNA plasmids is a highly useful tool in biology, used to analyse a huge variety of factors such as the effects of a proteins expression on a cell, its localisation, interactions and its effects on both a cellular and whole organism level. In this several cDNA plasmids were studied and the proteins they produce within cells. This will be of further use to the group in any future work into this area.

The Ero1α plasmids, Ero1α-wt, C394A and C397A, had been previously described and used by the group and the results for localisation in immunofluorescence (Chapter 3.2.1) and antibody detection with Western blotting (Chapter 3.1.3) was concordant with this data. The proteins were all detected by the Ero1α specific 2G4 antibody and the two mutant myc tagged versions of Ero1α were detectable with an anti-myc tag antibody (Chapter 3.1.3 Figure 3D). Localisation of all the Ero1α proteins to the ER was also as expected.

The two Jagged 1 plasmids and the encoded Jagged 1 protein, one V5 tagged the other not, were also successfully tested. Both the wild type human Jagged 1 and V5-tagged version, were detectable in immunoblotting with the C20 anti-Jagged 1 antibody (Chapter 3.1.3 Figure 3B) and only the V5-tagged Jagged protein was detectable with an anti-V5 antibody (Chapter 3.1.3 Figure 3C). In immunofluorescence studies (Chapter 3.2.1) due to constraints on stocks of the C20 antibody, only the V5-tagged version of Jagged 1 was used. Localisation in permeabilised cells was shown to place the Jagged 1 in the ER of the cell, with levels of detection higher than the background

levels of V5 staining in the mock transfected cells (Chapter 3.1.4). Non-permeabilised cells were used to see if there was any presentation of V5 tagged Jagged at the cell surface which was clearly seen (Chapter 3.2.2). Together this data allowed us to see that the Jagged 1 protein was being expressed by the cells, and that it is localised to the ER of the cell before being presented at the cell membrane.

Some problems were met when trying to confirm the identity of the Notch 1 plasmid and protein. Using anti-Notch 1 antibody from Dr. Carrie Ambler's group, no detection could be seen in either immunoblotting or immunofluorescence. As such it was not possible to confirm either the identity of the protein produced by the plasmid or indeed if there was any transcription of the protein at all. Whilst each experiment was attempted several times to try and optimise the protocol, no positive result was obtained. Personal communication with Dr. Ambler's group confirmed that they had also been having similar problems with the antibody. If more time had been available, commercially available antibodies could be investigated to continue the experiments in this area. Another alternative would have been to engineer the available Notch plasmid to give various tagged full length and domain Notch mutants.

The NICD domain of Notch presented some issues with confirmation of identity and localisation. No antibody against the active form of the Notch receptor was available and so no direct detection experiments could be carried out on both NICD plasmids/proteins. The non-inducible form of the protein was tagged with a myc sequence and this was instead used to identify this protein. In immunoblots (Chapter 3.1.3) the myc tag on FL-NICD*ni* could not be detected in transfected lysates. However, immunofluorescence localisation experiments (Chapter 3.2.1) allowed the detection of the protein around the ER of the cell, close to the nuclear membrane. As stated previously the NICD is the active form of the Notch receptor and moves to the

cell nucleus to alter transcription patterns within the cell (Schweisguth, 2004). Thus the excess of myc-tagged NICD seen at the nuclear boundary was expected and taken as confirmation of the NICD protein identity.

4.2 Characterisation of monoclonal anti-Ero1α antibody 2G4

Antibodies are highly useful tools for detecting proteins in immunoblots, immunofluorescence and immunoprecipitation studies. Here we have confirmed and extended the range of cell lines and conditions for the 2G4 antibody.

The 2G4 antibody recognised Ero1α in cell lysates of both transfected and non-transfected cell lines (Chapter 3.1.2) and was able to pull down Ero1α by immunoprecipitation (Chapter 3.2.3). The antibody was also able to localise Ero1α-wt and mutant Ero1α-C394A and Ero1α-C397A, dominant negative mutants used for controls in later experiments, in immunofluorescence studies (Chapter 3.1.4 and 3.2.1). This allowed us to isolate and detect Ero1α in this study and will also allow it to be used for any further work. In addition, we were able to confirm that 2G4 does not cross react with Ero1β proteins (Chapter 3.1.2 Figure 2) which is important to note as Ero1α and Ero1β potentially share some epitopes yet have differing functions and expression patterns within organisms.

4.3 Interaction between Jag1 and Ero1

After seeing that Jagged 1 was localised to the ER in permeabilised cells (Chapter 3.2.1) the possibility of an interaction between Jagged 1 and Ero1α was further

investigated. First we confirmed that the 2G4 antibody was capable of pulling down Ero1α and that Ero1α co-immunoprecipitated using PDI controls (3.2.3 Figure 14). Next we used 2G4 and anti-V5 antibodies to immunoprecipitate Ero1α and Jagged 1 from lysates transfected with V5-tagged Jagged 1. We saw that Ero1α was able to retrieve Jagged 1 and that the opposite was also true, with Jagged 1 pulling down Ero1α as well. This data would imply that we have found a previously uncategorised interaction between these two proteins. Jagged 1 contains a cysteine rich region in its structure at the base of the extracellular region (see diagram on page 20), and it is likely candidate for where Ero1 may bind to Jagged in order to facilitate disulphide bond formation. In addition to this the DSL region of Jagged 1 is also rich in disulphides required for Notch trans-activation or cis-inhibition (Cordle et al, 2008) and has a highly conserved structure required for notch binding. In the same way that Ero1 has been shown to be necessary for the function of the LNR region of Notch, Ero1 may be similarly needed for the correct formation of the disulphide bonds in the DSL region. If this is true then Ero1 may have a much wider role in ensuring the functionality of Notch signalling than previously thought, as the DSL region is present in all Notch ligands and is evolutionarily conserved across all studied species. Despite this, further work into this interaction is needed before any firm conclusions as to its biological significance can be made.

4.4 Future directions

Whilst this study has brought to light a potentially new protein interaction, it has raised still more questions to be further investigated. We were unable to successfully

demonstrate whether Ero1α and Notch 1 interact in mammalian cells and the novel interaction between Ero1α and Jagged 1 warrants further testing and exploration.

Firstly, we would need to acquire and rigorously test antibodies against both the Notch 1 receptor and the active NICD using immunoblotting, immunoprecipitation and immunofluorescence. With these antibodies we could build on the work started in this thesis, using immunofluorescence and immunoprecipitation to investigate if Notch 1 and Ero1α interact and co-localise, in the same manner as was used to elucidate the interaction between Jagged 1 and Ero1α.

The interaction between Ero1α and Jagged also needs further testing. Confirmation of the interaction could be obtained using further immunoprecipitation experiments, perhaps coupled with metabolic labelled and pulse chase. This would enable us to see whether the interaction occurred during the synthesis and folding of Jagged 1. Such experiments would also enable us to test whether post-translational modifications and hence maturation of Jagged I is under the control of Ero1a. Control experiments using transfection of the dominant negative mutants of Ero1 could also be used to determine whether the oxidoreductase activity of the protein is required for oxidative folding of Jagged proteins. Similar experiments could be conducted with PDI and its active site mutants to see whether any functional effects on Jagged required PDI activity. This would be of interest, because there are no examples in the literature of direct Ero1-client interactions being involved in quality control: the literature shows that Ero1a passes disulphides to clients via PDI. Thus another alternative is that Ero1a targets overexpressed or misfolded Jagged 1 to the ERAD system for degradation. This could be tested by determining whether Ero1a expression influences the half-life of Jagged 1 in pulse chase experiments. Use of different antibodies such as C20 or other commercially available antibodies against Jagged and the use of differently tagged

Ero1 proteins would affirm the results seen in Chapter 3.2.2. These experiments could also be carried out with other DSL containing proteins such as Jagged2, Delta, Delta-like 1 or Apx-1 to see if Ero1 is required for DSL formation in all these proteins.

The relationship between Ero1α, Notch and its clients could be investigated further using quantitative real time PCR. Using this technique we could test whether over-expression of Notch 1 leads to the induction of Ero1α/β and vica versa. Knock down experiments using siRNA could also serve a similar purpose, to see what the effects on Notch 1 production within the cell are when Ero1α/β are removed.

Biochemical assays could be preformed to look into the protein-protein interactions further. As described by Tien et al (Tien et al, 2008), the secretion efficiency of the different domains of the Notch or Jagged proteins, such as the LRNs or EGF-repeats can be measured. The domains can be tagged for detection in cell lines and then the efficiency of the secretion measured when Ero1α is manipulated within the cell. Should the secretion levels drop when Ero1α is reduced or knocked out it would be apparent that Ero1α is required for successful folding of the protein and secretion from the cell. Tien et al (Tien et al, 2008) suggested that in *Drosophila,* Ero1α was needed for a specific role in disulphide bridge formation of the LNR, and had a more general role in that of the EGF domains and a similar effect might also be seen in mammalian cells. To see if loss of Ero1α specifically affects disulphide bridge formation in either the LNR or EGF domains a trapping experiment can be performed, using a thiol conjugating reagent such as AMS. If there are extra free thiol groups on cysteine residues of the client domains due to lack of Ero1α, the reagent could bind to these thiol groups and increase the molecular mass of the protein by a set amount for each thiol group. This change in mass can then be detected in Western blot analysis.

The relationship between Notch and the Jagged protein could also be further looked into in the presence or absence of Ero1α or Ero1β, repeating some of the previous experiments using other variants of Jagged to see if they also form complexes with Ero1α. If these experiments show promise, one could investigate which disulphide bonds in Jagged are targeted by Ero/PDI by using mutant versions of Jagged where the bonds are disrupted by site directed mutagenesis give more insight into any potential binding of Jagged to Ero1α.

4.5 Concluding remarks

As well as characterising several antibodies not before used by the group, this thesis has laid the groundwork for investigating an entirely novel interaction between Ero1α and Jagged 1. Whilst constrains on time have not allowed a full analysis of this interaction, together the data collected has provided the opportunity for a great deal of further experimentation which could lead to new insights into both the action of Ero1α in the cell and the control and processing of the Notch pathway proteins.

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