Generation and analysis of \( p23 \)- and \( calnexin \)-deficient mice

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Abstract

This thesis provides in vivo information about two proteins - p23 and calnexin - which play major roles in the early secretory pathway of mammalian cells. The aim was to gain further understanding about the function of these proteins by generating mice deficient in the expression of p23 and calnexin.

p23 is a member of the integral p24 protein family. These proteins are highly abundant in the endoplasmic reticulum and Golgi apparatus and are thought to play a role in protein transport and vesicle formation. Disruption of both p23 alleles results in early embryonic lethality. Inactivation of one allele leads not only to reduced levels of p23 itself but also of other family members. The reduction in steady state protein levels also leads to an altered subcellular distribution of p23 as well as p26 (another family member) in p23 heterozygous cells. In addition, structural changes in the Golgi apparatus, in particular dilated saccules, were observed. These changes in p23 heterozygous mice have functional consequences, resulting in specific defects in the secretion of some plasma proteins.

Calnexin is a molecular chaperone molecule which is involved in the correct folding of newly synthesised polypeptide chains in the ER. 40% of the calnexin homozygous deficient mice died shortly after birth, the remaining 60% developed a severe neuronal phenotype and had to be sacrificed within 2 months. They were about one third smaller than their littermates and displayed motor disorders which included shaking of the body and a wobbly unsteady gait. Histological examination of brain, cerebellum, spinal cord, neuromuscular junctions and muscle did not reveal any abnormalities in the calnexin-deficient mice. Although these mice showed clear neurodegenerative symptoms, the molecular basis for the phenotype has not yet been characterised.
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List of Abbreviations

AA  amino acid
Ab  antibody
AchR acetylcholine receptor
APS ammonium persulfate
ATP adenosine 5'-triphosphate
BCR B cell antigen receptor
bp  base pair
β₂-m β₂-microglobuline
CD  cluster of differentiation
COP coat protein
cpm counts per minute
cDNA complementary DNA
CFTR cystic fibrosis transmembrane conductance regulator
Cre causes recombination
C-terminal carboxy-terminal
dATP deoxyadenosine 5'-triphosphate
dCTP deoxycytosine 5'-triphosphate
dGTP deoxyguanosine 5'-triphosphate
dTTP deoxythymidine 5'-triphosphate
DMEM Dulbecco's modified Eagle's medium
DMSO dimethyl sulfoxide
DN double negative CD4⁺CD8⁻ thymocytes
DNA deoxyribonucleic acid
DP double positive CD4⁺CD8⁺ thymocytes
DTA diphtheria toxin a
DTT dithiothreitol
d.p.c. days post coitus
EDTA diaminoethanetetra acetic acid
EGTA: [ethylene-bis(oxyethylenenitrilo)] tetra-acetic acid
FACS: fluorescence activated cell sorting
FCS: foetal calf serum
FITC: fluorescein isothiocyanate
g: unit of gravity
GFP: green fluorescent protein
HA: hemagglutinin
HC: MHC I heavy chain
HR: homologous recombination
HSV-tk: Herpes simplex virus thymidine kinase
LIF: leukaemia inhibitory factor
LoxP: locus of crossover (x) in P1 bacteriophage
Ig: immunoglobulin
IP_3R: inositol triphosphate receptor
kb: kilobase
kD: kilodalton
mAb: monoclonal antibody
MHC: major histocompatibility complex
min: minute
mRNA: messenger RNA
N-terminal: amino-terminal
OD: optical density
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PE: Pseudomonas exotoxin A
pfu: plaque forming units
pTα: pre TCR α chain
RAG: recombinase activating gene
RNA: ribonucleic acid
rpm: revolutions per minute
RPMI Rosewell Park Memorial Hospital Medium 1640
RT room temperature
SDS sodium dodecyl sulphate
SSC saline sodium citrate
SP single positive T cells, either CD4⁺CD8⁻ or CD4⁺CD8⁺
TAE tris/acetic acid/EDTA buffer
TBE tris/borate/EDTA
TCR T cell antigen receptor
TNT Tris/sodium/Tween-20
TEMED N,N,N,N,N-tetramethylethylenediamine
Tris tris(hydroxymethyl)aminomethane
Tween-20 polyoxyethylenesorbitan monolaurate
v volt
Chapter 1

General Introduction

Newly synthesised proteins in the endoplasmic reticulum (ER) are destined to travel along the early secretory pathway to the Golgi apparatus and subsequently to their final destination. To achieve this task the proteins have to be well prepared. Cotranslationally inserted polypeptide chains accumulate in the lumen and at the membranes of the ER. Some of them are folding intermediates or mature unassembled subunits of a protein complex and some are aggregates or misfolded proteins. A highly sophisticated machinery ensures that only correctly folded proteins leave the ER, whereas incorrectly folded proteins are retained within the ER for subsequent degradation. This surveillance is performed by molecular chaperones and known as the “quality control” mechanism.

Correctly folded secretory proteins are segregated from ER-resident proteins and have to be packaged into transport vesicles. This segregation may be mediated by specialised cargo receptors, which select proteins from the ER and escort them to the Golgi apparatus. These cargo receptors would probably be recycled within the early secretory pathway. Distinct subsets of transport vesicle mediate protein transport between the membranes of the early secretory pathway. Transport vesicles are fitted with different receptors to ensure delivery of proteins to the proper organelles.

1.1. Calnexin – a molecular chaperone of the endoplasmic reticulum

1.1.1. Independent identification of calnexin

In the early 90s three different research groups independently identified the same new protein. Degen and Williams found an 88kD protein, called p88, which was localised in the ER and transiently bound to newly synthesised heavy
chains of the major histocompatibility complex class I (MHC I) antigen. In murine cells binding of β₂-microglobulin to the heavy chain did not trigger release of the dimer from p88, although the binding resulted in conformational changes in the heavy chain (Degen and Williams, 1991). In parallel, Hochstenbach described an intracellular protein of 90kD (IP90) that associated with immature subunits of the T cell receptor (TCR), the MHC I complex and membrane-bound immunoglobulins but not with the fully assembled complexes (Hochstenbach et al., 1992). This led to the hypothesis that this new protein may be involved in folding and correct assembly of these molecules. At the same time Wada and co-workers isolated a 90kD phosphoprotein (pp90) from rough pancreatic microsomes. This protein was purified together with 3 other proteins (gp25L, gp25H and pgp35). Sequence comparison with known proteins revealed that pp90 showed high homology to the calcium-binding protein calreticulin (Michalak et al., 1992) and, therefore, was called calnexin (Wada et al., 1991). It became clear that p88, IP90 and calnexin were in fact the same protein (Ahluwalia et al., 1992; David et al., 1993; Galvin et al., 1992).

Shortly after discovering calnexin in mouse (Degen and Williams, 1991; Hochstenbach et al., 1992) and dog (Wada et al., 1991) calnexin homologues were found in human (David et al., 1993; Schreiber et al., 1994), rat (Tjoelker et al., 1994), *Xenopus leavis* (Treves et al., 1992), worms (Hawn et al., 1993), plants (Huang et al., 1993) and even in the yeast strains *S. pombe* and (Jannatipour and Rokeach, 1995) *S.cerevisiae* (de Virgilio et al., 1993). The overall organisation of the calnexins from the different species is quite similar. Mammalian calnexin is a non-glycosylated type I transmembrane protein. It consists of 573 amino acids (AA) the predicted size of which would be 65 kD, however on a SDS polyacrylamid gel calnexin runs at around 90kD. As shown in figure 1.1. calnexin consists of a short signal sequence, a large luminal region of 461 AA, a transmembrane segment (22 AA) and a 90 AA cytoplasmic domain. The luminal region contains a calcium binding site and a proline-rich region, which can be subdivided into two different motifs: motif 1 (I-DPD/EA-KPEDWDD/E) and motif 2 (G-W--P-I-NP-Y) both of which exist as tetra-repeats (Michalak et al., 1992; Tjoelker et al., 1994; Vassilakos et al., 1998). The interaction with glycoproteins occurs at the proline-rich sequence
repeats and depends on the presence of calcium (Vassilakos et al., 1998). The cytoplasmic domain of calnexin is phosphorylated by at least two different kinases. Casein kinase II phosphorylates Ser534 and Ser544 whereas Ser563 becomes phosphorylated by protein kinase C (Wong et al., 1998). Calnexin carries a RKPRRE sequence at its C-terminus which has been shown to be involved in protein ER retrieval (Jackson et al., 1990). This motif is responsible for the location of calnexin to the ER because truncated calnexin missing this cytoplasmic domain redistributed to the cell surface (Rajagopalan et al., 1994).

**Figure 1.1. Schematic representation of calnexin**

Schematic representation of the topology of mammalian calnexin. The areas indicated by A, B, C and D show region of homology to the luminal ER protein calreticulin. These areas show 49, 54, 78 and 42% identity, respectively, to murine calreticulin.

### 1.1.2. Molecular chaperones - the guides for protein folding in the ER

Proteins destined for various locations along the secretory pathway are translocated into the ER, where they are folded and assembled to acquire their final quarternary structure. The ER "provides" a range of molecular chaperones,
which support the correct folding of newly synthesised polypeptide chains. It is thought that the chaperones bind to new proteins thereby preventing aggregation and maintaining the immature folding intermediates in a conformation which allows their correct folding and assembly to mature proteins (reviewed Gething and Sambrook, 1992).

Such molecules include BiP (for binding protein) and the glucose-regulated protein 94 (GRP94), which are members of the heat shock protein (Hsp) 70 and Hsp90 family, respectively. BiP was initially identified in association with immunoglobulin (Ig) heavy chains (Haas and Wabl, 1983) but has since been shown to associate with numerous other proteins. GRP94 has been shown to interact with unassembled Ig, MHC II and viral proteins. Both chaperones are luminal ER resident proteins, however, it has been speculated that transmembrane forms may also exist (reviewed by Melnick and Argon, 1995). Binding of the folding intermediates to members of the Hsp70 and Hsp90 family is independent of ATP, however their release depends on ATP hydrolysis. The presence of ATP, therefore, drives a cycle of binding and release of immature proteins to these chaperones. If proteins achieve their correct folding they are not rebound after a transient release from the chaperone. This process is part of the “quality control” mechanism in the ER (Hurtley and Helenius, 1989). Incorrectly folded proteins are retained in the ER. Previously it was believed that incorrect folded proteins were degraded within the ER (Klausner and Sitia, 1990) but recently it has been demonstrated that proteins are transferred into the cytosol for degradation. In the cytosol, multiubiquitin chains are attached to the incorrectly folded proteins. Such proteins are targets for proteasomes which proteolytically cleave the ubiquitinated proteins (reviewed by Kopito, 1997). This mechanism has been shown for a variety of proteins such as $\alpha_1$-antitrypsin (Liu et al., 1997) and $\alpha$ chain of AChR (Keller et al., 1998).

1.1.3. Glycosylation of newly synthesised proteins

Various strategies exist to ensure correct folding of nascent proteins in the ER. One of them is N-linked glycosylation of newly synthesised polypeptide
chains. The majority of the ER localised soluble and membrane-bound proteins have oligosaccharides attached to their asparagine residues. 14-residue oligosaccharide chains (Glu<sub>3</sub>Man<sub>9</sub>GluNAc<sub>2</sub>) are attached to the polypeptide chains at the asparagine residue of an Asn-X-Ser/Thr consensus sequence by oligosaccharyl transferase. New proteins entering the ER are immediately glycosylated as the oligosaccharyl transferase is part of the translocation complex. Shortly after attaching the oligosaccharide chain elimination of the core glucose residues starts. The most distal glucose is removed by the glucosidase I, followed by removal of the two innermost glucose residues by glucosidase II. Glucosidase I is 92kD type II transmembrane protein, which does not share any homology with glucosidase II. Glucosidase II is a heterodimeric enzyme consisting of an α and a β subunit, which are localised in the lumen of the ER (Helenius, 1994; Helenius et al., 1997).

**Figure 1.2. De-and reglucosylation of the core oligosaccharide**

![Diagram of de-and reglucosylation](image)

Structure of N-linked core oligosaccharides and their processing/trimming in the ER. Glucosidase I removes the most distal glucose residue, while glucosidase II trims the two remaining glucose residues. Immature non-glycosylated proteins are reglucosylated by the glucosyltransferase. Monoglucosylated immature proteins interact with the molecular chaperones, calnexin and calreticulin, and dissociates from these proteins after removal of the last glucose residue.

The first two glucose residues are removed co-translationally, while the monoglucosylated forms usually exist up to 20 minutes. One reason why the monoglucosylated form appears to be more abundant is that the non-glycosylated mannose-rich side chains may become reglucosylated by UDP-Glc:glycoprotein glycosyltransferase (UGGT). The 170kD UGGT is a soluble protein, its C-terminus...
displays homology to other glycosyltransferases, suggesting that it contains the catalytic domain. UGGT senses the folding status of the proteins and only proteins which are incompletely folded or misfolded are reglucosylated while proteins which have achieved their native conformation are no longer substrates for UGGT (Helenius, 1994; Helenius et al., 1997).

1.1.4. Calnexin interacts with monoglucosylated proteins

On numerous occasions it has been reported that the interaction between newly synthesised proteins and calnexin depends on the glucose residues on these proteins. The cycles of re-and deglucosylation of the folding intermediates by UGGT correlate with association and release from calnexin and calreticulin.

Calnexin selectively binds to Glu$_1$Man$_9$GluNAc$_2$ oligosaccharide in a pool of Glu$_3$Man$_9$GluNAc$_2$ oligosaccharides and also preferentially selects Glu$_1$Man$_9$GluNAc$_2$ from a mixture containing Glu$_1$Man$_{5,7}$GluNAc$_2$ oligosaccharides (Spiro et al., 1996; Vassilakos et al., 1998; Ware et al., 1995). Identical substrate specificity has been shown for calreticulin, which also selectively binds to monoglucosylated proteins. The association between calnexin and newly synthesised proteins is transient and the half-life of the association varies between different proteins from 5 to 35 minutes (Li et al., 1996; Ou et al., 1993). The glycosylation-dependent association with calnexin has been shown for a large number of proteins as shown in table 1.1..

The requirement to be monoglucosylated for the folding intermediates to interact with calnexin has been confirmed in studies where the glycosylation machinery was inactivated. Glucosidases I and II are sensitive to α-glucosidase-inhibitors, such as 1-deoxynojirimycin and castanospermine. Treatment with these glucosidase inhibitors prevents removal or trimming of the glucose residues and, thereby, reduces the interaction between calnexin and the untrimmed Glu$_9$Man$_9$GluNAc$_2$ glucosylated folding intermediates (Balow et al., 1995; Hammond et al., 1994; Kearse et al., 1994). This is complemented by the finding that in a glucosidase I and II deficient cell line, in which the core glycans are not trimmed virtually no proteins were found to be associated with calnexin (Ora and
Helenius, 1995). Also addition of tunicamycin, which inhibits glucosylation, similarly reduces the interaction of folding intermediates with calnexin. The immature proteins are no longer glucosylated and their association with calnexin is greatly reduced (Hammond and Helenius, 1995; Ou et al., 1993). The inability to bind to calnexin resulted in rapid degradation of some of the immature proteins, while the stability of other nascent proteins was less affected (Kearse et al., 1994).

Figure 1.3. Interaction of monoglucosylated proteins with calnexin

Newly synthesised proteins are glycosylated while translated into the lumen of the ER. After trimming of the two outermost glucose residues the monoglucosylated folding intermediates interact with calnexin. Removing the remaining glucose residues initiates the release of the glycoproteins from calnexin.

It is widely accepted that, in general, removal of the remaining glucose residue initiates the dissociation from calnexin. Inhibition of glucosidase II prevents deglucosylation of proteins and their release from calnexin (Hebert et al., 1995; Hebert et al., 1996). This is in agreement with the data supporting the general idea that monoglucosylated folding intermediates interact with calnexin and upon deglucosylation dissociate from calnexin. The immature proteins are recognised by the UGGT, become reglucosylated and now reassociate with calnexin. The glycoproteins exit this cycle as soon as they have achieved their native conformations and no longer serve as targets for UGGT (Helenius et al., 1997).
However, in contradiction to this theory, evidence exists that the monoglucosylation of the folding intermediates is required only for initial association with calnexin but not for maintaining the interaction because proteins remained associated with calnexin after the last glucose residue was removed (Ware et al., 1995; Zhang et al., 1995). This would imply that the glycosylation is necessary to attach proteins to calnexin, but protein-protein interactions sustain the association of the unglucosylated protein to calnexin (Ware et al., 1995; Zhang et al., 1995). Additional conflicting data was obtained from an experiment investigating the dissociation of proteins from calnexin and calreticulin which revealed differences between the two chaperones. Glucosidase inhibition and therefore trimming of the glycoproteins after association to calnexin and calreticulin stabilised the protein-calreticulin interaction and prevented the release of glycosylated proteins from calreticulin. This effect was not observed for protein-calnexin interactions because immature monoglucosylated proteins were still able to dissociate from calnexin (van Leeuwen and Kearse, 1996a).

Table 1.1. Glycoproteins that interact with calnexin

<table>
<thead>
<tr>
<th>Ligand:</th>
<th>Reference:</th>
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</thead>
<tbody>
<tr>
<td>α₁-antitrypsin</td>
<td>(Le et al., 1994; Ou et al., 1993)</td>
</tr>
<tr>
<td>α₁-chymotrypsin</td>
<td>(Ou et al., 1993)</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>(Ou et al., 1993)</td>
</tr>
<tr>
<td>B cell receptor</td>
<td>(Hochstenbach, 1992)</td>
</tr>
<tr>
<td>CFTR</td>
<td>(Pind et al., 1994)</td>
</tr>
<tr>
<td>Coagulation factor VIII</td>
<td>(Kaufman et al., 1997)</td>
</tr>
<tr>
<td>Complement 3</td>
<td>(Ou et al., 1993)</td>
</tr>
<tr>
<td>Cytomegalovirus glycoprotein B</td>
<td>(Yamashita et al., 1996)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>(Roy et al., 1996)</td>
</tr>
<tr>
<td>GABAₐ receptor</td>
<td>(Connolly et al., 1996)</td>
</tr>
<tr>
<td>Glut-1 glucose transporter</td>
<td>(Oliver et al., 1996)</td>
</tr>
<tr>
<td>gp80 (MDCK glycoprotein)</td>
<td>(Wada et al., 1995)</td>
</tr>
</tbody>
</table>
hemagglutinin (HA) (Hammond et al., 1994; Hebert et al., 1995)
Hepatitis C virus E1/E2 (Dubuisson et al., 1996)
HIV gp160 (Otteken and Moss, 1996)
Inositol triphosphate receptor (IP$_3$R) (Joseph et al., 1999)
Insulin receptor (Bass et al., 1998)
Integrins (Lenter and Vestweber, 1994; Rigot et al., 1999)
Immunoglobulins Hochstenbach et al., 1992; Lassoued et al., 1996
Newcastle disease virus HN protein (McGinnes and Morrison, 1998)
MHC I (Degen and Williams, 1991; Jackson et al., 1994)
MHC II (Schreiber et al., 1994)
Nicotinic acetylcholine receptor (Gelman et al., 1995; Keller et al., 1996)
P-glycoprotein (Loo and Clarke, 1994)
Retinol binding protein (Bellovino et al., 1996)
Serotonin transporter (SERT) (Tate et al., 1999)
T cell receptor (Kearse et al., 1994; Rajagopalan et al., 1994)
Thyroglobulin (Kim and Arvan, 1995)
Transferrin Ou et al., 1993; Wada et al., 1995)
Tyrosinase (Toyofuku et al., 1999)
VSV-G (Hammond and Helenius, 1994; Peterson et al., 1995)

1.1.5. Calnexin and its lectin-like behaviour

As summarised in 1.1.4. calnexin association with newly synthesised proteins occurs in a monoglucosylation-dependent manner. Calnexin does not bind non-glycosylated proteins like albumin but all proteins binding to calnexin
also interact with the lectin concavalinA (Ou et al., 1993). These studies led to the hypothesis that calnexin (and calreticulin) themselves are lectins. As calnexin binds to monoglycosylated proteins it was always suspected that this interaction occurred with the glucose residues rather than with the protein backbone of the molecules. This hypothesis was further tested in two independent approaches where binding of ribonuclease B (RNase B) to calnexin was examined. Glucose residues were attached to correctly and completely folded and immature RNase B. Regardless of the confirmation of RNase B calnexin and calreticulin only bound to the monoglycosylated protein but not to the unglycosylated form. In addition non-glucosylated native or immature folding intermediates were neither bound by calnexin or by calreticulin (Rodan et al., 1996; Zapun et al., 1997). These studies imply that calnexin and calreticulin unlike other molecular chaperones select their substrates according to their glycosylation status and do not show any specificity for the conformation of the proteins (Rodan et al., 1996; Zapun et al., 1997).

However, several lines of evidence contradict the conclusion that calnexin and calreticulin are lectins and interact with proteins exclusively via their N-linked trimmed oligosaccharides because several proteins interact with calnexin independently of glycosylation. The CD3ε subunit clearly interacts with calnexin however, it is not N-glycosylated indicating that calnexin must recognise features of proteins other than glucose residues (Rajagopalan et al., 1994). More proteins, which also interact with calnexin in a glycosylation independent manner, have been identified. The human P-glycoprotein was still found associated to calnexin even after removing all glycosylation sites (Loo and Clarke, 1994). In addition large aggregates of non-glycosylated Vesicular Stomatitis Virus G protein (VSV-G) interacted with calnexin (Cannon et al., 1996). Surprisingly, the VSV-G proteins were even preferentially bound by calnexin when two glucose residues were present in contrast to the monoglycosylated form, which would have been predicted (Cannon et al., 1996). Furthermore the transmembrane domains of the folding intermediates have also been shown to be required for the association with calnexin. Mutating the transmembrane domain of a MHC I heavy chain abolished the association with calnexin (Margolese et al., 1993) and fusion of the MHC I heavy chain transmembrane domain to a non-calnexin-interacting protein
resulted in subsequent association of the chimeric protein to calnexin (Margolese et al., 1993). In a different experiment with subunits of MHC II it has even been reported that neither the N-linked glycans nor the transmembrane part are exclusively required for calnexin association, because neither truncation of the transmembrane domain nor inactivation of glycosylation abolished the interaction with calnexin (Arunachalam and Cresswell, 1995).

In summary many of the above mentioned data suggest that calnexin interacts with monoglucosylated newly synthesised proteins which are released from calnexin after removal of the last glucose residue. However there seem to be many exceptions to the rule and different proteins clearly exhibit different characteristic behaviour in their association with and dissociation from calnexin.

1.1.6. Calnexin as a molecular chaperone

Calnexin and calreticulin have been proposed to function as molecular chaperones because, as detailed in the previous section, they specifically interact with immature folding intermediates or with misfolded proteins but not with native proteins which have reached their final functional conformation (Degen and Williams, 1991; Hammond and Helenius, 1994; Hochstenbach et al., 1992).

The chaperone capacity of calnexin to retain incorrectly folded proteins in the ER has been reported for many different proteins. For example, misfolded forms of thyroglobulin, VSV-G protein, tyrosinase and α1-antitrypsin exhibit a prolonged association with the chaperone molecule or remain associated with calnexin until they become targeted for degradation (Hammond and Helenius, 1994; Kim and Arvan, 1995; Le et al., 1994; Ou et al., 1993; Toyofuku et al., 1999). This is consistent with the idea that only proteins, which have achieved their native conformation, are allowed to leave the ER. However, calnexin is not able to distinguish between functional and non-functional proteins, since misfolded but still functional proteins do not dissociate from calnexin (Pind et al., 1994).

In addition calnexin also acts as an indirect chaperone molecule. Thus calnexin interacts with subunits which are a part of a larger protein complex and prevents them from degradation and by doing so it enlarges the pool of available
folding intermediates which may be fully assembled to obtain their quaternary structure. This concept is supported by the fact that calnexin interacts with immature subunits of the T cell receptor (TCR), MHC I, and integrin complexes but not with the native functional complexes (Anderson and Cresswell, 1994; Jackson et al., 1994; Lenter and Vestweber, 1994). Furthermore, subunits of the TCR and MHC I complexes show a prolonged association with calnexin if their counterparts are missing (David et al., 1993; Jackson et al., 1994).

These studies illustrate the bona fide chaperone properties of calnexin, which participates in the correct assembly of folding intermediate and the retention of misfolded proteins within the ER.

1.1.7. The role of molecular chaperones in human diseases

The defective folding of proteins is the cause of a number of human diseases and molecular chaperones have been reported to be involved in these diseases (Thomas et al., 1995). In human patients suffering from juvenile emphysema, (proteolytic destruction of lung elastin fibres) the protein α1-antitrypsin is inefficiently secreted and incompletely folded. These patients express the naturally occurring mutated variant (null_{Hong Kong}) of α1-antitrypsin. However, the retention of the mutated protein in the ER by calnexin is not the primary cause of the disease, because the truncation of the C-terminus in the null_{Hong Kong} variant results in a misfolded non-functional protein (Le et al., 1994). The fatal disease cystic fibrosis is caused by mutation of a gene coding for a chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR). The most common deviation in the CFTR is the deletion of a phenylalanine at position 508 (ΔF508). This alteration causes structural changes but the CFTR protein is still functional. The mutated ΔF508 CFTR is retained within the ER by calnexin and not transported to the cell surface (Pind et al., 1994; Thomas et al., 1995). Although the ΔF508 CFTR is functional it does not escape the quality control mechanism and does not dissociate from calnexin.

Recently, calnexin has also been reported to be involved in viral disease. The Hepatitis B Virus contains of 3 different envelope proteins – S, L and M. The
M protein interacts with calnexin in a glycan-dependant manner, inhibition of glycosylation or incorrect trimming of the glucose residues greatly abolishes the association with calnexin and prevents assembly and secretion of subviral particles (Werr and Prange, 1998). This study was supported by the recent observation that woodchucks that were chronically infected with the Woodchuck Hepatitis Virus showed a dramatic decrease in the production of virus particles when the animals were treated with α-glucosidase inhibitors (Block et al., 1998). These studies show that inhibition of association of viral glycoproteins with calnexin could be a potential target to control and cure viral infections.

The loss of calmegin is discussed as a possible cause for otherwise unexplained fertility problems in male patients, since calmegin appears to be essential for the correct folding of proteins which mediate sperm adherence to the zona pelucida (Ikawa et al., 1997).

1.1.8. Differences between calnexin and calreticulin

In contrast to calnexin, calreticulin is a soluble ER protein carrying a KDEL ER-retrieval sequence at its C-terminus. In addition to its location to the ER, calreticulin has also been found on the cell surface, in the cytosol and in the nucleus. Calreticulin was first identified as a calcium binding protein in the sarcoplasmic reticulum of muscles, but later on it became clear that it is ubiquitously expressed. It appears that calreticulin is a multi-functional protein and is involved in chaperoning new proteins, calcium storage and signalling, cell-adhesion and gene expression (reviewed by Burns et al., 1992; Krause and Michalak, 1997; Michalak et al., 1992).

Calmexin and calreticulin are both molecular chaperones, they are localised in the ER and show lectin-like specificity for their substrates. In this respect their function and affinity is largely overlapping and, in this context, they have been found to associate simultaneously with the same glycoprotein (HA) (Hebert et al., 1997; Otteken and Moss, 1996). However, closer analysis of the two chaperones has revealed considerable differences, not only in their specificity for different
glycoproteins but also for different folding intermediates and even for distinct domains of one glycoprotein.

The VSV-G protein associates with calnexin via its two N-linked glycans but it does not interact with calreticulin (Hammond and Helenius, 1994). The coagulation factors V and VIII both interact with calreticulin but only factor VIII binds to calnexin (Pipe et al., 1998). The acetylcholine receptor (AChR) is a multimeric complex and interaction between calnexin and the AChR α chain has been observed, however, calreticulin fails to associate with the α chain (Keller et al., 1998). Another example for the different association behaviour of calnexin and calreticulin are the subunits for the TCR; while calreticulin exclusively interacts with the α and β subunits, calnexin also associates with the γ, δ and ε subunits (van Leeuwen and Kearse, 1996a).

In human cells calnexin binds to newly synthesised unassembled MHC I heavy chains. Upon assembly of β2-microglobulin the heavy chain is released from calnexin and associated with calreticulin (Sadasivan et al., 1996). The dissociation from calnexin and association with calreticulin is probably due to conformational changes in the heavy chain after assembly with the β2-microglobulin.

Calnexin and calreticulin have both been found to interact with influenza hemagglutinin (HA) (Hebert et al., 1997). Calreticulin only binds to the early folding intermediates, while calnexin association is maintained for a longer time. This difference could be explained by the different binding sites – calreticulin exclusively bind to the top/hinge domain, while calnexin preferentially binds to the membrane proximal stem domain of HA (Hebert et al., 1997). The top domain folds earlier than the stem domain, which may be reflected by the earlier dissociation of calreticulin from HA than calnexin. Given that calreticulin is a luminal protein and calnexin is anchored in the membrane, the top domain would be more accessible for calreticulin and the stem domain for calnexin (Hebert et al., 1997).
1.1.9. Calreticulin deficient cells and mice

Calreticulin-deficient mice and cells have been reported by two independent research groups. Embryonic stem (ES) cells deficient for calreticulin and mouse embryonic fibroblasts (MEFs) isolated from calreticulin knockout mice show a significantly reduced integrin-dependent cell adhesion in tissue culture (Coppolino et al., 1997). This is in agreement with previous studies which have shown that calreticulin interacts with the cytoplasmic domains of α-integrin subunits (Coppolino et al., 1995). Calreticulin has also been implicated in storage and release of intracellular Ca\(^{2+}\) (Burns et al., 1992), however calreticulin\(^{-/-}\) cells show no obvious alteration in Ca\(^{2+}\) storage and release compared to wild type cells. Ca\(^{2+}\) influx is one of the early responses to integrin-mediated cell adhesion. This influx is largely perturbed in calreticulin-deficient cells, and as a consequence cell adhesion is impaired. However, stimulating the intracellular pathway downstream of integrins resulted in the restoration the adhesion defects (Coppolino et al., 1997).

Figure 1.4. Schematic representation of calreticulin

Figure 1.4. shows a schematic representation of the murine calreticulin. Calreticulin is a soluble ER resident protein, retained within the ER by the KDEL sequence at its C-terminus. Calreticulin, like calnexin has been shown to act as a molecular chaperone.

The loss of both calreticulin alleles results in prenatal lethality with embryos dying between 12 and 18 days of gestation. Calreticulin deficient embryos suffer from a failure to absorb the umbilical hernia (omphalocele) and show severe
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misdevelopment of the heart, which is most likely the cause of death (Mesaeli et al., 1999). The use of a transgenic mouse model expressing the green fluorescent protein (GFP) driven by the calreticulin promoter revealed high expression of calreticulin in the developing heart, in contrast to adult heart where its expression is low. Employing a different Ca\(^{2+}\) releasing stimulus to that used by Coppolino and co-workers, the InsP\(_3\)-depentent Ca\(^{2+}\) release was disturbed in calreticulin deficient cells (Mesaeli et al., 1999). These studies show that the loss of calreticulin does not affect viability at the single cell level, but clearly compromises mammalian development and results in prenatal lethality.

1.1.10. Calmegin knockout mice

Calmegin was identified as a 93kD transmembrane protein, which is exclusively expressed during male germ cell meiosis. Comparison of its protein sequence reveals 58% homology to calnexin and calmegin also contains the proline rich region, with the repeated motifs. It possesses the ability to bind to calcium (Watanabe et al., 1994). Inactivation of both calmegin alleles in mice results in infertility (Ikawa et al., 1997). Sperm cells from calmegin knockout mice are unable to adhere to and penetrate the zona pelucida, although spermatogenesis is morphologically normal. This indicates that calmegin is required for the correct folding of at least one sperm surface protein which mediates the interactions between sperm and oocyte (Ikawa et al., 1997).

1.1.11. Calnexin-deficient yeast stains

Calnexin homologues have been identified in the budding yeast Saccharomyces cerevisiae (S. cerevisiae) and in the fission yeast Schizosaccharomyces pombe (S. pombe). The S. cerevisiae calnexin (CNE1) is 24% identical and 31% similar to its mammalian homologue and is located in the ER. However, unlike mammalian calnexin its luminal domain is glycosylated and does not possess Ca\(^{2+}\) binding capacities and the C-terminus ends with the transmembrane domain (Parlati et al., 1995). Inactivation of CNE1 does not interfere with the viability of yeast cells and with secretion levels of endogenous proteins. However,
transfection of mutated yeast proteins or mammalian proteins in CNE1 deficient cells resulted in an increased expression of the transfected proteins compared to wild type yeast cells. This indicates that Cne1p is involved in the machinery controlling correct protein secretion and transport in S. cerevisiae (Parlati et al., 1995).

A calnexin homologue has also been identified in S. pombe and called cnx1 (Jannatipour and Rokeach, 1995; Parlati et al., 1995). Cnx1 displays higher homology to the mammalian calnexin than to Cne1p because it contains a cytoplasmic domain and a calcium-binding site. However, it is stress inducible and glycosylated like Cne1p. Disruption of cnx1 leads to a lethal phenotype in S. pombe demonstrating that cnx1p fulfils essential functions and probably plays a key role in the protein quality control in the ER (Jannatipour and Rokeach, 1995; Parlati et al., 1995).

1.1.12. Analysis of a calnexin-deficient cell line

Howell et al. identified a sub-clone of the human T lymphoplastoid cell line CEM by co-culturing the cells with peripheral blood mononuclear cells (PBML). This sub-clone had lost its susceptibility for natural killer cell (NK) mediated lysis and was called CEM NKR for NK resistant. The molecular changes which caused the resistance initially remained unknown (Howell et al., 1985), but a decade later it was found that the CEM NKR cell line does not express calnexin (Scott and Dawson, 1995). The maturation and assembly of MHC I subunits have been extensively studied in this cell line. Although calnexin clearly associates with MHC I heavy chains no reduction in the expression levels of MHC I on the surface nor a decelerated transport rate to the cell membrane were detected (Prasad et al., 1998; Sadasivan et al., 1995; Scott and Dawson, 1995). Furthermore no significant differences in the HPLC profiles of peptides eluted from CEM and CEM NKR were obvious (Sadasivan et al., 1995). These studies suggest that calnexin may not be required for single cell viability. However, they do not allow any conclusions about the requirement for calnexin in mammalian development.
1.2. Protein transport in the early secretory pathway

Intracellular protein transport in the secretory pathway is mainly mediated by vesicles which bud off from one compartment and specifically fuse with the membranes of the target compartment. About 30 years ago a family of clathrin coated vesicles was discovered. They mediate protein transport between the trans-Golgi-network and are involved in endocytosis. In 1986 a new type of coated vesicle was identified that was covered by a characteristic “fuzzy” coat visible on electron microscopy images (Orci et al., 1986). The “fuzzy” coat consists of oligomeric protein complexes called coatomers. They contain seven different subunits named COPs for coatomer proteins. A few years later a second class of vesicles carrying a “fuzzy” coat was found. Although the two types of vesicles appear similar by electron microscopy, the coat components are completely different. To avoid confusion the two types of vesicles were called COPI and COPII coated vesicles (Barlowe et al., 1994).

1.2.1. Assembly and budding of COPI coated vesicles

The coatamer complex consists of seven subunits: \( \alpha \) (-140 kD), \( \beta \) (-107 kD), \( \beta' \) (-102 kD), \( \gamma \) (-97 kD), \( \delta \) (-57 kD), \( \epsilon \) (-35 kD) and \( \zeta \) (-20 kD) COP (Rothman, 1994). The corresponding yeast COP homologues have been identified and they share approximately 40% homology to the mammalian COPs (Gaynor et al., 1998).

The COPs are cytosolic proteins which assemble in equimolar volumes to form coatomer (Waters et al., 1991). Under non-physiological high-salt conditions the coatomers disassemble into an \( \alpha, \beta', \epsilon \)-COP trimer and \( \beta, \delta \)-COP and \( \gamma, \zeta \)-COP dimers, but the subunits will reassemble once they are returned to physiological conditions (Lowe and Kreis, 1995). The single COPI proteins assemble in the cytosol and fully formed coatomers then adhere as a complex to the membrane (Hara-Kuge et al., 1994).
Table 1.2. Coatomer subunits of mammalian and yeast cells

<table>
<thead>
<tr>
<th>Mammalian COPs</th>
<th>Yeast homologues</th>
<th>Mr (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-COP</td>
<td>Ret1p</td>
<td>~140</td>
</tr>
<tr>
<td>β-COP</td>
<td>Sec26p</td>
<td>~107</td>
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<tr>
<td>β'-COP</td>
<td>Sec27p</td>
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<tr>
<td>γ-COP</td>
<td>Sec21p</td>
<td>~97</td>
</tr>
<tr>
<td>δ-COP</td>
<td>Ret2p</td>
<td>~57</td>
</tr>
<tr>
<td>ε-COP</td>
<td>Sec28p</td>
<td>~35</td>
</tr>
<tr>
<td>ζ-COP</td>
<td>Ret3p</td>
<td>~20</td>
</tr>
<tr>
<td>ARF1</td>
<td>yARF 1, 2, 3</td>
<td>~20</td>
</tr>
</tbody>
</table>

Coatomer recruitment to the membrane occurs in a GTP-dependent manner and in the presence of an essential accessory cytosolic protein, the ADP-ribosylation factor 1 (ARF1) (Palmer et al., 1993; Serafini et al., 1991). Release of GDP from ARF and association of GTP with ARF1 is mediated by the guanidine nucleotide exchange factor (GEF). After isolating GEFs from yeast cells they were also identified in mammalian cells (Chardin et al., 1996). The exchange of GDP and GTP results in conformational changes within ARF and exposes the myristylated N-terminus which anchors ARF-GTP to the membrane (Chavrier and Goud, 1999). The coatomers adhere to ARF-GTP, β– and γ-COP mediating this interaction (Zhao et al., 1997; Zhao et al., 1999). In addition to their association with ARF the coatomers also interact with the transmembrane protein p23 for stable adherence to the membrane. The cytoplasmic domain of p23 (this protein will be extensively described in Chapter 5) is necessary to recruit coatomers to the membrane. p23 interacts with γ-COP of the pre-assembled coatomer, however, after dissociation of the coatomer complex it also interacts with other COPs (Harter and Wieland, 1998). The requirement for p23 in COPI vesicle formation has also been shown in an experiment where reconstituted phospholipid vesicles with only the cytoplasmic domain of p23 budded in an ARF and GTP dependent fashion (Bremser et al., 1999). Recently it has been shown that binding of coatomer to p23 triggers conformational changes within the coatomer. The coatomers
polymerise at the Golgi membranes and by doing so they cause the formation of a coated bud (Reinhard et al., 1999). The fission of the donor membrane is triggered by palmitoyl-CoA which initiates the release of COPI vesicles (Ostermann et al., 1993).

**Figure 1.5. Schematic representation of COPI vesicle formation**

A model for the formation of COPI coated vesicles. In a first step GTP-activated ARF1 inserts into the membrane. The coatomers are then recruited to the membrane γ-COPI attaches to the cytoplasmic domain of p24 family members and β-COPI and γ-COPI bind to ARF. The interaction between the p24 family members and the coatomers initiates polymerisation of the coatomers, which causes the formation a bud. The coated buds form into vesicles that pinch off the membrane.

The fusion of the COPI vesicle with the acceptor membrane requires shedding of the coat, as only uncoated vesicles are able to fuse with the
membranes of the target organelle. The disassembly of the COPI coat is initiated by hydrolysing the ARF1-bound GTP, which causes the dissociation of ARF1 from the COPI coat and results in detachment of coatomers from the membrane (Tanigawa et al., 1993). The GTPase-activating protein (GAP) that is specific for ARF1 stimulates GTP hydrolysis (Cukierman et al., 1995). In a recent work the crystal structure of ARF1 has revealed an unexpected location for ARF1-GAP binding site because, unlike most GTP-binding proteins, the GAP binding site in ARF1 does not cover the effector site (Goldberg, 1999). Thus, ARF1 is still able to interact with coatomer at the effector site, even in a GTP-bound state. Indeed, the tripartite complex consisting of ARF1, GAP and coatomer has been shown to catalyse GTP hydrolysis 1000-fold more efficiently than an ARF1-GAP dimer without coatomer (Goldberg, 1999).

In summary, assembly of the coatomers at the donor membrane is an ARF and GTP-dependent process. The coatomers are attached to the membrane via ARF and p23. Once the vesicle has budded, the COPI coat has to be dismantled to allow fusion with acceptor membrane. Hydrolysis of the ARF1-bound GTP preceeds disassembly of the coat.

1.2.2. Formation of COPII coated vesicles

Table 1.3. Coat proteins of COPII coated vesicles

<table>
<thead>
<tr>
<th>Yeast coat proteins</th>
<th>mammalian homologues</th>
<th>$M_r$ (kD)</th>
</tr>
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<tbody>
<tr>
<td>Sec13p</td>
<td>hSec13p</td>
<td>~34</td>
</tr>
<tr>
<td>Sec31p</td>
<td>hSec31p</td>
<td>~150</td>
</tr>
<tr>
<td>Sec23p</td>
<td>hSec23p</td>
<td>~85</td>
</tr>
<tr>
<td>Sec24p</td>
<td>hSec24p</td>
<td>~105</td>
</tr>
<tr>
<td>Sar1p</td>
<td>hSar1p</td>
<td>~21</td>
</tr>
</tbody>
</table>

The coat proteins of the COPII coated vesicles were first identified in yeast. A study analysing several temperature-sensitive yeast mutants, which showed
secretion abnormalities (*sec* mutants) allowed the classification of the mutant yeast strains into two different groups (Kaiser and Schekman, 1990). The class I mutants (*sec12, sec13, sec16 and sec23*) displayed deficiencies in the formation and budding of vesicles, while the class II mutants (*sec17, sec18 and sec22*) accumulated vesicles in the cytoplasm indicating a defect of these mutants in vesicle fusion (Kaiser and Schekman, 1990). Subsequently on it was demonstrated *in vitro* that in the presence of GTP the so-called Sec13p-, Sec23p complex and the protein Sar1p were the necessary and sufficient components for vesicle budding from membranes of the ER (Salama *et al.*, 1993). Sec13p complex, Sec23p complex and Sar1p were then identified as the coat proteins of isolated COPII coated vesicles (Barlowe *et al.*, 1994).

Sar1p is a small GTP binding protein (Nakano and Muramatsu, 1989) sharing a high amino acid sequence identity with its functional homologue ARF1, which itself is required for COPI vesicle formation. The 700kD Sec13p complex can be subdivided into 34kD Sec13p and 150kD Sec31p subunits. Both subunits contain WD-40 motifs that are implicated in protein-protein interactions. The 400kD Sec23p complex consists of 85kD Sec23p subunits that show Sar1p-specific GTPase activation and 105kD Sec24p subunits (Barlowe, 1998).

The formation of COPII coated vesicles starts with the recruitment of Sar1p to the membrane. There it interacts specifically with the guanine nucleotide exchange factor Sec12p which mediates both release of GDP from Sar1p and binding of GTP (Barlowe and Schekman, 1993). Upon activation, Sar1p attaches the Sec23p complex to the membrane, followed by binding of the Sec13p complex. Similarly to vesicle formation of COPI vesicles, GTP hydrolysis is not necessary for detachment of the vesicles from the donor membrane. However, GTP hydrolysis is essential for dissociation of the coat proteins from the vesicle, which is a prerequisite for fusion with the acceptor membrane (Barlowe, 1998; Barlowe *et al.*, 1994).
Model for assembly of COPII coated vesicles. Sec12 activates Sar1p at a transitional zone. A trimeric complex consisting of Sar1p, the Sec23 complex and cargo proteins is formed at the membrane. The Sec13 complex is then recruited to the membrane to drive vesicle budding.

1.2.3. Trafficking of COPI and COPII coated vesicles in the early secretory pathway

Membrane trafficking in the early secretory pathway is a well-balanced process. Anterograde transport from the ER to the ER-Golgi-intermediate compartment and to the Golgi depletes the ER of its membrane and essential accessory proteins that are required for protein transport. These components are
mainly reused and retrieved to the ER, from where they re-enter the anterograde pathway and thus constantly cycle between ER and Golgi. It has been discussed that interruption of either anterograde or retrograde transport would also lead to severe perturbation of the other transport branch and may probably result in a complete inhibition of the early secretory pathway. The membrane and protein transport between ER and Golgi is mediated by COPI and COPII coated vesicles (Gaynor et al., 1998; Lowe and Kreis, 1998).

1.2.3a. Anterograde transport

In yeast and mammalian cells, COPII coated vesicles bud exclusively from the membranes of the ER and mediate most, if not all, vesicle transport from the ER. The budding occurs at specialised exit sites at the ER, which are called transitional elements (TE). Inactivation of COPII proteins results in lethal phenotypes in yeast, which underlines their essential function in protein transport. The COPII proteins were first identified in yeast but it has been shown that they are functionally conserved between yeast and mammalian cells where they are also involved in ER to Golgi transport in mammals (Kaiser and Ferro-Novick, 1998).

The role of COPI in anterograde transport directly from the ER is less well established than that of COPII. Although it has been shown that COPI vesicles are able to bud off directly from the ER (Bednarek et al., 1995) and microinjection of antibody against β-COP inhibits protein exit from the ER (Pepperkok et al., 1993) it is unlikely that COPI is involved in protein exit from the ER to a large extent. Studies in yeast revealed that in strains with a mutation of sec21 (γ-COP), only a few proteins (CPY and α-factor) depend on COPI to exit the ER. Although, this effect could also be caused by an impaired retrieval of the cargo receptor for these proteins this would indicate that COPI is only indirectly involved in protein exit from the ER (Gaynor and Emr, 1997).

The current, widely accepted view is that COPI and COPII mediate "hand in hand" the membrane transport from the ER to the Golgi. COPII coated vesicles bud off the TEs, release the coat proteins and fuse with the vesicular clusters of
the intermediate compartment. At the intermediate compartment coatomers attach to the membranes and COPI coated vesicles then transport the proteins to the cis-site of the Golgi. In recent years much evidence has been provided which supports this hypothesis (Aridor et al., 1995; Griffiths et al., 1995; Presley et al., 1997). Recently, a study in which the transport from the ER to the Golgi of a chimeric protein of the VSV-G and the green fluorescent protein has been visualised provided convincing evidence for the coat exchange at the intermediate compartment (Scales et al., 1997). The reason for the early exchange of COPII to COPI could be that this is required for efficient retrieval of the ER resident proteins, which have escorted secretory proteins out of the ER.

1.2.3b. Retrograde transport

About ten years ago it was shown that transmembrane proteins which carry a KKXX-motif at their C-terminus are efficiently transported back from the Golgi to the ER. However, the mechanism responsible for retrograde transport remained obscure (Jackson et al., 1990). The first evidence that COPI vesicles may be involved in retrograde transport was provided when coatomer subunits were shown to bind to the cytoplasmic KKXX motif (Cosson and Letourneur, 1994). These findings were confirmed by studies in yeast which showed that mutations in the genes coding for COPII subunits had no influence on retrograde transport, while mutation of COPI subunits resulted in a defect in protein retrieval (Letourneur et al., 1994). A protein called Emp47p is found at steady state levels at the Golgi. However, it contains an ER-retrieval signal at its cytoplasmic domain, which allows Emp47p to exit the Golgi and cycle between the ER and the Golgi. In a temperature-sensitive sec12 mutant the cycling is blocked upon shifting to the restrictive temperature and Emp47p is mainly localised to the ER (Lewis and Pelham, 1996). In this mutant yeast strain where anterograde transport is inhibited, accumulation of Emp47p in the ER allows focusing on the retrograde transport. Additional mutation of sec21 in this yeast strain resulted in a reduction of Emp47p retrieval to the ER, suggesting that sec21 is required for retrograde transport of at least Emp47p (Lewis and Pelham, 1996).
Schematic representation of vesicle mediated protein transport in the early secretory pathway. COPII coated vesicles form exclusively at the transitional elements at the ER and fuse with membranes of the intermediate compartment. COPI coated vesicles are involved in anterograde and retrograde transport. They bud from the intermediate compartment and transport proteins to the Golgi and back to the ER. In addition COPI coated vesicles bud and fuse at every cisterna of the Golgi and thus mediate intra-Golgi transport.

Initially it was believed that COPI is mainly involved in retrograde transport, playing only a minor or no role in anterograde transport. However, as mentioned above, COPI vesicles also mediate anterograde protein transport. The
participation in anterograde and retrograde transport is in line with the finding that coatamer subunits bind a double phenylalanine (FF) motif in addition to the KKXX motif (Fiedler et al., 1996). This FF motif is present in members of the p24 protein family and has been shown to be necessary for anterograde protein transport (Dominguez et al., 1998; Sohn et al., 1996). Different COPI subunits exhibit different affinities for the FF and the KKXX motif – α-, β′- and ε- COP interact with the KKXX motif, while β-, γ- and ζ-COP associate with the FF motif (Fiedler et al., 1996). Preferential interaction with the different motif may be crucial for segregation of cargo that has to be secreted and cargo that has to be retrieved to the ER. This issue will be presented later in this chapter.

1.2.3c. Intra-Golgi transport

Initially it was believed that protein transport through the Golgi apparatus was achieved by “cisternal progression”. According to this model vesicles derived from the ER would fuse at the cis-side of the Golgi and form a new cisterna, while at the trans-side of the Golgi the most distal cisterna would disintegrate into vesicles which would then be transport to the cell surface (Glick and Malhotra, 1998). However, there are three major problems with this model. It would not explain why vesicles were found at the rims of the Golgi cisternae and why cargo proteins travel though the Golgi within minutes, while Golgi resident proteins may take hours for their transport from the cis- to the trans-Golgi. In addition many Golgi proteins are polarised, i.e. some proteins are found at the cis-side, while other are localised to the trans-side of the Golgi (Glick and Malhotra, 1998).

Orci and co-workers have isolated vesicles, which were claimed to be strong candidates for protein transport between Golgi cisternae (Orci et al., 1989). This finding was supported by their identification as COPI coated vesicles and the model of the “stable compartment” for the Golgi apparatus was proposed (Rothman, 1994). Recently it has been shown that COPI coated vesicles mediate anterograde and retrograde protein transport between single Golgi cisternae because COPI vesicles either carrying secretory proteins or containing cargo destined for the ER bud off from every cisterna of the Golgi (Orci et al., 1997). The
vesicles move in close proximity to the Golgi and probably only fuse with the
neighbouring cisternae. It is believed that a fibrous network tethers the vesicles to
the Golgi cisternae (Orci et al., 1998; Sonnichsen et al., 1998).

However, recently it was reported that procollagen fibres are transported
via cisternal progression through the Golgi because the fibres remain in the
cisternae during the transport through the Golgi (Bonfanti et al., 1998). Currently
both models have pros and cons and further work will determine which model
predominates, or whether different proteins choose different ways to travel
through the Golgi apparatus.

1.2.4. SNARE proteins direct the way

Membrane trafficking has to be controlled by a “key-keyhole” mechanism
to ensure that transport vesicles fuse with the membranes of the correct target
organelle and not with random membranes. According to the SNARE hypothesis
a complex of N-ethylmaleimide sensitive factor (NSF), soluble NSF attachment
proteins (SNAPs) and intra-membrane SNAP receptors (SNAREs) allows specific
attachment and fusion between vesicles and the target membranes (Sollner et al.,
1993b). It is widely accepted that the v-SNARE on the vesicle and the t-SNARE on
the target membrane interact and that NSF is required to separate the v- and t-
SNAREs again. However controversial opinions exist about the timing (Sollner et
al., 1993a). Initially it was believed that separating the SNAREs happened
simultaneously to membrane fusion but later it was suggested that the separation
occurs afterwards by which the membranes would be primed for the next fusion
event (Mayer et al., 1996).

A large number of site-specific SNAREs have been found predominantly in
yeast cells. Different intracellular organelles display different SNAREs. Therefore,
it is possible to direct specifically the vesicles to the target membranes (Ferro-
Novick and Jahn, 1994). The first mammalian SNAREs identified were the v-
SNARE synaptobrevin and the t-SNARE syntaxin. They pair to initiate fusion of
neurotransmitter-carrying vesicles to the nerve terminals (Sollner et al., 1993b).
Recently, it became clear that vesicles are also tethered to the target membrane by an additional subset of proteins (Waters and Pfeffer, 1999). Like the SNAREs the tethering factors are specific for distinct membranes and many interacting proteins have been identified (Waters and Pfeffer, 1999). The tethering factors have been studied in greater detail in yeast cells. However, one example in mammalian cells for tethering Golgi-derived vesicles to Golgi membranes is the interaction between p115, GM130 and giantin (Warren and Malhotra, 1998). Giantin is found on COPI coated vesicles, GM130 localises to membranes of the Golgi and p115 is a myosin-shaped factor, which by binding to both and linking giantin to GM130, tethers the vesicle to the membrane (Sonnichsen et al., 1998).

1.2.5. Cargo segregation and packaging

After leaving the ER, soluble and membrane proteins are transported to the Golgi apparatus. It has been the subject of extensive research how exit from the ER is mediated and how cells ensure that secretory proteins are secreted, while proteins which are required for membrane and protein trafficking are retained. In addition to segregating cargo proteins from the machinery which is involved in their transport, the cargo proteins themselves have to be sorted depending on their intended destination.

Two models have been put forward to explain how sorting is accomplished. The bulk flow model proposes that there are no signals on the proteins which selectively direct them to the cell surface. According to this model cargo moves exclusively by default (Wieland et al., 1987). Only proteins which have to be retained in the ER possess signals which either prevent their exit from the ER or lead to retrieval of proteins back from the Golgi. This model would also explain why very small proteins (in fact too small to encode a signal sequence) and bacterial proteins are usually efficiently secreted in eukaryotic cells (Warren and Mellman, 1999).

The receptor-mediated model proposes that all cargo proteins require a signal sequence to be efficiently sorted into COPII coated vesicles and to enter the exocytotic pathway (Kuehn and Schekman, 1997). Cargo proteins accumulate at
the ER exit sites and become packaged selectively into COPII vesicles (Balch et al., 1994; Kuehn et al., 1998).

Recently Warren and Mellman have reported that both models might be true and that acceptance of one model does not necessarily prove the other model wrong. It appears that highly abundant proteins do not require any receptors and become transported by default, while less abundant proteins rely on receptor-mediated transport to be efficiently transferred to their final destination (Warren and Mellman, 1999). This conclusion is based on evidence that for abundant secretory proteins like amylase the same concentration is measured in COPII coated vesicles as in the ER. Conversely, α-factor associates with transmembrane proteins of COPII coated vesicles suggesting that this protein is selectively sorted into COPII vesicles (Kuehn et al., 1998). The latter observation is in line with the discovery of the p24 protein family, whose members have been implicated to function as cargo receptors (Schimmoller et al., 1995). In addition, a di-acidic (DXE) motif has been found on transmembrane proteins as a potential ER exit motif (Nishimura and Balch, 1997). However, although mutation of this motif reduces ER exit significantly it does not abolish it completely (Nishimura et al., 1999). This indicates that should the receptor-mediated export fail proteins are still (possibly less efficiently) transported as described by the bulk flow model.

1.2.6. Selective cargo packaging into COPI and COPII coated vesicles

Several transmembrane cargo proteins have already been shown to interact directly with the coat proteins, for example proteins carrying a cytoplasmic KKXX motif (Cosson et al., 1996). For others it is highly likely that they are able to bind coat proteins, for instance it has been suggested that VSV-G is able to interact with COPII coat proteins (Aridor et al., 1998). In contrast to transmembrane cargo proteins soluble cargo proteins are not able to interact with the coat proteins. They need adapter molecules, which mediate interaction between soluble cargo and the cytosolic coat proteins. These adapters or cargo receptors have to fulfil at least two prerequisites: they have to possess a luminal domain to interact with the cargo and a cytoplasmic domain to associate with the coat proteins. Three
different proteins or protein families have been implicated as cargo receptors, the KDEL receptor, ERGIC-53 and the p24 family.

Resident luminal ER proteins carry a KDEL motif at their C-terminus that allows their retrieval to the ER. The KDEL receptor selectively promotes retrograde transport of such proteins which have escaped the ER. This receptor was initially found in yeast cells but a mammalian homologue has also been identified (Lewis and Pelham, 1992). The mannose-specific lectin ERGIC-53, which contains a KKXX motif at its cytoplasmic domain cycles between the ER and the cis-Golgi and has been suggested to be involved in the exchange of the COPII and COPI coat at the intermediate compartment (Tisdale et al., 1997). In addition, ERGIC-53 has been implicated to be a specific cargo receptor for blood clotting factors V and VIII since ERGIC-53-deficient patients do not secrete these factors and thus suffer from haemophilia (Nichols et al., 1998).

p24 family members are highly abundant in the early secretory pathway and have been proposed as receptors for cargo exit from the ER. This hypothesis was originally put forward as a consequence of disruption of two different family members in the budding yeast Saccharomyces cerevisiae (Emp24p and Erv25p) which are localised at COPII coated vesicles. Inactivation led in each case to a selective retardation in the rate of ER exit of the GPI linked protein Gaslp as well as invertase, while other secretory proteins remained unaffected (Belden and Barlowe, 1996; Schimmoller et al., 1995). Mammalian homologues have been identified and called p24 and p23, respectively, (Blum et al., 1996; Schimmoller et al., 1995; Stamnes et al., 1995). The cytoplasmic domain of p23, which contains a KKXX-like ER retrieval motif, also binds to coat proteins of both COPII and COPI coated vesicles and may, therefore, not only be important for anterograde protein transport but could also play a role in retrograde transport. Indeed, in recent studies protein transport from Golgi to ER was inhibited when antibodies specific for the cytoplasmic domain of p23 were microinjected into mammalian cells (Majoul et al., 1998).

Recently an additional double-phenylalanine (FF) motif has been identified in the cytoplasmic domain of the p24 family members and it is believed that interaction between COPII coat proteins and p24 family members occurs via this
FF motif (Dominguez et al., 1998). Interestingly, different COPI proteins displayed different specificities for the KKXX motif or the FF motif. While $\alpha$-, $\beta'$- and $\varepsilon$-COPI bound to the KKXX motif, $\beta$-, $\gamma$- and $\zeta$- COPI were found to associate with the FF motif (Fiedler et al., 1996). This study came to the conclusion that the FF motif functions in anterograde and the KKXX motif in the retrograde protein transport. In addition, these motifs could provide an explanation about cargo segregation into distinct COPI vesicles, which either transport proteins forward to the membranes of the Golgi apparatus or retrieve ER resident proteins back to the ER (Fiedler et al., 1996).

Recently in vitro studies investigated the importance of GTP for cargo packaging. This study showed that cargo targeted for anterograde and cargo targeted for retrograde transport were both efficiently packaged into COPI coated vesicles in the presence of GTP (Nickel et al., 1998). However, when the vesicles were generated in the presence of the slowly hydrolysable GTPγS analogue of GTP the vesicles contained significantly less proteins, indicating that GTP hydrolysis is required for efficient cargo packaging into COPI vesicles (Nickel et al., 1998). This observation led to the speculation that several cycles of GTP hydrolysis and thus ARF attachment and detachment allow the cargo molecules to accumulate and associate with p24 members before the coatomers are recruited to the membrane and the vesicle is formed (Wieland and Harter, 1999).

1.3. Aim of the thesis

Calnexin has been identified as a molecular chaperone and as such it is involved in the correct folding of many newly synthesised proteins in the ER. The requirement of calnexin for folding and assembly for many molecules responsible for a functional immune response has been demonstrated. A calnexin-deficient mouse strain would allow gaining further insights in the physiological role of calnexin during mammalian development in general and provides an in vivo model to study the necessity of calnexin for the developing immune system. In addition, evidence from yeast suggested that calnexin might be involved in the transduction of apoptotic signals. The physiological relevance of calnexin in
apoptosis in mammalian cells can be studied in calnexin-deficient mice and may provide further insights about ER resident proteins and their function in apoptotic cell death.

This thesis also describes a genetic approach by gene targeting to provide novel insights into the role of mammalian p23, a member of the p24 family of ER and Golgi integral proteins. This strategy permits the analysis of the functions of the commonly expressed p24 proteins both in the whole animal and at a single cell level, through the analysis of primary cell lines derived from mutant mice. The inactivation of p23 in a mammalian organism is particularly interesting in comparison to available inactivation studies in yeast, where disruption of the p23 homologue only causes mild secretory defects.
Chapter 2

Materials and Methods

2.1. Buffers and Solutions:

All chemicals were of analytical grade or equivalent, and were obtained from Sigma, UK or BDH, UK. All buffers and solutions were made in deionised water and sterilised by autoclaving or filtration where appropriate.

SSC (20x stock): 3M sodium chloride
0.3M sodium citrate
adjusted to pH 7.0 with 10M sodium hydroxide

Dulbecco's A phosphate buffered saline (PBSA) pH 7.4:
137mM sodium chloride
3.3mM potassium chloride
1.7mM potassium dihydrogen orthophosphate (anhydrous)
10mM disodium hydrogen orthophosphate (anhydrous)
adjusted to pH 7.4 with HCl

2.2. Bacteriological media

The following reagents were obtained from ICRF Central Services:

Lauria - broth: 0.5% bacto-yeast extract
1% bacto-tryptone
1% sodium chloride
adjusted to pH 7.6 with 10M potassium hydroxide
Lauria-agar : L-broth
15g/1 bacto-agar.

2.3. Estimation of nucleic acid concentration:

DNA solutions were diluted in distilled water by a factor of 500 and placed in a quartz cuvette. The optical density (OD) of the solution was measured, relative to water, at a wavelength of 260nm on an LKB spectrophotometer. The nucleic acid concentration, in µg/ml, was calculated by multiplying the absorbance reading by the dilution factor and then by 50 for DNA, 40 for RNA and 33 for oligonucleotides. In order to check the purity of the sample, the OD at 280nm was also measured. A ratio \((\text{OD}_{260}/\text{OD}_{280})\) of 1.7 - 1.95 indicated a protein free sample. In some cases, low concentrations of DNA were estimated visually by running DNA on agarose gels alongside standards of known concentration.

2.4. Restriction enzyme digestion

Enzymes were obtained from Boehringer or New England Biolabs. For digestion of cloned DNA, restriction endonucleases were used at a final concentration of 1 unit/µg DNA. Digestion was performed in the appropriate 1x buffer for 1-2 hours at 37°C. For digestion of high molecular weight DNA, 10µg DNA was digested using 30-40 units of enzyme in a total volume of 50µl. Spermidine was added to a final concentration of 4mM pH7.5 and the digest was performed overnight at 37°C.

2.5. Agarose gel electrophoresis

Resolution of DNA fragments for analytical or preparative purposes was by agarose gel electrophoresis, using a Gibco - BRL 'Horizon' 11.14 tank apparatus. All DNA samples were electrophoresed in standard agarose in 1x TAE buffer. Agarose (0.5-3%, Seakem) was melted in 1x buffer, allowed to cool, and
ethidium bromide added to 0.3μg/ml. The agarose was poured into a gel casting tray with an appropriate gel comb to form loading wells.

DNA samples were loaded into wells with one sixth volume of 6x loading buffer : 30% glycerol (v/v), 0.25% bromophenol blue (w/v). 1kb DNA ladder (Boehringer) was also loaded as a DNA molecular weight marker in the range of 0.1-12kb. Gels were electrophoresed at 1-10 volts/cm. DNA was visualised by ultra violet illumination and photographs were taken using a polaroid land camera.

**TAE (50x stock):**

- 2M Tris base pH 8.0
- 1M Glacial acetic acid
- 50mM EDTA pH 8.0

### 2.6. Small scale preparation of plasmid DNA ('minipreps')

For preparations from plasmids, single ampicillin resistant colonies were picked into 5ml L-broth plus appropriate antibiotic selection and shaken overnight at 37°C. 1.5 ml of this culture was centrifuged for 3 mins at 13,000rpm. Bacterial pellets were resuspended in 100μl of Solution I, to which 200 μl of Solution II was added and incubated at 4°C for 5 mins. 150 μl Solution III was then added to the lysate which was then vortexed vigorously, incubated for additional 5 mins at 4°C and centrifuged for 10 mins at 13,000rpm. The supernatant was transferred to another tube and DNA was precipitated with 2 volumes of ice-cold absolute ethanol, pelleted and washed once in 70% ethanol, before being resuspended in deionised water.

### 2.7. Large scale preparation of plasmid DNA ('maxipreps')

For maxipreps from plasmids, 1ml of a miniprep culture was added to 400ml L-broth containing 50μg/ml ampicillin and shaken overnight. Bacteria were pelleted by centrifugation at 5,000rpm and resuspended in 40ml of Solution I, followed by 80ml of Solution II and 40ml of Solution III. The lysate was separated from cell debris by centrifuging at 2,500rpm for 10 mins and the
plasmid DNA was precipitated from the resulting supernatant by the addition of 0.8 volumes of isopropanol and spinning at 10,000rpm for 15 mins. The DNA pellet was resuspended in TE pH 8.0 to a final volume of 9ml. 10g of caesium chloride and 500μl of 10mg/ml ethidium bromide were added to the solution. DNA was isolated after spinning at 45,000 rpm in a Beckman ultracentrifuge for 24 hours. Supercoiled DNA bands were removed by aspiration and ethidium bromide extracted with several changes of water saturated butan-1-ol. The DNA was then precipitated by the addition of 2 volumes of absolute ethanol and spinning at 10,000rpm at room temperature for 15 mins. Finally, the DNA pellet was resuspended in deionised water and the concentration and purity determined by spectroscopy. Maxiprep DNA was aliquoted and stored at -20°C.

2.7.a. Solutions for plasmid preparation:

Solution I:
- 50mM glucose
- 10mM EDTA pH 8.0
- 25mM Tris-HCl pH 8.0

Solution II:
- 0.2M sodium hydroxide
- 1% SDS

Solution III:
- 3M potassium
- 5M acetate (pH 4.8)

2.8. Polymerase chain reaction (PCR)

PCR was used to amplify cloned, genomic or cDNA. Short (20-30 mers) oligonucleotide primers (ICRF) were designed to have roughly 50% G/C content. Template DNA was mixed with 0.25μM primers, dATP, dCTP, dGTP and dTTP each at 200μM, in a final concentration of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl₂, 0.01% gelatin, and 2.5 units of Taq polymerase (ICRF), in a 0.2-ml-eppendorf tube. Amplification was carried out in a GRI thermal cycler. Standard PCR conditions were as follows:
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First cycle:
- denaturation at 94°C for 4 minutes
- annealing at Tm of primers for 30sec*
- extension at 72°C for 1 minute per Kb

Subsequent 29 cycles:
- denaturation at 94°C for 20 secs
- annealing at Tm of primers for 30secs min*
- extension at 72°C for 1 minute per kb

Final extension period:
- 72°C for 5 minutes
*Where Tm = n(A + T)x 2 + n(C + G) x 4

2.9. DNA Sequencing Reactions

A modified version of the dideoxy chain termination sequencing reaction was performed using the Sequenase Version 2.0 Kit (USB). 10µg of template DNA, sequencing buffer and 2.5ng of oligonucleotide primer were heated to 65°C for 2 mins and allowed to cool for 30 mins to room temperature. The following were added to the annealed template and primer: 1μl 0.1M DTT, 1μl 7.5% NP-40, 2μl 5x labelling mix (7.5μM each of dGTP, dCTP, and dTTP), 0.5μl [35S]-dATP, and 2μl of Sequenase enzyme (T7 DNA polymerase) diluted 1 in 8 in enzyme dilution buffer (10mM Tris-Cl pH7.5, 5mM DTT, 0.5mg/ml BSA). After 5 mins at room temperature, 3.5μl of the reaction was added to 2.5μl of each termination mix (containing either A, G, C or T dideoxynucleotides and a mixture of all four dNTPs at 80µM each) in a 96 well microtitre plate and incubated at 37°C for 5 minutes. The reaction was stopped by addition of 4μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) to each termination reaction. Samples were heated to 95°C for 5 mins immediately before loading.

2.10. Acrylamide gel separation of sequencing reactions

Biorad gel apparatus was used for DNA sequencing. Glass plates were washed thoroughly with distilled water and ethanol and the back plate
siliconized with sigmacote (Sigma). The apparatus was assembled with 0.4mm spacers and an acrylamide plug formed. The denaturing polyacrylamide gel solution was composed of 6% w/v acrylamide and 0.3% w/v bis-acrylamide at a ratio of 19:1, together with 7M urea, 1xTBE (Easigel). 50mls of 6% denaturing gel was polymerised by the addition of 300μl of 10% ammonium persulphate and 40μl of TEMED (Sigma). After pouring, the flat edge of a gel comb was used to indent the top of the gel, and the gel was left for at least 1 hour to set.

The gel was pre-run for half an hour in 1x TBE before loading to allow the gel to reach the 55°C running temperature. The samples were loaded using a 48 well shark's tooth comb and the gel was run at 1600volts. Sequencing reactions were generally loaded at split times allowing a short run of 2 hours and a long run of 4 hours to maximise readable sequence. The gel apparatus was dismantled and the gel then transferred onto a double layer of 3MM paper before drying on a vacuum drier at 80°C for 1 hour. Sequence gels were exposed overnight on Fuji or Kodak XAR5 film at room temperature.

**TBE (10xstock):**  
0.89M Tris base pH7.4  
0.89M Boric acid  
10mM EDTA pH 8.0

### 2.11. Oligo - labelling of DNA probes

OLB was made by mixing solutions A, B and C in proportions 10 : 25 : 15 respectively and stored at -20°C. 20 - 50ng of DNA was denatured by boiling for 5 mins. The DNA was placed on ice for 2 mins to prevent re-annealing and the following reagents added: 10μl OLB, 1μl 10mg/ml BSA, 5μl [\(^{32}\)P]-dCTP(Amersham), and 10U of Klenow fragment of DNA polymerase I, in a total volume of 50μl. After overnight incubation at room temperature, the reaction was centrifuged at 1,500rpm for 5 mins through a Sephadex G-50 medium grade column equilibrated in 3x SSC, to remove unincorporated label. The eluate was counted in an Oncor Probecount to determine specific activity. The probe was
then boiled for 5 mins, placed on ice for a further 2 mins prior to hybridisation. Probes used were routinely of specific activity of >10^8 dpm/μg DNA.

For those probes derived from genomic DNA and known to contain repetitive elements, the above protocol for labelling probes had to be modified to allow their use with genomic Southern blotting. In these cases, after probe purification through a sephadex G50 column, the probe solution was mixed with 40μl of 20x SSC, 20μl of 1mg/ml Cot1 DNA (Gibco BRL), 20μl of 10mg/ml cut placental DNA (Sigma) to a total volume of 200μl with H₂O. After boiling the probe for 10 mins, the reaction was left to anneal at 65°C for 1 hour and then added to the blot which had been pre-hybridising in Church solution for 2-6 hours.

**Reagents used to prepare oligonucleotide labelling buffer (OLB)**

*Solution A:* 1.25M Tris-HCl pH 8.0  
0.125M magnesium chloride  
18μl 2-mercaptoethanol per ml  
5μl per ml each of 0.1M dATP, dTTP, dGTP in TE (Pharmacia)

*Solution B:* 2M HEPES pH 6.6

*Solution C:* 90 OD units/ml random hexadeoxy-ribonucleotides (Pharmacia)

**2.12. Preparation of genomic DNA**

Mouse tail biopsies or tissue were placed immediately into 750μl DNA Lysis buffer. 2.5μl Proteinase K (10mg/ml) were added and incubated overnight at 56°C. The lysate was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol to remove denatured proteins. DNA was precipitated with 750μl of isopropanol, recovered by centrifugation in an eppendorf centrifuge for 10 mins at 14,000rpm, washed once in 70% ethanol, and left to dissolve in H₂O at 56°C for one hour.
DNA Lysis Buffer: 100mM sodium chloride, adjusted to pH 8.0
with 10M NaOH
10mM Tris-HCl
0.1% SDS
1mM EDTA pH 8.0

Phenol/chloroform solution
Tris equilibrated phenol/0.1% 8-hydroxyquinoline was added to an equal volume of a solution of chloroform:isoamyl alcohol (24:1).

2.13. Southern blot analysis of genomic DNA
10μg genomic DNA was digested overnight in a total volume of 30μl together with 30 units of the appropriate restriction enzyme, after which the samples were electrophoresed at 1.5V/cm overnight on a 0.7% TAE agarose gel. The gel tank was washed out prior to use and the running buffer was freshly prepared. Digested samples were loaded onto gels using fresh loading buffer to avoid any potential plasmid contamination. The gel was photographed with a ruler beside it, next to the DNA marker lane. To improve the efficiency of transfer of large restriction fragments from the gel to the membrane, the gel was placed in depurination solution for 15 minutes. Mild acid treatment of large restriction fragments facilitates their cleavage to smaller fragments which are more readily transferred from the gel to the membrane. After depurination, the gel was rinsed briefly in dH₂O, agitated in excess denaturing solution for 30 mins and then transferred to nylon membrane (Hybond N+) in denaturation solution by capillary action overnight. The blot was rinsed in 2x SSC, dried on 3MM paper, baked in an 80°C oven for 2 hours and UV cross-linked to immobilise the DNA (Stratagene UV crosslinker).
Solutions for Southern blotting:

Depurination solution: 0.25M HCl

Denaturing solution: 1.5M sodium chloride
0.5M sodium hydroxide

Neutralising solution: 1.5M sodium chloride
0.5M Tris-HCl pH 7.0

2.14. Hybridisation of Southern blots

Hybridisation reactions took place within pyrex glass roller bottles and were constantly rotated within an oven (Hybaid) equilibrated to the appropriate temperature. The filter was pre-hybridised in 10ml of Church buffer for 2-6 hours at 65°C. The boiled, labelled probe was added to Church hybridisation buffer. Hybridisation of the probe to the filter was performed overnight at 65°C. After hybridisation the filter was washed using varying levels of stringency. For low stringency washing of blots hybridised with cross-species probes, a solution of 3x SSC/0.1%SDS was used at 55°C. After each individual wash, blots were monitored to prevent complete removal of signal. For high stringency washing of blots hybridised with isogenic probes, two washes with 2x SSC/0.1%SDS were performed at 65°C for 15 mins each, followed by a single 15 min wash with 0.1%SSC/0.1%SDS at 65°C. Blots were exposed at -70°C on Kodak XAR5 film using intensifying screens.

Church Hybridisation Solution: 0.5M NaH₂PO₄
7% SDS
1mM EDTA pH 8.0
2.15. Membrane Stripping

After hybridisation, blots were incubated with gentle agitation in 0.4M NaOH for 30 mins at 45°C, before being transferred to a solution of 0.1xSSC, 0.1%(w/v) SDS, 0.2M Tris-HCl pH7.5 for a further 15 mins. Alternatively blots were stripped with boiling 0.1% SDS buffer. Probe removal was confirmed by autoradiography.

2.16. Colony hybridisation

For screening of recombinant bacterial colonies, after transformation by ligation products, colony 'lifts' were made by resting 9cm circular nylon membrane (Hybond N+) on each bacterial plate for 2 mins. To facilitate orientation, holes were pierced through the filter with a needle and their positions marked on the plates. The membranes were then soaked with 5ml of denaturing solution for 5 mins and then on 2 changes of neutralising solution (2 mins each). The filters were baked at 80°C for 30 mins, UV-fixed (Stratagene UV crosslinker), and then treated as Southern blot filters.

2.17. Dephosphorylation of vector

Calf intestinal phosphatase (CIP) treatment was employed to reduce the possibility of vector self ligation. After restriction digestion, vector DNA was diluted to 50μg/ml and mixed with 1 unit of CIP (Boehringer) for 1 hour. Vectors with blunt end or 3' overhanging ends were incubated with CIP at 50°C, whereas 5' overhanging ends were treated with CIP at 37°C. The CIP reaction was terminated by the addition of EDTA to 5mM and further incubation for 10 mins at 75°C, after which vector DNA was gel purified and used for subsequent ligation reactions.

2.18. Purification of DNA from agarose gels

Specific DNA restriction fragments were purified from agarose gels using the Gene-Clean system (Bio101), according to the manufacturers instructions.
Restriction fragments were briefly illuminated under UV light and excised from the gel using a fresh disposable scalpel. The agarose slice containing the restriction fragment of interest was melted by the addition of 3 volumes of 6M NaI and subsequent incubation for 10 mins in a 50°C water bath. Once the agarose had melted, the solution was incubated on ice for 5 mins with the addition of 10ml of resuspended silica matrix solution, which specifically binds single and double stranded DNA. The DNA bound silica matrix was pelleted briefly in an eppendorf centrifuge, after which the supernatant was discarded. Any contaminating protein, RNA or nucleotides from previous manipulations were eliminated by sequential resuspension with 500μl of ice cold “New Wash” solution, followed by centrifugation and removal of the supernatant. The resulting pellet was subjected to a further two rounds of similar washing. Elution of DNA was performed after the third wash step by resuspension of the silica matrix pellet in an appropriate volume of sterile distilled H₂O and incubation for 5 mins in a 50°C water bath. Eluted DNA was separated from silica matrix by a final centrifugation for 1 min. At this stage, DNA was ready for subsequent manipulations such as ligation.

2.19. Ligation Reactions

Ligation reactions consisted of a mixture of 10-50ng of linearised, CIP treated, gel purified vector and 30-150ng of restriction digested, gel purified insert in a total volume of 8μl of H₂O. To this mixture, 1μl of 10x ligase buffer and 1μl of 400U/ml T4 DNA ligase were added and reactions were incubated overnight at 16°C or at room temperature for 2 hours.

2.20. Shotgun Cloning

This protocol was specifically used to subclone restriction fragments from lambda phage to plasmid vectors. Phage DNA was digested with an appropriate restriction enzyme and ligated directly without any purification steps, to a similarly digested plasmid vector as described in the previous section. In this manner, all phage DNA fragments generated by the restriction digest were
ligated into the plasmid vector. The ligation reaction was then transformed into electrocompetent bacteria. Colony hybridisation from plates of the resulting bacterial transformants was used in conjunction with a variety of specific probes. Sequential hybridisation of such filters with specific probes lead to the subsequent cloning of a number of adjacent fragments along the length of the phage.

2.21. Cloning of PCR Products

PCR products were cloned using the Clonamp sytem (Gibco). Oligonucleotides were synthesised with the inclusion of dUMP residues at their 5' termini as follows:

Sense oligonucleotide 5' CUA CUA CUA CUA-Gene specific 21mer 3'
Antisense oligonucleotide 5' CAU CAU CAU CAU-Gene specific 21mer 3'

Use of the above oligonucleotides for PCR results in the incorporation of dUMP residues into the 5' termini of resulting products. Treatment of these products with Uracil DNA Glycosylase (UDG) renders dUMP residues abasic and unable to base-pair, generating 3' protruding termini. Cloning is achieved by incubating the PCR product with UDG and the linear pAMP1 vector possessing compatible 3' protruding termini. Selective deglycosylation of dUMP residues by UDG and annealing of the product to the vector occur simultaneously. Typically, 50ng of PCR product in a volume of 7µl of 1x PCR buffer was incubated with 1unit of UDG and 50ng of linear pAMP1 vector in a total volume of 10µl at 37°C for 30 mins. The reaction was then precipitated and transformed into bacteria as described in section 2.19 of this chapter.

2.22. Ligation of double stranded oligonucleotides to plasmid vectors

Double stranded oligonucleotides containing unique restriction sites were introduced into plasmid vectors to tailor such vectors to a specific cloning project. Complementary single stranded oligonucleotides were annealed to produce a double stranded oligonucleotide with overhanging restriction sites to facilitate cloning. Oligonucleotides were synthesised with 5' phosphorylated ends by the
ICRF Oligonucleotide laboratory, precipitated and resuspended at a concentration of 1mg/ml. 1µg of each complementary oligonucleotide was mixed with 1µl of 20x SSC and distilled water to a total volume of 10µl, boiled in a water bath for 5 mins and then allowed to re anneal slowly for 2 hours at room temperature. This mixture was then ready to use for ligation to an appropriately digested vector under standard ligation conditions.

2.23. Preparation of electrocompetent bacteria

Electrocompetent bacteria were prepared from the E Coli K12 derivative XL-1 Blue purchased from Stratagene which possesses tetracycline resistance. A 20ml overnight saturated bacterial liquid culture was prepared from an individual colony of the XL-1 Blue strain grown on LB solid agar supplemented with 50µg/ml tetracycline. 10ml of this original culture was used to seed two 400ml cultures of pre warmed LB broth plus 50µg/ml tetracycline. Cells were allowed to grow logarithmically and were regularly monitored until they reached an OD$_{600}$ of 0.5. At this stage, the cell suspension was pelleted at 5,000rpm for 15 minutes. From this point onwards, all steps were performed on ice and used pre chilled solutions and centrifuges. The initial cell pellet was subjected to three successive rounds of washing by resuspension in an equal volume of sterile dH$_2$O, spun down as before, then resuspended in a half volume of sterile dH$_2$O, spun down as before and finally resuspended in a half volume of sterile dH$_2$O, 20% glycerol and spun down. The approximate volume of the final pellet was estimated and the pellet was resuspended in two pellet volumes of sterile dH$_2$O, 20% glycerol. Cells were aliquoted in 100µl volumes and frozen at -70°C.

2.24. Bacterial transformation by plasmid products

Ligation reactions were incubated at 70°C for 5 mins to inactivate ligase activity. After cooling on ice for 2 mins, the reaction was precipitated by the addition of 1µl of 3M NaCl and 30µl of ice cold absolute ethanol and centrifuged for 10 minutes in an eppendorf bench top centrifuge. The resulting DNA pellet was washed with 50µl of 70% ethanol and finally dissolved in 20µl of dH$_2$O.
Bacteria were transformed with plasmid products using an electroporation protocol. Electrocompetent bacteria were thawed from -70°C on ice for 15 mins, after which 40μl of electrocompetent cells were mixed with 2.5μl of ligation reaction and transferred to a pre-chilled electroporation cuvette (with 0.1cm gap), making sure that no air bubbles were trapped across the electrode. The cuvette was then placed into the electroporation chamber and the cells were electroporated using the following settings: capacitance of 25μF, resistance of 2000Ohms and 1.8volts. Immediately after electroporation, 1ml of LB broth was added to the cells and mixed thoroughly. Due to the high level of competency of these cells, 10-fold dilutions of electroporated cells were made in sterile LB broth, which were then spread on 9cm L-agar plates containing 50μg/ml ampicillin and incubated overnight at 37°C. Ampicillin resistant colonies were analysed as potential recombinants. Where the number of colonies on background plates was high, colonies were first screened for the presence of inserts by colony hybridisation.

2.25. Total RNA isolation

Total RNA was prepared using the TRIzol reagent (Gibco) and the instructions recommended by the manufacturer. TRIzol consists of a mono-phasic solution of phenol and guanidine isothiocyanate. Approximately 100mg of frozen tissue were homogenised in 1ml TRIzol using a Polytron homogeniser. The homogenates were incubated at room temperature for 5 mins to allow complete dissociation of nucleoprotein complexes, after which 0.2ml of chloroform was added, mixed and left again at room temperature for a further 3 mins. The samples were then centrifuged for 15 mins at 4°C at 13,000 rpm. Following centrifugation, the RNA containing aqueous phase was transferred to a fresh tube and precipitated by the addition and mixing of 0.5ml of isopropyl alcohol. RNA was allowed to precipitate for 10 mins at room temperature and then centrifuged for 10 mins at 13,000rpm. The resulting pellet was washed with the addition of 1ml of 70% ethanol and then re-centrifuged for 5 mins at 7,500rpm. Pellets were finally dissolved in DEP-C treated distilled water. RNA purity was assessed by
the ratio OD 260/280, with a ratio of 1.8 or greater being suitable for subsequent downstream manipulation.

2.26. RNA agarose gels for Northern Blot

To avoid degradation of the RNA the gel tank and the gel tray were soaked in DEP-C treated H₂O over night. 3g agarose was boiled 240ml DEP-C H₂O, after cooling to ~60°C 8ml of 50x MOPS and 71ml formaldehyde (Fluka) were added and the gels was poured into a prepared gel tray in the fume cupboard. The RNA samples were lyophilised then resuspended in 40µl RNA denaturation buffer and incubated for 15 min at 56°C. Before loading the samples onto the gel 8µl of loading buffer was added and the RNA samples were separated in 1x MOPS buffer over night at 20V. The gel was rinsed 30 mins in 20x SSC and blotted onto an uncharged nylon membrane (Hybond N, Amersham).

Solutions for Northern blotting:

50x MOPS: 1M MOPS
0.25M Na acetate
50mM EDTA

running buffer: 1x MOPS

denaturation buffer: 2x MOPS
20% formamide
50% formaldehyde

6x loading buffer: 0.25% bromphenol blue
0.25% xylene-cyanole
30% glycerine
2.27. Membrane hybridisation for Northern blots

Filters were prehybridised for a minimum of 4 hours at 42°C rotating in a pyrex bottle (Hybaid). The prehybridisation buffer was replaced with hybridisation buffer containing the 32P-labelled cDNA probe which had been boiled for 5 min with salmon sperm DNA (50µg/ml final concentration; Sigma) and then quenched on ice. The final concentration of labelled probe was at least 3x10^6 dpm/ml. Hybridisation took place over night, at 42°C, in rotating pyrex bottles (Hybaid).

After hybridisation the blot was washed twice in 200ml 2x SSC, 0.1%SDS at room temperature to remove unbound surface probe and then given two 500ml washes of 0.1x SSC, 0.1%SDS at 50°C for 20 mins with shaking. The blot was briefly dried on 3MM to remove excess fluid and then wrapped into plastic film (Saranwrap) and subjected to autoradiography.

**Pre-/hybridisation solution:**

- 5x SSPE
- 10X Denhardt’s solution
- 100 µg/ml fresh denatured and sheared salmon sperm DNA
- 50% formamide
- 2% SDS

**20 x SSPE:**

- 3M NaCl
- 0.2M NaH₂PO₄.H₂O
- 0.02M Na₂EDTA
- adjust to pH 7.4 with 1M NaOH

**50x Denhardt’s solution:**

- 5g Ficoll (Type 400, Pharmacia)
- 5g Polyvinylpyrrolidone (Sigma)
- 5g BSA (fraction V, Sigma)
- add H₂O to 500ml
2.28. Total membrane preparations

The procedure was performed in the cold room and all equipment and solutions were prechilled. Isolated organs were immediately placed into sucrose buffer on ice and cut into small pieces. The tissue samples were then transferred into a loose dounce and homogenised with ten strokes. The homogenate was centrifuged at 1,800rpm (750g) for 10 mins at 4°C. The supernatant was carefully taken off and spun at 50,000rpm (200,000g) for 40 mins at 4°C. The supernatant was removed and the pellet resuspended in sucrose buffer.

**sucrose buffer:** 250mM sucrose

4mM imidazole pH 7.4

2.29. SDS-polyacylamide gel electrophoresis

The separation of proteins was carried out using SDS-PAGE. Two glass plates (18x16 cm) were assembled with 0.75 mm or 1.5 mm spacers and clamped together in a protein gel apparatus (Hoeffer). Two layers of parafilm were placed beneath the plates before clamping to ensure a tight seal. A 30 ml separating gel mix was made just before use and poured into a vertical gel apparatus, avoiding air bubbles and leaving ~3cm gap for the stacking gel and the comb.

<table>
<thead>
<tr>
<th>Reagents:</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide (37.5:1)</td>
<td>10ml</td>
<td>12ml</td>
<td>15ml</td>
<td>2ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.5ml</td>
<td>7.5ml</td>
<td>4.5ml</td>
<td>8.2ml</td>
</tr>
<tr>
<td>2M Tris pH 8.8</td>
<td>7.5ml</td>
<td>7.5ml</td>
<td>7.5ml</td>
<td></td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td></td>
<td></td>
<td></td>
<td>1.8ml</td>
</tr>
<tr>
<td>85% sucrose</td>
<td>2ml</td>
<td>2ml</td>
<td>2ml</td>
<td>3ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>150μl</td>
<td>150μl</td>
<td>150μl</td>
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<tr>
<td>TEMED</td>
<td>30μl</td>
<td>30μl</td>
<td>30μl</td>
<td>15μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>150μl</td>
<td>150μl</td>
<td>150μl</td>
<td>160μl</td>
</tr>
</tbody>
</table>
The mixture was overlaid with saturated butanol. After the gel had set the stacking gel was poured and a comb inserted. The protein samples were prepared by addition of half a volume of sample buffer followed by boiling for 5 min. 15μl of Rainbow markers (Amersham) were also loaded as protein standards. The gel was run at 20mA until the samples had passed through the stacking gel and then at 30mA for approximately 4-5 hours until the markers had separated sufficiently and the blue dye front had reached the bottom of the gel.

4x sample buffer: 62.5mM Tris-HCl, pH 6.8
10% Glycerol
2% SDS
5% β-mercaptoethanol
0.05% bromophenol blue

1x running buffer: 3g/l Tris
14.4 g/l Glycin
1g/l SDS (5ml 20% SDS)

2.30. Western Blot

SDS-PAGE gels were transferred using a Hoeffer Semi-Phor blotting apparatus. The stacking gel was removed and the size of the separating gel measured. Six pieces of blotting paper and a piece of Immobilon™-P PVDF 0.45μm membrane were cut to the size of the gel. The membrane was pre-wet in methanol and then soaked with the blotting paper in transfer buffer for 2 mins. Transfer buffer was 10mM CAPS pH11.0. A mylar mask was cut, 2mm smaller than the gel in width and length and placed over the electrode. Three sheets of blotting paper, followed by the membrane, then the gel and three more pieces of blotting paper were centred over the opening in the mylar mask and the edges rolled out ensuring there were no air bubbles which block the transfer of proteins. The proteins in the gel were transferred onto the membrane using a current of 0.8mA/cm² of gel for 45 min. The blot was removed and marked to identify on
which side the proteins were transferred, the coloured marker proteins gave an indication how efficient the transfer had taken place.

The membrane was rinsed once with water and placed into blocking solution for 5-10 min and incubated with the primary antibody in blocking solution overnight at 4°C on a spinning wheel. Excess primary antibody was removed by washing the membrane 3 times for 10 mins in wash buffer. The Western blot was then incubated with a secondary antibody in 10ml blocking solution for 1 hour at room temperature on an orbital shaker and washed 3 times in wash buffer and rinsed one with water to remove residual milk powder from the wash buffer. The secondary antibody was coupled with horseradish peroxidase and detected with ECL-kit (Amersham).

**blocking buffer:** 5% milk powder in 1x TNT

**wash buffer:** 1% milk powder in 1x TNT

**1x TNT:** pH 7.6

36g/4L NaCl

4.8g/4L Tris

20ml/4L Tween 20

### 2.31. 2D gel electrophoresis:

The 2 dimensional gel electrophoresis was performed by using the Immobiline® DryStrip Kit according to the manufacturer’s instructions (Pharmacia). Blood samples were taken from anaesthetised mice by cardiac puncture, the protein concentration was measured and the samples were pre-diluted to 20mg/ml. 5μl of the pre-diluted blood samples were added to 60μl loading buffer and the proteins were separated in the first dimension by isoelectric focusing on Immobiline® DryStrips. The strips allowed a non-linear separation in the range of pH 3-10, meaning that the pH gradient was flattened at the range of 5-7 to achieve higher resolution in this part of the gradient. The Immobiline® DryStrips were aligned in the electrophoration tank (Pharmacia)
and submerged with mineral oil. The protein samples were loaded onto the strips and the proteins were separated overnight at 2500V.

The next day the proteins were further separated on a SDS PAGE gel according to their molecular weight. This separation step is described under 2.29. To minimise the variations between the single SDS gels, twelve gels were cast together in a single casting chamber (Pharmacia) and gels were run simultaneously in a large gel tank (Pharmacia). This ensured that all gels were run under the exact same buffer conditions and for the same length of time. The glass plates were disassembled and the gels were stained with silver nitrate.

2.32. Silver Staining

The silver staining was performed according to the "Hochstrasse"-protocol.

1. wash gels for 5 mins in water
2. fix in 40%EtOH/10% glacial acetic acid for 1 hour
3. soak in 5%EtOH/5% glacial acetic acid for at least 3 hours
4. wash in water for 5 mins
5. soak in 0.5M sodium acetate/1% glutaraldehyde for 30 mins
6. wash 3 times for 10 mins in water
7. soak in 0.05% naphthalene sulphonyl acid
8. rinse 4 times for 15 mins in water
9. stain for 25 mins with silver stain solution
10. wash 4 times for 4 mins in water
11. develop staining in 0.005% citric acid/0.1% formaldehyde
12. stop developing reaction with 5% Tris base/2% glacial acetic acid
13. dry gels in "Easy Breeze" gel dryer overnight
Materials and Methods

Silver stain solution: 26.7 ml ammonium hydroxide
4 ml 10M NaOH
1760 ml water (mix first and then slowly add)
16g silver nitrate in 60 ml of water

2.33. Preparation of primary mouse embryonic fibroblasts (MEFs)

Pregnant mice were sacrificed at 13.5 dpc, the morning of the day the plug was found being defined as 0.5 dpc. The embryos were removed from the uterus, the yolk sacks were kept for genotyping and other extraembryonic tissue was discarded. The embryos were decapitated, eviscerated and the remaining tissue was minced with two sterile scalpels. Embryonic tissue was collected in PBS in a 15 ml Falcon tube and centrifuged for 1 min at 2500 rpm. The pellet was resuspended in 3 ml trypsin : versene (0.025% trypsin/0.5 mM EGTA) and incubated at room temperature for 15 mins. The supernatant was transferred to a fresh Falcon tube containing 6 ml of DMEM 10%FCS. This trypsin digestion step was repeated twice and the supernatants containing the MEFs were pooled, centrifuged for 3 mins at 1,000 rpm, and the pellet resuspended in 5 ml DMEM 10%FCS and plated on a 6 cm Petri dish. The cells were confluent the following day and were either transferred to a 10 cm dish or frozen down. MEFs were routinely cultured in DMEM 10%FCS in 10% CO₂ at 37°C.

2.34. Immunofluorescence

1x10⁵ MEFs/well were plated onto coverslips in a 6-well plate. The following day the coverslips were transferred to a new 6-well plate, washed twice with PBSA and fixed for 20' in 3% paraformaldehyde (PFA). The coverslips were washed for at least 15’ with 3 changes of PBS. Cells were permeabilised in 0.1% Saponin/PBS. All subsequent steps were carried out in 0.1% Saponin/PBS as the permeabilisation of the cell membrane is reversible. The coverslips were incubated for 20 min at room temperature with the primary antibody, then washed for 3 times 5 mins with 0.1% Saponin/PBS. Incubation times and
subsequent washing steps with the secondary antibodies (coupled with either FITC or Cy5) were identical to those used for the primary antibody.

2.35. Labelling procedure for pulse chase

For pulse chase experiments the MEFs were transferred to 3ml dishes at 5x10^5 cells/dish and one dish per time point. The cells were incubated in 3ml methionine-free medium after washing twice with PBS. All media used only contained 0.5% FCS because larger amount of FCS in the medium would lead to an overloading of the SDS gels and interfere with a proper separation of the secreted proteins. The FCS was dialysed overnight in PBS to remove methionine, which should be depleted as efficiently as possible before the labelling procedure. After preincubation at 37°C for 45 mins [35S]-methionine (200μCi/ml) was added. Incorporation was allowed to proceed for 5 min before the cells were washed once with E4 medium containing 0.2mM methionine (10x) and incubated in the same medium containing 100μg/ml cycloheximide. The medium was completely removed after defined time points and concentrated in Millipore spin columns to 100μl. The total amount of concentrated supernatant was loaded onto a 10% SDS polyacrylamide gel. The labelled MEFs were lysed, precipitated in equal volume 20% tricloroacetic acid (TCA), washed 3x with ice-cold acetone and the radioactivity of the pellet was analysed in a scintillation counter (Beckman).

2.36. Toxin assays

5x10^5 MEFs were incubated with either 20ng/ml or 100ng/ml Pseudomonas exotoxin A or either 5ng/ml or 12.5 ng/ml ricin for up to 5 hours. Cells were washed once and starved for 45 mins in methionine-free DMEM then labelled for 30 mins with [35S]-methionine 20μCi/ml and incubated in complete medium for further 30 mins. MEFs were then lysed and the lysates were transferred to eppendorf tubes. The proteins were precipitated in equal volume of 20% TCA, washed 3x with ice-cold acetone and radioactivity of the pellet was determined in a scintillation counter (Beckman).
2.37. Immunofluorescence analysis by flow cytometry

Single cell suspensions from thymus, spleen and lymph nodes were prepared by gently disaggregating the tissues on a piece of gauze with a syringe plunger in FACS buffer. Cell suspensions were stained for the detection of surface antigens and then analysed by flow cytometry. FACS buffer consisted of PBS, 5% FCS, 0.1% sodium azide. For the majority of FACS analysis, 10^6 cells were stained per sample. Antibodies (Pharmingen or Caltag) were generally diluted 1:100 in FACS buffer. Antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used at a final concentration of 5μg/ml. Alternatively, antibodies conjugated with biotin were visualised using a second layer of 0.02μg/ml streptavidin-Tricolour (Caltag). These fluorochromes are excited by a 488nm laser, with emission wavelengths of 525, 575 and 667nm respectively.

All incubations for FACS analysis were performed at 4°C. For staining, the appropriate number of cells were aliquoted into individual FACS tubes and pelleted by centrifugation at 1,000rpm for 5 mins. The pellet was resuspended in 100μl of FACS buffer together with the relevant antibody and incubated for 20 mins on ice in the dark. Cells were then washed free of unbound antibody by the addition of 2ml of FACS buffer, centrifuged at 1,000rpm for 5 mins and finally resuspended in 300μl of FACS buffer. If biotinylated primary antibodies were used, cells from the primary stain were washed as above but resuspended in 100μl of FACS buffer together with a 1:100 dilution of Streptavidin-Tricolour, incubated for a further 20 mins on ice in the dark and then finally washed and resuspended in 300μl of FACS buffer. Acquisition and analysis of stained cells by was performed using Cellquest software on a FACScan or FACSscalibur flow cytometer (Becton Dickinson). For routine staining, live cells were gated on forward and side scatter on a linear scale, and data for other parameters was collected in logarithmic scale. Unstained cells and cells stained with a single fluorochrome were used to set up electronic gating collection and compensation parameters. In general, 1-5x10^5 events were collected per sample. All events were saved and gates were introduced to allow analysis of specific cell populations.

For pre-TCR staining, double negative thymocytes were enriched by depleting double positive and single positive cells by complement lysis.
Chapter 2 Materials and Methods

Thymocytes were incubated in supernatants from RL172.4 (anti-CD4) and 3.168.8.1 (anti-CD8) cell lines for 10 mins at 37°C, rabbit complement was added and the cells were incubated for a further 45 mins at 37°C. The living cells were separated from the lysed cells by Ficoll density-gradient centrifugation. Recovered cells were stained with a primary antibody against the TCRβ chain (H57.597) which was conjugated with digoxigenin. The digoxigenin was then recognised by F(ab)₂ fragments which were coupled to liposomes (Bruno et al., 1999; Scheffold et al., 1995). There is no antibody commercially available against pTα, so pTα was detected indirectly using the antibody against TCRβ which is present as a heterodimer with pTα on double negative thymocytes.

2.38. Apoptosis assays

Single cell suspensions of thymocytes were adjusted to 4x10⁶ cells/ml and plated onto 6-well plated with 1ml per well. The thymocytes were either incubated with 1mM dexamethasone, 10µg/ml cycloheximide, 100µg/ml anti-Fas antibody, 10ng/ml Fas ligand. For apoptosis stimulation via CD3, the 6-well plates were coated with an anti-CD3ε antibody at a concentration of 10µg/ml in sterile PBS overnight. For UV stimulation the thymocytes were exposed to 40J/m² UV light (Stratalinker). The thymocytes were harvested 12, 24 and 36 hours after stimulation into FACS tubes, washed once in cold PBS and then fixed in ice cold 70% EtOH. The cells were incubated at 4°C at least for 30 mins to ensure complete fixation. The cells were centrifuged for 5 mins at 2,000rpm, resuspended in 250µl RNase A (100µg/ml) and incubated for 30 mins at 37°C. Propidium iodide (PI) (50µg/ml) was added and the cells were incubated for additional 15 mins at room temperature. PI intercalates in the DNA and the sub-G1 peak was analysed at a wavelength above 600nm with the FACScalibur (Beckton Dickinson).

2.39. Influenza infection of MEFs

MEFs were seeded onto 6-well plates and grown until they were confluent. Influenza viruses were harvested from infected eggs and titrated to 1x10⁷ pfu/ml. 200µl of the influenza virus was diluted in 300µl of serum-free medium and
added to the MEFs. After an initial incubation of 1 hour at 37°C 3ml of serum-free medium was added, and the MEFs were incubated for further 3 hours, 7 hours or overnight at the same temperature. The MEFs were washed once in medium and fixed for 1 hour at 37°C in 3% paraformaldehyde in PBS. Immunofluorescence was performed with a mixture of monoclonal antibodies against different HA epitopes.

2.40. Processing for electron microscopy

Kidney and liver samples were cut into very small cubes and fixed in 2.5% glutaraldehyde/0.1M sodium cacodylate buffer, pH 7.2. The samples were postfixed for 30 mins with 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.2. The samples were then washed in distilled water and a solution of 0.5% magnesium-uranyl acetate in distilled water was added for 1 hour. The samples were dehydrated in a series of graded ethanol solution, followed by propylene oxide, and finally embedded in Epon 812 (Taab Laboratories, Berkshire, UK). Silver sections were cut using a Leica ultramicrotome and were picked up on formvar coated grids. The sections were stained with a solution of lead citrate and uranyl acetate and examined in a Philips CM 10 electron microscope.

2.41. Antibodies

The anti-calnexin antibodies were purchased from STRESSGEN, the anti-CD4, anti-CD8, anti-CD3e, anti-MHC I (H2Kb) and anti-B220 antibodies were obtained from Pharmingen. The anti-MHC II (M5/114) antibody was a gift from Gitta Stockinger (Mill Hill). The antibodies against p23 and p26 were raised in rabbits according to standard procedures using peptides corresponding to amino acids 204-215 of murine p23 (QVFYLRRFFKAKKLIE), and the cytoplasmic domain of p26 (KQVLLLKSFFTEKRPISRAVHS), respectively. The antibody against GMP25 and COPI have been previously described by Dominguez et al., 1998 and Lowe and Kreis, 1995 respectively.
Chapter 3

Generation of p23- and calnexin-deficient mice

3.1. Introduction

This chapter describes the construction and use of gene targeting vectors to introduce specific mutations in the coding region of the murine p23 and calnexin genes. Gene targeting vectors integrate into the genome of embryonic stem cells (ES cells) by homologous recombination and inactivate the targeted gene leading to a mouse strain carrying germline mutations for that affected gene.

3.1.1. Homologous recombination

Homologous recombination (HR) is the term used to describe the exchange between two DNA strands, which share extended sequence homology. This event occurs naturally during meiosis and mitosis. The exchange of genetic material is not only a useful tool to repair damaged DNA but, as the newly recombined fragments may not be identical, HR acts as a mechanism to increase genome diversity – a necessity for evolution.

Over the last decade HR - or gene targeting - has become a powerful technique for introducing specific mutations into the genome and generating mouse strains deficient for targeted genes. Initially, gene targeting studies found that random integration occurred with much higher frequency than HR. Only one in a thousand integration events occurred at the homologous region. However, considering the size of the genome it was encouraging that a new technique could mutate a single gene of interest (Lin et al., 1985; Smithies et al., 1984).

Further experiments improved the conditions for HR resulting in an increased frequency of specificity. Various studies have shown that the ratio of
HR increases with the length of homology between the targeting vector and the targeted gene (Hasty et al., 1991; Thomas and Capecchi, 1987). The lower limit for HR is a region of about 500 bp on one arm of the targeting vector, longer targeting vectors integrate with much higher frequency, although the length of the targeting vector and the frequency of HR is not linearly proportional. Initial attempts to achieve gene targeting in strain 129/Sv ES cells and non-isogenic targeting vectors from Balb/c DNA resulted in a very poor HR frequency. The use of isogenic DNA for targeting vector construction greatly improved homologous recombination (Deng and Capecchi, 1992; te Riele et al., 1992). In addition, linearisation of the targeting vector also resulted in an increased frequency of HR. This has been supported by the finding that mammalian cells can tolerate non-homologous sequences, suggesting that the efficiency of HR depends on linear topology rather than homologous ends. Furthermore it has been speculated that chromatin structure and transcriptional activity of the targeted gene play a role but this hypothesis has not been further investigated.

3.1.2. Structure of gene targeting vectors

To select for integration of the targeting vector in the genome of the transfected cell the targeting vector carries a “selectable marker”. This marker gene facilitates the identification of HR. Firstly, a positive selectable marker (e.g. neomycin or hygromycin phosphotransferase) allows survival of ES cells which have stably integrated the targeting vector in their genome when appropriate antibiotics (Geneticin (G418)), are added to the culture medium. Secondly, the marker gene interrupts or replaces parts of the coding region and results in either truncated or no mRNA transcripts from the targeted locus. Thirdly, the selectable marker may contain additional endonuclease restrictions sites, which can then be used to confirm that the locus of interest was targeted.

Although the mechanism of HR is not well characterised, it is generally believed that integration occurs by reciprocal recombination or gene conversion; non homologous sequences and the free ends of the replacement vector are excised (Mansour et al., 1988). This feature of HR has been exploited to
incorporate negative selection markers like Herpes Simplex Virus thymidine kinase (HSV-tk) or diphtheria toxin A (DTA) at the end of replacement vectors to eliminate transfected cells in which random integration has taken place (Yagi et al., 1990).

**Figure: 3.1 Schematic representation of targeting vectors**

- **replacement vector**

  ![replacement vector diagram]

- **insertion vector**

  ![insertion vector diagram]

The targeting vector described above is known as a replacement vector because fragments of homologous DNA sequences are exchanged, and parts of the targeted locus are replaced, by the selectable marker. Another, less commonly used targeting vector, is the insertion vector. In contrast to replacement vectors, insertion vectors are linearised within the homologous sequence. The whole
targeting vector integrates in the targeted locus and interrupts the coding region by duplicating parts of the coding sequence and inserting the selectable marker. Figure 3.1. shows a schematic overview of the two different targeting vectors.

### 3.1.3. The Cre/loxP recombination system

Gene targeting experiments provide information about the function of the targeted gene in pre- and postnatal mammalian development. If the gene of interest has not been extensively characterised, the phenotypic analysis of mutant animals may prove a slow process, particularly if the mutation causes embryonic lethality.

Over recent years a powerful method has been developed to generate a tissue-specific gene targeting without compromising viability. The PI bacteriophage Cre recombinase recognises a 34 bp sequence called the “loxP” site which consists of a 8bp spacer region and two 13bp inverted repeats. The spacer region determines the orientation of the loxP site. If the loxP sites are in the same orientation the Cre recombinase excises DNA fragments located between two loxP sites with only one site remaining; if the loxP sites are in opposite orientations the Cre recombinase inverts the intervening DNA sequence as shown in figure 3.2. (Abremski et al., 1983; Hamilton and Abremski, 1984; Hoess and Abremski, 1984; Hoess et al., 1982).

**Figure 3.2. Schematic representation of Cre recombinase mediated excision events**

- **a)** loxP sites in same orientation

```plaintext
[Diagram showing Cre recombinase excising DNA between two loxP sites with one remaining]
```

- **b)** loxP sites in different orientation

```plaintext
[Diagram showing Cre recombinase inverting DNA between two loxP sites]
```

To generate a tissue specific inactivation, the targeting vector would not disrupt the coding sequence of the targeted gene but rather exchange parts of it
with the same part, now flanked by 2 loxP sites (floxed). Mice carrying such alleles appear phenotypically normal but have the potential to give rise to tissue specific knockout offspring when crossed with Cre recombinase transgenic mice. The Cre recombinase can be expressed as a transgene under the control of tissue specific or inducible promoters. Breeding of the knockout mice containing the gene of interest flanked by loxP sites with Cre recombinase transgenic mice would lead to offspring with the targeted gene inactivated in the specific tissues where the Cre recombinase is expressed (Kuhn and Schwenk, 1997; Kuhn et al., 1995; Lewandoski and Martin, 1997; Schwenk et al., 1995).

3.1.4. Derivation of embryonic stem (ES) cell lines

Embryonic stem cells were isolated nearly twenty years ago. Three-day-old blastocysts were cultured on a STO fibroblast feeder layer in embryonal carcinoma (EC) cell conditioned medium. These conditions led to the formation of EC cell-like colonies (Martin, 1981). EC cell colonies had been previously reported to be totipotent and had been used for in vitro differentiation studies. Nevertheless, EC cells display an abnormal karyotype and tumorigenic behaviour and were therefore unsuitable for in vivo experiments (Papaioannou et al., 1979).

ES cells possess two characteristics, which has facilitated their use as a convenient tool used in the study of mammalian development. Firstly, they are totipotent - once injected into blastocysts they are able to give rise to any tissue and organ of the embryo, including germ cells. Secondly, ES cells can be cultured in vivo for short periods without loosing their totipotency and can therefore be genetically manipulated. ES cells when injected into blastocysts were able to contribute to every tissue of the developing embryo even germ cells (Bradley et al., 1984), a progeny that has allowed the assessment of the percentage of ES cell contribution by the coat colour – the blastocysts give rise to different coat colours than ES cells. Some of the chimeras transmit the acquired mutation through the germ line onto their offspring. Most available ES cells show a male genotype for several reasons. Firstly male chimeric mice will produce more offspring over their reproductive life span than female chimeras. In addition, male ES cells, when
injected into female blastocysts tend to bias the development towards a male embryo. Finally, only XY germ cells will give rise to functional gametes in male chimeras, thus favouring the development of ES cell derived gametes (Patek et al., 1991).
3.2. Specific materials and methods

3.2.1. Mouse strain 129/Sv genomic DNA libraries

A lambda phage mouse genomic DNA library constructed with the EMBL-3A vector (Frischauf et al., 1983), was used to isolate the murine homologue of calnexin. The library was constructed with mouse 129/Sv genomic DNA consisting of Sau3A partial genomic restriction fragments ligated into the BamHI site of the vector polylinker. After ligation, recombinant phages contained partial genomic DNA inserts that replaced the stuffer fragment. This stuffer included the Red and Gam genes that prevented phage growth in bacterial hosts (e.g. NM538) with P2 prophages. Thus, non-recombinant phages that retained the stuffer fragment with the Red and Gam genes can be negatively selected within a P2 lysogenic host. To isolate the murine p23 gene a λ 2001 phage mouse 129/Sv genomic library was used (Karn et al., 1984). The λ 2001 phage uses the same principal to select for recombinant phages, i.e. it needs the NM539 bacterial host strain for growth.

Top agar: L-broth and L-agar mixed 1:1
10mM MgSO4

LB agar phage plates: L-agar
0.2% maltose
10mM MgSO4

Suspension medium: 100mM NaCl
10mM MgSO4.7H2O
50mM Tris-Cl pH 7.5
0.001% Gelatine

3.2.2. Preparation of host cells for phage infection

A 50ml culture of LB supplemented with 0.2% maltose and 10mM MgSO4 was inoculated with a single colony of the suitable bacterial host strain NM538 or
NM539 and grown overnight at 37°C whilst shaking at 200rpm. The presence of maltose in the culture medium induces the lamB gene within the maltose operon (which encodes the bacteriophage lambda receptor) and thereby greatly facilitates the efficient adsorption of phage particles to bacteria. Mg^{2+} ions are also thought to aid this adsorption process. The overnight culture was centrifuged for 10 mins at 2,000rpm at room temperature, gently resuspended in 20ml of filtered 10mM MgSO4 and stored at 4°C for up to 2 weeks.

3.2.3. Titration of a genomic library

Serial dilutions of phage library stock were made in 100μl of suspension medium (SM) buffer, mixed with 200μl of host cell suspension and incubated at 37°C for 15 mins to allow infection to take place. 3ml of LB top agar cooled to ~50°C was then added to the bacteriophage/bacteria mixture and quickly poured on top of dried 90mm LB-agar phage plates. Once the top agar had hardened, the plates were incubated inverted at 37°C overnight, after which the number of plaque forming units (pfu) for each dilution were counted and the phage titre (pfu per ml) of the library was calculated.

3.2.4. Screening of a genomic library

The representation of individual genomic DNA fragments within a library is dependent upon the size of the cloned fragments and the size of the genome. For a 99% probability of isolating a desired sequence from a mammalian genome using a typical phage lambda vector, at least $7 \times 10^5$ pfu should be plated out and screened. Once the titre of the phage library had been accurately determined, $10^6$ phages were plated out onto four large 225mm square bacterial petri-dishes, with $2 \times 10^5$ pfu aliquoted for 50ml of top agar per plate. Incubation of library plates at 37°C was carefully monitored to prevent too extensive lysis and generally lasted 8-12 hours. Plates were then stored overnight at 4°C to allow the top agar to solidify, before duplicate filter lifts and subsequent hybridisation were performed. Library plates were stored at 4°C with a small piece of Whatman
3MM filter paper soaked in chloroform and attached to the lid of each plate to reduce contaminant bacterial growth.

The filters were hybridised with a radioactive labelled probe (in most cases, cDNA of the gene of interest), excess probe washed away, and autoradiograph films exposed to the library filters. Once developed, films were aligned with the orientation marks on the filters and placed on a light box with the bacteriophage plates on top of the films to enable picking of positive plaques. The bacteriophages were picked by removing the area around the positive plaque with the wide bore end of a sterile blue tip and placing it for several hours at 4°C in 1ml of suspension medium containing 100μl of chloroform (to prevent any residual bacterial cell growth). Secondary and tertiary library screens were performed in a similar manner to facilitate enrichment of hybridisation positive plaques but using smaller 90mm plates and plating out dilutions of initial library screen positive plaques. Three rounds of plaque purification proved sufficient to isolate single hybridisation positive plaques.

3.2.5. Preparation of Phage Minipreps

A single hybridisation positive plaque was picked and placed into 50ml of LB phage broth and shaken vigorously overnight at 37°C. Together with the picked phage plaque host strain bacteria were transferred into LB phage broth. Cultures were checked visually the next day for efficient lysis, as judged by the presence of bacterial cellular debris. Chloroform (0.5ml) was added to the lysed cultures and shaken for a further 5 mins at 37°C was. The lysed culture was then transferred into a 50ml sterile Falcon tube and centrifuged at 4,000 rpm for 20 mins at room temperature. The supernatant was then harvested, incubated with 500μg each of DNase and RNase at room temperature for 30 mins, and then re-centrifuged for 2 hours at 16,000 rpm.

The supernatant was poured off carefully and the tubes were left inverted for 10 minutes on top of a tissue to dry. The pellet was resuspended in 0.3ml of 0.1M Tris HCl pH 7.9, 0.3M NaCl and stored overnight at 4°C. After resuspension, the phage solution was transferred to an Eppendorf tube and incubated with
500μg of Proteinase K and EDTA (10mM final concentration) for 30 mins at 37°C. Proteins were removed from the phage solution by three subsequent extractions with equal volumes of a phenol/chloroform mix followed by precipitation with 2 volumes of ice cold ethanol and centrifugation for 15 minutes in an microfuge. The resulting pellet was air dried for 10 mins, dissolved in 50 μl TE and stored at 4°C.

3.2.6. Restriction Mapping

Genomic clones derived from either phage or plasmid vectors were digested with a range of 15-18 restriction enzymes considered to be useful for subsequent subcloning. These restriction digests were separated on agarose gels blotted onto filters and sequentially hybridised with a series of gene specific probes. Filters were stripped of probe prior to re-hybridisation. Hybridisation with a vector specific probe was performed last, as such probes proved insensitive to stripping procedures. The above protocol allowed the calculation of the number of restriction sites present within a particular clone as well as assigning the location of probes to particular fragments, resulting in a basic restriction map.

**ES cell complete medium:**

DMEM (Gibco BRL) without sodium pyruvate, with 4500mg/ml glucose.

20% FCS (Gibco BRL), suitable for ES cell culture

1x Non essential amino-acids (Gibco BRL)

2mM L-Glutamine (Gibco BRL)

1mM 2-Mercaptoethanol (Gibco BRL)

100 units/ml Penicillin and Streptomycin (Gibco BRL)

10^3 units /ml ESGRO™

400μg/ml G418 (Geneticin, Gibco BRL) for culture of transfected ES cells.
STO medium:
DMEM (ICRF Cell Services)
10% FCS (Gibco BRL)
2mM L-Glutamine (Gibco BRL)

ESGRO™, recombinant murine Leukaemia Inhibitory Factor (LIF) was purchased from Gibco BRL as $10^7$ units/ml in PBS /1% BSA.

2X ES cell freezing medium:
Complete ES cell medium supplemented with 20% dimethyl sulphoxide (DMSO).

ES and feeder cell lines:
The male ES cell line GK129 derived from strain 129/Ola Hsd (agouti/chinchilla) (Philpott et al., 1992) was used.

STO fibroblasts are a neomycin resistant subline of the thioguanine and oubain resistant line of SIM mouse fibroblasts originally isolated by Dr. A. Bernstein (Martin, 1981).

ES cell DNA lysis buffer:
10mM Tris-HCl pH7.5, 10mM EDTA, 10mM NaCl, 0.5% Sarcosyl. Proteinase K was added immediately prior to use at a final concentration of 1mg/ml.

Restriction digest mixture:
1x optimal restriction buffer, 4mM spermidine pH7.4 and 10 units of each restriction enzyme per sample in a total volume of 30µl.

3.2.7. Routine ES cell culture:
ES cells were routinely grown upon mitotically inactivated feeder layers of neomycin resistant STO fibroblasts. Factors secreted by these cells are
uncharacterised but may influence the maintenance of ES cell totipotency in vitro. Cell-cell contact may also play a role in maintenance of ES cells in an undifferentiated state. Only low passage ES cells (less than 15 passages) were used, since longer culture times adversely affected ES cell totipotency. Medium changes were required every 24 hours as ES cells were seeded and kept at relatively high density to minimise induction of differentiation. ES cells were routinely passaged 1:3 during which the monolayer was disaggregated into a single cell suspension to minimise prolonged cell-cell contacts.

3.2.8. Preparation of STO cell feeder layers:

STO cells were routinely grown as a monolayer culture. Any STO cell cultures that exceeded the cell density required to maintain monolayer cultures were discarded. Tissue culture flasks or plates were coated with gelatin (0.1% w/v in sterile distilled water) to aid cell adhesion and incubated at room temperature for at least 1 hour. The gelatin was removed before plating the inactivated feeder cells. STO cells were grown to confluency in STO medium, after which they were mitotically inactivated by the addition of STO medium supplemented with 10μg/ml mitomycin C (Sigma) and incubation at 37°C for 2 hours (stocks of STO mitomycin C medium were used for up to one week after preparation and then discarded). The mitomycin C-containing medium was removed and plates were washed three times with PBS. STO cells were then trypsinised by incubating at 37°C for 10 mins with 2ml of 1x trypsin solution. Trypsin was inactivated with an equal volume of STO medium and cells were resuspended by vigorous pipetting, before transfer to a 50ml sterile centrifuge tube (Falcon) and centrifugation at 1,000 rpm for 5 mins at room temperature. The STO cells were resuspended in 10ml of STO medium, counted and plated at a concentration of 3.5x 10^5 cells/ml.

3.2.9. ES cell electroporation:

Targeting vector plasmid DNA was prepared using the “double banded” maxiprep technique: 100μg of targeting vector were linearised by restriction enzyme digestion. The completion of the restriction digest was assessed by
agarose gel electrophoresis of one hundredth of the digest. Linearised targeting vector was purified by phenol-chloroform extraction and ethanol/NaCl precipitation. DNA was recovered by centrifugation in a bench-top microfuge and washed with 70% ethanol. The DNA pellet was redissolved under sterile conditions in PBS at a concentration of 1mg/ml.

It is particularly important that ES cells are in log phase growth at the time of electroporation. To achieve this, actively growing ES cells (80% confluent) were passaged 1:3 one day before electroporation. ES cells were fed with fresh ES cell medium 4 hours before electroporation. Plates were washed twice with PBS and treated with 1x trypsin/versene solution for 10 mins at 37°C. Trysin was inactivated by the addition of 1 volume of ES cell medium. To prevent differentiation, cell clumps were well dissociated into a single cell suspension by vigorous pipetting. The cell suspension was centrifuged at 1,000rpm for 5 mins and the cell pellet was resuspended in 10ml sterile PBSA. Cell density was determined by counting with a Neubauer haemocytometer. Cells were recentrifuged and the cell pellet was resuspended in PBS at a final density of 1.1x 10^7 cells/ml. 25μg of linearised targeting vector were mixed with 0.9ml of ES cell suspension in an electroporation cuvette and incubated at room temperature for 5 minutes. The ES cells were electroporated using a Bio-Rad Gene Pulser at 230V, 500μF and then incubated at room temperature for 5 minutes. The entire contents of the cuvette was plated onto two 10cm plates with STO feeder cells in ES cell medium. G418 selection was applied 24 hours after electroporation. G418 selection was maintained with daily replacement of selective medium.

3.2.10. Picking and expansion of colonies after electroporation:

In general, colonies became macroscopically visible 5-6 days after electroporation. 8-9 days after electroporation colonies were large enough to be picked and manipulated. Medium was removed from each dish and the cells were gently washed twice with PBS. Finally 5ml PBS were added to cover the colonies to avoid drying out. A 96-well-U-bottomed plate containing 25μl of trypsin solution per well was prepared for disaggregating the colonies. The
original dish was placed on a dissecting inverted microscope. Colonies were picked using a Gilson P20 pipette with disposable sterile tips in a maximum volume of 5μl. Each colony was physically dislodged from the feeder layer with the tip, aspirated into the tip and transferred to a separate well in the 96-well plate containing the trypsin. Great care was taken not to disturb the surrounding feeder layer so that other colonies were not detached. Only those colonies displaying an absolute undifferentiated morphology were selected for picking. After 96 colonies had been picked, the plate was placed in an incubator (37°C, 5%CO₂) for 10 mins. During the incubation, medium was removed from a previously prepared 96-well plate with STO feeder layer and replaced with 200μl of ES cell medium per well which were added using a multi-channel pipette.

To each 96-well plate of trypsinised ES cell colonies, 25μl of ESC medium was added. ES cell colonies were then disaggregated by drawing the contents of the well up and down the pipette tip ten times. The cells were then transferred to the previously prepared 96-well feeder cell.

ES cell clones were grown in 96-well plates for 3-4 days (at 37°C, 5%CO₂) with ES cell medium being replaced every day. When almost reaching confluency, ES clones were split 1:2. Each well was washed twice with 200μl PBSA and trypsinised by the addition of 50μl of trypsin. 25μl of this cell suspension were seeded onto a separate fresh 96-well feeder layer plate and grown for another 4 days. This plate was then frozen at -70°C. The remaining 25μl of cell suspension were plated onto a previously treated gelatinised 96-well plate with ES cell medium. This plate served as a replica of the frozen clones and was grown to confluence to prepare genomic DNA for Southern blot genotyping.

3.2.11. Screening of transfected ES cell clones for homologous recombinant mutants using Southern blotting:

The procedure of Ramirez-Solis was utilised to prepare ES cell genomic DNA of sufficient quality to allow subsequent screening for homologous recombination events by Southern blotting (Ramirez-Solis et al., 1992). Replica 96-well plates were cultured for 5 days until reaching confluency. Plates were then
carefully washed twice with 200μl of PBS. Cells were lysed by the addition of 50μl of ES cell DNA lysis buffer per well and then incubated overnight at 60°C in a humid atmosphere. The following day 100μl of ice-cold 75mM NaCl/absolute Ethanol were added to each of the wells and allowed to stand at room temperature for 30 mins. The plate was then carefully inverted to discard the solution, leaving the precipitated DNA attached to the plate. The DNA was then washed three times with 150μl of 70% ethanol to remove any residual salt. After the final wash the plate was inverted and allowed to dry for 30 mins at room temperature. Precipitated genomic DNA was clearly visible as a filamentous network attached to the bottom of the well.

30μl of restriction digest mixture was dispensed into each well and mixed thoroughly by moving the tip in a circular motion around the bottom of the well. Tips were changed between wells to prevent any contamination. The plates were incubated at 37°C overnight in a humid atmosphere. The following day 6μl of 6x DNA loading buffer were added to each well and the samples separated on a 0.7% (w/v) agarose TAE gel at 70V for 6 hours. The gels were then prepared for Southern blotting as previously described in chapter 2.

3.2.12. Freezing and thawing ES cell clones in 96 well plates:

ESC medium was changed on each 96-well plate 4 hours prior to freezing. Medium was then removed by aspiration and washed twice with PBS. Cells were trypsinised with 50μl of trypsin solution per well and incubated for 10 mins in a 37°C incubator, after which colonies were dissociated with 50 μl of 2X freezing medium. Plates were then sealed with Parafilm, wrapped with 3 layers of tissue and stored in a Styrofoam box at -70°C. To thaw ES cell clones, 96-well plates were removed from the freezer and immediately transferred to a 37°C incubator for 10 mins. Wells containing recombinant clones were selected and the entire contents of each well transferred to a 24-well feeder plate with 2ml of ESC medium. The medium was replaced the next morning to remove any residual DMSO from the freezing medium.
3.2.13. Blastocyst injection:

All blastocyst injections were performed by Ian Rosewell at the ICRF Transgenic Unit, Clare Hall Laboratories using standard procedures. Briefly, C57BL/6 blastocysts were obtained by superovulation of 3-4 week old females and flushing the uterine horns at day 4 of pregnancy. Approximately 10-15 ES cells were micro-injected into each blastocyst. Twelve injected blastocysts were transferred to the uterine horns of each day 3 pseudopregnant C57BL/6 foster mother.
3.3. Results

3.3.1. Isolation of genomic calnexin fragments

At the beginning of this study the cDNA sequence of the dog homologue of calnexin had been determined (Wada et al., 1991). In order to generate a hybridisation probe specific for mouse, calnexin primer pairs were designed according to the canine homologue. These primers were used to perform a degenerate PCR with murine cDNA. A fragment of expected size was isolated and subcloned into a CloneAmp™ vector (GIBCO BRL). Sequencing of this PCR fragment confirmed that the amplified fragment was the murine homologue of canine calnexin.

The first 400bp of the coding region of the murine cDNA were used to screen an EMBL-3A library for phage plagues, which contained genomic calnexin DNA. The genomic phage library was isogenic to the ES cell line which was used for later electroporation since isogenicity between the targeting vector and targeted genomic DNA has been shown to increase the frequency of homologous recombination (Capecchi, 1989; te Riele et al., 1992). 12 clones were identified in the primary screen. Two additional hybridisation steps confirmed that 7 phage clones indeed contained genomic calnexin DNA. An initial restriction digest and Southern blotting of the digested phage DNA indicated that the phage clones contained identical genomic fragments. Only one of the clones (referred to as C1) was further characterised.

3.3.2. Characterisation of the calnexin \( \lambda \) phage clone C1

The C1 clone was digested with a variety of different restriction enzymes. Such restriction digests identify different smaller fragments which still contain the sequence of interest but are more suitable for further characterisation. Genomic phage DNA from clone C1 was digested with 18 different enzymes, as shown in figure 3.3. The restriction digests were separated on a 0.7% agarose gel, transferred to a positively charged nylon filter (Hybond N+ Amersham) and
hybridised with a probe to the 5' end of the calnexin cDNA. Several fragments between 2 and 12 kb were recognised by the probe (fig. 3.3.) and two of these fragments, the 2.5kb HindIII and the 9.5kb KpnI fragment, were subcloned into pBluescript vectors.

The 9.5kb KpnI and 2.5kb HindIII subclones, referred to as CK and CH, respectively, were subjected to further restriction mapping and identification of the exon-intron structure of the isolated genomic sequence. The CK and CH subclones were digested and hybridised in a similar way to the phage clone Cl. With this additional analysis a more detailed restriction map was generated (fig. 3.4.).

In order to identify the exon-intron structure in the subcloned genomic calnexin fragments, several primers were designed which annealed to the 5' region of the calnexin cDNA. These primers were used to sequence the genomic DNA subclones. The sequences were compared to the calnexin cDNA which helped to identify the exon-intron borders. The sequencing reactions were initially done manually and later by ABI Prism™ (Perkin Elmer) automatic sequencing. After identifying the exon-intron borders, PCR reactions were performed with primers which were located in two different exons to identify the size of the intervening introns. This analysis revealed the exon-intron structure shown in figure 3.4..

Although neither of the subclones contained the first exon, PCR reactions with genomic murine 129/Sv DNA indicated the relative size of the first intron and allowed an estimation of where the first calnexin exon is located. Table 3.1. shows the sequences at the exon-intron borders of the subcloned 5' region of genomic calnexin DNA.

Comparison of the known EMBL 3A restriction map suggests that the 5' region of the CK subclone contains phage DNA and that the SalI restriction site was derived from the EMBL 3A phage. This was confirmed by hybridisation of the CK subclone with EMBL 3A phage DNA.
Phage DNA prepared from clone C1 was digested with a variety of restriction enzymes (as indicated above each lane), separated on a 0.7% agarose gel, blotted onto positively charged filters and hybridised to murine calnexin cDNA. The 9.5kb KpnI and the 2.5kb HindIII fragments, both containing coding sequences of the murine calnexin gene, were subcloned into pBluescript vectors and further characterised.
Figure 3.4: Genomic organisation of the muine calnexin gene
Table 3.1. Exon-intron boundaries of murine calnexin

<table>
<thead>
<tr>
<th>exon (bp)</th>
<th>5' splice junction</th>
<th>intron (kb)</th>
<th>3' splice junction</th>
<th>exon</th>
</tr>
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<td>1 (174)</td>
<td>CCAAAG nd</td>
<td>1.2</td>
<td>cacagGTCACC</td>
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<td>2 (74)</td>
<td>GTCAGGgtaag</td>
<td>0.5</td>
<td>tacagGTGGAT</td>
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<tr>
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</tr>
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</tr>
<tr>
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<td>AGCCTG nd</td>
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<td>6</td>
</tr>
<tr>
<td>6 (193)</td>
<td>CATTAAgtaag</td>
<td>nd</td>
<td>nd TCTTGA</td>
<td>7</td>
</tr>
</tbody>
</table>

3.3.3. Isolation of a genomic p23 fragment

One of the first p24 family members (gp25L) was identified from rough pancreatic microsomes and found to be associated with calnexin. However, at this time the term “p24 family” did not exist, and was coined later with the identification of homologous proteins in yeast. In the following years, many more family members were found in yeast and mammalian cells and it became clear that the p24 family had been conserved throughout evolution. In a screen for genes involved in Alzheimer’s disease the human homologue of p23 was cloned and named s31.

The complete human s31 cDNA was used as a probe to screen the murine 129/Sv genomic DNA λ 2001 phage library. Out of approximately 1x10^7 phage clones, 14 positively hybridising clones were picked. All 14 phage clones were purified by two additional hybridisation steps, 9 clones remained positive and thus contained genomic p23 sequence.

Preliminary sequence analysis revealed that 8 of the 9 clones did not show any exon-intron structure. Their DNA sequence was ~70% homologous to the p23 cDNA but was missing any stop codons. Hybridisation of the first exon to a multiple restriction enzyme blot of genomic 129/Sv DNA revealed more than one band in most of the lanes, even when the restriction enzymes did not cut within
Figure 3.5. Hybridisation of genomic DNA from 129/Sv mice with the first exon of the murine p23 gene

<table>
<thead>
<tr>
<th>Apal</th>
<th>BamHI</th>
<th>ClaI</th>
<th>EagI</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>KpnI</th>
<th>NotI</th>
<th>Marker</th>
<th>PstI</th>
<th>SacI</th>
<th>SacII</th>
<th>SalI</th>
<th>SphI</th>
<th>XbaI</th>
<th>Xhol</th>
</tr>
</thead>
</table>

The first p23 exon hybridised to more than one fragment in the majority of lanes in genomic Southern blot of 129/Sv murine DNA, even when the enzyme used for the digest did not cut within the first exon, suggesting that a p23 pseudogene exists in the murine genome.
Figure 3.6. Characterisation of the phage $\lambda$ 2001 clone #10 that contains the first exon of the murine $p23$ gene

Phage DNA prepared from clone #10 was digested with a variety of restriction enzymes (shown above each lane), separated on a 0.7% agarose gel, blotted onto positively charged filters and hybridised to the first 400bp of the murine $p23$ cDNA. The 8.5kb HindIII, the 4.5kb Xhol and the 2.5kb Xbal fragments, all of which contained the first exon of the $p23$ gene, were subcloned into pBluescript vectors and further characterised.
Figure 3.7: Genomic organisation of the murine \textit{p23} gene

map of restriction enzymes
the first exon (fig. 3.5.). It is very likely that the additional bands arise from multiple integration sites of a non-functional p23 transcript into the genome.

The remaining clone, #10, which contained the first exon was further characterised (fig. 3.6.). Three overlapping fragments (a 8.5kb HindIII, a 4.5kb XhoI, and a 2.5kb XbaI fragment) were isolated from clone #10 and subcloned into pBluescript vectors. These subclones were subjected to further restriction mapping as shown in figure 3.7. None of the subcloned fragments, nor the initial phage clone contained additional exons.

### 3.3.4. Targeting strategy and vector design

Most gene targeting experiments aim to abolish completely gene function by introducing a null mutation into the gene of interest. The generation of a null mutation involves either insertion of a targeting vector into the reading frame, or replacement of parts of the genomic locus including coding sequence, by homologous recombination between the targeted locus and the targeting vector. In both cases it is desirable that the insertion takes place as close to the translational start site as possible to prevent the production of truncated proteins that may possess partial activity and therefore obscure the phenotypic analysis. However, many genes exhibit variability at their 5’ end (such as alternative transcriptional or translational start sites) and the targeting vector can be spliced out or protein translations could start 3’ of the integration site - in both scenarios the gene targeting would be ineffective.

To date, such alternative start sites for calnexin or p23 have not have been described. Both genes contain a signal sequence at their 5’end which determines the initial intracellular localisation and which is post-translationally removed. For efficient targeting, i.e. a high frequency of homologous recombination, 8-10 kb genomic sequence should ideally be isolated from a isogenic library and characterised. A typical targeting vector consists of two homologous regions the “short” and “long” arms located on either side of the selectable marker used to replace the coding region. The short arm consists of at least 500bp and the long
arm 4-5 kb of homologous DNA 5' and 3', respectively, of the coding section that will be interrupted or replaced.

Screening the ES cell clones for correct targeting is carried out using at least two different probes – an external probe which hybridises 5' or 3' to the homologous regions of the targeting vector, and an internal probe which in most cases recognises the selectable marker. The selectable marker introduces additional restriction enzyme sites into the targeted gene. The “new” restriction sites alter the size of particular restriction fragment length polymorphisms (RFLPs) and the alterations in the genome can be detected by Southern blotting. Alternatively, restriction sites can be removed by gene targeting but it has to be taken into consideration that larger restriction fragments could also occur if the genomic DNA is only partially digested. It is therefore preferable to detect introduced restriction sites which result in shorter DNA restriction fragments.

3.3.5. Constructing the calnexin gene targeting vector

Before designing the targeting vector, several probes were tested for their suitability for Southern blotting. A HindIII-Apal fragment (including the second exon) was chosen as the external probe. When hybridised to 129/Sv genomic DNA this fragment gave rise to a 11kb band in a KpnI digest and a 10kb band on an EcoRI digest. In general, it is useful if the probe contains some coding sequence because the coding sequence of a certain gene is normally only represented once in the genome and these probes are less likely to hybridise to unspecific repetitive elements.

As shown in figure 3.8, the calnexin targeting vector consisted of: a) a short and a long arm, b) a positive and negative selection marker between two loxP sites and an additional negative selection marker at the 5' end.

The 1.8 kb Apal-HindIII fragment, consisting of exon 3 and half of exon 4 was used as the short arm and a 3.8kb EcoRI-HindIII fragment (containing exon 6) was used as the long arm. The selectable markers, Neomycin/HSV-tk flanked by loxP sites were subcloned between the targeting vector arms. The positive selection marker, (Neomycin resistance gene (neo')) allows survival of those ES
Figure 3.8. *Calnexin* targeting vector

**Germline configuration**

**Targeting vector**

**Targeted allele**
cells which have integrated the targeting vector into their genome when G418 is added to the culture medium. The Herpes Simplex Virus - thymidine kinase (HSV-tk) is a negative selection marker, inducing the death of ES cells in the presence of gancyclovir in the culture medium. The diphtheria toxin A (DTA) at the short arm of the targeting vector (fig. 3.8.) decreases random integrations (Yagi et al., 1990). DTA irreversibly ribosylates the eukaryotic elongation factor (EF2) resulting in its inactivation. This reaction is very effective: a single molecule of DTA is able to kill a cell. In correctly targeted ES cells, DTA does not integrate into the genome and therefore no toxic DTA protein is expressed.

Linearisation of the targeting vector before electroporation into the ES cells increases the frequency of the gene targeting events but renders the targeting vector susceptible to exonucleases, which are present in the ES cells. To prevent this short hairpin loops were ligated to the 5’ and 3’ ends of the targeting vector before electroporation (Nehls et al., 1996).

3.3.6. Constructing the p23 targeting vector

Finding a suitable probe to detect homologous recombination by Southern blot proved to be more difficult for p23 than for calnexin. Apart from the first exon, no coding sequence was available and probes which only contain intron sequence can hybridise to repetitive sequence and give rise to high background signal. Several fragments were tested and a 500bp HindIII-XhoI DNA probe recognised a 8.5kb BamHI restriction fragment on a genomic 129/Sv Southern blot. To reduce unspecific hybridisation the probe was competed with human placental DNA and murine COT DNA which both compete out hybridisation of the probe to repetitive sequences.

The p23 targeting vector was designed to replace the first exon of the murine p23 gene. To construct the targeting vector the 5’ sequence between HindIII and EcoRV of the 8.5 HindIII subclone was removed, and the 500bp SmaI-ApaI fragment containing the first exon was replaced by the Neomycin resistance gene (neo'). The additional BamHI site present in the neo' resulted in an extra 3.3kb signal in addition to the wild type 8.5kb band from genomic ES cell DNA.
Figure 3.9. *p23* targeting vector

**Germline configuration**

**Targeting vector**

**Targeted allele**

![Diagram of germline configuration, targeting vector, and targeted allele with restriction enzyme sites and distances marked.](image)
3.3.7. Introduction of the calnexin targeting vector into ES cells

Previous studies in the lab had optimised the conditions for ES cell electroporation and cell culture. ES cells were electroporated with the targeting vector and selected for G418 resistance as described in the methods section. 6 days after transfection, the first ES cell colonies became macroscopically visible and 3 days later 400 ES cell colonies were picked and screened for homologous recombination by Southern blot. Only one of the ES cell clones was correctly targeted at the calnexin allele. This clone, called CNX-14 was expanded and injected into blastocysts. All ES cell injections into blastocysts were done by Ian Rosewell at the ICRF transgenics unit. More ES cells were electroporated and five additional ES cell clone heterozygously deficient for calnexin were obtained. One of these ES clones was also injected into blastocysts.

Table 3.2. Summary of ES injection record for ES cell clones CNX-14 and CNX 12-40

<table>
<thead>
<tr>
<th>ES cell clone</th>
<th>chimeric mice</th>
<th>male/female ratio</th>
<th>coat colour</th>
<th>germline transmitters</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNX-14</td>
<td>18</td>
<td>9:9</td>
<td>90-100%</td>
<td>3-partial transmitters</td>
</tr>
<tr>
<td>CNX 12-40</td>
<td>21</td>
<td>7:10</td>
<td>80-90%</td>
<td>1-partial transmitter</td>
</tr>
</tbody>
</table>

As shown in table 3.2, injection of the ES cells clones into blastocysts gave rise to 18 and 21 chimeras for clones CNX-14 and CNX 12-40, respectively. Of the 18 CNX-14 chimeras, 9 were male and 9 female, and all chimeras displayed 90-100% of the agouti 129/Sv coat colour encoded by ES cell DNA. 3 chimera breeding pairs passed the calnexin mutation on to their offspring, indicating that one or other of the parents was carrying the mutated calnexin allele in its germ cells. The CNX 12-40 chimeric mice (7 male and 10 female) showed an 80-90% ES
Figure 3.10. Genotyping of *calnexit* targeted ES cell clones

Southern blot genotyping of a representative panel of 5 ES cell clones after *calnexit* targeting vector electroporation and positive selection. Genomic DNA was digested with Asp718.

The left panel shows hybridisation of the Southern blot with the 5' external probe A. The Southern blot was stripped and rehybridised with a an internal neo probe (probe B), shown in the right panel. Lane 2 shows in both panels specific homologous recombination with the *calnexit* gene, while lanes 1, 3, 4 and 5 show only wild type alleles in the left panel and multiple non-specific integrations of the targeting vector in the right panel.
cell contribution to the coat colour and only one breeding pair transmitted the calnexin mutation through the germline.

### 3.3.8. Introduction of the p23 targeting vector into ES cells

GK129 ES cells were electroporated with the p23 targeting vector and 5 days after electroporation the first ES cell colonies became visible. 8 days after electroporation 600, colonies were picked out of a total of ~1500 colonies. Southern blot analysis revealed that in three ES cell clones specific homologous recombination had taken place resulting in the disruption of one p23 allele.

**Table 3.3: Summary of ES injection record for ES cell clones s1 and s2**

<table>
<thead>
<tr>
<th>ES cell clone</th>
<th>chimeric mice</th>
<th>male/female ratio</th>
<th>coat colour</th>
<th>germline transmitters</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1</td>
<td>9</td>
<td>8:1</td>
<td>~77%</td>
<td>2- 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-partial transmitter</td>
</tr>
<tr>
<td>s2</td>
<td>18</td>
<td>10:8</td>
<td>~90%</td>
<td>1- 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-partial transmitter</td>
</tr>
</tbody>
</table>

Two independent ES cell clones, s1 and s2, were injected into blastocysts and gave rise to 9 and 18 chimeric mice, respectively. As shown in table 3.3., 8 of the 9 s1 chimeras were male and showed an average 77% ES cell contribution as judged by coat colour. Two of the chimeras, with 100% ES cell contribution to the coat colour, transmitted the p23 mutation through the germline (one even to all of its offspring). The s2 chimeras showed about 90% ES cell contribution to the coat colour. One breeding pair consisting of chimeric parents passed the p23 mutation on to 70% of their offspring and one male chimera only gave rise to agouti offspring.
Figure 3.11. Genotyping of \( p23 \) targeted ES cell clones

Southern blot genotyping of a representative panel of 6 ES cell clones after electroporation with the \( p23 \) targeting vector and positive selection. Genomic DNA was digested with BamHI.

The left panel shows hybridisation of the Southern blot with a 3' external probe (probe A). The Southern blot was stripped and rehybridised with an internal neo probe (probe B) shown in the right panel. Lane 3 in both panels show specific homologous recombination with the \( p23 \) gene, while lanes 1, 2, 4, 5 and 6 show only wild type alleles in the left panel and non-specific integration of the targeting vector in the right panel.
3.4. Discussion:

The aim of this thesis was to generate two different mouse strains deficient for either p23 or calnexin to address their roles in vivo, in a multicellular mammalian organism. One of the first steps in such a gene targeting experiment involves the identification and cloning of the murine genomic DNA for the gene of interest. Once this has been accomplished, it is then possible to construct a targeting vector that will introduce specific mutations into the genes of interest upon homologous recombination. This chapter describes the required steps from identifying a suitable probe for screening a phage library, to breeding the chimeric mice to obtain mice which are heterozygous deficient for the gene of interest.

3.4.1. Characterisation and mutation of the murine calnexin gene

Seven phage clones containing the calnexin gene were isolated from a murine 129/Sv genomic DNA library. Initial restriction digests revealed that the isolated clones were largely overlapping, so that only one phage clone (C1) was further characterised. Subcloning of fragments of phage clone C1 allowed the generation of a detailed restriction map of the murine calnexin locus. Southern blotting of phage C1 restriction fragments and subsequent hybridisation to calnexin cDNA sub-fragments revealed the 5' to 3' orientation of the phage clone C1, as well as the crude localisation of calnexin exons to specific restriction fragments. This analysis showed that the C1 phage clone contained 5 calnexin exons (exons 2-6), but lacked the first 174bp, including the ATG initiation codon. The identified exons were called exons 2-6, under the assumption that the first 174bp would only compromise one exon.

Sequencing reactions using primers which anneal to the cDNA sequence of calnexin were performed on genomic subclones and comparison of the genomic sequence to the known cDNA sequence identified the exon-intron boundaries in the subcloned calnexin fragments, as shown in table 3.1.. The same primers were used in PCR reaction to amplify from one exon across the intervening intron to
the next exon. In this way exons could be accurately located within the overall calnexin restriction map.

Restriction mapping studies allowed the generation of a specific targeting vector for the calnexin gene where one and a half exons were replaced with genes coding for neomycin resistance (neo') and the Herpes Simplex Virus thymidine kinase (HSV-tk) upon homologous recombination. In addition, the restriction map was necessary to design a Southern blot strategy to identify ES cell clones that had undergone homologous recombination rather than random integration after electroporation of the calnexin targeting vector. Such a screening strategy predicted that after homologous recombination, the external probe A would detect both germline and recombinant Kpn I restriction fragments of 11kb and 5.5kb, respectively (fig. 3.10.). Rehybridisation of the Southern blot with the internal neo' probe gave rise to only one band of the expected size, suggesting that a single integration of the targeting vector had occurred in the genome. It is crucial that the targeting vector has only integrated at once (in the gene of interest) in the genome because a second integration may disrupt the coding region of an additional gene. Such an event may obscure the analysis of the phenotype resulting from disrupting the gene of interest. If the second integration had occurred on the same chromosome it is very difficult analyse the phenotype of the generated knockout mice.

Two separate electroporation experiments were performed with the calnexin targeting vector. The first electroporation resulted in only one correctly targeted ES cell clone but the second in 5 correctly targeted ES cell clones, from a total of 800 ES cell clones analysed. The high variation in correctly targeted ES colonies between the two electroporation experiments is due to inherited variation in culturing the ES cells. It has been observed in our lab, that although no reagents or the ES cell culture methods had been altered the number of correctly targeted ES clones varied significantly between two independent electroporations. To confirm that the phenotype in the KO mice resulted from the absence of the targeted gene and was not due to insertional mutagenesis after a non-specific integration event or an independent mutation, KO mice should be derived from two independent ES cell clones. It is very unlikely that the same
unspecified event takes place in two independent ES clones. Although diphtheria
toxin A (DTA) was used as negative selectable marker and should reduce the
number of ES cell clones with random integration of the targeting vector, the
overall frequency of homologous recombination was quite low with only 0.75% of
the screened ES cell clones were correctly targeted.

The use of the Cre-loxP system allows double transfection of ES cells with
the same targeting vector, thus generating homozygous deficient ES cells. In the
first step ES cells are electroporated with the targeting vector and selected for
homologous recombination in the presence of G418. ES cells with one successfully
targeted calnexin allele are then transfected with the Cre protein to remove the
selectable marker genes. The ES cells, now without any selectable marker, should
not be sensitive to gancyclovir and these ES cells (already heterozygous deficient
for calnexin) can then be electroporated with the same targeting vector to obtain
homozygous deficient calnexin targeted ES cells.

A promising aim of this project was to study the role of calnexin in T cell
development, but as calnexin is involved in the folding of a variety of newly
synthesised polypeptide chains the disruption of calnexin could possibly be lethal
at a very early embryonic stage. The advantages of “floxing” the selectable
marker are that multiple studies can be carried out with homologous calnexin-
deficient ES cells even if the knockout is embryonic lethal. Thus ES cells could be
injected into blastocysts from mice which are completely deficient for a non-
essential cell type, such as RAG-1 KO-mice which lack any T cells and B cells. If
the resulting chimeras develop T cells or B cells these lymphocytes must have
been derived from the calnexin-deficient ES cells and the resulting chimeric mouse
would allow the study of the role of calnexin in T cell and B cell development.
Deletion of selectable markers also would confirm that the phenotype seen in the
knockout mice is exclusively due to absence of the targeted gene and does not
result from expression of the marker genes.
3.4.2. Characterisation and mutation of the murine \( p23 \) gene

9 phage clones that hybridised with \( p23 \) cDNA sequences were isolated from a murine \( \lambda \) 2001 phage library. Initial characterisation revealed that 8 out of 9 phage clones did not show any exon-intron boundaries instead containing the genomic murine \( p23 \) cDNA sequence. Sequencing analysis of these phage clones revealed approximately 70% homology to the \( p23 \) cDNA and an absence of stop codons at the 3’ terminus of these \( p23 \) sequences. In addition, hybridisation of a multiple restriction enzyme blot with the first exon of \( p23 \) gave rise to more than one band in the majority of restriction digests, even when the enzymes used did not cut within the exon. It is therefore likely that a non-functional transcript of \( p23 \) had integrated randomly into the murine genome and thus created a \( p23 \) pseudogene. A non-functional pseudogene has recently been described for the human \( p23 \) homologue (Horner et al., 1999). It appears unlikely that the detected \( p23 \) pseudogene would give rise to a functional \( p23 \) protein because the C-terminus which has been shown as crucial for the specific function of this p24 family member was extensively altered (Dominguez et al., 1998; Sohn et al., 1996). At this state it could not be ruled out that this potential pseudogene is transcribed and would give rise to truncated \( p23 \) proteins. However, as \( p23 \) deficiency clearly results in a lethal phenotype it is highly unlikely that the potential pseudogene gives rise to functional protein which is closely related to \( p23 \).

One phage clone (#10) containing the intron-containing genomic sequence was further characterised. Although 11kb of genomic DNA were isolated and subcloned, Southern blotting and sequence analysis revealed that phage clone #10 only contained the first exon, including the ATG of the murine \( p23 \) gene. Most of the flanking sequence was 5’ of the first exon and about 2kb 3’ which indicates that the first intron is at least this size. Nevertheless, the obtained genomic sequence allowed the generation of a targeting vector replacing the first exon by homologous recombination into the genome of targeted ES cells.

The restriction map allowed the design of a Southern blot strategy to distinguish between homologous recombination and random integration. DNA of ES cell clones which were resistant to G418 after electroporation of the targeting vector was digested with BamHI and screened by Southern blot (fig. 3.11.).
Hybridisation with an external probe A resulted in an 8.5kb band for the wild type and a 3.5kb band for the mutated allele, as predicted from the restriction map (fig. 3.7).

600 ES cell colonies out of ~1500 which were obtained from one single electroporation were screened and 3 ES cell colonies in which specific homologous recombination had taken place were identified. The targeting frequency (0.5%) was low but the targeting vector did not contain any negative selectable markers which may have increased the frequency of homologous recombination. The initial Southern blot was confirmed by rehybridisation with the neo' probe which gave rise to a single band at the expected size, confirming that only one targeting vector had integrated into the genome and mutated one p23 allele.

3.4.3. Injection of targeted ES cells into blastocysts

Some general statements can be made regarding the ability of various ES cell clones to achieve germline transmission of the mutant allele. Germline transmission was positively correlated with a high male to female sex bias and production of high numbers of chimeric animals generated from a standardised number of 200 blastocysts injections. Furthermore, high level ES cell contribution to the chimeric animal correlated with increased probability of ES contribution to germ cells. The ability of ES cells to contribute to various tissues of the chimeric animal is thought to be purely random. It should therefore be theoretically possible for some chimeras with low levels of coat colour contribution to give rise to the germ cell compartment. However, in practice, this scenario did not occur. Thus, high level ES contribution to the coat colour of a chimera was a strong indicator of potential germline transmission, although many high level chimeric animals were unable to colonise the germline. This may be due to variability in either targeted ES cell clone totipotency or inability to compete with wild type host ES cells for the microenvironment of the inner cell mass of the host blastocyst.
The GK129 cell line which was used for all targeting vector electroporations was able to generate high level contribution to chimeric animals including the germline, as previously reported (Otto et al., 1997; Philpott et al., 1992). Once germline transmission of the mutant allele was achieved, initial phenotypic studies were undertaken to define the role of p23 and calnexin during the development of the mouse.
Analysis of calnexin-deficient mice

4.1. Introduction

Newly synthesised membrane and secretory proteins are translocated into the endoplasmic reticulum (ER). In the ER, the polypeptide chains are folded to their final secondary, tertiary and quaternary structures – and only correctly folded proteins leave the ER. Protein folding in living cells is a complex and error-prone process. To ensure that only correctly folded proteins leave the ER, the ER possesses an elaborate “quality control” system (Hurtley and Helenius, 1989). This “quality control” is performed by molecular chaperones, which are localised in the lumen (e.g. calreticulin, BiP) and the membranes (calnexin) of the ER. Given the very high protein concentration in the ER, (approx. 100mg/ml) the chaperones must work very efficiently to avoid the formation of protein aggregates; immature folding intermediates are particularly susceptible to misfolding and aggregation. Instead of being exported from the ER, incorrectly folded proteins are retained and subsequently become degraded. In the recent years the association of newly synthesised polypeptide chains with the molecular chaperone calnexin has been extensively studied.

4.1.1. The interaction of calnexin with molecules relevant for immune responses

Cell-cell communication of immunocompetent cells mostly takes place at the cell surface. However, correct folding is crucial for the function of the highly specific and sophisticated interactions that occur between the major histocompatibility complex (MHC) class I and II, T cell receptor (TCR) and B cell
Chapter 4 Analysis of calnexin-deficient mice

receptor (BCR). Surface expression of mal- or non-functional receptors could have fatal consequences for the immune response (e.g. autoimmune disease or lack of immune response to infectious agents). The folding of surface receptors that drive the immune response is strictly controlled in the ER by molecular chaperones such as calnexin, and only correctly folded proteins are allowed to leave the ER and to be presented at the surface. The involvement of calnexin in the assembly of MHC I, MHC II, TCR and BCR has been reported in the last few years.

4.1.1a. Assembly of the MHC I complex in the ER

The MHC I complex consists of a polymorphic α heavy chain (HC or H chain), β2-microglobulin (β2-m) and an 8 – 10 amino acid peptide which is “loaded” onto the heavy chain. The physiological function of the MHC I complex is to bind short endogenous peptide sequences in the ER and present them to cytotoxic CD8+ T cells (Krangel et al., 1979). The HC and β2-m assemble and then bind to a transmembrane molecule called the transporter associated with antigen processing (TAP) (Ortmann et al., 1994; Suh et al., 1994). TAP loads the processed peptide fragments, which are derived from endogenous proteins onto the heavy chain. The loading of the peptide causes conformational changes in the HC resulting in the release of the MHC I complex from TAP (Ortmann et al., 1994; Suh et al., 1994). The fully assembled MHC I complex is then transported via the Golgi apparatus to the cell surface. The presentation of endogenous peptides is a control mechanism of the immune system to check whether non-self proteins are synthesised which would indicate a viral infection. An infected cell presents non-self virus-derived peptides, which are recognised and killed by CD8+ T cells, thus controlling the spread of the virus.

Several reports suggested an involvement of calnexin in MHC class I H chains folding. Newly synthesised H chains associate with calnexin shortly after they enter the ER, thereby avoiding rapid degradation (Degen and Williams, 1991; Jackson et al., 1994). In addition calnexin has the ability to retain unassembled H chains within the ER - this capacity has been described in different studies. In the human β2m-deficient cell line, FO-1, neither MHC I molecules nor H chains are
expressed on the cell surface; the unassembled free H chains are stably bound to calnexin and retained within the ER (Romagnoli and Germain, 1994). Transfection of the FO-1 cells with a truncated calnexin protein missing the cytoplasmic domain which includes the ER retention/retrieval motif confirmed the interaction between calnexin and HC as both were now expressed together at the cell surface (Romagnoli and Germain, 1994). Overexpression of β2-m in FO-1 cells led to rapid dissociation of H chains from calnexin (Sugita and Brenner, 1994), suggesting that the presence of β2-m may be sufficient to trigger the release of H chains from calnexin. These results support the finding that the human H chain is either associated with β2-m or with calnexin, but not with both molecules (Nossner and Parham, 1995). In addition, dissociation rates of the H chain from calnexin were indistinguishable between a human peptide transporter-deficient cell line and a closely related control cell line, suggesting that in humans, peptide loading to the H chain is not crucial for its dissociation from calnexin (Sugita and Brenner, 1994).

Expression of murine H chains and β2-m in Drosophila melanogaster cells resulted in their rapid transport to the surface, where both free H chain and H chain β2-m complexes were found (Jackson et al., 1994). Drosophila cells do not possess a homologue of mammalian calnexin for retaining molecules within the ER. However, coexpression of murine calnexin with the H chain and β2-m remarkably reduced the rate of surface expression (Jackson et al., 1994). In contrast to human cells, binding of β2-m to the H chain in murine cells does not trigger its release from calnexin, because β2-m binds to the H chain while this is still associated with calnexin (Carreno et al., 1995). In addition dissociation rates of H chain-β2-m complexes from calnexin correlate with transport of fully assembled MHC I to the Golgi apparatus (Degen and Williams, 1991; Jackson et al., 1994).

Human H chains are possibly under tighter control by calnexin than their mouse counterparts, because in human cells lacking β2-m no H chain is found on the cell surface, whereas in mouse cells deficient for β2-m a small proportion of free H chains escapes to the surface (Raulet, 1994). This difference between mouse and human cells is not due to species specific variation of calnexin homologues, but rather due to species specific differences in the H chains because murine H
chains display mouse specific behaviour when transfected into human cells (Nossner and Parham, 1995).

**Figure 4.1. Schematic representation for the MHC I processing pathway**

Newly synthesised heavy chains are translocated into the ER where they associate with calnexin. The β2-m assembles with the heavy chain to form a dimer. Upon binding of small peptides, derived from endogenous proteins, the dimer is released from calnexin and the fully assembled MHC I complex is transported to the cell surface.

Association of calnexin with H chains is dependent on monoglucosylated residues of the H chains. However, while it appears that the Glu1Man4GlcNAc2 oligosaccharides are crucial for initial recognition, they are not necessary to maintain association (Ware et al., 1995; Zhang et al., 1995). In the murine RMA-S cell line (which lacks one of the TAP subunits) “empty” H chain and β2-m dimers
without the peptide are mainly retained in the ER by calnexin. The majority of such dimers do not show any glucosylation, indicating that they have matured beyond the stage at which glucose residues are removed but they are still associated with calnexin in a glucosylation-independent manner. The species-specific differences between the human and mouse H chain association properties with calnexin could be due to differences in their glucosylation (Parham, 1996). These differences may also in part explain the different affinities of calnexin for different mouse MHC I allotypes and the resulting variations between their transport rates out of the ER (Capps and Zuniga, 1994).

In addition to calnexin there are other molecular chaperones in the ER, which have been shown to interact with newly synthesised proteins, e.g. BiP and calreticulin. Of all known molecular chaperones, calreticulin is most closely related to calnexin. It also interacts with unfolded proteins in a glycosylation-dependent manner and is also involved in the folding of the MHC I complex subunits (Margolese et al., 1993; Nossner and Parham, 1995; Solheim et al., 1995; van Leeuwen and Kearse, 1996b). However, calnexin contains a transmembrane domain, which has also been implicated in the binding of nascent proteins and therefore could fulfil distinct functions from luminal chaperones like calreticulin (Margolese et al., 1993).

Surprisingly, the calnexin-deficient cell line CEM NK\(^R\) does not show any differences in MHC I assembly, transport, expression or peptide loading compared to the closely related CEM cell line which does express calnexin (Prasad et al., 1998; Scott and Dawson, 1995). CEM NK\(^R\) was derived from the human T lymphoblastoid cell line CEM, but has lost its susceptibility to natural killer (NK) cell-mediated lysis (Howell et al., 1985). It could be possible that other chaperones, like calreticulin take over the function of calnexin in the CEM NK\(^R\) cells. However, the association properties of calnexin and calreticulin are clearly distinct. In human cells, calreticulin appears to interact with H chains after their release from calnexin (Sadasivan et al., 1996). Similarly, in mouse cells calnexin binds newly synthesised proteins prior to calreticulin. This was shown in murine cells which lack \(\beta_2\)-m, in these cells H chain association with calreticulin was
clearly reduced, indicating that initial binding of newly synthesised peptides is calnexin dependent (van Leeuwen and Kearse, 1996b).

4.1.1b. Association of calnexin with subunits of the MHC II complex

The assembly of MHC II molecules consists of two distinct steps (fig. 4.2.). In the first step the \( \alpha \) and \( \beta \) chains are assembled to a dimer which then binds the invariant chain (Ii) - this step takes place in the ER (Lamb and Cresswell, 1992). The immature nonameric (three trimers) MHC II complex, consisting of 3 \( \alpha \), 3 \( \beta \) and 3 Ii chains, exits the ER and reaches the endosomal compartment, where the second assembly step takes place (Roche et al., 1991). In endosomes the Ii chain is removed from the \( \alpha \beta \) dimer and replaced by a short peptide antigen. The Ii chain stabilises the \( \alpha \beta \) dimer and occupies the peptide binding side to prevent inappropriate peptides from associating with the MHC II complex in the ER (Roche et al., 1991). The now fully assembled and functional MHC II complex is expressed at the cell surface, where it presents the processed antigens to CD4\(^+\) T helper cells.

Calnexin has been shown to be associated with partially assembled \( \alpha \), \( \beta \) and Ii chains (Schreiber et al., 1994). It remains associated with the immature MHC II until the nonameric transport complex is fully assembled and ready to leave the ER (Anderson and Cresswell, 1994). It is likely that calnexin facilitates the assembly of the nonameric complex and thus regulates MHC II exit to the Golgi. As is the case for MHC I subunits, the MHC II subunits interact with calnexin in a glucose residue-dependent fashion. Mutating the N-glycosylation site of the invariant chain inhibits its association with calnexin and results in its pre-endosomal degradation suggesting that calnexin prevents degradation of immature Ii chains (Romagnoli and Germain, 1994).

Newly synthesised \( \alpha \) and \( \beta \) chains transiently form aggregates before their assembly with the invariant chain (Marks et al., 1995). Calnexin does not interact with such aggregates but the molecular chaperone BiP does. Calnexin only interacts with \( \alpha \) and \( \beta \) chains released from aggregates suggesting that calnexin and BiP possess distinct functions in the assembly of the MHC II complex.
Alternatively, α and β chains which are not immediately able to associate with calnexin could form aggregates, which bind to BiP and thus wait either to bind to the Ii chain or to calnexin. The number of α and β aggregates is higher in Ii deficient cells indicating that Ii could be a molecular chaperone itself and prevent aggregation by binding to α and β chains (Romagnoli and Germain, 1994). Remarkably, calreticulin has not yet been reported to be involved in MHC II folding.

Figure 4.2. Schematic representation of the MHC II processing pathway

Newly synthesised subunits of the MHC II complex associate with calnexin. The α, β (independently and as dimers) and the invariant chain interact with calnexin. A nonameric complex consisting of three α, β and invariant chains exit the ER. In endosomes the invariant chain is cleaved and removed from the αβ dimer and peptides derived from exogenous proteins are loaded to the αβ dimer. The completely assembled MHC II complex is then expressed at the cell surface.
4.1.1c. The role of calnexin in the assembly of the T cell receptor

Assembly of the T cell receptor (TCR) takes place in the ER and proceeds in a stringent fashion. It starts with the non-covalent assembly of the CD3 ε and δ and ε and γ chains forming εδ and εγ dimers. The clonotypic α and β chains are then bound to εδ and εγ dimers, respectively, to form αεδ and βεγ sub-complexes. These sub-complexes rapidly assemble to generate αβεδεγ complexes and finally the ζζ homodimer is added to form the fully functional TCR complex (Exley et al., 1991; Klausner et al., 1990). Only fully assembled αβδεγζζ TCR and the incomplete αβδεγε TCR leave the ER, although the latter never reaches the cell surface because it is degraded in lysosomes (Klausner et al., 1990).

The majority of TCR subunits interact with calnexin (David et al., 1993; Hochstenbach, 1992; Kearse et al., 1994; van Leeuwen and Kearse, 1996a; Wiest et al., 1994). Only the ζ chain has never been reported to bind to calnexin (Melnick and Argon, 1995). Although, most subunits are glycosylated (which has been shown to be a prerequisite for the interaction with calnexin) the ε chain clearly associates with calnexin, although it is not N-glycosylated (Rajagopalan et al., 1994). Inhibition of the removal of glucose residues from α chains after castanospermine (which inhibits removal/trimming of glucose residues) treatment led to rapid degradation of α subunits and consequently to a reduced association of α and β chains (Kearse et al., 1994). In addition in a human cell line deficient for α chain expression shows a prolonged association of β chains with calnexin, indicating that β chains cannot form αβ heterodimers in the absence of α chains and are retained in the ER (David et al., 1993).

In immature T cells calnexin has been found on the cell surface together with clonotype independent CD3 (CD3γε and CD3δε) (Wiest et al., 1995; Wiest et al., 1994). It is believed that the cytoplasmic domain of the ε chain masks the ER retention/retrieval signal of calnexin and thus enables the “escape” to the cell surface (Wiest et al., 1995). Recently, calnexin has also been found in association with the pre Tα-β complex on the cell surface of immature T cells (Takase et al., 1997). Calnexin is not the only ER resident molecule “escaping” from the ER: other ER resident proteins and molecular chaperones have also been detected on the surface immature T cells (Wiest et al., 1997). It appears that the temporary
expression of complexes consisting of newly synthesised proteins plus ER resident molecules is a common and transient phenomenon in T cell development and may be involved in signal transduction (Takase et al., 1997; Wiest et al., 1997).

Calreticulin has also been reported to interact with subunits of the TCR, but in contrast to calnexin, calreticulin only associates specifically with TCR α and β chains and not with CD3 subunits (van Leeuwen and Kearse, 1996a).

4.1.1d. Calnexin is involved in the assembly of B cell receptors

The interactions of calnexin with subunits of the B cell receptor (BCR) are poorly studied. However, evidence exists that calnexin is involved in the correct folding of members of the BCR (Grupp et al., 1995; Lassoued et al., 1996; Nagata et al., 1997). The μ chain of immunoglobulins associates with calnexin in the ER and the expression of Igα initiates release of the μ chain (Lassoued et al., 1996). Similar to association of calnexin with subunits of the immature TCR (pTα) (Takase et al., 1997), calnexin is found on the cell surface of immature μ-negative pro B cells associated with Igα/β heterodimers (Nagata et al., 1997).

4.1.2. The influenza hemagglutinin protein interacts with calnexin

The influenza surface protein hemagglutinin (HA) has been shown to interact with calnexin during its maturation/folding in the ER (Hammond et al., 1994; Hebert et al., 1995; Tatu et al., 1995). The mature trimeric HA molecule does not interact with calnexin but all of its 6 folding intermediates do (Hebert et al., 1995; Hebert et al., 1996; Tatu et al., 1995). As expected, HA association with calnexin again occurs in a monoglucosylation-dependent manner and dissociation from calnexin was inhibited when trimming of the final glucose residue was inhibited (Hammond et al., 1994; Hebert et al., 1995; Hebert et al., 1996).

Calreticulin also interacts with HA, primarily with early folding intermediates. In contrast calnexin associates with all immature HA forms (Hebert et al., 1997; Peterson et al., 1995). HA, calnexin and calreticulin have been found in a trimeric complex whereby calnexin and calreticulin were associated with different domains of the HA molecule (Hebert et al., 1997). The luminal
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Chaperone, calreticulin, was exclusively bound to the top/hinge domain of HA, whereas calnexin was less selective and bound the top/hinge domain as well as the stem domains close to the membrane. Calnexin preferentially binds to the stem domains of HA (Hebert et al., 1997). Thus, calnexin and calreticulin promote folding and prevent degradation of HA folding intermediates. It appears however, that their function does not completely overlap because calnexin and calreticulin show distinct specificities for the intramolecular location of the glucose residues and the different HA folding intermediates (Hebert et al., 1997).

4.1.3 calnexin and the acetylcholine receptor

The nicotinic acetylcholine receptor (AChR) is a pentameric complex consisting of two α and one β, γ and δ chain each; the γ chain is replaced shortly after birth by the ε chain (reviewed by Hall and Sanes, 1993; Karlin and Akabas, 1995). Assembly of the AChR takes place in the ER and α, β and δ subunits have been shown to interact with calnexin (Chang et al., 1997; Gelman et al., 1995; Gelman and Prives, 1996; Keller et al., 1996; Keller et al., 1998). The folding intermediates assemble with calnexin in a glucose-dependent manner. Mature AChR subunits are released from calnexin prior to complete folding (Gelman et al., 1995). The α, γ and δ chains form αγ and αδ heterodimers (which do not associate with calnexin). The heterodimers are then associated with a β subunit and the fully functional pentameric complex is transported to the cell surface.

In summary calnexin associates with many newly synthesised proteins in a glycosylation dependent manner, stabilises them in their unfolded conformation and protects them from degradation. By doing so it enlarges the pool of subunits available for complete assembly to their tertiary and quaternary conformations. Despite the vast number of proteins that have been reported to interact with calnexin, this introduction has concentrated only on those proteins, which have been studied in the calnexin-deficient mice.
4.2. Results

4.2.1. Genotypic analysis of calnexin mutant mice

Germline transmission of the mutated calnexin allele resulted in mice which were heterozygously deficient for calnexin. Breeding of these mice produced wild type, heterozygous and homozygous offspring. To identify the different genotypes a PCR strategy was developed, using three different oligonucleotides (as shown in fig. 4.3a.). The oligonucleotide pair \( P_1 \) and \( P_2 \) amplified a 175bp fragment for the wild type allele and the oligonucleotide pair \( P_1 \) and \( P_{\text{neo}} \) gave rise to a 350bp fragment, from the targeted allele. Thus, a single PCR reaction distinguished between the three different genotypes: a 175bp band for wild type mice, a 350bp band for knockout mice and both bands for heterozygous mice. To confirm the PCR results, mice were also genotyped by Southern blotting. A typical Southern blot result and PCR reaction are shown in figure 4.4..

Figure 4.3a. PCR strategy to identify targeted calnexin alleles

PCR analysis of genomic DNA from calnexin mice using primers \( P_1, P_2 \) and \( P_{\text{neo}} \) will give rise to a 175bp band for the wild type allele, as \( P_1 \) and \( P_2 \) will anneal, and a 350bp band for the recombinant allele, as \( P_1 \) and \( P_{\text{neo}} \) will anneal.
The HSV-tk interferes with fertility of male and female mice

Two different ES cell clones (CNX 14 and CNX 12-40) which were used to produce chimeric mice transmitted the calnexin mutation through the germline. Crossing calnexin heterozygous male and female mice derived from the CNX 14 ES cell clone revealed that the majority of heterozygous male mice were sterile. In addition about 20% of the heterozygous female mice were infertile. Typically only one or two heterozygous breeding pairs out of ten produced any offspring. Litter sizes were very small with only 3-5 pups per litter and the parents usually stopped breeding after 4-5 litters. Breeding heterozygous mice derived from the CNX 12-40 clone has never produced any offspring, indicating that all males were sterile, because the majority of female mice have been shown to be fertile, when mated with C57BL/6 male mice.

It is likely that the fertility problems are caused by the selectable marker HSV-tk that has incorporated into the genome with the targeting vector, rather than the calnexin mutation. The expression of HSV-tk during spermatogenesis induces malformation of spermatids and thus results in infertility (Braun et al., 1990).

To overcome this problem, calnexin heterozygous mice were crossed with Zp3-cre transgenic mice. As described in 3.3.5. the calnexin targeting vector carried the selectable markers neo' and HSV-tk flanked by loxP sites. The loxP sites are recognised by the Cre protein, which combines two loxP sites and excises the intervening DNA sequence, leaving one loxP site at the targeted DNA region. Zp3-cre transgenic mice express the Cre protein under a zona pelucida specific promoter in oocytes (Lewandoski et al., 1997). Breeding Zp3-cre transgenic females with calnexin-mutant males resulted in F1 offspring, which carry the Zp3-cre transgene and the calnexin mutation. Female mice of the F1 generation produced offspring (F2), in which the selectable markers (neo' and HSV-tk) were completely deleted following excision in the oocytes. The F2 offsprings were genotyped by PCR (fig. 4.4c.). Theoretically, the use of the oligonucleotide pair P1 and P3 for the PCR reaction should have been sufficient to detect the three different genotypes (fig. 4.3b.). The oligonucleotide pair amplified a 1.3kb fragment for the wild type allele and a 300bp fragment for the recombinant allele,
which was missing ~1kb, the size of the deleted fragment of the genomic sequence between the two oligonucleotides (fig. 4.3b.). However, genomic DNA from heterozygous mice which should give rise to both fragments predominantly showed the smaller fragment (300bp), and the larger fragment (1.3kb) was not amplified by the PCR reaction in many DNA samples from heterozygous mice. This problem was solved by adding a third oligonucleotide (P₂) to the PCR reaction – the oligonucleotide pair P₁ and P₂ amplified a 175bp fragment from the wild type allele and the oligonucleotide pair P₁ and P₃ amplified a 300bp fragment from the recombinant allele. As shown in figure 4.4c. wild type mice were identified by a single 175bp fragment, knockout mice by a single 300bp fragment and DNA from heterozygous mice gave rise to both fragments.

Figure 4.3b. PCR strategy to identify targeted calnexin alleles after Cre transfection

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PCR analysis of genomic DNA from calnexin-targeted mice after excision of the selectable markers. Primer pair P₁ and P₂ will give rise to a 175bp band for the wild type allele and primer pair P₁ and P₃ amplifies a 300bp band for the recombinant allele.
Figure 4.4. Genotyping of *calnexin* targeted mice by Southern blot and PCR

Genomic DNA was extracted from tail biopsies and digested with KpnI. The Southern blot was hybridised with the 5' external probe. This Southern blot shows the genotyping of *calnexin* targeted mice carrying the HSV-tk in their genome.

PCR analysis was performed with three different primers as indicated in figure 4.3a, which amplified either the wild type (wt) or the mutated (mu) *calnexin* allele. These PCR results were obtained from mice which were carrying the HSV-tk gene in their genome.

This PCR analysis shows genotyping results from *calnexin* targeted mice which have the HSV-tk deleted from their genome. As schematically shown in figure 4.3b, the primers used amplify a 175bp wild type fragment and a 300bp fragment for the targeted *calnexin* allele which had the HSV-tk excised.
4.2.3. *Calnexin* knockout mice do not express calnexin protein

To confirm that *calnexin*-deficient mice did not express calnexin protein, Western blot analysis of liver protein lysates from wild type and calnexin knockout mice was performed (fig. 4.5.). Equal amounts of protein lysates from wild type and knockout mice were separated on a 12% SDS gel and blotted onto an immobilonP® membrane (Millipore). The Western Blots were incubated with two different anti-calnexin antibodies – the first antibody recognising the intraluminal N-terminal part (AA 30-48) of calnexin and the second antibody was specific for AA 555-573 of the cytoplasmic domain. Both antibodies were raised against canine peptides in rabbits and are both cross reactive with human, mouse, rat, pig, dog and hamster calnexin homologues. The polyclonal antibodies are commercially available from STRESSGEN.

Calnexin protein was only detectable in the liver lysates from wild type mice, while *calnexin* knockout mice did not show any calnexin protein or any truncated forms detectable by the anti-calnexin antibodies. In principle a truncated calnexin protein could have been made in *calnexin* knockout mice, because the targeting vector integrated in exon 4 leaving the first three exons intact. This would give rise to a 95 AA truncated protein, but such a protein would have been detected by the antibody against the N-terminus, but this was not the case.

4.2.4. Calnexin deficiency leads to postnatal death in ~50% of the new born mice

Mice were routinely genotyped 2 – 3 weeks after birth. Genotyping results from offspring of heterozygous parents did not show an expected Mendelian distribution between wild type : heterozygous : homozygous *calnexin* mice. Instead we identified 66 wild type, 126 heterozygous and 33 homozygous mice, suggesting that approximately half of the expected *calnexin* knockout mice had died (as shown in table 4.1a.).
Figure 4.5. Western blot analysis from wild type and calnexin deficient mice

Expression of calnexin analysed by Western blot analysis. Equal amounts of lysate from liver of wild type (wt) and calnexin knockout (ko) mice were separated on a 10% SDS gel. The two different lanes of each wt and ko sample are the supernatant and the pellet from the liver lysates. The upper blot has been incubated with an anti-calnexin antibody recognising the N-terminus (AA 30-48) and the lower blot with an anti-calnexin antibody against the C-terminus (AA 555-573). If a truncated calnexin protein is made in the calnexin deficient mice, the N-terminal antibody should detect a unique band in the which is present in liver lysates from knockout mice but not from wild type mice.
A large number of pups from heterozygous parents died within 24 hours after birth. Genotyping of the dead pups showed that the 28 out of 30 dead pups were \textit{calnexin} knockouts. With the genotyping results of the dead pups included into the statistics (table 4.1a.), the distribution of wild type : heterozygous : homozygous changes to 1 : 2 : 1 (table 4.1b.). This suggests that \textit{calnexin}-deficient embryos do not die before birth and are carried to full term. However, shortly after birth half of the new born \textit{calnexin} knockout pups die. The cause of death still remains to be determined.

At the beginning of our analysis, all the \textit{calnexin} knockout mice, which survived the first 24 hours after birth, died between two and three weeks of age. At birth, \textit{calnexin} knockouts were indistinguishable from their littermates. However, at 8-10 days after birth \textit{calnexin} knockouts could clearly be distinguished from wild type and heterozygous littermates by their size.

\textit{Calnexin} knockout mice display an obvious wobbly gait. They were less active in walking and the body shook during movements. They had difficulties balancing on their hind legs when reaching for food and water, which led us to the hypothesis that the early death could be caused or progressed by insufficient water and food uptake. Indeed, providing moist food in the cage so that the mice did not have to reach for they food remarkably prolonged the survival of some
Figure 4.6. Weight and length of calnexin knockout mice and wild type littermates at time of death

Panel a shows the weight in grams of calnexin deficient mice and wild type littermates when knockout mice had to be sacrificed or were found dead. In every case the calnexin-deficient mice were lighter than the wild type littermates. Panel b shows the length in centimetres of calnexin knockout mice and control littermates at time of death. Calnexin knockout mice appeared thinner that their littermates, which is supported by the observation that knockout mice were one third lighter but only about 10% shorter than their littermates.
calnexin mice. However, the average life span of a calnexin knockout mouse still was only 43.4 ± 31.3 days. The majority of calnexin-deficient mice died between 14 to 68 days of age, the high standard variation reflecting 3 mice, which lived 3, 4 and 5 months, respectively (fig. 4.6a.). The size difference between calnexin knockout mice and littermates was most prominent before weaning, however all calnexin knockouts were lighter (36.3%) and shorter (11.3%) than age- and sex-matched littermate controls as shown in table 4.2..

Table 4.2. Comparison of body weight and length of calnexin<sup>-/</sup> and wild type littermates

<table>
<thead>
<tr>
<th>weight/g (wt)</th>
<th>weight/g (ko)</th>
<th>ko/wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.66 ± 9.22</td>
<td>13.21 ± 6.49</td>
<td>63.7% ± 10.3%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>length/cm (wt)</th>
<th>length/cm (ko)</th>
<th>ko/wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.79 ± 1.01</td>
<td>7.81 ± 1.23</td>
<td>88.7% ± 8.0%</td>
</tr>
</tbody>
</table>

4.2.5. Characterisation of the calnexin knockout phenotype following the SHIRPA protocol

A full behavioural and functional analysis of the mice was performed following the SHIRPA protocol (Rogers <i>et al.</i>, 1997). The SHIRPA protocol provides a guideline which by the use of standardised evaluation methods allows a better comparative evaluation of any immediately visible abnormalities and does not rely on an exclusively descriptive assessment.

Calnexin-deficient mice and wild type littermates were analysed according to the SHIRPA protocol. All tested reflexes in the calnexin-deficient mice were normally developed and it appeared that the knockouts had no visual or acoustical deficiencies. However the calnexin<sup>-/</sup> mice suffered from truncal ataxia; the hind legs were abnormally splayed when walking and an intentional tremor of the forelimb was detected. Wild type mice that are placed head down on a vertical grid turn around and walk upwards. In contrast calnexin knockout mice
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4.2.6. Histopathological analysis of central- and peripheral nervous system

These observations suggested that calnexin-deficient mice show motoric disabilities similar to human patients suffering from neurodegenerative disease. Spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) are amongst the most frequent occurring diseases of that type. A characteristic phenotype of these diseases is the degeneration of large α-motor neurons in the anterior horn of the spinal cord. Serial section of the spinal cord from calnexin knockout and wild type mice showed some dark abnormally formed large cells in the spinal cord from calnexin knockout mice, likely to be degenerating α-motor neurons (fig. 4.7a.). SMA and ALS can be distinguished by anti-ubiquitin staining, while degenerative α-motor neurons from ALS patients are intensively stained by an antibody against ubiquitin, α-motor neurons from SMA patients appear largely negative (Urbanits and Budka, 1996). Preliminary results showed that the dark abnormal looking cells were negative for ubiquitin, although the background staining was high (fig. 4.7b.). This observation suggested that the calnexin knockout mice might suffer from a SMA-like disease. However, in a more detailed analysis the abnormal cells were also detected in spinal cord sections.
Figure 4.7. Spinal cord sections from wild type and *calnexin* knockout mice

**a.**

- Wild type
- *calnexin* knockout

- Anterior horn cells of spinal cord from wild type and *calnexin* knockout mice (indicated by arrow heads). The dark Nissl substance in the cytoplasm is well defined and distributed towards the periphery of the cells. Dark-staining Nissl substance obscured nuclear detail in the dark neurons (asterisk) noted predominantly in knockout mice. However, those cells were later also seen in wild type mice.

**b.**

- Wild type
- *calnexin* knockout

- Ubiquitin staining in wild type spinal cord sections showed staining of neurons (arrow heads) and moderate, relatively uniform cytoplasmic reactivity. Ubiquitin immunostaining of spinal cord sections from *calnexin* knockout mice showed that some neurons, especially dark appearing ones, were negative for ubiquitin (asterisk). However, a few ubiquitin negative cells were also noted in spinal cord sections from wild type mice.
Macroscopic comparison showed no obvious differences between brains from wild type and *calnexin* knockout mice.
from wild type mice indicating that these cells are highly unlikely to cause the phenotype in the \textit{calnexin} knockout mice. The tissue sections of spinal cord from \textit{calnexin} knockout mice were indistinguishable of those from wild type mice and did not show any further abnormalities.

Macroscopical comparison of whole brains did not show any obvious abnormalities in \textit{calnexin}\textsuperscript{1} mice (fig. 4.8.). Microscopical and analysis of sections from cerebellum did not show any structural differences between wild type and calnexin knockouts. In particular, the number and appearance of the Purkinje cells was very similar. Also sections through the cerebral cortex and mid brain showed identical structures between wild type and knockout mice. No morphological evidence of necrotic or apoptotic cell death in various organs of \textit{calnexin} knockout mice was detected either by light microscopy or TUNEL assays. The histological examinations and the SHIRPA analysis of \textit{calnexin} knockout mice were carried out in collaboration with Prof. Gordon Stamp, Hammersmith Hospital, London and with Prof. Jo Martin, Royal London Hospital, Whitechapel, London.

\textbf{4.2.7. Analysis of neuromuscular junctions in \textit{calnexin}-deficient mice}

Light microscopy of brain, cerebellum and spinal cord sections did not show any pathological alterations in the \textit{calnexin} knockout mice which would have explained the observed phenotype. The muscle compartment of the posterior lower hindlimb (gastrocnemius, soleus, plantaris), the quadriceps femoris and the diaphragm were analysed to investigate whether \textit{calnexin} knockout mice showed muscle degradation and/or denervation. The morphology of muscle biopsies from wild type and \textit{calnexin} knockout animals was analysed by light- and by electron microscopy. Immunohistochemistry was performed on muscle biopsies to investigate whether structural and functional proteins, which are affected in human neuromuscular diseases with a similar phenotype were affected by the loss of calnexin.

The acetylcholine receptor (AChR) subunits have been shown to associate with calnexin in the ER and calnexin is thought to mediate their folding (Gelman
Knockout mice, deficient for the AchR ε chain, die prematurely between two to three months after birth and are much lighter than their littermates (Witzemann et al., 1996), as observed in calnexin knockout mice. 10μm frozen muscle section were stained with an antibody specific for the AChR ε chain. Their analysis revealed that there was no obvious difference in AChR ε expression between wild type and calnexin knockout samples. These results were confirmed by counter staining with α-bungarotoxin, which binds to the acetylcholine binding site in the AChR α chain. Both stainings showed a physiological clustering of AChRs, indicating that the AChRs are functional and innervation of muscle fibre is not disturbed. The neuromuscular junctions did not show any apparent size alterations (fig. 4.10.).

Patients suffering from the muscle-wasting disease Duchenne muscular dystrophy show a lack of the protein dystrophin. Dystrophin is a component of the subsarcolemmal cytoskeleton and forms an oligomeric complex with the dystrophin-associated glycoproteins (DAG). Together these proteins link the extracellular matrix to the cytoskeleton of muscle fibres (Blake et al., 1994). Utrophin, an autosomally encoded homologue of dystrophin, is expressed during muscle development and is down regulated in adult muscle. It remains at the
Confocal microscopy on neuromuscular junctions on muscle sections from wild type and calnexin$^{-/-}$ mice. In panel a, the muscle sections were stained with antibodies against dystrophin (red) and α-bungarotoxin (green). Panel b, shows double immunofluorescence with utrophin (red) and α-bungarotoxin (green). In panel c the muscle sections were stained with N-CAM (red) and also counterstained with α-bungarotoxin (green) to identify the neuromuscular junctions.
neuromuscular junctions where it locates to the crest of postsynaptic folds, in contrast to dystrophin which is located at the base of the folds (Tinsley and Davies, 1993). Staining of muscle sections from wild type and calnexin-deficient mice with antibodies recognising dystrophin and utrophin, did not show any obvious abnormalities in calnexin knockout mice (fig. 4.10a&b.). Utrophin was also not detected in the outside line of the neuromuscular junction either, confirming that the muscle was not regenerating. In addition, the expression of neural cell adhesion molecule (N-CAM) was not significantly different between wild type and calnexin-deficient mice (fig. 4.10c.). N-CAM is down-regulated in the mature muscle but up-regulated on regenerating or denervated muscle fibres.

Recently, calnexin has been implicated in the folding of type I inositol triphosphate (IP₃) receptors (Joseph et al., 1999). One structurally similar protein, the ryanodine receptor, is found on the sarcoplasmic reticulum in muscles. The ryanodine receptor is responsible for efficient Ca²⁺ release from the sarcoplasmic reticulum and thus triggers muscle contraction (Barone et al., 1998). However, immunofluorescence staining with an antibody against the ryanodine receptor did not show any different expression between wild type and calnexin knockout mice (data not shown).

Electron microscopy showed a normal regular, striated appearance of the contractile apparatus with distinct I bands, A bands and M lines and no obvious signs of myofibrillar disruption. The morphology of mitochondria and triads (t-tubule and sarcoplasmic reticulum) appeared unaltered and normally located, suggesting that the machinery for excitation contraction coupling is preserved. Importantly, in diaphragm muscles which are necessary for respiration (and which are more severely affected in some muscular dystrophies) no ongoing regeneration or signs of muscle fibre degeneration were visible. The morphology of the postsynaptic membranes (with respect to the extent of folding) did not show any significant changes in all muscle samples examined (fig. 4.11.). The detailed analysis of the muscles and neuromuscular junctions was carried out in collaboration with Dr. Sue Brown, Neuromuscular Unit, Hammersmith Hospital, London.
Muscle samples of diaphragms were taken from three-week-old wild type and *calnexin*-deficient mice. At this age the neuromuscular junctions are immature and the folds are not yet fully developed. However, electron microscopy revealed that there are no obvious differences in the structural morphology of neuromuscular junctions from wild type and *calnexin* knockout mice.
4.2.8. Calnexin deficiency in the immature T cell compartment

Calnexin appears to be required for the correct folding of subunits of the TCR, BCR, MHC I and MHC II. To address the question whether the absence of calnexin has an effect on surface expression of these molecules thymus, spleen and lymph nodes from calnexin knockout mice and wild type littermates were analysed by FACS analysis. The expression of the surface molecules CD4 and CD8 reflects different stages of thymocyte development: thymocytes which do not express CD4 or CD8 are called double negative (DN) T cells. They develop to double positive (DP) thymocytes, which express both CD4 and CD8. DP T cells down regulate CD4 or CD8 and become mature single (SP) T cells. The mature T cells exit the thymus, enter the blood vessels and are now able to populate the lymphatic organs.

Thymocytes from calnexin knockout and wild type mice, stained with anti-CD4 and anti-CD8 antibodies, showed no abnormalities for these surface molecules (fig. 4.12a. middle panels). The ratios between DN : DP : SP thymocytes were normal, indicating that the relevant surface molecules required for T cell development were sufficiently expressed in calnexin knockout mice. This was consistent with surface staining for CD3e, which was indistinguishable between wild type and knockout mice (fig.4.12a. top panels). The pre-T cell receptor is only expressed on immature DN cells and has been found associated with calnexin on the cell surface (Takase et al., 1997). Surface staining for the pre-TCR is difficult because it is expressed at a very low density beyond detection levels by standard FACS analysis. To obtain a sufficient number of cells, 3 thymi from wild type and calnexin knockout mice were pooled and the cells were treated as described in 2.38. (chapter 2). Surface staining revealed that there was no significant reduction of the pre-TCR in calnexin knockout mice (fig. 4.12a. lower panels).

4.2.9. Consequences of calnexin deficiency for the B cell compartment

FACS analysis was also performed on the peripheral lymphoid organs, spleen and lymph nodes. The isolated cells were stained with antibodies against the B cell specific surface molecule B220 (CD45RA isoform) (which is already
Figure 4.12a. FACS analysis of thymocytes from wild type and *calnexin* knockout mice.

FACS analysis of thymocytes isolated from wild type and *calnexin*-deficient mice. In the upper panel the cells were stained with an antibody against the CD3ε chain of the TCR. The middle panel shows immunofluorescence staining with antibodies against CD4 and CD8. The lower panel shows staining for the pre-TCR of immature thymocytes.
Spleen cells were isolated from wild type and *calnexin*-deficient mice and the single cell suspensions were stained with antibodies against B220, MHC II, CD3ε and MHC I as indicated at each panel.
Figure 4.12c. FACS analysis of lymph node cells from wild type and \textit{calnexin} deficient mice

FACS analysis of lymph node cells isolated from wild type and \textit{calnexin} knockout mice. The single cell suspensions were stained with antibodies against B220, MHC I, CD3ε and MHC II as indicated at each panel.
expressed on immature B cells), the T cell specific CD3ε chain, MHC I and MHC II, respectively.

Staining with an anti CD3ε antibody revealed that the relative number of mature T cells isolated from spleen and lymph nodes is indistinguishable between wild type and calnexin knockout mice (fig. 4.12b. and 4.12c.; CD3ε panels). This observation is in agreement with the results obtained from surface staining of thymocytes with the same antibody, which also did not show any differences. Analysis of MHC I surface expression also revealed no significant differences between wild type and knockout cells (fig. 4.12b. and 4.12c.; MHC I panels). This result is consistent with the observation that the calnexin-deficient cell line CEM NKR does not show any alterations in MHC I expression (Prasad et al., 1998; Scott and Dawson, 1995).

However, FACS analysis of staining with antibodies against B220 and MHC II showed clear differences between wild type and calnexin-deficient mice (fig. 4.12b&c.; MHC II and B220 panels). The B cell population, identified by B220 positive staining, was significantly reduced in spleen and lymph nodes from calnexin-deficient mice as seen in figures 4.12b. and 4.12c.; B220 panels. In addition, the number of MHC II positive cells was also significantly reduced in calnexin−/− mice (fig. 4.12b. and 4.12c.; MHC II panels). It is likely that the B220 positive and MHC II positive cell population largely overlap, because B cells are the main population expressing MHC II in spleen and lymph nodes. Typically two thirds of lymphocytes found in lymph nodes are T cells and one third are B cells, as seen in the wild type controls (fig. 4.12b. left panels). In the calnexin knockout mice, two thirds of lymph node cells are still T cells, but only a quarter are B cells (fig. 4.12b., right panels), leaving about 10% of calnexin-deficient lymph node cells which are neither T nor B cells - this population has not yet been fully characterised.

4.2.10. Surface expression of hemagglutinin in calnexin-deficient cells

Calnexin has been shown to be involved in the folding of the influenza virus surface protein hemagglutinin (HA). To address the question whether HA
Figure 4.13. Hemagglutinin localisation in wild type and calnexin knockout fibroblasts

Wild type and calnexin deficient fibroblasts were infected with influenza virus and the expression and localisation of influenza hemagglutinin (HA) was assessed by immunofluorescence 4 hours (upper panel) and 8 hours (lower panel) after infection with antibodies against HA. Initial expression of HA on the cell surface is delayed in calnexin-deficient MEFs as seen 4 hours after infection, however 8 hours after infection the HA surface staining was indistinguishable between wild type and calnexin-deficient MEFs.
would still be folded as efficiently in the absence of calnexin, mouse embryonic fibroblasts (MEFs) were infected with influenza virus.

The morphology of MEFs allows a relatively easy determination of the subcellular localisation of HA and therefore MEFs are a useful tool to study HA expression and localisation. MEFs from wild type and calnexin knockout mice were infected with influenza virus and the expression of HA was assayed 4 and 8 hours after infection. The infected MEFs were fixed with paraformaldehyde at different time points after infection, permeabilised to detect not only surface but also intracellular staining and incubated with an antibody against HA. Four hours after infection HA was already localised to the cell surface in wild type MEFs, while in calnexin knockout MEFs the majority of HA was still localised within the cell (probably within the ER). Only very faint surface staining was detected (fig. 4.13. upper panels). 8 hours after infection no differences between wild type and calnexin knockout MEFs could be detected and both cell populations showed intensive HA expression on the surface (fig. 4.13. lower panels).

These results indicate that calnexin is involved in HA folding in the ER and that the absence of this molecule results in delayed surface expression of HA. Since that calnexin is not involved in the transport of HA to the cell surface, the delayed surface expression of HA in the calnexin-deficient MEFs indicates a prolonged stay of folding intermediates of HA in the ER in MEFs from these mice. This work was carried out in collaboration with Dr. Lin Yipu, NIMR, Mill Hill.

4.2.11. Apoptosis in calnexin-deficient MEFs

The fission yeast S. pombe expresses the calnexin homologue cnx1. Transfection of the pro-apoptotic gene bak into S. pombe results in a lethal phenotype (Torgler et al., 1997). However, transfecting bak in a S. pombe strain expressing only the transmembrane and luminal region of cnx1 rescues the yeast cell from Bak induced death (Torgler et al., 1997). In addition, a yeast-two-hybrid screen revealed that the cytoplasmic domain of human calnexin interacts with Bak (Torgler et al., 1997). These data led to the hypothesis that calnexin may be involved in apoptotic cell death.
To analyse whether calnexin-deficient cells have any survival advantage upon different apoptotic stimuli, thymocytes from wild type and calnexin knockout mice were challenged by UV exposure and by incubation with an anti CD95 antibody, respectively. 4x10^6 thymocytes from three wild type controls and three calnexin knockout mice were left either untreated to measure spontaneous cell death, treated with anti-CD95 antibody (100ng/ml) or exposed to UV light (40J/m²). 24 hours after treatment, apoptosis was assessed by analysing the subG1 peak by FACS analysis of fragmented DNA after propidium iodide staining. As shown in table 4.3. there were no significant differences in the rate of apoptosis in calnexin-deficient thymocytes compared to thymocytes from wild type littermates in untreated or treated samples.

Table 4.3. Survival rates of calnexin-deficient MEFs upon apoptotic stimuli

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>anti-CD95</th>
<th>UV</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>ko</td>
<td></td>
</tr>
<tr>
<td>% dead cells</td>
<td>17.2 ± 1.9</td>
<td>16.8 ± 4.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>ko</td>
<td></td>
</tr>
<tr>
<td>% dead cells</td>
<td>22.4 ± 4.3</td>
<td>22.7 ± 6.9</td>
<td>28.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>ko</td>
<td></td>
</tr>
<tr>
<td>% dead cells</td>
<td>31.5 ± 7.4</td>
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These results do not support the role for calnexin in the apoptotic process of naturally occurring cell death or induced by different stimuli in mammalian cells.
4.3. Discussion

4.3.1. Generation of calnexin knockout mice

During the last decade several studies have focused on the chaperone properties of calnexin and have demonstrated that calnexin is involved in the correct folding and assembly of newly synthesised proteins. The majority of these studies were performed in tissue culture systems and in many experiments the proteins of interest were artificially expressed in cell lines. These experimental approaches helped to characterise the function of calnexin in vitro, although they do not provide information about the requirement for calnexin in vivo. Calnexin knockout mice were generated to gain further insights into the physiological roles of calnexin.

Previously it has been reported that calnexin was not required for single cell viability because the calnexin-deficient cell line CEM NK^R^ does not show any growth abnormalities when compared to the closely related CEM cell line which expresses calnexin (Howell et al., 1985; Scott and Dawson, 1995). However, cell lines are not subject to the same complex changes and differentiation steps which occur during embryonic development and thus results obtained from cell lines may provide indications but would not allow conclusions about the requirement for calnexin in embryonic development. Given the existence of the closely related chaperone molecule, calreticulin, one may expect that calreticulin could take over essential functions of calnexin and calnexin knockout mice would only show subtle changes compared to wild type mice. However, it has been proven that calnexin cannot compensate for the loss of calreticulin - calreticulin knockout embryos die in utero due to defective heart development (Mesaeli et al., 1999). Although both chaperones bind to monoglucosylated proteins in the ER, calnexin and calreticulin clearly show distinct affinities for the proteins with which they interact (Hebert et al., 1997; van Leeuwen and Kearse, 1996a). Thus, the function of calnexin and calreticulin is largely overlapping but not identical.
4.3.2. Excision of the HSV-tk from *calnexin* mutated alleles

Breeding of calnexin heterozygous male and female mice derived from ES cell clone CNX 14 gave rise to offspring in only 10–20% of all breeding pairs and breeding pairs consisting of heterozygous mice derived from ES cell clone CNX 12-40 never produced any offspring. It is unlikely that the fertility problems are caused by heterozygous deficiency of calnexin because expression of HSV-tk in sperm cells results in an abnormal morphology, which has been reported to cause sterility (Braun *et al.*, 1990). All heterozygous mice carry the HSV-tk gene in their genome and thus only very few mice were obtained from heterozygous breeding pairs. The reduced number of mice severely limited the number of investigations, which could be performed with the *calnexin* knockout mice.

An immediate aim was, therefore, to cross the *calnexin* heterozygous mice with transgenic mice expressing the Cre recombinase to excise the HSV-tk from the *calnexin*-mutated allele. Excision of the HSV-tk occurs in the second generation of mice carrying the cre-transgene and the mutated *calnexin* allele. Heterozygous deficient *calnexin* male mice, which had deleted the HSV-tk are fertile and produce normal numbers of offspring. The *calnexin* knockout mice without the HSV-tk have no fertility problems and show the same phenotype as *calnexin* knockout mice with the HSV-tk, indicating that the observed phenotype resulted from the absence of calnexin and not the expression of HSV-tk. This was expected as heterozygous mice, which also express the HSV-tk, did not show any symptoms of the *calnexin* knockout specific phenotype.

4.3.3. Inactivation of *calnexin* results in premature death

Inactivation of one *calnexin* allele does not have any obvious consequences, these mice showing no detectable differences compared to wild type littermates. However, the inactivation of both *calnexin* alleles resulted in a clear phenotype and calnexin knockouts were immediately distinguishable from calnexin heterozygous and wild type littermates.

Calnexin knockout embryos are carried to full term and show no immediately obvious differences compared to wild type littermates. However,
approximately half of the newborn *calnexin* knockout mice died within 24 hours after birth. The cause of death remains to be solved. Since older *calnexin* knockouts suffer from motility problems, it is possible that the early postnatal death is caused by failure of essential muscles like heart and/or diaphragm or the failure of mice to suckle.

Newborn *calnexin* knockout mice which have not died perinatally do not gain weight as quickly as their littermates and all *calnexin* knockouts could be clearly distinguished from their littermates by size at about one week after birth. At about three weeks of age, the mice are weaned from their mothers and many calnexin knockouts had to be sacrificed at that stage because they lost weight even when moist food was provided in the cage. Even if the mice were initially able to feed and survive, 90% of the mice had to be sacrificed by the age of 10 weeks because the mice started to lose weight and showed hypoactive behaviour which indicated discomfort and stress.

4.3.4. *Calnexin* knockout mice show severe walking abnormalities

The *calnexin* knockout mice show motility abnormalities, demonstrating a wobbly unsteady gait and fall over when they walk. It appears that the hind limbs are more affected by the disorder than the front limbs. The analysis of a SHIRPA test revealed that *calnexin* knockout mice did not have sensoric abnormalities and that all provoked reflexes were fully developed. The initial grip strength was not reduced, but *calnexin* knockouts showed earlier signs of muscle fatigue and could not hold on as long as efficiently when placed on a pencil as littermate controls.

In addition the knockouts showed a negative geotaxis and retropulsion. These abnormalities are frequently seen in mice after administration of neurotoxins. However, this does not explain the phenotype seen in the *calnexin*\(^{-/-}\) mice. Negative geotaxis is most likely caused by vestibular defects which have not been formally excluded in *calnexin*-deficient mice, however vestibular defects are accompanied by an abnormal righting reflex which is normal in *calnexin*\(^{-/-}\) mice suggesting that there are no defects in the vestibular system. Retropulsion and tremor are also seen in human patients suffering from Parkinson's disease
indicating that the phenotype seen in calnexin-deficient mice is caused by insufficiencies in central neurotransmission.

4.3.5. Histological analysis of central and peripheral nervous system

Calnexin knockout mice showed symptoms which suggested that they suffered from motor neurone disease (MND), such as spinal muscular atrophy (SMA) or amyotrophic lateral sclerosis (ALS). Preliminary results from spinal cord sections appeared to reveal that large α-motor neurons in the anterior horn were degenerating, suggesting that the calnexin knockout mice suffered from a SMA like disease. However, further analysis showed that some of the α-motor neurons from wild type mice also appeared equally abnormal. It is not yet known what causes the changes in these cells, however it is very unlikely to be related to the phenotype of calnexin knockout mice because cells with identical morphological alterations were detected in wild type mice. Although, the mice clearly display a phenotype that suggests that these mice suffer neuronal lesions, no morphological abnormalities could be detected.

4.3.6. Neuromuscular junctions appear normal in calnexin-deficient mice

Microscopic histopathological analysis of the central and peripheral nervous system did not show any obvious changes in the calnexin knockout mice. However, the symptoms of these mice clearly implied alterations in the neuromuscular system.

One obvious molecule to investigate was the acetylcholine receptor (AChR) firstly because calnexin is involved in correct folding and assembly of AChR subunits (Gelman et al., 1995; Gelman and Prives, 1996; Keller et al., 1996; Keller et al., 1998) and secondly because AChRe chain knockout mice die prematurely and are much smaller than their littermates (Witzemann et al., 1996) a phenotype similar to that observed in calnexin knockout mice. However, calnexin knockout mice did not display any alterations in the surface expression and clustering of the AChR receptor. Another hypothesis was that there was a defect in dystrophin expression, because human patients suffering from Duchenne
Chapter 4  Analysis of calnexin-deficient mice

muscle dystrophy do not express dystrophin. However, calnexin knockout mice do not show any alteration in their dystrophin expression compared to wild type controls. Furthermore, utrophin and N-CAM are not upregulated in calnexin knockout mice. Both molecules are upregulated in regenerating muscle and alterations in their expression may have indicated muscle degeneration. Recently, calnexin has been shown to be involved in the folding of IP3 receptors, such as the ryanodine receptor on the sarcoplasmatic reticulum in muscle cells (Joseph et al., 1999). This receptor was detected in similar amounts in calnexin knockout mice and wild type controls. However, immunofluorescence does not reveal the functionality of the receptor and incorrectly folded receptor would still be localised to the same compartment as a functional receptor. Thus the subcellular distribution does not allow any conclusions about the functionality of the receptor. Interestingly, calreticulin does not interact with type I IP3 receptors, and is therefore unlikely to compensate for any loss of function due to the absence of calnexin (Joseph et al., 1999). Further experiments are necessary to measure Ca\(^{2+}\) influx in calnexin-deficient muscle cells. Electron microscopy also revealed a normal morphology in calnexin-deficient muscle and neuromuscular junctions.

In summary the obtained results from extensive the histological analysis of brain, cerebellum, spinal cord, neuromuscular junctions and muscle along with the results obtained from the SHIRPA assessments indicate that the phenotype of the calnexin knockout mice is caused by functional rather than structural abnormalities.

### 4.3.7. The effects of calnexin deficiency on the developing immune system

Calnexin has been reported to be involved in the correct folding of subunits of the TCR, BCR, MHC I and MHC II. To investigate their surface expression in calnexin-deficient mice lymphoid cells were isolated from various lymphatic organs (thymus, spleen and lymph nodes) and the surface expression of CD3\(\alpha\) (subunit of the TCR), B220 (B cell specific marker), MHC I and MHC II was analysed. FACS analysis revealed that the expression of T cell specific markers is indistinguishable between cells from calnexin knockout mice compared
Chapter 4 Analysis of calnexin-deficient mice

to wild type littermates. In the total thymocyte population (which is mainly represented by CD4⁺ and CD8⁺ double positive T cells) no differences in surface expression of CD4, CD8 and CD3ε were detectable. These results suggest that calnexin is not necessarily required for the correct folding of subunits of the TCR. It may be possible, that in the absence of calnexin, calreticulin assures correct folding of the TCR subunits. However, it is believed that calreticulin only interacts with the α and β chain of the TCR and not the CD3 subunits and that it interacts with more advanced folding intermediates than calnexin (van Leeuwen and Kearse, 1996a).

The association of MHC I with calnexin has been extensively studied in numerous reports. However, MHC I expression is indistinguishable between leukocytes isolated from spleen and lymph nodes from calnexin-deficient mice and wild type littermates. This result was not surprising as the calnexin-deficient cell line CEM NK⁸ does not exhibit any variations in MHC I surface expression, MHC assembly and peptide loading compared to the closely related CEM cells which express calnexin (Prasad et al., 1998; Scott and Dawson, 1995). It is possible that calreticulin, which also clearly interacts with MHC I subunits may take over the function of calnexin. Recently, the MHC I complex has been shown to interact with ERp57. This is a luminal ER protein and evidence exists that ERp57 is part of the glycan-dependent quality control mechanism (Hughes and Cresswell, 1998). Several ER chaperone proteins interact with MHC I subunits and they are likely to compensate for the loss of calnexin. The interaction of these chaperones with subunits of the TCR has not been as extensively studied as their interaction with the MHC I complex. However, one may speculate that, as the expression of the TCR is not altered in the absence of calnexin, these chaperones probably ensure proper folding of the TCR.

In contrast to T cells the proportion of cells positive for the B cell specific marker B220 was significantly reduced from spleens and lymph nodes of calnexin knockout mice compared wild type littermates. In addition, MHC II positive cells were also significantly reduced in calnexin-deficient mice, although no alteration was observed for MHC I. The expression of MHC II and B220 molecules on the cell surface was not reduced in the calnexin-deficient mice, but there were
significantly fewer cells carrying these markers. B220 staining and MHC II staining probably detect largely overlapping populations because B cells represent the majority of MHC II expressing cells in the organs analysed. Interestingly, calreticulin has only been reported to associate with subunits of the TCR and MHC I but not with subunits of the BCR and MHC II and only the latter complexes appear to be affected by the loss of calnexin. This would imply that calreticulin, or other ER chaperones, can partially compensate for the absence of calnexin and that correct folding of some proteins is more disturbed by the absence of calnexin than others.

Two thirds of the lymph nodes cells that fall in standard forward versus side scatter lymphocyte gates by FACS analysis, are T cells and one third are B cells. Although the relative number of B cells appears to be reduced in calnexin-deficient mice, the relative number of T cell did not change. This left about 10% of lymph node cells from calnexin-deficient mice which were neither expressing T cell- nor B cell specific markers - this population has yet not been characterised. In adult mice about two thirds of spleen cells are B cells and one third are T cells. As calnexin knockout mice die very young, FACS analysis has mainly been performed on two to three week old mice, at which age the immune system is still not fully developed and may explain the low percentages of B and T cells detected in wild type and calnexin knockout mice. Nevertheless, comparing the surface staining of spleen cells from the young knockout and wild type mice provided information on expression of CD3ε, B220, MHC I and MHC II. As in the lymph nodes, splenocytes from calnexin knockout mice also showed reduced numbers of B220 and MHC II positive cells, while surface expression of CD3ε and MHC I remained unaffected by the loss of calnexin. It may be possible that the yet uncharacterised population consists of immature B cell-precursor cells, which do not yet express any of the markers analysed on the cell surface. The absence of calnexin may result in a prolonged assembly process and an extended stay of the BCR or/and MHC II in the ER, as seen for HA in the calnexin-/- MEFs. The loss of calnexin could therefore have an impact on the B cell population and may compromise or delay B cell development.
4.3.8. Delayed surface expression of HA in calnexin-deficient MEFs

The correct folding of the influenza surface protein hemagglutinin is mediated by calnexin. MEFs from wild type and calnexin knockout mice were infected with influenza virus to investigate whether the loss of calnexin compromises efficient folding of HA in the ER. These experiments revealed that at early time points after infection (4 hours) HA was not as efficiently expressed on the surface of knockout MEFs as in wild type MEFs and the majority of HA remained inside the calnexin-deficient MEFs. However, at later time points (8 hours) HA expression was indistinguishable between wild type and calnexin knockout MEFs. This result indicates that calnexin may be required for quick and efficient folding of HA, but that HA folding is not absolutely dependent on calnexin. Calreticulin also interacts with the HA folding intermediates but preferentially associates with glycosylated regions at the top of the HA molecules while calnexin appears to be less specific (Hebert et al., 1997). Thus calreticulin is unlikely to compensate completely for the absence of calnexin. As calnexin is only localised in the ER it is assumed that calnexin does not influence anterograde protein transport beyond the ER and that the delayed expression of HA on the surface is due to a prolonged stay of HA in the ER.

The association of viral proteins with calnexin during their assembly in the ER may be a potential target in the development of anti-viral drugs. The data presented here indicate that in the absence of calnexin, assembly of HA in the ER is delayed and HA is not expressed as rapidly as in wild type MEFs. This may suggest that calnexin is required for efficient influenza infection and that following treatment with agents which abolish the interaction between HA and calnexin, influenza infections might be less severe. In agreement with this speculation, it has been shown that the M envelope protein of the Hepatitis B Virus interacts with calnexin. Upon treatment with glucosidase inhibitors, which prevent glycosylation of the M protein, M protein association with calnexin is dramatically reduced, very little M protein is exported from the ER and consequently very few viral particles are made (Block et al., 1998; Werr and Prange, 1998).
4.3.9. Apoptosis

Studies performed in yeast gave the first indications that calnexin may be involved in apoptosis. Yeast cells, which were transfected with the pro-apoptotic protein bak showed moderate survival if they expressed a truncated calnexin protein missing the cytoplasmic tail. In contrast, yeast cells expressing wild type calnexin did not survive bak expression, suggesting that the cytoplasmic domain of calnexin may be involved in triggering cell death. MEFs isolated from calnexin-deficient mice did not show any prolonged survival after inducing pro-apoptotic stimuli. However, the MEFs were given different pro-apoptotic stimuli than yeast the possibility remains that there would be differences in cell death between wild type and calnexin-deficient MEFs following bak expression.

4.3.10. Future directions

Although all calnexin knockout mice show an obvious phenotype and are clearly distinguishable from wild type and heterozygous littermates the molecular mechanism of this phenotype currently remains obscure. After extensive structural analysis of tissues from calnexin-deficient it seems more likely that the phenotype is based on functional alterations.

Considering that the ryanodine receptor, which is calcium release channel of the sarcoplasmic reticulum, which is a member of the IP₃R family. Recently, type I members of the IP₃R have been shown to interact with calnexin but not with calreticulin. Therefore, it might be possible that ryanodine receptors require calnexin for correct folding. It would be interesting to measure the calcium release from the sarcoplasmic reticulum in isolated muscle cells from wild type and calnexin-deficient mice to determine whether there are differences, such as a reduced or delayed Ca²⁺ release in the knockout mice.

Many subunits of receptors of the immune system such as MHC I, MHC II, TCR and BCR have been reported to interact with calnexin during their folding in the ER. Although, lymphocytes from calnexin-deficient mice show no reduction in cell surface of MHC I and TCR, the surface markers MHC II and B220 were expressed on fewer cells in the calnexin⁻/⁻ mice but the amount of surface staining
on individual cells was not reduced. Pulse chase experiments could answer the question whether the surface expression of these markers is delayed in the \textit{calnexin}^{−/−} lymphocytes and detect if immature folding intermediates of MHC I, MHC II, TCR and BCR subunits are retained longer in the ER compared to those of wild type cells.

The apoptosis assays were performed inducing different pro-apoptotic stimuli than those ones previously described to have reduced effects in \textit{calnexin} mutated yeast cells. To compare the results reported in yeast cells with results obtained from \textit{calnexin}-deficient mammalian cells, wild type and \textit{calnexin}^{−/−} MEFs should be transfected with bak and the survival rates of both cells should be compared.
Chapter 5

Analysis of p23-deficient mice

5.1. Introduction

5.1.1. The p24 protein family

A protein family of relatively small molecular weight (~24 kD) has recently been identified and called the p24 protein family. The first mammalian p24 family member, gp25L, was isolated from dog pancreatic rough microsomes and found to be associated with calnexin (Wada et al., 1991). p24 family members were subsequently also identified in yeast (Saccharomyces cerevisiae) (Schimmoller et al., 1995) and frogs (Xenopus leavis) (Holthuis et al., 1995), indicating that the p24 protein family is conserved throughout evolution. In the last few years several more p24 family members have been identified, there are between 5-8 family homologues per species (Belden and Barlowe, 1996; Blum et al., 1996; Dominguez et al., 1998; Holthuis et al., 1995; Marzioch et al., 1999; Rojo et al., 1997; Schimmoller et al., 1995; Sohn et al., 1996; Stamnes et al., 1995; Wada et al., 1991).

Figure 5.1. Schematic representation of a p24 family member

All p24 members are type I transmembrane proteins, consisting of a large luminal domain with a coiled-coil domain close to the membrane, a
transmembrane region and a short cytoplasmic domain (fig. 5.1.) (Blum et al., 1996; Sohn et al., 1996; Stamnes et al., 1995). The overall homology between single family members is approximately 30%, which is significantly higher at their cytoplasmic domain. However, some family members show a higher homology to members from other species than to members from the same species. This observation led the division of the p24 family members into different subgroups as shown in figure 5.2. (Dominguez et al., 1998; Fiedler et al., 1996; Stamnes et al., 1995).

Figure 5.2 Family tree of the p24 protein family

The apparent subgrouping and the order of discovery led to a nomenclature based on the species (e.g. h for human, y for yeast) followed by the p24 subfamily (α, β, γ, δ) and a number indicating the order of discovery (e.g.
hp24Δ, for p23) (Dominguez et al., 1998). However, at present the p24 members are still referred to by their original names rather than those given in this nomenclature.

5.1.2. Subcellular localisation of p24 family members

Studies using confocal and electron microscopy have located the p24 family members to the organelles of the early secretory pathway: the ER, the ER-Golgi intermediate compartment and the Golgi apparatus (Dominguez et al., 1998; Gommel et al., 1999; Rojo et al., 1997). Although, it appears that the p24 members do not exclusively colocalise, p23 and p24 are mainly found in the cis-Golgi-network and the intermediate compartment (Dominguez et al., 1998; Gommel et al., 1999; Rojo et al., 1997), while GMP25 is also found in the ER and gp25L was isolated in association with the ER resident chaperone calnexin (Dominguez et al., 1998; Wada et al., 1991).

5.1.3. Function of p24 proteins in the early secretory pathway

The precise function of the p24 proteins remains largely unknown. However, these abundant proteins have been proposed as receptors for cargo exit from the ER. This hypothesis was originally put forward because of the consequences of disrupting two different p24 family members, Emp24p or Erv25p (the closest homologues of the mammalian p24 and p23 proteins respectively), in the budding yeast Saccharomyces cerevisiae. Inactivation of Emp24p and Erv25p led in each case to a selective retardation in the rates of ER exit of the GPI linked protein, Gas1p, as well as invertase, while other proteins such as α-factor and carboxypeptidase Y (CPY) remained unaffected (Belden and Barlowe, 1996; Schimmoller et al., 1995). In addition Emp24p and Erv25p have been localised to COPII coated vesicles which would also argue for their participation in anterograde protein transport in the early secretory pathway (Belden and Barlowe, 1996; Schimmoller et al., 1995). COPII coated vesicles mediate anterograde protein transport and bud exclusively from membranes of the ER (Barlowe, 1998; Kuehn and Schekman, 1997).
In studies with a yeast mutant carrying a temperature sensitive NSF (N-ethylmaleimide-sensitive factor) gene (sec18-1), a shift to the restricted temperature (37°C) leads to accumulation of secretory vesicles due to attenuated vesicle fusion. The inactivation of Emp24p in such sec18-1 mutants reduces the number of accumulating vesicles to ~50% at the restricted temperature, indicating that Emp24p plays a crucial role in vesicle budding in yeast (Stamnes et al., 1995). In mammalian cells, microinjection of antibodies against p23 (hp24δ) or GMP25 (hp24α₂) inhibits cargo exit from the intermediate compartment and the generation of cargo exit sites at the ER, respectively (Lavoie et al., 1999; Rojo et al., 1997).

p23 and p24 have been found to be highly enriched in COPI coated vesicles (Sohn et al., 1996; Stamnes et al., 1995). COPI coated vesicles mainly mediate retrograde protein transport from the Golgi to the ER (Cosson and Letourneur, 1994; Letourneur et al., 1994) but are also involved in intra-Golgi protein transport (Orci et al., 1997). In addition they have been implicated in anterograde transport from the intermediate compartment (Presley et al., 1997; Scales et al., 1997) and from the ER (Bednarek et al., 1995; Pepperkok et al., 1993). The cytoplasmic domain of p23, which contains a KKXX-like ER retrieval motif, binds to coat proteins of both COPI and COPII coated vesicles (Dominguez et al., 1998; Sohn et al., 1996). It may, therefore, not only be important for anterograde protein transport but could also play a role in retrograde transport. Indeed, in recent studies protein transport from the Golgi to the ER was inhibited when antibodies to the cytoplasmic domain of p23 were microinjected into mammalian cells (Majoul et al., 1998). Inactivation of Emp24p in yeast cells also results in secretion of ER resident proteins (Kar2p and Pdi1p) into the culture medium. This suggests that such a mutation attenuates retention of ER resident proteins within the ER and their retrieval back to the ER (Elrod-Erickson and Kaiser, 1996). In reconstituted phospholipid vesicles with only the cytoplasmic domain of p23, COPI coatamer binding and budding was demonstrated to occur in an ARF-(ADP-ribosylating factor) and GTP-dependent fashion, indicating that these are the necessary and sufficient components of the mammalian machinery for COPI mediated membrane transformation events (Bremser et al., 1999).
5.1.4. Characteristics of the cytoplasmic domain of p24 family members

The cytoplasmic domain of the p24 proteins plays a crucial part in its interaction with coat proteins of transport vesicles and determines the subcellular localisation. All mammalian and the majority of the yeast p24 family members possess double phenylalanine residues at their cytoplasmic domain. In those yeast homologues which only possess a single phenylalanine residue the preceding amino acid can be a large hydrophobic or aromatic amino acid (Dominguez et al., 1998; Fiedler et al., 1996; Marzioch et al., 1999). In addition, many of the p24 members contain a dilysine motif (KKXX or KXXKK) at their C-terminus. The K(X)KKX motif has been shown to be involved in protein retrieval to the ER and it appeared to be crucial that the lysines are at position -3 and -4 or -3 and -5 respectively (Jackson et al., 1990; Nilsson et al., 1989). p23 does not possess the classic KKXX motif but a structurally very similar KKXXX motif as shown in table 5.1

Table 5.1. Relationship of cytoplasmic domains of some p24 family members

<table>
<thead>
<tr>
<th>p23 (hp24δ)</th>
<th>QVFYLRRFFKA</th>
<th>KKLIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>p24 (hp24β)</td>
<td>QIYYLRKFREEVRVRV</td>
<td></td>
</tr>
<tr>
<td>gp25L (hp24α)</td>
<td>MKSLNKFIAK</td>
<td>KKLV</td>
</tr>
<tr>
<td>GMP25 (hp24α)</td>
<td>MRHLKSFEEAK</td>
<td>KKLV</td>
</tr>
<tr>
<td>p26 (hp24γ)</td>
<td>VLLKLKFREETKRPI</td>
<td>SRAVHS</td>
</tr>
<tr>
<td>gp27 (hp24γ)</td>
<td>VFLLKSFFSDKRTTTRVGS</td>
<td></td>
</tr>
<tr>
<td>Emp24p (yp24β)</td>
<td>IYYLRKFREEVTS</td>
<td>L</td>
</tr>
<tr>
<td>Erv25p (yp24δ)</td>
<td>VNYLKNYFK</td>
<td>KT</td>
</tr>
<tr>
<td>Erp1p (yp24α)</td>
<td>MKHLGKFVVK</td>
<td>QKL</td>
</tr>
</tbody>
</table>

The FF motif has been shown to be important for binding of COPI and COPII coat proteins and is believed to be involved in anterograde and retrograde
protein transport (Dominguez et al., 1998; Fiedler et al., 1996; Harter and Wieland, 1998; Sohn et al., 1996). Exchanging the double phenylalanine motif with two hydrophobic alanine residues in a chimeric protein consisting of the extracellular domain of CD8 and the p24 cytoplasmic domain leads to a decrease in the exit rate of a chimeric CD8-p24 protein out of the ER (Fiedler et al., 1996). However, both the mutated chimeric protein and the CD8-p24 protein with the wild type p24 cytoplasmic domain are found at the cell surface (Fiedler et al., 1996). In studies where the extracellular domain of CD8 has been fused to the transmembrane and cytoplasmic domains of p23, such chimeric proteins predominantly localised to the ER (Nickel et al., 1997). Converting the FF motif to two alanine residues redistributed the chimeric proteins mainly to the Golgi apparatus, with some ER staining still detectable. Changing the dilysine motif to diserine located the chimeric CD8-p23 proteins exclusively to the Golgi apparatus (Nickel et al., 1997). Mutation of both the FF and the KK motif localises the chimeric protein to structures beyond the Golgi and even to the cell surface. This indicates that both motifs play a crucial role in protein retrieval to the ER and implies that the Golgi localisation of wild type p23 relies on its luminal domain (Nickel et al., 1997).

Studies have shown that conversion of the FF to AA in the cytoplasmic domains of p23 and GMP25 not only retained these proteins but also all other p24 family members within the ER. Furthermore, the mutation of KK to SS of p23 and GMP25 redistributed all p24 members to more distal structures including the cell surface (Dominguez et al., 1998). This suggests a role for the FF motif in the exit from the ER which is consistent with the finding that this motif interacts with the COPII coat protein Sec23 and may, therefore, recruit COPII coat proteins to the membranes of the ER (Dominguez et al., 1998).

Conflicting opinions exist about the necessity of the FF motif for COPI binding (Dominguez et al., 1998; Fiedler et al., 1996; Harter and Wieland, 1998; Sohn et al., 1996). On the one hand, it has been suggested that mutation of the FF motif in the p23 cytoplasmic domain completely abolishes the binding of all COPI coat proteins (Sohn et al., 1996). On the other hand it is claimed that coatamer binding appears to be reduced only to about 50% (Dominguez et al., 1998). The
KKXX ER retrieval signal also interacts with coatomers. However, it remains to be determined whether conversion of the KK to SS reduces coatomer binding to about 50% (Sohn et al., 1996) or completely abolishes adhesion (Dominguez et al., 1998). Nevertheless, mutation of both the FF and the KK motif clearly prevents any interaction between coatomers and the cytoplasmic domain of p23 (Dominguez et al., 1998; Sohn et al., 1996).

COPI coated vesicles are mainly involved in retrograde transport but are also required for anterograde transport (Cosson and Letourneur, 1994; Letourneur et al., 1994; Presley et al., 1997; Scales et al., 1997). Fiedler et al. have shown that cytoplasmic domains of different p24 members interact with different coatomers: p24 proteins with a KK and a FF motif interact with α, β', and ε subunits, while p24 proteins without a KK but with the FF motif bind to β, γ, ζ coatomer subunits. This selective binding would allow anterograde and retrograde specific packaging of distinct COPI coated vesicles (Fiedler et al., 1996). Mutation of the FF motif in the KKXX containing p24 members had no effect, while the FF to AA mutation in the p24 members without the KKXX motif dramatically reduced coatomer binding. In the same study Erv25p, the closest p23 homologue in yeast, did not show any significant binding to any of the coatomer (Fiedler et al., 1996). Lately, only γ COPI has been shown to interact with p23 under physiological conditions (Harter and Wieland, 1998).

5.1.5. Complex formation of p24 family members

Members of the p24 family have been found in close proximity and localised together in COPI and COPII coated vesicles, suggesting that they interact with each other (Belden and Barlowe, 1996; Stamnes et al., 1995). Inactivation of Emp24p and Erv25p led to indistinguishable phenotypes in Saccharomyces cerevisiae and overexpression of Erv25p could not rescue the Emp24p deletion (Belden and Barlowe, 1996). However, the yeast mutants are viable and even multiple deletions do not show a more severe phenotype than single knockouts (Belden and Barlowe, 1996; Marzioch et al., 1999). In mammalian cells redistribution of mutated p23 and GMP25 also affects the subcellular
localisation of p24 and p26/gp27 (Dominguez et al., 1998). In the yeast mutants the absence of the inactivated p24 member dramatically reduces the amount of other p24 members, suggesting that these p24 members are located in the same pathway and are functionally interdependent (Marzioch et al., 1999).

Coimmunoprecipitations have proven that p24 members interact in equimolar amounts with each other. It is, therefore, most likely that they form functional heteromeric complexes and that p24 proteins which are not associated in a complex will be degraded (Fullekrug et al., 1999; Gommel et al., 1999; Marzioch et al., 1999). Nevertheless, evidence exists that not all of the p24 family members are associated in complexes (Fullekrug et al., 1999). Further studies analysing the precise function of this intermolecular association need to be carried out.
5.2. Results

5.2.1. Development of a PCR based assay for genotyping p23 mutant mice

Once germline transmission of the mutated p23 heterozygous allele was achieved, a mutant p23 mouse colony was generated. Breeding pairs consisted of two p23 heterozygous animals, so that the litters from such crosses would give rise to wild type, p23 heterozygous and p23 homozygous deficient mice.

A PCR genotyping assay was developed which utilises three oligonucleotides within a single PCR reaction to amplify two products representing both the wild type and the recombinant allele of p23. The primers amplifying the recombinant allele are located within the targeting vector and, therefore, do not discriminate between random or homologous integrations. To prevent false positive PCR genotyping of a potential randomly inherited targeting vector integration, mice used to set up breeding pairs were initially genotyped by Southern blotting (fig. 5.3.) to provide unambiguous genotype results concerning the inheritance of a mutated p23 allele.

Figure 5.4. PCR strategy for genotyping p23 mutant mice

PCR analysis with genomic DNA from p23 mutant mice with primers Pa, Pb and Pneo will give rise to a 300bp band for the wild type allele as primers Pa and Pb will anneal and a 220bp band for the recombinant as Pa and Pneo anneal.

The PCR strategy for genotyping p23 mutant mice is shown in figure 5.4. The oligonucleotide pair Pa and Pb allowed amplification of a 300bp fragment
Figure 5.3. Generation of $p23$ mutant mice by gene targeting

Southern blot analysis showing correct 3' targeting of the $p23$ locus. Genomic tail DNA from wild type and heterozygous mice was digested with BamHI and hybridised to probe A. The resulting 8.5kb and 3.3kb fragments correspond to the wild type (wt) and mutated (mu) allele, respectively.

Wild type and $p23$ heterozygous mice were routinely genotyped by PCR. PCR analysis was performed with three different oligonucleotides as indicated in figure 5.4., resulting in a 300bp and 220bp fragment for the wild type or mutated allele, respectively.
which represents germline configuration of the wild type allele. Upon integration of the targeting vector the first exon of the \( p23 \) gene was replaced by the neomycin resistance gene (neo'). Addition of a third oligonucleotide, binding to the 5' end of neo' (Pneo) to the PCR reaction results in a 220bp amplification product with genomic DNA from \( p23 \) mutant mice. Thus, the \( p23 \) wild type genotype produces a single germline product of 300bp, while the \( p23 \) heterozygous genotype results in two products of 300bp and 220bp. In a \( p23 \) knockout genotype only a single recombinant product of 220bp would be amplified.

5.2.2. \( p23 \) deficiency leads to early embryonic lethality

A WT : HE : KO ratio according to the Mendelian distribution of 1 : 2 : 1 was expected. However, genotyping results revealed a generation of 72 \( p23^{+/+} \) and 145 \( p23^{+/} \) but no \( p23^{-/-} \) mice derived from ES cell clone s1. The \( p23-s2 \) strain yielded 53 \( p23^{+/+} \) and 102 \( p23^{+/} \) but again no \( p23^{-/-} \) mice. This represented a ratio of 1 : 2.01 : 0 and 1 : 1.92 : 0 between the WT : HE : KO mice derived from ES cell clones s1 and s2, respectively (as shown in table 5.2.).

Table 5.2. Ratio of \( p23 \) mutated mice

<table>
<thead>
<tr>
<th>mice:</th>
<th>wild type</th>
<th>heterozygous</th>
<th>homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1</td>
<td>72</td>
<td>145</td>
<td>0</td>
</tr>
<tr>
<td>s2</td>
<td>53</td>
<td>102</td>
<td>0</td>
</tr>
<tr>
<td>ratio:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1</td>
<td>1</td>
<td>2.013</td>
<td>0</td>
</tr>
<tr>
<td>s2</td>
<td>1</td>
<td>1.92</td>
<td>0</td>
</tr>
</tbody>
</table>

To investigate at which time point during development the inactivation of both \( p23 \) alleles would be lethal, embryos from time-mated inter-crosses of \( p23 \) heterozygous mice were genotyped. The embryos were sacrificed at 7.5 days of gestation. At this stage the embryo is about 1-2 mm of length, visible under the microscope and the organs have not yet started to develop. A total of sixty day 7.5
old embryos were genotyped but no p23 knockout embryos were detected. To assess if p23 may be required for implantation of blastocysts or even for their development, blastocysts were flushed out of the uterus horns at 3.5 days post coitus and genotyped. Again no p23-deficient blastocysts were found. The absence of p23\(^{-/-}\) blastocysts indicates that p23 is required at the earliest stages of mammalian development and that other p24 family members are unable to compensate for the loss of p23.

In order to generate homozygous p23-deficient ES cells the s1 ES cell clone which already had one inactivated p23 allele was electroporated with a different targeting vector that carries the hygromycin resistance gene (hygro\(^{r}\)) instead of the neo\(^{r}\). However, out of 400 screened neomycin and hygromycin resistant ES cell clones none was mutated at both p23 alleles.

5.2.3. p23 heterozygous mice show haploinsufficiency

p23 heterozygous mice appeared healthy and did not show any immediately obvious abnormalities when compared to wild type littermates. The mice were fertile, there were no premature losses and the body weight (n> 15 for each group) was comparable between p23 heterozygous and wild type mice as shown in table 5.3..

<table>
<thead>
<tr>
<th></th>
<th>wt female</th>
<th>he female</th>
<th>wt male</th>
<th>he male</th>
</tr>
</thead>
<tbody>
<tr>
<td>weight</td>
<td>25.1 ± 2.3 g</td>
<td>23.8 ± 1.9 g</td>
<td>36.6 ± 5.3 g</td>
<td>35.9 ± 2.1 g</td>
</tr>
</tbody>
</table>

To investigate whether the inactivation of one p23 allele would diminish the amount of p23 mRNA and the steady state p23 protein levels, mRNA and proteins were isolated from livers and kidneys of wild type and p23 heterozygous mice. The isolated mRNA samples were run on an agarose/formaldehyde gel, blotted onto an uncharged nylon membrane (Hybond N (Amersham)) and
Figure 5.5. Northern and Western blot analysis of wild type and p23 heterozygous mice

a. Northern blotting with a p23 murine cDNA probe and Western blotting with an antibody against the cytoplasmic domain of p23 indicate that p23 mRNA and p23 protein are reduced to about one third in liver and kidney of p23 heterozygous mice compared to wild type littermates. The intensity of protein and mRNA bands on Western and Northern blots, respectively, were quantified and compared using the "NIH image" programme.

b. Northern blotting with a GMP25 murine cDNA probe shows that GMP25 mRNA levels are identical in wild type and p23 heterozygous mice. In contrast, Western blotting with an antibody against GMP25 shows a reduction to approximately 30% of GMP25 protein in liver and kidney from p23 heterozygous mice compared to wild type mice.

c. Western blot of wild type and p23 heterozygous MEFs shows that also in these cells the level of p23 and GMP 25 is significantly reduced in the p23 heterozygous MEFs compared to wild type MEFs.
hybridised to the full length murine \textit{p23} cDNA. Quantification of the signals on
the autoradiograph showed that in organs from \textit{p23} heterozygous animals only
about 30\% of the \textit{p23} wild type mRNA could be detected (fig. 5.5a. lower panel).

The amount of \textit{p23} protein in the \textit{p23} heterozygous mice was analysed by
Western blots. To enrich transmembrane proteins (amongst them members of the
\textit{p24} family) and to separate them from cytosolic proteins, protein isolation was
performed according to the protocol for total membrane preparation as described
under 2.28. in the Material and Methods chapter. Equal amounts of membrane
fractions were separated by SDS PAGE followed by Western blotting with a
polyclonal antibody raised against the cytoplasmic domain of \textit{p23}. Although
identical amounts of transmembrane proteins from liver and kidney preparations
of wild type and \textit{p23} heterozygous mice were loaded on the SDS gels, both tissue
samples from \textit{p23} heterozygous mice showed remarkably weaker signals than the
tissue samples from wild type mice. \textit{p23} protein levels were also reduced to only
one third in the \textit{p23} animals (fig. 5.5a. upper panel) compared to wild type mice.
Obviously inactivation of one \textit{p23} allele leads to the reduction of \textit{p23} mRNA and
consequently to reduction of \textit{p23} protein which is not compensated by
upregulation of transcription of the untargeted allele.

The Northern blot was rehybridised with the \textit{GMP25} cDNA (a different
\textit{p24} family member). The levels of \textit{GMP25} mRNA were identical in the tested
organs from wild type and \textit{p23} heterozygous mice (fig. 5.5b. lower panel). This
suggests that the disruption of one \textit{p23} allele resulting in the decrease of \textit{p23}
mRNA does not affect the transcription of related \textit{p24} family members. However,
on a Western blot with liver and kidney membrane proteins isolated from wild
type and \textit{p23} heterozygous mice the level of \textit{GMP25} appeared dramatically
reduced in \textit{p23} heterozygous mice to approximately the same extent that the \textit{p23}
protein was diminished (fig. 5.5b. upper panel).

Mouse embryonic fibroblasts (MEFs) were used as \textit{in vitro}-system for
further analysis (confocal microscopy, pulse chase experiments and toxin assays)
which will be presented later in this chapter. Western blot analysis with MEFs
isolated from wild type and \textit{p23} heterozygous mice confirmed that \textit{p23} is also
reduced in fibroblasts from p23 heterozygous mice compared to cells from wild type littermate controls (fig. 5.5c.).

In addition, a Western blot probed with an antibody against the γ4 family member p26 showed that also the level of p26 was reduced by 60-70% in liver samples from p23 heterozygous mice compared to wild type liver (kidney was not tested). These findings indicate that the stability of GMP25 and p26 is dependent of wild type amounts of p23 protein and suggests that these p24 members function together in a heteromeric complex.

5.2.4. Structural changes of the Golgi apparatus in p23 heterozygous mice

The Golgi apparatus consists of flattened saccules or cisternae, which are tightly arranged on top of each other and which are able to communicate with one another. The saccules are slightly dilated at the rim, the main area in which vesicle budding and fusion occurs. Depending on the cell type, the number of Golgi stacks and the shape of the Golgi apparatus varies. Hepatocytes contain several Golgi stacks in their cytoplasm and each Golgi stack typically consists of 3-7 saccules. In other cell types that do not show a polarised secretion, the Golgi may form a web-like structure around the nucleus. The Golgi is often located near the nucleus and shows a convex curvature. The convex site facing the nucleus is the more immature part of the Golgi, with only a few small vesicles surrounding this area. At the more mature concave site the formation of secretory granules takes place.

Members of the p24 protein family accumulate in highest abundance in the cis or peri Golgi apparatus (Dominguez et al., 1998; Rojo et al., 1997; Sohn et al., 1996). To analyse whether the apparent reduction of several members of the p24 family in p23 heterozygous mice results in structural alterations of the cell organelles where the p24 proteins are localised, the structural morphology was examined by electron microscopy (EM). This was carried out in collaboration with Rose Watson, Cell Biology lab at the ICRF. Animals were starved over night to deplete the glycogen from liver cells because large glycogen containing vesicles
The top panel shows a section of kidney from wild type mice. The saccules of the Golgi apparatus are flat, slightly curved and only slightly dilated at the rims as indicated by the arrow heads. The lower panel shows a kidney section from p23 heterozygous mice. Here the morphology of the Golgi stacks is altered. The saccules are dilated, specially at the rims (arrow heads) and an increased amount of surrounding vacuoles is observed, marked by asterisks.
Figure 5.6b. Electron microscopy on liver sections from wild type and p23\textsuperscript{+/-} mice

The top panel shows a liver section from wild type mice and the bottom panel a liver section from p23 heterozygous mice. The liver section from p23\textsuperscript{+/-} mice showed very similar alterations as observed for kidney. The rims of the Golgi stacks are much dilated (arrow heads) and the number of vacuoles surrounding the Golgi apparatus appears increased (asterisks).
interfere with the fixation of tissue sections. Liver and kidney from wild type and
$p23$ heterozygous mice were cut in small cubes and fixed in 2% glutaraldehyde/0.1M cacodylate buffer in PBS. The fixed cubes were further processed into thin sections which were analysed by electron microscopy. EM pictures of liver and kidney cells from wild type mice showed a typical morphology of the Golgi apparatus. The saccules were flat, slightly curved and showed slight dilation at the rim (fig. 5.6a. upper panel and fig. 5.6b. upper panel). The Golgi saccules in liver and kidney cells from $p23$ heterozygous mice on the other hand appeared to be more dilated when compared to the same organelles from wild type littermates (fig 5.6a. lower panel and fig 5.6b. lower panel). This alteration was visible to various degrees. In some Golgi stacks the majority of saccules appeared to be dilated, while in others only one or two saccules were torn apart. The dilation seemed to be more prominent at the rims of the Golgi cisternae, although in some Golgi stacks the dilation was also detectable throughout the entire cisternae. It also seemed that the number of vacuoles surrounding the Golgi was increased in $p23$ heterozygous cells.

As shown in figures 5.6a. and 5.6b., the dilation of the Golgi cisternae was visible in liver and kidney from wild type and $p23$ heterozygous mice. It is unlikely that this effect is due to fixation artefacts because the cristae of the mitochondria, which serve as an indicator for the quality of the fixation, are well preserved in tissue from wild type and $p23$ heterozygous mice.

5.2.5. Subcellular distribution of $p23$ and $p26$

In previous studies members of the $p24$ family have been localised to the intermediate compartment and the cis Golgi apparatus by using immunofluorescence microscopy and different cell lines, including CHO, BHK, HeLa or pancreatic acinar cells and fibroblast lines such as Vero or COS cells (Blum et al., 1999; Dominguez et al., 1998; Rojo et al., 1997; Sohn et al., 1996). To compare the subcellular distribution of $p23$ in wild type and $p23$ heterozygous mice and to determine whether the reduction of $p23$ steady state levels in the $p23^{+/-}$ mice has any effect on its subcellular distribution, immunofluorescence was
Chapter 5 Analysis of p23-deficient mice

performed with isolated mouse embryonic fibroblast (MEFs). MEFs were chosen because they can easily be isolated, they may be cultured in vitro for several passages and due to their spread morphology they are a convenient cell type for confocal microscopy.

MEFs isolated at day 13.5 of gestation were stained with a polyclonal antibody raised against the cytoplasmic domain of p23. In MEFs from wild type mice a perinuclear structure, likely to be the Golgi apparatus, stained positively for the p23 antibody. In addition, several peripheral punctated structures also appeared to be recognised by the p23 antibody (fig. 5.7a. upper left panel). Although the perinuclear staining was detected in MEFs isolated from p23 heterozygous mice, it seemed less intense compared to wild type MEFs. The amount of peripheral punctated fluorescence was dramatically reduced to approximately 10-15% of the wild type level (fig. 5.7a. upper right panel). The apparent higher background staining in the p23 heterozygous MEFs compared to the wild type MEFs was due to an inherent limitation of the technique used. Since the p23 staining was less intense in the p23 heterozygous MEFs (dramatic reduction of p23), the sensitivity of the microscope to detect the fluorescence had to be increased in order to obtain comparable images of wild type and p23+/− MEFs.

In addition to p23, the protein levels of other p24 family members, like GMP25 and p26 were reduced in the p23 heterozygous mice (as shown in fig. 5.5b.). The MEFs were, therefore, stained with an antibody against p26 and very similar changes in the subcellular distribution to those observed for p23 were seen. The antibody against the cytoplasmic domain of p26 stained the perinuclear, Golgi-like structure and many peripheral punctated structures in wild type MEFs, comparable to the p23 staining (fig. 5.7b. upper left panel). The upper right panel of figure 5.7b. shows that p26, like p23, mainly localises to the perinuclear structure and that only very few peripheral punctated structures were positive for p26 staining in p23+/− MEFs. Similarly to the p23 staining, the sensitivity of the microscope had to be increased for p26 staining in the p23 heterozygous mice in order to detect the fluorescence, because of the overall reduction of p26 protein in the MEFs from p23+/− mice.
Wild type and $p_{23}$ heterozygous fibroblasts were fixed and double stained for immunofluorescence with antibodies against $p_{23}$ and COPI. Fluorescein and Cy5 channels were adjusted to similar levels and overlayed. $p_{23}$ displays a perinuclear staining in wild type and $p_{23}^{+/−}$ MEFs. In wild type MEFs peripheral punctate structures are also positively labelled, whereas in $p_{23}^{+/−}$ MEFs only very few of the peripheral punctate structures are stained for $p_{23}$. COPI shows a very similar pattern of staining for the Golgi apparatus and peripheral punctate structures in MEFs from wild type and $p_{23}^{+/−}$ mice. Overlay of the channels indicate that a large number of peripheral punctate structures are either stained for $p_{23}$ or COPI but not for both.
Wild type and p23 heterozygous MEFs were fixed and double stained for immunofluorescence with antibodies against p26 and COPI. p26 shows a very similar intracellular distribution compared to p23 in p23+/− MEFs. In the latter, many fewer peripheral punctate structures are positively labelled for p26 compared to wild type MEFs and only the perinuclear staining is visible. The overlay of the two channels shows again, that COPI is absent from a large number of p26 positive punctuated structures and vice versa.
5.2.6. COPI distribution in MEFs from wild type and p23 heterozygous mice

COPI coated vesicles mediate anterograde and retrograde protein transport in the early secretory pathway between the ER and Golgi and are, therefore, mainly localised to the same organelles as the members of the p24 family. Recent studies have reported that the cytoplasmic domain of p24 family members, especially those members containing a C-terminal KKXX motif (GMP25) or a KKXX-like motif (p23), can interact with COPI subunits in vitro (Dominguez et al., 1998; Sohn et al., 1996). However, conflicting data exists as to whether p23 is enriched in COPI coated vesicles (Sohn et al., 1996) or whether it is large absent from COPI coated vesicles (Blum et al., 1999; Rojo et al., 1997) with the localisation of p23 and COPI to the same intracellular structures being coincidental.

Double immunofluorescence staining experiments with antibodies against p23 and β′COP (COPI subunit) in wild type and p23 heterozygous MEFs were performed to provide further information on whether p23 and COPI colocalise and whether the observed altered subcellular distribution of p24 family members in the p23 heterozygous MEFs would also have an influence on COPI distribution. The staining with the anti-β′COP antibody showed that in MEFs from both wild type and p23 heterozygous mice, β′COP localised to the perinuclear and the peripheral punctated structures. In contrast to the situation for p23 and p26, the distribution of β′COP was indistinguishable between wild type and p23 heterozygous MEFs and did not show a reduction of peripheral punctated structures in p23+/− MEFs (fig. 5.7a. middle panel and 5.7b. middle panel). The overall COPI distribution in wild type and p23+/− MEFs appeared similar to the subcellular distribution of p23 and p26 in wild type MEFs. It seems that the reduction and altered subcellular distribution of p23 in p23+/− MEFs does not affect the β′COP distribution.

An overlay of p23 and β′COP staining of wild type MEFs showed that both p23 and β′COP localised to the perinuclear structure (fig. 5.7a. lower left panel). Nevertheless, it appeared from the p26 and β′COP double immunofluorescence that there are areas in the Golgi-like structure which are predominately stained.
for either p26 or β’COP (fig. 5.7b. lower left panel). In wild type MEFs the majority of peripheral punctated structures were stained for either p23 or β’COP and only few peripheral punctated structures were stained for p23 and βCOP (fig. 5.7a. lower left panel). The same observation was made for double staining with p26 and βCOP. β’COP seemed largely absent from a large number of the peripheral punctated structures which were positive for p26 and it seemed that β’COP rather stained a different subset of these structures (fig. 5.7b. lower left panel).

5.2.7. Protein secretion in wild type and p23 heterozygous MEFs

Studies in Saccharomyces cerevisiae mutants deficient for Emp24p and Erv25p, (yeast homologues of the p24 family) revealed selective protein transport defects in the mutated yeast cells. The majority of proteins were secreted in comparable amounts as in wild type cells but a small subset of proteins was secreted less efficiently (Belden and Barlowe, 1996; Schimmoller et al., 1995).

MEFs were used to establish if the reduction of p23 protein levels influences the efficiency of the anterograde secretory pathway in p23 heterozygous mice. Total protein secretion was assayed in [35S]-methionine pulse chase experiments and the levels of proteins secreted from wild type and p23+/- MEFs were compared as described in chapter 2, section 2.34. Equal numbers of wild type and p23+/- MEFs were plated onto 3cm petri-dishes and incubated at 37°C overnight. The cells were then depleted of methionine in methionine-free medium for 45 minutes before the addition of radioactively labelled [35S]-methionine. The incorporation of [35S]-methionine into newly synthesised proteins was allowed for 5 mins after which time the cells were washed and incubated in medium with a 10-fold excess of unlabelled methionine. In this way the [35S]-methionine was diluted and its incorporation during protein biosynthesis stopped. The supernatants were collected from the wild type and p23+/- MEFs, (one dish for each time point) and the secreted proteins were concentrated and separated by SDS PAGE (fig. 5.8.).
Figure 5.8. Pulse chase analysis of wild type and p23 heterozygous MEFs

MEFs from wild type and p23 heterozygous mice were starved in methionine-free medium, labelled for 5 minutes with $[^{35}\text{S}]$-methionine and chased with excess of "cold" methionine. The culture supernatant was harvested after different time points and proteins were analysed on a SDS gel.
Chapter 5 Analysis of p23-deficient mice

The earliest protein secretion was detected 15 min after $[^{35}\text{S}]$-methionine labelling in both wild type and $p23$ heterozygous MEFs (fig. 5.8). The autoradiograph showed a very similar secretion pattern in wild type and $p23^{+/\cdot}$ MEFs with the appearance of five major bands. Comparison of the pulse chase SDS gels on a phospho-imager (Molecular Dynamics) revealed no obvious quantitative differences between the protein amounts secreted from wild type and $p23$ heterozygous MEFs. Thus, the kinetics of secretion were comparable, with no additional or missing proteins and no quantitative changes observed between wild type and $p23$ heterozygous MEFs.

5.2.8. Quantitative differences between blood plasma proteins from wild type and $p23$ heterozygous mice

As no obvious secretory defects could be detected in the $p23$ heterozygous MEFs studied by pulse chase experiments in vitro, blood plasma samples from wild type and $p23$ heterozygous mice were analysed by comparative 2D gel electrophoresis to investigate if the overall secretion would be compromised under physiological conditions in vivo.

Plasma samples were taken from six-week-old wild type and $p23$ heterozygous mice. To allow for strain differences between the plasma proteins, plasma samples from age matched BALB/c and C57BL/6 mice were used as controls. In the first dimension the proteins were separated according to their isoelectric point. To minimise the variations which naturally occur during the technical procedures the SDS gels for the second dimension were cast together in the same tray (12 gels) (Pharmacia) and running the SDS gels together in the same gel tank (Pharmacia), so that the gels and buffer conditions were as similar as possible. The proteins on the 2D gels were visualised by silver nitrate staining following the "Hochstrasser" protocol as described (2.32.).

Analysis of the plasma samples separated on the 2D gels showed subtle differences in plasma protein secretion between wild type and $p23$ heterozygous mice (fig. 5.9.). Although the overall pattern of secreted proteins appears to be identical with no major proteins missing, some plasma proteins seem to be
Blood plasma from wild type and p23 heterozygous mice was separated by 2D gel electrophoresis and revealed subtle changes. The differences between wild type and \( p23^{+/-} \) samples are indicated by arrow heads. The protein marked by an open arrow head only appears in the \( p23^{+/-} \) mice and the spots marked by asterisks are due to strain differences.
secreted less efficiently in p23 heterozygous mice when compared to littermates and mice of either BALB/c and C57BL/6 strain. The protein spots indicated by the arrow heads appear less prominent on the 2D gels of plasma samples from p23 heterozygous mice compared to wild type mice. Only the spots which appeared consistently weaker were marked and therefore it is unlikely that the differences in the intensity are due to technical variation between the different gels (fig. 5.9). The protein indicated by an open arrow head is only seen in plasma samples from p23 heterozygous but not in wild type controls. Intensity differences of the protein spot marked by an asterisk are due to variations between different mouse strains.

Although no secretion differences could be detected between wild type and p23 heterozygous MEFs by pulse chase experiments in vitro, the 2D gel maps of plasma protein samples from wild type and p23+/− mice indicate that a small subset of plasma proteins appears to be secreted less efficiently in p23 heterozygous mice.

5.2.9. Retrograde protein transport in wild type and p23 heterozygous MEFs

Recent studies have provided evidence that p23 is involved in retrograde protein transport. Thus, microinjection of antibodies against the cytoplasmic domain of p23 inhibited retrograde transport of a cholera toxin mutant from the Golgi to the ER (Majoul et al., 1998).

To address the question of whether the retrograde transport is perturbed in p23 heterozygous MEFs the transport rate of two different toxins, Pseudomonas exotoxin A (PE) and ricin, was analysed in MEFs. If the reduction of the protein level of p23 has consequences for retrograde transport we would predict a prolonged survival for the p23 heterozygous MEFs, as measured by [35S]-methionine incorporation. Both toxins are transported from the cell surface, via the Golgi, to the ER and to those sites in the cell where they interfere with cell viability (Jackson et al., 1999; Lord and Roberts, 1998). PE is internalised by receptor mediated endocytosis is cleaved intracellularly. The 37kD C-terminal subunit is translocated via the Golgi to the ER, where it exits into the cytoplasm.
Figure 5.10. Toxin assays

a. *Pseudomonas* exotoxin A

![Graph showing the effect of *Pseudomonas* exotoxin A on MEFs.]

b. Ricin

![Graph showing the effect of ricin on MEFs.]

MEFs were either incubated with *Pseudomonas* exotoxin A (a) or ricin (b). The cells were starved in methionine-free medium, labelled with $^{35}$S-methionine and subsequently lysed. The amount of incorporated $^{35}$S, which reflects the survival rate of cells after toxin treatment, was determined by scintillation counting.

The solid line shows the survival of the wild type MEFs, while the dashed line represents the p23 heterozygous MEFs. The squares and triangles indicate the different concentrations at which the toxins were preincubated:

- a) squares: 20ng/ml; triangles 100ng/ml
- b) squares: 5ng/ml; triangles 12.5ng/ml
and irreversibly ADP-ribosylates elongation factor 2 of the eukaryotic translation machinery, thereby inhibiting protein biosynthesis. Ricin, however, once transported to the nucleus via the Golgi and ER blocks protein biosynthesis by hydroglycosylation of RNA (Montecucco, 1998).

Equal numbers of wild type and p23 heterozygous MEFs were seeded onto 6-well plates in duplicates for the different toxin concentrations, time points and controls. The cells were preincubated with or without the different concentrations of PE or ricin for defined time periods, then starved in methionine-free medium and labelled with [35S]-methionine. They were washed twice and then lysed. The lysates were precipitated with 20% TCA and the level of incorporation of [35S]-methionine was measured using a scintillation counter (Beckman). This strategy allowed us to determine the rate of protein biosynthesis as an indication of the relative number of viable, toxin treated cells compared to untreated controls. As shown in figure 5.10, no significant differences between the survival rates of wild type and p23 heterozygous MEFs were observed at the various time points between wild type and p23 heterozygous MEFs with two different concentration of either PE or ricin, respectively. These results indicate that the reduction of p23 does not severely effect the retrograde transport machinery.
5.3. Discussion

5.3.1. Inactivation of p23 leads to early embryonic lethality in mice

The inactivation of p23 by gene targeting was undertaken to provide new insights in the role of p23 in vivo. Mice deficient in the expression of either one or both p23 alleles show a clear phenotype. Inactivation of both p23 alleles is lethal and other family members cannot compensate for the loss of p23. Mice were routinely genotyped between two and three weeks after birth but crosses from p23 heterozygous mice never resulted in p23 homozygously deficient mice. These results were obtained from both p23 heterozygous strains derived from two independent p23+/− ES cell clones. It is, therefore, highly unlikely that the lethality is due to random integration of the targeting vector into another essential gene.

To determine at which stage in mammalian development the disruption of p23 is lethal, embryos were genotyped at 7.5 days post coitus (d.p.c.). At this age the embryo is easily visible under the microscope but no p23 knockout embryos were found. In addition, blastocysts, consisting of about 64 cells were isolated at 3.5 d.p.c. and genotyped, but again only wild type and p23 heterozygous blastocysts could be identified. An attempt to generate p23+/− ES cells by electroporating p23+/− ES cells with another targeting vector failed – of 400 ES cell clones screened none was p23-deficient.

The observation that the inactivation of p23 is lethal suggests that p23 is either required at the earliest stages of mammalian development possibly even for single cell viability. This is surprising given that previous studies on p24 family members in S. cerevisiae have demonstrated that, although deletion of Erv25p, the closest p23 homologue in yeast, results in decreased secretion of some proteins, notably Gas1p and invertase, the yeast cells remain viable and do not show any growth abnormalities (Belden and Barlowe, 1996). Interestingly, yeast strains simultaneously deficient for four p24 family members (Emp24p, Erv25p, Erp1p and Erp2p) are also viable and do not display a phenotype more severe than that of the single knockout yeast cells (Marzioch et al., 1999).
The early lethality of p23 inactivation in mammals could be due to secretory defects of proteins involved in cell-cell interactions. These include the impaired transport of adhesion molecules which are required to form cell clusters such as blastocysts or ES cell colonies or an impaired transport of growth factors required in early development. This could explain the milder effects observed in a single cell organism like yeast which does not rely on an elaborate system of cell-cell interaction to the same extent as mammalian organisms do.

Since breeding of heterozygous mice led to a ratio of 1:2:0 between the wild type: heterozygous: homozygous mice, male mice must have p23-deficient sperm cells and female mice must have p23-deficient oocytes; since sperm and oocytes are haploid, half of each germ cell population must be p23-deficient to achieve this ratio. This indicates that p23 is not required for single cell viability and does not play an essential part in general mammalian intracellular membrane trafficking. One may speculate that p23 could be involved in the mitotic disassembly and reassembly of the Golgi apparatus, because firstly sperm and oocytes undergo mitosis only after fertilisation and secondly the Golgi apparatus in yeast cells is not subject to comparable disassembly and reassembly during mitosis as in mammalian cells (Warren, 1993). This would explain why p23 is essential in mammalian cells but not in yeast. p23 may be necessary to maintain the structural organisation of organelles (fig. 5.6a. and 5.6b.) in the early secretory pathway in mammalian cells in addition to its secretory functions.

5.3.2. p23 heterozygous mice show haploinsufficiency

The inactivation of one p23 allele leads to decreased levels of p23 mRNA in liver and kidney which then results in a dramatic reduction of p23 protein in these organs in p23 heterozygous mice to approximately 30% of wild type levels. Neither the transcription of the functional p23 allele nor translation of p23 mRNA is upregulated to compensate for the loss of the targeted p23 allele. Interestingly, reduction of p23 protein simultaneously leads to decreased protein levels of GMP25 and p26, two other p24 family members. Although GMP25 protein is reduced in p23 heterozygous mice by about the same extent as p23, the mRNA
levels of GMP25 are indistinguishable between wild type and p23 heterozygous mice. This indicates that inactivation of one p23 allele does not interfere with transcription of other family members, as shown for GMP25. The reduced protein levels of GMP25 and p26 in the p23 heterozygous mice provide a genetic indication that the p24 family members are stabilised by interacting with each other and are likely to form functional heterooligomers.

First indications that p24 family members are interdependent were obtained from yeast showing that Erv25p were significantly lower in a strain deleted for the Emp24 gene (Belden and Barlowe, 1996). Furthermore, absence of Emp24p and Erv25p from yeast cells, resulted in virtually no detectable protein levels of two other p24 family members (Marzioch et al., 1999). The notion that p24 family members form functional complexes is also supported by recent studies, in yeast and in mammalian cells which have demonstrated a specific coimmunoprecipitation of different p24 family members (Fullekrug et al., 1999; Gommel et al., 1999; Marzioch et al., 1999). The steady state levels of one p24 family member could thereby limit the number of functional complexes which may be assembled and family members which are not associated in such a complex would subsequently be degraded.

5.3.3. Reduction of p23 results in structural changes of the Golgi apparatus

The p24 family members are localised to the early secretory pathway and p23 is a, if not the, major protein in the Golgi apparatus. Electron microscopy provided evidence that the reduction of p24 family members in p23 heterozygous mice might have consequences for the structural integrity of the organelles in which they are localised. Electron microscopy of liver and kidney sections from wild type and p23 heterozygous mice revealed subtle changes in the structure of the Golgi apparatus, with the normally flattened cisternae appearing dilated in p23 heterozygous mice. This effect was seen to varying degrees and ranged from only one affected cisternae, to dilation of all cisternae of one Golgi apparatus. It is not yet determined whether, in the Golgi stacks with only one cisternae dilated, the dilation has occurred at the cis or the trans side of the Golgi. Considering that
the p24 family members are highly abundant in the cis Golgi one would expect that changes become more obvious at the cis Golgi. In addition the dilation was particularly obvious at the rims of the Golgi, the main areas where vesicle fusion and budding occurs. It seems as if the organisation of Golgi saccules are less stable in p23 heterozygous mice and that the membranes of the saccules are more easily torn apart. At the moment it cannot be excluded that this effect is due to fixation but even if this was the case it shows that the Golgi morphology is much more fragile in the p23 heterozygous mice compared to wild type controls.

5.3.4. Altered subcellular distribution of p24 family members

Confocal microscopy on MEFs isolated from wild type and p23 heterozygous mice indicates that the reduction of p23 and other p24 family members, GMP25 and p26, in p23 heterozygous mice also leads to an alteration of their subcellular distribution. In MEFs from wild type mice p23 localises to a perinuclear structure, likely to be the Golgi apparatus, and to peripheral punctate structures, which may be vesicular structures of the intermediate compartment. In p23 heterozygous MEFs the perinuclear staining with the anti-p23 antibody was similar to the staining observed in wild type MEFs but, interestingly, only very few peripheral punctated structures were stained for p23 in the p23+/− MEFs. A similar change in subcellular distribution was seen for p26. Again, in wild type MEFs p26 localised to the perinuclear Golgi-like structure and peripheral punctate structures and - as seen for p23 - in the p23+/− MEFs mainly the Golgi-like structure shows p26 localisation while the amount of p26 positive peripheral structures was much reduced. This observed redistribution of both p23 and p26 in p23 heterozygous fibroblasts supports the idea that the p24 family members not only form functional complexes but are also interdependent in their subcellular distribution (Dominguez et al., 1998; Gommel et al., 1999).

In mammalian cells expression of GMP25 and p23 carrying mutations in their KKXX retrieval signal causes not only a redistribution of GMP25 and p23 to the cell surface but also of p24 and p26/gp27 (Dominguez et al., 1998). Recently, it has been reported that p26 seemed largely absent from a heteromeric complex of
p23, p24, GMP25 and gp27 (Fullekrug et al., 1999). However, the reduced amount of p26 assayed by Western blotting and the observed changes in subcellular distribution in p23 heterozygous mice clearly suggest that p26 interacts at some stage with p23 and that p26 is not independent from p23.

5.3.5. The influence of p23 reduction on COPI distribution in MEFs

In vitro binding assays have shown that the cytoplasmic domains of p23 and GMP25, which both consist of an ER retrieval motif, are able to interact with coat proteins of COPI coated vesicles (coatomers) and might, therefore, function as a receptor for coatomers on the membrane (Dominguez et al., 1998; Fiedler et al., 1996; Harter and Wieland, 1998). Furthermore, p23 has been shown to be ~20-fold enriched in COPI coated vesicles (Sohn et al., 1996). However, other reports concluded from studies in BHK and HeLa cells that p23 and COPI are unlikely to colocalise (Blum et al., 1999; Rojo et al., 1997). Recent studies support the distinct and not exclusive localisation of COPI and p24 family members (shown for gp27) in Vero cells (Fullekrug et al., 1999).

To investigate whether the altered subcellular distribution of p23 in p23+/− MEFs affects COPI distribution, double immunofluorescence analysis was performed with antibodies against p23 and COPI or p26 and COPI. In wild type MEFs p23 and COPI localised to the Golgi-like structure and both antibodies stained peripheral punctate structures. However, it appeared as if the punctate staining for p23 and COPI did not exclusively overlap and a significant number of punctate structures was either stained for p23 or for COPI but not for both. The same effect was observed for p26 and COPI double staining with COPI appearing to be absent from many p26 positive peripheral punctate structures. In addition there were areas in the Golgi which appeared predominantly stained for p26 or COPI. However, it is difficult to determine colocalisation by confocal microscopy because of the lack of resolution. Interestingly, the subcellular distribution of COPI was indistinguishable between wild type and p23 heterozygous MEFs and did not show any reductions as observed for p23 and p26 in the p23+/− MEFs.
The antibody use to detect p23 recognises its cytoplasmic domain which is the part that interacts with the coatomers. Thus, the p23 proteins which have bound coatomer would be expected to be “invisible” to the antibody because the epitope is hidden. The reduction of p23 positive punctate structures could reflect the reduction of p23 in the coatomer-unbound state because in relation to p23 coatomers are in about 3-fold excess in the $p23$ heterozygous mice. This would mean that the ratio between free and coatomer-bound p23 is shifted towards the coatomer-bound state, which further decreases the amount of p23 recognised by the antibody. It could, therefore, be possible that the observed reduction of p23 positive punctate structures in $p23$ heterozygous MEFs is simply due to efficient binding of coatomers to the available free p23 and thus giving the impression that the actual number of p23 positive punctate structures is reduced. However immunofluorescence with an antibody against p26 showed a very similar reduction of p26 positive punctate structures in $p23$ heterozygous mice. p26 does not interact with coatomers and thus the epitope on p26 is not blocked by coatomer, nevertheless, p26 positive punctate structures are reduced. Given that p24 family members form oligomeric complexes (Marzioch et al., 1999) it is likely that the number of p26 positive structures correlates to the number of p23 positive punctate structures. This would argue that the reduced number of p23 positive punctate structures in $p23$ heterozygous mice is not simply caused by masking of epitopes.

Given that the antibody against p23 cannot access the p23 protein on COPI coated vesicles and that vesicles have to shed their coat before fusing with the target membranes; it is tempting speculate that COPI positive punctate structures are coated vesicles that just have budded from the donor membrane and that p23 positive punctate structures are vesicles which have already shed their coat are ready to fuse with membranes of the Golgi.

5.3.6. Protein secretion in $p23$ heterozygous mice

Inactivation of the yeast p24 family members, Emp24p or Erv25p, results in a delayed transport of some secretory proteins (Gas1p and invertase), while
secretion of other proteins is not affected (Belden and Barlowe, 1996; Marzioch et al., 1999; Schimmoller et al., 1995). In addition, microinjection of an antibody raised against the cytoplasmic domain of p23 has been reported to interfere with protein transport to the surface (Rojo et al., 1997). Together, these studies imply that the reduction of p23 in p23 heterozygous mice may show selective defects in protein secretion.

Pulse chase experiments were performed with wild type and p23 heterozygous MEFs and total protein secretion into the culture medium was analysed. Comparison of the amounts of secreted proteins and the onset of secretion revealed no significant differences between wild type and p23+/− MEFs. Autoradiography showed the same proteins in comparable quantities and earliest secreted proteins in the culture medium were detected at the same time points from wild type and p23 heterozygous MEFs.

To analyse protein secretion under more physiological conditions in vivo, blood plasma proteins from wild type and p23 heterozygous mice were separated by 2D gel electrophoresis. The protein maps obtained from these separations revealed that the majority of proteins was secreted at comparable amounts and no major proteins were missing in plasma samples from p23 heterozygous mice. However, some proteins seemed to be less efficiently secreted in the p23 heterozygous mice. The protein spots marked by arrow heads (fig. 5.9.) appeared consistently weaker in blood samples from p23+/− mice than in blood samples from wild type littermates or mice from different mouse strains which were used as genetic background controls. The proteins which were less efficiently secreted in p23+/− mice have not yet been identified. The higher sensitivity and the use of physiological blood samples for the 2D gel electrophoresis - as compared to the in vitro pulse chase experiments - could explain the observation that differences were observed in plasma samples but not in protein secretion of fibroblasts.

The results obtained by 2D gel analysis are consistent with the findings in yeast that inactivation of Erv25p (the closest p23 homologue) results in a selective delay in the transport of specific secretory proteins (Belden and Barlowe, 1996). The lack of ERGIC-53, which is also localised in the early secretory pathway and which plays a role in vesicle trafficking has been shown to be involved in the
specific transport of blood clotting factors V and VIII and ERGIC-53 deficient patients suffering from haemophilia (Nichols et al., 1998). Thus, ERGIC-53 probably functions as a selective cargo receptor for these molecules (Nichols et al., 1998). In the light of these studies, the observed selective defects in protein secretion in $p23$ heterozygous mice is not unexpected.

5.3.7. Retrograde transport in $p23$ heterozygous MEFs

The experimental data suggest that the retrograde protein transport is unaffected by the reduction of $p23$ protein levels in $p23$ heterozygous mice. MEFs from wild type and $p23$ heterozygous mice were preincubated with two toxins which have to be transported from the cell surface into the ER to be able to kill the cells. A prolonged survival of $p23$ heterozygous MEFs in the presence of these toxins would suggest that the toxin is transported more slowly to the ER. Since no differences in the rates of survival of wild type and $p23$ heterozygous MEFs could be detected, the reduction of $p23$ protein levels does not seem to affect the retrograde transport machinery.

Nevertheless, recent studies suggest that $p23$ might very well be involved in retrograde transport as microinjection of antibodies against $p23$ inhibited the transport of cholera toxin A subunit from the Golgi to the intermediate compartment (Majoul et al., 1998). One possible explanation for the difference between these studies and the present one could be that even low toxin concentrations are efficiently transported in $p23^{+/−}$ mice and that defects would only become visible upon overloading the retrograde transport machinery. However, even usage of very low toxin concentrations did not reveal a prolonged survival of $p23$ heterozygous MEFs and thus a delay in retrograde transport (data not shown). As observed from confocal microscopy, the reduction of $p23$ positive punctate structures (likely to be transport clusters of the intermediate compartment) was more prominent than a reduction of $p23$ in the Golgi apparatus. This may suggest that retrograde transport from the Golgi is less affected by reduction of $p23$ than the anterograde transport.
5.3.8. Future directions

The studies presented here show that gene disruption of p23 in mice leads to early embryonic lethality, with the loss of p23 homozygous blastocysts. The early lethality in a mammalian organism is surprising, considering that a yeast strain deficient for four p24 family members are viable and does not show any growth abnormalities. In contrast to earlier speculation that the abundance of p24 family members would be likely to accommodate the loss of one family member, p23 is essential for viability in mice. p23 heterozygous deficient mice appear grossly normal. However, the decrease of p23 protein in $p23^{+/\cdot}$ mice results in a reduction of other family members, structural changes in the Golgi apparatus and an altered subcellular distribution of p23 itself and other p24 family proteins.

The physiological roles of p23 remain still unknown. Half the germ cell population of male and female p23 heterozygous mice is p23-deficient. Therefore, it is unlikely that p23 is required for single cell viability. It would be interesting to analyse the morphology of the Golgi apparatus of p23-deficient oocytes or sperm. These studies could be carried out by electron microscopy or even confocal microscopy using antibodies against p23 and Golgi markers, respectively. The hypothesis that p23 is necessary for disassembly or reassembly of the Golgi could be tested in in vitro experiments, where this process might be compromised after adding synthetic peptide corresponding to the cytoplasmic domain.

The protein spots which appeared weaker in the 2D gel from p23 heterozygous mice could be isolated and sequenced. This may give information for which mammalian proteins p23 is potentially functioning as a cargo receptor. The toxin assays were performed with very potent toxins; the use of less powerful toxins may show inefficiencies in retrograde transport in the p23 heterozygous mice. Time-lapse microscopy after internalisation of dye-coupled agents may provide information of the transport rates in p23 heterozygous cells.
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