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Investigation into the assembly of adiponectin as a target for countering obesity related diseases

Lutz Hampe

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

The University of Auckland

August 2016
Abstract

Adiponectin, a collagenous hormone secreted abundantly from adipocytes, possesses potent anti-diabetic and anti-inflammatory properties. Mediated by the conserved Cys39 located in the variable region of the N-terminus, the trimeric (low molecular weight (LMW)) adiponectin subunit assembles into different higher order complexes, e.g. hexamers (middle molecular weight (MMW)) and 12-18-mers (high molecular weight (HMW)), the latter being mostly responsible for the insulin-sensitizing activity of adiponectin. The endoplasmic reticulum (ER) chaperone ERp44 retains adiponectin in the early secretory compartment and tightly controls the oxidative state of Cys39 and the oligomerization of adiponectin. Biologically active, recombinant and pure adiponectin oligomers are difficult to produce hampering the analyses of the oligomerisation process. To mitigate this production problem, we engineered and synthesized a model peptide of the N-terminal domain of adiponectin. We demonstrated that the peptide could be used for probing the influence of the variable domain on the multimerization of this important circulating hormone as well as the interaction between the adiponectin and ERp44.

Using cellular and in vitro assays, we showed that ERp44 specifically recognizes the LMW and MMW forms but not the HMW form. Binding assays with short peptide mimetics and the aforementioned peptide model of the N-terminal domain of adiponectin suggest that ERp44 intercepts and converts the pool of fully oxidized LMW and MMW adiponectin, but not the HMW form, into reduced trimeric precursors. In vivo, these ERp44-bound precursors in the cis-Golgi may be transported back to the ER and released to enhance the population of adiponectin intermediates with appropriate oxidative state for HMW assembly, thereby underpinning the process of ERp44 quality control.

Obesity-induced ER stress causes dysregulation of ER chaperone activity in vivo including ERp44 action, resulting in a decreased level of secreted HMW, which is associated with insulin resistance and type 2 diabetes. Using adipocyte cells and genetically obese mice we demonstrated that designed peptide mimetics derived from ERp44 clients can restore dysregulated ERp44 activity and in turn facilitated adiponectin assembly into HMW form and promote adiponectin release from the ER.
Therefore, these peptides can act as reagents to counteract impaired adiponectin multimerization caused by dysregulation in ER chaperone activity.
Acknowledgements

First and foremost, I would like to thank my principal supervisor Professor Alok K. Mitra who provided me the opportunity to undertake my Ph.D. under his supervision. I am very grateful for his expertise and excellent mentorship and feel privileged for having been one of his students.

I want to express my gratitude to my co-supervisor Dr. Mazdak Radjainia for his great mentorship. He has contributed to many aspects of my research and I have greatly benefited from his knowledge and experience. Mazdak also introduced me to this great country and showed me how to enjoy life in New Zealand. Thank you for all the great moments in the past four years.

Many thanks go to Professor Yu Wang and Dr. Cheng Xu (Aaron) for a very fruitful collaboration. I have greatly benefited from the helpful scientific discussions throughout this project. Thanks for hosting me in Hong Kong and teaching me how to work with cell-based assays, as well as show how to conduct mice experiments and providing some of the results shown in chapter four and six of this thesis.

I was also fortunate to work with Dr. Ghader Bashiri. I have greatly benefited from the helpful discussions. Also thanks for all the help with crystallography-related work. I would like to thank Professor Margaret Brimble, Dr. Meder Kamalov and Dr. Paul Harris for introducing me to the world of peptide synthesis and for all the helpful discussions.

I had the pleasure to work with a lot of great people throughout the last four years. I would like to thank all members of the Structural Biology Laboratory for their support and help that contributed to my work. In particular, I want to thank Hariprasad Venugopal; Dr. Ambroise Desfosses and Dr. Joe Bartho for being such great labmates; Ben Rushton for a great year working together on ERp44 dimerization; Dr. David Goldstone for advising me on SEC-MALS experiments; Martin Middleditch
for conducting the mass spectroscopy experiments; Dr Kien Ly for helping out with lab supplies; and James Dickson for advising me on molecular cloning.

This work would have not been possible without the support of my friends in New Zealand and back home. Thanks to all of you for being there for me and special thanks to Jully Pinheiro for supporting me and her companionship.

Lastly, I would like to thank my parents and sister, who believed in me and have always been encouraging and supportive despite being so far. I am very grateful to have you all.

This work was generously sponsored by the German Academic Exchange Service (DAAD) and the University of Auckland.
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<tr>
<td>AdipoR1</td>
<td>adiponectin receptor 1</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>adiponectin receptor 2</td>
</tr>
<tr>
<td>AHD</td>
<td>adiponectin hypervariable domain model peptide</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>BiP</td>
<td>chaperone Binding Protein</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CNX/CRT</td>
<td>calnexin/calreticulin</td>
</tr>
<tr>
<td>co-IP</td>
<td>co-immunoprecipitation</td>
</tr>
<tr>
<td>COPII</td>
<td>Coat Protein complex II</td>
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<tr>
<td>CPPs</td>
<td>cell penetrating peptides</td>
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<tr>
<td>DsbA-L</td>
<td>disulfide-bond A oxidoreductase-like protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERDA</td>
<td>ER associated degradation</td>
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<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
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<tr>
<td>ERGIC-53</td>
<td>ER–Golgi intermediate compartment-53</td>
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<tr>
<td>Ero1-Lα</td>
<td>ER membrane associated oxidoreductase – like α</td>
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<td>ER resistant 44 kD protein</td>
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<td>early secretion compartment</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
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<td>GPx7/8</td>
<td>glutathione peroxidases-7 and -8</td>
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<td>GSH</td>
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<td>GSSG</td>
<td>oxidized glutathione</td>
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<td>glutathione transferase Kappa</td>
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<td>HMW</td>
<td>high-molecular –weight</td>
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HPLC  High Performance Liquid Chromatography
HSe  Homoserine
I/R  ischaemia/reperfusion
IgM  Immunoglobulin M
IL  interleukin
IRE1α  inositol-requiring enzyme 1α
KDEL-R  KDEL recognizing receptors
LC-MS  liquid chromatography-coupled with mass spectrometry
LMW  low-molecular-weight
MMW  middle-molecular-weight
MS  metabolic syndrome
NAFLD  non-alcoholic fatty liver disease
NASH  non-alcoholic steatohepatitis
NCL  native chemical ligation
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NMR  nuclear magnetic resonance
OST  oligosaccharyltransferase complex
p38MAPK  P38 mitogen-activated protein kinases
PAI-1  plasminogen activator inhibitor-1
PCR  polymerase chain reaction
PDI  protein disulfide-isomerase
PERK  protein kinase RNA-like endoplasmic reticulum kinase
PI3K  phosphatidylinositol 3-kinases
PPAR  peroxisome proliferator-activated receptors
Prx4  Peroxiredoxin 4
PTM  post-translational modifications
QC  quality control
RI  refractive index
RP  reversed-phase
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-MALS  size-exclusion chromatography with multi-angle light scattering
SERT  Serotonin Transporter
SPPS  solid phase peptide synthesis
SRP  signal recognition particle

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<table>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAT</td>
<td>trans-activating transcriptional activator</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin-like</td>
</tr>
<tr>
<td>TUDCA</td>
<td>tauroursodeoxycholic acid</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinediones</td>
</tr>
<tr>
<td>UGGT</td>
<td>UDP-glucose glycoprotein glucosyltransferase</td>
</tr>
<tr>
<td>URP</td>
<td>unfolded protein response</td>
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Co-Authorship Forms

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**chapter 4: Regulation and quality control of adiponectin assembly by ERp44**


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<td>Margaret A. Brimble</td>
<td>devised peptide synthesis experiments.</td>
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**Certification by Co-Authors**

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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Chapter 4 - Regulation and quality control of adiponectin assembly by ERP44

| Nature of contribution by PhD candidate | Planned, designed and executed most of the experiments, manuscript write up. |
| Extent of contribution by PhD candidate (%) | 85% |

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Last updated: 19 October 2015

XIX
Chapter one - Introduction

1.1 Obesity and type 2 diabetes mellitus
Over the last three decades, diabetes mellitus has developed into one of the major threats to human health. Mainly pronounced changes in the environment, behavior and lifestyle have led to an escalation in the rates of both obesity and diabetes. Two forms of diabetes are primarily distinguished: Type 1 and Type 2 (T2DM) [1]. Type 1 diabetes is characterized by complete absence of insulin production due to autoimmune-mediated destruction of insulin producing β-cell in the pancreas [2]. Compared to Type 2, cases of Type 1 diabetes are rare; in fact, Type 2 diabetes accounts for over 90% of the ca. 350 million cases globally [3]. Insulin resistance and/or abnormal insulin secretion are the predominant causes of Type 2 diabetes. While patients who suffer from Type 1 diabetes depend on the administration of exogenous insulin for survival to prevent the development of ketoacidosis, Type 2 diabetes patients are not dependent on exogenous insulin [3]. However, if control of the blood glucose level can not be achieved with diet alone or with oral hypoglycemic agents, treatment with exogenous insulin is required [1]. The escalating global epidemic of T2DM is strongly connected to rising rates in obesity. In fact, around 44% of diabetes burden can be attributed to obesity. The World Health Organization estimates that the rate of overweight (body mass index (BMI) of 25–30 kg/m²) or obesity (BMI of ≥30 kg/m²) in the global population will rise from 33% in 2005 to 57.8% in 2030. This rise is predicted to also raise the number of diabetic patients [4]. Thus, unsurprisingly the World Health Organization predicts diabetes to become the number one cause of death. In addition, obesity is attributable to other non-communicable diseases such as cardiovascular disease of which 23% of the worldwide burden can be linked to obesity [1, 4].

‘It is abundantly clear that obesity and diabetes have already imposed a huge burden on health-care systems and this will continue to rise in the future. Hence, the interest in diabetes and obesity linked disease is now mounting rapidly and provides exciting opportunities for researchers and clinicians involved in such studies’[1].
1.2 Adipose tissue and adipokines

Adipose tissue has long been believed to serve only as energy storage. Its primary function during the lifecycle of animals and humans is to store energy when food is available in excess, which may be released when food resources are rare and energy is required. Energy homeostasis is achieved in adipocytes, an energy reservoir, through expansion in response to food intake exceeding energy needs and releases of lipids in response to energy deficit [5].

In the late 1980s, a number of studies reported that adipocytes can secrete a number of factors and hormones involved in metabolic regulation. Despite this exciting discovery, it took a decade until adipocytes were cast as endocrine cells. From this point onwards, adipocytes have been subjected to active research and a whole new family of hormones was discovered; which are now called ‘adipokines’ [6]. Amongst those adipokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, plasminogen activator inhibitor (PAI)-1, adipin and resistin are well studied. These hormones are involved in many different systemic phenomena and act on physiological processes in multiple organs, such as brain, muscles and liver [7]. Many adipokines are also expressed in other organs and only few are exclusively produced in adipocytes. Of those, adiponectin is the most prominent member [8].

In the case of obesity, important changes in production and secretion of adipokines, as well as metabolic rearrangements occur, resulting in a disorder known as the metabolic syndrome (MS). MS is accompanied by increased waist circumference, dyslipidaemia, increased serum triglyceride levels, hypertension, and insulin resistance [9].

1.3 Adiponectin

1.3.1 Adiponectin source and target tissue

Adiponectin, also termed Acrp30, ADIPOQ, apM1 or GBP28, is a 30 kDa protein and was first described in 1995 by Scherer and colleagues [10-12]. Abundantly expressed and produced in white adipocytes, and also in skeletal muscle endothelial cells and cardiac myocytes[13], this serum protein has attracted much attention due to its multiple beneficial functions. Adiponectin acts on various target tissues such as, skeletal muscle, liver and heart and can protect against almost all of the major
obesity-related pathologies [14]. Well-studied for its insulin sensitizing properties, adiponectin has also been shown to protect against hypertension [15], atherosclerosis [16], NAFLD (non-alcoholic fatty liver disease) and NASH (non-alcoholic steatohepatitis) [17], heart failure [18], airway inflammation [19] and several types of cancer (Figure 1.1)[14, 20-22]. Unlike most adipokines, adiponectin serum concentrations are decreased in case of obesity, resulting in loss of its protective function, thus consequentially leading to medical complications [22].

Figure 1.1: Summary of multiple physiological actions of adiponectin.
Adiponectin has been associated with multiple physiological actions. Although adiponectin is most popular for its insulin sensitizing function, the hormone was also found to protect against almost all of the major obesity-related pathologies; this includes hypertension, atherosclerosis, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis, heart failure, airway inflammation and several types of cancer. (I/R, ischaemia/reperfusion; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.) This Figure was adapted from [22]

1.3.2 Adiponectin structure and polymorphism
Adiponectin belongs structurally to the TNF-α/complement factor C1q superfamily [23]. The primary sequence of adiponectin is composed of four domains. At the N-terminal end a 21 amino acid long signal peptide ensures its translocation into the endoplasmic reticulum (ER). The signal sequence is followed by a region that lacks any commonly recognizable structural motif and is referred to as the hyper-variable
domain [24-26]. This region shows no sequence similarity to any other known protein and includes a 12 amino acid long stretch of highly conserved residues that plays a critical role in the oligomerisation of the protein. Upstream of this hyper-variable domain is a collagenous-like domain comprised of 22 Gly-Xaa-Yaa repeats (where Xaa and Yaa can be any other amino acid, however, Xaa is frequently occupied by proline and Yaa by hydroxyproline). A fourth domain, which is globular, is located at the C-terminus of the protein. This domain accounts for approximately 55 % of the total number of amino acid residues in the entire molecule [27-29]. Structurally this domain is similar to the C1q-like globular domain of other TNF-like superfamily members and folds into a ten-beta-stranded jellyroll topology analogous to the TNF-α protein (Figure 1.2 A and Figure 1.3)[24].

The smallest adiponectin unit observed outside adipocytes is trimeric [30]. Trimerization is driven by extensive hydrophobic interactions between the globular domains of the three individual monomers [23, 31]. This interaction serves as the seed for the collagen-like stretch leading to the formation of a stable triple helical structure [24]. The architecture of the trimeric building block as visualized by electron microscopy (EM) reveals a lollypop-like appearance (Figure 1.3)[32].

The trimer, which is also termed low-molecular-weight (LMW) adiponectin, can further assemble into hexameric (middle-molecular-weight;MMW) and/or 12-18 (high-molecular -weight) isoforms (Figure 1.2 B)[30, 33, 34]. The hexamer forms through disulphide linkage of two homotrimers mediated by a cysteine residue (C36 in human and C39 in mouse adiponectin) in the highly conserved region of the hyper variable domain (Figure 1.2B)[27, 33, 35]. As demonstrated by mutational studies, this residue is essential for the formation of hexamers as well as the HMW isoform [24, 33, 35-37]. The hexamer typically appears in the shape of a Y on electron micrographs with the globular domains clearly delineated in a head to head manner (Figure 1.3)[36]. The HMW form appears as an arrangement of three to six trimers. The octadecameric form is the most prominent and elaborate in the form of a ‘bouquet’ or ‘fan’.

Due to its heterogeneous nature, structural characterisation of the HMW form remains difficult. Sedimentation equilibrium and dynamic light scattering studies showed
octadecamers for HMW adiponectin purified from NIH 3T3-L1 adipocytes and bovine serum [36]. In contrast, gel electrophoresis of human adiponectin shows multimers in a range from 18 to 30mers and even higher complexes [38].

Figure 1.2: Schematic showing the structural domains, post-translational modifications, species alignments and multimerisation of adiponectin.

A: The four structural domains of adiponectin are shown in block form (amino acid numbers are those for human adiponectin). The expanded region, encompassing the variable and collagenous domains, shows the various post-translational modifications (PTM). Conservation between species is shown by Clustal X alignment. B: Organisation and multimerisation of adiponectin. This figure was reproduced from [25].
Chapter 1 – Introduction

Low-resolution EM reconstructions of the octadecamer suggest a dynamic structure of HMW that can be subdivided into two main structural arrangements, the ‘fan-shaped’ and ‘bouquet-shaped’ adiponectin oligomer. The asymmetric ‘bouquet-like’ architecture of adiponectin and the complement C1q resemble each other in appearance. Both consist of six trimers held together by interactions between the globular head domains, which are arranged in a tight ring atop a thin stack, consisting of the six collagenous domains and further stabilized by extensive contact at the N-terminal end of the protein (Figure 1.3)[32].

1.3.3 Functional polymorphism of adiponectin

Heterogeneity in the multimeric state of adiponectin is closely related to its multiple biological functions [39]. For example, the HMW form is believed to be mainly responsible for adiponectin’s insulin sensitizing and anti-diabetic properties [22]. The first evidence for this was provided by Scherer and colleagues, who found that a shift in the ratio of HMW to total adiponectin levels towards the HMW form rather than the absolute adiponectin concentration is correlated with improved insulin sensitivity in patients with T2DM. This was also the case in mice after treatment with thiazolidinediones (TZD), an insulin sensitizing drug [40]. This observation was further supported by the fact that HMW blood levels increased, whereas LMW and MMW levels remained unchanged after weight reduction due to calorie restriction in human patient and mice [41, 42]. Furthermore, obesity-induced metabolic abnormalities are better correlated with HMW levels than to the absolute adiponectin blood concentration. Thus, several studies suggest HMW to be a better biomarker for insulin resistance, MS and T2DM rather than the total adiponectin concentration [43, 44].

Currently, it is believed that different adiponectin forms target different tissues and elicit distinct functions. The major target tissue for HMW has been shown to be the liver. Here, HMW adiponectin acts as an insulin sensitizing hormone [45-48] and activates AMP-activated protein kinase (AMPK) leading to improved fatty acid oxidation and glucose uptake [30, 44, 47]. In addition, HMW adiponectin was shown to exert insulin-sensitizing effects in the liver following an AMPK-independent pathway. However, the exact underlying mechanism remains unknown [44, 49, 50].
In addition, the HMW form has also been shown to be the form of adiponectin most protective against fatty liver disease [51].

**Figure 1.3: Images of different adiponectin oligomers**

Electron micrographs of adiponectin oligomers (A trimer, B hexamer, C 18mer; round boxes mark bouquet-shaped particles and square boxes mark fan-shaped particles)[32].
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The trimeric globular form of adiponectin by itself was shown to be active in skeletal muscle improving fatty acid oxidation and glucose uptake [52, 53]. The LMW and MMW, but not the HMW form act on the central nervous system to regulate appetite and energy expenditure [48, 54, 55]. Different adiponectin oligomers are also attributed anti-cancer properties, for example HMW adiponectin inhibits cell growth in prostate cancer and various oligomers are connected with cell apoptosis [56] in breast cancer (Figure 1.1)[22, 57].

Several in vivo studies in mice suggest beneficial effects of adiponectin in cardiovascular disease. For example, low adiponectin levels are associated with coronary artery disease [58, 59]. Vice versa, patients with higher adiponectin levels exhibited decreased risk of myocardial infraction [60]. Mice studies further support these findings, apolipoprotein-E knockout mice with characteristically higher blood concentration of globular adiponectin were found to have a lower propensity to form atherosclerotic lesions [16]. Furthermore, adiponectin knockout mice demonstrated increased neointimal hyperplasia and smooth muscle cell hyperplasia, and impaired neovascularization after acute vascular injury [6, 61].

Adiponectin also exhibits anti-inflammatory properties by decreasing expression and secretion of inflammatory cytokines such as TNF-α and IL-6. Both HMW and LMW blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in endothelial and monocytic cells, and thus counteract inflammation and metabolic alteration in obesity, caused by NF-κB activity [62, 63].

1.3.4 Adiponectin receptors and molecular signalling in insulin resistance and T2DM

Each of the different adiponectin oligomers shows different affinity to one of the three receptors namely, adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2) and T-cadherin [62, 64]. AdipoR1 and AdipoR2 are 7-transmembrane proteins with opposite topology to that for G-protein-coupled receptors (GPCRs) and are believed to exhibit a unique mode of action [65]. Expression levels of Adipo1 and Adipo2 vary from cell type to cell type - AdipoR1 being the one mainly expressed in muscle tissue, while AdipoR2 is mainly abundant in hepatocytes [66]. Globular adiponectin has high affinity to AdipoR1 whereas full-length adiponectin shows low
affinity to AdipoR1. AdipoR2 has higher affinity to HMW and MMW but low affinity to LMW. MMW and LMW also bind to T-cadherin [66]. Adiponectin receptor binding activates several intracellular signaling pathways leading to the activation of multiple molecules including, phosphatidylinositol 3-kinases (PI3K), P38 mitogen-activated protein kinases (p38MAPK), peroxisome proliferator-activated receptors (PPAR) and AMPK. Among those the AMPK pathway involving PPARs plays a dominant role leading to modification of lipid and carbohydrate metabolism and in turn improvement in insulin resistance and T2DM [48, 62, 64].

T-cadherin was shown to bind to adiponectin and plays an essential role in adiponectin-modulated protection against cardiac stress in mice [67, 68]. T-cadherin is also believed to participate in adiponectin binding to AdipoR1 and AdipoR2. In addition, T-cadherin can initiate adiponectin signal transduction, however its exact role and mechanism of action remains unknown [45, 69].

1.3.5 The hyper-variable domain regulates adiponectin multimerization

The N-terminal domain of mature adiponectin shows a stretch of 12 highly conserved amino acids (18-44 in murine adiponectin) that are involved in adiponectin multimerization. Within this stretch cysteine residue 39 in murine adiponectin is conserved throughout all species (Figure 1.2 A). Several groups demonstrated that mutation of this residue to serine or alanine impairs adiponectin multimerization into the MMW or HWM form [30, 34, 37]. Furthermore, multiple lines of evidence support that the formation of disulfide bonds between cysteine residues of homotrimers facilitates multimerization into the HMW form. Although it is known that almost all cysteine residues within the MMW and HMW are disulfide bonded to each other, the exact disulfide linkage arrangement of HMW adiponectin still needs to be elucidated [30, 34, 37]. For the MMW form it is known that two trimers are held together by a disulfide link between two cysteine 39 residues within two different homotrimers [30, 34, 37].

In vitro, reassembly experiments of purified bovine adiponectin showed that adiponectin can spontaneously assemble into the HMW form [35]. Furthermore, [70, 71] showed that the spontaneous self-assembly strongly depends on the redox environment and can be influenced by metal ions, such as zinc.
Tsao and colleagues also observed that the MMW form can exist in different redox states. All hexamers possess at least one intermolecular disulfide link between two homotrimers. The remaining cysteine residues can either be reduced or form disulfide bonds with each other. The resulting disulfide bond network can include multiple intermolecular disulfide bonds between two trimers or intramolecular disulfide bridges within a trimer. The redox state of the hexamer is important since only hexamers, which possess a single disulfide bridge, can further assemble into higher order complexes [72]. ‘Blocked’ intermediates, i.e. hexamers, that exhibit more than one disulfide bond, are referred to as ‘assembly trapped’ and cannot form higher order complexes.

In addition to cysteine bonds, non-covalent interactions between trimeric subunits stabilize the HMW complex. This is because even when all cysteine residues are reduced in the HMW form, the complex remains intact. On the other hand the HMW complex collapses at a pH below pH 6. Such a drop in pH leaves only hexameric species [35].

In addition, a highly conserved tryptophan residue (W42 in murine adiponectin) is known to play a critical role in the assembly of HMW by presumably acting as a modulator of oxidation and disulfide bond formation of C39 [73].

Careful investigation of the EM reconstruction of the HMW form reveals a well-contrasted clear volume of electron density at the N-terminal end of the complex especially distinguished in the bouquet-like arrangement, suggesting a compact structure of the clearly positioned N-terminal domains. This observation is again in line with the observation that the conserved N-terminal region is important in the assembly pathway of the HMW form [32].

1.3.6 Post-translational modifications stabilize the trimeric structure of adiponectin and are required for the formation of HMW adiponectin

Mammalian adiponectin, particularly the HMW form, shows multiple post-translational modifications including various proline hydroxylations and lysine glycosylations (Figure 1.2 A)[74, 75]. Two-dimensional gel electrophoreses illustrates the polymorphous nature of adiponectin [76]. Crude purified serum adiponectin is
composed of various oligomers, of which each oligomer includes multiple different species that exhibit various post-translational modifications. The polymorphism in post-translational modifications is believed to contribute to the diverse functions of adiponectin although a direct link between the two still needs to be established [14].

Several different groups demonstrated the contribution of post-translational modifications in the formation of the HMW form. Recombinant adiponectin expressed in E. Coli only forms LMW, likely due to the lack of post-translational modifications [14, 75]. Also, purified LMW adiponectin from normal human plasma has only approximately 30% of the carbohydrate content of the HMW purified from the same plasma sample [77].

Despite the strong evidence that post-translational modifications affect HMW formation the biochemical basis of how these modifications contribute to the HMW formation remains speculative. In one possible scenario, the post-translational modification restrict flexibility of the collagen triple helix and/or contribute to a compact packing of the collagen like structure, which might be required for the formation of HMW adiponectin, i.e. enable the compact clustering of six triple helix of the octadecamer. Another possibility is that interactions between lysine and/or proline residues within separate trimers of the HMW play a role in the stability of the octadecamer. In this case, post-translational modification might play an important role in these interactions [24].

In addition to lysine glycosylation, adiponectin also possesses sialic acid modifications, which contribute to the half-life of adiponectin in circulation [78, 79]. Using mass spectrometry Kitajima identified two sialic acid-containing glycopeptides near the N-terminus of mature adiponectin [78]. However, sialylation appears not to influence oligomerization [79].

1.3.7 ER chaperones and oxidoreductases control adiponectin assembly and secretion

The different adiponectin oligomers do not interchange once released from adipocytes into circulation. This means adiponectin oligomer distribution in circulation is controlled by the assembly/secretion level in adipocytes [30]. Given the importance of
adiponectin oligomer distribution for health as well as various disease states, understanding the molecular machinery behind the assembly process is of great importance [14].

Three main players have been identified that tightly regulate adiponectin secretion and assembly in the endoplasmic reticulum (ER). The ER resident 44 kD protein (ERp44) [80] and ER membrane-associated oxidoreductase – like α (Ero1-Lα) [81] have been demonstrated to modulate adiponectin secretion in a Ying and Yang like fashion [22], whereas the disulfide-bond A oxidoreductase-like protein (DsbA-L), also named glutathione transferase Kappa (GST-kappa), acts on adiponectin multimerization via an independent pathway (Figure 1.4)[82].

A significant portion of *de novo* produced adiponectin is retained in adipocytes for a prolonged period via thiol-mediated retention. The retention can be reversed by treatment with reducing agents and was shown to involve ERp44 binding. ERp44 covalently binds to Cysteine 39 of adiponectin in the ER and prevents adiponectin from proceeding along the secretion pathway. It is believed that this thiol-mediated retention by ERp44 maintains adiponectin in an oxidizing environment, namely the ER, where maturation of adiponectin oligomers and extensive post-translational modifications can occur. The extended residence in the ER provides a better chance for adiponectin to properly fold into its desired multimeric state. Accordingly, overexpression of ERp44 decreases the rate of adiponectin secretion, and vice versa, knockdown of ERp44 by si-RNA enhances secretion of this adipokine [80].

In contrast, overexpression of Ero1-Lα, ERp44’s foremost partner, results in increased levels of adiponectin secretion and down regulation of Ero1-Lα inhibits adiponectin secretion. Furthermore, Ero1-Lα can disturb adiponectin retention caused by ERp44. Co-expression of both partners, Ero1-Lα and ERp44 demonstrated Ero1-Lα competes with adiponectin to covalently bind ERp44 and therefore counters the thiol-mediated retention of adiponectin through ERp44 binding. However, siRNA-mediated down-regulation of Ero1-Lα promotes LMW release from adipocytes and inhibits secretion of HMW [80, 81].
Several different mechanisms have been proposed for Ero1-Lα stimulation of adiponectin release and production of HMW. The first mechanism includes Ero1-Lα action as an oxidoreductase, i.e. Ero1-Lα utilizes its ability to oxidize intramolecular cysteine pairs [83]. Through disulfide bond exchange, Ero1-Lα subsequently oxidizes the protein disulfide-isomerase (PDI), which in a later process exchanges its disulfide bonds with adiponectin and promotes formation of disulfide linked adiponectin in higher oligomeric arrangements [84](Figure 1.4).

Secondly, Ero1-Lα competes with adiponectin for ERp44 binding. Hence, Ero1-Lα can potentially displace an ERp44 client and facilitate the release of ER trapped adiponectin and in turn enhances secretion of adiponectin. Although it is obvious that maturation and secretion of adiponectin oligomers is tightly controlled by the interplay between ERp44 and Ero1-Lα in a Ying and Yang fashion the mechanism underlying these observations is only poorly understood [22, 80].

DsbA-L is a 25-kDa ER resident protein and was recently found to modulate adiponectin secretion and multimerization in a manner independent of ERp44 and Ero1-Lα [81]. Overexpression of DsbA-L enhances the production and secretion of HMW adiponectin. In contrast si-RNA-mediated down regulation of DsbA-L expression inhibits secretion of HMW adiponectin in 3T3-L1 adipocytes, leading to lower blood HMW levels. The modulation of DsbA-L expression does not affect secretion and assembly of any other adipokines such as TNFα, leptin or resistin suggesting that DsbA-L selectively regulates adiponectin assembly and secretion [82].

Noteworthy, DsbA-L expression is not adipocyte specific, however expression levels of this protein in liver, kidney, pancreas and heart are remarkably lower than in adipocytes, where adiponectin is synthesized and secreted. In the case of obesity, levels of DsbA-L are significantly reduced in adipose tissues of human subjects and in obese mice, which is believed to be a cause of reduced adiponectin levels in these patients and mice [22, 82]. Taken together, these findings suggest DsbA-L plays an important role in the formation and secretion of HMW adiponectin. However, further investigations will be needed to reveal the mode of action of this protein in the complex assembly pathway of adiponectin [22, 48, 82, 84, 85].
1.3.8 Defective adiponectin secretion and assembly during ER stress and the beneficial effects of TZD

Although the cause for down-regulation of adiponectin in obesity and its precise underlying mechanisms remain elusive, strong evidence supports the idea of a direct link between ER stress [86], obesity and inhibition of adiponectin production in adipose tissue [34]. Most secretory proteins reach their extracellular destination by following the secretory pathway from the ER through the Golgi apparatus to their final destination. Here, the ER serves multiple important biological purposes (also see chapter 1.4.1) such as undergoing crucial post-translational modifications, folding, disulphide bond formation, glycosylation—just to name a few. To facilitate these processes, the ER possesses a cohort of catalysts, folding enzymes and chaperones. These proteins guarantee protein maturation and generate an optimal redox and ionic milieu [87]. Obesity causes a disruption of this fine-tuned ensemble, which causes an accumulation of unfolded proteins in the ER. This obesity-mediated ER dysfunction is called ER-stress. The cell react to this situation with a demand of increased ER function and triggers the evolutionarily conserved unfolded protein response (UPR), which maintains viability and cell function by removing incorrectly folded proteins from the ER [48, 88].

Several groups have investigated the influence of ER stress on adiponectin secretion and have observed that chemically inducing ER stress [89] decreases adiponectin levels in 3T3-L1 adipocytes [90]. Furthermore, ER stress can be partially restored by the treatment with a “chemical chaperone” tauroursodeoxycholic acid (TUDCA). Treatment of genetically obese db/db mice and diet-induced obese mice, whose adipose tissue is chronically subjected to ER stress conditions, with this chemical at least partially restored adiponectin levels [48, 90]. These results indicate that ER stress potentially contributes to decreased adiponectin levels in obesity [48]. Furthermore, ob/ob obese mice showed a reduction in the ratio of HMW to total adiponectin in the circulation [48]. At the same time altered expression levels of ERp44 and Ero1-Lα in adipose tissue were found, which suggested their involvement in the ER stress induced down-regulation of adiponectin [80, 81]. Further support is provided by a large number of studies that have established a relationship between peroxisome-proliferator-activated receptor γ (PPARγ, a transcription factor that positively regulates adiponectin gene transcription) agonists thiazolidinediones (TZD)
treatment, a class of insulin sensitizing drugs, and selectively induce secretion of HMW adiponectin [91]. It was also shown that TZDs induce Ero1-Lα and ERp44 expression in 3T3-L1 adipocytes and adipose tissue of ob/ob obese mice [80], which raises the possibility of their involvement in the observed beneficial effect of TZDs on adiponectin assembly/secretion [22, 48].

Another report revealed that ER stress-induced adiponectin downregulation [90, 92] can be reduced by over-expression of DsbA-L [85, 88, 92]. It is believed that DsbA-L action counteracts high fat diet-induced ER stress and therefore recovers proper adiponectin multimerization, thus, restoring total levels of adiponectin [85]. Also DsbA-L levels in adipose tissue were increased following TZD treatment showing its significance for the oligomer assembly process of adiponectin. However, the precise mechanisms by which DsbA-L promotes adiponectin multimerization in ER stress conditions remains unknown [48].

A possible mechanism for obesity-induced inhibition of adiponectin assembly and secretion includes PPARγ [93-96]. During ER stress PPARγ is suppressed and as a result adiponectin mRNA levels are reduced. However, a discrepancy between adiponectin mRNA levels and adiponectin levels in circulation was demonstrated by multiple groups [24, 97-99], which makes it questionable whether or not the reduction of adiponectin mRNA levels is the cause for inhibition of adiponectin assembly and secretion during ER stress. In contrast, PPARγ regulates the expression of ER chaperones, namely ERp44, DsbA-L and Ero1-Lα [80-82, 84]. Hence, the dysregulation of ER chaperone expression as a consequence of PPARγ suppression is more likely to be involved in obesity-induced reduction of adiponectin secretion. At the same time, PPARγ agonists, such as rosiglitazone and pioglitazone are believed to upregulate the expression of ERp44, DsbA-L and Ero1-Lα, restore the adiponectin assembly and folding machinery in the ER and enhance the secretion of HMW adiponectin, which in turn results in improved insulin sensitivity [22, 24, 48, 84](Figure 1.4).
1.4 Protein folding and quality control

In multicellular organisms, it is essential for survival, that the cells can exchange information with one another in order to adapt to environmental changes, undergo differentiation or apoptosis. Transmembrane receptors and secretory proteins mainly mediate this communication. The fidelity of the interaction between the two partners (receptor and ligand) requires both molecules to adopt their correct three-dimensional structure before being secreted into the extracellular space or integrated into the cell membrane. Misfolded proteins and/or aggregation may prevent proper transmission of signals and can become toxic to cells. Therefore, all eukaryotic cells have developed a quality control mechanism to ensure proper folding of proteins. Folding can occur in three different cellular compartments, the cytosol, mitochondria and the endoplasmic reticulum, each of which is equipped with its own assembly of folding enzymes and chaperones [100].

About one third of all proteins enter the ER and undergo folding and maturation while traversing the secretory pathway [101]. Amongst those proteins the majority are transmembrane receptors and secretory proteins. Also enzymes and chaperones designated to function in specific organelles, such as the ER, ER-Golgi intermediate compartment (ERGIC), Golgi, endosomes and lysosomes start the journey to their final destination by first entering the ER [102].
The ER lumen is physically separated from the cytosol and provides an environment that is optimized for protein folding. Firstly, the ER lumen contains a chaperone and folding enzyme machinery that assist unfolded polypeptide chains to fold into their correct three-dimensional structure. Secondly, the ER lumen exhibits a more oxidizing environment when compared with the cytosol, facilitating the formation of disulfide bridges. These covalent linkages between cysteine residues contribute to the stability of correctly folded proteins. Furthermore, the ionic milieu in the ER is more similar to the extracellular space when compared to the cytosol. Lastly glycosylation, the attachment of sugars to side chain nitrogen of residues like asparagine occurs in the ER. This post translational modification also plays an important role in the stabilization of proteins as well as determines the correct secretion pathway [103].

Eukaryotes have evolved a complex quality control system to monitor the success of protein folding and maturation to prevent potentially toxic aggregation or protein dysfunction [104]. After release into the ER, the polypeptide chain advances along the early secretion compartment (ESC) and undergoes folding and assembly into its desired conformation. At each step of this process, the cell preforms extensive quality control checks and ensures that only native conformations are transported further downstream [105]. Immature or aberrant proteins are retained for refolding. In cases where these attempts are unsuccessful, the intermediates are eventually dispatched from the ER and degraded (Figure 1.5)[106, 107].

The ESC is divided into multiple subcompartments. Each of these subcompartments is dedicated to the retention, quality control and biogenesis of a subset of proteins [108]. In this way, the cell can cope with the large diversity of proteins in the proteome. Furthermore, the ESC is able to sense the accumulation of incorrectly folded proteins as well as any overload of the folding machinery with unfolded intermediates. So far, three sensors that recognize the overload of unfolded polypeptides in the ER have been identified, namely inositol-requiring enzyme 1α (IRE1α)[109], protein kinase RNA-like endoplasmic reticulum kinase (PERK) [110] and activating transcription factor 6 (ATF6) [111]. Those sensors can activate different responses to the overload of misfolded or partially folded polypeptides in the ER by activating different signal cascades. The stimulation of those sensors is generally referred to as ‘unfolded protein response’ (UPR). As a result, the UPR initiates the overexpression of specific ER
chaperones, accelerates protein degradation or blocks proteins from entering the ER [112].

1.4.1 Protein folding and quality control in the ESC

The translation of secretory proteins starts in the cytosol. In the next step, a N-terminal signal sequence is recognized by the signal recognition particle (SRP) as soon as it merges from the ribosome exit tunnel and initiates the translocation into the ER. For this propose, the ribosome nascent chain complex is transported to the ER membrane and binds to the translocon, a transmembrane channel. This assembly allows the nascent protein chain to enter the ER lumen co-translationally via the translocon channel [113]. As soon as the nascent protein enters the ER, the polypeptide chain folds into its native structure before continuing its journey to the Golgi apparatus and downstream compartments [103]. A second post-translational route into the ER lumen has been reported. This pathway is mainly used by smaller proteins (<75 amino acids) and those with so called ‘weak’ signal sequence. The post-translational translocation into the ER has been studied in yeast and the exact underlying events in mammalian cells are only poorly understood [114].

The ER is an unique environment that is optimized to allow proteins to fold into their unique native conformation and to acquire covalent modifications such as glycosylation, disulfide bond formation, addition of glycosylphosphatidylinositol (GPI anchor) and removal of signal sequences [108]. A plethora of catalysts, chaperones and folding enzymes orchestrate these modifications and maintain an ideal environment for protein folding by fine tuning the ionic milieu and oxidizing potential (i.e., a defined ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG)[115]. As a protein chain enters the ER through the translocon channel co-translationally it is immediately recognized and bound by folding helpers. This is particularly important since the exposure of even short hydrophobic patches to the crowded environment of the ER lumen can cause irreversible aggregation and protein misfolding. Subsequently, the protein is transported along different ‘ER-platforms’, where different post-translational modification steps are executed. This platform arrangement is analogous to a manufacturing assembly line in which products undergo sequential manipulation until the final product is obtained. The transport from one to the other platform is precisely timed to guarantee efficiency and prevent
jams along the ‘assembly line’ in the ER. The intermediates are only passed to the
next platform when the given manipulation has been successfully completed. This
also implies the existence of a quality control (QC) mechanism that verifies correct
assembly and identifies aberrant proteins; as reviewed in [116]. In this connection, the
sequential addition of sugar molecules to glycoproteins is the most studied ‘assembly
line’ system and serves as prototypic example of the labor organization in the ER
protein factory. In fact, the stepwise addition and manipulation of polysaccharides
displays an elegant way to time and control the fabrication of cargo glycoproteins. In
close proximity to the translocon channel the oligosaccharyltransferase complex
(OST) executes the addition of triglucosylated high-mannose-type tetradecasaccharide
to the side chain of asparagine residues in the sequence NXS/T (X: Is any amino acid
other than proline)[117]. The glycoprotein is subsequently transported along different
‘platforms’ of the ESC and the sugar motifs are progressively modified by ER
resident enzymes while being monitored by the quality control mechanisms in the
ESC [108, 118].

Currently, two QC and folding pathways have been characterized; one pathway is
based on the chaperone Binding Protein (BiP) [119, 120]; the second pathway is
restricted to glycoproteins and is based on the calnexin/calreticulin cycle [121].
The calnexin/calreticulin (CNX/CRT) system mainly relies on the enzyme UDP-
glucose glycoprotein glucosyltransferase (UGGT). UGGT identifies unfolded, non-
native conformers and adds a glucose moiety to those. The addition of the sugar
molecule initiates calnexin and/or calreticulin binding [122, 123]. In the next step, the
isomerization of disulfide bonds occurs through the binding of ERp57 [124, 125]. At
the same time, calnexin and/or calreticulin prevent aggregation of exposed
hydrophobic regions and aid in the maturation of the substrate protein [125]. The
cycle ends when the terminal glucose is removed from the N-glycans and the enzyme
glucosidase II dissociates the substrate from CNX/CRT complex [124]. Subsequently,
the substrate undergoes another round of inspection by UGGT and either re-enters the
calnexin/calreticulin system or is given ‘green light’ to move further downstream in
the secretory pathway [123].

The second well-characterized quality control system depends on an abundantly
present chaperone of the hsp70 family, namely BiP (also called GRP78) and is
glycosylation independent. Besides its chaperone activity, BiP plays a key regulatory role in ER signaling, regulates intracellular Ca\(^{2+}\) storage [126] and participates in ER associated degradation (ERAD) [120, 127]. The C-terminal domain of BiP senses and binds hydrophobic patches of immature or misfolded proteins [128, 129]. The N-terminal domain of BiP exhibits ATPase activity and regulates substrate binding by ATP/ADP exchange. Hydrolysis of ATP to ADP triggers a conformational change within BiP and activates substrate release [87]. This process is catalysed by hsp40-like proteins (ERdj) [130-132], since BiP by itself has only weak ATPase activity. Thus, ERdj play a key regulatory role in BiP activity. Similar to the calnexin/calreticulin system substrates can undergo multiple cycles of BiP binding and release until properly folded [133].

Very large protein and protein complexes can bind simultaneously to BiP and calnexin/calreticulin. Furthermore, the two pathways are not mutually exclusive. In the case, where glycoproteins fail to fold into their native conformation while going through calnexin/calreticulin cycles, they may also participate in the BiP system and vice versa. When both processes fail to generate the native conformation, the protein is retro-translocated back into the cytosol to be degraded by the 26S proteasome [134].

Before successful QC and subsequent ER exit, some proteins need to assemble into heteromeric multi protein complexes, achieve appropriate glycosylation, associate with cofactors (particularly calcium), and/or form proper disulfide bond linkages [108, 116]. At the end of successful quality checks, exit from the ER to the Golgi occurs via Coat Protein complex II (COPII)-coated vesicles [135]. The COPII machinery drives vesicle formation using the ER membrane until the vesicle is cleaved and released from the ER membrane to form the ER-Golgi intermediate compartment (ERGIC) [136, 137].

In addition to the so-called ‘proximal quality control’ in the ER, proteins are subjected to ‘distal quality control’ checkpoints in ERGIC and Golgi as follows: Many chaperones and enzymes reside stably in the ER to execute proximal QC. In contrast, proteins such as ERP44 can cycle through the earlier stacks of the Golgi, i.e. between
ERGIC and cisGolgi via the KDEL-receptors. While moving up and down the ESC these enzymes execute distal QC [108, 116]. The active motion occurs either in complex with cargo proteins, or in an unbound state [103, 138, 139]. Whereas proximal QC often relies on simple retention of substrates at specific ‘platforms’, distal QC implies substrate retrieval to the ER, either for further attempts to fold, or for retro-translocation and degradation. The level of cargo concentration in specific compartments and selective binding between different protein species depending on the surrounding environment are important features of the distal QC mechanism [100].

![Protein quality control in the early secretory compartment.](image)

Proteins destined to the extracellular space or ESC resident enzymes are co-translationally translocated into the ER. Here, they start their folding journey under strict quality control. Once entered into ER, nascent proteins can bind BiP or enter the calnexin/calreticulin cycle. The initial choice is dictated by N-glycan position. In addition, quality control can act in sequential steps. After the proximal QC, proteins (especially multimeric) can undergo checks at distal points in ERGIC- cisGolgi. This ‘later’ quality control can regulate protein concentration and selective export of oligomerized species. While the proximal QC is based on protein retention, the distal QC can imply substrate retrieval to ER for another round of folding or for degradation. This figure is adapted from [140].

One of the best-characterized examples of distal QC is the ER–Golgi intermediate compartment-53 (ERGIC-53), a hexameric transmembrane lectin that derives its name
from being particularly abundant in the ERGIC. This enzyme can cycle between the ER and the ERGIC where it binds cargoes with high mannose content and facilitates their forward transport [141]. Cargo binding and release depends on Ca^{2+} concentration and pH [142]. Both of these environmental parameters vary in different ESC compartments (as described below). In addition, modifications of the sugar motif attached to the cargo proteins determine whether the substrate is degraded or transported further downstream the ESC. One hypothesis is that the altered sugar motif reduces the hydrodynamic volume of the substrate glycoproteins and in this way facilitates the retro-translocation and degradation [143, 144].

1.4.2 Oxidative Folding
One feature of protein folding in the ER is the oxidative linkages of free cysteine thiols in disulfide bridges. This process is known as oxidative protein folding [145]. A relatively low ratio of reduced to oxidized glutathione (GSH:GSSG in the ER between 1:1 and 3:1, compared to the cytosol GSH:GSSG between 30:1 and 100:1) [146] creates an oxidative environment in the ER and supports disulfide bond formation [115]. However, the higher ratio of reduced to oxidized glutathione by itself is not sufficient to ensure correct oxidative folding and linkages between appropriate pairs of cysteines. More often than not, the oxidative folding process implies de novo disulfide bond formation as well as isomerization [147]. In fact, the hyper-oxidizing environment in the ER lumen can interfere with the correct folding of proteins containing multiple disulfide bonds. Hyper-oxidation may promote mispairing of cysteines and in turn decrease protein stability and induce protein misfolding [108]. Therefore, transformation of free thiols into native disulfide bridges in the ER is a progressively refined procedure that requires a dynamic and specific S-S bridge formation, reduction, and reshuffling of disulfide bonds eventually resulting in the introduction of native and functional disulfide bridges. Furthermore, when free thiols are exposed to a competing H_{2}O_{2}-mediated oxidative process oxygenated thiol derivatives can be produced, as it is the case for sulfenylated proteins [147]. Such derivatives can act as transient intermediates in the formation of more stable disulfides, serve as precursors for higher oxidized sulfur oxides (sulfinic and sulfonic acids) or react with GSH to produce S-glutathionylated proteins [146, 148].
To prevent such unwanted oxidation of free thiols and to provide the connections of an intricate disulfide relay within a protein, the cell has developed machinery that supports the disulfide bond formation process. The efficient formation of native disulfides is promoted by a class of specialized chaperones, named protein disulfide isomerase (PDI) [149]. PDIs can directly oxidize thiols in client proteins and so introduce disulfide bonds. The unifying feature of the PDIs is the presence of a thioredoxin-like (Trx) domain possessing the characteristic catalytic amino acid motif CXXC, which transfers disulfide bonds to reduced substrates and as a consequence is reduced itself [149]. The second class of enzymes involved in the oxidative folding pathway are endoplasmic oxidoreductin like proteins (Ero1 α and β), which reactivate the CXXC motif of PDIs through re-oxidation [150]. Thus, the PDI chaperone can undergo a new cycle of disulfide bond transfer. The endoplasmic oxidoreductin like proteins subsequently utilize a co-factor flavin adenine dinucleotide (FAD) to transfer the excess electrons to molecular oxygen. Consequently, PDI/Ero1 activity generates one H₂O₂ molecule for each formed disulfide bond (Figure 1.6)[151, 152].

PDI, the most prominent member of the PDI protein family, is comprised of four thioredoxin domains organized in an a-b-b’-a’ arrangement. a and a’ domains perform the actual redox-function, whereas the b and b’ domain provide a hydrophobic surface, which bind hydrophobic stretches of nascent proteins and guide their active cysteines to the CXXC motif in the a and a’ domain [153, 154]. Accordingly, PDI functions as an oxidoreductase as well as a chaperone [149].

In different PDIs, the redox-potential of the CXXC motif changes depending on the amino acids composition surrounding the active site, in particular the amino acids X [154]. Therein, some PDI members catalyze the formation of disulfide bonds and others reduce non-native disulfide bonds [153]. The reductive cleavage of non-native cysteine pairs enables the client protein to again attempt correct oxidative folding or aids in the unfolding of proteins that failed quality control and are subjected to dislocation to the cytosol for proteasomal degradation [155-157].

H₂O₂ is a highly reactive molecule, a potentially dangerous by-product of enzyme catalysed disulphide linkage and can damage vital cellular components. However, it is also a secondary messenger molecule in a plethora of metabolic reactions. Therefore,
the regulation of \( \text{H}_2\text{O}_2 \) concentration is necessary and high concentration of \( \text{H}_2\text{O}_2 \) in the cell and/or the ER is toxic [158]. For the disposal of \( \text{H}_2\text{O}_2 \) the cell is endowed with multiple ER peroxidases. As their name suggests, these proteins are dedicated to the degrading of \( \text{H}_2\text{O}_2 \). At present, three peroxidase family members have been identified that reside in the mammalian ER, Peroxiredoxin 4 (Prx4), glutathione peroxidases-7 and -8 (GPx7/8) [159-161]. Prx4 is the most widely distributed of the three and most abundant in secretory tissues. Compared with other ER peroxidases members Prx4 shows a high \( \text{H}_2\text{O}_2 \) turnover rate (a rate constant of \( 2.2 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1} \)) [162]. This makes Prx4 an efficient scavenger of low concentrations of \( \text{H}_2\text{O}_2 \) [146].

In addition to Prx4’s characteristic hydrogen peroxide catabolism activity the enzyme participates in oxidative protein folding. The enzyme uses \( \text{H}_2\text{O}_2 \) as a substrate to streamline protein metabolism [146]. As such, oxidized Prx4 (Prx4 after \( \text{H}_2\text{O}_2 \) degradation) can transfer disulfide bonds efficiently to PDI [163], ERp46 [164] and possibly other ER oxidoreductases [165]. Prx4 itself leaves this electron transfer reaction in a reduced state ready for another cycle of hydrogen peroxide catabolism [162].

Among eight mammalian GPxs only three contain an active cysteine (GPx5, 7 and 8) and two are bona fide ER resident proteins (GPx7 and 8) [160]. Whereas GPx7 is a monomeric soluble ER enzyme, GPx8 is anchored in the ER membrane and possesses only a short N terminal cytoplasmic tail [160]. Both Gpx7 and 8 contain a RDEL and KDEL motif, which allows them to travel in the ESC utilizing the KDEL-receptor system (described in detail below) and prevents their secretion across the cell membrane [166]. In analogy to Prx4, GPx7 and 8 accept electrons from PDIs and re-oxidize their active cysteines, in other words they recharge PDIs for further cycles of oxidative folding. In this model, GPxs reduce Ero1\( \alpha \) derived \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \), simultaneously oxidizing a PDI family member. In turn, this PDI can oxidize its cargo protein [161]. Furthermore, it was shown that Prx4 preferentially oxidizes the CXXC motif in the a’ domain of PDI while GPx7 recharges the active site in the a domain of the enzyme [163, 167]. In contrast to GPx7 and 8, Prx4 lacks a C-terminal KDEL sequence and cannot utilize the KDEL-receptor system to prevent secretion from the ESC [168]. Thus, Prx4 depends on ERp44 for its retention in the distal ESC. Taken together, Prx4, Gpx 7 and 8 couple the regulation of potentially toxic \( \text{H}_2\text{O}_2 \)
concentrations with the oxidation of PDIs to execute cycles of oxidative folding (Figure 1.6)[146].

In the last two decades, many new ER-resident PDI-like oxidoreductases have been discovered and characterized. Altogether, over twenty different human proteins are now assigned to the PDI family. Chaperones of this protein family contain up to five thioredoxin domains and from none to four redox-active CXXC motifs [149, 169]. The mechanisms, which control their redox state and activity as well as their exact roles in the ER during oxidative folding remain unclear. Nonetheless, the high number of PDI family members residing in the ESC illustrates the importance of proper disulfide bond formation for protein folding, protein QC as well as for protein complex formation/oligomerisation (for example in adiponectin) [108, 116].

![Figure 1.6: H₂O₂ metabolism and oxidative folding mediated by Prx4.](image)

Ero1-mediated oxidative folding produces H₂O₂. Prx4 can use the produced H₂O₂ to catalyze another cycle of oxidative folding. PDI leaves step I in a reduced state. In step II PDI is reoxidized by Prx4. Next, Prx4 is partly reoxidized by reacting with H₂O₂ resulting in the release of one molecule of water. In step III regenerating of the disulfide of PRx4 is achieved and the second molecule of water is generated [146].
1.5 Retention and selective secretion: the role of chaperones and the environment.

As apparent above, secretory proteins need to pass the strict proximal QC checks based on BiP and calnexin/calreticulin cycles in order to exit the ER. However, oligomerization and formation of protein complexes occurs in the ERGIC. Therefore, the oligomeric species, whose biogenesis demands more sophisticated folding strategies, have to pass additional examination in distal ESC stations. The migration from the ER along the ERGIC and later into the Golgi apparatus exposes assembling proteins to different environments with specific ion concentrations (e.g. those of Ca$^{2+}$, Zn$^{2+}$ and H$^+$) as well as different chaperones and enzymes. Mechanistically, the retention and assembly of higher oligomers depend on the different properties and localizations of key chaperones in specialized ESC compartments. As a result, some proteins are held back in certain compartments to undergo assembly and folding. Once those species assemble into their desired conformation they pass the distal QC checkpoint and are transported further downstream towards the Golgi apparatus. Incorrectly assembled intermediates are recognized by transporter proteins and retrieved to the ER for degradation or to repeat assembly. Most of those transport proteins are characterized by a C-terminal KDEL sequence. Specific KDEL recognizing receptors (KDEL-R) continually move up and down the secretory pathway, capturing KDEL-bearing client proteins in the ERGIC and Golgi complex in order to retrieve and release them into the ER. This process enables the ESC to execute selective retention, retrieval and export to ensure and assist proper protein folding and oligomerization (Figure 1.5) as reviewed in [108, 116].

1.5.1 Role of ESC pH gradient

For all living cells, it is a constant challenge to maintain an appropriate pH within the different organelles [170]. Therefore, cells have evolved a variety of proton pumps, which can actively transport protons across membranes to create and maintain a suitable pH within organelles [171]. The proton gradient so produced can then be used to regulate protein degradation, activity and ATP production [172, 173].

The pH decreases along the ESC from a more alkaline environment in the ER to a more acidic pH in lysosomes. The pH of the ER is in fact near neutral (pH: 7.0) [174], which is similar to the cytoplasmic pH. This value progressively decreases along the
ERCIG-Golgi complex (cisGolgi: pH 6.7) and reaches its minimum at the transGolgi network (TGN, pH 6.0-5.8) [173, 175, 176] (Figure 1.7).

The acidic pH in the different ESC compartments is achieved and maintained through the active transport of $\text{H}^+$ into the organellar lumen by vacuolar $\text{H}^+$-ATPases. The transport requires the presence of $\text{Mg}^{2+}$ ions and consumes ATP [177]. ‘Pumping’ of protons into the organelle lumen creates an electric potential due to the electrogenic nature of $\text{H}^+$. This transmembrane voltage may limit speed and intensity of the proton gradient. To compensate for the electrogenic displacement and to sustain rapid acidification of ESC compartments counterions like $\text{Cl}^-$ are transported across the organelle membrane into the cytosol. For example, a $\text{Cl}^-$ channel named Mid-1-related chloride channel allows chloride ions to cross the membrane of the ER and Golgi apparatus [173, 178].

The pH of each one of the secretory organelles along the ESC has critical implications for protein trafficking, processing, glycosylation and recycling [179-181]. The importance of the pH gradient along the secretory pathway for distal ESC function is simply explained as follows: Through the employment of weak bases or ionophores the luminal proton concentration in ESC compartments may be changed. Such an imposed modification of the pH environment in the ESC results in impaired protein processing and traffic [182].

Another analogous example occurring in nature is the M2 proton channel of influenza viruses. It could be shown that upon infection the M2 proton channel raises the pH value of the Golgi apparatus. As a consequence, protein delivery to the plasma membrane is delayed. This step plays a crucial role in the virus assembly [183, 184]. Despite its importance, the exact underlying mechanisms and molecular events of the above described observations are poorly understood [173].

As described in section 1.4.1, ERGIC-53 is a transporter protein that traverses along the ERGIC and delivers cargo from the ER to downstream compartments. ERGIC-53 release and binding occurs in a tightly regulated $\text{Ca}^{2+}$ concentration and pH-dependent manner. This observation suggests that ERGIC-53 exploits the pH gradient along the ESC to execute its transport function demonstrating an example of how the pH
environment promotes ER enzyme function [185, 186]. It is likely that such mechanisms also hold true for multiple other ER chaperones.

In sum, despite the obvious importance of the pH gradient along the ESC for protein folding and assembly, our knowledge about the involved molecular processes is not yet sufficient for a complete understanding.

![Figure 1.7: pH of secretory compartments.](image)

**Figure 1.7: pH of secretory compartments.**
Organelles rapidly acidify as they progress along the secretory and endocytic pathway. Here, the pH gradient is represented with a color scale from blue to yellow. The nascent protein (in light violet) is co-translationally translocated into the ER lumen at pH 7.0-7.1 (in blue) and continues its journey to secretory vesicles (in purple, pH 5.5) or to lysosomes (in yellow, pH<5.5). This figure is reproduced from [140].
1.5.2 KDEL- receptors

It was estimated that between 1.5 - 5 % of the total ER content exists the organelle per minute, which based on the surface/volume ratio, translates to one of largest flow rates of biosynthetic endomembranes [187]. Therefore, the traffic flow across the ESC must be precisely controlled and coordinated to avoid risking structural and functional disruption of the system. The so-called ‘endomembrane’-signaling pathway is responsible for the control of the protein flow within the ESC. Multiple organelles receptors and signaling components take part in this signaling pathway. In endomembrane signaling a stimulus is generated in a distant intracellular organelle, however, the signaling target is located in a different compartment within the same cell [188]. For the secretory pathway, this implies that protein traffic itself can be used for endomembrane signaling, i.e. for communication between different ESC compartments. In principle two different types of signaling molecules have been identified: I) Signaling proteins that fulfill their signaling role while traveling through the ERGIC- Golgi complex before being directed to the plasma membrane. II) A group of signal proteins that are retained in the ESC exploiting a defined recycling mechanism [189, 190].

For the second group of signaling proteins, a C-terminal signal sequence was discovered, which is carried by many ESC resident soluble and membrane proteins. Molecules bearing such a signal sequence avoid secretion across the plasma membrane by retro-transport from downstream organelles back to the ER [191]. The shared carboxyl terminal motif consists of four amino acids, namely ‘KDEL’[191]. The KDEL sequence is recognized by a group of seven transmembrane proteins (KDEL-receptors) at the ERGIC-Golgi level [192]. Structurally, these 26kDa membrane proteins resemble the topology of GPCRs [193]. After a KDEL-receptor molecule binds a KDEL-bearing protein client, the receptor is activated and dimerizes. The homodimer, subsequently, recruits the COPI-trafficking machinery and initiates the retro-transport of the receptor cargo complex to the ER, where the client protein is released [193, 194]. From here the KDEL-receptor can again migrate downstream through the ER-Golgi complex and start a new cycle of retro-transport [193]. Multiple lines of evidence showed that KDEL like motifs are only recognized by KDLE receptors in acidic conditions [195]. This observation led to a model of KDEL receptor function as follows: At lower pH in the Golgi complex the cargo
protein is bound by KDEL receptors. After retrieval to the ER, where the pH is higher, the environment triggers the release of the client protein [196].

Recent studies indicate the existence of two groups of KDEL-bearing proteins in the ESC. One group of proteins interacts with the ER matrix and seldom reaches the Golgi apparatus. However, some members of this protein family can diffuse more easily and exit the ER. ERp44, an oxidoreductase predominantly present in the ERGIC-Golgi region, is likely to be a member of the latter group [140].

Beside their transport function KDEL-receptors can also act as signaling molecules. In the Golgi apparatus KDEL-Rs can activate a pool of Gq and Gs signaling proteins [193, 197]. This interaction, in turn, regulates Golgi traffic and input from the ER. Thus, KDEL-receptors fulfill a role as traffic sensors and can activate signaling pathways that regulate transport in the ESC. In addition, molecules that are involved in cell functions such as in cell motility and growth are resident in the Golgi apparatus. Hence, KDEL-Rs activation and signaling might also have implications in a wider range of cellular processes and may not be limited to ESC traffic control.[198]

1.6 ERp44
In 2002, ERp44 was first discovered as a covalent binding partner of Ero1α and identified as a 44 kDa ER resident protein [199]. Sequence-based structure prediction suggested that ERp44 structurally belongs to the thioredoxin/PDI protein family. Although the amino acid sequences show significant differences between ERp44 and PDI, the 3D-model of ERp44 could be largely superimposed on PDI active domains. Notably, the active site in the α domain of ERp44 is endowed with an active CRFS motif, analogous to the CXXC motif of PDI, but with the second cysteine residue being replaced with a serine (Figure 1.8 A). This feature distinguishes ERp44 from other typical PDIs. However, an additional cysteine residue (C63) can be found further upstream of the binding motif, which is not present in any other PDI family members. Based on the close proximity to the active C29, a putative role as accessory-binding residue was proposed for C63 [199].
Later analysis of the X-ray crystal structure of human ERp44 [200] revealed that it resembles a clover like structure composed of 3 thioredoxin-like domains a, b and b’. Furthermore, a C-terminal tail (residue 236-377) composed of random coils and a short beta sheet, bridges the b’ and a domain. The amino acid sequence RDEL at the tip of the tail is not resolved in the structure. Nonetheless, it is clarified that this segment plays an important role in preventing ERp44 secretion by binding to KDEL-receptors [200].

ERp44 contains in total six cysteine residues (29, 63, 160, 212, 272 and 289). In the crystal structure, C272 and C289 in the domain b’ are covalently linked. C160 and C212 in the domain b are separated from each other by 4.3 Å indicating a potential to form a disulfide bridge upon appropriate conformational change [200]. In fact, in vivo, the presence of this covalent link was observed in some cases [83, 201]. Furthermore, the crystal structure of ERp44 confirmed the location of C63 to be in close vicinity to C29 in the CSRF motif [200].

In the crystal structure, which adopts the ‘closed’ conformation of ERp44, the C-terminal tail covers the active site around C29 and the hydroxyl of T369 in the tail appears to interact with C29 via a H-bond (Figure 1.8 B). This interaction is possibly important for the stability of the closed conformation, i.e. is involved in shielding the active site. Also the sulfhydryl of C29 is within hydrogen bonding distance of S32, which occupies the position of the second cysteine residue in the corresponding active site of the CXXC motif of PDI. The active CRFS motif is surrounded by a hydrophobic patch (that includes residues F31, H63, M34, P37, I38, V45, V100, W28, A70, I75, W78, P79, L81 and Y94), which is believed to be involved in client binding (Figure 1.8 C)[200].

The b’ domain of ERp44 exhibits a similar fold as the b’ domain of PDI (Figure 1.9)[200]. In PDI the b’ domain is thought to be the primary binding site and a hydrophobic patch located in this domain binds hydrophobic regions of PDI substrates [202]. In ERp44, a similar hydrophobic pocket in the b’ domain with a solvent-accessible surface area of about 200 Å can be found and is comprised of I219, G224, F234, A270, F275, P278, F297, I236, F238 and A293. Sequence alignment
based on the crystal structures of both enzymes (ERp44 and PDI) indicates high similarity in residues of the hydrophobic pocket in the b’ domain as well as the adjacent hydrophobic patch in the a domain. Hence, Wang et al. 2008 proposed that the hydrophobic pocket in the b’ domain serves as docking site for ERp44 clients. However, this patch is partly covered by the carboxyl-terminal end of ERp44. Therefore, the accessibility of this region for substrates is likely to be limited. At least when the enzyme adopts the closed confirmation; as in the crystal structure (Figure 1.9)[200].

Figure 1.8: Features of the CRFS motif in domain a of ERp44.
A: Structural superposition of the a domains from ERp44 (pink) and yPDI (cyan) with a close up view of the CRFS region (right). B: Stereo view of the CRFS motif region with the straight 2Fo-Fc omit map (contoured at 1.5σ). Hydrogen bonding interactions are depicted as a black dotted line with distance indicated. C: The hydrophobic patch (yellow) around the CRFS motif (red) and the C-tail (orange ribbon). ERp44, human ERp44; yPDI, yeast protein disulphide isomerase. This Figure is adapted from [200].

In vivo experiments showed that ERp44 can exist as a covalently linked homodimer [83]. However, the nature and function of the homodimer has not been clarified. When the active C29 in ERp44 is mutated to a serine, non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses showed no band for the homodimeric protein. In contrast, for a mutant in which C29 is replaced with by
an alanine residue, a significant amount of covalently linked dimer is observed. This dimer is likely generated through a bisulfide bridge between corresponding C63 residues of each individual ERp44 molecule [116].

Figure 1.9: Hydrophobic pockets in the b’ domains of human ERp44 and yeast protein disulphide isomerase.

The pockets are indicated by circles, with the surface in green for ERp44 (left) and dark blue for yPDI (right). The C-terminal tail of ERp44 is indicated by the orange ribbon. The middle shows a close-up superposition view of the hydrophobic pockets from ERp44 (green) and yPDI (dark blue) using stick models to represent the residues that compose the hydrophobic pockets. ERp44, human ERp44; yPDI, yeast protein disulphide isomerase. This figure is reproduced from [200].

Although ERp44 utilizes its ability to bind KDEL-receptors for shuttling between Golgi and ER, the majority of ERp44 is localized in the ERGIC-cisGolgi region [203]. However, when the protein is over-expressed the primary localization of ERp44 changes from the ERGIC region to the ER. This peculiar scheme of compartmentalization suggests a specific role of ERp44 in distal QC. It was also shown that interactions between ERGIC-53 and ERp44, at least in part, function to control ERp44 localization in the ESC [203].

In general, high expression levels of ERp44 are primarily found in secretory tissue like pancreas, urinary bladder, salivary gland, stomach, duodenum, testis, prostate, seminal vesicles, ovary, placenta, and bone marrow (see www.proteinatlas.org for detailed information). However, in specific cell types, like pre-adipocyte, the localization of ERp44 depends on the state of differentiation and can change from the ERGIC to the ER. This observation might reflect different needs of preventing
misfolded or unfolded protein secretion during the life cycle of the cells that are subjected to ERp44 action [203].

ERp44 seems to fulfill diverse functions in the ESC. For example, ERp44 binds and retains a number of client proteins by disulfide bond linkage between C29 present in the a domain of ERp44 and a free cysteine residue in the client protein [83, 200]. These ERp44 binding partners usually undergo disulfide-mediated assembly into large complexes while traversing along the ESC [87]. Hence, the secretion inhibition by ERp44 is believed to assist formation of disulfide linkages between unassembled subunits of such substrates and is called ‘thiol-mediated retention’ [80, 81]. In addition, ERp44 interacts with soluble ER enzymes that are retained in the ESC for the purpose of maintaining redox homeostasis. Examples of these clients are: Ero1α and β [199, 204] and Prx4 [205, 206].

Several studies demonstrated a crucial regulatory role of the C-terminal tail for ERp44 function. It was shown that the tail regulates exposure of the substrate-binding site around C29 and the RDEL motif [138, 200]. Sitia and co-workers engineered an ERp44 variant that contains a cysteine residue in position 369 of the ERp44 tail (T369C). By forming a disulfide bond between C369 and C29 the tail is ‘locked’ in the closed conformation. Consequently, the accessibility of clients to the active site of ERp44 is hindered and KDEL-receptor binding is impaired [200]. On the other hand, a mutant lacking the tail shows increased binding to Ero1α in vitro and in human cell lines [200].

When C29 in ERp44 was substituted with an alanine residue (C29A) a decrease in ERp44 activity was detected mostly due to its inability to covalently link with substrates. However, ERp44 was still able to retain proteins in the ESC by non-covalent interactions. Therefore, it was suggested that ERp44 retains clients in the ESC using a combination of covalent and non-covalent interactions [140].

A recently published study shed further light on ERp44 activity and the regulatory role of the C-terminal tail. Sitia et al. 2013 showed that the tail at the C-terminus of ERp44 features a certain degree of flexibility. At higher pH, as it can be found in the ER (pH 7.2), the C-terminal tail shields C29. At this pH the buried C29 is
deprotonated (deactivated) and in addition the RDEL motif is inaccessible. In contrast, at lower pH, similar to the pH in the cisGolgi region (pH 6.7), C29 becomes protonated, which promotes the reactivity of the thiol. Also the C-terminal tail rearranges to make the active site accessible. This movement also exposes the RDEL motif (Figure 1.10 A)[138].

These observations led to a model for ERp44 chaperone function as follows: In more distal ESC compartments, where an environment of lower pH is maintained, ERp44’s C-terminal tail exist in an open conformation, exposing the KDEL-receptor binding site and the reactive protonated thiol of C29. Here, ERp44 patrols for unassembled client proteins such as IgM subunits. After recognition and binding to one of those clients, ERp44 engages with a KDEL-receptor transport molecule. Subsequently, the cargo protein complex is retro-transported to the ER, where the more neutral pH triggers release of the substrate. From here, the substrate can attempt a new cycle of folding and ERp44, now existing in its closed conformation, can travel to downstream compartments, where it patrols for unassembled client protein. In this way, ERp44 executes distal QC on oligomerization of secretory proteins (Figure 1.10 B)[138].
Figure 1.10: A model for the pH-mediated conformational changes in ERp44 regulating its activity in vivo.

A: ERp44 consists of three thioredoxin-like domains (a, b, and b’) and a C-terminal tail (shown in red) that contains the ER-retention motif RDEL (shown in green). Upon lowering the pH, C29 becomes protonated, leading to a conformational change that simultaneously exposes a hydrophobic site in domain b’ (hydrophobic sites in domain a and b’ are shown in yellow), the reactive C29, and the RDEL motif. 

B: ERp44 and its substrates (e.g., an IgM monomer) leave the ER independently for the ERGIC. There, the lower pH activates ERp44 (see A), allowing formation of a covalent ERp44-substrate disulfide bond and exposure of the RDEL motif. This links the incompletely assembled protein subunit:chaperone complex to the KDEL ER-retention system, providing a mechanism to return the substrate to the ER for further assembly or degradation. Red arrows indicate steps that are not yet resolved mechanistically. This figure is adapted from [207].
1.6.1 ERp44 involvement in IgM polymerization and secretion

Antibodies are produced by activated B-lymphocytes, which are specialized in their secretion. The largest antibody produced by the human body is Immunoglobulin M (IgM). IgM oligomerizes in the ESC into planar hexa- or pentamers. In these arrangements disulfide bridges covalently link multiple IgM chains. For the antibody to be functional, each monomeric subunit must correctly fold and expose the complementary binding site. Unassembled, partially assembled or non-polymerized IgMs are not secreted, due to their potential to inhibit lytic antibody activity. Moreover, misshapen polymers could activate an antibody response in the absence of a real antigen. However, plasma cells need to produce and secrete a large amount of antibodies to respond to infections, which creates a high demand on the secretory machinery of B-lymphocytes. Thus, cells evolved a highly sophisticated QC system for this task as reviewed in [87, 208, 209].

The first step of IgM assembly takes place in the ER. The so-called ‘heavy chain’ (µ) as well as the light chain (L) fold separately before pairing via disulfide bond formation to form a tetramer (µ2L2) comprised of two heavy and two light chains. This process is facilitated by Bip and other folding helpers (a detailed description of the tetramer assembly can be found elsewhere [209]).

After the tetramer has formed successfully and passed the QC checkpoints in the ER, oligmerization of the IgM hexamer occurs [103]. ERGIC53 drives the oligmerization process by providing a planar hexameric template for the monomeric µ2L2 subunits to assemble into IgM [203]. A highly conserved 18-amino-acid long stretch at the carboxyl terminus of the secretory µ chain plays a crucial structural and functional role in the multimerization process [210, 211]. In particular, the tripeptide NVS (amino acid 563-565) and a cysteine residue at position 575 are implicated in the assembly process. ERGIC-53 recognizes preferentially µ2L2 subunits which carry a glycan on residues N563 [212] and C575 provides the covalent link between different chains of the oligomer (Anelli et al, 2007a). Furthermore, in free µ2L2 subunits, C575 acts as binding site for ERp44, which prevents secretion of those unassembled intermediates by thiol-mediated retention in favor of oligomer formation [213]. Also ERp44 can directly interact with ERGIC-53[213]. Based on these facts, ERp44 likely facilitates IgM oligomerization by increasing concentration of subunits ready to
polymerize in the correct ESC compartments, guides IgM monomers to the ERGIC-53 assembly platform and by Ero1α recruitment, which provides oxidative power for disulfide bond formation [87, 210, 213].

1.6.2 ERp44 and calcium flux via (1,4,5-trisphosphate receptors) IP3R1
Calcium ions play a virtual role in biological systems as well as fundamental signal transduction pathways[214]. Furthermore, large amounts of Ca\(^{2+}\) are stored in the ER and the plasma membrane. The release of Ca\(^{2+}\) from intracellular storage sites such as the ER, is mediated by Inositol 1, 4, 5-trisphosphate receptors (IP3R), which also act as secondary messengers [215]. So far, three IP3R isoforms IP3R1, IP3R2 and IP3R3 are known, with different tissue distributions [215]. IP3Rs are also found in the ER membrane. Calcium flux via those channels can be regulated from both sides of the membrane, i.e. the cytosol and the ER lumen. In the ER lumen, one of the regulatory molecules is ERp44, which can inhibit inositol 1,4,5-trisphosphate receptor type 1 (IP3R1)-dependent Ca\(^{2+}\) fluxes in a redox-dependent way [216]. ERp44 binds to a free cysteine residue in a variable loop region of the 3rd luminal loop of IP3R. The interaction is pH, redox and Ca\(^{2+}\)-dependent [216]. Although ERp44 interacts with soluble client proteins through its binding site around the conserved C29, IP3R1 binding involves C160 and C212 located in the b domain of ERp44 [217]. Furthermore, Ero1α, ERp44’s foremost binding partner, also regulates IP3R1 activity by competing with ERp44 for IP3R1 binding. Furthermore, Ero1α can oxidize IP3R1 and decreases its affinity to ERp44. In any of those events, Ero1α activity prevents ERp44 binding to IP3R1 and allows Ca\(^{2+}\) flux from the ER into the cytosol. In this manner, both proteins are important players in calcium homeostasis [218, 219].

1.6.3 ERp44 and adiponectin oligmerization
ERp44 plays a crucial role in the formation of HMW adiponectin. As summarized in section 1.3.7, adiponectin oligomerization is tightly controled by the interplay between ERp44 and Ero1-Lα in a Ying and Yang fashion. However, the underlying mechanism is only poorly understood [80, 81].

1.6.4 Unsolved problems of ERp44 function
The discovery of pH-regulated ERp44 activity and shuttling of substrates between the ER and cisGolgi is of marked significance for understanding the thiol-mediated
Chapter 1 – Introduction

retention and the quality control cycle. However, several aspects of the underlying mechanism remain unclear. For example, what provides the oxidative power for ERp44 to form mixed disulfide bonds with clients and how does the ERp44-cargo complex dissociate when retrieved to the ER? Whether ERp44 can act alone or requires assistance for the substrate release? How does the ERp44 homodimer form and what is its function in the ESC? [210]

1.7 Aims and objectives

The anti-diabetic, anti-atherogenic and anti-inflammatory actions of adiponectin suggest a potential therapeutic role for this protein in the treatment of diabetes mellitus Type 2 and related obesity-induced disease. In particular, the HMW form of adiponectin was shown to improve insulin sensitivity, promote vascular health and exhibit anti-diabetic properties [22]. HMW adiponectin serum concentrations are decreased in obesity and in diabetic individuals accompanied with the loss of adiponectin’s beneficial properties [22]. For these reasons, an understanding, at the molecular level, of the oligmerization process leading to the HMW form is of high interest. As described earlier, the in vivo multimerization process involves the action of multiple (ER) chaperones, in this context, obesity-induced ER stress causes a dysregulation in chaperone action resulting in decreased levels of circulating HMW. However, the role of chaperones in the molecular mechanism that promotes and controls HMW formation is poorly understood.

In this thesis we aimed to investigate the molecular mechanism that drives and regulates the oligomerization of adiponectin into its HMW form by focusing on the role of the important chaperone ERp44. In addition, we sought a refined way of intervening in the intercellular interaction between ERp44 and adiponectin with a goal of replenishing diminished adiponectin levels in circulation.

I) Detailed understanding of the assembly process of adiponectin has been hampered by the polymorphism of adiponectin and its heterogeneity in multimeric state (see section 1.3.3). Biologically active, recombinant and pure adiponectin oligomers, in particular of the HMW form, are difficult to produce in milligram amounts which are required to study the assembly process by biophysical methods [220]. For this reason,
we decided to design model peptides that could help examine the assembly process of adiponectin in vitro.

The N-terminal hyper-variable domain of adiponectin has been demonstrated to play a critical role in the assembly process of adiponectin. In particular, a cysteine residue within a 12 amino acid long stretch of highly conserved residues modulates adiponectin assembly through disulphide bond linkage [35]. Furthermore, a tryptophan residue located three amino acids upstream of the cysteine regulates disulfide bond formation and oligomeric assembly of HMW [73]. Further support for the importance of the hyper variable domain is given by the EM reconstruction of the HMW form. The analysis of the EM electron density map of the ‘bouquet-shape’ HMW reveals a well-contrasted clear volume of electron density at the N-terminal end of the complex. This observation suggests that the conserved N-terminal region is structurally important in the organization of the HMW form [32].

Informed by these facts, we decided to design a model peptide of this N-terminal region of adiponectin. The idea was that the examination of the oligomerization propensity of the designed peptide model of the N-terminal region can report on the assembly process of full length adiponectin. This would further our understanding of the mechanism that drives the assembly of adiponectin into the clinically important HMW form.

Since it was necessary to produce the model peptides in milligram amounts for in vitro studies we decided to adopt solid phase peptide synthesis (SPPS) for the production of the peptide. This decision was based on the fact that SPPS was previously used to generate peptide segments of the collagen domain of adiponectin in milligram scale [221]. Once the design, synthesis and purification of the model peptides were completed functional and structural characterization would be followed. To this end, we applied a range of biophysical techniques including mass spectrometry, circular dichroism (CD) spectroscopy and size-exclusion chromatography with multi-angle light scattering (SEC-MALS).

II) In the cell, adiponectin assembly is tightly regulated in the ER. A significant portion of de novo produced adiponectin is retained in the ER for a prolonged period
of time via thiol-mediated retention involving binding to ERp44. ERp44 covalently binds to C39 of adiponectin preventing it from proceeding along the secretory pathway. It is believed that this thiol-mediated retention by ERp44 maintains adiponectin in an oxidizing environment, where maturation of adiponectin oligomers and extensive post-translational modifications can occur. The extended residence in the ER increases the probability of adiponectin to properly fold into its desired multimeric state [80]. However, current knowledge of the molecular processes involved does not provide a complete understanding of this process. As stated before, in obesity ER stress causes a dysregulation of ERp44 action resulting in down regulation of assembly as well as secretion of HMW adiponectin accompanied with important ramification [84]. Therefore, we decided to further analyze the quality control mechanism of ERp44 in adiponectin oligomerization in the ER. To this end, we applied a multi-disciplinary approach. Firstly, recombinant ERp44 protein and the designed model peptide system was employed to analyze the interaction between the partners by various biochemical and biophysical methods. In parallel, a combination of in vivo and cell-based assays involving full-length and wild type adiponectin were used to confirm our in vitro findings under physiological conditions.

III) After we were able to provide a mechanism for the regulation of adiponectin assembly and shed light on ERp44 function in the ER we wanted to understand the mechanism underlying the multifunctional activity of ERp44 in adiponectin assembly at a structural and molecular level. We attempted to exploit X-ray crystallography to visualize the interaction between ERp44 and adiponectin at atomic resolution. Due to the heterogenic and flexible nature of the full-length adiponectin molecule a complex comprised of adiponectin and ERp44 is unlikely to form three-dimensional and well-ordered protein crystals. To mitigate this problem, we designed and generated a complex of ERp44 and an adiponectin-derived peptide. The designed peptide featured a short stretch of the region of adiponectin, which binds to ERp44 and was produced by SPPS. The peptide was modified so that it irreversibly linked to its target protein (ERp44) in a rapid chemical reaction. We characterized the generated protein peptide complex for its chemical stability before subjecting it to crystallographic analyzes.

IV) Based on our current knowledge of the secretory pathway of endogenous adiponectin and ERp44’s involvement in that process, we sought for an intervention
in the ER chaperone machinery that could in turn favorably modify plasma adiponectin levels. This approach could help restore aberrantly diminished HMW adiponectin levels in serum, especially in patients with abnormal ER function caused by obesity-induced ER stress. To this end, we aimed to develop heterologous cell penetrating peptides (CPPs) derived from the sequence of ERp44 clients that can intervene with critical ERp44 adiponectin interactions.

Drugs that are currently used for the treatment of obesity-related disease, such as TZDs activate PPARγ and increase plasma concentrations of adiponectin by enhancing ER function [40, 84, 85].

In ER stress, ER function is dysregulated and an abundant pool of adiponectin is trapped inside the ER by thiol-mediated linkage of adiponectin to ERp44 [80]. We wondered if a short peptide derived from the sequence of ERp44 client proteins would be able to disrupt the interaction of adiponectin with ERp44. This interaction may in turn have an impact on adiponectin release similar to TZD-induced transcriptional repression of ERp44, which was shown to increase adiponectin secretion [222].

To test our hypothesis, we designed and tested two sets of CCPs. One group derived from the adiponectin sequence and a second set was based of the sequence of the IgM binding site to ERp44. Lastly, we assessed the activity of our CCP constructs using cell-based assays and animal models.
Chapter two - General Material and Methods

General materials and methods used in the experiments of this thesis are described in this chapter. Project-specific descriptions of experimental procedures are also presented in each results chapter.

2.1 DNA sequences and protein data

Table 2.1: List of proteins used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Uniprot ID</th>
<th>Protein name</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipoq</td>
<td>Q60994</td>
<td>Adiponectin</td>
<td><em>Mus musculus</em> (Mouse)</td>
</tr>
<tr>
<td>ERp44</td>
<td>Q9D1Q6</td>
<td>Endoplasmic reticulum</td>
<td><em>Mus musculus</em> (Mouse)</td>
</tr>
</tbody>
</table>

2.2 Bioinformatics

Physical and chemical parameters (i.e. molecular weights, extinction coefficients, isoelectric points, and other biochemical data) for proteins and peptides were calculated using the online ProtParam tool [223], and are listed in Table 2.2. The extinction coefficient assumes that all cysteine residues are reduced. Nucleic acid and protein sequences were searched against the GenBank database using BLAST [224].
Chapter 2 - General Material and Methods

Table 2.2: Physical and chemical parameters of proteins and peptides used in this study

<table>
<thead>
<tr>
<th>Protein/Peptide</th>
<th>No. of amino acids</th>
<th>Molecular weight (Da)</th>
<th>Extinction coefficient (M⁻¹ cm⁻¹)</th>
<th>Calculated pI</th>
</tr>
</thead>
<tbody>
<tr>
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<td>247</td>
<td>26809</td>
<td>31985</td>
<td>5.3</td>
</tr>
<tr>
<td>ERp44</td>
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<td>46853</td>
<td>33725</td>
<td>5.09</td>
</tr>
<tr>
<td>AHD</td>
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<td>6072</td>
<td>5500</td>
<td>4.49</td>
</tr>
<tr>
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<td>924</td>
<td>5500</td>
<td>8.21</td>
</tr>
<tr>
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<td>908</td>
<td>5500</td>
<td>8.75</td>
</tr>
<tr>
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<td>0</td>
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<td>5500</td>
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<tr>
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<td>-</td>
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<td>5500</td>
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<td>5500</td>
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<tr>
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<td>6990</td>
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<tr>
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<td>6990</td>
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<tr>
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<td>-</td>
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<tr>
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<td>2980</td>
<td>11.38</td>
</tr>
</tbody>
</table>

2.3 Materials

2.3.1 Chemicals reagents and general buffer preparation

Unless otherwise stated, all chemicals, reagents and solvents were supplied by Merck Chemicals and Laboratory supplies, Sigma Aldrich Chemical Company, Merck, Panreac, AppliChem, Apollo Scientific, New England Biolabs (NEB) and Serva.

All buffers, solutions and media were prepared with ultra-pure water de-ionised by Milli-Q water system (Millipore). All buffers and solutions were sterile filtered using 0.2 µm Supor-200 filter (Pall) or 0.2 µm Minisart syringe filter (Sartorius). Size exclusion, metal affinity and ion exchange chromatography as well as solutions for crystallization experiments were degased prior to the experiment.
DNA ladder, protein size markers and enzymes used for molecular cloning (polymerases, ligase, digestion enzymes and restriction endonucleases) were purchased from Roche, NEB, Invitrogen or Bio-Rad. Oligonucleotides for polymerase chain reaction (PCR) were order from Integrated DNA Technologies.

2.3.2 Bacterial strains
In this work several different *E. Coli* strains were used. For plasmid propagation, *E. Coli* TOP10 or DH5α strains were utilized. Protein expression was conducted in *E.coli* BL21 (DE3), *E.coli* Rosetta 2 (DE3), *E.coli* chaperone competent Cell pG-KJE8/BL21 or *E.coli* Chaperone competent Cell pGro7/BL21 (#9122) cells.

2.3.3 Antibiotics
Antibiotic solutions were prepared as a 1000 times stock, sterile filtered and diluted into the medium to a final concentration of 50 µg/ml (Kanamycin), 35 µg/ml (Chloramphenicol) and 100 µg /ml (Ampicillin) after the medium was autoclaved. All stock solutions were stored at -20 °C until used.

2.3.4 Media
Media used for protein expression, plasmid selection/maintenance and preparation of competent cells are listed in table 2.3.
Table 2.3: List of culture media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysogeny broth (LB)</td>
<td>10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl</td>
</tr>
<tr>
<td>LB Agar</td>
<td>10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, 8 g/l agar</td>
</tr>
<tr>
<td>Terrific broth (TB)</td>
<td>12 g/l Bacto-tryptone, 24 g/l yeast extract, 4 ml/l Glycerol</td>
</tr>
<tr>
<td>ZYM 5052</td>
<td>1% tryptone, 0.5% yeast extract, 0.5% glycerol, 50 mM NH4Cl, 25 mM Na2HPO4, 25 mM KH2PO4, 5 mM Na2SO4, 2 mM MgSO4, 1X metal mix, 0.05% glucose, 0.2% lactose</td>
</tr>
<tr>
<td>1000X Metal Mix</td>
<td>50 mM FeCl3, 20 mM CaCl2, 10 mM MnCl2, 10 mM ZnSO4, 2 mM CoCl2, 2 mM CuCl2, 2 mM NiCl2, 2 mM Na2MoO4, 2 mM Na2SeO3</td>
</tr>
<tr>
<td>Super optimal broth (SOB)</td>
<td>20 g/l Bacto-tryptone, 5 g/l yeast extract, 2.5 mM Cl3PO4, 10 mM MgCl2</td>
</tr>
</tbody>
</table>

2.4 Molecular biology and molecular cloning

2.4.1 Preparation of competent cells

2.4.1.1 Chemically competent cells

Chemically competent *E. coli* cells were prepared for over-expression of proteins using the following strains: *E. coli* BL21 (DE3), *E. coli* Rosetta 2 (DE3), chaperone competent Cell pG-KJE8/BL21 or Chaperone competent Cell pGro7/BL21 (#9122). A single colony from a freshly streaked plate was selected to inoculate a 5 mL LB starter culture and the culture was incubated overnight at 37 °C on a shaker. The next morning, 1 ml of the starter culture was used to inoculate 500 mL SOB in a 2-L baffled flask. The culture was placed on a shaker running at 180 rpm in a 37 °C environment. After 4 h an optical density OD600 of 0.6 was reached. The culture was chilled on ice for 20 min and the cells were harvested by centrifugation (4000 g, 4 °C, 20 min) (Sorvall RC6 Plus centrifuge, Thermo Scientific). Cells were resuspended in ice cold 100 mM calcium chloride solution and incubated for 40 min on ice. Subsequently, the cells were pelleted by centrifugation (4000 g, 4 °C, 20 min) and
resuspended in 4 ml of sterile ice cold 100 mM calcium chloride containing 15 % DMSO. 50 µL aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

2.4.1.2 Electro-competent cells

For plasmid propagation and maintenance, E. Coli TOP10 or DH5α cells were prepared. A single colony of the E. Coli strain was used to inoculated 5 ml of SOB medium and incubated overnight at 37 °C on a shaker (180 rpm). 1 ml of the overnight culture was transferred to 500 ml of SOB medium and incubated at 37°C on a shaker (180rpm) until an optical density OD₆₀₀ of 0.6 was reached. The flask was rapidly cooled to 4°C and stored on ice for 20 min. The cells were harvested by centrifugation (4000 g, 4 °C, 20 min). The supernatant was discarded, the cells were resuspended in 250 ml of 10 % glycerol on ice and cells were harvested again as above. This procedure was repeated three times. In the final step, the harvested cell pellet was resuspended in 1 ml of 15 % glycerol, 2 % sorbitol and aliquots of 50 µl were snap frozen in liquid nitrogen before being stored at -80 °C.

2.4.2 Transformation

2.4.2.1 Transformation by heat shock

The transformation of chemically competent cells was conducted as follows: 50 - 100 ng of plasmid DNA in 0.5 - 1.5 µl of water were added to 50 µl of competent cells suspension and incubated on ice for 30 min. Subsequently, cells were transferred to a water bath at 42 °C and incubated for 45 sec. After incubation cells were immediately returned on ice for 2 min, followed by the addition of 450 µl of SOB media. The suspension was placed on a shaker (160 rpm) for 45 min at 37 °C. 50 µl of the cell suspension was plated directly on an LB agar plate supplemented with the appropriate antibiotic. Colonies appeared on the plate after incubation at 37 °C overnight.

2.4.2.1 Transformation by electroporation

Electrocompetent cells were transformed by electroporation. To this end, a MicroPulse electroporator (Bio-Rad) and glass cuvettes with a 0.2 mm gap between two electrodes were used. 50- 100 ng of plasmid DNA in 0.5-1.5 µl water was added to 50 µL of competent cells. A cuvette was pre-chilled on ice and the cell suspension was transferred to the cuvette before electroporation was performed. 0.5 ml of SOB
media was added, cells were transferred to a 1.5-ml microfuge tube and cells were incubated at 37°C for 45 min on a shaker (160 rpm). 50 µl of cell suspension was subsequently plated on LB agar plates with the appropriate antibiotic. Colonies appeared on the plate after incubation at 37 °C overnight.

2.4.3 Cloning of ERp44

2.4.3.1 Gene sequences and Primer design
cDNA encoding for mouse ERp44 without the signal sequence was cloned into a pET28b vector carrying an N-terminal His6-tag using NheI and XhoI cloning sites as described below. pET28b vector was purchased from EMD Biosciences. The gene of interest was purchased from OriGENE and the DNA template was amplified by the polymerase chain reaction using primers designed based on gene-specific DNA sequences with flanking nucleotide sequences specific for the selected restriction enzyme sites. The primer pair (Table 2.4) was designed to match a corresponding melting temperature. Physical properties of the oligonucleotides, such as melting temperature (T\textsubscript{m}) were calculated using online oligonucleotide properties calculator [225]. This online tool was also used to check for potential self-complementarity sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>T\textsubscript{m}</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERp44fwd</td>
<td>GCGGCGGCGCTAGCGAAATAGCAAGTCTTGATTCAGAGAAT</td>
<td>70.5°C</td>
<td>NheI</td>
</tr>
<tr>
<td>ERp44rev</td>
<td>TTACAGCTCATCTCGATCCCTCAAATTAGTATACCTATACTCGCTGGG</td>
<td>69.5°C</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

2.4.3.2 PCR amplification of ERp44 gene
The ERp44 gene was amplified using PrimeStar DNA polymerase (Takara Bio) according to the manufacturer's directions. The protocol was optimized and conditions as listed below were used to amplify the desired DNA template (Table 2.5 and 2.6).
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Table 2.5: Composition of PCR Reaction Mixture (total volume of 50 µl)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X PrimeSTAR Buffer (Mg2+ Plus)</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP Mixture (2.5 mM each)</td>
<td>200 µM each</td>
</tr>
<tr>
<td>ERp44fwd</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>ERp44rev</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>50 ng</td>
</tr>
<tr>
<td>PrimeSTAR HS DNA Polymerase (2.5 units/µl)</td>
<td>1.5 units</td>
</tr>
</tbody>
</table>

Table 2.6 PCR conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>98 °C</td>
<td>60 sec</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>98 °C</td>
<td>20 sec</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>62 °C</td>
<td>20 sec</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72 °C</td>
<td>45 sec</td>
</tr>
<tr>
<td>5. Final extension</td>
<td>72 °C</td>
<td>120 sec</td>
</tr>
<tr>
<td>6. End</td>
<td>4 °C</td>
<td></td>
</tr>
</tbody>
</table>

Step 2-4 was repeated for 30 cycles

At the end of the reaction the PCR product was analyzed by agarose gel electrophoresis (see section 2.5.1).

2.4.3.3 Purification of PCR products and DNA quantification

PCR products were purified using the PCR purification kit (Axygen) as described in the manufacturer’s manual. DNA concentrations were measured by using absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.4.3.4 Amplification and purification of plasmid DNA

The target plasmid pET28b was transformed into TOP10 E.coli cells as described in section 2.4.1.2. Subsequently, 5 ml LB with the appropriate antibiotic were inoculated with a single colony from the freshly transformed plate and incubated at 37 °C overnight on a shaker (160 rpm). The next morning, the cells were harvested by
centrifugation (4000g, 4°C) (Tabletop centrifuge, Eppendorf) and the plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) or AxyPrep Plasmid Miniprep Kit (Axygen) according to the manufacturer’s protocol. The yield and purity of the amplified plasmid was analyzed by agarose gel electrophoresis and UV-visible spectroscopy (see Section 2.5.1 and 2.4.3.3).

2.4.3.5 Restriction enzyme digestion
Restriction digest was performed with the PCR product and the target plasmid (pET28b) using restriction enzymes (Roche). In two separate reactions, 1.2 µg of the PCR product and 5 µg of the plasmid were digested in a total volume of 50 µl in the presence of 10 of units XhoI and NdeI in buffer M (Roche) for 3 h at 37°C. To avoid re-circularization of the plasmid and to enhance the yield following ligation, 5 units of Antarctic Phosphatase (NEB) was added to the digestion of the plasmid as well as 10X Antarctic Phosphatase Reaction Buffer was added to a final concentration of 1X. The mixture was incubated for additional 30 min. The completion of the digestion reactions was then verified by agarose gel together with undigested plasmid as the control. Products of the double-digestion were purified using the protocol described in section 2.4.3.3 prior to setting up of the ligation reactions.

2.4.3.6 Ligation
Ligation of plasmid and insert was performed with T4 DNA Ligase from NEB following the manufacturer’s protocol. Ligation was performed with a 3:1 molar ratio of insert to the vector in a total volume of 10 µl containing 100 ng of plasmid DNA. Control reactions without insert or without enzyme were also performed. The composition of the ligation reaction is shown in Table 2.7. The ligation cocktails were incubated for 16 h at 18°C. Following incubation, the ligation reaction mix was directly used for transformation into electrocompetent E. coli TOP10 cells as described in section 2.4.2.1.
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<table>
<thead>
<tr>
<th>Component</th>
<th>Amount added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>100 ng</td>
</tr>
<tr>
<td>Insert: plasmid ratio</td>
<td>3:1</td>
</tr>
<tr>
<td>10X T4 DNA Ligase buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 µl</td>
</tr>
<tr>
<td>Water</td>
<td>Up to 10 µl</td>
</tr>
</tbody>
</table>

2.4.3.7 Selection of positive clones

To select for plasmids carrying the desired ERp44 DNA sequence, five colonies were selected to inoculate reaction mixtures in a tube containing 5 ml LB with the appropriate antibiotic. The tubes were incubated at 37 °C on a shaker (160 rpm) overnight. The next morning, the cells were harvested by centrifugation (4000g, 4°C) and the plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s protocol (section 2.4.2.1). The purified plasmids were analyzed by agarose gel electrophoresis together with the pET28b vector lacking the desired DNA sequence. On the agarose gel the empty vector migrates at a different position when compared with the variants carrying an insert. Clones showing such a shift in the agarose gel analyses were submitted for sequencing to confirm the success of the cloning procedure.

2.4.3.8 DNA sequencing

The DNA Sequencing Facility, Centre for Genomics and Proteomics, University of Auckland performed all sequencing reactions in the following manner: Sequencing reactions were performed using the ABI Prism Big Dye Terminator Sequencing Kit version 3.1 in an Applied Biosystems GeneAmp PCR System 700. Agencourt CleanSEQ magnetic beads were used to removed the unincorporated fluorescent dyes. The sequencing products were separated by capillary electrophoresis on a ABI Prism 3130XL Genetic Analyser with 50 cm capillaries and POP (PERFORMANCE OPTIMIZED POLYMER) 7 polymer (Thermo Fischer).
2.4.4 Mutagenesis and generation of ERp44 clones (ΔtailERp44 and ERp44C29S)

2.4.4.1 Creation of ERp44C29S clone

The plasmid coding for the ERp44C29S mutant was created by Ben Rushton in our laboratory and is described in detail in [226]. The point mutation was performed on the pET28b plasmid carrying the DNA sequence coding for murine full length ERp44 without signal sequence using the QuikChange® mutagenesis protocol (Agilent).

2.4.4.2 Generation of ΔtailERp44 by inverse PCR site-directed mutagenesis

Clones of ΔtailERp44 were generated by site directed mutagenesis. The pET28b plasmid carrying the DNA sequence coding for murine full length ERp44 without signal sequence was used as template. A stop codon (TAA) was introduced after the DNA triplet (GAA) coding for E331 in the ERp44 sequence with the following pair of mutagenic primers:

Table 2.8: Primer used for site directed mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δtailfwr</td>
<td>TAATTCCATCACGGACCTGACCC</td>
<td>57.1 °C</td>
</tr>
<tr>
<td>Δtailrev</td>
<td>TTCTCTGGTTGTAATTTTCAATGTTAGGTCAATAC</td>
<td>58.6 °C</td>
</tr>
</tbody>
</table>

Primers were designed according to Q5® Site-Directed Mutagenesis Kit Quick Protocol (NEB). The forward primer carries the mutated sequences at the 5’-end. The reverse primer is complementary to the gene sequence immediately following the 5’ end of the forward sequence. Both primers were phosphorylated at the 5’-end. The linearized DNA of the entire pET28b vector and insert with the desired mutation at the 5’-end was produced by PCR using PrimeStar DNA polymerase (Takara Bio) according to the manufacturer's directions. The protocol was optimized and the conditions listed below were used to amplify the desired DNA template (Table 2.9 and 2.10).
Table 2.9: Composition of PCR Reaction Mixture (total volume of 50 µl)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X PrimeSTAR Buffer (Mg2+ Plus)</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP Mixture (2.5 mM each)</td>
<td>200 µM each</td>
</tr>
<tr>
<td>Δtailfwr</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Δtailrev</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>200 ng</td>
</tr>
<tr>
<td>PrimeSTAR HS DNA Polymerase (2.5 units/µl)</td>
<td>1.5 units</td>
</tr>
</tbody>
</table>

Table 2.10 PCR Conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>98°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>98°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>55°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72°C</td>
<td>330 sec</td>
</tr>
<tr>
<td>5. Final extension</td>
<td>72°C</td>
<td>120 sec</td>
</tr>
<tr>
<td>6. End</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

Step 2-4 was repeated for 15 cycles

At the end of the reaction the PCR product was analyzed by agarose gel electrophoresis (section 2.5.1) and the DNA was purified as described in section 2.4.3.3. Subsequently, the template vector was removed by DpnI (NEB) digestion. The purified DNA was subjected to digestion in 50 µl of reaction mixture containing 5 units of DpnI at 37°C for 3 h. The composition of the DpnI (NEB) digestion is given in Table 2.11

Table 2.11: Composition of the DpnI (NEB) digestion

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>2 µg</td>
</tr>
<tr>
<td>CutSmart® Buffer (NEB)</td>
<td>5 µl</td>
</tr>
<tr>
<td>DpnI</td>
<td>5 µl</td>
</tr>
<tr>
<td>deionized water</td>
<td>up to 50 µl</td>
</tr>
</tbody>
</table>
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After digestion, the PCR product was purified as described in section 2.4.3.3 and the linear DNA was ligated to allow (section 2.4.3.6) re-circularization of the vector DNA. Subsequently the DNA was transformed into E. coli DH5α cells (section 2.4.1.2), the plasmid was propagated (see section 2.4.3.4) and purified (see section 2.4.3.3). Lastly, the mutation was verified by DNA sequencing (see section 2.4.3.8).

2.5 Electrophoresis

2.5.1 Agarose gel electrophoresis

Analysis of DNA was performed by agarose gel electrophoresis. For this purpose, 0.8 g of agarose were added to 50 ml of 1X TAE buffer (40 mM Tris-HCl, 1 mM EDTA, 10 mM glacial acetic acid, pH 8.5), the mixture was heated in a microwave for 45 sec and a horizontal agarose gels was cast. The agarose gel was supplemented with 0.5 µg/ml ethidium bromide and allowed to set before transferred to an electrophoresis apparatus (Hoefer HE 33). Next, the electrophoreses chamber was filled with TAE buffer. DNA samples (50-200 ng of DNA) were mixed with 1.66 µl of 6X loading dye (0.25 % (w/v) bromophenol blue and 30 % (w/v) glycerol). The volume was adjusted to 10 µl with water and the samples were loaded into wells. Electrophoreses was conducted at 80 V for 45 min. DNA bands were visualized on an ultraviolet (UV) trans-illuminator with an imaging system (Gel Doc, Biorad). The DNA fragments were analyzed according to their migration in gels when compared to standard DNA molecular markers (1 kb ladder, BioRad).

2.5.2 Denaturing SDS-polyacrylamide gel electrophoresis

Reducing or non-reducing denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [227] was performed to separate and analyse proteins based on their size. A SE260 Mighty Small II Deluxe Mini Vertical Electrophoresis Unit (Hoefer Inc, USA) was used to carry out electrophoresis. SDS-PAGE gels were prepared in two steps. First, the separating gel was made by pouring separating gel solution containing 10 - 15 % (w/v) acrylamide mix into a gel caster (Hoefer Inc, USA). After 45 min the separating gel was solidified and the stacking gel solution containing 5 % (w/v) acrylamide mix and combs were inserted to form wells. Protein samples (1 – 20 µl) were prepared by mixing protein sample with 4X SDS loading buffer to a final concentration of 1X SDS loading buffer, heated at 95 °C for 5 min,
cooled, and loaded onto the gel. The electrophoresis unit was filled with running buffer and electrophoreses was carried out at 220V (20 mA) using molecular weight standards; either pre-stained or unstained SDS-PAGE page molecular markers. Following electrophoresis (usually after 60 min), the SDS-PAGE gel was removed from the electrophoresis unit, washed with water and stained with staining solution. The gel was stained at room temperature for 45 min on a shaker, the Coomassie solution was then removed and the gel was washed with sterile water followed by incubation in destaining solution on a shaker until the gel was destained. All solutions used are shown in Table 2.12.

Table 2.12: SDS PAGE Solutions

<table>
<thead>
<tr>
<th>Separating gel</th>
<th>Stacking gel</th>
<th>Reducing SDS-gel loading buffer</th>
<th>Non-reducing SDS-gel loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 mM Tris-HCl pH 8.8</td>
<td>125 mM Tris-HCl pH 6.8</td>
<td>50 mM Tris-HCl pH 6.8</td>
<td>50 mM Tris-HCl pH 6.8</td>
</tr>
<tr>
<td>10 – 15 % (w/v) acrylamide</td>
<td>5 % (w/v) acrylamide</td>
<td>100 mM DTT</td>
<td>2 % (w/v) SDS</td>
</tr>
<tr>
<td>1 % (w/v) SDS</td>
<td>1 % (w/v) SDS</td>
<td>2 % (w/v) SDS</td>
<td>1 % (w/v) bromophenol blue</td>
</tr>
<tr>
<td>1 % (w/v) (NH4)2S2O8</td>
<td>1 % (w/v) (NH4)2S2O8</td>
<td>1 % (w/v) bromophenol blue</td>
<td>10 % (v/v) glycerol</td>
</tr>
<tr>
<td>0.04 % (v/v) Tetramethylethlenediamine</td>
<td>0.1 % (v/v) Tetramethylethlenediamine</td>
<td>10 % (v/v) glycerol</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Running buffer</th>
<th>Staining solution</th>
<th>Destaining solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris</td>
<td>0.125 % (w/v) Brilliant blue R-250</td>
<td>30 % (v/v) % methanol</td>
</tr>
<tr>
<td>250 mM glycine</td>
<td>30 % (v/v) % methanol</td>
<td>10 % (v/v) acetic acid</td>
</tr>
<tr>
<td>0.1 % (w/v) SDS</td>
<td>10 % (v/v) acetic acid</td>
<td></td>
</tr>
</tbody>
</table>

2.6 Protein production and purification

2.6.1 Protein expression

Recombinant proteins were over-expressed in *E. Coli* using medium and expression strain as indicated for each protein in the following chapters. A 5 ml LB starter culture supplemented with appropriate antibiotics was inoculated with a single colony
picked from a fresh LB-agar plate and incubated overnight at 37 °C. The next morning, the starter culture was used to inoculate 500 ml of expression medium (Table 2.3) in a 2 l baffled Erlenmeyer flasks and incubated on a shaker (180 rpm) at 37 °C until the desired optical density (OD$_{600}$) was reached. The culture was then transferred to an incubator set at appropriate temperature for expression (depending on the protein) and the culture was allowed to adjust to the desired temperature before induced with IPTG and incubated on a shaker (180 rpm) until the cells were harvested by centrifugation (4000 g, 4 °C, 20 min). Lastly, the cell pellets were stored at -20 °C.

2.6.2 Solubility and expression screening

Expression levels and solubility of target proteins in different media and expression strains as well as at various expression temperatures were assessed prior to large scale protein expression. This assessment was executed by a small scale expression test experiment. To this end, expression was carried in a volume of 25 ml using the protein expression protocol outlined in section 2.6.1. 5-10 ml aliquots of the expression culture were taken at different time points, cells were harvested (4000 g, 4°C, 20 min), resuspended in 3 mL of lysis buffer (50 mM Tris, 500 mM NaCl, 5% Glycerol, 5 mM 2-mecaptoethanol at pH 8) and lysed by sonication using a sonictor (Sonicator 3000, Misonix). The cellular debris was separated from the soluble fraction by centrifugation (20,000g, 4 °C for 45 min), the supernatant was separated from the pellet and analysed on SDS-PAGE (see section 2.5.2) The cellular debris was also analysed by SDS-PAGE. The condition showing largest amount of soluble protein in the supernatant was selected for large scale expression.

2.6.3 Lysis

Cell pellets were thawed and resuspended on ice in lysis buffer supplemented with 20 mM imidazole (approximately 5 ml buffer per 1 g wet cell mass). Subsequently, cells were lysed either using a cell disruptor (Microfluidizer M-110P , Microfluidics) at 17-19 kPa or by sonication (Sonicator 3000, Misonix). The lysate was cleared of cellular debris by centrifugation (20,000g, 4°C, 45 min). The lysate was then manually filtered using a syringe through a 0.45 mm syringe filter (Sartorius) before protein purification.
2.6.4 Immobilised metal affinity chromatography (IMAC) protein purification

His-tagged recombinant proteins were purified by IMAC using a 5 mL Hi-Trap column (GE Healthcare). Prior to use, the Hi-Trap column was regenerated as follows: Using a peristaltic pump the column was washed with 10 column volumes (CV) of ultra-pure Milli-Q water, stripped with 5 CV of stripping buffer (5 mM EDTA and 500 mM NaCl) and again washed with 10 CV Milli-Q water. Next, the column was charged with 10 CV of 50 mM NiCl₂, washed with 10 CV of Milli-Q water and lastly, equilibrated with 10 CV of lysis buffer. The protein was bound to the resin by running the cleared and filtered lysate over the column resin at a flow rate of 1-2 ml/min. Next, the column was transferred to an Akta FPLC system (Prime, Explorer or Purifier, GE Healthcare) and bound protein was washed with 50 ml of wash buffer (lysis buffer + 25 mM imidazole) to remove non-specifically bound proteins. The protein was then eluted using a continuous gradient of imidazole (typically over 30 ml from 25 to 500 mM imidazole in lysis buffer) and fractions of 1 ml were collected during the experiment. The elution was monitored by following the absorbance at 280 nm. At the end of the gradient, the column was washed with 10 CV water and 10 CV of 20 % ethanol (EtOH) before being stored at 4 °C. Fractions were analysed by SDS-PAGE as described in section 2.5.2 and fractions containing the desired protein as main component were pooled.

2.6.5 Protein dialysis

For further purification, the proteinsample was dialysed against ion exchange buffer buffer (20 mM Tris, 5 mM 2-mecaptoethanol at pH 8). To this end, a cellulose membrane with a 3.5 kDa molecular weight cut-off (Sigma-Aldrich) was prepared by boiling the membrane for 15 min in water containing 5 mM EDTA. Subsequently, the membrane was washed three times in boiling Milli-Q water for 15 min before being soaked the in ion exchange buffer. A sufficient length of dialysis tubing was cut off, fold over at one end of the tubing and closed with a dialysis clip. The protein sample was loaded through the open end of the tubing and a second clip was used to close the other end. The sample was then dialysed against 1 l of ion exchange buffer over night at 4 °C and the buffer was stirred using a magnetic stir bar. The next morning, dialysis tubing containing the protein sample was removed from the reservoir and the sample was harvested in a 50 ml falcon tube.
2.6.6 Ion Exchange Chromatography (IEX)

Ion exchange chromatography was employed to further purify the crude IMAC purified proteins. IEX separates proteins based on their charge and in this work anion exchange chromatography, which binds negatively charged proteins, was employed. A high resolution MonoQ 5/50 GL column (GE Healthcare) was used for this purification step and was coupled with an Akta FPLC system (Explorer, GE Healthcare) equipped with a UV monitor (GE Healthcare). At the start of the IEX experiments, the MonoQ column was washed with 10 CV of filtered Milli-Q water, 10 CV of the IEX buffer containing 1 M NaCl and equilibrated with 10 CV IEX buffer without NaCl. Prior to the experiment, the protein solution (~3 - 4 mL) was manually filtered through a 0.2 µm syringe filter and spun down (4000g, 4°C, 15 min) in a table top centrifuge (Eppendorf) to remove particulate matter. The sample was then injected into a sample loop connected with the Akta system. The sample was loaded onto the MonoQ column, washed with 5 CV IEX buffer and eluted typically using a gradient over 50 ml from 0 to 1 M NaCl. Protein fractions were collected with the Akta fractionator in a 96-well plate. The elution was monitored by following the absorbance at 280 nm. At the end of the gradient, the column was washed with 10 CV water and 10 CV of 20 % EtOH before stored at 4°C. The protein fractions were analysed by SDS-PAGE (see section 2.5.2) and fractions containing the desired protein were pooled.

2.6.7 Concentrating proteins and protein concentration determination

Protein samples were concentrated using a Vivaspin centrifugal concentrator (Sartorius) with appropriate molecular weight cut-off. The membranes of the concentrator were first washed with Milli-Q water by centrifugation and then rinsed. Centrifugation was subsequently used to equilibrate the membranes with appropriate protein buffer before the protein solution was added to the Vivaspin concentrator. The sample was concentrated by centrifugation at 3000 g at 4°C with a tabletop centrifuge (Eppendorf) until the desired concentration was reached.

The protein concentration was determined by measuring the absorbance at 280 nm with a Nanodrop spectrophotometer (Nanodrop technologies) or an Ultrospec 2100
pro spectrometer (Amersham biosciences). In this case, the protein solution (undiluted or diluted when necessary) was applied onto the pedestal of the spectrophotometer and the extinction coefficient and molecular weight calculated as in table 2.2 were input. The protein concentration was calculated using Beer’s law:

\[ A = \varepsilon l c, \]

where \( A \) is absorbance at 280 nm, \( \varepsilon \) is the molar extinction coefficient (1/(M*cm)(see table 2.2), \( l \) is the path length of the sample (cm) and \( c \) is the concentration of the protein solution (M).

The Ultrospec 2100 pro spectrometer was used to measure protein concentrations with higher accuracy than possible for the Nanodrop spectrometer. To this end, a quartz cuvette (50 µl) was filled with buffer and the spectrometer was blanked. Subsequently, the blanking buffer was replaced with protein solution and the absorbance at 280 nm was measured. The protein sample was diluted if necessary, so that an absorbance value between 0.1-1.0 was reached and the protein concentration was calculated as outline above.

2.6.8 Size exclusion chromatography (SEC)

In some cases, proteins were further purified after IMAC by SEC, or SEC was used as a final purification step after the IEX chromatography step. SEC separates proteins based on their size. Columns were chosen depending on the size of the target protein and the amount of protein purified. Columns used included HiLoad 16/60 Superdex 75 or 200 and Superdex S75 or S200 10/300 GL (all GE Healthcare). The appropriate column was attached to an Akta FPLC system (Prime or Explorer, GE Healthcare) equipped with a UV monitor (GE Healthcare). Prior to the SEC experiment, the SEC column was washed with 1–2 CV of filtered and degassed Milli-Q water and equilibrated with the desired buffer by washing the column with 1-2 CV of buffer. Protein solutions were concentrated to a volume appropriate for injection (0.5 ml or 5 ml for 10/300 and 16/60 columns, respectively), manually filtered through a 0.2 µm syringe filter and spun down (4000g, 4°C, 15 min) in a tabletop centrifuge (Eppendorf) to remove any particulate matter, before being injected onto the column.
through a sample loop. During the SEC experiment, fractions were collected with an Akta fractionator and the absorbance at 280 nm was monitored. At the end of the SEC run, the column was washed with 1–2 CV of Milli-Q water and 1 CV of 20 % EtOH before stored at 4 °C. The protein fractions were analysed by SDS-PAGE (see section 2.5.2) and fractions containing the desired protein were pooled.

2.6.9 Size exclusion chromatography coupled to multi angle laser light scattering (SEC-MALS) experiments
Samples (100 µl) were applied to a Superdex 200 increase 10/300 GL column mounted on a Dionex HPLC at a flow rate of 0.5 ml/min. The scattered light intensity and protein concentration in the column eluate were recorded using a PSS SLD7000 MALS detector and a Shodex RI-101 differential refractometer (dn/dc = 0.186), respectively. The weight-averaged molecular mass of material in chromatographic peaks was determined using the PSS winGPCUnichrom software.

2.7 Peptide synthesis and purification
Commercially available reagents were purchased as ‘reagent grade’ from the following companies: Polypeptides Group, ChemPep, Peptides International, Chem-Impex, Acros Organics, AK Scientific, Alfa Aesar, GL-Biochem, Merck Millipore, Sigma-Aldrich, Bio-Rad, Pure Science, MP Biomedicals, ECP and were used without further purification.
Solvents for RP-HPLC were purchased as HPLC grade and used without further purification.

2.7.1 Analytical reversed-phase (RP) - High Performance Liquid Chromatography (HPLC)
During solid phase peptide synthesis (SPPS) analytical RP-HPLC was performed to analyze purity of intermediate products as well as the final products. To this end, a Dionex (Sunnyvale, CA) UltiMate 3000 system equipped with a four-channel UV detector was employed. A Gracesmart C18 (5 µm; 2.0 x 50 mm) column (Grace Discovery Science; Columbia, MD) was attached to the HPLC system and a linear gradient of 1% to 61% B over 60 min (1% B per minute) at a flow rate of 0.2 ml/min unless otherwise indicated was applied.
2.7.2 Liquid chromatography (LC) coupled with mass spectrometry (MS)
Identity of peptides as well as purity of either the intermediate or final reaction products were confirmed by LC-MS using an Agilent (Santa Clara, CA) 1260 infinity system equipped with an Agilent 6120 quadruple mass spectrometer using Electrospary ionization (ESI) in the positive mode. LC-MS was performed using a Zorbax 300-SB C3 (5 µm; 3.0 x 150 mm) column (Agilent) or a Gracesmart C18 (5 µm; 2.0 x 50 mm) column (Grace Discovery Science; Columbia, MD) and a linear gradient of 1% to 61% B over 21 min (3% B per minute) at a flow rate of 0.3 ml/min. The solvent system used was A (0.1% formic acid in H2O) and B (0.1% formic acid in acetonitrile). In some cases, the system was used for ‘flow injections’ to acquire a mass spectrum of the injected sample. For this propose, the Agilent system was used without column and the sample was directly injected into the Agilent 6120 quadruple mass spectrometer using a flow rate of 0.3 ml/min with 50% B.

2.7.3 Semi-preparative HPLC peptide purification
Peptides were purified using a Dionex UltiMate 3000 system equipped with a Foxy Jr fraction collector (Teledyne Isco) using a Gemini C18 (5 µm; 10.0 x 250 mm) column (Phenomenex) at a flow rate of 5 ml/min and eluted using a one-step slow gradient protocol unless otherwise indicated. The solvent system used was A (0.1% trifluoroacetic acid in H2O) and B (0.1% trifluoroacetic acid in acetonitrile). Fractions were collected, analyzed by RP-HPLC and LC-MS, pooled and lyophilized.

2.7.4 Ninhydrin test/Kaiser test
The Ninhydrin or Kaiser test is a qualitative test for the presence or the absence of free primary amino groups and was used to monitor the completion of each coupling and/or deprotection step during SPPS. A small portion of resin was separated from the reaction vessel, washed with CH2Cl2 and dried on air. A drop each of 5% w/v ninhydrin in EtOH, 80% w/v phenol in EtOH and 0.2 mM KCN in pyridine solutions were added to the dried resin. Subsequently, the mixture was heated at 100°C for 5 min. Blue coloured beads and a deep blue solution indicated the presence of free primary amine groups, and a yellow solution indicated no amino groups were present.
2.7.5 Fmoc - SPPS

Fmoc-amino acids were used as purchased from GL Biochem with the following side chain protection: Fmoc-Arg(Pbf)-OH (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Asn(Trt)-OH, (Trt = triphenylmethyl), Fmoc-Asp(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Trp(Boc)-OH. Fmoc-Homoser(Trt)-OH was purchased from Sigma Aldrich.

Fmoc- SPPS was typically conducted at a 0.1 mmol scale either manually using a fritted glass reaction vessel or solid-phase peptide synthesis was performed on a Tribute synthesizer (Protein Technologies, Tucson, Az).

At the start of the reaction 100 mg aminomethyl resin (1mmol/g binding capacity, Rapp Polymer Tuebingen) was swollen in Dichloremethane (DCM) for an hour and washed three times with DCM. 2 eq. of both Fmoc-L-(X)-O-CH₂-phi-OCH₂-CH₂-COOH-linker (X first amino acid of the desired peptide sequence) and N,N'-diisopropylcarbodiimide (DIC) were dissolved in DCM, added to the resin and incubated for 1 h. The completion of the reaction was confirmed by Kaiser test. The resin was washed three times with DCM, dried and swollen in DMF.

Long peptide sequences (>10 amino acids) were assembled using Tribute synthesizer (Protein Technologies, Tucson, Az) using 20% piperidine/DMF (v/v) as the deblocking reagent (2 × 5 min) and HCTU/N-methyl morpholine (1 × 20 min) as the coupling reagents according to the manufacture’s instructions.

Short peptides (<10 amino acids) were assembled manually in a fritted glass reaction vessel and SPPS was carried out by repetitive cycles of deprotection and coupling steps as follows.

Deprotection:
The Fmoc group was deprotected with 20% v/v piperidine in DMF for 10 min followed by a second deprotection for 10 min, both shaking at room temperature.
Coupling:
Coupling of individual amino acids was performed with 5.5 equivalents of Fmoc protected amino acid in DMF (0.2 M), 5 equivalents of O-(benzotriazol-1-yl)-N,N,N’,N’’-tetramethyluroniumhexafluorophosphate (HBTU) in DMF (0.45 M) and 10 equivalents of diisopropylethylamine (DIPEA) in N-methylpyrrolidine (NMP) (2 M). Couplings were performed for 30 min on a shaker at room temperature.

Cycles of deportation and coupling were repeated for each residue in target sequences.

Peptide cleavage:
Upon completion of the synthesis, the peptide was released from the resin with simultaneous removal of protecting groups by treatment with cleavage cocktail (94:1:2.5:2.5 mixture of TFA/triisopropylsilane/H₂O/EDT (v/v/v/v)) at room temperature for 3 hours.

The crude peptide was precipitated with ice cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% trifluoroacetic acid (TFA) and lyophilised. The crude and purified peptides were analysed for purity by analytical LC-MS as described in 2.7.2. The crude peptide was purified by semi-prep RP HPLC as described above (section 2.7.3).

2.7.6 BOC – SPPS
Unless otherwise specified, the following N-Boc amino acids and the equivalent N-Boc-D amino acids were used for synthesis experiments: Boc-Ala-OH, Boc-Cys(4-MeBzl)-OH, Boc-Asp(OcHex)-OH, Boc-Glu(OcHex)-OH, Boc-Phe-OH, Boc-Gly-OH, Boc-His(DNP)-OH/iPrOH, Boc-Ile-OH.1/2H₂O, Boc-Lys(2-Cl-Z)-OH, Boc-Leu-OH, Boc-Met-OH, Boc-Met(O)-OH, Boc-Asn(Xan)-OH, Boc-Pro-OH, Boc-Gln-OH, Boc-Arg(Tos)-OH, Boc-Ser(Bzl)-OH, Boc-Thr(Bzl)-OH, Boc-Val-OH, Boc-Trp(Formyl)-OH, Boc-Tyr(2-Br-Z)-OH.
Chapter 2 - General Material and Methods

BOC-SPPS was typically conducted manually at a 0.1 mmol scale using a fritted glass reaction vessel. At the start of the reaction, PAM linker (0.3 mmol) was coupled to AM-PS resin (0.3 g, 0.1 mmol, loading 1 mmol/g) with DIC (0.3 mmol) and Cl-HOBt (0.4 mmol) in CH$_2$Cl$_2$ (5 mL) for 1 h, drained and washed with CH$_2$Cl$_2$. The Kaiser test was negative. Boc(X)-OH (X: first amino acid of the desired sequence) (0.5 mmol) DIC (0.5 mmol) and DMAP (0.01 mmol) were added and the mixture shaken for 1 h. The resin was drained, washed with DMF and the acylation repeated twice more. Any remaining resin-bound hydroxyl groups were capped by shaking the resin with Ac$_2$O (5 mmol) and DMAP (0.1 mmol) in DMF for 15 min.

Subsequently, in situ neutralisation Boc-SPPS was carried out by repetitive cycles of deprotection and coupling steps as follows.

Deprotection:
The drained resin was subjected to a 10 sec neat TFA flow wash followed by a 2 min batch wash. The resin was drained and washed with DMF (30 s flow wash).

Coupling:
The drained resin was coupled with a mixture of Boc-AA-OH (5 eq.), iPr$_2$NEt (12 eq.) in 0.475 M HATU/DMF (2 ml) for 5 min, with a pre-activation of 1 min. The resin was washed with DMF (30 s flow wash) and drained.

Cycles of deportation and coupling were repeated for each residue in target sequences. After the coupling of the final residue in a target sequence, resin was washed with DMF, and a final deprotection carried out. The peptidoresin was then washed with DMF, EtOH and dried in vacuo.

Hydrofluoric acid (HF) cleavage
$p$-Cresol (0.5 ml) was added to dry peptidoresin (up to ~1.0 g) in a specialised Teflon reaction vessel attached to a Teflon cleavage apparatus (Peptide Institute, Inc., Osaka, Japan). The reaction vessel was cooled to -78°C (using a dry ice/ethanol bath) and HF (10 ml) was condensed into the vessel under vacuum. The reaction was stirred for 1 h at 0 °C, after which the HF was removed in vacuo and quenched through a CaO trap.
Ice-cold Diethyl ether (Et₂O) was added to the residue and the resulting suspension was recovered by centrifugation. The supernatant was removed, ether was added, and the peptide recovered by centrifugation. The crude peptide was dissolved in 50% aq. MeCN containing 0.1% v/v TFA and lyophilised. The crude and purified peptides were analysed for purity by analytical LC-MS as described in 2.7.2. The crude peptide was purified by semi-prep RP HPLC as described above (section 2.7.3).
Chapter three - Design, synthesis and characterization of a peptide model for the N-terminal domain of adiponectin

[Parts of this chapter have been published in [228]. The synthesis strategy and the chemical synthesis of the various peptides was designed and carried out by Dr Paul W.R. Harris at the University of Auckland, School of Chemical Sciences.]

3.1 Introduction

Adiponectin assembles into trimers, hexamers and so called ‘High-Molecular-Weight forms’ (as described in chapter 1.3.2) the latter being of particular interest due to its potent biological functions [22]. However, the assembly pathway of HMW adiponectin remains poorly understood mainly due to the heterogeneity in the assembled adiponectin oligomers. Adiponectin undergoes diverse post-translational modifications and exhibits polymorphism in oligomeric state making the isolation of biologically active and pure oligomers difficult [22]. Although several groups have reported the successful large-scale production of adiponectin trimers and globular adiponectin, the accessibility of homogeneous adiponectin oligomers to probe and investigate the assembly process remains a limiting factor for researching both the assembly of adiponectin and the role of agents that facilitate the assembly process [31, 220]. To address that limitation, we decided to develop model peptides of adiponectin for the region thought to be important in adiponectin assembly as well as for interaction with chaperones, namely the variable domain as described in chapter 1.3.5. This approach allows for the large-scale production of these model peptides. These can subsequently be subjected to extensive biophysical and biochemical characterization to help gain further insight into the assembly of adiponectin oligomers. The model peptides also enabled us to probe the interaction between ER chaperones, such as ERp44 and adiponectin.
Chapter 3 - A peptide model for the N-terminal domain of adiponectin

3.2 Material and methods

[Part of this subchapter are taken from [228]. Experiments described in 3.2.1 and 3.2.4 to 3.2.9 were conducted by Dr. Paul W.R. Harris at the School of Chemical Sciences at the University of Auckland]

3.2.1 Circular Dichroism spectroscopy

CD measurements were carried out on a PiStar-180 spectrometer (Applied Photophysics, Leatherhead, Surrey, UK) using peptide solutions at 50 µM (by weight) in 10 mM potassium phosphate 150 mM KF pH 7.4 that had been incubated at 4 ºC for a minimum of 16 hours. The spectra were recorded at 0.5 nm increments in wavelength with a 4 s averaging time at 6 ºC and averaged over 10 scans. Thermal transition experiments were recorded for peptide solutions at 50 µM (by weight) in 10 mM potassium phosphate 150 mM potassium fluoride (KF) pH: 7.4 that had been incubated at 4 ºC for a minimum of 16 hours. The solutions were heated from 6 to 94 ºC in 0.3 ºC steps with a 30 sec equilibration time at each step. The ellipticity at 225 nm was monitored at each temperature with a 5 s averaging time.

3.2.2 Size exclusion chromatography coupled to multi angle laser light scattering (SEC-MALS) experiments

SEC-MALS was used to determine the solution molecular weight composition of Adiponectin^{39}C^{-53}N (GPO)_9 and Adiponectin^{18}E^{-53}N (GPO)_9 peptide. Adiponectin^{39}C^{-53}N (GPO)_9 and Adiponectin^{18}E^{-53}N (GPO)_9 peptide were dissolved in 20 mM Tris, 150 mM NaCl at pH 7.4 at a concentration of 20 mg/ml in the absence and presence of 5 mM H_2O_2 or 5 mM beta-mercaptoethanol as described in Results. After incubation at 4°C for 16 hours, samples (100 µl) were applied to SEC-MALS analysis as described in 2.6.9. To analyze pH stability of Adiponectin^{18}E^{-53}N (GPO)_9, 0.5 mg of the peptide was separately dissolved in 100 µl of 20 mM Mes, 150 mM NaCl at pH 6.5 and 20 mM Tris, 150 mM NaCl at pH 7.4 at pH 7.5 and 8.0, respectively. After incubation for 16 hours, samples were analyzed by SEC-MALS.

3.2.3 Fmoc SPPS of Adiponectin^{39}C^{-74}A

Adiponectin^{39}C^{-74}A was synthesized using Fmoc SPPS as described in section 2.7.5 with the addition that Fmoc 4(R)-hydroxyPro(O'Bu)-OH was used for residues 47Pro, 50Pro and 56Pro. Fmoc-Asp(OtBu)-(Dmb)-Gly-OH was used for 59Asp-60Gly and
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62Asp-63Gly. Synthesis of Adiponectin39Cys-74Ala yielded 5.25 mg of pure peptide as confirmed by ESI-MS (M+4H)4+, calc. 898.9, found 898.3.

3.2.4 [59E, 62E, 73E] Adiponectin39C-74A
[59E, 62E, 73E] Adiponectin39C-74A as synthesized using Fmoc SPPS as described in section 2.7.5. Synthesis of [59E, 62E, 73E] Adiponectin39C-74A yielded 10.65 mg of pure peptide as confirmed by ESI-MS (M+4H)4+, calc. 909.5, found 909.3.

3.2.5 Boc SPPS (GPO)9 O:hydroxyproline
(GPO)9 was synthesized as described in section 2.7.6. The synthesis of (GPO)9 yielded 5.1 mg of pure peptide as confirmed by ESI-MS (M+3H)3+, calc. 808.5, found 808.3.

3.2.6 Adiponectin39C-53N (GPO)9
Resin-bound (GPO)9 was elongated using Boc in situ neutralisation procedure as described in 2.7.6 with a minor modification. To minimise deletion impurities, a solution of acetic anhydride in DMF (20% v/v) (1 mL) was added immediately following the coupling reaction to cap any unreacted amino groups. A 2 min reaction time was used. The synthesis of Adiponectin39C-53N(GPO)9 yielded 20.2 mg of pure peptide as analysed ESI-MS (M+3H)3+, calc. 1304.1, found 1304.2. and RP-HPLC tR = 12.2 mins, (Zorbax 300SB-C3 3.5µ; 3.0 x 150 mm) using a linear gradient of 5% to 65%B over 21 mins at 75 °C at 0.3 ml/min.

3.2.7 Adiponectin18E-38T-COS-CH2CH2CO-Gly-OH
Adiponectin18E-38T-COS-CH2CH2CO-Gly-OH was synthesised as described in section 2.7.6. The crude product (147 mg, ESI-MS (M+2H)2+, calc. 1163.3, found 1163.1) was used without any further purification.

3.2.8 Adiponectin18E-53N (GPO)9
To a degassed solution of 6.0 M guanidine hydrochloride and 200 mM Na2HPO4 (2.5 mL), 4-mercaptophenylacetic acid (200 mM) and tris(2-carboxyethyl)phosphine hydrochloride (20 mM) was added. The pH of the resulting solution was adjusted to 6.9 by addition of 10 M NaOH and then 2 M NaOH. The two reactants, peptide
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Adiponectin$^{39}$C-$^{53}$N (GPO)$_9$ (7.5 mg, 3.22 µmol, final peptide conc. 1.23 mM) and peptide Adiponectin$^{18}$E-$^{38}$T-COS-CH$_2$CH$_2$CO-Gly-OH (9.0 mg, 2.3 µmol, final peptide conc. 0.92 mM) were then added, the vial flushed with argon and the reaction mixture was left at room temperature. After 24 h the reaction was quenched by addition of 5 M HCl to pH = 2, diluted with water to 10 mL, isolated by solid phase extraction and lyophilised to afford crude ligation product (12.9 mg) which was purified by RP-HPLC. The yield was 6.11 mg, 44% based on Adiponectin$^{39}$C-$^{53}$N (GPO)$_9$. ESI-MS (M+9H)$^9+$, calc. 675.6, found 675.2; (M+8H)$^8+$ calc. 759.4, found 759.3; (M+7H)$^7+$ calc. 868.4, found 868.0; (M+6H)$^6+$ calc. 1012.9, found 1012.3; (M+5H)$^5+$ calc. 1215.3, found 1214.6. RP-HPLC $t_r = 13.6$ mins, (Zorbax 300SB-C3 3.5µ; 3.0 x 150 mm) using a linear gradient of 5 % to 65 % B over 21 mins at 75 °C at 0.3 ml/min.
3.3 Results and Discussion

3.3.1 Design of model peptide

A model peptide suitable for analyses of the oligomerization of adiponectin should comply with the following requirements:

1. The model peptide should include the regions in adiponectin considered to be involved in oligomerization.
2. Prior to the assembly into higher order structures, adiponectin spontaneously folds into a trimeric arrangement. Therefore, a suitable model peptide should also spontaneously adopt a trimeric structure.

**Adiponectin Trimer**

<table>
<thead>
<tr>
<th>Signal peptide</th>
<th>Variable domain</th>
<th>Collagen domain</th>
<th>Globular domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-17</td>
<td>18-44</td>
<td>45-110</td>
<td>111-247</td>
</tr>
</tbody>
</table>

**Primary sequence of mouse adiponectin:**

18EDDVTTTEELAPALVPPPKGTCAVGMAIPGHPGHNGTPRDRGDRGDTPGEGKEKGD 74

[^9E, ^62E, ^73E]Adiponectin[^9C-24A]:

39CAGWMAGIPGHPGHNGTPRDEGREGTPGDKGDGEA74

Adiponectin[^9C-24A]:

39CAGWMAGIPGHPGHNGTPRDRGDGTPGEGKEKGD 74

Adiponectin[^18E-38T; 18EDDVTTTEELAPALVPPPKGT38](GPO)₉:

GPOGPOGPOGPOGPOGPOGPOGPOGPO

Adiponectin[^9C-53N(GPO)₉]:

39CAGWMAGIPGHPGHN(S3)(GPO)₉

Adiponectin[^18E-53N(GPO)₉; 18EDDVTTTEELAPALVPPPKGTCAVGMAIPGHPGHN(S3)(GPO)₉

**Figure 3.1: Illustration of adiponectin model peptides**

The top panel shows a representation of the adiponectin trimer. Below a portion of the primary structure of native murine adiponectin is shown (variable domain is colored in light blue and the collagen-like domain in black). The amino acid sequences of the various model peptides are listed in the bottom panel.
Adiponectin trimers form through hydrophobic interaction of the globular domain and are stabilized by the triple helical structure of the collagen-like domain. The assembly into MMW and HMW occurs via oxidative folding and is mediated by intermolecular disulfide bond formation between cysteine residues located in position 39 in the variable domain [35]. It was also observed that a conserved tryptophan residue three amino acids upstream of the critical C39, namely in position 42 is involved in the assembly process of adiponectin oligomers, presumably by controlling the oxidative state of C39 [73]. In addition, these two residues were shown to be critical for binding of adiponectin to ER chaperones, such as ERp44 and DsbL-A [14].

The analyses of the reported low-resolution EM reconstructions of HMW adiponectin indicate that the N-terminal regions (which also contain C39 and W42) of the adiponectin trimers cluster together in a compact bundle. This observation suggests an important role for the N-terminal variable domain in the stability of the HMW form as well as for the assembly process leading to higher-order structures [32]. Given the important role of this region for adiponectin oligomerization, we decided to include the complete variable domain (residues 18-44 of the murine adiponectin sequence) in the model peptide.

The variable domain of adiponectin by itself shows no sequence homology with any known protein and is likely to be unstructured [26]. To ensure the model folds into a trimeric structure we decided to include a stretch of the trimeric collagen-like domain in our design. It was previously reported that ten repeats of the GXY collagen sequence pattern are sufficient to form a stable collagen triple helix [229]. Hence, we included a N-terminal portion of the collagen-like domain covering the first ten repeats of the GXY motif in the model peptide design. As result the model peptide consisted of amino acid 18-74 of the murine adiponectin sequence (Figure 3.1).

The on resin solid phase synthesis of such a 56 amino acid long peptide as a single peptide chain is challenging. However, this challenge can be overcome by separate synthesis of two smaller fragments of the desired sequences and subsequent native chemical ligation (NCL) of these two fragments. NCL is achieved by the chemoselective reaction of an N-terminal cysteiny1 peptide and a C-terminal thioester
Chapter 3 - A peptide model for the N-terminal domain of adiponectin

peptide, [230]. Examination of the amino acid composition of mouse adiponectin collagen-like domain and the variable region showed only a single cysteine (C39) which we considered as being a suitable site for NCL. Our initial synthesis strategy was therefore, to synthesis the N-terminal portion composed of ten GXY repeats of the collagen like domain (30 amino acids) and additional six residues CAGWMA (C39 to A74) of the variable domain (hereafter called Adiponectin$^{39\text{C}-74\text{A}}$ (see Figure 3.1). In the second synthesis the remaining part of the variable domain (G$^{18\text{T}-38\text{T}}$) (hereafter called: Adiponectin$^{18\text{E}-38\text{T}}$ (see Figure 3.1) would be synthesised and the two fragments would be ligated. This strategy would enable the synthesis of the appropriate stretch of the collagen domain and the complete variable domain of adiponectin using a single ligation step.

3.3.2 Characterization of Adiponectin$^{39\text{C}-74\text{A}}$ and $[^{59\text{E}},^{62\text{E}},^{73\text{E}}]\text{Adiponectin}^{39\text{C}-74\text{A}}$

After chemical synthesis of Adiponectin$^{39\text{C}-74\text{A}}$ using microwave Fmoc solid-phase synthesis on suitably functionalized aminomethyl polystyrene resin, a coeluting by-product was detected during HPLC-MS analysis of the crude product. A discrepancy of 18 Da between the expected mass for Adiponectin$^{39\text{C}-74\text{A}}$ and the ‘undesired’ by-product was measured by mass spectrometry. Separation of the two species by HPLC purification failed due to similar retention times of both constructs. Presumably deportation of the Fmoc protection group with 20% piperidine during elongation cycles of the peptide chain caused formation of aspartimide by cyclization of the Asp-Gly or Asp-Ala motif resulting in a loss of 18 Da [231].

To mitigate this problem all three aspartic acid residues ($^{59\text{D}},^{62\text{D}}$ and $^{73\text{D}}$) were replaced with glutamic acid residues in our peptide design resulting in a glutamyl analogue named $[^{59\text{E}},^{62\text{E}},^{73\text{E}}]\text{Adiponectin}^{39\text{C}-74\text{A}}$ hereafter (see Figure 3.1). It was reasoned that glutamyl peptides are unlikely to undergo aspartimide formation. Moreover, glutamic acid and aspartic acid differ only in the number of CH$_2$ groups in their side chain. Glutamic acid contains two methylene groups, whereas the side chain of aspartic acid includes only one methylene group. Therefore, the replacement of residue $^{59\text{D}},^{62\text{D}}$ and $^{73\text{D}}$ with E should still maintain the overall charge properties as well as the structural integrity of the peptide.
Solid phase synthesis using Fmoc protection groups followed by HPLC purification yielded pure \([^{59}\text{E},\,^{62}\text{E},\,^{73}\text{E}]\text{Adiponectin}^{39}\text{C}-^{74}\text{A}\) in multi-milligram amounts and no aspartimide by-product was detected.

We then tested the chemically synthesised peptide \([^{59}\text{E},\,^{62}\text{E},\,^{73}\text{E}]\text{Adiponectin}^{39}\text{C}-^{74}\text{A}\) for its ability to form the expected trimeric collagen structure. To this end, the peptide was dissolved in 10 mM potassium phosphate, 150 mM KF, pH 7.4 at a concentration of 50 \(\mu\)M and incubated at 4 ºC for 16 h before analysed by Circular dichroism (CD) spectroscopy. Collagen triple helices are unique structures, which have three polyproline chains, in which every third residues is replaced by a glycine. The three individual chains wrap together in a helical arrangement. Each single strand of the trimer exhibits a conformation similar to the confirmation of poly-L-proline in an extended helical form, in which all of the peptide bonds are trans to each other [232]. This unique feature gives the CD spectrum of the collagen structures a distinct pattern with strong positive bands at around 225 nm and a pronounced negative signal at about 200 nm [233]. However, the signal for \([^{59}\text{E},\,^{62}\text{E},\,^{73}\text{E}]\text{Adiponectin}^{39}\text{C}-^{74}\text{A}\) exhibits a minimum at 195 nm and very low ellipticity for wavenumbers over 210 nm indicating a disordered arrangement of the construct (Figure 3.2) [233].

Although unlikely, we wanted to exclude the possibility that the absence of the collagen-like helix is caused by the D to E replacement. Hence, we synthesized the native sequence, using a more advanced synthesis strategy, where a protected dipeptide building block Fmoc-Asp(OtBu)-(Dmb)-Gly-OH is integrated into the peptide chain during synthesis and milder deportation procedures were applied to prevent cyclisation by aspartimide as described in [228]. The new synthesis strategy gave us milligram amounts of Adiponectin\(^{39}\text{C}-^{74}\text{A}\) after HPLC purification and no aspartimide formation was detected.

After purification, Adiponectin\(^{39}\text{C}-^{74}\text{A}\) was subjected to CD spectroscopic analyses using the same protocol as for \([^{59}\text{E},\,^{62}\text{E},\,^{73}\text{E}]\text{Adiponectin}^{39}\text{C}-^{74}\text{A}\) and revealed also a minimum at 195 nm and low signal for wavelength higher than 210 nm (see Figure 3.2). The graph is almost identical to the curve observed for \([^{59}\text{E},\,^{62}\text{E},\,^{73}\text{E}]\text{Adiponectin}^{39}\text{C}-^{74}\text{A}\) and suggests the polypeptide of Adiponectin\(^{39}\text{C}-^{74}\text{A}\) also
exist predominantly as a random coil. In other words, the isolated native sequence of adiponectin from residues 39 to 74 does not fold into a collagen-like structure confirming that the E to A mutations did not change the overall structural properties of the peptide chain.

The inability of the peptides to generate a collagen-like triple helix might due to the fact that:

1. The trimeric structure of the collagen-like triple helix requires close proximity of the three chains to fold, which is provided by the trimeric arrangement of the globular head domains in the full-length protein.

2. The 30 amino acid (ten GXY repeats) long stretch of the collagen domain is too short for the trimer to form and the complete length of the collagen-like domain is required for the peptide to assume a collagen triple helix.
3. Post-translational modifications present in the endogenous protein contribute to stabilize the trimeric structure of the collagen helix and are necessary for the trimeric structure to form.

3.3.3 Design of the second generation of model peptides \((GPO)_9\), Adiponectin\(^{39}C-^{53}N\) \((GPO)_9\) and Adiponectin\(^{18}E-^{53}N\) \((GPO)_9\)

Based on the fact that the synthesized native sequence did not assume a collagen-like triple helix we sought structural elements that initiate the folding of trimeric helices. We looked for a peptide sequence that exhibits similar characteristics to the C-terminal domain of the endogenous protein as described above and in chapter 1.3.2. A peptide sequence traditionally termed the ‘canonical collagen sequence’ has been extensively researched and has been used previously as an analogue to investigate the biophysical properties of naturally occurring collagen. The canonical model sequence is comprised of multiple repeats of GPO \((O: \text{Hydroxyproline})\) and organizes in a stable triple helical structure \([234]\). We anticipated that the fusion of such a trimeric peptide to the native peptide sequence of the variable domain of adiponectin will facilitate the generation of a trimeric collagen-like arrangement. The trimeric helix of the canonical peptide brings together the three chains of the native peptide. In addition, the collagen fold of the canonical collagen sequence might induce folding of the portion belonging to the native collagen-like sequence of adiponectin into a collagen-like arrangement. Therefore, we fused nine GPO repeats to the C-terminus of the model peptide as shown in Figure 3.1. Nine GPO repeats have previously been demonstrated to form a stable trimer \([234]\).

For the synthesis we chose a stepwise approach, where we first synthesized the nine GPO repeats and analyzed its structural characteristics. In the next step, we added a stretch of the native adiponectin sequence, namely from \(^{53}N\) to the \(^{39}C\). This stretch was chosen since a N-terminal cysteine is required for NCL in the next synthesis step. In this construct, we included two GXY repeats of the collagen-like domain of adiponectin, which contain H50 and H53 since these histidine residues have been implicated in stabilizing adiponectin oligomers by chelating Zinc (Figure 3.1)[71]. The canonical collagen peptide sequence replaced the part of the collagen-like domain further upstream of the two histidine residues, which were included in the initial
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model peptides Adiponectin$^{39}C^{-74}A$ and $[^{59}E, ^{62}E, ^{73}E]$ and Adiponectin$^{39}C^{-74}A$. The primary sequence for the resulting model peptide is shown in Figure 3.1 and hereafter called Adiponectin$^{39}C^{-53}N(GPO)_{10}$. After we showed that Adiponectin$^{39}C^{-53}N(GPO)_{10}$ folds into a trimeric collagen structure, we synthesized Adiponectin$^{18}E^{-38}T$. In the last step we obtained the final model (Adiponectin$^{18}T^{-53}N(GPO)_{10}$) through NCL of Adiponectin$^{18}E^{-38}T$ and Adiponectin$^{39}C^{-53}N(GPO)_{10}$ (Figure 3.1).

3.3.4 Characterization of model peptide (GPO)$_{10}$

(GPO)$_{10}$ was prepared using 	extit{in situ} neutralization Boc SPPS, a method to synthesize so called “difficult” sequences. This is because the routinely-used Fmoc synthesis yielded less product and contained several impurities as described in [228].

Liquid chromatography coupled with mass spectrometry (LC-MS), which is routinely used to check for the correct synthesis of peptides and/or HPLC purification, revealed that (GPO)$_{10}$ existed in various multimeric states (Figure 3.3). Besides peaks of 1212.1 amu and 808.6 amu representing the doubly charged and triply charged state of the monomer, respectively, peaks at 1616.0 amu corresponding to the triply charged dimer and 1454.5 amu representing the penta-charged trimer of (GPO)$_{10}$ were detected by MS (Figure 3.3). Subsequently, CD spectroscopy of (GPO)$_{10}$ displayed a spectrum expected for a collagen triple helix with a defined maxima at 225 nm and a pronounced minimum near 200 nm (Figure 3.4).

In the next experiment, we assessed thermal stability of the triple helix using thermal denaturation. To this end, a peptide solution at 50 uM in 10 mM potassium phosphate and 150 mM KF at pH 7.4 was incubated at 4 °C for 16 h. The unfolding of the triple helix was analyzed in a thermal transition experiment by heating the peptide solution from 6 °C to 94 °C and monitoring the maximum at 225 nm in the CD spectrum. The melting curve showed a distinct decrease in signal at around 55 °C establishing a thermal transition temperature (Tm) of 55 °C. This confirmed the presence of a trimeric conformation at room temperature (Figure 3.5).
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Figure 3.3: Mass spectrum of crude (GPO)$_9$

(GPO)$_9$ was analysed by LC-MS. The spectrum shows peaks at monomeric (GPO)$_9$ Calc. (M+2H)$^{2+}$ 1212.3, Found 1212.1, Calc. (M+3H)$^{3+}$ 808.5, Found 808.6. Dimeric (GPO)$_9$ Calc. (M+3H)$^{3+}$ 1616.0, Found 1615.9. Trimeric (GPO)$_9$ Calc. (M+5H)$^{5+}$ 1454.5, Found 1454.7. This result suggests that the (GPO)$_9$ peptide at least partially exist in a trimeric arrangement.

Figure 3.4: CD spectra of (GPO)$_9$, Adiponectin$^{13}C$-$^{53}N$ (GPO)$_9$ and Adiponectin$^{18}E$-$^{53}N$ (GPO)$_9$

A 50 µM solution of (GPO)$_9$, Adiponectin$^{13}C$-$^{53}N$ (GPO)$_9$ and Adiponectin$^{18}E$-$^{53}N$ (GPO)$_9$ in 10 mM potassium phosphate 150 mM KF at pH 7.4 was incubated at 4 °C for a minimum of 16 hours. Subsequently, the CD spectra were recorded at 0.5 nm increments in wavelength with a 4 s averaging time at 6 °C and averaged over 10 scans. All three spectra show a distinct minimum at 200 nm and a maximum at 225 nm characterizing a collagen-like secondary structure for the three peptides.
Figure 3.5: CD thermal transition curves of (GPO)$_9$, Adiponectin$^{39}$C-$^{53}$N (GPO)$_9$ and Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$

Thermal transition experiments of (GPO)$_9$, Adiponectin$^{39}$C-$^{53}$N (GPO)$_9$ and Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$ were recorded at a peptide concentration of 50 µM in 10 mM potassium phosphate 150 mM potassium fluoride at pH: 7.4 that had been incubated at 4 °C for a minimum of 16 hours. The solutions were heated from 6 to 94 °C in 0.3 °C steps with a 30 sec equilibration time at each step. The ellipticity at 225 nm was monitored at each temperature with a 5 s averaging time. All three spectra show a $T_m$ of $\sim$55°C indicating that the thermal stability of the collagen-like secondary structure is not disturbed by the fusion of the N-terminal segment of adiponectin.

3.3.5 Characterisation of Adiponectin$^{39}$C-$^{53}$N(GPO)$_9$

We then continued synthesis of Adiponectin$^{39}$C-$^{53}$N(GPO)$_9$ in a similar manner to (GPO)$_9$. Adiponectin$^{39}$C-$^{53}$N(GPO)$_9$ also showed evidence of higher oligomers in the LC-MS profile (Figure 3.6) [228]. Peaks found in the mass spectrum at 1955.8 amu and 1304.2 amu can be attributed to the doubly and triply charged states of the monomer, respectively, whereas signals at 1676.6 amu and 1467.1 amu originate from the seven and eight fold charged state of the trimer, respectively (Figure 3.6).

Furthermore, the monomeric and the trimeric species could be separated by HPLC and both species eluted in two separate peaks by applying a linear gradient from 5% to 65% acetonitrile in water in the presence of 0.1% formic acid over a period of 60 min (Figure 3.6).
Figure 3.6: LC-MS analyses of purified of Adiponectin$^{39}\text{C}-^{53}\text{N}(\text{GPO})_9$

A: Adiponectin$^{39}\text{C}-^{53}\text{N}(\text{GPO})_9$ was analysed by LC-MS. A slow linear gradient from 5% to 65% B over 60 mins was performed, where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile. The LC-MS profil shows that Adiponectin$^{39}\text{C}-^{53}\text{N}(\text{GPO})_9$ elutes in two separate peaks 1 and 2 .

B: Peak 1 is attributed to the monomer species Calc. (M+3H)$^{3+}$ 1304.1, Found 1304.2 and

C: Peak 2 is attributed to the trimer, Calc. (M+6H)$^{6+}$ 1955.6, Found 1955.5, (M+7H)$^{7+}$ 1676.4, Found 1676.6, Calc. (M+8H)$^{8+}$ 1467.0, Found 1467.1, Calc. (M+9H)$^{9+}$ 1304.1, Found 1304.3.
Followed analysis of Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} for its triple-helix forming properties by CD spectroscopy confirmed the presence of a trimeric collagen structure in solution (Figure 3.4). Next, we repeated the thermal transition experiment for the construct Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} to determine whether the addition of the native adiponectin sequence to the canonical collagen sequence has any effect on the stability of the collagen triple helix. The ellipticity of the maxima at 225 nm was monitored over a temperature range from 6ºC to 95ºC and established that the T\textsubscript{m} of Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} was 55ºC (Figure 3.5). This suggests that the addition of the native adiponectin N-terminal sequence to the N-terminus of the canonical collagen peptide has no significant impact on the thermal stability of the triple helical structure of the construct.

Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} carries a cysteine residue at the very N-terminal end of the sequence. This cysteine allows for the possibility of the trimeric peptide model to assemble into higher order structures via disulfide bond linkage in a fashion similar to adiponectin. Such a situation cannot be detected by CD spectrometry. To analyse the oligomeric state of the model peptide we conducted SEC-MALS experiments to determine the average molecular mass of various species present in the sample. For this purpose 2 mg of peptide was dissolved in 100\,µl of 20 mM Tris, 150 mM NaCl at pH 7.4 and the sample incubated at 4ºC for 16 h. Subsequently, the sample was applied onto a Superdex 200 column connected to a MALS setup. The elution profile showed multiple peaks with average molecular masses ranging from 24 kDa to 4 kDa (Figure 3.7). The main peak eluted after ca.17 ml representing the trimer. A second species eluted at ca. 14.5 ml consisting of hexamers. The elution profile also showed that more trimer than hexamer was present. In addition, earlier eluting species (likely representing higher oligomers) and some monomers were also observed (Figure 3.7). These results indicated that Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} spontaneously assembles into trimers which can undergo multimerisation through disulfide bond formation. This oxidative multimerisation is likely driven by atmospheric oxygen.
Figure 3.7: SEC-MALS of Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9}

SEC-MALS of Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} after 24 h incubation at 4 °C in 20 mM Tris, 150 mM NaCl at pH: 7.4 (top panel), in the presence of 5 mM beta-mercaptoethanol (middle panel) and in the presence of 5 mM H\textsubscript{2}O\textsubscript{2} (bottom panel). The UV\textsubscript{280} nm trace (green, purple or blue) is reported for each sample. The average molecular mass across the protein peaks as determined by MALS (black circles) are shown in the plot. In the absence of any redox-environment-controlling reagents (top panel) Adiponectin\textsuperscript{39}C-\textsuperscript{53}N(GPO)\textsubscript{9} elutes in multiple peaks attributed to trimers (theoretical mass: 11727 Da), hexamers (theoretical mass: 23454 Da), and some higher oligomers of trimer. In the presence of 5 mM beta-mercaptoethanol the peptide forms trimers (theoretical mass: 11727 Da) (middle panel) and in the presence 5 mM H\textsubscript{2}O\textsubscript{2} the peptide forms oxidized multimers of trimers, hexamers (theoretical mass: 23454 Da), and fewer amounts of oxidised trimers (theoretical mass: 11725 Da).
To confirm whether the multimerisation was due to disulfide bond formation between trimers and not induced by hydrophobic interactions between the trimeric building blocks we repeated the experiment in the presence of 5 mM beta-mercaptoethanol, in order to preserve the cysteines in a reduced state. When the experiment was repeated under reducing conditions, Adiponectin$^{39-53}N(GPO)_9$ eluted with a mass of around 12 kDa corresponding to the trimer and a small peak representing monomers was also seen (Figure 3.7) confirming Adiponectin$^{39-53}N(GPO)_9$ exist predominantly as trimer in a non-oxidising environment and generates multimers of trimers under oxidising conditions.

Next, we tested the behaviour of Adiponectin$^{39-53}N(GPO)_9$ under oxidising conditions. To this end, we dissolved 2 mg of Adiponectin$^{39-53}N(GPO)_9$ in 100 nl of 20 mM Tris, 150 mM NaCl at pH: 7.4 in the presence of 5 mM H$_2$O$_2$. Under such an oxidising condition Adiponectin$^{39-53}N(GPO)_9$ generated oxidised trimers, hexamers and higher oligomers. In addition, a significant amount of peptide with an average molecular mass less than the hexamer but higher than the trimer was detected.

3.3.6 Characterisation of Adiponectin$^{39-53}N(GPO)_9$

Having established the oligomeric state of Adiponectin$^{39-53}N(GPO)_9$, we next synthesised Adiponectin$^{18-38}T$ [228] and ligated this peptide to Adiponectin$^{39-53}N(GPO)_9$ to obtain Adiponectin$^{18-53}N(GPO)_9$. Ligation was judged complete after a period of 24h and the final product was purified by HPLC yielding 44% pure product [228]. Analagous to the situation for the two previous peptide constructs (Adiponectin$^{39-53}N(GPO)_9$ and (GPO)$_9$), LC-MS also revealed a trimeric state for Adiponectin$^{18-53}N(GPO)_9$ (Figure 3.8). Signal in the mass spectrum at 1401.5 amu, 1655.9 amu and 1821.4 amu correspond to different charged states of the trimer and 675.2 amu, 759.9 amu, 867.9 amu, 1012.2 amu, 1214.5 amu and 1518.2 amu to various charges states of the monomer as described in [228](Figure 3.8).
Figure 3.8: LC-MS of purified Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$

LC-MS analyses of Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$ revealed that the peptide at least partially exists as trimer. The calculated mass of Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$ is 6071.6 Da. Deconvolution yields a mass of 6068.5 ± 0.8. The masses in italics are attributed to the trimeric oligomerization state of Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$. Conditions: linear gradient of 5–65% B over 21 min where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile.

We analyzed the triple helix forming properties of Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$ with CD spectroscopy and the thermal transition assay using the same protocol as for the previous constructs. The CD spectrum displayed a profile consistent with a collagen triple helix (Figure 3.4). The thermal transition temperature of Adiponectin$^{18}$E-$^{53}$N (GPO)$_9$ was determine to be 55°C (Figure 3.5), the same detected for the two shorter peptide sequences establishing that the variable domain does not influences the thermal stability of the collagen triple helix.
Figure 3.9: SEC-MALS analysis of Adiponectin\textsuperscript{18E-53N} (GPO)\textsubscript{9}

SEC-MALS analysis of Adiponectin\textsuperscript{18N-53N}(GPO)\textsubscript{9} after incubation in 20 mM Tris, 150 mM NaCl at pH: 7.4 (top panel) and in the presence of 5 mM H\textsubscript{2}O\textsubscript{2} (bottom panel) for 24 h at 4 °C. The UV\textsubscript{280} nm trace (green or blue) is reported for each sample. The average molecular weights across the protein peaks as determined by MALS (black circles) are shown in the plot. In the absence of any redox-environment-controlling reagents (top panel) Adiponectin\textsuperscript{39C-53N}(GPO)\textsubscript{9} elutes in as trimer (theoretical mass: 18225 Da) and in the presence 5 mM H\textsubscript{2}O\textsubscript{2} the peptide forms fewer amounts oxidized multimers of trimers, hexamers (theoretical mass: 36450 Da) and oxidised trimers (theoretical mass: 18223 Da).

Next, we analyzed the oligomeric states of Adiponectin\textsuperscript{18E-53N}(GPO)\textsubscript{9} after incubation at 4°C for 16 h in 20 mM Tris, 150 mM NaCl, pH 7.4 (in the absence of agents that regulate the redox-environment) by SEC-MALS. The peptide eluted as a single peak with an average mass of 18 kDa corresponding to an Adiponectin\textsuperscript{18E-53N}(GPO)\textsubscript{9} trimer (Figure 3.9). This was surprising since for Adiponectin\textsuperscript{39C-53N}(GPO)\textsubscript{9} the trimeric building block had oligomerized into hexamers and higher oligomeric structures under identical conditions (Figure 3.7). This had suggested that
the N-terminal portion downstream of C39 may regulate the oligomerization process of the peptide, which might also apply for full-length adiponectin. In the second experiment, we oxidized the Adiponectin\textsuperscript{39C-53N(GPO)}\textsubscript{9} over a period of 16 h with 5mM hydrogen peroxide before loading it onto the superdex 200 column. In this case, the major species found was the hexamer with a molecular mass of 36 kDa (Figure 3.10). A peak of 18 kDa corresponding to the trimer was also detected and only very small amounts of higher oligomers (>36kDa) were seen (Figure 3.9). The outcome of this experiment significantly differed from the result for Adiponectin\textsuperscript{39C-53N(GPO)}\textsubscript{9} which lacked residues \textsuperscript{18}E to \textsuperscript{38}T. Here, following incubation under oxidizing conditions larger amounts of higher oligomers were seen, with hexamers, oxidized trimers and a species with an average molecular mass between trimer and hexamer (Figure 3.7). This observation supported a regulatory role of the N-terminal region of the variable domain in the oligomerization of the peptide.

Adiponectin reaches the extracellular space by transit along the secretion pathway, where it undergoes oxidative multimerisation [108]. Along the secretion pathway the pH ambient is strictly controlled and changes from a more alkaline value (pH 7.2) in the ER to a more acidic milieu (pH 6.1) in the Golgi apparatus [138]. It should be noted that the redox potential of cysteine residues varies under different pH conditions [235]. Hence, we examined whether the oligomeric state of Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9} is influenced by pH. For this purpose aliquots containing 0.5 mg of Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9} peptide were suspended in 100 µl buffer at pH 6.5 (20 mM MES, 150 mM NaCl), 7.5 (20 mM Tris-HCl, 150 mM NaCl) or 8.0 (20 mM Tris-HCl, 150mM NaCl). After incubation at 4°C for 16 hours, the samples were analyzed by SEC-MALS. The elution profiles for each of the experiments displayed essentially similar profiles consisting of one major peak with an averaged mass of ~18-19 kD, which corresponded to the mass of the Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9} trimer (Figure 3.10). In addition, a minor peak with an averaged mass of ~36 kDa and a smaller peak at ~6 kDa appeared in each elution profile (Figure 3.10). These masses correspond to an Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9} hexamer and an Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9} monomer, respectively. Thus, pH has no noticeable effect on the oligmerization property of Adiponectin\textsuperscript{18E-53N(GPO)}\textsubscript{9}.

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Figure 3.10: pH dependence of the oligomeric states of the Adiponectin$^{18}$E$^{53}$N(GPO)$_9$ peptide determined by SEC-MALS.

0.5 mg amounts of Adiponectin$^{18}$E$^{53}$N(GPO)$_9$ were suspended in 100 µl buffer at pH 6.5 (20 mM MES, 150 mM NaCl) (top panel), 7.5 (20 mM Tris-HCl, 150 mM NaCl) (middle panel) or 8.0 (20 mM Tris-HCl, 150 mM NaCl) (bottom panel). After incubation at 4°C for 16 hours, the samples were analyzed by SEC-MALS experiments. The UV$_{280}$ nm traces (black line pH 6.5, blue line pH 7, orange line pH 8) are shown. The elution profiles for each of the samples shows one peak eluting at 14 ml with an averaged mass of ~18-19 kD, the mass of the AHD trimer.
3.4 Conclusion and Summary

Characterization of the various peptide constructs led to the following conclusions:

1. The stretch from 39C to 74A of the adiponectin collagen domain in isolation does not assemble into a trimeric collagen-like structure.

2. The ligation of the variable domain of adiponectin to the ‘canonical collagen sequence’ does not influence the ability of the collagen-like structure of the canonical collagen peptide sequence to form.

3. The variable domain of adiponectin plays an important role in the regulation of the assembly of Adiponectin18E-53N(GPO)9 into higher oligomers by controlling disulfide bond formation between C39 residues in the trimers.

4. pH has no noticeable effect on the oligomerization of the Adiponectin18E-53N(GPO)9 peptide.

5. The redox environment tightly controls the multimerization of Adiponectin18E-53N(GPO)9.

We thus have been able to generate a robust trimeric model of the N-terminal region of adiponectin, namely Adiponectin18E-53N(GPO)9 (in the following chapters of this thesis this peptide is called AHD ‘adiponectin hyper-variable domain model peptide’). The peptide allowed us to analyze the influence of this subunit on the multimerization of the triple helical building block of adiponectin into higher order structures as well as for probing the interaction between ER chaperons and the variable domain of adiponectin using biophysical approaches.
Chapter four-Regulation and quality control of adiponectin assembly by ERp44

[This chapter have been published in Hampe et al., 2015. Experiments presented in Figure 4.1 were carried out by Dr. Cheng Xu in Prof. Yu Wang’s group at the University of Hong Kong.]

4.1 Introduction
Adiponectin is a collagenous adipokine possessing direct anti-diabetic, anti-atherogenic, anti-inflammatory and anti-tumour properties [21, 45, 46, 57, 236-244]. It acts on a wide range of target tissues and is present at high plasma concentrations [22, 245]. Adiponectin monomers consist of four domains - an N-terminal secretion-signaling segment followed by a variable domain, a triple helical collagenous domain and a C-terminal globular head domain [27, 33]. Adiponectin monomers spontaneously form trimers (Low Molecular Weight; LMW) that are held together by inter-monomer hydrophobic interactions between the globular head domain and stabilized by the trimeric collagen helix [23]. Via the trimeric building block, adiponectin can further oligomerize into hexamers (Middle Molecular Weight; MMW) or 12-18 mers (High Molecular Weight; HMW) [30, 32, 36]. Pleomorphism in its oligomeric state together with post-translational modifications appear to be intimately linked to the functional diversity of adiponectin [22, 25, 39]. Studies provide strong evidence that the most biologically potent form of adiponectin is the HMW species, which is markedly reduced in obesity concomitant with loss of protective properties of adiponectin [246]. Despite its importance, the maturation process of the various adiponectin oligomeric species is not fully understood.

Assembly of the MMW and HMW form is mediated by the formation of inter-trimer disulfide bonds by the conserved cysteine residue (C39) in the variable domain [30, 33]. In vitro studies [22, 35] have demonstrated the importance of intra-trimer disulfide bonds en route to the formation of HMW from its precursors. In this context, our earlier study showed that the conserved tryptophan (W42) controls the oxidation state of C39 and promotes the formation of HMW adiponectin [73]. In addition, this study further supported the previously reported [80] importance of endoplasmic reticulum (ER) chaperone ERp44 in controlling oxidative maturation of HMW adiponectin [73].
ERp44 is a member of the protein disulfide-isomerase (PDI) family of proteins. It belongs to an ensemble of ER chaperones, including Ero1L-α and Dsb1A [81, 82, 247], which mediate assembly of adiponectin and also act on other cysteine-rich client proteins such as IgM antibodies and Serotonin Transporter (SERT) [203, 248]. Unlike other PDI family members, which are mainly found in the ER, the majority of ERp44 is localized in the ERGIC-cisGolgi region [83, 201, 203, 249]. The X-ray crystal structure of ERp44 [200] and recent findings [138], have revealed new insights into ERp44 action.

ERp44 consists of an amino-terminal thioredoxin domain a followed by two thioredoxin-like domains b, and b’arranged in a clover leaf-like structure containing 6 distinct cysteine pairs, namely, 29, 63; 160, 212; 272, 289. For instance, the C160/C212 pair has been implicated in the regulation of binding to the IP3R1 channel [217] whereas the biological roles of C289/C272 pair and that of residue C63 have not yet been assigned. C29 was shown to be responsible for thiol-mediated retention and release of ERp44 substrates, such as IgM antibodies and oxidoreductase Ero1α and also suggested to be implicated in a similar process with adiponectin [138, 200, 201]. Binding and release of ERp44 substrates is believed to be pH dependent occurring in the following manner: A flexible tail at the C-terminus of ERp44 shields C29 contained in the CRFS motif at the binding site in the a domain. At higher pH in the ER (pH 7.2), the buried C29 is then deprotonated and in addition, an RDEL motif facilitates the transport of ERp44 substrates from the Golgi back to the ER upon binding to the KDEL receptors, is inaccessible. It is believed that at lower pH in cisGolgi (pH 6.7) C29 becomes protonated, a state that exhibits higher affinity to target Cys residues of ERp44 substrates, and the C-terminal tail rearranges to make the active site accessible. This exposes the RDEL motif at the end of the C-terminal tail, which can facilitate binding of the ERp44-client-complex to KDEL receptors [138].

The discovery of pH-regulated ERp44 activity and shuttling of substrates between the ER and cisGolgi is of marked significance for understanding the thiol-mediated retention and the quality control cycle i.e. establishment of the correct disulfide linked oligomers of client secretory proteins. However, several aspects of the underlying mechanism remain unclear. For example, what provides the oxidative power for ERp44
Chapter 4 - Adiponectin assembly regulated by ERp44

to form mixed disulfide bonds and how does the ERp44-cargo complex dissociate when retrieved to the ER [210]? In this study, we investigated factors that underpin the role of ERp44 action in adiponectin assembly. To further our understanding of this process, we used short peptide mimetics derived from the variable domain of adiponectin for revealing the mode of adiponectin complexation with ERp44. Our findings provide a mechanism for the regulation of adiponectin assembly and shed light on ERp44 function.

4.2 Material and methods

[Experiments presented in 4.2.1, 4.2.7, 4.2.8 and 4.2.9 were carried out by Dr. Cheng Xu in Prof Yu Wang’s lab at the University of Hong Kong.]

4.2.1 Production of adiponectin

The production and purification of murine adiponectin was as described [73]. The expression vector encoding murine adiponectin with a FLAG epitope tag at the C-terminus was transfected into HKE293 cells. Single colonies overexpressing FLAG-tagged adiponectin were selected for large-scale expansion. The cells were incubated in serum-free Dulbecco's Modified Eagle Medium (DMEM) containing 0.2% vitamin C and 0.2% BSA for 48 h. The FLAG-tagged recombinant protein was purified from the conditional medium using the monoclonal anti-FLAG affinity gel as previously described [74].

4.2.2 Production of mouse ERp44

The recombinant form of ERp44 was overexpressed in E. coli and purified as described [138] with one additional purification step to separate ERp44 monomers and dimers. For this purpose, purified ERp44 was loaded onto the Superdex-200 10/300 GL column (GE Healthcare) pre-equilibrated with 20 mM MES 150 mM NaCl pH 6.5 (buffer A). The fractions containing monomeric ERp44 or dimeric ERp44 as the main component were pooled.

4.2.3 Production of 9-amino acid residue peptides

WT36-44, C39S36-44 and control peptides were synthesized as described in section 2.7.5.
WT 36-44: NH$_2$-KGTCAGWMA-CO$_2$H (22.1 mg, yield 24.0%, purity>95%), observed mass 925.3 (M+H)$^{+}$, calculated mass 925.0.

C93S 36-44: NH$_2$-KGTSAGWMA-CO$_2$H, (16.5 mg, 18.3% yield%, purity>95%), observed mass 909.3 (M+H)$^{+}$, calculated mass 909.02.

Control peptide: NH$_2$-AGACGMWTK-CO$_2$H (18.3 mg, yield 19.9%, purity>95%), observed mass 925.2 (M+H)$^{+}$, calculated mass 925.0.

### 4.2.4 AHD peptide

The peptide was synthesized and purified as previously described in section 3.2. and [228].

#### 4.2.5 Production of dimeric WT36-44 peptide

WT36-44 at a concentration of 2 mg/ml was dissolved in buffer A containing 5 mM H$_2$O$_2$ and incubated for 2 h on ice. Subsequently, the sample was extensively dialyzed against buffer A using a membrane with a molecular weight cut off of 100 Da.

#### 4.2.6 Production and separation of oxidized AHD trimer and hexamer

AHD peptide was dissolved in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (buffer B) containing 5 mM H$_2$O$_2$ at a concentration of 20 mg/ml and incubated for 16 h at 4°C. The sample was subsequently loaded onto a Superdex-200 10/300 GL column (GE Healthcare) pre-equilibrated with (buffer A), and the trimeric AHD was thereby separated from the hexameric form. The fractions containing trimeric AHD peptide or hexameric AHD peptide as a main component were pooled. In SEC-MALS experiments, these pooled fractions exhibited single peaks with corresponding masses for the trimer and hexamer, respectively.

#### 4.2.7 Co-immunoprecipitation (IP) and Western blot

For detection of different oligomeric adiponectin complexes, cell lysates or purified adiponectin were incubated with a non-reducing sample buffer (1% SDS, 5% glycerol, 10 mM Tris-HCl, pH 6.8) at ambient temperature for 10 min, separated by a 4–20% gradient SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting with an in-house anti-adiponectin antibody [250] or anti-ERp44 antibody (Santa Cruz Biotechnology, CA, USA).
For the co-immunoprecipitation studies, cells were washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, pH 7.4) and solubilized in a RIPA buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM NaF, 1% Na deoxycholate, 1% NP-40, 0.1% Triton-X 100 plus protease inhibitor cocktails). 100 µg cell lysate was pre-cleared with protein G beads, and then incubated with the antibody and protein G beads overnight at 4°C with shaking. The beads were precipitated and washed with RIPA buffer three times, and the immune-precipitated complexes were eluted by incubation with non-reducing SDS-PAGE sample buffer and analyzed by Western blot.

4.2.8 Far-Western blot
Purified adiponectin (1 µg) was first separated by non-reducing SDS-PAGE and transferred to PVDF membranes and incubated with or without ERp44 (10 µg) in control buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20, pH 7.5) for 24 h at 4°C. After incubation, membranes were washed three times with control buffer and probed with anti-ERp44 antibody.

4.2.9 Co-incubation experiments with adiponectin and ERp44
Purified murine adiponectin (1 µg) was incubated with purified ERp44 (10 µg) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, pH 7.4). As a control, adiponectin and ERp44 was incubated independently under the same condition. Subsequently, the samples were separated by non-reducing SDS-PAGE. In two independent experiments either anti-adiponectin antibody or anti-ERp44 antibody was used for identification of the protein bands. For the co-incubation assay at varying pH, experiments were conducted as described above using buffer containing 50 mM HEPES, 150 mM NaCl at pH 6.5, pH 7.5 and pH 8.0. For the pH stability test an aliquot of the sample incubated at pH 6.5 was taken and pH was adjusted to 8.0 by adding NaOH. After incubation for 48 h the sample was separated by non-reducing SDS-PAGE and anti-adiponectin antibody was used for identification of the protein bands.
4.2.10 Size exclusion chromatography coupled to multi angle laser light scattering (SEC-MALS) experiments

SEC-MALS was used to determine the solution molecular weight, stability, composition of AHD peptide (oxidized and reduced trimer and hexamer) and to identify the formation of ERp44-peptide complexes. AHD peptide was dissolved in buffer B at a concentration of 20 mg/ml in the absence and presence of 5 mM H$_2$O$_2$. After incubation for 24 h, 100 µl aliquot was subjected to SEC-MALS as described below.

The binding assay between ERp44 and AHD peptide oligomers was carried out at the stated peptide concentration in the presence of 0.5 eq. (26.5 µM), 1 eq. (53 µM) and 2 eq. (106 µM) of ERp44 monomer or dimer. After incubation at 4°C for 16 h, samples (100 µl) were analysed as described in section 2.6.9.

4.2.11 Electrospray ionization mass spectroscopy (ESI-MS)

The mass spectroscopy assay was used to analyze the amount of disulfide bond formation between ERp44 and the various peptides. To this end, ERp44 (43 µM) was incubated with ten -fold excess (430 µM) of peptide (WT36-44 monomer, WT36-44 dimer, C39S36-44, control peptide, AHD reduced trimer, AHD oxidized trimer and AHD hexamer). All samples were incubated for 7 days in buffer A unless stated otherwise and applied to mass spectroscopy. Samples at pH 7.5 and 8.0 were incubated in 20 mM Tris-HCl, 150 mM NaCl pH 7.5 or pH 8.0. In order to analyze the effects of a reducing condition, the WT36-44 sample was incubated in buffer A in the presence of 5 mM β-ME.

For LC-MS analysis, samples were diluted in 0.1% formic acid and 10 µl injected onto a 0.32x100mm 3 µm Discovery Bio Wide pore C5 column (Supelco, Bellefonte, PA, USA) and separated using the following gradient at 6 ml/min: 0-4min 10% B, 24min 70% B, 27min 97% B, 30min 97% B, 32 min 10% B and 35 min 10% B where A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The column eluate was ionized in the electrospray source of a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). A TOF-MS scan from 400-1600 m/z was performed. The resulting data was deconvoluted into
protein molecular weights using the Bayesian Protein Reconstruct Tool within Analyst QS 1.1 (Applied Biosystems).

4.2.12 Tryptophan fluorescence quenching
Each peptide (30 µM) was incubated at ambient temperature in the presence or absence of 15 µM ERp44 in buffer A for 5 min at a total volume of 300 µl. Fluorescence was recorded between 340 nm and 450 nm on an EnSpire Multimode Plate Reader 2300-001M. Data were analyzed using EnSpire Manager software package. The ratio of the fluorescence intensities was used as a measure of quenching. The maximum of fluorescence intensities at 388 nm was used and the ratio \( f/f_0 \) calculated as described [251], where \( f \) is fluorescence of the mixture (peptide + ERp44) and \( f_0 \) is the fluorescence signal of the peptide alone.

4.2.13 Non-reducing SDS-PAGE
Disulfide bond formation between ERp44 monomer and AHD peptide oligomers were analyzed by non-reducing SDS PAGE. ERp44 monomer (15 µM) was incubated with 1 eq. (15 µM), 2eq. (30 µM) or 5 eq. (75 µM) of the particular AHD peptide. After incubation for 24 h at 4°C, samples were loaded onto a non-reducing SDS-PAGE (12.5%). To determine pH dependence of disulfide bond formation between ERp44 and AHD peptide, ERp44 monomer (15 µM) was incubated with 1 eq. (15 µM), 2eq. (30 µM) or 5 eq. (75 µM) of the reduced trimeric AHD peptide for 5 days at 4°C in buffer A at pH 6.5 and 20 mM Tris-HCl, 150 mM NaCl pH 7.5 or 20 mM Tris-HCl, 150 mM NaCl pH 8.0. Subsequently the samples were analyzed by non-reducing SDS-PAGE (12.5%). To analyze pH stability of the ERp44-AHD peptide complex, ERp44 monomer (15 µM) was incubated separately with 30 µM of the reduced trimeric, oxidized trimeric and hexameric AHD peptide. After incubation for 5 days at 4°C, the pH of 10 µl aliquots of each sample was set to 8.0 by the addition of 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 solution. The samples were subsequently incubated for further 16 hours at 4°C prior to loading onto a non-reducing SDS -PAGE (12.5%). GelQuant.NET software (biochemlabsolutions.com) was used to quantify intensity of the gel bands by densitometry.
4.3 Results

4.3.1 ERp44 binds LMW and MMW but not the HMW form of adiponectin

We investigated the adiponectin/ERp44 interaction by (i) co-immunoprecipitation using HEK293 cells stably expressing adiponectin (co-IP), (ii) far-Western blot analysis and (iii) Western blot analysis using purified recombinant ERp44 and adiponectin. In the immunoprecipitated complex milieu generated by using anti-adiponectin antibody, two species were recognized by anti-ERp44 antibody (Figure 4.1 A) migrating at ~130 kDa, likely corresponding to the ERp44-adiponectin LMW complex, and a species migrating at around 190 kDa, likely corresponding to ERp44-adiponectin MMW complex. Far-Western blot using purified recombinant adiponectin also showed complexes with ERp44 corresponding to LMW and MMW adiponectin (Figure 4.1 B), but again no signal corresponding to the HMW form was detected (Figure 4.1 B).

Experiments i and ii suggest that ERp44 does not associate with the HMW form, which instigated experiment iii. Here, we wanted to reveal what happens to the different adiponectin oligomers in solution after binding to ERp44, while not being constrained and localized on the PVDF membrane as is the case for Far-Western blots. Towards this end, we performed a direct co-incubation of purified murine adiponectin and ERp44 and subjected this to Western blot analysis after separation by non-reducing SDS-PAGE (Figure 4.1 C). The Western blot showed no band corresponding to HMW or MMW adiponectin in complex with ERp44. When compared to the controls, irrespective of whether anti-ERp44 or anti-adiponectin antibody was used, an additional band was detected, which may be assigned to the ERp44-LMW complex (Figure 4.1 C). The co-incubated sample, also features a change in the relative levels of the various adiponectin oligomers. As can be seen (Figure 4.1 C), when compared to the ratio of LMW to MMW in the control, the co-incubated sample displays enhanced level of LMW after ERp44 treatment (Figure 4.1 C). We also note that in addition to the ERp44 monomer band, another band consistent with ERp44 existing as a dimer was also observed. This is due to the presence of both monomers and dimers in the purified ERp44 sample, which is in line with previously observed in vivo data [83].
Given that regulation of ERp44 activity has been indicated to be pH dependent [138], we queried whether pH dependence also applies to the binding of full-length adiponectin to ERp44. Co-incubation of ERp44 and adiponectin at pH 6.5, 7.5 and 8.0 showed the largest complexation at pH 6.5 and very little at pH 8.0 with an intermediate amount of complex seen at pH 7.5 (Figure 4.1 D). In agreement with this observation, when the pH of an aliquot of such a sample incubated at 6.5 to generate the ERp44-LMW complex was raised to 8.0, the complex disassembled. Western blot analysis of the sample showed that most of the released adiponectin was trimeric whereas only a small increase of hexameric species was seen (Figure 4.1 E).

Taken together, these results using full-length adiponectin for complexation with ERp44 established distinctive binding profiles for the various oligomeric states of adiponectin. However, our results described in the paragraph above also raised questions. These were a) why the ERp44/MMW adiponectin interaction was observed by co-IP and Far-Western blot experiments but not by Western blot and b) what factors lead to the apparent increase of LMW adiponectin level after co-incubation in solution?
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Figure 4.1: Examination of interactions between ERp44 and different adiponectin oligomers.

A: Co-immunoprecipitation (IP) profiles of cell lysates of transfected HEK293 cells stably expressing murine adiponectin. The immune complexes, separated by non-reducing SDS-PAGE, and probed using anti-adiponectin (left panel) and anti-ERp44 antibody (right panel), for the presence of adiponectin and ERp44, are shown. Non-immune-IgG was used as the control antibody. The species migrating at ~130 kDa corresponds to the ERp44-adiponectin LMW complex, whereas the species migrating at around 190 kDa corresponds to the ERp44-adiponectin MMW complex.

B: Purified murine adiponectin (1 µg) was separated by non-reducing SDS-PAGE and transferred to PVDF membranes. After incubating with control buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) with (10 µg) or without ERp44 for 24 hours at 4°C, the membrane was washed with the control buffer and probed by anti-ERp44 antibody (Panel 1 and Panel 2) and anti-adiponectin antibody to show the distribution of oligomers of adiponectin (Panel 3). The red arrows indicate the locations of LMW and MMW adiponectin (molecular weights of ~110 kDa and ~170 kDa) that bind ERp44.

C: ERp44 (10 µg), purified murine adiponectin (1 µg) or the mixture of two recombinant proteins were incubated for 24 hours at 4°C in PBS. After separation by non-reducing SDS-PAGE, the membranes were probed with antibodies against adiponectin and ERp44. Anti-adiponectin antibody recognizes the different adiponectin oligomers and a complex consisting of ERp44 and LMW (at ~130 kDa). The estimated amounts as reflected by densitometry of the band intensity in adiponectin-only control were 2% HMW, 89% MMW and 9% LMW. These for the ERp44-adiponectin co-incubated sample were 2% HMW, 62% MMW, 23% LMW and 13% of the ERp44-adiponectin complex showing an increase of trimeric adiponectin after ERp44 co-incubation. Anti-ERp44 antibody recognizes ERp44-LMW complex (at ~130 kDa) as also ERp44 monomers and
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dimers. D: The same experiment as in C) carried out using HEPES buffer at pH 6.5, 7 and 8. The amount of complex formed decreased with increasing pH and was almost abolished at pH 8.0 as confirmed by densitometry, which showed complex formation at a level of 30% at pH 6.5, 18% at pH 7.5 and 6% at pH 8.0 when adiponectin antibody was used for detection. When detected with ERp44 antibody, the measured complex formation was at the level of 30% at pH 6.5, 18% at pH 7.5 and <1% at pH 8.0. E: The pH of an aliquot of the sample incubated at pH 6.5 as in D) was adjusted to 8. Subsequently, the sample was analyzed as in C). After incubation at pH 8.0 no ERp44-LMW was detected. Quantitation of the gel bands for the pH 8.0 experiment indicated 75% of complex at pH 6.5 and <1% at pH 8.0, whereas the amount of the uncomplexed LMW increased from 4% to 46% so also the amount of uncomplexed MMW from 21% to 56%.

4.3.2 Investigation of ERp44 interaction with adiponectin N-terminal model peptides

In order to answer these questions and in the process to reveal details of the mode of ERp44 interaction with the various adiponectin oligomers, we focused on small peptides derived from the variable domain of adiponectin. Our strategy of using peptide mimetics mitigates the complexity of a biophysical study as posed by the post-translational modifications and the various oligomeric states when working with the full-length adiponectin [22]. We are informed by the knowledge that apart from C39, the aromatic residue W42 in the conserved WMA triplet is also involved in adiponectin assembly by influencing the redox state of C39 [32, 73, 80]. Previously, we have shown that the N-terminal region of adiponectin cluster together into a compact mass in the HMW form [32, 73] signifying an important role for this region of the sequence in the oligomerization process.

4.3.3 Design of adiponectin N-terminal model peptides

For this purpose, we utilized two types of model peptides. The first consist of a set of short peptides only comprising residues of the variable region. Peptides of the second set span the variable region and collagen-like repeats as a bona fide trimeric adiponectin mimetic for the N-terminal region (Figure 4.2 A).

As a minimal model for ERp44 target region in adiponectin, we first synthesized a 9-amino acid long peptide wherein C39 and W42 are flanked by three and two amino acids of the murine sequence at the N- and C-terminal ends, respectively (36KGTCAGWMA44, WT36-44) (Figure 4.2). For the full N-terminal adiponectin
mimetic, we made use of the previously reported trimeric model for the N-terminal domain of adiponectin (see chapter 3 and [228]). This peptide, termed AHD (adiponectin hyper-variable domain), constitutes the entire variable domain and the N-terminal part of the collagen-like domain of murine adiponectin (residues 17-53) fused to nine canonical collagen repeats (GPO, O: hydroxyproline) at the C-terminus (Figure 4.2 and section 3.3.6) [228].

As reported in section 3.3.6 oxidation of AHD followed by SEC enabled us to isolate both the oxidized trimer and hexamer of AHD and allowed us to query how the oligomeric and oxidative state of AHD may affect its interaction with ERp44.

![Figure 4.2: Illustration of the model peptide ensemble.](image)

Top panel - The three domains of mature adiponectin and the secretion signal domain are shown for murine adiponectin. The N-terminal stretch of the primary sequence of mouse adiponectin encompassing the hyper-variable domain and the first seven repeats of the collagenous domain is indicated below the various domains of the polypeptide chain. Also shown are the amino acid sequences of the WT36-44 peptide (Middle panel) as well as that for the AHD (adiponectin hyper-variable domain) peptide mimetic (O: hydroxyproline) (Bottom panel).

### 4.3.4 ERp44 binds WT36-44 in a sequence specific manner

Optimal complex formation between ERp44 and the WT36-44 peptide appears to occur at pH 6.5 as evidenced by electrospray ionization mass spectroscopy (ESI-MS). Thus, for exploring the mode of interaction of ERp44 with various model peptides the following experiments were carried out at pH 6.5 (20 mM MES, 150 mM NaCl) using ERp44 monomers (see below) unless stated differently.
To test whether WT36-44 peptide is an informative model in binding ERp44, we used ESI-MS and tryptophan fluorescence quenching experiments. After incubation of ERp44 with ten-fold excess of WT36-44 for 16 hours, ESI-MS detected ~15% of ERp44 to be covalently linked to the peptide, whereas after 7 days of incubation, this value had increased to ~65% (Figure 4.3 A). The mass for the complex was increased by 923 Da, which is the mass of the model peptide less one hydrogen atom, giving evidence that the peptide has formed a disulfide bond with ERp44 (Figure 4.3 A). The slow formation of disulfide bond suggests that oxidation is mainly driven by atmospheric oxygen. Since the peptide contains a tryptophan (corresponding to W42 in adiponectin), this residue can be used as probe for fluorescence quenching experiments. However, W28 in ERp44, located next to C29 also contributes to the fluorescence signal albeit at a much smaller level. Despite this ‘background’ signal, significant decrease in fluorescence was still observed giving evidence of binding (Figure 4.3 B). Fluorescence quenching occurs on a much shorter time scale compared to disulfide bond formation monitored by ESI-MS (i.e. minutes vs. days), suggesting that electrostatic and/or hydrophobic factors are likely to be responsible for initiating the peptide-protein recognition. This was confirmed by tryptophan quenching experiments in the presence of a reducing agent (Figure 4.3 A). Furthermore, we also observed interaction of ERp44 with an analogous peptide construct in which C39 was substituted by serine-(C39S36-44: 36KGTSAGWMA44) (Figure 4.3 B). Compared to WT36-44, the quenching for C39S36-44 and WT36-44 under reducing conditions was less pronounced but still detectable, whereas ESI-MS experiments did not detect covalently linked complexes (Figure 4.3 A). Taken together, these results indicate that disulfide bond formation between C39 of adiponectin and a thiol group of ERp44, likely C29, is the key driver for binding but additionally, possibly sequence specific factors, are also likely at play. We were unable to detect non-covalent interactions by ESI-MS, which may be due to dissociation of a complex upon ionization.

Next, we asked whether non-covalent interactions between ERp44 and WT36-44 are influenced by the sequence of the peptide. For this purpose, we scrambled the sequence of WT36-44 yet maintaining length and composition to be used as control (control peptide: WGAMAKGCT). ESI-MS and tryptophan quenching assay for this control
peptide showed markedly reduced level of complex (<10%) and no detectable quenching, respectively (Figure 4.3 A and B). This result suggests that, indeed ERp44 recognition is sensitive to the sequence with the location of the C39 residue in the peptide being crucial for proper binding. As an aside, we noted that monomeric form of the peptide binds more readily to ERp44 than its dimeric counterpart (produced by treatment with 5mM H₂O₂ (Figure 4.4)) as assayed by ESI-MS (Figure 4.3). This was confirmed by fluorescence quenching, which also showed higher affinity of reduced peptide monomers relative to oxidized dimers (Figure 4.3 A and B).
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Figure 4.3: Binding experiments of ERp44 with the 9-amino acid model peptide

A: ERp44 was incubated with WT36-44 for 1 day and 7 days and the mixture analyzed by ESI-MS (top panel). The spectra show two peaks - one at 46074 amu representing free ERp44 and another at 49997 amu representing the disulfide linked ERp44-peptide-complex. The corresponding spectra for C39S36-44, the control peptide, WT36-44 dimer and WT36-44 under reducing condition are also shown (top and bottom panel). The satellite peaks of the primary ERp44 peak are likely due to adducts with the recombinantly purified ERp44 and appeared in some of the ERp44 overexpression exercises. The existing ESI MS database failed to detect identities, for instance, for the extra ~100 Da and ~150 Da species in the primary satellite peaks.

B: ERp44 was incubated with 2-fold access of WT36-44 (under reducing and non-reducing conditions), C39S36-44, WT36-44 dimer and the control peptide. Fluorescence emission spectra from 340 nm to 450 nm were recorded. In each case a spectra for the peptide alone was recorded in the same wavelength range. Quenching was determined as described in [251] with $f/f_0 = 0.78$ for WT $f/f_0 = 0.91$ for C39S, $f/f_0 = 0.84$ for WT under reducing conditions.
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condition, $f/f_0 = 1.04$ for the control peptide and $f/f_0 = 0.92$ for WT36-44 dimer. C: ERp44 was co-incubated with WT36-44 at pH 6.5, 7.5 and 8.0 and analyzed by ESI-MS (first three plots, as indicated). The pH of an aliquot of the sample incubated at pH 6.5 was set to 8 using 100 mM Tris-HCl pH 8.0. After incubation for 16 hours, the sample was analyzed by ESI-MS. The peaks for ERp44 (mass 46074 amu) and that for the ERp44-WT36-44 complex (mass 46997 amu) are indicated in the deconvoluted spectrum.

Figure 4.4: MS Analyses of monomer and dimer of model peptide WT 36-44. WT36-44 was incubated in the presence and absence of 5 mM H$_2$O$_2$ and subjected to ESI MS. The mass spectrum of the peptide not treated with H$_2$O$_2$ shows one peak at of 923 amu, which is the mass of the monomer. The mass spectrum of the peptide treated with H$_2$O$_2$ shows one peak at a of mass 1844 amu, which is the mass of the disulfide linked dimer.

4.3.5 ERp44 binds WT36-44 in a pH dependent manner

Next, we queried whether similar pH dependence binding as demonstrated for full length adiponectin (see Figure 4.1) applies to the binding of our model peptides to ERp44. ESI-MS analysis of the formation of ERp44-WT36-44 complex for a range of
pH (6.5, 7.5 and 8) showed 65% of complex at pH 6.5, 40% of complex at pH 7.5 and very minor amount of complex at pH 8.0 (Figure 4.3C). The ERp44-WT36-44 complex can be disassembled by raising the pH to 8 with an efficiency of over 95% (Figure 4.3C). This is analogous to what we observed for the ERp44-LMW complex.

4.3.6 ERp44 can distinguish between the oligomeric and oxidative states of AHD

Given the binding results for the full length adiponectin (described Figure 4.1) and to gain possible molecular-level insight into ERp44-mediated adiponectin assembly, we queried whether ERp44 can distinguish between different oligomeric states and disulfide linked configurations of AHD.

We generated and subjected reduced and oxidized trimers as well as hexamers of AHD to same treatment and ESI-MS binding assay as outlined in Figure 4.3. In the case of the reduced AHD trimers, 50% of the peptide was found to be complexed with ERp44 after one-week of incubation (Figure 4.5 A). On the other hand, in the case of the oxidized forms of trimer and hexamer, nearly 100% of the peptide was complexed with ERp44 (Figure 4.5 A). Due to the sizable mass difference of 6072 Da, ERp44 and ERp44-AHD complexes could be resolved by non-reducing SDS-PAGE, unlike in the case of the short WT36-44 peptides. Non-reducing SDS-PAGE analysis of a titration series using ERp44 and AHD at ratios of 1:1, 1:2, 1:5 revealed largest level of binding for the oxidized hexamers, followed by oxidized trimers with the reduced trimers binding the least (Figure 4.5 B).

Analogous to the experiments in Figure 4.3, we assessed any pH dependence of AHD binding to ERp44. First, we confirmed that the influence of pH on the oligomeric state of the AHD peptide was minimal and therefore did not notably influence the binding assays (chapter 3.3.6). Non-reducing SDS-PAGE analysis of ERp44-AHD complexes showed most efficient complex formation at pH 6.5, with lesser amounts of complex formed at pH 7.5 and almost no detectable complex at pH 8.0 (Figure 4.5 C). These results are in line with those of the WT36-44 peptides. Likewise, the pH stability assay showed that ERp44-AHD complexes formed at low pH disassemble (at a level of ~90%) when incubated at pH 8.0 for 12 hours (Figure 4.5C). In the case of reduced trimers, the dissociation of the complex at pH 8.0 is most pronounced and goes to completion.
Figure 4.5: ESI-MS and non-reducing SDS-PAGE experiments showing different binding affinity of ERp44 to AHD peptide mimetics

A: Reduced trimeric AHD, oxidized trimeric AHD and hexameric AHD peptides were co-incubated with ERp44 at a 10:1 ratio (peptide:protein) and analyzed by ESI-MS measurements. The peak for ERp44 (mass 46074 amu) and that for the AHD-ERp44 complex (mass 52145 amu: 46074 Da + 6071 Da) are indicated in the deconvoluted mass spectra – the latter indicates the formation of S-S link. The ‘harsh’ ionization process applied in this method disassembles all but the covalent linked complexes. Hence, only complex consisting of ERp44 and peptide monomer was detected even though ERp44 was incubated with the AHD trimer or hexamer.

B: Non reducing SDS-PAGE experiment of ERp44 co-incubated with different amounts of reduced AHD trimer, oxidized AHD trimer and AHD hexamer (protein to peptide ratios 1:1, 1:2, 1:5), respectively. SDS denatures and disassembles all but not covalently linked complexes. As a result, we detected ERp44-peptide monomer complex for all samples irrespective of the nature of the peptide complex (trimer, oxidised trimer or hexamer) used in the incubation experiments. However, uncomplexed AHD appeared to be somewhat SDS-resistant, so that AHD trimers, dimers and monomers are all detected in the gel. Due to its linear shape, the peptide apparently moves slower through the gel and migrates at effectively higher than the true molecular weight (as indicated by the marker). Based on quanitation by densitometry, ERp44 co-incubated with AHD hexamer resulted in the largest level of complexation (22% at a 1:1 ratio; 49% at a 1:2 ratio and 63% at a 1:5 ratio), somewhat lower level of complexation was observed with the oxidized trimer (11% at a 1:1 ratio; 22% at a 1:2 ratio and 54% at a 1:5 ratio) and very low level of complexation was observed with the reduced trimer (4% at a 1:1 ratio; 4% at a 1:2 ratio and 3% at a 1:5 ratio).

C: ERp44 and reduced AHD peptide was co-incubated at different ratios (1:1, 1:2, 1:5) at pH 6.5, pH 7.5 and pH 8.0, respectively. After 5 days, the samples were analyzed by non-reducing SDS-PAGE. The amount of complex formed decreased with increasing pH.
Thus the amount of complex detected was 17% at a 1:1 ratio; 25% at a 1:2 ratio and 26% at a 1:5 ratio at pH 6.5, 11% at a 1:1 ratio; 13% at 1:2 ratio and 19% at a 1:5 ratio at pH 7.5 and 3% at a 1:1 ratio 7% at a 1:2 ratio and 9% at a 1:5 ratio at pH 8.0. D: ERp44 was incubated for 24 hours with 5-fold excess of reduced trimer, oxidized trimer and hexamer of the AHD peptide at 4°C. The pH of an aliquot of the sample incubated at pH 6.5 was set to 8 using 100 mM Tris-HCl pH 8.0. After incubation further for 16 hours the samples were analyzed by non-reducing SDS-PAGE. An aliquot of the sample before and after treatment with 100 mM Tris-HCl pH 8.0 was examined. At pH 8 the complex disassembled into free ERp44 and AHD peptide. The effect was most pronounced for the reduced AHD trimer with a decrease in the amount of complex from 48% to 3%. For the oxidized AHD trimer, the amount of complex decreased from 50% to 6% and for the AHD hexamer from 70% to 38%.

4.3.7 ERp44 disrupts AHD hexamers to form ERp44-AHD trimer complex
SEC-MALS was used for determining the molecular weights of ERp44/AHD complexes. Prior to these experiments, we confirmed that the different AHD oligomers (reduced and oxidized trimer as well as hexamer) were stable over time (Figure 4.6 A) and that the exchange of monomeric to dimeric ERp44 had only very minor affect on the interpretation of our results (chapter 5.3.2).
When ERp44 was mixed with hexameric AHD, surprisingly, SEC-MALS showed predominantly an ERp44-AHD trimer complex and only a small amount of ERp44-AHD hexamer complex (Figure 4.6 B and 4.7). The SEC-MALS elution profile, in fact, consisted of multiple peaks. The largest peak was an overlap of the peaks for two species, one attributed to the ERp44-AHD trimer complex (57 kDa) and, the other to an unbound AHD hexamer. This peak resolved entirely to the ERp44-AHD trimer complex in a titration experiment where the amount of ERp44 used was varied. The three other smaller peaks could be attributed to 1) a free AHD trimer, 2) ERp44-AHD hexamer complex and 3) an AHD hexamer in complex with two ERp44 molecules (Figure 4.7).
Next, we repeated the same set of experiments using oxidized AHD trimers instead. Here also, we observed the formation of ERp44-AHD trimer complexes, but in addition and interestingly, we found the generation of species comprised of two AHD trimers complexed with either one or two ERp44 molecules (Figure 4.7), even though in the incubated sample AHD hexamers were initially absent. In fact, the relative amounts of these species were actually larger than those observed in the case of the AHD hexamer experiment.
Figure 4.6: SEC-MALS assay of AHD peptide oligomers and binding of ERp44 to the AHD hexamer.

A: Purified AHD trimer (oxidized or reduced) or hexamer retain their oligomeric states over time. SEC-MALS of reduced AHD trimer, oxidized AHD trimer and AHD hexamer after aliquots of each were incubated for 3 days at 4°C. Each AHD oligomer still eluted as a single peak after 3 days, at their respective masses and therefore do not interconvert. B: Analytical SEC-MALS analysis of AHD hexamer incubated with ERp44 at 1:2 ratio (top panel). The RI trace (green) is reported for the sample. The average molecular weight across each peak determined by MALS (black circle) is shown in the plot. 0.25 ml fractions were taken over the course of the experiment and analyzed by reducing SDS-PAGE (bottom panel). The result for the experiment using AHD hexamer and ERp44 monomer in a 1:2 ratio is shown. The analysis confirms that the peaks eluting at 10.9 ml, 11.4 ml and 12.4 ml in fact contain both ERp44 and AHD peptide.
Figure 4.7: SEC-MALS assay of ERp44 binding to various AHD peptide oligomers

The AHD hexamer A, oxidized AHD trimer B and reduced AHD trimer C were incubated with varying relative amounts of ERp44 (peptide-ERp44; 1:0.5 (in blue), 1:1 (in red) and 1:2 (in green)) for 16 hours at 4°C and analyzed by SEC-MALS. The RI trace (colored line as indicated) is reported for each sample. The average molecular weight for one representative measurement across each peak as determined by MALS (black circle) is shown in the plot. The identity of each peak is illustrated in the top panel and theoretical molecular weight of each ‘complex’ is indicated.
A: The location and therefore the mass for the major peak changed as a function of the ratio of ERp44 to AHD hexamer used in the experiment. The titration experiment showed that this major peak is an overlap of the peak associated with the free AHD hexamer and a species attributed to the ERp44-AHD trimer complex. The relatively small peaks in the SEC-MALS run eluting at 10.9 ml, 11.4 ml and 13.9 ml correspond to two ERp44 in complex with AHD hexamer, one ERp44 in complex with AHD hexamer and free AHD trimer, respectively. B and C: The SEC-MALS elution profile of oxidized (B) and reduced (C) AHD trimer consisted of multiple peaks eluting at 10.9 ml, 11.4 ml, 12.4 ml, 13.9 ml and 14.4 ml corresponding to two ERp44 in complex with AHD hexamer, one ERp44 in complex with AHD hexamer, ERp44 in complex with AHD trimer, free AHD trimer and monomeric ERp44, respectively.

Lastly, we performed SEC-MALS experiments using reduced AHD trimers. The elution profiles in these experiments were identical to that of the oxidized AHD trimer with somewhat lesser amounts of complexes formed and also a slight shift in mass towards ERp44-AHD trimer complex (Figure 4.7). The complex formation appeared to be driven by non-covalent interactions rather than through disulfide bonds as evidenced by non-reducing SDS-PAGE (Figure 4.5).

4.4 Discussion

Very little is currently known about how ERp44 exerts quality control on the oligomeric assembly of adiponectin, one of its client proteins. Based on our current results, we propose a model of how ERp44 executes this quality control, which we describe below.

4.4.1 ERp44 binding depends on the relative location of the C39 in peptide mimetics.

We observed that recognition of peptide WT36-44 by ERp44 is sequence specific, especially the relative location of the Cys in this peptide (analogous to C39 of adiponectin), which also established the fidelity of the designed 9-amino acid model peptide system. Additionally, we observed that the ERp44-peptide interaction is also driven by electrostatic and hydrophobic interactions.

We compared adiponectin sequences, especially the N-terminal region, with other ERp44 clients such as, Ero1α, IgM antibody and SERT to examine whether we could reveal any consensus ERp44 binding motif. There appears, however, no apparent consensus binding motif, with only the 3-amino acid stretch (GTC) in the C-terminus of IgM showing an overlap with the corresponding region in adiponectin [208],[83, 204,
248]. It is interesting to note that most of these binding, cysteine residues are found to be proximal to an aromatic residue W, Y or F in the sequence (Figure 4.8).

**Adiponectin**  
C39 ELAPALVPPPKCLAGWMAGIG  
IgM  
C575 YNVSLIMSDTGCTCY  
**SERT:**  
C109 AVDLGNIWRFPYICYQNGGGAF  
C200 LISSTFDQLPWTSDKNSWNTGN  
C209 PWTSDKNSWNTGNCTNYFAQDN  
**Ero1α**  
C35 HGEERRPETAQQRCFCQVSGY  
C39 EERRPEAAQRCFCQVSGYLD  
C46 QRRCFCQVSGYLDCTCDVETI  
C48 CFCQVSGYLDCTCDVETIDK  
C85 DYFRRYYKVNKKPCPFWNDIN  
C94 LKKPCPFWNDINQCGRRDCAV  
C99 INQCGRRDCAVKPCHSDEVPD  
C104 INQCGRRDCAVKPCHSDEVPD  
C130 ASYKYEEANRIEEIEQQAERLG  
C165 LQWTKHDDSDDSPECIDDIQS  
C207 DAWRIWSVIYENCFKPOQTIQ  
C237 SKENTFYNWLEGLOVEKRAFY  
C387 FRLHRFRNISRIMDVCFCFKCR  
C390 HFRNISRIMDVCFCFKCRILWG  
C393 NIRISRIMDVCFCFKCRILWGKLQ

**Figure 4.9: Examination of suggested ERp44 binding sequence on client proteins**

Sequence alignment of active cysteines in ERp44 clients. The active cysteine 39 in adiponectin shows sequence similarity to the active cysteine 575 of IgM. In comparison to the region around the active Cys39 in adiponectin no sequence similarities are observed for the corresponding regions around cysteine residues in SERT or Ero1α.

**4.4.2 Electron transfer drives discriminative binding of ERp44 to oxidized AHD**

Binding experiments with the AHD peptide in various redox states demonstrated that ERp44 could interfere and redistribute the pattern of the disulfide bonds in the model peptide in a manner as follows. Binding of ERp44 to AHD hexamer disrupts the intermolecular disulfide bond between C39 residues linking the two trimeric AHD moieties of the AHD hexamer by donating an electron to one of the trimeric units.
Concurrently, ERp44 covalently links to the other trimeric moiety by forming a disulfide bond between presumably C29 and C39 in AHD. This process results in a disulfide linked ERp44-AHD trimer complex and a free trimeric unit with a thiol group. On the other hand, when ERp44 interacts with the oxidized AHD trimer, ERp44 reduces the trimer by donating an electron to the trimer.

We also observed that ERp44 binds reduced AHD trimer; albeit, at a considerably slower rate compared to the oxidized AHD trimers and hexamers. In this case, however, no inter-molecular disulfide bonds are formed suggesting that binding to the reduced trimer is triggered by hydrophobic and/or electrostatic interactions. We therefore surmise that the oxidation equivalent (i.e. the electron acceptor) necessary for the formation of a disulfide bond between ERp44 and the oxidized AHD trimer is supplied by the oxidized peptide itself. On the other hand, when ERp44 was incubated with the oxidized, dimeric WT36-44, disulfide bond formation between ERp44 and the peptide was substantially reduced when compared to that in the case of the monomeric species. This observation and in view of the fact that, as mentioned in section 4.3.7, there is an increased disulfide bond formation between ERp44 and the oxidized AHD peptides compared to the reduced AHD trimer suggests that residues further up or downstream of the 9 amino acids of the WT36-44 peptide also contribute to the process of exchange of disulfide bonds. This needs to be clarified from further examination.

4.4.3 ERp44 promotes HMW formation by converting the pool of oxidized LMW and MMW to reduced LMW
A key question remains as to the mechanism behind the promotion of HMW adiponectin formation by ERp44. Previous studies have reported [35, 70] that fully oxidized LMW and MMW adiponectin fail to assemble into the HMW form, whereas reduced trimers could assemble into HMW through oxidative formation of disulfide bonds in vivo and in vitro [35, 70, 72]. These observations indicate that the MMW comprised of oxidized trimers are assembly trapped. Our SEC-MALS data with AHD suggest that ERp44 transforms the AHD hexamer and the oxidized AHD trimer into reduced AHD trimers. These results are in line with the in vitro co-incubation experiment of ERp44 and adiponectin. When ERp44 was co-incubated with the full-length adiponectin, only a complex of ERp44 with adiponectin trimer was detected.
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(Figure 4.1 C, 4.1 D). In addition, the level of LMW form was increased upon co-incubation. These results strongly suggest that the action of ERp44 on AHD mimetics parallels that for the full-length adiponectin hexamers and trimers. Thus, the observed disulfide bond exchange between the AHD and ERp44 may in part be similar to that happening for the full-length adiponectin in the transition from the LMW or MMW forms to the HMW form through an analogous disulfide bond exchange. Far Western blot and co-IP experiments demonstrated that ERp44 binds MMW and LMW adiponectin, whereas the HMW form does not interact with ERp44. We hypothesize that ERp44 is not able to disrupt/modify the compact structure at the N-terminus that has been visualized for the HMW form [32]. In the case of MMW and LMW adiponectin, the N-terminus is likely to be more flexible and/or accessible for ERp44 binding.

4.4.4 The pH environment tightly regulates binding and release of AHD by ERp44

We observed pH dependence of ERp44 binding to our model peptides and full-length adiponectin. This situation is analogous to the results reported in recent studies [138]. We also investigated the pH-stability of the ERp44-peptide complexes to answer the question of ‘how the ERp44-cargo complexes dissociates when retrieved to the ER’ [210]. Remarkably, raising the pH 6.5 to 8.0 ruptures the covalently linked complex consisting of ERp44 and the binding moiety. This result is particularly interesting, since a disulfide bond between two polypeptide segments requires a reducing equivalent, i.e. an electron, to transform the disulfide bond into two sulfhydryl groups, which cannot be achieved by just a pH change. The crystal structure of ERp44 [200] has shown that the cysteine pair C289/C272 in the b’ domain is disulfide bonded whereas the two cysteines in the b domain (C160 and C212) are reduced but are in close proximity. Furthermore, ERp44 contains a reduced cysteine (C63) in the a domain that is proximal to C29 [200]. It is possible that the observed pH-influence on the disulfide bond stability is due either to the donation of electrons from the aforementioned cysteine pair in the ERp44 b domain or because the disruption of the ERp44-reduced trimer complex leads to a disulfide bond between C29 and C63. This phenomenon however needs further examination.
4.4.5 Model for ERp44 quality control on adiponectin oligomerization

On the basis of our results discussed in this chapter and those from earlier studies [138, 200], we developed a model for the ERp44-mediated quality control of adiponectin oligomerization as described below (Figure 4.9). As in the case of all secretory proteins, adiponectin is synthesized in the ER where an array of ER resident chaperones and oxidoreductases assist the adiponectin monomers to fold into a trimeric unit. The trimeric building blocks further oligomerize in transit along the early secretion pathway from the ER to the Golgi and are monitored by the quality control mechanisms of the ER. Further downstream, in the ERGIC/cisGolgi region, ERp44 is thought to scrutinize the assembly of HMW adiponectin [203]. In the acidic pH environment of the cisGolgi, the hydrophobic active site around the free cysteine C29 of ERp44 is exposed and allows ERp44 to bind to its substrates [138]. Here ERp44 scans for incorrect disulfide-inked adiponectin intermediates, namely oxidized trimers and hexamers, preventing these from subsequent secretion. Concomitantly, completely assembled HMW adiponectin is not recognized and continues down the secretion pathway to the Golgi where maturation through post-translational modification occurs. Our model posits that ERp44 traps incorrectly oxidized intermediates through the donation of an electron to the oxidized trimeric building block and by the simultaneous formation of a disulfide bond with the adiponectin intermediate (oxidized trimer or oxidized hexamer). In the case of the adiponectin hexamer, interaction with ERp44 results in a partly reduced trimer with one free cysteine and an ERp44-adiponectin trimer complex. The so released, partly reduced trimeric unit may subsequently be prevented from secretion by another ERp44 oxidoreductase molecule. Subsequently, the covalently linked ERp44-adiponectin complex utilizes the RDEL sequence at the ERp44 C-terminus to form an adiponectin-ERp44-KDEL receptor cargo complex that is brought back to the ER. In the ER, the neutral pH triggers the release of the reduced trimeric unit from ERp44 and this trimer may participate again for assembly into HMW adiponectin. Our results show that the pH change by itself may be sufficient to cause release of the adiponectin trimer from ERp44. However, in order for ERp44 to continue its role in adiponectin oligomerization, ERp44 needs to be reduced. Most likely Ero1α, ERp44’s foremost partner delivers the required reducing equivalents either by binding the ERp44-adiponectin-complex directly thereby setting free the reduced trimer or by subsequently reducing oxidized ERp44 after the reduced trimer has been released. We noted that
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ERp44 could bind reduced adiponectin through an interaction that is likely to be hydrophobic in nature, which is unlikely, however, to survive the ERp44-mediated transport from the cisGolgi to the ER [138]. Such a scenario raises the possibility that effectively, retention of reduced adiponectin trimer in the cisGolgi region caused by ERp44, allows the reduced trimer to participate again in the formation of the HMW form. These HMW assemblies can then traffic to the Golgi to undergo further maturation.

![Diagram of adiponectin assembly and regulation by ERp44](image)

Figure 4.9: Model for the quality control of adiponectin oligomerization executed by ERp44.

Adiponectin is synthesized in the ER, where it forms trimers. From the ER, adiponectin trimer is engaged in the secretion pathway and undergoes oxidative oligomerization into disulfide-linked trimers, hexamers, and HMW adiponectin. In the ERGIC, ERp44 exists in its active state and screens for incorrectly assembled adiponectin intermediates, namely oxidized trimers and hexamers. Via its free Cteine (C29) ERp44 binds and reduces these intermediates. At the same time, ERp44 forms a mixed disulfide bond with its client, yielding a stable ERp44 adiponectin complex. Subsequently, the ERp44-adiponectin complex is retrieved to the ER by the KDEL receptor by engaging the C-terminal RDEL sequence. Once transported to the ER, the ERp44-adiponectin-complex disassembles triggered by the neutral pH in the ER and potentially facilitated by ERo1α. This process results in the release of a reduced adiponectin trimer, which in turn can participate in the cycle of the oxidative assembly pathway leading to the formation of HMW adiponectin.

In conclusion, in this chapter we have explored the interaction of ERp44 with adiponectin through a combination of in vitro and in cellular experiments both with wild-type adiponectin as well as representative, synthesized adiponectin mimetics in vitro. Our results provide a molecular level insight into the mechanism of differential
binding of ERp44 to the various adiponectin oligomeric states. Moreover, our findings reveal how ERp44 executes quality control on adiponectin assembly.
Chapter five - Design, generation and characterization of a stable complex of ERp44 with adiponectin-derived peptide for structural analyses

5.1 Introduction
The active cysteine (C29) in domain a of ERp44 is surrounded by a hydrophobic patch. The C-terminal end of ERp44, also known as the C-terminal tail (residues 331-377), shields this hydrophobic binding site containing C29 [200]. In a pH-dependent manner, the C-terminal tail swings out and unmask the binding site at lower pH. At neutral pH, however, the C-terminal tail moves towards the surface of the protein and occludes the binding site, and hence blocking client proteins from binding to ERp44. This movement is believed to tightly regulate the function and activity of ERp44 in vivo [138]. However, no structural evidence has been reported to support this proposed hypothesis. In addition, there is no data available for the interaction of ERp44 with client proteins at a molecular level. Therefore, investigations into the structure and dynamic aspects of the ERp44 tail movement, as well as ERp44 interaction with client proteins should significantly contribute to the understanding of ERp44 and its role in the quality control of the assembly of client proteins in the early secretory pathway.

Structural studies of the functionally important flexible C-terminal tail of ERp44 could potentially be performed using nuclear magnetic resonance (NMR) spectroscopy [252] or, for low resolution information, with fluorescence-based techniques [253]. These tools are well suitable for analysis of conformational heterogeneity and dynamics of proteins, as it is the case for the tail movement of ERp44. Given the size of ERp44 (44 kDa), its structural investigation by NMR spectroscopy is hampered by the size limitation of the classical NMR techniques, which is usually limited to proteins with molecular masses of no more than ca. 20 kDa [254]. Fluorescence-based methods, such as Förster resonance energy transfer (FRET), could also be technically challenging in the case of ERp44. Site-specific labelling using routinely applied fluorescent reagents that target lysine or cysteine residues may be complicated, given that ERp44 contains nine lysine residues and six cysteine residues. Therefore, it is challenging to achieve labelling of specific residues using these reagents [254].

X-ray crystallography can provide high-resolution information on the static states of
macromolecules and their interaction with other molecules. We, therefore, decided to explore the interaction of ERp44 with client proteins, including the mobile C-terminal tail, using X-ray crystallography. However, X-ray crystallography also poses several challenges. Protein structure determination requires high-quality protein crystals diffracting to high-resolution. Well-ordered crystals with high-resolution diffraction pattern are more likely to be produced from homogenous and mono-dispersed protein samples. In addition, large amounts of pure protein samples are required to conduct extensive crystallization screening [255].

We produced the recombinant ERp44 protein for crystallization experiments with high yields using standard protocols. ERp44 forms a complex with a short peptide model derived from the binding motif of adiponectin (see chapter 4.3.4), which is easily accessible through chemical synthesis. However, our initial results indicated that the formation of a homogenous ERp44-peptide complex for crystallization purposes would be challenging using the methods described in chapter 4.2.1. That strategy involved formation of a disulfide bond between C29 of ERp44 and a free cysteine in the peptide mimetic in the presence of atmospheric oxygen, resulting in only 65% complex formation after one week (see chapter 4.3.4). Attempts to accelerate the formation of complex by adding oxidizing reagents such as H₂O₂ resulted in polymerization of ERp44. ERp44 contains two additional free cysteine residues, which can form intermolecular disulfide bridges, likely leading to the oxidation of the ERp44 molecules to form polymers.

Co-crystallization experiments using ERp44 and the peptide mimetics, as well as soaking pre-formed crystals of apo ERp44 with the peptides, failed to generate high-quality diffracting crystals of ERp44-peptide complex, see appendix 8.3. Given the shortcomings of this approach, a robust protocol for the production of milligram amounts of a chemically stable, pure and homogeneous peptide-ERp44 complex likely to be more susceptible to crystallization is highly desired.

5.2 Material and methods

5.2.1 Production of Δtail ERp44

The truncated Δtail ERp44 variant was tested for expression in different E. coli strains, including BL21 (DE3) and Rosetta 2 (DE3), and in the presence of plasmids (pG-KJE8 and pGro7) that encode for different chaperones as described in 2.6. Large-scale expression and purification of Δtail ERp44 construct was carried out using similar protocols described for the
wild-type protein (section 4.2.2) with some modifications. The best condition for expression of soluble Δtail ERp44 was established as follows and used to produce and purify the protein: *E.coli* Rosetta 2 (DE3) were grown at 37°C in liquid TB medium to OD$_{600nm}$ = 0.6 and induced with 500 µM IPTG. Expression of the recombinant gene was carried out at 18°C for 2h. After IMAC purification (section 2.6.4), Δtail ERp44 was purified by size exclusion chromatography (section 2.6.8).

5.2.2 Reducing and non-reducing SDS-PAGE
Fraction of the full-length and Δtail ERp44 constructs, collected during IMAC, IEX and size-exclusion chromatography steps were analysed by reducing and non-reducing SDS-PAGE as described in section 2.5.

5.2.3 SEC-MALS analysis
SEC-MALS was used to determine the molecular mass of the ERp44 monomer and dimer in solution, as well as to analyze complex formation between ERp44 and the AHD peptide oligomers as described in section 2.6.9 and section 4.2.10.

5.2.4 AHD peptide production
The AHD peptide was produced as described in chapter 3.2.10. Production and separation of oxidized AHD trimer and hexamer was conducted as described in section 4.2.4.

5.2.5 Preparation of WT-Br
Resin bound (KGT(HSe)AGWMA, Hse: homoserine) was synthesized manually using Fmoc/Bu solid phase synthesis as described in section 2.7. In the next step, the peptide (KGT(HSe)AGWMA, Hse: homoserine) grafted on resin was treated by 15 cycles of 2 min washing with a mixture of TFA/triisopropylsilane/dichloromethane, (1/5/94, v/v/v). Deprotection of the trityl group was subsequently confirmed as follows: an aliquot of the peptide was treated with 20% acetic anhydride in DMF for 2 h to acetylate the free alcohol. Subsequently, the peptide was released from the resin with concomitant removal of protecting groups by treatment with trifluoroacetic acid /triisopropylsilane/H$_2$O/ethane dithiol (94:1:2.5:2.5, v/v/v/v) at room temperature for 1 h. The crude peptide was precipitated with ice-cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% trifluoroacetic acid and completion of the reaction was confirmed by LC-MS(section 2.7.2).
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After deprotection of the trityl group, the resin was dried and subsequently swollen in tetrahydrofuran (3 mL), and bromination was achieved with carbon tetrabromide (30 equiv.) and triphenylphosphine (30 equiv.) at room temperature for 12 h. Washing the resin with tetrahydrofuran, chloromethane, and methanol eliminated the precipitate. This procedure was repeated three times. The peptide was released from the resin with concomitant removal of protecting groups by treatment with trifluoroacetic acid /H₂O (94:5, v/v) at room temperature for 3 h and purified as described in 2.7.3.

WT-Br: NH₂-KGT(Hse-Br)AGWMA-CO₂H (4 mg, 3.8% yield), observed mass 1028.3 (M+H)⁺, calculated mass 1028.5 Da.

5.2.6 LC-MS analysis

LC-MS was performed as in section 2.7.2.

5.2.7 Complex formation between ERp44 and WT-Br

Purified monomeric ERp44 or Δtail ERp44 was buffer exchanged into 20 mM MES, 150 mM NaCl at pH 6.5. Simultaneously, the WT-Br peptide was dissolved at a concentration of 10 mg/ml in the same buffer and imminently mixed with the protein solution to a final protein concentration of 43 µM, resulting in five time excess of peptide (215 µM). Following incubation at 4°C for 12 h, the reaction was either quenched by adding 2-mecaptoethanol to a final concentration of 1 mM or the complex was separated from the excess peptide by size-exclusion chromatography.

5.2.8 Analyses of ERp44 WT-Br complex by electrospray ionization mass spectroscopy (ESI-MS)

A mass spectroscopy assay was used to analyze the complex formation between ERp44 and WT-Br, pH stability of the ERp44-WT-Br complex and also binding of C29S ERp44 to WT-Br. To this end, ERp44 variants (full length ERp44 and Δtail ERp44)(43 µM) were incubated with five-fold excess (215 µM) of the WT-Br peptide. The samples were incubated at 4°C for 16 h (20 mM MES, 150 mM NaCl, pH 6.5) and applied to mass spectroscopy. To examine pH stability of the ERp44-WT-Br complex, 50 ul of 200 mM Tris-HCl pH 8.8 was added to 50 ul of ERp44-peptide solution to increase the pH to 8.5. The sample was then incubated at 4°C for 16 h before being analyzed by LC-MS (section 4.2.11).
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5.2.9 Crystallization and X-ray diffraction
Initial crystallization screening was performed using sitting-drop vapour diffusion method in 96-well plate format at 18°C (Intelliplate™, Art Robbins Instrument). 576 conditions from an in-house library were tested in the initial screening ([256] and appendix 8.1). Protein samples after IEX (wild-type ERp44) or size-exclusion chromatography (Δtail ERp44) were used to form a protein-peptide complex as described in section 4.2.6 and concentrated to 10 mg/ml before being screened against the entire crystallization library. A Multiprobe II HT/EX liquid-handling robot (PerkinElmer) was used to dispense 85 µL of the each of the crystallization solution into the reservoirs of the 96-well crystallization plates. Using an Oryx4 robot (Douglas Instrument), 250 nL of the protein solution and 250 nL of the crystallization reservoir solution (1:1 ratio) were mixed on a stage. Finally, the plates were sealed with clear plastic film and incubated at 18 °C. Crystallization was monitored daily using a standard light microscope (Leica).

Once a lead crystallization condition was identified by the initial screening, the conditions were optimized by fine screening. Fine screens were usually performed using the same protocol as for the initial screening (as described above) with the exception that conditions with varying precipitant concentration, varying pH or additive concentration were used as stated in appendix 8.2.

Crystals obtained in various conditions were fished using a cryoloop (Hampton Research) and transferred to 5 µL crystallization reservoir solution containing 30% sucrose or sorbitol as cryoprotectant. After 30 sec, the crystal was mounted in a cryoloop and flash-cooled in liquid nitrogen before X-ray diffraction analysis at MX1 beamline at the Australian synchrotron.

5.3 Results and discussion
5.3.1 ERp44 production and purification
The wild-type murine ERp44, without the signal sequence, was expressed in E. coli cells with an N-terminal His$_6$-tag. The recombinant ERp44 was first purified by nickel affinity chromatography, followed by ion-exchange chromatography. During ion-exchange chromatography, ERp44 eluted as two separate peaks, at 250 mM and at around 300-350 mM NaCl (Figure 5.1 A). Subsequent analyses of the peaks using SDS-PAGE revealed that both peaks consist of pure ERp44 protein (Figure 5.1 A). Next, we used SEC-MALS to analyse the nature of the two peaks. The first IEX peak eluted as one major peak in the SEC-MALS
profile with an average molecular mass of ca. 100 kDa and can therefore be assigned as ERp44 dimer (Figure 5.1 B). The second IEX peak also featured one major peak in the SEC-MALS experiment corresponding to a molecular mass of monomeric ERp44 (47 kDa) (Figure 5.1 B).

**5.3.2 ERp44 exist in a monomer-dimer equilibrium**

SEC-MALS results indicated the presence of monomeric and dimeric ERp44 in solution. The same experiments were conducted after storing separate monomeric and dimeric protein samples at 4°C for a week. The results showed that only a minor portion of the corresponding protein sample is converted to the other form, suggesting a slow exchange of these protein species (Figure 5.2 A).

The nature of the ERp44 dimer and monomer was further analyzed by non-reducing SDS-PAGE (Figure 5.2 B). The ERp44 monomer appeared as a single band in the gel suggesting this species exist in a reduced state, whereas the dimer protein migrated in three separate bands. One of these bands can be attributed to reduced monomeric ERp44 while the two other bands correspond to dimeric ERp44 suggesting that the dimeric species is composed of a mixture of reduced and disulfide-linked ERp44. This finding indicates that a small portion of the ERp44 dimers form through covalent disulfide linkage between free cysteine residues in the protein, presumably C29, as reported by for ERp44 dimers *in vivo* [83, 116]. However, the majority of the dimer seem to not be covalently linked suggesting that non-covalent interactions are responsible for the formation of the dimer. (Figure 5.2 B).
Figure 5.1: ERp44 exist as monomer and dimer in solution.

A: IEX chromatogram of ERp44 after IMAC purification. The UV absorbance profile ($A_{280}$) is shown in black. ERp44 was eluted using a linear gradient of NaCl (0-1 M, 48 ml) 0% to 100% buffer B as indicated in green. ERp44 elutes as two overlapping peaks, both comprised of pure ERp44 protein as evidenced by SDS-PAGE. The inset indicates protein fractions collected over the elution of both peaks, as indicated by a red bar in the chromatogram.

B: SEC-MALS analyses of pooled fractions collected from peak 1 (top panel) and peak 2 (bottom panel) of the IEX experiment. The differential refractive index (RI) trace is reported for each sample. The weight-average molecular mass across the protein peaks, as determined by MALS, are shown in the plot (black circles). These results indicate that the ERp44 protein in the first IEX peak is monomeric (~45 kDa), whereas the second IEX peak constitutes dimeric ERp44 (~90 kDa).
Figure 5.2: ERp44 exists in a slow monomer/dimer equilibrium.
A: SEC-MALS analyses of ERp44 fractions from peak 1 (top panel) and peak 2 (bottom panel) of the IEX experiment after incubation for 7 days. The differential refractive index (RI) trace is reported for each sample. The weight-average molecular mass across the protein peaks as determined by MALS are shown in the plot (black circles). The majority of ERp44 protein remain in the same oligomeric state over a period of 7 days at 4°C. B: Non-reducing SDS-PAGE analyses of ERp44 monomers and dimers. The monomeric ERp44 migrates as a single band at ~45 kDa, whereas the dimeric species shows three bands. A band at ~45 kDa representing reduced monomeric ERp44 molecules. The other two bands at ~70 kDa and at ~100 kDa representing covalently linked ERp44 dimers. The two species of dimers are likely to differ in disulfide bond pattern between the two ERp44 molecules (including C29 and/or C63) and, therefore, migrate in two distinct bands.

5.3.3 ERp44 binds AHD peptides in a one to one complex

After examining the oligomeric state of ERp44, we asked whether the ERp44 dimer shows binding properties to adiponectin different to that of the monomeric species. This is relevant for the design of an ERp44 adiponectin derived peptide complex. In chapter 4.3.7 we analyzed binding of monomeric ERp44 to adiponectin, in which we used our previously designed robust trimeric model peptide of the hyper-variable domain of adiponectin (AHD).

SEC-MALS was used to assay the interaction between ERp44 and the various oligomers of the AHD peptide. For this task, ERp44 (monomer or dimer) was mixed with AHD model peptide (reduced trimer, oxidized trimer or hexamer) and incubated for 16 h. Subsequent analyses by SEC-MALS showed that for all experiments ERp44 predominantly forms complexes comprised of ERp44 monomer and AHD trimer, similar to the results described in section 4.3.7. Irrespective of the use of ERp44 monomer or dimer in the SEC-MALS
experiments, the elution profiles for all runs appeared similar with relative changes in the intensity of the peaks. In addition to the ERp44 AHD trimer peak, four smaller peaks were detected and could be attributed to 1) a free ERp44 monomer, 2) a free AHD trimer, 3) ERp44-AHD hexamer complex, and 4) an AHD hexamer in complex with two ERp44 molecules (Figure 5.3 A).

In the case where ERp44 dimer is mixed with reduced AHD trimer, the detected amount of ERp44 in complex with one AHD trimer decreased. Simultaneously, the amount of ERp44 bound to two AHD trimers increased compared to the same experiment using the monomeric ERp44 protein.

When oxidized AHD trimers were incubated with ERp44 monomers or dimers, SEC-MALS demonstrated almost identical elution profiles for both samples.

Probing ERp44 monomer with AHD hexamers generated predominantly one to one adducts of ERp44 and AHD trimer. In contrast, incubation of ERp44 dimers with AHD hexamers produced mainly one or two ERp44 molecules associated with two copies of the AHD trimer. At the same time, the amount of the one to one ERp44 AHD trimer complex was reduced (Figure 5.3 A).

In the following experiment, we asked whether or not the amount of disulfide-linked complex generated between ERp44 and the model peptide varies depending on the use of reduced monomeric or partially oxidized dimeric ERp44 in the assay. To answer this query, ERp44 (monomer or dimer) was incubated with AHD model peptide (reduced trimer, oxidized trimer or hexamer) for 16 h and analyzed by non-reducing SDS-PAGE. In all these experiments, profiles of the samples for both the dimers and monomers are very similar suggesting that ERp44 dimers and monomers produce comparable amounts of disulfide-linked complexes. SEC-MALS showed the formation of complexes comprised of ERp44 and AHD hexamer and two ERp44 proteins complexed with two AHD trimers. Non-reducing SDS-PAGE however, detects only adducts of one ERp44 molecule bound with one peptide chain suggesting that ERp44 covalently links with only one AHD peptide. (Figure 5.3 B).
Figure 5.3: ERp44 monomer and dimer species display similar profiles of complex formation with AHD peptides.

A: SEC-MALS traces after co-incubation of ERp44 monomer (red) and dimer (green) with reduced AHD, oxidized AHD and hexameric AHD (1:1 ratio) display closely similar peak positions. However, there are varying degrees of alterations in the amount of formed complexes. The identity of each peak is illustrated in the top panel, and the theoretical molecular weights of each “complex” are indicated. B: Non-reducing SDS-PAGE profiles of the samples used in (A). Non-reducing SDS-PAGE detects only adducts of one ERp44 molecule bound with one peptide chain suggesting that ERp44 covalently links with only one AHD peptide.
These findings show the heterogeneous binding profile of the ERp44 peptide complex and established the need of a homogeneous and simplified ERp44 peptide model complex for structural investigation of ERp44 client binding. In addition, the fact that we see ERp44 monomer in complex with one AHD peptide trimer suggests that a design of a stable ERp44 model peptide complex could be comprised of one ERp44 and one peptide ligand.

5.3.4 Design of a covalently linked ERp44 adiponectin derived peptide complex

In previous chapters 3 and 4, we introduced several model peptides (varying in length and oligomeric state) to gain molecular level insight into ERp44 binding to client proteins, adiponectin in our case. We designed and generated a nine amino acid long peptide derived from the murine adiponectin sequence surrounding the active C39 (residues 36-44, so called WT36-44). We showed that ERp44 specifically recognizes the peptide and established the suitability of this peptide system as an appropriate ERp44 ligand (section 4.3.4).

As part of our experimental plan, we aimed at crystallizing ERp44 with the WT36-44 peptide, to investigate the atomic details of the interactions between these molecules upon complex formation. We anticipated this short model peptide would be suitable to form a stable ERp44-peptide complex aimed for crystallization. This was based on the assumption that the peptide sequence would be long enough to reveal important details on the interactions between ERp44 and its client, adiponectin. However, there are major challenges for crystallization and structural investigation of the ERp44-WT36-44 peptide complex;

1. Formation of a disulfide-linked complex is slow and results in only 65% complex formation after 7 days.
2. ERp44 binding to the WT36-44 peptide is pH dependent and only a small amount of complex forms at pH 7.4 or above. Furthermore, the ERp44-WT36-44 complex disassembles once pH rises above 8.0.
3. Separation of the ERp44-WT36-44 complex from the free ERp44 protein is difficult due to similar biophysical characteristics.

Given these limitations, we sought an alternative route to obtain a stable, covalently-linked ERp44-peptide complex. We aimed to design a specific and irreversible linkage that forms via a fast chemical reaction. There is such an approach in the literature, in which the authors report using a homoalanine thioether bond instead of a labile cysteine disulphide bond to
create a DsbA protein in complex with DsbB-derived peptide [257]. Thiols are well-known to react readily with electrophiles such as iodoacetamide. The authors synthesized a novel electrophilic compound, a homoalanine cation equivalent 4-bromohomoalanine, which is able to react with thiols [257]. Encouraged by this study, we decided to substitute the cysteine residue in the sequence of the WT36-44 peptide with a bromohomoalanine (called WT-Br hereafter), to improve the chances of stable complex formation with ERp44. As shown in Figure 5.4, the WT-Br peptide could establish a thioether linkage with the active C29 of ERp44 in a specific and irreversible manner. In the resulting ERp44-WT-Br complex, the S-cysteinylhomoalanyl motif displays a legitimate mimic for the cysteine linkage that exists between native adiponectin and ERp44 [257, 258]. In addition, the thioether bond is resistant to acidic and basic hydrolysis, proteolytic degradation, and most reducing agents [259, 260].
5.3.5 Synthesis of a covalently linked ERp44 adiponectin derived peptide complex

Synthesis of the brominated peptide derivative was based on a previously published report [257]. We devised a strategy to synthesize WT-Br, as outlined in Figure 5.5, which is based on a two-step procedure. First, the precursor peptide of the brominated moiety is assembled on functional polystyrene resin using standard Fmoc SPPS and in the second step the peptide is brominated.

For the precursor peptide we used a protected homoserine in position 4 of the sequence (KGT(HSe)AGWMA; HSe: homoserine), replacing the cysteine in the native sequence. The commercially available Fmoc-Hse(-trityl)-OH was employed since the trityl-group can be selectively removed using 1% TFA in dichloromethane [261], whereas under these conditions
all other protection groups remain intact. This deprotection of the O-tritylhomoserine can be carried out on the resin-bound peptide. Next, the peptide is brominated by treatment with carbon tetrabromide (CBr$_4$) and triphenylphosphine in tetrahydrofuran as described previously [257, 262, 263], before final cleavage and HPLC purification.

The synthesis of the intermediate peptide KGT(HSe)AGWMA resulted in large amounts of pure precursor with only minor side products as judged by cleavage and LC-MS analyses of a small aliquote of the crude resin-bound product (Figure 5.6 A). The quantitative and selective removal of the O-trityl group of the homoserine was assessed in the following manner. A small portion of the total product was treated with 20% acetic anhydride in DMF to acetylate the free alcohol of the homoserine followed by cleavage of the peptide. LC-MS analysis of the test reaction revealed that over 90% of the peptide was acetylated at a single site, verifying the quantitative and selective deprotection of O-tritylhomoserine (Figure 5.6 B).

Attempts to brominate the HSe residue using the brominating conditions described by [257], using 10 eq. of carbon tetrabromide and triphenylphosphine in tetrahydrofuran at room temperature for 3 h, did not result in an efficient formation of the brominated peptide. The condition were therefore optimized for reaction time, equivalents of CBr$_4$ and triphenylphosphine employed in the reaction, as well as different solvents. The best conditions obtained were as follows: 30 eq. of CBr$_4$ and triphenylphosphine dissolved in tetrahydrofuran and immediately applied to the resin-bound peptide. The reaction was continued for 12 h and the procedure was repeated twice. This reaction yielded in ca. 10% of brominated peptide which was then purified by HPLC (Figure 5.6 C).
In the next step, we aimed to form the ERp44-WT-Br complex and only used the purified monomeric ERp44 species for this purpose to produce a one to one ERp44 peptide conjugate. Since ERp44 is purified under reducing conditions in the presence of 5 mM 2-mecaptoethanol, the reducing agent needed to be eliminated prior to the complex formation to avoid any side reactions with the brominated peptide. The ERp44 protein was therefore buffer exchanged into 20 mM MES, 150 mM NaCl, pH 6.5 immediately before treatment with the brominated peptide to avoid dimer formation or oxidation of C29 of ERp44 by atmospheric oxygen. Given the instability of bromohomoalanine group in aqueous solutions and its tendency to hydrolyses [257], the brominated peptide was dissolved in the same buffer at a concentration of 10 mg/ml and immediately added to the protein solution. Complex formation was carried out at a protein concentration of 43 µM with five fold excess of the WT-Br peptide (215 µM) for 12 h. The reaction was quenched by adding 2-mecaptoethanol to a final
concentration of 1 mM. Alternatively, the complex was separated from the excess peptide by size-exclusion chromatography.

Figure 5.6: LC-MS of the WT-Br peptide and its precursors.

A: LC-MS of the precursor peptide (KGT(HSe)AGWMA; HSe: homoserine). The mass at 964.4 amu indicates the desired peptide, carrying an alcohol group at the homoserine side chain. B: LC-MS of the product after quantitative removal of the O-trityl group and subsequent acetylation of the free alcohol, followed by cleavage of the peptide. The mass at 1121.4 amu corresponds to the acetylated peptide in complex with one TFA molecule. C: LC-MS of the final product (WT-Br). The mass of 1028.3 amu corresponds to the WT-Br peptide.
5.3.6 Analyses of a covalently linked ERp44 adiponectin derived peptide complex
The reaction between the WT-Br peptide and ERp44 resulted in over 95% substitution yield after incubation for 12 h as analyzed by LC-(ESI)-MS (Figure 5.7 A). As a comparison, the complex formation between the WT36-44 peptide and ERp44 yielded 15% complex after incubation for 12 h and 65% after one week (chapter 4.3.4). Next, we explored the pH stability of the thioether bond. For this purpose, 50 ul of 200 mM Tris-HCl pH 8.8 were added to 50 ul of ERp44-WT-Br peptide complex in 20 mM MES, 150 mM NaCl at pH 6.5, resulting in a pH excess of 8.5. After incubation at 4ºC for 16 h, the sample showed no detectable decomposition of the conjugate (Figure 5.7 A). In comparison, the ERp44-WT36-44 peptide complex disassembles when the reaction pH rises to a value of 8 or higher (chapter 4.3.5).

In addition to the active C29, ERp44 possesses a second free cysteine residues (C63) in the a domain, which is presumably solvent accessible [139, 200] and may potentially undergo undesired covalent linkage with the brominated peptide. Therefore, in the next set of experiments we aimed to establish that the WT-Br peptide specifically binds to C29 in ERp44. The initial ESI-MS experiments (Figure 5.7 A) showed one major peak for the ERp44-WT-Br peptide complex, as well as a second small peak for the free protein. However, no signal at a mass corresponding to a complex of ERp44 and two copies of the peptide was observed, indicating that only one peptide chain covalently links with one ERp44 molecule (Figure 5.7 A). To verify the specific binding of the brominated peptide to C29 in ERp44, we repeated the LC-(ESI)-MS assay using an ERp44 mutant in which C29 was mutated to a serine residue. The obtained mass spectrum showed only a single signal corresponding to the mass of the free protein and no complex formation was detected (Figure 5.7 A).

Lastly, we established that the ERp44-WT-Br peptide complex exists as a monomeric entity in solution. To this end, the ERp44 peptide conjugate was subjected to SEC-MALS analyses and the elution profile confirmed the existence of the complex as a single species with a mass representing a monomeric ERp44 WT-Br peptide complex (Figure 5.7 B).
Figure 5.7: Analyses of a covalently linked ERp44 adiponectin derived peptide complex.

A: Binding experiment of ERp44 and WT-Br. ERp44 or C29S was incubated in the presence or absence of WT-Br for 16 h and the mixture was analysed by ESI-MS. The spectra show two peaks, one at 46,074 atomic mass units (amu) representing free ERp44 (46053 amu for C29S) and another at the mass of ERp44 WT-Br peptide complex as indicated in the spectra. To test the complex for its pH stability the complex was incubated at pH 8.5 for further 16 h and analysed by ESI-MS. B: SEC-MALS analyses of ERp44 WT-Br peptide complex after co-incubation of ERp44 and WT-Br for 16 h. The differential refractive index (RI) trace is reported as black line. The weight-average molecular weights across the protein peak as determined by MALS is shown in the plot (black circles). The ERp44 WT-Br peptide complex elutes as one peak corresponding to the ERp44 monomer WT-Br complex (~ 47 kDa).
5.3.7 Crystallization of a covalently linked ERp44 adiponectin derived peptide complex

For initial crystal screening, the ERp44-WT-Br peptide complex was formed overnight at a concentration of 2 mg/ml and subsequently purified by size-exclusion chromatography. The purified complex was concentrated to 10 mg/ml and initial crystallization screening was carried out using the sitting-drop vapour diffusion method in 96-well plate format. An in-house library of 576 conditions based on the scientific literature and on commercially available crystallisation conditions was tested in the screening experiment ([256] and appendix 8.1). Protein crystals were observed in some conditions after ~2-3 days (Table 4). All conditions were subsequently subjected to fine screening (appendix 8.2), which resulted in the appearance of typically one to two crystals in some of the conditions. Crystals suitable for X-ray diffraction (Figure 5.8 A) were fished out using cryoloops and cryoprotected by soaking into the crystallisation condition containing 30% sucrose or glucose prior to flash-cooling in liquid nitrogen. Crystals were exposed to the X-ray beam on MX1 beamline at the Australian Synchrotron (Figure 5.8 B). The diffraction patterns confirmed that the tested crystals were indeed protein crystals (Figure 5.8 C). Unfortunately, none of the tested crystals showed high-quality and/or high-resolution diffraction patterns to attempt data collection and processing.

Table 5.1: Crystallisation conditions resulting in crystals for the ERp44-WT-Br complex.

<table>
<thead>
<tr>
<th>Precipitant composition</th>
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<tbody>
<tr>
<td>20% (w/v) PEG 3350, 0.2 M tripotassium citrate monohydrate, pH 8.3</td>
</tr>
<tr>
<td>20% (w/v) PEG 3350, 0.2 M potassium nitrate, pH 6.9</td>
</tr>
<tr>
<td>20% (w/v) PEG 3350, 0.2 M trisodium citrate, pH 8</td>
</tr>
<tr>
<td>14% (w/v) PEG 6000, 0.2 M TAPS/KOH, pH 8.5</td>
</tr>
<tr>
<td>10% (w/v) PEG 4K, 20% glycerol, 0.02M AA, pH 7.5</td>
</tr>
</tbody>
</table>

AA: Amino acid mix; including 0.02 M sodium L-glutamate, 0.02 M DL-alanine, 0.02 M glycine, 0.02 M DL-lysine HCl, 0.02 M DL-serine.
5.3.8 Crystallization of a truncated ERp44 variant in complex with WT-Br.

Following the unsuccessful attempts to produce high-quality diffracting crystals of the ERp44-WT-Br peptide complex, we decided to modify the protein construct in order to produce a more suitable protein-peptide complex for crystallization. We anticipated that the C-terminal tail of ERp44 might interfere with the formation of a tight and regular crystal lattice due to its presumably flexible and disordered nature. Hence, we generated a version of the ERp44 protein lacking the C-terminal tail as previously reported in [200], hereafter called Δtail ERp44. Expression and solubility tests of Δtail ERp44, applying the same protocols as the ones used for the wild-type construct, revealed low expression levels with mostly insoluble protein. Expression conditions were therefore optimized for the expression strain, media, temperature and duration. The highest expression of soluble Δtail ERp44 was obtained in terrific broth (TB) medium when cells were grown at 37°C to OD₆₀₀ = 0.6 and then induced with IPTG at 18°C for 2h. Cells were harvested and mechanically lysed, centrifuged and the lysate was used for purification by IMAC chromatography as described for the wild-type protein. This was followed by size-exclusion chromatography to separate protein aggregates and other contaminating proteins using 20 mM MES, 150 mM NaCl, pH 6.5. The size-exclusion profile showed one major peak containing pure Δtail ERp44 protein (Figure 5.9 B).
Figure 5.9: Purification of Δtail ERp44.
Chromatograms and accompanying SDS-PAGE gels of (A) IMAC and (B) size-exclusion chromatography of Δtail ERp44. IMAC was carried out using a linear gradient of 10-300 mM imidazole over 30 ml (A: 50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, 5 mM 2-mecaptoethanol and 10 mM imidazole; B: 50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, 5 mM 2-mecaptoethanol and 300 mM imidazole). The fractions containing Δtail ERp44 from IMAC purification were pooled, concentrated and further purified on the size-exclusion column.
Fractions containing Δtail ERp44 were pooled and concentrated to 43 μM, and the complex of Δtail ERp44 and the WT-Br peptide was formed using the procedure described for the wild-type construct (chapter 5.3.6). After concentrating the sample to 10 mg/ml, initial crystallization screening was carried out using the same in-house library of 576 conditions, as described in chapter 5.9. Crystallization trials, however, did not result in the appearance of any crystal and due to time constrains the project was suspended at this stage.

5.4 Conclusions and Summary

During this study, we successfully designed and generated a stable and covalently linked ERp44-adiponectin peptide mimetic complex. Although our ERp44-WT-Br complex crystals did not diffract to high quality/resolution, this result is a promising first step towards the determination of a high-resolution crystal structure of ERp44 in complex with a peptide mimetic derived from one of its binding partners. Based on our body of results, we can outline a detailed optimization strategy for the protein and peptide design that are more suitable for structural study by X-ray crystallography. This can, in the future, enable a molecular level analyses of the interaction between ERp44 and its client proteins, as well as the tail movement involve in the binding event.

In summary, biophysical characterization of the ERp44 complex formation with diverse model peptides revealed the following insights into ERp44 function:

1. ERp44 exists in a slow monomer-dimer equilibrium in vitro.
2. ERp44 dimers exist as a mixture of reduced and disulfide linked dimers.
3. In addition to disulfide linkage, non-covalent interactions contribute to the dimer formation of ERp44.
4. ERp44 dimers and monomers bind in a similar manner to the AHD peptide mimetic of the adiponectin variable domain.
5. One ERp44 molecule covalently links with only one AHD peptide chain.
6. The brominated peptide derivative from WT36-44 specifically recognizes C29 in ERp44, further underpinning high sequence selectivity for ERp44 client interactions.
Chapter six - Modulation of adiponectin production and oligomerization by designed peptide mimetics

[Experiments presented in Figure 3, 4 and 5 were carried out by Dr. Cheng Xu in Prof. Yu Wang’s group at the University of Hong Kong. Experiments presented in Figure 7, 8 and 9 were carried out under the supervision and in collaboration with Dr. Cheng Xu in Prof. Yu Wang’s group at the University of Hong Kong.]

6.1 Introduction

Adiponectin, a hormone secreted abundantly from adipocytes, possesses potent anti-diabetic and anti-inflammatory properties [21, 45, 46, 236-239, 242-244]. Adiponectin assembles into a variety of oligomeric forms, including trimeric, hexameric and the higher-molecular-weight (HMW) oligomers [23, 27, 30, 32, 33, 36]. These oligomeric forms do not interconvert once secreted into the circulation [39, 264]. The various oligomeric forms harbor diverse levels of post-translational modifications, such as glycosylation, and are involved in distinct biological functions [14, 22, 25, 39, 74]. The HMW adiponectin is implicated in improving insulin sensitivity, decreasing blood glucose levels, and protecting against liver injuries [40, 51, 246, 265, 266]. Dysregulation in the assembly of HMW adiponectin is associated with various metabolic complications, such as obesity, insulin resistance, type 2 diabetes (T2DM), and arteriosclerosis [16, 30, 34, 41]. Patients with T2DM and/or cardiovascular disease have depressed serum levels of HMW adiponectin [12, 18, 267-269]. Increase in the ratio of HMW to total adiponectin, rather than the overall adiponectin concentration, is associated with improved insulin sensitivity in diabetic mice and can be achieved upon treatment of T2DM patients with thiazolidinediones (TZDs) [40, 44, 270-272]. Drugs targeting PPARγ, such as TZD’s, promote the release of HMW adiponectin by enhancing the functions of the endoplasmic reticulum (ER), which is disturbed under obese conditions known as ER stress (chapter 1.3.8) [80-82, 84, 247]. PPARγ agonists stimulate adiponectin biosynthesis, multimerization and secretion through regulation of the expression of ER-associated proteins, such as, Ero1-Lα, ERp44 and DsbA-L, which play important roles in adiponectin assembly [82, 92]. However, the exact mechanisms underlying adiponectin multimerization as well as the beneficial clinical effects of TZDs remain unclear. These facts underpin the importance of revealing how the secretion of various complexes of adiponectin is regulated and how the multimerization process is controlled in the ER.

Previous studies [80, 81] have revealed that ERp44 retains adiponectin in the ER through
Chapter 6 - Designed peptides modulate adiponectin assembly and secretion

binding to the highly conserved cysteine residue 39 (C39) in the N-terminal non-collageneous domain of adiponectin and thereby acts in regulating and controlling adiponectin assembly to the HMW form [138]. Under stress conditions, there is an overload of unfolded proteins in the ER, causing an upregulation of chaperones such as ERp44 [48, 80, 86, 273]. As a result, a large pool of adiponectin is trapped inside the ER by thiol-mediated linkage between adiponectin and ERp44 [80]. Relevant to this phenomenon, our (chapter 4) and other studies have highlighted the roles of ERp44, Ero1-Lα and DsbA-L in adiponectin assembly [80-82, 85, 92, 138, 274].

In this chapter we investigated whether designed peptide mimetic(s) derived from the N-terminal variable domain of adiponectin or IgM antibodies, which interact with ERp44, are able to disrupt the interactions between adiponectin and ERp44, in turn facilitating adiponectin release from the ER. This approach to enhance the level of secreted adiponectin by rescuing aberrantly trapped adiponectin is distinct from TZD-induced transcriptional repression of ERp44 leading to increase in adiponectin production (Figure 6.1 B) [222].

6.2 Material and Methods
[Experiments presented in Figure 3, 4 and 5 were carried out by Dr. Cheng Xu in Prof. Yu Wang’s group at the University of Hong Kong. Experiments presented in Figure 7, 8 and 9 were carried out under the supervision and in collaboration with Dr. Cheng Xu in Prof. Yu Wang’s group at the University of Hong Kong.]

6.2.1 Production of peptides
WT, C39S, W42A, TAT-WT, TAT-C39S, TAT-W42A, M1, M2, TAT-M1 and TATM2 peptides were synthesized manually using Fmoc/Bu solid phase synthesis and purified as described in section 2.7.
WT: NH₂-KGTCAGWMA-CO₂H (22.1 mg, yield 24.0%, purity>95%), observed mass 925.3 (M+H)⁺, calculated mass 925.0.
C39S: NH₂-KGTSAGWMA-CO₂H, (16.5. mg, yield18.3%, purity>95%), observed mass 909.3 (M+H)⁺, calculated mass 909.02.
W42A: NH₂- KGTCGWMA-CO₂H-(18.3mg, yield 22%, purity>95%), observed mass 809.2 (M+H)⁺, calculated mass 809.0.
TAT-WT: NH₂-YGRKKRRQRRKKGTCAGWMA-CO₂H (45.3mg, yield 18.3%, purity>95%), observed mass 2465 (M+H)⁺, calculated mass 2466.
Chapter 6 - Designed peptides modulate adiponectin assembly and secretion

TAT-C39S 36-44: NH2-YGRKKKRRQQRKRGTSAGWMA-CO2H, (52.2. mg, yield 21.3%, purity>95%), observed mass 2449.5 (M+H)+, calculated mass 2450.

TAT-W42A: NH2-YGRKKKRRQQRKGTAGWMA-CO2H (40.3mg, yield 17.1%, purity>95%), observed mass 2351.2 (M+H)+, calculated mass 2351.

M1: NH2-GTCY-CO2H (14.3mg, yield 32.4%, purity>95%), observed mass 440.3 (M+H)+, calculated mass 441.0.

M2: NH2-DTGGTCY-CO2H, (21.8mg, 30.4% yield%, purity>95%), observed mass 716 (M+H)+, calculated mass 715.

TAT-M1: NH2- GTCYYGRKKKRRQQR-CO2H (32.1mg, yield 16.2%, purity>95%), observed mass 1984.2 (M+H)+, calculated mass 1984.

TAT-M2: NH2- DTGGTCYYGRKKKRRQQR-CO2H (35.8mg, yield 14.0%, purity>95%), observed mass 2558.1 (M+H)+, calculated mass 2557.

To remove trifluoroacetic acid from the lyophilized peptide, the peptide was dissolved in 10 mM hydrochloric acid at a concentration of 1 mg/ml and again lyophilized. This procedure was repeated three times.

6.2.2 Electrospray Ionization Mass Spectroscopy assay

Mass spectrometry assay was used to analyse the amount of disulfide bond formation between ERp44 and various peptides. To this end, ERp44 (43 µM) was incubated with a 10-fold excess (430 µM) of the peptides (WT, TAT-WT, W42A, TAT-W42A, C39S, TAT-C39S, M1, TAT-M1, M2 and TAT-M2). All samples were incubated for 4 days in 20 mM MES, 150 mM NaCl at pH 6.5 and applied to mass spectrometry as in 4.2.11.

6.2.3 Cell culture

Murine 3T3-L1 pre-adipocytes (ATCC No.CL-173, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-fungisone (PSF) at 37°C in 5% CO2 95% humidified air. Prior to differentiation, cells were sub-cultured at 1x10^6 per 35 mm culture plate. When attachment to the plate was complete, confluent 3T3-L1 preadipocytes were first supplemented with 4 mM insulin, 0.25 µM dexamethasone and 0.5 mM isobutylxanthine (IBMX) in culture medium for the first two days. After this incubation, the cells were supplemented with 4 mM insulin in the culture medium for another two days. The differentiation medium was replaced by normal culture medium at day 4 and changed every two days until day 8.
For peptide treatment, differentiated 3T3-L1 cells were treated with 200 nM peptide for 24 h in FBS-free medium. Cell lysate and medium were collected after treatment.

6.2.4 Co-immunoprecipitation and Western Blotting
For detection of different oligomeric adiponectin complexes, cell lysates, medium, serum or purified adiponectin were incubated with a non-reducing sample buffer (1% SDS, 5% glycerol, 10 mM Tris-HCl, pH 6.8) at room temperature for 10 min, separated by 4–20% gradient SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting with an in-house anti-adiponectin antibody or anti-ERp44 antibody (Santa Cruz Biotechnology). For the co-immunoprecipitation studies, cells were washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and solubilized in radioimmune precipitation assay buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM NaF, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% Triton X-100 plus protease inhibitor mixture). 100 µg of cell lysate was precleared with protein G beads and incubated with the antibody and protein G beads on a shaker overnight at 4 °C. The beads were precipitated and washed three times with radioimmune precipitation assay buffer, and the immunoprecipitated complexes were eluted by incubation with nonreducing SDS-PAGE sample buffer before analyzed by Western blotting.

6.2.5 Animals
All procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong and carried out in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) as well as institutional guidelines for the care and use of laboratory animals. Mice were housed in a room under controlled temperature (23±1°C) and 12-h light-dark cycles, with free access to water and food. Obese db/db mice were fed with normal diet. For high fat diet feeding, C57/BL6, wildtype mice had free access to high fat diet from an age of four weeks. For peptide injection experiments, mice were injected with the peptides (10mg/kg body weight). The serum was collected at 0, 1, 2, 4, 8, and 24 h from the tail veins.

6.3 Results
6.3.1 Design and synthesis of adiponectin peptide mimetics
In order to facilitate the internalization of a peptide mimic to subcellular compartments for potential interaction with ERp44 we conjugated the adiponectin-derived peptides to the cell
penetrating peptide (CPP) sequence YGRKKRRQRRR of trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus (Figure 6.1 A) [275]. We previously found that ERp44 specifically recognizes a highly conserved region of the adiponectin variable domain, which includes amino-acid residues 36 to 45 (chapter 4.3.4) (Figure 6.1 A). We synthesized, a fusion of this 9-amino acid stretch (KGTCAGWMA) to the carboxy-terminus of the CPP sequence (TAT-WT) (Figure 1A). In addition, two other variants KGTSAGWMA and KGTCAGAMA with the cysteine 39 and tryptophan 42 replaced by serine (C39S) and alanine (W42A), respectively, were also similarly prepared (TAT-C39S and TATW42A) (Figure 6.1 A). In parallel, for control experiments we synthesized variants with adiponectin derived peptide sequence without the aforementioned CPP segments (WT) (Figure 6.1 A).

6.3.2 Adiponectin-derived TAT fusion peptides bind ERp44
First, binding of peptides (WT, W42A and C39S) to ERp44 in vitro was assessed by mass spectroscopy. Peptides were mixed at a ratio of 1:10 with the recombinant mouse ERp44 (overexpressed and purified from E.coli) and incubated for 4 days. The mixture was subsequently subjected to ESI-MS analysis similar to the experiments described in chapter 4.3.4. In line with our previously reported result (chapter 4.3.4), ESI-MS detected ~35% of ERp44 to be covalently linked to the WT peptide (Figure 6.2). Interestingly, the peptide carrying a W42A mutation showed an elevated level of complex formation with ERp44 (Figure 6.2), whereas the C39S mutant failed to show any detectable level of complex formation, consistent with the fact that the ESI mass spectrometry only detects covalently linked complexes (via disulfide bonds) (Figure 6.2).

Next, we examined the interactions between ERp44 and TAT-conjugated peptides TAT-WT, TAT-W42A and TAT-C39S. TAT-WT showed comparable amount of disulfide linkage with ERp44 as was seen for WT (Figure 6.2). TAT-W42A displayed a larger amount of complex in line with the results obtained for the shorter peptides WT and W42A (Figure 6.2). Interestingly, the addition of TAT at the N-terminus caused a drastic increase in intermolecular disulfide bond formation, as deducted from the results for TAT-W42A and W42A. As expected, replacement of Cys by Ser (TAT-C39S) led to undetectable complex formation as was the case for the corresponding shorter analogue C39S (Figure 6.2). These results suggest that the N-terminal fusion of TAT leads only to a small change in the degree of binding of adiponectin-derived peptides to ERp44. Therefore, we used these peptides as good
starting models to test their possible intervention into adiponectin-ERp44 binding in cell and animal studies as described below.

**Figure 6.1: Adiponectin secretory pathway and peptides to exploit the pathway**

A: Sequences of designed peptides and the sequences of adiponectin variable domain (mouse and human) are shown. Sequence of the CPPs containing Tat sequence is also shown. B: Schematic representation of proposed peptide activity. Adiponectin is retained in the ER-Golgi Intermediate Compartment (ERGIC) via thiol-mediated retention by ERp44. CCP binding triggers adiponectin release from ERp44 and allows assembly of adiponectin into its HMW form and subsequent secretion into circulation. Upon peptide binding, the ERp44 peptide complex is retrieved to the ER where the peptide is released potentially mediated by Ero1-Lα. ERp44 can now undergo another cycle of adiponectin retention. In this model, small model peptides are able to extenuate ERp44-mediated retention of adiponectin in a distinct mode of action from TZDs.
Figure 6.2: ESI-MS analysis of adiponectin derived peptides.

Binding experiment of ERp44 and the adiponectin derived peptides. ERp44 was incubated with WT, C39S, W42A, TAT-WT, TAT-C39S or TAT-W42A for 4 days, and the mixture was analyzed by ESI-MS. The spectra show two peaks, one at 46,074 atomic mass units (amu) representing free ERp44 and a second peak representing the ERp44 peptide complex as indicated.

6.3.3 Adiponectin derived peptide mimetics disturb ERp44-adiponectin interactions in 3T3-L1 adipocytes

Next, we analyzed the ability of the peptides (WT, TAT-WT, W42A, TAT-W42A, C39S and TAT-C39S) to perturb ERp44-adiponectin interactions in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells were treated with peptides at 200 nM for 24 h and were subsequently harvested for co-immunoprecipitation (co-IP) to determine the amount of adiponectin associated with ERp44. The results in Figure 6.3 A showed that TAT-WT and TAT-W42A were able to suppress ERp44-adiponectin interactions, whereas TAT-C39S had no effect suggesting a critical role of the cysteine residue for the action of the peptide. It is reasonable to assume that the peptides without TAT fusion did not interfere with ERp44-adiponectin interactions (Figure 6.3 A), because of their inability to gain access into the ER compartment. These peptides served as controls in subsequent experiments.
6.3.4 Treatment of 3T3-L1 adipocytes with adiponectin derived peptide mimetics induces modulation in adiponectin multimerization

First, mRNA levels of adiponectin were quantitated by qPCR in differentiated 3T3-L1 cells treated with peptides. This assay indicated that the mRNA levels for all the examined peptides (TAT-WT, TAT-W42A and TAT-C39S) and the control groups (WT, W42A, and C39S) (Figure 6.3 B) were indistinguishable. Second ELISA was performed to measure the total amount of adiponectin in adipocytes after peptide treatment and again this experiment showed no significant alteration of total adiponectin levels (Figure 6.3 C). These results suggest that the peptide treatment did not influence the process of adiponectin synthesis in 3T3-L1 cells.

![Figure 6.3](image)

**Figure 6.3 Effect of adiponectin derived peptides on cellular adiponectin metabolism.**

A: Co-immunoprecipitation of differentiated 3T3-L1 cell treated with TAT-WT, TAT-W42A, TAT-C39S and their corresponding control peptide (WT, W42A and C39S, respectively). Cells were incubated with peptides (200 nM) for 24 h and the cell lysate and the condition medium were collected. The immune-complex was precipitated by anti-ERp44 antibody and probed by anti-ERp44 and anti-adiponectin antibody. B: Expression of adiponectin mRNA was evaluated by qPCR. C: The total adiponectin content in the cell lysate was evaluated by ELISA and normalized to total protein content. D: Distribution of adiponectin oligomers in cells after 24 h peptide treatment was determined by non-reducing SDS-PAGE. The relative intensity of each oligomer was analyzed using ImageJ software package. Results were normalized to the corresponding control (non-TAT peptide) group and presented as fold change. *, p<0.05 compared with corresponding control.

In the next step, we investigated adiponectin oligomer distribution in cell lysate by non-reducing SDS-PAGE. At the intracellular level, we observed changes in the relative
distribution of adiponectin oligomers when the cells were treated with TAT-WT and TAT-W42A relative to those for the controls (WT and W42A). MMW levels were drastically reduced and the level of HMW adiponectin was also reduced (Figure 6.3 D). Thus, accompanying these alterations, the distribution of oligomeric composition shifted towards LMW adiponectin (Figure 6.3 D). TAT-C39S did not cause any alteration in the intracellular distribution of the adiponectin oligomers as for the corresponding peptide lacking the TAT sequence (Figure 6.3 D) further establishing that residue C39 plays a crucial role in the propensity to generate complexes observed for TAT-WT and TAT-W42A.

6.3.5 Level of adiponectin secretion and multimerization in 3T3-L1 adipocytes are affected by peptide treatment
In cultured medium, the total amount of adiponectin was significantly reduced in samples incubated with TAT-WT or TAT-W42A, as determined by ELISA (Figure 6.4 A). Non-reducing SDS-PAGE was performed to analyze the oligomeric distribution of adiponectin. The amount of trimeric and HMW adiponectin were significantly reduced in the medium from both the TAT-WT- and TAT-W42A-treated cells, whereas the level of hexamers remained unaltered after peptide treatment (Figure 6.4 B and C). TAT-C39S treatment did not alter the oligomeric distribution of adiponectin in the cultured medium (Figure 6.4 D).

6.3.6 Level of adiponectin production in mice changes upon treatment with adiponectin-derived peptide mimetics
Genetically-obese db/db mice were used for in vivo treatment with the peptide mimetics. The serum was collected over a period of 24 h after injection of the peptides (10 mg/kg). Analogous to the adipocyte experiments, the total (all oligomers) adiponectin concentration in mouse serum decreased after treatment with TAT-WT and TAT-W42A when compared to the controls WT and W42A (Figure 6.5). Treatment with TAT-WT caused a shift of oligomer distribution towards the MMW form (Figure 6.5) with almost no LMW adiponectin detected on the Western blots when the same volume of serum collected after 24 h were examined for each sample. Also, the level of HMW adiponectin in serum decreased over the period of the experiment. Injection of TAT-W42A also raised the amount of MMW in mice serum and a decrease in the levels of both LMW and HMW adiponectin were noted (Figure 6.5). Not surprisingly, no differences in adiponectin distribution and total concentration were observed for peptides C39S and TAT-C39S (Figure 6.5).
6.3.7 Design of IgM-derived peptide mimetics

In order to accrue more knowledge on the mode of interaction of client proteins to ERp44, we chose the IgM system as another example. IgM antibodies have been extensively examined with regard to their hexameric assembly in the ER and the involvement of ERp44 in this process [116]. A highly conserved 18 amino-acid long tail-segment located in the C-terminus of the monomeric secretory µ chains of IgM harbors the ERp44 binding site, namely cysteine residue in position 575, and presumably controls the IgM assembly [212]. Blast analyses of the tail-segments of Ig-µ and Ig-α chains (Figure 6.6 A) demonstrate high conservation of a GTCY motif [116]. Directed by this observation, two peptides derived from the C-terminal tail of IgM were synthesized. Both included a cysteine residue, 1) a four-amino acid long peptide GTCY (M1) which corresponds to the C-terminal end of the IgM antibody sequence and 2) a longer seven-amino acid long sequence DTGGTCY (M2) (Figure 6.6). Both of these were also conjugated to the TAT sequence (YGRKKRRQRRRGTGY; TAT-M1 and YGRKKRRQRRR DTGGTCY; TAT-M2).
Chapter 6 - Designed peptides modulate adiponectin assembly and secretion

Figure 6.5: Synthetic adiponectin derived peptides alter adiponectin concentrations in mouse serum.

The distribution of adiponectin oligomers in serum was determined in 20-weeks old db/db obese mice after intraperitoneal injection of control and TAT peptide (WT, W42A and C39S, 10mg/kg), respectively.

A: Adiponectin content in serum was evaluated by ELISA. B: The serum (1µl) was collected at different time intervals and evaluated by non-reducing SDS-PAGE. The relative intensity of HMW was quantified using ImageJ software package. Results were normalized to the corresponding control (non-TAT peptide) group and presented as fold change. *, p<0.05 compared with corresponding control.
6.3.8 IgM-derived peptides bind ERp44 in vitro and interfere with ERp44 adiponectin binding in adipocyte cells

First, we examined the in vitro binding of M1, M2, TAT-M1 and TAT-M2 to ERp44 using the same ESI-MS protocol as described above. This assay indicated strong affinity of M1 and M2 to ERp44 as was observed for adiponectin-derived peptides WT and W42A (Figure 6.6 B and 6.2). The amount of complex observed for M2 was larger (Figure 6.6 B) and, interestingly, the TAT fusion elicited marked reduction of disulfide bond formation (Figure 6.6 B and C).

Next, we probed whether the IgM peptides are able to perturb ERp44-adiponectin interactions in 3T3-L1 adipocytes ex vivo. Differentiated 3T3-L1 cells were treated with TAT-M1 and TAT-M2 peptides and the corresponding controls (M1 and M2) for 24 h. The co-IP experiment for TAT-M1 and TAT-M2 showed less amount of adiponectin complexed with ERp44 when compared to those in the case of M1 and M2. This observation demonstrated perturbation of ERp44 adiponectin interaction in the presence of TAT fused peptides (Figure 6.7 A). We also noted that the levels of adiponectin mRNA was unaltered for all the IgM derived peptides, as were the case for the adiponectin-derived peptides (Figure 6.7 B).

Furthermore, ELISA assays detected similar adiponectin concentrations in the cell lysate for all four peptides (Figure 6.7 B), which is in agreement with the results described in Figure 6.3 C. When the relative amounts of the different adiponectin oligomers in cell lysates were mutually compared, it was clear that the amount of HMW in the presence of TAT-M1 and TAT-M2 were more compared to the case of M1 and M2. Under the same situation, the amount of MMW was less for TAT-M1 and TAT-M2 relative to that for the case of M1 and M2. Interestingly, this perturbation in adiponectin oligomerization was more pronounced for the shorter TAT-M1 than TAT-M2 (Figure 6.7 C and D).
Figure 6.6: Design of IgM derived peptides and their binding properties to ERp44 in vitro.

A: IgM chain derived peptide sequences. Top panel shows the amino acid sequence of the IgM antibody derived peptides. In the bottom panel, the blast analysis of the tailpieces of Ig-µ and Ig-α chains is shown. The conserved penultimate cysteine is highlighted in red and the conserved residues at the C-terminal end of the tailpiece are colored in green. B: Binding experiment of ERp44 and the IgM derived peptides. ERp44 was incubated with M1, M2, TAT-M1 or TAT-M2 for 4 days, and the mixture was analyzed by ESI-MS. The spectra show two peaks, one at 46,074 atomic mass units (amu) representing free ERp44 and another at 46514 amu for M1, 46787 amu for M2, 48056 amu and 48329 amu for TAT-M2 representing the disulfide-linked ERp44-peptide complex.
6.3.9 IgM-derived peptides enhance secretion of (HMW) adiponectin in culture medium

We examined culture medium for the relative oligomer population of secreted adiponectin, as also the total concentrations of the adiponectin. The treatment of 3T3-L1 adipocyte cells with TAT-M1 and TAT-M2 strongly modulated the level of adiponectin in the medium, albeit in a manner distinct from that observed in the case of the adiponectin derived peptides.

ELISA results showed that the concentrations of total adiponectin in cultured medium collected from cells treated with TAT-M1 or TAT-M2 were significantly increased, when compared to the corresponding controls (Figure 6.8 A). Oligomeric analyses revealed that all three oligomers were increased by IgM peptides treatment (Figure 6.8 B and C).

Figure 6.7: Effect of IgM derived peptides on adiponectin metabolism

A: Co-IP profiles of differentiated 3T3-L1 cells treated with TAT-M1, TAT-M2 and their corresponding control peptides (M1 and M2). Cells were incubated with peptide (200 nM) for 24 h and the cell lysate and the condition medium were collected. The immune-complex was precipitated by anti-ERp44 antibody and probed by anti-ERp44 and anti-adiponectin antibody. B: Expression of adiponectin mRNA in differentiated 3T3-L1 cells treated with TAT-M1, TAT-M2 and their corresponding control peptides (M1 and M2), as evaluated by qPCR.

C: The total adiponectin content in cell lysate was evaluated using ELISA. D: Distribution of adiponectin oligomers in cells after 24 h treatment with IgM derived peptides was determined by non-reducing SDS-PAGE. The relative intensity of each oligomer was analyzed using ImageJ software package. Results were normalized to the corresponding control (non-TAT peptide) group and presented as fold change. *, p<0.05 compared with corresponding control.
Figure 6.8: IgM derived peptides enhanced HMW adiponectin secretion and release from adipocyte cells.

A: Adiponectin content in condition medium after 24 h peptide treatment was evaluated by ELISA. B and C: Adiponectin oligomer distribution in culture medium after peptide treatment for 0 h and 24 h was evaluated by non-reducing SDS-PAGE. Same volume (5 µl) of medium was loaded onto the gel. The relative intensity of each oligomer was analyzed using ImageJ software package. Results were normalized to the corresponding control (non-TAT peptide) group and presented as fold change. *, p<0.05 compared with corresponding control.

6.3.10 IgM-derived peptides enhance secretion of (HMW) adiponectin in obese mice

WT obese mice (fed with high fat diet) were used for in vivo treatment with IgM derived peptide mimetics. The serum was collected 24 h after injection of the peptides (10mg/kg). The adiponectin oligomer distribution was analyzed using the same volume (1 µl) of serum (Figure 6.9). The total amount of adiponectin in mice serum increased over the 24 h period. In particular, we detected an increase of the HMW form when TAT-M1 and TAT M2 are
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compared to the controls. The increase for the HMW form was more pronounced for TAT-M1 in comparison to that for the TAT-M2.

![Graphs showing adiponectin concentration and oligomer distribution](image)

**Figure 6.9: IgM peptides enhance HMW adiponectin secretion in mice.**
The distribution of adiponectin oligomers in serum was determined in 20 weeks old C57/BL6 mice fed with high fat diet. Intraperitoneal injection was performed for control and TAT peptides (M1 or M2, 10 mg/kg). 

A: Adiponectin concentration in serum was evaluated by ELISA. B: Adiponectin oligomers distribution was analyzed by non-reducing SDS-PAGE. The same volume of serum (1 μl) was load onto the gel. The relative intensity of HMW was quantified using ImageJ software package. Results were normalized to the corresponding control peptide group (at time zero) and presented as fold change. *, p<0.05 compared with corresponding control.

**6.4 Discussion**
In case of obesity lowered production and secretion of adiponectin is, at least in part, responsible for the pathogenesis of the insulin resistance syndrome and diabetes [16, 30, 34, 41]. Therefore, replenishment of adiponectin could represent a novel treatment strategy for insulin resistance and type 2 diabetes [46]. In this connection, the adiponectin protein has therapeutic implications as an anti-obesity drug [268]. It was shown that administration of
adiponectin increased glucose uptake and fat oxidation in muscle, and improved insulin sensitivity [276]. However, recombinant production of polymorphic protein and generation of pure HMW form remains challenging. In particular, this hormone exhibits high circulation concentration and high amounts of this hormone would be required for supplement therapy [220]. To mitigate the production problem, the regulation/manipulation of pathways controlling its production, secretion or release represents a promising avenue for managing obesity, hyperlipidemia, insulin resistance, type 2 diabetes, and vascular inflammation [277]. ERp44 is critically involved in regulating the assembly process of adiponectin oligomers in vivo [80]. ERp44 specifically binds assembly trapped adiponectin oligomers in the ERGIC, retrieves those to the ER, where it releases them as reduced trimer to attempt again HMW assembly (chapter 4.4.5). Under ER stress precipitated by obesity there is dysregulation of these processes and hence adiponectin assembly and release is impaired. Our goal was to explore means by which this dysregulation is mitigated, possibly by rescuing aberrantly trapped adiponectin (figure 6.1 B).

We first synthesized a set of rationally designed peptides derived from the adiponectin variable domain and conjugated these with the TAT sequence from HIV to generate CPPs. We demonstrate that the CPPs are able to interfere/modulate ERp44 adiponectin interaction in adipocyte cells ex vivo and furthermore show that this interference leads to modulation of adiponectin assembly and/or release in adipocytes and obese mice in vivo. Next, the IgM sequence was used as template for the design of a second generation of peptides. This set of CPPs is able to modulate adiponectin assembly and release. This effect was similar as with treatment with TZDs, namely enhancement of adiponectin secretion and simultaneous shifting of the ratio of HMW to total adiponectin towards the HMW form. Therefore, our approach, in which we targeted ERp44 by designed peptide mimetics to interfere in adiponectin-ERp44 interaction to replenish HMW adiponectin levels in circulation, opens a novel avenue to explore potential drug candidates for the treatment of obesity related disease such as Type 2 diabetes mellitus.

A key question remains as to the mechanism behind the action of the peptide mimetic in the observed modulation of adiponectin assembly and release. Our observation demonstrated that the group of adiponectin and the group of IgM derived peptides lead to different profiles for adiponectin oligomer distribution and secretion levels of these oligomers. In case of TAT–WT, peptide treatment drastically reduces the amount of intracellular adiponectin hexamer
and the HMW form, whereas the amount of LMW increases. In turn, the concentration of LMW and HMW in cell medium is reduced but MMW remains unchanged. The reduction of LMW and HMW secretion also leads to a decline in total adiponectin medium levels. These results indicate that the adiponectin derived CPPs predominantly modulate ERp44 interaction with hexameric adiponectin in cells, leading to the release of the hexamers into the medium as well as conversion of the intracellular hexamers to trimers. At the same time, adiponectin derived CPP action also seems to reduce HMW assembly and LMW secretion. The latter might be due to the lower formation of HMW within adipocyte cells.

On the contrary, IgM derived peptide treatment leads to increased concentration of HMW and LMW in cells and only minor decrease of the MMW form. In fact, close examination of the Western blot of the intracellular adiponectin samples reveals that the MMW consists of two different species, of which IgM derived peptide treatment only affects one. As we reported previously, ERp44 selectively recognizes fully oxidized adiponectin hexamers and trimers, which cannot further assemble into HMW (chapter 4.3.7). We had shown earlier that a tryptophan residue in position 42 of adiponectin regulates the oxidation of C39 and thereby prevents full length adiponectin from forming oxidized trimeric or hexameric states, which are unable to assemble into higher order complexes. Unsurprisingly, this control of adiponectin oxidation in turn regulates ERp44 binding to adiponectin in adipocytes [73]. Hence, the two populations of MMW as detected in figure 6.9 are likely to differ in their oxidative state, which determines its interaction with ERp44. Therefore, it is possible that ERp44 specifically recognizes the fully oxidized species and converts it into trimers. IgM CPP activity appears to modulate this process and accelerate the rate of the conversion. At the same time, IgM CPP action enhances the formation of the HMW form. As a consequence, we observe an increase in HMW adiponectin within adipocyte cells and a higher abundance of HMW in the cell also increases secretion of this oligomer.

The changes in profiles for adiponectin oligomer distribution and secretion levels of these oligomers after peptide treatment might be due to the difference in affinity between adiponectin and ERp44 [80] compared to IgM and ERp44 [213]. The covalent linkage of adiponectin to ERp44 appears to be tight in vivo [80] whereas IgM and ERp44 undergo fast cycles of binding and release [213], which is also reflected in our ESI-MS in vitro studies (Figure 6.2 and 6.6). Hence, we speculate that adiponectin derived peptides compete in binding to ERp44 with endogenous adiponectin in the ESC and may silence the enzyme. The
more transient binding of IgM derived peptides potentially elevates/accelerates ERp44 adiponectin turnover and, therefore, increases secretion of total and/or HMW adiponectin. ERp44 and Ero1-Lα [81] have been demonstrated to modulate adiponectin secretion in a Ying and Yang like fashion [22] whereas ERp44 acts in synergy with ERGIC-53 to control IgM assembly. Therefore, it is possible that the secretory pathway followed by the ERp44 adiponectin-derived peptide complex is distinct from that followed by the ERp44 complexed with IgM derived peptides. As a result, differential effects on adiponectin assembly and release in the two cases are generated.

A third scenario to explain the observed modulations in adiponectin assembly and release is based on the site of location where interaction between ERp44 and the CCPs take place. Assembly of adiponectin and oligomerisation of IgM occur in different subcompartments of the ESC [87, 116]. Thus, the two groups of peptides may interfere with ERp44 action in different places of the ESC. As a consequence, the two different families of peptides modulate the adiponectin assembly differently.

To conclude, this study explores the potential of therapeutic invention in the ER by using CPPs to directly target ERp44, a member of the largest group of chaperones, the disulfide isomerase family. We used small peptides derived from ERp44 client proteins to modulate adiponectin assembly and secretion, which can therefore act as effective reagents to counteract impaired adiponectin multimerization caused by dysregulation in ER chaperone expression during ER stress.
Chapter seven - Discussion and Future Directions

Adiponectin is believed to be a major modulator of insulin action and therefore, has therapeutic implications as an anti-obesity drug [277]. The mechanisms by which adiponectin is synthesized and secreted are poorly understood. In addition, the process behind the regulation of adiponectin oligomerization in the ER by multiple chaperones is not fully understood.

This thesis has presented insight into the adiponectin assembly pathway in the ER and the involvement of the ER chaperone machinery, in particular ERp44. This study led to a rational exercise of exploring means to replenish the decreased circulating adiponectin levels in type 2 diabetic model (ob/ob mice) by modulation of ERp44 adiponectin interactions through the use of CPPs.

7.1 Model peptides to study adiponectin assembly

In the third chapter of this thesis, we presented the successful design, production and characterization of an engineered model peptide system for the N-terminal region of adiponectin.

The variable domain of adiponectin appeared to play an important role in the regulation of the assembly of AHD (the model peptide including the complete variable domain) into higher oligomers by controlling disulfide bond formation between C39 residues in the trimeric building block. Oligomerization of AHD can be chemically controlled; under oxidizing conditions AHD assembles mainly into hexamers with some oxidized trimers and only small amounts of tetramer, whereas in the presence of reducing agent it remains as reduced trimeric subunit. In contrast, assembly of the trimeric peptide model lacking residues E18-T38 of the variable domain, which carries a cysteine residue at the very N-terminus of the sequence, cannot be controlled. In the presence of 5 mM hydroperoxide, this peptide assembles in a ‘uncontrolled’ manner into hexamers, higher oligomers and intermediates comprised of four to five peptides chains. The mechanism behind these observations remains speculative. It is possible that the exposure of the cysteine as the N-terminal residue causes unregulated linkage of thiols. The simple addition of a stretch of residues may be efficient to shield the exposed sulfur and prevent random formation of disulfide linkages. However, it is also
possible that residue-specific interaction between different trimers guide the formation of the hexamer, as well as the higher oligomeric species. Therefore, the role of the residues E18-T38 in the assembly process requires further investigation.

The adiponectin sequence features two conserved histidines, which are both located near the N-terminal end of the collagenous domain in a HXXH motif; a sequence pattern associated with metal-binding [71, 278]. In addition, it was demonstrated that a significant amount of zinc is associated with HMW adiponectin, whereas no zinc is present in the trimeric form. In the same study, the authors also describe that the presence of zinc promotes the assembly of HMW adiponectin. These observations led to the hypothesis of a potential metal-chelating role of the histidine residues [71]. The model peptide system could be utilized to shed further light on this hypothesis. For example, assembly of the peptide in the presence of various metal ions could be investigated. In the next step, with histidine residues substituted by alanine residues the oligomerisation profiles under various conditions could be compared with those for the wild type sequence. Also, different redox environments (GSH:GSSG ratios) as well as salt and other additives may influence the assembly pathway. All those parameters could be analyzed using the model peptides and the assembly assay described in chapter three.

We also discovered that the isolated native peptide sequence of the variable domain together with the N-terminal stretch of the collagen-like domain is not able to form a trimeric structure. To overcome this problem, we fused a collagen peptide model, to the stretch of the native sequence of adiponectin, leading to generation of a trimeric model. We argued that the collagen-like triple helix might require close proximity of the three chains to fold, which is provided by the trimeric arrangement of the globular head domains in the full-length protein. Another reason could be that the 30-amino acid (ten GXY repeats) long stretch of the collagen domain is be too short for the trimer to form and the complete length of the collagen like domain is required for the peptide to assume a collagen triple helix. Furthermore, post-translational modifications present in the endogenous protein might stabilize the trimeric structure of the collagen helix and may therefore be crucial for the trimeric structure to form.

A recent study from [279], which was published after the work presented in chapter three of this thesis was finished, reports the synthesis and characterization of a peptide covering the complete variable and collagen-like domain of adiponectin. The group prepared different peptides, one of which is comprised of amino acids E18-G107 and a second one is made up of
the same residues with post-translational modifications. The authors demonstrated that the first peptide forms a collagen-like structure and assembles into trimers and hexamers, whereas the construct bearing post-translational modifications is able to form in addition HMW species. Moreover, the second model peptide with post-translational modifications assembles into the HMW form in the presence of reducing agents, suggesting that non-covalent interactions between trimers mediate the assembly into HMW for this peptide. These results confirm that the complete collagen domain (or more than ten repeats of the GXY motif) is required for a trimeric arrangement to occur and that the presence of the globular domain of adiponectin appears to be unnecessary for the assumption of the collagen-like trimeric structure. In addition, these observations indicate that interactions between sugar molecules stabilize the formation of the HMW form. It also, based on the results reported, appears that post-translational modifications are not required for the collage-like structure to develop.

In future, it will be interesting to identify the minimal length of the collagen domain required for the timer to form, as well as to identify any key residues that drive the formation of the trimer. Also, the role of the variable domain in the trimer assembly would be interesting to investigate. We showed that the addition of the variable domain to the trimeric collagen model peptide does not alter its thermal stability. It would be interesting to analyze the effect of the variable domain on the stability of the trimeric collagen-like structure of the native sequence. Furthermore, the exact role of the PTMs still remains elusive. Is a peptide of the full-length collagen domain bearing PTMs able to adopt HMW forms or is the presence of the variable domain indispensable for HMW assembly? These questions still await answers.

7.2 Role of ERp44 in adiponectin assembly

The experiments described in chapter four, explored the interaction of ERp44 with adiponectin and revealed how ERp44 exerts QC on the oligomeric assembly of adiponectin. We established that ERp44 specifically recognizes a highly conserved nine amino acid long sequence of adiponectin, which includes cysteine residue 39. Interestingly, none of the known ERp44 binding partners show any overlap in their proposed binding segment with this binding motif. This observation raises the following questions: is the nature of sequence specificity in binding to ERp44 also preserved in the case of other client proteins? If so, what signals in the binding motif allow for specific recognition of different client proteins by ERp44? Structural studies of the complex between ERp44 and clients might be able to provide further insight into this phenomenon in future. In this context, it will be very
informative to examine how these different interactions at a molecular level influence ERp44 action \textit{in vivo}. Earlier work \cite{87, 210} suggested that ERp44 is able to distinguish between clients and can act on different client proteins in individual modes. In other words, binding of adiponectin to ERp44 triggers a different cellular cascade than Ero1α binding to ERp44.

We also observed that ERp44 binds to adiponectin in a pH dependent manner confirming earlier findings of \cite{138}. Also, we discovered that the ERp44 adiponectin complex is pH labile. This implies that the covalently linked complex between ERp44 and adiponectin dissociates when the pH is raised to eight or higher. This result is particularly interesting, since a disulfide bond between two polypeptide segments requires a reducing equivalent, i.e. an electron, to transform the disulfide bond into two sulfhydryl groups, which cannot be achieved just by a pH change. It is known that the cysteine pair (C160 and C212) in the b domain of adiponectin can exist as oxidized disulfide bridge or as two separate reduced thiols \cite{200}. Furthermore, ERp44 contains a reduced cysteine (C63) in the a domain in close proximity to C29 \cite{200}. We speculated that electrons might be donated from the aforementioned cysteine pair in the ERp44 b domain or the disruption of the ERp44-reduced trimer complex leads to a disulfide bond between C29 and C63. This phenomenon, however, needs further examination. In future, the study of various mutants, in which the listed cysteine residues are replaced with serine or alanine residues and application of a release assay (described in chapter 5) could give further insight into this process.

For the \textit{in vivo} situation, a hitherto unidentified oxidoreductase, possibly Ero1α, ERp44’s foremost partner, might deliver the required reducing equivalents. In this case, the release could occur either by direct binding of Ero1α to the ERp44 adiponectin complex thereby setting free the reduced trimer or by subsequently reducing of the oxidized ERp44 after the reduced trimer has been released. Here, an \textit{in vitro} co-incubation experiments of ERp44-AHD complex and Ero1α can be developed to dissect the underlying mechanisms. Also mass spectroscopy can help to understand the disulfide bond array in ERp44 before peptide binding, as well as after peptide release.

\textbf{7.3 Structural characterization of adiponectin ERp44 interaction}

The work described in chapter five aimed to understand ERp44 client interactions at a structural and molecular level, which would enable us to answer some of the questions raised above. To this end, we designed and generated a covalently linked and chemically stable
complex of ERp44 and an adiponectin-derived peptide suitable for X-ray crystallography studies. However, the obtained crystals did not show diffraction patterns of good enough quality to attempt structure solution. Therefore, we decided to modify the construct. We posited that the flexible C-terminal end of ERp44 might interfere with the formation of a tight and regular crystal lattice. Hence, we generated a version of the ERp44 protein lacking the C-terminal tail. However, we were unable to produce any crystal of this construct. Expression levels of the truncated construct of ERp44 were very low compared to the full-length protein, making the production and purification of the construct more challenging. For this reason, the purified protein sample of the truncated ERp44 molecule contained more impurities after purification than the sample of the full-length protein. Given that impurities can often interfere with the crystallization process, optimization of the purification protocol would be the next step towards the achievement of a crystal structure of the ERp44 peptide complex. In addition, strategies like surface entropy reduction (a strategy where small clusters of two to three surface residues characterized by high conformational entropy are replaced with alanine residues) [280] or methylation of surface lysine residues could be explored to generate a complex more amenable to crystallization [281]. Moreover, the length of the peptide used for complexation could potentially mediate crystal contacts. Hence, peptides of different length as well as peptides derived from various client proteins (such as IgM or Ero1α) could be tested for their ability to aid crystallization.

Nonetheless, the results in chapter five demonstrated a promising first step towards the determination of a high-resolution crystal structure of ERp44 in complex with a peptide mimetic of one of its binding partners. Based on the outlined strategy, a more detailed optimization of the design can now be undertaken to generate a construct suitable for the structural study by X-ray crystallography.

7.4 CPPs, potential drug candidates for the treatment of obesity related disease

Based on our finding on the role of ERp44 in the regulation of adiponectin assembly, we sought for an intervention in the ER chaperone machinery that could favourably modify plasma adiponectin levels. We decided to target ERp44 by designed peptide mimetics to interfere with adiponectin-ERp44 interaction. We showed that designed peptides fused with CPP sequences for the delivery of the cargo to the ER/cis-Golgi are effective in perturbing adiponectin oligomer composition in cell medium (ex vivo) as well as in serum (in vivo) by directly interacting with ERp44. In addition, we demonstrated that treatment of adipocytes
and obese mice with CCPs derived from the IgM binding motif to ERp44 modulate adiponectin assembly and secretion in a manner that leads to similar effects as treatment with TZDs, namely enhancement of HMW secretion. These results set the basis for further development of potential peptide-based drug candidates for the treatment of obesity-related disease such as T2DM.

Furthermore, the presented strategy of restoring adiponectin levels overcomes challenges in the existing approaches for adiponectin-based therapies include 1) severe side effects of thiazolidinediones that exert antidiabetic effects through action at the transcription level, 2) expensive cost of direct administration of recombinantly produced adiponectin and 3) adiponectin receptor agonists eliciting adiponectin-like physiological effects have not yet been proven effective in a clinical setting.

Currently, the development of orally active small-molecule agonists for both AdipoR1 and AdipoR2 display the most promising treatment modality for obesity-related diseases [282]. The adiponectin receptor agonist ‘AdipoRon’ that works through both AdipoR1 and AdipoR2 in vivo was shown to exert multiple effects very similar to those of adiponectin [283]. As result, reduced tissue triglyceride content in the liver and muscle, improvement in insulin sensitivity and glucose tolerance, as well as suppression of cardiovascular diseases were detected in obese diabetic mice on a high-fat diet [284]. However, safety, toxicity and potential long-term side-effects of this molecule have not yet been determined in human studies. In fact, concerns about the safety and efficacy of such agonists were raised in recent reports [284]. The presented approach of rescuing aberrantly trapped adiponectin potentially overcomes the above-mentioned limitations; thus establishing a novel and promising angle for the development of a biotherapeutic.

However, the mechanisms behind the action of the peptide mimetic in the observed modulation of adiponectin secretion and/or assembly need further examination. For example, one of the key questions remains as to whether the peptides modulate assembly of adiponectin or the secretion of the different oligomers. Also treatment of adipocytes with two sets of adiponectin and IgM derived peptides resulted in different profiles for adiponectin oligomer distribution and levels of secretion of these oligomers. These finding suggest a fundamentally different biological action between the two groups of peptides and the processes behind these observations remain to be understood. Therefore, elucidation of the action mechanisms
whereby CPPs modulate adiponectin assembly/secretion will be an important objective for future research. In this context, the molecular level understanding of the specificity of the binding pocket of ERp44 can provide detailed insight into the process by which ERp44 action is controlled. Also, analysis of the molecular mechanism of ERp44 client binding and release cycle can help to understand CPP action on ERp44. In this connection, cell-based experiments to analyze how peptide binding affects ERp44 binding to its retrotransport (KDEL) receptor and how this in turn influences ERp44 retention and secretion, localization as well as retro-transport could be carried out.

Lastly, and more importantly, the designed CPPs remain to be tested for their anti-diabetic, cardiovascular protective and anti-inflammatory function in cells (ex vivo), mice models (in vivo) and evaluated for use in human patients.
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<td>20% (w/v) PEG 3530, 0.2 M ammonium chloride pH 6.3</td>
<td>40% (v/v) MDP 8000, 0.1 M NaAc pH 7.0</td>
<td>20% MDP, 0.02 M CaCl$_2$, 0.1 M NaAc pH 4.5</td>
<td>20% ethanol, 0.1 M Tris pH 8.5</td>
<td>30% (w/v) PEG 8000, 0.2 M Li$_2$SO$_4$, 0.1 M NaAc pH 4.5</td>
<td>16% (w/v) PEG 8000, 0.04 M KH$_2$PO$_4$, 20% glycerol</td>
<td>1.0 M (NH$_4$)$_2$HPO$_4$, 0.1 M NaAc pH 4.5</td>
<td>20% Jeffamine M-600, 0.1 M HEPES pH 6.5</td>
<td>0.2 M sodium chloride, 20% PEG 3350</td>
<td>0.2 M magnesium nitrate, 20% PEG 3350</td>
</tr>
<tr>
<td>B</td>
<td>20% (w/v) PEG 3530, 0.2 M potassium formate pH 7.3</td>
<td>20% (w/v) PEG 3530, 0.2 M potassium formate pH 7.3</td>
<td>40% ethanol, 5% (w/v) PEG 1000, 0.1 M phosphate-citrate buffer pH 5.2</td>
<td>20% (w/v) PEG 8000, 0.2 M NaCl, 0.1 M phosphate-citrate buffer pH 4.2</td>
<td>25% (v/v) 1.2-propanediol, 0.1 M Na$_2$PO$_4$, 10% (v/v) glycerol pH 6.8</td>
<td>70% (v/v) MDP, 0.2 M MgCl$_2$, 0.1 M HEPES pH 7.5</td>
<td>1.0 M sodium citrate, 0.1 M citrate pH 6.5</td>
<td>1.6 M MgSO$_4$, 0.1 M MES pH 6.5</td>
<td>50% (v/v) ethylene glycol, 0.2 M MgCl$_2$, 0.1 M Tris pH 8.5</td>
<td>0.2 M calcium chloride, 20% PEG 3350</td>
<td>0.2 M magnesium nitrate, 20% PEG 3350</td>
</tr>
<tr>
<td>C</td>
<td>20% (w/v) PEG 3530, 0.2 M diammonium hydrogen citrate pH 5.0</td>
<td>50% MDP, 0.2 M (NH$_4$)$_2$PO$_4$, 0.1 M Tris pH 8.5</td>
<td>10% (w/v) PEG 4000, 0.1 M NaAc pH 4.6</td>
<td>20% (w/v) PEG 6000, 1.0 M LiCl, 0.3 M citric acid pH 4.0</td>
<td>10% (w/v) PEG 20,000, 2% dioxane, 0.1 M bicine pH 9.0</td>
<td>20% (w/v) PEG 8000, 0.1 M Tris pH 8.5</td>
<td>2.0 M Li$_2$SO$_4$, 0.2 M NaCl, 0.1 M citrate pH 6.5</td>
<td>10% (w/v) PEG 6000, 0.1 M bicine pH 9.0</td>
<td>10% MDP, 0.1 M Tris pH 8.5</td>
<td>0.2 M potassium formate, 20% PEG 3350</td>
<td>0.2 M magnesium sulfate, 20% PEG 3350</td>
</tr>
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<td>D</td>
<td>30% (v/v) PEG 3530, 0.2 M potassium nitrate pH 6.9</td>
<td>20% (w/v) PEG 3530, 0.2 M ammonium nitrate pH 6.3</td>
<td>10% (w/v) PEG 8000, 0.2 M MgCl$_2$, 0.1 M Tris pH 7.0</td>
<td>20% (w/v) PEG 3530, 0.2 M ammonium nitrate pH 6.3</td>
<td>2.0 M (NH$_4$)$_2$SO$_4$, 0.1 M MDP, 0.1 M citrate pH 6.6</td>
<td>40% (v/v) PEG 4000, 0.2 M Li$_2$SO$_4$, 0.1 M citrate pH 7.8</td>
<td>10% (v/v) PEG 8000, 0.1 M citrate pH 7.0</td>
<td>14.4% PEG 3000, 0.16 M Ca(OAc)$_2$, 0.08 M citrate pH 7.5</td>
<td>0.2 M sodium fluoride, 20% PEG 3350</td>
<td>0.2 M sodium iodide, 20% PEG 3350</td>
<td>0.2 M magnesium acetic acid, 20% PEG 3350</td>
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<td>E</td>
<td>20% (w/v) PEG 3530, 0.2 M magnesium nitrate formate pH 5.9</td>
<td>0.8 M (NH$_4$)$_2$SO$_4$, 0.1 M citric acid pH 4.0</td>
<td>20% (w/v) PEG 6000, 0.1 M citric acid pH 5.0</td>
<td>10% (w/v) PEG 8000, 0.1 M citrate pH 7.0</td>
<td>10% (w/v) PEG 1000, 0.1% MDP, 0.1 M citrate pH 7.0</td>
<td>40% (v/v) PEG 8000, 0.1 M Tris pH 8.0</td>
<td>1.26 M (NH$_4$)$_2$SO$_4$, 0.2 M Li$_2$SO$_4$, 0.1 M citrate pH 8.5</td>
<td>10% (w/v) PEG 8000, 0.1 M imidazole pH 8.0</td>
<td>0.2 M potassium fluoride, 20% PEG 3350</td>
<td>0.2 M potassium iodide, 20% PEG 3350</td>
<td>0.2 M zinc acetate, 20% PEG 3350</td>
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<td>F</td>
<td>20% (w/v) PEG 3530, 0.2 M sodium thioacetate pH 6.9</td>
<td>20% (w/v) PEG 3350, 0.2 M sodium thioacetate pH 6.6</td>
<td>50% (w/v) PEG 3350, 0.2 M PEG 5000, 0.8 M KH$_2$PO$_4$, 0.1 M HEPES pH 7.5</td>
<td>24% (v/v) PEG 5000, 0.1 M Tris pH 8.0</td>
<td>24.5% (v/v) PEG 4000, 0.1 M MDP, 0.15% MDP, 0.1 M citrate pH 7.0</td>
<td>40% (v/v) PEG 3000, 0.2 M NaCl, 0.1 M HEPES pH 7.5</td>
<td>20% (w/v) PEG 3000, 0.2 M Zn(OAc)$_2$, 0.1 M HEPES pH 8.0</td>
<td>3.2 M (NH$_4$)$_2$SO$_4$, 0.1 M citric acid pH 5.0</td>
<td>0.2 M lithium chloride, 20% PEG 3350</td>
<td>0.2 M potassium thiocyanate, 20% PEG 3350</td>
<td>0.2 M calcium acetate, 20% PEG 3350</td>
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<td>G</td>
<td>20% (w/v) PEG 8000, 0.1 M CHES pH 9.5</td>
<td>20% (w/v) PEG 3350, 0.2 M ammonium formate pH 7.5</td>
<td>1.6 M sodium citrate pH 6.5</td>
<td>40% (v/v) PEG 3000, 0.1 M phosphate-citrate buffer pH 7.2</td>
<td>30% (w/v) PEG 3000, 0.2 M MgCl$_2$, 0.1 M HEPES pH 7.5</td>
<td>40% (v/v) PEG 3000, 0.2 M Ca(OAc)$_2$, 0.1 M HEPES pH 7.5</td>
<td>20% (w/v) PEG 3350, 0.2 M Zn(OAc)$_2$, 0.1 M citrate pH 9.5</td>
<td>3.2 M Li$_2$SO$_4$, 0.1 M citric acid pH 5.0</td>
<td>0.2 M lithium chloride, 20% PEG 3350</td>
<td>0.2 M magnesium nitrate, 20% PEG 3350</td>
<td>0.2 M magnesium chloride, 20% PEG 3350</td>
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<td>20% (w/v) PEG 3350, 0.2 M ammonium formate pH 7.5</td>
<td>10% (w/v) PEG 8000, 8% ethylene glycol, 0.1 M HEPES pH 7.5</td>
<td>20% (w/v) PEG 3350, 0.2 M ammonium formate pH 8.3</td>
<td>10% (w/v) PEG 3350, 0.2 M NaCl, 0.1 M phosphate-citrate pH 8.3</td>
<td>14% 2-propanol, 0.14 M CaCl$_2$, 0.07 M citrate pH 4.6, 30% glycerol</td>
<td>10% 2-propanol, 0.2 M ZnOAc, 0.1 M MDP, 0.1 M citrate pH 6.5</td>
<td>20% MDP, 0.1 M Tris pH 8.0</td>
<td>1.0 M ammonium chloride, 20% PEG 3350</td>
<td>0.2 M sodium chloride, 20% PEG 3350</td>
<td>0.2 M lithium nitrate, 20% PEG 3350</td>
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### Robot Screen II

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<td>0.2 M tri-sodium citrate pH 7.2, 1 M NaCl, 0.1 M Tris pH 7.5, 40% PEG 3500</td>
<td>0.01 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.1 M calcium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.01 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
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<tr>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.1 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.1 M calcium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.01 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
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<tr>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.1 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.1 M calcium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M Tris pH 7.5, 5% PEG 4000</td>
<td>0.1 M sodium acetate pH 5.6, 25% (w/v) dextrose</td>
<td>0.01 M HEPES pH 7.5, 5% MDP</td>
<td>0.1 M sodium chloride, 0.1 M Tris pH 7.5, 5% PEG 4000</td>
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Note: The table continues with similar entries for other conditions.
### Robot Screen III

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**Chapter 8 - Appendix**
### Chapter 8 - Appendix

**Robot Screen IV**

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## Robot Screen V

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<td>0.3 M NaCl</td>
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<td>2 M NaSO₄</td>
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<td>20% PEG</td>
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<td>2 M NaSO₄</td>
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<td>2 M NaSO₄</td>
<td>0.3 M NaCl</td>
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<tr>
<td>I</td>
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<td>2 M NaSO₄</td>
<td>0.3 M NaCl</td>
<td>100% PEG</td>
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*Note: Concentration is expressed in % volume.*
The MORPHEUS protein crystallization screen [285]

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<td>10% PEG 3350, 0.02M MOPS, pH 7.5</td>
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Chapter 8 - Appendix
### 8.2 Crystallization fine screen formulations

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<td>A</td>
<td>6% PEG 4K,20% Glycerol, 0.02M AA, pH 7.1</td>
<td>8% PEG 4K,20% Glycerol, 0.02M AA, pH 7.3</td>
<td>10% PEG 4K,20% Glycerol, 0.02M AA, pH 7.5</td>
<td>12% PEG 4K,20% Glycerol, 0.02M AA, pH 7.7</td>
<td>14% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>15% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>15% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>15% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>15% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>10% PEG 4K,20% Glycerol, 0.02M AA, pH 7.5</td>
<td>12% PEG 4K,20% Glycerol, 0.02M AA, pH 7.7</td>
<td>14% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>20% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>20% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>20% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>10% PEG 4K,20% Glycerol, 0.02M AA, pH 7.5</td>
<td>12% PEG 4K,20% Glycerol, 0.02M AA, pH 7.7</td>
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<td>23% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>25% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>12% PEG 4K,20% Glycerol, 0.02M AA, pH 7.7</td>
<td>14% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
<td>0.15 M tri-sodium citrate, 15% PEG 3350</td>
<td>0.2 M tri-sodium citrate, 15% PEG 3350</td>
<td>0.25 M tri-sodium citrate, 15% PEG 3350</td>
<td>0.3 M tri-sodium citrate, 15% PEG 3350</td>
<td>30% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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<td>F</td>
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<td>8% PEG 4K,15% Glycerol, 0.02M AA, pH 7.3</td>
<td>10% PEG 4K,20% Glycerol, 0.02M AA, pH 7.5</td>
<td>10% PEG 4K,25% Glycerol, 0.02M AA, pH 7.7</td>
<td>0.15 M tri-sodium citrate, 20% PEG 3350</td>
<td>0.2 M tri-sodium citrate, 20% PEG 3350</td>
<td>0.25 M tri-sodium citrate, 20% PEG 3350</td>
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<td>G</td>
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<td>8% PEG 4K,20% Glycerol, 0.02M AA, pH 7.3</td>
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<td>0.15 M tri-sodium citrate, 25% PEG 3350</td>
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<tr>
<td>H</td>
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<td>0.2 M tri-sodium citrate, 25% PEG 3350</td>
<td>0.2 M tri-sodium citrate, 25% PEG 3350</td>
<td>0.25 M tri-sodium citrate, 25% PEG 3350</td>
<td>0.3 M tri-sodium citrate, 25% PEG 3350</td>
<td>34% PEG 4K,20% Glycerol, 0.02M AA, pH 7.9</td>
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8.3 Crystallization of recombinant mouse ERp44, soaking experiments with ERp44 crystals and WT36-45 as well as co-crystallization experiments of ERp44 and WT36-45

In order to solve the structure of ERp44 in complex with a short peptide mimetic of adiponectin (WT36-45), we first crystallized the apo ERp44 protein. In the next step, these crystals were soaked with peptide (WT36-45) in order to form complex with ERp44. Mouse ERp44 was produced in E. Coli, and purified as described in chapter 5.2.1. Crystallization screening was carried out (as described in chapter 5.2.9) and crystals were obtained after 2 - 3 days in the following conditions:

1) 12.5% PEG 1K, 12.5 % PEG 3350, 12.5% MPD, 0.02M AA, pH 7.5 [285]

2.) 1.4 M NaKPO$_4$, 10 % PEG 3350, 0.1 M Hepes pH 7.5

Since condition (1) contained 12.5 % MPD, these crystals were flash-cooled in liquid nitrogen without any additional cryoprotection. For condition (2), 10 different cryoprotectants were screened and the best diffraction was observed using 30% Sorbitol, 1.4 M NaKPO$_4$, 10 % PEG 3350, 0.1 M Hepes pH 7.5 as cryo-protectant. Crystals were exposed to the X-ray beam on MX1 beamline at the Australian Synchrotron. For both conditions, a full dataset was collected using the following data-collection parameters - 1° oscillation and 1 s exposure time. Diffraction patterns extended to approximately 3.30 Å. The diffraction data were processed using XDS [286]. Data were scaled using the program SCALA [287] and the integrated reflections were re indexed by POINTLESS [288], which suggested the space group P3$_2$121 and unit cell dimensions a = 84.79 Å, b = 84.79 Å, c = 128.98Å, α = 90°, β = 90°, γ = 120°. The structure of mouse ERp44 was solved by molecular replacement with PHASER [289], using the human ERp44 structure (PDB code: 2R2J) as a reference model. The structure was automatically build with Phenix [290] and the initial model was visualized in COOT [291]. The unrefined structure was compared with the structure of human ERp44 (PBD code: 2R2J) (Figure 8.3.1). Both structures appeared similar. This result established that we were able to produce well diffracting crystals of ERp44, which may be used to determine the structure of mouse ERp44, possibly at a better, sub 3Å resolution after improvement of crystal order. In the next step, we used these crystals for soaking experiments with the WT36-45 peptide. For this purpose, crystals were transferred into crystallization solution (1) or (2) supplemented with 5mM peptide and incubated for 16 h. Subsequently crystals were
subjected to the same procedure as the crystals obtained for the apo ERp44 protein and the initial structure was solved as described above. However, in none of the cases, any additional electron density around the peptide-binding site (C29) could be observed suggesting that the peptide did not form any complex with ERp44 even though ERp44 binds the peptide in solution (chapter 4.3.2).

![Figure 8.1: Comparision of the overall structure of human and mouse ERp44](image)
Structural superposition of human ERp44 (green) (PDB code: 2R2J) and mouse ERp44 (light blue). Both structures appeared to be similar.
Next we attempted co-crystallization experiments with the peptide. For this purpose, the purified ERp44 protein (213 µM) was mixed with WT36-45 (2.13 mM) before being subjected to the initial crystallization screening. After 2-3 days crystals with similar morphology as for the apo protein were obtained in the following conditions:

1) 3 M sodium formate, 4% PEG 8000, 0.1 M Imidazole pH 6.5

2) 1.6 M sodium citrate pH 6.5

Crystals were subjected to the same procedure as the crystals obtained for the apo protein. The crystals typically diffracted to a resolution of 3.2 - 3.8 Å. The initial structure was solved by molecular replacement using the same procedure as described above for the apo protein. However, again no additional electron density around the peptide-binding site of ERp44 (C29) could be observed indicating in contrast to the results from solution studies that the crystals were comprised of only apo ERp44.
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## References


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