Pro Gradu

Disulfide bond formation – the missing link between the HR and the UPR?

Marja Luukas

University of Oulu
Department of Biochemistry
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This work was done at the Department of Biochemistry, University of Oulu.
Oulu, Finland.

Supervisor:
Professor Lloyd Ruddock
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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
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<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BACE</td>
<td>Beta-secretase 1</td>
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<tr>
<td>BCL2</td>
<td>Apoptosis regulator Bcl-2</td>
</tr>
<tr>
<td>BiP</td>
<td>Immunoglobulin heavy chain binding protein</td>
</tr>
<tr>
<td>BNIP3</td>
<td>BCL2/adenovirus E1B 19 kDa protein-interacting protein 3</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>c-myc</td>
<td>Transcriptional regulator Myc-1</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydroascorbate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco/Vogt modified Eagle's minimal essential medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td><em>Escherichia coli</em></td>
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<td>EDEM1</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>eIF2α</td>
<td>Eukaryotic translation initiation factor 2</td>
</tr>
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<td>EPO</td>
<td>Erythropoietin</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERAD</td>
<td>ER-associated degradation</td>
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<tr>
<td>Ero1</td>
<td>ER oxireductin 1</td>
</tr>
<tr>
<td>ERp18</td>
<td>Endoplasmic reticulum resident protein 18</td>
</tr>
<tr>
<td>ERSEI</td>
<td>ER stress response element I</td>
</tr>
<tr>
<td>ERSEII</td>
<td>ER stress response II</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>FIH1</td>
<td>Factor-inhibiting HIF1</td>
</tr>
<tr>
<td>GLO</td>
<td>Gulonolactone oxidase</td>
</tr>
<tr>
<td>GRP78</td>
<td>Immunoglobulin heavy chain binding protein</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HERP</td>
<td>Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HR</td>
<td>Hypoxia response</td>
</tr>
<tr>
<td>HRD1</td>
<td>ERAD-associated E3 ubiquitin-protein ligase</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia responsive element</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IPAS</td>
<td>Inhibitory PAS domain protein</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol requiring kinase 1</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun amino terminal kinase</td>
</tr>
<tr>
<td>Luc</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Nuclear transcription factor Y</td>
</tr>
<tr>
<td>NIX</td>
<td>BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like</td>
</tr>
<tr>
<td>$\text{O}_2$</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>PHDs</td>
<td>Prolyl hydroxylases</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Prx IV</td>
<td>Peroxiredoxin-4</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>p300</td>
<td>Histone acetyltransferase p300</td>
</tr>
<tr>
<td>QSOX</td>
<td>Quiescin-sulfhydryl oxidase</td>
</tr>
<tr>
<td>RLuc</td>
<td>Renilla luciferase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RNase</td>
<td>RNA endonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RTP801</td>
<td>DNA damage-inducible transcript 4 protein</td>
</tr>
<tr>
<td>Sd</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Sd%</td>
<td>Percentage standard deviation of the average</td>
</tr>
<tr>
<td>SDA</td>
<td>Semihydroascorbate</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>SOX</td>
<td>Sulphydryl oxidase</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 serine protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 protease</td>
</tr>
<tr>
<td>Th</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>Tu</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UPRE</td>
<td>Unfolded protein response element</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau protein</td>
</tr>
<tr>
<td>VKOR</td>
<td>Vitamin K epoxide reductase</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein</td>
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1 REVIEW OF THE LITERATURE

1.1 Introduction

Aerobic organisms need oxygen (O$_2$) for energy production as well as other metabolic processes and hence hypoxia is the cause of severe stress to the cell. A hypoxic cell uses two routes to react to the demands of its newly hostile environment: It has to adjust itself to its environment and it will also try to signal to the organism to increase environmental oxygenation. Both of these responses happen simultaneously and form the initial hypoxia response (HR) of the cell. An activated HR can also trigger apoptosis, perhaps when the adaptive responses fail (Greijer & van der Wall 2004).

For the cell to survive in low oxygen concentrations it has to relinquish its aerobic high energy metabolism and shift to anaerobic glycolysis. Also, to accommodate this new low energy metabolism the cell has to reduce its energy consumption by slowing down its cellular processes. Any high energy demanding cellular processes like cell proliferation, DNA replication and protein synthesis are reduced or put on hold. The general slowdown of cellular processes increases the cells tolerance to hypoxia by aiding the maintenance of energy levels (Koumenis et al. 2002). For example, apart from the use of ATP for active ion pumps, protein production is the biggest energy sink of the cell and needs to be significantly slowed down. Indeed, a dramatic drop in the rate of protein synthesis has been observed in hypoxic cells – under severe hypoxia the ATP demand for protein synthesis has been recorded to drop to 7% of that of normoxic cells (Hochachka et al. 1996). In addition to the shift towards a low energy existence, the hypoxia response activates a wide variety of genes including VEGF (vascular endothelial growth factor) which is one of the cells attempts to better its environmental conditions (Koumenis et al. 2002).

The essential adaptations to hypoxic conditions are coordinated by various cellular pathways, including gene regulation by hypoxia-inducible factors (HIFs), the unfolded protein response (UPR) and signaling through the mammalian target of rapamycin (mTOR). mTOR acts as a central regulator of protein synthesis and ribosome biogenesis at transcriptional and translational levels by sensing and integrating signals from mitogens and nutrients (Wouters & Koritzinsky 2008). The UPR is a key response to cellular stress that is normally activated by the accumulation of unfolded proteins to the ER, but extreme hypox-
ia has also been demonstrated to activate the UPR (Koumenis et al. 2002). The activation of the UPR contributes to the slowing down of protein translation, as translation attenuation is one of the main functions of the PERK-branch of the UPR. This helps in the maintenance of energy levels of the cell and promotes survival, however the mechanism of UPR activation under hypoxia is not known (Wouters & Koritzinsky 2008; Majmundar et al. 2010).

Disulfide bond formation is one of the three most common post-translational modifications in human proteins. These covalent linkages formed between the side chains of cysteines residues provide stability for protein structure and are especially important for secreted proteins (Hatahet & Ruddock 2009). In eukaryotes catalyzed disulfide bond formation occurs in the lumen of the endoplasmic reticulum (ER) and the inter-membrane space of the mitochondria, the ER however being the main location (Riemer et al. 2009). A disulfide bond is formed in a catalyzed reaction where two dithiols form a covalent link by transferring two protons and electrons to the catalyst (Hatahet & Ruddock 2009). There are multiple potential catalysts and small molecules that can contribute to the formation of disulfide bond formation in vivo. All of them however use molecular oxygen as their terminal electron acceptor. Thus the availability of oxygen could affect disulfide bond formation and provide a link for a classical activation of the UPR under hypoxia via the accumulation of unfolded proteins.

1.2 Oxidative protein folding in eukaryotes

The formation of protein disulfide bonds is an oxidative reaction that is essential for the folding and maturation of many secreted and membrane proteins. It is a key rate limiting step in protein folding and one of the most common post translational modifications in human proteins. In vitro, disulfide bond formation is an extremely slow reaction that can take hours to form. However, such reactions are fast in vivo, where disulfide bond formation is a catalyzed reaction. In eukaryotes catalyzed disulfide bond formation occurs in the lumen of the endoplasmic reticulum (ER) and in the mitochondria, the ER however being the main location (reviewed in Hatahet & Ruddock 2009; Riemer et al. 2009).
1.2.1 The chemistry of disulfide bond formation

Disulfide bonds are covalent linkages formed between the thiol groups of two cysteine residues. Redox reactions are the driving force behind the formation of this bond. Redox reactions or reduction-oxidation reactions, primarily involve the transfer of electrons between two chemical species. In the reaction one chemical species goes to a higher oxidation state through the loss of electrons or gain of oxygen (is oxidized), while the other goes to a lower oxidation state through the gain of electrons or a loss of oxygen (is reduced). In the formation of a disulfide bond, the two thiol groups involved need to go from the -2 oxidation state of the sulphur atom to the -1 oxidation state. In the reaction two electrons and two protons are transferred to an oxidant (see figure 1A.). The simplest oxidant that can be used in the reaction is molecular oxygen which in the process of forming the disulfide is itself reduced to hydrogen peroxide (see figure 1B). The formation of disulfide bonds using molecular oxygen as the oxidant is a thermodynamically favoured process, and hence, a disulfide bond will form spontaneously as long as the two thiol groups are accessible. However non-catalyzed disulfide bond formation is very slow (Hatahet & Ruddock 2009).

\[
\text{A. } \quad 2\text{-SH} \quad \xrightarrow{\text{oxidant}} \quad -\text{S-S-} \\
\text{B. } \quad 2\text{-SH} + \text{O}_2 \quad \xrightarrow{} \quad -\text{S-S-} + \text{H}_2\text{O}
\]

Figure 1. Schematic representation of the formation of a disulphide bond from two cysteine thiol groups.
A. The sulphydryl groups need to go from the -2 oxidation state of the sulphur atom to the -1 oxidation state to form a disulfide bond. An oxidant is needed as an electron acceptor.
B. Using molecular oxygen as the electron acceptor results in the formation of hydrogen peroxide.

Disulfide bonds require a sufficiently oxidizing environment to form, which in eukaryotes is provided by the lumen of the ER. The ER is a eukaryotic organelle specialized to fold secretory proteins. Compared to the cytosol, the ER is equipped with a greater calcium concentration, a more oxidizing redox potential, and dedicated enzymes for protein folding and glycosylation (van Anken & Braakman 2005).

*In vivo* disulfide bond formation is a catalyzed process where multiple enzymatic oxidants co-operate with peptides and molecular oxidants to form an efficient oxidative machinery.
A disulfide bond can be formed between any two cysteine residues in a protein. With multiple cysteine residues, there are multiple different patterns of thiol/disulfide redox states that can be adopted by a folding protein. In order to attain the native pattern of disulfide bonds and free thiols for any protein containing more than two cysteines residues it is imperative to have a pathway to convert any non-native disulfide bonds into native ones. This is achieved by the isomerase function of protein disulfide isomerase (PDI) an enzyme that is also central in the oxidative reactions of disulfide bond formation. PDI is the only known enzyme involved in disulfide bond formation in which disruptions or knockouts are not tolerated. This is probably due to its double function in oxidation and isomerisation of disulfide bonds (reviewed in Hatahet & Ruddock 2009).

1.2.2 Oxidizing pathways in vivo

Until recently the main pathway in disulfide bond formation was thought to be driven by Ero1 (ER oxireductin 1), a conserved FAD-dependent enzyme, and PDI. In the model involving Ero1 and PDI Ero1 is oxidized by molecular oxygen forming hydrogen peroxide ($H_2O_2$) in the process (Tu & Weissmann 2004). Oxidized Ero1 then acts as a specific oxidant of PDI, which in turn directly oxidizes disulfide bonds in folding proteins (see figure 2). This protein relay will lead to the formation of one disulfide bond per oxygen molecule consumed (Baker et al. 2008). The coupling of molecular oxygen to disulfide bond formation via Ero1 has one potentially lethal side-effect – the production of hydrogen peroxide. Ero1 activity thus constitutes a potential source of ER-derived oxidative stress. The production of hydrogen peroxide combined with the need for the redox potential of the ER to be optimal for disulfide bond formation, implies that the activity of Ero1 must be tightly regulated. Indeed in oxidizing conditions the activity of Ero1α, an isoform of the Ero1 protein, is downregulated through the formation of regulatory disulfide bonds between non-catalytic cysteines and cysteines located within the first active site. The formation of this inactive Ero1α is controlled by the availability of reduced PDI providing a substrate for the active Ero1α (Appenzeller-Herzog et al. 2008; Bakker et al. 2008).
Figure 2. Schematic representation of the Ero1 – PDI pathway of disulfide bond formation.
Ero1 is oxidized by molecular oxygen and in turn acts as a specific oxidant of PDI, which then directly oxidizes dithiols to disulfide bonds in folding proteins. Because Ero1 activity generates H$_2$O$_2$, a powerful oxidant and a potential source of oxidative stress, Ero1 activity needs to be tightly regulated. Ero1 activity is regulated through the formation of regulatory disulfide bonds between non-catalytic cysteines and cysteines located within the first active site. The availability of reduced PDI affects the activity of Ero1. (Bakker et al. 2008; Appenzeller-Herzog et al. 2008)

Mammals have two Ero1 paralogs, Ero1α and Ero1β, and approximately 20 PDI family oxidoreductases, many of which can be oxidized by Ero1 (Schulman et al. 2010). The Ero1 driven pathway is a major contributor in disulfide bond formation in yeast (Frand & Kaiser 1998) but was recently shown to be dispensable in mammals. Knockout mice of both Ero1α and Ero1β are diabetic due to defects in insulin production and secretion, but numerous other essential functions requiring disulfide bond formation work normally (Zito et al. 2010). Thus other potential pathways contributing to disulfide bond formation must be considered. H$_2$O$_2$

There are multiple other enzymes, including peroxidases, peroxiredoxins, members of the sulfhydryl oxidase (SOX) family and vitamin K epoxide reductase (VKOR), that have the potential to act as a specific oxidant of PDI and thus promote disulfide bond formation. SOX, which include Ero1 family members, couple molecular oxygen to their cycle and produce one molecule of H$_2$O$_2$ per disulfide bond (Sevier et al. 2001; Thorpe et al. 2002.). The quiescin-sulphydryl oxidases (QSOX), a human family member of SOX, are able to introduce disulfide bonds directly into substrate proteins without the need to interact with additional proteins (Chakravarthi et al. 2007; Hecklera et al. 2007). The lumen of the ER is an oxidative environment where oxidants are generated and accumulated. These compounds can also act as electron acceptors receiving electrons from folding proteins or PDI to promote oxidative protein folding. Unknown relative contributions of the multiple potential pathways must contribute to the normal physiological pathway of disulfide bond formation.
1.2.3 Small molecules in oxidative protein folding

Glutathione (GSH) is a tripeptide that is composed of three amino acids connected in tandem: glycine, cysteine, and in an unusual γ-amide bond to its acidic group, glutamate (Meister & Tate 1976). Glutathione is the major endogenous antioxidant produced by cells. It participates directly in the neutralization of free radicals and reactive oxygen species (ROS), as well as in maintaining exogenous antioxidants such as ascorbate in the reduced forms. Glutathione exists in reduced and oxidized states, but is found almost exclusively in its reduced form in the cytoplasm due to a cytosolic NADPH-dependent reaction catalysed by glutathione reductase (Chakravarthi et al. 2006). While in the cytoplasm less than 1% of glutathione is present in its oxidized state GSSG, in the ER approximately 25% of glutathione is present as GSSG (Hwang et al. 1992; Bass et al. 2003). This makes the ER a more oxidizing environment and ideal for oxidative protein folding. In addition to functioning as a buffering system glutathione may have functions in disulfide bond formation. In vitro GSSG has been shown to efficiently oxidize the active site of PDI and the disulfide bond in GSSG can also be directly transferred to a folding protein (see figure 3) (Jessop & Bulleid 2004).

\[
2\text{-SH} + \text{GSSG} \rightleftharpoons \text{-S-S-} + 2\text{GSH}
\]

**Figure 3. Formation of a disulfide bond in folding proteins using glutathione.**
The reaction is a thiol-disulfide exchange reaction and there is no net increase in disulfide bonds.

In this reaction however, the dithiol is transferred from GSSG to the folding protein and there is no net increase of disulfide bonds. Before the discovery of Ero1 GSSG was thought to be the primary oxidant of PDI in vivo. Reduced glutathione on the other hand functions as a net reductant in the ER reducing non-native disulfide bonds in the folding proteins (Jessop & Bulleid 2004; Chakravarthi et al. 2006).

Another small molecule that has potential in the disulfide bond forming pathway is hydrogen peroxide (H₂O₂). Hydrogen peroxide is a potent reactive oxygen species produced as a by-product in normal oxidative metabolism as well as a result of sulfhydryl oxidase function discussed before. H₂O₂ is usually viewed as a harmful by-product leading to oxidative stress. However, H₂O₂ is a potent oxidant that can be used in the formation of disulfide
bonds via the formation of a sulphenic acid intermediate (see figure 4) (Cumming et al. 2004; Saurin et al. 2004).

\[
\begin{align*}
A. \quad & -\text{SH} + \text{H}_2\text{O}_2 \longrightarrow -\text{SOH} + \text{H}_2\text{O} \\
B. \quad & -\text{SOH} + -\text{SH} \longrightarrow -\text{S-S-}
\end{align*}
\]

**Figure 4. Hydrogen peroxide in the formation of a disulfide bond.**

A. The initial reaction between hydrogen peroxide and a cysteine thiol group is to form a cysteine sulphenic acid intermediate
B. The cysteine sulphenic acid intermediate formed in the previous reaction can undergo an intra- or intermolecular reaction with another cysteine thiol to generate a disulphide bond

It has been discovered that the addition of exogenous H\textsubscript{2}O\textsubscript{2} to cells results in the formation of sulphenic acids and disulfide bonds (Cumming et al. 2004; Saurin et al. 2004). In addition H\textsubscript{2}O\textsubscript{2} is able to directly oxidize the active site of PDI and also less efficiently GSH to GSSG (Karala et al. 2009).

There is a catalyzed pathway for H\textsubscript{2}O\textsubscript{2} production in the ER via the Ero1 pathway discussed before. H\textsubscript{2}O\textsubscript{2} can also be produced by ascorbate biosynthesis (not in humans) and other pathways, including mitochondrial metabolism (Sohal & Brunk 1992; Linster & Van Schaftingen 2007). By utilising the H\textsubscript{2}O\textsubscript{2} produced by Ero1 it is possible to make two disulfide bonds from one molecule of oxygen. This utilisation would make the system more efficient in disulfide bond formation and also eliminate the potential threat of H\textsubscript{2}O\textsubscript{2} as the source of oxidative stress (Nguyen et al. 2010). Recent results demonstrate that disulfide bond formation itself does not generate oxidative stress (Malhotra et al. 2008). This implies that there must be a way to use H\textsubscript{2}O\textsubscript{2} produced by Ero1, or extremely efficient control of enzyme function. Because H\textsubscript{2}O\textsubscript{2} is a potent oxidant that has been shown to oxidize the active site of PDI, the simplest utilization of H\textsubscript{2}O\textsubscript{2} involves Ero1 and the two active sites of PDI. Ero1 makes H\textsubscript{2}O\textsubscript{2} while oxidizing an active site of PDI, but PDI has two active sites that both can be in the reduced state ready to be oxidized. The H\textsubscript{2}O\textsubscript{2} molecule produced in the process could increase the probability of the second active site of PDI to be oxidized due to the high local concentration of an oxidant. There are however 20 human PDI family members that include single active site species such as ERp18 that interact with Ero1. Oxidation of such a single active site species would require an alternative mechanism for peroxide utilisation (Nguyen et al. 2010). Recently published PDI peroxidases and Prx IV could provide an efficient alternative mechanism for H\textsubscript{2}O\textsubscript{2} utilisation (Nguyen et al. 2010;
Tavender & Bulleid 2010; Zito et al. 2010). These proteins are able, using different mechanisms, to use \( \text{H}_2\text{O}_2 \) as an oxidant and by cycling via PDI promote disulfide bond formation. It has also been suggested that PrxIV acts as a monitor of the oxidative burden within the ER. It has been shown that PrxIV is able to utilize \( \text{H}_2\text{O}_2 \) produced by Ero1 and protect the ER from oxidative stress. PrxIV can however be hyperoxidized by an excess of \( \text{H}_2\text{O}_2 \) and inactivate. This inactivation would result in a sudden increase in the levels of \( \text{H}_2\text{O}_2 \) leading to oxidative stress in the ER. This could lead to an activation of the ER stress response and the UPR, but also mediate apoptosis through ER generated peroxidases (Tavender & Bulleid 2010).

Dehydroascorbate must also be included in the potential pathways for disulfide bond formation. Ascorbate is an important cellular antioxidant that has two physiologically relevant higher oxidation states, semihydroascorbate (SDA) and dehydroascorbate (DHA). DHA can be generated by the reaction of ascorbate with ROS or in a reaction of two SDAs. In humans there is no ascorbate synthesis, but in most other vertebrate species ascorbate is synthesised from lactone by L-gulonolactone oxidase (GLO). In humans ascorbate is a nutrient and recycling of the oxidized forms back to ascorbate plays an important role in maintaining adequate levels of the antioxidant (Linster & Van Schaftingen 2007). DHA is unstable in aqueous solutions and at a physiological pH is rapidly hydrolyzed to 2,3-diketogluconate. In vivo however, DHA can be nonenzymatically reduced to ascorbate by GSH, resulting in disulfide bond formation in the process. The reaction is thermodynamically favorable but kinetically slow. In addition DHA is able to react rapidly with dithiols found in unfolded or partially folded proteins. However no active processes are known to make disulfide bonds through the oxidation of ascorbate to DHA. It may be that disulfide bond formation is a passive byproduct linked to the role of ascorbate as an antioxidant in the ER (Saaranen et al. 2010).

Another vitamin potentially linked to disulfide bond formation is vitamin K, or more specifically an enzyme involved in its recycling, vitamin K epoxide reductase (VKOR). VKOR catalyzes the reduction of vitamin K so that it can be used as a co-factor in \( \gamma \)-carboxylation, an essential PTM for example in many clotting factors (Rost et al. 2004). In prokaryotes a homologue of VKOR is able to catalyze disulfide bond formation by substituting DsbB, an enzyme similar in function to Ero1 in the periplasm of bacteria (Dutton et al. 2008). The eukaryotic VKOR has been recently found to interact with PDI and thiore-
doxin (Trx)-like proteins thus providing the link between $\gamma$–carboxylation and disulfide bond formation (Wajih et al. 2007; Schulman et al. 2010). If the physiological reductant of VKOR is PDI VKOR present another alternative contributor to disulfide bond formation. However $\gamma$–carboxylation is not a high capacity pathway such as disulfide bond formation and cannot be fully accountable for oxidative protein folding (Margittai & Bánhegyi 2010). The eukaryotic VKOR has been poorly characterized, but other enzymes in the family use quinones in their redox reactions (Unden & Bongaerts 1997). Quinones are a class of cyclic organic compounds that are involved in a wide variety of biological and chemical processes, including the electron transport chain in aerobic respiration. Quinones are highly reactive compounds often using molecular oxygen as their terminal electron acceptor generating ROS in the process (Wang et al. 2005). If VKOR could utilize ER resident quinones in a reaction not coupled to $\gamma$–carboxylation as is done in prokaryotes, a high-capacity alternative route to oxidative formation of disulfides could be established.

Catalyzed disulfide bond formation in vivo can occur via the well established pathway involving Ero1 and PDI, in which molecular oxygen is used to oxidize Ero1 and subsequently PDI which in turn will catalyze disulfide bond formation. However there are multiple alternative pathways that could contribute in PDI oxidation and disulfide bond formation (summarized in figure 5). Unknown relative contributions of the multiple potential pathways must contribute to the normal physiological pathway of disulfide bond formation. However all of the pathways discussed lead to the conclusion that oxidative protein folding requires molecular oxygen and since inhibition of this process leads to a UPR, this could provide a direct link between hypoxia and UPR induction.
1.3 Unfolded protein response

Protein folding in the ER is a carefully controlled process in which a correctly oxidizing environment, chaperones and folding catalysts come together to give the folding proteins just the right push on their way towards a native conformation. However with this many moving parts and a delicate balance to keep there needs to be mechanisms in place to help the cell adapt to changes in the protein folding load of the ER and also to respond to any damage to the cell or the ER that will impair protein folding. The Unfolded Protein Response (UPR) is a cellular stress response that helps the ER fine tune its protein folding machinery and allows it to slow down and correct the situation when damage has occurred. There are three ER localized transmembrane signal transducers that activate to mediate the UPR: PKR-like endoplasmic reticulum kinase (PERK), inositol requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6). These three branches of UPR can affect the general level of translation as well as the expression of a number of folding catalysts and parts of the protein degradation pathway (Malhotra & Kaufman 2007).

Figure 5. Multiple oxidants in the formation of disulfide bonds.
The established pathway for disulfide bond formation involves the oxygen dependent Ero1 and PDI. That pathway can however be supplemented or replaced with multiple other potential oxidants including proteins and low molecular weight compounds. Abbreviations: Ero, ER oxireductin, PDI, protein disulfide isomerase, SOX, sulfhydryl oxidase, $H_2O_2$, hydrogen peroxide, $O_2$, molecular oxygen, DHA dehydroascorbic acid, ROS, reactive oxygen species, VKOR, vitamin K epoxide reductase. Adapted from Hatahet & Ruddock 2009.
The classical UPR results from the accumulation of unfolded proteins to the lumen of the ER, but it can also be activated by a wide variety of changes in the ER and the cellular environment. These changes include changes in the redox status and calcium concentration of the ER, nutrient deprivation of the cell, pathogen infections and hypoxia. There are still some ambiguity regarding how the accumulation of unfolded proteins is detected, but the general model involves the immunoglobulin heavy chain binding protein (BiP, GRP78) and the three transmembrane signal transducers of the UPR: PERK, IRE1 and ATF6. BiP is the most abundant molecular chaperone in the ER lumen and in nonstressed conditions is associated with all three ER stress sensors. This binding of BiP inhibits either dimerization needed for activation (IRE1 and PERK) or transport from ER to the Golgi, which is necessary for activating modifications (ATF6). BiP however is a chaperone able to bind the exposed patches of unfolded proteins. When there is an excess of unfolded proteins they are able to sequester BiP away from the three stress sensors enabling activation of the UPR. There are however indications that there is an active component in the release of ATF6 from BiP as well as involvement of unfolded proteins in the activation of IRE1. (Malhotra & Kaufman 2007; reviewed in Kohno 2010)

The most immediate response to ER stress is a transient attenuation of mRNA translation which will inhibit further crowding of the ER with unfolded protein. This attenuation of translation is mediated by PERK. Release of PERK from BiP results in dimerization and autophosphorylation. The phosphorylated PERK will subsequently phosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) inhibiting the formation of the ternary translation initiation complex required during the initiation phase of polypeptide chain synthesis. A lower level of active ternary complex will result in lower levels of translation (Harding et al. 1999; Harding et al. 2000; Scheuner et al. 2001). However, phosphorylation of the eIF2α is also required for the selective translation of activating transcription factor 4 (ATF4) which is involved in restoring ER homeostasis by promoting the expression of a number of chaperones, genes for amino acid transport and synthesis and anti-oxidative stress responses (Harding et al. 1999; Scheuner et al. 2001; Ron 2002).

IRE1 is the other kinase mediating the UPR. It is structurally similar to PERK and also undergoes dimerization and autophosphorylation upon BiP release. There are two homologues of IRE1 in mammals IRE1α and IRE1β that differ in tissue specificity (Tirasophon
et al. 1998; Wang et al. 1998). The activated IRE1 has site specific endonuclease (RNase) activity targeting the transcription factor X-box binding protein (XBP1). Upon activation IRE1 cleaves a 26 nucleotide intron from XBP1 mRNA causing a translational frameshift producing a larger form of the XBP1, protein with a novel transcriptional activation domain in its C-terminus (Yoshida et al. 2001; Calfon et al 2002; Lee et al. 2002). The spliced XBP1 is a transcription factor promoting the expression of a number of UPR target genes, mainly components of the ER-associated degradation (ERAD), chaperones and folding catalysts (reviewed in Malhotra & Kaufman 2007). IRE target genes are recognized via cis-acting elements called ER stress response element I and II (ERSEI, ERSEII) and the unfolded protein response element (UPRE) in the promoter regions of the target genes (Yoshida et al 1998; Mori et al. 2000; Patil & Walter 2001).

The third mediator of the UPR is ATF6, a transcription factor itself. In mammals there are two forms of ATF6 named ATF6α and ATF6β. They are both constitutively expressed as ER transmembrane proteins and bound to BiP under nonstressed conditions. Binding with BiP interferes with the signal responsible for their transport to Golgi. Activation of the transcription factor function requires release from BiP and transport to the Golgi where the cytoplasmic domains are released by two proteases, site-1 serine protease (S1P) and site-2 protease (S2P). The processed forms can then translocate to the nucleus and activate their target genes (Haze et al. 1999; Li et al. 2000; Yoshida et al. 2001). ATF6α and ATF6β activate their target genes in association with a constitutive nuclear transcription factor Y (NF-Y) (Li et al. 2000). ATF6α contributes significantly to UPR induced genes, whereas no target genes of ATF6β are known (Malhotra & Kaufman 2007). ATF6β has been implicated as a negative regulator of ATF6α (Thuerauf et al. 2004). The regulatory elements ERSEI, ERSEII and UPRE also mark the transcriptional targets of ATF6α (Yoshida et al 1998; Yoshida et al. 2001).
Figure 6. Activation of the unfolded protein response.
Three ER transmembrane proteins IRE1, ATF6 and PERK sense ER stress and mediate the UPR through their respective signalling cascades. BiP binds the luminal domain of IRE1, ATF6 and PERK under non-stressed conditions keeping them inactive. Accumulating unfolded protein in the ER lumen can sequester BiP away from the three stress sensors. Released from BiP IRE1 and PERK both undergo dimerization and auto-phosphorylation to activate their kinase activities. IRE1 also has RNase activities resulting in the selective splicing of XBP1 and the creation of a transcription factor targeting multiple UPR target genes including chaperones and components of ERAD. PERK activation results in phosphorylation of eIF2α leading to general translational attenuation and paradoxically induction of ATF4 translation. ATF4 is a transcription factor targeting multiple chaperones and proteins involved in maintaining ER homeostasis (see figure 6). The released cytoplasmic ATF6 is a transcription factor that is transported to the nucleus where it induces the expression of multiple UPR target genes including chaperones. All the ER stress sensors also contribute to ER stress induced apoptosis if the primary UPR fails to correct the protein-folding defect. Adapted from Malhotra & Kaufman 2007.

The primary response to ER stress involves translational attenuation and expression of UPR target genes all designed to help alleviate the stress on the protein folding machinery. Translation attenuation prevents further crowding of the ER by unfolded proteins, ERAD degrades permanently misfolded proteins and the expression of chaperones and various components of the ER help in protein folding and re-establishment ER homeostasis (see figure 6). The secondary response however is initiated to protect the organism, not the individual cell: If the UPR fails to resolve the protein-folding defect, apoptosis is initiated. The apoptotic signal is generated as a combination of the three UPR mediators. Induction of the proapoptotic transcription factor C/EBP homologous protein (CHOP) is induced via the PERK/eIFα pathway. The CHOP promoter also receives positive input from the ATF6 component of the UPR. An activated IRE1 can contribute to the proapoptotic signal by...
activating the apoptosis signal-regulating kinase 1 (ASK1)/c-Jun amino terminal kinase (JNK) leading to the activation of a proapoptotic caspase signalling cascade. The ER can also participate in proapoptotic signalling independent of the three branches of the UPR by a stress-induced Ca\(^{2+}\) release regulated by the BCL2 family of apoptosis regulator proteins. This can lead to a depolarization of the mitochondrial inner membrane and a mitochondrially induced proapoptotic caspase signalling cascade (reviewed in Malhotra & Kaufman 2007).

The UPR is a central cellular stress response that can be activated by a wide variety of changes ER and cellular environment in addition to the accumulation of unfolded proteins to the lumen of the ER. Severe hypoxia has been shown to induce activation of the UPR and the following translation attenuation has been linked to an increased viability in hypoxic conditions by reducing the energy requirement of the cell and thus aiding in the metabolic transition of the hypoxia response (Feldman et al. 2005; Wouters & Koritzinsky 2008). The molecular basis for UPR activation during hypoxia is not known. However if oxidative protein folding requires molecular oxygen and the inhibition of this process leads to a UPR, there is a potential direct link between hypoxia and UPR induction.

1.4 Hypoxia response

The hypoxia response (HR) is a cellular stress response initiated when organisms are subjected to a low oxygen environment. The HR leads to the activation of multiple cellular signalling pathways aiding in cell survival and re-establishment of viable environmental oxygenation. The HR activates a large array of glycolytic genes that switch cellular metabolism from aerobic to anaerobic. At the tissue level the HR attempts to re-establish tissue oxygenation by enhancing angiogenesis, the formation of new blood vessels, and by improving the oxygen-carrying capacity of the blood by increasing the expression of erythropoietin (EPO) (reviewed in Weidemann & Johnson 2008).

In mammalian cells the master regulator of the HR is the hypoxia-inducible factor (HIF). HIF is a heterodimeric transcription factor that consists of an oxygen-regulated α-subunit (HIF\(\alpha\)) and a constitutively expressed β-subunit (HIF\(\beta\) also known as ARNT, the aryl hydrocarbon receptor nuclear translocator). There are three paralogs of the α-subunit; HIF1\(\alpha\),
HIF2α and HIF3α. The three HIFα paralogs have different transcriptional functions creating important diversity to the HR. HIF1α is associated with acute responses to hypoxia whereas HIF2α has an important role in long term hypoxia (Holmquist-Mengelbier et al. 2006). HIF2α has also been implicated in cellular differentiation and stem cell control. The role of HIF3α is not well understood since no HIF target genes regulated by HIF3α have been recognized. It has been suggested however that HIF3α has a role as a negative regulator of the HR because a splicing variant of HIF3α, inhibitory PAS domain protein (IPAS), has been recognized as a strong dominant negative regulator of HIF-mediated signalling. All three HIFα proteins show similar oxygen regulated stability and they function in a complex with the constitutively expressed HIF subunit ARNT to activate their target genes in response to hypoxia (reviewed in Rankin & Giaccia 2008; Weidemann & Johnson 2008).

The oxygen regulated protein stability of the HIFα subunits is mediated by two mechanisms. In normoxic conditions soluble prolyl hydroxylases (PHDs) target the HIFα subunits for ubiquitylation and proteosomal degradation by hydroxylating two proline residues in the HIFα proteins. This increases the affinity of the HIFα subunits for the von Hippel-Lindau protein (VHL), which is the substrate recognition component of an E3 ubiquitin ligase mediating protein degradation. The PHDs contain Fe(II) in the active site and the hydroxylation process utilized by the enzymes depends on the availability of O2 and 2-oxoglutarate. Therefore the availability of molecular oxygen regulates the rate at which HIFα is hydroxylated and degraded (Kallio et al. 1999; Cockman et al. 2000; Tanimoto et al. 2000; Jaakola et al. 2001). The other mechanism regulating the activity of HIFα is mediated by factor-inhibiting HIF1 (FIH1). FIH1 mediates the hydroxylation of an asparagine residue in the C-terminal transactivation domains of HIF1α and HIF2α. In normoxia this hydroxylation correlates with repression of the HIF transactivation function. The hydroxylation function of FIH1 is also dependent on O2 and 2-oxoglutarate and it contains Fe(II) in its active site (Lando et al. 2002; Zhang et al. 2010). In hypoxic conditions HIFα protein is stabilized and transported to the nucleus where it forms a heterodimeric complex with ARNT. The HIFα transactivation domain binds two central co-activators CREB-binding protein (CBP) and p300 that are needed for the complex to function. An additional level of regulation is achieved by potential post-translational modification, sumoylation, of the HIF1α and ARNT subunits modulating HIF1α binding to VHL and the ability of ARNT to interact with additional partner proteins altering the function of the complex. The
important co-activators CBP and p300 can serve as a platform to recruit additional co-activators harbouring histone acetyltransferase and potentially histone deacetylase functions (reviewed in Lendahl et al. 2009).

In the nucleus the HIFα-ARNT complex will bind a specific hypoxia responsive element (HRE) in the promoter region of HR target genes (Wenger et al. 2005). The targets of HIF1α in the acute HR include genes involved in glycolytic metabolism, including glucose transporters, glycolytic enzymes, lactate production and pyruvate metabolism. In acute HR cell proliferation is stopped by inhibiting c-myc activity. A general downregulation of cellular energy consumption is needed when adapting to the low energy metabolism of glycolysis in the early stages of hypoxia. C-myc promotes cellular proliferation by regulating the expression of genes involved in cell cycle control including cyclins and cyclin kinase inhibitors. In chronic, long term hypoxia the situation is changed as HIF2α starts to promote c-myc activity to re-establish proliferation. Another important group of HIF target genes promote angiogenesis and blood oxygenation to improve the hypoxic environment of the cell. These targets include the vascular endothelial growth factor (VEGF-A) and erythropoietin (EPO) (reviewed in Rankin & Giaccia 2008). Under prolonged or extreme hypoxia apoptosis can also be initiated by the HR via induction of several proapoptotic target genes, including RTP801 and the BCL-2 family members of proapoptotic factors BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and Bnip3-like protein X (NIX) (Bakker et al. 2007).

The ability of the HIFs to promote cell proliferation and angiogenesis makes them advantageous in tumourgenesis. Angiogenesis is a prerequisite for the growth of a tumour beyond 1-2 mm in diameter and for tumour metastasis (Bast et al. 2000). The expression of HIF1α and HIF2α are in fact commonly increased in human tumours. HIF activity in tumours can be influenced by the tumour microenvironment: It has been estimated that 50-60% of solid tumours contain hypoxic areas because of an imbalance between oxygen supply and consumption in proliferating tumours. Mutations in several tumour-suppressor genes have also been implicated in HIF activation in cancer. The clearest example is the loss of VHL function in the von Hippel-Lindau disease. The connection between HIF and tumour growth makes the HIFs attractive targets for cancer therapy (Rankin & Giaccia 2008).
1.5 Monitoring the HR and the UPR

1.5.1 HRE and UPRE

One of the most well known targets of HIF is the erythropoietin (EPO) gene which regulates red blood cell production. Hypoxia can increase serum EPO levels up to several hundred-fold (Ebert & Bunn 1999). By studying EPO expression HIF1α and the hypoxia responsive element were first recognized (Semenza & Wang 1992). The hypoxia responsive element (HRE, 5’-TACGTGCT) is a consensus binding site for HIF and in EPO it is situated in the 3’-end of the gene. Functional HREs have been found in the promoter sequences of many hypoxia-inducible genes indicating a general role for this binding sequence (Miyazaki et al. 2002). HIF1α has the highest affinity for the HRE sequence, but HIF2α is actually responsible for endogenous EPO activation (Rankin et al. 2007). It has been suggested that HIF2α binding to the EPO HRE is modulated by additional nuclear factors that associate with the EPO gene. Consequently an increase in HIF2α binding has been obtained from a EPO-HRE-luciferase construct after the addition of a 223-bp enhancer fragment containing additional transcription factor binding sites (Warnecke et al. 2004). EPO HRE-based luciferase reporter assays, in which a linear combination of multiple HREs drives the expression of a luminescent protein firefly luciferase, have been the method of choice to monitor the activation of the cellular hypoxic response (Kimura et al. 2000; Rius et al. 2008). EPO expression itself is not a particularly good choice for monitoring activation of the HR, since its expression is highly cell-type specific with the primary expression sites being the liver and kidney. These are also the tissues where highest inducibility is achieved. Hence the reporter construct, often contains only a fragment of the EPO promoter sequence, including three copies of the HRE 5’-TACGTGCT and the supporting sequence 5’-CACAG. This makes the EPO HRE-based luciferase reporter a good indicator of the hypoxia response and HIF1α activation. Another widely used assay for HR activation is the detection of HIF1α protein by western blotting.

Similar assays exist to monitor the UPR. The complex cellular response of the UPR is mediated by a collaboration of three proteins: PERK, ATF6 and IRE1. ER-stress induced activation of ATF6 and IRE1 results in the release or formation of two transcription factors (ATF6 p50 and XBP1) that recognize their target genes by three binding sequences called the ER Stress Response Element I (ERSEI), ER Stress Response Element II (ERSEII) and
the Unfolded Protein Response Element (UPRE). The consensus binding sequence of ERSE I is CCAAT<sub>N9</sub>CCACG. For ERSE II the sequence is ATTGG<sub>N1</sub>CCACG and for UPRE TGACGTGG/A. For the activation of gene expression via ERSE I or ERSE II the CCAC side of the binding sequence must be bound by the activated ATF6/XBP1 while the other side of the response element is bound by NF-Y. These cis-acting response elements have been identified by mapping the promoters of a number of ER stress responsive genes such as Grp78 (BiP), calreticulin, HERP, EDEM1 and HRD1 (Yoshida et al. 1998; Roy & Lee 1999; Wang et al. 2000; Kokame et al. 2001; Yamamoto et al. 2004). There is some debate about which transcription factor (and in which combination) binds which binding sequence, but in general all are considered specific targets of an active UPR and suitable for luciferase-based reporter assays for activation. Other approaches for the detection of an activated UPR are XBP-1 splicing, in which the existence of XBP-1 mRNA (a spliced variant of IRE that acts as a transcription factor) is detected via total RNA extraction and RT-PCR, and by monitoring, by techniques such as western blotting, the expression levels of known UPR target proteins such as CHOP, ATF4 and Grp78 (Samali et al. 2010).

1.5.2 Luciferases

Firefly and renilla luciferase are two bioluminescent proteins that are widely used in reporter assays to study cellular responses. Both proteins produce light in a reaction with their substrate D-luciferin (firefly) or coelenterazine (renilla) and the intensity of the light produced is directly proportional with the concentration of the protein in the sample. In the experimental assays, expression of the reporter gene – here firefly or renilla luciferase – is put under the control of a promoter of a natively expressed protein of interest or a sequence that signals of the cellular response in question. In this way the expression of the reporter gene is directly proportional to the expression of the protein of interest and changes in the expression can be monitored. The assay is very sensitive and small changes in expression patterns and the intensity of responses can be monitored – percentual changes instead of on/off or large fold difference changes that some of the other commonly used methods like western blots or GFP-based methods are able to examine. Firefly and renilla luciferase and their substrates are not natively found in mammals so there is no threat of signal confusion or corruption. Another advantage of the luciferase assay is that the two proteins use different substrates and the reaction of firefly luciferase can be efficiently quenched allowing for
the use of both of these proteins in the same sample – one as a reporter of cellular responses and the other as a control reporter (Fan & Wood 2007; Thorne et al. 2010).

The experimental vectors used in our study consist of the firefly luciferase gene fused together with different promoters to create reporters exposing activity in the UPR and HR pathways. The UPR reporter promoter consists of six UPREs in tandem. The HR reporter has a modified promoter of the EPO gene containing three copies of a HRE fused with firefly luciferase. EPO is a downstream target of HIF1α, the primary regulator of the hypoxic response. For both assay systems a renilla luciferase reporter was used as an internal control for transcription efficiency and overall systemic effects on transcription and translation. This is especially important as both the HR and UPR down regulate protein production. The renilla luciferase gene is placed under a minimal SV40 promoter to create a constitutively expressing reporter construct.

Firefly luciferase (Uniprot ID P08659) is a single polypeptide chain of 550 amino acids, 60.7 kDa. It catalyses a monooxygenase reaction in which D-luciferin is converted to oxy-luciferin using ATP, O₂ and Mg²⁺ as a cofactor. The reaction produces green light with a wavelength of 562 nm. The protein is folded into two compact domains. The large N-terminal domain consists of a beta-barrel and two beta-sheets. The sheets are flanked by alpha-helices to form an αβαβα five-layered structure. The C-terminal portion of the molecule forms a distinct domain, which is separated from the N-terminal domain by a wide groove. There are four cysteines in the protein but none of them are surface exposed and should not pose a problem related to the redox altering treatments used in this study. No disulfides are formed and no PTMs are required for enzyme activity – functional protein can be produced in E. coli (Wood et al. 1984, de Wet et al. 1985).

Renilla luciferase (Uniprot ID P27652) is a monomeric protein of 310 amino acids, 36 kDa. The enzyme catalyzes the emission of visible light in the presence of oxygen and the luciferin coelenterazine or one of its analogues. This luciferase produces light with a wavelength of 480 nm. The protein structure demonstrates a α/β-hydrolase fold at its core. There are three cysteines in the protein and when purified from its natural source the protein is composed of 3% carbohydrate. However no PTMs are required for enzyme activity. (Matthews et al. 1977, Lorenz et al. 1991, Loening et al 2007)
In previous studies the importance of the three cysteines in renilla luciferase to enzyme function has been studied (Liu & Escher 1999). Each residue was individually mutated to alanine and one mutant contained all three mutations: wtRluc, C24ARluc, C73ARluc, C124ARluc and triple mutant were studied. C73A mutation abolished enzyme activity – in activity measurements C73ARluc and the triple mutant showed levels of light emission near background. In contrast C24A and C124A mutants were functional, with the C124A mutant showing bioluminescence levels higher than the wild type Rluc: a 10-fold increase in bioluminescence activity was found in C124ARluc compared with wtRluc at 37°C 48 hours after transfection. The increased level of luminescence seemed to be due increased stability of the protein; luminescence of the wtRluc started to decline 30 hours after transfection while light emission from C124ARluc remained relatively constant. An explanation for the stabilizing effect of the C124A mutation was presented by Loening et al. in 2006 & 2007 who, using computational predictions and later a crystal structure of the protein, discovered that the C124 residue is buried; in the case that the C124 residue is buried the stabilizing effect of the C124A mutation likely results from a better packing of the hydrophobic core of the protein. The C124A mutation is among the eight stabilizing mutations in the construct used for the crystal structure (Loening et al 2007). Since the role of the C124A mutation seems to be significant in the stability of renilla luciferase it was decided to make this mutation in the control reporter. From the crystal structure of Rluc it was clear that the second and third cysteines are buried, but for the first cysteine, the situation was not as clear. The C24 residue is close to the protein surface but pointing towards the centre of the protein. If it is able to rotate it may be solvent accessible and could be oxidized potentially altering enzyme activity. It was also decided to mutate the C24 cysteine to alanine to prevent any possible detrimental effects to enzyme activity due to redox sensitivity in the protein.
2 OUTLINES OF THE STUDY

Previously a link has been established between the hypoxia response and the unfolded protein response, with the discovery of a hypoxia induced UPR (Wouters & Koritzinsky 2008). Despite active research in the field the mechanism of UPR activation under hypoxia remains controversial. We hypothesized that the UPR activation under hypoxia, results from the same reason as the classical UPR – accumulation of unfolded protein in the ER. If oxidative protein folding requires molecular oxygen and the inhibition of this process leads to a UPR, there is a direct link between oxygen levels and hence hypoxia and UPR induction.

In the experimental work the effect of increased disulfide bond formation on the HR and the UPR was investigated in vivo. If disulfide bond formation utilises molecular oxygen, then the amount of disulfides or disulfide-bonded protein should influence oxygen consumption of the cell and the strength of the hypoxia response. To address this issue disulfide bond formation in the ER was increased via low DTT concentrations and by over-expressing a highly disulfide bonded protein. The effects of these treatments on the HR and the UPR in normoxia and hypoxia were quantified by using a luciferase reporter assay. Examination of other effects of UPR induction on the HR were also made by using other UPR inducers such as tunicamycin, thapsigargin or unfolded protein.
3 MATERIALS AND METHODS

3.1 Cell lines and culture conditions

Cell lines used in these experiments were HeLa (American Type Culture Collection CCL2), Hek293 (a gift from Thomas Kietzmann, University of Oulu) and HepG2 (European Collection of Cell Cultures 85011430). Cells were grown in DMEM-high glucose medium supplemented with Glutamax, 10% FCS, penicillin 100 units/ml and streptomycin 100 µg/ml. Cells were grown at 37°C in a normoxic atmosphere of 16% O₂, 79% N₂, and 5% CO₂ (by volume). Hypoxia treatment was performed at 37°C in an atmosphere of 5% or 3% O₂ with 5% CO₂ corrected to 100% with N₂ (by volume). For the luciferase assay cells were grown on 24-well plates. Cells seeded one day earlier were transfected according to the manufacturers’ protocol with 1.5 µl of Fugene6™ -transfection reagent and 400 ng of plasmids. After optimization 200 ng of reporter construct plasmid (UPR or HR) and 10 ng of control reporter plasmid were used for all transfections reported. When necessary 100 ng of protein expression plasmid (HSA or BACE) was used. Plasmid amount in all transfections was corrected to 400 ng by the addition of empty pcDNA3.1 plasmid. For each individual experiment four parallel samples were measured and compared with control cells transfected only with the reporter and control plasmids.

3.2 Treatments

All chemical treatments of cells were done 19 hours after transfection by changing the cell culture media to a treated one. Control cells also underwent a media change. Treatment lasted for five hours after which cells were harvested making the total time after transfection 24 hours. Dithiothreitol (DTT) was used to promote a UPR and to increase disulfide bond formation. A stock solution of DTT was made (1M) in sterile water and frozen in aliquots. An experimental concentration of 2 mM – 0.016 mM was used (see section 4.5). 5 µg/ml tunicamycin (1000x stock in DMSO) and 200 µM thapsigargin (1000x stock in DMSO) were also used as UPR inducers (see section 4.3). Antimycin A (1µg/ml) and rotenone (0.5µM) were used as mitochondrial respiration inhibitors (see section 4.8).
3.3 Plasmids

Three different luciferase-containing reporter plasmids were used in this study. The pGL3-Epo-HRE-Luc construct consists of three HREs from the promoter region of EPO inserted in front of a minimal SV40 promoter driving the expression of a firefly luciferase gene. The pGL3-Epo-HRE-Luc construct was a generous gift from Thomas Kietzmann and has been described previously (Kietzmann et al. 2001). The pGL3-5xATF6-UPRE-Luc construct consists of five UPREs in front of a minimal c-fos promoter driving the expression of a firefly luciferase gene. The plasmid was also a gift from Thomas Kietzmann. The pGL4.73[hRluc/SV40] vector puts a renilla luciferase gene behind a minimal SV40 promoter to create a consistently expressing control plasmid to check transfection efficiencies and general transcription/translation effects. The pGL4.73[hRluc/SV40] vector was acquired from Promega. In order to make the control reporter less redox sensitive two variants of the renilla gene were made by mutating one or two cysteines (C124A – the single mutant and C124A, C24A – the double mutant) of the renilla gene to alanine.

A beta-secretase 1 (BACE), isoform C containing plasmid was used as the native UPR-inducer in these experiments. This isoform of BACE has a deletion of amino acids 146-189 and does not fold properly. As a result it is forced to stay in a futile cycle of folding attempts in the ER constituting a massive natural UPR signal (Molinari et al. 2002; Molinari et al. 2003). The plasmid was a generous gift from Maurizio Molinari (Institute for Research in Biomedicine, Bellinzona Switzerland).

3.4 Cloning and mutagenesis

3.4.1 Renilla luciferase variants

Two mutants of the control reporter pGL4.73[hRluc/SV40] were made to improve protein stability and to reduce potential redox sensitivity. The single mutant containing a C124A mutation was made using the following primers:
Forward: GGGCTGCTCTGGCCTTTCACTACTCCTACGAG
Reverse: CTCGTAGGAGTAGTGAAAGGCCAGAGCAGCCC
Tm 78°C
The PCR reaction mixture contained in 1x Pfu reaction buffer 125 ng of forward and reverse primers, 200 μm of each dNTP, 30ng of template DNA and 2.5 U of PfuTurbo polymerase. The PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>30 s</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>1 min</td>
<td>18</td>
</tr>
<tr>
<td>68</td>
<td>8 min</td>
<td></td>
</tr>
</tbody>
</table>

To eliminate the unmutated template DNA the PCR product was treated with 10 U of DpnI restriction enzyme which cleaves methylated DNA. The treatment was done at 37° for one hour after which 3 μl of the DpnI treated DNA was used for transformation into XL-Blue CaCl₂ competent *E. coli*. Selection of transformed cells was done on Tetracycline-Thiamine-Ampicillin-plates. Colonies were picked for 2 ml overnight LB cultures containing the appropriate antibiotics and plasmid-DNA was isolated using Qiagens Miniprep Kit according to manufacturers’ protocol. DNA was sequenced and checked for the correct mutation and any unwanted extra mutations.

The mutant was sequenced by using the following four primers:
Sequencing 1: GTTAAATTGCTAACGCAGTCAGTGGGC
Sequencing 2: GATCTGATCGGAATGGGTAAGTCC
Sequencing 3: GACCATGCTCCCAAGCAAGATCATG
Sequencing 4: CTATTGTCGAGGGAGCTAAGAAGTTCC

The first sequencing primer attaches to the vector and the others follow about 400bp apart. The sequencing reaction mixture contained 0.16 μM of the primer, approximately 500 ng of template DNA and Big Dye Terminator Premix in 1x Big Dye terminator buffer. The PCR program was as follows:
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>20s</td>
<td>30</td>
</tr>
<tr>
<td>47</td>
<td>15s</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1min</td>
<td></td>
</tr>
</tbody>
</table>

To concentrate and de-salt the resulting sequencing products standard Ethanol precipitation procedure was followed before separating the fragments on an acrylamide gel for sequence detection. Precipitation was done by adding 1/10 volume of Sodium Acetate (3M) and 2.5x volume of absolute ethanol to the sample. Precipitation was done overnight at -20°C after which DNA was pelleted by centrifugation (4° 13 000 rpm). The supernatant was removed and the pellet was washed with cold 70% ethanol and finally dried in a vacuum. Fragment separation and sequence detection was done with an automated ABI sequencer. GeneDoc software was used for sequence alignment and Chromas for the visualization of fluorescence peaks from the sequencing reaction.

After a successful single mutation the second mutation (C24A) was done according to the same protocol in the C124A containing plasmid, but with the following primers:
Forward: GGGCCTCAGTGGTGGGCTCGCGCTAAGCAAATG
Reverse: CATTTGCTTAGCGCGAGCCCACCACCTGAGGCC
Tm 78°C

3.4.2 HSA

Human serum albumin (HSA) was used in this study to increase disulfide bond formation in the human cell by over expressing a heavily disulfide bonded protein. HSA contains 17 disulfide bonds. It was cloned from an image-clone (4734617) using the following primers:
Forward: TTTTTTTTGGATCCACCATGAAGTGGGTAACCTTTATTTCCCTTC
Annealing temperature of the complementary region = 63°C
Reverse: TTTTTTTTAAGCTTTTATTATAGCCCTAAGGCAGCTTGACTTG
Annealing temperature of the complementary region = 63°C

BamHI was used as a restriction site in the forward primer and HindIII in the reverse. The restriction enzymes were chosen to clone the gene to the multiple cloning site of
pcDNA3.1. The forward primer contains the Kozak consensus sequence CCACC to improve eukaryotic translation of the gene. The reverse primer contains a stop codon.

PCR reaction mixture contained in 1x Expand PCR buffer, 300 nM of both primers, 200 μM of each dNTP, 50 ng of the template DNA and 2.5 U of Expand enzyme. The PCR program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>15 s</td>
<td>25</td>
</tr>
<tr>
<td>60</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1 min 48 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Unlimited time</td>
<td>1</td>
</tr>
</tbody>
</table>

PCR product was checked by running a 30 μl sample of the product (with 1x loading dye and 1x SybrGreen for detection) on a 1% agarose gel. Product of the right size (around 1800 bp) was excised from the gel and purified with the Qiagen gel purification kit according to the manufacturers’ protocol. In order to clone the multiplied gene into the eukaryotic expression vector pcDNA3.1 both the insert and vector were cut with BamHI and HindIII:

<table>
<thead>
<tr>
<th></th>
<th>HSA (μl)</th>
<th>pcDNA3.1 (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>25.75</td>
<td>2 (2 μg)</td>
</tr>
<tr>
<td>10x Roche Buffer B</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>BamHI (10u/μl)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HindIII (40u/μl)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>H₂O</td>
<td>-</td>
<td>23.75</td>
</tr>
</tbody>
</table>

Reactions were done at 37°C for two hours after which products were separated on a 1% agarose gel. Products of the expected size were cut from the gel (about 1800 bp for the insert and 5.5 kbp for the digested vector) and purified according to the manufacturers’ protocol with the Qiagen gel purification kit. Ligation of the cut products was done in a 10 μl reaction with 5 μl of the cut insert, 3 μl of the cut vector and 1U of L4 ligase in 1x L4 ligase buffer. The reaction was done at room temperature for two hours after which it was
stopped by heat inactivation of the enzyme by incubating the samples at 65° for 15 minutes. The whole reaction mixture was used in a transformation to XL-Blue CaCl competent E. coli. Selection of transformed cells was done on Tetracycline-Thiamine-Ampicillin-plates. Colonies were picked for 2 ml overnight LB cultures containing the appropriate antibodies. Plasmid-DNA was isolated using the Qiagen Miniprep Kit according to the manufacturers’ protocol. A test restriction of the subsequent clones was done in a 10 μl volume to screen for correct sized products. 1 μl of plasmid DNA was cut with 5U of BamHI and HindIII enzymes in 1x Roche Buffer B. The reaction was done at 37°C for one hour after which products were separated on a 1% agarose gel.

Clones with the correctly sized bands (1800 bp and 5.5 kbp) were sequenced using the following primers:
Sequencing 1: GCACAGTTGCAACTCTTCGTGAAAC
Sequencing 2: CTTCTGCTGCACAGAGACTCAAG
Sequencing 3: GCAAGAAGGCATCCTGATTACTCTGTC
Sequencing 4: CACCAAATGCTGACAGAATCCTTGG

To sequence the beginning and the end of the gene primers specific for the vector were also used: pcDNA3.1F and pcDNA3.1R.

pcDNA3.1F: CTGGCTAACTAGAGAACCCACTGC
pcDNA3.1R: GCAAACAACAGATGGCTGGCAACTAG

These attach to the start of the multiple cloning site of the vector and after the multiple cloning site. Sequencing was done according to the same reaction mixture and PCR program as before. Samples were ethanol precipitated and air-dried before separation of the fragments on the ABI sequencer.

3.4.3 Subcloning renilla luciferase variants to pET23b

To investigate the effects of the C124A and C24A mutations on the renilla luciferase protein the three variants were cloned to a bacterial expression vector pET23b that has an N-terminal His-tag in frame with the NdeI site to attempt expression and purification of the protein as well as an investigation of the properties of the protein. To insert restriction sites compatible with the expression vector the following primers were used:
Forward: TTTTTTTTCATATGGCTTCCAAGGTGTACGACCCC
Annealing temperature of the complementary region = 66.5°C
Reverse: TTTTTTTTGGATCCTTATTACTGCTCGTTCTTCAGCAGCGC
Annealing temperature of the complementary region = 68.3°C

NdeI was used as a restriction site in the forward primer and BamHI in the reverse. The PCR reaction mixture contained 1x Expand PCR buffer, 300 nM of both primers, 200 μM of each dNTP, 50 ng of the template DNA and 2.5 U of Expand enzyme. The reaction program was as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>30 s</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Unlimited time</td>
<td>1</td>
</tr>
</tbody>
</table>

PCR product was checked by running a 30 μl sample of the product (with 1x loading dye and 1x SybrGreen for detection) on a 1% agarose gel. Product of the right size (around 1000 bp) was excised from the gel and purified with the Qiagen gel purification kit according to the manufacturers' protocol. In order to clone the multiplied gene into pET23b both the insert and vector were cut with NdeI and BamHI:

<table>
<thead>
<tr>
<th></th>
<th>Insert (μl)</th>
<th>pET23b (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>25</td>
<td>25 (2 ug)</td>
</tr>
<tr>
<td>10x Roche Buffer H</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NdeI (10u/μl)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BamHI (10u/μl)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Reactions were done at 37°C with BamHI added 30 minutes after NdeI to allow better cutting with NdeI. Total reaction time was two hours after which products were separated on a 1% agarose gel. Products of the expected size were cut from the gel (about 1000 bp for the insert and 3.5 kbp for the digested vector) and purified with the Qiagen gel purification kit.
according to the manufacturers’ protocol. Subsequent ligation and transformation were done as before. Selection of transformed cells was done on Tetracycline-Thiamine-Ampicillin-plates. Colonies were picked for 2 ml overnight LB cultures containing the appropriate antibodies. Plasmid-DNA was isolated and a test restriction of the subsequent clones was done in a 10 μl volume to screen for correct sized products. 1 μl of plasmid DNA was cut with 5U of NdeI and BamHI enzymes in 1x Roche Buffer H. The reaction was done at 37°C for one hour after which products were separated on a 1% agarose gel. Clones with the correctly sized bands (1000 bp and 3.5 kbp) were sequenced using the following primers specific for renilla luciferase:

Sequencing 2: GATCTGATCGGAATGGGTAAGTCC
Sequencing 3: GACCATGCTCCCAAGCAAGATCATG
Sequencing 4: CTATTGTCGAGGGAGCTAAGAAGTTCC

To sequence the beginning and the end of the gene primers specific for the vector were also used: pET23bF and pET23bR.

pET23bF: GCTGCCCGAGATCTCGATCCCGCG
pET23bR: GCCAACTCAGCTTCCTTTCGGGC

These attach to the start and the end of the multiple cloning site of the vector. Sequencing was done according to the same reaction mixture and PCR program as before. Samples were ethanol precipitated and air-dried before separation of the fragments on the ABI sequencer.

3.4.4 Cloning firefly luciferase to the renilla luciferase reporter plasmid

Finally to investigate firefly luciferase folding and function in the different cell lines used, the firefly luciferase gene from the UPR reporter was subcloned to the renilla luciferase reporter backbone. By cutting the plasmids with NcoI and BamHI the genes can be interchanged between the two. The plasmid backbones are highly similar with differences mainly in the promoter region. NcoI (ccatgg) includes the starting methionine of the luciferase genes in both plasmids thus enabling an in-frame exchange of the genes. BamHI cuts after the terminator sequence (an identical SV40 PA). Both plasmids were cut with NcoI and BamHI to isolate the firefly and renilla luciferase genes.
<table>
<thead>
<tr>
<th></th>
<th>UPR firefly luciferase (µl)</th>
<th>RLuc plasmid (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1 (1 ug)</td>
<td>2 (2 ug)</td>
</tr>
<tr>
<td>10x Roche Buffer H</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NcoI (10u/ul)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BamHI (10u/ul)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>H₂O</td>
<td>24</td>
<td>23</td>
</tr>
</tbody>
</table>

Restrictions were done at 37°C for two hours and the products were separated on a 1% agarose gel. Products of expected sizes (1600 bp for firefly luciferase and 3300 bp for the renilla reporter backbone) were cut out and DNA was isolated with the Qiagen gel extraction kit according to the manufacturer’s protocol. For the ligation 2 µl of the cut plasmid and 6 µl of the firefly luciferase gene insert was used. Otherwise ligation was done as before in a 10 µl reaction volume. The following transformation and isolation of plasmid DNA for clone screening was also done as described before. Positive clones were further checked by sequencing the plasmid-gene interface with the following plasmids that bind to the start of the multiple cloning site and after the gene of the renilla luciferase plasmid, now FFiR reporter.

Sequencing 1: GTTAAATTGCTAACGCAGTCAGTGCGGTC
Sequencing 4.2: CTTATCATGTCTGCTCGAAGCG
3.5 Recombinant protein production in *E. coli*

To investigate the redox sensitivity of the three renilla luciferase variants, the genes were cloned to a bacterial expression vector pET23b and transformed to the expression strain BL21 (DE3) pLysS RARE. A single colony was used for the starter culture which was done in 20 mls of LB with chloramphenicol-ampicillin selection. The cultures were grown overnight at 37°C, 200 rpm. The following morning bacteria were inoculated to a starting OD600 of 0.05 in 200 mls of LB with the correct antibiotics. Cultures were grown at 37°C, 200 rpm. The OD600 of the cultures were monitored regularly. At OD600 of 0.4 expression of the recombinant protein was induced with 1 mM of IPTG. Induction was allowed to proceed for four hours after which the final OD600 was recorded and the cells pelleted by centrifugation at 12 000 g for 10 minutes. The cells were resuspended to the Ni-NTA spin kit lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole pH 8.0) to an equivalent of OD600 of 10. 1x Dnase and 0.1 mg/ml lysozyme were also added and the cells were frozen at -20°C.

To try and increase the expression levels of soluble protein the protocol above was modified in the following ways: The cultures were grown at a reduced temperature of 30°C and when this did not help expression was tried in minimal media instead of LB at two different temperatures 37°C and 30°C.

3.6 Purification of His-tagged protein

Purification of the His-tagged recombinant renilla variants was done with the Qiagen Ni-NTA Spin Kit according to the manufacturers’ protocol for protein purification under native conditions from *E. coli* lysates. The cell lysates stored at -20°C were thawed at room temperature, 100 rpm for 30 min. A 1 ml sample of the total lysate was saved for further analysis. The soluble fraction was separated by centrifugation at 16 000 g for 15 minutes at 4°C. A 1 ml sample was saved for later analysis and the remaining lysate was loaded to the Ni-NTA spin columns.
3.7 SDS-PAGE

To analyse the recombinant protein produced in *E. coli* 12.5% SDS-PAGE gels were cast. Samples were prepared by adding 1x of the sample buffer (25 mM Tris pH 6.8; 10% glycerol; 1% SDS; 0.01% bromphenol blue). For reducing samples 5% of β-mercapto-ethanol was also added to the samples. The samples were boiled for 5 minutes at 95°C before loading on to the gel. For the redox sensitivity test of the purified renilla variants the protein samples were treated with 1 mM H₂O₂ or 1 mM GSSG for 15 minutes at room temperature before the addition of the sample buffer and boiling of the samples. At least two empty lanes were left between non-reducing and reducing samples.

3.8 Luciferase assay

Firefly luciferase and renilla luciferase use different substrates in their luminescent reaction. In addition, the luminescence of firefly luciferase can be quenched without affecting the activity of renilla luminescence. Thus the amount of each active protein can be individually defined. For the luciferase assay cells were seeded to 24-well plates and transfected with 200 ng of the firefly luciferase reporter and 10 ng of the renilla luciferase reporter 24 hours after seeding. The total amount of DNA was 400 ng for each individual well – empty pcDNA3.1 plasmid was used to adjust the amount of DNA to 400 ng. 24 hours after transfection the cells were washed with PBS and lysed with 1x passive lysis buffer of the Dual-luciferase reporter assay system from Promega. The cells were lysed for 20-30 minutes at room temperature with rocking on a platform shaker. The lysate was collected to sterile eppendorf-tubes and snap-frozen in liquid nitrogen to improve lysis. 10 μl of the lysate was used for luminescence measured on a white 96-well plate. To induce the first luminescent reaction, 50 μl of Luciferase Assay Reagent II containing the luciferin substrate of the firefly luciferase was added to the sample, the sample was mixed and firefly luminescence quantified. The Stop & Glo Reagent of the kit quenches firefly luminescence and provides the coelentenzine substrate for renilla luciferase enabling quantification of renilla luminescence without disturbance from firefly luminescence. After quantifying luminescence of firefly luciferase, 50 μl of the Stop & Glow reagent was added to the sample, the sample was mixed and renilla luminescence quantified. Renilla luminescence is much stronger than firefly luminescence. To avoid interference from adjacent renilla sam-
ples, firefly luminescence of all samples on the 96-well plate were measured first before inducing renilla luminescence.

3.9 Flow-cytometry

For flow cytometric analysis HeLa and HepG2 cells were grown on 24-well plates. Hek293 cells were excluded from this experiment because they detach easily from the culture plate making the multiple washes involved in probe loading difficult. 24 hours after seeding, the cells were rinsed three times with PBS and loaded with the fluorescent probe Phen Green (20 μM) or FluoZin (10 μM) at 37°C for 30 minutes in the following loading buffer: 137 mM NaCl, 5.7 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 0.4 mM KH2PO4, 0.4 mM MgSO4, 0.3 mM Na2HPO4, 25 mM HEPES, pH 7.3 (Ma et al. 2006). The cells were then washed five times with PBS and detached from the plate by incubating them with 500 μl Trypsin-EDTA solution (0.5 mg/ml Trypsin and 0.02% EDTA in PBS) for 5 min at 37°C. Finally 0.5 ml of PBS containing 10% FCS and any experimental additives (DTT or bipyridine) was added and the cells incubated on ice for 15 minutes before measurements.

For each sample, the green (emission maxima of Phen Green and FluoZin 532 nm and 516 nm) fluorescence of 5000 cells was analyzed using a CyFlow flowcytometer (Partec). Nontreated cells, without probe loading, were used as a negative control. Nontreated samples were included in all sample sets at least in duplicates. To analyze the mean fluorescence intensity of cells containing the fluorescent probe gates were assigned using FloMax software (Partec). The concentrations of probes used for loading were determined with a preliminary screen of different concentrations: 1 μM; 5 μM; 10 μM; 15 μM and 20 μM for Phen Green and 0.1 μM; 1 μM; 10 μM and 15 μM for FluoZin. Separation of autofluorescence and probe fluorescence was best in the 15 μM and 20 μM samples of Phen Green and 20 μM was used in further experiments. For FluoZin no adequate separation of the two peaks could be made making reliable detection of changes impossible.
3.10 Statistical significance

Variation within a sample set was tested by estimating standard deviation (sd) or percentage standard deviation of the average (sd%) in Microsoft Office Excel. Statistical significance of an effect was tested with a two-tailed heteroscedastic Student's t-Test in Microsoft Office Excel. A statistically significant value was defined as $p < 0.05$. 
4 RESULTS AND DISCUSSION

4.1 Establishing the renilla luciferase control reporter

The purpose of this study was to establish a link between two central cellular stress responses the UPR and the HR via disulfide bond formation. Changes in UPR and HR were detected with a luciferase based assay with experimental firefly luciferase reporters specific for each stress response and a renilla luciferase based control reporter to detect changes in transfection efficiency and any general effects on protein transcription/translation. Renilla luciferase contains three cysteines of which one (C73) is important for structure and/or function. Protein folding in the ER and hence the UPR are sensitive to changes in the redox environment. As part of our studies on UPR activation we wanted to study this, including the use of reducing agents such as DTT. It is also likely that the redox state of the cell will change in response to hypoxia. Hence the control protein used should be redox insensitive. From the literature it is evident that a C124A mutation increases protein stability and hence luminescence. For this reason a C124A mutant was made. The other cysteine residue in renilla luciferase that is not important to function is located on the surface of the protein and as such is potentially redox sensitive. To eliminate this potential problem a second mutant (C124A, C24A) was made in the C124A background. The effects of these mutations on the luminescence of the reporter were checked with and without co-expression of the experimental UPR reporter firefly luciferase.

Table I. Control experiments for firefly and renilla luciferase signals and the effects of mutations on renilla luminescence.

<table>
<thead>
<tr>
<th>Firefly reporter</th>
<th>Renilla construct</th>
<th>Firefly signal, mean (% sd)</th>
<th>Renilla signal, mean (% sd)</th>
<th>Relative renilla signals ±sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>wt</td>
<td>43 (19)</td>
<td>563*10^3 (9)</td>
<td>1.00 ±0.09</td>
</tr>
<tr>
<td>-</td>
<td>C124A</td>
<td>41 (20)</td>
<td>2430*10^3 (10)</td>
<td>4.31 ±0.85</td>
</tr>
<tr>
<td>-</td>
<td>C24A, C124A</td>
<td>45 (14)</td>
<td>1740*10^3 (7)</td>
<td>3.10 ±0.80</td>
</tr>
<tr>
<td>UPR</td>
<td>wt</td>
<td>760*10^3 (6)</td>
<td>989*10^3 (5)</td>
<td>1.00 ±0.05</td>
</tr>
<tr>
<td>UPR</td>
<td>C124A</td>
<td>940*10^3 (4)</td>
<td>4810*10^3 (4)</td>
<td>4.86 ± 0.68</td>
</tr>
<tr>
<td>UPR</td>
<td>C24A, C124A</td>
<td>869*10^3 (7)</td>
<td>3660*10^3 (8)</td>
<td>3.71 ± 0.52</td>
</tr>
<tr>
<td>UPR</td>
<td>-</td>
<td>1180*10^3 (10)</td>
<td>897 (17)</td>
<td>-</td>
</tr>
</tbody>
</table>

The effect of two mutations C124A and C24A on the luminescence of the control reporter renilla luciferase was tested with and without co-expression of the experimental UPR reporter firefly luciferase. The reporter was also expressed on its own as a control of signal separation. Values given are the mean values of eight separate measurements with the standard deviation expressed as a % of the mean (% sd). Also reported are renilla signals normalized to the wild type variant and the standard deviations of these values.
The results presented in table I provide an important control for the dual-luciferase reporter assay. The results indicate that there is no signal interference between the two reporters; in the absence of a firefly reporter the background luminescence recorded is 0.007 – 0.002 % of renilla luminescence and in the absence of a renilla reporter the luminescence recorded is 0.076 % of the firefly luminescence. From the results presented in table I it is also evident that renilla luminescence significantly improves after the addition of the C124A mutation – there is a four to five fold increase in luminescence with the mutation in question. This is most likely due to the increased stability of the protein as reported in the literature (Liu & Escher 1999; Loening et al. 2006). The second mutation, C24A, designed to reduce the redox sensitivity of the reporter seems to reduce the maximal luminescence slightly, but the increase to the wild type renilla is still three to four fold. There is also a slight increase in the overall luminescence with the addition of a second reporter, but since there is no statistically significant difference in the relative values and since the controls show minimal cross-talk, the increase in signal is not likely to be caused by interference by the firefly luciferase. The increase in the overall signal might result from an increase in cellular protein production brought on by the introduction of the second expression vector.

Since the second renilla mutant (C24A, C124A) was made to reduce the potential for redox sensitivity in the protein, the effect of the reducing agent DTT on the luminescence of the double mutant was tested (see table II). In the experiments with DTT as a inducer of disulfide bond formation and the UPR the maximal concentration of DTT to be used was 2 mM (see section 4.5). Here, the direct effect of 2 mM DTT on renilla luminescence was tested by adding the agent post-lysis, to eliminate any cellular responses affecting the luminescence. A non-treated, renilla and UPR-firefly co-transfected sample was divided into two after the lysis of the cells and 2 mM DTT was added to one of the samples. A comparison was made with cell lysates from two cell lines HeLa and Hek293. There was no statistically significant effect on renilla luminescence or the renilla normalized luciferase with 2 mM DTT.
Table II. Renilla double mutant under reducing conditions.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sample</th>
<th>Firefly signal mean (%sd)</th>
<th>Renilla signal mean (%sd)</th>
<th>Renilla normalized signal (%) ±sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>NT</td>
<td>3280 (8)</td>
<td>1030*10^3 (6)</td>
<td>0.316 ±0.20</td>
</tr>
<tr>
<td>HeLa</td>
<td>DTT</td>
<td>3560 (8)</td>
<td>1020*10^3 (10)</td>
<td>0.350 ±0.10</td>
</tr>
<tr>
<td>Hek293</td>
<td>NT</td>
<td>675*10^3 (7)</td>
<td>1240*10^3 (8)</td>
<td>54.4 ±0.49</td>
</tr>
<tr>
<td>Hek293</td>
<td>DTT</td>
<td>750*10^3 (7)</td>
<td>1380*10^3 (7)</td>
<td>54.4 ±2.55</td>
</tr>
</tbody>
</table>

The effect of DTT on the luminescence of a renilla double mutant (C24A, C124A) was tested. The protein was expressed in two human cell lines and the cell lysates were divided into two samples. One of the samples was treated with 2 mM DTT, while the other remained non-treated (NT) before recording luminescence. The experimental reporter used was UPR. Values given are mean values of eight measurements with the standard deviation expressed as a % of the mean (%sd). A renilla normalized firefly luciferase (UPR) signal is also presented expressed as a percentage of renilla luminescence with values of standard deviation (±sd).

To examine the potential redox sensitivity of the different renilla mutants at the molecular level the genes were cloned into a bacterial expression vector, protein was expressed in *E. coli* and purified. Purified protein variants were treated with the oxidants GSSG or H$_2$O$_2$ and nonreducing and reducing samples were visualized on SDS-PAGE. Nonreducing and reducing samples without any additional treatments were used as controls. Images of the purification process and the tests are presented in figures 6 and 7.

Figure 6. Bacterial expression of renilla luciferase variants and purification.
The three variants of renilla luciferase were subcloned into a bacterial expression vector where the protein was fused with a His-tag. The variants were expressed and purified with Ni-NTA columns for further testing. The molecular weight of the markers (in KDa) is indicated.
A. Total and soluble samples of the wt, double mutant and C124A renilla luciferase
B. Purification fractions of the double mutant: total, soluble, flow through, wash, and the elution fractions of the wt, double mutant and C124A
Renilla luciferase is not expressed in *E. coli* as a fully soluble protein. In fact, the wild type protein is mostly insoluble (figure 6A, lanes 1&2) which indicates serious folding issues related to expression in a prokaryotic expression system. This could be due to the lack of glycosylation in the *E. coli* expressed protein that would normally assist in keeping the protein soluble: renilla luciferase is composed of 3% carbohydrate when purified from natural sources (Matthews *et al.* 1977). However according to the literature it is possible to attain functional protein from *E. coli* so any potential PTMs that are missing upon heterologous expression should not affect protein function (Lorenz *et al.* 1991, Liu & Escher 1999).

The two mutations tested here affected the amount of soluble protein produced (see figure 6). The C124A mutation increased the yields of soluble protein considerably. The C24A mutation on the other hand reduced the yields of soluble protein counteracting any advantage brought by the C124A mutation. It is possible that by changing the surface exposed cysteine to an alanine the hydrophobicity of the protein surface was increased. This could increase inter molecular interactions resulting in aggregation. It is also possible that a folding intermediate of the protein was destabilized resulting in increased aggregation. As the signal acquired from the eukaryote expressed double mutant was three to four fold higher than that of the wild type variant (see table I) it can be assumed that the folding issues encountered in *E. coli* are not present in the eukaryotic system.

The soluble wild-type and mutant proteins were then purified using Ni-NTA spin columns. These did not give the expected level of purity (figure 6B) suggesting interactions between the renilla protein and *E. coli* proteins. This is often observed for non-native or incorrectly folded proteins. The reduced solubility of the double mutant also affected purification as can be seen from the yields from figure 6B. Since there was much less soluble protein to concentrate and purify the relative purity of the elution fraction of the double mutant is inferior to that of the two other variants. Attempts were made to increase the yield of the soluble protein, such as reducing the expression temperature and expression in minimal media. However, these were not successful and so these samples were used to examine the redox sensitivity of the protein.
Figure 7. Testing the effect of reducing conditions on the renilla variants.
A. Elution fractions of the original, double mutant and C124A under non-reducing and reducing conditions.
B. Elution fractions of the original, double mutant and C124A under non-reducing and reducing conditions, having been previously treated with H2O2. Regions with change due to treatment are indicated.
C. Elution fractions of the original, double mutant and C124A under non-reducing and reducing conditions, having been previously treated with GSSG.
The molecular weight of the markers (in KDa) is indicated.
The renilla variants expressed in *E. coli* were purified with Ni-NTA columns and the resulting protein fractions subjected to different reducing conditions. Due to problems with protein purity (figure 6B) it is difficult to estimate the significance of the larger molecular weight compounds observed in the samples. The purified renilla protein exists mostly as a monomeric protein running just below its expected weight marker of 35 kDa (see figure 7). However there are signs of higher molecular weight bands reacting to treatment with H$_2$O$_2$ (see figure 7B, the area marked with lines). There is also a slight increase in the strength of the monomeric protein band in the wild-type and single mutant renilla in the reducing SDS-PAGE for samples previously treated with H$_2$O$_2$ (see figure 7B, WT and C124A samples). This could indicate that the wild-type and single mutant proteins interact with *E. coli* proteins or themselves through mixed disulfides. The double mutant renilla was made to reduce such interactions. However, the quality of the double mutant sample does not allow for the investigation of potential protein-protein interactions.

### 4.2 Cell line differences in the UPR and the HR

In order to investigate the relationship between the UPR and the HR the normal cellular responses needed to be determined. Using the renilla control reporter it became evident that the strength of the resting level UPR varied greatly between different cell lines (see table III). The UPR signal normalized to renilla is 54% in Hek293 but only 0.3% in HeLa suggesting a difference of more than 100-fold. To find out more about the relative strengths of the UPR and the HR between different cell lines, the resting levels were determined in three cell lines; HeLa, Hek293 and HepG2. The results (table III) are presented as relative signals normalized to Hek293.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HR/Renilla ±std</th>
<th>UPR/Renilla ±std</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.014 ±0.004</td>
<td>0.003 ±0.001</td>
</tr>
<tr>
<td>Hek293</td>
<td>1.00 ±0.12</td>
<td>1.00 ±0.21</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.114 ±0.06</td>
<td>0.154 ±0.06</td>
</tr>
</tbody>
</table>

The resting level of HR and UPR responses was investigated between the three cell lines by co-expressing the experimental reporters HR and UPR with the renilla luciferase control reporter. The results are presented as relative values normalized to Hek293 signal with standard deviations. Reported values are the average of eight independent samples.
The cell line differences for the renilla normalized HR and UPR signals are considerable (table III). There is a 9-fold difference in the resting level of the HR between Hek293 and HepG2. For the UPR the difference between Hek293 and HepG2 is fairly similar with a 6-fold difference in signal. For HeLa however the differences are huge: a 71-fold difference in the resting level of the HR between Hek293 and HeLa and a 300-fold difference in the UPR. To find out if the difference in signal results from folding and function issues of firefly luciferase or if there really is a big difference in the basal level of these central stress responses a new reporter was constructed. To form the new reporter firefly luciferase was subcloned to the renilla reporter backbone in order to make a constitutively expressing firefly luciferase reporter. As renilla and firefly luciferase are two different luminescent proteins with different size and luminescent properties the signal from the two reporters is not expected to be the same even if they are expressed from the exact same vector backbone. However, the ratio of firefly luciferase signal to renilla luciferase might be expected to remain fairly similar between different cell lines, unless there are cell-specific effects. The relative signals of the two constitutively expressing reporters were investigated in the three cell lines. The results (table IV) are shown as relative signals normalized to Hek293.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative FFiR/Renilla ±std</th>
<th>Normalized FFiR/Renilla ±std</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.016 ±0.004</td>
<td>0.061 ±0.01</td>
</tr>
<tr>
<td>Hek293</td>
<td>0.266 ±0.03</td>
<td>1.00 ±0.10</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.074 ±0.02</td>
<td>0.280 ±0.06</td>
</tr>
</tbody>
</table>

The relative expression of firefly luciferase to renilla luciferase was investigated in three cell lines by co-expression of two constitutively expressing control reporters (200ng of FFiR and 10 ng Renilla reporter per sample). The results are presented as relative values and relative values normalized to Hek293 with standard deviations. Reported values are the average of eight independent samples.

From the absolute values presented in table IV it can be seen that firefly and renilla luciferase give very different signals even though they are expressed from the same vector backbone: firefly luciferase gives only 1.3% of renilla signal in Hek293 (table IV, relative Hek293 sample has 200 ng of firefly luciferase but only 10 ng renilla luciferase transfected 0.266/20 = 1.3%). It is also clear that the ratio between firefly luciferase and renilla luciferase is not the same between the three cell lines. From the Hek293 normalized FFiR/Renilla values it can be calculated that there is a 16-fold difference in the relative active levels of firefly luciferase and renilla between HeLa and Hek293 from the same plasmid. The difference between Hek293 and HepG2 is four fold. This could be due to
differential folding or activity of the luciferases in the different cell types. However, the differences in renilla normalized HR and UPR signal are bigger than the differences found here in firefly luciferase folding and/or function (compare tables III and IV) indicating that there are cell typical differences in the resting levels of the HR and the UPR. To confirm this, parallel single transfections of the HR, UPR and FFiR reporters were made in the three cell lines (table V). Relative signals were calculated and are presented as relative signals normalized to Hek293.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HR/FFiR ±std</th>
<th>UPR/FFiR ±std</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.154 ±0.01</td>
<td>0.03 ±0.05</td>
</tr>
<tr>
<td>Hek293</td>
<td>1.00 ±0.27</td>
<td>1.00 ±0.15</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.826 ±0.25</td>
<td>0.521 ±0.01</td>
</tr>
</tbody>
</table>

To further investigate the cell line differences in basal HR and UPR signals parallel single transfections of the experimental firefly reporters (HR & UPR) were made with the constitutively expressing firefly luciferase reporter (FFiR). Relative expression levels were calculated and are presented here as relative signals normalized to Hek293 with standard deviations. Reported values are the average of eight independent samples.

By comparing the firefly luciferase control reporter to an experimental vector it is evident that there is a level of signal repression that is not due to the reporter: There is a 6-fold difference in the HR signal between HeLa and Hek293 (HR/FFiR) but only a slight difference between Hek293 and HepG2. With the UPR the differences are again bigger: there is a 30-fold difference in HeLa compared to Hek293 (UPR/FFiR) but a modest two fold difference between Hek293 and HepG2. By taking into account that firefly luciferase is expressing or folding differently in the three cell lines similar levels of repression can be calculated from the UPR/Renilla and HR/Renilla values presented in table III. The differences found could be due to different relative strengths of the UPR and HR promoters between the cell lines. To get comparative values of the cell lines percentual differences in signal were compared between the minimal sv40 promoter in the FFiR reporter and the experimental UPR and HR promoters in the experimental reporters (table VI).
Table VI. Relative strengths of UPRE and HRE promoters compared to minimal sv40.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% HR/sv40 ±std</th>
<th>% UPR/sv40 ±std</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>24.9 ±2.96</td>
<td>4.17 ±0.29</td>
</tr>
<tr>
<td>Hek293</td>
<td>161 ±17.7</td>
<td>128 ±16.1</td>
</tr>
<tr>
<td>HepG2</td>
<td>133 ±41.6</td>
<td>66.8 ±13.2</td>
</tr>
</tbody>
</table>

The relative expression of firefly luciferase to firefly luciferase with a UPR or HR sensitive promoter was investigated in three cell lines with parallel single transfections of a constitutively expressing control reporter (FFiR) and an experimental reporter (UPR or HR). The relative signals (UPR/FFiR and HR/FFiR) are presented as % values with standard deviations. Reported values are the average of eight independent samples.

Finally basal activity for the UPR and HR was calculated from the absolute UPR/FFi and HR/FFi values (see table VI). Firefly luciferase is expressed 28% better with a UPR promoter than with the control promoter minimal sv40 in Hek293. The basal signal for HR seems to be even bigger in Hek293 as well as in HepG2 with relative strengths hitting 161% and 133% respectively. HeLa signal for HR is bigger than for UPR but both are still very small compared to the other two cell lines.

In conclusion the basal level of UPR and HR activity was found to differ greatly between the three cell lines used in this study: HeLa, Hek293 and HepG2. By using a combination of experimental firefly luciferase reporters and constitutively expressed firefly and renilla luciferase reporters it was discovered that HeLa has very little basal level activity of both stress responses compared to the other two cell lines. Hek293 holds strong basal levels of both responses and HepG2 has a strong basal level for the HR but only half of the activity of Hek293 when it comes to the UPR.

4.3 Induction of the UPR

In order to study changes in the UPR and HR signals basal levels and reproducible induction methods needed to be established. Toxins are the most commonly used approach for induction of the UPR. Thapsigargin (Th) is an inhibitor of SERCA (sarco/endoplasmic reticulum Ca$^{2+}$ ATPase) type enzymes and induces a UPR by depleting Ca$^{2+}$ storages of the ER (Lytton et al. 1991; Sagara Y & Inesi G 1991; Lodish et al. 1992; Haze et al. 1999). Tunicamycin (Tu) is another commonly used toxin that blocks the synthesis of N-linked glycoproteins causing them to accumulate to the ER (Olden et al. 1979; Guarnaccia et al. 1983; Højmann et al. 2001; Apostolou et al. 2008). UPR however naturally results from
the accumulation of unfolded protein to the ER. To mimic this and to avoid the undesirable additional effects a toxin may have, a folding defective variant of BACE (Beta-secretase 1, isoform C) was also tested as a UPR inducer. Thapsigargin (200 μM) and tunicamycin (5 μg/ml) were tested for UPR induction in concentrations already established in the host group. The results of these experiments are presented in table VII. Due to the different mechanisms and administration of the three inducers used, different times of induction were necessary. BACE is introduced to the cells by transfection at the same time as the experimental reporters. Cells were gathered and responses measured 24 hours after transfection so the induction period of BACE was longer than that of the two toxins used. Induction of a UPR with the two toxins was done in the last 5 hours before the cells were gathered. This time period was chosen because the use of 2-8 hour induction with these toxins has been used in the literature and similar experiments with 5 hour induction were widely used in the host group (Haze et al. 1999; Arai et al. 2006; Apostolou et al. 2008; Gjymishka et al. 2009). Larger incubation times with Th or Tu result in cell death.

Table VII. Different UPR inducers.

<table>
<thead>
<tr>
<th>UPR induction</th>
<th>HeLa (%sd)</th>
<th>Hek293 (%sd)</th>
<th>HepG2 (%sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th (200 μM) 5h</td>
<td>180 (34)</td>
<td>381 (21)</td>
<td>224 (29)</td>
</tr>
<tr>
<td>Tu (5 μg/ml) 5h</td>
<td>209 (32)</td>
<td>302 (41)</td>
<td>147 (36)</td>
</tr>
<tr>
<td>BACE 24h</td>
<td>432 (4)</td>
<td>3860 (6)</td>
<td>762 (10)</td>
</tr>
</tbody>
</table>

In order to study the range of UPR induction, the effects of different ER stressors, thapsigargin (Th), tunicamycin (Tu) and a folding defective protein (BACE), were tested as UPR inducers. The experimental firefly reporter UPR was co-expressed with the constitutively expressing renilla control reporter in the three cell lines indicated. Results are presented as a % increase in the renilla normalized UPR signal with the standard deviation expressed as a % of the mean (%sd). Reported values are the average of eight independent samples.

Out of the three tested methods for UPR induction expression of the folding defective variant of BACE proved to be the most effective and the most reproducible. Thapsigargin and tunicamycin did induce a statistically significant UPR in the three cell types tested but the size of the induction varied greatly. BACE produced a maximal induction of the UPR very reliably with less than 10% standard deviation of the average. It also is the single UPR inducer that shows how different the maximal induction of UPR is for the different cell lines; Fold increase in the UPRE signal of HeLa is 430-fold while in HEk293 it is nearly four thousand. BACE induction of the UPR was used in all of the later experiments.
4.4 Induction of the HR

Normally the HR is induced by lowering the oxygenation of the environment from 16% to between 1-8% O₂ for several hours. 1% O₂ however, is a lower concentration than found under physiological conditions and was not considered for these experiments. The use of additional treatments (DTT, Th and Tu) with hypoxia required a fast induction of the HR since these compounds are all toxic with prolonged exposure. At 8% O₂ full induction of the HR can only be achieved by prolonged exposure so 5 and 3% O₂ were chosen for further experiments. The magnitude of HR induction and the time needed for induction at the two different O₂ concentrations was tested in all the three cell lines (table VIII).

<table>
<thead>
<tr>
<th>HR induction</th>
<th>HeLa (%sd)</th>
<th>Hek293 (%sd)</th>
<th>HepG2 (%sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 hour, 5% O₂</td>
<td>192 (15)</td>
<td>129 (12)</td>
<td>373 (36)</td>
</tr>
<tr>
<td>5 hour, 5% O₂</td>
<td>125 (6)</td>
<td>104 (6)</td>
<td>150 (11)</td>
</tr>
<tr>
<td>20 hour, 3% O₂</td>
<td>252 (8)</td>
<td>189 (10)</td>
<td>662 (9)</td>
</tr>
<tr>
<td>5 hour, 3% O₂</td>
<td>145 (10)</td>
<td>128 (9)</td>
<td>156 (16)</td>
</tr>
</tbody>
</table>

In order to study the range of HR induction, the effects of 5 hour in 5% or 3% O₂ and 20 hour in 5% or 3% O₂ were tested on the HR signal. The experimental firefly reporter HR was co-expressed with the constitutively expressing renilla control reporter in the three cell lines indicated. Results are presented as a % increase in the renilla normalized HR signal with the standard deviation expressed as a % of the mean (%sd). Reported values are the average of eight independent samples.

The liquid environment of the cell is responsible for transmitting the change in environment to the cell. For this reason, the amount of liquid media was also tested as a factor in generating the HR (table IX).

<table>
<thead>
<tr>
<th>HR induction</th>
<th>HeLa (%sd)</th>
<th>Hek293 (%sd)</th>
<th>HepG2 (%sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml media</td>
<td>166 (11)</td>
<td>106 (7)</td>
<td>263 (20)</td>
</tr>
<tr>
<td>1 ml media</td>
<td>192 (15)</td>
<td>129 (12)</td>
<td>368 (40)</td>
</tr>
</tbody>
</table>

The effect of the liquid environment of the cell on HR induction was tested with by measuring HR induction in different amounts of cell culture media with 20 hour induction at 5% O₂. The experimental firefly reporter HR was co-expressed with the constitutively expressing renilla control reporter in the three cell lines indicated. Results are presented as a % increase in the renilla normalized HR signal with the standard deviation expressed as a % of the mean (%sd). Reported values are the average of eight independent samples.

In order to reproducibly induce the HR in all the cell lines used the most important factor turned out to be the liquid environment of the cell i.e. the amount of cell culture media. A maximal induction of the HR was achievable in both O₂ concentrations tested (3% and 5%
O$_2$) but in the more severe hypoxia the time it took to produce a full HR was reduced: With 5 % atmospheric oxygen the fastest growing cell line Hek293 did not show signs of hypoxia after five hours of treatment, but reached a 1.3 fold induction in the HR signal after 20 hours. At a lower oxygen concentration of 3 % a statistically significant fold induction of 1.3 was achieved after a five hour treatment. A similar trend can be seen with the other two cell lines – a stronger induction can be achieved in less time at 3 % O$_2$ compared to the 5 % O$_2$ (table VIII). However this was only true if the cells were grown in an adequate amount of culture media. If grown in 0.5 ml of cell culture media Hek293, was unable to induce a HR. The other two cell lines were able to activate a HR in the 0.5 ml culture volume but they also showed improvement in their induced signal in the larger culture volume (table IX). A 2 ml volume of cell culture media was also tested, but no statistically significant effects were seen.

The reason behind the weakened response to hypoxia in 0.5 ml of culture media is most likely due to different levels of oxygen consumption between the cell lines. Because cell culture plates are not oxygen permeable oxygen can only reach the cell by diffusion through the cell culture media. Hence oxygen gradients may form depending on the volume of media on top of the cells (Doege et al. 2005). The amount of oxygen consumed by the cells will also affect the formation of oxygen gradients and the actual oxygen concentration available for the cells. Cell type, activity of the cells i.e. what phase of the cell cycle the cells are in and cell density are some of the factors contributing to the rate of oxygen consumption. In this experiment cell density was the same between the different samples so at the start oxygen consumption would have been the same. A difference was in the volume of cell culture media, the distance of oxygen diffusion. In a bigger volume the formation of oxygen gradients could cause mildly hypoxic conditions even before the actual hypoxic stimulus. Hence the actual time in hypoxia would be different between the samples. Evidently with the shorter diffusion distance in the 0.5 ml media the hypoxic stimulus is considerably smaller for the cells and not enough to induce the HR of Hek293. Because the other two cell lines do show an induction in the 0.5 ml culture the difference must be accounted to oxygen consumption connected to metabolic activity and cell type. Because a reproducible induction of the HR was achieved in 1 ml cell culture media in all the cell lines and chemical treatments will be performed in a five hour period, induction at 3% O$_2$ for five hours in 1 ml cell culture media was used in all of the later experiments.
4.5 DTT has multiple effects on the cell

Two methods were used in order to increase disulfide bond formation in the cells: expression of a highly disulfide bonded protein human serum albumin and treatment with a redox reagent dithiothreitol (DTT). DTT has been widely used as a UPR inducer. According to the literature treatment with DTT causes a UPR by reducing disulfide bonds of ER resident folding intermediates making them unable to exit the ER (Tatu et al. 1993). This will lead to crowding and ER stress. The reduction of disulfide bonds of folding intermediates in the ER would also rather counter intuitively increase disulfide bond formation, because the cell would have to reform any reduced disulfide bonds in order for the protein to exit the ER as a fully native protein. DTT has not been previously reported of having any adverse effects on the cell and has been considered a relatively specific inducer of a UPR (Lodish & Kong 1993; Tatu et al. 1993; Leber et al 2004; Liang et al. 2005; Yoshida et al. 2006).

A fully induced UPR will reduce translation of new proteins via the PERK arm of the UPR. This will give the cell time to properly fold or degrade proteins crowding the ER. This will also normalize disulfide bond formation. In order for DTT to work optimally in increasing disulfide bond formation a concentration that will stress the cells, but not induce a fully induced UPR, needed to be found. For this reason, dose dependency of DTT induced UPR was tested. Hypoxia can potentially alter the redox status of the cell and thus affect the function of DTT in the cells. The lack of molecular oxygen to spontaneously oxidize DTT can also affect the effective concentration of DTT in solution. Therefore the effects of DTT must also be checked under normoxic and hypoxic conditions. The most commonly used concentration of DTT used to induce a UPR ranges from 2 to 10 mM (Bertolotti & Ron 2001; Leber et al 2004; Liang et al. 2005; Shen et al. 2005; Dai et al. 2009). The concentrations tested here were 2 mM, 400 μM, 80 μM and 16 μM – five fold step-decreases from 2 mM DTT. BACE was used as a positive control for full UPR induction. The effects of the four concentrations of DTT were tested on UPR in all three cell lines under normoxic and hypoxic conditions (see figure 8).
Figure 8. Effects of DTT on the UPR under normoxic and hypoxic conditions.
The level of UPR induction with different concentrations of DTT (2 mM, 400 μM, 80 μM and 16 μM) was tested by co-expressing the experimental firefly reporter UPR with the constitutively expressing renilla control reporter. Effects on the UPR were tested in normoxic and hypoxic conditions with a 5 hour DTT (and 5 hour hypoxic at 3% O₂) treatment.
A., C. and E. Fold change in the UPR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 12 independent samples.
B., D. and F. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 12 independent samples.
As expected, DTT induces a UPR in a concentration dependent manner, with a threshold value between 400 μM and 2 mM for induction (see figure 8, renilla normalized luciferase values). In all the cell lines a statistically significant UPR (P < 0.05) was induced with 2 mM DTT. This effect however is much smaller than achievable with the expression of BACE, a protein that cannot fold properly and accumulates to the ER inducing the UPR. In Hek293 and HepG2 there is a four-fold difference in the UPR signal between the 2 mM DTT and BACE-treated samples. In HeLa the difference is three fold. There also appears to be an additional core DTT dependent effect on renilla. At 2 mM DTT this could, in part be due to UPR induction because an induced UPR will cause translational attenuation. However DTT and BACE are not equal as UPR inducers: there is a four-fold difference in the UPR signal between the 2 mM DTT and BACE-treated samples. In contrast the renilla values of these samples show similar drops. Also there are clear effects on renilla at 400 μM or less where there is no significant UPR induction. HepG2 is most sensitive to this additive effect with a statistically significant difference even between non-treated and 16 μM DTT samples.

4.6 Non-UPR related DTT effects

The additional effect of DTT on general transcription/translation could arise from the fact that in addition to being a strong reducing agent DTT is also a strong chelator with high affinity for Zn(II) and Fe(II) (Krężela et al. 2001). Metal ions and transition metals are required for the activity of a large number of enzymes and proteins. Zinc in particular is important for general cellular functions as it is incorporated into zinc-finger proteins, one of the main class of DNA binding proteins functioning as transcription factors. Protein kinase C (PKC) is one central zinc-finger transcription factor involved, for example, in cell growth and immune responses. Recently it was also reported that the addition of zinc is able to down regulate HIF1α levels by increasing proteosomal degradation in normoxia and hypoxia (Nardinocchi et al. 2009 & 2010). With the log Kc value, concentration equilibrium constant, of 6.9 DTT could, when in high excess over protein, compete for Zn(II) against weaker zinc finger motifs (Krężela et al. 2001). This competition for zinc could affect the function of general transcription factors leading to the response observed (figure 8). Competing zinc away from proteins could also increase the cellular labile pool of zinc leading to a HIF1α specific inhibition of the HR.
Iron is another important metal ion found in proteins, especially in proteins mediating redox reactions in cellular metabolism: NADH dehydrogenase, cytochrome c reductase and succinate reductase as well as the Complex I and Complex II of oxidative phosphorylation all contain iron-sulphur clusters mediating electron transport. In addition the PHDs and FIH1 involved in repression of the HR in normoxic conditions are iron dependent enzymes. If the additional effect of DTT on the system would be through chelation, DTT treatment might modulate the HR by either zinc or iron. To investigate this additional effect of DTT the effects of the different concentrations of DTT on the HR were tested under normoxic and hypoxic conditions (see figure 9).
Figure 9. Effects of DTT on the HR under normoxic and hypoxic conditions.
The effect of different concentrations of DTT (2 mM, 400 μM, 80 μM and 16 μM) on the HR was tested by co-expressing the experimental firefly reporter HR with the constitutively expressing renilla control reporter. Effects on the HR were tested in normoxic and hypoxic conditions with a 5 hour DTT (and 5 hour hypoxic at 3% O₂) treatment.
A., C. and E. Fold change in the HR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 12 independent samples.
B., D. and F. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 12 independent samples.
As expected, the same trend of DTT decreasing renilla luciferase signal can be seen from the HR-experiments shown in figure 9 as in the UPR-experiments shown in figure 8. The HeLa and HepG2 renilla values do not differ with statistical significance (pairwise t-tests) from the renilla values of the UPR test before. Hek293 values however differ in the hypoxic samples with statistical significance (p < 0.05) indicating that there was perhaps a difference in cell density between the two tests contributing to a difference in the hypoxic response. Here the difference between the normoxic and hypoxic samples is smaller indicating a smaller induction of the HR. The HR signal is not as conclusive in the three cell lines. The HR seems to be inhibited by DTT, but the effects vary between the cell lines. In HeLa there is a concentration dependent inhibition of the HRE signal by DTT and this is emphasized under hypoxia. In normoxia only the 2 mM DTT induces a statistically significant drop in HR signal, but in hypoxia, 400 μM induce a statistically significant drop in the signal. The maximal level of repression is 10% in normoxia (2 mM DTT) and 30% in hypoxia (2 mM DTT). The increased severity can be accounted to the increased effective concentration of DTT under hypoxia due to reduced availability of oxygen to oxidize DTT to a state in which it cannot bind metal ions or reduce disulfide bonds. In Hek293 and HepG2 there however is no such trend to be observed. In HepG2 there is no statistically significant effect of DTT on HR signal, but a general fluctuation in the signal between different concentrations. In Hek293 there is a statistically significant drop in HR with 80 μM DTT in normoxia and hypoxia, while 2 mM DTT induces a statistically significant drop in the signal in hypoxia. The maximal level of repression is 10% in normoxia (80 μM and 2 mM DTT) and 20% in hypoxia (2 mM DTT). These results suggest that zinc chelation maybe the dominant additive effect of DTT influencing both HIF1α and general levels of transcription/translation.

BACE was also included in the assay as a positive control for fully induced UPR and in all cell lines it seems to influence the HR signal, but again responses are different between the cell lines. In HeLa BACE represses the HR signal whereas in the other two cell lines HR is induced by BACE. Inhibition of the HR in HeLa is statistically significant under normoxic and hypoxic conditions but the induction of HR by BACE in Hek293 and HepG2 is only significant under normoxia (p < 0.05). BACE induces a UPR because it is a folding defective variant that will end up in a futile cycle of folding attempts in the ER. There are three disulfide bonds in BACE that could contribute to a considerable load on disulfide bond formation during this recycling. Assuming that disulfide bond formation consumes mo-
lecular oxygen this increase in disulfide bond formation could contribute to an intracellular
decrease in O₂ concentration and activation of the HR. The strength of induction with
BACE is at 10-20% quite moderate, suggesting a subtle change in oxygen availability. In
addition the fact that the BACE-induced HR is not additive in hypoxia suggests that the
induction is a result of a slight decrease in O₂ concentration and is overrun in true hypoxia.
In HeLa however there is a clear inhibition of the HR of about 30% that cannot be ac-
counted for by a general effect on transcription/translation of an activated UPR since the
HR specific reporter shows a greater response than the control reporter. The fact that the
level of repression is slightly more severe in hypoxia suggests that BACE induced repres-
sion is strong enough to prevent the HR from activating at all. It is noteworthy that in this
experimental set-up the UPR is activated long before the hypoxic stimulus: BACE expres-
sion is initiated by transfection whereas hypoxic stimulation takes place during the last five
hours of the 24 hour experiment. It is possible that the effect witnessed here is an attribute
of the aggressive HeLa cell line. The activation of two strong cellular stress responses has
the potential to initiate apoptosis. One of the defining characteristics of a cancerous cell is
the ability to avoid apoptosis. It is possible that amongst the many alterations to normal
cellular function HeLa has a mechanism in place to inhibit the activation of a second stress
response that might initiate an apoptotic signal.

4.7 DTT chelates metal ions in vivo

The potential of DTT as a chelator of iron and zinc was also tested by the use of two fluo-
rescent probes, Phen Green and FluoZin (see figure 10). Phen Green SK is a fluorescent
probe that responds to both major oxidation states of iron, providing a 93% reduction in
signal in response to Fe²⁺ and a 51% reduction in signal in response to Fe³⁺. However other
divalent metal and transition metal ions including Cu²⁺, Cd²⁺ and Zn²⁺ can elicit a nonspe-
cific quenching response, making selectivity a problem (Domaille et al. 2008). There is no
information available regarding the affinity of Phen Green to the bound metal ion, but it
has been shown that with a high excess, millimolar concentrations, of an specific chelator
it is possible to compete the Phen Green bound ion away (Petrat et al. 2000). FluoZin-3 is
another fluorescent probe suitable for detection of intracellular Zn²⁺ with relatively little
signal interference from other ions. Fluorescence of the probe increases upon binding Zn$^{2+}$ (Domaille et al. 2008).

Figure 10. DTT as a chelator of iron.
In the cell Phen Green will bind the labile iron pool of the cell quenching the fluorescent signal. Cells were loaded with the fluorescent probe (20 μM) by incubation for 20 minutes in HBSS (37°C). Cells were then washed with PBS, detached from the plate with TE and suspended in PBS with 10% FCS. Cells were incubated for 15 minutes on ice before measurements. Fluorescence of 5000 cells was registered using a flow cytometer. DTT was added in the appropriate concentration (2 mM, 400 μM, 80 μM or 16 μM) to the suspended cells 15 minutes before measurement. 100 μM bipyridyl was used as a control for a specific iron chelator.

A.-C. Controls of the flow cytometry measurements; A. Particles that were gated form measurements as cells are shown in region 1 (R1). B. Autofluorescence from cells not labeled with the fluorescent tag PhenGreen. C. Fluorescent signal received from cells labeled with 20 μM PhenGreen.

D. and E. Effect of DTT on the free iron pool of the cell. The results are presented as % change compared to the non-treated control with error bars for standard deviation. The results are the average of 12 measurements (4 parallel samples on 3 days) where in each 5000 cells were registered. 100 μM bipyridyl (Romeo et al. 2001) was used as a control for a specific iron chelator.

In most cells the intracellular concentration of free Zn$^{2+}$ is extremely low (<1 nM), with the remainder being bound to proteins or nucleic acids (Simons 1991). In the two cell lines tested here (HeLa and HepG2) the labile pool of zinc was too low to reliably differentiate auto fluorescence from probe fluorescence and to accurately track changes in the concentration. As the zinc specific measurements were unsuccessful, specific in vivo chelation of zinc could not be demonstrated. Nevertheless in the literature DTT is reported to have high affinity for zinc (Krëžela et al. 2001).
In contrast for iron detection, the situation was better. Auto fluorescence was distinct from the fluorescence of the probe (see figure 10 panels B and C) and an effect was seen with the addition of bipyridyl, an iron specific chelator. Bipyridyl releases protein bound iron and affects intracellular iron storages thus increasing the concentration of free iron (100 μM, Romeo et al. 2001). Free iron can be bound by the probe hence leading to a decrease in probe fluorescence. There was a statistically significant decrease in fluorescence of the probe with bipyridyl up to 20% in HeLa and 10% in HepG2. The addition of DTT had a similar effect with probe fluorescence reduced up to 25% in HeLa and 15% in HepG2 at concentrations as low as 16 μM. This indicates that DTT is able to chelate iron *in vivo*.

4.8 Increasing disulfide bond formation affects the hypoxia response

The other method we used to increase disulfide bond formation *in vivo* involved over-expression of a highly disulfide bonded protein, human serum albumin (HSA). HSA is a 69 kDa protein that contains 17 native disulfide bonds. It folds very efficiently in the ER and has been used in protein folding studies for decades (Kragh-Hansen 1981; Mao et al. 2000; Zunszain et al. 2003). Serum albumin is also a natural constituent of the cell culture media making adverse effects due to its expression unlikely.

In preliminary experiments (see figure 11) the effects of HSA expression on the UPR and HR were tested. In HeLa and Hek293 a small, but statistically significant UPR was induced. Induction was approximately 30%. In HepG2 however, no induction of the UPR occurred. In the HR signal a 10% increase followed the addition of HSA in HeLa and Hek293. This increase was however not statistically significant in normoxic or hypoxic conditions. HepG2 again showed no change in the signal. A statistically significant induction might have been achievable using a much larger sample size than the eight data points here.
Figure 11. Increasing disulfide bond formation with HSA.

The HR reporter and the UPR reporter were co-expressed with a constitutively expressing renilla luciferase control reporter in the three cell lines HeLa, Hek293 and HepG2. Cells were grown in normoxic and hypoxic (3% O₂, 5 hours) conditions. HSA was co-expressed as an inducer of disulfide bond formation in a parallel sample set.

A., C. and E. Fold change in the HR or the UPR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 8 independent samples for the HR and 6 samples for the UPR.

B., D. and F. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 8 independent samples for the HR and 6 samples for the UPR.

However it was not unexpected that the induction accomplished with HSA was small. The main sink of consumed oxygen in the cell is mitochondrial respiration, with 80% of oxygen consumed coupled to ATP production (Rolfe & Brown 1997). Thus even with maximal induction of disulfide bond formation the increase in consumed oxygen is a small fraction of the total. In an attempt to amplify the proportion of the total signal resulting from disulfide bond formation, inhibitors of mitochondrial complex I and III were used to re-
duce mitochondrial oxygen consumption. This would increase the proportion of oxygen used in the ER and potentially increase the induction of HR seen before.

Aerobic respiration is a cellular process where the energy of food (glucose, fatty acids, glycerol and amino acids) is converted to adenosine triphosphate (ATP) in the presence of oxygen. It involves the catabolism of nutrients to form pyruvate and subsequently acetyl-CoA, which is oxidized in the citric acid cycle to form some ATP and reducing equivalents in the form of NADH and FADH$_2$. These reducing equivalents are used further in oxidative phosphorylation to drive ATP synthesis. In the process of oxidative phosphorylation reducing equivalents (\(-H\) or \(e^+\)) are transferred down a redox gradient of carrier proteins to their final reaction with oxygen to form water. The electron carriers in this process are the four respiratory chain complexes (Complex I-IV) in the inner mitochondrial membrane. Three of the four complexes use the energy released in the redox gradient to pump protons to the outside of the membrane, creating an electrochemical potential between the matrix and the intramembrane space. ATP synthase is the final component of oxidative phosphorylation utilizing the generated electrochemical gradient to drive ATP synthesis (Murray et al. 2003).

Antimycin A and rotenone are both inhibitors of mitochondrial respiration acting directly on Complex III (antimycin A) and Complex I (rotenone) of the electron transport chain (Murray et al. 2003; Chen et al. 2003). With rotenone it is possible to inhibit respiration efficiently and to reduce endogenous oxygen consumption to 20% (Barrientos et al. 1998; Barrientos & Moraes 1999). The residual activity could be through Complex II of the electron transport chain, which does not require input from the Complex I, and any other oxygen consuming process in the cell, including disulfide bond formation. With antimycin A inhibition of oxygen consumption to 20-30% of the normal has also been observed (Reiss & Roos 1978; Tzung et al. 2001). Inhibition of mitochondrial respiration will cause depletion of ATP levels forcing the cell to initiate glycolysis for energy production (Dickman & Mandel 1990; Sherer et al. 2003). Defects in mitochondrial respiration are also behind aerobic glycolysis (Warburg effect) evident in many human tumours (reviewed in Seyfried & Shelton 2010). This chemical intervention to cellular respiration thus models the metabolic shift occurring in pre-cancerous cells. The ability to increase glycolysis due to intermittent respiratory damage is a prerequisite to malignant cell growth. All of the cell lines
used in this study are cancer cells (HeLa and HepG2) or immortalized cell lines with cancer-like attributes (Hek293) and hence should have undergone this transition.

Inhibitors of mitochondrial respiration were intended to be used to try and amplify the effect of HSA on the HR. As a control experiment to rule out any direct interactions with the luciferases, a sample co-expressed with HR and renilla luciferase was divided into two and one aliquot treated with the experimental concentration of antimycin A or rotenone for 15 minutes before recording luminescence. The experiment was done in four parallel samples. No change was seen between the nontreated and treated samples implying no effects of these compounds on renilla or firefly luciferase. Thus the effects of antimycin A and rotenone on the HR were tested in the three cell lines (see figure 12 and section 4.9).

Figure 12. Mitochondrial respiration inhibitors and the HR
Two inhibitors of mitochondrial respiration, antimycin A (1 μg/ml) and rotenone (0.5 μm), were used to down regulate overall oxygen consumption of the cell. The HR sensitive reporter HR was co-expressed with a constitutively expressing renilla luciferase control reporter in the two cell lines Hek293 and HepG2. Samples were treated with antimycin A (1 μg/ml) or rotenone (0.5 μm) for the last five hours before sample collection. Cells were grown in normoxic and hypoxic (3% O₂, 5 hours) conditions.

A. and B. Fold change in the HR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.

C. and D. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.
The three cell lines used have very different responses to depletion of mitochondrial respiration; HeLa is discussed later. Hek293 and HepG2 show a 25-30% reduction in the HR signal in normoxia. Under hypoxia antimycin A and rotenone treatment inhibited any induction of the HR and maintained the signal level within 5% of the values in normoxia. There is also a drop in the renilla control values with a 40% drop in HepG2 in normoxia and hypoxia and a 5-10% drop in Hek293 at normoxia and a 20-30% drop in hypoxia. The drop in HR can be accounted for by a decrease in cellular oxygen consumption once aerobic respiration has been inhibited.

It has been reported that confluent cultures with an active metabolism experience a pO₂ of less than 2 mm Hg (160 mm in air) when incubated in a gas atmosphere containing 20% oxygen (Metzen et al. 1995). Reducing oxygen consumption will bring the oxygen concentration of the cells closer to that of the incubator and increase availability of oxygen to the PHDs leading to increased HIF degradation. A moderate hypoxia (8-2% O₂) might not be able to reduce oxygen availability enough to impair the function of the PHDs. The HIF-prolyl hydroxylases are effective oxygen sensors, with K_m values for O₂ close to atmospheric oxygen concentrations (Hirssilä et al. 2003). Thus even small decreases in O₂ are likely to influence their activities. However, if the normal mitochondrial respiration is able to deplete the environment of oxygen, the threshold level of significantly impaired PHD activity must be low. In the elevated oxygen concentration of the mitochondrially impaired cells, severe hypoxia might be needed to activate the HR. Also the drop in the control reporter indicates that the Hek293 and HepG2 are under considerable stress due to the treatment. Evidently these cell lines, especially HepG2, have trouble adapting to deficiencies in mitochondrial respiration at least in this timeframe where complete respiration deficiency is induced in a short timeframe. UPR induction was also tested with the respiration inhibitors, but HepG2 and Hek293 showed no signs of induction, rather a slight repression (see figure 13).
Two inhibitors of mitochondrial respiration, antimycin A (1 μg/ml) and rotenone (0.5 μm), were used to downregulate overall oxygen consumption of the cell. The UPR sensitive reporter was co-expressed with a constitutively expressing renilla luciferase control reporter in the two cell lines Hek293 and HepG2. Samples were treated with antimycin A (1 μg/ml) or rotenone (0.5 μm) for the last five hours before sample collection.

A. and C. Fold change in the UPR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 6 independent samples.

B. and D. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 6 independent samples.

The inhibitors of mitochondrial respiration were successful in reducing cellular oxygen consumption, but they also seemed to cause significant stress for Hek293 and HepG2. Regardless, the effect of HSA was tested with the mitochondrial toxins in Hek293 and HepG2 (see figure 14).
Figure 14. Increasing disulfide bond formation with HSA
Two inhibitors of mitochondrial respiration, antimycin A (1μg/ml) and rotenone (0.5 μm), were used to down regulate overall oxygen consumption of the cell. The HR sensitive reporter was co-expressed with a constitutively expressing renilla luciferase control reporter in the two cell lines Hek293 and HepG2. Samples were treated with antimycin A (1μg/ml) or rotenone (0.5 μm) for the last five hours before sample collection. HSA was co-expressed as an inducer of disulfide bond formation in a parallel sample set.

A., C., E. and G. Fold change in the HR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.

B., D., F. and H. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.
In HepG2 HSA expression did not induce the HR; in contrast it became another stress factor for the cell further dropping the HR signal. The addition of HSA did not change the control renilla signal. In Hek293 the addition of HSA to the nontreated cells increased the HR signal by 11%, but due to a large standard deviation this increase was not statistically significant (p = 0.06). In the antimycin A treated samples the expression of HSA added to a 15% increase in the HR signal in hypoxia, but again variation turned out to be a problem (p = 0.103). Treatment with rotenone did not allow for further induction of the HR.

4.9 Increasing disulfide bond formation induces the HR in HeLa

Unlike the two cell lines discussed before, HeLa has underlining mitochondrial defects that cause it to have truncated citric acid cycle and low intrinsic oxygen consumption (Piva & McEvoy-Bowe 1998). It has also been shown that mitochondrially depleted HeLa have an essentially normal response to hypoxia with an increase in HIF1α accumulation in strong hypoxia (less than 2% O₂ Enomoto et al. 2002). The effects of antimycin A and rotenone were tested on the HR and the UPR in HeLa (figure 15).
Figure 15. Mitochondrial respiration inhibitors in HeLa
Two inhibitors of mitochondrial respiration, antimycin A (1μg/ml) and rotenone (0.5 μm), were used to down regulate overall oxygen consumption of the cell. The HR reporter and the UPR reporter were co-expressed with a constitutively expressing renilla luciferase control reporter in HeLa. Samples were treated with antimycin A (1μg/ml) or rotenone (0.5 μm) for the last five hours before sample collection. Cells were grown in normoxic and hypoxic (3% O2, 5 hours) conditions.

A. and C. Fold change in the HR and the UPR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 8 independent samples for the HR and 6 samples for the UPR.

B. and D. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 8 independent samples for the HR and 6 samples for the UPR.

In HeLa antimycin A and rotenone do not inhibit the activation of the HR under hypoxia; rather both mitochondrial toxins induce the HR in normoxic and hypoxic conditions (figure 15). The HR signal is induced nearly two fold in normoxia by these toxins and more than three fold in hypoxia. The potential mechanisms behind this induction of the HR are discussed later. However there is a small induction of the UPR as well with the inhibitors, indicating that they might have some unspecific effects in HeLa. Finally, the effect of HSA in the mitochondrially inhibited HeLa was tested (figure 16).

Interestingly the expression of HSA gives an additional increase in the HR in the respiratory inhibited HeLa (see figure 16). In the nontreated HeLa the increase in HR signal with HSA is approximately 10% but with antimycin A and rotenone the difference is boosted up
to 20-30%, a statistically significant increase in the HR. The additional induction of the HR can be traced to increased oxygen consumption.

**Figure 16. Increasing disulfide bond formation with HSA in the mitochondrially inhibited HeLa**

Two inhibitors of mitochondrial respiration, antimycin A (1μg/ml) and rotenone (0.5 μm), were used to down regulate overall oxygen consumption of the cell. The HR sensitive reporter was co-expressed with a constitutively expressing renilla luciferase control reporter in HeLa. Samples were treated with antimycin A (1μg/ml) or rotenone (0.5 μm) for the last five hours before sample collection. HSA was co-expressed as an inducer of disulfide bond formation in a parallel sample set.

A. and C. Fold change in the HR signal shown as renilla normalized luciferase (%) with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.

B. and D. Fold change in the control reporter renilla luciferase signal normalized to the nontreated control sample with error bars for standard deviation of the average. Reported values are the average of 8 independent samples.

PHDs and FIH1 are all oxygen requiring enzymes that target HIF1α for degradation and limit its function. An increase in oxygen consumption would translate to a lowered intracellular oxygen concentration limiting the function of the PHDs and FIH1. This would result in increased protein stability of the HIFα and an induction of the HR. The only factor differentiating the two antimycin A or rotenone treated samples is the expression of a heavily disulfide bonded protein, HSA (see figure 16). Protein expression requires raw material (nucleotides and amino acids) and energy in the form of ATP. In the respiring cell this extra requirement for ATP could translate to increased mitochondrial respiration and thus increased oxygen consumption, but in the respiratory deficient cell, that uses glycolysis for energy production, no such extra requirement for oxygen exists. The only oxygen requiring component of HSA expression in the respiratory deficient cell is disulfide bond formation.
Thus this result establishes a link between intracellular consumption of molecular oxygen and disulfide bond formation. It also provides a molecular mechanism responsible for UPR induction under strong hypoxia; the accumulation of unfolded protein due to insufficient disulfide bond formation. The expression of a non-disulfide bonded HSA would further exemplify the role of disulfide bond formation in the evidenced induction of the HR, but since disulfide bonds are essential for proper protein structure, such variant would most likely cause problems in protein folding and result in ER-stress and an induced UPR.

The mechanism behind antimycin A and rotenone induced up regulation of the HR however proves puzzling. The two inhibitors of mitochondrial respiration will reduce cellular oxygen consumption and should lead to a reduced activity of the HR, as was seen in the case of Hek293 and HepG2. HeLa however has mutations in the mitochondrial DNA leading to a deficient complex I of the electron transport chain and consequently different mitochondrial metabolism with a truncated citric acid cycle (Herrnstadt et al. 2002; Piva & McEvoy-Bowe 1998). As we see an increase in the effect of HSA with the mitochondrial toxins, it seems clear that there is some function left in the normal electron transport chain of HeLa to be inhibited. Thus antimycin A and rotenone do act as respiratory inhibitors in HeLa. However in order to up-regulate the HR these toxins must have other unspecific effects. The small induction seen in the UPR as a response to the inhibitors also suggests unspecific effects of the inhibitors. The stabilization of HIF1α in normoxia can occur among other things by inhibition of the prolyl hydroxylases and FIH1 by transition metals, iron chelators, nitric oxide or 2-oxoglutarate analogs (Doege et al. 2005). Also, certain citric acid cycle intermediates have been recognized as in vivo inhibitors of the PHDs and FIH1 with succinate and fumarate inhibiting the PHDs and citrate inhibiting the FIH1 and PHD-3 (Koivunen et al. 2007). The effects of respiratory inhibition in HeLa are difficult to predict due to the natively altered state of the metabolism. However if further disruption would lead to an increase in for example citrate transport, the result might be a slight increase in the HR. It has also been reported that ROS can cause oxidative damage to the catalytic domain of the PHDs and FIH1 (Gerard et al. 2004). This would also lead to impaired HIF degradation and lead to an increase in the HR. Increased ROS production has been reported as a result of antimycin treatment in HeLa (Park et al. 2007).
5 CONCLUSIONS

In this study on mechanisms of activation of the UPR and the HR, the studies were primarily based on cell culture approaches. Indeed cell lines are used extensively in research as model systems to investigate mechanisms that occur in living organisms. There are however considerable differences between normal cells and immortalized or cancerous cell lines that should be considered when interpreting results from such in vivo research. For example the HeLa cell line used in this study has an abnormal chromosome number of 82 with multiple copies of chromosomes 12, 6, 8, and 17 (American Type Culture Collection entry CCL2). HeLa also has abnormal mitochondrial metabolism due to mutations in the gene coding complex I of the electron transport chain (Piva & McEvoy-Bowe 1998; Herrnstadt et al. 2002). Other cancer cell lines have similar alterations: HepG2 has a modal chromosome number of 55 while Hek293 has 64, HT-29 71 and MB 157 62 chromosomes to name a few (American Type Culture Collection entries HB-8065; CRL-1573; HTB-38; CRL-7721). These changes in chromosome number together with the resulting genomic, proteomic and metabolic changes alter the normal physiological status of a cell beyond recognition. The data presented here, should be viewed with this caveat in mind.

Previously a link was established between the hypoxia response and the unfolded protein response, with the discovery of a hypoxia induced UPR (Wouters & Koritzinsky 2008). Despite active research in the field the mechanism of UPR activation under hypoxia remains controversial. We hypothesized that the UPR activation under hypoxia, results from the same reason as the classical UPR – accumulation of unfolded protein in the ER. If, as currently thought, oxidative protein folding requires molecular oxygen in vivo and the inhibition of this process leads to a UPR, there would be a direct link between oxygen levels and hence hypoxia and UPR induction.

In this work the link between the HR and the UPR was investigated via an induction of disulfide bond formation in the ER. The main sink of oxygen consumption in the cell is the mitochondria with 90% of total oxygen consumption happening in the mitochondria and 80% of oxygen consumption coupled to ATP production (Rolfé & Brown 1997). That leaves 10% of total cellular oxygen consumption unaccounted for and available for other cellular processes. This also exemplifies how small changes are expected to occur in cellular oxygen consumption as a result of disulfide bond formation or other oxygen consuming
processes in the cell. Such small expected changes require a very sensitive method of observation. Here the hypoxia response of the cell was used to observe changes in intracellular oxygen availability. The cell uses oxygen dependent prolyl hydroxylases and the hydroxylase FIH1 to monitor changes in oxygen availability and to activate the adaptive responses of the HR. The HIF prolyl hydroxylases have a Michaelis constant (Km) of 200 μM enabling sharp regulation of enzyme turnover at low oxygen concentrations (Hirssilä et al. 2003). This provides the cell a sensitive but flexible mechanism for oxygen sensing with gradual adaptations in HIF protein stability and function. Here, changes in the HR were monitored with a sensitive luciferase based assay with experimental firefly luciferase reporters and a constitutively expressing renilla luciferase reporter. The renilla luciferase gene was mutated for increased stability and to prevent any detrimental redox reactions during experimental conditions. Reliability of the luciferase assay system and its suitability for our experimental set-up was established in a set of control experiments and the assay system was found to be highly sensitive providing an effective tool to investigate factors affecting the UPR and the HR.

In order to investigate changes in the UPR and the HR the basal level of activity as well as a normal physiologically relevant induction of the stress responses needed to be determined. The basal level of UPR and HR activity was found to differ greatly between the three cell lines used in this study indicating differences connected to cell type or perhaps altered state of the cell lines. Induction of the two stress responses was also investigated and optimized for our purposes.

Two methods were chosen for the in vivo induction of disulfide bond formation: expression of a highly disulfide bonded protein HSA and chemical treatment with the reducing agent DTT. In the literature DTT is considered a specific inducer of the UPR through its ability to reduce disulfide bonds of folding intermediates, but not those in mature proteins, in the concentration range of 1-5 mM (Braakman et al. 1992 & 1993; Lodish et al. 1992; Lodish & Kong 1993). Reduction of disulfide bonds in folding intermediates would cause the accumulation of unfolded protein to the ER and an induction of the UPR. DTT is widely used as a UPR inducer in the concentration range of 1-5 mM. The secretory pathway has been found to be intact in DTT treated cells and there are no reports of adverse effects of DTT on cellular function (Lodish & Kong 1993; Yoshida et al. 2006). The reduction of disulfide bonds in folding intermediates would also lead to an increase in net
disulfide bond formation by forcing the protein folding machinery to remake any reduced disulfide bonds. For this reason DTT was included in our investigation as an inducer of *in vivo* disulfide bond formation. However, we found evidence of severe effects on the general level of transcription/translation in DTT treated cells in addition to a statistically significant induction of the UPR. The additional effect of DTT on transcription/translation of the cell was more prominent than its effect on the UPR and occurred at lower concentrations of DTT. According to Krëžela *et al.* (2001) DTT is a strong chelating agent with high affinity for iron, zinc and copper. Here we showed *in vivo* chelation of iron at micromolar concentrations of DTT and found indirect evidence suggesting that zinc chelation is also a significant factor *in vivo*.

The additional effect of DTT on the general level of transcription/translation made the use of DTT as a specific inducer of intra-cellular disulfide bond formation unfeasible. However it also brought new evidence of *in vivo* effects of DTT. In the light of this new evidence the use of DTT as a UPR inducer should be reconsidered. It should be also noted that many UPR target genes have been recognized with a chemical induction of the UPR either with DTT, thapsigargin and/or tunicamycin (McCracken & Brodsky 2000; Lee *et al.* 2003; Park *et al.* 2003; Hollien & Weissman 2006). If there are additional effects on the cell with these chemicals, a number of UPR target genes could have been incorrectly recognized. In *Aspergillus niger* DTT has been found to induce gene expression independent of the UPR and differential activation of XBP1 has been found in human lymphocytes by DTT and tunicamycin (MacKenzie *et al.* 2005; Lemin *et al.* 2007). We also investigated two other commonly used chemical inducers of the UPR, thapsigarin and tunicamycin, compared with the effect of expression of a protein (BACE), with a folding defect. We found that the UPR induced by BACE was several fold higher than with the two chemicals and the results were less variable. These results combined with the DTT results accumulate evidence against chemical induction of the UPR. The induction achievable with chemicals does not reveal the true potential of the response, they are toxic to the cells depending on the exposure and they have potentially misleading additional effects on the cell, including in the case of DTT severe effects on general transcription/translation.

The other method chosen for the *in vivo* induction of disulfide bond formation is the expression of HSA, a highly disulfide bonded protein. The expression of disulfide bonded protein has been previously used in the investigation of the UPR and disulfide bond forma-
tion in the ER. Malhotra et al. (2008) used differently folding variants of coagulation factor VIII (factor VIII contains eight disulfide bonds) to investigate the relationship of protein miss folding and oxidative stress. The study provided evidence that oxidative stress in the ER is not caused by disulfide bond formation, but is rather an attribute of unfolded proteins in the ER. HSA was chosen for our investigation because it folds very efficiently and should not induce a UPR but still causes a considerable load for the protein folding machinery of the ER with its 17 disulfide bonds. Here we saw a small induction of the HR in response to HSA expression which was emphasized in respiration deficient HeLa to a statistically significant induction of the HR. This result needs replication in another cell line and conformation through alternative methods such as oxygen consumption measurements of HSA expressing cells. However it indicates that the in vivo pathway for disulfide bond formation does require molecular oxygen. Hence the hypoxic activation of the UPR probably results from the accumulation of unfolded, insufficiently disulfide bonded protein in the ER. This result has great significance by connecting the two central stress responses of the cell. It underlines the cellular requirement for molecular oxygen and the adaptive mechanisms that have evolved to work in concert as a response to acute hypoxic stress. A working UPR improves the viability of hypoxic cells by aiding in the transition to a low energy metabolism through PERK-mediated attenuation of translation (reviewed in Wouters & Koritzinsky 2008). The extent of oxidative protein folding can also affect the strength of the HR, which could be physiologically meaningful in the actively secreting cells such as B lymphocytes of the immune system, that can make 100 000 disulfide bonds per second and are also exposed to low oxygen tensions as they develop and migrate between blood and different tissue (Cenci & Sitia 2007; Kojima et al. 2002).

Our results suggest that disulfide bond formation utilizes molecular oxygen in vivo and thus provides a direct link for hypoxic induction of the UPR. However, there are other ER resident pathways that could also contribute to UPR induction under hypoxia. The immediate activation of the PERK branch of the UPR upon anoxic exposure suggests a direct role for oxygen in ER localized protein maturation. Disulfide bond formation is a high capacity pathway that, if affected, can quickly activate the UPR. Other oxygen requiring pathways in the ER include prolyl hydroxylation of collagen, which is a potential high capacity contributor in fibroblasts (Myllyharju 2003; Loenarz & Schofield 2011) and many steps of steroid biosynthesis are also ER resident and strictly dependent on molecular oxygen (Espenshade & Hughes 2007). The last steps of ascorbate biosynthesis, in mammals
capable of ascorbate synthesis (not human or primates), are also ER resident and dependent on molecular oxygen (Linster & Van Schaftingen 2007).

In this work the role of disulfide bond formation in the activation of the UPR and the HR has been investigated. New evidence was found that questions the established methods of UPR induction via chemicals such as DTT, thapsigargin and tunicamycin. Evidence was also found to support our hypothesis that disulfide bond formation utilises molecular oxygen \textit{in vivo} and can thus influence oxygen levels of the cell. More work is however required to solidify the role of disulfide bond formation in the hypoxic activation of the UPR.
REFERENCES


