A ROLE FOR THE N-TERMINAL DOMAIN IN MODULATING THE ACTIVITIES OF THE NUCLEOTIDE EXCHANGE FACTOR SIL1

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Excessive cellular reactive oxygen species (ROS), or oxidative stress, can lead to cell damage and is implicated in diseases such as cancer, aging and neurodegenerative disorders. Yet, although these highly reactive molecules can be damaging, ROS can also elicit beneficial effects in cells as signaling molecules. In the endoplasmic reticulum (ER), where ROS are produced from protein folding, the Hsp70 BiP plays a role in cell protection during ER oxidative stress. BiP is a chaperone that binds and release substrates, and these activities are regulated by its ATPase cycle. We have previously discovered that yeast BiP’s conserved cysteine gets modified by the ROS peroxide during oxidative stress conditions. Our lab discovered that the protein Sil1 functions in cells as a reductant that can remove the modification on BiP’s cysteine. The reductant activity of Sil1 is mediated through two N-terminal cysteines. Sil1 has been well-characterized as BiP’s nucleotide exchange factor (NEF) that facilitates ADP release. Sil1 is active as a NEF even in the absence of its N-terminal region. A molecular picture as to how the N terminus, which contains the redox-active cysteines, influences interaction with BiP remains unclear.

Our biochemical characterization of the full-length Sil1 has revealed that the N-terminal domain plays a role in modulating Sil1 activities. We uncovered that the presence of the N-terminal domain decreases Sil1 NEF activity and impacts the structural conformation adopted by full-length Sil1. We have mapped the region of the N terminus that accounts for the auto-inhibition of Sil1 NEF activity to a stretch of 36 amino acids of highly conserved sequence. Mutations in human SIL1 have been found in patients with Marinesco-Sjogren syndrome (MSS). One such mutation, R92W, is in the conserved N-terminal region. Through the characterization of the yeast
version, Sil1-R84W, we have demonstrated that the mutation increases NEF activity, impacts reductant activity, and changes the apparent conformation of Sil1. Our overall findings suggest that the N-terminal domain’s conserved region is important in regulating Sil1 activities. We predict that this region may undergo post-translational modifications in cells to modulate NEF and reductant functions of Sil1 under specific physiological conditions.
BIOGRAPHICAL SKETCH

Kristeen Alcaide Pareja was born on an island in Occidental Mindoro, Philippines, on July 17th, 1986. She is the second oldest out of the five children of Elaida Alcaide Pareja and Nestor Bautista Pareja. Kristeen has valued education at an early age, especially when she and her siblings were forced to drop out of school for two years due to poverty. In 2001, her family was extremely fortunate to immigrate to the United States with the help of her grandparents, Sofia and Anastacio Pareja. Kristeen then went to Richmond High School where her passion for science led to a research internship at Lawrence Berkeley National Laboratory for two summers where she studied chromium reduction in wastewater. Kristeen obtained her Bachelor of Science degree from UC Davis majoring in Biochemistry and Molecular Biology. During her undergraduate career, she became very interested in biochemistry and realized that going to graduate school will lead to more experience and will help her decide on future career plans. To gain more research experience before coming to graduate school, she did a summer internship at Novozymes where she worked in the protein chemistry department and studied biomass hydrolysis. She then became a research assistant in Dr. Alan Miller’s laboratory at Touro University where she investigated the potassium channel called HERG. After having various research experience, Kristeen decided to apply to the Biological and Biomedical Sciences program at Cornell University where she saw a variety of research laboratories she was interested in. She joined Dr. Carolyn Sevier’s laboratory as her rotation project on the molecular chaperone BiP sparked her curiosity on the molecular mechanisms involved in ER oxidative stress signaling. On her second year of graduate school, Kristeen received the NSF Graduate Research Fellowship.
Dedicated to my son, Leonel Pareja Navarro
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CHAPTER 1. INTRODUCTION

This dissertation is centered on the endoplasmic reticulum (ER) protein called Sil1 and how its activities are impacted by its N-terminal domain. Sil1 has gained attention over the last 13 years as mutations in the $SIL1$ gene have been found in patients with Marinesco-Sjogren syndrome (MSS). Sil1 is the nucleotide exchange factor (NEF) of BiP, a very well-characterized chaperone that is engaged in every aspect of ER protein quality control. We have shown that BiP and Sil1 are involved in ER oxidative stress signaling (Siegenthaler et al., 2017; Wang and Sevier, 2016; Wang et al., 2014). A conserved cysteine in BiP gets modified under ER oxidative stress, and this modification changes BiP’s behavior, making it protective against oxidative stress and important for cell survival. We have discovered that Sil1 has a reductant function that can remove the modification on BiP’s cysteine through its redox-active N-terminal cysteines, thus providing a mechanism for the reversal of BiP’s cysteine modification. The goal of my project was to characterize Sil1 with its N-terminal domain that contains the cysteines that can remove the modification on BiP’s cysteine. Because Sil1 does not require its N-terminal domain as a NEF (Rosam et al., 2018; Yan et al., 2011), functional studies exploring the N terminus have been limited. Through biochemical assays, I have found that the N-terminal domain of Sil1 impacts the conformation and both its NEF and reductant functions.

In the first part of the introduction, I will provide general information on reactive oxygen species (ROS) from protein folding in the ER. Then, I will discuss the role of the ER Hsp70 BiP in responding to ROS. The second part will be focused on what is known about Sil1 and its functions, including its role in ER oxidative stress signaling through its
interaction with BiP. I will also discuss what is known about the disease MSS which is caused by mutations in \textit{SIL1}.

**ER OXIDATIVE STRESS SIGNALING AND THE ROLE OF THE HSP70 BIP**

**The ER is a source of Reactive Oxygen Species (ROS)**

ROS such as superoxide anion (O\textsuperscript{−}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radical (HO\textsuperscript{•}) are highly reactive molecules that can damage our DNA, lipids and proteins (D'Autréaux and Toledano, 2007). These molecules can come from environmental factors such as smoking as well as from our intracellular metabolism. In particular, H\textsubscript{2}O\textsubscript{2} molecules are known to be produced by the mitochondria (Boveris and Chance, 1973; Brand, 2010), peroxisomes (De Duve and Baudhuin, 1966; Fransen and Lismont, 2018) and the endoplasmic reticulum (Tu and Weissman, 2002). One of the well-known biological processes that produces ROS is oxidative protein folding in the ER (Tu and Weissman, 2002). Disulfide bond formation in the ER involves the proteins called protein disulfide isomerase (PDI) and ER oxidoreductin 1 (Ero1) (Frand and Kaiser, 1998; Pollard et al., 1998). Ero1 oxidizes PDI and transfers the electrons to molecular oxygen through its FAD cofactor, producing H\textsubscript{2}O\textsubscript{2} (Tu and Weissman, 2002) (**Fig. 1.1**). Oxidized PDI then accept the electrons from nascent folding proteins, thus catalyzing the formation of disulfide bonds (**Fig.1.1**) (Tu and Weissman, 2004).

The accumulation of ROS can lead to oxidative stress and is implicated in many diseases such as cancer, diabetes, aging and neurodegenerative disorders. The damaging impact of ROS and oxidative stress highlight the importance of anti-oxidants (e.g. Vitamin C, reduced glutathione, catalase) that control the amounts of, or detoxify,
The proteins PDI and Ero1 catalyze the formation of disulfide bond formation during oxidative protein folding through an electron transport chain (curved arrows). The flavoprotein Ero1 drives the reaction through its FAD cofactor using oxygen as the electron acceptor resulting into the production of H$_2$O$_2$ (a type of ROS).
ROS molecules for cell protection. However, it is also known that ROS are important molecules in signal transduction (Veal et al., 2007). For example, we have shown that the yeast Hsp70 in the ER called BiP gets modified by peroxide. The modification of BiP’s cysteine changes its chaperone activity, leading to protection of cells under ER oxidative stress (Wang et al., 2014).

**The Hsp70 BiP and its functions in the ER**

The molecular chaperone BiP (immunoglobulin heavy-chain binding protein), also known as Grp78, belongs to the family of Hsp70s (70-kDa heat shock proteins) and is considered to be the master regulator of the endoplasmic reticulum (ER) proteostasis (Haas and Wabl, 1983; Wang et al., 2017). The Hsp70 family of proteins are highly conserved and ubiquitously expressed. About a third of the genome encodes proteins that are processed through the secretory pathway and molecular chaperones like BiP are important for their successful folding, assembly, maturation and maintenance. BiP is the most abundant Hsp70 in the ER lumen and a well-characterized molecular chaperone.

BiP has been demonstrated to be important in protein folding, protein translocation, mediating activation of the unfolded protein response (UPR), ER-associated degradation (ERAD) and maintaining Ca$^{2+}$ homeostasis. BiP is known to bind to hydrophobic stretches of newly-synthesized peptides, keeping them from premature folding or aggregation, thus promoting proper folding (Bole et al., 1986; Gething and Sambrook, 1992; Hammond and Helenius, 1994). BiP is also required for both co- and posttranslational translocation of proteins into the ER lumen (Brodsky et al., 2005). In addition, BiP is a target as well as a regulator of the UPR. The UPR is a response that cells undertake to adapt and cope when stress or proteostatic imbalance occurs in the ER (Ron and Walter, 2007). The UPR
results into processes that will help restore balance and maintain homeostasis such as attenuation of translation to decrease protein load and induction of chaperones to increase folding capacity. In higher eukaryotes like humans, there are three pathways for UPR signaling mediated by the membrane proteins inositol-requiring enzyme 1 (IRE1), protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Chakrabarti et al., 2011). When there is an accumulation of misfolded proteins, BiP dissociates from the lumenal domains of IRE1, PERK and ATF6, thus activating the UPR signaling pathways (Chakrabarti et al., 2011; Wang et al., 2017). Another pathway that BiP is involved in is the process known as ERAD where BiP assists in retro-translocation of aberrant or misfolded proteins to target them for proteasomal degradation in the cytosol (McCracken et al., 1998; Plemper et al., 1997). BiP is also important for binding Ca^{2+} and preventing its leakage through the Sec61 protein conducting channel (Schäuble et al., 2012). The numerous pathways that BiP is involved in demonstrate the importance of this chaperone in maintaining ER proteostasis.

**BiP structure and its ATPase cycle**

Like other Hsp70s, BiP has two conserved domains, the nucleotide binding domain (NBD) (also known as the ATPase domain) and the substrate binding domain (SBD), connected by a flexible linker (Fig. 1.2) (Alderson et al., 2016; Yang et al., 2017). Numerous structures of BiP and its homologs have been solved (Bertelsen et al., 2009; Kityk et al., 2018; Yang et al., 2017). These structures together with biochemical assays have provided insights into how BiP performs its functions as a chaperone. The NBD of BiP is divided into four subdomain lobes: IA, IIA, IB, IIB (Fig. 1.2). The nucleotide binds
Figure 1.2. Crystal structure of full-length BiP with ATP.

(A, B) Crystal structure of full-length human BiP when bound to ATP (pink) shows that the NBD (teal) makes contact with SBDα (green). PDB: 6ASY (C) Cartoon depiction of BiP with labels of subdomains of NBD (IA, IIA, IB, IIB) and SBD (α, β).
in the central pocket of the NBD. The SBD is also subdivided into alpha-helical SBDα lid and SBDβ which consists of beta-sheets (Fig. 1.2). The NBD and SBD domains are allosterically coupled and known to adopt multiple conformations (Mayer and Bukau, 2005; Zuiderweg et al., 2012). Crystal structures of BiP and its bacterial homolog, DnaK, bound to ATP have shown that the protein goes through a dramatic conformational change with the linker and SBDα lid making contacts with the NBD (Fig. 1.2) (Kityk et al., 2018; Yang et al., 2017). When ATP gets hydrolyzed to ADP, the two domains separate and the SBD subdomains are in a closed conformation (Bertelsen et al., 2009). The conformations of SBD from these structures support biochemical assays that show Hsp70s have high on and off rates for peptide substrates when the NBD is bound to ATP (Bukau et al., 2000; Greene et al., 1995; Schmid et al., 1994). The ATPase cycle regulates the conformational changes of BiP’s NBD and SBD, allowing the chaperone to bind and release substrates.

**Co-chaperones stimulate the ATPase cycle of BiP**

BiP and Hsp70s in general have low intrinsic ATPase activity and co-chaperones are known to stimulate this cycle: J proteins trigger ATP hydrolysis while nucleotide exchange factors (NEFs) promote the release of ADP (Fig. 1.3) (Behnke et al., 2015; Kampinga and Craig, 2010). These co-chaperones have also been characterized and some have been shown to impact distinct Hsp70 functions. One type of Hsp70 co-chaperones is referred to as J domain proteins or J proteins. J proteins share a conserved 70 amino acid residue region, referred to as J domain after the bacterial DnaJ, which
Figure 1.3. BiP’s ATPase cycle regulates its activity as a chaperone.

BiP has two conserved domains, the nucleotide binding domain or NBD also known as ATPase domain (teal) and the substrate binding domain or SBD (green) that change conformations based on its ATPase cycle. ATP binding leads to docking of the SBDα on the NBD and opening of the SBD subdomains. This conformation of BiP has a lower affinity for peptide substrates. ATP hydrolysis (ADP-bound BiP) causes the two domains to be separated. The SBD domain adopts a closed-conformation and has a higher affinity for peptides. J proteins stimulate ATP hydrolysis while nucleotide exchange factors, Sil1 and Lhs1 (Grp170), promote the release of ADP from BiP’s nucleotide binding domain.
stimulates the ATP hydrolysis of bacterial Hsp70 (DnaK) (Kampinga and Craig, 2010; Karzai and McMacken, 1996). Crystal structures of Hsp70 bound to a J domain show that the J domain binds to NBD’s lobes IA and IIA as well as to the flexible linker, destabilizing the NBD and SBD interface of ATP-bound Hsp70 (Jiang et al., 2007; Kityk et al., 2018). In the mammalian ER, there are currently 8 known J proteins (ERdj1-8), some of which are transmembrane proteins with luminal J domains while some are soluble (Kampinga and Craig, 2010). Furthermore, some J proteins also bind to client proteins and deliver them to their Hsp70 partners. For example, ERdj3-6 have all been shown to bind to client proteins (Behnke et al., 2016). It is widely accepted that the variety of J proteins allow modulation of BiP activities in performing distinct functions. For example, BiP and the J domain containing transmembrane protein, Sec63, work together for efficient translocation of polypeptides into the ER (Matlack et al., 1999).

Grp170 (glucose regulate protein of 170 kD), also known as Lhs1 (lumenal Hsp seventy), and Sil1 (suppressor of the ire1Δ lhs1Δ) are the two known NEFs in the ER. Although Lhs1 and Sil1 are structurally different, both bind in distinct but overlapping regions of BiP. Lhs1 is similar to BiP in that it also has a nucleotide binding domain and a substrate-binding domain. A crystal structure of the cytosolic homolog of Lhs1, Sse1, in complex with the Hsp70, Ssa1, demonstrates that the NBD and C-terminal helix bundle of Sse1 make contacts with the Hsp70’s lobe IIB. Comparing the structure of the Sse1-Ssa1 with ADP-bound Ssa1 shows that Sse1 binding leads to rotation of Ssa1’s lobe IIB by 27° (Polier et al., 2008). Similarly, Sil1 binds to BiP’s NBD lobe IIB. However, Sil1 binding only leads to approximately 13.5° rotation of BiP’s lobe IIB but also causes rotation of lobe IB by about 3.7° (Yan et al., 2011).
J proteins and NEFs bind to different subdomains of BiP’s NBD. It is possible that they may interact with BiP at the same time even though they prefer either ADP or ATP-bound BiP. Sil1 for example can still bind to ATP-bound BiP although not as pronounced as binding to ADP-bound (Hale et al., 2010).

**BiP modifications**

As mentioned above, the ROS hydrogen peroxide produced in the ER is used by cells as a signaling molecule that can modify BiP and alter its activities. Using a hyperactive Ero1 mutant, we can specifically induce oxidative stress in the ER (Sevier et al., 2007). By inducing oxidative stress in the ER, we have uncovered that a highly conserved cysteine residue in the NBD domain of BiP gets modified by peroxide in yeast BiP, Kar2. Yeast BiP C63 is susceptible to modification by peroxide (sulfenylated) and glutathione (glutathionylated). Modification/oxidation of the conserved cysteine allosterically decouples the two domains of BiP in that it causes enhanced holdase activity and decreased ATPase activity. We propose that this modification may lead to protection of cells under ER oxidative stress as it prevents aggregation of proteins (Fig. 1.4) (Wang and Sevier, 2016; Wang et al., 2014). Another function of oxidized BiP has recently been demonstrated by Ponsero et al., (2017). They have shown that oxidized BiP (Kar2) blocks the import of glutathione into the ER through the Sec61 channel (Ponsero et al., 2017). The authors have argued that Ero1 activation and glutathione transport are coupled, which help maintain ER redox homeostasis. Oxidation of BiP through Ero1 activation, which increases during ER stress, leads to negative regulatory feedback loop through BiP blocking the import of reduced glutathione. These results suggest that oxidized BiP may also help with cell protection under ER oxidative stress by inhibiting the activation of
Figure 1.4. Cysteine modification of yeast BiP by peroxide.

The modification of conserved cysteine decouples the two domains of BiP leading to enhanced holdase activity and decreased consumption of ATP. We have proposed that modified BiP protects cells from ER oxidative stress as it may lead to prevention of protein aggregation.
of Ero1 and further production of peroxide (Fig. 1.1). It will be interesting to see how oxidation of BiP impacts its many other functions in the ER and how it would impact its interactions with its cofactors.

BiP modifications have also been seen in other orthologs. For example, mouse BiP’s cysteines, C41 and C420, get oxidized by the glutathione peroxidase, NPGPx, under oxidative stress. This modification also leads to enhanced holdase function of BiP (Wei et al., 2012). The authors suggest that the interaction of NPGPx with BiP is important for oxidative stress signaling as loss of NPGPx in mice showed increased oxidative stress and shortened lifespan (Wei et al., 2012). Several research laboratories have also shown that BiP’s activity changes based on fluctuations of protein load in the ER. For example, drosophila, human and hamster BiP have been demonstrated to be AMPylated, a posttranslational modification that involves the addition of AMP to hydroxyl side chains of proteins (Ham et al., 2014; Preissler et al., 2015a; Sanyal et al., 2015). The AMPylation of mammalian BiP by the enzyme FICD at residue T518 has been demonstrated to occur when there is less unfolded protein load, thus inactivating its chaperone activity. Removal of the modification by the same enzyme leads to reactivation of BiP and occurs when the unfolded proteins increase, making AMPylation an important posttranslational modification for modulating BiP’s activity (Preissler et al., 2015a). Furthermore, BiP is also known to form oligomers through interactions between SBD of one BiP molecule and the hydrophobic linker of another. Oligomerized BiP are thought to be inactive and can be disassembled when there is an increase in unfolded protein load (Preissler et al., 2015b). Thus, BiP’s chaperone functions can be modulated by inactivating it through posttranslation modifications and through the physiological state of the cells. A pool of inactive
BiP that can be activated may be beneficial when there is a sudden increase of protein load.

THE HSP70 NUCLEOTIDE EXCHANGE FACTOR SIL1

Identification of Sil1 and its homologs in model systems

Sil1 is an ER-resident glycoprotein originally known for binding the Hsp70 BiP, stimulating its ATPase cycle as an exchange factor, and for playing a role in protein translocation. *Y. lipolytica* Sls1, the first Sil1 ortholog identified (Table 1.1), was discovered from a genetic screen that aimed to find suppressors of mutations in the 7S RNA component of the signal recognition particle (SRP) important for SRP-dependent translocation (Boisramé et al., 1996a). Thus, Sls1’s suppressor phenotype suggests that it may be important for translocation. This observation is further supported by a study of an ortholog in *S. cerevisiae*, termed Sc Sls1 (Table 1.1) that showed synthetic lethality of *Sc sls1* deletion strains in combination with strains already defective in translocation (Kabani et al., 2000). A yeast two-hybrid screen showed that Sc Sls1 and yeast BiP (Kar2) physically interact, and biochemical assays indicated that Sc Sls1 is able to stimulate BiP’s ATPase hydrolysis, especially in the presence of GST-Sec63J, a J protein known to stimulate ATP hydrolysis of BiP. Further investigation suggested that Sc Sls1 may be acting as a nucleotide exchange factor as BiP binds less to both ATP and ADP when Sc Sls1 is present and Sc Sls1 preferentially binds to ADP-bound BiP (Kabani et al., 2000). Meanwhile another lab identified the identical *S. cerevisiae* ER NEF and coined it Sil1 when it appeared from a genetic screen as a suppressor of the *ire1△ lhs1△* double mutant (Table 1) (Tyson and Stirling, 2000). Ire1 is the activator of the UPR in yeast and Lhs1 (Grp170) is the other known NEF of BiP. Even though yeast *SIL1* is non-essential, both
Table 1.1 Sil1 orthologs and homologs.

<table>
<thead>
<tr>
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<td></td>
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<td><em>S. cerevisiae</em></td>
<td>Kabani et al., 2002</td>
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*SIL1* and *LHS1* are important for translocation and loss of both NEFs is lethal, implying that they play similar roles in the cell. The authors also found that BiP’s ATPase domain is sufficient for interacting with Sil1 (Tyson and Stirling, 2000). The mammalian version of Sil1, called BAP for BiP-associated protein (Table 1.1), was discovered through a yeast-two hybrid system using the ATPase domain of hamster BiP as bait (Chung et al., 2002a). BAP was shown to stimulate the ATP hydrolysis activity of BiP two-fold by itself and even more so (four-fold) when combined with the J protein, ERdj4. Moreover, BAP preferentially binds to ADP-bound BiP and causes BiP to release ADP, thus also providing evidence that BAP is a NEF of BiP (Chung et al., 2002a).

As there are Hsp70s throughout the cell, NEFs for these Hsp70s also exist. Human HspBP1 and yeast Fes1 are nucleotide exchange factors in the cytosol that have similar structures but share low sequence identities with yeast and human Sil1 (Table 1.1, Fig. 1.5). HspBP1 was pulled from a yeast two-hybrid screen of a human heart cDNA library using a human Hsp70 ATPase domain as bait (Raynes and Guerriero, 1998). Pull-down results indicated that Hsp70 and HspBP1’s ATPase domain bind in vivo as well and biochemical assays showed that high concentrations of HspBP1 (about five to six-fold greater than Hsp70) slightly stimulate Hsp70’s ATP hydrolysis. However, the stimulation of Hsp70’s ATP hydrolysis by Hsp40, a J domain protein, decreased when HspBP1 was added. Moreover, the ability of Hsp70 to refold denatured luciferase was explored in the presence of Hsp40 and HspBP1 and results indicated that adding higher concentrations of HspBP1 inhibited luciferase refolding (Raynes and Guerriero, 1998). Another research group reports this inhibition of Hsp70’s refolding activity by showing that HspBP1 inhibits luciferase refolding in vitro. Intriguingly, HspBP1 was able to act on both yeast and
Figure 1.5 Alignment of Sil1 orthologs and homologs.

Sequence alignment of the ER NEFs (S. cerevisiae Sil1, H. sapiens BAP) and cytosolic NEFs (S. cerevisiae Fes1, and H. sapiens HspBP1) shaded at 50% homology shows very little sequence conservation between Sil1 orthologs and homologs.
mammalian cytosolic Hsp70. Their in vitro assays also show that HspBP1 inhibits ATP hydrolysis of cytosolic Hsp70, Ssa1 (Kabani et al., 2002a). Additionally, a study investigated the impact of the N terminus of HspBP1 in its ability to refold denatured luciferase. Interestingly, they found that HspBP1 showed less inhibitory activity when the N terminus was truncated (McLellan et al., 2003).

Fes1, named for factor exchange for Ssa1, was identified from homology search of Sls1/Sil1 in the yeast genome using PSI-BLAST. Fes1 was shown to be important for heat shock response and translation as evidenced by sensitivity of the FES1 deletion strain to higher temperatures and the translation inhibitor drug cycloheximide. Like its homologs, Fes1 prefers binding to ADP-bound Ssa1, and its binding results into more dissociation of ATP and ADP from the Hsp70. Unlike HspBP1 which can promote nucleotide release from yeast Ssa1, the interaction between Ssa1 and Fes1 is specific as Fes1 is unable to bind to Kar2 in pull-down assays. Fes1 also seems to inhibit ATPase activity of Ssa1 (Kabani et al., 2002b).

It is important to note that the in vitro assays that show inhibition of Hsp70's ATPase activity and refolding activity by either HspBP1 and Fes1 may be influenced by the excess amount of the nucleotide exchange factors relative to the Hsp70.

Sil1 functions and expression

The highly abundant chaperone BiP performs many functions in the ER and some of these functions have been shown to be influenced by Sil1. As mentioned above, Sil1 has been shown to be important for protein translocation. It has also been suggested that Sil1 may play a role in ER-associated degradation (ERAD) as deleting Sil1 in yeast strains
defective in ERAD causes reduced viability (Kabani et al., 2000). Additionally, a study investigating the relationship between UPR and ERAD has shown that deleting Sil1 leads to slower clearance or degradation of an ERAD substrate called CPY* (Travers et al., 2000). BiP plays an important role in insulin secretion by beta cells as reducing BiP expression also leads to reduced insulin secretion. Sil1 has been shown to play a role in this process as Sil1 knockout mice also exhibit decreased insulin secretion (Ittner et al., 2014). Recently, we have demonstrated that Sil1 also plays a role in ER oxidative stress signaling by reducing oxidized BiP (Siegenthaler et al., 2017). This novel function of Sil1 suggests new possibilities for other Sil1 substrates or interactors and new pathways that Sil1 may be involved in.

Because Sil1 is BiP’s co-chaperone and it has an N-terminal signal sequence as well as a C-terminal KELR retention signal sequence, it is generally accepted that Sil1 is localized in the ER lumen. It has also been shown that Sil1 co-localizes with BiP in the ER through immunofluorescence staining (Boisramé et al., 1996; Chung et al., 2002). However, there has been a debate whether the sequence KELR is a true retention signal as disrupting this sequence does not cause Sil1 to be secreted (Howes et al., 2012). It is possible that what keeps Sil1 in the ER is its interaction with a much more abundant BiP, which has been observed to be a thousand times more abundant than Sil1 in canine pancreas (Weitzmann et al., 2007). However, the expression levels of Sil1 and relative abundance with BiP is not the same in all tissues. Examination of a human multiple tissue Northern blot has shown that although Sil1 is ubiquitously expressed like BiP, it is more abundant in placenta, liver, kidney, skeletal and heart tissues (Chung et al., 2002). The expression of Sil1 may also vary in different species. For example, Sil1 has been shown
to be highly expressed in mouse pancreas but not in liver or muscle tissues (Ittner et al., 2014).

Sil1 localization and expression levels may also be influenced by the physiological state of the cells. Heat shock and treatment with the N-glycosylation inhibitor tunicamycin caused two-fold induction of Sil1 in *Y. lipolytica* (Boisramé et al., 1996), and Sil1 in *S. cerevisiae* has been shown to be a UPR target (Travers et al., 2000; Tyson and Stirling, 2000).

**Sil1 structure and the mechanism of NEF activity**

The crystal structure of yeast Sil1 in complex with yeast BiP’s ATPase domain has been solved (Fig. 1.6), and this structure provides mechanistic details into how Sil1 can facilitate the release of ADP from BiP’s nucleotide binding pocket. The structure of the complex includes Sil1 residues 125-406 and BiP’s NBD or ATPase domain (residues 47-426). Sil1 is composed of 16 α-helices and the 14 central helices form four armadillo repeats (ARM1-ARM4) (Fig. 1.6). Sil1 acts like a clamp that opens up BiP’s NBD lobes, weakening the interaction with the bound ADP (Yan et al., 2011). The structure for HspBP1 has also been solved in complex with the cytosolic Hsp70’s IIB subdomain. HspBP1 has a very similar structure to Sil1 with the core domain consisting of four armadillo repeats. The co-crystal structure illustrates that HspBP1 also binds to lobe IIB of Hsp70, but it shows some steric conflict between HspBP1’s N terminus and Hsp70’s lobe IB (Shomura et al., 2005). Both structures of Sil1 and HspBP1 do not have the N-terminal domain as these domains are thought to be too flexible and disordered for crystallization. This is in line with CD spectra of full-length and truncated Sil1 or HspBP1, which show that the N-terminal domain forms a random coil while the core domain
Figure 1.6 Diagram of Sil1 sequence and co-crystal structure of yeast Sil1 and yeast BiP’s ATPase domain.

(A) Diagram of Sil1 sequence showing the solved structure of the protein consisting of four armadillo repeats (ARM). (B) Co-crystal structure of *S. cerevisiae* Sil1 (125-406) and BiP’s NBD or ATPase domain (47-426) shows that Sil1 core domain binds to BiP’s lobe IIB. (PDB:3QML)
consists of \(\alpha\)-helix secondary structures (McLellan et al., 2003; Rosam et al., 2018). Nevertheless, the NEFs without their N-terminal domains are effective at promoting the release of ADP from the Hsp70’s nucleotide binding domain (Shomura et al., 2005; Yan et al., 2011).

**The role of the N-terminal domain of Sil1**

The solved structure of Sil1 does not contain the purportedly flexible N-terminal domain (Yan et al., 2011) and because the truncated protein is functional as a NEF, the N-terminal domain has been ignored. However, recent studies suggest that this domain has several functions. We have shown that Sil1 has a pair of N-terminal redox-active cysteines, C52 and C57, that are able to remove the modification on BiP’s cysteine (Siegenthaler et al., 2017). Either of the two cysteines can form a mixed-disulfide with oxidized BiP. These data show that Sil1 has another function as a reductant, and it is possible that Sil1 has other substrates that it can reduce other than oxidized BiP.

The N-terminal domain of human Sil1 has been demonstrated to bind to BiP’s substrate binding domain (SBD), thus behaving as a pseudo-substrate and play a role in regulating BiP’s ATPase activity. Rosam et al. (2018) suggests that the N-terminal domain binds to BiP’s SBD and promotes the release of an already bound peptide or substrate, thus resetting BiP’s ATPase cycle. They used a prediction server BiPPred (Schneider et al., 2016) to identify sequences that will bind BiP and predicted that the residues in the N-terminal domain of Sil1, EFALTNP and HVRLNLQ, will bind strongly to BiP’s substrate binding domain. ATPase assays reveal that the predicted peptide, EFALTNP, stimulates BiP’s ATPase cycle with the same magnitude as a known substrate of BiP. Their spFRET studies also demonstrate many interesting conformational changes that occur in apo or
nucleotide-bound BiP when mixed with either the N-terminal domain alone, Sil1 without the N-terminal domain, or with full-length Sil1. For example, the SBD of ADP-bound BiP are mostly in closed conformation. When Sil1 is added, some BiP SBDs are in open conformations. However, adding Sil1 without its N-terminal domain causes about half of BiP SBDs to be open, and adding only the N-terminal domain results in most of BiP SBDs to be in open conformations (Rosam et al., 2018). These data give more insights into how the N-terminal domain of Sil1 influences BiP’s ATPase cycle by not only causing conformational changes in BiP’s nucleotide binding domain but also its SBD. These findings are further supported by another group’s research on Fes1 and HspBP1. Through photo-crosslinking experiments, they provided evidence that the N-terminal domain of Fes1 directly binds to the substrate binding domain of the Hsp70 Ssa1 (Gowda et al., 2018). These new insights on the role of N-terminal domain may explain why previous studies have shown that HspBP1 and Fes1 display inhibitory activities as adding high concentrations of the NEFs may compete with the substrates of the Hsp70s in vitro. The N-terminal domain is important for Sil1 activities and it will be valuable to obtain the structure of this part of the protein to understand the mechanisms involved in performing its functions as BiP’s nucleotide exchange factor and reductant.

**Sil1 modification**

Unlike BiP, there are not that many post-translational modifications known for Sil1. Human and yeast Sil1 have both been shown to be glycosylated as treatment of cells with the glycosylation inhibitor, tunicamycin, leads to a lower molecular weight protein (Chung et al., 2002a; Stevens et al., 2017). Intriguingly, it has been shown that treatment of cells with DTT leads to accumulation of unglycosylated Sil1. Further investigation reveals that
Sil1 undergoes N-glycosylation at amino acid residue N181 by one of the subunits of the oligosaccharyltransferase, Ost3 (Stevens et al., 2017). The lumenal domain of Ost3 contains a CxxC thioredoxin-like motif that is important for redox-dependent binding of peptides. Ost3’s oxidoreductase activity is important for N-glycosylation of Sil1. The group was able to show that mutating Sil1-N181 to Q (which prevents N181 glycosylation) protects against reductive stress and seems to increase Sil1 function as the mutant better compensates for the loss of Lhs1 in ire1Δ lhs1Δ double mutant. They hypothesize that reductive stress results into structural changes in Ost3, preventing it from glycosylating Sil1. As unglycosylated Sil1 seems to be more functional in the presence of the reducing agent, DTT, it seems that under non-stress conditions, glycosylation of Sil1 negatively regulates aspects of its functions (Stevens et al., 2017). It will be interesting to identify other enzymes that post-translationally modify Sil1 and determine how these modifications impacts Sil1 activities as both a NEF and a reductant.

**Mutations in SIL1 cause Marinesco-Sjögren syndrome**

Mutations in SIL1 have been associated with a very rare autosomal recessive disease called, Marinesco-Sjögren syndrome (MSS). MSS is a multisystemic disorder that impacts the eyes, brain and muscle tissues of patients. The three most common features of MSS are congenital or early-onset cataracts, cerebellar atrophy and myopathy. Some MSS patients also display varying degrees of mental, skeletal, and motor abnormalities (Krieger et al., 2013). Several laboratories around the world focus on trying to understand and explain how disruption in SIL1 cause MSS. However, more research on Sil1 activities and behavior is necessary to consider it a therapeutic drug target to combat MSS.
A link between Sil1 and neurodegeneration was first established when a research group identified a recessive mouse mutation, called woozy (wz) (Zhao et al., 2005). The wz mice display adult-onset ataxia and degeneration of Purkinje cells. Immunostaining of wz mice brain sections show protein accumulation or aggregation and signs of ER stress. Genome mapping of the wz mutation reveals truncated Sil1 mRNA as the wz-causing phenotype. The same research group used gene-trapping to generate mice with truncated Sil1 mRNA, Sil1GT, which show similar phenotype as the wz mice (Zhao et al., 2005).

In the same year that the wz mice was discovered, two separate research groups established that patients with MSS have mutations in their SIL1 genes (Anttonen et al., 2005; Senderek et al., 2005) The authors identified and characterized different mutations leading to frame shifts and truncations of SIL1 in MSS patients (Table 1.2). These patients display the phenotypic hallmarks of MSS such as myopathy, congenital or infantile cataracts and cerebellar atrophy. Thus far, no other genes have been implicated in MSS other than SIL1. Many other research groups have since identified MSS-causing SIL1 mutations in about 100 patients and most of these mutations lead to a significant portion of Sil1 truncated (Table 1.2) (Annesi et al., 2007; Anttonen et al., 2005, 2008; Byrne et al., 2015; Cerami et al., 2015; Eriguchi et al., 2008; Ezgu et al., 2014; Gai et al., 2016; Goto et al., 2014; Hasegawa et al., 2014; Horvers et al., 2013; Karim et al., 2006; Krieger et al., 2013; Nair et al., 2016; Noreau et al., 2015; Riazuddin et al., 2009; Senderek et al., 2005; Takahata et al., 2010; Terracciano et al., 2012). Furthermore, only several point mutations have been identified (Table 1.2). Meanwhile there are some MSS patients without SIL1 mutations that display the same clinical features, but have no known genetic
Table 1.2. List of known MSS-causing Sil1 mutations.
Highlighted in red are point mutations that lead to single amino acid change.

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Table 1.2 List of known MSS-causing Sil1 mutations continued.

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mutations, suggesting genetic heterogeneity of MSS (Anttonen et al., 2008; Goto et al., 2014; Schulz et al., 2007; Singhi et al., 2014; Yiş et al., 2011). Anttonen and colleagues also investigated other candidate genes (HSPA5, HYOU1, AARS, genes coding for BiP, Grp170, alanyl t-RNA synthetase respectively) in SIL1 mutation-negative MSS patients but did not identify any mutations responsible for the disease phenotype (Anttonen et al., 2008).

Since disruption of SIL1 has been discovered to be a cause of MSS, there has been ongoing research on how disfunction or loss of the protein can lead to disease. Some research groups have characterized known MSS-causing mutations. For example, three mutations that impact the retention signal, KELR, have been characterized. It was postulated that these mutations cause MSS by disrupting Sil1 retention in the ER (Anttonen et al., 2008; Senderek et al., 2005). However, it has been shown that two of these mutants, L456fs and L457P, do not cause Sil1 to be secreted from the ER, but instead the mutations cause aggregation and accumulation of Sil1 (Howes et al., 2012). Several other groups have investigated MSS phenotypes through animal models. Analysis of the motor function of the wz mice ages 5 to 71 weeks show that they develop muscle weakness at 11 weeks (Hayashi et al., 2017). Further characterization of the wz mice phenotype suggest that loss of Sil1 function leads to changes in the nuclear envelope in muscles and loss of body weight (Buchkremer et al., 2016). Another group investigated how loss of SIL1 impacts ER homeostasis ultimately leading to defects in muscle physiology in Sil1GT mice (Ichhaporia et al., 2018). Zebrafish with disrupted sil1 mRNA has also been used as animal models for MSS. A zebrafish MSS model has smaller eyes, shows a loss of Purkinje cells and abnormal muscle formations, which are
all hallmarks of the disease (Kawahara and Hayashi, 2016). The animal models are great tools for understanding MSS and display great potential for drug screening tools. Studies on both MSS patients and animal models have shown a decrease or complete loss of Sil1 expression. To further investigate how loss of Sil1 can lead to disease, a recent study investigated the consequences of deleting *SIL1* in human embryonic kidney 293 (HEK293) cells (Roos et al., 2014). Their proteomics data suggest that Sil1 depletion impacts the expression of proteins in the ER, Golgi, mitochondria, plasma membrane, cytoplasm and the nucleus and that loss of Sil1 function causes the induction of the UPR and ERAD.

So far, most *SIL1* mutations found in MSS patients lead to non-functional proteins. In addition, MSS animal models that have disrupted *sil1* mRNA phenocopy the human disease patients. Since BiP is a critical chaperone and a major regulator of many processes in the ER, it is not hard to imagine how losing its co-chaperone known to regulate its activity will be detrimental to cells. However, we know that Sil1 is not only a NEF for BiP but also play a role as a reductant that can remove BiP’s cysteine modification. Even though reductant activity for human Sil1 has not been shown, the protein also contains two N-terminal cysteines that may be redox-active (Fig. 1.5). It is possible that loss of Sil1 is harmful as it inhibits the removal of the modification on oxidized BiP.

**Protective roles of Sil1 in neurodegenerative disorders**

There have been reports showing that Sil1 has protective roles in neurodegenerative disorders using mouse models for Amyotrophic Lateral Sclerosis (ALS) and Alzheimer’s Diseases (AD) (Filézac de L’Etang et al., 2015; Liu et al., 2016).
To elucidate the role of Sil1 in these diseases, Labisch and colleagues studied the consequences of overexpressing Sil1 in mammalian cells and investigated Sil1 expression under ER stress (Labisch et al., 2018). Increased Sil1 expression induced the UPR, ERAD and autophagy. Their results also show that under ER stress, Sil1 expression is increased and stabilized but interestingly, its interaction with BiP is reduced. Additionally, immunohistochemical staining of Sil1 in autopsies of Alzheimer’s disease cases show increased levels of Sil1 in surviving neurons as well as increased cytoplasmic population of Sil1. Overexpression of Sil1 in mammalian cells also showed increased cell proliferation with or without ER stress, further supporting protective role of Sil1. These findings suggest that Sil1 may have functions other than BiP’s co-chaperone as ER stress results in Sil1 induction but reduced affinity with BiP (Labisch et al., 2018).

To summarize, this dissertation focuses on the characterization of Sil1 with the N-terminal domain that has been left out in previous biochemical and structural studies. Sil1 is a nucleotide exchange factor of BiP that is known for stimulating the release of ADP to regulate BiP’s ATPase cycle. We have found that Sil1 also has another function as a reductant which depends on its two N-terminal cysteines. The structure of Sil1 in complex with BiP ATPase domain lacks the N-terminal domain that is important for its reductant activity. Our findings indicate that the N-terminal domain of Sil1 impacts the conformation and Sil1 activities as a NEF and reductant. A conserved region in the N-terminal domain may be going through post-translational modifications that can control or tune the movement of the N terminus, thus regulating its activities. Modulation of Sil1 activities may be another way of regulating the chaperone functions of BiP in a dynamic cellular environment. Understanding how the N-terminal domain of Sil1 regulate its function as a
NEF and reductant may lead us to better understand how loss of function of this protein may cause the disorder MSS and how increasing its levels may be protective in ALS and AD.
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Takahata, T., Yamada, K., Yamada, Y., Ono, S., Kinoshita, A., Matsuzaka, T., Yoshiura,


Tyson, J.R., and Stirling, C.J. (2000). LHS1 and SIL1 provide a lumenal function that is essential for protein translocation into the endoplasmic reticulum. EMBO J. 19, 6440–6452.


CHAPTER 2. A ROLE FOR THE N-TERMINAL DOMAIN IN MODULATING THE ACTIVITIES OF THE NUCLEOTIDE EXCHANGE FACTOR SIL1

ABSTRACT

Sil1 is a well-known nucleotide exchange factor (NEF) of the endoplasmic reticulum (ER) Hsp70 BiP. The structure of the Sil1 and BiP complex reveals that Sil1 binding results into the opening of BiP’s nucleotide binding domain, thus facilitating ADP release (Yan et al., 2011). However, the structure does not include the N-terminal domain of Sil1 that has been recently suggested to be important in its functions and interaction with BiP. We have previously shown that Sil1 has redox-active N-terminal cysteines that can reduce oxidized BiP (Siegenthaler et al., 2017). In addition, the N terminus has been suggested to be important for oligomerization (Yan et al., 2011) and has been demonstrated to bind to the substrate binding domain of BiP (Gowda et al., 2018; Rosam et al., 2018). Additionally, one of the point mutations identified in a patient with Marinesco-Sjögren syndrome (MSS), a very rare multisystemic disease, is within this region of the N terminus of Sil1 (Riazuddin et al., 2009). We have focused on biochemically characterizing full-length Sil1 to gain a better understanding of the role of the N-terminal domain. Here we demonstrate that the conserved N-terminal region of Sil1 is important for its apparent conformation and activities as a NEF and reductant. Furthermore, our findings suggest that the NEF activity of Sil1 is autoinhibited by the N terminus. Characterization of an equivalent mutation of the MSS-associated mutation in yeast, Sil1-R84W, showed that replacement of this conserved arginine changes its apparent conformation and influences its reductant function. We hypothesize that the conserved N-terminal of Sil1 region modulates its activities and may be a place of regulation.
INTRODUCTION

The Hsp70 BiP is a molecular chaperone important for maintaining ER proteostasis. BiP has two conserved functional domains, a nucleotide binding domain and a substrate binding domain, that are allosterically coupled. The domains adopt multiple conformations dictated by the ATPase cycle (Mayer et al., 2003; Zuiderweg et al., 2012). Sil1 is a nucleotide exchange factor (NEF) that stimulates BiP’s ATPase cycle by facilitating ADP release. The co-crystal structure of yeast Sil1 without its N-terminal domain (residues 113 to 421) and yeast BiP’s ATPase domain (residues 43-426) has been solved (Yan et al., 2011). The structure reveals that Sil1 acts like a clamp that causes the opening of BiP’s nucleotide binding domain. Sil1 binding facilitates ADP release by weakening the interactions of the bound nucleotide with the nucleotide binding pocket of BiP (Yan et al., 2011). The co-crystal structure and biochemical studies show that the N-terminal domain of Sil1 is dispensable for NEF activity (Rosam et al., 2018; Yan et al., 2011). However, a growing number of evidence suggests that the N-terminal domain has several roles. We have recently shown that yeast Sil1, has a pair of N-terminal cysteines that work in tandem to remove the modification on conserved cysteine of yeast BiP (Siegenthaler et al., 2017), making the N-terminal domain necessary for reductant activity. It has recently been shown that the N-terminal domain of Sil1 and its homologs, Fes1 and HspBP1, can also bind to the substrate binding domain of BiP (Gowda et al., 2018; Rosam et al., 2018). In addition, the N-terminal domain has been suggested to be important for Sil1 dimerization (Yan et al., 2011). The present study demonstrates that the N-terminal domain is important for apparent conformation as well as NEF and reductant functions of Sil1. Prediction servers suggest that the purportedly
flexible N-terminal domain is anticipated to form secondary structures (Drozdetskiy et al., 2015; Källberg et al., 2012). We hypothesize that the conserved region in the N-terminal domain may play a role in stabilizing these secondary structures. Our biochemical assays show that disrupting the highly conserved region by mutating certain conserved residues in the N-terminal domain increases NEF activity of Sil1. These data imply that the N-terminal domain of Sil1 behaves as an inhibitor of NEF activity. Notably, mutations in the SIL1 gene have been shown to cause an autosomal recessive disease called Marinesco-Sjogren syndrome (MSS) (Anttonen et al., 2005; Senderek et al., 2005). MSS is a disease characterized by congenital or infantile cataracts, cerebellar atrophy and myopathy. Sil1 mutations found in MSS patients range from point mutations to deletions, mostly leading to non-functional proteins (Anttonen et al., 2005; Horvers et al., 2013; Krieger et al., 2013; Senderek et al., 2005). How disruption of SIL1 causes MSS is not well-understood. Intriguingly, one of the MSS mutations maps to the N-terminal region, Sil1-R92W (Riazuddin et al., 2009). Biochemical characterization of an equivalent mutation in yeast, Sil1-R84W, revealed that the disease mutation also increases NEF activity, affects the conformation and reductant function of the protein. We constructed additional Sil1 mutants with point mutations in the N-terminal region (N-terminal point mutants) and these mutants behaved similarly as Sil1-R84W. In cells, we observed that the Sil1-R84W and other N-terminal mutants, show lower protein expression than wild-type Sil1. We suggest that the increased activity of our mutant alleles in vitro may not translate to increased function in cells and that the decreased protein levels in cells may relate to the pathologies observed for the R92W mutation in MSS. Our findings suggest that the N-terminal domain modulates Sil1 activities. We postulate that the conserved region within
the N terminus may be a place of regulation for activities of Sil1, allowing for tuning of NEF or reductant activities in a dynamic cellular environment.

RESULTS

The N terminus inhibits yeast Sil1 NEF activity

We employed stopped-flow spectroscopy to assess the NEF function of Sil1. The fluorescent ADP analog MABA-ADP shows enhanced fluorescence when bound to an Hsp70 (Mayer et al., 2003; Preissler et al., 2017; Theyssen et al., 1996). To follow the ability of Sil1 to stimulate ADP release from BiP, we monitored the decrease in MABA-ADP fluorescence upon mixing of a BiP-MABA-ADP complex with an excess of unlabeled ADP in the presence or absence of Sil1. We observed that the release of MABA-ADP was accelerated ~5-fold when Sil1 was present in the ADP solution (Fig. 2.1, A and B); addition of Sil1 resulted in an ADP exchange rate of 0.35 s⁻¹ versus a rate of 0.07 s⁻¹ in the absence of Sil1 (Fig. 2.1B). For these assays, near full-length recombinant yeast BiP (residues 42-682) and Sil1 (residues 19-421) proteins were used; both proteins lack only the N-terminal hydrophobic signal sequences.

We observed Sil1 lacking additional N-terminal sequence (Sil1 residues 113-421; Sil1-NΔ) further stimulated the rate of MABA-ADP release relative to the stimulation observed with wild-type Sil1 (Fig. 2.1, A and B). Sil1-NΔ stimulated ADP exchange at an average rate of 0.68 s⁻¹ (Fig. 2.1B). The increased rate of ADP release stimulated by Sil1-NΔ, relative to Sil1, was maintained across a range of Sil1 concentrations (Table 2.1). Although initially unexpected, these data are consistent with a recent report focused on
Figure 2.1 Sil1 NEF activity is auto-inhibited by its N-terminal region.

(A-B) The ability of Sil1 to catalyze the exchange of MABA-ADP bound to BiP was monitored by stopped-flow. BiP (1 µM) preloaded with MABA-ADP (1 µM) was chased with 125 µM ADP in the absence or presence 0.5 µM Sil1. Panel A depicts the mean fluorescence values and the non-linear fit for three technical replicates from one experiment; error bars indicate the data range. Panel B shows the rates determined from the non-linear fit for three independent experiments (each including a minimum of three technical replicates). Error bars represent the standard deviation. (C) ATPase activity of BiP (1 µM) was monitored by following the accumulation of free phosphate in the presence of Sec63 J protein (2 µM) with or without Sil1 (0.5 µM). (D) BiP (1 µM) ATPase activity in the presence of Sec63 J protein (2 µM) was monitored across a range of Sil1 concentrations (0-8 µM). Initial phosphate release rates were determined from a linear fit of the phosphate generated in the first 10 min of the reaction; these values were plot relative to the corresponding Sil1 concentration. Graphs (C-D) show the mean of three independent experiments; error bars depict the range.
Table 2.1 Observed rates of nucleotide release.

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<th>( BiP + Sil1 ) ( k_{obs} ) (sec(^{-1}))</th>
<th>( BiP + Sil1-N\Delta ) ( k_{obs} ) (sec(^{-1}))</th>
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human Sil1, wherein a similarly truncated recombinant human Sil1 protein (referred to as Bap-C) was demonstrated to be more active than full-length human Sil1 (Bap) in its ability to stimulate MABA-ADP release from BiP (Rosam et al., 2018). We reasoned that the increase in NEF function observed for Sil1-NΔ would result in an augmented ability of Sil1-NΔ to stimulate BiP ATPase activity under steady state conditions. Indeed, we observed an accelerated rate of ATP hydrolysis by BiP in the presence of Sil1-NΔ, relative to Sil1 (Fig. 2.1C). A comparison of the BiP ATP hydrolysis rate across a range of Sil1 concentrations indicates that the N-terminal domain behaves as a non-competitive inhibitor; the presence of an intact N-terminal domain reduced the maximal rate of ATP turnover by BiP without changing the apparent binding affinity for the reaction (Fig. 2.1D).

The maximal velocity observed with Sil1-NΔ was 3-to-4-fold greater than observed with Sil1. A fit of the data suggests that the apparent affinity for the reaction with Sil1 or Sil1-NΔ were both ~0.6 µM (Fig. 2.1D). Isothermal titration calorimetry (ITC) measurements confirmed that the removal of the N terminus does not impact the affinity of Sil1 for BiP. The measured dissociation constants (K_d) for Sil1 and the BiP ATPase domain (Fig. 2.2, A and B) were measured. Sil1 and Sil1-NΔ showed a K_d of 110 nM and 120 nM for the ATPase domain of BiP, respectively.

**A conserved cluster of amino acids within the Sil1 N terminus confers the auto-inhibition of Sil1 NEF activity.**

Despite sharing the same functions, Sil1 orthologs show poor primary sequence homology. The full-length human and yeast Sil1 show only 20% sequence identity. Intriguingly, prior studies highlighting this poor overall sequence conservation also note a
Figure 2.2 Removal or mutation of the Sil1 N terminus does not change Sil1 affinity for BiP.

Isothermal titration calorimetry (ITC) was used to monitor the interaction of full-length Sil1 (A) and Sil1-NΔ, (B) with the BiP ATPase domain (residues 43-426). Data fitting show similar $K_d$ values for wild-type Sil1 and Sil1-NΔ, ranging between 110-166 nM for the BiP ATPase domain.
region of strong homology within the N-terminal domain that is shared between human and yeast Sil1 (Chung et al., 2002b; Tyson and Stirling, 2000). Here we aligned 19 Sil1 sequences, spanning from diatom to primate (Fig. 2.3, A and B). We observed that this noted region of N-terminal sequence conservation, residues 61-97 in *S. cerevisiae*, is maintained across these diverse Sil1 orthologs.

Making use of various yeast Sil1 truncation mutants, we determined that the disruption of this strikingly conserved region relieves the inhibition of Sil1 NEF activity. Truncated versions of Sil1 were tested for their ability to stimulate BiP’s ATPase activity. Sil1 missing the first 58 N-terminal residues, Sil1-(59-421), showed a similar activity as full-length Sil1, suggesting that the poorly conserved sequence prior to residue 59 does not influence Sil1 activity towards BiP (Fig. 2.3C). Disruption of the conserved region, by removal of an additional 20 amino acids (deletion of residues 1-78), resulted in a Sil1 protein that displayed increased activity similar to that observed with Sil1-NΔ (Fig. 2.3C). These data suggest an importance for the conserved region in the relatively dampened NEF function observed for full-length Sil1.

To further define the inhibitory region, we generated single amino acid changes for nine residues within the conserved sequence. Guided by our sequence alignment (Fig. 2.3A and 4A), we selected several of the most highly conserved residues spanning across this region to mutate. The selected residues include an absolutely conserved glycine (G80) as well as sites consistently showing a non-polar (F62, W68) or polar residue (D67, Q75, R84, K95) in all the aligned orthologs. We also chose two amino acids to mutate that demonstrated a strong preference for a specific amino acid at that position,
Figure 2.3. Disruption of a conserved cluster of amino acids within the Sil1 N terminus relieves the auto-inhibition of Sil1 NEF activity.

(A) Sequence alignment and (B) phylogenetic tree data for a subset of Sil1 orthologs. Protein sequences were aligned, and tree data were generated using Clustal Omega (Li et al., 2015; Sievers et al., 2011). The sequence alignment was formatted using Multiple Align Show with a consensus shading setting of 70% (Stothard, 2000). Lines and numbers denote the truncation sites for the recombinant *Saccharomyces cerevisiae* (Sc) Sil1 mutants analyzed in panel C. Alignment shows Sc Sil1 residues 1-113; Sil1-NΔ corresponds to residues 113-421. The Sc Sil1 signal sequence is predicted to end at residue 19; wild-type Sil1 corresponds to residues 19-421. (C) ATPase activity of BiP (1 µM) was monitored in the presence of J protein (2 µM) and various Sil1 mutants (0.5 µM). Data shown are the mean of at least three independent assays. Error bars depict the range.
but also showed less conservative substitutions in some orthologs: G74 (a D in one, or an N in two, of the 19 orthologs) and P78 (a D in the diatom sequence). Residues were substituted with alanine in the context of our full-length Sil1 (residues 19-421), and the recombinant proteins were assayed for their ability to stimulate BiP ATPase activity. We found that the majority of mutants showed increased activity (Fig. 2.4B), similar to what was observed with Sil1-NΔ. Only one mutant, Sil1-D67A showed lower NEF activity, similar to wild-type Sil1 (Fig. 2.4B). Although demonstrating a preference for a negatively-charged polar residue at this site (D or E in 15/19 sequences), Sil1 orthologs with amino acids containing a polar neutral side chain (Q in 3/19) or a polar positive residue (K in 1/19) were identified, and we suggest that this position may be able to accommodate a variety of amino acid side chains without significantly altering Sil1 activity. Wild-type Sil1 and the Sil1-D67A mutant both increase the rate of ATP turnover by BiP, relative to that observed in the absence of Sil1 (Fig. 2.4B). These data indicate that the lack of robust activity for the Sil-D67A mutant was not a consequence of gross misfolding of the mutant protein.

Intriguingly, a Marinesco-Sjögren syndrome (MSS) patient mutation corresponds to an arginine-to-tryptophan substitution within this conserved section of the Sil1 N terminus (Riazuddin et al., 2009). The substituted arginine in human MSS patients (R92) corresponds to R84 in yeast. Given our observation that a yeast Sil1-R84A mutant showed increased activity as a NEF, we sought to determine if a substitution comparable to that observed in the MSS patients would also alter Sil1 activity. Thus, we mutated yeast Sil1 R84 to tryptophan. In addition, we generated three more substitutions at R84: (1) an R84Q mutation (a Q residue is present at this site in 2 of the 19 analyzed sequence), (2)
Figure 2.4. Individual point mutations within Sil1 residues 62-95 relieve the auto-inhibition of Sil1 NEF activity.

(A) Sequence logo generated by WebLogo (http://weblogo.berkeley.edu/ Crooks et al., 2004; Schneider and Stephens, 1990) from the 19 Sil1 sequences aligned in Fig. 2.3A. The logo depicts the relative conservation of the protein sequence corresponding to Sc Sil1 residues 61-96; the sequence for Sc Sil1 is shown below the logo in grey. Underscored Sc Sil1 amino acids and colored numbers highlight the residues mutated in recombinant Sil1 mutants analyzed in panels B-D. Symbols refer to panel B, which depicts the activity of Sil1 with the indicated residues changed to alanine. (B-D) BiP ATPase activity (1 µM) in the presence of J protein (2 µM) and various Sil1 mutants (0.5 µM) was measured. Data for panel B, and the left panel of C, represent a minimum of two independent experiments. Data in the right panel of panel C depict a minimum of three independent experiments. (D) The catalyzed exchange of MABA-ADP from BiP by Sil1-R84W was monitored by stopped-flow, and the rate of release is shown for three independent experiments. The calculated rates for ADP release stimulated by wild-type Sil1, and the basal ADP rate of exchange in the absence of Sil1, are re-plotted from Fig. 2.1B as a point of reference. All error bars depict the data range.
an R84E charge-substitution mutant, and (3) a conservative R84K replacement. We observed that mutation of R84 to Q, E, or W each resulted in a Sil1 with an increased ability to stimulate BiP ATPase activity (Fig. 2.4C). The activity of these mutants was comparable to the majority of the alanine point mutants generated across the conserved N terminal region (Fig. 2.4C). In contrast, the more conservative R84K replacement resulted in a Sil1 with only a modest increase in its ability to stimulate BiP ATPase activity relative to wild-type Sil1 (Fig. 2.4C). Overall, these data suggest that a positively charged reside at position 84 helps maintain lower Sil1 NEF activity. The increased activity we observed with the Sil1-R84Q mutant suggests that specific positional amino acid conservation across species is not sufficient to predict the impact of a given Sil1 substitution allele.

Further analysis of the Sil1-R84W allele demonstrated that the increased ability of Sil1-R84W to stimulate BiP ATPase activity correlates with an increase in the ability of Sil1-R84W to facilitate ADP exchange by BiP; addition of Sil1-R84W resulted in a mean ADP exchange rate of 0.52 s$^{-1}$ versus a rate of 0.35 s$^{-1}$ for Sil1 (Fig. 2.4D). Similar to the Sil1-N∆ mutant, the increased activity of Sil1-R84W was observed across a range of Sil1 concentrations (Fig. 2.5 and Table 2.1). Similarly, the R84W mutation showed little impact on the affinity of Sil1 towards BiP as measured by ITC (Fig. 2.5).

**Sil1’s N-terminal domain adopts an extended conformation that is impacted by mutations in the conserved region.**

When analyzed by size exclusion chromatography (SEC), recombinant wild-type Sil1 (46 kD) shows an elution profile consistent with that of a much larger (~75 kD) protein (Fig. 2.6A). In contrast, the N-terminal deletion mutant migrates as expected for its 36 kD
Figure 2.5. A Sil1-R84W mutant facilitates enhanced turnover of ATP by BiP but does not alter Sil1 affinity for BiP.

(A) BiP (1 µM) ATPase activity was monitored in the presence of Sec63 J protein (2 µM) and a range of Sil1 concentrations (0-8 µM). Initial phosphate release rates were determined from a linear fit of the phosphate generated in the first 10 min of the reaction; these values were plotted relative to the corresponding Sil1 concentration. Graphs show the mean of three independent experiments; error bars depict the range. Wild-type Sil1 data are the same as shown in Fig. 2.1D; the Sil1, Sil1-NΔ, and Sil1-R84W activities shown here and in Fig. 2.1D were all assayed together in the same experiment(s). (B) Isothermal titration calorimetry (ITC) was used to monitor the interaction of Sil1-R84W with the BiP ATPase domain (residues 43-426). Data fitting show a $K_d$ value of 166 nM.
size (Fig. 2.6A). The distinct gel filtration profiles for Sil1 and Sil1-NΔ have been reported previously (Yan et al., 2011). The larger apparent size of full-length Sil1 was interpreted as the formation of a Sil1 dimer, facilitated by the presence of the N terminus (Yan et al., 2011). We sought to establish whether full-length Sil1 forms dimers in solution, and (if so) if Sil1 dimerization might contribute to the lower NEF activity associated with full-length Sil1. Using the combination of size exclusion chromatography and multi-angle light scattering (SEC-MALS), we determined that the mass of Sil1 is consistent with that of a Sil1 monomer (not dimer) (Fig. 2.6B). Given that Sil1 behaves as a larger protein on a size exclusion column, we propose that the N-terminus of Sil1 adopts a conformation that increases the relative hydrodynamic volume for Sil1. Such an elongated conformation for Sil1 would account for the apparent increase in mass observed for Sil1 analyzed by SEC. We suggest that in the absence of the N terminus, Sil1 behaves as a globular monomeric protein, which is consistent with the characterized Sil1-NΔ structure (Yan et al., 2011).

We also analyzed the Sil1 point mutants by SEC-MALS. All proteins were monomeric in solution (Fig. 2.6C). Intriguingly, all the N-terminal mutants that showed an increase in NEF activity (Fig. 2.4) displayed an even further leftward shift in their SEC profile, relative to the already faster migrating Sil1 (Fig. 2.6C). In contrast, Sil1-D67A, which displayed NEF activity identical to wild-type Sil1 (Fig. 2.4B), maintained the same profile as wild-type Sil1 on SEC-MALS (Fig. 2.6C). A CD spectrum of the purified N-terminal domain of human Sil1 corresponds to a random coil (Rosam et al., 2018). Yet, prediction programs suggest the potential for some secondary structure; specifically, DISOPRED3 forecasts that, unlike the rest of the N terminus, amino acids 48-99 are not intrinsically disordered (the same region that is inhibitory) (Fig. 2.7). We suggest that the
Figure 2.6 Removal or mutation of the Sil1 N-terminal domain affects the mobility of Sil1 on size exclusion chromatography (SEC).

(A) Overlaid gel filtration (Superdex 200) profiles for Sil1 Sil1-NΔ. (B) SEC-MALS data for Sil1 and Sil1-NΔ. The absorbance signal at 280 nm (left, y-axis) was used to follow the timing of protein elution from the size exclusion column. The absorbance values for Sil1 and Sil1-NΔ are shown as dotted or solid lines, respectively. The average molecular weight, as calculated for every second across the protein elution peak, is depicted as a dotted line (right, y-axis). The theoretical molecular weights for Sil1 and Sil1-NΔ based on primary sequence (46.3 kD and 36.1 kD) are indicated as horizontal dashed grey lines. (C) SEC-MALS data for wild-type Sil1 and various Sil1 mutants (residues 19-421). Representative traces are shown from a single day. SEC-MALS results were reproduced for one (or two) additional independent run with Sil1-D67A, G80A, R84W, and K95A (or Sil1 and BSA).
Figure 2.7 Structural predictions for the Sil1 N terminus.

Prediction of protein disorder within yeast Sil1 residues 19-113 using (A) the DISOPRED3 prediction method through the PSIPRED server (Buchan et al., 2013) or (B) the RaptorX (Källberg et al., 2012) server. (C) Secondary structure prediction output from the JPred 4 server (Drozdetskiy et al., 2015) for yeast Sil1 residues 1-113. Residues that increase Sil1 NEF activity when mutated are noted in light blue. H, helical. E, extended. -, other secondary structure. (D) Predicted model for yeast Sil1 residues 19-113 generated using RaptorX (28). Residues that increase Sil1 NEF activity when mutated are shown as cyan sticks. A portion of the minor pseudophilin EPSH (PBD 4dq9A) was found to be the best template with a p-value of 3.29e-02.
structure in this ordered section is disrupted by our point mutations. We propose that these structural changes generate an even more elongated Sil1 structure, which accounts for the altered mobility observed by SEC. Our data imply that a change in secondary structure may relieve the inhibitory action of this region.

**Mutations in the Sil1 N-terminal domain impact reductant activity.**

In addition to its NEF activity, we have shown that yeast Sil1 acts as a reductant (Siegenthaler et al., 2017). The reductant activity of Sil1 relies on a pair of cysteines within the N-terminal region; these cysteines are found just upstream of the conserved N-terminal section that appears to dampen Sil1 activity as a NEF. To investigate whether perturbing the conserved N-terminal region also influences Sil1 reductant activity, we compared the ability of wild-type Sil1 and two of the N-terminal mutants (Sil1-R84W and Sil1-K95A) to reduce and remove a TNB adduct from BiP. We specifically selected Sil1-R84W based on the connection of the mutation to MSS. We also analyzed Sil1-K95A based on its distant position (in primary sequence) from the redox-active Sil1 cysteines (C52 and C57), the increased activity shown for this mutant in BiP ATPase assays (**Fig. 2.4B**), and the pronounced shift in mobility observed for Sil1-K95A using SEC (**Fig. 2.6C**). Making use of an assay we previously designed to monitor Sil1 activity as a reductant (**Fig. 2.8A**), we followed the ability of Sil1 to release a TNB anion from BiP that has been oxidized at C63 by Ellman’s reagent (DTNB) (Siegenthaler et al., 2017). We observed that Sil1, Sil1-R84W, and Sil1-K95A were able to reduce (remove) the TNB adduct from BiP, and all three mutants showed similar kinetic profiles (**Fig. 2.8B**). These data suggest that the initial attack and displacement of the TNB anion are unaffected by the R84W or K95A mutations.
Figure 2.8 Point mutations within the Sil1 N-terminal domain retain reductant activity but show a slower resolution of the BiP-Sil1 disulfide-bonded reaction intermediate.

(A) Schematic for following reduction of BiP-TNB adduct by Sil1. (B) BiP (residues 42-682) modified (oxidized) with DTNB was mixed with reduced wild-type Sil1 or a Sil1 mutant. Release (reduction) of the BiP-TNB adduct by Sil1 was monitored by measuring the absorbance of released TNB at 412 nm. Graphs show the average from three independent experiments; error bars depict the range. (C) Coomassie-stained gels follow the BiP-TNB reaction with wild-type and mutant Sil1 proteins. Samples were quenched at the indicated time with N-ethylmaleimide (NEM) and separated by non-reducing SDS-PAGE. BiP-Sil1 mixed-disulfide species accumulate with the Sil1-R84W or Sil-K95A mutants. Gels shown are representative of a minimum of 2 and 3 independent experiments for Sil1-R84W and Sil1-K95A respectively. (D) Quantification of the BiP-Sil1 mixed-disulfide species from panel C. The intensity of the BiP-Sil1 species band is expressed relative to total sum of the band intensities within a single gel lane. Bars reflect the mean; circles show individual value from a single experiment. A dotted line highlights the maximal signal for wild-type Sil1.
Upon displacement of the TNB anion, a transient disulfide-linked (mixed-disulfide) complex is formed between BiP and Sil1 (Fig. 2.8A). The formation and resolution of this intermediate species can be easily monitored using SDS-PAGE, wherein the Sil1-BiP complex is observed as a higher mobility species under non-reducing conditions (Siegenthaler et al., 2017). Consistent with their efficient release of TNB from BiP (Fig. 2.8B), wild-type Sil1 and the N-terminal mutants all formed a detectable mixed-disulfide intermediate with BiP (Fig. 2.8C). An increased amount of the BiP-Sil1 disulfide-linked complex was recovered for both the Sil1-R84W and Sil1-K95A mutants, relative to wild-type Sil1 (Fig. 2.8C, D). In addition, we found that the Sil1-BiP intermediate persisted for a longer time with the Sil1 mutants (Fig. 2.8C, D). These results indicate a slower resolution of the mixed-disulfide intermediate formed between modified BiP and Sil1-R84W or Sil1-K95A during electron transfer, which suggests that the enhanced activity as a NEF correlates with a decreased effectiveness as a reductase.

**Lower expression levels for the N-terminal Sil1 mutants may confer a loss-of-function phenotype for the hyperactive mutants in cells.**

In humans, the autosomal recessive disorder MSS is associated with Sil1 mutations that results in a decrease in cellular Sil1 activity. Characterized human Sil1 MSS-associated mutations have been linked to a lack of synthesized full-length Sil1 as well as the destabilization and/or aggregation of mutant Sil1 proteins (Ezgu et al., 2014; Howes et al., 2012). Given the expectation that an MSS-linked Sil1 mutation results in a loss of NEF function, we were intrigued whether the yeast Sil1-R84W mutant, which shows increased NEF activity in vitro, is active as a NEF in cells. Or, if the R84W change generates a loss-of-function Sil1 allele in cells, as might be expected from the association
of the analogous mutation (human Sil1-R92W) in patients with MSS. Yeast deleted for both ER NEFs (*lhs1Δ sil1Δ*) are inviable (Tyson and Stirling, 2000). Consistent with their established NEF activities in vitro, each of the generated Sil1 point mutants, including Sil1-R84W, could rescue the inviability of a *lhs1Δ sil1Δ* strain (**Fig. 2.9A**). Given that the N terminal mutants appear to perturb the structure of Sil1 in vitro, we sought next to determine if disturbing the N terminus impacts Sil1 expression in cells. Upon analysis of the Sil1 protein levels in cell lysates, we observed that the N-terminal point mutants result in lower steady-state expression, in comparison to wild-type Sil1 (**Fig. 2.9B**). In particular, the Sil1-W68A showed strikingly lower expression levels in yeast, and the Sil1 F62A, R84A, and R84W mutants showed markedly decreased levels of Sil1 (**Fig. 2.9B**). The relatively low expression of the Sil1 mutants in a *lhs1Δ sil1Δ* strain does provide sufficient activity to function as the sole ER-localized cellular NEF (**Fig. 2.9A**). We speculate that the sufficiency of these low levels of Sil1 may relate to the enhanced NEF activity observed for these mutants in vitro. We propose that the decreased levels of the yeast Sil1-R84W mutant seen in yeast may explain how this apparently hyperactive Sil1 NEF mutant (based on in vitro work described here) could phenotypically behave as a loss of function allele in patients.

**DISCUSSION**

We have described an unexpected role for the Sil1 N-terminal region in modulating Sil1 NEF activity. We identified a region of sequence in yeast Sil1 (amino acids 61 and 96) that – when intact – dampens Sil1 NEF activity. The auto-inhibitory N-terminal sequence we uncovered in yeast Sil1 shows striking sequence conservation between Sil1 proteins across species, despite a poor overall primary sequence homology between Sil1
Figure 2.9 Mutations within the N-terminal domain result in decreased levels of Sil1 protein in yeast cells.

(A) A sil1Δ lhs1Δ strain covered with an URA3-marked SIL1 plasmid was transformed with a LEU2-marked plasmid encoding the indicated Sil1 proteins. The ability of the various Sil1 mutants to substitute for wild-type Sil1 was assessed after counter-selection for the URA3-plasmid on 5-FOA. (B) Lysates were prepared from mid-log cultures of yeast (sil1Δ lhs1Δ) transformed with a low-copy plasmid coding for wild-type Sil1 or a Sil1 mutant. Protein levels were analyzed by western blotting with Sil1 and Pgk1 antisera.
orthologs. We suggest that the negative impact of the N-terminal region is a feature shared between divergent Sil1 proteins. Consistent with our expectation, data with human Sil1 support a role for the N-terminal domain in inhibiting Sil1 NEF activity; human Sil1 lacking its entire N terminus stimulates an increased rate of nucleotide exchange from mammalian BiP, relative to a full-length Sil1 (Rosam et al., 2018). However, before a clearly conserved auto-inhibitory role for the Sil1 N terminus is established, these data showing an increased NEF activity for an N-terminal human Sil1 mutant will need to be reconciled with additional data reported in the same study that demonstrate a lack of stimulated BiP ATPase activity by an N-terminally truncated human Sil1 (discussed further below) (Rosam et al., 2018).

A CD spectrum of the human BiP N terminus indicates a protein domain devoid of significant alpha-helical or beta-sheet content (Rosam et al., 2018). Nevertheless, we propose that the inhibitory region within the Sil1 N terminus adopts some degree of secondary structure. Consistent with our model, structure prediction and protein modeling servers indicate the inhibitory region within the N terminus is not disordered and could potentially form short beta-sheets (Fig. 2.7). We suggest a change in the structure of N-terminal region (induced herein by individual amino acid mutations) can confer an increase in Sil1 activity as a NEF. A correlation between an increased NEF activity and a change in protein conformation is consistent with the altered relative mobility we observe for hyperactive Sil1 proteins, relative to wild-type Sil1, when analyzed by SEC (Fig. 2.6C).

We speculate that the inhibitory domain we identified in our mutational analysis of Sil1 may be a site of post-translational modification, which would allow for the modulation of Sil1 NEF activity in cells in response to physiological need. For the 19 orthologous Sil1
sequences we analyzed, only one residue within the N-terminal region of interest was completely conserved, yeast Sil1 G80 (Fig. 2.4A), and it is possible that this internal glycine undergoes modification through a post-translational event. There are also several basic and threonine residues that are highly conserved, and perhaps these residues a subject to methylation, acetylation, or phosphorylation. Lysine acetylation of lumenal ER proteins has been established in mammals (Pehar and Puglielli, 2013). Although ER lumen is not widely recognized as a site for phosphorylation, tyrosine-kinase activity has been described in in the mammalian ER (Frasson et al., 2009). The lower Sil1 levels we observed for several of the N-terminal point mutants in cells (Fig. 2.9) may suggest also a means for the down-regulation of 'activated' Sil1. It is possible that that lower steady-state levels of the mutant Sil1 reflect an increased turnover of active Sil1. Alternatively, the lower levels of these mutants in cells may reflect aberrant folding and/or a decreased thermo-stability of the N-terminal domain that results in the recognition of these proteins as misfolded, triggering their degradation. As noted above, the lower steady-state levels we observe for the N-terminal mutants are also noteworthy in that they suggest a possible explanation as to how a Sil1 mutant that is more active as a NEF in vitro could result in a loss of Sil1 function in cells.

How a change in structure for the inhibitory region influences Sil1 activity as a NEF remains unclear. At present, we have established that the N terminus acts as a non-competitive inhibitor of Sil1 NEF activity, and thus we do not expect that the conformation adopted by the N terminus changes the affinity of BiP for ATP. Moreover, we found that the presence or absence of the N terminus does not dramatically influence the affinity between BiP and Sil1 (Fig. 2.2). Structural studies indicate that the binding of the C-
terminus of Sil1 (Sil1-N△) facilitates BiP NBD subdomain rotations that disrupt BiP-ADP hydrogen bonds; a disruption in these hydrogen bonds is expected to weaken the affinity of BiP for ADP and facilitate ADP release (Yan et al., 2011). It seems reasonable to suggest that when full-length Sil1 binds to BiP, contacts formed between BiP and the Sil1 N terminus may limit the opening of the BiP NBD and slow the release of ADP; such a mechanism would be consistent with the lower NEF activity we observe with full-length Sil1.

Data with human Sil1 indicate the presence of the N-terminal domain may influence also more global conformational changes in BiP. Experiments using various BiP spFRET sensors imply that the human Sil1 N-terminal domain keeps the BiP ATPase and peptide-binding domains separated (Rosam et al., 2018). This conclusion is based on an increased distance observed between BiP NBD and SBD FRET probes in the presence of human Sil1 with an intact N terminus (Bap), relative to an N-terminal truncation (Bap-C). In addition, an intact N-terminal region appears to inhibit the ability of Sil1 to facilitate a separation of the lid and SBD. Addition of full-length Sil1 and ADP results in an increased distance between the SBD and lid, relative to what is observed with ADP alone; the shift towards a more open lid is even is more pronounced upon addition of Sil1-N△ (Bap-C) (Rosam et al., 2018). Altogether, these data are consistent with a dampened ability of Sil1 to mediate conformational changes in BiP when the Sil1 N-terminus is intact. These data are consistent also with an increased capacity for human Sil1-N△ as a NEF, which allows for the release of ADP, a change in proximity of the NBD and SBD, and an opening of the SBD lid (to facilitate peptide release from the SBD). It will be interesting to determine how the N terminus impacts these observed movements within BiP. The N
terminus may serve to physically limit the BiP domain movements. Alternatively, the changes in BiP states may be a downstream consequence of a decrease in nucleotide exchange stimulated by the full-length Sil1.

Until recently, how the presence (or absence) of the N terminal domain impacts Sil1 activity remained largely unexplored. Yet several recent works have begun to highlight an importance for the N terminal region. In the future, it will be necessary to define if and/or how these new emergent functions come together to modulate Sil1 activity. It will be important to determine also which features are unique to distinct Sil1 orthologs or conserved across species.

Our data suggest that the reductant activity we recently described for yeast Sil1 (Siegenthaler et al., 2017) may be influenced by the status of the inhibitory region we define herein. We observed that the disruption of the inhibitory region of the yeast Sil1 N terminus concomitantly increased NEF activity and dampens Sil1 activity as a reductant (Fig. 2.4 and 2.8). Structural studies have established extensive molecular contacts occur between the Sil1 core (C-terminal region) and the ATPase domain of BiP (Yan et al., 2011), and we anticipate that the association of BiP and Sil1 through these contacts that positions the Sil1 N terminus in proximity to the NBD cysteine, allowing for reductant activity (Siegenthaler et al., 2017). We envision that the disruption of secondary structure of the inhibitory region within the N terminus likely alters the positioning of the cysteines relative to the Sil1 core, and that the changes may decrease the efficiency of electron exchange between Sil1 and BiP. Of note, the kinetic delay we observe in the resolution of the BiP-Sil1 intermediate formed during the reduction process is modest (Fig. 2.8). We propose that a more significant effect on reduction may be observed in a more
physiological system when Sil1 levels are likely substoichiometric to BiP (versus equimolar as in our assay). We speculate that a tuning of Sil1 reductant and/or NEF activity under certain physiological conditions may be beneficial to cells; for example, slowing the activity of Sil1 as a reductant during conditions of oxidative stress may allow for prolonged oxidation of the BiP cysteine and increased holdase activity (Wang et al., 2014). During the same conditions, the increased NEF activity of Si1 could be beneficial by facilitating the ER-associated degradation of misfolded polypeptides (Williams et al., 2015). BiP is an abundant protein within the ER lumen, and we anticipate only a portion of BiP is oxidized (and inactivated as an ATPase) even under stress conditions (Wang et al., 2014). An increase in NEF activity may act to increase ADP turnover from non-modified BiP (which is active as an ATPase) and facilitate protein turnover, while the decreased reductant activity of Sil1 may (simultaneously) serve to prolong the existence of oxidized BiP holdase activity during stress.

A compelling model wherein the human Sil1 N-terminal domain serves as a pseudo-substrate that stimulates BiP ATPase activity and substrate release was recently outlined (Rosam et al., 2018). A similar model was put forth also for the yeast cytoplasmic homologs of Sil1, yeast Fes1 and human HspBP1 (Gowda et al., 2018). We suggest that altering the structure of the N-terminal domain inhibitory region could act to augment the ability of the N-terminus to act as a pseudo-substrate. The region we have identified as inhibitory overlaps with 7 of the 11 human Sil1 N-terminal peptides that were noted for their high scoring in a BiP peptide-substrate prediction algorithm (Rosam et al., 2018). Hsp70s show a preference in binding unstructured polypeptides (Bole et al., 1986; Gething et al., 1986); perhaps a change in structure within the inhibitory region not only
increases NEF activity but also acts to 'unfold' this region and facilitate its recognition as a pseudo-substrate. An extended conformation within the N terminus could also broaden the reach of this region and enable the association of the N terminus with the BiP substrate-binding pocket.

As noted above, a disparity between our data with yeast Sil1 and those reported for human Sil1 is the observed inability of human Sil1 lacking its N terminus to stimulate BiP ATPase activity (Rosam et al., 2018). The lack of ATPase stimulation by a truncated human Sil1 is strikingly distinct from the increased activity we observed with yeast Sil1-NΔ (Fig. 2.1). This discrepancy could reflect a distinction between the activities of human and yeast Sil1 proteins. Yet given the same report revealed an enhanced ADP release for the human Sil1-NΔ (Bap-C) mutant relative to wild-type Sil1 by stopped flow (similar to our observations; Fig. 2.1A), we think it is likely the human and yeast proteins are not so different. We speculate that our conflicting data may relate to the prior observation that increased NEF activity can allow for rapid release of ATP prior to hydrolysis, which can manifest as a loss of ATPase activity (Kabani et al., 2002b; Raynes and Guerriero, 1998; Xu et al., 2016). Consistent with our speculation, when the activity of yeast Sil1 is assayed in the absence of J protein, we observe that yeast Sil1-NΔ shows an apparent decrease in its ability to stimulate ATP turnover by BiP (data not shown), similar to what was reported for human Sil1 (Rosam et al., 2018). In our assays we have driven ATP hydrolysis with a J protein (specifically, a Sec63 J protein domain that has no peptide-binding activity). In the future, it will be interesting to observe how the addition of J protein influences BiP ATPase activity in the presence of an N-terminally deleted human Sil1, relative to full-length Sil1.
METHODS

Plasmid construction

A list of plasmids used are listed on Table 2.2. In experimental procedures, yeast BiP is referred to as Kar2, the standard name for yeast BiP. The plasmids pCS817 and pCS675 are the same as previously described (Wang et al., 2014). pKP148 was made by cloning the DNA sequence coding for Kar2 residues 43-426 with BamHI and XhoI restriction sites into pET28-SUMO (Addgene). All SUMO-tagged constructs will have an additional serine residue at the N terminus after cleavage of the SUMO tag. Yeast Sil1 sequences coding for residues 20-421, 79-421, 59-421, and 113-421 were amplified with BamHI and XhoI restriction sites and cloned into pET28-SUMO to make pKP136, pKP147, pKP159 and pKP144 respectively. To construct SUMO-tagged Sil1 mutants, amino acid substitutions were made with QuikChange site-directed mutagenesis (Agilent Technologies) using pKP136 as a template. pKS115 was made by cloning the DNA sequence coding for mouse BiP residues 25-654 with NdeI and XhoI restriction sites and ligating with pET-15b vector (Addgene). pHS116 and pCS925 are the same as (Siegenthaler et al., 2017). Plasmids pHS128, pHS246, pH247, pHS248, pHS249, pHS250, pHS251, pHS253, pHS254, pKP198, pKP199 and pKP200 were made with QuikChange site-directed mutagenesis (Agilent Technologies) using pHS116 as a template. All plasmids and mutations generated were confirmed by sequencing. Strains CSY448 (MATalpha GAL 2 ura3 leu2 sil1::KanMX) and CSY647 (MATa GAL2 ura3 leu2 sil1::KanMX lhs1::KanMX [pJW7]) were made as described previously (Siegenthaler et al., 2017).
Table 2.2 List of plasmids.

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Recombinant protein expression and purification

Plasmids expressing SUMO-tagged Sil1 proteins were transformed into BL21 (DE3) cells and plated on LB with 15 µg/ml kanamycin and incubated at 37°C overnight. Colonies containing plasmids encoding for SUMO-tagged proteins were grown overnight at 37°C in Luria-Bertani (LB) medium with 15 µg/ml kanamycin. Cells were then diluted 1:75 in Terrific Broth (TB) with 15 µg/ml kanamycin and grown to an OD\textsubscript{600} of 0.9 at 37°C. Cells were shifted to 18°C for 1 hr and induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 20 hrs. Cells were harvested, washed with water and pellets were stored in -80°C. Cells from a 1 L culture were resuspended in lysis buffer (20 mM HEPES, pH 8, 0.5 M NaCl, 10 mM Imidazole, 10% glycerol) with added ½ tablet of EDTA-free protease inhibitor (Pierce), 1 mM betamercaptoethanol (BME), 250U benzonase (Pierce) and 20 mg lysozyme (VWR). Resuspended cells were sonicated using a microtip sonicator 5 times for 30 seconds at 70% duty cycle, each with at least 1 min rest in between sonications. Insoluble materials were removed by centrifugation at 23,700 g for 35 min at 4°C. Soluble materials were loaded onto a HiTrap chelating column (GE Healthcare) charged with nickel. The column was washed with at least 40 column volumes (cv) of wash buffer 1 (20 mM HEPES, pH 7.4, 0.5 M NaCl, 10 mM imidazole, 10% glycerol) and 20 cv of wash buffer 2 (20 mM HEPES, pH 7.4, 0.5 M NaCl, 25 mM imidazole, 10% glycerol). The SUMO-tagged protein was eluted with elution buffer (20 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5 M imidazole, 10% glycerol). Purified SUMO-tagged protein was cleaved with 50 µg/ml of ULP, incubated at 4°C for at least 30 min. After removing imidazole by running on HiTrap desalting column (GE Healthcare) equilibrated with SUMO binding buffer (20 mM HEPES, pH 7.4, containing 0.5 M NaCl, 10% glycerol),
protein cleaved with ULP was loaded on to HiTrap chelating column charged with nickel. Untagged protein was eluted with wash buffer 1. Untagged protein was further purified by running on HiLoad™ 16/60 Superdex 200 pg (GE Healthcare) equilibrated with freshly made gel filtration buffer (20 mM HEPES, 0.1 M NaCl, 10 mM TCEP, pH 7.4). Peak fractions eluted starting at 70 ml were collected and concentrated for storage.

**His₆**-tagged Kar2 protein induction and the initial Ni-purification step were completed as described above for Sil1. **His₆**-tagged Kar2 was buffer exchanged using a HiTrap desalting column equilibrated with low salt buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 10% glycerol) and Kar2 was loaded onto a HiTrap Blue HP (GE Healthcare) column and washed with low salt buffer for five cv and then eluted with wash buffer 1. Kar2 was further purified by gel filtration using a HiLoad™ 16/60 Superdex 200 pg (GE Healthcare) equilibrated with 10 mM Tris, pH 7.4, 50 mM NaCl, 10% glycerol. Peak fractions eluted starting at 70 ml were collected and concentrated for storage.

SUMO-tagged Kar2 was expressed and purified with HiTrap chelating column (GE Healthcare) charged with nickel the same way as SUMO-tagged Sil1. SUMO-tagged Kar2 was cleaved with ULP, desalted and purified with HiTrap chelating column charged with nickel the same way as SUMO-tagged Sil1. Pure fractions were collected and desalted using PD-10 column (GE Healthcare) pre-equilibrated with 10 mM Tris, pH 7.4, 50 mM NaCl, 10% glycerol.

All protein concentrations were determined by measuring the absorbance at 280 nm. Concentrated proteins were flash frozen with liquid nitrogen before storing at -80°C. All Kar2 and BiP proteins were checked for presence of nucleotides with the HPLC.
ADP release NEF assay

Concentrated BiP (pCS817) were stored in 10 mM Tris, pH 7.4, 50 mM NaCl, 10% glycerol and concentrated Sil1 were stored in 20 mM HEPES, 0.1 M NaCl, 10 mM TCEP, pH 7.4. Experiments were carried out in 25 mM HEPES-KOH, pH 7.4, 50 mM KCl, 5 mM MgCl₂. 300 to 500 µM BiP and 5 mM stock of MABA-ADP (Jena Bioscience) were each diluted to 2 µM in 2 ml of the reaction buffer and incubated for 30 seconds at 25°C. 300 µM to 1 mM concentrated Sil1 were diluted to 0.2, 0.5, 1, 1.5 or 2 µM in 2 ml of the reaction buffer with 250 µM of ADP. BiP with MABA-ADP was mixed with ADP in the presence or absence of Sil1. Final reactions consist of 1 µM BiP, 1 µM MABA-ADP, 125 µM ADP, and 0, 0.1, 0.25, 0.5, 0.75 or 1 µM Sil1. The nucleotide release was measured at 0.05 seconds interval and monitored for 60 seconds using a Hi-Tech SFA-20 stopped-flow coupled to a FluoroMax-4 Spectrofluorometer with excitation of 335 nm, emission of 440 nm and 3 nm slits. GraphPad Prism (version 7.03) was used for data fitting to get \( k_{obs} \) of each reaction. Data were obtained from three injections with at least three technical replicates for each injection. Each protein used were from a minimum of two different preps.

Steady state ATP hydrolysis assay

BiP’s ATP hydrolysis was measured using the EnzCheck Phosphate Assay Kit (Thermo Fisher Scientific) as described previously (Siegenthaler et al., 2017). For experiments using yeast BiP and Sil1, 1 µM BiP (pCS817), 2 µM GST-Sec63J (pCS765) and different Sil1 concentrations (0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 µM) were diluted in ATPase buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT). Reactions were carried out in ATPase buffer with added 200 µM 2-amino-6-mercapto-7-methylpurine riboside (MESG) and 0.2 U/ml purine nucleoside phosphorylase (PNP) to a
final reaction volume of 200 µl. The reactions were started by adding a final concentration of 5 mM ATP and the phosphate released was measured at 360 nm for 1 hour using BioTek Synergy 2 plate reader. For Sil1 titration experiments, the rates (mol Pi released per mol BiP per min) were measured by plotting the phosphate released from 1 µM BiP for the first 10 minutes (linear range). Experiments with Sil1, Sil1 N-terminal truncations, Sil1-N∆ and Sil1-R84W are from three independent experiments using at least two different protein preps for each protein. Experiments with Sil1 N-terminal mutants are from two independent experiments using one protein prep.

**Isothermal titration calorimetry (ITC)**

The apparent dissociation constants (Kd) and stoichiometry (molar ratio) of interactions between BiP and Sil1 were measured using MicroCal VP-ITC (GE Healthcare). BiP protein (pKP148) and Sil1 proteins were exchanged into ITC buffer (20 mM HEPES, 0.15 M NaCl, 1 mM TCEP, pH 7.4) using a NAP-5 column (GE Healthcare) and degassed for 20 min at 25°C using a vacuum. The reference cell of the ITC was filled with degassed ITC buffer and the sample cell was filled with 15 µM BiP. 150 µM of Sil1 was titrated at 25°C with a delay of 240 seconds between injections. The dissociation constant (Kd) was obtained from curve fitting the integrated heat released using the single-site binding model from the Origin software (GE Healthcare). Replicates were conducted with slightly different concentrations of BiP and Sil1 and obtained similar values (data not shown).
SEC Coupled to Multi-Angle Light Scattering (SEC-MALS)

The molecular weights of purified Sil1 proteins were measured using SEC-MALS. 5 mg/ml of Sil1 proteins were injected onto Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with gel filtration buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl). Molecular weights were measured through light scattering detector (DAWN HELEOS-II) and refractive index detector (Optilab T-rEX). The software ASTRA was used to analyze data and BSA (Sigma) was used for normalization of the detectors.

In vitro reduction assays

100 µM BiP (pCS817) was oxidized with 10-fold excess of DTNB in TNE buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA) for 2 hours at room temperature. 100 µM Sil1 was reduced using 10-fold excess of DTT for 1 hour at room temperature. BiP was diluted in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol and Sil1 was diluted in 20 mM HEPES, pH 7.4, 0.1 M NaCl. Unreacted DTNB and DTT were removed from the BiP-TNB and reduced Sil1 samples using NAP-5 columns (GE Healthcare) equilibrated in TNE buffer. Equimolar amounts of oxidized BiP and reduced Sil1 were mixed together and the release of the TNB-adduct from BiP was measured at 412 nm for 15 min using a Beckman Coulter DU730 UV/Vis spectrophotometer. The Abs at 412 nm was plotted against time using GraphPad Prism (version 7.03). Three independent experiments were conducted with at least two protein preps for BiP, Sil1 and Sil1-R84W. Only one prep was used for Sil1-K95A. To follow the mixed-disulfide formation, oxidized BiP and reduced Sil1 (10 µM each) were mixed at room temperature and quenched at indicated time points with 100 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.1% bromophenol blue and a 10-fold molar excess of N-ethylmaleimide (NEM) to number of
total cysteines. Protein samples were separated on non-reducing SDS-PAGE and reducing samples were supplemented with 5% BME. Proteins were visualized by staining with Coomassie brilliant blue. Data shown is a representative image of two independent experiments with Sil1 and Sil1-R84W. One experiment was done comparing Sil1 and Sil1-K95A.

**Growth assay**

A *sil1Δ lhs1Δ* strain (CSY647) covered with *URA3-SIL1* plasmid [pJW7], were transformed with *LEU*-marked plasmids pRS315, pHS116, pHS128, pKP198, pKP199, pKP200 and pCS925, and plated on SMM-Leu at 30°C for 2 days. pJW7 in CSY647 were counter-selected on SMM containing 5-fluoroorotic acid (5-FOA). Data shown is a representative image of three independent experiments.

**Sil1 expression westerns**

CSY647 cells were transformed with plasmids pRS315, pHS116, pHS246, pHS247, pKP198, pHS248, pKP199, pHS249, pHS250, pHS253, pHS251, pHS254, pHS128, pKP200. Transformants were grown overnight at 30°C in SMM-Leu liquid media, diluted and grown to mid-log phase. 4 OD$_{600}$ of cells were harvested after 5 hours of growth at 30°C. Lysates were resuspended in 100 μl of sterile water and lysed with 0.2 M NaOH at 25°C for 5 min. Pelleted cells were resuspended in 50 μl SDS sample buffer (60 mM Tris-HCl [pH 6.5], 5% glycerol, 2% SDS, 0.0025% bromophenol blue) with 4% BME, boiled at 100°C for 5 min and pelleted for 1 min at max speed. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose. Sil1 proteins were detected with anti-Sil1 antibody and the loading control phosphoglycerate kinase (PGK1)
was detected by anti-PGK1. Signals from secondary antibodies were detected with a ChemiDoc MP system (Bio-Rad). A representative image of three independent experiments is shown.
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CHAPTER 3. SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The reductant function of Sil1 depends on the cysteines located within the N terminus (Siegenthaler et al., 2017). Yet, the N terminus has been shown in previous studies to be dispensable for the NEF activity of Sil1. (Rosam et al., 2018; Yan et al., 2011). We set out to further determine the impact of the N-terminal domain on activities of Sil1. We were motivated in part by a previous suggestion for a role of the N-terminal domain in the oligomerization state of Sil1 (Yan et al., 2011), which we thought might be important for reductant activity. We were also curious how the MSS-associated mutation, Sil1-R92W, that maps within the N-terminal domain, would impact reductant activity. Based on the prior reports, we expected that this mutation might selectively impact Sil1 reductant activity, but not its NEF activity. By biochemically characterizing full-length Sil1, we uncovered an unexpected role for the conserved N-terminal region. We discovered that the N-terminal domain does influence Sil1 NEF activity and impacts the apparent conformation and reductant function of Sil1. We believe that NEF activity of Sil1 is autoinhibited by the N terminus as the absence of this region increases the ability to release ADP and stimulate BiP’s ATPase cycle.
FUTURE DIRECTIONS

Structure of the N-terminal domain

Several open questions remain pertaining to the actions of the Sil1 N terminus. We do not know how the presence of the N terminus physically acts to decrease Sil1 NEF activity. Also, we still do not understand how the presence of the N terminus might influence the interaction with BiP at the molecular level. We have shown that the presence of the N terminus does not change the affinity of Sil1 for BiP, but we still expect that the N terminus still likely influences the association between these two proteins. Obtaining the structure of full-length Sil1 may give us better information on the role of the N-terminal domain in modulating the activities of Sil1. To this end, we attempted to crystallize the BiP-Sil1 complex as well as full-length Sil1. Although our attempts to obtain the structure were unsuccessful, we had promising crystals that have room for optimization.

Crystallization of the BiP-Sil1 complex

The most promising crystals were obtained with untagged *S. cerevisiae* BiP ATPase domain (Kar2 residues 43-426) in complex with untagged *S. cerevisiae* Sil1 (residues 19-407). The tagged proteins were purified separately using metal-affinity chromatography. Proteins were expressed as fusions with a SUMO-his₆ tag. The SUMO-his₆ tag portion was cleaved and separated from the untagged proteins by metal-affinity chromatography. The untagged BiP and Sil1 proteins were mixed at a 1:1 ratio and was run on size exclusion column pre-equilibrated with 20 mM HEPES, 0.15 M NaCl, 1 mM MgCl₂, 5 mM TCEP, pH 7 (Fig. 3.1 A). The peak fractions were collected (lanes 4-8) (Fig. 3.1 B), concentrated to 17 mg/ml, and used for crystallization trials using the sitting-drop
Figure 3.1 Crystallization of the BiP-Sil1 complex.

(A) Size exclusion chromatography profile of the BiP-Sil1 complex using HiLoad 16/60 Superdex 200 pg. (B) Fractions from size exclusion column visualized on a 12% SDS-polyacrylamide gel stained with Coomassie blue. BiP and Sil1 are very similar in size (41.8 kDa and 44.7 kDa respectively) (C) Washed crystals were run on an 8% SDS-polyacrylamide gel (lanes 1-3, lane 5 shows input) and stained with SYPRO Ruby. (D) Picture of one of the optimized crystals sent to beamline. Crystals formed in 0.2 M ammonium sulfate, pH 6, 23% polyethylene glycol (PEG) 1,500, 10 mM MgCl$_2$ after 3 days at 4°C. Crystals were flash-frozen in a cryoprotectant solution (crystallization buffer supplemented with 20% xylitol). Crystals were frozen by John O’Donnell, data were obtained by Toshi Kawate and Kevin Michalski, data were analyzed by John O’Donnell and Holger Sondermann.
vapor diffusion technique. The crystallization condition consisting of 0.2 M ammonium sulfate, pH 6, 20% polyethylene glycol (PEG) 3,350 (Peglon screen tray) yielded a crystal hit after 2 weeks at 4°C. The crystal hit condition was optimized using the hanging-drop vapor diffusion technique. To ensure that the crystals were not salt and to see if both BiP and Sil1 were present, several crystals from optimization trays were washed, solubilized and were run on an 8% SDS-polyacrylamide gel. Crystals show a doublet very similar in size suggesting the presence of both BiP and Sil1 (Fig. 3.1 C). We also used additive screen for further optimization of the initial hit. Several optimized crystals were frozen and sent to Cornell High Energy Synchrotron (CHESS) (Fig. 3.1 D). Crystals diffracted X-ray at 1.7 Å. However, solving the structure only showed the presence of BiP's ATPase domain.

Our attempts to crystallize a Sil1-BiP complex resulted in BiP crystals. We expect that the BiP ATPase domain more easily forms crystals, which is consistent with the many Hsp70 ATPase domain structures that have been solved. Purifying the BiP-Sil1 mixed-disulfide (formed by disulfide bond between BiP C63 and Sil1 C52) may overcome this problem. This process involves purifying BiP, modifying it with DTNB, mixing it with reduced Sil1-C52A (trapping mutant) (Siegenthaler et al., 2017), and finally purifying the complex. We have previously attempted to purify the BiP-Sil1 complex linked with a mixed-disulfide bond but faced several issues: (1) unlike full-length BiP, modification of the BiP ATPase domain with DTNB results in aggregates forming (2) the reaction of complex formation was difficult to get to completion (3) the mixed-disulfide was hard to separate from unreacted BiP-TNB and Sil1 using size exclusion chromatography.
**Crystallization of full-length Sil1**

We also attempted crystallization of Sil1 alone. *S. cerevisiae* Sil1 (19-421) was expressed with a SUMO-his\textsubscript{6} tag and purified using metal-affinity chromatography. The SUMO-his\textsubscript{6} tag was cleaved and separated from the untagged Sil1 with metal-affinity chromatography. Sil1 was further purified using the size exclusion column, HiLoad 16/60 Superdex 200 pg, pre-equilibrated with 20 mM HEPES, 0.15 M NaCl, 1 mM MgCl\textsubscript{2}, 5 mM TCEP, pH 7. Peak fractions corresponding to 43 kDa molecular weight (Fig. 3.2 A and B) were collected, concentrated to 25 mg/ml and used for crystallization trials using sitting-drop vapor diffusion technique. Needle-like crystals formed after 2 weeks at 4°C in 0.2 M L-Proline, 0.1 M HEPES, PH 7.5, 24% PEG 1,500 (Peglon screen tray). This crystal hit was optimized using the hanging-drop vapor diffusion technique. Because it has been suggested the N terminus is prone to cleavage and is not suitable for crystallization (Yan et al., 2011), we checked if the crystals were not salt crystals and if they retained the N-terminal domain. A crystal from optimization tray was washed, solubilized and ran on a 12% SDS-polyacrylamide gel. Compared to the input Sil1 (19-421) material (lane 1), Sil1 solubilized from crystals (lane 4) shows the same mobility, suggesting that the N-terminal domain is intact. Truncated Sil1 without its N terminus would run past the 37 kDa ladder (Fig. 3.2 C). Optimized crystals were sent to both CHESS and Advanced Photon Source (APS) and diffracted X-ray at 8 Å. Crystals were further optimized by seeding. Although seeding yielded slightly bigger needles (Fig. 3.2 D), the crystals still diffracted at 8 Å.
Figure 3.2 Crystallization of Sil1 with intact N-terminal domain.

(A) Size exclusion chromatography (SEC) profile of *S. cerevisiae* Sil1 (19-421). (B) Fractions from SEC visualized on a 12% SDS-polyacrylamide gel stained with Coomassie blue. (C) Washed crystal (lane 4) run on a 12% SDS-polyacrylamide gel and stained with SYPRO Ruby. (D) Picture of one of the optimized crystals from seeding sent to CHESS. Crystals from optimization trays were frozen by Holger Sondermann and Carl Schiltz. Data collected from CHESS were analyzed by Holger Sondermann. Data collected from APS were analyzed by Carl Schiltz.
Several truncated *S. cerevisiae* Sil1 protein and the Sil1-R84W mutant were also used for crystallization trials. These proteins all yielded crystals with similar thin needle-like morphology. We also used a different Sil1 ortholog, *S. arboricola* Sil1 (19-407), for crystallization. The initial crystal hits showed promising results as not only were they thicker needles (Fig. 3.3 A and B), but the unoptimized crystal (Fig. 3.3 A) diffracted at 6 Å. However, these crystals took 2 months to grow. Optimization of the *S. arboricola* Sil1 crystals is worth trying. Another approach worth pursuing is using NMR to get the structure of the N-terminal domain alone. We have successfully purified GFP-Sil1 (19-113) and it may be possible to use this protein for NMR studies.

We believe that obtaining the structure of full-length Sil1 will provide insights into how the N-terminal domain modulates its activities. The prediction server JPRED forecasts the formation of β-sheets in the conserved N-terminal region of *S. cerevisiae* Sil1 at residues 61-64, 69-71, 82-86 and 92-94. It is possible that the conserved residues are forming a structured conformation. The residues with positive charges and aromatic rings may be forming a noncovalent cation-π interaction. A cation-π interaction, generally characterized as an electrostatic interaction, is known to occur in biological systems when a cationic side chain (e.g. Lys, Arg or a positively charged histidine) is in proximity of an aromatic side chain (e.g. Phe, Tyr, or Trp) (Burley and Petsko, 1986; Gallivan and Dougherty, 1999; Ma and Dougherty, 1997). A statistical analysis of a set of structures from protein data bank has revealed that an average of one energetically significant cation-π interaction for every 77 amino acids exists in a protein and that about 26% of all tryptophans are involved in at least one cation-π interaction (Gallivan and Dougherty, 1999). It is possible that in the N-terminal domain, the positively-
Figure 3.3 Crystallization of *S. arbicola* Sil1.

(A) Crystal formation from 2% tacsimate pH 5, 0.1 M sodium citrate tribasic, pH 5.6, 16% PEG 3,350 solution incubated at 4°C. The crystal was sent to beamline and diffracted at 6 Å. (B) Crystals from 0.2 M sodium thiocyanate, pH 6.8, 20% PEG 3,350 solution incubated at 20°C. Freezing crystals, data collection and analysis were done by Holger Sondermann.
charged residues Lys or Arg are interacting with the aromatic side chains of Phe or Trp. As the conserved N-terminal region is predicted to form β-sheets, it is possible that cation-π interactions are involved in formation or stabilization of a β-hairpin secondary structure. Studies have suggested that cation-π interactions help stabilize β-hairpin structures (Hughes et al., 2007; Tatko and Waters, 2009). Additionally, it has been demonstrated that mono, di- and tri- methylation of lysine residues impacts the stability of cation-π interaction involving lysine residues, with more methylation leading to more stability and favorable interaction with an aromatic residue (Hughes et al., 2007). It will be interesting to see if the N-terminal domain does form a β-hairpin structure stabilized by cation-π interactions and whether changes (i.e. methylation) will disrupt or strengthen this conformation, thus providing a mechanism to tune the activities of Sil1.

We are interested in exploring the possibility that the N-terminal domain of Sil1 undergoes post-translocation modification(s) (Fig. 3.4). Modification at the N terminus may relieve its autoinhibition that can then increase NEF activity. For example, there are several lysine residues at the conserved N-terminal region of Sil1, one of which is highly conserved (K95 in S. cerevisiae Sil1), that may be acetylated or methylated. The Nε-amino group of lysine can be modified with an acetyl group, a modification known as lysine acetylation. This process requires a donor of the acetyl group and an enzyme that will catalyze the addition of the acetyl group to a lysine substrate (Pehar and Puglielli, 2013). Although acetylation is well-known to occur in the nucleus and the cytosol, there have been evidence of acetylation of ER proteins. Additionally, an acetyl-CoA ER transporter and two ER acetyltransferases have been discovered (Pehar and Puglielli,
Modification of the conserved residues may disrupt the structured conformation of the N-terminal domain (green). Addition and removal of post translational modifications may allow for regulation of Sil1 activities by tuning NEF activity and modifying the positions of the redox-active cysteines (yellow circles).
Acetylation of an ER protein was demonstrated to be important in stabilization and maturation of the nascent ER-based form of human BACE1 (Costantini et al., 2007). Furthermore, a proteomic study looking for lysine acetylation in the ER revealed that 60 out of 143 proteins identified from the screen were ER resident proteins (Pehar et al., 2012). Although Sil1 was not identified as a hit, the authors point out that other previously identified ER proteins that are known to undergo transient lysine acetylation did not show up on the screen (Pehar et al., 2012). It will be interesting to see if Sil1 is susceptible to acetylation and deacetylation and how this modification might impact its activities. Further studies on Sil1 acetylation may involve detecting the modification through antibodies and mass spec.

Our studies on the N-terminal domain of Sil1 led to many interesting questions. For example, we are interested in exploring the potential role of post-translational modifications, such as methylation and acetylation, on the N-terminal domain. In addition, our findings raised some questions relating to recent models on the N-terminal domain of Sil1 binding to the substrate binding domain of BiP (Rosam et al., 2018). Does the N-terminal domain of Sil1 also regulate the ability of BiP to release substrates and restart the ATPase cycle? It is possible that disrupting the conformation of the N-terminal domain may allow it to extend and have more access to the substrate binding domain of BiP. Using fluorescent peptides that can measure peptide release from BiP or FRET assays that can monitor conformational changes of BiP domains will give information on whether the conformation of the N-terminal domain of Sil1 also influences BiP peptide binding.
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Our lab has previously shown that BiP’s conserved cysteine (C63 in yeast) located in its ATPase domain gets modified by peroxide and glutathione during ER oxidative stress. This modification is necessary for the cell’s viability under ER oxidative stress conditions, as changing the conserved cysteine to alanine, C63A, causes the cells to be inviable under stress. We have found that modification of BiP cysteine decouples the two domains, allowing for enhanced peptide binding while decreasing ATPase activity. We proposed that modified BiP leads to cell survival under ER oxidative stress by preventing aggregation. To understand how the modification of cysteine impacts BiP activity to protect the cell from oxidative damage and to find other features of BiP that are protective, we screened for mutations in BiP that would allow for cell protection under ER oxidative stress. The KAR2 gene, which encodes yeast BiP, was randomly mutagenized using PCR. The PCR products were transformed into a yeast strain, which can be induced to go under ER oxidative stress, to identify previously unknown BiP functional interactions that are important in mitigating ER oxidative stress. We expected to pull two classes of mutants from the screen: (1) mutants that behaved similarly to the modified form of BiP and (2) mutants that stabilized the modified form of BiP. The screen isolated 12 different mutants that rescued cell viability of kar2-C63A mutant cells that normally die during ER oxidative stress (Fig. A1).
Figure A1. Screening for BiP mutants that are alive under ER oxidative stress.

The KAR2 gene was PCR-amplified and randomly mutagenized. The PCR products were then transformed with cut vectors into a kar2-C63A yeast strain, which is inviable under stress. Colonies that were alive under conditions of oxidative stress should contain plasmids with mutant kar2 that confer protection against oxidative stress. Isolation and sequencing of the plasmids containing mutated kar2 showed mutants contained three to ten missense mutations in both ATPase and substrate binding domains. Mutants from the screen were serially diluted and plated on glucose and galactose plates (where oxidative stress is induced through $P_{GAL}^{Ero1^*}$). kar2-Mutant(Mut)3 shows protection against oxidative stress like the mimetic of oxidized form kar2-C63W. Mutant with a single amino-acid change, kar2-K314E, is also able to protect cells from oxidative stress.
The rescuing BiP mutants contained mutations in both the ATPase domain and the substrate-binding domain. We paired down and characterized six of the 12 mutants to a single amino acid change responsible for protection. The six mutants that have been characterized are R118G, R201S, D244V, K314E, S321G and E489V. A secondary screen was performed to test for cysteine dependence of the BiP mutants (Fig. A2). The secondary screen revealed that the protective phenotype of K314E is dependent on the cysteine (second class of mutant), while the rest of the mutants showed similar phenotype without the cysteine (first class of mutant). The residue K314 is known to form a salt bridge with S202 of Sil1 (Yan et al., 2011). GST-pulldown assays showed that the K314E charge reversal mutation significantly disrupted binding of BiP to Sil1 (Fig. A3). The K314E mutant has been published (Siegenthaler et al., 2017) and a manuscript describing the characterization of the mutants R118G, R201S, D244V, E258G and E489V is currently in preparation with the help of Heather Marsh.
Figure A2. Screening for cysteine dependence of BiP mutants that protect under ER oxidative stress.

The cysteine of each BiP mutant from the initial screen was mutated to alanine and tested for their ability to survive under ER oxidative stress. The kar2-K314E mutant which showed enhanced growth on galactose plates (oxidative stress) lost its protective phenotype when the cysteine is mutated to alanine.
To study the interaction of BiP mutants with its accessory proteins, Sil1, Lhs1 and J proteins were fused to GST. The GST-tagged proteins were then purified using glutathione agarose beads and incubated with BiP or BiP-mutant lysates. Only BiP that interacted with the accessory proteins should be pulled down with the GST-fusion protein. Shown is the result for the assay using GST-Sil1. The mutants did not show difference in binding Lhs1 and J proteins (data not shown). The mutant K314E was unable to bind to Sil1 while the rest of the mutants showed the same binding to Sil1 as wild-type BiP.
REFERENCES
