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Role of FtsH proteases in the cyanobacterium *Synechocystis* sp. PCC 6803

Ph.D. Thesis

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- **Annotation**

This thesis focuses on the functional and structural characterization of FtsH proteases in *Synechocystis* PCC 6803. One of the aims was to determine localization and subunit organization of FtsH homologues in *Synechocystis* cells using GST and GFP tagged FtsH derivatives. The main result of the thesis is identification of two FtsH hetero-oligomeric complexes and one homo-oligomeric complex in *Synechocystis* cells. The large part of the thesis is aimed at establishing the role of the first hetero-oligomeric complex, FtsH2/FtsH3, in quality control of Photosystem II and at identification of a mechanism, how its substrate proteins D1 and D2 are recognized. Another part is dedicated to characterization of the second hetero-oligomeric complex, FtsH1/FtsH3, which consists of two essential FtsH homologues and which is here identified as an important regulatory element in maintaining iron homeostasis.

- **Declaration [in Czech]**

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 15. 10. 2015

Vendula Krynická

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List of papers and author's contribution

The thesis is based on the following papers and manuscript (listed chronologically):

Papers:

I. **Komenda, J., Knoppová, J., Krynická, V., Nixon, P.J., and Tichý, M., (2010). Role of FtsH2 in the repair of photosystem II in mutants of the cyanobacterium *Synechocystis* PCC 6803 with impaired assembly or stability of the $CaMn_4$ cluster. Biochimica et Biophysica Acta 1797: 566–575**.

VK was responsible for collecting samples of Synechocystis mutant strains and isolation of RNA from them; she performed reverse transcription and quantitative PCR and then she analyzed and evaluated the data

II. **Boehm, M., Yu, J., Krynicka, V., Barker, M., Tichy, M., Komenda, J., Nixon, P.J, and Nield, J., (2012). Subunit organization of a *Synechocystis* hetero-oligomeric thylakoid FtsH complex involved in Photosystem II repair. Plant Cell 24: 3669–3683.**

VK characterized the phenotype of a GST-Tagged FtsH2 derivative from Synechocystis 6803, performed all experiments with mutant deficient in FtsH3, performed all two-dimensional Clear Native/SDS-PAGE analyses and contributed to writing the manuscript.

III. **Krynická, V., Tichy, M., Krafl, J., Yu, J., Kaňa, R., Boehm, M., Nixon, P.J., and Komenda, J., (2014). Two essential FtsH proteases control the level of the Fur repressor during iron deficiency in the cyanobacterium *Synechocystis* sp. PCC6803. Molecular Microbiology 94: 609–624.**

VK participated in designing most of the experiments, performed all of them with exception of confocal microscopy, processed and evaluated the data, wrote most of the manuscript

Manuscript:

I. **Krynická, V., Shao, S., Nixon, P.J., Komenda, J. Accessibility controls selective degradation of photosystem II subunits by FtsH protease (manuscript accepted in Nature Plants**)

VK carried out all experiments, participated in data processing and evaluation and contributed to writing the manuscript.

Josef Komenda and Peter Nixon, the corresponding authors of listed papers, approve the contribution of Vendula Krynická in these papers as described above.

Prof. RNDr. Josef Komenda, CSc., DSc.

Prof. Peter Nixon

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- **List of abbreviations:**

A. thaliana	*Arabidopsis thaliana*
aa	Amino acid
AAA+	ATPases associated with various cellular activities
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosintriphosphatase
CM	Cytoplasmic membrane
Cox2	Cytochrome c oxidase 2
Ctp	C-terminal processing carboxypeptidases
Deg	Degradation of periplasmic proteins
DNA	Deoxyribonucleic acid
E. Coli	*Escherichia Coli*
FtsH	Filamentous temperature sensitive H
Fur	Ferric uptake regulator
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HSP	Hereditary spastic paraplegia
HtrA	High temperature requirement A
Chl	Chlorophyll
LPS	Lipopolysaccharide
Nde1	NADH dehydrogenase 1
OM	Outer membrane
PCC	Pasteur Culture Collection
PL	Phospholipids
PSI	Photosystem I
PSII	Photosystem II
RC	PSII reaction center
RNA	Ribonucleic acid
SP	Signal peptidases
Spp	Signal peptide peptidases
TEM	Transmission electron microscopy
TM	Thylakoid membrane
Tsp	Tail specific protease
WT	Wild type

1. Overview

1.1 Cyanobacteria

Cyanobacteria are gram-negative bacteria performing oxygenic photosynthesis. As other oxygenic phototrophs, they are able to acquire energy from sun light for converting inorganic carbon to organic molecules needed for basic cellular functions. A secondary effect of the photosynthetic process is production of oxygen. Fossil evidence has revealed that cyanobacteria are likely more than 3.5 million years old (Schopf, 1996). Hence, cyanobacteria, as one of the earliest organisms contributing to the oxidizing atmosphere, dramatically changed the composition of life forms in nature (Schopf, 2014). Even today, cyanobacteria are responsible for a large portion of the photosynthetic production in aquatic environments and thus play an important role in global CO_2 assimilation and oxygen recycling (Geider *et al.*, 2001).

These prokaryotic autotrophs are able to acclimate to almost all aquatic but also terrestric habitats. Except of the water and soil, they survive in hot springs, arctic ice or desert rocks and even in volcanos (Makhalanyane *et al.*, 2015, Hagemann *et al.*, 2015, Sciuto and Moro, 2015). Due to their extreme adaptability and simplicity they became one of the most successful group of microorganisms in the world. They also became very popular in biological research starting from evolutionary studies (Shi and Falkowski, 2008) to biotechnological applications, including biofuel production or drug discovery (Ruffing, 2011).

1.1.1 *Synechocystis* sp. PCC 6803 as a model organism

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is one of the most frequently used model cyanobacteria. The strain was isolated from a fresh water lake and was deposited in the Pasteur Culture Collection (PCC) in 1968 (https://research.pasteur.fr/en/team/biological-resources-center). It is an unicellular organism with spherical cells of about 1.5–2 µm in diameter. As in other gram-negative bacteria, the cell envelope consists of an outer membrane and a plasma membrane (PM), separated by the peptidoglycan containing periplasmic space. The cell interior harbours the thylakoid membranes (TM), which are organized in largely concentric layers around the periphery of the cell. In the central part are present circular fibrils of DNA and carboxysomes, microcompartments important for carbon fixation. Ribosomes are dispersed throughout the cell but are present in the highest density in the central region around the nucleoplasm (Fig. 1). Thylakoid membrane system forms the basis for processes of photosynthesis, but also for respiration, making the coordination of the biochemical reactions even more complex (Peschek *et al.*, 2004, Vermaas, 1994).

Synechocystis grows fast in both liquid and solid media. Apart from autotrophic cultivation, it is also able to grow in a wide range of conditions including mixotrophy and heterotrophy, where the only energy source is organic carbon in the form of glucose. Its cultivation is very cheap and undemanding. Another fundamental advantage of that organism is the fact that *Synechocystis* was the first photosynthetic organism for which the entire genome sequence was determined (Kaneko *et al.*, 1996). Moreover, in contrast to higher plants, gene inactivation, modification and regulatable expression became routine in this organism due to natural transformation and efficient homologous recombination. Thus, this organism provides an ideal tool for targeted mutagenesis. *Synechocystis*, as a close relative of progenitors of plant plastids, contains photosynthetic components and maintenance systems that are similar to those of chloroplasts (Deusch *et al.*, 2008, Falcon *et al.*, 2010). For all these reasons, this is one of the most frequently used organisms for studies on biogenesis of photosynthetic complexes and quality control of photosynthetic apparatus (Komenda *et al.*, 2010, Xu *et al.*, 2004). Apart from that, it became sought for study of function of various essential proteins and mechanisms underlying diverse, universally conserved cellular processes.

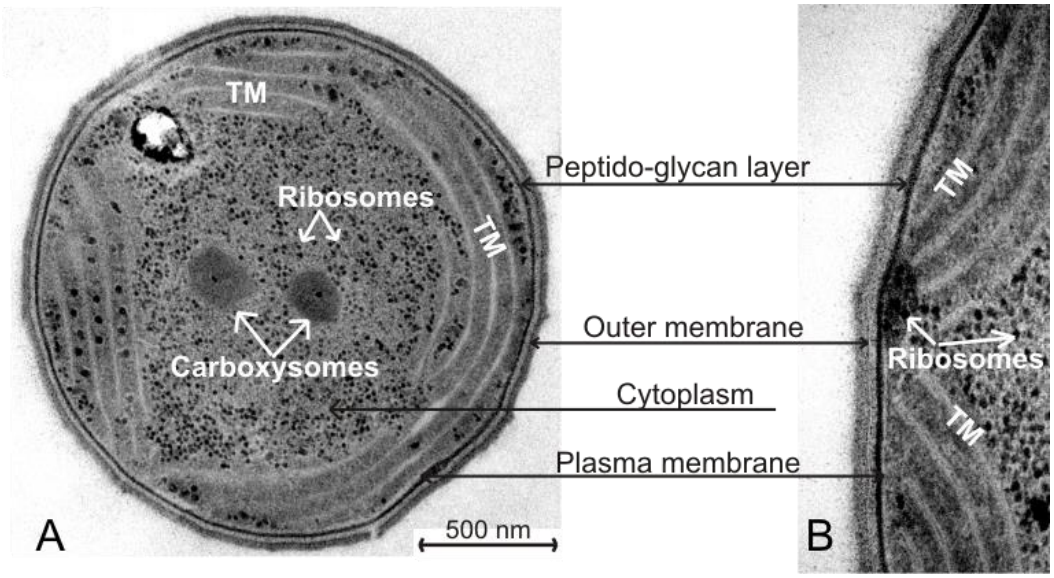

Fig.1.Transection electron microscopic picture of WT cell of Synechocysits PCC6083 (A) and the detail of membrane organization (B); TM: thylakoid membranes; Photo made by Lenka Bučinská

1.2 Proteases

Proteins, the products of gene expression, are key macromolecules guaranteeing most of the cellular processes in all living organisms. Protein molecules are continuously synthesized and degraded. The desired level of individual cellular proteins is maintained by a balance between rates of synthesis and degradation. Both phenomena, synthesis and degradation, are controlled by a series of regulated biochemical processes. Regulated proteolysis is a form of posttranslational mechanism with a direct impact on the amount of certain proteins to facilitate the immediate responses to external influences (Pruteanu and Baker, 2009, Gur *et al.*, 2011, Andersson and Aro, 1997, Adam, 2000).

The enzymes performing proteolysis are known as proteases or peptidases. They catalyse protein digestion by hydrolysis of the peptide bonds that link amino acids together inside a target protein molecule. Proteases can be divided into two large groups according to their substrate specificities: endopeptidases and exopeptidases. Exopeptidases remove single or several amino acid residues from N- or C-termini (Naqvi *et al.*, 2005, Cadel *et al.*, 2001). Endopeptidases, on the other hand, attack internal peptide bonds of a protein. The general mechanism of proteolysis involves a nucleophilic attack on the peptide carboxyl group. Depending on the nucleophile, the proteases are currently classified into six broad groups: serine, threonine, cysteine, aspartic, glutamic and metallo-proteases**.** Serine, cysteine and threonine proteases use a functional group (usually hydroxyl or thiol group) within the enzyme itself, while aspartic acid, glutamic acid and metallo-proteases activate a water molecule to serve as a nucleophile (Rawlings and Barrett, 1999, Rawlings *et al.*, 2014).

Protein turnover in both eukaryotes and prokaryotes play a crucial role in the key cellular processes, such as a protein quality control by removal of mistranslated or aberrant polypeptides expressed from defective RNAs (Goldberg and Dice, 1974, Luciakova *et al.*, 1999, van Wijk, 2015) or of post-translationally modified proteins that cannot return to their native, functional form. Some proteases control gene expression by direct or indirect interaction with transcription through regulation of sigma factors or transcription repressors (Herman *et al.*, 1995a, Herman *et al.*, 1995b, Zhang *et al.*, 2007b, Krynicka *et al.*, 2014). Other proteases play important role in maturation of non-active protein forms through processing (Anbudurai *et al.*, 1994, Teixeira and Glaser, 2013). Apart from that, they are involved in adaptive response to various stress conditions by regulating the level of some short-lived cytoplasmic proteins. To such stress conditions belong extreme irradiance (Adamska *et al.*, 1996, Porankiewicz *et al.*, 1998, Malnoe *et al.*, 2014), heat shock (Lipinska *et al.*, 1990, Spiess *et al.*, 1999, Xu *et al.*, 2015), high salinity (Gerth *et al.*, 1998), nutrient

starvation (Alexander *et al.*, 1993, Collier and Grossman, 1994) or treatment with chemical reagents (Andersson and Aro, 1997).

In eukaryotic cells, the turnover occurs in all organelles including mitochondria, chloroplasts, peroxisomes, lysosomes and vacuoles (Burgess, 1978, Hicke and Lippincott-Schwartz, 2001). Above that, a considerable part of the degradation processes also occurs in cytoplasm. A major cytoplasmic proteolytic pathway is mediated by an ubiquitin system (Ciechanover *et al.*, 1980, Ciechanover, 1998, Hershko, 1983). In this pathway substrate proteins are selectively marked for destruction with a poly-ubiquitin tag and then degraded by a proteasome (Hershko, 1983, Hershko, 1988). Ubiquitin is small polypeptide of 76 amino acids that binds covalently to lysine residues on a target protein (Hershko, 1983). Proteasome represents a highly conserved cylindrical complex with multiple protease active sites in their interior chamber digesting proteins in an ATP-dependent fashion (Glickman, 2000, De Mot *et al.*, 1999, Ciechanover and Schwartz, 1989, Ciechanover, 1998).

Unlike eukaryotes, prokaryotic cells do not comprise organelles, but the activity of their major proteolytic systems is, similarly to proteasome, spatially sequestered inside cylinder-shaped complexes forming nanocompartments, into which the selected substrates are delivered for degradation, usually under ATP consumption. The sequestration of proteolytic activity to prevent unspecific degradation of unwanted substrates is also achieved by directing proteases to specific cellular regions, such as the periplasmic space of gram-negative bacteria or the lumen of cyanobacteria. The other way how to better target the degradation process is anchoring the protease in the plasma membrane and in the case of cyanobacteria also in thylakoid membranes.

1.2.1 Characterization of cyanobacterial proteases

Similar to other prokaryotic organisms, cyanobacteria control many regulatory circuits or metabolic pathways by proteolysis to facilitate the immediate responses to external stimuli. Many proteases function in ATP-dependent manner although proteolysis needs not necessarily consume energy.

1.2.1.1 ATP independent proteases

In cyanobacteria there exist four main families of ATP-independent proteases: Deg, Ctp, SP and SppA.

The Deg (Degradation of periplasmic proteins)/HtrA (high temperature requirement A) proteases (Strauch *et al.*, 1989, Lipinska *et al.*, 1989), found in almost all living forms, are ATP-independent serine endopeptidases with a trypsin-type catalytic domain. They also contain up to three PDZ domains, a common structural domain of 80-90 amino-acids found in the signaling proteins of prokaryotes and eukaryotes, that interact with nonpolar C-terminal residues of their ligands and are important for substrate recognition (Iwanczyk *et al.*, 2007). Structural studies showed that Deg/HtrA proteases form oligomeric complexes with a trimer as the basic unit (Clausen *et al.*, 2002, Kley *et al.*, 2011). Deg proteases are well-characterized in *Escherichia coli* and human. The *E. coli* DegP protease has dual chaperone and protease functions, which are regulated by temperature (Clausen et al., 2002). As a protease it is involved in degrading irreversibly damaged proteins, while as a chaperone it assists in refolding of denaturated proteins (Spiess et al., 1999). *E. coli* DegS can release the stress response sigma factor sigE (RpoE) by degrading its inhibition factor (Clausen et al., 2002). Human HtrA2/Omi contributes to the regulation of programmed cell death (Clausen et al., 2002). In higher plants, several Deg members were shown to be localizes in the chloroplast stroma and lumen. It was reported that *Arabidopsis thaliana* Deg2 is part of a larger network of enzymes that ensure protein quality control in PSII (Huesgen *et al.*, 2009). The cyanobacterial Deg/HtrA was proposed to participate in the response to light and heat stress (Cheregi *et al.*, 2007, Lucinski *et al.*, 2011). Genome of *Synechocystis* encodes three Deg proteases known as HhoA (Sll1679), HhoB (Sll1427) and HtrA (Slr1204) which were found to be attached to the thylakoid and plasma membranes (Roberts *et al.*, 2012). Very recently, RbcS has been identified as a natural substrate for HhoA, PsbO for HhoB and HtrA, and Pbp8 for HtrA (Tam *et al.*, 2015). Whether Deg/HtrA proteases participate also in degradation of photoinhibited D1 protein in cyanobacteria is still under the debate (Barker et al., 2006, Huesgen et al., 2009).

Ctp proteases belong to C-terminal processing carboxypeptidases functioning as serine proteases and containing a PDZ domain (Paetzel and Dalbey, 1997). Their physiological function is less understood with the exception of CtpA which is involved in maturation of D1 protein in photoautotrophs (Oelmuller *et al.*, 1996), and the bacterial Tsp (tail-specific-protease) which is involved in removing improperly translated proteins after their SsrA tagging (Spiers *et al.*, 2002). In *Synechocystis*, there are three Ctp orthologs, encoded by *ctpA* (*slr0008*), *ctpB* (*slr0257*) and *ctpC* (*slr1751*) (Jansen *et al.*, 2003). CtpA, like in other photoautotrophs, is involved in biogenesis of Photosystem II (PSII) complex by C-terminal processing of the D1 precursor (pD1) of PSII (Anbudurai et al., 1994) and had been

originally located in association with plasma membrane (Zak *et al.*, 2001). However, newer data show that this protease is located in lumen similarly to plant chloroplast (Selao and Knoppová, unpublished results). On the other hand CtpB has been found in periplasmic space and CtpC in association with plasma membrane and their function is unknown.

Other protease families not possessing the ATPase activity belong to the signal peptidases SP and the signal peptide peptidases, SppA. They are transmembrane proteases with a large C-terminal domain protruding into the periplasmic space (Wang *et al.*, 2008, Zhbanko *et al.*, 2005). SPs serve a crucial role in the removal of the signal peptide after translocation of secretory protein precursors from the cytoplasmic membrane (van Roosmalen *et al.*, 2004). This removal is essential for protein release into the periplasmic or lumenal space, transport of proteins to the outer membrane in gram-negative bacteria, and secretion of proteins into the extracellular medium. In cyanobacteria were found two types of SP, SPI with serine-type peptidase activity and SPII, lipoprotein signal peptidases with aspartic-type endopeptidase activity. SppA, on the other hand, are responsible for further degradation of the signal peptide cleaved by SP. This degradation is important, because signal peptides may be harmful to the cell, as they can interfere with membrane integrity and block protein translocation via the Sec machinery (Dalbey *et al.*, 2012).

1.2.1.2 ATP dependent proteases

ATP-dependent proteases belong to a broader AAA+ superfamily of proteins (ATPases associated with various cellular activities) which combine an ATP-driven unfolding activity with the proteolysis. AAA+ proteases are self-compartmentalizing proteolytic complexes. Their active centres are sequestered in the interior of barrel or ring-shaped assemblies. The access is granted only through a narrow pore, passage of even small-sized folded proteins is not permitted. A key feature of the entry pore is the occurrence of aromatic amino acids (aa) that are important for substrate recognition and translocation (reviewed in Langklotz *et al.*, 2012, Yamada-Inagawa *et al.*, 2003, Makino *et al.*, 1999). Targeted proteins carry a degradation signal that is recognized either directly by these pore residues or indirectly via adaptor molecules. ATP hydrolysis triggers conformational changes in the AAA ring pulling the substrate towards the narrow pore, eventually resulting in unfolding and subsequent translocation events (Suno *et al.*, 2006, Bieniossek *et al.*, 2009). Proteins are processively degraded into small peptides of about 6–25 aa in length (Hersch *et al.*, 2005). Three main groups of AAA+ proteases: Clp family, FtsH proteases and Lon proteases, were found in

cyanobacteria (Kegg database - Aoki-Kinoshita and Kanehisa, 2007, CyanoBase - Nakao *et al.*, 2010).

Clp proteases are widespread soluble multisubunit protein complexes belonging to the family of serine endopeptidases (Rawlings *et al.*, 2014). The basic structure of the Clp machinery consists of a cylinder-like or barrel-like protease core and an AAA+ chaperone ring complex. The chaperone complex serves as a molecular gate that controls substrate access, and recognizes, unfolds and translocates protein substrates into the core cavity in an ATP-dependent manner. The cyanobacterial Clp system comprise two types of tetradecameric proteolytic core with catalytically active ClpP and inactive ClpR subunits, hexameric ATP-dependent chaperones consist of ClpX or CplC and adaptor proteins ClpS enhancing delivery of subsets of substrates (Nishimura and van Wijk, 2015). Most cyanobacteria contain an additional component, NblA, which is a specific adaptor protein to deliver phycobilisome proteins to the ClpCPR system (Baier *et al.*, 2014). In cyanobacteria Clps are important for the steady-state growth and acclimation of cells to various stresses like high light, cold stress and UV-B (Clarke, 1999, Porankiewicz *et al.*, 1998). Cyanobacterial ClpC can prevent aggregation of unfolded proteins (Andersson *et al.*, 2006) and in plants the Clp system appears to be essential for chloroplast biogenesis and proteostasis (Shikanai *et al.*, 2001, Ramundo *et al.*, 2014).

Lon proteases are ATP-dependent serine endopeptidases found in archaea, bacteria and eukaryotic mitochondria and peroxisomes. The Lon activity was ascribed to be essential for cellular homeostasis, mediating protein quality control and metabolic regulation (Barakat *et al.*, 1998, Lee and Suzuki, 2008). In some cyanobacteria the gene encoding Lon protease was annotated but further information regarding the gene product is missing (Kegg database - Aoki-Kinoshita and Kanehisa, 2007, CyanoBase - Nakao et al., 2010).

In contrast to other bacteria, the existence of a homologue of HslV/U proteases was not described in cyanobacteria (Kegg database - Aoki-Kinoshita and Kanehisa, 2007, STRING database - Szklarczyk *et al.*, 2015). The heat shock proteins HslV and HslU (also known as ClpQ and ClpY, respectively are analogous to Clp proteases (Ramachandran et al., 2002). They are expressed in many bacteria in response to cellular stress. HslU proteins are ATPases, while HslV proteins are proteases with high similarity to β subunits of proteasomes. Therefore, the HslVU complex has been envisaged as a precursor or ancestral type of proteasome (Gille *et al.*, 2003, Ruiz-Gonzalez and Marin, 2006).

The last group of cyanobacterial energy-dependent proteases belongs to the family of FtsH proteases described in detail in the following chapter.

1.3 FtsH proteases

FtsHs (Filamentous temperature sensitive H), in case of mitochondrial enzymes also termed AAA proteases, are membrane-bound metalloproteases universally conserved in bacteria, chloroplasts and mitochondria, where they have essential function in protein turnover and processing (Langklotz *et al*., 2012). Among the AAA+ proteases, FtsHs are unique owing to its anchoring to the membrane. They preserved more than 40% sequence identity in various organisms like *E. coli*, *Saccharomyces cerevisiae*, *Homo sapiens*, *A. thaliana* and cyanobacteria (NCBI Blast program). FtsHs are essential in many, but not all, gram-negative bacteria (Akiyama *et al.*, 1995). Although the *ftsH* gene is not essential in gram-positive bacteria, *ftsH* deletions lead to severe pleiotropic effects. Most bacteria possess a single gene encoding FtsH, only cyanobacteria are known to encode four FtsH homologues (CyanoBase - Nakao *et al.*, 2010, Boehm *et al.*, 2012). Three genes are present in yeast and human mitochondria (Arnold and Langer, 2002) while 12 orthologues were found in the genome of model higher plant *A. thaliana* (Yu *et al.*, 2004, Sokolenko *et al.*, 2002). Cellular abundance of FtsHs increases in response to cellular stress (Ito and Akiyama, 2005). FtsH proteases are active toward both membrane and soluble substrates, and their activities can be divided into protein quality control: degradation of unassembled, unfolded and damaged proteins, and regulatory function: degradation of transcription regulators and short-lived regulatory proteins (Ito and Akiyama, 2005, Zhang *et al*., 2007b). As a member of AAA family, FtsHs convert the chemical energy stored in ATP via conformational rearrangements into the mechanical force that is used for substrate unfolding and translocation into the proteolytic chamber, where the proteins are degraded. They have a unique ability to dislocate substrate membrane proteins out of the membrane (Ito *et al.*, 2005). However, the unfoldase activity of FtsH is very weak and data obtained *in vitro* using mutated apoflavodoxin proteins as a substrate for *E. coli* FtsH protease indicate, that FtsH, rather than unfolding the protein, acts on the fraction that is already unfolded (Ayuso-Tejedor *et al.*, 2010). In addition, a chaperone-type activity of the FtsH complex has been reported for the yeast mitochondria homologue Yme1 protein (Leonhard *et al.*, 1999). In agreement with their function as quality control enzymes, FtsHs have degenerate substrate specificity and appear to mainly recognize the folding state of substrate proteins, not the amino acid sequence (Chiba et al., 2000). They are able to recognize a variety of apolar sequences becoming exposed in unfolded states to cytosol. Current results indicate that FtsH-dependent processive degradation of membrane proteins can be initiated either on N-terminal or C-terminal cytosolic tails. However, an internal region of a polypeptide, an extended cytosolic loop, can also sensitize

the protein to FtsH (Shotland *et al.*, 2000, Chiba *et al.*, 2002). The genetic manipulation of N-terminal cytosolic tails of several substrates revealed that FtsH recognized a cytosolic region of sufficient length - about 20 amino acids (Chiba *et al.*, 2000), while only ten C-terminally-exposed amino acids are sufficient for degradation (Fuhrer *et al.*, 2006). Although recognition of free ends is a common principle, the molecular basis of target interaction and membrane dislocation might differ from case to case (Chiba *et al.*, 2002). The enzyme works as endopeptidase, since its degradation products are oligopeptides containing several residues (Asahara *et al.*, 2000). FtsH complexes may interact with other proteins, forming the large membrane spanning supercomplexes. In the plasma membrane of *E. coli* FtsH exists as an exceptionally large complex containing HflC/HflK, the prohibitin-like proteins (Saikawa *et al.*, 2004). Prohibitins also regulate the activity of the m-AAA proteases in mitochondria (Langer, 2000).

1.3.1 Structural features of the FtsH protease

1.3.1.1 Primary structure of FtsH

Contrary to the most other AAA+ proteases, FtsHs are harbouring both ATPase and proteolytic domains on the same polypeptide chain (Ito and Akiyama, 2005) that is about 650 aa in length in most eubacteria (Fig. 2). The N-terminus contains one or two helices that anchor the protein to the respective membrane. In *E. coli*, a small periplasmic region is located between the two transmembrane helices. The membrane-spanning part is connected to the cytosolic part of the enzyme via a glycine-rich linker of about 15–20 aa in length (reviewed in Langklotz et al., 2012). Cytosolic moiety, the main body of the enzyme, comprises two domains, AAA module connected to the glycine-rich linker and protease domain at the C-end. The AAA moiety contains the characteristic sequence motifs of the AAA family, namely Walker A and B, necessary for binding and hydrolysis of ATP; the second region of homology (SHR) fingerprint which carries conserved arginine residues (‘‘arginine fingers’’) important for oligomerization and ATP hydrolysis; and the conserved FGV pore motif required for substrate recognition and translocation (Bieniossek *et al.*, 2009) (Fig. 2). The characteristic ‘zincin’ HEXXH motif (frequently HEAGH) identifies the protease active center. The active zinc ion is coordinated by the two imidazole side-chains of the HEXXH motif on a strictly conserved aspartic acid, thus classifying FtsH as a novel Asp-zincin (Bieniossek *et al.*, 2006) (Fig. 2). At the C terminus there is a leucine-rich motif that has been

implicated in co-recognition of certain substrates (Graef *et al.*, 2007, Suno *et al.*, 2006). The C-terminal end is of variable length and is predicted to be mostly unstructured. *E. coli* FtsH has been reported to auto-catalytically clip its C terminus but the significance of this modification is unknown (Akiyama, 1999).

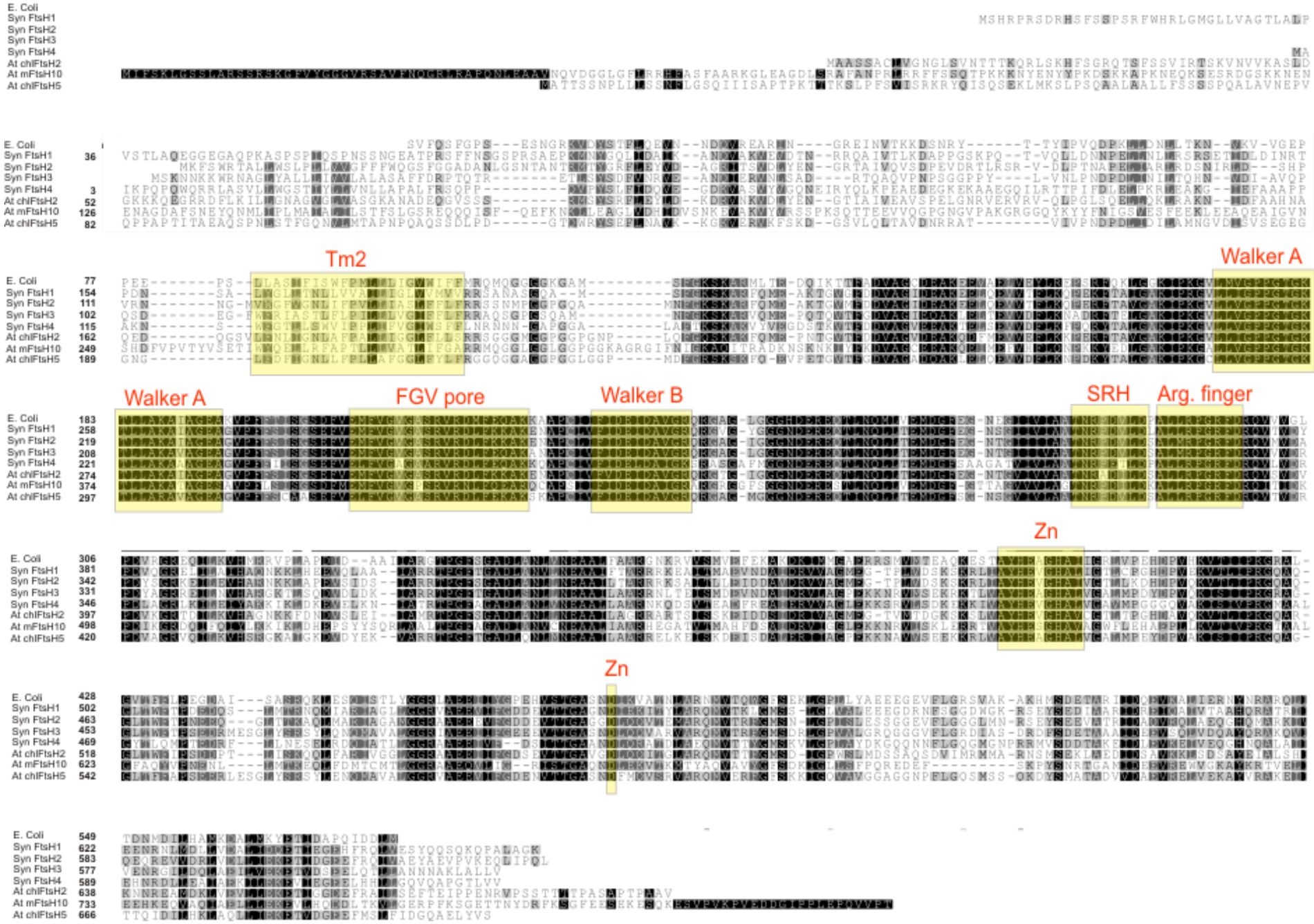
E. Coli
Syn FtsH1
Syn FtsH2
Syn FtsH3
Syn FtsH4
At chlFtsH2
At mFtsH10
At chlFtsH5
Tm2
Walker A
Walker A
FGV pore
Walker B
SRH
Arg. finger
Zn
Zn

Fig. 2: Sequence alignment of FtsH homologues. Consensus is highlighted with a black background. Key elements (adopted from Langklotz *et al*., 2012) are indicated by yellow rectangle with description above the sequences in orange: Tm2, transmembrane helix 2 (predicted); Walker A and B, motifs responsible for ATP binding and hydrolysis; Pore, FGV pore motif; SRH, second region of homology; Arg-finger, arginine finger; Zn, zinc-ligands; FtsH homologues were abbreviated as follows and Kegg entries are given: SynFtsH1-4, *Synechocystis* FtsH homolog 1,2,3 and 4 respectively; *E.coli* FtsH - FtsH homolog from *E. coli* str. K-12 substr. Mg1655; At chl FtsH2: chloroplastic ATP-dependent zinc metalloprotease FtsH 2 from *A.thaliana*; At chl FtsH5: chloroplastic ATP-dependent zinc metalloprotease FtsH 5 from *A.thaliana*; At m FtsH10: mitochondrial ATP-dependent zinc metalloprotease FtsH10 from *A.thaliana*.

1.3.1.2 Tertiary structure

The hydrophobic properties of the apoprotein make difficult crystallization of the entire FtsH complex. Therefore, little is known about the transmembrane N-terminal regions which are dispensable for protease activity (Ito and Akiyama, 2005). Recently, the structural analysis of recombinant periplasma region of FtsH from *E. coli* (aa residues 25–96) revealed a compact monomeric α + β fold comprising two α-helices and five β-strands. The hexameric ring has been constructed by crystallographic symmetry from monomers having a disk-like shape of approximately 5–7 nm in diameter and 2.5 nm in height (Scharfenberg *et al.*, 2015). More detailed structural data were obtained using crystallized soluble part of the protein containing both ATPase and protease domain and lacking transmembrane segment. Initially, by analogy with the soluble ATP-dependent proteases a hexameric ring–like structure has been constructed by homology modelling (Krzywda *et al.*, 2002). More recently, other crystal structures of isolated FtsH AAA domains from *Thermus thermophilus*, *Helicobacter pylori* and human paraplegin have been published (Suno *et al.*, 2006, Karlberg *et al.*, 2009, Kim *et al.*, 2008). All these structures agree in monomer fold and a general hexameric assembly with dimensions of about 10 nm in diameter and 6.5 nm in height. The fold of the AAA moiety is canonical with a wedge-shaped N-terminal subdomain consisting of five stranded parallel beta sheets surrounded by α-helices on both sides and a C-terminal four-helix bundle. The proteolytic domain is nearly all-helical, comprising 6 α-helices and a short piece of β structure (reviewed in Langklotz *et al*., 2012). The crystal of the ATPase domain of *Thermus thermophilus* FtsH shows inter-subunit contacts, involving both electrostatic and

hydrophobic interactions of subunits, to form a hexameric structure (Niwa *et al.*, 2002). The cytosolic region fully retains an ATP-dependent protease activity and adopts a three-fold-symmetric hexameric structure. The protease domains displayed a six-fold symmetry, while the AAA domains, each containing ADP, alternate two orientations relative to the protease domain, making ‘‘open’’ and ‘‘closed’’ interdomain contacts. Apparently, ATPase is active only in the closed form, and protease operates in the open form. The protease catalytic sites are accessible only through a tunnel following from the AAA domain of the adjacent subunit, raising a possibility of translocation of polypeptide substrate to the protease sites through this tunnel (Suno *et al.*, 2006). Nucleotides bind to the Walker A motif (also called P-loop) located close to the cleft between the two subdomains. ATP hydrolysis is coupled to up and down movements of conserved loops contacting the substrate in the central pore of the ATPase ring (Hinnerwisch *et al.*, 2005, Martin *et al.*, 2008). These conformational changes could be responsible for an inward movement of the substrate into the proteolytic part of protease in which it is degraded (Bieniossek *et al.*, 2009).

Only recently, a low resolution electron cryomicroscopic structure of the whole mitochondrial FtsH protein complex from yeast was obtained (Lee *et al.*, 2011) (Fig. 3). This enzyme consists of hetero-hexamer Yta10 and Yta12 subunits. Its transmembrane part localized in the inner membrane of mitochondria, as well as its intermembrane space segments are clearly separated from the main body of the complex. The main soluble part of the enzyme faces mitochondrial matrix. The detailed structure model answers the question, how soluble and membrane-integral protein substrates enter the protease. This study revealed a 13 Å wide gap between the main hexamer body and the N-terminal transmembrane segment, which is large enough to accommodate unfolded region of polypeptides. After binding the substrate to the AAA domain, this gap might enable a guided transfer of the unfolded region to the central pore of the AAA ring (Cha *et al.*, 2010). Minimal length of 20 amino acid residue of unfolded segment corresponds to the distance between the initial substrate binding site and the central pore loops of the AAA ring. Unfolded segment is then threaded into the proteolytic chamber of protease domain, where it is degraded, and the cleaved peptides are released through nearby lateral openings (Lee *et al.*, 2011, Cha *et al.*, 2010).

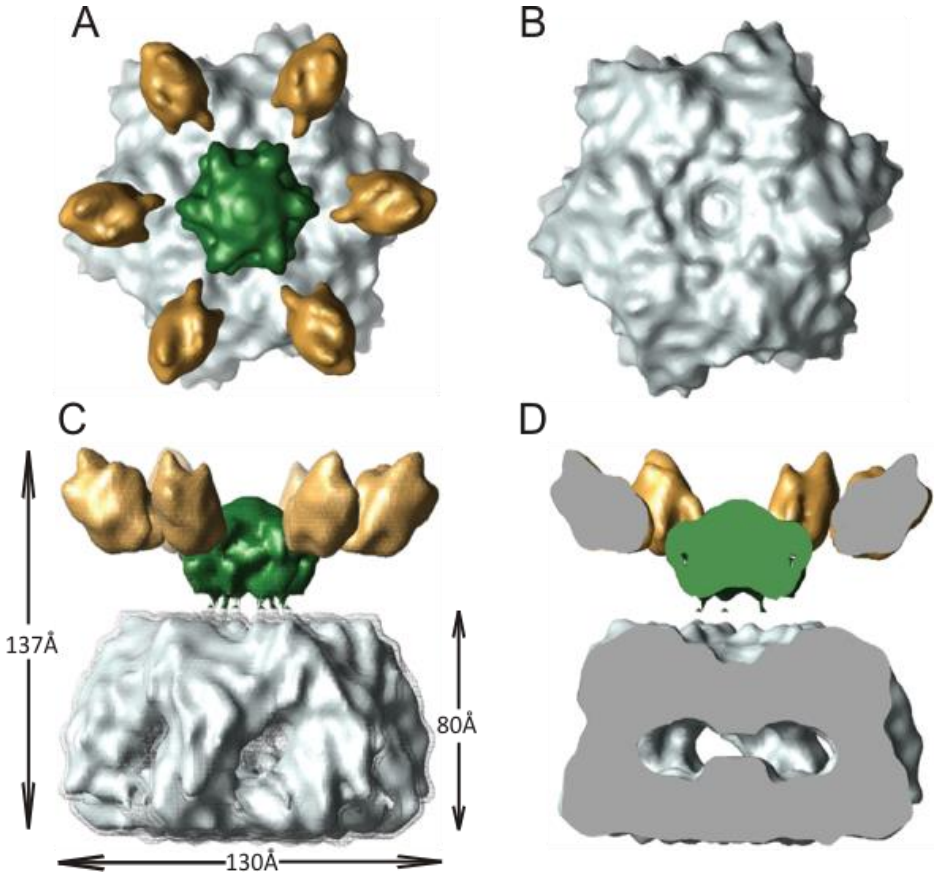

Fig. 3. Cryo-EM structure of an intact yeast m-AAA protease. The structure of the Trap-ATP hexamer is shown as isosurface representation with the transmembrane domain coloured green, the intermembrane space domain gold, and the matrix domain gray. A: top down view, B: top down view of the main body only, C: side view, and D: vertical central section. The meshed density is contoured at a lower threshold to show the connectivity between the transmembrane segments and the AAA ring.

Adopted from Lee et al., 2011, Journal of Biological Chemistry

1.3.2 Function of FtsH proteases in different organisms and organelles

FtsHs exert versatile activities in almost all living organisms on the planet, starting with bacteria, ending with mammalian cells. They were found to degrade non-native proteins to peptides, regulate level of some short-lived cytosolic proteins, mediate the specific processing on certain substrates to promote their biogenesis and were even found to exert non-proteolytic functions (reviewed in Langklotz *et al.*, 2012). Mutations in different

mitochondrial FtsH subunits are associated with different neurodegenerative disorders in human.

1.3.2.1 FtsH in Escherichia coli

The first available information on FtsH proteases comes from *Escherichia coli.* Its genome possesses a single *ftsH* gene, which is crucial for cell viability. The gene product assembles into a homo-hexameric complex that interacts with HflK–HflC membrane protein complex forming a large complex, termed FtsH holo-enzyme (Saikawa *et al.*, 2004). This complex can be separated from membrane protein fraction after detergent solubilization and velocity sedimentation. It appears to have molecular mass of around 1000 kDa with an ability to bind one or a few substrate molecules (Ito and Akiyama, 2005).

FtsH holo-enzyme plays a crucial role in the membrane protein quality control. It degrades the membrane α subunit of F_0 sector of the ATP synthase and SecY membrane proteins when they are not assembled with their partner proteins to form the ATP synthase or the SecYEG translocase, respectively (Akiyama *et al.*, 1996, Schumann, 1999). SecY degradation by FtsH also occurs when the translocase is blocked (van Stelten *et al.*, 2009) and in this way FtsH may affect the biosynthesis of membrane components. *E. coli* FtsH holoenzyme also degrades YccA, a protein of unknown function that seems to be linked to biofilm formation (Beloin *et al.*, 2004, Kihara *et al.*, 1998).

The FtsH protease is also required for degradation of many cytoplasmic substrates in *E. coli*. It regulates level of cytosolic short lived proteins like a heat shock sigma factor RpoH (σ32). Upon heat shock the bacterial cell has to cope with unfolded and aggregated proteins. This initializes the expression of genes coding for molecular chaperones, which in *E. coli* are tightly controlled by the RpoH (Guisbert *et al.*, 2008). Upon physiological temperature, the RpoH protein is destabilized by attachment of DnaK/J chaperons and in this form it is destined for degradation by FtsH (Guisbert *et al.*, 2008). Upshifts in temperature are indirectly sensed by the amounts of denatured proteins, which titrate DnaK/J away from RpoH. Released RpoH is bound into stable complex with RNA polymerase which initiates the transcription of heat shock genes (Nonaka *et al.*, 2006).

FtsH also plays a role in phage λ infection since it degrades a short lived λCII transcriptional factor (Nonaka *et al.*, 2006, Schumann, 1999) and in this way starts the phage lytic pathway (Shotland *et al.*, 1997). Another phage λ protein, λIXis, is also degraded by the FtsH protease and is responsible for excision of phage DNA from the bacterial genome (Leffers and Gottesman, 1998).

FtsH also participates in the degradation of SsrA-tagged cytosolic proteins in *E. coli*. To enable ribosome recycling and removal of aberrant proteins from the cell, the SsrA-tag is added to stalled nascent chains during translation. The SsrA-tag consists of 11 residues and represents a general degradation signal for ClpAP/XP, Lon and FtsH (Herman *et al.*, 1998).

Finally, FtsH helps to maintain a balance in the lipid composition of the membrane by degrading LpxC, the regulatory enzyme in the pathway of bacterial lipid A biosynthesis. This FtsH function is essential for viability of *E. coli* (Ito and Akiyama, 2005). As other gram-negative bacteria, *E. Coli* is surrounded by outer membrane containing lipopolysacharides (LPSs) which form permeable barrier of the membrane. LPSs consist of a polysaccharide O-antigen, a core oligosaccharide and lipid A (Wang *et al.*, 2010). Lipid A is a hydrophobic anchor of LPS in the outer membrane. Both excess and deficiency of lipid A are toxic for *E. coli*, thus the rate of LPS synthesis is strictly controlled by FtsH via proteolysis of the LpxC enzyme. Initial precursor for lipid A is also a precursor for the synthesis of phospholipids (PL). Impaired regulation of the LpxC level thus enhances LPS synthesis at the expense of phospholipids. Viability of Δ*ftsH* cells is permitted by a suppressor mutation, which rebalances the LPS to PL ratio. This mutation compensates for LpxC stabilization by increased activity of FabZ, the key enzyme of PL biosynthesis (Ogura *et al.*, 1999). Moreover, FtsH has a second function in regulation of LPS biosynthesis as it also degrades the KDO glycosyl transferase KdtA, another enzyme of LPS biosynthetic pathway, which is anchored in the plasma membrane. In any case, the above mentioned enumeration of functions executed by FtsH in *Escherichia Coli* demonstrates the important role of FtsH in diverse cellular processes.

1.3.2.2 Mitochondrial FtsHs (AAA proteases)

Two types of AAA protease complexes with different topology, i-AAA and m-AAA, are found in the inner membrane of yeast (Leonhard *et al.*, 1996), mammalian (Coppola *et al.*, 2000) and plant (Lindahl *et al.*, 1996) mitochondria. The i-AAA proteases span the inner membrane once and expose their catalytic domains to the mitochondrial intermembrane space. On the other hand, the m-AAA proteases with two transmembrane regions have their active sites directed toward the matrix. In yeast mitochondria two AAA complexes are present: the homo-oligomeric i-AAA complex composed of Yme1 subunits and the hetero-oligomeric m-AAA complex built of homologous Yta10 (Afg3) and Yta12 (Rca1) subunits (Leonhard *et al.*, 1996, Arlt *et al.*, 1996). Mammalian mitochondria also contain a single homo-oligomeric i-AAA complex (Stiburek *et al.*, 2012) but, unlike yeast, they have multiple m-AAA complexes. Two such complexes from human mitochondria have been described: a hetero-oligomeric

one composed of paraplegin and AFG3L2 subunits, and a homo-oligomeric one assembled from AFG3L2 subunits alone (Atorino *et al.*, 2003, Koppen *et al.*, 2007). In mice three types of m-AAA complexes have been found: one hetero-oligomeric AFG3L1/paraplegin and two homo-oligomeric composed of AFG3L1 or AFG3L2 (Koppen *et al.*, 2007). All mammalian isoenzymes are able to complement for the loss of the m-AAA protease in yeast, showing their functional conservation and functional redundancy between mammalian m-AAA proteases (Atorino *et al.*, 2003). Similarly to the mammals, two *A. thaliana* m-AAA proteases, AtFtsH3 and AtFtsH10, are able to form hetero-oligomeric as well as homo-oligomeric complexes (Piechota *et al.*, 2010). Based on the transcript and protein abundance, the homo-oligomeric complex of AtFtsH3 is predominant in *A. thaliana* mitochondria. While in yeast and mammals only one homo-oligomeric i-AAA complex exists, in *A. thaliana* two mitochondrial i-AAA proteases, AtFtsH4 and AtFtsH11, form two independent homo-oligomeric complexes (Urantowka *et al.*, 2005, reviewed in Janska *et al.*, 2013).

The *m*-AAA protease assembles with prohibitins into a large supercomplex in yeast and mammals (Steglich *et al.*, 1999, Metodiev, 2005). Prohibitins are multimeric complexes embedded in the mitochondrial inner membrane composed of the highly conserved subunits prohibitin 1 (Phb1) and prohibitin 2 (Phb2) (Steglich *et al.*, 1999, Nijtmans *et al.*, 2000, Artal-Sanz *et al.*, 2003). Deletion of prohibitins in yeast results in accelerated protein degradation by the *m*-AAA protease suggesting a function of prohibitins in regulation of *m*-AAA activity (Steglich *et al.*, 1999). However, neither the molecular mechanism of this regulation nor conservation of this regulatory function in mammals is known.

The main function of mitochodrial AAA/FtsH proteases is selective degradation of non-assembled, incompletely assembled and/or damaged membrane-anchored proteins. (Nolden *et al.*, 2005, Koppen and Langer, 2007). A limited number of substrates of the protein quality control have been identified for yeast and human i- and m-AAA proteases. Unassembled subunits of the respiratory chain complexes and non-native inner membrane proteins, which expose unfolded segments either to the inner membrane space or to the matrix, belong to this class of substrate proteins (Nakai *et al.*, 1995, Weber *et al.*, 1996). The *m*-AAA protease was shown to degrade a number of non-assembled mitochondrially encoded respiratory chain subunits of Complex II, IV and V and a peripheral membrane subunit of Complex V, ATP7 (Arlt *et al.*, 1996, Korbel *et al.*, 2004). Deletion of the *m*-AAA protease in yeast results in respiratory deficiency due to the loss of assembled respiratory chain and ATP synthase complexes (Arlt *et al.*, 1998, Galluhn and Langer, 2004). So far, only a few quality control substrates of the *i*-AAA protease have been identified. These include the

unassembled integral membrane proteins cytochrome *c* oxidase subunit 2 (Cox2), prohibitin 1 and prohibitin 2 as well as the soluble NADH dehydrogenase (Nde1) (Pearce and Sherman, 1995, Weber *et al.*, 1996, Kambacheld *et al.*, 2005). These substrates are completely degraded to oligopeptides, which are subsequently degraded to amino acids by mitochondrial oligopeptidases or released from mitochondria. No substrates of the protein quality control of the plant mitochondrial AAA proteases have been identified so far. However, it has been suggested that the oxidatively damaged mitochondrial proteins are substrates of the i-AAA proteases in *A. thaliana* mitochondria (Kicia *et al.*, 2010).

Apart from quality control executed by means of protein degradation, m-AAAs can also activate cellular regulatory proteins by proteolytic processing (Rape and Jentsch, 2004). m-AAA is required for maturation of mitochondrial ribosomal protein MrpL32, allowing its association with preassembled ribosomal particles and completion of ribosome assembly in close proximity to the inner membrane (Nolden *et al.*, 2005). Consequently, inactivation of m-AAA proteases causes mitochondrial-translation defect resulting in a reduced synthesis of mitochondria-encoded proteins, such as a proteins of respiratory chain. Genetic experiments demonstrated that growth defects associated with the loss of the m-AAA protease in yeast as well as in mouse are caused by impaired processing of MrpL32 which thus represents the central function of the m-AAA protease in mitochondria (Nolden *et al.*, 2005).

In generall, defects in genes encoding mitochondrial m-AAA proteases cause pleiotropic phenotype in various organisms. Mutations in different m-AAA protease subunits are associated with different neurodegenerative disorders in human. For instance, several loss-of-function in paraplegin cause autosomal recessive hereditary spastic paraplegia (HSP) complicated by optic, cerebellar and cerebral atrophy. Although muscle biopsies from severely affected patients revealed defects in mitochondrial oxidative phosphorylation (Casari *et al.*, 1998, McDermott *et al.*, 2001, Wilkinson *et al.*, 2004), the molecular basis of these phenotypes is still not fully understood and remains to be explored.

1.3.2.3 FtsH in photosynthetic membranes

Photoautotrophic organisms, as cyanobacteria and green plants, encode FtsH homologues targeted to the thylakoid membrane. Due to their localization these FtsH proteases are expected to be responsible for quality control of protein complexes required in photosynthesis (Lindahl *et al.*, 2000). Photosynthesis is a unique process that utilizes energy of photons to convert carbon dioxide into organic compounds with concomitant release of

oxygen as a very important side product (Whitmarsh and Govindjee, 1999). During the light-dependent phase of photosynthesis, energy of photons is transferred onto special chlorophyll (Chl) molecules located in photosystem II (PSII) and photosystem I (PSI), large pigment-protein complexes embedded within thylakoid membrane. Excitation of these pigments drive photosynthetic electron flow used for synthesis of ATP and reduced compounds. Due to unusual photochemical properties of PSII reaction center (RC) needed for extraction of electrons from water, PSII is permanently damaged by light and especially excessive light may limit growth by irreversibly inactivating PSII complexes causing phenomenon called photoinhibition of photosynthesis. Photochemical activity of PSII in light is maintained via so called repair cycle, which involves several steps resulting in the restoration of photochemical activity of the photodamaged PSII (Nixon *et al.*, 2005, Bailey *et al.*, 2002). The main target for photodamage in PSII is the RC D1 protein and its inactive form needs to be quickly replaced by a newly synthesized intact copy. This fast replacement is demonstrated by high turnover of the protein (Bottomle.W *et al.*, 1974) and FtsHs were shown to play key role in this replacement (Bailey et al., 2002, Silva *et al.*, 2003, Komenda *et al.*, 2007). Light-induced inactivation of PSII is accompanied by a signal, which triggers degradation of the damaged D1. Nature of this signal is not known but before the degradation, destabilization of the binding of the PSII antenna protein CP43 must also occur to allow access of the protease to the D1 protein. After the "old" D1 protein is replaced, a complete reassembly of PSII including re-binding of CP43 follows and finally the photochemical activity and oxygen evolution is activated (reviewed in Nixon *et al.*, 2010).

1.3.2.4 Plants

The nuclear genome of *Arabidopsis thaliana* contains 12 genes encoding FtsH proteins, eight of them are targeted exclusively to chloroplasts (AtFtsH1, 2, 5, 6, 7, 8, 9, 12) whereas other three are mitochondrial (AtFtsH 3, 4, and 10) (reviewed in Janska *et al.*, 2010). AtFtsH11 is double targeted to both organelles (Urantowka *et al.*, 2005). In addition, five *Arabidopsis* FtsH-like proteins (FtsHi1 to FtsHi5) targeted to the chloroplast envelope lack the conserved zinc binding motif HGXXH and are presumably inactive as protease (reviewed in Wagner *et al.*, 2012, see also Arabidopsis proteome databases - Ferro *et al.*, 2010). Eight genes encoding active chloroplast targeted FtsH homologues can be divided into four highly-conserved pairs (Sakamoto *et al.*, 2003, Sakamoto *et al.*, 2010) of which only four isomers FtsH1, 2, 5 and 8 accumulate in *Arabidopsis* leaves grown under optimal conditions. AtFtsH2

is by far the most abundant species and is followed by AtFtsH5, 8 and 1 (Sinvany-Villalobo *et al.*, 2004). Characterization of FtsH homologues in *Arabidopsis* indicates that they most probably form hetero-oligomeric complexes consisting of FtsH5/FtsH1- type A and FtsH2/FtsH8 - type B homologues (Yu *et al.*, 2004). AtFtsH2 and AtFtsH5 represent major isoforms of the hetero-complex, AtFtsH1 and AtFtsH8 appear to function as auxiliary minor components. The members of one type have sequence identity of about 90%, and can partly substitute each other in the complex. The identity between the members of type A and type B is close to 50%. (NCBI/Blast program). Depletion or mutation in genes encoding major isoforms causes the variegation phenotype typical by a combination of green sectors containing normal appearing chloroplast and white sectors containing abnormal plastids, that lack pigments and organized lamellae (Sakamoto *et al.*, 2002). The variegation phenotype implies that certain threshold level of FtsHs is necessary for proper chloroplast development, primarily in early stages of thylakoid formation (Yu *et al.*, 2005). Complementation of variegation phenotype with an inducible FtsH expression suggests that the overall FtsH level predominantly determines the chloroplast development when the protease activity is in excess (Sakamoto *et al.*, 2010). The threshold depends on the developmental stage and environmental conditions and the fate of cells is determined at an early stage of plastid development (Zaltsman *et al.*, 2005, Kato *et al.*, 2007). Several suppressor-of-variegation mutants were described to compensate for the lack of AtFtsH2 during early chloroplast biogenesis (Liu *et al.*, 2010b, Liu *et al.*, 2010a, Liu *et al.*, 2010c). Interestingly, most of these suppressors are involved in the coupled processes of chloroplast ribosomal RNA (rRNA) processing and translation. This has given rise to the hypothesis that reductions in chloroplast translation are able to compensate for a lack of AtFtsH2 by lowering the threshold demand for FtsH in the population of developing chloroplasts (Miura *et al.*, 2007, Liu *et al.*, 2010a). Interestingly, it was demonstrated that leaf variegation of plants lacking both Type B isomers (AtFtsH2, AtFtsH8) could be rescued by expressing a proteolytically inactive AtFtsH2, suggesting dispensability of protease activity of these isomers for proper chloroplast development (Zhang *et al.*, 2010). This results can imply that variegated phenotype is independent of proteolysis and only the chaperone function of the complex is responsible for the correct thylakoid formation (Wagner *et al.*, 2012). Except of variegation phenotype, inactivation of AtFtsH proteases also causes inhibition of the D1 turnover after PSII photodamage (Bailey *et al.*, 2002). Other substrates have also been reported for AtFtsH1, 2, 5 and 8, like Rieske protein of the pea cytochrome b6-f complex (Ostersetzer and Adam, 1997) and cytochrome b6 in *Chlamydomonas* (Malnoe *et al.*, 2014).

Four active FtsH homologues (AtFtsH7, 9, 11 and 12), and five inactive FtsH homologue were found in the *A. thaliana* chloroplast envelope membrane (Wagner *et al.*, 2012). Not much is known about the function of AtFtsH7, AtFtsH9 and AtFtsH12. AtFtsH7 and AtFtsH9 derived from recent gene duplication (Garcia-Lorenzo *et al.*, 2006), therefore it seems likely that these proteases have similar functions and they even might form a complex (Wagner *et al.*, 2012). Single deletion mutants of both proteases do not cause any phenotype. Based on co-expression studies, the existence of other complex consisting of AtFtsH12 and one of the inactive FtsHs (FtsHi) is speculated (Wagner *et al.*, 2012). The oligomeric state of the remaining chloroplast FtsH proteases in *A. thaliana*, AtFtsH11 and AtFtsH6, as well as the thylakoid location and function is unknown for these proteases. Deletion of corresponding genes resulted in no significant phenotype (Wagner *et al.*, 2011). Sequence analysis showed that AtFtsH6 homologues also exist in other plant species (Garcia-Lorenzo *et al.*, 2006). This evolutionary conservation indicates that FtsH6 most probably play an important, but so far unknown role in the chloroplast.

1.3.2.5 Cyanobacteria

Unlike other gram-negative bacteria, cyanobacteria encode more than one, usually four, FtsH homologues that are located both in plasma and thylakoid membrane. The most intensive studies of the cyanobacterial FtsH have been performed on cyanobacterium *Synechocystis* PCC 6803. The genome of *Synechocystis* encodes four FtsH homologues designated FtsH1-4 (corresponding genes *slr1390, slr0228, slr1603 and sll1463,* respectively). FtsH1 and FtsH3 are crucial for cell viability, so it is not possible to delete their genes. In contrast, FtsH2 and FtsH4 are dispensable proteins. Deletion of FtsH4 have not resulted in any apparent phenotypic change of the mutant grown under standard conditions (Mann *et al.*, 2000). On the other hand, inactivation of FtsH2 protease have induced very significant phenotypical changes in the mutant like a decrease in the level of PSI and related lower chlorophyll content (Mann *et al.*, 2000) and non-functional PSII repair reflected by inhibited D1 turnover and high light sensitivity (Mann *et al.*, 2000, Silva *et al.*, 2003). For that reason, great effort has been devoted to further studies of the FtsH2 role in this organism. The obtained results confirmed that the most relevant physiological function of the FtsH2 homologue is related to the above mentioned degradation of the D1 protein during PSII repair cycle in vivo. Using several *Synechocystis* mutants with the truncated N-terminus of D1, a model for FtsH mediated D1 degradation has been developed (Komenda *et al*., 2007).

This model assumes that the PSII photodamage leads to conformational changes within PSII which allow better access of FtsH to the N-terminus of D1. In agreement with the mechanism described above for *E. coli* (Chiba *et al.*, 2000), interaction of FtsH with the sufficiently long N-terminus initiates the degradation process which then occurs processively up to the C-terminus of D1. Indeed, truncation of its N-terminus by 20 aa residues inhibits the D1 degradation (Komenda *et al*., 2007). The effect of the FtsH2 absence on the accumulation and degradation of PSII proteins was analyzed in WT and also in PSII mutants that are not able to assemble the functional PSII complex. In these mutants the process is stopped at a certain stage of the assembly and cannot proceed further due to a specific mutation. In these strains PSII subunits are typically inserted into the membrane and can be detected either in the unassembled state or are assembled into assembly intermediate sub-complexes, which are usually rapidly degraded and fail to accumulate to appreciable levels (Vermaas *et al.*, 1988). The results showed that FtsH2 is needed for the removal of both unassembled PSII subunits and non-functional partially assembled PSII sub-complexes (Vermaas *et al.*, 1988, Komenda *et al.*, 2006).

Apart from the function in PSII repair-related D1 degradation and quality control of other PSII membrane proteins, characterization of FtsH2-less mutant also showed that the protease might be involved in acclimation of the cell to various stress conditions. During salt stress, it controls the level of glucosylglycerol phosphate synthase involved in osmoregulation (Stirnberg *et al.*, 2007). FtsH2 is also involved in acclimation to low carbon conditions via regulation of the level of NdhR which is a transcriptional repressor controlling transcription of genes required for induction of carbon-concentrating mechanisms (Zhang *et al.*, 2007a). Unlike in WT, in the FtsH2 protease mutant the CCM complexes like NDH dehydrogenase, SbtA and CmpA, all inducible by low CO_2 concentration, were not induced upon shift to low CO_2. Also the transcripts of the inducible CCM genes and their regulator *ndhR* failed to accumulate upon shift of FtsH2 mutant cells from high to low CO_2, indicating that the regulation by the FtsH2 protease is upstream of NdhR (Zhang *et al.*, 2007a, Zhang *et al.*, 2007b). We assume that the above mentioned list of various processes in which FtsH2 participates is still incomplete, and that not only FtsH2 but also other not yet studied homologues may be implicated in regulating other cellular processes.

Interestingly, recent research indicated a large increase in the levels of FtsH1, FtsH2 and FtsH3 upon iron depletion in *Synechocystis* (Wegener *et al.*, 2010). For cyanobacteria, iron belongs to one of the essential elements because it is a constituent of many proteins involved in photosynthetic electron transport chain. In spite of the fact that iron is very

abundant element on the Earth, the iron limitation is very common phenomenon due to highly oxidizing environment. High concentration of oxygen in the atmosphere causes oxidation of soluble ferrous ions to ferric ones, which are nearly insoluble under normal conditions (Breitbarth *et al.*, 2010). While iron limitation has obvious consequences, bacteria also have to maintain a tight control over iron acquisition because free intracellular iron can react with superoxide and hydrogen peroxide forming highly reactive hydroxyl radicals (Guerinot and Yi, 1994). Thus, cyanobacterial iron homeostasis is tightly controlled, both at the protein level through incorporation of free metal ions into metallo-proteins (Lewin *et al.*, 2005) and more importantly at the transcriptional level through the regulated expression of genes encoding metal trafficking proteins, including uptake and storage proteins (Ghassemian and Straus, 1996, Hernandez-Prieto *et al.*, 2012).

This mechanism is mediated by transcriptional factor Fur, ferric uptake regulator. (Escolar *et al.*, 1999, Gonzalez *et al.*, 2012). Fur protein binds iron as a cofactor under iron sufficiency which enhances its affinity for AT-rich DNA elements termed Fur-boxes, present in the core promoters of iron-regulated genes (Escolar *et al.*, 1997, Yu and Genco, 2012). Binding to DNA causes either repression or activation of DNA transcription which slows down the process of iron uptake. On the contrary, under iron limitation iron-free Fur (apo-Fur) has a reduced affinity for the Fur-boxes allowing the RNA polymerase to initiate transcription of genes encoding metal-trafficking proteins and hence iron uptake. This regulation responds rapidly to change in environmental conditions and allows maintaining stable concentration of intracellular iron (Hernandez *et al.*, 2006, Yu and Genco, 2012). Whether the increase in the level of FtsHs under iron depletion could indicate involvement of FtsHs in the acclimation to iron deficiency remains an interesting topic for further research.

The current information about localization, architecture and composition of FtsH complexes in *Synechocystis* is also spare. The preliminary data showed that FtsH2, as well as FtsH4, are localized in the thylakoid membrane (Komenda *et al.*, 2006). The location of essential FtsH3 and FtsH1 homologues also remains to be clearly specified. Previous investigations based on mass spectrometric analysis of cytoplasmic and thylakoid membranes purified by a combination of sucrose-gradient centrifugation and two-phase separation (Huang *et al.*, 2002) indicated that FtsH3 is present in both membranes while FtsH1 was found just in the cytoplasmic membrane fraction (Pisareva *et al.*, 2007, Pisareva *et al.*, 2011). On the other hand, the architecture and composition of high-molecular FtsH complexes in *Synechocystis* as well as the function of the three FtsH homologues has been

completely unknown. However, FtsHs appear to be important players in diverse cellular processes and therefore they provided a valuable object for our research.

2. Summary

2.1 Summary

FtsHs/m-AAAs are transmembrane metalloproteases universally conserved in bacteria, chloroplasts and mitochondria, having an essential function in protein turnover and processing, and maintaining cellular homeostasis. In many bacteria, they are crucial for cell viability. In various eukaryotic organisms, defects in genes encoding mitochondrial FtsH proteases cause pleiotropic phenotype including morphology defects and respiratory deficiencies. In humans, these defects are especially associated with different neurodegenerative disorders. In plants, FtsHs are necessary for proper chloroplast development, primarily in early stages of thylakoid formation. They are also key players in ongoing photosynthesis, specifically in quality control of Photosystem II in many phototrophic organisms. Some molecular mechanisms, by which FtsHs control other cellular processes like response to stress conditions, remain to be clarified.

My thesis was dedicated to the research of cellular function of FtsH proteases in the model cyanobacterium *Synechocystis* sp. PCC 6803. As other cyanobacteria, this strain contains four FtsH homologues, FtsH1-FtsH4. Most of the previous studies in *Synechocystis* were focused on the FtsH2 homologue playing an important role in quality control of PSII during photosynthesis (Komenda et al., 2006). However, the mechanism of PSII quality control as well as a structural arrangement of the FtsH2 in a complex was still unclear. It was also unknown whether the FtsH2 forms a homo-oligomeric complex or, more probably, sets up hetero-oligomeric complexes with other FtsH homologues as proposed for chloroplasts. Moreover, the information on the remaining homologues was completely missing. The aim of my thesis was to clarify structural relationship between the FtsH homologues and to elucidate their localization and function in the cells of *Synechocystis*.

The first publication specifies in more detail the role of FtsH2 in quality control of PSII. It focuses on the analysis of the synthesis, assembly and degradation of PSII proteins in mutants of *Synechocystis* either lacking the important extrinsic oxygen-evolving enhancer proteins or unable to completely process the precursor of the D1 protein. The lack of the extrinsic proteins accelerated the turnover of the D1 protein and decreased the accumulation of PSII complexes in the mutant cells. Additional inactivation of the ftsH2 gene resulted in a drastic slowing down in the degradation of D1 and other PSII proteins and an increase in the level of PSII complexes. Overall we concluded that FtsH2 plays a major role in the degradation of D1 assembled into larger core complexes during donor-side photoinhibition. However, in mutants blocked at an early stage of PSII assembly,

unassembled D1 protein was still efficiently degraded in the absence of FtsH2 pointing to the involvement of other protease(s).

The second article clarifies structural relationship between the FtsH homologues including architecture and composition of the FtsH complexes in vivo. We analyzed the composition of FtsH complexes using 2D electrophoretic protein analysis of membranes isolated from various FtsH mutants in combination with immunoblotting using FtsH-specific antibodies. Moreover, we employed GST-tagged derivatives of the FtsH homologues to purify and analyze their native complexes. The existence of three oligomeric complexes in vivo, one homo-oligomeric and two hetero-oligomeric complexes, was revealed. We focused namely on the study of the FtsH2/FtsH3 hetero-oligomeric complex. It was purified using GST-tagged FtsH2 and was subjected to single particle analysis which confirmed its hexameric architecture. Finally, using a mutant limited in expression of the FtsH3 homologue, we demonstrated the role of FtsH3 in D1 turnover. We concluded that the FtsH2/FtsH3 hetero-complex is responsible for the quality control of PSII, the role previously attributed only to the FtsH2 homologue.

In the third article we studied the role of the second hetero-oligomeric complex existing in *Synechocystis* in vivo, the essential FtsH1/FtsH3 complex. As both FtsH1 and FtsH3 are indispensable for cell viability, inactivation of their genes and subsequent characterization of resulting null mutants was not possible. To overcome this problem, we prepared conditional knock-down mutants, in which we were able to down-regulate the expression of desired proteases. As the mutants conditionally depleted in FtsH1 or FtsH3 were unable to induce normal expression of the IsiA chlorophyll-protein and FutA1 iron transporter upon iron deficiency we focused on the role of FtsH1/FtsH3 complex in the response of *Synechocystis* cells to iron deficiency and in maintaining iron homeostasis. We demonstrated that FtsH1/FtsH3 complex regulates transcription of iron depletion-induced genes by controlling the level of the transcriptional regulator Fur (Sll0567). We also analyzed location of various FtsH homologues in cells expressing GFP (green fluorescent protein) - tagged versions of FtsHs by the assessment of GFP fluorescence (in comparison with chlorophyll fluorescence) using confocal microscopy. This analysis confirmed location of both FtsH1 and FtsH3 in the cytoplasmic membrane. Our preliminary results obtained from genome-wide expression profiling and two-dimensional protein analyses of mutants conditionally depleted in FtsH1 and/or FtsH3 show that the FtsH1/FtsH3 complex could also be implicated in the acclimation of cells to phosphate stress as well as in the transcriptional regulation of the level of some house-keeping proteins like ribosomal proteins (unpublished

results). We assume that by controlling the level of certain transcriptional factors the FtsH1/FtsH3 complex works as a transcriptional supervisor, which is crucial for cellular homeostasis and response of the organism to various environmental stresses.

The last study included in my thesis explores the mechanism of substrate recognition in PSII quality control. Our experiments employed *Synechocystis* mutants blocked at specific stages of PSII assembly incubated in the dark to prevent protein photodamage and in the presence of lincomycin to prevent possible protein resynthesis. We concluded that the principal prerequisite for the recognition of D1 and D2 proteins by protease is their accessibility induced by incomplete PSII assembly or its partial disassembly and to the protein damage itself. We suggest that damaged D1 is recognised and selectively degraded because of partial or complete detachment of CP43 from damaged PSII complexes which thereby directs the proteolytic machinery towards D1 rather than D2 degradation. Using FtsH2/FtsH3-lacking versions of the employed mutants we also confirmed the crucial role of this protease complex in the dark PSII degradation.

3. Results

3.1 Published results

3.1.1 Role of FtsH2 in the repair of photosystem II in mutants of the cyanobacterium *Synechocystis* PCC 6803 with impaired assembly or stability of the CaMn(4) cluster.

Reprint of: Komenda, J., Knoppová, J., Krynická, V., Nixon, P.J., and Tichý, M. (2010). Biochimica et Biophysica Acta 1797: 566–575.

Abstract:

The FtsH2 protease, encoded by the *slr0228* gene, plays a key role in the selective degradation of photodamaged D1 protein during the repair of Photosystem II (PSII) in the cyanobacterium *Synechocystis* sp. PCC 6803. To test whether additional proteases might be involved in D1 degradation during high rates of photodamage, we have studied the synthesis and degradation of the D1 protein in ΔPsbO and ΔPsbV mutants, in which the CaMn4 cluster catalyzing oxygen evolution is less stable, and in the D1 processing mutants, D1-S345P and ΔCtpA, which are unable to assemble a functional cluster. All four mutants exhibiteda dramatically increased rate of D1 degradation in high light compared to the wild-type. Additional inactivation of the ftsH2 gene slowed the rate of D1 degradation dramatically and increased the level of PSII complexes. We conclude that FtsH2 plays a major role in the degradation of both precursor and matureforms of D1 following donor-side photoinhibition. However, this conclusion concerned only D1 assembled into larger complexes containing at least D2 and CP47. In the ΔpsbEFLJ deletion mutant blocked at an early stage in PSII assembly, unassembled D1 protein was efficiently degraded in the absence of FtsH2 pointing to the involvement of other protease(s). Significantly, the ΔPsbO mutant displayed unusually low levels of cellular chlorophyll at extremely low-light intensities. The possibilities that PSII repair may limit the availability of chlorophyll for the biogenesis of other chlorophyll-binding proteins and that PsbO might have a regulatory role in PSII repair are discussed.

3.1.2 Subunit organization of a *Synechocystis* hetero-oligomeric thylakoid FtsH complex involved in Photosystem II repair.

Reprint of: Boehm, M., Yu, J., Krynicka, V., Barker, M., Tichy, M.,Komenda, J., Nixon, P.J, and Nield, J. (2012). Plant Cell 24: 3669–3683

Abstract:

FtsH metalloproteases are key components of the photosystem II (PSII) repair cycle, which operates to maintain photosynthetic activity in the light. Despite their physiological importance, the structure and subunit composition of thylakoid FtsH complexes remain uncertain. Mutagenesis has previously revealed that the four FtsH homologs encoded by the cyanobacterium *Synechocystis* sp PCC 6803 are functionally different: FtsH1 and FtsH3 are required for cell viability, whereas FtsH2 and FtsH4 are dispensable. To gain insights into FtsH2, which is involved in selective D1 protein degradation during PSII repair, we used a strain of *Synechocystis* 6803 expressing a glutathione S-transferase (GST)–tagged derivative (FtsH2-GST) to isolate FtsH2-containing complexes. Biochemical analysis revealed that FtsH2-GST forms a hetero-oligomeric complex with FtsH3. FtsH2 also interacts with FtsH3 in the wild-type strain, and a mutant depleted in FtsH3, like ftsH22 mutants, displays impaired D1 degradation. FtsH3 also forms a separate heterocomplex with FtsH1, thus explaining why FtsH3 is more important than FtsH2 for cell viability.We investigated the structure of the isolated FtsH2-GST/FtsH3 complex using transmission electron microscopy and single-particle analysis. The three-dimensional structural model obtained at a resolution of 26 Å revealed that the complex is hexameric and consists of alternating FtsH2/FtsH3 subunits.

3.1.3 Two essential FtsH proteases control the level of the Fur repressor during iron deficiency in the cyanobacterium *Synechocystis* sp. PCC 6803.

Reprint of: Krynická, V., Tichy, M., Krafl, J., Yu, J., Kaňa, R., Boehm, M., Nixon, P.J., and Komenda, J. (2014). Molecular Microbiology 94: 609–624

Abstract:

The cyanobacterium *Synechocystis* sp. PCC 6803 expresses four different FtsH protease subunits (FtsH1-4) that assemble into specific homo- and heterocomplexes. The FtsH2/FtsH3 complex is involved in photoprotection but the physiological roles of the other complexes, notably the essential FtsH1/FtsH3 complex, remain unclear. Here we show that the FtsH1 and FtsH3 proteases are involved in the acclimation of cells to iron deficiency. A mutant conditionally depleted in FtsH3 was unable to induce normal expression of the IsiA chlorophyll-protein and FutA1 iron transporter upon iron deficiency due to a block in transcription, which is regulated by the Fur transcriptional repressor. Levels of Fur declined in the WT and the FtsH2 null mutant upon iron depletion but not in the FtsH3 downregulated strain.Asimilar stabilizing effect on Fur was also observed in a mutant conditionally depleted in the FtsH1 subunit. Moreover, a mutant overexpressing FtsH1 showed reduced levels of Fur and enhanced accumulation of both IsiA and FutA1 even under iron sufficiency. Analysis of GFP-tagged derivatives and biochemical fractionation supported a common location for FtsH1 and FtsH3 in the cytoplasmic membrane. Overall we propose that degradation of the Fur repressor mediated by the FtsH1/FtsH3 heterocomplex is critical for acclimation to iron depletion.

3.2 Unpublished results

3.2.1 Accessibility controls selective degradation of photosystem II subunits by FtsH protease.

Krynická, V., Shao, S., Nixon, P.J., Komenda, J, manuscript accepted in Nature Plants

Abstract:

The oxygen-evolving photosystem II (PSII) complex located in chloroplasts and cyanobacteria is sensitive to light-induced damage which unless repaired causes reduction in photosynthetic capacity and growth. Although a potential target for crop improvement, the mechanism of PSII repair remains unclear. The D1 reaction center protein is the main target for photodamage, with repair involving the selective degradation of the damaged protein by FtsH protease. How a single damaged PSII subunit is recognised for replacement is unknown. Here, we have tested dark stability of PSII subunits in strains of the cyanobacterium *Synechocystis* PCC 6803 blocked at specific stages of assembly. We have found that when D1, which is normally shielded by the CP43 subunit, becomes exposed in a photochemically active PSII complex lacking CP43, it is selectively degraded by FtsH even in the dark. Removal of the CP47 subunit, which increases accessibility of FtsH to the D2 subunit, induced dark degradation of D2 at a faster rate than that of D1. In contrast CP47 and CP43 are resistant to degradation in the dark. Our results indicate that protease accessibility induced by PSII disassembly is an important determinant in the selection of the D1 and D2 subunits to be degraded by FtsH.

4. Conclusions

4.1 Conclusions

This PhD thesis contributed to characterization of the FtsH complexes present in the cyanobacterium *Synechocystis* sp. PCC 6803. It elucidates the localization and composition of these complexes and further specifies their role. The thesis consists of one first-author publication, two co-author publications and one first-author manuscript accepted for publication. The main conclusions are as follows:

- One homo-oligomeric complex consisting of FtsH4 and two hetero-oligomeric complexes, FtsH2/FtsH3 and FtsH1/FtsH3, exist in *Synechocystis* PCC 6803 (Boehm *et al.*, 2012, Krynicka *et al*., 2014). Although the existence of any other hetero-oligomeric or homo-oligomeric complex was not excluded completely, we did not obtain any experimental support for this possibility.

- The most abundant FtsH2/FtsH3 complex is located in the thylakoid membranes, whereas the less abundant, essential FtsH1/FtsH3 is located in plasma membrane (Krynicka *et al*., 2014). The FtsH4 homo-oligomeric complex is located in well-defined spots within the thylakoid membrane (unpublished data).

- The FtsH2/FtsH3 complex is a hexamer consisting of regularly alternating FtsH2 and FtsH3 subunits. This holoenzyme is responsible for the quality control of PSII previously ascribed just to FtsH2 (Boehm *et al*., 2012). As there is no evidence that FtsH2 subunit is assembled in other functional complex in vivo, we suggest that FtsH2/FtsH3 hetero-oligomer is responsible also for other functions previously attributed to FtsH2 (Fig. 4).

- FtsH2/FtsH3 complex plays a major role in the degradation of both precursor and mature forms of D1 assembled into larger complexes containing at least D2 and CP47 (Komenda *et al*., 2010). However, the unassembled D1 forms present in the cell of the D2-less strain are still quickly degraded even in the absence of the FtsH2/FtsH3 complex suggesting the role of additional protease. However, the photodamage of the D1 and D2 subunits within PSII is not necessary for their recognition by FtsH complex. When they become accessible to the protease, both subunits can be degraded in the dark even in a photochemically active PSII complex. It means that accessibility of D1 and D2 determined by PSII conformation and/or partial disassembly, is an important determinant in the selection of these subunits for degradation by FtsH. Unlike D1 and D2, the CP47 and CP43 subunits of PSII are resistant to degradation in the dark.

- FtsH1/FtsH3 is involved in the acclimation of cells to iron deficiency. It regulates transcription of iron depletion-induced genes by controlling the level of the transcriptional regulator Fur (Sll0567) (Fig. 5) (Krynicka *et al*., 2014).

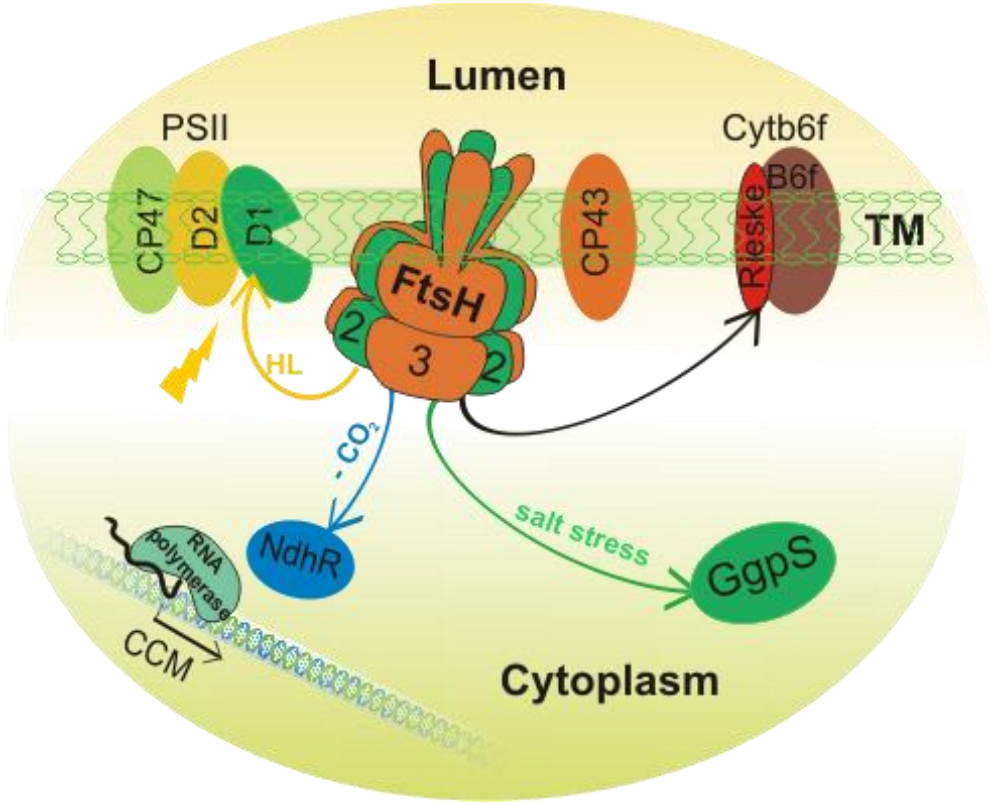

Fig. 4. Scheme of FtsH2/FtsH3 complex and its role in the cell. TM, thylakoid membrane; PSII, photosystem II; CCM, carbon concentrating mechanism; -CO_2, CO_2 limitation; HL, high light, Cytb6f, cytochrome b6f complex; Rieske - Rieske complex.

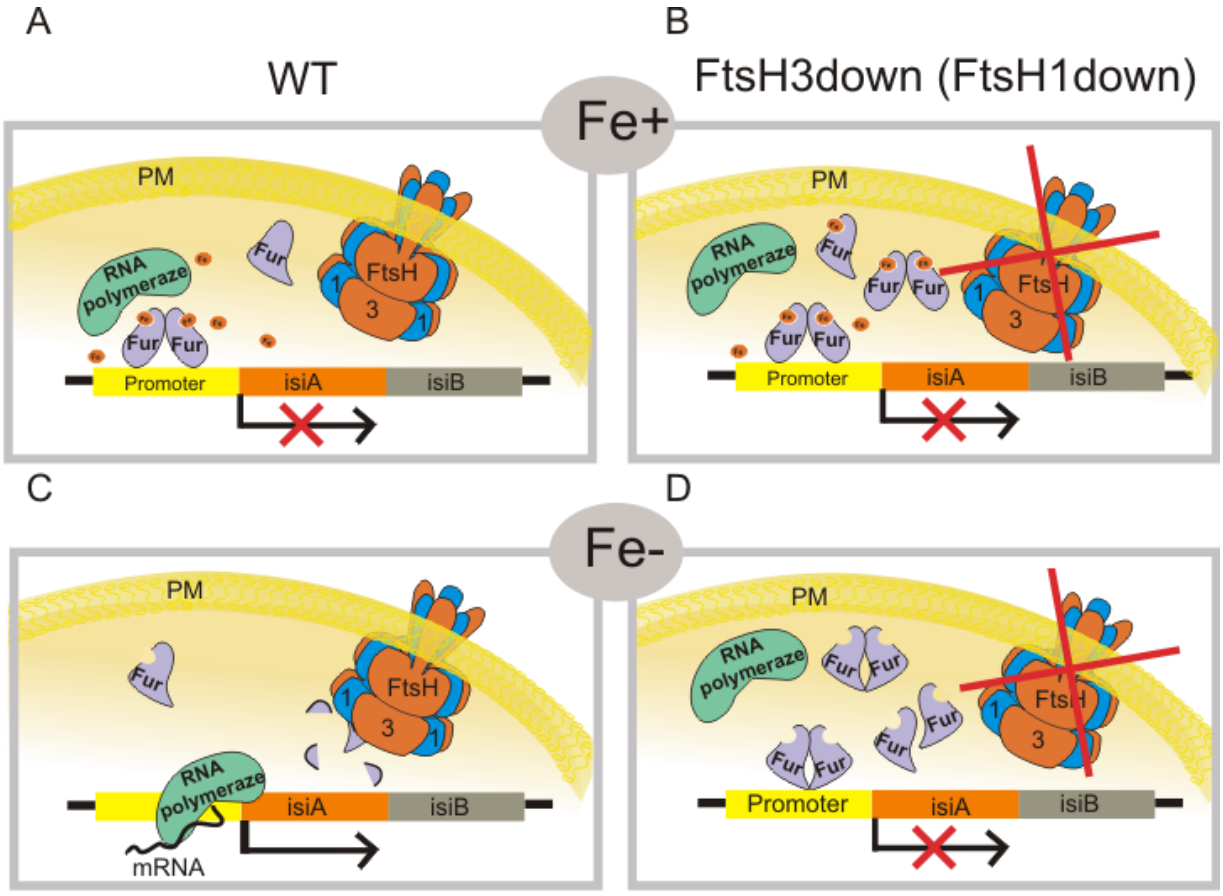

Fig. 5. Model for regulation of *isiA /isiB* expression by FtsH1/FtsH3 complex via controlling the level of apo-Fur. A, B: Repression of *isiA/isiB* transcription under iron sufficiency by binding of Fur-Fe dimer to promoter in WT (A) and in FtsH3down and/or FtsH1down, mutants limited in the level of FtsH3 and/or FtsH1proteases, respectively (B). C: Trancription of *isiA/isiB* under iron deficiency in WT. Fur loses iron and resulting apo-Fur with lower affinity to DNA is released to cytoplasm, where it is degraded by FtsH1/FtsH3. Concurrently, RNA polymerase binding site is not blocked and *isiA/isiB* is transcribed. D: Repression of *isiA/isiB* transcription under iron deficiency in mutants. Fur loses iron, becomes released to cytoplasm where it is accumulated due to missing FtsH1/FtsH3 complex. Accumulated apo-Fur can dimerize even without iron and may, although with lower affinity, bind to promoter inhibiting transcription of *isiA/isiB* gene by RNA polymerase.

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