



## LABORATORIES

# THE PAST AND PRESENCE OF PROTEIN CRYSTALLOGRAPHY AT THE INSTITUTE OF MOLECULAR BIOLOGY OF THE SLOVAK ACADEMY OF SCIENCES

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### Abstract

For years, the Institute of Molecular Biology of the Slovak Academy of Sciences has been involved in the leading trends of molecular biology, including genomics and proteomics, especially in the sequencing, cloning and expression of genes as well as in the isolation and purification of proteins using biochemical methods. The proteins studied here have been used as research models and some of them were also used in industrial applications. To better understand the structure-function relationship of these proteins we focused our attention on protein crystallography. The Institute of Molecular Biology was the first institution in the former Czechoslovakia that used the methods of protein crystallization and structure determination. Ribonuclease from the microbial strain *Streptomyces aureofaciens*, determined in 1988, was the first protein structure from the Czechoslovak Republic. Several of the laboratories of this Institute are focused on protein crystallography, making it the leading Slovak institution in structural biology.

### Laboratory of protein crystallography (head Ing. Jozef Ševčík, DrSc.)

Approximately in 1980 I read one of the earliest papers dealing with the crystallization of proteins. Because of my original education in electronics I had been working primarily in the sphere of instrumentation. However, having worked in a biological environment, the crystallization of proteins became a challenge for me. I started with crystallization experiments, initially secretly, with a few commercially available proteins, obviously not overlooking lysozyme. At that time I had no experience with protein crystallization and the interest of the Czechoslovak scientific community in the determination of the tertiary structures of proteins was minimal, a circumstance which certainly did not create an encouraging atmosphere. What was encouraging it was the appearance of some protein crystals, which paved the way for the crystallization of a guanylspecific ribonuclease from *Streptomyces aureofaciens* (RNase Sa), which was discovered and well-characterized at our Institute [1, 2, 3]. Although the isolation of this protein was very difficult (from 50 litres of fermentation media, only ~4 mg of protein could be obtained), we were able to prepare the enzyme in high purity and in sufficient quality for crystallization experiments [4]. After

countless crystallization experiments in Petri dishes without present-day equipment and knowledge, large, beautiful, high-quality crystals of RNase Sa finally appeared (Fig. 1). These results were appreciated by Prof. Zelinka, the director of the Institute, who provided funds for the purchase of the only equipment available at that time: a Mikrometa 2 X-ray generator with a sealed 1.2 kW tube and a precession camera, which was constructed by Dr. Hanic at the Institute of Chemistry, SAS. Given the high quality and large size of the RNase Sa crystals I was able to get X-ray diffraction patterns on a film (Fig. 2) in spite of the very low power of the generator. These patterns were used to determine the essential characteristics of the native crystals and to verify the quality of the isomorphous crystals. Heavy atom substitution was clearly seen in the differences in intensities as shown for the mersalyl (Hg) derivative (Fig. 3). At this stage our capabilities in the process of structure determination had been exhausted.

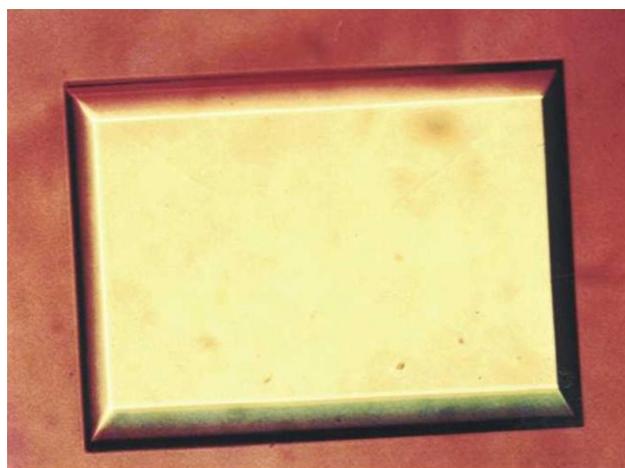


Figure 1. RNase Sa crystal (1.2 x 0.8 x 0.5 mm).

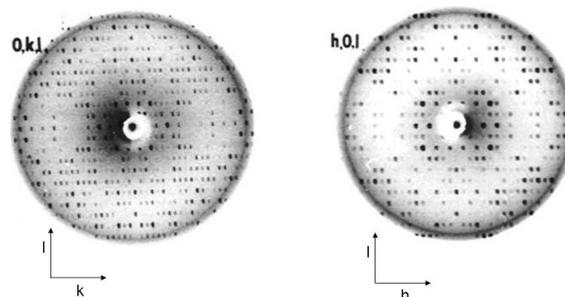
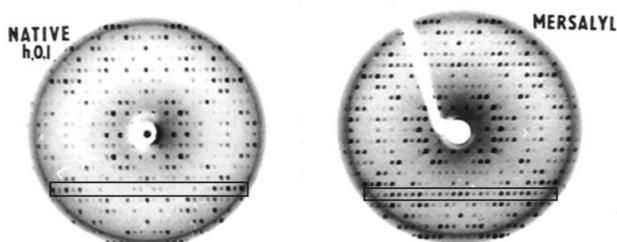


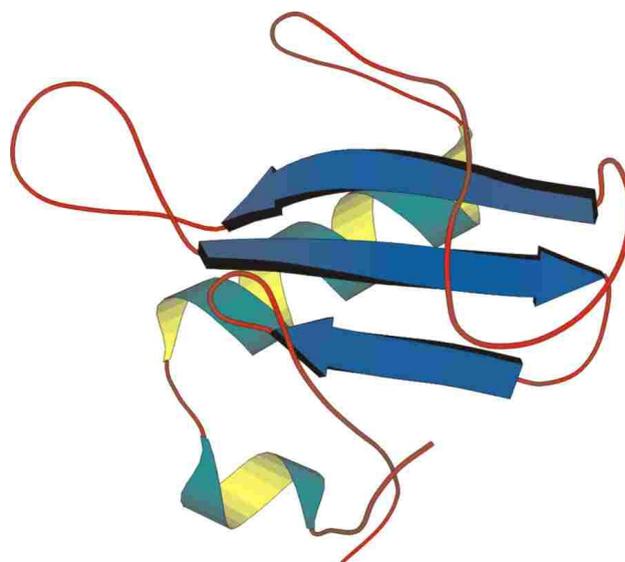
Figure 2. Precession photographs taken from RNase Sa crystals.



**Figure 3.** Intensities from native and isomorphous crystals containing mersalyl (Hg). Differences in intensities are clearly visible in the boxes.

By a coincidence of favourable circumstances, the well-known structural biologist Prof. David Phillips from Oxford University visited a number of biological institutions in Czechoslovakia, including our Institute. He appreciated our results and recommended us to contact Prof. G. Dodson at the University of York for cooperation. The response was favorable; I received funding from the British Council for two short visits to Prof. Dodson's laboratory. The diffraction power of the RNase Sa crystals, as well as those of its complex with guanosine-3'-monophosphate (3'-GMP) and the isomorphous crystals were tested on a Hilgert-Watts diffractometer. The results confirmed the quality of the crystals and resulted in my stay at the University of York for one year. Diffraction data from crystals of RNase Sa and a complex with 3'-GMP were measured using synchrotron radiation at Daresbury laboratory with a resolution of 1.8 Å. One set of data with 1.8 Å resolution contained about 270 films. We also collected data at 1.2 Å resolution, but these were not used owing to a lack of time. Data from three isomorphous crystals containing Hg, Pt and I were collected using a laboratory source with an Arndt-Wonacott rotation camera. The measurement of intensities using Joyce-Loebel Scandig 3 densitometer was a very time consuming process and required a large amount of computer memory, which was not available at that time, therefore we had to use magnetic tapes. The positions of the heavy atoms were determined by direct methods using MULTAN [5]. They were also identified using the Patterson function. Electron density maps were plotted on translucent plastic sheets and the polypeptide chains were constructed by manually connecting electron density maxima. The coordinates of about 50 identifying positions in the backbone of each molecule were recorded and used to label the backbone when displaying the map on an Evans & Sutherland PS300 graphics system using the program FRODO [6]). The atomic positions were refined by the stereochemically restrained least-squares method [7]. The structures of RNase Sa (Fig. 4) and its complex with 3'-GMP were completed in 1988 [8, 9]. The coordinate file was deposited with the PDB as the 589<sup>th</sup> structure. This was followed by structures of a complex with the cyclic nucleotide guanosine 2',3'-cyclophosphorothioate [10], a complex with guanosine-2'-monophosphate [11] and the atomic resolution structure of RNase Sa [12].

Based on these results and on the possibility of implementing structural biology in our country, our application for a grant from the Howard Hughes Medical Institute

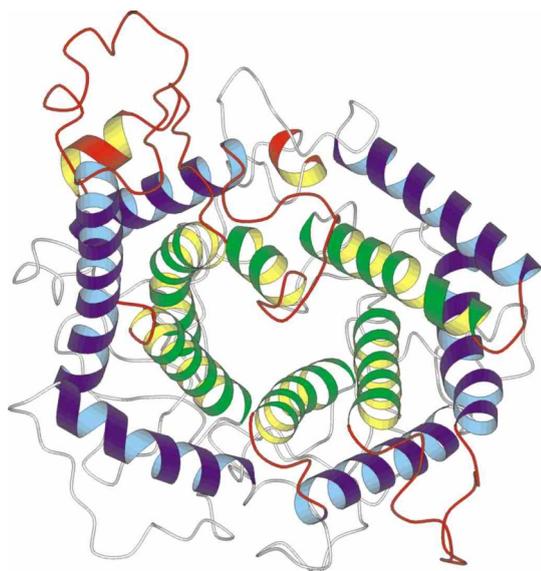


**Figure 4.** Structure of RNase Sa (96 amino acids).

(1995) in cooperation with Prof. Wilson was successful. The money from the grant helped us to create a protein crystallography laboratory and to become independent from foreign laboratories by purchasing what was at the time a top computer graphics station (Silicon Graphics). Since then, we have been able to carry out all activities related to structural work except for data collection, which still requires the use of foreign facilities.

Later, RNases Sa2 and Sa3 from *Streptomyces aureofaciens* were also isolated and purified. The properties of these proteins (small size, similar function, high stability, high production of recombinant products) have made them the object of intense structural studies in our laboratory and in the laboratories of our co-workers (EMBL Hamburg, Texas A&M University, University of Wisconsin) for more than two decades. These enzymes are highly specific endoribonucleases which hydrolyse the phosphodiester bond of a single-stranded RNA at the 3'-side of guanosine nucleotides [2, 13]. They have been invaluable in shedding light on the mechanism of catalytic action [8, 10, 14, 15, 16, 17], molecular recognition [18], the conformational stability and folding of proteins [13, 19, 20, 21, 22], cytotoxicity [23] and they have also provided solid evidence of the flexibility of protein molecules [24, 25, 26, 27]. Some structures of RNase Sa have been solved at truly atomic resolution, 0.85 Å.

Another class of proteins that has become the subject of structural research are hydrolytic enzymes and enzymes active on carbohydrates. The most thoroughly studied so far is glucoamylase from *Saccharomycopsis fibuligera*. This enzyme was isolated and crystallized in the laboratory of protein chemistry. The structure of this enzyme was determined at 1.8 Å resolution [28], and it was the first report of the three-dimensional structure of a yeast family 15 glucoamylase. The enzyme has the fold of an ( $\alpha/\beta$ )<sub>6</sub> barrel, which is composed of six antiparallel helices (Fig. 5). Later the resolution was extended to 1.1 Å and the structure of a complex with acarbose [29] was solved. The structure of the complex revealed the presence of the raw starch binding site directly on the catalytic domain. It is the only en-



**Figure 5.** Structure of glucoamylase (490 amino acids).

zyme so far known that does not contain a separate raw starch binding site domain.

We have also been involved in a NASA project for the crystallization of proteins in a microgravity environment. One of our proteins, glucoamylase, was crystallized in the space shuttles Columbia and Atlantis in 1988, Fig. 6. Before the flight the crystal growth conditions were optimized in ground-controlled experiments with the prototype hardware for the laboratory. The crystallization experiments in the shuttle were successful.

In cooperation with Mendel University in Brno we have also studied the structure-function relationship of  $\alpha$ -1,4-glucosidase from *Zea mays*. The enzyme has a  $(\alpha/)_8$  barrel structure and according to the CAZy classification belongs to the glycoside hydrolases family 30. Because the structure of the enzyme with a free active site is known [30], we focused on structural studies of mutants and complexes with flavonoid inhibitors. At present, five structures of enzyme-ligand complexes at 1.6-2.5 Å resolution are being finished.

The structure-function relationship and molecular recognition are also the main topics of the study of xylanase A from the plant pathogen *Erwinia chrysanthemi* being done in cooperation with the Chemical Institute, SAS in Bratislava. This enzyme belongs to the glycoside hydrolases family 1 and its structure consists of a catalytic domain with a  $(\alpha/)_8$  barrel structure and an additional domain with an immunoglobulin like structure [31]. The structure of a complex with the product of the enzymatic reaction was determined at 1.4 Å resolution [48].

We have also recently begun to cooperate with the Biological Research Center, HAS, Szeged on the structural study of enzymes taking part in the photosynthesis of purple sulphur bacteria. Crystals of the first enzyme under study, flavocytochrome c4, have been obtained.

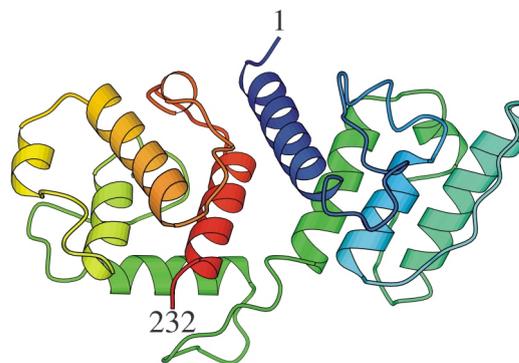
In the last decade we have focused primarily on proteins that are important in human medicine: plectin, tau protein, the ryanodine receptor and the vascular endothelial growth factor receptor.



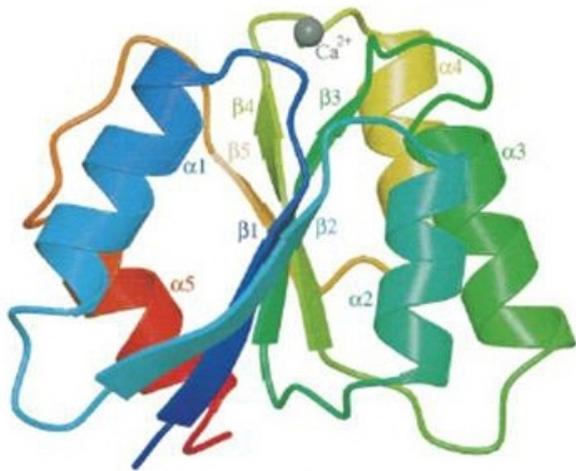
**Figure 6.** Poster showing our participation in the NASA project.

In collaboration with the University of Vienna, we were involved in the study of plectin. It is a widely expressed cytolinker protein, composed of several subdomains that harbor binding sites for a variety of different interaction partners. Plectin contains more than 5000 amino acids. Defects in the plectin gene cause a severe skin blistering disease combined with muscular dystrophy. We determined the structure of the actin binding domain of plectin at 2 Å resolution [32], Fig. 7. The actin binding domain of plectin is structurally similar to that of fimbrin, both having closed conformations which differ from the open conformation of dystrophin and utrophin.

Tau protein, responsible for Alzheimer's disease and its related dementia, is one of the naturally disordered proteins. Neuronal damage in AD is accompanied by self-assembly of tau protein molecules into paired helical



**Figure 7.** Structure of the actin binding domain of plectin (232 amino acids).



**Figure 8.** Structure of the phosphorylated N-terminal domain of Spo0A.

filaments. The core of these filaments has a clearly defined tertiary structure, which can be mapped by binding monoclonal antibodies to them followed by structure determination of the complexes of antibodies with their epitopes. In cooperation with the Institute of Neuroimmunology, SAS, we determined the structure of the complex of monoclonal antibody MN423 with the C-terminal hexapeptide of the core PHF tau at a resolution of 1.65 Å [33].

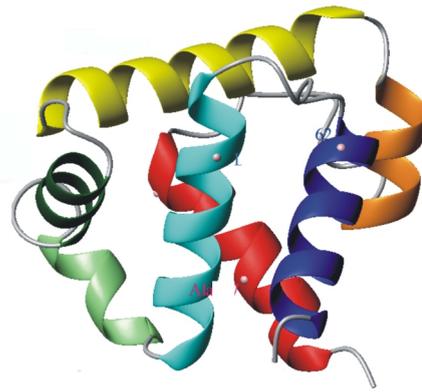
The ryanodine receptor is the largest known ion channel and consists of four subunits, each containing ~5000 amino acid residues. The receptor is expressed in the heart muscle. Mutations in this protein cause arrhythmia and sudden cardiac death. We have prepared three domains of this protein in soluble monomeric form, which are being subjected to biophysical measurements and crystallization. CD spectra and secondary structure predictions suggest that the domains have a globular fold [34]. This study is being performed in cooperation with the Institute of Molecular Physiology and Genetics, SAS.

The vascular endothelial growth factor receptor (VEGFR-2) is considered to be crucial in the process of endothelial cell activation which results in tumor neoangiogenesis and its metastasis, or in diseases connected with pathological vascularisation such as macular degeneration. Here we cooperate with Department of Chemistry, Comenius University. The isolation and purification of the recombinant protein as well as its crystallization is under way.

### Department of Microbial Genetics (head Dr. I. Barák, DrSc.)

During the last decade, the Department of Microbial Genetics has focused on studying the function and structure of proteins involved in the cell division and sporulation of the Gram-positive bacterium *Bacillus subtilis*, an internationally-recognized model organism, whose physiology, biochemistry and genetics have been studied for many years.

In response to nutrient limitation, *B. subtilis* can differentiate and form a highly resistant endospore within a mother cell. This spore is then released and can lie dormant indefinitely and germinate into new vegetative cell when



**Figure 9.** Structure of the C-terminal domain of Spo0A.

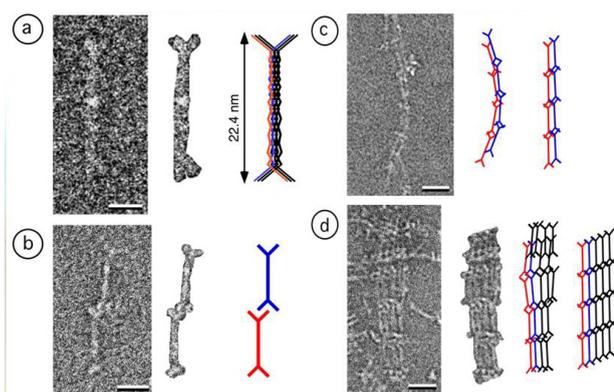
favorable conditions for growth are restored. The sporulation process is regulated by complex regulatory circuits. The regulation of gene expression during sporulation in *B. subtilis* is well-understood and provides a textbook example of gene regulation by alternate RNA polymerase sigma factors. Sporulation begins when a threshold concentration of phosphorylated Spo0A is reached. Phosphorylated Spo0A causes profound changes in the global pattern of gene expression in the cell. Spo0A~P activates the transcription of sporulation specific genes and, at the same time, represses the transcription of many stationary phase specific genes.

Our collaboration with York University led to the determination of a series of crystal structures of the regulatory and trans-activation domains of Spo0A [35, 36, 37]. The structure of the N-terminal receiver domain in its phosphorylated form was determined at 2.5 Å resolution (Fig. 8) and in its unphosphorylated form at 1.65 Å resolution. A comparison of these structures revealed the structural changes associated with phosphorylation and suggested a possible mechanism of Spo0A activation upon phosphorylation.

The structure of the C-terminal DNA binding domain was determined at 2 Å resolution (Fig. 9). The high-resolution of the crystal structures of both Spo0A domains provide a framework for further understanding Spo0A function and interpreting its rich array of mutational data.

Later we studied the *B. subtilis* cell division protein DivIVA. It has a curious dual role in FtsZ ring placement during vegetative growth and chromosome segregation during sporulation. It appears to have an intrinsic capacity to recognize cell poles. In vegetatively growing cells it localizes at the cell poles following the association of the cell division apparatus. Interestingly, DivIVA localizes to cell poles also in heterologous systems such as *E. coli* or the yeast *Saccharomyces pombe*. We solved the TEM (transmission electron microscopy) structure of DivIVA [38]. Fig. 10 shows doggy-bone shaped particles of length 28.5 nm (a). These particles serve as building blocks in the formation of higher order assemblies giving rise to strings (b,c), wires and two-dimensional lattices (d).

In our recent work we have focused on the SpoIISA–SpoIISB toxin-antitoxin system which is involved in programmed cell death in *B. subtilis*. The



**Figure 10.** TEM structure of DivIVA.

*spoIIISA* gene encodes a 248-residue protein containing three putative trans-membrane domains with the last two-thirds predicted to be located in the cytoplasm. On the other hand, the *spoIIISB* gene encodes a basic, hydrophilic, 56-residue protein.

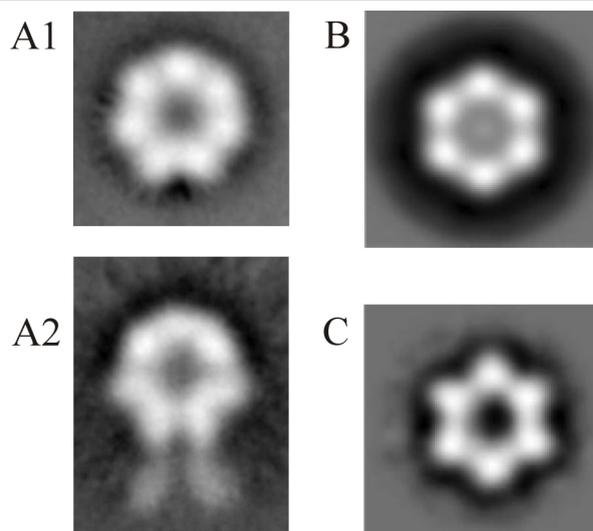
We prepared a complex containing the C-terminal region of SpoIIISA (C-SpoIIISA) and SpoIIISB, successfully crystallized it, and determined its structure.

### Department of Biochemistry (head Dr. E. Kutejová, CSc.)

The ATP-dependent protease Lon is one of the key components of mitochondrial homeostasis. It is responsible for the degradation of oxidatively damaged, misfolded, misassembled and also short-lived proteins in the mitochondrial matrix. In the yeast *S. cerevisiae* Lon was found to be important for mitochondrial DNA stability and in human mitochondria Lon is bound to the mtDNA coding region. The cryoelectron microscopy studies of *S. cerevisiae* Lon protease that were done in close cooperation with the University of Basel showed that Lon forms a heptamer ring-like structure, Fig. 12 [39]. On the other hand, crystallographic studies of the proteolytic domain of human mitochondrial Lon, done in close cooperation with the University of York, suggested a hexamer ring-like assembly, Fig. 13 [40]. These crystallographic studies enabled us to hypothesize that oligomer formation allows a conformational shift to occur, which converts an inactive active site conformation to an active one.

### Laboratory of Molecular Microbiology (head Dr. M. Farkašovský, CSc.)

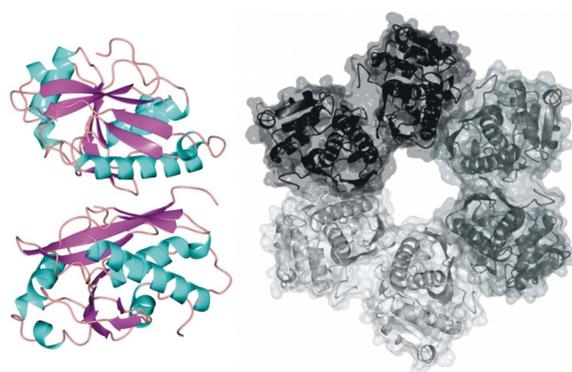
Regular chromosomes segregation during cell division is a crucial process, which depends on spatial and temporal coordination of cytokinesis with nuclear migration. The cleavage apparatus is composed of two major components: septins and the actomyosin contractile ring. Septins are conserved GTP-binding proteins present in most eukaryotic organisms and were first discovered in screens for temperature sensitive mutations that control budding yeast cell cycle (41). *CDC3*, *CDC10*, *CDC11*, *CDC12* and *SHS1* mutants were found to cause cell cycle arrest and defects in the cytokinesis. These proteins were isolated from yeast as a 340 kDa protein complex, which is capable to



**Figure 12.** EM analysis of Lon proteases. (A1) (A2) *Saccharomyces cerevisiae* Lon non-symmetrized average images with diameters of the ring and central hole (11 and 2.5 nm, respectively). The leg-like structures are less contrasted in the average image because of their high flexibility. (B) (C) Correlation averages of *E. coli* Lon wild type and *E. coli* Lon E614K mutant with the diameters of the ring and central hole of EcLon (11 and 3 nm, respectively) and of EcLon E614K mutant (12 and 4 nm, respectively) (adapted from Park *Mol. Cell.* **21**,

form filaments in vitro [42]. Using electron and immunofluorescence microscopy, yeast septins were localized to the bud neck region, where they form higher order filamentous structures (Fig. 14). The molecular function of septins in the bud formation, in cytokinesis and membrane trafficking in yeast and higher organisms is not well understood. One hypothesis on the common role of septins is that they function as polymeric, GTP-regulated scaffolds recruiting other proteins. Septin cortex also serves as a diffusion barrier for integral membrane proteins, which is highly selective for proteins exclusively localized to the bud or mother compartment [43].

14 septins were found in human cells that assemble along existing actin bundles into higher-order structures, which provide scaffold for other proteins and help to organize actin-based structures. Septins have also been found to



**Figure 13.** Crystal structure of the human Lon proteolytic domain. Left: Human Lon proteolytic domain dimer observed in the crystal structure. The asymmetric unit contained two dimers and two independent monomers. Right: A hexamer constructed using the human Lon dimer and the *E. coli* Lon hexamer.

play a role in some kinds of cancers and found to accumulate into pathological structures in neurodegenerative disorders in humans e.g. Alzheimer's disease [44]. In collaboration with Prof. Wittinghofer (MPI fuer molekulare Physiologie, Dortmund, Germany) we previously solved the crystal structure of the SEPT2 G domain in its GDP-bound state and the low-resolution structure of a human oligomeric septin complex consisting of SEPT2/6/7 (Fig. 15). X-ray structures revealed that filament formation involves conserved interactions between adjacent nucleotide-binding sites (G interface) and N- and C-terminal extensions (NC interface) of the protomers. These results, complemented by electron microscopy studies of septin complexes from human, *Caenorhabditis elegans* and *S. cerevisiae* demonstrated a universal principle for the assembly of septins into nonpolar linear filaments ([45, 46] and Fig. 16).

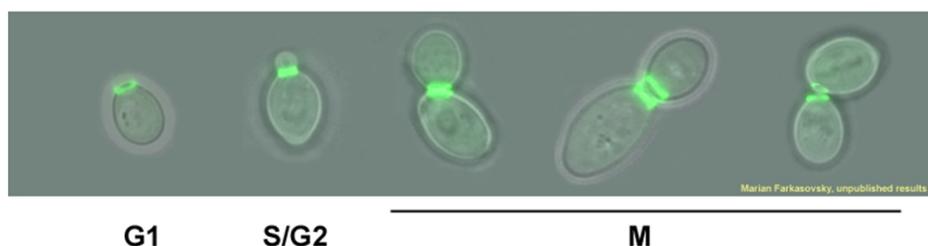
Very recently, we reported the crystal structure of SEPT2 bound to GppNHp at 2.9 Å resolution. GTP binding induces conformational changes in the switch regions at the G interfaces, which are transmitted to the N-terminal helix and also affect the NC interface. Based on our biochemical and structural studies, we propose that GTP binding/hydrolysis influence the stability of interfaces in heterooligomeric and polymeric septins and are required for proper septin filament assembly [47]. In spite of recent development, it is still not well understood how these specific protein-protein interactions lead to changes in the polymerization of septin filaments or assembly of higher-order structures *in vivo*. Detailed characterization of the dynamics of septin assembly and the involved regulatory mechanisms will be important in understanding septin function.

## Acknowledgment

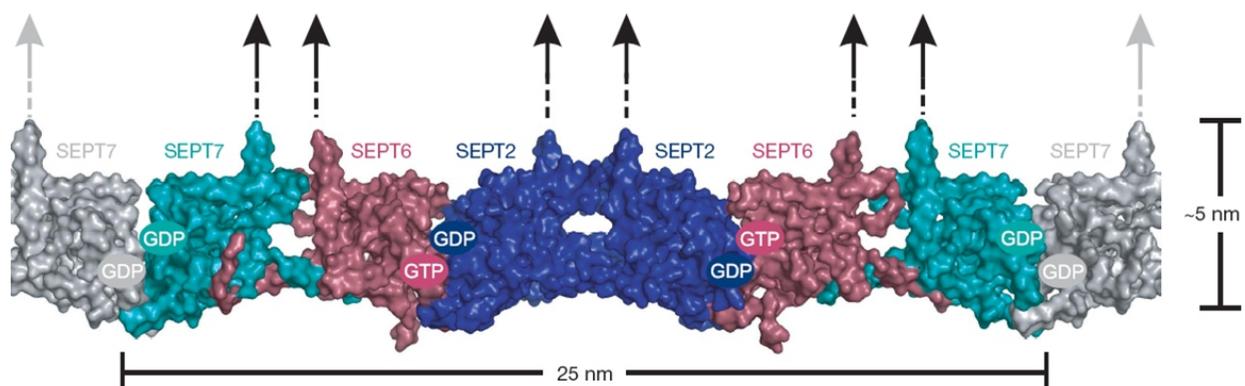
*As the list of cooperating laboratories would be quite long, all co-authors in papers listed in References should be acknowledged. All of them significantly contributed to the quality of published results. In particular our thanks have to be expressed to Chemistry Department of York University, EMBL Hamburg, University of Basel, ESRF Grenoble, and others, who helped us with the know-how and allowed us to use their technical equipment which was not available in our Institute.*

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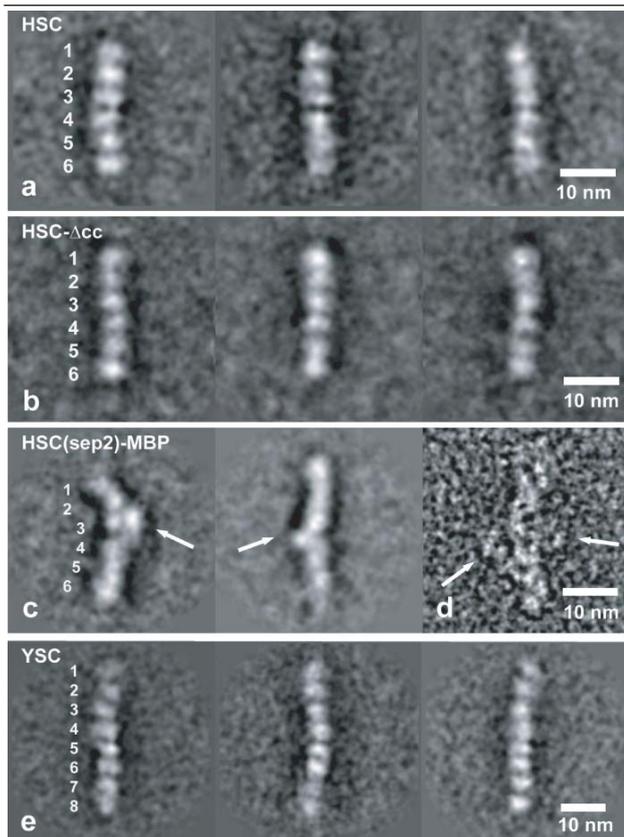
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**Figure 14.** Dynamic of the septin structures during cell cycle.



**Figure 15.** Modular assembly of septin filaments.



**Figure 16.** EM septins - single particle analysis.

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