Posudek na habilitační práci Dr. Romana Sobotky „Biosynthesis of chlorophyll-binding proteins"

Habilitační práce dr. Sobotky je koncipována jako komentovaný soubor devíti uveřejněných vědeckých publikací. Jedná se o velmi hodnotné publikace v prestižních mezinárodních impaktovaných vědeckých časopisech (např. Nature – Chemical Biology, Plant Cell, Journal of Biological Chemistry, Plant Physiology). Jak sám autor uvádí, na 4 publikacích byl v pozici „key researcher", na 4 publikacích byl korespondujícím autorem a na jedné publikaci byl supervizorem. Gratuluji dr. Sobotkovi k těmto publikovaným pracím. Jsou opravdu špičkové i v mezinárodním měřítku a svědčí o velmi vysoké kvalitě jeho vědecké práce.

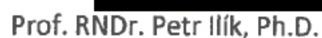
Komentář k tomuto souboru publikací tvoří 13 stran textu, přičemž 3 strany představují citace 40 článků. V této části práce zasadil autor výsledky přiložených prací do širšího kontextu a její součástí je i přehledné sumární schéma pracovního modelu biosyntetických drah pigment-vázajících proteinů u sinic. K odborné stránce textu nemám zásadních připomínek. Autor prokazuje opravdu hlubokou znalost problematiky. Pro větší přehlednost a zhodnocení přínosu přiložených prací (označených I – IX) v komentáři bylo vhodnější se odkazovat na jednotlivé přiložené práce těmito římskými čísly.

Čím jsem však byl poněkud zaskočen, byl samotný název habilitační práce, který je velmi široký a který slibuje široké pojednání o biosyntéze chlorofyl-vázajících proteinů napříč celým fylogenetickým stromem fotosyntetických organismů. V práci se však autor omezuje pouze na biosyntézu chlorofyl-vázajících proteinů u sinic a tedy na biosyntézu proteinů fotosystému II a fotosystému I. Například o biosyntéze významných světlosběrných antén LHC vázajících chlorofyly u řas a vyšších rostlin není pojednáno.

K práci má následující dotazy:

1) Ve schématu biosyntézy chlorofylů u sinic je v práci uvedeno, že protochlorofylid se mění na chlorofylid díky enzymu protochlorofylid oxidoreduktáze (POR). Jedná se o enzym, jehož fungování je závislé na světle a proto se označuje také jako LPOR („light-dependent" POR). U sinic však funguje také enzym se stejnou funkcí, který je nezávislý na světle (DPOR – „dark-operative" POR). Má DPOR u sinic nějakou regulační funkci? Je známo, že jeho fungování je částečně inhibováno za aerobních podmínek. Jak vlastně funguje regulace biosyntézy chlorofylu a chlorofyl-vázajících proteinů u sinic za anaerobních podmínek?
2) Autor v práci prezentuje hodnotný článek publikovaný v časopise Nature – Chemical Biology, který je v komentáři habilitační práce zmíněn jen okrajově. Jedná se o článek odhalující mechanismus nefotochemického zhášení excitací v ochranném proteinu HliD. O tomto významném výsledku není v komentáři habilitační práce pojednáno. Proč byl mechanismus studován právě na proteinu HliD a ne na jiných ochranných proteinech?

Závěrem konstatuji, že i když komentář k přiloženým článkům považuji za relativně krátký, práce Dr. Sobotky splňuje požadavky kladené na habilitační práci a **doporučuji ji k obhajobě**.



Prof. RNDr. Petr Ilík, Ph.D.

V Olomouci dne 29. 8. 2017

LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

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Ihr Zeichen, Ihre Nachricht vom

Unser Zeichen
Habilitation Roman Sobotka

München, den 13.07.2017

Assessment of the habilitation thesis of Dr. Roman Sobotka

It was a pleasure to assess the habilitation thesis of Dr. Roman Sobotka entitled "Biosynthesis of chlorophyll-binding proteins", with whom I have not collaborated before and whom I never met before to my knowledge. So, there are no conflicts of interest.

According to his CV, which is attached to the abbreviated version of the habilitation, Dr. Sobotka has finished his PhD thesis at the age of 27. After his military service (not mentioned in the CV but on the website http://kmb.prf.jcu.cz/en/people/people-at-dep/labhead/en-ing-roman-sobotka-phd.html) he continued his career which also included stays abroad, most notable a postdoc period with Neil Hunter.

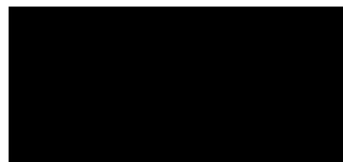
The habilitation thesis of Dr. Sobotka comprises an abbreviated version with an overview of his topic – the biosynthesis of chlorophyll-binding proteins – and the nine papers selected for the thesis.

The abbreviated version covers 20 pages and does very nicely introduce into the topic of the habilitation. It is clearly written, fully referenced and contains 5 figures. It concludes with an outlook for future work. I have only one suggestion for improvement which could be implemented in future habilitation studies. It would be helpful, if the author's own papers would be always highlighted such that the contribution of the habilitant to the topic is immediately clear. Doing this on my own I was impressed by the continuous contribution of Dr. Sobotka to this research field. Taken together, this summary/overview is a very nice piece of work which fulfils all expectations.

The second part of the habilitation consists of reprints of the nine manuscripts selected for the habilitation. Also this part of the habilitation fulfils very high expectations. It most important part contains 2 manuscripts published in *JBC*, 2 in *Plant Physiology*, 2 in *Plant Cell* and 1 in *Nature Chemical Biology*. These are all very good or top journals, underlining the high scientific impact of these publications. In all of these nine publications, Roman Sobotka is either the key experimenter (first author), supervisor or corresponding author. A closer inspection of these nine manuscripts clearly shows that they all deserved to be published in such prestigious journals, representing well planned, well executed and well interpreted pieces of work.

Taken together, the abbreviated version and the nine publications selected for the habilitation thesis clearly demonstrate that Dr. Roman Sobotka has significantly contributed to the research field of thylakoid biogenesis. He has focused on the biosynthesis of chlorophyll-binding proteins in the model cyanobacterium Synechocystis. He has learned from renowned experts, to name some of them - Josef Komenda, Martin Tichy and Neil Hunter - and has developed his own individual profile. He is on a good way to become one of the leading scientists in his research field and is internationally recognized for his work.

In conclusion, I fully support the habilitation of Dr. Roman Sobotka. His habilitation thesis is outstanding and was a pleasure to read and to assess.



Prof. Dr. Dario Leister

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Review of the habilitation thesis by Roman Sobotka, PhD

5 SEPTEMBER 2017

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On the following pages you will find my review of the habilitation thesis.

Here is my overall conclusion:

The central question of how photosynthetic cells regulate the synthesis of chlorophylls and their concomitant incorporation into protein complexes avoiding or limiting toxicity caused by photo-excited Chl-pigments or precursors hereof has been the central theme of the nine submitted publications included for the habilitation of Roman Sobotka.

The nine publications represent a significant, original, novel and independent contribution by Dr. Roman Sobotka to the field of photosynthesis, in particular our understanding of the intricate mechanism of inserting the pigment-cofactors into the proteins during insertion into the thylakoid membranes as well as its regulation. The identification of novel complexes consisting of biosynthetic enzymes, novel assembly factors and translocations proteins is the work of an outstanding, dedicated and international recognized scientist. I therefore recommend that the habilitation thesis by Dr. Roman Sobotka is accepted.

Yours sincerely,

Poul Erik Jensen
Professor, Head of Copenhagen Plant Science Centre, Head of Section

Review of the habilitation thesis of Ing. Roman Sobotka, PhD, entitled "Biosynthesis of chlorophyll-binding proteins" by Prof. Poul Erik Jensen, University of Copenhagen, Denmark.

The habilitation thesis consists of a short introduction (10 pagrs; 5 Figures) to the photosynthetic apparatus in cyanobacteria, chlorophyll biosynthesis, and insertion of chlorophylls into apoproteins and regulation of the machinery that synthesizes chlorophyll-proteins. This is followed by nine publications authored or co-authored by Dr. Sobotka. The nine selected publications represent part of his work in the 10 year period from 2005 till 2016. The publications cover a theme around biosynthesis of chlorophyll-binding proteins that collectively constitute photosystem I and II in oxygenic phototrophs.

A central question of the thesis is how cells regulate the synthesis of chlorophylls, which are efficient photo-synthesizers with the potential to react with molecular oxygen and cause toxicity, and their concomitant incorporation into protein complexes avoiding or limiting this toxicity.

The nine selected papers are published in high quality, high-profile journals; Journal of Biological Chemistry, Plant Physiology, Molecular Microbiology, the Plant Cell, Nature Chemical Biology and Frontiers in Plant Science.

Each publication represents an important, independent and original contribution to the field of molecular understanding of the photosynthetic complexes and in particular to their assembly and regulation of their synthesis.

Original contributions to the field

In paper #1, the experimental work is focused on accumulation of chlorophyll and expression of the chlorophyll (Chl)-binding CP47 protein that serves as the core antenna of photosystem II (PSII) and which is indispensable for the assembly of a functional photosystem II complex. Sobotka and co-workers characterized a CP47 mutant with an impaired photosystem II assembly and two spontaneous pseudorevertants with improved photoautotrophic growth. The complementing mutations in these pseudorevertants were previously mapped to the ferrochelatase (FeCH) gene. In this work it was demonstrated that complementing the mutations dramatically decrease ferrochelatase activity in pseudorevertants and that this decrease is responsible for their improved photoautotrophic growth. This is indeed an interesting and somewhat puzzling result. Photoautotrophic growth of the CP47 mutant was also restored by in vivo inhibition of ferrochelatase by a specific inhibitor (N-methylprotoporphyrin). The decrease in ferrochelatase activity in

pseudorevertants was followed by increased steady-state levels of Chl precursors and Chl, leading to CP47 accumulation and photosystem II assembly. Similarly, supplementing of the CP47 mutant with the Chl precursor Mg-protoporphyrin IX increased the number of active photosystem-II centers, suggesting that synthesis of the mutated CP47 protein is enhanced by an increased availability Chl. The probable role of ferrochelatase in the regulation of Chl biosynthesis is presented in a model in Fig. 8 of paper #1. The model describes how the ferrochelatase activity controls PSII assembly by controlling the availability of chlorophyll. This was, at the time, a very valuable contribution to the field and our understanding of how PSII assembly is regulated at the cellular level.

In paper#2 the work is focused on Gun4, which is a porphyrin-binding protein that activates magnesium chelatase, a multimeric enzyme catalyzing the first committed step in chlorophyll biosynthesis. In the experimental work, Sobotka and co-workers present the functional analysis of Gun4 in the cyanobacterium Synechocystis sp. PCC 6803. FLAG-tagged Gun4 is used to co-purify interactors. The ChlH subunit of the magnesium chelatase was shown to one of these protein interactors. This confirmed the association of Gun4 with the magnesium chelatase enzyme in cyanobacteria. Inactivation of the gun4 gene abolished photoautotrophic growth of the resulting mutant strain that also exhibited a decreased activity of magnesium chelatase, thus proving that Gun4 is needed for full optimal activity and function of the cyanobacterial magnesium chelatase. In the gun4-mutant, the cellular content of chlorophyll-binding proteins was highly reduced, especially those of photosystem II. Methods like immunoblot analyses, blue native polyacrylamide gel electrophoresis, and radiolabeling of the membrane protein complexes suggested that the availability of the photosystem II antenna protein CP47 is a limiting factor for the photosystem II assembly in the gun4 mutant. Paper #2 contributed valuable knowledge on Gun4 and its essential role in the magnesium branch of tetrapyrrole biosynthesis, dramatically affecting the Chl content in the cell. Again, the extensive deficiency of PSII could be related to the insufficient accumulation of CP47. The results suggested that this Chl-binding protein is most sensitive to changes in the Chl availability. This paved the way for new experiments on mutual regulation of tetrapyrrole biosynthesis and formation of new PSII complexes.

In paper 3, the involvement of ferrochelatase (FeCH) that catalyzes the insertion of Fe^{2+} into protoporphyrin, forming protoheme is expanded. In photosynthetic organisms, FeCH and magnesium chelatase lie at a biosynthetic branch point where partitioning in the heme and the chlorophyll (Chl) pathways occurs. Cyanobacterial and algal FeCHs as well as FeCH2 isoform from plants possess a carboxyl-terminal Chl a/b-binding

(CAB) domain with a conserved Chl-binding motif. The CAB domain is connected to the FeCH catalytic core by a proline-rich linker sequence (region II). In this work the regulatory, catalytic, and structural roles of the region II and CAB domains are dissected, by analyzing a FeCH mutant that retains region II but lacks the CAB domain. This is compared with another FeCH mutant that lacks both these domains. The results show that the CAB domain is not required for catalytic activity but is essential for dimerization of FeCH and when absent it causes aberrant accumulation of Chl-protein complexes under high light accompanied by high levels of the Chl precursor chlorophyllide. The work suggested that the CAB domain serves mainly a regulatory function, possibly in balancing Chl biosynthesis with the synthesis of apoproteins eventually binding the Chl-pigments. It was found that, region II is essential for the catalytic function of the plastid-type FeCH enzyme, although the low residual activity of the mutant FeCH is more than sufficient to furnish the cellular demand for heme. It is proposed that the apparent surplus of FeCH activity in wild type cells is critical for cell viability under high light due to a regulatory role of FeCH in the distribution of Chl into apoproteins. Overall the work demonstrated both regulatory and structural roles for the FeCH CAB domain and quite unexpectedly, revealed a critical role of region II for the catalytic function of the plastid-type FeCH enzyme. This work also provided new knowledge on ferrochelatases.

In paper 4 details work on how cyanobacteria acclimate to high-light conditions by adjusting the photosystem stoichiometry through a decrease of photosystem I (PSI) abundance in thylakoid membranes. PSI complexes bind the majority of chlorophyll (Chl) in cyanobacterial cells. It is assumed that the mechanism controlling PSI level/synthesis is tightly associated with the Chl biosynthetic pathway. However, how Chl is distributed to the two photosystems under different light conditions was unknown at this time. Using radioactive labeling with ^{35}S and ^{14}C combined with native/two-dimensional electrophoresis, it was shown how the synthesis and accumulation of photosynthetic complexes in parallel with the synthesis of Chl in Synechocystis sp. PCC 6803 cells acclimated to different light intensities. The results of this paper suggested that PSII subunits are mostly synthesized using recycled Chl molecules released during the PSII repair-cycle involving D1 protein degradation. In contrast, most of the newly synthesized Chl is utilized for synthesis of PSI complexes supporting a mechanism that maintain a constant level of PSI during cell divisions. The outcome of this work also had implications the interplay between Chl biosynthesis and protein complex biogenesis in higher plant chloroplasts.

In paper 5, Type IV pilins, which are small bacterial proteins that have a broad range of functions, including motility, transformation competence and secretion. Pilins vary in sequence, but they possess a characteristic signal

peptide that has to be removed by a prepilin peptidase PilD during pilin maturation. Sobotka and co-workers generated a pilD (slr1120) null mutant in the cyanobacterium Synechocystis 6803 that accumulates an unprocessed form of the major pilin PilA1 (pPilA1) and its non-glycosylated derivative (NpPilA1). The pilD strain had aberrant membrane ultrastructure and did not grow photoautotrophically because the synthesis of photosystem II subunits was abolished. Interestingly, other membrane components such as PSI and ATP synthase were synthesized at levels comparable to the control strain. Proliferation of the pilD strain was rescued by elimination of the pilA1 gene, demonstrating that PilA1 prepilin inhibited the synthesis of Photosystem II, i.e. processing of the protein is essential for its function. The non-glycosylated derivative of PilA1 (NpPilA1) co-immunoprecipitated with the SecY translocase and the YidC insertase, and both of these essential translocon components were degraded in the (pPilA1) mutant. The authors proposed that unprocessed prepilins inactivate a pool of translocons that function in the synthesis of both pilins and the core subunits of Photosystem II. In other words a model is proposed where the abundant and large machinery engaged in PSII biogenesis also produces high levels of pilins when motility becomes important. In this model, accumulation of PilA1 prepilin, or its non-glycosylated form, induces destruction of this machinery resulting in the lack of essential PSII subunits. However, there are other interpretations as well. Overall, this work is pioneering in the sense that molecular details involving both PSII assembly and pillins are provided and is yet another example that the work published by Dr. Sobotka is pioneering and opens novel research questions.

In paper 6, the delivery of chlorophylls to the newly synthesized photosystem apoproteins was investigated in Synechocystis PCC 6803 using a tagged version of the last enzyme of the chlorophyll biosynthetic pathway, chlorophyll synthase (ChlG). This tagged version was used as bait in pull-down experiments. An enzymatically active complex comprising the tagged ChlG and the high-light-inducible protein HliD, associated with the Ycf39 protein, a putative assembly factor for photosystem II, and with the YidC/Alb3 insertase was retrieved in the pull-down experiments. Techniques like 2D electrophoresis and immunoblotting provided additional evidence for the presence of SecY and ribosome subunits in this complex. The isolated complex also contained chlorophyll, chlorophyllide, and carotenoid pigments. Deletion of hliD elevated the level of the ChlG substrate, chlorophyllide, more than 6-fold suggesting that HliD is required for assembly of the FLAG-ChlG protein into larger complexes with other proteins such as Ycf39. The data revealed, probably for the first time, a link between chlorophyll biosynthesis and the Sec/YidC-dependent co-translational insertion of nascent photosystem polypeptides into membranes. The authors envisage that this physical linkage between the above

mentioned proteins coordinates the arrival of pigments and nascent apoproteins to produce photosynthetic pigment-protein complexes with minimal risk of accumulating phototoxic unbound chlorophylls. This indeed makes sense and the experimental evidence is very convincing.

Paper 7 addresses how chlorophyll is delivered to PSII during protein-complex assembly and how these vulnerable pigment-protein assembly complexes are protected from photodamage. A chlorophyll and beta-carotene binding protein complex was identified in the cyanobacterium Synechocystis PCC 6803 which is important for formation of the D1/D2 reaction center assembly complex. This complex was found to be composed of putative short-chain dehydrogenase/reductase Ycf39 and two members of the high-light-inducible protein (Hlip) family, HliC and HliD, which are small membrane proteins related to the light-harvesting chlorophyll binding complexes found in plants. Perturbed chlorophyll recycling in a Ycf39-null mutant and co-purification of chlorophyll synthase (ChlG) and unassembled D1 with the Ycf39-Hlip complex indicate a role of this complex in the delivery of chlorophyll to newly synthesized D1. A related complex in chloroplasts of plants is suggested based on similarities in proteins found both in this complex and proteins in chloroplasts.

Paper 8 involves biophysical methods and addresses mechanism of photoprotection in the cyanobacterial ancestor of plant antenna proteins and continues the pioneering work on the role of Hlips in paper #5 and #6. LHC antennae as well as other members of the LHC superfamily evolved from cyanobacterial ancestors high light–inducible proteins (Hlips). A purified Hlip family member HliD isolated from the cyanobacterium Synechocystis sp. PCC 6803 was characterized. It was found that the HliD binds chlorophyll-a (Chl-a) and beta-carotene and exhibits an energy-dissipative conformation. Using advanced spectroscopic techniques including femtosecond spectroscopy it was demonstrated that the energy dissipation is achieved via direct energy transfer from a Chl-a Qy state to the b-carotene S1 state. Cations of beta-carotene that would accompany Chl-a quenching was not detected. The results provide proof of principle that this quenching mechanism operates in the LHC superfamily and also shed light on the photoprotective role of Hlips and the evolution of LHC antennae. The work presented is indeed pioneering and settles a long-standing question about the role of the high light–inducible proteins. A very thorough and interesting study that will impact future work which is also indicated by the fact that this paper already is cited more than 45 times 2-years after publication.

Paper 9 addressed on of the least characterized steps in the chlorophyll (Chl) biosynthetic pathway, namely the formation of protochlorophyllide catalyzed by Mg-protoporphyrin IX methyl ester (MgPME) cyclase. The Ycf54 protein was recently shown to form a complex with another

component of the oxidative cyclase, Sll1214 (CycI), and partial inactivation of the ycf54 gene leads to Chl deficiency in cyanobacteria and plants. However, the exact function of the Ycf54 is not fully known, and further progress depends on construction and characterization of a mutant cyanobacterial strain with a fully inactivated ycf54 gene. In this paper the complete deletion of the ycf54 gene in the cyanobacterium Synechocystis 6803 was reported. The resulting Δycf54 strain accumulates huge concentrations of the cyclase substrate MgPME together with another pigment, which was identified using nuclear magnetic resonance as 3-formyl MgPME. The detection of a small amount of Chl in the Δycf54 mutant provides clear evidence that the Ycf54 protein is important, but not essential, for activity of the oxidative cyclase, i.e. Chls can be synthetized in the absence of Ycf54. The reduced formation of protochlorophyllide in the Δycf54 strain provided an opportunity to use ^{35}S protein labeling combined with 2D electrophoresis to examine the synthesis of all known Chl-binding protein complexes under drastically restricted availability of de novo biosynthesized Chls. It was shown that although the Δycf54 strain synthesizes very limited amounts of photosystem I and the CP47 and CP43 subunits of photosystem II (PSII), the synthesis of PSII D1 and D2 subunits and their assembly into the reaction centre (RCII) assembly intermediate were not affected. The levels of other Chl-containing complexes such as the cytochrome b_6f and the HliD–Chl synthase (ChlG) complex remained comparable to wild-type. These data demonstrate that the requirement for de novo synthesized Chl molecules differs for each Chl-binding protein. The work in addition has contributed to the role of Ycf54 in the MgPME-cyclase complex. The work shows that Ycf54 is required for stabilization of Cyc1, one of the known catalytic components of the MgPME-cyclase, but also that Ycf54 is unlikely to be one of the catalytic subunits. Another interesting find is that Ycf54 is not directly implicated in Chl phytolation or Chl insertion into proteins. As with so many of the mutants generated in Dr. Sobotka's work also this fully segregated ycf54 mutant has and can in the future be used in further investigations of the regulation and biogenesis of photosynthetic complexes.

Methods and interdisciplinary approaches

The organism of choice in all the papers submitted for this habilitation has been the cyanobacterium Synechocystis PCC6803. This organism has served as a model for numerous studies of oxygenic photosynthesis, in particular molecular understanding of the thylakoid processes including the PSI, PSII, Cyt b6f etc. The tools, i.e. transformation, neutral sites, vectors, promoters and other parts, for this organism are well-developed. In this habilitation the use of this organism to generate valuable and relevant mutants has been even further optimized, progressed and expanded. The fact

that fully segregated mutants, affected in critical functions, have been generated and used for biochemical, molecular, physiological and spectroscopic studies are unique and overall fascinating. Clearly this is one of the crucial strengths of the work presented in the nine papers by Dr. Sobotka. In addition, he has implemented and optimized biochemical methods such as exploiting expression of tagged versions of proteins (i.e. Gun4 or ChlG) to be used for co-localization of proteins in complexes. This has allowed identification of novel protein complexes and identification of several novel proteins involved in these complexes. This has provided new ground breaking knowledge about membrane protein insertion concomitantly with pigment co-factor insertion and how it is regulated at the molecular level. Reading through the 9 papers submitted it is impressive how elegant molecular genetics, biochemistry, enzyme work, physiology, TEM and spectroscopy has been combined and used to address the research questions. This requires an extraordinary skillset, dedication, intelligence and hard work.

International collaborations and visibility

In continuation of the above it is impressive to see how Dr. Sobotka has managed to collaborate with a number of senior colleagues in his home country, in Germany and the United Kingdom. Besides expanding the access to research infrastructure, discussion of ideas and testing these, this also creates international visibility and network which is reflected in for instance conference invitations etc.

Conclusion

The central question of how photosynthetic cells regulate the synthesis of chlorophylls and their concomitant incorporation into protein complexes avoiding or limiting toxicity caused by photo-excited Chl-pigments or precursors hereof has been the central theme of the nine submitted publications included for the habilitation of Roman Sobotka.

The nine publications represent a significant, original, novel and independent contribution by Dr. Roman Sobotka to the field of photosynthesis, in particular our understanding of the intricate mechanism of inserting the pigment-cofactors into the proteins during insertion into the thylakoid membranes as well as its regulation. The identification of novel complexes consisting of biosynthetic enzymes, novel assembly factors and translocations proteins is the work of an outstanding, dedicated and international recognized scientist. I therefore recommend that the habilitation thesis by Dr. Roman Sobotka is accepted.

Professor Poul Erik Jensen Copenhagen 5th of September 2017