Leibniz-Gemeinschaft

Final report

Title of the project:

Role of proteostasis in cellular aging

Leibniz-Institute: Leibniz Forschungsinstitut für Molekulare Pharmakologie (FMP)

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1. Executive summary

Many common human diseases including Alzheimer's or Parkinson's disease display strikingly higher incidences during aging. Frequently, aging-related diseases are caused by the accumulation of defective or aggregated proteins or the malfunction of entire organelles or cells. The project was therefore built on the hypothesis that alterations in protein homeostasis underlie aging-related disorders, thereby providing a handle for pharmacological interference. The research program was conducted in part in collaboration with researchers from the Leibniz Institute for Neurobiology (LIN) and from the Freie Universität Berlin (FUB).

In **WP1** we determined age-related changes in synaptic protein levels and have begun to dissect the role of autophagy in the maintenance of neuronal function during aging. We found that neuronal aging is accompanied by an imbalance of pre- and postsynaptic protein levels, in particular of synaptic scaffolding proteins, that correlates with changes in the autophagy/ lysosomal pathway. Our preliminary data have further unraveled a key function of the autophagy pathway in the regulation of the reticular/ tubular endoplasmic reticulum. These surprising and entirely novel findings are of possible key relevance for our understanding of age-related neuronal diseases.

In **WP2** we successfully established various stable or transiently transfected cell models for the functional expression of plasma membrane-targeted mutant ClC-7 together with its required β-subunit Ostm1 that allow the development of functional assays for the identification of specific CLC-7 inhibitors by medium-to-high throughput screening as a novel approach to treat age-related osteoporosis. In addition, we have set up functional assays for the development of isoform-specific VRAC inhibitors, which is of potential medical importance for aging-related pathologies such as stroke.

Furthermore, in **WP3** we explored the limits of NMR technology to characterize the lysosomal metabolome in wild-type and different CLC-7 mutant mouse strains. These studies will be followed up by mass spectroscopy and RNAseq experiments to elucidate the effects of loss or modification of ClC-7 on lysosomal metabolism and storage.

Finally, in **WP4** we found, using sensitive solid-state NMR spectroscopy analyses, that αB-crystallin interacts much more strongly with mutant γS- and βB2-crystallin variants associated with cataract formation in humans. The identification of potential "binding hotspots" within the core-domain of αB-crystallin provides important information for the future development of novel treatment strategies against cataracts in aging humans.

In summary, we have been able to obtain novel insights into aging-related changes in synaptic protein levels and the role of the autophagy/ lysosomal pathway in neuronal protein turnover and have identified molecular mechanisms underlying cataract formation in humans.

Final Report

2. Starting point and aims of the project

Developed countries currently undergo a dramatic demographic change characterized by a sharp increase in the elderly population. Impairments in organ maintenance and the development of aging associated diseases reduce the quality of life in this population and for many of these conditions there are currently no efficient therapies available.

Aging is associated with a wide range of functional alterations that affect diverse organs and cell types including the brain, sensory organs such as the eye, and bones. Consistent with this many common human diseases, including neurodegeneration or osteoporosis, display strikingly higher incidences during aging. Frequently, aging-related diseases are associated with the accumulation of defective or aggregated proteins or the malfunction of entire organelles or cells. Based on these observations we have pursued the **hypothesis that alterations in protein homeostasis (proteostasis) underlie aging-related disorders**. We have tested this hypothesis using several models including the nervous system, bone, and key proteins of the eye lens. To this aim we have combined studies at the systems level *in vivo* with biochemical and NMR-based structural studies and with high throughput small molecule screening.

The **specific aims of the project** were to:

1.) determine age-related changes in synaptic protein levels and to dissect the role of autophagy in the maintenance of neuronal function during aging

2.) develop ClC-7 inhibitors as tools to investigate lysosomal function and as potential treatment of age-related osteoporosis

3.) analyze lysosomal dysfunction in mice with changed ClC-7 activity

4.) determine the role of molecular chaperones for proteostasis using the eye lens as a model.

We have achieved significant new insights with respect to most of these questions that are summarized in more detail below.

3. Development of the project and deviation from originally proposed plans

Overall, the project has been conducted largely as originally planned and has yielded exciting, though partially still preliminary data on the role of the various proteolytic systems in maintaining neuronal and brain function. As much of our research has focused on **mouse models** to study brain aging, experiments are conducted over a time frame of > 3 years to allow comparison of young (6-7 weeks-old) and old mice (> 20 months). This has led to **delays in publication** of significant parts of the results. Publication of the majority of data obtained is expected to be completed within the forthcoming 12-18 months.

Within **WP1** we have decided, following discussions with leading researchers in autophagy, to use conditional knockout mice lacking the essential autophagy component ATG5 instead of the originally planned ATG4B dominant-negative strain. ATG5 is an essential gene and a core component of the E3 ligase complex that conjugates LC3 to phosphatidylethanolamine to enable autophagophore closure. Loss of ATG5 is, thus, a more stringent means to stall autophagosome formation and thereby protein turnover via the autophagy/ lysosome pathway. Moreover, as gross proteomic changes in synaptic protein levels may differ between different brain areas, we have decided to focus our biochemical and cell biological analysis of synaptic protein levels and localization on the mossy fiber CA3 area of the hippocampus. This area displays forms of presynaptic plasticity amenable to electrophysiological analyses and, thus, allows us to correlate biochemical and morphological changes in pre-

synaptic composition with functional readouts that are often difficult to achieve in the field of aging research. These experiments are currently being completed.

In **WP2** we have been facing difficulties in establishing a reliable assay for HTS for ClC-7 inhibitors, which might not only be useful as laboratory tools, but may eventually lead to the development of drugs for the treatment of osteoporosis, a major and frequent health problem in the aging population. We are continuing this effort with modified experimental strategies and have successfully devoted about half of our effort on finding inhibitors and activators of the volume-regulated anion channel VRAC which we have recently identified. Novel VRAC inhibitors have potential applications in other aging-related, frequent pathologies such as stroke.

In **WP3** we realized that the initial strategy to identify lysosomal metabolites in different ClC-7 mouse models by NMR is, unfortunately, not sensitive enough. We therefore changed our experimental approach to mass spectroscopy, which we complement with RNAseq experiments that may detect secondary transcriptional changes caused by lysosomal pathology.

In **WP4** structural investigations on systems involved in protein homoeostasis were undertaken. We investigated the interaction of alpha B-crystallin with mutant beta- and gamma crystallins as well as the respective wildtypes. Additionally, we investigated the structural properties of the nascent chain within the ribosome tunnel.

4. Results and discussion

The original project has been conducted in four work packages led by different PIs (**WP1-4**), the results of which are briefly summarized below.

4.1. Work Package 1 (WP1): Dissection of the role of autophagy in the maintenance of neuronal function during aging in vivo [PI: Haucke (FMP), in collaboration with Gundelfinger, Fejtova (LIN Magdeburg) and Sigrist (FU Berlin)]

The primary goal of this WP was to determine age-related compositional alterations that occur during aging in synapses and to analyze the role of the autophagy machinery in synaptic and neuronal protein turnover.

4.1.1. Aging-related changes in synaptic protein composition

As gross proteomic changes in synaptic protein levels may differ between different brain areas, we have focused our biochemical and cell biological analysis of synaptic protein levels and localization on the mossy fiber CA3 area of the hippocampus [1]. To this aim we have generated cohorts of young (6-7 weeks-old) and old (24 months-old) mice. Animals were perfused and synaptic protein levels and localization were determined by confocal imaging of cryosections immunostained with specific antibodies against selected pre- and postsynaptic proteins as well as markers of the endolysosomal and autophagy system.

We initially analyzed key components of the machinery for presynaptic neurotransmitter release such as active zone (AZ) proteins, large presynaptic scaffolds that couple release-ready synaptic vesicles to presynaptic calcium channels at release sites. These include the giant proteins piccolo and bassoon as well as the recently identified RIM binding protein 2 (RIM-BP2), an ortholog of *Drosophila melanogaster* RBP [2]. Quantification of AZ protein levels in the mouse fiber CA3 area identified by co-labeling with the Zinc transporter ZnT3 (not shown) revealed an elevation of the levels of Piccolo, Bassoon, and RIM-BP2 in old mice (Figure 1A,B). Analysis by dual-color super-resolution STED microscopy further showed that these AZ proteins remained closeley clustered at AZ release sites labeled by antibodies against presynaptic calcium channels (not shown).

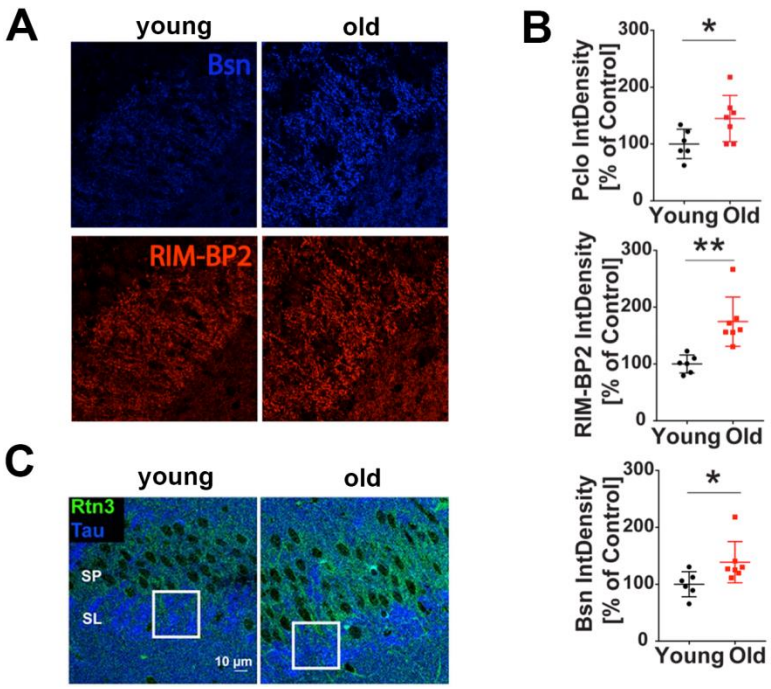


*Figure 1: (A) Representative images showing expression of the active zone proteins bassoon (Bsn) and RIM binding protein 2 (RIM-BP2 in the hippocampal CA3 area of young (6-7 weeks old) and old (24 months old) mice.) (B) Quantification of the levels as shown in A of the active zone proteins Piccolo (Pcl, top), Bsn (middle), and RIM-BP2 (bottom). *p<0.05; **p<0.01. (C) Representative images showing expression of RTN3 and the axonal marker Tau in the hippocampal CA3 area of young (6-7 weeks old) and old (24 months old) mice. During aging the levels of RTN3 (boxed areas in images) tend to increase in mossy fiber axons (young: 43.9, 18.7 – 181.3; old: 120.1, 53.8 – 170.7; n=4/group).*

Interestingly, these alterations were specific to AZ scaffold proteins that contribute to the determination of presynaptic release probability as the levels of SV proteins including synaptotagmin 1, the SV calcium sensor, and synaptophysin, were significantly reduced in mossy fiber terminals from old animals, in agreement with earlier studies using whole brain samples [3,4].

We were then interested to see whether and how such presynaptic changes correlate with altered postsynaptic protein levels. As mossy fiber boutons are excitatory synapses we used antibodies against the postsynaptic scaffold PSD95 to analyze alterations at the level of the postsynapse. We found PSD95 levels to be reduced in the CA3 area (Figure 2), while no overt effects on the pre- and postsynaptic apposition were seen at either the confocal or STED microscopy levels (not shown).

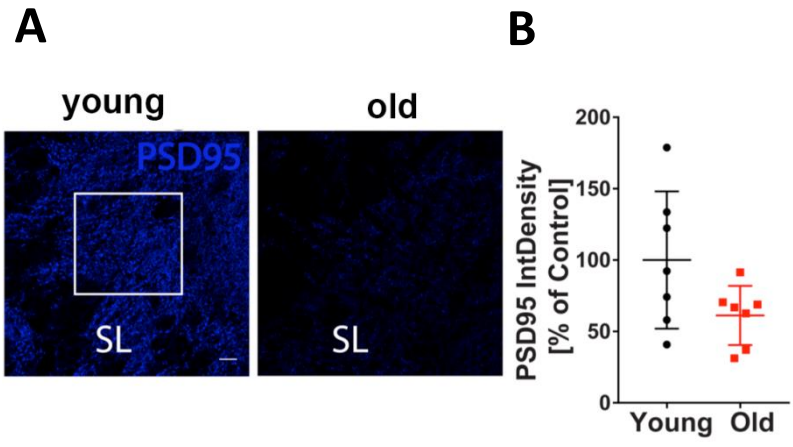


Figure 2: Postsynaptic PSD95 levels tend to decline during aging at mossy fiber CA3 synapses. (A) Representative images on the left show expression of the postsynaptic marker PSD95 in the hippocampal CA3 area of young (6-7 weeks old) and old (24 months old) mice. (B) Quantification of data as exemplarily shown in A. PSD95 levels tend to decrease in CA3/ mossy fibers from old mice.

These alterations in pre- and postsynaptic proteins important for neurotransmission in old mice were not due to an overall change in synapse number or density determined by the number of pre- and postsynaptic appositions per area (not shown).

According to the working hypothesis, changes in pre- and/ or postsynaptic protein composition might be caused by or directly or indirectly related to alterations in the autophagy/ lysosomal pathway for protein turnover [5]. Confocal microscopy analysis of cryosections from young and old mice revealed a significant reduction in the levels of the late endosomal/ lysosomal marker protein Lamp1 (Figure 3B,C). In contrast, no significant changes were seen for the endosomal markers CHMP4, a component of ESCRT-III, or syntaxin-13, an endosomal SNARE protein (not shown). However, we found a striking accumulation of dystrophic neurites immunopositive for the autophagy receptor p62 (Figure 3A).

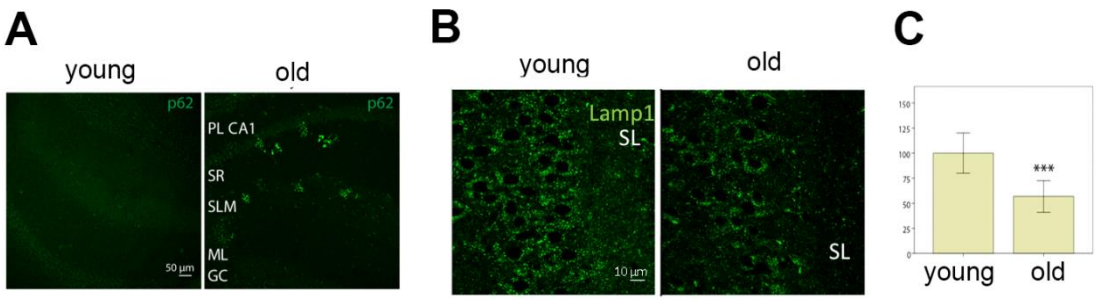
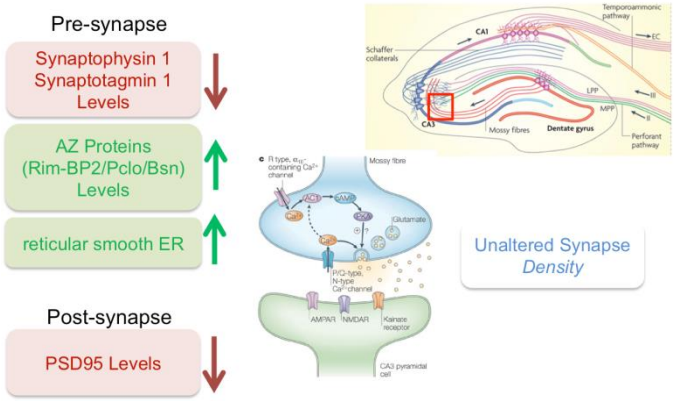


*Figure 3: Age-dependent alterations in late endosomal compartments in mossy fiber CA3 synapses. (A) Dystrophic neurites accumulate in the CA3 area of old mice. Representative images using antibodies against the autophagy receptor p62 are shown (n=5-7 mice for each age-group). (B) Representative images showing expression of the late endosomal/ lysosomal marker Lamp1 in the hippocampal CA3 area of young (6-7 weeks old) and old (24 months old) mice. (C) Quantification of data as shown in B. ***p<0.001.*

These collective data summarized in Figure 4 show that neuronal aging is accompanied by an imbalance of pre- and postsynaptic protein levels, in particular of synaptic scaffolding proteins, that correlates with changes in the autophagy/ lysosomal pathway.

Figure 4: Summary of age-related presynaptic alterations in mossy fiber CA3 synapses in mice.



4.1.2. Role of autophagy in synaptic function and aging-induced memory impairment

It is possible that the observed alterations in the levels of pre- and postsynaptic scaffolds that accompany aging in mossy fiber synapses in mouse brain are a consequence of altered proteolysis via the autophagy/ lysosomal pathway. We therefore set out to quantitatively determine changes in neuronal protein levels and turnover in the absence of the essential autoph-

agy component ATG5, a subunit of the E3 ligase, that is required for LC3 lipid conjugation and, thereby, for autophagosome formation in mammals [5].

To this aim we generated conditional knockout (KO) mouse lines, in which the ATG5 gene can be acutely inactivated either upon addition of tamoxifen (ERT2-Cre) or is selectively deleted in postmitotic neurons in the brain (Emx1-Cre). Acute inactivation of ATG5 in cultured neurons allows the analysis of neuronal protein levels and turnover using SILAC based quantitative proteomics (conducted in-house by Dr. Eberhard Krause, FMP). Neurons were cultured in medium with heavy (H), medium (M) or light (L) labelled amino acids and H/M ratios (fold change) for each protein were calculated as a measure of protein degradation (Figure 5A). This analysis revealed a surprising enrichment of proteins localized to the reticular/ tubular endoplasmic reticulum (ER) (Figure 5B,C). In addition to the tubular ER, a subset of mitochondrial proteins also displayed altered degradation. The levels of pre- and postsynaptic proteins including SV markers, AZ proteins or postsynaptic components appeared to be unaltered, at least within the time course of the chase experiment (5 days).

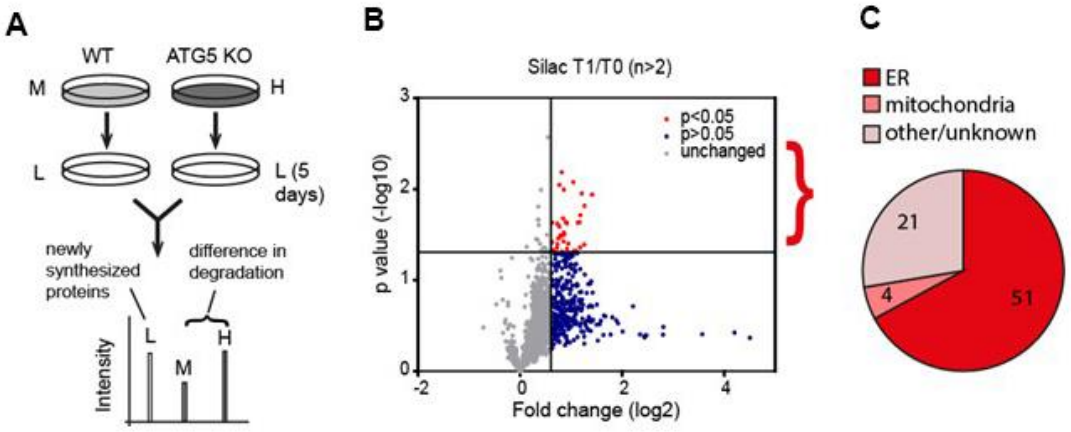


Figure 5: (A) Changes in protein degradation rates measured by SILAC/MS. Neurons are cultured in medium with heavy (H), medium (M) or light (L) labeled amino acids. (B) H/M ratios (fold change) for each protein are calculated as a measure of degradation. Red dots show proteins with significantly decreased degradation (76 hits) in ATG5 KO neurons. (C) Most (51/76) of the identified proteins are primarily localized to the endoplasmic reticulum (ER).

To determine whether altered levels of reticular/ tubular ER proteins such as Reticulon 3 (RTN3) are also reflected in elevation of morphologically discernable smooth ER structures we analyzed wild-type and ATG5 cKO neurons by confocal imaging and electron microscopy. In agreement with the proteomic data we observed a striking accumulation of reticular/ tubular ER components, most notably RTNs, in neurons from ATG5 cKO mice (Figure 6A). The accumulation of reticular/ tubular ER was only seen in axons but not in dendrites (Figure 6B), consistent with the published observation that autophagosomes primarily form in distal axons and move retrogradely to the neuronal cell body [6,7]. Accumulation of reticular/ tubular ER structures in ATG5 KO axons and presynaptic boutons was confirmed by electron microscopy (not shown).

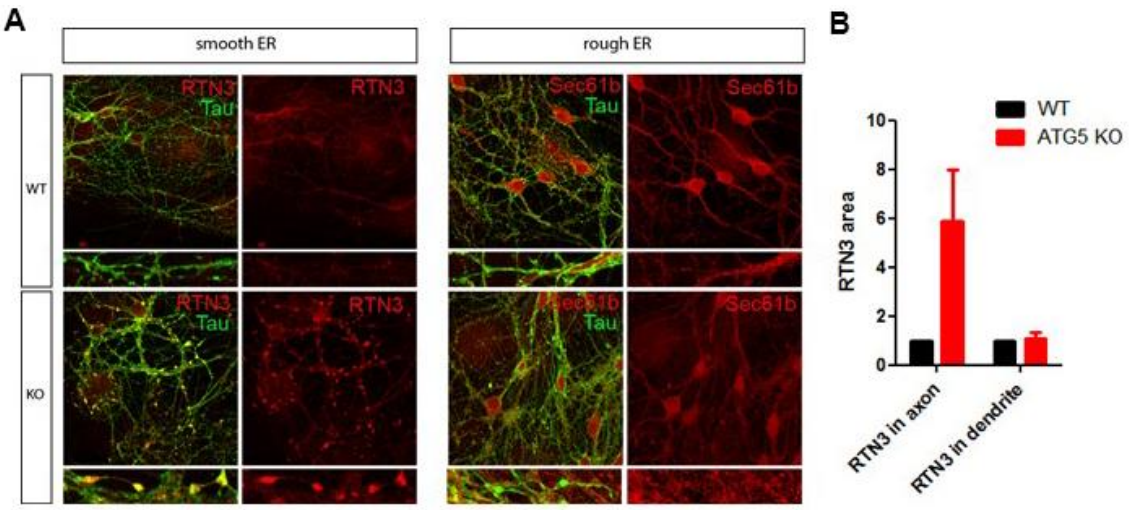


Figure 6: (A) Representative images showing expression of RTN3 (tubular/ reticular ER marker) or Sec61b (rough ER marker) and the axonal marker Tau in wildtype (WT) and ATG5 KO cultured hippocampal neurons. (B) Levels of RTN3 quantified at Tau-positive axons or Map-positive dendrites.

To determine whether ATG5/ autophagy also controls the levels and morphology of the reticular/ tubular ER *in vivo*, we deleted ATG5 expression in forebrain neurons (via Emx1-Cre). Mice conditionally lacking ATG5 in postmitotic neurons were born at Mendelian ratios but displayed severely reduced postnatal viability within the first 2-5 months (Figure 7A), suggesting a key physiological role of the autophagy system for neuronal health. Loss of ATG5 in young mice was accompanied by a prominent accumulation of reticular/ tubular ER markers such as RTN3 in axons and a striking accumulation of p62 puncta (an autophagy receptor and substrate for autophagy) in neuronal cell bodies (Figure 7B), similar to our observations in cultured neurons *in vitro*.

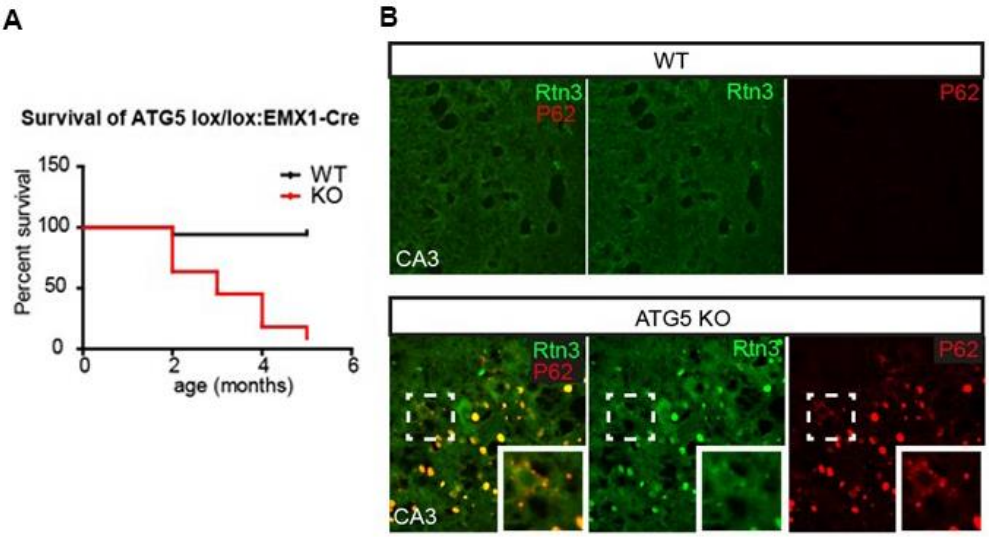


Figure 7: (A) Survival curves of WT and brain specific ATG5 KO mice. (B) Representative confocal images showing expression of RTN3 and p62 in the hippocampal CA3 area of WT and ATG5 KO mice.

These collective data unravel a key function of the autophagy pathway in the regulation of the reticular/ tubular ER.

Major physiological roles of the reticular/ tubular ER include lipid transfer and the regulation of axonal and synaptic calcium levels. Preliminary data indeed indicate defective calcium homeostasis in ATG5 KO neurons (not shown).

We therefore hypothesize that altered synaptic protein levels observed in aged mice might be an indirect consequence of defective calcium homeostasis due to the accumulation of the reticular/ tubular ER when autophagy flux is reduced during aging [8]. Consistent with this hypothesis, our preliminary analysis suggests an accumulation of reticular/ tubular ER in the CA3 mossy fiber area of old mice (Figure 1C).

We expect to complete these studies (e.g. by additional slice electrophysiology and electron microscopy as well as functional optical imaging experiments) within the forthcoming 6-12 months with two major publications emanating from this work.

4.2. Work Package 2 (WP2): Development of ClC-7 inhibitors as tools to investigate lysosomal function and as potential treatment of age-related osteoporosis [PIs: Jentsch (FMP); von Kries (FMP)].

We have shown previously that mutations in the lysosomal $2Cl^-/H^+$-exchanger ClC-7 lead to severe osteopetrosis and neurodegeneration associated with lysosomal storage disease in mice and man [9]. Osteopetrosis is associated with hypercalcified, brittle bone and can be regarded as mirror image of osteoporosis, which is associated with demineralized bone and which is a major, frequent health issue in the aging population, in particular with elderly women. Drugs specifically inhibiting the ClC-7 anion transporter are predicted to increase bone mineralization and hence might be developed into clinically useful drugs for osteoporosis. The lysosomal storage disease and neurodegeneration observed with a total loss of ClC-7 activity is not of major concern for this therapeutical strategy because patients with the clinically milder autosomal dominant osteopetrosis (ADOII), which is due to dominant negative, heterozygous *CLCN7* mutations, lack lysosomal storage and any neurological symptoms. Hence there is an apparently large therapeutic window in which reduction of ClC-7 activity is sufficient to reduce osteoclast activity and thereby increase bone calcification without impairing lysosomal function to a degree sufficient to cause lysosomal storage.

In WP2 we successfully established an assay based on transient transfection of HeLa cells with a plasma membrane (PM)-targeted ClC-7 mutant ($ClC\text{-}7^{PM}$) [10] together with its required β-subunit Ostm1 [9] and the snail FaNaC channel (a homomeric Na^+-channel gated by the peptide FMRF-amide). Depolarizing the HeLa cells by opening FaNaC with the addition of FMRF-amide opened the voltage-gated ClC-7/Ostm1 $2Cl^-/H^+$-exchanger, leading to an intracellular alkalinization that could be detected optically with the BCECF pH indicator that was previously loaded into the cell (Figure 8A,B). However, transiently transfected cells are poorly suited for HTS because of variable transfection efficiencies. We therefore proceeded to generate stably transfected cell lines using a variety of different transfection and selection protocols and DNA constructs (e.g. for inducible expression because toxicity of stably transfected channels can be a concern). Unfortunately, none of the cell lines gave sufficiently high stable (or inducible) expression of all three proteins as determined by Western blots and functional assays (Figure 8C,D). As a next step, we have been trying several other approaches: Transient transfection with a plasma-membrane targeted ClC-7/E²GFP fusion protein together with Ostm1 (on the same plasmid) and FaNaC, based on the idea that variable transfection efficiencies would be less problematic because, in contrast to BCECF measurements, only transfected cells will give optical signals as pH readout. Additionally, we are generating cell lines in which we express a pore mutant of PM-expressed ClC-7 (together with Ostm1) that abolishes its voltage-dependence and converts it into a pure chloride conductance. This strategy obviates the need for cell depolarization, but carries the intrinsic risk

that the (even though transient, since tetracyclin-induced) expression of a constitutively open channel is toxic for the cell even though it is not associated with pH changes. Detection of channel activity will be performed by iodide-influx experiments that use an iodide-sensitive YFP mutant as optical reporter as in our previous siRNA screen for the VRAC channel. This strategy requires again stable expression of three proteins in HEK cells and has the disadvantage that the pore mutation might influence the binding or the effect of compounds. In addition, we are currently generating cell lines expressing, together with Ostm1, a C-terminal mutant of PM-expressed ClC-7 that is less rectifying and activates faster. This mutant promises to result in a more sensitive assay for ClC-7 exchange activity.

Although this project turned out to be more difficult than anticipated, **we will continue with our efforts to identify ClC-7 inhibitors because of the potential relevance for human health.** However, the graduate student involved in this work has performed, together with Dr. Jens von Kries, **in parallel screens for inhibitors and activators of the recently identified volume-regulated VRAC channel** using well-established assays. We have identified several interesting compounds that are currently being modified by the FMP Medicinal Chemistry group to increase their efficacy. Importantly, some of these compounds display preference for certain LRRC8 subunit combinations of the heteromeric VRAC channel, which will be needed if specific functions of VRAC will be targeted. **Development of isoform-specific VRAC inhibitors is of potential medical importance for aging-related pathologies such as stroke.**

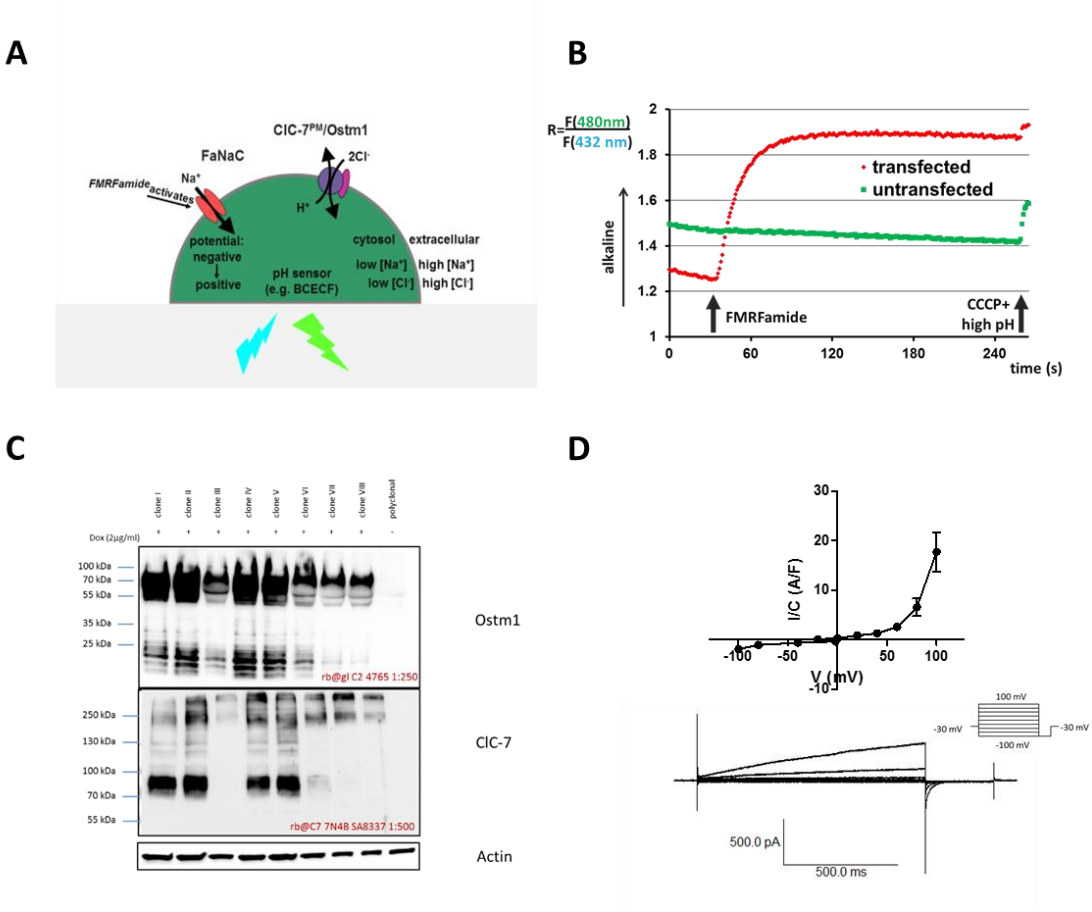


Figure 8: Establishment of functional assays suitable for identifying ClC-7 inhibitors in high-throughput screens. (A) Principle of assay. FMRFamide opens FaNaC Na^+ channels, leading to sodium influx and plasma membrane depolarization that is required to activate plasma membrane-targeted ClC-7^{PM}/Ostm1 transporters. At intracellular potentials more positive than +20 mV, ClC-7 is activated and transports chloride ions into and protons out of the cell. This results in intracellular alkalinization that can be measured by the ratiometric pH sensor

BCECF in high-throughput screens. (B) Proof-of-principle experiment comparing WT HEK cells and cells transiently transfected with ClC-7/Ostm1 and FaNaC. Treatment with 30µM FMRFamide leads to an alkalization in transfected, but not in non-tranfected cells, as deteremined by the ratio of BCECF fluorescence at 480 nm/ 432nm. Addition of the protonophore CCCP at the end of the experiment is used as control. (C) Cell lysates prepared from Flp-In HeLa stably transfected with a tetracycline-inducible ClC-7^{PM}-T2A-Ostm1 (C72AO) construct after treatment with 2µg/ml doxycycline (Dox) for 48h. Lysates were subjected to SDS-PAGE and Western blotting to detect expression of ClC-7 and Ostm1. The mature, processed Ostm1 polypeptide migrates at ~20 kDa, whereas ClC-7 runs at ~90 kDa. (D) Electrophysiological analysis of ClC-7^{PM}/Ostm1 currents from one of the most promising clones (clone V in panel C). Consistent with the expression of small amounts of ClC-7^{PM}/Ostm1 at the plasma membrane, typical ClC-7 currents, which slowly activate upon strong depolarization, were observed in whole-cell patch-clamp experiments, but the magnitude of these currents was insufficient for yielding significant depolarization-induced pH changes.

4.3. Work Package 3 (WP3): Analysis of lysosomal dysfunction in mice with changed ClC-7 activity [PIs: Jentsch (FMP); Oschkinat (FMP)].

Initially, we and others hypothesized that the lysosomal storage disease and the osteopetrosis observed with disruption of ClC-7 are caused by a failure of lysosomes and the osteoclast resorption lacuna to be acidified. The mechanism proposed stated that ClC-7 provides neutralizing currents that are needed for an efficient operation of the electrogenic H^+-ATPase that ultimately lowers the pH of those compartments. However, we found that, probably owing to a lysosomal cation conductance, lysosomal pH was not changed in cells lacking ClC-7, but that lysosomal Cl^- concentration was significantly diminished. We had also generated *knock in* mice, in which ClC-7 $2Cl^-/H^+$-exchange was converted to a pure Cl^- conductance or was completely transport-deficient. The pathologies of these strongly supported the importance of lyosomal Cl^- accumulation and additionally suggested a role of so far unknown interactions of ClC-7 with other proteins. In essence, however, the mechanism leading to lysosomal dysfunction, which includes impaired degradation of endocytosed proteins and lysosomal storage, remains enigmatic.

In WP3 we therefore aimed at determining the content of lysosomes, with a particular focus on metabolites, obtained from our various ClC-7 mouse models in comparison to WT lysosomes. Quantitative changes in the pattern of these compounds may give valuable insights into the pathomechanism. These changes may hint, for instance, at changes in lysosomal enzymatic activities, which might be influenced by lysosomal Cl^-; or at impaired transport of metabolites across the lysosomal membrane, which may use Cl-dependent transport proteins. As a high-risk part of this project, we applied NMR to lysosomal preparations from our diverse mouse models to quantitatively investigate the inventories of metabolites. This approach was relying on the recently accessible dynamic nuclear polarization (DNP) technology that promises an increase in signal-to-noise-ratio (S/N) by factors in the range of 10 - 100. The measurement time needed for achieving a certain S/N is determined by the square of this factor.

We therefore purified lysosomes from mouse liver using several centrifugation methods, including self-generated Percoll density gradients and discontinuous Optiprep gradients. We chose liver as a relatively large and uniform organ, and mice because we wanted to investigate the impact of ClC-7 on lysosomal metabolism using our various *Clcn7* mouse models. The enrichment of lysosomes and the relative purity of the preparation were evaluated using markers for lysosomes and other compartments, and preparations used typically livers from 5 mice each. Lysosomal preparations were prepared for solution and solid-state NMR analysis, which was performed by Hartmut Oschkinat's group. The standard procedure for preparation was modified to minimize signals from buffer and gradient components, and investigated in standard solution and solid-state NMR experiments. Despite these optimization steps, however, DNP NMR spectrum was still dominated by components of the buffer and the gradient, with no lysosomal metabolite being identified. We concluded that the amount of

lysosomal material was insufficient for NMR analysis at the current state of the development of the DNP methodology and at the given background signals of buffer components; a problem that might possibly not be solved without, maybe, drastically increasing the number of mice used for each experiment, in combination with improving resolution or S/N. We therefore abandoned the NMR approach at the time of the first series of NMR measurements and changed to mass spectroscopy and RNAseq experiments to elucidate the effect of the loss or modification of ClC-7 on lysosomal metabolism and storage. In parallel, the DNP NMR approach was improved by introducing new radicals in collaboration with Snorri Sigurdsson, Rejkjavik [11], yielding much higher S/N, and introducing the high-temperature DNP approach [12] assuming that higher resolution will help to distinguish signals from metabolites. We are planning to revisit the approach in the near future on the basis of these improvements.

4.4. Work Package 4 (WP4): Regulation of proteostasis by molecular chaperones [PI: Oschkinat (FMP)]

The original plans comprised investigations on molecular chaperones that play a role in protein homeostasis and that are often involved in aging-related developments. In the eye-lens, αB-crystallin is involved in the formation of cataracts at old age. We investigated the interactions of αB-crystallin with clients that are present in the eye lens, especially those that suffer modifications with aging. Furthermore, αB-crystallins play a role as heat-shock proteins in prevention of protein aggregation in many kinds of cells [13,14]. In addition, we investigated a second system connected to protein homeostasis, the ribosome, and in particular the structure of the nascent chain in its exit tunnel. For this purpose, and also related to the work done for WP3, we improved the DNP technology in order to obtain higher enhancement factors.

4.4.1. Interaction studies of αB-crystallins with substrate proteins

In the course of this study we investigated the interaction of αB-crystallin with wild-type γS- and βB2-crystallin, and their aggregating variants, G18V γS- and Q70E/Q162E βB2-crystallin (in part generated by post-translational modification). Upon the transient interaction of isotope labeled αB with unlabeled wild-type γS-crystallin, effects in the ssNMR are only marginal and we cannot specify a binding site. In contrast, when binding to G18V mutant γS-crystallin, strong perturbations for residues in a hydrophobic patch of the β7-β7 dimer-interface are observed, which has previously been suspected to be a chaperone-binding site. ssNMR spectra of labeled αB in complex with unlabeled wild-type βB2 showed relatively strong signal perturbations from residues of the β4-β8 groove. However, signal perturbations for the β7-β7 dimer-interface were weak, therefore we do not consider this region as being affected upon binding βB2-crystallin. Interestingly, upon interaction of αB with the Q70E/Q162E variant of βB2-crystallin, we see stronger signal perturbations throughout the entire sequence as compared to interaction with wild-type βB2-crystallin (Figure 9). In summary, it was found that the mutant clients trigger a stronger binding response than the wild-type protein. Several potential “binding hotspots” were identified within the core-domain of αB-crystallin, located in the β4-β8 groove and in the β7-β7 dimer-interface. Due to the substrate-dependent behavior of signal perturbations, we consider the involvement of the β7-β7 dimer-interface and the β4-β8 groove for substrate protein binding to function independently from one another.

For these studies, we employed a strategy to study αB in complex with substrate proteins by FROSTY solution-MAS NMR spectroscopy. αB was expressed as reported previously with a labelling scheme with specific incorporation of amino-acids ^{2}H, ^{15}N IWY and ^{2}H, ^{13}C, ^{15}N V in an otherwise deuterated background with 20% ^{1}H back-exchange on labile sites.

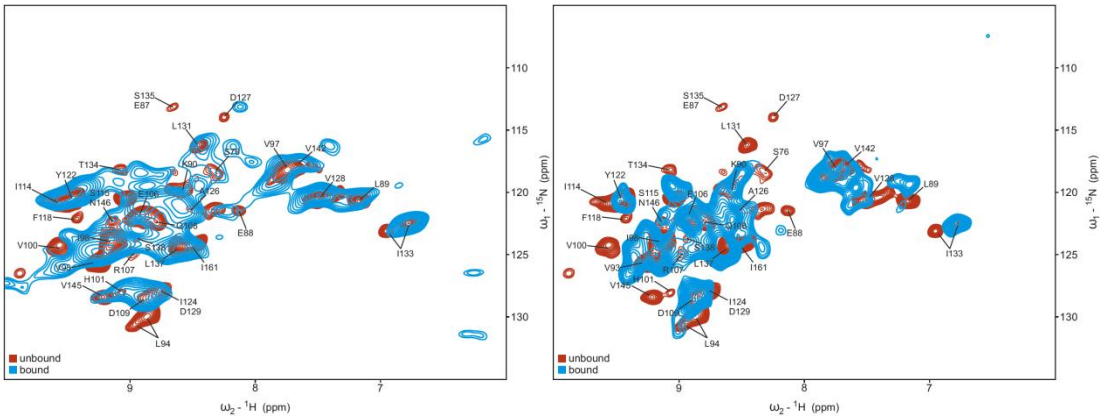


Figure 9: Interactions of αB-crystallin (red) with βB2-crystallin (blue) wildtype (left) and the variant Q70E/Q162E (right). The shown superposition of HSQC spectra represents an NMR-based binding assay. Spectra that show peaks at similar positions indicate similar structural arrangements, i.e. no distortions in the environment of individual αB-crystallin molecule and thus their chemical shifts by binding of βB2. Large differences between the red and blue spectra indicate a change in the chemical environment of the individual protein molecules, induced by binding. The blue spectrum of the complex of αB crystallin with wildtype βB2 crystallin shows most blue peaks in places very similar to red peaks, whereas the spectrum to the right shows stronger differences and lacking signals, indicating stronger binding of the variant.

4.4.2. Analysis of a nascent chain in the ribosome

In another study we used DNP MAS NMR in a structural analysis of the DsbA signal peptide inside the ribosome tunnel. As newly synthesized peptides emerge from the ribosome, certain signal sequences may recruit the signal recognition particle (SRP) onto ribosome protein L23. In this, the early adaptation already in the exit tunnel of α-helical secondary structure may potentially contribute to the SRP targeting. We used DNP to be able to observe the small amount of isotope-labeled nascent chain stalled in the otherwise unlabeled ribosome. From two-dimensional DQ-SQ correlation spectra of the stalled nascent chain we could analyze chemical shifts that may be indicative for specific types of secondary structure (Figure 10). The absence of typical α-helical chemical shifts called into doubt that any secondary structure already exists within the ribosome for the chosen DsbA signal peptide.

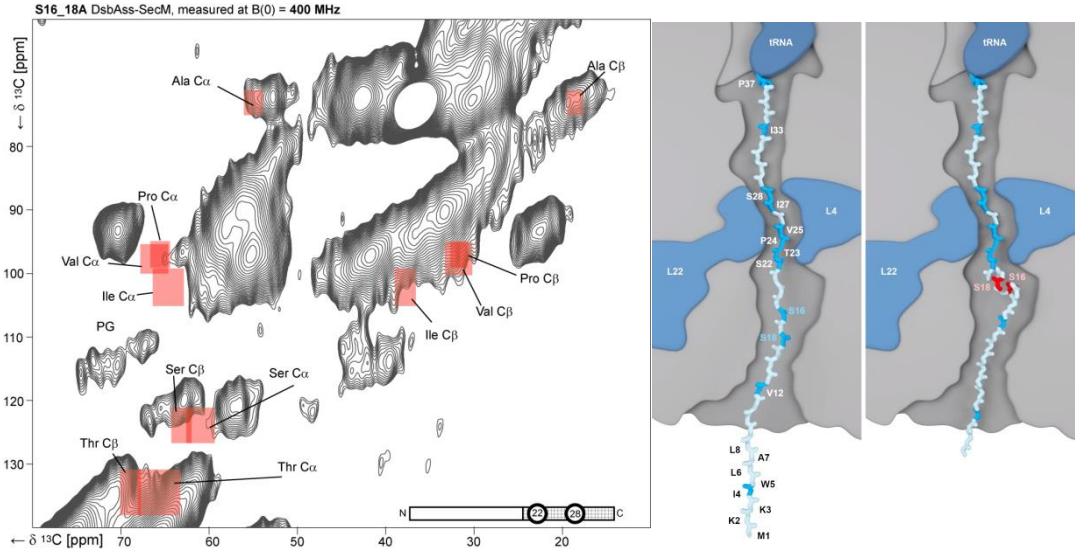


Figure 10: Left: DNP MAS NMR spectra of the selectively labeled nascent chain of the DsbA signal peptide fused to the SecM stalling sequence (MKKIWLALAGLVLAFS$_{16}$AS$_{18}$AA-FS$_{22}$TPVWIS$_{28}$QAQGIRAGP), mutant S16A, S18A, in the otherwise unlabeled ribosome. The red frames indicate chemical shift areas where signals from valines, isoleucines, prolines, serines and alanines in helices are expected. We detect only a small portion of alanines in a helical environment, and serines in the spectra of the mutant S22A, S28A, where only the signals of S16 and S18 are seen in the serine region of the spectrum. Right: Nascent chain structure. The detected chemical shifts indicate strand-like or random-coil arrangements (blue residues), with a small portion of helix in the area of S16-S18 (red).

4.4.3. Improvement of DNP technology

To achieve better DNP enhancements, we explored new bi- or multi-radicals and improved existing bi-radicals. For example, by increasing the electronic relaxation time by use of ^{2}H labeling, we could achieve a 50% gain in enhancement. Furthermore, attempts to increase the water-solubility of the radical resulted in a new molecule called bcTol that has led to a DNP enhancement of ~250. Furthermore, improving the temperature-dependence of these radicals is being explored.

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5. Patents, industry cooperation

For the duration of the project funding the focus has been on the understanding of basic mechanisms of aging and the role of proteostasis by the autophagy/ lysosome pathway. Aspects of the project have led to a proposal funded by the German Ministry of Science (BMBF) (participating PI: Volker Haucke) with the aim to test whether autophagy induction by polyamines can counteract aging-induced memory impairment. This project may well eventually lead to patent applications and cooperation with industry.

A patent on the method used to screen for ClC-7 inhibitors has recently been discontinued.

6. Cooperation partners

The project has involved intense collaborations with researchers within the Leibniz Association and outside. Prominent cooperation partners - as outlined in the original proposal - have included:

- Prof. Stephan Sigrist (FU Berlin) and Dr. Anna Fejtova / Prof. Eckart Gundelfinger (LIN, Magdeburg) with major contributions to WP1
- Prof. Bernd Bukau (ZMBH Heidelberg) in the context of investigations on ribosomes
- Prof. Snorri Sigurdsson (Rejkjavik), synthesis of biradicals for DNP studies

Collaboration within the FMP in WP2 (Jentsch/von Kries) and WP3 (Jentsch/Oschkinat)

7. Theses conducted in the course of the project

VH: none (two ongoing)

TJJ and HO: Dustin Vomund: Metabolomics analysis of lysosomes employing NMR spectroscopy; Master thesis, Freie Universität Berlin

TJJ: Ph.D. two theses on lysosomal biology and ClC-7/VRAC inhibitors ongoing

HO: Ph.D. thesis on activation and substrate binding of alphaB-crystallin ongoing, was interrupted by pregnancy and parental leave.

8. Project-related publications

Azarnia Tehran, D.*, Kuijpers, M.*, **Haucke., V. (2017)** Presynaptic endocytic factors in autophagy and neurodegeneration. *Curr. Op., Neurobiol.*, in revision [Review]

Lange, S., Franks, W.T., Rajagopalan, N., Döring, K., Geiger, M.A., Linden, A., van Rossum, B.-J., Kramer, G., Bukau, B., **Oschkinat, H. (2016)** Structure analysis of a signal peptide inside the ribosome tunnel by DNP MAS NMR. *Sci Adv* **2(8)**, e1600379.

Geiger, M.-A., Orwick-Rydmark, M., Märker, W.T., Akhmetzyanov, D., Stöppler, D., Zinke, M., Specker, E., Nazaré, M., Diehl, A., van Rossum, B.-J., Aussenac, F., Prisner, T., Akbey, Ü., **Oschkinat, H. (2016)** Temperature dependence of cross-effect dynamic nuclear polarization in rotating solid: advantages of elevated temperatures. *PCCP*, **18(44)**, 30696-30704.

Jagtap, A.P., Geiger, M.-A., Stöppler, D., Orwick-Rydmark, M., **Oschkinat, H.**, Sigurdsson, S.Th. **(2016)** bcTol: A highly water-soluble biradical for efficient dynamic nuclear polarization of biomolecules. *ChemCommun* **52(43)**, 7020-7023.

related publications not directly funded from this project:

Jentsch, T.J. (2015). Discovery of CLC transport proteins: Cloning, structure and pathophysiology. *J. Physiol*. **593,** 4091-4109. [Review]

Jentsch T.J. (2016). VRACs and other ion channels and transporters in the regulation of cell volume and beyond. *Nature Rev. Mol. Cell. Biol*., **17:** 293-307. [Review]

Lutter D., Ullrich F., Lueck J.C., Kempa S., **Jentsch T.J. (2017).** Selective transport of neurotransmitters and –modulators by distinct volume-regulated LRRC8 anion channels. *J. Cell Sci.* **130**, 1122-1133.

Kononenko, N.L., Claßen, G.A., Kuijpers, M., Puchkov, D., Maritzen, T., Tempes, A., Malik, A.R., Skalecka, A., Bera,S., Jaworski, J., **Haucke, V. (2017)** Retrograde transport of TrkB-containing autophagosomes via the endocytic adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration. *Nature Communications*, **8**, 14819. [doi: 10.1038/ncomms14819]

Kuijpers, M., **Haucke, V. (2016)** Autophagosome formation by endophilin keeps synapses in shape. *Neuron* **92**, 675-677. [Review]

Planells-Cases R., Lutter D., Guyader C., Gerhards N.M., Ullrich F., Elger D.A., Kucukosmanoglu A., Xu G., Voss F.K., Reincke S.M., Stauber T., Blomen V.A., Vis D.J., Wessels L.F., Brummelkamp T.R., Borst P., Rottenberg S., **Jentsch T.J. (2015).** Subunit composition of VRAC channels determines substrate specificity and cellular resistance to Pt-based anticancer drugs. *EMBO J.*, **34**, 2993-3008.

9. Data management and safety

Research data obtained in the course of this project will eventually be disseminated through publication in peer-reviewed international journals. The institute encourages open access publication, e.g. via selecting the open access option in journals not generally openly accessible or by publication in open access journals. Major results that have emanated from this project are still to be published.

The FMP pursues an institutional policy to store all primary resarch data related to published research on internal servers that are backed up for long-term storage and kept for 10 years.

10. List of project-related press releases or media coverage

None.