CHAPTER 6

Summary and General Discussion

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The molecular mechanisms underlying frontotemporal dementia (FTD) and Parkinson's disease (PD) are still poorly understood and the progress in promising therapeutic targets is limited. Genetics has identified pathogenic mutations in genes that cause these neurodegenerative diseases, which increased our understanding and provided new starting points for developing therapeutics. New genetic technologies such as next-generation sequencing (NGS) of whole-exome DNA and NGS-based RNA sequencing are replacing the classical "positional cloning" approaches and consequently, the attention is shifting from studying single genes to gene networks for genetically complex diseases.[1] In FTD and PD, these technological advancements have already contributed to identifying a number of genetic risk factors for sporadic forms of disease. [2,3] However, there is an urgent need to understand the molecular networks that link these genetic risk factors to disease phenotypes and the traditional reductionist approach, focusing on individual components, would be time-consuming and insufficient to interpret the full complexity of the gene networks. Multi-"omics" approaches are now being explored to understand how genetic risk factors contribute to the pathogenic mechanisms underlying neurodegenerative diseases. In this thesis I focused on functional genomics, transcriptomics and cellomics approaches to investigate pathogenic aspects of FTD and PD. The overall aim was to gain insight into gene regulation and molecular mechanisms underlying these neurodegenerative diseases. To this end, I studied the regulation of *C9orf72* expression in **Chapter 2**. In an attempt to find splicing regulators and to examine the potential of two RNA-based technologies to specifically decrease endogenous 4R tau levels, splicing of *MAPT* exon 10 was studied in **Chapter 3 and 4**. In addition, I prioritized candidate genes for PD in **Chapter 5** with RNA-interference (RNAi) screens in human cell culture and experimental animal models.

SUMMARY

In **Chapter 1**, I reviewed the literature regarding cell-based screening approaches in the context of neurodegeneration and outlined the current progress in the field. The advancements in automation of human cell culture, in combination with methods to systematically modify gene expression on a genome-wide scale, makes high-throughput cell-based screens feasible and has the potential to bridge the gap between *in vitro* and animal models. [4-6] I discussed the genetics of FTD with a focus on the disease-causing genes *MAPT* [7-9] and *C9orf72* [10,11] which, together with granulin (*GRN*) [12,13], explain the majority of familial FTD cases. [29,50] Furthermore, I reviewed the genetics of PD and outlined the general aim of this thesis.

In **Chapter 2**, I studied the regulation of *C9orf72* expression. Cap Analysis of Gene Expression sequence (CAGEseq) data was used and we observed that *C9orf72* expression is mostly low with the exception of a subset of myeloid cells, especially CD14+ monocytes,

which are involved in innate and adaptive immunity. This suggests that *C9orf72* might play a role in immune-related processes. Transcription at the *C9orf72* locus showed a complex architecture and the annotated *C9orf72* transcripts are differentially expressed across samples. We detected novel TSSs in both sense and antisense strands of the *C9orf72* locus. Notably, *C9orf72* coding transcripts show a consistent decrease not only in brain tissue and monocytes from *C9orf72*-HRE patients, but also in several brain regions from *MAPT* and *GRN* mutation carriers together with an increase in antisense transcripts. This suggests that the reduction of *C9orf72* expression is not entirely dependent on the repeat expansion and might play a more general role in neurodegeneration. Moreover, I explored the possibility of a functional interaction and performed knockdown experiments in BE(2)-M17 cells with lentiviral constructs targeting *C9orf72*, *MAPT* and *GRN*. I observed a clear influence on expression of *C9orf72* after knockdown of *MAPT* and *GRN* separately compared to a non-targeting scrambled control, which implicates that *MAPT*, *GRN* and *C9orf72* might interact in a yet unknown common pathway.

In **Chapter 3**, we performed a shRNA-based RNAi screen of the human spliceosome to determine alterations in *MAPT* exon 10 inclusion with a qPCR assay to specifically detect endogenous 4R and 3R tau levels in BE(2)-M17 neuroblastoma cell lines. To optimize the assay, knockdown of known modulators of tau exon 10 inclusion were tested. Knockdown of *STOX1* led to a consistent 2-fold increase in 4R tau transcript levels and with an average robust Z' factor of 0.57, our assay was considered "excellent". The library contained 324 shRNA pools targeting splicing-related factors and we identified 27 pools that significantly changed 4R tau transcript levels compared to non-targeting scrambled control wells, without changing total tau. Notably, we found several splicing factors previously identified to modify tau exon 10 splicing, demonstrating the strength of our approach. We then reconstructed a splicing network and revealed a total of 51 interactions among 27 genes with high confidence through a protein-protein interaction network analysis. Validation experiments must be devised to further validate and prioritize candidate genes.

In **Chapter 4**, we explored the potential of two RNA-based technologies to specifically decrease the levels of 4R tau. We designed several partly overlapping antisense oligonucleotides (AONs) to cover exon 10 and the exon/intron and exon/exon boundaries with the help of the Human Splicing Finder algorithm. The potential of both AON and short hairpin RNA (shRNA) was assessed at the endogenous level in BE(2)-M17 cells. We identified 7 AONS and 3 shRNAs decreasing 4R tau transcript levels specifically. The RNAi findings were translated to the protein level and primary cortical neurons of humanized *MAPT* mice were used to select 2 shRNAs for further studies. As proof-of-principle, we demonstrated the effect of one of the shRNAs targeting the exon 10/ exon 11 boundary in cortical neurons derived from human induced pluripotent stem cells (iPSCs).

In **Chapter 5**, we used whole exome sequencing (WES) to identify rare PD susceptibility variants. Following Sanger sequencing confirmation, we identified a total of 27 candidate genes all predicted to cause a loss of gene function. To define potential links between the 27 candidate genes and well-established mechanisms of PD susceptibility and pathogenesis, we deployed RNAi-based functional studies in both mammalian cells and experimental animal models, including *Drosophila* and *C. elegans*. We quantified mitochondrial morphology after gene knockdown in BE(2)-M17 cell lines and used the well-established Parkin translocation assay based on neuroblastoma cell lines stably expressing Parkin-GFP. Furthermore, developmental lethality and survival were tested in *C. elegans* and α-synuclein mediated toxicity was tested in *Drosophila*, the latter recapitulating features of PD-related neurodegenerative pathology. Taken together, we identified genetic evidence consistent with replication of 7 genes that were implicated by WES analysis, of which 5 were further validated based on functional screening in PD-relevant model systems. By integrating human genetic and functional evidence, interesting PD susceptibility gene candidates were identified for further investigation.

DISCUSSION AND FUTURE PERSPECTIVES

Towards understanding the regulation of *C9orf72* expression and its role in FTD pathogenesis

We studied the transcriptional activity of the *C9orf72* locus using CAGEseq, a technique providing high-resolution strand-specific profiling of transcription start sites (the 5' end of capped transcripts) in a quantitative manner. [14] This technique allowed us to identify coding, non-coding and novel transcripts. The strength of CAGEseq data is its ability to perform high-throughput gene expression profiling with simultaneous identification of tissue and/or cell specific transcriptional start sites.[15] The library preparation we used for CAGEseq includes a size selection and is not suitable for short transcripts (<200bp) and therefore some transcripts might have been missed in our study. Furthermore, we used post-mortem brain as input and consequently we might have missed transcripts prone to RNA degradation. We found that the expression profile at the *C9orf72* locus is more complex than previously described and we observed multiple new sense and antisense TSSs. To fully understand how *C9orf72* transcription is regulated and what this means on a functional level, further detailed analysis will be necessary. For example, it would be interesting to explore the role of the novel sense and antisense transcripts we described. Antisense transcription has been described as an important regulator of gene expression and these transcripts could, for example, interfere with transcription, translation or mRNA stability of other transcripts at the *C9orf72* locus or elsewhere in the genome. [16] It has been shown

previously that both sense and antisense *C9orf72* transcripts can be translated to proteins and that both sense and antisense repeat-containing RNA can accumulate in nuclear foci in the brain. [17,18] Interestingly, Cooper-Knock and colleagues found that antisense foci in motor neurons of *C9orf72*-ALS patients correlated with mislocalized TDP-43, a hallmark of ALS and some FTDs.[19] They examined different types of neurons and observed a consistent relationship between sense and antisense foci in each cell type. For example, purkinje and motor neurons always contained more antisense than sense foci, whereas granule neurons consistently had more sense foci. This pattern was similar for dipeptide repeat (DPR) proteins in granule and motor neurons. Further experiments, focused on extending the findings from post-mortem tissue to model systems, will be necessary to fully understand these correlations. For therapeutic development, it will be important to consider strategies that target both sense and antisense repeat-containing RNAs.

Recently, several new transgenic mouse models have been developed, which will be valuable for studying the pathogenic mechanisms of FTD/ALS and testing potential therapeutics.[20] Two BAC transgenic mouse models carrying (part of) the human *C9orf72* gene, including expansion, showed formation of RNA foci and DPR proteins but no neurodegeneration or behavioral deficits. [21,22] On the other hand, two different BAC transgenic mouse models that use patient-derived *C9orf72* gene constructs showed FTD/ALS related phenotypes. The mice developed cognitive and motor abnormalities and showed reduced survival. [23,24] The phenotypic differences in these new mouse models can have several reasons, such as the specific promoter and transgene/flanking sequences used, size of the expansion, integration site and/or genetic background of the mouse strain.[25] Further research, comparing the models thoroughly and focusing on cellular as well as behavioral phenotypes, should determine which mouse model best recapitulates pathogenic mechanisms of FTD/ALS. The mouse model developed by Jiang et al. has already been used to test AONs targeting repeat-containing RNAs and showed reduced formation of RNA foci and DPR proteins and decreased behavioral abnormalities.[23] Several other antisense oligonucleotides lowering GGGGCC repeat-containing RNA have been tested as well.[26,27] In a recent publication, it was found that targeting transcription elongation factor Spt4 decreased production of both sense and antisense *C9orf72* transcripts with expanded repeats.[28] Spt4 knockdown also decreased DPRs and mitigated degeneration in animal models. Knockdown of SUPT4H1 (the mammalian ortholog of Spt4) with small interfering RNAs decreased production of both sense and antisense RNA foci and DPR proteins in patient fibroblasts. Originally, this gene was found in a phenotypic screen for modifiers of expanded polyglutamine stretches in yeast and further studies showed that AONs reducing Supt4h in mouse models of Huntington's disease decreased huntingtin protein aggregation,

prolonged lifespan and delayed motor abnormalities. [29,30] This finding demonstrates that phenotypic screening in combination with functional follow-up is a promising strategy to identify potential therapeutic targets for neurodegeneration.

In our study, we observed a decrease in *C9orf72* expression in material from carriers with pathogenic *MAPT* and *GRN* mutation carriers and explored the possibility of an interaction between these genes. Interestingly, we observed a reduction of total *C9orf72* after knockdown of *MAPT*, while knockdown of *GRN* led to increased expression of *C9orf72*. On the other hand, *C9orf72* knockdown led to a reduction in *MAPT* expression and an increase of *GRN* expression. This might suggest that *C9orf72*, *MAPT* and *GRN* interact in a common pathway. The current experiment was done in BE(2)-M17 neuroblastoma cells and should be repeated in a model system closer to patients, such as human iPSCs. Furthermore, experiments are necessary to confirm our findings and to investigate whether the observed interaction is direct or indirect. Nonetheless, in transcriptome profiles of *C9orf72* cases with clinical diagnosis of ALS it has been observed that exon 10 of *MAPT* was more prone to be spliced out of transcripts, which would disrupt the ratio of 4R tau and 3R tau proteins and might be an interesting finding to support this interaction. [31] In a recent study, Ferrari and colleagues characterized 12 known FTD-genes including *C9orf72*, *MAPT* and *GRN* by providing insight into their brain region-specific co-expression patterns and they revealed that *MAPT* and *GRN* were included in the same module (where *MAPT* was a hub gene).[32] This work is a nice example of shifting from a reductionist cell biology strategy to a gene network approach.

In addition, our results suggest a possible role of *C9orf72* in the immune response. In our analysis, expression of *C9orf72* is high in a subset of myeloid cells and low in the remaining cell types and tissue samples we tested, including CNS. Recently, three studies found that mouse lines lacking *C9orf72* in all tissues develop a robust immunological phenotype. [33-35] One of the studies reported defects in late endosomal/lysosomal trafficking and it has been shown that depletion of *C9orf72* in neuronal cell lines shows inhibition of endocytosis and dysregulation of autophagy.[34,36] This will have important implications for therapeutic strategies reducing *C9orf72* levels. Previous reports using AONs in mice revealed an upregulation of immune markers in the nervous system.[27] Specific targeting of transcripts containing the repeat would be a promising strategy to avoid an *C9orf72*-related immune response.

Towards the modulation of endogenous *MAPT* exon 10 splicing and therapeutic development

In this thesis, I focused in two chapters on *MAPT* exon 10 splicing, as mutations in *MAPT* are a major cause of FTD and a large subgroup affects alternative splicing of exon 10. [7-9] Alternative splicing is carried out by the spliceosome and regulated by a network of

trans-splicing factors that bind to *cis*-elements.[37] We systematically analyzed the effect of knockdown of 324 components of the splicing machinery on *MAPT* exon 10 inclusion. The 27 potential modifiers of *MAPT* exon 10 inclusion we identified might include new targets for therapeutic development. The inclusion of known regulators of *MAPT* exon 10 splicing suggests that the approach captures important aspects of *MAPT* exon 10 splicing regulation. The major limitation of this study is the use of shRNA pools; validation experiments will therefore be necessary to further confirm and prioritize candidate genes. We used multiple shRNAs to improve the chance of having at least one potent shRNA per gene and to increase the effectiveness of gene silencing. On the other hand, this approach also increases the possibility of seed matches and (microRNA-like) off target effects. [38] To follow up this study we could perform deconvolution experiments or use independent shRNA reagents in a secondary screen and determine whether the level of knockdown correlates with the level of *MAPT* exon 10 inclusion. Furthermore, rescue experiments still represent the gold standard for validation of RNAi screening results and therefore we might use shRNA-resistant cDNA to rule out off-target effects.[38] If available, it would be valuable to test small molecule inhibitors targeting selected splicing factors and determine how these correlate to the level of *MAPT* exon 10 inclusion, as this would be a good starting point for therapeutic development. To further explore the selectivity of the candidate genes for *MAPT* exon 10 splicing, we could test the effect of knockdown on gene expression and splicing of other genes with RNA sequence analysis, as has been done by Naryshkin and colleagues for survival of motor neuron-2 (*SMN2)* splicing modifiers. [39] For promising candidate genes we should also determine potential (toxic) off-target effects of gene perturbation, which can be tested with RNA sequence analysis as well (e.g. to investigate deregulated expression of gene families involved in cell survival) and with cell viability, proliferation and apoptosis assays. In addition to sequence-specific off-target effects, double-stranded RNA can also activate innate immune receptors, triggering inflammatory and interferon responses, but it has been reported that specific chemical modifications can abrogate binding of these receptors.[40] As previously mentioned, small molecules (indirectly) altering *MAPT* exon 10 splicing would be a good alternative approach for short hairpins. For example, recent advances in designing small molecules targeting RNA would be interesting to further explore.[41] Ultimately, a detailed knowledge of the splicing factors that are crucial for *MAPT* exon 10 splicing and how they cooperate with other spliceosomal components will help the design of new therapeutics. If I would re-design the study, targeting the splicing reaction remains an attractive strategy for therapy because it is an early step in gene expression and intervention does not alter the genome. In addition, the dynamics of the transcriptome allow for a rapid effect on a specific target and (iPS) cell lines derived from patients can identify potential off-target effects at an early stage. For example, several small molecule inhibitors are currently in preclinical development for the treatment of spinal muscular atrophy (SMA),

a motor neuron disease caused by the loss of both copies of the *SMN*1 gene. Naryshkin and colleagues used HEK cells containing an *SMN2* minigene construct to screen a library of small molecules for compounds that promoted the inclusion of exon 7 into *SMN2* mRNA transcripts.[39] They identified three orally available compounds that increased SMN protein biosynthesis in patient fibroblasts and iPSC-derived motor neurons. [39,42] They also found a substantial benefit of these compounds in animal models of SMA. Furthermore, *SMN2* exon 7 inclusion has been restored using small interfering RNAs reducing splicing factors hnRNP-A1 and -A2 in cells expressing a transfected minigene, indicating the therapeutic potential of RNAi strategies.[43]

In addition to the shRNA library screen of the human spliceosome, we tested a more directed approach and used two RNA-based technologies to specifically decrease the levels of 4R tau. Several AONs and shRNAs effectively decreased 4R tau transcript levels in BE(2)M17 cells and for shRNAs we extended our findings in primary cortical neurons from htau mice and human iPSC-derived cortical neurons. In our shRNA-based experiments we used lentiviral-based vectors, which have been tested previously in clinical trials for neurodegenerative diseases. However, recombinant adeno-associated virus (AAV) has become the vector of choice for several therapeutic applications due to its serotype-dependent cell tropism, non-pathogenicity and low immunogenicity. They are promising vectors and have been shown in a number of human clinical trials to deliver therapeutic genes to a variety of organs and tissues, including the CNS. Nonetheless, it remains important to tailor expression and mimic physiological conditions to minimize side effects, such as transgene toxicity and (capsid directed) immune response. [44,45] Notably, an AAV9-based gene therapy for SMA has shown great promise in an ongoing Phase 1 clinical trial (NCT02122952).[46,47] In follow-up experiments it would therefore be preferable to switch to an AAV-backbone, especially when we would like to perform *in vivo* experiments as well.[48,49] Very recently, the first therapeutic using RNAi was headed for review by the Food and Drug Administration (FDA). The drug "patisiran" is developed for the rare nerve disease familial amyloid polyneuropathy and recently succeeded in a Phase 3 clinical trial. [50] This is very promising and will pave the way for other future therapeutics based on RNAi.

In our AON-based experiments, the validation of the selected sequences has been performed in human neuroblastoma cells only. We tried transfection in human "iCell" neurons as well, but the efficiency of FAM-labeled AON ranged between 5 and 15% and this was not sufficient to detect changes in 4R tau transcript levels. We tested the transfection of these human neurons in a pilot experiment with Lipofectamine and nucleofection using the Amaxa 4D-Nucleofector and we think that optimizing the parameters for electroporation-based transfection will improve the efficiency. Notably, nucleofection has been described in several publications to deliver AONs to iPSC-derived neurons.[51,52] Optimizing the parameters for chemical modification of oligonucleotides, which has been done for other

AONs before advancing to clinical development,[53] might improve transfection efficiency of AONs in human neurons as well. In general, it has been described that the delivery of AONs to target tissues and specific cell types is challenging.[54] Proposed delivery systems include liposomes, polymers, cell-penetrating peptides and nanoparticles. [55] AAV vectors have also been described as an alternative delivery strategy for antisense sequences. For example, in pre-clinical work for Duchenne muscular dystrophy (DMD), AONs have been delivered using AAV vectors.[56-58] An important example for AONs in clinical trials is nusinersen, the first treatment for SMA. Nusinersen is an AON that restores production of full-length SMN protein in motor neurons by correcting *SMN2* splicing. In previous studies the AON rescued a severe SMA mouse model and the drug has been approved by the FDA. [59,60] For the treatment of DMD, a fatal muscle degenerative disorder arising from mutations in the dystrophyn gene, AON-based therapy eteplirsen has been approved by the FDA. Eteplirsen targets splicing elements in DMD pre-mRNA and induces exon skipping, restores the open reading frame and thereby restores the production of functional dystrophin directly translating into a slower rate of decline.[61-63]

Translating these studies to *MAPT* will be challenging due to the tight regulation of the 4R/3R tau ratio; expression and splicing is developmentally regulated and shows regional differences. [64,65] However, also *SMN* expression is developmentally regulated and the first AON-based therapy has been approved by the FDA to treat both children and adults with SMA.[66] Recently a potent AON targeting *MAPT* has been designed in collaboration with the pharmaceutical company Ionis, which decreases human tau gene expression in transgenic mice expressing mutant P301S human tau. [67] Mice treated with the AON showed less neurodegeneration, less behavioral deficits and an increase in life-span compared to control animals. Furthermore, intrathecal delivery of the tau AON was successful in nonhuman primates and significantly reduced the levels of tau in the brain and CSF. In a recent publication, Schoch and colleagues used AONs to manipulate the 4R/3R tau ratio in humanized *MAPT* (htau) mice and showed that increasing 4R tau in the htau mouse model increases tau phosphorylation and behavioral abnormalities. [68] Decreasing 4R tau was also achieved and suggests application of the AON-based strategy described in this thesis in human tauopathies. In contrast to a total tau reduction approach as published by DeVos and colleagues, the *MAPT* splicing strategy described in this thesis would not affect total tau levels, thereby avoiding potential side-effects associated with a loss of tau.[69-71] In future experiments we could test whether, using (a combination of) the potential splicing modifiers identified in our screen and the RNA-based sequences specifically reducing 4R tau transcript levels, we can diminish tau pathology in one of the mouse models for human tauopathies.

Functional screening and prioritization of disease candidate genes

In our study we coupled WES analysis in PD to functional studies in both mammalian cells and experimental animal models. Exome sequencing targets protein-coding regions comprising approximately 1% of the human genome, but accounting for about 85% of mutations identified in Mendelian diseases.[72,73] WES technologies accelerated the genetic studies of Mendelian diseases and is being extended to complex diseases as well. [74] In combination with functional screening I think this is a promising approach for future studies. For functional screening we used human neuroblastoma cell lines and *C. elegans* and *Drosophila* experimental model systems. Invertebrate animal models provide a simple and powerful tool to test the potential impact of our candidate genes on molecular processes underlying PD, although not all molecular pathways exist in these animal and a fraction of human orthologs are missing.[75,76] Neuroblastoma cell lines are a robust model system but the cells are undifferentiated and immortalized, which is a disadvantage for modelling human neurons. Besides this, neuroblastoma cells proliferate in culture and this inevitably leads to increased rates of genetic and epigenetic changes.[77] More recently, developments in reprogramming and transdifferentiation made functional screening in patient-derived neurons possible.[78] This allows the use of cells with the same genetic background as the patients' own cells. In future studies we could differentiate iPSCs from individuals with PD into, for example, dopaminergic neurons or any other relevant cell type.

In our experiments, we focused on a well-established Parkin translocation assay and several phenotypes related to mitochondrial morphology.[79-83] Future studies could examine potential genetic interaction in iPSCs from *Parkin* mutation carriers [84-86] and/or PINK1 mutation carriers,[87-90] in which several mitochondrial abnormalities have been reported. [91] In addition, iPSCs from a PD patient with a triplication of the *SNCA* locus also show several interesting phenotypes for functional screening, such as, delayed maturation and dysregulation of autophagy.[92] Already, several models have been developed from patient-derived neurons that show disease-related phenotypes and functional screens can be conducted in these cells.[78,93] Imaging analysis pipelines are developing rapidly and it is now possible to define phenotypic signatures of iPSCs based on live-imaging data. [94] Furthermore, it is possible to generate stable, highly expandable iPSC-derived neural progenitor cells and rapidly differentiate them into cortical-like neurons to perform high-content automated microscopy assays.[95] Another exciting development is the use of stem-cell-derived three-dimensional cultures that recapitulate aspects of cortical development, also known as "organoids". [96-98] These organoids can be grown from human stem cells and patient-derived iPSCs, which can help to understand brain development, the causes of human disease and the potential of new therapeutics. In combination with CRISPR/Cas9 this development could have a great impact on functional modeling of human brain disease in a patient-specific manner.[99] For example, this approach has been used to generate "cerebral

organoids" and model human brain development, recapitulating features of human cortical development. RNA interference and patient-specific iPSCs were already used to model microcephaly [100] and another study used forebrain organoids derived from patient iPSCs to study autism. [101]

FINAL REMARKS

In this thesis, I showed that the regulation of *C9orf72* transcription is complex and it is likely that this gene plays a role in immune-related processes. Detailed analysis will be necessary to study the exact function of *C9orf72* and in particular the molecular mechanisms underlying FTD/ALS. In addition, we performed a shRNA library screen of the human spliceosome and used two RNA-based technologies to find regulators of *MAPT* exon 10 splicing and decrease endogenous 4R tau levels, respectively. Notably, antisense technology has great potential with the first therapies for DMD and SMA recently approved by the FDA and also RNAi-based therapy is promising with the first therapy heading for FDA review. We also provided an integrated approach to identify and prioritize novel PD genes. We believe that the combination of high-throughput sequencing and functional screening is a promising strategy for future genomic studies in PD and other neurodegenerative diseases. Together, the data presented in this thesis contributes to our understanding of mechanisms involved in neurodegeneration and ultimately may provide new targets and strategies for therapeutic development.

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