**Protein changes in hnRNPA1-diseased muscle guide toward the identification of the underlying molecular genetic defect and provide insights into the underlying pathophysiology:**

Despite the considerable advances in sequencing technologies, a significant proportion of patients with rare genetic disorders including neuromuscular disorders do not receive a genetic diagnosis after exome sequencing which became a molecular genetic standard in the diagnostic work-up of patients. Proteogenomics is being frequently established in the pre-clinical research of Mendelian disorders and provides opportunities regarding the understanding of molecular interplay between DNA variants and protein dysregulations.[9] The relevance of this aspect for the evaluation of ambiguous variants such as amino acid substitutions of unknown pathogenic significance is evident and thus a proteomic signature might even hold the potential to predict or pinpoint the underlying defective gene. Here, by overcoming the limitation of availability of native muscle for protein extraction via utilization of proteins lysates containing Laemmli sample buffer and beta-mercaptoethanol, we generated a proteome profile which guided toward the identification of a defective gene causative for neuromyopathy in a toddler: we identified an increase of hnRNPA1 as a pathobiochemical finding which has been linked to the presence of dominant disease-causative variants in the corresponding gene before. Indeed, a careful re-analysis of the exome data unravelled the presence of an in-frame deletion affecting exon 7 of the H*NRNPA1* gene. Successful utilization of muscle protein lysates originally targeted for immunoblot analysis for our proteomic study opens new avenues in the diagnostic management of (myopathic) patients where frozen biopsies or formalin-fixed and paraffin-embedded material is no longer available.

However, apart from the identification of the molecular genetic defect based on the proteomic signature, our mass spectrometric findings also enabled to obtain insights into the overall neuromyopatholgy:

Indication of membrane leakage in hnRNPA1-related neuromyopathy

Creatine kinase M-type (CK) is commonly assayed in blood tests as a marker of membrane-damage of CK-rich tissue such as skeletal muscle. Interestingly, it has been described that muscular CK-upregulation might represent a compensatory strategy to antagonize the leakage of the protein in muscular dystrophies.[8] This protein is moderately elevated in blood (up to 400 U/l) as well as in the skeletal muscle of our patient (Tab. 1). In addition, lesion of the sarcolemma in terms of a pathophysiological permeability is indicated by increase of TRIM72, a muscle-specific protein that plays a crucial role in cell membrane repair by nucleating the assembly of the repair machinery at injury sites.[2]

Indication of oxidative stress in hnRNPA1-related neuromyopathy

TRIM72 not only functions as a membrane repair protein but thereby also as a sensor of oxidation and our proteomic data moreover suggest increased oxidative stress burden of myofibers by increase of AKR1B1, GSTM2, MDH1, NDUFA4 as well as PRDX1 and 6 (Table 1). Also, changes of mitochondrial architecture – along with the altered abundance of 20 mitochondrial proteins (Table 1) – accord with the conjecture of oxidative stress burden as part of the hnRNPA1-myopathology. Of note, Suzuki and colleagues already reported that a low-grade excess of hnRNPA1 expression causes cytotoxicity by activating the mitochondrial apoptosis pathway.[11]

Indication of protein aggregation in hnRNPA1-related neuromyopathy

Given that hnRNPA1 function is crucial for proteasome assembly[10], one might speculate that the intragenic deletion of exon 7 (reducing the pool of functionally active hnRNPA1) impacts on the cellular protein clearance machinery. Indeed, increase of polyubiquitin-B and ubiquitin-conjugating enzyme E2 N (Table 1) supports this assumption along with the pathomorphological build-up of massive vacuolar protein aggregates (Figure 1). In addition, oxidative stress burden (see above) triggers the modification of protein thiol side chains that may affect protein folding and lead to the build-up of misshaped proteins[12]. Interestingly, different chaperones such as HSPA1B/A2/A5/A8/A9/B3, HSP90AA1/AB1, PPIA and VAPA are also increased most likely acting towards the antagonization of protein aggregate formation. It is very likely that the impaired proteolysis (associated with the build-up of protein aggregates as demonstrated by our combined microscopic studies) reflects a pathophysiological event arising from reduction of functional hnRNPA1 and build-up of protein aggregates triggered by expression of the mutant allele (as proven by our transcript studies). However, further studies such as examinations on cultured muscle cells depleted for *HNRNPA1* expression are needed to address the impact of reduced availability of hnRNPA1 on proper proteasomal function.

Indication of cytoskeletal vulnerability in hnRNPA1-related neuromyopathy

The presence of tubulofilamentous deposits might correlate with the increased abundance of a variety of cytoskeletal proteins including different myosin heavy chains and myosin-binding proteins (MYBPH, MYH1, MYH11, MYH3, MYH8, MYH9, MYL4, MYL5), actin and actin-related proteins, tubulin chains (TUBA4A, TUBB, TUBB4B, TUBB6), and myopalladin that tethers nebulin together (Table 1). Interestingly, mutations in myosin heavy chain proteins (MYH2 and 7) as well as in desmin cause a distinct class of myopathies that are characterized by deposition of tubulofilamentous deposits and the build-up of marked autophagic vacuoles filled with electron-dense material and pathological desmin immunoreactivity was identified by our immunohistochemistry studies. Decreased abundance of synaptopodin (SYNPO) - an actin binding protein, which regulates synaptic plasticity[5] , might hint toward the nervous system abnormalities present in our patient. Given that abnormal hnRNPA1 fibrilization has been described in the context of different dominant variants of clinical significance[1], one might speculate that the observed tubulofilamentous deposits may also (partially) correlate with this effect based on the presence of a mono-allelic in-frame deletion affecting exon 7 of the *HNRNPA1* gene in our case. Further functional studies would be needed to address this speculation.

Indication of activated rescue mechanisms in hnRNPA1-related neuromyopathy

Remarkably, altered expression of different proteins with known protective functions in muscle fibers or the nervous system such as GOT1, GOT2, VAPA, KLHL41 and RYR1 hint towards the activation of compensatory/ protective mechanisms in our hnRNPA1-patient[3],[4] (Table 1). Interestingly, the proteomic signature of hnRNPA1-diseased muscle also indicatives an enhanced glycolysis which might in turn reflect a further antagonizing strategy of diseased muscle fibers (Table 1). Decrease of general protein production is a common hallmark of neurological disorders associated with build-up of misfolded proteins[7] and indicated by our proteomic findings: as a total of four different histone-types were upregulated in the patient muscle fibres – most likely leading to chromatin condensation – altered transcriptional regulation of proteins is suggested. This might be a protective consequence of impaired pre-mRNA packaging into hnRNP particles and disturbed myonuclear export of mRNA due to the hnRNPA1 mutation. Along this line, increase of NCL, a well-known binding partner of an hnRNPA1 inhibitor[6] as well as elevation of eIF2α and decrease of eIF4β support this assumption.

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