Hsp70 machinery vs protein aggregation
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Chapter 4

DNAJB6 mutants causing limb-girdle muscular dystrophy diversely alter its anti-aggregation activity

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Abstract

DNAJB6 is a co-chaperone of the HSP70 system that can suppress aggregation of various aggregation prone proteins including polyglutamine and mutant parkin. Several mutations in the DNAJB6 gene have been identified as the cause of limb-girdle muscular dystrophy type 1D (LGMD1D), an autosomal dominant myopathy. Both isoforms expressed from DNAJB6 gene, the nuclear DNAJB6a and the cytoplasmic/nuclear DNAJB6b, carry the LGMD1D-associated mutations within a conserved G/F-rich region of uncertain function. Previous studies reported conflicting results regarding the impact of these mutations on the function of DNAJB6 against protein aggregation. Here, we show that DNAJB6b mutants exhibit only moderate loss of activity against polyQ or mutant parkin aggregation, a defect that is only detectable under high aggregation conditions. Surprisingly, we found that a LGMD1D-associated mutant of DNAJB6a, the nuclear isoform, could suppress cytoplasmic polyQ aggregation better than the wild type DNAJB6a. Our data confirm previous studies showing that LGMD1D mutations on DNAJB6 gene can have a negative impact on the function of DNAJB6b but not DNAJB6a.
Altered anti-aggregation capacity of DNAJB6 LGMD mutants

**Introduction**

DNAJB6 belongs to the DNAJ family of molecular chaperones that mainly act as co-factors of HSP70s, the central chaperones involved in protein quality control, by aiding and modulating the activity of HSP70s and recruiting unfolded or misfolded substrates to them (Kampinga and Craig, 2010). Although little is known about its endogenous substrates, DNAJB6 has been identified as a very effective suppressor of protein aggregation, including polyglutamine (polyQ) (Gillis et al., 2013; Hageman and Kampinga, 2009; Kakkar et al., 2016b; Månsson et al., 2014b), mutant parkin (Kakkar et al., 2016a), amyloid (Månsson et al., 2014a) and -synuclein (Aprile et al., 2017b).

DNAJB6 is ubiquitously expressed in human tissues (Hageman and Kampinga, 2009) in two splice variants: a 326 amino acid long DNAJB6a, that harbours a C-terminal nuclear localization signal (NLS) and resides in the nucleus, and a 241 amino acid long DNAJB6b, that is both nuclear and cytoplasmic (Hanai and Mashima, 2003). All DNAJ family members, and hence also DNAJB6, are characterized by a conserved J domain, within which a crucial His-Pro-Asp (HPD) motif is responsible for interaction with HSP70s and stimulation of their ATPase activity (Tsai and Douglas, 1996). Besides a J domain, DNAJB6 contains a Gly/Phe (G/F) -rich region of unknown function and a distinct C-terminal substrate binding domain, in which a Ser/Thr (S/T) -rich stretch was found to be required for binding to polyQ proteins (Kakkar et al., 2016b) (Fig 1).

Recently, several mutations were identified in the gene that encodes DNAJB6 as the cause of limb-girdle muscular dystrophy type 1D (LGMD1D), a dominant late-onset muscle disease (Couthouis et al., 2014; Harms et al., 2012; Nam et al., 2015; Palmio et al., 2015; Ruggieri et al., 2015; Sarparanta et al., 2012; Sato et al., 2013; Suarez-Cedeno et al., 2014; Tsai et al., 2017; Yabe et al., 2014), reviewed in (Ruggieri et al., 2016). The disease pathology

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**Figure 1. DNAJB6 mutants causing LGMD1D.** Scheme of the functional domain of the two DNAJB6 isoforms, a and b. In the G/F-rich domain, exon 5 deletion and the single amino acid substitutions (red) that cause LGMD1D are indicated.
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is characterized by large rimmed vacuoles and cytoplasmic protein aggregates in muscle cells, including DNAJB6 itself (Harms et al., 2012; Sandell et al., 2016). All of the LGMD1D-related mutations reside in the G/F-rich region of DNAJB6. Most of these are point mutations that lead to a substitution of one of the (usually Phe) residues (F89I, F91I/L, F93I/L, P96R/L, F100V) and there is one splicing defect causing-mutation that results in exon 5 skipping and a complete loss of the G/F-rich domain (ΔG/F) (Fig 1, Table 1). This G/F-rich region, which is found in all DNAJAs and DNAJBs (Kampinga and Craig, 2010), is a structurally disordered flexible region (Pellecchia et al., 1996). Although its purpose has not been clearly determined yet, it has been previously suggested to be critical for activity of certain DNAJs in yeast (Yan and Craig, 1999) or play a role in substrate recruitment or transfer to HSP70s (Perales-Calvo et al., 2010; Stein et al., 2014; Wall et al., 1995).

As stated above, the polyQ anti-aggregation activity of DNAJB6 is highly dependent on a S/T-rich region which serves as a polyQ peptide binding site (Hageman et al., 2010; Kakkar et al., 2016b) and interestingly enough, DNAJB6b mutants lacking the entire G/F-rich region were found to be largely unaffected in suppressing polyQ aggregation (Hageman et al., 2010). Contradictory to these findings though, other studies showed that the disease-associated G/F-rich region mutants do cause a defect in DNAJB6b activity to prevent aggregation of either polyQ aggregates (Palmio et al., 2015; Sarparanta et al., 2012; Tsai et al., 2017) or TDP-43 inclusions after heat shock (Stein et al., 2014).

Moreover, despite both splice variants bearing the mutations, only expression of DNAJB6b isoform’s F93L mutant has been shown to generate an abnormal muscle phenotype in LGMD1D animal models; animals expressing DNAJB6a^F93L mutation did not show any disease phenotype (Bengoechea et al., 2015; Sarparanta et al., 2012). Therefore, DNAJB6a mutants have not been considered as contributing factors to the disease development. It is, however, unclear if the function of DNAJB6a is altered by the mutations.

Our aim was to unravel this apparent controversy on DNAJB6b mutants, concerning their effect on polyQ aggregation, and to further elucidate the impact of LGMD1D-related mutations on DNAJB6b and DNAJB6a functions. Hereto, we investigated whether these mutations disturb DNAJB6a or DNAJB6b anti-aggregation activity using polyQ and mutant parkin aggregation models. We confirmed that some LGMD1D-associated DNAJB6b mutants show an impaired aggregation suppressing activity, suggesting that the G/F-rich region does contribute to normal DNAJB6b function. Unexpectedly, we found that a LGMD1D-associated mutant of the nuclear isoform, DNAJB6a, led to a decrease of cytoplasmic polyQ aggregation.
Results

DNAJB6b mutations only moderately impair its anti-aggregation function

Mutations in DNAJB6b were previously reported to cause impairment in the protein’s ability to suppress polyQ (Q109/Q120) aggregation but the magnitude of defect seemed to vary between these studies as well as between the different mutational variants (Palmio et al., 2015; Sarparanta et al., 2012; Tsai et al., 2017, summarized in Table 1).

Table 1. Disease severity and anti-aggregation activity impairment of DNAJB6b LGMD1D-associated mutants.

<table>
<thead>
<tr>
<th>DNAJB6b mutant</th>
<th>Disease severity</th>
<th>PolyQ120 anti-aggregation activity impairment</th>
<th>Parkin anti-aggregation activity impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Previously published</td>
<td>Previously published Current study</td>
<td>Previously published Current study</td>
</tr>
<tr>
<td>F89I</td>
<td>Intermediate 1,2,3</td>
<td>Intermediate 1,2,3 Mild</td>
<td>Mild</td>
</tr>
<tr>
<td>F91I</td>
<td>Severe 4,5</td>
<td>Mild 4</td>
<td>Mild</td>
</tr>
<tr>
<td>F91L</td>
<td>Severe 4,5,6</td>
<td>Intermediate 4</td>
<td>Mild</td>
</tr>
<tr>
<td>F93I</td>
<td>Mild 1,2,3</td>
<td>Mild 1,2,3</td>
<td>Intermediate 10</td>
</tr>
<tr>
<td>F93L</td>
<td>Mild 1,2,3</td>
<td>Mild 1,2,3</td>
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</tr>
<tr>
<td>P96R</td>
<td>Intermediate 8</td>
<td>Intermediate</td>
<td>Mild</td>
</tr>
<tr>
<td>P96L</td>
<td>Intermediate 11</td>
<td>Severe 11</td>
<td>Mild</td>
</tr>
<tr>
<td>ΔG/F</td>
<td>Severe 5</td>
<td></td>
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</table>

*Anti-aggregation activity impairment level was calculated according to average % of activity lost relative to WT activity. Mild <20%, Intermediate 20-50%, Severe >50%. Data used are from the above-mentioned references or the current study.

We directly compared some of these mutants, DNAJB6b\textsuperscript{F89I} and DNAJB6b\textsuperscript{F93L}, and also included DNAJB6b\textsuperscript{P96R} mutant that has not been previously tested. Firstly, using fluorescence microscopy, we examined the subcellular distribution of GFP-tagged mutants; DNAJB6b\textsuperscript{WT} and all the mutants showed similar expression pattern throughout the cell in HEK293 cells (Fig S1A). Since these mutants are associated with muscle defects, we additionally expressed DNAJB6b mutants in C2C12, a muscle-derived cell line. Similar to what we observed in HEK293 cells, DNAJB6b\textsuperscript{WT} and the LGMD1D-associated mutants did not show any divergence in their subcellular localization (Fig S2B).

To assess the capacity of DNAJB6b mutants to suppress polyQ aggregation, we co-expressed V5-tagged DNAJB6b, wild type or the three mutants, with exon-1 fragment of the huntingtin protein that contains an expansion of 74 glutamines (GFP-polyQ\textsuperscript{74}). Subsequent analysis of GFP-polyQ\textsuperscript{74} aggregation by filter trap assay demonstrated the great potential of DNAJB6b\textsuperscript{WT} to suppress polyQ aggregation, as previously described (Hageman et al., 2010). In contrast to our expectations, all DNAJB6b mutants exhibited suppressive activity of polyQ\textsuperscript{74} aggregation similar to DNAJB6b\textsuperscript{WT} (Fig 2A). Similar results were obtained using an exclusively nuclear polyQ\textsuperscript{74} (GFP-polyQ\textsuperscript{74}-NLS): DNAJB6b\textsuperscript{F93L} and DNAJB6b\textsuperscript{F89I} were equally good in suppression aggregation as DNAJB6b\textsuperscript{WT} while DNAJB6b\textsuperscript{P96R} on average showed a trend (albeit not significant) to be less effective (Fig 2A), suggesting that nuclear function of the mutants was also largely unaffected. Interestingly, the expression levels of DNAJB6b\textsuperscript{F93L} and DNAJB6b\textsuperscript{P96R} levels were significantly elevated compared to DNAJB6b\textsuperscript{WT} (Fig 2B), suggesting that these mutations lead to DNAJB6 accumulation.

As polyQ aggregation propensity is proportionally linked to polyQ expansion size (Bennett et al., 2002; Chen et al., 2002; Georgalis et al., 1998; Scherzinger et al., 1999), we used a longer polyQ expansion with 119 glutamines (polyQ\textsuperscript{119}-YFP) to examine whether increased aggregation would reveal some defects for the DNAJB6b mutants. Whereas the average aggregation suppressing performance of DNAJB6b\textsuperscript{F89I} was lower than that of DNAJB6b\textsuperscript{WT}, there was no statistically significant difference from DNAJB6b\textsuperscript{WT}. In the case of DNAJB6b\textsuperscript{F89I}, average anti-aggregation activity was even lower than DNAJB6b\textsuperscript{P96R} but still not significantly different from DNAJB6b\textsuperscript{WT} activity (Fig 2C). Only, the newly tested DNAJB6b\textsuperscript{P96R} mutant, that causes a more severe disease phenotype than the DNAJB6b\textsuperscript{F93L} mutant (Ruggieri et al., 2015), showed a significant loss of function in terms of preventing polyQ\textsuperscript{119}-YFP aggregation, although it still retained significant activity when compared to control condition without any chaperone (Fig 2C). Taken together, these data suggest that, regarding their polyQ aggregation suppressive activity, DNAJB6b\textsuperscript{P96R} is significantly affected but DNAJB6b\textsuperscript{F89I} and DNAJB6b\textsuperscript{F93L} display only minor defects.
DNAJB6b was previously shown to be capable of suppressing also parkin<sup>C289G</sup> aggregation and the DNAJB6b<sup>F93L</sup> mutant was found to be, if anything, only moderately impaired regarding this function (Kakkar et al., 2016a). Therefore, we aimed to examine whether other LGMD1D-related DNAJB6b mutations affected their ability to prevent parkin<sup>C289G</sup> aggregation. We used FLAG-parkin<sup>C289G</sup> and V5-tagged DNAJB6b<sup>WT</sup> or the mutants DNAJB6b<sup>F93L</sup>, DNAJB6b<sup>F89I</sup> and DNAJB6b<sup>P96R</sup> and monitored mutant parkin aggregation with Triton X-100.

Figure 2. DNAJB6b mutants show minor defects in their ability to suppress polyQ and mutant parkin aggregation. (A) Filter trap assays (FTA) with serial 5-fold dilution of HEK293 cells co-expressing cytoplasmic or nuclear GFP-Q<sub>74</sub> and DNAJB6b<sup>WT</sup> or disease-causing mutants DNAJB6b<sup>F93L</sup>, DNAJB6b<sup>F89I</sup> or DNAJB6b<sup>P96R</sup>. a-GFP is used for FTA blots. The percentage of aggregation of GFP-Q<sub>74</sub> in the presence of DNAJB6b<sup>WT</sup> or the mutants DNAJB6b<sup>F93L</sup> (n=8), DNAJB6b<sup>F89I</sup> (n=2) or DNAJB6b<sup>P96R</sup> (n=2) is indicated on the left graph and percentage of aggregation of GFP-Q<sub>74</sub>-NLS in the presence of DNAJB6b<sup>WT</sup> or DNAJB6b<sup>F93L</sup> (n=5), DNAJB6b<sup>F89I</sup> (n=2) or DNAJB6b<sup>P96R</sup> (n=2) on the right graph. Both graphs represent quantification of GFP-polyQ blots normalized to DNAJB6b<sup>WT</sup> (100%). (B) Western blot of GFP-Q<sub>74</sub> or GFP-Q<sub>74</sub>-NLS and DNAJB6b mutants. a-GFP is used for polyQ, a-V5 for DNAJB6b and a-GAPDH as loading control. Graphs show ratio of DNAJB6b mutants relative to DNAJB6b<sup>WT</sup> (100%) from quantification of DNAJB6b (V5) blots (n=11 for DNAJB6b<sup>F93L</sup>, n=4 for DNAJB6b<sup>F89I</sup> and DNAJB6b<sup>P96R</sup>). (C) FTA as described in (A) but with co-expression of polyQ<sub>119</sub>-YFP (n=18 for DNAJB6b<sup>F93L</sup>, n=6 for DNAJB6b<sup>F89I</sup> and DNAJB6b<sup>P96R</sup>). (D) Western blots of 1%TX100 fractionation of cells co-expressing FLAG-parkin<sup>C289G</sup> and V5-DNAJB6b<sup>WT</sup> or mutants. a-FLAG is used for parkin<sup>C289G</sup>, V5 for DNAJB6b and GAPDH as loading control. Graphs represent quantification of FLAG-parkin<sup>C289G</sup> blots normalized to DNAJB6b<sup>WT</sup> (100%). In the upper graph, percentage of aggregation is calculated as insoluble (P)/soluble (S) percentile ratios. In the lower graph, soluble (S) and insoluble (P) fractions are presented separately (n=5 for DNAJB6b<sup>F93L</sup> and n=3 for DNAJB6b<sup>F89I</sup> and DNAJB6b<sup>P96R</sup>). Mean values are given with s.e.m.; * = p<0.05.
fractionation. Overall, similarly to what we observed for polyQ<sup>119</sup>, average parkin<sup>C289G</sup> aggregation was increased when the disease-related mutants were expressed but none of them showed a statistically significant difference compared to DNAJB6b<sup>WT</sup> (Fig 2D).

**Nuclear isoform DNAJB6a<sup>F93L</sup> mutant decreases polyQ aggregation in the cytoplasm**

The nuclear DNAJB6a isoform, although not effective against cytoplasmic polyQ, can suppress nuclear polyQ aggregation as efficiently as DNAJB6b (Hageman et al., 2010). However, it has not been examined whether DNAJB6a mutants show any difference in terms of aggregation suppressing action. Therefore, we tested whether F93L mutation has any effect on the anti-aggregation function of DNAJB6a and we co-expressed it with cytoplasmic polyQ (GFP-Q<sup>74</sup>) or nuclear polyQ (GFP-Q<sup>74</sup>-NLS). As expected, DNAJB6a<sup>WT</sup> was equally effective as DNAJB6b<sup>WT</sup> in protecting against nuclear GFP-Q<sup>74</sup>-NLS aggregation (Fig 3A). Similar to what we observed for DNAJB6b<sup>F93L</sup>, DNAJB6a<sup>F93L</sup> did not seem to be significantly affected in this function; although, on average, more Q<sup>74</sup>-NLS aggregation was observed with DNAJB6a<sup>F93L</sup>, there was no significant difference with DNAJB6a<sup>WT</sup> (Fig 3A).

Unexpectedly, however, we found a protective effect of the nuclear DNAJB6a<sup>F93L</sup> on cytosolic polyQ<sup>74</sup> aggregation (Fig 3A). Unlike DNAJB6b<sup>F93L</sup>, which exhibited significantly higher protein levels compared to DNAJB6b<sup>WT</sup> (Fig 2B), the nuclear DNAJB6a<sup>F93L</sup> mutant was expressed to the same level as DNAJB6a<sup>WT</sup> (Fig 3B), excluding the possibility that differential expression levels are responsible for the observed effect. For cytosolic Q<sup>119</sup>-YFP, however, DNAJB6a<sup>F93L</sup> was as ineffective as DNAJB6a<sup>WT</sup> (Fig 3C), meaning that this gain of function in the cytosol is only moderate. More importantly, though, these data imply that the G/F-rich region mutation does not directly lead to a loss of capacity to suppress polyQ aggregation; they rather suggest that changes on nuclear-cytoplasmic distribution of the mutant could underlay the observed difference in its chaperoning capacity.

**DNAJB6a<sup>F93L</sup> shows normal nuclear localization**

As stated above, DNAJB6b mutants showed overall normal distribution, similar to the DNAJB6b<sup>WT</sup> (Fig S1), but DNAJB6a localization has not been investigated. To test whether the improved chaperone efficiency of DNAJB6a<sup>F93L</sup> to suppress cytoplasmic polyQ aggregation is due to an altered nuclear/cytoplasmic distribution, we used fluorescence microscopy to detect DNAJB6a<sup>WT</sup> or DNAJB6a<sup>F93L</sup> when co-expressed with GFP-Q<sup>74</sup>. Both DNAJB6a<sup>WT</sup> and DNAJB6a<sup>F93L</sup> were indistinguishable and almost exclusively expressed in the nucleus (Fig 4A, 4B). Only a small percentage of cells showed partial cytoplasmic localization for both DNAJB6a<sup>WT</sup> and DNAJB6a<sup>F93L</sup> (Fig 4B) and this mostly included cells
with abnormal nuclear staining (dividing, dead or necrotic/apoptotic cells). Therefore, within the resolution of our current imaging experiments, we find no direct evidence for the hypothesis that the increased cytoplasmic activity of DNAJB6a<sup>F93L</sup> can be attributed to an altered nucleocytoplasmic shuttling of the protein.

Figure 3. DNAJB6a<sup>F93L</sup> mutant suppresses cytoplasmic polyQ<sup>74</sup> aggregation. (A) Filter trap assays with serial 5-fold dilution of cells co-expressing cytoplasmic or nuclear GFP-Q<sup>74</sup> constructs and DNAJB6a<sup>WT</sup> or disease-causing mutant DNAJB6a<sup>F93L</sup>. Graphs show the percentage of aggregation of GFP-Q<sup>74</sup> (left) or GFP-Q<sup>74</sup>-NLS (right) in the presence of DNAJB6a<sup>WT</sup> or DNAJB6a<sup>F93L</sup> mutant. Both graphs represent quantification of polyQ-GFP blots of DNAJB6a<sup>F93L</sup> (n=8 for GFP-Q<sup>74</sup>, n=5 for GFP-Q<sup>74</sup>-NLS) normalized to DNAJB6a<sup>WT</sup> (100%). (B) Western blot of GFP-Q<sup>74</sup> or GFP-Q<sup>74</sup>-NLS and DNAJB6a<sup>WT</sup> or DNAJB6a<sup>F93L</sup>. a-GFP is used for polyQ, a-V5 for DNAJB6a and a-GAPDH as loading control. Ratio of DNAJB6a<sup>F93L</sup> relative to DNAJB6a<sup>WT</sup> (100%) from V5 blot quantification is shown on the graph (n=14). (C) As described in (A) but using Q<sup>119</sup>-YFP (n=3). Mean values are given with s.e.m., * = p<0.05.
Discussion

Several DNAJB6b LGMD1D-associated mutants have been already assessed by different labs for their ability to suppress protein aggregation but results were variable (Table 1), possibly due to different cell lines or other experimental conditions used. Consistent with most studies, we also found that DNAJB6b<sup>F93L</sup> mutation only mildly affects its anti-aggregation function, both in the case of polyQ and mutant parkin (Fig 2). We also found DNAJB6b<sup>F89I</sup> to be only mildly affected in anti-aggregation capacity (Fig 2), although in literature this mutation was found to cause a more pronounced effect (Table 1). However, the newly tested DNAJB6b<sup>P96R</sup> caused a significant increase in polyQ aggregation, that could only be detected when a long polyQ<sup>119</sup> fragment was expressed (Fig 2C), implying that its defects are minor and only become apparent under increased proteotoxic stress conditions.

Although two out of the three mutants tested showed no significant functional impairment compared to the DNAJB6b<sup>WT</sup>, the average aggregation protection loss positively correlates with the disease severity (Table 1), which still suggests that a (minor) impairment in anti-aggregating function could accumulate over years into a significant defect and thus be an important factor contributing to the disease.

But what is the molecular defect caused by these LGMD1D-associated mutants that all reside in the G/F-rich region of DNAJB6? This region is unlikely the prime substrate binding...
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site for polyQ, as evidenced by our previous studies with G/F-rich domain deletions
mutants that were mostly unaffected in their polyQ suppressive activity (Hageman et al.,
2010). Rather, as demonstrated recently, an S/T-rich region at the C-terminus of DNAJB6
has been identified as the polyQ substrate interacting region (Hageman et al., 2010;
Kakkar et al., 2016b). Our unanticipated data about nuclear DNAJB6a F93L even reducing
cytoplasmic polyQ further support the notion that the G/F-rich region mutations, most
likely, do not directly impede on polyQ binding. We, however, cannot exclude that the
G/F-rich domain may play a role in binding of different conformations of substrates, as
it was previously proposed (Perales-Calvo et al., 2010; Stein et al., 2014). Especially since
phenylalanines are known to form aromatic-aromatic interactions that are, among others,
implicated in protein folding and stability (Makwana and Mahalakshmi, 2015) and have
even been proposed to stabilize aggregation-prone beta-strand structures in proteins,
possibly to avoid aggregation (Budyak et al., 2013). Therefore, this region may act as a
support in order to prevent aggregation or stabilize certain (conformations of) substrates
together with the S/T-rich region.

Alternatively, the G/F-rich region mutants could impede DNAJB6 communication with the
HSP70s. Interestingly, in vitro experiments using the bacterial DnaJ revealed that a G/F-
region mutant was impaired in transferring substrates to DnaK (HSP70), although it could
still bind to and stimulate ATPase activity of DnaK (Wall et al., 1995). This potential client
transfer defect of the LGMD1D mutants may explain why the defects on polyQ aggregation
are only marginal, as DNAJB6b dependency on HSP70 is only limited regarding polyQ
suppression (Kakkar et al., 2016b). Moreover, it can be further supported by our findings
that these mutants had no significant effect on suppression of parkin C289G aggregation (Fig
2D). As suggested before (Kakkar et al., 2016a), in the case of mutant parkin, the mode of
action of DNAJB6b (and several other DNAJs) is not specific and is mostly depending on
the ability of DNAJs to stimulate HSP70 ATPase activity, an ability that, for bacterial DnaJ,
was shown not to be influenced by the G/F-rich region (Wall et al., 1995). Impaired client
transfer could also relate to our observations that the expression levels of the DNAJB6b F93L
and DNAJB6 b P96R mutants, were increased compared to that of DNAJB6b WT (Fig 2B). Such
increased levels of DNAJB6b F93L have been also reported in a mouse model of LGMD1D,
where it was found that the degradation rate of mutant DNAJB6b was lower than that of
the wild type (Bengoechea et al., 2015). Due to a possible inefficient transfer, clients may
bind longer to DNAJB6, on one hand preventing its normal turnover and, on the other
hand, increasing the chance of co-aggregation. Consistent with this model, DNAJB6b F93L
was found in aggregates in the muscles of the patients along with other proteins that are
possible DNAJB6 substrates (Harms et al., 2012; Sandell et al., 2016).
Finally, our results about the nuclear DNAJB6a isoform, that was found marginally weaker than DNAJB6a\textsuperscript{WT} against nuclear Q\textsuperscript{74} aggregation but showed an unexpected (minor) protection against cytoplasmic Q\textsuperscript{74} aggregation, compared to DNAJB6a\textsuperscript{WT} (Fig 3A), suggested some possible localization changes. Even though our microscopy data (Fig 4) did not reveal any gross alterations in the intracellular distributions of the DNAJB6 mutants (both a- and b- isoforms), the idea that the mutations affect nucleocytoplasmic shuttling and hereby function of DNAJB6 cannot be fully discarded yet and should be further investigated. Nevertheless, based on our preliminary data and under the conditions tested, a displacement of DNAJB6a\textsuperscript{F93L} towards the cytoplasm does not seem to be the reason for the decrease in cytoplasmic Q\textsuperscript{74} aggregation. An alternative scenario could be the shift of Q\textsuperscript{74} aggregation towards the nucleus, for example by a distorted import/export to/from the nucleus caused by DNAJB6a\textsuperscript{F93L} mutation. However, we did not observe any obvious accumulation of GFP- Q\textsuperscript{74} at the nucleus (Fig 4a) to further support this hypothesis. In any case, regardless of the underlying mechanism, these data strengthen the previously proposed idea that DNAJB6a mutations do not contribute to the disease pathology, considering that nuclear DNAJB6a\textsuperscript{F93L} did not induce any muscle defect in animal models (Bengoechea et al., 2015; Sarparanta et al., 2012). We could speculate that DNAJB6 levels in the cytosol are more critical to muscle fitness and this is not negatively affected by mutations in the nuclear isoform.

To conclude, LGMD1D-associated mutations tested so far, lead to defects that impede cytosolic protein homeostasis. Whether these are due to altered substrate interaction, mislocalisation or HSP70 communication still remains unclear but experiments are ongoing to further substantiate these ideas.

**Materials and Methods**

*Cell cultures, transfections and plasmids*

Human embryonic kidney (HEK293) stably expressing the tetracycline repressor (Flp-In T-REx HEK293, Invitrogen) and mouse myoblast (C2C12) cells were cultured using standard protocols in DMEM (Gibco) supplemented with 10% fetal bovine serum (Greiner Bio-One) and penicillin/streptomycin (Gibco). HEK293 cells were transiently transfected with Lipofectamine (Invitrogen) and C2C12 cells with Lipofectamine 2000 (Invitrogen), according to manufacturer’s instructions. Expression in HEK293 was induced by tetracycline. pcDNA5-FRT/TO-V5 or pcDNA5-FRT/TO-GFP plasmids containing DNAJB6b wild type, F93L, F89I or P96R mutants and DNAJB6a wild type or F93L mutant were a kind gift of Dr. Chris Weihl (Washington University, USA). The Q\textsuperscript{119}-YFP, pEGFP-Q\textsuperscript{74}, pEGFP-Q\textsuperscript{74}-NLS were
Altered anti-aggregation capacity of DNAJB6 LGMD mutants described previously (Hageman et al., 2010). The pcDNA3-FLAG-parkin constructs (wild type and C289G) were a kind gift from Dr. Michael Cheetham and previously described (Kakkar et al., 2016a).

Filter trap assays
24 or 48 hours after transfection, cells were recovered by washed and recovered by either trypsinization or scraping in cold PBS, centrifuged at 3800 g for 3 minutes. Cell pellets were lysed in RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40 (Igepal CA-630, Sigma), 1% sodium deoxycholate, 2% SDS, complete protease inhibitors cocktail (Roche) and sonicated (50% input for 5 seconds). Protein concentrations were determined using DC protein assay (Bio-Rad). Concentrations were equalized and part of the samples was kept separate and diluted 1:1 with 2x Laemmli buffer (125mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 20% 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes for western blot analysis. The remaining part of the samples was diluted in FTA buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 50 mM dithiothreitol, 2% SDS), boiled for 5 minutes and prepared in three 5-fold serial dilutions into a final of 1x, 5x and 25x diluted samples for filter trap assay (FTA). FTA samples were loaded onto a 0.2 µm pore size cellulose acetate membrane prewashed with 0.1% SDS-containing FTA buffer. Membranes were washed three times with 0.1% SDS-containing FTA buffer, blocked with 10% non-fat milk and blotted with anti-GFP/YFP (JL-8, Clontech). After HRP-conjugated secondary antibody (Amersham) incubation, visualization was performed using enhanced chemiluminescence and Hyperfilm (ECL, Amersham).

Triton X-100 fractionations
24 hours after transfection, cells were washed once in cold PBS before adding TX100-lysis buffer [PBS, 1% Triton X-100, complete protease inhibitor cocktail (Roche)]. Cell lysates were scraped and centrifuged at 20000 g for 15 minutes at 4 °C. Supernatants with TX100-soluble proteins were transferred to new tubes (S fraction). Pellets with TX100-insoluble proteins were further resuspended in SDS-buffer [PBS, 1% SDS, complete protease inhibitors cocktail (Roche), sonicated (50% input for 5 seconds) and centrifuged at 20000 g for 15 minutes at 4 °C. Supernatants were collected to a new tube (P fraction). Both S and P fraction samples were diluted with 2x Laemmli buffer (125mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 20% 2-mercaptoethanol, 0.001% bromophenol blue) and samples were used immediately or kept frozen at −20 °C until western blot analysis.

Western blots
Equal amounts of proteins were loaded into 12% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and blotted with the primary antibodies: anti-GFP/
YFP (JL-8, Clontech); anti-V5 (R960-25, Invitrogen); anti-FLAG (M2, Sigma), anti-GAPDH (10R-G109A, Fitzgerald). After incubation with the appropriate HRP-conjugated secondary antibody (Amersham), visualization was performed with enhanced chemiluminescence and Hyperfilm (ECL, Amersham). Quantification of western blots was performed with ImageJ (NIH, https://imagej.nih.gov/ij/) and graphs were prepared with GraphPad Prism (GraphPad Software).

**Immunofluorescence and microscopy**

48 hours after transfection, cells grown on coverslips were fixed using 3.7% formaldehyde for 15 minutes, washed three times with PBS, permeabilized with 0.2% Triton X-100, incubated with 10mM glycine for 10 minutes and blocked with 3% BSA for 30 minutes. Coverslips were incubated with anti-V5 (Invitrogen) or anti-FLAG (Sigma) primary antibodies at 4°C overnight, washed again three times and incubated with Cy3-conjugated (Amersham) secondary antibody for 1-2 hours. Nuclei were stained using 0.2 µg/ml DAPI in PBS. Microscopy was performed with a Leica DM6000 M fluorescence microscope and image processing was done using ImageJ (NIH, https://imagej.nih.gov/ij/).

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**Author contributions**

DS, RH & EFEK performed the experiments. HHK designed and supervised experiments. DS & HHK analyzed all the data and wrote the manuscript.
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References


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Figure S1. Subcellular distribution of DNAB6b mutants. (A-B) Fluorescence microscopy of GFP-tagged DNAJB6b^wt or mutants DNAJB6b^F93L, DNAJB6b^F89I and DNAJB6b^P96R in (A) HEK293 and (B) C2C12 cells. GFP is shown in green and DAPI (nuclear staining) in blue in the merged image.