

NEURODEVELOPMENTAL DISORDERS

PSMC3 proteasome subunit variants are associated with neurodevelopmental delay and type I interferon production

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A critical step in preserving protein homeostasis is the recognition, binding, unfolding, and translocation of protein substrates by six AAA-ATPase proteasome subunits (ATPase-associated with various cellular activities) termed PSMC1-6, which are required for degradation of proteins by 26S proteasomes. Here, we identified 15 de novo missense variants in the *PSMC3* gene encoding the AAA-ATPase proteasome subunit PSMC3/Rpt5 in 23 unrelated heterozygous patients with an autosomal dominant form of neurodevelopmental delay and intellectual disability. Expression of *PSMC3* variants in mouse neuronal cultures led to altered dendrite development, and deletion of the *PSMC3* fly ortholog Rpt5 impaired reversal learning capabilities in fruit flies. Structural modeling as well as proteomic and transcriptomic analyses of T cells derived from patients with *PSMC3* variants implicated the *PSMC3* variants in proteasome dysfunction through disruption of substrate translocation, induction of proteotoxic stress, and alterations in proteins controlling developmental and innate immune programs. The proteostatic perturbations in T cells from patients with *PSMC3* variants correlated with a dysregulation in type I interferon (IFN) signaling in these T cells, which could be blocked by inhibition of the intracellular stress sensor protein kinase R (PKR). These results suggest that proteotoxic stress activated PKR in patient-derived T cells, resulting in a type I IFN response. The potential relationship among proteasome dysfunction, type I IFN production, and neurodevelopment suggests new directions in our understanding of pathogenesis in some neurodevelopmental disorders.

INTRODUCTION

Proteasomes are large multi-protein complexes whose structure is adapted to the function of regulated protein degradation, thereby controlling many cellular processes (1, 2). Together with the autophagosomal-lysosomal system, proteasomes maintain protein homeostasis by counterbalancing the synthesis of new proteins by the translational machinery (3–6). The proteasome is part of the ubiquitin-proteasome system (UPS), which counts numerous enzymes acting upstream of the proteasome (7, 8). Aged or unstructured proteins are preliminary ubiquitin-tagged by the ubiquitination machinery through a cascade of enzymatic reactions for degradation by the 26S proteasome (9, 10). The 26S proteasome

consists of two parts, the 20S core proteolytic particle and the 19S regulatory particle, which caps the 20S particle at one or both ends (11, 12). Polyubiquitinated proteins are recognized by the 19S regulatory particle, which comprises two parts: a base and a lid. In the base, four regulatory particle non-adenosine triphosphatase (ATPase) (Rpn) subunits (Rpn1, Rpn2, Rpn10, and Rpn13; encoded by the proteasome genes *PSMD2*, *PSMD1*, *PSMD4*, and *ADRM1*, respectively) ensure recognition and capture of ubiquitin-modified substrates (13, 14). The lid contains eight additional non-ATPase subunits, such as Rpn3, Rpn5-9, Rpn12, and Rpn15, which serve as scaffolds for binding of other subunits (15, 16) and the deubiquitinating enzyme Rpn11 (encoded by *PSMD14*)

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(17). Six AAA-ATPase (ATPase-associated with various cellular activities) subunits (Rpt1 to 6), encoded by the genes *PSMC1-6* in the base, use the energy provided by adenosine triphosphate (ATP) hydrolysis to unfold and translocate the substrate into the barrel-shaped 20S proteolytic core particle by gate opening. The 20S complex comprises heptameric α - and β -rings with an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ architecture encoded by proteasome alpha subunit *PSMA1-7* or beta subunit genes *PSMB1-7*. The β -ring may be subjected to variations, thereby giving rise to two major proteasome isoforms, namely, standard and immunoproteasomes. Standard proteasomes typically contain the catalytic $\beta 1$, $\beta 2$, and $\beta 5$ subunits with caspase-, trypsin-, and chymotrypsin-like activities, respectively (18, 19). In immunoproteasomes, the $\beta 1$, $\beta 2$, and $\beta 5$ subunits are replaced by the inducible $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits, encoded by the *PSMB9*, *PSMB10*, and *PSMB8* genes, respectively (20). Although standard proteasomes are expressed in virtually all types of tissues, the expression of the inducible β subunits is restricted to immune cells and nonimmune cells exposed to type I or II interferons (IFNs) (21, 22).

Pathogenic variants in proteasome subunit genes cause rare proteasomopathies with a broad spectrum of symptoms (23, 24). So far, with the exception of the *PSMB1* ($\beta 6$) subunit (25), all pathogenic variants related to the 20S core particle have been shown to provoke immune dysregulation. Several genes encoding β subunits (*PSMB4*, *PSMB8*, *PSMB9*, and *PSMB10*), α subunits (*PSMA3*), or assembly

chaperone genes [proteasome maturation protein (*POMP*) and proteasome assembly chaperone Pac2 gene (*PSMG2*)] of the 20S proteasome complex have been involved in autosomal recessive proteasome-associated autoinflammatory syndromes (PRAAS) typically characterized by persistent type I IFN signaling (26–33). By contrast, genetic disorders involving genes of the 19S regulatory particle such as the Stankiewicz-Isidor syndrome [STISS; Mendelian Inheritance in Man (MIM): 617516] caused by truncating variants of *PSMD12* (also referred to as Rpn5) are neurodevelopmental polymalformative syndromes (34) with subclinical activation of type I IFN signaling (35, 36). These observations place both PRAAS and STISS in the category of type I interferonopathies, a recent family of genetically determined rare autoinflammatory syndromes with dysregulated type I IFN signaling that includes Aicardi-Goutières syndrome and familial chilblain lupus (37, 38). Clinically, these diseases are complex, demonstrating multiple organ involvement (often brain and skin), encompassing a broad range of phenotypes, and being associated with high morbidity and mortality (39). In this work, we identified 15 dominant de novo variants in the *PSMC3* gene coding for the AAA-ATPase *PSMC3/Rpt5*. These rare missense variants were detected in 23 individuals presenting with neurodevelopmental delay (NDD), intellectual disability (ID), or both, together with various congenital malformations. Together, our data highlight interferonopathy as a potential contributor to the pathogenesis of NDD/ID in patients

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carrying loss-of-function variants in subunits of the 19S proteasome regulatory particle and identify protein kinase R (PKR) as a major player in disease pathogenesis.

RESULTS

Identification of PSMC3 variants

The first *PSMC3* variant was detected in patient #2, a female newborn presenting with severe cardiac, gastrointestinal, inflammatory, and immune issues. Whole-exome sequencing (WES) highlighted the de novo nonsynonymous c.523A>G p.(M175V) variant (GenBank ID: NM_002804.4), which was absent in any public variant databases [Genome Aggregation Database (gnomAD, >246,000 chromosomes; NHLBI Exome Variant Server, >13,000 alleles; Bravo, 125,568 alleles] and predicted to be pathogenic by bioinformatics programs, including Sorting Intolerant from Tolerant (SIFT), PolyPhen-2n CADD, REVEL, and MetaDome, as well as all programs compiled by MobiDetails (40). Our overall strategy described in Materials and Methods allowed us to identify a total of 15 distinct rare de novo missense *PSMC3* variants in 23 unrelated children presenting with a syndrome characterized by NDD and various congenital anomalies (Table 1).

As shown in Fig. 1, most of the *PSMC3* substitutions were localized in the AAA domain that was predicted to be intolerant to variations (fig. S1). Two distinct regions of the AAA domain were particularly prone to substitutions. The first hotspot was centered on the recurrent variant c.910C>T p.(R304W) detected in four unrelated children and encompassed variants c.910C>G p.(R304G),

c.915G>T p.(E305D), and c.929 T>C p.(M310T) (Fig. 1). The second region—enriched in rare variants [c.775A>G p.(M259V), c.776 T>C p.(M259T), c.782 T>C p.(I261T) (seen six times), c.784G>A p.(G262R), and c.806G>C p.(R269P)]—was more N-terminally located (Fig. 1). All 13 affected residues were highly conserved across species from mammals down to fission yeast (Fig. 1). One major phenotypic hallmark of all individuals carrying *PSMC3* variants was the predominance of neurodevelopmental or neuropsychiatric symptoms (table S1). In more detail, apart from patient #2, all affected children exhibited developmental delay (DD) (22 of 22; 100%) characterized by speech delay (19 of 19; 100%) alone or with ID (16 of 18; 89%) and motor delay (15 of 19; 79%). Brain magnetic resonance imaging highlighted frequent anomalies (11 of 15; 73%), whereas the occurrence of abnormal behavior (9 of 18; 50%) and seizures (5 of 21; 24%) varied. Nine of 19 (47%) individuals experienced growth failure, most with feeding difficulties (8 of 18; 44%). Malformations were frequently observed in the skeleton [11 of 15; 73%; scoliosis, acetabular dysplasia, and brachymetatarsy], heart (10 of 18; 56%; ventricular or septal defects, patent ductus arteriosus, pulmonary hypertension, and atresia), kidney (4 of 15; 27%; horseshoe shape, pelvicalyceal dilatation, nephrocalcinosis, and multi-cystic dysplastic kidney), and head [microcephaly in 6 of 17 (35%); relative to severe macrocephaly in 2 of 16 (13%)]. Tumors were noted in 2 of 19 (11%) individuals (craniopharyngioma and neuroblastoma). Hearing loss was detected in 9 of 19 individuals (47%) and labeled as sensorineural in two and conductive in one of them, respectively. Most of the children (18 of 20; 90%) displayed dysmorphic facial features (fig. S2),

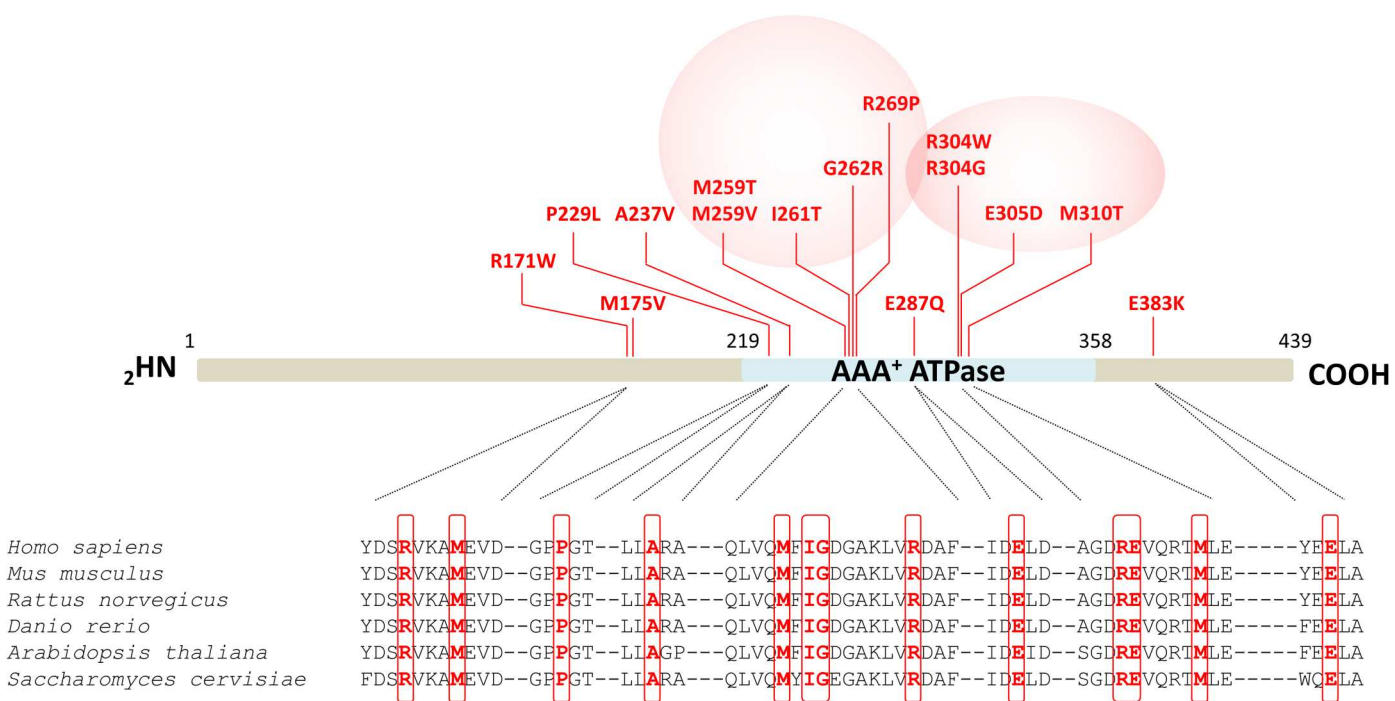


Fig. 1. Distribution of the de novo heterozygous *PSMC3/Rpt5* variants identified in patients. Shown are the locations of the 15 NDD-causing missense variants (indicated in red) along the *PSMC3/Rpt5* protein. The AAA-ATPase domain of the *PSMC3/Rpt5* proteasome subunit of the 19S regulatory particle is depicted in blue. Pink circles indicate the presence of variants hotspots. A sequence alignment of regions immediately adjacent to the amino acids subjected to missense substitutions is also shown. Comparison of the *PSMC3/Rpt5* primary structure across six eukaryotic organisms indicates the high conservation of the missense variant residues identified in patients with NDD/ID, which are highlighted by red boxes.

Table 1. Main characteristics of the PSMC3 de novo variants* identified in the patients included in the study. ND, not determined; IFN, interferon; Metadome: I, intolerent; HI, highly intolerent; N, neutral.												
Chromosomal localization (Chr11/GRCh37)	cDNA change†	Protein change	Variant database‡	CADD Phred score (v1.6.)	Metadome	MobiDetails§	Effect on neuronal development	Effect on PSMC3 stability	Effect on proteasome assembly	Effect on mitophagy	IFN signature	Number of patients with the variant
g.47445677G>A	c.511C>T	R171W	rs775517283	27.3	I	45364	Improvement	Decrease	ND	ND	ND	1
g.47445665T>C	c.523A>G	M175V	Absent	23.4	I	45365	ND	None	ND	ND	ND	1
g.47444430G>A	c.686C>T	P229L	Absent	25.5	I	195979	ND	ND	ND	ND	ND	1
g.47444406G>A	c.710C>T	A237V	Absent	26.9	HI	45366	ND	Decrease	ND	ND	ND	1
g.47444234T>C	c.775A>G	M259V	Absent	23.4	I	45367	ND	None	ND	ND	ND	1
g.47444233A>G	c.776T>C	M259T	Absent	24.7	I	45368	ND	Decrease	ND	ND	ND	1
g.47444227A>G	c.782T>C	I261T	Absent	24.6	I	45369	ND	Decrease	ND	ND	ND	6
g.47444225C>T	c.784G>A	G262R	Absent	26.8	I	45370	ND	None	None	Increase	Moderate	1
g.47444203C>G	c.806G>C	R269P	Absent	24	I	45371	ND	Decrease	ND			1
g.47444150C>G	c.859G>C	E287Q	Absent	26.7	I	45372	ND	Decrease	ND			1
g.47442253G>A	c.910C>T	R304W	rs1363348500	31	I	45373	Impairment	None	Impairment	Increase	Strong/very strong	4
g.47442253G>C	c.910C>G	R304G	Absent	28.2	I	45374	ND	None	ND			1
g.47442248C>A	c.915G>T	E305D	Absent	23.1	I	45375	Impairment	None	Impairment	Increase	Moderate	1
g.47442234A>G	c.929T>C	M310T	Absent	26.2	HI	45376	ND	None	ND			1
g.47440729C>T	c.1147G>A	E383K	Absent	27.8	N	45377	Impairment	None	ND			1

*All variants presented are de novo, except variant c.775A>G, which is suspected de novo. †RefSeq transcript used for PSMC3 is NM_002804.4. ‡gnomAD V3, dbSNP v154, ClinVar v20210828. §The access to detailed predictions for variant XXXXX (5364, 45365...) is as follows: <https://mobidetails.iurc.montp.inserm.fr/MD/api/variant/XXXXX/browser/>

including tall or broad forehead (7 of 19; 37%), thin upper lip with down-turned corners of mouth (6 of 19; 32%), abnormal palate (5 of 19; 26.5 of 19; 26%), epicanthal folds (5 of 19; 26%), and orofacial clefts (2 of 19; 10%). Computational analysis of facial morphology by GestaltMatcher (41) revealed that facial dysmorphism among the patients carrying PSMC3 variants was rather heterogeneous, with similarities only observed between patients carrying identical variants (fig. S2).

Silencing of the PSMC3 Drosophila ortholog Rpt5 in adult flies fails to reverse stimulus contingencies

Given the neuronal nature of the phenotype of patients carrying PSMC3 variants, we next sought to address the potential involvement of PSMC3 in cognitive function by evaluating the learning performance of Drosophila melanogaster fruit flies with an RNA interference (RNAi) knockdown of Rpt5 (Drosophila ortholog of human PSMC3) expression by two different small interfering RNAs (siRNAs), namely, Rpt5³²⁴²² and Rpt5⁵³⁸⁸⁶ targeting Rpt5 transcripts at two different sites. To this end, we used a standard conditioning of odor-avoidance paradigm, in which animals were exposed to two different odors [3-octanol (OCT) or 4-methylcyclohexanol (MCH)], only one of which resulted in the simultaneous application of a foot shock (OCT⁺, MCH⁻), as previously described (Fig. 2, A and B) (42). Silencing of Rpt5 using Rpt5 RNAi under control of the neuron-specific embryonic lethal abnormal visual

system (Elav) promoter resulted in no substantial differences in learning performance for Rpt5³²⁴²² [wild type (WT) versus Elav:RPT5³²⁴²², P = 0.6435, n = 4] or Rpt5⁵³⁸⁸⁶ [WT versus Elav:RPT5⁵³⁸⁸⁶, P = 0.5282, n = 6] siRNAs (Fig. 2C). We next determined the reversal learning performance of Rpt5-silenced flies by training with an initial odor shock pairing (OCT⁺, MCH⁻) immediately followed by training with a reversed odor shock pairing (OCT⁻, MCH⁺) (Fig. 2B, bottom). Reversal learning performance was significantly poorer with pan-neuronal Rpt5 RNAi expression of Rpt5³²⁴²² (WT versus Elav:RPT5³²⁴²², P < 0.0001, n = 4) or Rpt5⁵³⁸⁸⁶ (WT versus Elav:RPT5⁵³⁸⁸⁶, P = 0.0022, n = 6) siRNAs. The three control groups once again did not significantly differ from each other (Fig. 2C). These data suggest that PSMC3 ortholog Rpt5 appears as a prerequisite for the changes in learned associations in D. melanogaster.

Ectopic expression of PSMC3/Rpt5 or its variants differentially affects neuronal development

In view of the negative impact of PSMC3/Rpt5 gene silencing on reversal learning, we next asked whether PSMC3 was involved in the regulation of hippocampal neuron dendritic development. We therefore ectopically expressed WT PSMC3/Rpt5 in murine primary hippocampal neurons before neurite length quantification as previously described (43). As shown in fig. S3, expression of WT PSMC3/Rpt5 at an early developmental time point in vitro [day in

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vitro (DIV 3] resulted in significantly reduced neurite lengths of the neurons (empty vector versus PSMC3 WT, $P = 0.0089$). These results suggested that ectopic expression of WT PSMC3/Rpt5 may be detrimental to neurite outgrowth. We next sought to determine whether the different PSMC3/Rpt5 variants identified in patients with NDD/ID behaved differently compared with WT PSMC3/Rpt5 in neurons when ectopically expressed. Expression of the R304W, E305D, and E383L PSMC3/Rpt5 variants resulted in similar neuronal morphological changes as those seen with WT PSMC3/Rpt5 (fig. S3). By contrast, expression of the M175V variant did not affect neuronal morphology when compared to the empty vector control and showed significant improvement when compared with WT PSMC3/Rpt5 (fig. S3). The positive effects exerted by the M175V PSMC3/Rpt5 variant on neurite length and arborization are intriguing but do not reflect an increased ability of the mutant subunit to incorporate into 19S-capped proteasome complexes (fig. S4). In addition, these observations do not preclude a milder pathogenicity of this variant, because the morphological changes seen are not necessarily beneficial for neurite outgrowth. Together, these results suggest that PSMC3/Rpt5 participates in the regulation of neurite development and that any alteration of this gene might affect this process positively or negatively.

PSMC3 gene variants differentially affect PSMC3/Rpt5 steady-state protein expression

Because missense variants may cause haploinsufficiency by affecting mRNA and/or protein turnover, we next sought to determine the impact of the identified PSMC3 variants on PSMC3/Rpt5 steady-state protein expression. To this end, 13 of the PSMC3 variants were expressed in the SHSY5Y neuroblastoma cell line with a fused N-terminal hemagglutinin (HA) tandem repeat before Western blot analysis. As shown in Fig. 3A, the four PSMC3/Rpt5 variants carrying the R171W, A237V, M259V, and I261T mutations exhibited lower PSMC3/Rpt5 protein expression than their WT counterpart. Densitometric analysis of the HA-PSMC3 bands (Fig. 3A, bottom left) and of PSMC3 bands (Fig. 3A, bottom right) emerging from these constructs revealed that PSMC3/Rpt5 protein expression was reduced by about 90% when compared with WT HA-PSMC3 (Fig. 3A). However, all PSMC3 variants generated equivalent amounts of HA-PSMC3 transcripts in SHSY5Y cells in a 24-hour plasmid-driven expression, as determined by reverse transcription polymerase chain reaction (RT-PCR) and densitometric quantification (Fig. 3B), thereby indicating that reduced protein expression was due to increased protein turnover, decreased translation efficiency, or both. To determine whether these effects could be caused by nonpathogenic PSMC3 mutations as well, we next analyzed the steady-state expression of three HA-tagged single-nucleotide polymorphisms (SNPs) PSMC3/Rpt5 variants (I77N, I291V, and P355L) reported in the gnomAD. As shown in

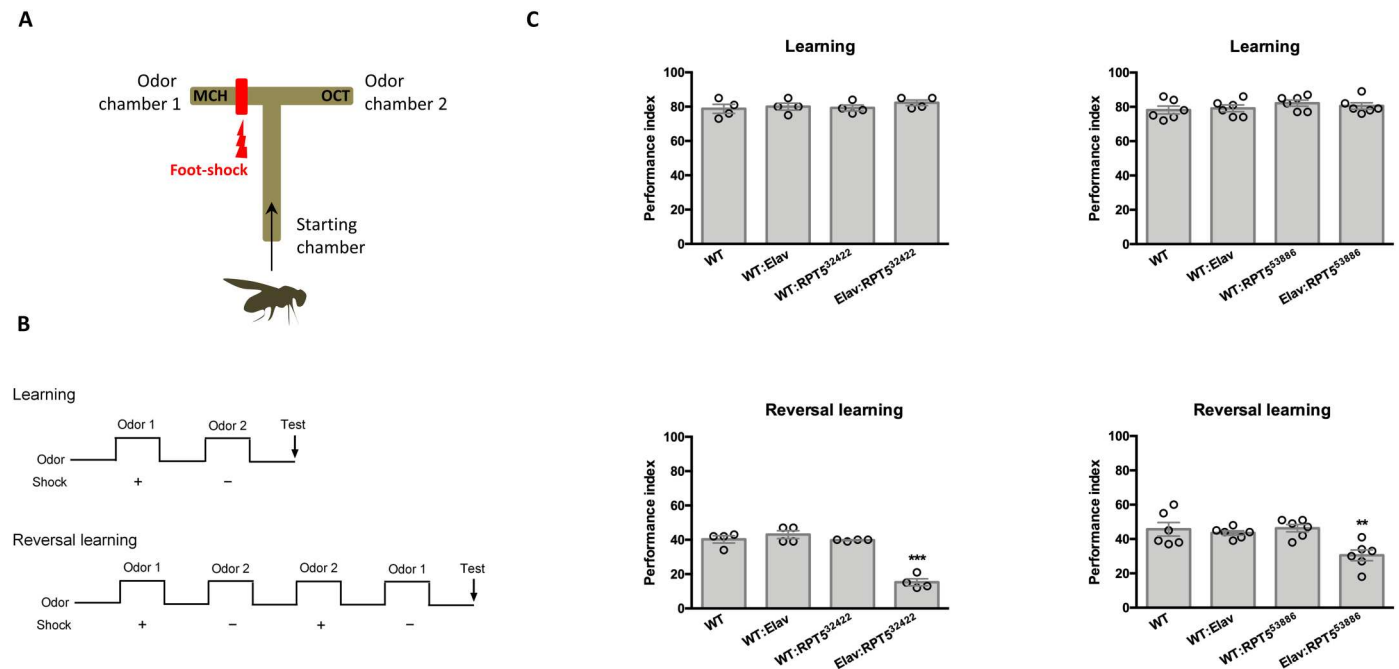


Fig. 2. Pan-neuronal RNAi-mediated knockdown of PSMC3/Rpt5 results in normal learning performance but defective reversal learning performance. (A) An illustration of a T-maze used for conditioning of odor avoidance in *Drosophila* is shown. Flies were trained to avoid one particular odor chamber that was associated with a foot shock (in this example, odor chamber 1). (B) Time course of the learning and reversal learning protocols used in these experiments is illustrated. Reversal learning was assessed by reversing odor shock pairing [4-methylcyclohexanol (MCH[−]) and 3-octanol (OCT⁺)], as indicated. (C) Top left: Learning performance index of wild-type (WT) flies, flies expressing Elav (WT:Elav), flies with full expression of Rpt5³²⁴²² RNAi (WT:Rpt5³²⁴²²), and flies with pan-neuronal expression of Rpt5³²⁴²² RNAi (Elav:Rpt5³²⁴²²) ($P = 0.6435$, $n = 4$) is shown. Top right: Learning performance index of WT flies, flies expressing Elav (WT:Elav), flies with full expression of Rpt5⁵³⁸⁸⁶ RNAi (WT:Rpt5⁵³⁸⁸⁶), and flies with pan-neuronal expression of Rpt5⁵³⁸⁸⁶ RNAi (Elav:Rpt5⁵³⁸⁸⁶) ($P = 0.5282$, $n = 6$; top right) is shown. Bottom left: Reversal learning performance of all groups described in (C) (top left) ($P < 0.0001$, $n = 4$) is shown. Bottom right: Reversal learning performance index for all groups described in (C) (top right) ($P = 0.0022$, $n = 6$) is shown. Statistical analysis was performed using ANOVA and then Tukey's tests using JMP statistical software (SAS). ** $P < 0.01$ and *** $P < 0.001$.

fig. S5, the three investigated SNPs behaved similarly to their WT counterpart, suggesting that decreased PSMC3/Rpt5 protein expression may be a specific feature of some pathogenic variants.

Ectopic expression of HA-PSMC3/Rpt5 fusion proteins in SHSY5Y cells was accompanied by expression of untagged PSMC3/Rpt5 (Fig. 3A and fig. S5). One potential explanation for this observation implies a partial destruction of the HA epitope, as previously described (44). This assumption is, however, not supported by the fact that such a phenomenon was not observed when other proteasome AAA-ATPases such as PSMC5/Rpt6 were N-terminally tagged with the same HA epitope (fig. S6). Rather, it may be that the increased pools of untagged PSMC3/Rpt5 detected in cells expressing HA-PSMC3/Rpt5 might reflect a stabilization of endogenous PSMC3/Rpt5. Overexpressed and endogenous PSMC3/Rpt5 proteins might undergo self-association, resulting in the formation of homomers protecting them from degradation. To address this point, HA-PSMC3/Rpt5 fusion proteins were pulled down from SHSY5Y cells using HA antibody followed by Western blot analysis for PSMC3/Rpt5. As shown in fig. S7, HA-PSMC3 coprecipitated with untagged PSMC3/Rpt5, thereby confirming physical interaction between overexpressed and endogenous PSMC3/Rpt5. However, although the regulation of the PSMC3/Rpt5 subunit in response to concentration changes is potentially interesting, the observation that this feature does not vary between WT and stable *PSMC3* variants suggests that this is not relevant to disease pathogenesis. Together, these data showed that the *PSMC3* missense variants identified in patients with NDD/ID differentially affect protein expression and stability.

Structural modeling predicts that *PSMC3* substitutions affect inter- and intramolecular interactions between proteasome subunits

We next attempted to predict the structural consequences of each of the 14 *PSMC3* substitutions by assessing the localization of the mutated residues in the human 26S proteasome structure generated by Dong *et al.* (45) [Protein Data Bank (PDB) entry code: 6MSK]. Most of the affected amino acids emerged within the N-terminal α/β domain of PSMC3/Rpt5 with five residues (G262, I261, M259, R304, and E305) residing in two loops adjacent to the substrate tunnel pointing toward the center of the AAA-ATPase ring (Fig. 3, C and D). Specifically, on one loop, G262 was fixed by a main chain hydrogen bond to E305, thereby promoting flexibility of the preceding loop containing M259. Besides, E305 itself was held through a salt bridge by R308, with its preceding residue R304 involved in a polar network stabilizing the neighboring loop (fig. S8). Because these six residues stabilized or were part of the tertiary structure of the loops, any alteration of these amino acids is predicted to affect substrate trafficking and interactions with other AAA-ATPase subunits. As shown in fig. S8, the A237V variant was more difficult to classify and did not reveal itself structurally at first sight. However, one cannot exclude that the slight increase in residue size at position 237 might lead to structural changes. The overexpression assays in SHSY5Y cells suggest that such substitution does affect side chain packing and protein stability (Fig. 3A). The E383L missense variant was the only substitution lying within the C-terminal α -helical domain of PSMC3/Rpt5 adjacent to the 19S-20S interface. E383 held Q166 from the PSMA1/ $\alpha 6$ subunit for polar interactions with both of the R169 and R386 residues (Fig. 3E). Changing the negatively charged E383 to a

positively charged K383 is therefore predicted to disrupt such a hydrogen bond network and affect the association of the 19S complex with the 20S core particle. E287 was also located in close proximity to the ATP binding site (fig. S8), and its substitution with Q287 presumably generated additional polar bonds with N333 likely to affect ATP binding, hydrolysis, or both. R171 was positioned at the PSMC3/Rpt5-PSMC2/Rpt1 interface and was part of a polar network involving the neighboring D169 and Q258 residues (fig. S9). As such, the substitution of positively charged arginine to hydrophobic tryptophan at this position is predicted to disrupt these interactions and a fortiori to affect the contact between the two subunits (fig. S9). The change of M175 to V175 resulted in the loss of a polarized thiol group and hydrophilic environment, which was likely to destabilize the tertiary structure of this protein region (fig. S9). Together, these data suggest that the complex 26S proteasome structure may be strongly affected by the identified *PSMC3* missense variants, although an incorporation of the dysfunctional PSMC3/Rpt5 subunit with no detrimental effects of proteasome structure cannot be fully excluded.

PSMC3 variants differentially affect proteasome assembly

To further address the pathogenicity of the *PSMC3* variants, T cells from patients #13, #18, and #21 were analyzed for their proteasome contents. The intracellular expression of the $\alpha 7$ proteasome subunits and PA28- α did not vary between relative controls (father, mother, or both) and index cases (Fig. 4A). Likewise, the abundance of the 19S subunit PSMD12/Rpn5 in mutant T cells was comparable to that detected in their control counterparts. T cells expanded from controls and patients exhibited two prominent PSMC3/Rpt5 immunoreactive bands migrating at about 50 and 40 kDa (Fig. 4A). Although the upper band running at ~50 kDa corresponded to the PSMC3/Rpt5 expected size, the nature of the lower band running at ~40 kDa was unclear. It is, however, unlikely that this band may be nonspecific, because it was detectable in T cells using another anti-PSMC3/Rpt5 antibody or in other cell types (fig. S10). This additional species might reflect a shorter, as-yet-undescribed PSMC3/Rpt5 isoform or a processed form arising from the PSMC3/Rpt5 full-length protein. Nevertheless, the unchanged protein expression profile of PSMC3/Rpt5 between controls and patients indicates that none of these phenomena is affected by any of the *PSMC3* variants. Likewise, none of *PSMC3* variant T cells showed reduced expression of the PSMC3/Rpt5 full-length protein, suggesting that proteasome dysfunction in these affected individuals was not caused by haploinsufficiency.

Next, T cells from affected individuals and relative controls were analyzed for proteasome complex formation and activity by in-gel fluorescence followed by Western blotting on native polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 4B, the chymotrypsin-like activity of the 26S and 20S proteasome complexes [fluorogenic peptide-aminomethyl coumarin (LLVY-AMC)] was reduced for the majority of comparisons between patients and controls. Subsequent Western blot analysis revealed that the decreased 20S activity observed in patient #21 was associated with a decreased pool of 20S-PA28 complexes, as determined by reduced band intensity for the $\alpha 6$ and PA28 proteins. The amounts of unbound PA28- α/β complexes in T cells were increased in patient #13 and decreased in patient #18 (Fig. 4B). The reasons for these contrasting data between these two patients are unclear but might reflect distinct abilities to compensate for proteasome dysfunction. It should be

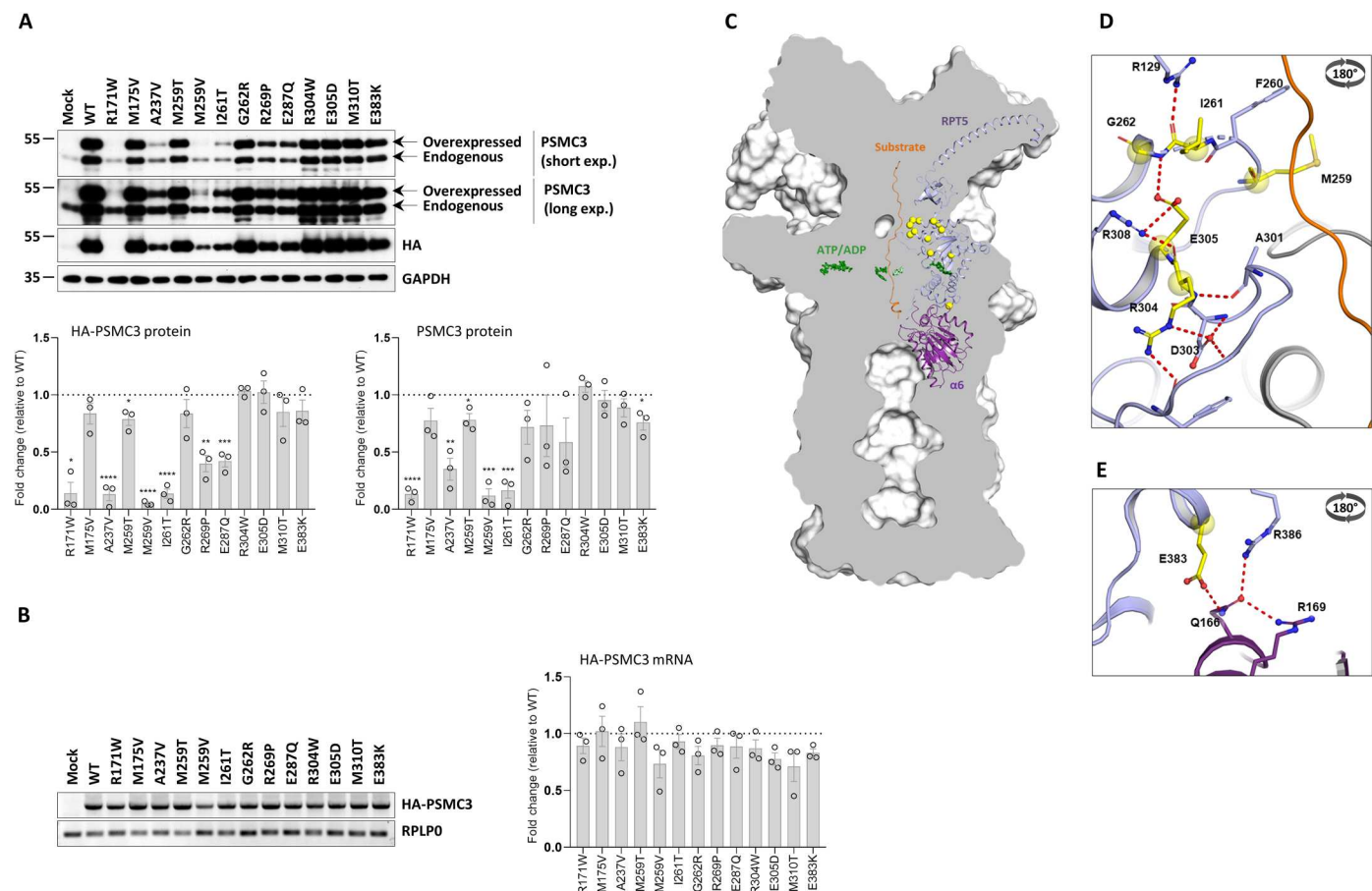


Fig. 3. PSMC3/Rpt5 protein variants do not behave similarly at the molecular level. (A) Top: SHSY5Y cells were transfected with HA-tagged *PSMC3* mutants for 24 hours before protein extraction and Western blotting using antibodies specific for PSMC3/Rpt5 and HA, as indicated. Nontransfected and mock-transfected cells served as negative controls. Equal protein loading was ensured by probing the membranes with an anti- α -tubulin monoclonal antibody (two exposure times are shown). Arrows indicate overexpressed and endogenous PSMC3/Rpt5. One representative experiment of three is shown. Bottom: Quantification of HA-tagged and untagged PSMC3/Rpt5 proteins in transfected SHSY5Y cells by densitometry. Data are presented as protein fold changes to WT PSMC3/Rpt5 proteins whose densitometry measurements were set to 1 (grid line) after normalization with GAPDH. Mean values $\pm$ SEM from three independent experiments are shown. Statistical significance was assessed by unpaired Student's *t* test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001). (B) Left: SHSY5Y cells that were transfected with HA-PSMC3 variants were subjected to total RNA extraction followed by semi-quantitative PCR using primer located in *PSMC3* and the bovine growth hormone (BGH) polyadenylation signal of the pcDNA3.1/myc-HIS expression vector. Equal loading between the samples was ensured by amplifying the *RPLP0* gene, as indicated. Right: Quantification of HA-tagged *PSMC3* transcripts in transfected SHSY5Y cells by densitometry. Data are presented as mRNA fold changes to WT HA-PSMC3 mRNA whose densitometry measurements were set to 1 (grid line) after normalization with *RPLP0*. Mean values $\pm$ SEM from three independent experiments are shown. (C) A sliced surface view of the 26S proteasome (gray) was superimposed with a cartoon representation of the subunit PSMC3/RPT5 (blue) and PSMB1/α6 (purple) as well as the substrate (orange). The ATP/adenosine diphosphate (ADP) molecules of the AAA-ATPase ring are shown as green sticks, whereas the positions of the investigated missense variants are indicated as bright yellow spheres. (D) Detailed representation of the missense variants in the loop region of the N-terminal α/β domain. The residues affected by these variants are involved in a polar interaction network close to the substrate tunnel (view rotated by 180° around the x axis). (E) Close-up view on the RPT5-α6 interface affected by the E383L variant. Residues affected by this variant are shown as bright yellow balls and sticks with atoms colored by polarity (oxygen in red, nitrogen in blue, and sulfur in dark yellow; view rotated by 180° around the x axis).

noted, however, that, in contrast to PA28-bound proteasomes whose amounts did not change in these patients, free PA28-α/β are not equipped with protease activity and as such have no impact on intracellular proteolysis. Densitometric analysis of the PSMC3/Rpt5 signals revealed impaired subunit incorporation into 26S, hybrid and 30S proteasome complexes in patients when compared with controls (*P* = 0.0329) (Fig. 4B, bottom). Diminished expression of the α6 and PSMC3/Rpt5 subunits was observed in the 26S proteasomes of patient #18 (Fig. 4B), indicating that the decline in 26S activity detected in this patient was likely to be attributed to

decreased amounts of 26S complexes. Likewise, the PSMC3/Rpt5 contents in 26S complexes were also reduced in patient #21 (Fig. 4B). Unexpectedly, 20S and 26S proteasome pools of patient #13 did not substantially vary when compared with those of the related control (Fig. 4B). The minor effects exerted by the G262R substitution in patient #13 may be partially explained by the fact that G262 is surrounded by a large amount of empty space (fig. S11), thereby allowing this region of the PSMC3/Rpt5 protein to accommodate mutations to some extent. Nevertheless, these data indicated that both of the R304W and E305D PSMC3/Rpt5 variants

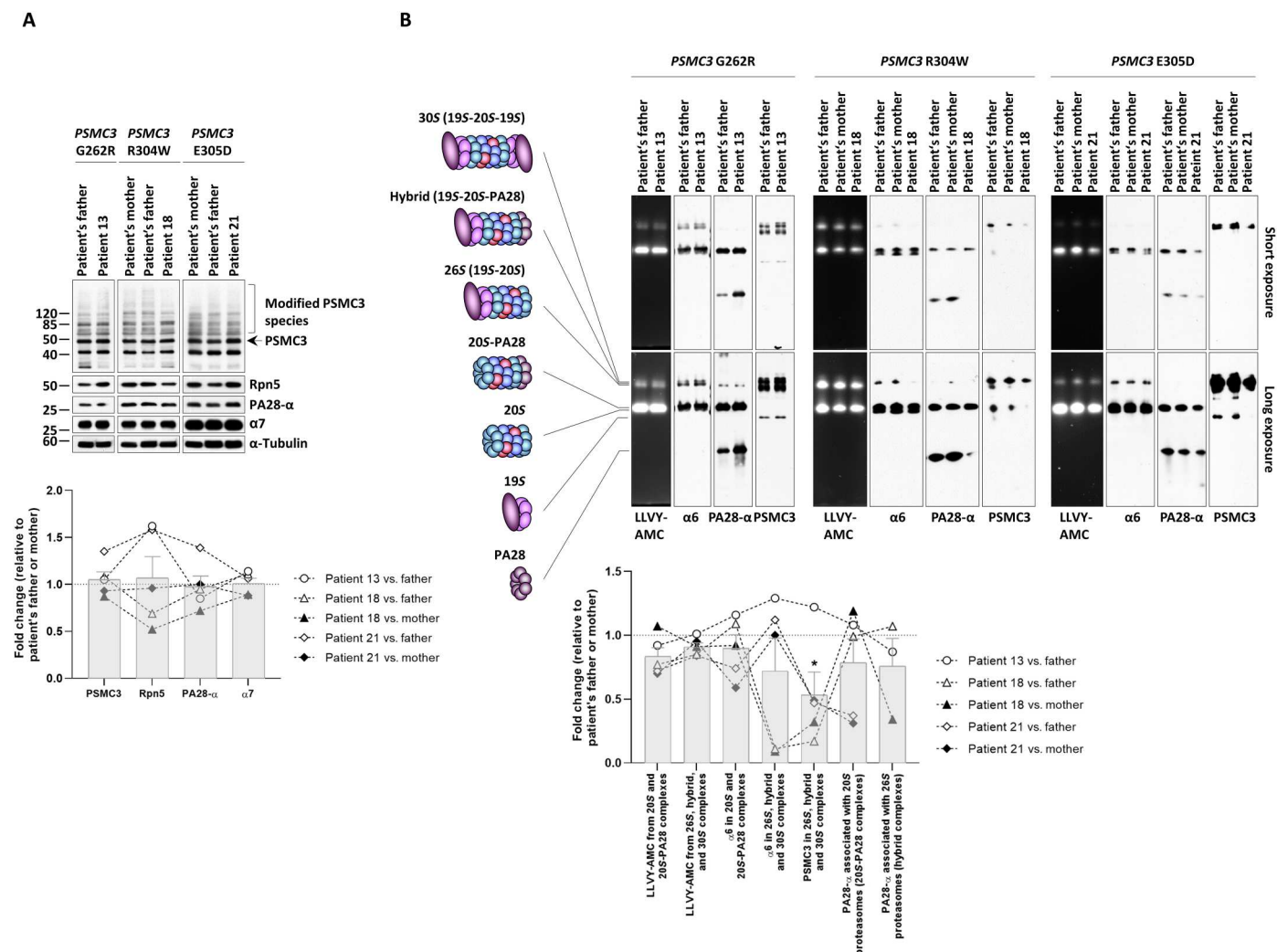


Fig. 4. PSMC3/Rpt5 protein variants lead to proteasome assembly defects in patient T cells. (A) Top: Five to 20 µg of radioimmunoprecipitation assay (RIPA) lysates from T cells isolated from patients #13, #18, and #21 and related controls (index case's father and/or mother) were separated by SDS-PAGE, followed by Western blotting using antibodies directed against PSMC3/Rpt5, PSMD12/Rpn5, PA28-α, and α7, as indicated. Equal protein loading was ensured by probing the membrane with an anti-α-tubulin antibody. Arrow indicates full-length PSMC3/Rpt5 migrating at the predicted size of about 50 kDa. High-molecular weight (HMW)-modified PSMC3/Rpt5 species are marked by a bracket. Bottom: Quantification of the Western blots by densitometry. Data are presented as fold changes in patients #13, #18, and #21 versus their father, mother, or both, whose densitometric measurements were set to 1 (grid line), as indicated. Columns indicate the fold change mean values ± SEM calculated from the five normalizations. (B) Top: Twenty micrograms of T cell lysates from patients #13, #18, and #21 and their parents (mother and/or father) were separated by 3 to 12% native PAGE. Proteasome chymotrypsin-like activity was assessed in gels using the LLVY-AMC fluorogenic peptide, as indicated. Gels were subsequently subjected to Western blotting using antibodies specific for α6, PSMC3/Rpt5 and PA28-α, as indicated. The schematic to the left depicts the proteasome complexes (30S, hybrid, 26S, 20S-PA28, and 20S) and free regulators (19S and PA28) detected by the three antibodies. Bottom: Quantification of the LLVY-AMC fluorescent signals and α6, PA28-α, and PSMC3 immunoreactive bands in 20S (short exposure), 20S-PA28 (short exposure), 26S (long exposure), hybrid (long exposure), and/or 30S (long exposure) proteasome complexes by densitometry, as indicated. Data are presented as activity (LLVY-AMC) and protein (α6, PA28-α, and PSMC3) fold changes in patients #13, #18, and #21 versus their father and/or mother, whose densitometric measurements were set to 1 (grid line), as indicated. Columns indicate the fold change mean values ± SEM calculated from the five normalizations. Statistical significance was assessed by unpaired Student's test (*P < 0.05).

affect 20S proteasome assembly, 26S proteasome assembly, or both in individuals with NDD/ID, whereas the G262R variant has little impact in this process (Table 1).

Quantitative proteomics identifies cellular pathways affected by PSMC3 loss of function in patients with NDD/ID

To better understand the cellular consequences of PSMC3 loss of function, we next performed a mass spectrometry-based

comparative analysis of the T cell proteomes of patient #17 and patient #21 (R304W and E305D, respectively) with that of their relative controls. As shown in Fig. 5, our data identified a protein signature consisting of 17 ribosomal proteins of the small 40S (RPS) or large 60S (RPL) ribosomal subunits that were specifically up-regulated in both investigated patients. This suggests that mRNA translation is a major affected pathway upon PSMC3 loss of function, a notion that is further supported by the fact that components of the

mRNA processing machinery such as CUG-binding protein Elavl-like family member 1 (CELF1), a member of the LSM family of RNA binding proteins (LSM1), RNA polymerase II CTD phosphatase (SSU72), and inosine triphosphatase were also differentially expressed between patients and controls (Fig. 5). Other notable proteins whose abundances varied in patients carrying *PSMC3* variants included components of the immune system such as MX dynamin-like GTPase 1 (MX1), a typical IFN-stimulated gene (ISG) product, and the α chain of the IL3 receptor (IL3RA). These proteins were regulated in opposite directions, with both patients exhibiting higher amounts of MX1 but reduced amounts of IL3RA (Fig. 5). Our analysis further revealed that *PSMC3* loss of function was also associated with increased protein expression of the H1.5 and H1.2 linker histone H1 variants, a finding that is in line with a role of the UPS in chromatin regulation (46). Protein set enrichment analysis uncovered that most of the significant ($P < 0.05$) proteomic changes between control and patients carrying *PSMC3* variants were related to mRNA metabolism and translation (fig. S12), confirming that protein synthesis was dysregulated in these patients. Other differentially expressed proteins found to be enriched in T cells with *PSMC3* variants belonged to the category of viral processes (fig. S12), unveiling a potential relationship between proteasome loss of function and innate immunity. Collectively, these data suggested that patients with *PSMC3* variants exhibit alterations in basic cellular processes, including mRNA translation, immune signaling, and chromatin remodeling.

***PSMC3* variants cause proteotoxic stress in T cells from patients**

Proteasome dysfunction has been shown to be accompanied by accumulation of ubiquitin-protein conjugates and proteotoxic stress (24). As shown in Fig. 6A, all four investigated patients exhibited typical features of unbalanced protein homeostasis, as evidenced by the increased accumulation of ubiquitin-modified species when compared with their respective related controls using immunoblotting and densitometric analysis. Proteotoxic stress is known to induce the unfolded protein response and integrated stress response (UPR and ISR, respectively) (24, 47). To address this point, we quantified the expression of the glucose-regulated protein 94 chaperone protein [GRP94; the heat shock protein 90 kDa in the endoplasmic reticulum (ER)], whose up-regulation is understood to be a major hallmark of the UPR (48). The expression of GRP94 was increased in all patients (Fig. 6A). However, the activation of the UPR was only partial, because the phosphorylation and activation status of two other UPR markers, namely, serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α (IRE1) and eukaryotic translation initiation factor 2 (eIF2) α , were not changed between controls and patients carrying *PSMC3* variants (Fig. 6A). The failure to detect increased phosphorylated eIF2 α , although the upstream kinase PKR was consistently activated in all patients (Fig. 6A), can be explained by up-regulation of both eIF2 α phosphatases GADD34 (growth arrest and DNA damage-inducible protein 34) and CREP (constitutive repressor of eIF2 α phosphorylation) across all four patients. Immunoblotting and densitometric analysis revealed that T cells of the patients carrying *PSMC3* variants were also endowed with increased protein expression of microtubule-associated protein 1A/1B light chain 3 phosphatidylethanolamine conjugate LC3b-II (Fig. 6B), suggesting that the inability of these cells to eliminate ubiquitin-protein

aggregates cells via their 26S proteasomes triggers a compensatory mechanism mediated by activation of the autophagy system. Consistently, the mitochondrial proteins PINK1 (PTEN-induced putative kinase protein 1) and Bnip3L/NIX (BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like/NIP3-like protein X) were found to be decreased in the patients carrying *PSMC3* variants (Fig. 6B and Table 1), supporting the notion that selective autophagic processes, including mitophagy, were activated upon *PSMC3* disruption. Because proteasome impairment typically results in the release of the TCF11/Nrf1 (transcription factor 11/nuclear factor erythroid-derived 2 like 1) protein from the ER membrane (49, 50), we next sought to determine the TCF11/Nrf1-processing pattern in NDD/ID-affected individuals. However, no differences in the TCF11/Nrf1-processing pattern could be detected between controls and patients carrying *PSMC3* variants (Fig. 6B), suggesting that *PSMC3* variants associated with NDD/ID do not lead to activation of the TCF11/Nrf1 signaling pathway.

T cells from patients with *PSMC3* variants exhibit a type I IFN signature

Because proteasome loss of function results in the generation of a typical type I IFN response in patients with PRAAS (30), we next sought to determine whether alterations in the *PSMC3* gene would induce type I IFN signatures as well. To this end, we undertook a comparative examination of the mRNA expression of 750 predefined immunologically relevant genes in T cells from patients carrying *PSMC3* variants and relative controls (father and/or mother) using the NanoString nCounter platform. A total of 30 differentially expressed genes could be identified, including 11 ISGs that were specifically up-regulated in all patients carrying *PSMC3* variants (Fig. 7A), suggesting that *PSMC3* loss of function is associated with type I IFN responses. The transcriptomic analysis of control and patient T cells further revealed that *PSMC3* disruption resulted in the up-regulation of genes of the notch signaling pathway such as NOTCH2 (neurogenic locus notch homolog protein 2) and JAG2 (protein jagged-2) involved in developmental pathways, including neurodevelopment (Fig. 7A) (51). To validate the type I IFN gene signature revealed by our omics profiling, we next evaluated the expression of seven ISGs (*IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *MX1*, *RSAD2*, and *IFI44*) in samples of these four families with *PSMC3* missense variants. As shown in Fig. 7B, all four affected children's samples (patients #13, #17, #18, and #21) exhibited much higher ISG expression than their parents (father and/or mother; Table 1). Calculation of the fold change median of the seven ISGs revealed that a significant increase was detected in all *PSMC3* index cases when compared with their respective controls (patient #13 versus father, $P = 0.0148$; patient #17 versus mother, $P = 0.0008$; patient #18 versus father, $P = 0.0002$, patient #18 versus mother, $P = 0.0011$; patient #21 versus father, $P = 0.0012$; and patient #21 versus mother, $P = 0.0142$) (Fig. 7B). The strongest type I IFN signature was observed in patient #18, whose ISGs were up-regulated by approximately 40- or 10-fold when compared with the father or mother, respectively. Patients #13, #17, and #21 exhibited a milder type I IFN induction characterized by a two- to sixfold increase in ISG transcripts compared with their respective controls. Among the seven ISGs tested, *IFIT1* and *IFI44L* were the genes that underwent the most pronounced up-regulation in all four affected individuals.

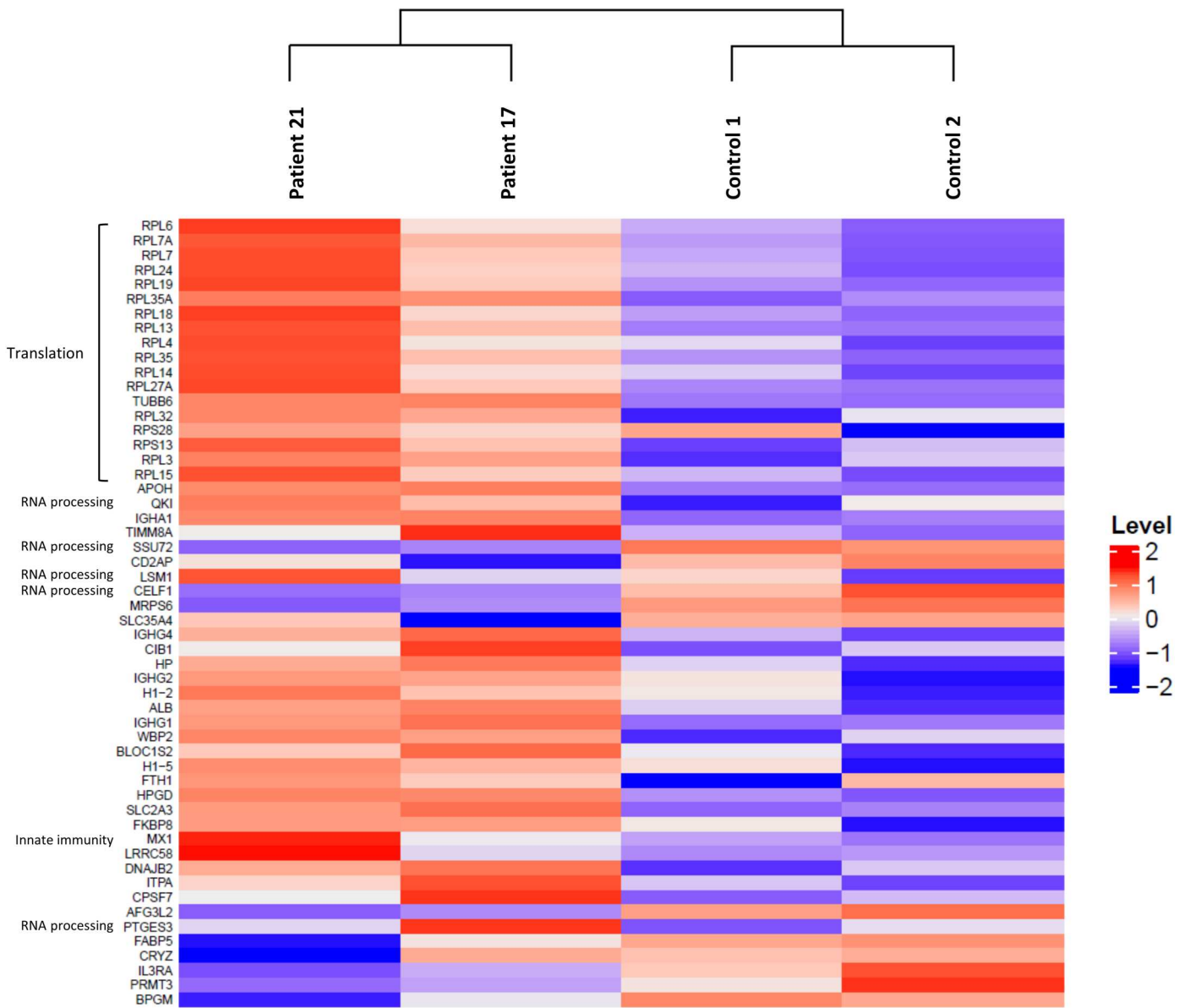


Fig. 5. Proteomic signatures of patients carrying *PSMC3*/*Rpt5* variants. Heatmap cluster analysis showing the similarities in the protein expression profiles of the patients #17 and #21 (carrying the R304W and E305D *PSMC3* variants, respectively) compared with their related controls (father and/or mother of the proband), as indicated. The heatmap indicates the normalized and scale expression value of proteins in the individual samples. Only the differentially expressed proteins with an absolute value of log₂ fold change greater than 2 were selected for the clustering analysis.

The type I IFN signature in T cells from patients with *PSMC3* variants is PKR dependent

We next calculated and compared the IFN scores of both patients carrying *PSMC3* variants and their related controls with those of T cells isolated from six healthy donors. As shown in Fig. 8A, three of the related controls had an IFN score slightly above the cut-off value of 2.466 defined by Rice *et al.* (52) to be abnormal. However, the IFN scores of all related and unrelated controls remained significantly lower than those of the four tested patients ($P = 0.0845$ and $P = 0.0044$), thereby confirming that these *PSMC3* variants were associated with enhanced type I IFN signaling. A second independent assessment of patient #18 at 14 months after enrollment

revealed that this patient still exhibited a very high type I IFN score (fig. S13). Because all three inducible immunoproteasome subunits— $\beta 1i$ (*PSMB9*), $\beta 2i$ (*PSMB10*), and $\beta 5i$ (*PSMB8*)—are encoded by genes typically stimulated by type I and II IFN (20), we next asked whether *PSMC3* loss of function was accompanied by a switch from standard proteasomes to immunoproteasomes. As illustrated in fig. S14, the steady-state expression of these subunits in T cells did not substantially change between control and patients, as determined by Western blotting. This may be because T cells, as immune cells, express high amounts of immunoproteasomes, a feature that renders any further protein up-regulation of the inducible subunits very difficult (53).

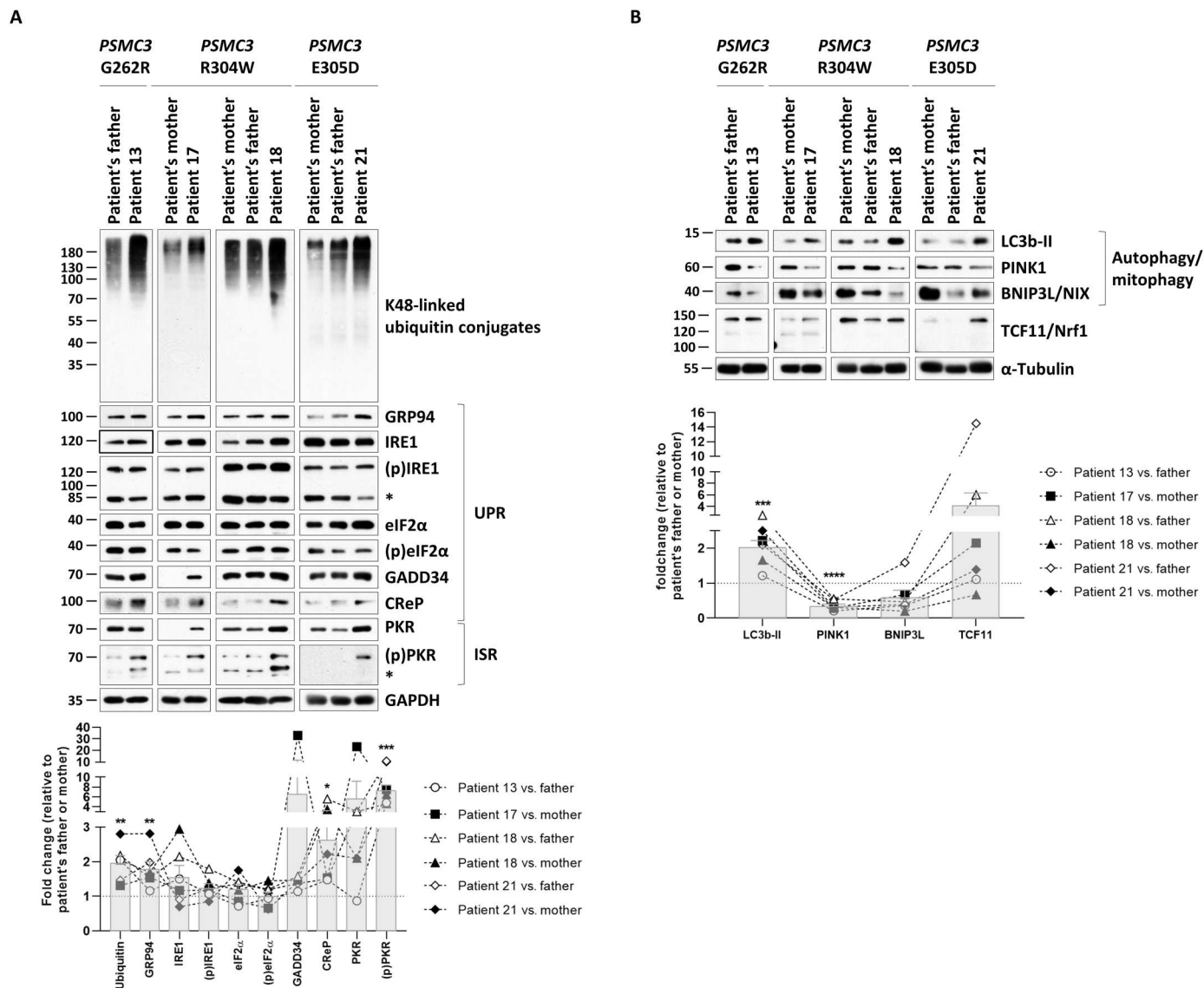


Fig. 6. T cells from patients with *PSMC3/Rpt5* missense variants exhibit signs of protein homeostasis perturbations and alterations of the UPR, ISR, and autophagy/mitophagy pathways. (A) Top: Five to 20 µg of RIPA lysates from T cells isolated from patients #13, #17, #18, and #21 and related controls (*PSMC3* index case's father and/or mother) were separated by SDS-PAGE followed by Western blotting using antibodies directed against K48-linked ubiquitin-modified proteins, such as GRP94, IRE1, phospho-IRE1, eIF2α, phospho-eIF2α, GADD34, CREP, PKR, phospho-PKR, and GAPDH (loading control), as indicated. Bottom: Quantification of the Western blots by densitometry is shown. Data are presented as fold changes in patients #13, #17, #18, and #21 versus their father and/or mother, whose densitometric measurements were set to 1 (grid line), as indicated. Columns indicate the fold change mean values ± SEM calculated from the six normalizations. Statistical significance was assessed by unpaired Student's test (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). (B) Top: RIPA cell lysates from patients #13, #17, #18, and #21 and their related controls (index case's father and/or mother) were subjected to SDS-PAGE/Western blotting using antibodies specific for LC3b-II, PINK1, BNIP3L, and α-tubulin (loading control), as indicated. Bottom: Quantification of the Western blots by densitometry. Data are presented as protein fold changes in patients #13, #17, #18, and #21 versus their father and/or mother, whose densitometric measurements were set to 1 (grid line), as indicated. Columns indicate the fold change mean values ± SEM of the six normalizations. Statistical significance was assessed by unpaired Student's test (*** $P < 0.001$ and **** $P < 0.0001$).

We next attempted to unravel the mechanisms by which type I IFN responses were initiated in patients carrying *PSMC3* loss-of-function variants. Having shown that all four affected patients exhibited marked alterations in the mitophagy, UPR, and ISR pathways (Fig. 6), we postulated that the dysregulation of either one of these pathways might act as a danger signal triggering innate immunity. To address this point, we inhibited key players of the UPR and/or ISR, including PKR, IRE1, and GADD34 (24), by treating

patients' T cells with the C16, 4µ8C, or Guanabenz inhibitors, respectively. In addition, the potential implication of mitophagy-mediated mitochondrial DNA in this process was investigated by treating the cells with H-151, an inhibitor of the cytosolic DNA sensor STING (stimulator of IFN genes protein) (54). Of the four inhibitors used, only C16, targeting PKR, could substantially reduce the type I IFN response associated with *PSMC3* variants in these patients (Fig. 8B). These data thus suggest PKR as a sensor of

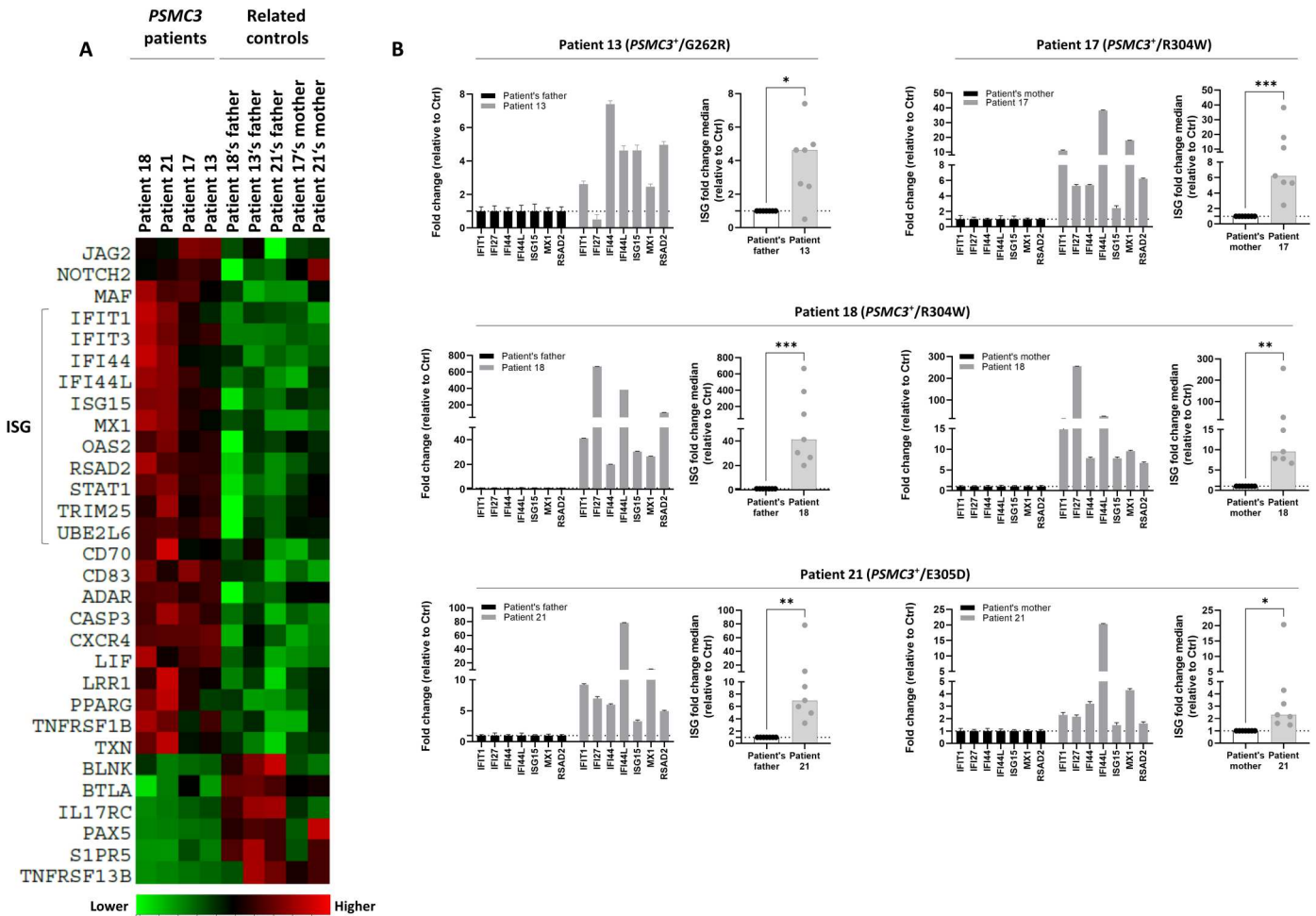


Fig. 7. T cells from patients with *PSMC3/Rpt5* missense variants exhibit a type I IFN signature. (A) Heatmap clustering of gene expression in T cells isolated from patients carrying a *PSMC3* variant and their relative controls (father, mother, or both). Each column represents one individual patient or related control, and each row represents one gene. Clustering of genes and samples was carried out by centered Pearson correlation. Color indicates normalized counts of each transcript, with green representing higher expression and red representing relatively lower expression. (B) Gene expression of seven typical IFN-stimulated genes (*IFIT1*, *IFI27*, *IFI44*, *IFI44L*, *ISG15*, *MX1*, and *RSAD2*) was assayed by RT-qPCR on T cells derived from patients #13, #17, #18, and #21 and their respective controls (the index case's father and/or mother). Expression levels were normalized to *GAPDH*, and relative quantifications are presented as fold change over controls. The median fold expression of the seven ISGs over relative controls is also shown. Statistical significance was assessed by ratio paired t test (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

proteasome dysfunction triggering a type I IFN signature in individuals carrying *PSMC3* variants.

DISCUSSION

In this study, we identified 15 missense variants in the *PSMC3* gene in 23 unrelated individuals with NDD/ID (Fig. 1, Table 1, and table S1) and showed that the 19S AAA-ATPase proteasome subunit *PSMC3/Rpt5* is a critical protein for the development of the central nervous system (CNS). This notion is in line with previous reports showing that conditional inactivation of other 19S proteasome subunits (*Psmc2/Rpt2* and *Psmc4/Rpt3*) in mice results in severe neuronal phenotypes with features of neurodegeneration and locomotor dysfunction (55, 56). Recently, a homozygous deep intronic variant creating a cryptic exon in the *PSMC3* gene was linked to a familial recessive neurosensory syndrome (57). However, given their distinct modes of inheritance and

pathogenesis, this recessive disorder observed in a single family and the dominant variants, which we describe, are likely two different clinical entities with only partial overlap of clinical and molecular disease phenotypes (57). This dichotomy between dominant and recessive disorders may, nevertheless, be too reductive, because our data show that the dominant *PSMC3* variants do not necessarily exert the same effects on proteasome expression, assembly, or both (Figs. 3A and 4B and Table 1). These variations make it difficult to classify dominant *PSMC3* variants according to their impact on cellular function without any in vitro functional studies.

There are several limitations to this study. Given the limited number of biological samples investigated, a correlation between genotype and cellular phenotype could not be established. In this regard, it should also be noted that the gene silencing in the *Drosophila* model does not completely reflect disease biology patients, given that gene product is still available. We can furthermore not fully exclude a recruitment bias in our patient dataset.

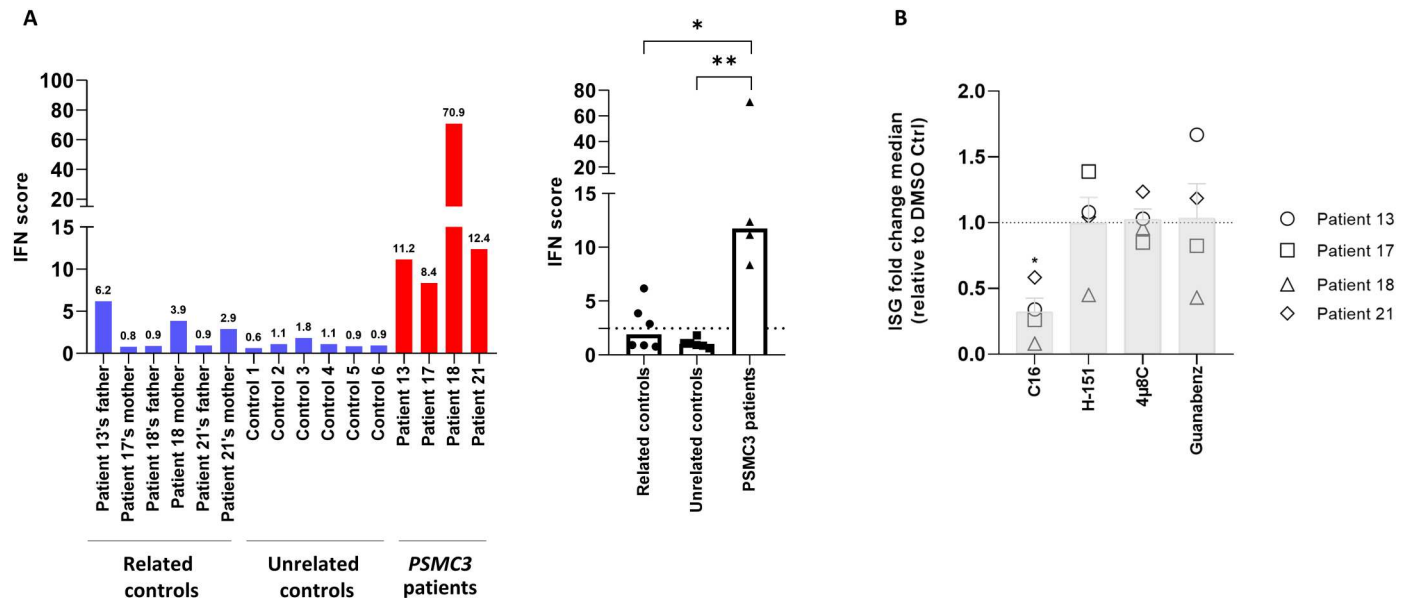


Fig. 8. T cells from patients with *PSMC3/Rpt5* missense variants exhibit high PKR dependent type I IFN scores. (A) Left: IFN scores for patients #13, #17, #18, and #21 and related controls as well as for six unrelated controls (1 to 6) were calculated as the median of the relative quantifications of the seven ISGs over a single calibrator control. The IFN scores of each sample (left) and the sample groups, namely, parents, unrelated healthy donors, and patients carrying *PSMC3* variants are shown, as indicated. Right: Box plot of concatenated data. Statistical significance was assessed by unpaired t test (* $P < 0.05$ and ** $P < 0.001$). (B) T cells isolated from individuals carrying *PSMC3* variants were subjected to a 6-hour treatment with DMSO (vehicle), C16 (500 nM), H-151 (2 μ M), 4 μ 8C (100 μ M), or Guanabenz (50 μ M) inhibitors before RNA extraction and RT-qPCR for expression analysis of *IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *RSAD2*, *IFI44*, *OASL*, and *MX1*. Transcript expression was normalized to *GAPDH*, and data are presented as the fold change median values of the eight ISGs relative to DMSO (grid line) for each patient in each treatment. Columns indicate the fold change mean values $\pm$ SEM of the patient group ($N = 4$) for each treatment. Statistical significance was assessed by ratio paired t test (* $P < 0.05$).

Cognitive flexibility is an important aspect of typical brain function, which allows adaptation to both physical and social environmental changes (58, 59). This may be assessed by evaluating reversal learning performance, a process that was initially identified in *Drosophila* models (60) and whose dysfunction has been associated with the pathogenesis of various neuropsychiatric disorders (61–65). Although *Rpt5* gene silencing in flies had no discernible effect on learning performance, it led to compromised reversal learning (Fig. 2). Although proteasomes have been shown to regulate long-term potentiation (LTP) (66, 67), their involvement in reversal learning has not previously been shown. Our data identify *PSMC3/Rpt5* as a key regulator of this process, whose molecular landscape was initially limited to a few molecules related to the cytoskeleton and GABAergic system (68–70). One cannot exclude that *Rpt5* gene silencing in *Drosophila* may result in global depletion of 26S complexes, which, in turn, impairs reversal learning. However, some of the missense mutations identified in this study led to decreased *PSMC3/Rpt5* expression (Fig. 3A), reduced incorporation into 26S complexes (Fig. 4B), or both, suggesting that a shortage of the *PSMC3/Rpt5* subunit may reflect disease pathogenesis. The mechanisms by which proteasomes regulate reversal learning are unclear but may imply the degradation of specific neuronal proteins to control some synaptic connections and “reset” learning associations. It is tempting to speculate that one of these substrates could be Arc (activity-regulated cytoskeleton-associated protein), a key mediator of synaptic plasticity whose persistent expression has recently been shown to interfere with the reversal learning process (71). Additional evidence in favor of a critical role of *PSMC3/Rpt5* in behavioral flexibility emerged from our experiments in primary

hippocampal neurons, showing that ectopic expression of *PSMC3/Rpt5* affected dendrite growth (fig. S3). The observation that the R304W, E305D, and E383L *PSMC3* variants did not differ from their WT counterpart in this process is intriguing but may have several explanations. First, WT *PSMC3* overexpression may exert ceiling effects that overshadow any potential detrimental impact of the *PSMC3* variants on neuronal morphology. Second, it may be that *PSMC3* variants, with the exception of M159V, have no substantial effect on neuronal morphology. The reasons why the M159V *PSMC3* variant behaves differently are unclear, but these findings might be explained by distinct half-life, intracellular localization, or posttranslational modifications. One could also argue that the adverse effects of *PSMC3/Rpt5* on this process might be due to extra-proteasome functions as a consequence of an excess of “free” subunits after transfection. However, our investigations on patient T cells showed that *PSMC3* missense variants were associated with an increased accumulation of ubiquitin-modified proteins (Fig. 6A), suggesting that these alterations give rise to proteasome loss-of-function variants associated with perturbed protein homeostasis. Future functional studies involving a larger number of biological samples are required to evaluate a possible correlation between *PSMC3* variants and the extent of intracellular proteolysis dysfunction as a prerequisite for predicting disease severity.

Our proteomic analysis revealed that T cells from patients carrying *PSMC3* variants were enriched with ribosomal proteins such as RPL4, RPL6, RPL7A, and RPL7 (Fig. 5 and Table 2). These proteins may be specifically targeted for degradation, and their accumulation may occur as a consequence of impaired intracellular protein

clearance. Consistent with this notion, proteasome inhibition has been recently shown to result in the aggregation of ubiquitin-modified ribosomal proteins (72). Our data therefore support the recent view that ribosome dysregulation defines a key feature of NDD/ID phenotypes (73, 74) and the concept of translational arrest upon proteotoxic stress via the action of eIF2 α kinases (Fig. 6A).

One key finding is the observation that patients with *PSMC3* variants generate a type I IFN gene signature (Figs. 7 and 8A). Although it is well established that proteasome loss-of-function variants cause interferonopathies in patients with PRAAS (27–33), it was only recently that pathogenic mutations in the 19S regulatory particle subunit *PSMD12/Rpn5* were reported to engage constitutive type I IFN signaling in patients with this NDD disorder (35, 36) sharing similarities with the patients described in this manuscript. Although patients with PRAAS and individuals with NDD/ID and *PSMD12* or *PSMC3* variants carry genomic alterations that affect the same multisubunit enzyme (26S proteasome), their clinical phenotypes do not entirely overlap. For instance, patients with NDD/ID and *PSMC3* variants did not develop recurrent fever, lipodystrophy, and/or skin lesions, which are usually detected in patients with PRAAS (Table 1). One could argue that such differences may reflect distinct localizations of the affected subunits within the 26S proteasome complex, thereby suggesting that alterations of the 19S regulatory particle promote the generation of NDD/ID, whereas those of the 20S core particle or assembly chaperones favor the development a PRAAS phenotype. This assumption is, however, challenged by the fact that *PSMB1/* $\beta 6$ variants of the 20S core particle lead to the acquisition of a neuronal phenotype very similar to that observed in patients with NDD/ID and *PSMC3* variants (25). The lack of systemic autoinflammation in patients with NDD/ID and *PSMC3* variants mounting a constitutive type I IFN response may seem surprising at first sight, but it is not totally unexpected, because this inconsistency is found in other NDDs, including Aicardi-Goutières (75, 76) and Down syndrome (77, 78). This is particularly well exemplified in patients with Down syndrome who, similar to patients with NDD/ID and *PSMC3* dominant variants, exhibit a constitutive activation of type I IFN signaling (79, 80). To what extent type I IFN actively contributes to the pathogenesis of these disorders remains to be fully determined, although a growing body of evidence supports the notion that IFN has detrimental effects on CNS function (81–84) as well as stem cell function and differentiation (85, 86). Because proteasome dysfunction typically engages stress responses involving compensatory mechanisms such as autophagy (87), ISR, and UPR (24, 88), we reasoned that the type I IFN response detected in patients with *PSMC3* variants might be triggered by sustained activation of either one of these pathways. As anticipated, high expression of autophagy and ER stress markers was detected in affected individuals, as evidenced by increased expression of the LC3-II and GRP94 proteins (Fig. 6, A and B). Both PINK1 and NIX mitochondrial proteins were found to be decreased in affected individuals (Fig. 6B), suggesting that *PSMC3* variants increase autophagy-driven elimination of mitochondria (mitophagy). This observation supports the growing consensus that mitochondrial dysfunction is a key determinant of the pathogenesis of neurodevelopment (89) and the cause of interferonopathies (90). The activation status of PKR, a protein of the ISR that intersects with the UPR (91), was substantially increased in all investigated patients carrying the *PSMC3* variant. Both ISR and UPR have the ability to counterbalance proteotoxic stress by inducing a global translational

arrest via eIF2 α phosphorylation. This is accompanied by concomitant accumulation of nontranslated mRNAs, the formation of stress granules recruiting different RNA species, and RNA-processing enzymes, and IRE1-dependent mRNA decay (RIDD) (92). Although PKR typically responds to viral double-stranded RNA (93), it also may undergo activation under sterile conditions upon different stresses, including ER stress involving PKR-associated activator (PACT) and its modulator TAR RNA binding protein 2 (TRBP), a protein required for microRNA biogenesis (94–96). Both PACT and TRBP were observed to be increased in patient cells along with several RNA-processing factors. Our inhibition experiments suggested PKR as the inducer of type I IFN in these patients (Fig. 8B). The mechanisms by which *PSMC3* variants activate PKR in affected individuals remain unclear, but our data open the possibility that PKR may sense a broader spectrum of danger signals than initially assumed, including perturbations of protein homeostasis. This concept is in line with the observation that activated PKR was found in the CNS of patients with neurodegenerative diseases (97–100) and that neurodegeneration is associated with neuroinflammation (101, 102). Together, our work demonstrates that heterozygous *PSMC3* dominant variants result in a neurodevelopmental syndrome associated with a specific type I IFN gene signature and suggests treatment options targeting type I IFN signaling or PKR.

MATERIALS AND METHODS

Study design

The aim of the study was to determine the involvement of the human *PSMC3* gene in a neurodevelopmental disorder hitherto unreported. Affected individuals were recruited, and data were collected on an ongoing basis. Affected individuals were identified via the data sharing platform GeneMatcher (103) and direct requests in variants databases whose access was authorized to the University of Washington School of Medicine. Clinical and molecular data were provided by the referring geneticists following the patients. Facial recognition by GestaltMatcher (41) was used to measure the similarities of the facial phenotypes between affected individuals (fig. S2). Prediction of variant pathogenicity was done using the bioinformatics programs indicated in Table 1, and their frequency was determined in public variant databases [gnomAD; (104), Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (<http://evs.gs.washington.edu/EVS/>) (accessed March 2023); Bravo powered by TOPMed Freeze 8 on GRCh38 (105)]. The effects of the *PSMC3* variants on proteasome function, protein homeostasis, proteotoxic stress sensors (UPR and ISR), autophagy/mitophagy, and inflammation status (type I IFN) were studied in vitro by Western blotting, NanoString analysis, and quantitative PCR (qPCR) using T cells expanded from whole-blood samples of patients ($N = 4$) and their parents ($N = 6$) who agreed to provide blood specimens. The first available T cell samples (patients $N = 2$, related controls $N = 2$) were used for proteomics analyses to explore deregulated biological pathways. The impact of the variants on neuronal function was assessed in two models: (i) upstream activation sequence–Rpt5 RNAi *D. melanogaster* lines targeting *psmc3* for evaluating the effect of Rpt5 knockdown on behavior (Fig. 2) and (ii) primary hippocampal neuronal cultures overexpressing cDNA constructs containing the first identified variants ($N = 4$) for evaluating their effects on neuronal

Table 2. List of proteins enriched in patients #17 and #21 versus their controls (mother and father/mother, respectively).					
PG.Protein Groups	Protein name	Gene name	Mut/Co signal_log2_ratio	Mut/Co raw_p_value	Mut/Co adjusted_p_value
P02749	Beta-2-glycoprotein 1	APOH	3.90875773	3.9233 × 10 ⁻⁰⁵	0.01752392
P01861	Immunoglobulin heavy constant gamma 4	IGHG4	3.41081457	3.9313 × 10 ⁻⁰⁷	0.0003951
Q02878	60S ribosomal protein L6	RPL6	3.09519982	8.1085 × 10 ⁻⁰⁵	0.02173071
P62424	60S ribosomal protein L7a	RPL7A	2.97520531	2.4946 × 10 ⁻⁰⁶	0.00143263
P18124	60S ribosomal protein L7	RPL7	2.90834996	5.5264 × 10 ⁻⁰⁵	0.01815176
P83731	60S ribosomal protein L24	RPL24	2.78639617	5.8732 × 10 ⁻⁰⁵	0.01815176
P16403	Histone H1.2	H1-2	2.72232694	4.4042 × 10 ⁻⁰⁵	0.0177049
P02768	Serum albumin	ALB	2.64236248	3.3722 × 10 ⁻¹⁸	1.3556 × 10 ⁻¹⁴
P36578	60S ribosomal protein L4	RPL4	2.50022766	3.276 × 10 ⁻⁰⁷	0.0003951
P01876	Immunoglobulin heavy constant alpha 1	IGHA1	2.32067402	3.007 × 10 ⁻⁰⁵	0.01511011
P20591	IFN-induced GTP-binding protein Mx1	MX1	2.20162951	6.3215 × 10 ⁻⁰⁵	0.01815176
Q6P2Q9	Pre-mRNA-processing-splicing factor 8	PRPF8	1.33941179	1.5549 × 10 ⁻⁰⁶	0.00125012
Q01469	Fatty acid-binding protein 5	FABP5	-2.29088412	5.7962 × 10 ⁻⁰⁵	0.01815176

morphology (fig. S3). Details about data replication are provided in the figure legends. Experimenters were not blinded.

Human participants

All affected participants were initially referred for unexplained DD and/or ID together with various congenital malformations. They underwent extensive clinical examination by at least one clinical geneticist participating in the study. Routine genetic testing was performed whenever clinically relevant, including copy number variation analysis by high-resolution array-based comparative genomic hybridization. Because these tests failed to establish the diagnosis of a specific disease, trio-based WES was performed in a diagnostic or research setting whenever parental samples were available. Because disorders associated with *PSMC3* are very rare, the reported patient cohort is representative of cases identified worldwide over the past 6 years and is not, for example, a selection of a subgroup of a larger patient population. This study was approved by the CHU de Nantes ethics committee (Research Programme “Génétique Médicale DC-2011-1399). Probands 2, 5 to 10, 12 to 14, 16 to 18, and 20 to 23 were enrolled by one of the participating centers [Washington University in St. Louis, University Medical Centre of Utrecht, University Hospital Center of Nantes, Arnold Palmer Hospital, Seattle Children’s Hospital, St. Luke’s Hospital, Health San Antonio, Vanderbilt University Medical Center, Sydney Medical School, Children’s Hospital of Philadelphia, Technical University of Munich, Pitié-Salpêtrière University Hospital, Children’s Hospital of Orange County, Ambry Genetics, Geisinger Medical Center, Guy’s and St Thomas’ NHS Foundation Trust, McGill University Health Centre, Nottingham University Hospitals NHS Trust, Hôpital Universitaire Necker-Enfants Malades, University Children’s Hospital, Salzburger Landeskliniken (SALK), and Paracelsus Medical University] after approval of genetic studies by local ethics committees. Written informed consent was obtained from all study participants, including probands and healthy

parents. All affected individuals were initially referred for unexplained DD and/or ID together with various congenital malformations. They underwent extensive clinical examination by at least one expert clinical geneticist. Individuals 1, 4, 11, 15, and 19 participated in “Simons Foundation Powering Autism Research” (SPARK) or “Deciphering Developmental Disorders” (DDD) initiative; clinical information about individuals 1, 3, and 14 was retrieved by members of the University of Washington School of Medicine in agreement with SPARK and DDD.

Cell culture

Peripheral blood mononuclear cells (PBMCs) used in this paper were isolated from blood draws from patients and related healthy controls (father and/or mother of the proband). Briefly, PBMCs were isolated by PBMC spin medium gradient centrifugations (pluriSelect), washed three times with phosphate-buffered saline (PBS), frozen in fetal bovine serum with 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen for further use. In some experiments, collected PBMCs were expanded in U-bottom 96-well plates together with feeder cells using RPMI 1640 supplemented with 10% human AB serum (both purchased from PAN-Biotech GmbH) in the presence of IL-2 (150 U/ml; Miltenyi Biotec) and L-PHA (1 µg/µl; Sigma-Aldrich) following the procedure of Fonteneau *et al.* (106). After 3 to 4 weeks of culture, resting T cells were washed and frozen as dry pellets for further use.

SDS-PAGE and western-blot analysis

Cell pellets from resting T cells isolated from patients and related controls were lysed in equal amounts of standard radioimmunoprecipitation assay buffer [50 mM tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM N-ethylmaleimide, 10 µM MG-132, 1% NP-40, and 0.1% SDS] and separated by 10 or 12.5% SDS-PAGE before transfer to polyvinylidene difluoride membranes (200 V for 1 hour). After blocking (20-min exposure to 1X Roti-Block at room temperature),

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membranes were probed with relevant primary antibodies overnight at 4°C under shaking. The anti- $\alpha 6$ (clone MCP20), anti- $\alpha 7$ (clone MCP72), anti- $\beta 1$ (clone MCP421), anti-PSMC2 (BML-PW8315), and anti-PSMC3 (BML-PW8310) primary antibodies were purchased from Enzo Life Sciences. Primary antibodies specific for TCF11/Nrf1 (clone D5B10), ubiquitin (clone D9D5), glyceraldehyde phosphate dehydrogenase (GAPDH) (clone 14C10), PINK1 (clone D8G3), BNIP3L/NIX (clone D8G3), LC3b (#2775), eIF2 α (#9722), phospho-eIF2 α (Ser⁵¹, #119A11) were obtained from Cell Signaling Technology. The anti-PSMD12 antibody (clone H3) was a purchase from Santa Cruz Biotechnology Inc. The anti-PA28- α (K232/1) is laboratory stock and was used in previous studies (31). Antibodies directed against $\beta 5$ (ab3330), α -tubulin (clone DM1A), and phospho-PKR (Thr⁴⁴⁶, clone E120) were purchased from Abcam. After incubation with primary antibodies, membranes were washed three times with PBS/0.2% Tween and subsequently incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 hour at room temperature. Proteins were then visualized using an enhanced chemiluminescence detection kit (Bio-Rad).

RNA isolation, RT, and PCR analysis

Total RNA was isolated from resting T cells using the kit from Analytic Jena AG following the manufacturer's instructions. For subsequent real-time PCR, 100 to 500 ng of the isolated total RNA were reverse-transcribed using the M-MLV reverse transcriptase (Promega). qPCR was performed using the Premix Ex Taq (probe qPCR purchased from TaKaRa) and in duplicates to determine the mRNA expression of each ISG using FAM-tagged TaqMan Gene Expression Assays obtained from Thermo Fisher Scientific according to the manufacturer's instructions. TaqMan probes used in this study for ISG quantification included *IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *RSAD2*, *IFI44*, *MX1*, *OASL1*, *CXCL9*, and *CXCL10*. The cycle threshold (Ct) values for target genes were converted to values of relative expression using the relative quantification method ($2^{-\Delta\Delta C_t}$). Target gene expression was calculated relative to Ct values for the GAPDH control housekeeping gene.

Statistical analyses

Figures were created with PyMOL version 2.0 (pymol.org) using the human 26S proteasome structure. Data are typically median or means $\pm$ SEM and were analyzed by unpaired and pair ratio *t* test between two groups. Neuronal morphology data were analyzed using a one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test for multiple comparisons. All charts and statistical analyses were generated using GraphPad Prism version 8. A *P* value < 0.05 was considered significant.

Supplementary Materials

This PDF file includes:

Materials and Methods

Fig. S1 to S14

Table S1

References (107–116)

Other Supplementary Material for this

manuscript includes the following:

Data files S1 and S2

MDAR Reproducibility Checklist

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