



Published in final edited form as:

J Allergy Clin Immunol. 2019 May ; 143(5): 1939–1943.e8. doi:10.1016/j.jaci.2018.12.1012.

Novel Proteasome Assembly Chaperone mutations in *PSMG2/PAC2*, cause the autoinflammatory interferonopathy, *CANDLE/PRAAS4*

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Summary:

Chronic Atypical Neutrophilic Dermatitis with Lipodystrophy and Elevated temperature (CANDLE) is a rare autoinflammatory interferonopathy caused by additive loss-of-function mutations in proteasome genes. Mutations in the proteasome chaperone, *PSMG2/PAC2* are a novel cause of CANDLE.

Keywords

Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature; Proteasome Associated Autoinflammatory Syndrome; Type I IFN; Interferonopathy; Whole exome sequencing; Pediatrics; Autoinflammatory diseases

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[ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02974595) identifier: NCT02974595

Conflicts of Interest

The authors declare no relevant conflict of interest. Dr. Goldbach-Mansky has received grant support from SOBI, Regeneron, Novartis and Eli Lilly.

To the Editor:

Chronic Atypical Neutrophilic Dermatositis with Lipodystrophy and Elevated temperature (CANDLE) or proteasome associated autoinflammatory syndrome (PRAAS) is a rare autoinflammatory interferonopathy¹ caused by additive loss-of-function (LOF) mutations in proteasome genes, [PRAAS1 MIM#256040 (recessive *PSMB8* and digenic with *PSMB4* and *PSMA3*,^{23,E1-E3} PRAAS2 MIM#618048 (*POMPHaploinsufficiency*)^{3,4} and PRAAS3 MIM#617591 (recessive *PSMB4* and digenic with *PSMB9*)].³ We report a patient with clinical features of CANDLE and an episode of autoimmune hemolytic anemia (AIHA), who has two novel mutations in the proteasome assembly gene *PSMG2*, identifying the earliest proteasome assembly chaperone PAC2, as critical in human proteasome assembly and as a novel gene causing CANDLE, PRAAS4.

The patient, an 8-year, 10-months-old Caucasian girl at diagnosis, presented since 4-months of age with edema of the extremities (Fig 1, *A, B*). At 7-months she developed erythematous, violaceous nodules on the upper and lower extremities and was diagnosed with panniculitis. During a hospitalization for recurrent fever flares at 2 yrs of age, generalized lipodystrophy (Fig 1, *C*), mild gross motor and speech delay, in the context of elevated acute phase reactants, hepatosplenomegaly and diffuse lymphadenopathy were noted; height and weight were normal and an immunodeficiency work up was unremarkable. At 5 years of age, she developed severe direct antigen test (DAT) (or Coombs) positive autoimmune hemolytic anemia (AIHA), with a hemoglobin of 3.1 g/dL, requiring 3 blood transfusions, pulse doses of corticosteroids, 4 infusions of rituximab, and a 19-month-course of azathioprine plus IVIG every 4 weeks. Prednisolone, which was started at the age of 5 years, improved panniculitis and hepatosplenomegaly but lipoatrophy and joint contractures progressed. An evaluation at 7 yrs of age revealed active myositis on MRI (Fig E1), and significant muscle atrophy in her upper and lower extremities with marked fatty infiltration of thigh muscles. A brain CT showed basal ganglia calcifications (Fig 1, *D*) and a clinical diagnosis of possible CANDLE was made. A clinical genetic panel for known autoinflammatory diseases (including then known CANDLE genes) was negative. WES performed on a research protocol revealed 2 novel variants in conserved amino acids in *PSMG2* (Chromosome 18p11.21, NM_020232) encoding the proteasome assembly protein, PAC2, a frameshift mutation, c.666_667delGT, p.Y223Sfs*2, inherited from her father and a missense mutation c.675 T>G, p.N225K, from her mother; both were confirmed by Sanger sequencing (Fig 1, *E* and see Fig E2, *A,B*). The variants were not found in public (gnomAD, ESP6500, 1000G) or local (308 exomes) databases. An interferon signature assessed at the time of active disease was elevated comparable to patients with genetically confirmed CANDLE and the interferonopathy SAVI (Fig 1, *F*).

The ubiquitin-proteasome system (UPS) is the primary homeostatic degradation system for ubiquitinated proteins; and proteasome dysfunction is associated with several human diseases including CANDLE /PRAAS.^{5, E4, E5} PAC2 is a proteasome assembly chaperone that dimerizes with PAC1, encoded by *PSMG1*, to form the PAC1/PAC2 complex.^{E6} Studies in different model systems showed a pivotal role of the PAC1/PAC2-complex in incorporating the proteasome α -subunits into the full 20S proteasome (Fig E3).^{E7-E10} To assess whether the novel mutations confer a loss-of-function, we compared the proteolytic

activity (chymotrypsin-, trypsin- and caspase-like activities) of the proteasome in the patient's fibroblast lysates to lysates from disease controls of genetically confirmed CANDLE patients (DC) and healthy controls (HC). The patient had decreased proteolytic activities similar to the two DCs (Fig 2, A) which leads to similar accumulation of ubiquitin conjugates as in the 2 DCs (Fig 2, B). Proteasome-subunit expression showed a strong reduction of PAC1 and PAC2 protein subunits in the patient's sample and no apparent changes in the expression of other proteasome subunits (Fig 2, C). However, the amount of assembled proteasome complexes was decreased ($\alpha 6$ staining), even when compared to the 2 DCs. As only small amounts of mutant *PSMG2*/PAC2-variants were incorporated into nascent proteasome complexes, the impaired proteasome assembly (Fig 2, D), is conferred by reduced binding of the mutant PAC1/PAC2 heterodimer to the α -subunit of the proteasome core (Fig 2, D) that results in reduced proteasome assembly and activity (Fig 2, E). To exclude extensive non-sense mediated RNA decay, we assessed messenger RNA (mRNA) expression of the mutant alleles (frameshift and missense) in construct-transfected HeLa cells (Fig 2, F). Compared to wildtype constructs, the proteasome-incorporation of each mutant protein (shown for $\alpha 6$) was reduced with more severe impairment for the frameshift mutation (Fig 2, G), thus confirming an independent contribution from each mutant allele.

Most CANDLE-causing mutations reported so far occur in genes encoding α - or β - subunits of the 20S core particle (CP) proteasome which is composed of 2 α -rings, and 2 β -rings. Each α -ring is composed of 7 α -subunits (encoded by *PSMA1-7*) each β -ring is composed of 7 β -subunits (encoded by *PSMB1-7*).^{E11} The mutations are hypomorphic and inherited in a recessive or digenic mode, conferring additive LOF. PAC2, is one of 3 assembly chaperones including POMP ("human Ump1")^{E8-E10} and the PAC3/PAC4 heterodimer.^{E7} The 3 reported hypomorphic disease-causing *POMP* mutations are dominant due to haploinsufficiency (Fig E4),^{3,4} whereas the hypomorphic, disease-causing *PSMG2* mutations are recessive conferring additive LOF. As both parents are asymptomatic, the wildtype allele in the heterozygous state can rescue function.

In contrast to patients with IL-1-mediated autoinflammatory diseases, patients with interferonopathies have elevated autoantibody titers, including ANA and ENA, c-ANCA, anti-thyroid antibodies, lupus anticoagulant, anti-cardiolipin and beta-2-glycoprotein I [$\beta 2$ GPI] antibodies which is consistent with the prominent role of Type-1 IFN in driving autoantibody formation. In many instances the antibodies are transient and not associated with clinical pathology.^{E12-E14} However, our patient developed DAT (or Coombs) positive AIHA caused by antibodies against self-erythrocyte antigens. Most AIHA patients have IgG warm antibodies that trigger erythrocyte phagocytosis, a smaller subset has IgM antibodies that are cold activated, fix complement and cause cold-type AIHA.^{6,E15, E16} AIHA has been reported in two CANDLE patients,^{7,8} and is frequently seen in a spectrum of patients with immune dysregulatory diseases (eg, systemic lupus erythematosus, lymphoproliferative diseases including B-cell malignancies, primary immunodeficiencies and infections^{E17} and can present as hemolytic anemia and thrombocytopenia (Evans syndrome), which suggests that common genetic variants influence the development of AIHA.^{E15}

Lastly, the diagnosis of CANDLE/PRAAS led to treatment with the JAK inhibitor ruxolitinib, which inhibits the transduction of IFN-mediated signals on the IFN-receptors and of other class I and class II cytokine receptors.^{E18} JAK inhibitors have shown benefit in reducing the symptoms and the IFN score in CANDLE patients,⁹ and treatment resulted in marked improvement and halted progressive lipoatrophy (Fig E5). Ruxolitinib was started at 8 years at 5mg twice a day and increased with weight gain. Ruxolitinib treatment enabled a rapid corticosteroid taper from 0.6 mg/kg/d to discontinuation after 14 months on JAK inhibition without a need to restart corticosteroid treatment (Fig E6). Hepatosplenomegaly and diffuse lymphadenopathy disappeared, joint contractures improved dramatically (with the exception of the left ankle) and inflammatory markers normalized.

In summary, we describe two novel compound heterozygous, loss-of-function mutations in *PSMG2* that encodes the proteasome assembly chaperone, PAC2, as a novel genetic cause of CANDLE/PRAAS. Given the advance of emerging treatments targeting Type-I interferon signaling for CANDLE, the recognition of new genetic causes of CANDLE/PRAAS will facilitate a rapid clinical diagnosis and early treatment.

Acknowledgements

We would like to acknowledge Daniela Ludwig for excellent technical assistance. This work was supported by the Intramural Research Program of the NIH, NIAID and NIAMS and by grants of the DFG (SFB740, SFBTR186, SFBTR167) to EK and the Fritz-Thyssen-Foundation (10.16.2.022MN) to EK and AB.

Funding: This research was supported by the Intramural Research Program of the NIH, NIAID and NIAMS. EK and AB were supported by the Fritz-Thyssen-Foundation and the DFG.

Supplementary Appendix

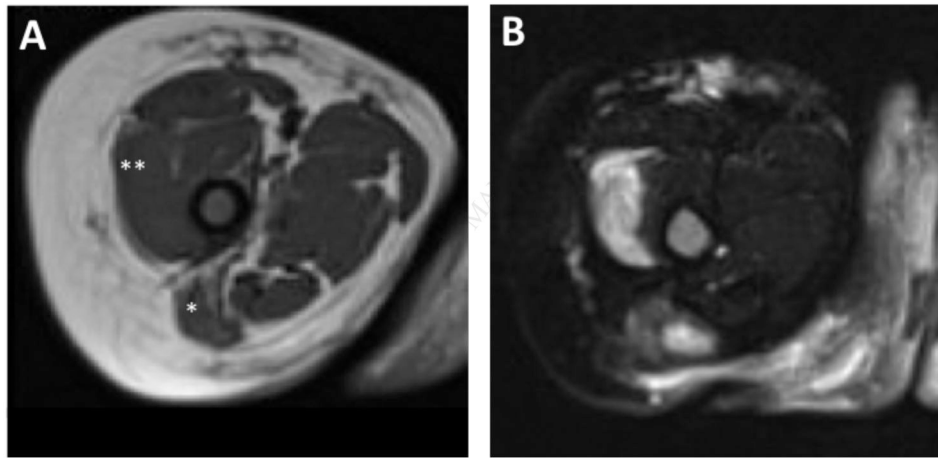
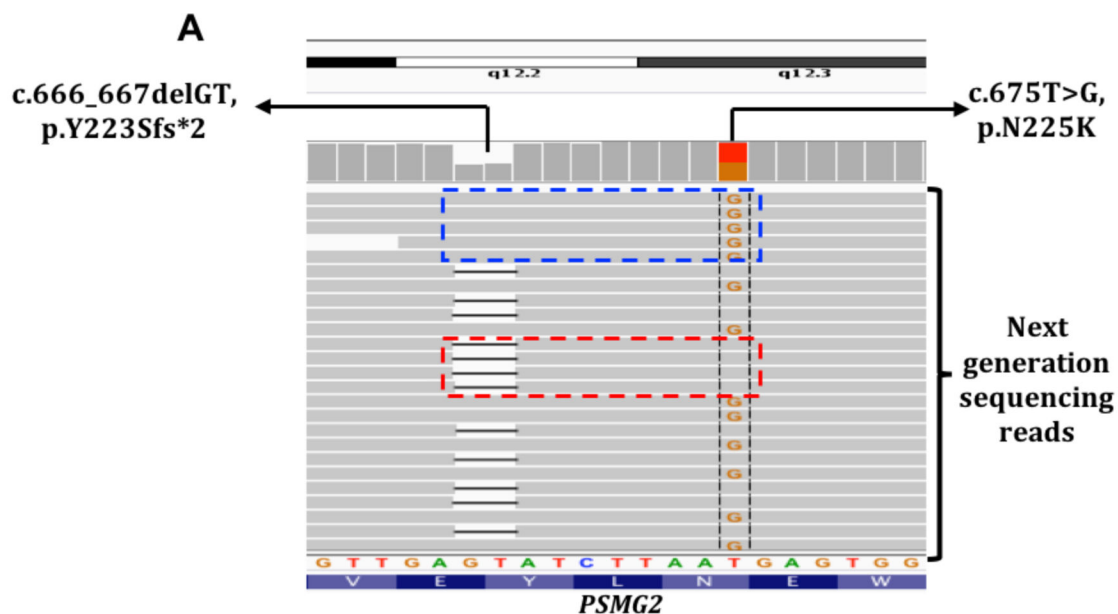


FIG E1. Magnetic resonance imaging (MRI) of upper thighs showing muscular atrophy and patchy myositis.

(A) T1 weighted axial MRI of right and left thigh shows atrophy and fatty replacement of the gluteus maximus and vastus medialis muscles. (B) The corresponding T2 weighted fat suppressed axial image shows patchy enhancement in these areas suggestive of active inflammation.



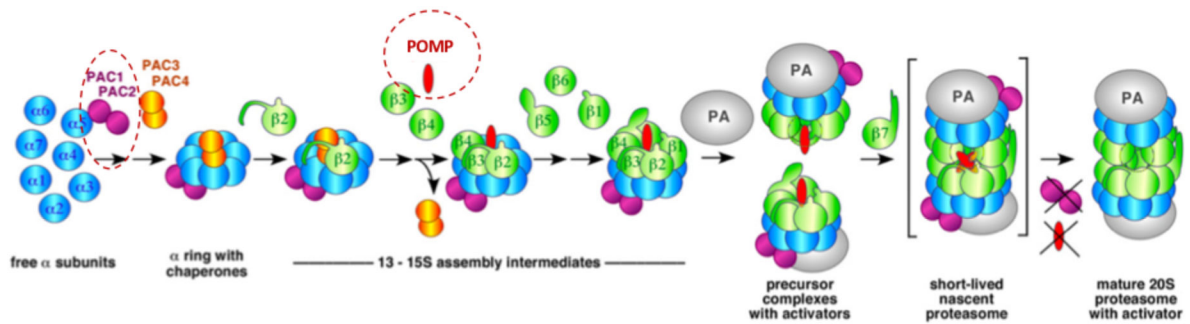
B

p.Y223Sfs*2 and N225K

H. sapiens	208	VSEGDNIPDALGLVVEYLNEWLQILKPL--SDDP	238
P. troglodytes	208	VSEGDNIPDALGLVVEYLNEWLQIIKPL--SDDP	238
M. mulatta	208	VSEGDNIPDALGLVVEYLNEWLQIIKPL--SDDP	238
F. catus	208	VSEGDNIPDALGLVVEYLNEWLQIIKPLqCDDP	240
M. musculus	208	VSEGDNIPDAVSLVVEYLNEWLQIIKPC--NDGP	238
B. taurus	208	VSEGDNIPDALGLVVEYLNEWLQIIKPH--CEDP	238
S. scrofa	208	VSEGDNIPDALGLVVEYLNEWLQIIKPC--CEDT	238
R. norvegicus	208	VSEGDNIPDAVSLVVEYLNEWLQIIKPC-----	234
G. gallus	208	CSEGDNIPDAFGLVNYLNEWLQIIKIR-rNSST	239
X. tropicalis	208	CSEGDNVPDAFSLLNQVNEWLHLVAS--tNGDV	238
D. rerio	208	CSEGDNIPDAFALINCLNWLHLLEKP-----	234

FIG E2. Whole Exome Sequencing (WES) and PSMG2 Multispecies Alignment

(A) Visualization of the binary alignment map (bam) using Integrative Genomics Viewer (IGV) revealed that the two variants are in *trans* configuration. The sequencing reads containing the 2 base-pair deletion lack the missense mutation (red dashed box), whereas the sequencing reads containing the missense mutation lack the 2 base-pair deletion (blue dashed box), thus suggesting that each variant was inherited from a different parent which was confirmed by Sanger sequencing. (B) Multispecies alignment of the PSMG2 protein region affected by the compound heterozygous variants confirms conservation across species for both variants.



Adapted from Ramos, PC and Jürgen Dohmen, R

FIG E3. Model of chaperone-aided proteasome assembly.

The PAC1/PAC2 heterodimer initiates α -subunit-ring assembly by binding to the 20S core protein subunits, $\alpha_5/PSMA5$ and $\alpha_4/PSMA4$ ^{E6} and prevents dimerization of the α -rings and promotes the assembly of the hetero-heptameric $\alpha\beta$ -subunit-ring (Le Tallec et al 2007). The PAC3/PAC4 complex binds later and dissociates from the maturing proteasome complex, at the “half-mer ($-\beta_7$) intermediate” stage when only the β_7 -subunit is lacking. At that point, POMP is recruited to mediate incorporation of either standard $\beta_5/PSMB5$ or the $\beta_5i/LMP7/PSMB8$ immunoproteasome subunit and dimerization of two “half-proteasome precursor complexes” to form the short-lived “nascent proteasome”.^{E6} This conformational change triggers the release of the PAC1/PAC2 complex from the nascent proteasome and enables binding of the 19S regulatory particle.^{E6,E7,E11} In the red circles: Loss of function mutations in *PSMG2/PAC2* (compound heterozygous, disease-causing mutations lead to additive loss of function) and *POMP/POMP* ((alias UMP1), heterozygous, disease-causing mutations cause haploinsufficiency), mutations in both genes cause CANDLE/PRAAS.

CANDLE Patients*	Gender	Age of First Symptoms	Age at Time of Report	Country of Origin	Clinical Manifestations	Mutation
Patient 1	Male	Day 1 of life	10 yrs	Palestine	Fever, panniculitis, arthralgia, lipodystrophy, increased inflammatory markers	<i>POMP</i> heterozygous c.344_345insTTGA, p.E115Dfs*20
Patient 2	Male	Week 2 of life	2 yrs	NR	Neutrophilic dermatosis, pulmonary nodules, thrombocytopenia, circulating autoantibodies, combined immunodeficiency	<i>POMP</i> heterozygous c.342_348delinsACC, p.F114Lfs*18
Patient 3	Male	Week 2 of life	2 yrs	NR	Neutrophilic dermatosis, thrombocytopenia, seizures, circulating autoantibodies, combined immunodeficiency	<i>POMP</i> heterozygous c.334_335delAT, p.I112Wfs*3
Patient 4	Female	Fifth week of life	9 yrs	France	Fever, neutrophilic panniculitis, lipodystrophy, muscle atrophy, hepatosplenomegaly, lymphadenopathy, basal ganglia calcification, increased inflammatory markers, autoimmune hemolytic anemia	<i>PSMG2</i> compound heterozygous c.666_667delGT, p.Y223Sfs*2 and c.675 T>G, p.N225K

FIG E4. Reports of patients with CANDLE/PRAAS who have mutations in proteasome assembly chaperones^{4,5}.

*denotes CANDLE patients with currently known chaperone mutations in *POMP* and including our patient with *PSMG2* mutations. NR: not reported (in this figure, cases from refs. 4 and 5 were combined)

Laboratory Analysis	Pre-treatment (2 yrs)	14 months after JAK inhibitor treatment (9yrs)	Normal range
Hemoglobin (g/dL)	9.0 (L)	11.0	(10.2 - 12.7)
MCV (fL)	NA	72.8	(72.3-85.0)
Platelet (K/uL)	188 (L)	235	(214 - 459)
White blood cell (WBC) count (K/uL)	2.75 (L)	3.5 (L)	(6.48 - 13.02)
Neutrophil Absolute (K/uL)	1.63	2.0	(1.27 – 7.18)
Lymphocyte Absolute (K/uL)	NA	1.27 (L)	(1.52 – 8.09)
Monocyte Absolute (K/uL)	NA	0.19 (L)	(0.26 – 1.08)
CRP (C-reactive protein) (mg/L)	66 (H)	21 (H)	<5
ESR (erythrocyte sedimentation rate) (mm/hr)	85 (H)	13	<20

FIG E5. Laboratory parameters before and 14 months after treatment with the Jak inhibitor ruxolitinib.

H: high; L: low; NA: not available

Date	Ruxolitinib dose	Pt weight	Prednisone dose in mg/kg/d
2/14/17	10 mg/d (divided bid)	21.5kg	0.6 mg/kg/d
7/17/17	10 mg/d (divided bid)	22.0kg	0.22 mg/kg/d
9/?/2017	15 mg/d (divided bid)	22.5kg	0.17 mg/kg/d
1/?/2018	15 mg/d (divided bid)	25.0kg	0.12mg/kg/d
4/9/18	20 mg/d (divided bid)	27.0kg	0.05 mg/kg/d → stop
11/6/18	15 mg/d (divided bid)	31.0kg	0

FIG E6.
Ruxolitinib dose, weight changes and steroid taper on JAK inhibitor baricitinib.

METHODS

Patient

The parents provided consent and the patient was enrolled into an IRB approved NIH natural history protocol, “Studies of the Natural History, Pathogenesis, and Outcome of Autoinflammatory Diseases (NOMID/CAPS, DIRA, CANDLE, SAVI, NLRC4-MAS, Still’s-like Diseases, and other Undifferentiated Autoinflammatory Diseases”, 17-I-0016, NCT02974595).

Genetic Analysis

Whole exome sequencing (WES) was performed on Illumina® HiSeq sequencers as previously reported (Liu et al. 2014). In brief, exomes were captured with the Agilent SureSelect® Human 51 Mbp All Exon Kit (Agilent Technologies, Santa Clara, CA). Variants were called using a pipeline with the Genome Analysis Toolkit (GATK). Variants were annotated with functional impact and allele frequency from public and local datasets. Potential disease-causing mutations were selected and prioritized based on quality score, allele frequency, functional impact, probable inheritance model (*de novo*, autosomal recessive or autosomal dominant), and expert evaluation. The *PSMG2* variants detected by

WES in the patient were confirmed by Sanger sequencing of exon 6 and the reference sequence used for primer design and variant annotation was *PSMG2* NM_020232.

Cell lines

Primary fibroblasts cell lines from patients and healthy controls were generated from superficial skin biopsies after obtaining consent. Standard protocols were used to generate the cell lines. In brief, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1× penicillin/streptomycin and stable glutamine.

Proteasome assay, immunoblot

The proteolytic activity assays were performed as described. Equal amounts of protein extracts from patients' fibroblasts, or transfected HeLa cells were separated on SDS-Laemmli gels (15%) or native PAGE (3%–12%, Invitrogen) and immunoblotted for proteasome subunits using published protocols³. The following primary antibodies were used: β 35i (LMP7), and POMP are from laboratory stocks (1, 2); PAC1 (Cell Signaling, 13378S); PAC2 (Enzo, EX-6); α 6 (Enzo, MCP20); PA28 β (Cell Signaling, 2409); RPT6 (Enzo, p45-110); PA200 (Novus, NBP2-22236); Flag (Sigma, M2 F1804); β -tubulin (Covance, MMS-410P); Neomycin-Phosphotransferase (Millipore, AC113). Ubiquitin blots were performed with cell lysates from fibroblasts in native buffer. The insoluble fraction was lysed in urea lysis buffer (7 M urea, 4 M thiourea, 4% m/v chaps) and separated on SDS-Laemmli gels (10%) and immunoblotted for ubiquitin (Dako, Z0458).

Transfection

HeLa cells (ATCC) were transiently transfected with expression vectors for wt Flag-Pac2 and mutant-Pac2 variants using Lipofectamine2000 (Invitrogen) for 24h. Transfection efficiency was monitored by immunoblot analyses of Neomycin-Phosphatase expression, encoded by the pcDNA3.1 vector backbone. Ectopic expression of the Flag-PAC2 mRNA from the expression vector in HeLa cells was monitored by semi qRT-PCR as described³ using the following primers:

Gapdh_for: (cagacacatggggaaggtg);

Gapdh_rev: (CAGCAGTGAGGGTCTCTCTC);

T7_for: (TAATACgACTCACTATggg);

PAC2_rev: (gAAAAgTgCAgggggAAgAC),

Density gradient fractionation

A linear 10%-40% Glycerol gradient (BIOCOMP) was generated in ultracentrifugation tubes and loaded with 1 mg protein; gradient ultracentrifugation was performed at 4°C at 30 000 rpm for 18 hrs. Gradients were fractionated in 0.5 ml aliquots from the top of the gradient; TCA precipitated pellets were resuspended in 1.5x sDs-PAGE loading buffer for immunoblot analyses.

BACKGROUND

Proteasome defects have also been associated with neurologic diseases (Zheng Q et al. 2016). Copy-number variant (CNV) deletions or single-nucleotide variants (SNVs) in *PSMD12*, encoding the RPN5/*PSMD12* subunit of the 19S regulatory protein cause an autosomal dominant syndromic form of intellectual disability. In murine models, loss of *Psm12* led to development of microcephaly, renal tubular atrophy, and aberrant rostrocaudal patterning of the branchial arches, suggesting a role of some proteasome components in organ development (Kury S et al. 2017). Whether the mild cognitive delay that is reported in some CANDLE/PRAAS patients is due to proteasome effects on neurological development or multifactorial remains to be assessed.^{E12}

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Abbreviations:

CANDLE	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature
PRAAS	Proteasome Associated Autoinflammatory Syndrome
PAC2	Proteasome Assembly Chaperone 2
PSMG2	Gene encoding PAC2

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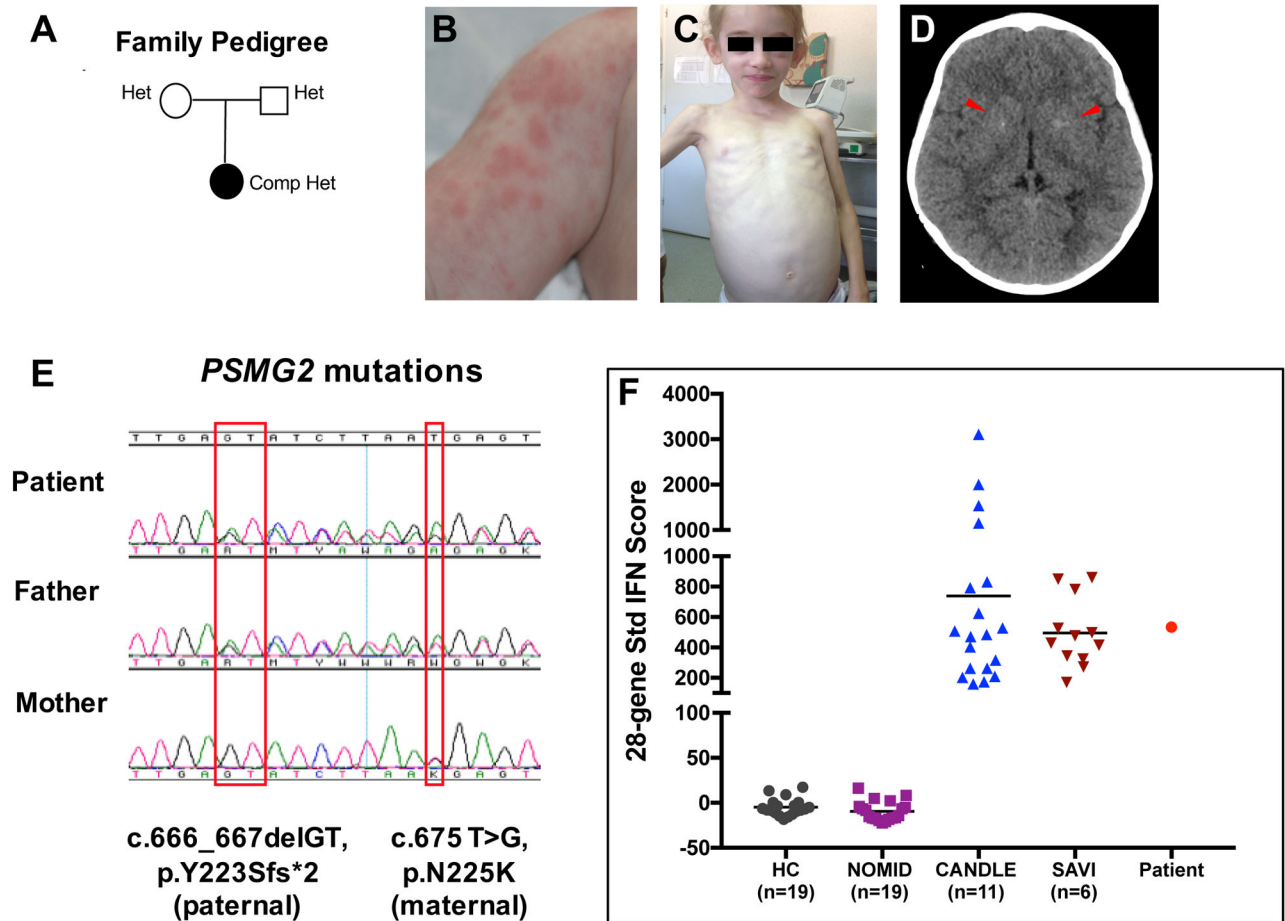


FIG 1. Clinical presentation and genetic findings in CANDLE/PRAAS4.

A, Family tree depicting that both patient's parents are heterozygous for a different *PSMG2* mutation. **B**, Nodular skin rash at the age of 7 months. **C**, Generalized lipoatrophy involving the face and upper extremities at the age of 7 years. **D**, A brain CT showing faint bilateral basal ganglia calcifications (arrow heads). **E**, Sanger sequencing electropherogram from patient and parents showing that the frameshift deletion is on the paternal allele and the missense variant is on the maternal allele. **F**, 28-gene interferon (IFN) score performed with a customized Nanostring assay demonstrated that our patient has a high IFN score, similar to the observed in patients with the interferonopathies CANDLE and SAVI⁹. Mean ± SD of 28-gene IFN score: healthy controls (HC): -4.8 ± 9.53 ; neonatal onset multisystem inflammatory disease (NOMID): -9.37 ± 10.97 ; CANDLE: 738.2 ± 754.4 ; SAVI: 495.6 ± 225.9 ; Patient: 533.6

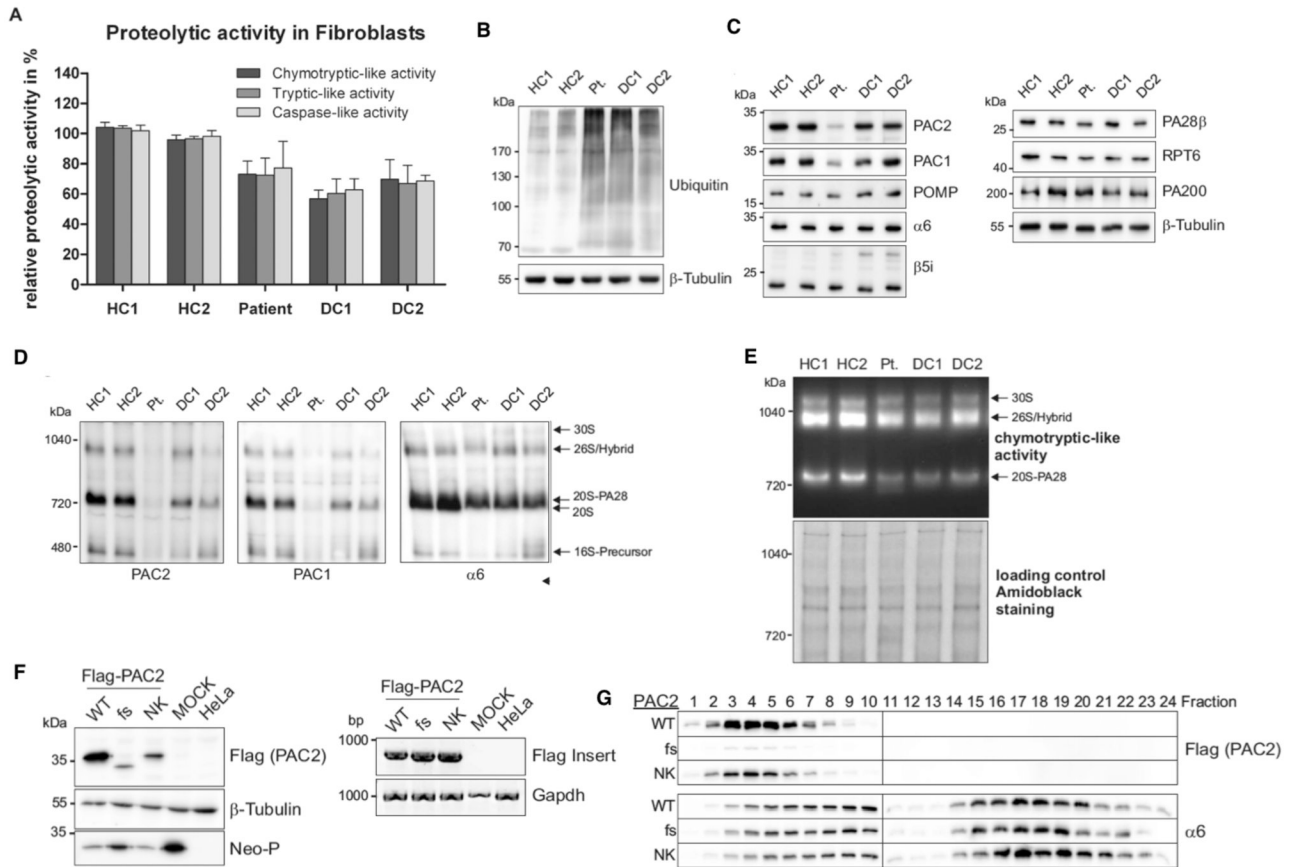


FIG 2. Fibroblasts from the patient with mutant PAC2 had reduced proteolytic activity and proteasome amounts with accumulation of ubiquitin aggregates.

A, Fibroblasts lysates from the patient (Pt.) and CANDLE/PRAAS disease controls (DC) had approximately 75% of proteasome activity compared to healthy controls (HCs). Means \pm SD estimated from triplicates and normalized against HC1+2 ($n=3$). **B**, PRAAS patients accumulated insoluble ubiquitin aggregates. **C**, Reduced PAC1 and PAC2 expression in patient's sample compared to HCs and DCs. Expression of subunits POMP/*POMP*, α 6/*PSMA1*, β 5i/*PSMB8*, PA28b/*PSME2*, RPT6/*PSMC5*, PA200/*PSME4* was normal. β -Tubulin: loading control. **D**, Native PAGE-immunoblot-analyses shows a reduced PAC1 and PAC2 incorporation into the proteasome complexes. Reduced α 6 staining indicates less proteasome complexes in all CANDLE/PRAAS patients **E**, Overlay of chymotrypsin-like activity shows reduced proteolytic activity in the main proteasome complexes for CANDLE/PRAAS patients. **F**, The epitope-tagged PAC2 variants (wildtype (WT); fs (frameshift mutation); NK (N225K mutation) were expressed in HeLa cells and detected by a Flag-specific antibody. MOCK, empty vector backbone; HeLa, non-transfected control. β -Tubulin: loading control. Neomycin-Phosphatase (Neo-P): transfection efficiency control. All PAC2-variant mRNAs were equally expressed in HeLa cells (RT-PCR). *GAPDH*: loading control. Panels A-F: representative results from $n=3$. **G**, Density gradient fractionation (panel F) shows incorporation of both mutant Pac2 variants into proteasome complexes, without impacting total proteasome formation (α 6 staining).