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Mutations in *AP3D1* associated with immunodeficiency and seizures define a new type of Hermansky-Pudlak syndrome

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Abstract

Genetic disorders affecting biogenesis and transport of lysosome-related organelles are heterogenous diseases frequently associated with albinism. We studied a patient with albinism, neutropenia and immunodeficiency, neurodevelopmental delay, generalized seizures and impaired hearing, but no mutation in genes so far associated with albinism and immunodeficiency. Whole exome sequencing identified a homozygous mutation in *AP3D1*, leading to destabilization of the adaptor protein 3 (AP-3) complex. AP-3 complex formation and the degranulation defect in patient T cells were restored by retroviral reconstitution. A previously described hypopigmented mouse mutant with an *Ap3d1* null mutation (mocha strain) shares the neurological phenotype with our patient and shows a platelet storage pool deficiency characteristic of HPS that was not studied in our patient due to lack of bleeding. HPS-2 caused by mutations in *AP3B1A* leads to a highly overlapping phenotype without the neurological symptoms. The AP-3 complex exists in a ubiquitous and a neuronal form. *AP3D1* codes for the AP-3δ subunit of the complex, which is essential for both forms. In contrast, the AP-3β3A subunit, affected in HPS-2 patients, is substituted by AP-3β3B in the neuron-specific heterotetramer. AP-3δ deficiency thus causes a severe neurological disorder with immunodeficiency and albinism, which we propose to classify as HPS-10.

Introduction

Syndromes of albinism associated with immunodeficiency include Chediak-Higashi Syndrome (CHS), Griscelli Syndrome (GS) type 2, Hermansky-Pudlack Syndrome (HPS) type 2 and type 9 and endosomal-adaptor protein p14 deficiency 1. The immunodeficiency is characterized by variable infection susceptibility associated with impaired lymphocyte degranulation and cytotoxicity and neutropenia. The cytotoxicity defect is linked to a risk of developing hemophagocytic lymphohistiocytosis (HLH) mainly in CHS, GS-2 and HPS-2 2–4. Chronic neutropenia is characteristic in HPS2 5 and MAPBPIP-deficiency 6, while it is usually transient in CHS and GS2. Apart from immunodeficiency, albinism syndromes can also present with other systemic manifestations including bleeding due to a storage pool disorder (HPS, CHS)7 and progressive lung fibrosis (HPS)8 as well as neurological disorders (CHS, GS)9,10.

The pathophysiological basis of these partly overlapping syndromes is a disturbed biogenesis and transport of secretory lysosome-related organelles 11,12. These secretory organelles are found in several cell types for secretion of specific, cell-type-dependent proteins and peptides. They are important for very diverse physiological processes including pigmentation of eyes, hair and skin 13, release of small molecules by delta granules from platelets at site of blood vessel damage which facilitate platelet adhesion and activation7, secretion of perforin and granzyme by lytic granules in cytotoxic lymphocytes 11,14, secretion of surfactant by lysosome-related lamellar bodies in pulmonary alveolar type II cells 15. A distinct, but in its protein components partly overlapping molecular machinery is responsible for the biogenesis and transport of secretory lysosome-related organelles in different cell types. This explains the variable combination of different clinical manifestations in genetic diseases interfering with this pathway.

A key protein complex involved in lysosome biogenesis is the AP-3 complex. This is an adaptor protein complex involved in the budding of vesicles from early endosomal compartments and for correct sorting of integral membrane proteins from the endosome to the secretory lysosom16,17. This includes the sorting of LAMP-1 and LAMP-2 to secretory lysosomes 18, of tyrosinase to melanosomes 13 and of neutrophil elastase to neutrophil granules 19. In the absence of AP-3, missorting of these proteins to the cell membrane has been observed. Two AP-3 heterotetramers can be distinguished: The ubiquitous AP-3 consists of two large subunits, β3A and δ, an intermediate subunit μ3A and a small subunit σ3, which can be one of two isoforms A or B20. A second form of AP-3 is specific for the central nervous system and consists of β3B, μ3B, one of the two σ3 isoforms, and the shared δ subunit 21. Deficiency of the AP-3β3A subunit, which is encoded by *AP3B1*, results in HPS-2 5, while mutations in the other subunits of the AP-3 complex have so far not been associated with human disease.

Here we describe a patient with albinism and immunodeficiency with some characteristic features of HPS-2 and additional neurological symptoms from birth including microcephaly, severe neurodevelopmental delay, generalized seizures and hearing impairment. An overt tendency for bleeding was not observed. We detected a homozygous frameshift deletion in *AP3D1.* Reconstitution of patient T cells with wild-type *AP3D1* corrected the degranulation defect. In mocha mice, *Ap3d1* mutations cause an HPS-like phenotype with severe seizures and hearing loss 22. Thus, AP-3δ deficiency causes a new severe neurological disorder with immunodeficiency and albinism, which we propose to classify as HPS-10.

Methods

Informed consent

Informed consent was obtained from the patients' family according to the guidelines of the local ethics committee (Ethics committee University of Freiburg, approval number 192/08).

Genetic analysis

For exome sequencing, 3 μg of DNA was fragmented using sonication technology (Diagenode, Seraing, Belgium). The fragments were end-repaired and adaptor-ligated. After

size selection, the library was subjected to the enrichment process using the Nimblegen SeqCap EZ Human Exome Library v2.0 (Roche, Madison, WI) enrichment kit and sequenced using the Illumina HiSeq2000 instrument (Illumina, San Diego, CA). Data analysis of filter-passed reads was done with BWA-short in combination with GATK and SAMTOOLS as implemented in Varbank (Cologne Center for Genomics). In-house scripts were applied for filtering against dbSNP, the 1000 Genomes Project and our database of exome variants. We focused on rare missense, nonsense, frameshift and splice site mutations assuming autosomal recessive inheritance. Further criteria for variant selection were coverage of more than six reads, minimal quality score of 10, minor allele frequency <1%.

Selected variants were confirmed by conventional sequencing. Respective exons were PCR amplified and products directly sequenced using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 3730 DNA Analyzer (Applied Biosystems). Primer sequences are available upon request.

Protein analysis

Lysates were prepared from MACS-purified CD8 T cells or virally reconstituted PHA/IL-2 blasts, sorted for GFP expression, using NP-40 (Igepal, Sigma) lysate buffer with protease inhibitor cocktail (Roche). Lysates were diluted with 1x sample buffer (Novex™ NuPAGE™ LDS Sample Buffer (4X)) and 1x reduction agent (Novex™ NuPAGE™ Sample Reducing Agent (10X). Commercial gels were used (NuPAGE Novex 4-12% Bis-Tris Protein Gels, 1.0 mm) with 1X 0.1% SDS running buffer (NuPAGE® MES SDS Running Buffer (20X). Protein transfer was done on nitrocellulose membrane over night with constant 220 mA and 1x running buffer plus 10% methanol, 0,1% SDS (NuPAGE® Transfer Buffer (20X)). Membranes were blocked with 5% milk in 1x PBS-T (0,05% Tween-20) for 1 hour. Antibodies specific for AP-3δ (rabbit-Dinah (1:200) or delta2 (1:500)), AP-3β (rabbit-Marlene (1:100)), AP-3μ (rabbit – Diane (1:500)) or AP-3σ (rabbit-Buttercup (1:500)) in 1% milk in 1xPBS-T were incubated over night at 4°C. All antibodies were provided by MS Robinson and A Peden and raised against fusion proteins encoding the AP-3δ subunit 23,24. Secondary anti-rabbit IgG antibody (Jackson) was incubated in 5% milk with 1x PBS-T for 30min.

Degranulation and cytotoxicity assays

NK-cell assays were performed as described previously 25. Degranulation of cultured transduced T cells was analyzed after resting cells for 24 hours in medium without IL-2. T cells were stimulated with 3 μg/ml plate-bound anti-CD3 (OKT3, eBioscience) or left in medium. Anti-CD107a-PE (BD Pharmigen) was added for the 3-hour stimulation time and was again used in combination with anti-CD8-APC (BD pharming) for surface staining. Cells were analyzed with flow cytometry (NaviosTM flow cytometer, BeckmanCoulter) and FlowJo software.

Functional reconstitution of T cells

For the generation of an *AP3D1* encoding retrovirus, Phoenix- Ampho cells were transfected with the wildtype *AP3D1* gene using PromoFectin (PromoKine) with the packaging vector

pML and pMX-AP3δ1-IRES-GFP. Viral supernatant was harvested after 48 hours of culture and used for viral transduction. T cell cultures were prepared for transduction by stimulating PBMC with PHA (1,25 μg/ml, Remel) and ~100u/ml hIL2 (produced from a transfected cell line) in the presence of irradiated (30 Gy, 5min) allogeneic PBMC as feeder cells. Cells were cultured in IMDM (gibco) with 10% human serum (Sigma) and 1% Penicillin and Streptamycin (gibco). Cells were restimulated, with fresh IL-2 and feeder cells every 14-18 days. Two days after restimulation, cells were resuspended in viral supernatant (containing pMX-AP3δ1-IRES-GFP or pMX-IRES-GFP as a negative control) and plated in a 24 well non-culture tissue plate coated with retronectin (10μg/ml, TaKaRa), followed by 2 hours of centrifugation at 32°C. T cell cultures were further propagated by repeated restimulation until the day of analysis.

Immunofluorescence staining

PHA/IL-2 blasts ($5x10^4$cells/spot) from the patient reconstituted with either pMX-AP3δ1-IRES-GFP or pMX-IRES-GFP were adhered to multispot slides (multispot microscopy slides, Hendley essex) in serum free medium (DMEM, Gibco) for 25 min at 37°C and fixed with 3%PFA for 15min at RT. Cells were blocked for 1 hour in PBS, 1% BSA, 0.4% Saponin in 1xPBS and incubated with anti-AP-3δ (mouse-SA4, provided by A Peden 18) in blocking buffer for 1 hour at RT, followed by extensive washes and incubation with secondary antibody (Alexa Fluor® 568 Goat Anti-Mouse IgG, Invitrogen, A-11031) for 35min at RT. Nuclei were stained with Hoechst in 1x PBS for 10min at RT Samples were washed prior to mounting with non-setting mounting medium (Vectashield, Vectalabs) and were imaged using an Andor Revolution Spinning disk system with CSU-X1 spinning disk (Yokogawa) with Borealis system, 1024 x 1024, pixel size 13x13μm camera (iXon Ultra DU888, Andor) IX81 microscope (Olympus) using the 100x objective (numerical aperture 1.45) and 2.0x magnification lens. Laser excitation was at 405, 488, and 561 nm. Images were acquired using IQ3 (Andor) and processed using Imaris software (Bitplane).

Results

A patient with early onset seizures, severe developmental delay, dysmorphic features and infection susceptibility associated with neutropenia

The index patient was the first child of consanguineous Turkish parents (first cousins). The mother had secondarily generalized focal epilepsy that was treated until 18 years of age, the father was healthy. A younger brother was affected by osteopetrosis, caused by a homozygous *TCIRG1* (OC116) mutation. At birth, the patient was microcephalic (length 50^{th} percentile, head circumference 3^{rd} percentile) with large, low set ears, retrognatia with a Pierre-Robin sequence, hypotelorism and a flat philtrum. At one week of age, he developed recurrent apneas followed by a focal seizure at the age of 11 days and increasingly dystonic movement patterns with truncal hypotonia. Within the first years of life, he developed spasms, tonic and myoclonic focal seizures as well as generalized tonic-clonic seizures that occurred up to 30 times daily and were poorly controlled with multiple anti-epileptic drug regimes. The EEG showed multifocal spike slow wave activity and no physiological differentiation. A hearing test revealed bilaterally reduced otoacustic potentials and a reduced brainstem evoked response audiometry. He made little

developmental progress until his death at 3.5 years and maintained general hypotonia, lack of head control with little spontaneous movement and no ocular fixation. He had significant feeding difficulties. Oculocutaneous albinism was associated with poorly pigmented hair and intermittent nystagmus. Brain MRI at the age of 3.5 years showed atrophy of the telencephalon and enlarged external and internal cerebrospinal fluid spaces as well as a large arachnoidal cyst in the posterior fossa. Furthermore, the myelination was insufficient for age corresponding rather to a 15 months old child: while cerebellar white matter was hypointense indicating myelin, only projection fibres and central parts of the supratentorial white matter were myelinated involving some subcortical domains, but not U-fibres (Fig. 1A). A hip radiograph showed flat acetabulae (Fig. 1B).

From the age of 8 months he developed recurrent bronchitis and pneumonia. Chest X-rays revealed chronic interstitial pneumonia (Fig. 1C). He had persistent hepatosplenomegaly and febrile episodes that were frequently associated with severe neutropenia (Fig. 1D), but did not develop thrombocytopenia or anemia. Bone marrow showed moderate dysplasias (Fig. 1E) with full maturation of all lineages. He had asymptomatic seroconversion to EBV and CMV. Abnormal bleeding was not noted. The patient died at the age of 3.5 years of a septic pneumonia.

Immunologic investigation and exclusion of hemophagocytic lymphohistiocytosis

In the context of his infection susceptibility and hepatosplenomegaly associated with albinism, he received an immunological assessment. He had normal immunoglobulin levels and vaccine responses, IgE was elevated (3990 U/ml). Lymphocyte counts were normal with an inverted CD4/CD8 ratio (0.53) during episodes of pneumonia. Since a number of immunodeficiencies with albinism are associated with impaired lymphocyte cytotoxicity, we analyzed expression of the degranulation marker CD107a on fresh (Fig. 2A) and IL-2 stimulated NK cells (Fig. 2B) after incubation with the NK sensitive target cell K562. NK cell degranulation was clearly abnormal. These results were confirmed in a ^{51}Cr release assay (Fig. 2C). T cell degranulation was impaired, but not absent (Fig 2D). A bone marrow aspiration performed in one of the febrile neutropenic episodes showed no evidence of hemophagocytosis. Also, fibrinogen, triglycerides and ferritin were normal, sCD25 was below 2.400 U/ml. Criteria for HLH 26 were not fulfilled.

An unknown homozygous mutation in the gene coding for the delta subunit of the AP-3 complex

The clinical and immunological findings suggested a genetic disorder of lysosomal trafficking associated with oculocutaneous albinism and immunodeficiency. The combination with facial and acetabular dysplasia, neutropenia and interstitial lung disease was most consistent with HPS-2, which is caused by mutations in *AP3B1* 5, the gene encoding AP-3β3A. However we did not find mutations in the gene. There were also no mutations in the genes *LYST* (mutated in CHS) 27, *RAB27A* (GS2) 28 and *LAMTOR2* (p14 deficiency) 6. We then performed whole exome sequencing using DNA extracted from peripheral blood. The patient's exome was covered with a mean depth of 103 reads, 97% of the exons were covered >10x and 95% were covered >20x. 84 M. unique reads were aligned to the reference human genome, and 78 M. reads were uniquely mapped to target

sequences. Filtering revealed 29 homozygous autosomal variants. Assuming homozygosity by descent, we further concentrated on larger homozygous intervals, which covered 440 Mb in total. 19 variants resided in these regions, including 17 missense variants, one potential splicing variant in *AOC1* and one frameshift deletion. Among these variants, the mutations in *AP3D1* had the most plausible relationship to the clinical phenotype, especially in synopsis with the *AP3d1* mouse mutant and its HPS-like phenotype (supplementary table 1). The deletion of GT at positions c.3565-3566 in exon 32 of *AP3D1* (c.3565_3566delGT; NM_001261826; Fig. 3A) leads to a frameshift and a termination codon after seven residues (p.Val1189Leufs*8) (Fig. 3A). The deletion was not identified in the 1000 Genomes Project or the NHLBI Exome Sequencing Project. The mutation affects both expressed isoforms of *AP3D1*: In isoform 2, which is the shorter splicing variant lacking exons 23 and 24, the mutation is found in exon 30 (c.3379_3380delGT; NM_003938). *AP3D1* is not only expressed in the ubiquitous AP-3 complex but also in the neuronal form (Fig. 3B).

The mutation allows strongly reduced AP-3δ protein expression, and impairs stable formation of the AP-3 complex

Next, we analyzed the presence of the four subunits of the AP-3 complex in patient CD8 T cells by Western blot analysis. AP-3δ protein was significantly decreased compared to cells from a healthy control and an AP-3β3A deficient HPS-2 patient. Consistent with an instable heterotetramer formation, protein levels of other subunits (AP-3β3A, AP-3σ and AP-3μ) of the AP-3 complex were clearly reduced (Fig. 4). Consistent with published data 24, the control cells from the HPS-2 patient only showed reduced levels of AP-3β3A and AP-3μ, whereas AP-3δ and AP-3σ were present at normal levels. Note that the molecular sizes of the four AP-3 subunits observed in these experiments showed slight differences to the sizes originally reported 23, although the same antibodies were used. We ascribe these differences to the use of different gel systems and markers.

Retroviral reconstitution of primary T cells restores AP-3 complex formation and T cell degranulation

To address the question whether the mutant AP-3δ is directly responsible for the destabilization of the AP-3 complex and the impaired lysosomal trafficking in patient cells, we generated a T cell culture from patient PBMC, which was maintained with PHA, IL-2 and feeder cells. The T cells were transduced with a retrovirus expressing either a pMX-AP3D1-IRES-GFP construct with wildtype *AP3D1* or pMX-IRES-GFP as a negative control. Following transduction, the culture was maintained for another 2 weeks followed by expression studies and functional tests. Successful reconstitution of GFP positive cells with *AP3D1* could be demonstrated by immunofluorescence (Fig.5A), showing the expression of AP-3δ as red dots in GFP positive cells only. Furthermore, Western Blot analysis of reconstituted and GFP+ sorted T cells revealed that retroviral transfection of T cells with the *AP3D1* wildtype gene restored the presence of AP-3δ, AP-3β3A, AP-3σ and AP-3μ to normal levels (Fig.4). These data confirmed that the patient mutation *AP3D1* was responsible for the destabilization of the AP-3 complex.

To test whether re-expression of the AP-3 complex in patient cells was also associated with functional reconstitution, we analyzed CD8+ T cell degranulation. Figure 5B shows the

flow cytometric analysis of retrovirally transduced cells. For degranulation, we incubated the transduced T cell cultures either with plate-bound anti-CD3 or with medium and measured the CD107a expression in successfully transduced GFP+ CD8+ T cells (Fig. 5C). As expected from previous experiments with AP-3B1 deficient T cells 29, the degranulation of patient's T cells transduced with the empty vector was reduced, but not absent. Importantly, transduction with the *AP3D1* containing vector fully restored the degranulation response of patient cells to levels of wild-type cells in two independent experiments (Fig.5D+E), while transduction with the empty control vector had no effect. These data support the notion that the lytic granule secretion defect results directly from the loss of AP-3 delta.

Discussion

Here we describe a hitherto unknown human disorder of lysosomal trafficking that shares several clinical and immunohematologic features of Hermansky-Pudlak syndrome type 2 5,29, but also manifests with severe epilepsy and neurodevelopmental delay. Using whole exome sequencing we have identified a homozygous mutation in *AP3D1* in the patient. Four independent lines of evidence support our conclusion that this is the causative mutation for the phenotype.

First, the complex clinical and cellular phenotype of the described patient shares many features with that of patients with HPS-2, which is caused by defects in AP-3β3A 5,30, a subunit of the AP-3 protein complex and an interaction partner of AP-3δ 16,31. The features in common include the oculocutaneous albinism, mild facial dysmorphism, microcephaly, hip dysplasia, chronic neutropenia, persistent hepatosplenomegaly, reduced cytotoxic lymphocyte degranulation, and susceptibility to airway infections. Of note, an overt bleeding disorder was not noticed, which is a characteristic feature of Hermansky-Pudlak syndromes 12,32. However, despite the unambiguous presence of a significant platelet degranulation defect, the clinical bleeding tendency is also moderate and non-spontaneous and its manifestation in other forms of HPS depends on the physical insult 33. Unfortunately, since we were not able to analyze platelet function in our patient ex vivo, it is unclear whether dense granule deficiency is associated with this disorder. We speculate that the patient had a platelet granule defect that was overlooked because it was a minor part of the clinical phenotype. The most striking difference to HPS-2 was the neurological phenotype including significant neurodevelopmental delay, severe epilepsy and a hearing disorder. Only mild neurodevelopmental delay was reported in one HPS-2 patient 29. Neurological involvement ranging from mild sensory deficits to mental retardation is more prominent in CHS 10.

Second, the phenotype of our patient showed striking similarities to the mocha mouse strain, a naturally occurring mouse mutant with a null mutation in the *Ap3d1* gene 22 (Table 1). These mice have oculocutaneous albinism and reduced coat pigmentation and, importantly, they show a functional platelet defect 22, similar to AP-3β3A deficient pearl mice 34. To our knowledge, neither the skeletal phenotype nor investigations on neutrophil counts or lymphocyte degranulation have so far been published in mocha mice. Thus, the phenotypic overlap of human and murine deficiency in the AP-3β3A or AP-3δ subunits of the AP-3 complex remains incompletely defined. Most relevant, however, mocha mice show

a distinct cortical excitability phenotype with brief epileptic discharges as well as balance problems due to otolith defects and eventual deafness 22,35. These features specifically mirror the severe neurological phenotype observed in our patient. The analogy to the mouse model suggests that both epilepsy and deafness in our patient were causally related to the AP-3δ deficiency. It should be noted that the initially described strain of mocha mice additionally carried grizzled (gr) and retinal degeneration 1 (Pde6b^{rd1}) alleles 36. However, the neurological phenotype and severe hearing loss are also observed in other *Ap3d1* mutant strains not carrying these additional mutations37.

Third, we show that the identified mutation significantly reduces expression levels of the AP-3δ protein. Apparently this has severe consequences for the stability of the AP-3 complex since the other subunits AP-3β3A, AP-3σ and AP-3μ were also decreased in protein expression. This is in line with observations in mocha mice 22, where analysis of brain extracts revealed that the in the complete absence of AP-3δ expression was associated with degradation of all other subunits. Notably, the physiology of the heterotetrameric AP3-complex offers an explanation for the severe neurological manifestations in *Ap3d1* but not in *Ap3b1* deficiency. The δ subunit is central to both the neuronal and the ubiquitous form of AP-3 (Fig.3B), and therefore its absence cannot be compensated. In contrast, the absence of β3A can be largely compensated in neurons but not in other tissues, because of the expression of another isoform (*AP3B2*). The pathophysiological link from neuronal AP-3 deficiency to the severe neurological phenotype remains to be explored. Based on the observation of an interaction between the neuronal AP-3 complex and a zinc transporter (ZnT3) in the brain, it was previously postulated that the absence of AP-3 may lead to missorting of ZnT3 resulting in zinc deficiency 38. In addition, in the absence of AP-3, various synaptic vesicle membrane proteins are mistargeted, including synaptic vesicle chloride channel 3 (ClC-3) 39, vesicular glutamate transporter 1 (VGLUT1)40, PI4KIIa 41, the vesicular GABA transporter (VGAT) 42, and the synaptobrevin-1-like SNARE VAMP7 21,43. These alterations may be highly relevant for the development of the epilepsy.

Fourth, introduction of the wild-type *AP3D1* gene into CD8+ T cells restored the stability of the whole AP-3 complex. While all subunits were highly reduced in unmanipulated patient cells, they were all detected in normal levels after retroviral reconstitution with the wild-type *AP3D1* gene, revealing that the patient mutation of *AP3D1* was indeed responsible for the AP-3 complex instability. Importantly, the genetic correction also led to a functional reconstitution of the granule exocytosis defect in cytotoxic lymphocytes, proving that *AP3D1* deficiency is responsible for this phenotype. Overall, the impaired degranulation response of lymphocytes in our patient was similar to that of patients with HPS-2 2,29. It was most evident in fresh NK cells, where the response was very low and indistinguishable from that of patients with familial HLH, GS2 or CHS25,44. However, in contrast to these conditions and similar to our observations in HPS-2, the response of PHA/IL-2 stimulated CTL was less markedly impaired. This could possibly be due to an effect of IL-2 on the indirect vesicular transport pathway45,46, which is independent of the AP-3 complex and has a recycling function 5,18.

Although these arguments provide a plausible link of AP-3 delta deficiency to all aspects of the patient phenotype, a contribution of other homozygous alleles (suppl. table 1)

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cannot be formally excluded. The description of further patients will help to resolve this issue. Based on the preliminary definition of this complex phenotype in a single patient, what are the clinical implications of a diagnosis of AP-3δ deficiency? AP-3δ deficiency appears to be mainly a neurological disorder. In our patient as well as in mocha mice22, the severe epilepsy associated with a significant neurodevelopmental delay dominated the clinical picture. However, the immune-hematological aspects of the disease are probably of similar importance and can be life-limiting. The resulting infection susceptibility is at least in part favored by a defect in neutrophil development and – in analogy to HPS-2 – probably also by functional defects in other immune cell populations 47 This includes an impaired degranulation and cytotoxicity of lymphocytes 2,25,48 and could also imply impaired dendritic cell function, as demonstrated in a mouse model of HPS type 2 49. We have previously shown that HPS-2 patients carry a small, but relevant risk of developing HLH2. Asymptomatic seroconversion to EBV, CMV and HSV in our patient indicates that this risk is also limited in AP-3δ deficiency. However, we interpret the persistent hepatosplenomegaly and recurrent fevers in our patient as subclinical HLH-associated manifestations. In the context of the poor neurological prognosis, the hematological manifestations may not justify hematopoetic stem cell transplantation, but monitoring of neutropenia and hepatosplenomegaly should be part of the clinical management of affected patients. This should also include evaluation for chronic lung disease as the interstitial lung disease can determine long-term prognosis in some HPS variants 1,8.

In summary, AP-3δ deficiency further adds to the growing spectrum of human genetic disorders of biogenesis and trafficking of lysosome-related organelles and illuminates the importance of the cell-type specific synthesis of AP complex subunits. Although we could not analyze the characteristic storage pool deficiency of HPS in our patient, we suggest classifying AP-3δ deficiency as a novel type of HPS. From a clinical point of view, bleeding appears to be a minor aspect of the disease (as in HPS-2), but the AP-3 related pathophysiology favors classification as HPS rather than as a new congenital neurodevelopmental disease, a new variant of congenital neutropenia or a novel primary immunodeficiency. Hence, we suggest giving the disorder the acronym HPS-10, but this has to be confirmed by an appropriate taxonomy committee.

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Key points

- A phenotype with albinism, early onset seizures, neurodevelopmental delay, infection susceptibility and neutropenia is caused by *AP3D1* mutations
- AP-3δ deficiency destabilizes the AP-3 complex and defines a novel type of Hermansky-Pudlak syndrome with severe neurological involvement

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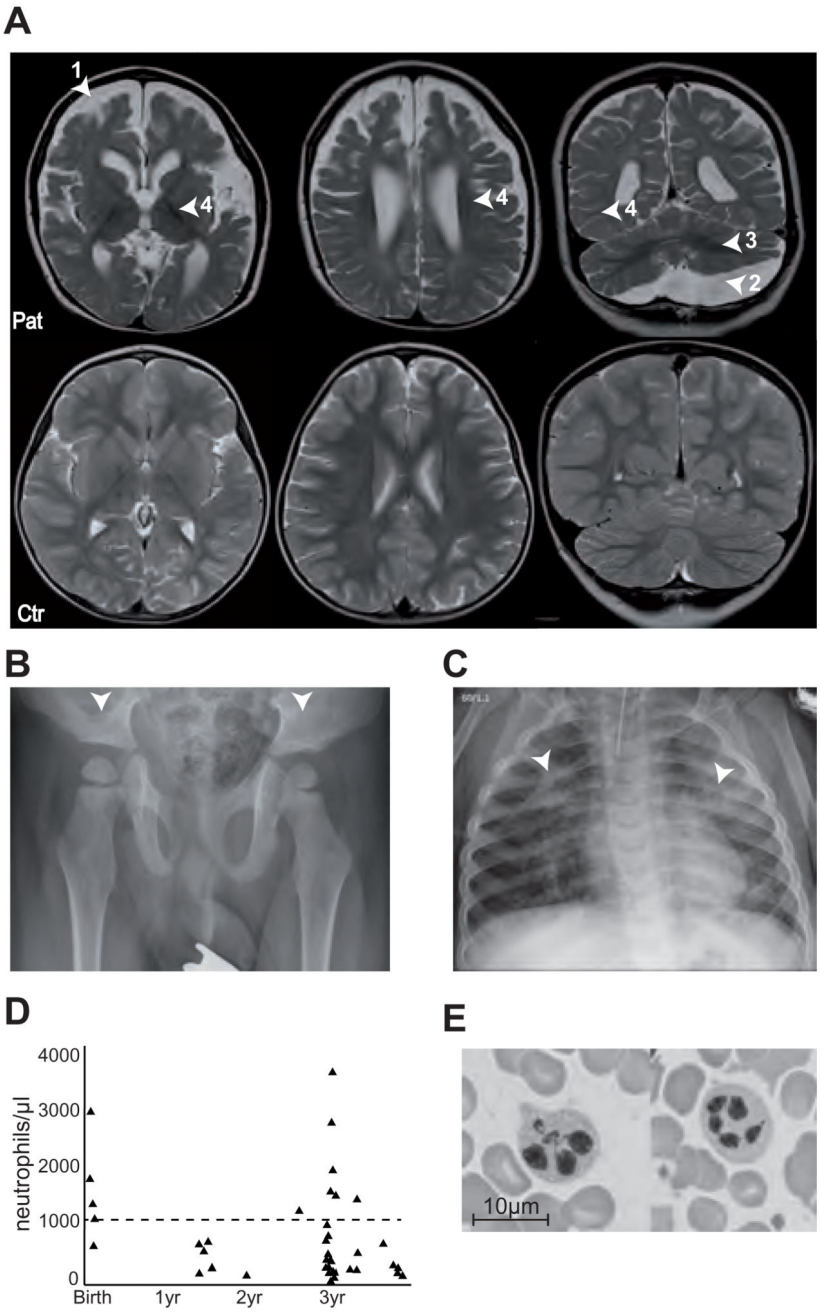

Figure 1. Clinical phenotype of the patient

(A) MRI scan of the brain at 3.5 years (upper panel), showing atrophy of the telencephalon (1), enlarged external and internal cerebrospinal fluid spaces (4), arachnoidal cyst (2) in the posterior fossa and insufficient myelination (3) indicated by arrow heads. The lower panel shows an MRI scan of a healthy 3.5 year old child. (B) X-ray of the pelvis showing flat, dyplastic acetabulae. (C) X-rays of the chest showing chronic interstitial pneumonia. (D) Neutrophil counts over time. Dashed line at 1000 neutrophiles/µl represent limit for neutropenia. (E) Bone marrow smear showing hypersegmented neutrophils.

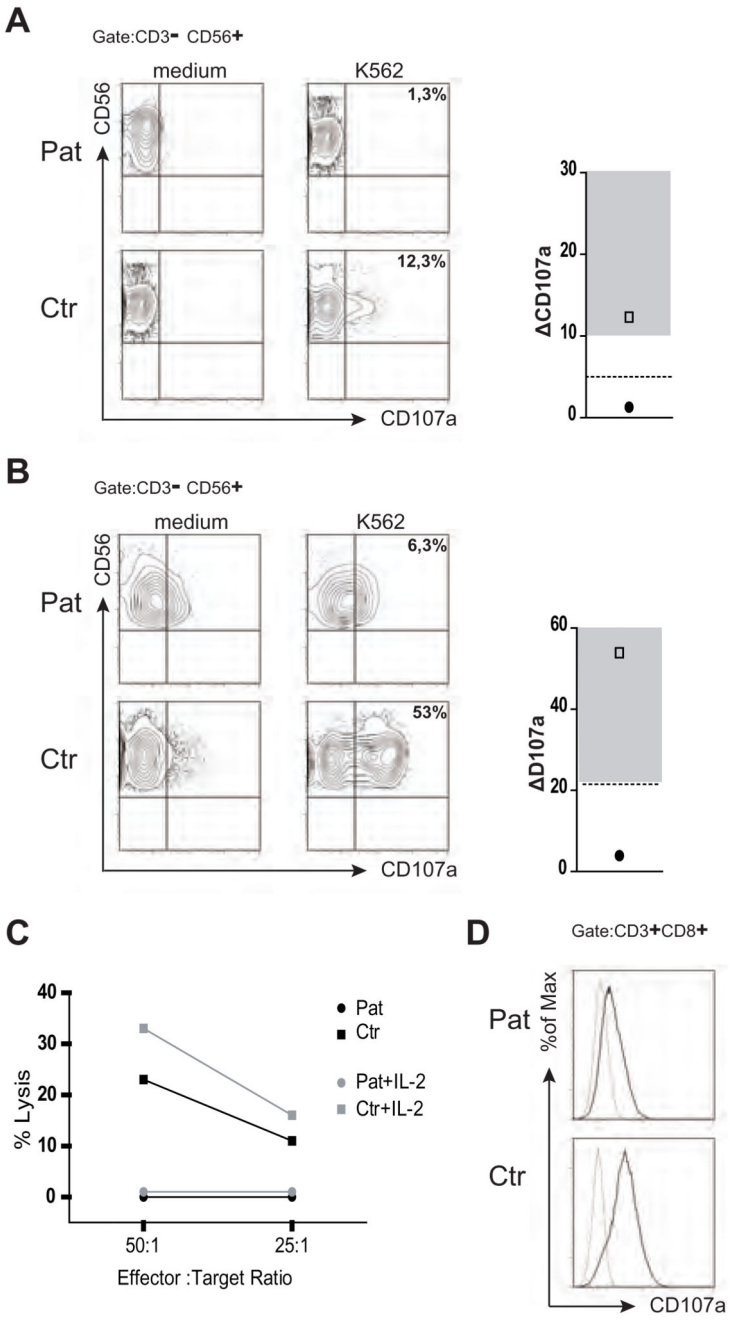

Figure 2. Impaired degranulation and cytotoxicity of patient NK cells

(A) *Ex vivo* degranulation of NK cells (CD3-CD56+) from the patient (Pat) and a healthy donor (Ctr) after incubation with medium (left panel) or with K562 cells (middle panel) as assessed by flow cytometric analysis of CD107a surface expression. The right panel shows the difference in CD107a expression between unstimulated and stimulated cells of the patient (closed symbol) and a day control (open symbol) relative to healthy controls (gray area). The dashed line represents the level below which NK cell degranulation has the best positive and negative predictive value for a mutation in a gene relevant for cytotoxicity

in an unfiltered cohort of patients with HLH. (B) Degranulation of NK cells prestimulated with IL-2 and PHA for 48 hours. (C) Cytotoxicity of patient (circle) and control (squares) PBMC on K562 target cells without (black symbols) or after overnight pre-incubation with IL-2 (grey symbols). (D) T cell degranulation. Cultured T cell of the patient and a healthy control were incubated in medium or stimulated with 3μg/ml of plate-bound anti-CD3. The increase in CD107a expression upon stimulation is shown as an overlay of histograms of unstimulated (dotted line) and stimulated (solid line) cells.

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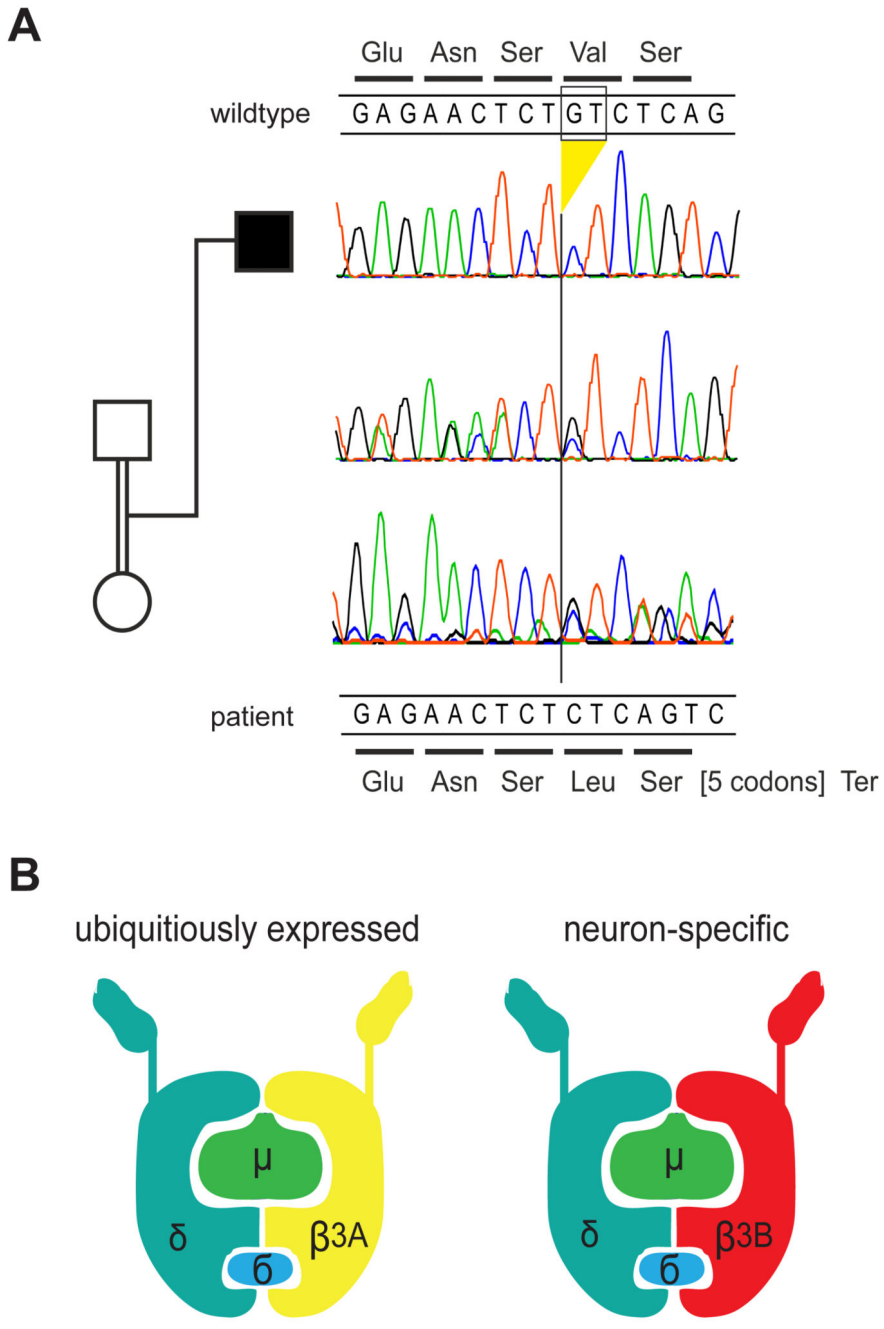

Figure 3. Genetic analysis and model of the AP-3 complex

(A) Electropherograms of the section of exon 32 harboring the homozygous deletion in the patient. The deletion leads to a frameshift at codon 1189 and a termination codon after seven residues. The encoded wild-type peptide sequence is shown at the top, the codons of the mutant sequence are shown below the DNA sequences. The parents are heterozygous for the deletion as expected. For technical reasons the reverse strand was sequenced in the DNA sample of the father but the complementary sequence is depicted here, i.e. it is shown in the same direction as the other samples. (B) Model of the AP-3 protein complex in the

ubiquitous and the neuronal form (adapted from 31). AP-3δ is an essential part of both complexes, while AP-3β3A, mutated in HPS-2, is substituted by AP-3β3B in neuronal cells.

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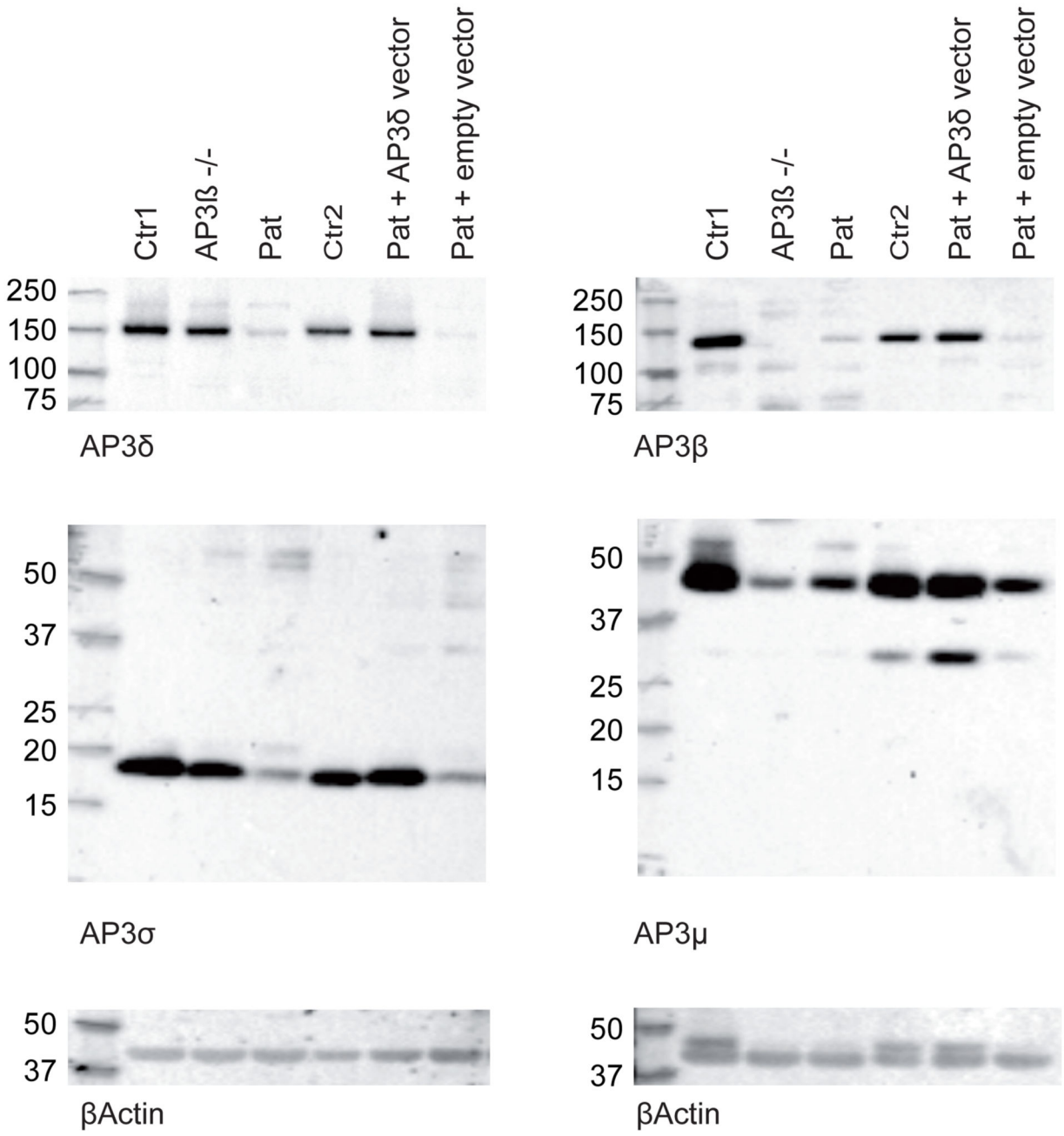

Figure 4. Reduced expression of AP-3δ affects the stability of the AP-3 complex

Western blot analysis of lysates from Macs-purified CD8 T cell of two healthy controls, an AP-3β3A deficient patient and the index patient (lane 1-4). Two separate blots are using rabbit antibodies against AP-3δ and AP-3σ (left) and AP-3β and AP-3μ (right). β-actin was used as a loading control. AP-3δ protein was analysed using two different antibodies23,24 in three independent experiments each, each giving the same results. Lanes 5 and 6 show blots from patient PHA/IL-2 blasts retrovirally transduced with an *AP3D1* expressing or empty

vector analysed in the same experiments. These cells were FACS sorted for GFP positive cells to enrich for successfully transduced cells.

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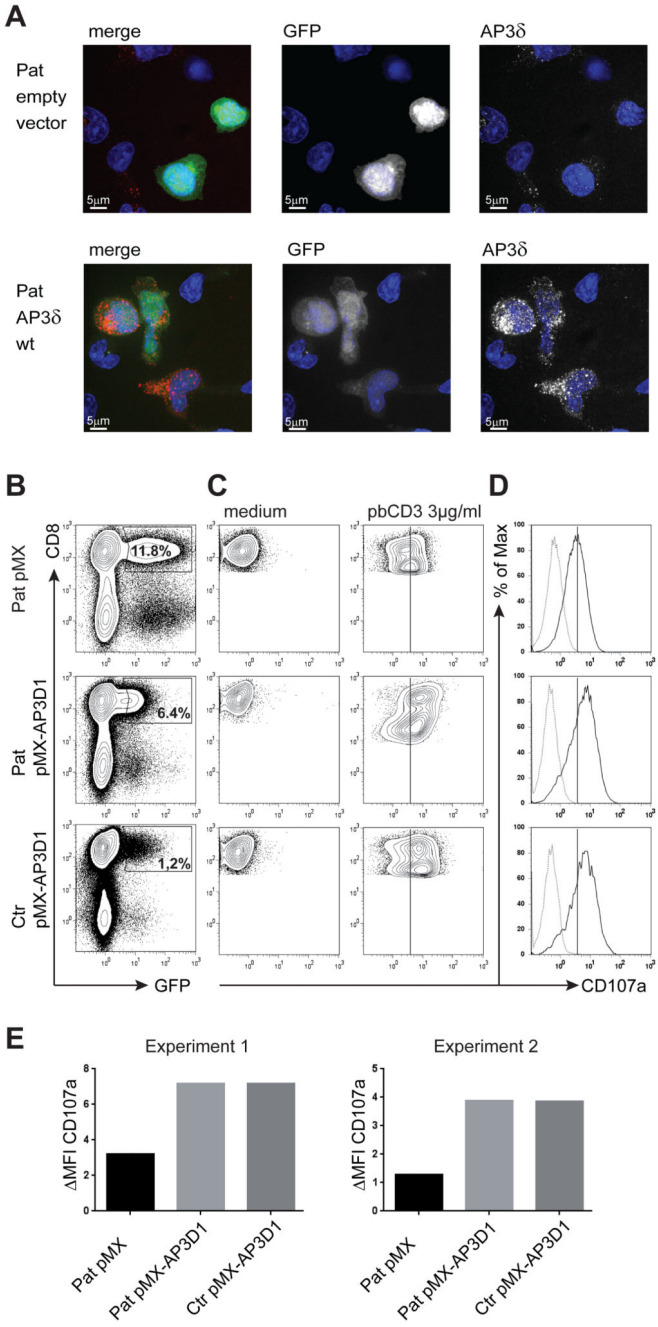

Figure 5. Genetic reconstitution with wild-type *AP3D1* showing expression and function of the AP-3 complex

(A) Immunofluorescence analysis of patient PHA/IL-2 blasts reconstituted with either empty vector or wild-type *AP3D1* using mouse anti-AP-3δ (SA4). Cells are shown in one 3D stack picture of 28 slices. Cells successfully transduced with the vector are GFP+ (white in the single stains, green in the merged picture), the nucleus is stained with Hoechst (blue) and AP-3δ is stained with SA4 provided by A. Peden 18 (white in the single stains, red in the merged picture). The variable GFP intensity of reconstituted cells is explained by the use of an IRES-GFP construct where *AP3D1* is placed before and *GFP* is placed

after the IRES sequence. (B) Transduced T cells were rested in medium free of IL-2 for 24 hours and then incubated in medium or stimulated with 3μg/ml pb CD3 for 3 hours. Transduction efficiencies varied between 1 and 12%. (C) CD107a expression after incubation of transduced cells with medium or plate-bound anti-CD3. Plots are gated on successfully transduced GFP+ CD8+ cells. The solid line serves indicates peak CD107a expression in patient cells transduced with the empty vector. (D) Overlay of CD107a expression of transduced T cells kept in medium (dotted line) and stimulated stimulated with anti-CD3 (solid line) (E) The difference in mean fluorescence intensity (delta MFI) of CD107a expression between cells incubated in medium versus stimulated with pb anti-CD3 is shown for patient (pat) and ctonrol (ctr) GFP+CD8+ T cells transduced with the indicated vector. Results are shown from two independent experiments.

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Table 1

Comparison of the clinical manifestations of the index patient, mocha mice and AP-3β3A deficiency (HPS-2) in humans.

	HPS-2[a]	patient[b]	mocha mice[c]
oculocutaneous albisim	yes	yes	yes
hair hypopigmentation	yes	yes	yes
microcephaly	yes	yes	Unknown
Low set ears	yes	yes	unknown
Retrognathia	Yes	yes	unknown
Flat philtrum	Yes	yes	unknown
Flat acetabula	Yes	yes	unknown
infection susceptibility	yes	yes	unknown
Epilepsy	no	yes	yes
Truncal hypotonia	No	Yes	unknown
Deafness	no	yes	yes
impaired platelet degranulation	yes	unknown	yes
neutropenia	yes	yes	unknown
impaired NK/CTL degranulation	yes	yes	unknown

[a] Enders et al 2006 29

[b] this report

[c] Kantheti et al 1998 22