Published in final edited form as:

Nat Genet. 2003 September ; 35(1): 84–89. doi:10.1038/ng1229.

Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1)

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Abstract

Hermansky-Pudlak syndrome (HPS; MIM 203300) is a genetically heterogeneous disorder characterized by oculocutaneous albinism, prolonged bleeding and pulmonary fibrosis due to abnormal vesicle trafficking to lysosomes and related organelles, such as melanosomes and platelet dense granules^{1–3}. In mice, at least 16 loci are associated with HPS^{4–6}, including sandy (*sd*; ref. 7). Here we show that the *sd* mutant mouse expresses no dysbindin protein owing to a deletion in the gene *Dtnbp1* (encoding dysbindin) and that mutation of the human ortholog *DTNBP1* causes a novel form of HPS called HPS-7. Dysbindin is a ubiquitously expressed protein that binds to α - and β -dystrobrevins, components of the dystrophin-associated protein complex (DPC) in both muscle and nonmuscle cells⁸. We also show that dysbindin is a component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1; refs. 9–11), which regulates

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Note: Supplementary information is available on the Nature Genetics website.

Competing Interests Statement: The authors declare that they have no competing financial interests.

trafficking to lysosome-related organelles and includes the proteins pallidin, muted and cappuccino, which are associated with HPS in mice. These findings show that BLOC-1 is important in producing the HPS phenotype in humans, indicate that dysbindin has a role in the biogenesis of lysosome-related organelles and identify unexpected interactions between components of DPC and BLOC-1.

We previously showed⁷ that the *sd*y mutant mouse is a valid model for human HPS and localized the gene *sd*y to mouse chromosome 13. Here we genotyped 20 microsatellite markers in 1,250 progeny of *sd*y backcrosses to localize *sd*y to the 2.2-cM interval (42.73–43.29 Mb) between *D13Mit244* and *D13Mit267* (Fig. 1). We identified the *sd*y interval within a 28-Mb scaffold (Celera Discovery System) containing two known genes, *Jmj* and *Dtnbp1* (Fig. 1b). We used PCR products of *D13Mit179* and the *Dtnbp1* cDNA as probes to generate a BAC contig covering the *sd*y interval (Fig. 1b).

Northern-blot analysis and sequencing of RT-PCR products of *Jmj* identified no abnormalities in *sd*y mutants, but truncated genomic PCR products (Fig. 2a) and mRNA (Fig. 2b) of *Dtnbp1* were apparent in *sd*y tissues. Sequencing of RT-PCR products showed that exons 6 and 7 (156 bp) of *Dtnbp1* were deleted in mutant mice, resulting in the loss of 52 amino acids from position 119–172 of the dysbindin protein (Supplementary Fig. 1 online). Genomic sequencing showed that this results from a large deletion (38,129 bp) from nucleotide 3,701 of intron 5 to nucleotide 12,377 of intron 7. This deletion was not found in twelve other inbred mouse strains (Fig. 2a), including coisogenic DBA/2J, indicating that it was not a strain-specific polymorphism. This in-frame deletion creates a 1.5-kb mutant dysbindin transcript (Fig. 2b) and abolishes expression of the 51-kDa dysbindin⁸ protein in *sd*y/*sd*y mice (Fig. 2c). Expression of dysbindin is restored in *sd*y/*sd*y transgenic mice containing BAC54F9 (Fig. 2c). Platelet serotonin levels of six of these transgenics were normal (>1.12 µg per 10⁹ platelets), whereas all five *sd*y/*sd*y litter-mates without BAC54F9 had platelet serotonin levels of <0.06 µg per 10⁹ platelets. *sd*y/*sd*y progeny containing the BAC transgene had darker coats than *sd*y/*sd*y progeny lacking the transgene. These results confirmed that mutation of dysbindin causes the *sd*y phenotype and that dysbindin is important for normal platelet dense granule and melanosome biogenesis.

We screened for mutations in the 10 exons of the human ortholog *DTNBP1* in 22 unrelated non-Puerto Rican individuals with HPS who did not have mutations in *HPS1*, *HPS3*, *HPS4*, *HPS5* or *HPS6*. We found six non-pathological polymorphisms (162A→G, IVS5+21G→C, IVS6-8—9delTT, IVS8+2C→T, 814C→T, 874A→G) in these individuals and the nonsense mutation 307C→T (resulting in the amino acid substitution Q103X; Supplementary Fig. 2 online) in the homozygous state in a 48-year-old Portuguese woman with oculocutaneous albinism, ease of bruising and a bleeding tendency. Bleeding time was 13 min, and platelet aggregation indicated a storage-pool deficiency. This woman has mild shortness of breath on exertion and reduced lung compliance but otherwise normal pulmonary function and high-resolution computed-tomography (CT) chest scans with no muscle weakness or ataxia. Her parents were first cousins.

Destabilization of the pallidin protein in the muted mutant (*mu*) and of the muted protein in the pallid mutant (*pa*) has been shown, consistent with their association in the BLOC-1 complex^{10,11}. We confirmed these results (Fig. 3) and observed similar destabilization of dysbindin in kidney extracts of both *pa* and *mu* mutants and destabilization of both muted and pallidin proteins in *sd*y kidney extracts. These observations suggested that dysbindin, like pallidin, muted and cappuccino, is a component of the BLOC-1 complex. We also observed the reported^{10,11} smaller reductions in the amounts of the muted and pallidin proteins in the reduced pigment (*rp*) mutant, although we saw no change in levels of the dysbindin protein in *rp* kidney extracts. We observed no changes or minimal changes in the

amounts of α 1- or β -dystrobrevin in tissues of any HPS mutant (data not shown) or in the amounts of pallidin, muted or dysbindin in 11 other mouse HPS mutants (Fig. 3).

To further identify proteins that directly interact with dysbindin, we used yeast two-hybrid analyses. We found interaction between dysbindin and pallidin and confirmed the interaction⁸ between dysbindin and β -dystrobrevin (Fig. 4a). We also found that β -dystrobrevin interacted with muted but did not directly interact with either pallidin or pearl (also called Ap3b1) and that the muted and pallidin BLOC-1 proteins interacted (Fig. 4a). We confirmed the association of dysbindin, pallidin, muted and β -dystrobrevin by coimmunoprecipitation (Fig. 4b). We found dysbindin and pallidin in common fractions in both size-exclusion chromatography (Fig. 5a) and sedimentation velocity (Fig. 5b) analyses, consistent with coresidence in BLOC-1. In contrast, the bulk of α 1- and β -dystrobrevins and another DPC component, Dp71 (a dystrophin isoform found in liver¹²), were separable from the BLOC-1 components dysbindin and pallidin. Together, these experiments indicate that dysbindin is a subunit of BLOC-1 and that the dystrobrevins are not subunits of BLOC-1 although they interact with BLOC-1 components. The dystrobrevins and dysbindin may have been separable in cytosolic liver extracts (Fig. 5) because the intact DPC complex was not extracted from liver membranes.

To characterize the impact of the *sd*y mutation on melanosome biogenesis, we carried out electron microscopic analysis of eye sections (Fig. 6). Relative to control DBA/2J mice, *sd*y mice had much fewer melanosomes in the retinal pigment epithelium and choroid, and choroidal melanosomes were generally smaller and irregular, further illustrating the importance of dysbindin in melanosome biogenesis.

The human disorders HPS-1 (ref. 13), HPS-2 (ref. 14), HPS-3 (ref. 15), HPS-4 (ref. 16), HPS-5 (ref. 17) and HPS-6 (ref. 17) result from mutations in genes that encode both known vesicle trafficking proteins and novel proteins. In the mouse, seven additional genes^{4,5,9,18–21} associated with HPS have been identified. We found that mutations in *DTNBP1/Dtnbp1* cause HPS-7/*sd*y, providing the first evidence to our knowledge that a mutation affecting a BLOC-1 complex component causes HPS in humans. The phenotypes of the four BLOC-1 mutants (pallid, muted, cappuccino and sandy) are the most severe of the mouse HPS mutants^{6,22}, emphasizing the importance of BLOC-1 in the biogenesis of lysosome-related organelles, and their similar phenotypes^{6,22} are consistent with their encoded proteins forming a common complex. One interpretation of the fact that dystrobrevin interacts with components (dysbindin and muted) of the BLOC-1 complex is that DPC components are involved in vesicle trafficking in nonmuscle tissues. Alternatively, dysbindin may be independently part of both BLOC-1 and the DPC complex, with its vesicle trafficking function in BLOC-1 distinct from its DPC function. In muscle, the DPC complex connects the actin cytoskeleton with the extracellular matrix²³. Interaction of the pallidin subunit of the BLOC-1 complex with actin¹¹ suggests the BLOC-1 complex and the actin cytoskeleton similarly interact to control biogenesis of lysosome-related organelles.

Methods

Mice and backcrosses

We obtained mutant *sd*y and coisogenic DBA/2J mice from The Jackson Laboratory and subsequently bred them at Roswell Park Cancer Institute. We established a backcross between homozygous *sd*y mice and PWK wild-type mice (subspecies of *Mus musculus musculus*) as described²⁴. *sd*y mutant mice have no obvious muscle abnormalities. Despite the reported genetic association of haplotypes involving the gene encoding dysbindin with schizophrenia^{25,26}, preliminary tests of *sd*y mice, which included the prepulse inhibition test, have identified no behavioral abnormalities, in agreement with the fact that there are no

apparent behavioral abnormalities in the individual with HPS-7. All procedures (mouse protocol 125M) were reviewed and approved by the Roswell Park Institutional Animal Care and Use Committee and adhered to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Genetic mapping, physical mapping and BAC sequencing

We generated high-resolution genetic and BAC-based physical maps of the *sdv* critical region as described²⁷. Altogether, we typed 1,250 backcross progeny at 6 weeks of age for the *sdv* phenotype and for crossovers in the region surrounding *sdv* using the flanking microsatellite markers *D13Mit138* (proximal) and *D13Mit39* (distal). Because coat color alone was unreliable, we classified the *sdv* phenotype by determination of flashing, on ultraviolet illumination, of platelet dense granules loaded with the fluorescent dye mepacrine²⁸. We further genotyped informative mice for markers *D13Mit164*, *D13Mit165*, *D13Mit244*, *D13Mit179*, *D13Mit64*, *D13Mit245*, *D13Mit277*, *D13Mit267*, *D13Mit180*, *D13Mit181*, *D13Mit155*, *D13Mit308*, *D13Mit91* and *D13Mit92* to derive a genetic map spanning the *sdv* interval.

We selected BACs by screening the RPCI-23 BAC library (Roswell Park Cancer Institute) with probes in the *sdv* interval, including microsatellite markers and mouse *Dtnbp1* cDNA and based BAC alignment on the Celera database and direct sequencing of BAC ends. We sequenced BAC 257B17 completely at the University of Oklahoma Genome Center using standard optimized BAC shotgun sequencing, closure and finishing methods²⁹. We annotated BAC sequences based on GenBank nr and dbest homologies using the BLAST programs after masking repetitive sequences.

Mutation screening of *Dtnbp1* in *sdv* mice and *DTNBP1* in individuals with HPS

We isolated total RNA from mouse tissues with RNAzol B reagent (Tel-Test) and carried out reverse transcription of RNA with the Superscript Preamplification System for RT-PCR (Gibco BRL). Primers 13282F and 13282R (sequences available on request) amplified a region spanning exons 5–10 of mouse *Dtnbp1* cDNA. We designed 22 sets of primers (sequences available on request) to amplify overlapping regions of introns 4–8 in genomic DNA and used primer IN5F4 (sequence available on request) as a sequencing primer for the breakpoint between intron 5 and intron 7.

We used primers (sequences available on request) spanning the 10 exons of *DTNBP1* for mutational analysis and subjected PCR products to single-strand conformation polymorphism/heteroduplex analysis³⁰ and then sequencing. The homozygous Q103X mutation in *DTNBP1* in the woman with HPS-7 was not observed in genomic DNA of 61 normal white controls. These experiments were approved by the Colorado Multiple Institutional Review Board. Informed consent was obtained from all individuals.

We tested genomic DNA samples of various inbred mouse strains (The Jackson Laboratory) for the deletion of exon 7 in *Dtnbp1* found in *sdv/sdv* mice with a duplex PCR procedure (primer sequences available on request) designed to produce PCR products of 472 bp (from exon 7, deleted in *sdv*) with normal DNA and 274 bp (spanning introns 5–7) with *sdv* DNA.

Western-blot analysis

We rapidly homogenized and froze fresh mouse tissues in Protease Inhibitor Cocktail Set I (Calbiochem) and stored extracts at -20°C . We boiled extracts in Laemmli sample buffer with 5% mercaptoethanol and separated 40 μg of extract protein on 12% SDS-PAGE gels or 4–20% gradient gels (Gradipore) and blotted gels to a PVDF membrane. We probed the blots with rabbit polyclonal antisera to dysbindin⁸ (1:250 dilution), muted¹¹, pallidin¹¹ and

dystrobrevin⁸. We purchased rabbit polyclonal antibody to Rab4 (D-20) from Santa Cruz Biotechnology and mouse monoclonal antibody to α -tubulin (Clone B512) from Sigma. Film was developed after treatment with the ECL Plus kit (Amersham; ref. 20).

Electron microscopy

We fixed eye tissues for 18 h at 4 °C in 3% glutaraldehyde and 0.1 M phosphate buffer, pH 7.2, and embedded and stained them with uranyl acetate and lead citrate²⁰.

Coimmunoprecipitation of dysbindin with other proteins associated with HPS

We transfected COS-7 cells with different combinations of expression constructs encoding dysbindin⁸, dysbindin lacking amino acids 120–171 (*sdys/sdys* dysbindin) in pCMVTag-4A, β -dystrobrevin⁸, pallidin in pCMV-Myc (Clontech), muted in pCMV-Myc and pearl (Ap3b1) in pCMVTag-3B (Stratagene) using Fugene-6 (Roche). Twenty-four hours after transfection we scraped the cells into 4 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 1 mM EGTA), clarified the supernatant by centrifugation and precipitated proteins with 5 μ g of antibody for 6 h at 4 °C. We captured immune complexes by overnight incubation with goat anti-rabbit MagnaBind beads (Pierce), washed them extensively in RIPA buffer and resuspended them in 200 μ l of SDS/urea buffer (4 M urea, 3.8% w/v SDS, 20% v/v glycerol, 75 mM Tris pH 6.8, 5% v/v 2-mercaptoethanol). We resolved proteins on 4–15% SDS–PAGE gradient gels (BioRad) and detected immunoprecipitated proteins with antibody to Myc 9E10 (1:200, pallidin and muted; Covance), antibody to β -dystrobrevin¹² β 521 (1:200) or a goat polyclonal antibody to Myc (1:100, pearl; Santa Cruz). We included controls in which the antibody to dysbindin was replaced by an unrelated rabbit polyclonal antibody in parallel on the same lysates.

Yeast two-hybrid interactions

We used the Matchmaker Gal4 Two-Hybrid System 3 kit (CLONTECH) for two-hybrid analyses. We fused cDNA sequences containing the entire protein coding regions of mouse wild-type and *sdys/sdys* dysbindin (from kidney of *sdys* mice), β -dystrobrevin, pallidin and muted in-frame to the DNA binding (pGBKT7) and activation (pGADT7) domains of the Gal4 transcription factor and verified resulting fusion constructs by sequencing. We cotransformed fusion constructs into *Saccharomyces cerevisiae* strain AH109 grown at high and low stringency as described¹⁷, incubated plates at 30 °C for 5 d and monitored growth and development of blue color.

Size-exclusion chromatography and sedimentation velocity analyses

We fractionated mouse liver cytosol by size exclusion chromatography on Superose 6 columns and linear 5–20% sucrose gradients as described¹¹ and analyzed these fractions by western blotting.

BAC rescue of *sdys/sdys* in transgenic mice

We injected BAC RP23-54F9 into pronuclei derived from C3H/HeJ $\times$ C57BL/6J F₁ females and transferred the transgene to the *sdys/sdys* background by mating with DBA/2J *sdys/sdys* mice to produce F₁ progeny. We mated those F₁ progeny that contained BAC RP23-54F9 to *sdys/sdys* mice to produce F₂ progeny, which were typed by PCR techniques (primer sequences available on request) for the presence of the BAC transgene, the *Dmbp1* deletion and polymorphisms in microsatellite markers closely linked to *Dmbp1*. We compared platelet serotonin concentrations⁷ and coat colors in *sdys/sdys* F₂ progeny containing and lacking the BAC transgene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. Reddington, L. Zhen, Y. Jiang, D. Poslinski, D. Tabaczynski, M.K. Ellsworth, J. Tan, H. Chen and X. Hu for technical assistance. M. Pagan contributed to the construction of the *sdly* genetic map. This work was supported in part by grants from the US National Institutes of Health (R.T.S., R.W.E., R.A.S., E.C.D. and B.A.R.) and by the National Cancer Institute, US Department of Health and Human Services (N.G.C. and N.A.J.). D.J.B. is a Wellcome Trust Senior Fellow. This research used core facilities supported in part by Cancer Center Support Grant to Roswell Park Cancer Institute funded by the National Cancer Institute.

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Figure 1. High-resolution genetic and physical maps of the *sdy* gene region of mouse chromosome 13. (a) High-resolution genetic map of the *sdy* gene region. Numbered *D13Mit* markers are listed above (without the prefix). The centromere is represented by a solid circle. (b) High-resolution physical map based on the BAC contig and Celera database. Two known genes in the *sdy* critical region are in bold with arrows indicating directions of transcription and relative sizes. A BAC contig (with RPCI-23 library designations) spans the *sdy* genetic interval (~570 kb). Arrows, proportional in length to the BAC sizes and oriented with the SP6 end at the arrowhead and the T7 end opposite, represent BACs.

Figure 2.

Dtnbp1 mRNA is smaller and dysbindin is undetectable in tissues of *sdy* mutant mice. (a) Genomic PCR. We amplified genomic DNA from control DBA/2J, *sdy*/+, *sdy*/*sdy* and various inbred strains by a duplex PCR method targeting exon 7 of *Dtnbp1* and designed to produce PCR products of 472 bp from wild-type DNA and 274 bp from *sdy*/*sdy* DNA. *sdy* arose in the DBA/2J strain. (b) Northern-blot analysis. We hybridized total RNA (20 µg) from kidney, brain and heart of wild-type (DBA/2J), heterozygous (*sdy*/+) and homozygous (*sdy*/*sdy*) mice with *Dtnbp1* (upper panels) and *Gapd* (lower panels) cDNA probes. *Dtnbp1* mRNA in *sdy* tissues was 1.5 kb in size compared with 1.65 kb in control DBA/2J; mRNA of heterozygous *sdy*/+ mice contains both ~1.5-kb and 1.65-kb *Dtnbp1* mRNAs. (c) Western-blot analysis. We resolved kidney extracts of control DBA/2J, *sdy*/+ and *sdy*/*sdy* mutant mice together with transgenic progeny containing (+) or lacking (-) BAC54F9, which contains the entire *Dtnbp1* genomic region, in denaturing gels, blotted gels and incubated them with polyclonal antibody to dysbindin 3111A (top). The blots were reprobed with antibody to Rab4 as a loading control (bottom). Immunoblot analyses of brain, heart, liver and skeletal muscle (data not shown) also showed lack of expression of dysbindin in *sdy*/*sdy* mutants.

Figure 3.

Destabilization of the dysbindin, pallidin and muted proteins in extracts of three HPS mutants (*sdv*, *pa* and *mu*). We separated kidney extracts from 16 mouse HPS or HPS-related mutants and 4 control strains (DBA/2J, *mu/+*, C57BL/6J and C3H/HeJ) on denaturing gels and probed them with antibodies to dysbindin, muted and pallidin as indicated at left.

Dystrobrevins were similarly analyzed in the brain as expression of α-dystrobrevin is weak in kidney. Inbred strain DBA/2J served as the control for *sdv/sdv*, *mu/+* for *mu/mu*, C3H/HeJ for *sut/sut*, *ash/ash* and *ru^{6J}/ru^{6J}* and C57BL/6J for the remaining mutants. Antibodies to Rab4 and α-tubulin served as loading controls.

Figure 4.

Association of dysbindin with muted, pallidin and β -dystrobrevin. **(a)** Yeast two-hybrid interactions. We transformed yeast strain AH109 with constructs expressing the entire coding regions of the indicated proteins fused to binding domains (left) or activation domains (top) and spotted cotransformants on plates containing high-stringency and low-stringency media. All combinations grew vigorously on low-stringency plates, indicating that no construct was lethal to the cells. Cotransformants pGBKT7-53 and pGADT7-T are positive controls for interacting proteins; pGBKT7-LAM and pGADT7-T are negative controls. AD (N) and BD (N) represent ‘empty’ activation and binding domain constructs, respectively. *sdyl/sdy* dysbindin is the *sdyl* mutant form of dysbindin with the 52-amino-acid deletion. We were unable to test for interactions among wild-type dysbindin, *sdyl/sdy* dysbindin and muted because they autoactivated in the binding domain. *sdyl/sdy* dysbindin did not interact with dystrobrevin but did interact with pallidin. **(b)** Coimmunoprecipitation shows direct association of dysbindin with components of BLOC-1. We cotransfected COS-7 cells with either dysbindin or *sdyl/sdy* dysbindin (the *sdyl* mutant form of dysbindin with the 52-amino-acid deletion) and Myc-pallidin, Myc-muted, β -dystrobrevin or Myc-pearl. Proteins were immunoprecipitated with antibody to dysbindin or an unrelated polyclonal antibody (control IgG) and detected with an antibody to Myc. Muted and pallidin were detected when immunoprecipitated with dysbindin but not with an unrelated IgG, indicating that both muted and pallidin form a complex with dysbindin. The cell lysate is shown for comparison. In control experiments, β -dystrobrevin coimmunoprecipitated with dysbindin as described previously⁸ but pearl did not. Pallidin and muted immunoprecipitated with *sdyl/sdy* dysbindin, but to a lesser extent. β -dystrobrevin, wild-type dysbindin and pearl did not coimmunoprecipitate with *sdyl/sdy* dysbindin. The asterisk indicates the IgG heavy chain that is detected by the secondary antibody.

Figure 5. Dysbindin and pallidin are components of a common protein complex in mouse liver. **(a)** Size exclusion chromatography. We fractionated mouse liver cytosol on a calibrated Superose 6 column and analyzed the resulting fractions by immunoblotting with antibodies to dysbindin, pallidin, α 1-dystrobrevin, β -dystrobrevin and the dystrophin isoform Dp71 (ref. 12). The exclusion volume (V_0) and the elution positions of standard proteins (Stokes radii given in Angstroms) are indicated at the top. **(b)** Sedimentation velocity analysis. We fractionated mouse liver cytosol by ultracentrifugation on a 5–20% (w/v) sucrose gradient and analyzed the resulting fractions by immunoblotting as in **a**. Fractions 1 and 28 correspond to the top and bottom ends of the gradient, respectively. The positions of standard proteins (sedimentation coefficients given in Svedberg units) are indicated at the top. Taken together, the size-exclusion chromatography and sedimentation velocity analyses indicate a native molecular mass of ~230 kDa (221–247 kDa) and a frictional ratio of ~2.4 for mouse BLOC-1. Both numbers are in close agreement with those calculated for bovine BLOC-1 (ref. 11), indicating that mouse BLOC-1 is similarly a large asymmetric protein complex.

Figure 6. Retinal melanosomes are deficient and abnormal in *sd*y mice. Retinal sections of DBA/2J (**a**) and *sd*y/*sd*y (**b**) mice. In each case, the hyphenated partial line at the left indicates the interface between the retinal pigment epithelium (above) and the choroid (below). Scale bars = 2 μ m.