

Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11

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Familial hemophagocytic lymphohistiocytosis (FHL) is a rare autosomal recessive disorder characterized by hyperactive phagocytes and defects in natural killer cell function. It has been shown previously that mutations in the perforin 1 gene (*PRF1*) and in *UNC13D* are associated with FHL2 and FHL3, respectively, indicating genetic heterogeneity. We performed genome-wide homozygosity mapping in a large consanguineous Kurdish kindred with five children affected with FHL. Linkage to a 10 cM region on chromosome 6q24 between D6S1569 and D6S960 defined a novel FHL locus. By screening positional candidate genes, we identified a homozygous deletion of 5 bp in the syntaxin 11 gene (*STX11*) in this family. We could demonstrate that syntaxin 11 protein was absent in the mononuclear cell fraction of patients with the homozygous 5 bp deletion. In addition to this family, we found homozygous mutations in *STX11* in five consanguineous Turkish/Kurdish FHL kindreds including two families with the 5 bp deletion, one family with a large 19.2 kb genomic deletion spanning the entire coding region of *STX11* (exon 2) and two families with a nonsense mutation that leads to a premature stop codon in the C-terminal end of the protein. As both *STX11* and *UNC13D* are involved in vesicle trafficking and membrane fusion, we conclude that, besides mutations in perforin 1, defects in the endocytotic or the exocytotic pathway may be a common mechanism in FHL.

INTRODUCTION

Familial hemophagocytic lymphohistiocytosis (FHL) is a genetically heterogeneous disorder typically first manifesting in early childhood with a rapidly fatal outcome if untreated (1). At diagnosis, patients present with a hyperinflammatory syndrome characterized by persistent fever, hepatosplenomegaly, cytopenia and, less frequently, central nervous system involvement. Common laboratory findings include low levels of fibrinogen and high concentrations of triglycerides, ferritin and the α -chain of the soluble interleukin 2 receptor (sCD25) secreted by activated T-cells. Defective natural killer (NK)

cell activity is a hallmark of the disease (2). Hemophagocytosis in bone marrow, cerebrospinal fluid or lymph nodes by activated histiocytes may be absent initially but can be observed later in most of the cases. Chemoimmunotherapy based on corticosteroids, epipodophyllotoxins, cyclosporin A and antithymocyte globulins results in control of the disease in the majority of patients; however, remission is rarely sustained (3,4). Most patients suffer an early death unless treated by hematopoietic stem cell transplantation (HSCT), which is the only curative approach so far (5).

Linkage analysis in FHL patients has identified a genomic region with a defect in a still unknown gene on chromosome

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Table 1. Two-point LOD scores for each analyzed marker from the *FHL4* interval and FHL in the large, consanguineous Kurdish family used for the genome-wide scan

| Marker | Recombination fraction | | | | | | | |
|------------|------------------------|--------|--------|--------|--------|--------|--------|--------|
| | 0.000 | 0.001 | 0.010 | 0.050 | 0.100 | 0.200 | 0.300 | 0.400 |
| D6S1009 | -1.873 | -0.321 | 0.623 | 1.121 | 1.164 | 0.930 | 0.570 | 0.221 |
| D6S1569 | -∞ | 1.257 | 2.197 | 2.626 | 2.576 | 2.127 | 1.487 | 0.747 |
| D6S308 | 1.516 | 1.512 | 1.481 | 1.339 | 1.157 | 0.779 | 0.403 | 0.105 |
| D6S971 | 0.902 | 0.901 | 0.888 | 0.828 | 0.751 | 0.587 | 0.409 | 0.216 |
| D6S1649 | 3.038 | 3.033 | 2.990 | 2.794 | 2.541 | 2.001 | 1.409 | 0.751 |
| GATA184A08 | 3.663 | 3.656 | 3.596 | 3.322 | 2.966 | 2.213 | 1.420 | 0.644 |
| D6S311 | 4.891 | 4.883 | 4.813 | 4.495 | 4.079 | 3.179 | 2.184 | 1.105 |
| D6S960 | -∞ | -4.084 | -2.133 | -0.948 | -0.598 | -0.430 | -0.380 | -0.259 |
| D6S2436 | -∞ | -1.325 | -0.357 | 0.200 | 0.326 | 0.297 | 0.187 | 0.084 |

9q21 (*FHL1*; MIM 603552) (6), and a defect in the perforin 1 gene (*PRF1*) on chromosome 10q21 (*FHL2*; MIM 603553) (7,8). In *FHL2*, perforin mutations lead to significant reduction or complete absence of protein synthesis resulting in a defective cytolytic activity of T-cells and NK cells (9) [reviewed by Arico *et al.* (10)]. The incidence and type of *PRF1* mutations vary in different ethnic groups (11–15). Very recently, *FHL3* was identified on chromosome 17q25 with mutations in the gene *UNC13D* (16). The gene product of *UNC13D*, hMunc13-4, is a member of a protein family involved in vesicle priming, which prepares cytolytic granules at the plasma membrane for vesicle membrane fusion. Lymphocytes of FHL patients with *UNC13D* mutations were shown to lack the ability to deliver cytotoxic substances like perforin or granzyme B to the target cells. Both proteins, perforin and granzyme B, are directly involved in the cytotoxic response of the immune system to exo- or endogenous pathogens (17). It can be assumed that defects in other genes involved in the release of cytotoxic proteins could also lead to the clinical picture of FHL. In fact, two other related disorders characterized by hemophagocytosis and immune defects are the Chédiak–Higashi Syndrome (CHS) and the Griscelli-Syndrome (GS2) with defects in the genes *CHS1* and *RAB27A*, respectively (18,19). Patients with these disorders have defective NK and/or T-cell cytotoxicity and, besides the overall secretion deficiency in immune cells, defects in the transport of pigment proteins that result in a partial albinism, not seen in the classical FHL forms (20). In all types of genetically based hemophagocytosis syndromes, the immune defect leads to the same severe clinical picture as in FHL. As all primary forms of FHL should rapidly undergo HSCT, early identification of the underlying genetic defect is crucial for patients without family history.

In the present study, we excluded mutations in *PRF1* by genomic sequencing of the entire coding region, and performed a genome-wide homozygosity analysis in a large consanguineous FHL kindred of Kurdish descent. Linkage analysis led to identification of a novel FHL locus on chromosome 6q24, designated *FHL4*. By analyzing several genes residing in this region, we identified a homozygous 5 bp deletion in *STX11* in all affected patients of this family. We could show that the 5 bp deletion, resulting in a premature stop codon, leads to abrogation of syntaxin 11 protein synthesis. We identified three different mutations in *STX11* in a

total of 10 patients from six different families with FHL, all of them with a common ethnic background.

RESULTS

Identification of *FHL4* by linkage and homozygosity analysis

A genome-wide homozygosity analysis was performed with 19 members of the large consanguineous FHL family of Kurdish origin described earlier (family 1). By analyzing 380 markers, all known loci for FHL, namely *FHL1*, *FHL2* (*PRF1*) and *FHL3* (*UNC13D*) were clearly excluded. We obtained a maximum two-point LOD score of 4.89 at $\Theta = 0.00$ to D6S311 on chromosome 6q24 representing the only region with an LOD score significant for linkage (Table 1). We confirmed the localization by construction of likely haplotypes and demonstrated homozygosity in a 10 cM interval between D6S1569 and D6S960 in all affected individuals, and a heterozygous mutant haplotype in all obligate carriers (Fig. 1). A maximum multi-point LOD score of 4.94 was obtained between D6S1649 and D6S311. We have designated this novel locus for FHL *FHL4*.

Detection of homozygous mutations in *STX11*

The candidate genomic region is 11 Mb in length and harbors more than 50 different known or predicted genes (Fig. 2A). Among other candidates, we analyzed the gene encoding syntaxin 11 (*STX11*) as syntaxins are known for their function in vesicle transport. The *STX11* gene consists of two exons and covers a genomic interval of 37 kb (Fig. 2B), of which exon 2 contains all of the coding sequence. We identified a homozygous deletion of two plus three nucleotides, c.369_370delAG and c.374_376delCGC, in exon 2 of *STX11* (Fig. 2C). Both deletions completely cosegregated in the pedigree. The total deletion of five basepairs leads to a frameshift and a premature termination codon after 59 altered residues (Fig. 2D). The homozygous mutation, Val124fsX60, was found in all affected children of the family, the heterozygous mutation was present in the obligate carriers. Neither the complete mutation nor the 2 bp or the 3 bp deletion alone was identified in samples from more than 200 control subjects of Kurdish or Turkish descent. Subsequently, additional FHL patients of Turkish or Kurdish

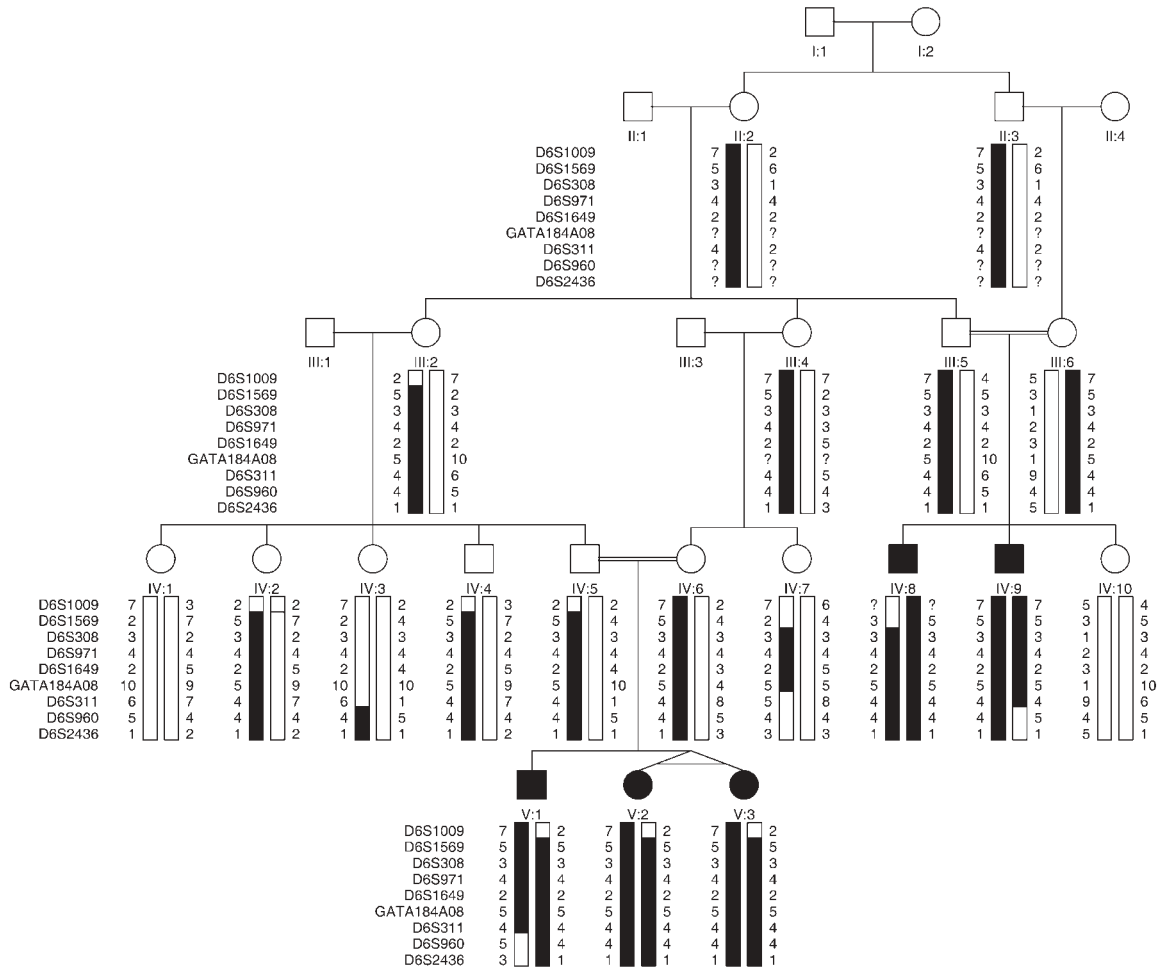


Figure 1. Pedigree of the large Kurdish FHL family used for the mapping study and construction of haplotypes in the *FHLA* region on chromosome 6. The filled box denotes the mutant haplotype completely cosegregating with the *FHLA* mutation. Oblivatory recombination events and lack of homozygosity, respectively, define the critical interval between D6S1569 and D6S960.

descent without *PRF1* mutations were analyzed for mutations in *STX11*. In two patients from different families of Kurdish origin (families 2 and 3, respectively), the homozygous mutation Val124fsX60 was also present (Table 2).

The analysis of marker loci around *STX11*, namely GATA129G07, D6S1569, D6S308, D6S971, D6S1649, GATA184A08 and D6S311, revealed the same mutant haplotypes in the patients from families 1, 2 and 3 in a 5.9 cM interval between D6S1569 and D6S311. As these families had the same ethnic background and originated from the north eastern part of Turkey, mutation Val124fsX60 probably had a single, common origin.

In family 4, mutations in *PRF1* or *UNC13D* were excluded by direct sequencing of the entire coding regions. Amplification of genomic DNA from the patient yielded no product for exon 2 of *STX11*. Normal amplification was observed with DNA from other family members (father, mother and one sister; data not shown). To characterize a possible homozygous deletion within *STX11*, we established a semi-quantitative DHPLC assay to detect heterozygosity in obligate carriers. In a first approach, duplex PCR was performed with

an interchromosomal control (exon 3 of *VWF* on chromosome 12) and fragment 1 of exon 2 (Ex2-1) of *STX11*. To detect differences in the amount of PCR product between individuals, reactions were stopped in the logarithmic phase after 27 cycles. DHPLC analysis revealed no *STX11* PCR product in the sample from the patient and ~50% DNA amount in parents when compared with controls (Fig. 3A). Amplification of the internal control was at comparable levels in all four samples.

Several fragments, each 300–400 bp in length, were analyzed in order to identify the deletion breakpoints. They covered a total of 37.6 kb genomic DNA from exon 2 and adjacent regions (Fig. 3B). Whereas all reactions from a control DNA resulted in expected products, reactions c–f from DNA of the patient failed, indicating a homozygous genomic deletion in this region (Fig. 3B, upper panel). PCR with primers flanking this interval and subsequent sequence analysis identified a breakpoint spanning fragment, which revealed a deletion of 19 189 bp including part of intron 1 and the entire coding region of *STX11*. The breakpoint was located between nucleotide 25560 and nucleotide 44750 of

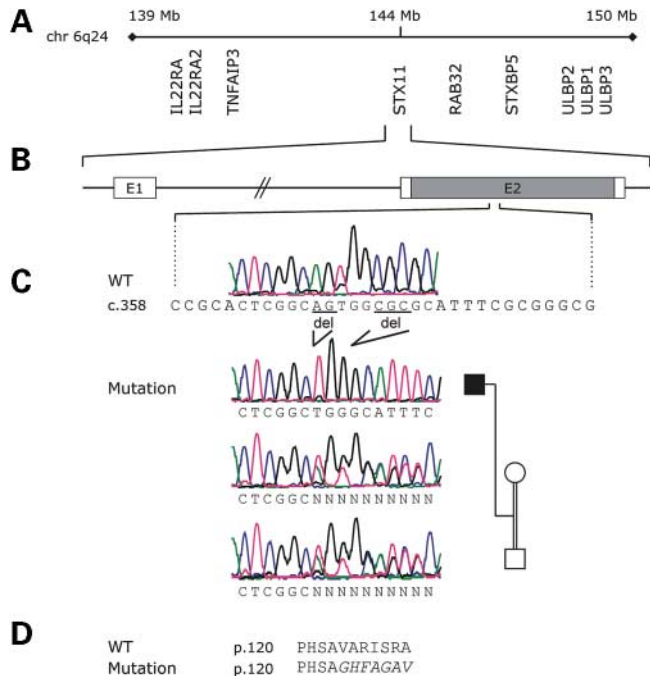


Figure 2. Sequence analysis and detailed map of *STX11*. (A) The 11 Mb candidate region on chromosome 6q24 containing several positional candidate genes including *STX11*. (B) Genomic organization of *STX11*. The coding region (shaded) comprises only exon 2. (C) DNA sequence of *STX11* in patient V:1 and his parents (IV:5 and IV:6) from the large Kurdish family with FHL. The homozygous mutation c.369_370delAG and c.374_376delCGC in exon 2 was detected in samples from the patients, the respective parents showed the heterozygous mutation. (D) Corresponding section of the peptide sequence. The mutation leads to a frameshift in codon 124 (altered residues shown in italics) and a premature termination codon. WT, wild-type.

the corresponding genomic clone (Fig. 3B, lower panel). Both parents and the brother of the patient were heterozygous carriers of this deletion. In families 5 and 6, mutation screening revealed a C to T exchange at nucleotide 802 that leads to a premature termination codon at amino-acid 268 (Table 2). No *STX11* mutation was detected in more than 30 FHL patients of non-Kurdish/Turkish origin.

Synthesis and distribution of syntaxin 11

The gene product of *STX11*, syntaxin 11, consists of 287 amino acid residues, completely encoded by exon 2. It has previously been demonstrated that *STX11* is predominantly expressed in cells of the immune system, including thymus, spleen and lymph nodes (21), and is strongly associated with intracellular membranes (21). In control samples, we detected syntaxin 11 in the Triton X-100 soluble membrane fraction from monocytes but not from lymphocytes (Fig. 4A, left and middle panels). Syntaxin 11 was not detectable in any cytosolic fraction. In membrane fractions from B- or T-cell lines only weak or absent signals were found (Fig. 4A, right panel, lanes 2 and 3). Syntaxin 11 was clearly present in the myelo-monocytic cell line HL-60 (Fig. 4A, right panel, lane 1). In mononuclear cells (MNCs) from two FHL families with mutations in *STX11* (Fig. 4B, panels 1 and 2), we

could detect syntaxin 11 in membrane lysates from the heterozygous parents (samples M and F), as well as in lysates from healthy donors (HD). However, lysates prepared from patients (Pt) carrying the homozygous mutation in *STX11* were entirely negative. As a control, we also investigated *STX11* expression in a patient with GS2 (19) without mutations in either syntaxin 11 or perforin 1 (Fig. 4B, panel 3). This patient presented with a clinical picture of FHL and carried a 27.8 kb intragenic deletion in combination with an A87P missense mutation in *RAB27A* (zur Stadt, unpublished data). Moreover, we investigated SNAP23 (22), the syntaxin 11-binding synaptosomal-associated protein 23 that is essential for membrane fusion and protein transport (23). We found SNAP23 at comparable levels in all samples tested, regardless of whether the syntaxin 11 (or Rab-27A) mutation was present or not (Fig. 4B). Thus, our studies revealed that *STX11* was expressed in peripheral MNCs from healthy controls, heterozygous parents and a patient with a related disease (i.e. Griscelli syndrome), but was negative in samples from individuals carrying the homozygous mutation. At least by western blot analysis, a consequence of Val124fsX60 on the expression of *STX11* in heterozygous carriers could not be detected. Western blot analysis in families 3–6 was not feasible because of insufficient material. However, the genomic deletion of exon 2 in family 4 should obviously result in complete absence of the protein and the premature termination codon in families 5 and 6 should lead to a loss of regular protein function. These findings demonstrate that the mutations have a truly recessive effect and can be supposed to directly cause the lack of syntaxin 11 in type-4 FHL patients.

DISCUSSION

FHL is a severe disease of uncontrolled inflammation characterized by fever, hepatosplenomegaly, cytopenia, hemophagocytosis and characteristic laboratory values. Diagnostic guidelines were published by the Histiocyte Society in 1991 (24), and revised criteria were summarized in 2004 by Janka and Schneider (2). The uncontrolled activation of immune cells is thought to occur because of an incomplete eradication of infectious organisms or an inadequate response to endogenous factors like tissue damage, autoantigens or other stimuli. Nearly all FHL patients exhibit a complete deficiency of NK cell activity as measured in a standard 4 h chromium release assay against K562 cells (25).

Two different molecular defects have been identified earlier, indicating genetic heterogeneity. Mutations in *PRF1* (FHL2) and *UNC13D* (FHL3) disrupt the ability of cytotoxic cells to kill their targets. Whereas mutations in *PRF1* lead to reduced amounts or absent perforin 1 in cytotoxic granules, defects in *UNC13D* inhibit the priming step of these granules prior to vesicle membrane fusion.

In addition to mutations in *PRF1* (FHL2) and *UNC13D* (FHL3), we here present a novel genetic defect in FHL, in *STX11* on chromosome 6q24, which we identified after homozygosity mapping in a large Kurdish FHL family with altogether six affected individuals. The homozygous mutation, Val124fsX60, found in all five investigated patients from this family was predicted to result in a premature termination

Table 2. Characteristics of patients reported with mutations in syntaxin 11

| Family (patient) ^a | Mutation in <i>STX11</i> | Deduced peptide change | Sex | Age at onset (months) | Age at HSCT ^d (months) | Outcome |
|-------------------------------|--------------------------------|------------------------|-----|-----------------------|-----------------------------------|-------------------------|
| 1 (IV:8) ^b | c.369_370delAG/c.374_376delCGC | Val124fsX60 | M | 12 | 125 | Alive (>10 years) |
| 1 (IV:9) ^b | c.369_370delAG/c.374_376delCGC | Val124fsX60 | M | 39 | 113 | Alive (>10 years) |
| 1 (V:1) | c.369_370delAG/c.374_376delCGC | Val124fsX60 | M | 24 | 43 | Alive (>1 year) |
| 1 (V:2) ^c | c.369_370delAG/c.374_376delCGC | Val124fsX60 | F | 5 | 16 | Dead (graft rejection) |
| 1 (V:3) ^c | c.369_370delAG/c.374_376delCGC | Val124fsX60 | F | 17 | 27 | Alive (>1.5 years) |
| 2 | c.369_370delAG/c.374_376delCGC | Val124fsX60 | M | 13 | 30 | Dead (Liver failure) |
| 3 | c.369_370delAG/c.374_376delCGC | Val124fsX60 | F | 7 | 16 | Alive (~2 months) |
| 4 | AL135917:g.25561_44749del | deletion | F | 33 | 41 | Dead (TRM) ^e |
| 5 | c.802C → T | Gln268X | M | 16 | Not transplanted | Alive |
| 6 | c.802C → T | Gln268X | F | 1.5 | Not transplanted | Alive |

^aNumbering according to Figure 1.

^bFirst described by Henter *et al.* (36).

^cV:2 and V:3 were monozygotic twins.

^dHSCT, hematopoietic stem cell transplantation.

^eTRM, transplantation related mortality.

codon leading to complete loss of syntaxin 11. We further detected the 5 bp deletion, a 19 kb deletion including exon 2 or a nonsense mutation in five additional families with FHL, all of them with the same ethnic background.

Syntaxin 11 is a member of soluble *N*-ethylmaleimide sensitive factor attachment protein receptors present on target membranes (t-SNAREs) (26,27). In contrast to other syntaxins, it harbors a short cystein-rich C-terminal tail instead of a hydrophobic membrane anchor that serves as a putative palmitoylation site (21,28). Despite the lack of this membrane anchor, syntaxin 11 is retained in membrane protein fractions solubilized by Triton X-100 treatment (23). As expected from the SNARE hypothesis of regulated intracellular vesicle transport, which specified the complex organization within different intracellular membrane fusion processes (29), the non-neuronal SNAP25 homolog SNAP23 was clearly identified as one of the binding partners *in vivo* (23). A SNARE complex harbors a four-helix bundle including a vesicle-associated v-SNARE (synaptobrevin/VAMP), a t-SNARE (syntaxin) and two molecules of either SNAP25 involved in neuronal complexes or SNAP23 in complexes associated with fusions in non-neuronal cell types (26). All syntaxin family members harbor an N-terminal H_{ABC} domain that regulates SNARE complex formation (30).

In FHL3, the priming step at the immunological synapse during exocytosis of cytotoxic vesicles is abrogated because of mutations in *UNC13D* as recently described by Feldmann *et al.* (16). It is unlikely that syntaxin 11 is involved in these steps during exocytosis because of the different intracellular localization. Confocal immunofluorescence microscopy analysis showed a strong association of syntaxin 11 with the intermediate compartment, a region between late endosomal areas and the trans-Golgi network suggesting a regulatory role in cycling between these two compartments. Syntaxin 11 may actively be involved in the transport of yet unknown vesicles from intracellular regions to the cell surface rather than being directly involved in exocytotic processes as other homologues such as syntaxins 1–4.

As we do not know the exact role of syntaxin 11 in the intracellular vesicle transport of the phagocytic system, a

clear functional link to the characteristic features of the disease is still elusive. The genes known previously to underlie FHL, *PRF1* and *UNC13D*, are directly associated with the effector cells, cytotoxic T, or NK-cells. The *PRF1*/granzyme dependent cytotoxic pathway is essential for the elimination of target cells transformed or infected with viral proteins (31). On the other hand, NK cells are stimulated by cell-to-cell interactions with DCs. This direct DC to NK cell contact seems to have an impact on the activation of the cytolytic activity of NK cells and their IFN- γ production (32). Thus, a reduced or absent NK cell activity may be caused not only by mutations in *PRF1* or *UNC13D* but also indirectly by an incomplete activation of NK cells by DCs (33). Future studies have been initiated to investigate these complex interactions.

Clinically, the age at onset of the disease and the time between diagnosis and time point of transplantation in the seven patients from three different families with homozygous mutation Val124fsX60 varied markedly (Table 2). The patient from family 4 with a complete deletion of the *STX11* coding region had the first signs of FHL at the age of 33 months (Table 2). This is strikingly different from our findings regarding the most common *PRF1* mutation detected in patients who originated from Turkey. All patients with a homozygous mutation 1122G>A (Trp374X) had a very early onset of the disease between 1 and 3 months of age (34). These Trp374X patients should also undergo HSCT as soon as possible to avoid an early death. Both types of mutations result in a premature stop codon in either *PRF1* or *STX11*, and both groups of patients were otherwise clinically very similar. Insufficient defense against a pathogen may result more efficiently and more rapidly in the typical signs of FHL in perforin 1-deficient patients than in those with a defect in *STX11*, because syntaxin 11 is not directly involved in the 'killing' machinery. Nevertheless, the described patient groups are too small to make a statistically significant conclusion. The identification of *STX11* as a novel gene involved in the development of FHL will provide new insights into the mechanisms of the disease and will also further facilitate

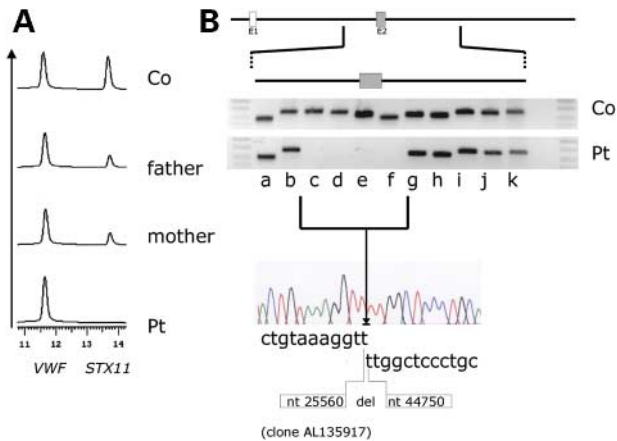


Figure 3. Identification of a large homozygous deletion including exon 2 of syntaxin 11. (A) Inheritance from father and mother was shown by a semi-quantitative DHPLC assay. Duplex amplification with an interchromosomal control (exon 3 of *VWF* on chromosome 12) and part one of *STX11* was stopped in the logarithmic phase after 27 cycles. (B) Deletion boundaries were mapped by amplification of various fragments of 300–400 bp around exon 2, from nucleotide 22500 (sample a) to nucleotide 60140 (sample k). Amplification and subsequent sequencing of a fragment spanning the deletion identified the breakpoint.

genetic counseling and prenatal diagnosis (35) in affected families.

MATERIALS AND METHODS

Families

All patients specified in this study are children of Kurdish origin with consanguineous parents. They all fulfilled the diagnostic criteria for FHL including decreased NK cell activity in the standard 4 h ^{51}Cr release assay (24). Patients' characteristics are given in Table 2 and the index family analyzed for linkage (family 1) is described in more detail subsequently.

In family 1, FHL was first diagnosed in three members of the family, two of whom were included in the study described here (IV:8, IV:9 in Fig. 1). The age at onset of the disease in the three brothers was 3, 12 and 39 months, respectively. The oldest brother died shortly after diagnosis, and samples were not available for further studies. The other two underwent HSCT after several relapses, and they are both alive. Clinical data from these patients have been described previously (36). The first signs of FHL in one of the monozygotic female twins (V:2) occurred at 5 months of age. Haploidentical HSCT with the father as a donor was performed at the age of 15 months. After primary graft failure and no engraftment of the autologous back-up, the child died. At the age of 25 months, the brother (V:1) was referred to the hospital with a history of fever and diarrhea. In the other twin sister (V:3), onset of disease did not occur until the age of 17 months. Both children received HSCT from unrelated donor and are presently doing well.

Samples

For linkage analysis, DNA was extracted from peripheral whole blood. In case of patients who had undergone HSCT,

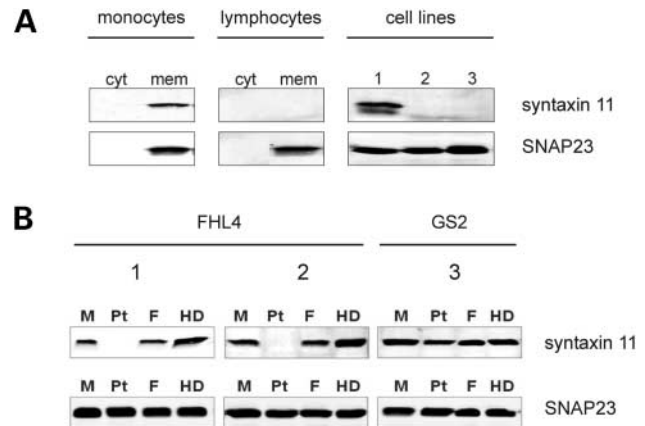


Figure 4. Synthesis and distribution of syntaxin 11 and SNAP23 in different cell types. (A) Analysis of cytosolic (cyt) and Triton-soluble membrane proteins (mem) from monocytes and lymphocytes of normal controls, and of membrane proteins from three different cell lines (1, HL-60; 2, Jurkat; 3, B cell line). Proteins (50 μg) were separated by SDS-PAGE and detection was performed by western blot analysis with anti-syntaxin 11 or anti-SNAP23 antibodies. (B) Analysis of membrane proteins from peripheral MNCs of various probands. The left two panels (1 and 2) represent two families with *STX11* mutations, the right panel (3) shows a family with Griscelli syndrome type 2 and mutations in *RAB27A*. M, mother; Pt, patient; F, father; HD, healthy donor.

we prepared DNA from mouth wash material and from fibroblast cultures. Once the gene had been identified, additional patients with FHL from Turkey were investigated. We obtained written informed consent from all patients and family members before the study was performed.

Homozygosity mapping and linkage analysis

A whole-genome scan was performed with 380 microsatellite markers (37), with an average distance of 11 cM. Markers were amplified in singleplex reactions in a final reaction volume of 10 μl containing 6 ng of genomic DNA. DNA amplification was carried out in PTC-225 thermal cyclers (MJ Research). Products were then pooled, and semi-automated genotyping was performed with MegaBACE-1000 analysis systems (Amersham Biosciences). Data were analyzed with Genetic Profiler software, version 1.5. Two-point LOD score calculations were performed with the program package LINKAGE version 5.2 (38) using an autosomal recessive model with full penetrance. Haplotypes were constructed either manually or with the program SIMWALK (39). SIMWALK was also used for the calculation of multi-point LOD scores, assuming equal allele frequencies.

Mutation analysis

The genomic structure of *STX11* was determined by alignment of the cDNA sequence (NM_003764) with the appropriate sequence of a genomic chromosome 6 contig (AL135917). Primers used for amplification of the coding exon were as follows: Ex2F (5'-ACTTATTGCCACACCGAGGAATAC) and Ex2R (5'-TGGGCTTCTGTCAAGACGGTAAGAG) with two internal sequencing primers, Ex2-2F and Ex2-3F. For subsequent analysis of additional samples and for DHPLC

analysis of control samples, exon 2 of *STX11* was divided into three PCR fragments using the following primer pairs:

Ex2-1F (5'-ACTTATTGCCACACCCGAGGAATAC) and Ex2-1R (5'-TTGGCGATGGAGTTGGTGTCGC);
Ex2-2F (5'-AGAACGCCCGCTTCCTCACGTCC) and Ex2-2R (5'-TGGCGGCTCTCGATCTCGTTGAG);
Ex2-3F (5'-ACCAGATCGAGGACATGTTTCGAG) and Ex2-3R (5'-AGAGCTCCCGCTTTGGTGCGTC).

DHPLC analysis

As control, for the presence or absence of the 5 bp deletion in *STX11* 230 samples of healthy blood donors from Turkey were analyzed. General DHPLC conditions were described elsewhere (40). DHPLC-based mutation analysis in fragments 1–3 of exon 2 (Ex2-2) was performed at 66 and 68°C column oven temperature. DHPLC-based semi-quantitative PCR analysis was performed at a column oven temperature of 50°C. DHPLC was performed on a WAVE system (Transgenomic, Crewe, UK).

Antibodies and inhibitors

Polyclonal rabbit antiserum raised against human SNAP23 was purchased from Novus Biochemicals (Littleton, CO, USA), and monoclonal antibody directed against amino acids 12–41 of human syntaxin 11 (clone 32) was from BD Transduction Laboratories (Heidelberg, Germany). Alexa680-conjugated goat anti-mouse IgG was obtained from MoBiTec (Göttingen, Germany) and IRDye800-conjugated goat anti-rabbit IgG was from Biotrend (Köln, Germany). Complete and Pefabloc SC were obtained from Roche (Mannheim, Germany) and okadaic acid from Alexis (Grünberg, Germany).

Preparation of mononuclear cells

MNC were routinely isolated from ethylenediaminetetraacetic acid (EDTA) blood of healthy controls and patients, respectively, by dextran sedimentation (Plasmasteril; Fresenius, Oberursel, Germany) followed by Ficoll-Hypaque (Pharmacia; Freiburg, Germany) density centrifugation as described previously (41). More than 98% of the cells were viable as assessed by trypan blue exclusion.

Isolation of detergent-soluble membranes

MNC were collected by rapid centrifugation. Cells were lysed in hypotonic lysis buffer (42 mM KCl, 10 mM HEPES, pH 7.4, 5 mM MgCl₂) supplemented with inhibitors (2 mM sodium *ortho*-vanadate, 2 mM NaF, 500 nM okadaic acid, 4 mM Pefabloc, and 1× Complete). Lysates were incubated for 30 min on ice, followed by sonication. Thereafter, lysates were cleared by centrifugation at 250g for 10 min at 4°C to remove nuclei and intact cells. The supernatant was additionally centrifuged at 150 000g for 30 min at 4°C to separate the cytoplasm from the membrane fraction. The membrane fraction was then lysed for 30 min on ice in the same volume of

1% Triton X-100 in TNE buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with inhibitors. Lysates were centrifuged at 150 000g for 30 min at 4°C to remove Triton-insoluble material, and protein concentrations were determined in the supernatant by the method of Bradford (42), using BSA as a standard (Biorad, München, Germany). For western blotting, 50 µg protein were diluted in 3-fold concentrated sample buffer, and the samples were boiled for 5 min prior to electrophoresis.

Western blot analysis

Proteins derived from cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (43) using a 10% polyacrylamide gel and blotted onto polyvinylidene fluoride (PVDF) membranes (Biorad, München, Germany) with a tank-blot transfer unit (Transphor II; Amersham Pharmacia Biotech, Freiburg, Germany). Immunodetection was performed as described in detail elsewhere (44) with some modifications. Briefly, membranes were blocked in Roti-ImmunoBlock (Roth, Karlsruhe, Germany), incubated with the respective primary antibodies and Alexa680-conjugated goat anti-mouse IgG or IRDye800-conjugated goat anti-rabbit IgG secondary antibodies according to the manufacturer's recommendations. Bands were visualized by Odyssey infrared imaging system (LICOR, Bad Homburg, Germany).

ELECTRONIC DATABASE INFORMATION

GenBank, [http://www.ncbi.nlm.nih.gov/Genbank/Center for Medical Genetics, Marshfield Clinic Research Foundation](http://www.ncbi.nlm.nih.gov/Genbank/Center%20for%20Medical%20Genetics,%20Marshfield%20Clinic%20Research%20Foundation), [http://research.marshfieldclinic.org/genetics/Online Mendelian Inheritance in Man \(OMIM\)](http://research.marshfieldclinic.org/genetics/Online%20Mendelian%20Inheritance%20in%20Man%20(OMIM)), <http://www.ncbi.nlm.nih.gov/Omim/>

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