The American Journal of Human Genetics, Volume *93*

Supplemental Data

Mutations in *BCAP31* Cause a Severe X-Linked Phenotype with Deafness, Dystonia, and Central Hypomyelination and Disorganize the Golgi Apparatus

Pierre Cacciagli, Julie Sutera-Sardo, Ana Borges-Correia, Jean-Christophe Roux, Imen Dorboz, Jean-Pierre Desvignes, Catherine Badens, Marc Delepine, Mark Lathrop, Pierre Cau, Nicolas Lévy, Nadine Girard, Pierre Sarda, Odile Boespflug-Tanguy, and Laurent Villard

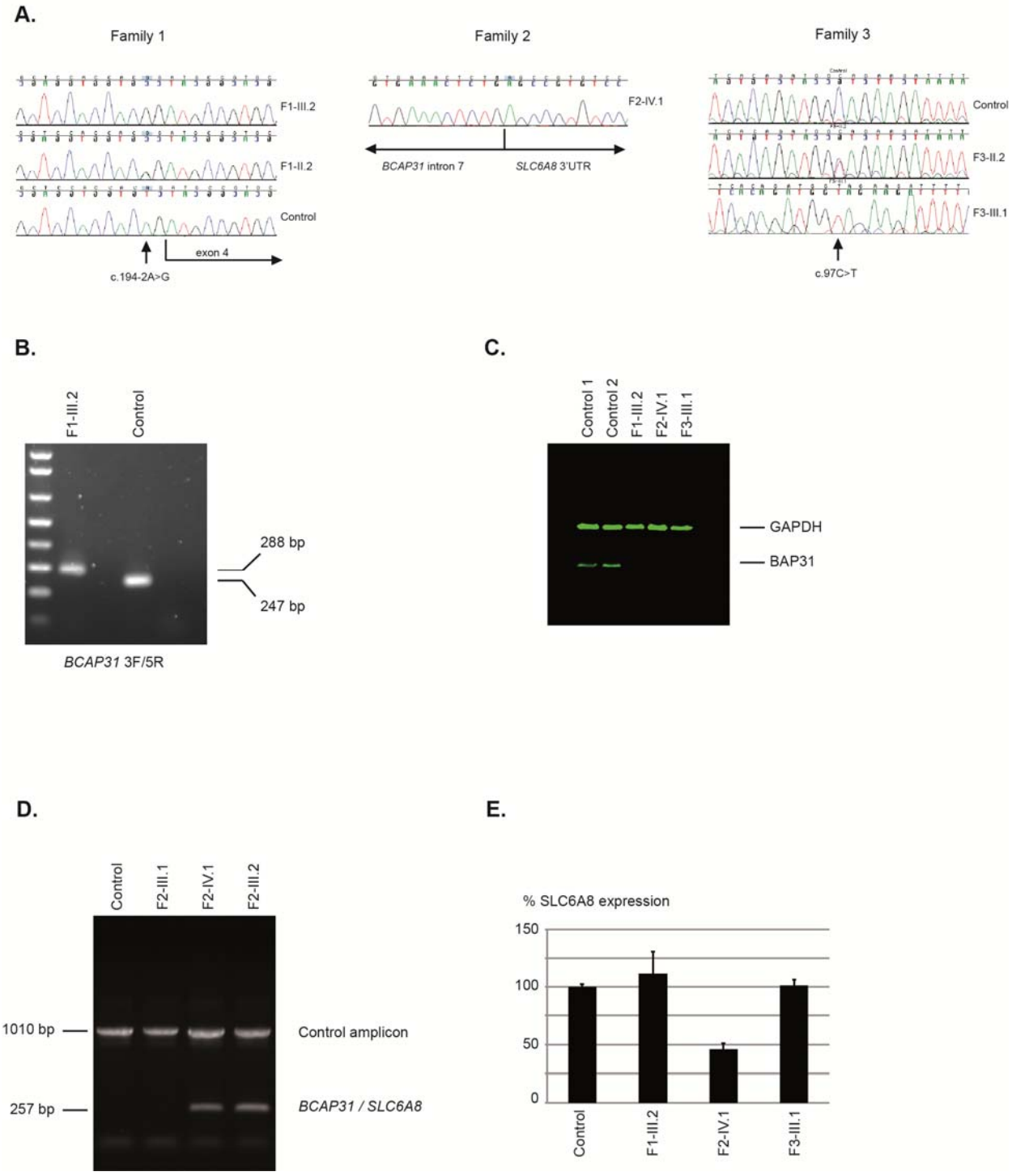

Figure S1. Identification and Molecular Characterization of *BCAP31* Mutations

(A) Electropherograms showing the mutations detected in the three families. (B) Detection of an abnormal *BCAP31* transcript in an affected male from family 1 (F1-III.2). Sequencing of the abnormal PCR product showing the abnormal splicing of *BCAP31* exon 4 in the cDNA of individual F1-III.2 was performed using primers located in exon 3 of the gene (forward, 5'- AGTTGTTAGTGTCCTATGGC -3') and exon 5 (reverse, 5'- GAAATGAGAGTCACCAGGC -3') giving a 247 bp PCR product in control cDNAs and 288 bp in the affected individual cDNA. (C) Western blot analysis demonstrates the absence of full-length BAP31 in the cells of affected individuals from the three families. GAPDH was used as a control. Proteins were extracted by sonication and isolated in a lysis buffer containing 20mM Tris-HCL (pH=7,5), 150mM NaCl, 2mM EGTA, 0,1% Triton X-100 and complete protease inhibitor tablet (Roche). Protein concentrations were determined by the bicinchoninic acid method. After a denaturating step at 95°C for 5min, proteins (20 µg) were separated on 8% SDS-polyacrylamide gel

and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) by liquid electroblotting (Bio-Rad) for 1h30 at 200mA. The membrane was preincubated with 5% nonfat dry milk in PBS 1X for 1h at room temperature. The primary antibodies for BAP31 (rat, 1:200, Santa Cruz Biotechnology, sc-56007) and GAPDH (1:200, Santa Cruz Biotechnology, sc-48167) were diluted in 1% nonfat dry milk in PBS Tween 0,1% and incubated overnight at 4°C. After extensive washing of the membrane with PBS Tween 0.1%, appropriate IRDye 800CW and 680RD secondary antibodies (LI-COR Biosciences) were incubated 2 h at room temperature. IR dye signals were revealed using the LICOR Odyssey Imager (LI-COR Biosciences). (D) An abnormal 257 bp junction fragment detected in family 2 was amplified using primers located on both sides of the deletion. (E) Quantification of *SLC6A8* transcripts using Q-PCR in two controls and in affected individuals fibroblasts revealed a decrease of *SLC6A8* expression in individual F2-IV.1. The percentage of *SLC6A8* expression in control fibroblasts was arbitrarily set to 100. Error bars represent the SEM.

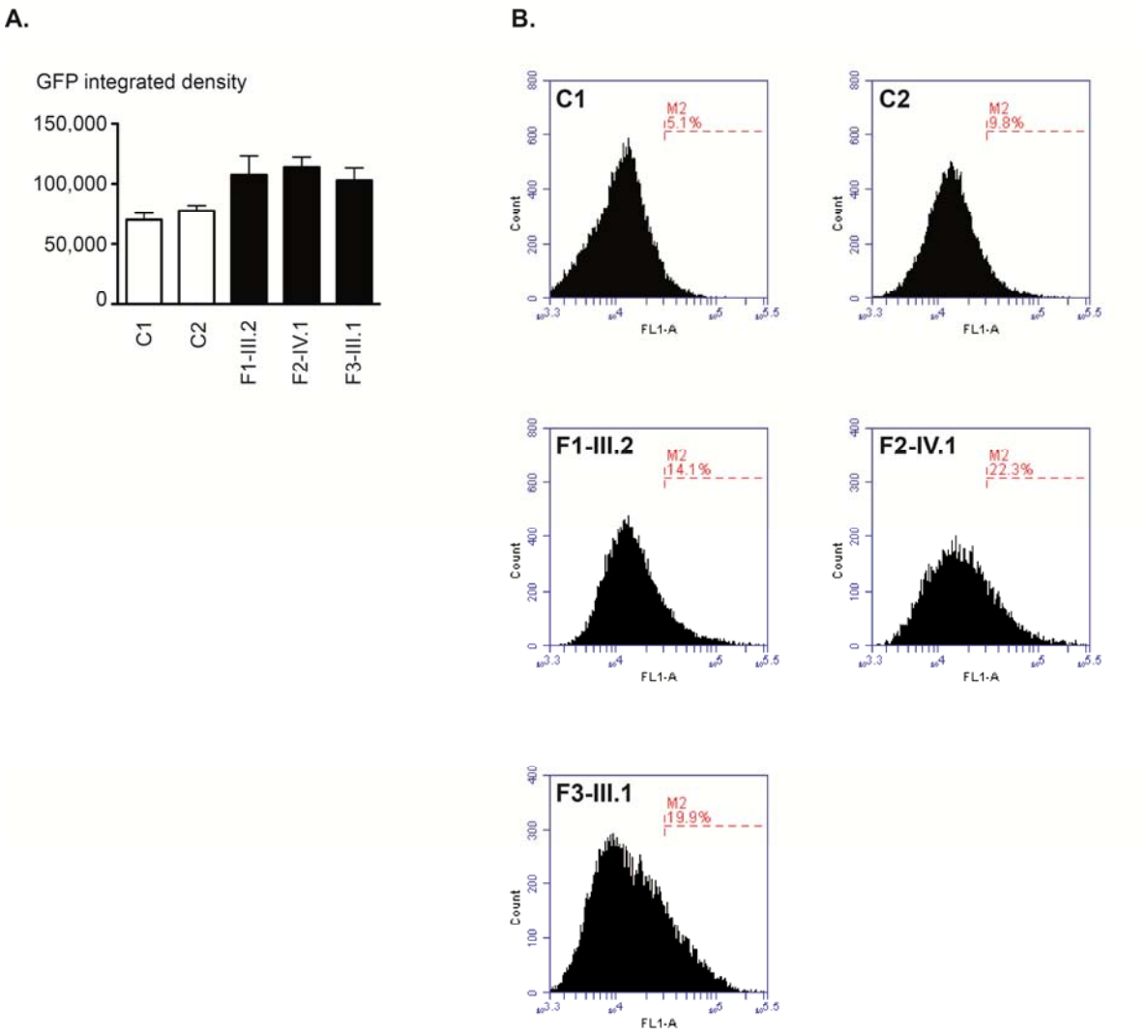

Figure S2. **Measure of UPR Efficacy Using ΔF508CFTR**

(A) Quantification of fluorescence intensity in the transfected primary fibroblasts of two control individuals (C1, C2), individual III.2 from family 1 (F1-III.2), individual IV.1 from family 2 (F2-IV.1) and individual III.1 from family 3 (F3-III.1). Although increased GFP fluorescence is measured in the affected individuals fibroblasts, indicating a higher production of mutant (misfolded) ΔF508CFTR, it does not reach statistical significance. Transfection, fixation and scanning of the images were performed under the same conditions, using the same solutions and timing, and by alternating fibroblasts coming from the control and affected individuals. The linearity of the camera response was verified, and the fluorescence intensity was carefully selected in order to avoid reaching saturation. Densitometric analysis of the staining level was performed on 8-bit images using ImageJ software (http://rsb.info.nih.gov). The integrated density was calculated as the sum of the values of the pixels in the region of interest. We measured the staining level of at least 40 fibroblasts in each group. For each fibroblast, we subtracted the mean of the background from the specific GFP-staining. Integrated density was calculated as the sum of the values of the pixels in the region of interest and data are expressed in arbitrary units. Error bars represent the SEM. (B) Analysis of the fibroblasts of the three affected individuals and two controls, 24 hours after transfection with pEGFP-ΔF508CFTR using flow cytometry. The plots present the cell count (Y axis) and the GFP fluorescence intensity (X axis). For each condition, an arbitrary fluorescence threshold was set at $3x10^4$ and the percentage of cells displaying a fluorescent signal above the threshold was measured (M2 portion of the graph). In these conditions, increased fluorescence is indicative of reduced EGFP-ΔF508CFTR degradation by the UPR system. A larger proportion of cells have a GFP fluorescence signal above the threshold in affected individuals samples (14.1%, 22.3% and 19.9%) compared to controls (5.1% and 9.8%). However, these differences are not statistically significant. Flow cytometry experiments were performed using a Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Fibroblasts transfected with pEGFP-ΔF508CFTR were grown for 24 hours in 25 cm^2 flasks. Fluorescence intensity at 488 nm and cell count were measured using 20,000 fibroblasts injected at medium flow in the cytometer.

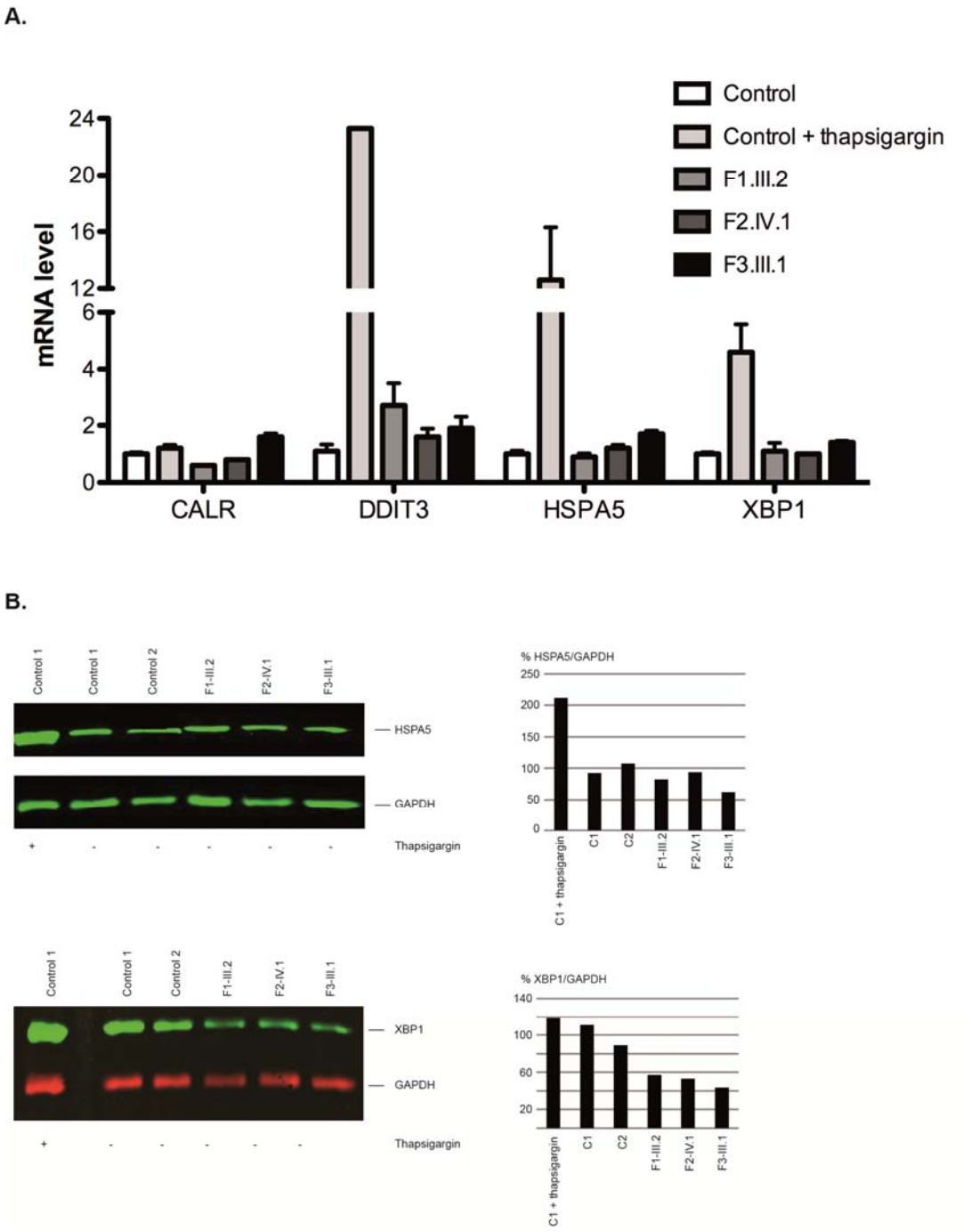

Figure S3. Quantification of UPR Response at the mRNA and Protein Level in BAP31-Deficient Fibroblasts

(A) Quantification using quantitative PCR of the expression of an ER marker (*CALR*) or transcripts encoding proteins involved in the unfolded protein response (DDIT3, HSPA5 and XBP1) using cDNA prepared with DNaseI-treated mRNA extracted from the fibroblasts of affected individuals and controls. Thapsigargin (2 μg/ml for 4 hours) was used as a positive control to trigger the UPR response. The cycle of threshold value (Ct) was used to calculate the relative expression of the genes of interest normalized to Tata-box binding protein (*TBP)*. Error bars represent the SEM. (B) Western blot analysis of XBP1 and HSPA5 in the cells of affected individuals and controls. No significant differences were found at the protein level. To induce ER stress before protein preparation, cells were treated with thapsigargin at 2 μg/ml for 24 hours. Western blots were performed as described above (Figure S1C), with primary antibodies against HSPA5 (1:1000, Abcam, ab151269) or XBP1 (1:1000, Abnova, H00007494-M04).

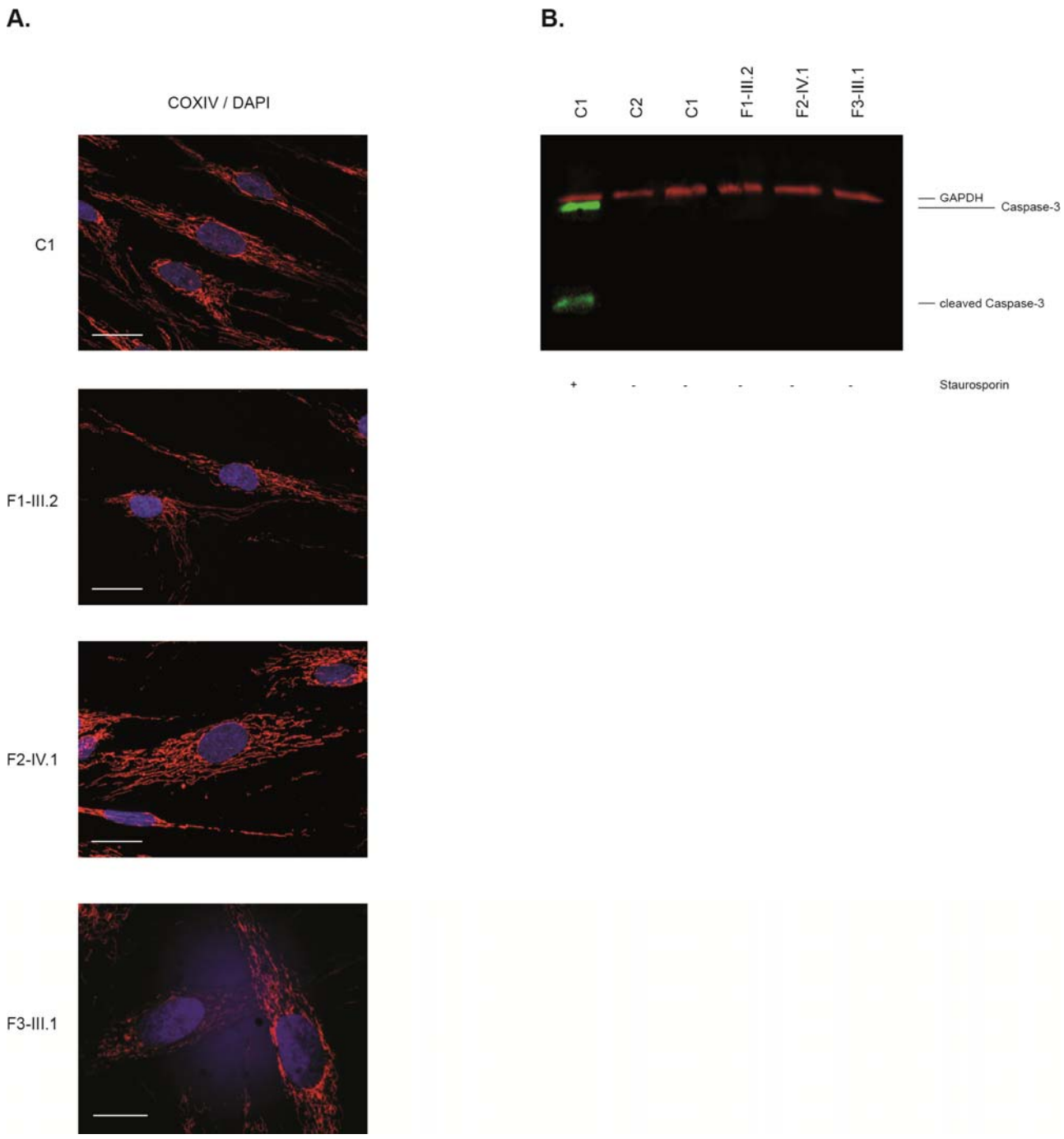

Figure S4. Observation of the Mitochondrial Network and Measurement of Caspase-3 Activation in BAP31-Deficient Fibroblasts

(A) Immunohistochemistry performed on fibroblasts of a control individual or affected individuals from family 1 (F1-III.2), family 2 (F2-IV.1) or family 3 (F3-III.1) using an antibody against cytochrome C oxidase subunit IV (COX IV) (red) (1:1000, Abcam ab33985) to label the mitochondrial network. All cells were stained with DAPI (blue) to reveal nuclear DNA. There are no significant differences between control and BAP31-deficient cells. Scale bar = 25 μm. (B) Western blot analysis performed using a cleaved caspase-3 antibody in control and affected individuals fibroblasts. Staurosporin triggers apoptotic cell death and was used as a positive control (1 μM treatment for 16 hours). Cleaved caspase-3 is absent in the BAP31-deficient cells. GAPDH was used as a control. Western blots were performed as described above (Figure S1C), with a cleaved caspase-3 primary antibody (rabbit, 1:1000, Cell Signaling Technology, 9661s).

Table S1. Summary of X Chromosome Exome Sequencing

Mapped reads	978,606				
Reads in aligned pairs	878,032				
Variants by position					
downstream	3				
exonic	243				
splicing	6				
intergenic	415				
intronic	438				
ncRNA	26				
upstream	7				
UTR3	46				
UTR5	15				
Exonic variants by subtype		**Not found in dbSNP132**	**Quality filter**	**UMD-HTS**	**Segregation**
Frameshift deletion	3	0	-	-	-
Frameshift insertion	5	0	-	-	-
Nonframeshift substitution	4	0	-	-	-
Nonsynonymous	110	9	Pass for 5 variants	Non pathogenic	Unaffected individuals
Splicing	6	1	Pass	Pathogenic	Affected individuals only
Stop gain	3	0	-	-	-
Stop loss	0	-	-	-	-
Synonymous	118	9	Pass for 1 variant	Non pathogenic	Unaffected individuals

Although whole exome was captured, we focused our analysis on the X chromosome only. Exonic variants were considered for pathogenicity if they were absent from dbSNP132, had a minimum quality of 30 and an average quality of surrounding bases of 20 (Quality filter), were predicted to be pathogenic using UMD-HTS and segregated with the disease in affected individuals.