Mutations in STAMBP, encoding a deubiquitinating enzyme, cause microcephaly–capillary malformation syndrome

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Microcephaly–capillary malformation (MIC-CAP) syndrome is characterized by severe microcephaly with progressive cortical atrophy, intractable epilepsy, profound developmental delay and multiple small capillary malformations on the skin. We used whole-exome sequencing of five patients with MIC-CAP syndrome and identified recessive mutations in STAMBP, a gene encoding the deubiquitinating (DUB) isopeptidase STAMBP (STAM-binding protein, also known as AMSH, associated molecule with the SH3 domain of STAM) that has a key role in cell surface receptor-mediated endocytosis and sorting. Patient cell lines showed reduced STAMBP expression associated with accumulation of ubiquitin-conjugated protein aggregates, elevated apoptosis and insensitive activation of the RAS-MAPK and PI3K-AKT-mTOR pathways. The latter cellular phenotype is notable considering the established connection between these pathways and their association with vascular and capillary malformations. Furthermore, our findings of a congenital human disorder caused by a defective DUB protein that functions in endocytosis implicates ubiquitin-conjugate aggregation and elevated apoptosis as factors potentially influencing the progressive neuronal loss underlying MIC-CAP syndrome.

MIC-CAP syndrome was recently described in six children, including one brother-sister pair, who all presented with small scattered capillary malformations, severe congenital microcephaly, early onset intractable epilepsy, profound global developmental delay, spastic quadriaparesis, hypoplastic distal phalanges and poor growth¹–⁴. Capillary malformations, sometimes referred to as port-wine stains, are nonregressing cutaneous vascular abnormalities⁵ that are seen in a growing number of congenital syndromes linked to dysregulated RAS-MAK (RAS-mitogen activated protein kinase) function; these are collectively termed ‘RASopathies’. For example, mutations in RASA1, encoding p120-RasGAP, a negative regulator of the RAS pathway, have been found in patients with capillary malformation–arteriovenous malformation syndrome⁶, and mutations in KRIT1, encoding a RAS-related protein 1A interactant, cause hyperkeratotic cutaneous capillary–venous malformations associated with cerebral capillary malformations⁷. Sequencing of RASA1 in two patients with MIC-CAP syndrome did not show any mutations, and sequencing of KRIT1 was not pursued⁸. Until now, the genetic mechanism responsible for this devastating disorder has been unknown.

We studied ten affected individuals from nine families with MIC-CAP syndrome (Fig. 1 and Table 1). Brain magnetic resonance imaging scans of the affected individuals showed enlarged extra-axial spaces and other changes suggesting prenatal-onset cerebral atrophy with relative sparing of the cerebellum (Fig. 1a–c). The gyral pattern was universally simplified and was associated with variable degrees of diffuse hypomyelination and hippocampal hypoplasia. We found

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that all individuals with MIC-CAP syndrome had intractable epilepsy, severe developmental delay and profound intellectual disability. Other distinguishing features of MIC-CAP syndrome include infantile spasms, hypoplasia of the distal phalanges characterized by variable degrees of nail and toe hypoplasia and capillary malformations (Fig. 1d–f). The capillary malformations were striking in appearance and visible at birth in all patients. They were also generalized in distribution and tended to vary from small (2–3 mm) to large (15–20 mm) lesions. Interestingly, limited evidence suggests that the vascular anomalies are not restricted to skin capillary malformations; one patient (designated P3.1 in this study) had a cerebellar angioma\(^1\), and another patient (9.1) had possible vascular malformations of the liver as determined by ultrasound (data not shown).

To establish the genetic cause of MIC-CAP syndrome, we performed exome sequencing on DNA samples from five individuals (Table 1) diagnosed with MIC-CAP syndrome. The two affected children in family 1, from nonconsanguineous parents, suggested a recessive mode of inheritance for this disorder. Therefore, we focused on identifying genes in which a maximal number of patients had two rare protein-altering variants that were absent from dbSNP131, the 1000 Genomes Project and 159 in-house control exomes. In four of the five patients studied by exome sequencing (P1.1, P1.2, P2.1 and P3.1), including the two siblings from family 1, we identified two variants in STAMBP in each individual (Fig. 2a, Supplementary Figs. 1a, 2 and Supplementary Table 1). Analysis of an additional three affected individuals (P6.1, P8.1 and P9.1) by Sanger sequencing identified two coding STAMBP variants in each patient (Supplementary Table 2). Co-segregation analysis confirmed an autosomal-recessive mode of inheritance in all families (Supplementary Fig. 3). Protein blot analysis of whole-cell extracts from patient-derived lymphoblastoid cell lines (LCLs) did not detect STAMBP expression in patient 1.2 (p[Glu42Gly];[Arg178*]) (Fig. 2b). Patient 3.1 (p[Phe100Tyr];[Arg424*]) showed a reduction of STAMBP expression compared to wild-type (WT) controls (Fig. 2b).

We identified one coding mutation in STAMBP in patient 7.1. Analysis by protein blotting did not detect STAMBP expression in this individual (Fig. 2b), and further sequencing of the gene revealed an intronic mutation (c.203+5G>A) believed to lead to an increase in skipping of the first coding exon (Table 1 and Supplementary Figs. 1b and 4a–d).

In patient 5.1, we did not identify any coding mutations using exome sequencing. The depth of coverage across the exons of \(STAMBP\) did not suggest a deletion. However, analysis of SNP data from an Illumina Human Omni2.5 array, which contains 25 probes within \(STAMBP\), suggested a 40-Mb region of copy-neutral homozygosity spanning \(STAMBP\). Protein blotting revealed a severe reduction in STAMBP expression in this patient (Fig. 2b), suggesting that P5.1 has MIC-CAP syndrome secondary to noncoding mutations in \(STAMBP\). Sequencing of patient-derived complementary DNA showed the presence of a 108-bp pseudoexon containing a premature stop codon (Supplementary Fig. 4e,f). Deep intronic sequencing identified a homozygous mutation (c.1005+358A>G). Application of a computational model of splicing regulation\(^8\) predicted that this mutation would activate a new donor site, as well as a cryptic AG acceptor site 114 bp upstream (\(P = 8.7 \times 10^{-7}\), sign test). We believed that this mutation caused the leaky splicing of the full-length transcript and showed that patient cells have a threefold reduction of full-length transcript expression (Supplementary Figs. 1c and 4g).

Sanger sequencing in patient 4.1 identified a homozygous stop mutation encoding p.Arg424*. Co-segregation analysis was not consistent with the suspected autosomal-recessive mode of inheritance in this nonconsanguineous family, as only the mother was heterozygous for the mutation causing p.Arg424*. We analyzed ten microsatellite markers spanning chromosome 2 and found all markers to be homozygous; a diagnostic array performed using DNA extracted from whole blood showed no evidence of copy number variation across chromosome 2. Therefore, we suspect that the mechanism of MIC-CAP syndrome in this patient is secondary to maternal isodisomy (Supplementary Fig. 5). In summary, we identified two mutations in \(STAMBP\) in a total of ten patients: six missense variants, two nonsense mutations, two translational frameshift mutations predicted to cause a premature truncation of the \(STAMBP\) protein and three intronic mutations leading to alternative splicing of the \(STAMBP\) transcript (Fig. 2a).

\(STAMBP\) is a JAMM-family DUB containing a microtubule-interacting and transport (MIT) domain and a STAM-binding domain, both of which interact with the endosomal sorting and trafficking machinery (Fig. 2a and Supplementary Fig. 6a)\(^9–11\). \(STAMBP\) is recruited to the endosomal sorting complexes required for transport (ESCRTs), a group of distinct macromolecule assemblies that mediate the sorting and trafficking of ubiquitin-tagged proteins from endosomes to lysosomes. \(STAMBP\) functions in regulating endosomal sorting of ESCRT machinery and ubiquitin-tagged receptor cargo\(^9,12–17\). Endosomal sorting is a highly dynamic process that is fundamental to regulating protein homeostasis through the active regulation of receptor-mediated signal transduction and enabling processes such
as autophagy\(^{18,19}\). Impaired ESCRT function is associated with the intracellular accumulation of ubiquitinated proteins. Brain lesions containing ubiquitinated protein aggregates have been noted in Stambp\(^{-/-}\) mice\(^{20}\), suggesting this to be a probable mechanism influencing microcephaly and its progression in MIC-CAP syndrome. Consistent with this, we observed elevated amounts of conjugated-ubiquitin aggregates after short interfering RNA (siRNA)-mediated silencing of STAMBp in the human medulloblastoma line T98G using indirect immunofluorescence with an antibody that specifically detects conjugated ubiquitin (FK2) and not free ubiquitin (Fig. 3a and Supplementary Fig. 6b). Notably, we also observed elevated amounts of conjugated-ubiquitin aggregates in several LCLs

### Table 1 Clinical characteristics and molecular findings in patients with MIC-CAP\(^{a}\)

| Patient | P1.1 | P1.2 | P2.1 | P3.1 | P4.1 | P5.1 | P6.1 | P7.1 | P8.1 | P9.1 |
|---------|------|------|------|------|------|------|------|------|------|------|
| Exome sequencing | + | + | + | + | + | + | + | − | − | − |
| Validated mutations\(^{b}\) (protein alteration (cDNA)) | p.Glu42Gly (c.125A>G) | p.Glu42Gly (c.125A>G) | p.Arg38Cys (c.532C>T) | p.Phe100Tyr (c.299T>A) | p.Arg424* (c.1270C>T) | c.1005+358A>G | p.Lys378Asnfs*2 (c.1134_1138delACTAA) | p.Arg38Cys (c.112C>T) | p.Arg38Cys (c.112C>T) | p.Tyr63Cys (c.188A>G) |
| Gender | F | M | M | M | M | F | M | F | M | M |
| Age at assessment | 2 y | 9 mo | 12 mo | 2 y | 5 y 4 mo | 2 mo | 28 mo | 8 mo | 15 mo | |
| Ethnicity | African American | African American | European descent | European descent | European descent | European descent | European descent | European descent | Polynesian | European descent |
| Gestational age (weeks) | 39 | 39 | 36±5 | 36 | 37 | 36 | 37+2 | 37+6 | 35 | |
| Clinical features | + | + | + | + | + | + | + | + | + | + |
| Progressive congenital MIC | + | + | + | + | + | + | + | + | + | + |
| Small for gestational age | + | + | + | + | + | + | + | + | + | + |
| Birth OFC (s.d.) | −6 | −4 | −2 | −2 | −4 | −1.8 | −2 | −8 | −5 | −2 |
| Birth weight (s.d.) | −1.5 | −1.5 | −1.5 | −2 | −2 | +1.8 | −1.5 | −4 | −1.5 | −1.5 |
| Birth length (s.d.) | −4 | −2 | 0.5 | ND | −1.5 | 0 (mean) | −2 | −4 | −1 to −2 | −2 |
| Later age | 2.5 y | 9 mo | 12 mo | 17 mo | 22 d | 2 y 10 mo | 2 mo | 28 mo | 8 mo | 18 mo |
| Later OFC (s.d., age) | −8 | −6 | −6 | −4 | −3 | −2.5 | −6 | −8 | −4 | −4 |
| Later weight (s.d.) | 1.5 to +2 | −4 | +1 to +2 | −1 to −2 | −3 | +2 | −2 | −2.5 | +1 | −3 |
| Later length (s.d.) | ND | ND | −1 to −2 | −2 | −3 | 0 (mean) | −3 | −4 | ND | −3 to −4 |
| Generalized capillary malformations | + | + | + | + | + | + | + | + | + | + |
| Early onset intractable seizures | + | + | + | + | + | + | + | + | + | + |
| Infantile spasms | + | − | + | − | − | − | ND | + | + | + |
| Hypoplastic distal phalanges | + | + | − | + | + | + | + | + | + | + |
| Global DD | + | + | + | + | + | + | + | + | + | + |
| Spastic quadripareisis | + | + | + | + | + | − | + | + | + | + |
| Myoclonus | + | − | + | − | − | − | + | + | + | + |
| Optic atrophy | + | + | + | + | + | ND | ND | + | − | − |
| Neuroimaging features | + | + | + | + | + | ND | + | + | + | + |
| Simplified gyral pattern | + | + | + | + | + | ND | + | + | + | + |
| Increased extra-axial space | + | + | + | + | + | ND | + | + | + | + |
| Hippocampal hypoplasia | + | + | + | ND | + | ND | − | + | ND | + |
| Hypomyelination | + (ref. 3) | + (ref. 3) | + (ref. 3) | + (ref. 1) | + (ref. 1) | + (ref. 2) | − | − | − | − |

\(^{a}\)This table summarizes the clinical findings in the study participants. \(^{b}\)The numbering of the mutations and alterations is relative to NM_006463.4 (gene) and NP_006454.1 (protein), respectively. OFC, occipitofrontal circumference; s.d., values are shown by their s.d. value from the mean; ND, not determined; DD, developmental delay.
from patients with STAMBP alterations compared to WT control LCLs after serum starvation (Fig. 3b). This phenotype was reversed after stable lentiviral transduction of patient LCLs with STAMBP (Supplementary Fig. 6c,d). Furthermore, this phenotype was also associated with apoptosis induction, denoted by elevated amounts of cleaved caspase 3 (Fig. 3c) and annexin V staining (Fig. 3d) in the LCLs of patients with STAMBP alterations compared to WT LCLs. STAMBP functions with the ESCRT machinery to facilitate autophagy. Autophagic flux can be monitored by detection of the expression of autophagy-associated phosphatidylethanolamine (PA)-conjugated conjugates, such as LC3-II, which then phosphorylates and activates ERK1 and ERK2 (ERK1/2) which then phosphorylates and activates MEK1/2 kinase, leading to the proteolysis and termination of receptor signaling21. STAMBP interacts with key components of receptor signaling pathways, such as Grb2 (Fig. 4a)10,22. Considering the known role of STAMBP in regulating receptor-mediated endocytosis, sorting and trafficking, we investigated aspects of the interconnected RAS-MAPK and PI3K-AKT-mTOR signal transduction pathways in our MIC-CAP LCLs, as mutations in components of these networks are associated with congenital capillary malformation disorders6,7,23. We found elevated amounts of GTP-bound RAS (active RAS) in extracts from LCLs of patients with STAMBP alterations compared with WT LCLs, which is suggestive of elevated signaling through this pathway (Fig. 4b). Similarly, we found elevated amounts of phosphorylated active phosphoinositide 3-kinase (PI3K) in cell extracts from LCLs from patients with STAMBP alterations relative to WT cells, even after serum starvation (Fig. 4c). Collectively, these data suggest elevated and insensitive active signal transduction in these interconnected pathways associated with defective STAMBP in patient LCLs.

To further characterize signaling abnormalities, we examined the response of patient LCLs to serum starvation for both of these pathways using a selection of substrates. Serum starvation induced a substantial reduction in C-RAF phosphorylation at Ser338 in WT LCLs, consistent with inhibition of C-RAF activity under these conditions (Fig. 5a). LCLs from patients with STAMBP alterations maintained C-RAF phosphorylation at Ser338 in the absence of serum, indicating persistent activation and insensitivity of this pathway. Further evidence suggesting insensitive signal transduction in the RAS-MAPK pathway in STAMBP-mutated LCLs is given by the relative insensitivity of these cells to the MEK1 and MEK2 (MEK1/2) inhibitor U0126. Active C-RAF phosphorylates and activates MEK1/2 kinase, which then phosphorylates and activates ERK1 and ERK2 (ERK1/2) (Fig. 4a). We repeatedly found elevated amounts of phosphorylated ERK1/2 in exponentially growing STAMBP-mutated LCLs compared to WT LCLs after a short treatment (1 h) with U0126 (Fig. 5b and Supplementary Fig. 6e). The excess of phosphorylated ERK1/2 in
STAMBP-mutated LCLs under these robust inhibition conditions is further supportive of a hyperactive and insensitive RAS-MAPK pathway in these cells. Analysis of several endpoints in the PI3K-AKT-mTOR pathway under identical conditions indicated a similar insensitivization of this pathway. Serum starvation of WT LCLs reduced the phosphorylation of AKT at Thr308, of the AKT-dependent Thr1462 of TSC2 and of Ser240 and Ser244 of S6 protein. This is consistent with pathway inactivation under these conditions in WT LCLs (Fig. 5c,d). In contrast, LCLs from patients with STAMBP alterations maintained phosphorylation of all three proteins under these conditions (Fig. 5c,d). Furthermore, stable lentiviral transduction of patient LCLs with STAMBP resulted in the reconstitution of a normal signaling response to serum starvation (Supplementary Fig. 6f).

The RAS-MAPK and PI3K-AKT-mTOR pathways regulate crucial cellular processes, including cell growth, cell-cycle progression and differentiation. Disorders characterized by hyperactivity of the RAS-MAPK network, including Noonan and Costello syndromes, present with growth delay. Considering the marked postnatal growth retardation and capillary abnormalities seen in MIC-CAP syndrome, hyperactive RAS-MAPK signaling may be a major biological
Figure 5 Elevated and insensitive RAS-MAPK and PI3K-AKT-mTOR signaling in MIC-CAP syndrome. (a) Serum starvation (24 h) inhibits C-RAF activation in WT LCLs, in contrast to LCLs from P7.1 and P1.1. p-C-RAF, phosphorylated C-RAF. (b) LCLs were either treated (+) or not treated (−) with 10 μM U0126, a specific MEK1/2 inhibitor, for 1 h (Fig. 4a). Cells were harvested, and whole-cell extracts were probed for phosphorylation of ERK1/2 (pERK1/2), which is mediated by MEK. Insensitivity to this treatment (as measured by relative amounts of pERK1/2 remaining after treatment with the MEK inhibitor) would reflect the magnitude and intensity of signal transduction from RAF to MEK to ERK (Fig. 4a). Residual pERK1/2 (Thr202 and Tyr204) signal (MEK-dependent phosphorylation) was seen in MIC-CAP LCLs in contrast to WT LCLs. This phenotype is underscored after titration of U0126 in various MIC-CAP LCLs compared to WT LCLs (Supplementary Fig. 6e). Collectively, these data indicate a greater strength of MEK1/2 activity in MIC-CAP LCLs compared to WT cells. (c) Serum starvation (24 h) reduces phosphorylation (activation) of AKT at Thr308 (pAKT) and of TSC2 at Thr1462 (pTSC2) and AKT-dependent inhibitory phosphorylation of TSC2 in WT LCLs in contrast to LCLs from P7.1, P1.1 and P3.1. The TSC1 and TSC2 complex is the principal negative regulator of the mTOR kinase complex (Fig. 4a). These data are consistent with active signal transduction from PI3K-AKT-mTOR in MIC-CAP cells under these conditions. (d) S6 protein is phosphorylated by S6 kinase in an mTOR-dependent fashion (Fig. 4a). Consistent with active signal transduction in this pathway under serum starvation conditions, LCLs from P7.1 and P1.1 maintained S6 phosphorylation (p-S6) at Ser240 and Ser244 in contrast to WT LCLs.

consequence induced by impaired STAMBP function in humans, suggesting that STAMBP-mutated MIC-CAP syndrome may have an overlapping pathomechanism with the RASopathies. Furthermore, as the PI3K-AKT-mTOR pathway also has a role in angiogenesis and vascularization, and considering the interconnectivity between these networks (Fig. 4a), it is possible that the combined insensitive activation of both these networks may contribute to the MIC-CAP phenotype.

In summary, we identify mutations in STAMBP in MIC-CAP syndrome, a recently described severe developmental disorder. Analysis of LCLs from patients with MIC-CAP syndrome demonstrated elevated ubiquitin-conjugated protein aggregation and apoptosis activation. These data are consistent with elevated ubiquitin-conjugated protein aggregate–induced progressive apoptosis as a potential underlying mechanism for the microcephaly in this disorder. This is consistent with brain imaging and human pathological analysis of MIC-CAP syndrome1 and of the knockout mouse model of Stammbp25. Furthermore, we document elevated autophagosome content and active and insensitive RAS-MAPK and PI3K-AKT-mTOR pathways as previously unidentified consequences of defective STAMBP, potentially contributing to the vasculature and growth characteristics of MIC-CAP syndrome. This work presents the first example, to our knowledge, of a human disorder caused by a congenitally defective protein aggregate–induced progressive apoptosis as a potential consequence induced by impaired STAMBP function in humans, suggesting that STAMBP-mutated MIC-CAP syndrome may have an overlapping pathomechanism with the RASopathies. Furthermore, as the PI3K-AKT-mTOR pathway also has a role in angiogenesis and vascularization, and considering the interconnectivity between these networks (Fig. 4a), it is possible that the combined insensitive activation of both these networks may contribute to the MIC-CAP phenotype.

In summary, we identify mutations in STAMBP in MIC-CAP syndrome, a recently described severe developmental disorder. Analysis of LCLs from patients with MIC-CAP syndrome demonstrated elevated ubiquitin-conjugated protein aggregation and apoptosis activation. These data are consistent with elevated ubiquitin-conjugated protein aggregate–induced progressive apoptosis as a potential underlying mechanism for the microcephaly in this disorder. This is consistent with brain imaging and human pathological analysis of MIC-CAP syndrome1 and of the knockout mouse model of Stammbp25. Furthermore, we document elevated autophagosome content and active and insensitive RAS-MAPK and PI3K-AKT-mTOR pathways as previously unidentified consequences of defective STAMBP, potentially contributing to the vasculature and growth characteristics of MIC-CAP syndrome. This work presents the first example, to our knowledge, of a human disorder caused by a congenitally defective DUB isopeptidase functioning in the endocytosis pathway, providing important new insights into the pathophysiology of human microcephaly and capillary malformation.

URLs. National Heart, Lung, and Blood Institute (NHLBI) Exome variant server, http://evs.gs.washington.edu/EVS/; FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/; Picard tools, http://picard.sourceforge.net/; SAMTools, http://samtools.sourceforge.net/.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
K.M.B., M.O., W.B.D. and D.E.B. directed the study. M.T.C., L.L., C.L.C., J.M.G., D.J.M.-R., T.P., G.A., S.T., A.H., B.I., A.D., C.D.S., A.R.P., M.W., J.W., S.D., D.A. performed the protein biochemistry and cell biology studies, which were directed by M.O. J.S. and J. Majewski performed exome variant calling analysis. The manuscript sequencing, genotyping studies and variant analysis supervised by K.M.B. and D.E.B.

D.A. performed the protein biochemistry and cell biology studies, which were directed by M.O. J.S. and J. Majewski performed exome variant calling analysis. The manuscript was written by L.M.M., G.M.M., M.O. and K.M.B. FORGE Canada Consortium provided the clinical and bioinformatic infrastructure under the direction of K.M.B. assisted by C.L.B. and J. Marcadier. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Study participants. All families provided written informed consent, and this study was approved by the ethics review boards at the Children’s Hospital of Eastern Ontario, the University of Chicago and Seattle Children’s Hospital. We studied a cohort of ten affected individuals from nine families with MIC-CAP syndrome. Genomic DNA was extracted from the whole blood of affected subjects and their family using standard techniques.

Sequencing technology and variant calling pipeline. Using target capture with the Agilent SureSelect 50 Mb All Exon kit (Agilent Technologies, Santa Clara, CA) and sequencing of 100-bp paired-end reads on Illumina HiSeq, we generated over 15 Gb of sequence for each sample such that approximately 90% of the coding bases of the exome defined by the consensus coding sequence (CCDS) project were covered by at least 20 reads. Reads were first quality trimmed from the 3’ end using the Fastx toolkit and were then aligned to hg19 with BWA. Duplicate reads were marked using Picard and excluded from downstream analyses. For each sample, single nucleotide variants (SNVs) and short insertions and deletions (indels) were called using SAMtools pileup and varFilter with the base alignment quality (BAQ) adjustment disabled and were then quality filtered to require that at least 20% of reads supported the variant call. Coverage of the exome was determined using the Genome Analysis Toolkit (GATK). Variants were annotated using both Annovar and custom scripts to identify whether they affected protein-coding sequence and whether they had previously been included in dbSNP131 or in the 1000 Genomes pilot release (Nov. 2010).

Genetic analysis. To elucidate the molecular mechanism of MIC-CAP syndrome in family 4, we PCR amplified ten polymorphic microsatellite markers spanning the length of chromosome 2, four on the short arm and six on the long arm. The amplification products were resolved using the IR2 DNA Analyzer and interpreted using SAGA software (LI-COR). Analysis of the intronic mutations was performed using the computational model of splicing Analyzer and interpreted using SAGA software (LI-COR). Analysis of the exome was determined using the Genome Analysis Toolkit (GATK). Variants were annotated using both Annovar and custom scripts to identify whether they affected protein-coding sequence and whether they had previously been included in dbSNP131 or in the 1000 Genomes pilot release (Nov. 2010).

Functional analysis. All antibodies used in this section can be found in Supplementary Table 3. STAMBP expression in patient-derived LCLs (P1.1, P7.1 and P3.1) was assessed by protein blotting using anti-STAMBP (H-4) with an epitope directed to amino acids 131–270 of STAMBP (Santa Cruz Biotechnology, Santa Cruz, CA). The caspase 3 antibody was from Cell Signaling Technology (Beverly, MA). For annexin V apoptosis assessment, we used the Single Channel Annexin V Apoptosis Kit (Alexa Fluor 488–conjugated anti-annexin V with SyTOX Green) from Life Technologies LTD (Paisley, UK) according to the manufacturer’s instructions. The anti-LC3 was from Cell Signaling (DSG08 XP(R)), and bafilomycin A was from Sigma-Aldrich (Poole, UK). Amounts of active RAS-GTP were determined using the RAS activation assay kit (17-218) from Millipore according to the manufacturer’s instructions.

For siRNA-mediated silencing of STAMBP, we used ONTARGET plus SMARTpool human STAMBP (L-012202-00-0005) from Dranharma-Thermo Fisher Scientific (UK) and performed transfection using Metafectene-Pro from Canbio (Cambridge, UK) according to the manufacturer’s instructions. Cells were analyzed 24 h after transfection. The SMARTpool is a mixture of four oligonucleotides with distinct target sequences (Supplementary Table 2).

For indirect immunofluorescence, LCLs were pelleted, swollen in 75 mM KCl (10 min), immobilized onto polylysine-coated slides by cytopinning (CytoSpin, Shandon), permeabilized (0.1% Triton X-100 in 5% BSA and PBS for 2 min) and blocked in 5% BSA and PBS (10 min) before sequential incubation with primary and secondary antibodies. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and preserved in antifade mounting medium (Vectashield). Slides were analyzed using the Zeiss AxioPlan platform, and images were captured using SimplePCI software at constant exposure times. Anticonjugated ubiquitin mouse monoclonal clone FK2 was from Enzo Lifesciences UK LTD (Exeter, UK).

To interrogate RAS-MAPK pathway function, patient-derived LCLs were grown exponentially in complete medium in the presence or absence of fetal bovine serum for 24 h. Antibodies, including phospho-specific antibodies to p-C-Raf (Ser33) and pMAPK and pERK1/2 (Thr202 and Tyr204, respectively), along with their corresponding native antibodies, were from Cell Signaling Technology (Beverly, MA). The MEK1/2 inhibitor U0126 was used at 10 μM for 1 h. Whole-cell extracts were prepared by sonication in urea buffer (9 M urea, 50 mM Tris-HCl, pH 7.5, and 10 mM β-mercaptoethanol).

For lentiviral transduction of LCLs, high-titer Precision LentiORF viral particles derived from the pLOC system were obtained from Thermo Scientific (Open Biosystems) and used according to the manufacturer’s instructions. Stable STAMBP-expressing clones were obtained after blastidicin S selection of transduced populations.

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