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Supplemental information

Rare germline heterozygous missense variants in BRCA1-associated protein 1, *BAP1*, cause a syndromic neurodevelopmental disorder

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Supplemental figure

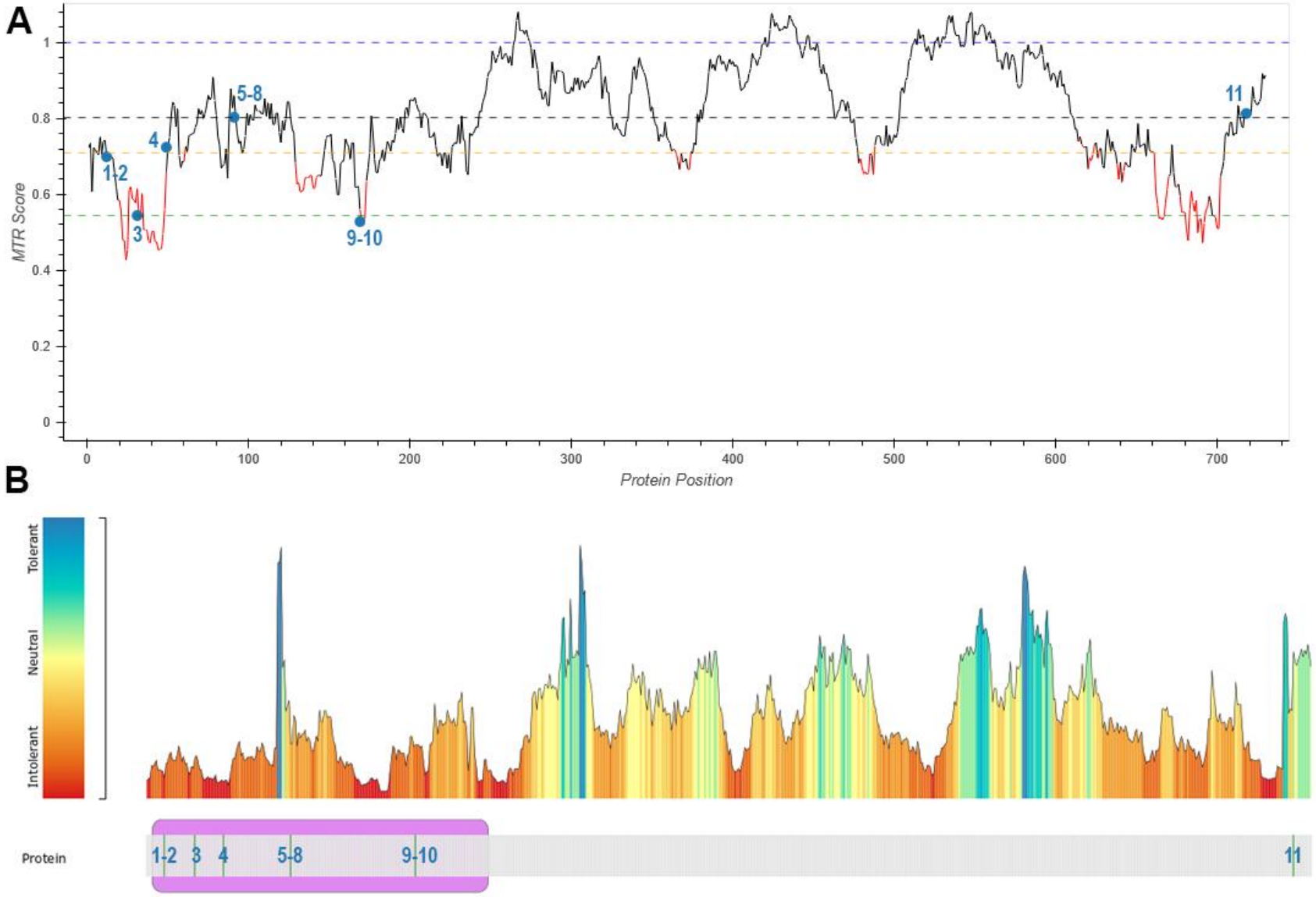

Figure S1. Tolerance predictions of the amino acid residues of BAP1 (GenPept: NP_004647.1) affected by the variants reported in the study. A., Analysis by Missense Tolerance Ratio (MTR; v1)[1] suggests that all the variants harbored by affected individuals participating in the study, and more especially those harbored by individuals 3, 9 and 10, affect residues intolerant to missense variants and are thus expected to significantly impact BAP1 function. Horizontal lines show gene-specific MTR percentiles 5th (in green), 25th (in yellow), 50th (in black), and neutrality (in blue; MTR = 1.0) MTR calculated using exome sequencing (ES) component of gnomAD v2.0. **B.**, MetaDome web tool[2] indicates overall intolerance of BAP1 regions, inducing a classification of the residues of interest from highly intolerant to highly tolerant to missense variants. The catalytic domain, where lie most variants, is particularly intolerant to missense variants.

Supplemental methods

Cell culture

Human haploid HAP1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco) and 1X non-essential amino acid (Gibco). Cells were passaged when reach around 80% confluence. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA whole-blood samples using spin medium gradient centrifugations (PluriSelect) following the manufacturer's recommendations. PBMCs were then plated on irradiated feeder cells in RPMI 1640 medium supplemented with 10% human AB serum (PAN-Biotech GmbH) in the presence of 150 U/ml IL-2 (Miltenyi Biotec) and 1 µg/µl L-PHA (Sigma) to promote T-cell expansion, as previously described [3]. After 3-4 weeks of culture, resting T cells were collected, washed twice with PBS and stored as dry pellets at -70°C until further use.

Generation of BAP1 KO cells and rescue of WT and mutant *BAP1*

BAP1 was inactivated in HAP1 cells using CRISPR-Cas9 technique as previously described (Campagne 2019). Reintroduction of wild-type or mutant forms of *BAP1* was performed by infection of *BAP1* KO cells with a pBABE retrovirus. Production of retroviral particles was performed in HEK293 cells. Transduction was performed by incubating the cells with viral particles mixed with Polybrene (final concentration, 8 µg/ml) for 3 h at 37 °C and subsequently selected with puromycin for 5 days. Re-expression of BAP1 proteins was evaluated by western blot.

Preparation of nuclear extract and western blot (Figure 2)

Cells were washed once with PBS and resuspended with Buffer A (10 mM HEPES pH 7.9, 5 mM MgCl2, 0.25 M sucrose and 0.1 % NP-40, 1 mM DTT, 200 µM PMSF, and protease

inhibitors). After 10 min incubation on ice, cells were pelleted by centrifugation at 8000 g for 10 min. Supernatant was removed and pellets were resuspended with Buffer B (25 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.1 mM EDTA pH 8.0, 20 % glycerol, 700 mM NaCl, 1 mM DTT, 200 µM PMSF and protease inhibitors. Nuclei were sonicated, then centrifuged at 14000 g for 15 min at 4 °C. Protein concentration was measured by Bradford assay (Biorad). Primary antibodies targeting against BAP1 (sc-28383, Santa Cruz), HDAC1 (5358S, Cell Signalling Technology), H2Aub (8240S, Cell Signalling Technology), total H3 (39763, Active Motif) were used in this study. Western Blot analysis of protein extracts was performed by StarBright Blue 700 fluorescent secondary antibodies (Biorad) and DyLight 800 secondary antibody (Biorad). Imaging was carried out by ChemiDoc System (Biorad).

SDS-PAGE/western blotting (Figure 3)

Control and patient T cell pellets were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM N-ethylmaleimide, 10 µM MG-132, 1% NP40, 0.1% SDS) prior to protein quantification using a standard BCA assay following the manufacturer's instructions (Thermo Scientific). Ten to forty micrograms of whole-cell lysates were separated by 10 or 12% SDS-PAGE and subsequently transferred to PVDF membranes for western-blotting. Primary antibodies used in this study include antibodies specific for Rpt1 (BML-PW8315), Rpt2 (BML-PW0530), Rpt3 (clone TBP7-27), Rpt4 (clone p42-23), Rpt5 (BML-PW8310), Rpt6 (clone p45-110), β1 (clone MCP421), β2 (clone MCP165, all purchased from Enzo Life Sciences), β5 (ab3330, Abcam), β5i (clone A12, Santa Cruz Biotechnology, Inc), Ub-H2A (clone E6C5, Millipore) and GAPDH (clone 14C10, Cell Signaling Technology, Inc).

RT-qPCR

Cells were seeded on 12-well plates. After reaching confluence, RNA was extracted using RNeasy kit (Qiagen) following manufacture's instruction and was quantified by Nanodrop. 1µg of cDNA was prepared using High capacity cDNA reverse transcription kit (Thermofisher). Quantitative PCR (qPCR) was performed in biological triplicates. TBP was used for internal control.

ChIP-Seq

PBMCs from patients and their family members were isolated by ficoll gradient on EDTA-anticoagulated blood. Cells were lysed and chromatin was fragmented with 300 units of Micrococcal nuclease (M0247S, New England Biolabs) per well for 10 minutes at 37°C. 2µg of anti-H3K27ac (catalog number 39133, Active Motif) were added to samples and rotated at 15 rpm at 4°C overnight. ChIP and input samples were digested with proteinase K (Eurobio) and purified using Phenol Chloroform. Libraries were verified and equimolar pools were sequenced on NovaSeq 6000 (75bp single-end). See detailed methods in [4]. Single-end reads were mapped to the hg19 genome by the BWA algorithm and duplicate reads (read-pairs mapping to the same genomic location) were collapsed. Reads mapped to non-canonical and mitochondrial chromosomes were also removed. For each sample, ChIP-seq peaks were detected using MACS2 standard parameters, and peaks were selected only if they are present in both duplicates. Differentially acetylated (DA) peaks were determined using DESeq2 after a median of ratios normalization. DA peaks were defined with a Benjamini-Hochberg Q-value ≤10%. GREAT was used to link peaks to their nearest genes and to test for gene ontology enrichment[5].

Immunofluorescence

Cells were seeded on glass coverslip and were fixed in 4 % paraformaldehyde in PBS for 10 minutes. After PBS wash, cells were permeabilized with 0.5 % triton in PBS for 5 min. After 3 washes in PBS, cells were blocked with 4 % BSA in PBS for 1 hour, then proceeded with antibody incubation (BAP1; 1:100 dilution) overnight at 4 °C. Following 3 washes with PBS, cells were incubated with secondary antibody Alexa 488 in 1:500 dilution for 1 hour. Following 3 washes, glass slides were mounted with Vectashield with DAPI counterstain (Vector laboratories), sealed with nail polish and stored at 4 °C in dark.

Supplemental notes

Predicted BAP1 Missense Variant Effects Suggest Selective Loss of Deubiquitinase Activity

Many of the *de novo* missense variants reported here occur at critically important functional residues and the rest are localized to a small region of the protein. Cys91 is the critical active site residue of this enzyme and changes from Cys have been functionally confirmed to disrupt activity[6-10]. This residue provides the "thiol" which defines BAP1 as being a "thiol-dependent deubiquitinase". Variants at this site have been confirmed to eliminate activity while maintaining protein-protein interactions. His169 is also part of the enzyme active site and interacts directly with Cys91 as a proton donor - chemical activity that likely only His could provide, making any variant at this site highly disruptive. R718 falls within the Nuclear Localization Signal (NLS) from Arg717-Arg722[7; 11]. This region matches the Eukaryotic Linear Motif (ELM) Database pattern for an NLS Bipartite 1 motif. NLS motifs can be robust however p.(Arg718Gln) eliminates the ELM match for this region while several other plausible variants, such as p.(Arg717Gln), do not. The variant p.(Arg718Gln) may also disrupt BAP1 regulation by interfering with the nearby phosphorylation target of UBE20 (positions 691-

711)[11]. The other four variants [p.(Pro12Ala), p.(Pro12Thr), p.(Glu31Lys), p.(Leu49Pro)] occur very early within the protein, however they are contained within regions predicted to be ordered in MobiDB (https://mobidb.bio.unipd.it/) and are not within degenerate/low complexity regions.

Supplemental references

1. Traynelis, J., Silk, M., Wang, Q., Berkovic, S.F., Liu, L., Ascher, D.B., Balding, D.J., and Petrovski, S. (2017). Optimizing genomic medicine in epilepsy through a gene-customized approach to missense variant interpretation. Genome Res 27, 1715-1729.
2. Wiel, L., Baakman, C., Gilissen, D., Veltman, J.A., Vriend, G., and Gilissen, C. (2019). MetaDome: Pathogenicity analysis of genetic variants through aggregation of homologous human protein domains. Hum Mutat 40, 1030-1038.
3. Fonteneau, J.F., Larsson, M., Somersan, S., Sanders, C., Munz, C., Kwok, W.W., Bhardwaj, N., and Jotereau, F. (2001). Generation of high quantities of viral and tumor-specific human CD4+ and CD8+ T-cell clones using peptide pulsed mature dendritic cells. Journal of immunological methods 258, 111-126.
4. Latypova, X., Vincent, M., Molle, A., Adebambo, O.A., Fourgeux, C., Khan, T.N., Caro, A., Rosello, M., Orellana, C., Niyazov, D., et al. (2021). Haploinsufficiency of the Sin3/HDAC corepressor complex member SIN3B causes a syndromic intellectual disability/autism spectrum disorder. American journal of human genetics 108, 929-941.
5. McLean, C.Y., Bristor, D., Hiller, M., Clarke, S.L., Schaar, B.T., Lowe, C.B., Wenger, A.M., and Bejerano, G. (2010). GREAT improves functional interpretation of cis-regulatory regions. Nat Biotechnol 28, 495-501.
6. Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y., et al. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene 16, 1097-1112.
7. Ventii, K.H., Devi, N.S., Friedrich, K.L., Chernova, T.A., Tighiouart, M., Van Meir, E.G., and Wilkinson, K.D. (2008). BRCA1-associated protein-1 is a tumor suppressor that requires deubiquitinating activity and nuclear localization. Cancer Res 68, 6953-6962.

8. Nishikawa, H., Wu, W., Koike, A., Kojima, R., Gomi, H., Fukuda, M., and Ohta, T. (2009). BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. Cancer Res 69, 111-119.

9. Bott, M., Brevet, M., Taylor, B.S., Shimizu, S., Ito, T., Wang, L., Creaney, J., Lake, R.A., Zakowski, M.F., Reva, B., et al. (2011). The nuclear deubiquitinase BAP1 is commonly inactivated by somatic mutations and 3p21.1 losses in malignant pleural mesothelioma. Nat Genet 43, 668-672.

10. Machida, Y.J., Machida, Y., Vashisht, A.A., Wohlschlegel, J.A., and Dutta, A. (2009). The deubiquitinating enzyme BAP1 regulates cell growth via interaction with HCF-1. J Biol Chem 284, 34179-34188.

11. Mashtalir, N., Daou, S., Barbour, H., Sen, N.N., Gagnon, J., Hammond-Martel, I., Dar, H.H., Therrien, M., and Affar el, B. (2014). Autodeubiquitination protects the tumor suppressor BAP1 from cytoplasmic sequestration mediated by the atypical ubiquitin ligase UBE2O. Molecular cell 54, 392-406.