Identification of disease-linked hyperactivating mutations in UBE3A through large-scale functional variant analysis

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The mechanisms that underlie the extensive phenotypic diversity in genetic disorders are poorly understood. Here, we develop a large-scale assay to characterize the functional valence (gain or loss-of-function) of missense variants identified in UBE3A, the gene whose loss-of-function causes the neurodevelopmental disorder Angelman syndrome. We identify numerous gain-of-function variants including a hyperactivating Q588E mutation that strikingly increases UBE3A activity above wild-type UBE3A levels. Mice carrying the Q588E mutation exhibit aberrant early-life motor and communication deficits, and individuals possessing hyperactivating UBE3A variants exhibit affected phenotypes that are distinguishable from Angelman syndrome. Additional structure-function analysis reveals that Q588 forms a regulatory site in UBE3A that is conserved among HECT domain ubiquitin ligases and perturbed in various neurodevelopmental disorders. Together, our study indicates that excessive UBE3A activity increases the risk for neurodevelopmental pathology and suggests that functional variant analysis can help delineate mechanistic subtypes in monogenic disorders.
The identification of genes linked to human disorders traditionally focuses on lesions that can be interpreted easily as reducing gene function. Although this method has been effective in establishing causality, a simple loss-of-function model is insufficient to account for the broad phenotypic heterogeneity observed in many neurodevelopmental disorders. This is especially important for the millions of uncharacterized coding variants identified in the human genome, some of which represent pathogenic mutations that may bi-directionally alter the functional valence of a protein (loss or gain-of-function). However, the clinical significance for the vast majority of known variants remains undetermined, representing an immensely understudied area of human genetics.

UBE3A encodes an E3 ubiquitin ligase that promotes the proteasomal degradation of proteins. Abnormal changes in UBE3A activity are associated with various human conditions including human papilloma virus-mediated cancer and neurodevelopmental disorders. In neurons, UBE3A expression is epigenetically modified such that transcription of paternal UBE3A is silenced and only the maternally-inherited copy is expressed. It is well established that loss of maternal UBE3A causes Angelman syndrome (AS), a severe form of intellectual disability characterized by epilepsy, motor deficits, dysmorphic facial features, and a unique happy demeanor. In contrast, excessive UBE3A activity resulting from duplication of maternal chromosome 15q11-13, the region where UBE3A resides, is linked to an autistic disorder known as Dup15q syndrome. However, there are only two known examples that specifically link UBE3A gain-of-function to neurodevelopmental disease. This includes one de novo hyperactivating missense mutation identified in a child with autism, and a microduplication of UBE3A that segregates with neuropsychiatric phenotypes in one family. These limited observations have raised questions about the extent to which excessive UBE3A activity contributes to neurodevelopmental pathology.

UBE3A belongs to the HECT (Homologous to E6-AP C-terminus) domain superfamily of E3 ubiquitin ligases which are essential to account for the broad phenotypic heterogeneity observed in many neurodevelopmental disorders. This included constitutively self-targeting mutations whose severity was demonstrated previously to be strong (T106P, 8.45% ± 1.8) and mild (T106K, 65.09% ± 5.1 and I130T, 76.31% ± 6.1), indicating that BAR responses can distinguish the relative severity of UBE3A dysfunction. Together, these results demonstrate the utility of the BAR assay as a general and accurate reporter of UBE3A activity across different mechanisms that perturb enzyme function.

UBE3A variants define a broad landscape of functional effects. We cloned nearly all of the UBE3A missense variants present in the ClinVar database and assessed their effect on UBE3A activity (Fig. 2a). Our library contained 152 variants in total, comprised of 133 amino acid substitutions, 18 in-frame deletions, and one in-frame duplication. Whereas the activity of ~23% of variants did not change from WT UBE3A, indicating they are likely benign, we classified ~61% as strong loss-of-function mutations (≤50% of WT UBE3A activity) and ~4% as weak loss-of-function mutations (>50% of WT UBE3A activity; Fig. 2b and Supplementary Data 1). Surprisingly, our screen also found that ~9% of all variants are strong gain-of-function mutations (>150% of WT UBE3A activity; Fig. 2b and Supplementary Data 1).

Some of the variants tested in our screen disrupted residues in domains known to be important for UBE3A function. One such domain was an N-terminal zinc (Zn2+) finger known as the AZUL domain (amino-terminal Zn-finger of UBE3A ligase) that dictates UBE3A localization. Six variants were located within the AZUL domain including a loss-of-function mutation that disrupts a Zn2+ chelating residue (C21Y, 1.07% ± 0.001 of WT UBE3A activity) and the adjacent amino acid (G20V, 2.07% ± 0.79; Fig. 2c). Our screen also included variants in the E6 binding domain (E6BD; amino acids 378–396), a site where the human papilloma virus (HPV) E6 protein binds to UBE3A. Two hyperactivating mutations were found within this region including a L390F mutation that elevated UBE3A activity 229.81% ± 9.33 above the WT BAR response (Fig. 2d), suggesting the E6BD is an important regulator of UBE3A function even in

**Results**

An efficient cell-based screen to characterize UBE3A variant function. There are hundreds of non-truncating UBE3A missense variants contained in the Genome Aggregation (gnomAD v2.1.1) and ClinVar databases. Most variants are exceedingly rare (average allele frequency <0.0001) and nearly two-thirds of variants in ClinVar have conflicting interpretations or are classified as a variant of uncertain significance (VUS; Fig. 1a). Moreover, variants in UBE3A occur both inside and outside of known functional domains, making it impossible to predict their functional significance without empirical assessment (Fig. 1b).

We utilized the luciferase-based β-catenin activity reporter (BAR)²⁰, which is activated in a dose-dependent way by UBE3A (Fig. 1c), to determine if it can accurately assess the activity of UBE3A variants. We tested a variety of variants clinically and functionally validated in previous studies. Among these were the benign variants R39H and A178T, the autism-linked hyperactivating mutation T485A, as well as numerous AS-linked mutations known to cause loss-of-function through various mechanisms. We introduced mutations into a plasmid encoding human UBE3A isoform 2 and measured the BAR response in HEK293T cells. For every mutant, the BAR assay correctly identified changes in the valence and severity of the mutation in relation to wild type (WT) UBE3A (Fig. 1d).

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F690C)32, and loss-of-function mutations at the catalytic cysteine function mutations in the E2 enzyme binding domain (T656I and mutations in the HECT domain of UBE3A, including loss-of-the absence of HPV E6. Finally, we identified 18 additional hyperactivating mutations (Fig. 3a) with ed 18 additional hyperactivating mutations (Fig. 3a) with hyperactivating mutations in general, are hyper-modulatory sites that mediate the active and inactive states of UBE3A. To test this hypothesis, we substituted every possible amino acid at leucine 781 (L781) and T787, two positions that contained the strongest hyperactivating mutations in our screen (L781H and T787M; Supplementary Fig. 1b). We anticipated that mutation of these sites would produce multiple strong loss and gain-of-function enzymes reflecting their central role in enzyme regulation. Indeed, we found an extensive range of effects in our BAR assay that spanned from 7.48 to 656.55% of WT UBE3A activity for L781I and 1.33 to 1245.07% of WT UBE3A activity for T787 (Supplementary Fig. 1b and Supplementary Table 1). Taken together, our analyses demonstrate that variants in UBE3A impart highly heterogeneous effects to protein function and suggest that sites of hyperactivating mutations signify amino acids that are critical for UBE3A regulation.

Hyperactivating mutations alter UBE3A ubiquitin ligase activity. To date, the UBE3A T485A mutation, which abolishes a regulatory PKA phosphorylation site, is the only disease-linked gain-of-function mutation reported for UBE3A12. Our analysis identified 18 additional hyperactivating mutations (Fig. 3a) with many exhibiting much higher activity than UBE3A T485A (Fig. 3b). We sought to validate whether these variants represented a true gain-of-function of the ubiquitin ligase activity of...
UBE3A. Western blot analysis showed that all gain-of-function mutants were present at comparable abundance or were less abundant than WT UBE3A in HEK293T cells (Supplementary Fig. 2a, b), indicating hyperactivity did not result from a simple increase in enzyme levels. Moreover, when we introduced a ligase-dead mutation (C820A) into each gain-of-function mutant, there was a complete loss of the BAR response (Fig. 3c), demonstrating that reporter activation resulted from increased ubiquitin ligase activity.

We next tested the ability of gain-of-function mutants to self-ubiquitinate, a process shown to be accelerated for the hyperactive UBE3A T485A mutation as well as for a variety of active HECT domain ubiquitin ligases. We selected several strong gain-of-function mutations in the HECT domain and performed in vitro ubiquitination reactions using recombinant proteins purified from bacterial cells. We found that mutant proteins accelerated self-ubiquitination resulting in the formation of higher molecular weight bands and the eventual loss of the monomeric UBE3A band (Fig. 3d). This result was consistent in HEK293T cells where gain-of-function mutants increased self-ubiquitination compared to WT UBE3A (Supplementary Fig. 2c). Finally, we measured the ability of gain-of-function mutants to target a substrate protein for degradation in cells. We performed this experiment with a ligase-inactive mutant of RING1B, a polycomb repressor complex protein used widely as an in vitro substrate of UBE3A. Western blot analysis showed that whereas RING1B abundance increased in the presence of ligase-dead UBE3A (176% ± 0.13 relative to WT), every hyperactivating mutation tested in our assay decreased RING1B protein levels. Indeed, R516Q (67.48% ± 3.68), R516W (60.16% ± 4.63), A521T (55.16% ± 4.71), Q588E (60.58% ± 4.44), L787A (52.31% ± 4.30), T787A (52.96% ± 4.31), and T787M (52.37% ± 4.91) all reduced RING1B abundance to a fraction of WT UBE3A levels (Supplementary Fig. 2d, e). Collectively, these results provide direct evidence that gain-of-function mutations identified in our study elevate the ubiquitin ligase activity of UBE3A.
Structure-function analysis reveals a degenerate exosite in UBE3A. The rich functional information provided by our screen allowed us to perform extensive structure-function analyses of UBE3A. When we mapped the spatial distribution of all loss and gain-of-function mutations identified in our screen, we found they localized to several structural features in the UBE3A HECT domain (Fig. 4a). This included the C-lobe, which contains the catalytic cysteine required for ubiquitin transfer\(^{25}\), a number of mutations in the E2 binding region\(^{32}\), a group in a region of unknown function, and one group that demarcated a recently characterized non-covalent ubiquitin binding site in UBE3A known as the exosite\(^{39}\). The exosite is best characterized in NEDD4 subfamily HECT domain enzymes, where studies showed that binding of monomeric or dimeric ubiquitin mediates ubiquitin ligase activity\(^{36,40-43}\). Previous structural and mutational analyses determined that ubiquitin binding at the exosite requires two principal contact points in NEDD4\(^{42}\): a hydrophobic patch formed by phenylalanine 707 (F707) in NEDD4 and isoleucine 44 (I44) in ubiquitin, and another hydrophobic contact formed by two tyrosines (Y605 and Y634) that interact with leucine 73 (L73) near the C-terminus of ubiquitin (Fig. 4b and Supplementary Fig. 3a). Sequence alignment of the ubiquitin
contacting domain in UBE3A showed a high degree of conservation across species (Supplementary Fig. 3b). However, whereas the NEDD4 F707 position is conserved in UBE3A (F665 in UBE3A), UBE3A lacks the aromatic residues at Y605 and Y634 (I564 and Q588, respectively) that are present in every NEDD4 subfamily enzyme (Supplementary Fig. 3c).

Our observations raised the possibility that UBE3A possesses a degenerate exosite that binds ubiquitin through mechanisms that diverge from NEDD4 subfamily enzymes. To explore this possibility, we used the crystal structure of the UBE3A HECT domain and the co-crystal structure of ubiquitin bound to the NEDD4 exosite (Fig. 4b; PDB: 4BBN) to perform constrained rigid-body docking with the Rosetta molecular modeling program.44. Modeling showed that F665 in UBE3A contacts I44 of ubiquitin, and in functional experiments, we found that mutation of F665 to alanine (F665A) caused a near-complete loss of UBE3A activity in our BAR assay (Supplementary Fig. 3d), demonstrating that F665 is indispensable for UBE3A activity. In contrast, there were large structural changes at the second ubiquitin binding site. Y605 and Y634 in NEDD4 contribute to form a deep hydrophobic pocket that permits a stable interaction with the side chain of L73 in ubiquitin (Fig. 4d). However, this pocket is shallow in UBE3A and superimposing the orientation of ubiquitin bound to NEDD4 resulted in steric clashing between atoms in the sidechains of Q588 in UBE3A and L73 in ubiquitin (Fig. 4c).
Position Q588 is critical for ubiquitin binding at the UBE3A exosite. Intriguingly, there were three mutations at the Q588 position tested in our screen including a substitution to glutamate (Q588E), proline (Q588P), and arginine (Q588R), all of which altered the overall charge in the ubiquitin-binding pocket of UBE3A (Supplementary Fig. 3e). The Q588E mutation was a gain-of-function mutation that elevated UBE3A activity 389% ± 35.8 above WT UBE3A levels (Fig. 3b and Supplementary Data 1) whereas Q588P and Q588R abolished UBE3A ubiquitin ligase activity (Supplementary Data 1). These functional observations suggested that Q588 is a critical residue that mediates exosite function in UBE3A.

To further interrogate how Q588 impacts ubiquitin binding, we used the Relax application in the Rosetta Macromolecular modeling suite45, starting from the orientation of ubiquitin in the NEDD4:ubiquitin co-crystal structure. High confidence models placed ubiquitin in a similar conformation relative to its starting position, and key contacts typically made by ubiquitin when bound to NEDD4 subfamily exosites were preserved in UBE3A. Further analysis revealed an ensemble of closely-aligned conformations in which the C-terminus of ubiquitin, including L73, bound UBE3A through a series of interactions surrounding the shallow hydrophobic pocket, including contacts with Q588 (Fig. 4e). Root mean square deviation (RMSD) versus Rosetta score plots showed a broad funnel that corresponds to this ensemble of similar C-terminal tail conformations (Fig. 4g). We repeated this modeling with the Q588E variant and found that RMSD versus Rosetta score plots revealed two divergent populations of high-confidence structures that corresponded to distinct ubiquitin tail conformations docked to the Q588E exosite (Fig. 4f, h). The first population was similar to ubiquitin bound to WT UBE3A (Fig. 4f). In contrast, the second set had an alternative ubiquitin tail conformation in which the positive charge from the adjacent arginine 72 (R72) residue in ubiquitin contacted the negative charge resulting from the Q588E mutation in UBE3A. Reanalysis of the WT modeling with this alternative tail revealed there were four structures out of ~30,000 that had this similar tail. Together, our analysis indicated that Q588E mutation alters the mechanism by which ubiquitin binds to UBE3A.

The Q588E mutation enhances ubiquitin binding to the exosite. Previous studies showed that ubiquitin binding to the exosite of HECT domain enzymes facilitates its ubiquitin ligase activity36,39,41,43,46. We performed a series of experiments to explore if the charge at amino acid position 588 alters the affinity of ubiquitin binding to UBE3A. We began by performing comprehensive mutation analysis at position Q588 in UBE3A (Fig. 5a). We tested a series of mutations at the Q588 site and assessed UBE3A activity using the BAR assay (Fig. 5a and Supplementary Table 1). Consistent with a charge-dependent interaction between Q588E of UBE3A and R72 of ubiquitin, mutation to a positively-charged aspartate (Q588D) resulted in an inactive enzyme. Hydrophobic or uncharged residues, including mutation to aromatic residues present in NEDD4 sub-family enzymes (Q588Y and Q588W), caused UBE3A to exhibit little activity (5.10% ± 0.97 and 11.99% ± 0.62, respectively), further suggesting the mechanism of ubiquitin binding to the UBE3A exosite is divergent from NEDD4 sub-family enzymes. Finally, we found that polar residues such as serine (Q588S; 112.62% ± 11.27), threonine (Q588T; 125.72% ± 10.74), or asparagine (Q588N; 153.53% ± 11.91) all produced an enzyme with comparable activity to WT UBE3A, suggesting that Q588 participates in hydrogen bonding with ubiquitin in its native state.

We next tested whether the Q588E mutation enhances ubiquitin binding to UBE3A. We first accomplished this task using fluorescence polarimetry. Our experiments demonstrated that ubiquitin bound the WT HECT domain with a dissociation constant (K_D) of 65.7 ± 28.5 μM (Fig. 5b), which was in line with a previous report39, but bound with higher affinity to the Q588E HECT domain with a K_D of 18.0 ± 1.5 μM (Fig. 5c). Second, we utilized a new technique termed the mechanically transduced immunosorbent (METRIS) assay47. In brief, this system measures weak protein interactions by utilizing a protein that is covalently conjugated to ferromagnetic particles placed on coverslips pre-coated with another protein of interest (Fig. 5d). A rotating external magnetic field is applied, which causes the beads to spin in place. If there is an interaction between the two proteins of interest, the bead migrates along the coverslip (Fig. 5e), and the extent of this linear displacement is dependent on the strength of the protein–protein interaction in the system. This displacement is captured as the rolling parameter (R), and the value of the rolling parameter scales in proportion with the affinity of the interaction (see “Methods” section for detailed information). We performed this experiment by conjugating ubiquitin to ferromagnetic particles and calculating its rolling parameter on UBE3A HECT domain-coated coverslips. We observed that WT UBE3A yielded a rolling parameter of 0.178 ± 0.0056, whereas the Q588E mutation increased the rolling parameter to 0.247 ± 0.0107, indicating enhanced affinity between the two proteins, and the Q588R mutation reduced the rolling parameter to 0.114 ± 0.0021 (Fig. 5f). These results confirmed our fluorescence polarimetry results that Q588E mutation enhances ubiquitin binding.

Our findings provide the first characterization of disease-relevant mutations in the exosite of any HECT domain enzyme. When we examined reported variants for other HECT domain proteins, we found additional variants classified as VUS and likely pathogenic/pathogenic in various disease contexts, including several neurodevelopmental disorders (Supplementary Fig. 4). Taken together, our experiments provide strong evidence that ubiquitin binding to the UBE3A exosite is essential for proper enzyme activity, and dysfunction at the exosite is a disease-relevant mechanism that is shared across several human disorders.

Excessive activity of maternal UBE3A is sufficient to cause neonatal behavioral impairments in mice. We next considered the biological impact of UBE3A hyperactivating mutations and their potential to contribute to pathology. We used CRISPR-Cas9 recombination to create a line of knock-in mice possessing a Q588E mutation in UBE3A (Fig. 6a). All mutant animals were born at expected Mendelian ratios and possessed indistinguishable body weights compared to WT littermates (Fig. 6b), indicating the absence of overt physical defects or delays in growth. We began our characterization using heterozygous Ube3a^mQ588E/pWT mice (mQ588E; Supplementary Fig. 5a), which possess the Q588E mutation on the maternally-inherited allele, and is thus expressed in neurons. Western blot analysis of the cortex in mQ588E animals showed reduced mutant UBE3A protein levels (Supplementary Fig. 5b). These observations were consistent with enhanced self-targeted ubiquitination and reduced steady-state levels observed previously for highly active ubiquitin ligases36,37, including UBE3A12,48 (also see Supplementary Fig. 2a–c). We tested early life behaviors in our animals using Fox’s battery of tests, which are designed to assess sensorimotor development in juvenile mice49,50. We first
performed the surface righting reflex which measures the ability of pups to right themselves when placed in a prone dorsal position. In postnatal day 10 (P10) animals, we found that mQ588E pups accomplished this task just as effectively as their respective WT littermates (WT = 0.79 s ± 0.04, mQ588E = 0.88 ± 0.09, Supplementary Fig. 5c). Moreover, we found no differences between WT and mutant animals when testing forepaw and hindpaw grasping reflexes (Supplementary Fig. 5d, e). Next, we used the negative geotaxis assay which measures how quickly an animal repositions itself vertically after being placed facing down a 45° slope. Although we found no differences between WT (56.59 ± 6.46) and mQ588E (62.65 ± 8.94) animals in their ability to rotate (Supplementary Fig. 5f), we observed that a much larger fraction of mQ588E mice consistently fell from the testing apparatus as compared to WT mice (Fig. 6c). This effect was specific to mQ588E mice as Ube3amWT/pQ588E animals possessing a paternally-inherited mutation (pQ588E; Supplementary Fig. 5a) were indistinguishable from WT littermates (Fig. 6d).

Our observations suggested that reflexive responses remained largely intact, but raised the possibility that mQ588E animals possessed deficits in strength and motor coordination. This led us to perform two additional tests in P10 mice. First, we performed the front-limb suspension test and found that mQ588E on average remained suspended on an elevated wire half as long as WT littermates (WT = 7.73 s ± 1.34, mQ588E = 3.82 ± 0.42) whereas no effect was seen in pQ588E animals (WT = 4.90 ± 0.52, pQ588E = 4.13 ± 0.38; Fig. 6e, f). Next, we tested four-paw grip strength by measuring the ability of pups to remain on a wire mesh rotated slowly from a horizontal position (0°) to an inverted position (180°; Fig. 6g). At P10, mQ588E animals performed poorly and fell from the mesh at an average tilt angle of 129.48° ± 7.33 compared to 158.47° ± 3.34, for WT littermates (Fig. 6h). When we re-tested these animals at P14, we found that mQ588E mice drastically improved in their performance (171.56 ± 2.65) and were nearly indistinguishable from WT littermates (177.33 ± 1.31; Fig. 6j), suggesting that motor development was delayed in mQ588E mice. Meanwhile, pQ588E mice showed no significant differences from WT littermates at either
Fig. 6 UBE3A Q588E mutation perturbs neurological function in a mouse model. a The genomic region containing the coding sequence for position Q588 in UBE3A was amplified and sequenced from WT and mQ588E mice. The arrow marks the mutated nucleotide. b Mean ± range of body weights of WT and mutant mice at postnatal (P) day 5, 7, 10, and 14. Solid black: WT males (WT M), Open black: WT females (WT F), Solid gray: mQ588E males (mQ588E M), Open gray: mQ588E females (mQ588E F). The following numbers of mutant mice at postnatal (P) day 5, 7, 10, and 14. Solid black: WT males (WT M), Open black: WT females (WT F), Solid gray: mQ588E males (mQ588E M), Open gray: mQ588E females (mQ588E F). The following numbers of animals were used for each group: WT M: P5, P7, P9, P14; WT F: P5, P7, P9, P14; mQ588E M: P5, P7, P9, P14; mQ588E F: P5, P7, P9, P14. c Mean ± range of body weights of WT and mQ588E mice. The arrow marks the mutated nucleotide. d Mean ± range of body weights of WT and mQ588E mice. The arrow marks the mutated nucleotide. e, f Individual times and the mean ± SE for mQ588E (e, gray) and pQ588E (f, blue) mice in the front limb suspension test are shown. Each animal was tested three times and the average of the three trials was used in for analysis. WT, n = 29 animals; mQ588E, n = 16 animals in e, WT, n = 21 animals; pQ588E, n = 26 animals in d. e, f Individual times and the mean ± SE for mQ588E (e, gray) and pQ588E (f, blue) mice in the front limb suspension test are shown. Each animal was tested three times and the average of the three trials was used in for analysis. WT, n = 29 animals; mQ588E, n = 16 animals in e, WT, n = 21 animals; pQ588E, n = 26 animals in f. **p = 0.001, Mann-Whitney U-test, exact significance (two-tailed). g, k Schematic (g) and individual and mean ± SE values for the four-paw grip strength test at mQ588E (h, i gray) and pQ588E (j, k blue) animals. Animals were tested at P10 (h, j) and at P14 (i, k) as indicated. **p = 0.001 (h), *p = 0.026 (j), Mann-Whitney U-test, exact significance (two-tailed). WT, n = 29 animals; mQ588E, n = 16 animals (h, j); WT, n = 21 animals; pQ588E, n = 26 animals (i); WT, n = 17 animals and pQ588E, n = 22 animals (k). l Mean ± SE values showing the number of calls at P5, P7, and P9 for WT (black) and mQ588E (gray) littermates (l), and in WT (black) and pQ588E (blue) littermates (m). **p = 0.001 at P5, *p = 0.038 at P9, Repeated measures ANOVA. WT: P5-P7, n = 31 animals; mQ588E: P5-P9, n = 23 animals (l); WT: P5 and P7, n = 21 animals, P9, n = 15 animals; pQ588E: P5, n = 32 animals; P7, n = 27 animals, P9, n = 26 animals (m).
timepoint (P10: WT = 157.70 ± 4.26, pQ588E = 148.81 ± 4.64; P14: WT = 180.0 ± 0, pQ588E = 178.64 ± 1.36; Fig. 6i, k).

Finally, we measured ultrasonic vocalizations (USVs) to determine whether early-life communication is affected in mQ588E animals. We found that on average, the number of USVs produced by WT animals increased gradually from 68.11 ± 12.67 calls at P5, to 80.81 ± 15.53 at P7, and 95.03 ± 19.10 at P9. In contrast, mQ588E animals produced dramatically fewer calls at P5 (12.57 ± 3.32) and at P9 (42.04 ± 11.55; Fig. 6l). Intriguingly, these results were opposite to neonatal Angelman syndrome model mice, which were shown to produce increased vocalizations. We also observed higher pitched vocalizations, shorter call durations, and longer pause durations (Supplementary Fig. 5g–i) to varying degrees in mQ588E animals. In contrast, there were no differences in the number of vocalizations at any timepoint between pQ588E mice and their WT littermates (Fig. 6m) and a very subtle difference in pitch frequency at P5 (Supplementary Fig. 5j) with no differences in call duration or pause duration (Supplementary Fig. 5k, l). Taken together, our observations provide direct evidence that hyperactivation of maternal, but not paternal UBE3A, is sufficient to delay motor development and produce deficits in early life communication.

Hyperactivating mutations in UBE3A may contribute to neurodevelopmental phenotypes that are distinct from Angelman syndrome. Although AS is clinically recognizable, variants caused by excessive UBE3A activity are not well documented. Thus, when we compared our functional screening results with variant classifications in the ClinVar database, we predicted that a greater number of loss-of-function variants would be classified as pathogenic whereas gain-of-function mutations would be classified as VUS. Consistent with our hypothesis, our data showed that about half (50.6%) of loss-of-function mutations identified in our screen were deemed pathogenic whereas most gain-of-function mutations were classified as VUS (84%) with a few designated as benign (16%) and none designated as pathogenic (Fig. 7a).

To gain further clarity into human phenotypes, we collaborated with clinical centers to collect data from 17 individuals possessing hyperactivating mutations identified in our study. A summary of their clinical phenotypes is presented in Supplementary Table 2 and a detailed account for each individual is presented in our Supplementary Information (Supplementary Note 1). Our data indicated that hyperactivating mutations are typically maternally inherited, and individuals generally exhibit intellectual disability, seizures, and autism. In some cases, phenotypes distinct from either classic AS or Dup15q were reported. However, none of the individuals examined in our study were diagnosed with AS.

Here we highlight two families in which mutations segregated with affected individuals. Individual 1 was an adult who possessed a T787A mutation that raised UBE3A activity 517.81% ± 49.3 above WT levels. The individual was diagnosed with failure to thrive, hypotonia, feeding difficulties, and global developmental delay as a child. Autistic behaviors, intellectual disability, and epilepsy were also observed. In addition, Individual 1 was noted to possess small hands and feet, which are typical characteristics seen in Prader–Willi syndrome patients, but no methylation abnormalities in chromosome 15 were detected. The observed phenotypes were not consistent with AS. Individual 1 also had two siblings: one who was typically developing and did not possess the T787A mutation. The other sibling inherited the T787A mutation (noted here as Individual 2) and displayed autistic behaviors and was non-verbal until the age of 4 (Fig. 7b). It was not known at the time of this report if Individual 2 exhibited seizures. Sequencing analysis confirmed the T787A mutation was maternally inherited, however, we were not able to establish the parent-of-origin or the mutation in the mother.

Individual 5 was an adolescent who possessed an in-frame deletion that resulted in the removal of leucine 726 (L726Δ) and raised UBE3A activity 180.75% ± 9.9 above WT levels. The individual presented with global developmental delay, microcephaly, and intractable epilepsy requiring vagal nerve stimulation placement. Delayed motor and language milestones were evident in early childhood and the proband was later diagnosed with quadriplegic cerebral palsy and severe intellectual disability. Individual 5 is currently G-tube dependent for feeding, possesses generalized muscle atrophy and spasticity in the lower extremities, pectus carinatum deformity, severe thoracolumbar scoliosis and thoracic kyphosis. Osteoporosis in the femoral head and neck was also evident. Individual 5 was found to be heterozygous for a pathogenic ADARI variant that was inherited from the mother. However, as ADARI mutation causes an autosomal recessive disorder, this variant was not determined to be causative of disease phenotypes. Individual 5 had three siblings. One adolescent sibling (Individual 6), possessed the same L726Δ variant and exhibited intellectual disability and ADHD. The cognitive ability of Individual 6 was estimated at 4 years of developmental age and required special resources at school (Fig. 7c). Individual 6 was not able to read or count and had limited speech. The proband did not exhibit seizures and clinical and behavioral phenotypes, as well as facial features, were not consistent with a diagnosis of AS. A second adolescent sibling did not possess the L726Δ mutation and was typically developing. The final sibling was an adolescent maternal half-sibling who...
inherited the L726Δ mutation (noted here as Individual 7). Individual 7 was diagnosed with ADHD and was noted for possessing learning difficulties. The proband required an individualized education program (IEP) at school, including special classes for math and social studies. No other abnormalities were noted. Sequencing analysis confirmed the L726Δ mutation was maternally inherited, but the parent-of-origin was unknown for the mother.

Discussion

Historically, the need to infer the impact of mutations strictly from sequence changes has biased the discovery of disease-causing genes toward lesions that produce loss-of-function alleles. Our functional analyses of missense variants in UBE3A revealed that ~12% of variants cause a gain of enzyme function. We found that hyperactivating mutations segregate with affected phenotypes in two unrelated families, and consistent with the neuronal imprinting of UBE3A, our mouse modeling and patient data both strongly suggest that hyperactivating mutations confer an increased risk for pathogenicity when present on the maternally inherited allele.

Our functional variant analysis allowed us to identify a group of mutations in the UBE3A exosite, thereby implicating its dysfunction in UBE3A-dependent disorders. In general, the exosite is thought to facilitate ubiquitin chain elongation by binding the distal ubiquitin on a growing chain and stabilizing ubiquitinated target proteins to the E3 enzyme. However, this mechanism appears context-dependent as ubiquitin binding to the exosite can also inhibit chain elongation and ubiquitin variants that bind the exosite with high affinity can alter enzyme activity in complex ways. We also found that additional HECT domain enzymes possess VUS or pathogenic/likely pathogenic variants within their exosites (Supplementary Fig. 4). Examples include NEDD4-2, which is implicated in periventricular nodular heterotopia, HECW2, which is implicated in a neurodevelopmental disorder with hypotonia, seizures, and absent language, and HUWE1, which contains a mutation (Y4106C) at a position that is analogous to Q588 in UBE3A and is associated with an X-linked form of intellectual disability (Supplementary Fig. 4). Although the functional impact of these variants awaits further study, our observations suggest that exosite dysfunction is a common mechanism that underlies additional neurodevelopmental disorders.

We found that UBE3A hyperactivity causes a general reduction in steady-state enzyme levels due to enhanced self-targeted degradation both in vitro and in vivo (Supplementary Figs. 2 and 5b), a result that is contrary to UBE3A duplication, which leads to increased amounts of protein. These observations paint a complex picture about the pathogenic mechanisms of gain-of-function UBE3A variants, and our study does not rule out the possibility that such variants may actually cause a loss-of-function state in vivo by lowering overall UBE3A levels in the cell. However, we note that enhanced self-targeting is an enigmatic process that can reflect both enzyme loss and gain-of-function states for UBE3A as well as for other ubiquitin ligase enzymes. Previous studies with various HECT domain enzymes have shown that gain-of-function mutations cause a reduction in steady-state enzyme levels through enhanced self-targeted degradation and AS-linked missense mutations in UBE3A known to cause constitutive self-targeted degradation, produced a loss-of-function response in our BAR assay (Fig. 1d and Supplementary Data 1). In addition, UBE3A is known to undergo self-targeted degradation after the depletion of its substrates, suggesting that heightened self-targeted degradation may be a predicted outcome in the context of enzyme gain-of-function. Additional studies will be required to distinguish these intriguing possibilities and will provide greater insight into the mechanisms of UBE3A gain-of-function variants in disease.

Our work strongly suggests that gain-of-function mutations in UBE3A confer an increased risk for neurodevelopmental pathology, however, there were variables in our clinical data that limited our ability to elucidate the extent to which excessive UBE3A activity contributes to disease phenotypes. First, our mouse modeling experiments indicated that gain-of-function of maternal UBE3A is required to alter behavioral phenotypes (Fig. 6 and Supplementary Fig. 5). We were able to establish maternal inheritance for some individuals, however, we were unable to determine the parent-of-origin for Individuals 3, 4, 10, 13–17 (Supplementary Table 2). Second, our study could not resolve the contribution of additional genetic and environmental influences on patient phenotypes. For example, Individuals 8 and 10 both possessed variants in STXBP1, a gene linked to pediatric seizures and intellectual disability, and Individual 10 was diagnosed with fetal alcohol syndrome (Supplementary Table 2). Moreover, several patients in our cohort possessed skeletal malformations not typically associated with UBE3A dysfunction, reflecting the presence of additional contributing factors for disease. This was particularly notable for Individuals 5–6, who were siblings who possessed the same L726Δ variant. Individual 5 was more severely affected and presented with quadriplegic cerebral palsy, pectus carinatum deformity, severe thoracolumbar scoliosis, and thoracic kyphosis, which were all phenotypes that were absent in the siblings.

Seizures were a prevalent phenotype observed in our patient cohort, and this observation was consistent with the high degree of penetrance of epilepsy in UBE3A-dependent disorders. However, we stress that careful consideration must be given when attributing this phenotype to gain-of-function mutations in UBE3A. A previous study noted that individuals who possess a microduplication encompassing only maternal UBE3A exhibited developmental delay, intellectual disability, and behavioral abnormalities, but none of these individuals presented with seizures. In the current study, we observed that seizures were not always a shared phenotype among siblings with the same UBE3A gain-of-function mutation (Fig. 7b). For example, seizures were a prominent phenotype in Individual 5, but they were absent in the siblings who possessed the same maternal L726Δ mutation (Individuals 6 and 7). Although we cannot determine at this time whether excessive UBE3A activity is a contributing factor for seizure susceptibility, our study suggests that UBE3A gain-of-function alone is likely insufficient to produce this phenotype.

Finally, our results have broad implications for the nosology of developmental disorders. The results of our study add to the emerging literature suggesting that functional valence can provide a method to distinguish mechanistic subtypes of monogenic disorders. For example, a recent series of studies found that gain-of-function mutations in the sodium channel gene SCN2A result in infantile seizures whereas loss-of-function mutations produce autistic phenotypes without seizures. Although large-scale functional studies of missense variants remain scarce, there are many examples of copy number variations in well-established disease genes that produce distinct disease phenotypes. These include the Rett syndrome gene MeCP2, the Fragile X syndrome gene FMR1, and NSD1, whose deletion causes Sotos syndrome and duplication results in a poorly characterized growth disorder. With the increasing development of genetic therapies, understanding such mechanistic differences will be critical for the design and safe implementation of these approaches.
Methods

Growth conditions and bicistronic constructs

Bicistronic constructions were performed using a human Hsit Ubiquitin-activating enzyme/UBE1 recombinant protein (R&D Systems, #E304050), 10× 13 ligase reaction buffer (R&D Systems, #B-71), UbcH7 recombinant protein (R&D Systems, #E2640100), human FLAG-ubiquitin recombinant protein (R&D Systems, #U12002M), and ATP. (Sigma). Assays were performed as described previously11. In brief, a single reaction contained 13 ligase reaction buffer, 0.1 µM UBE1, 0.5 µM UBE2L3 (UbcH7), 12.5 µM FLAG-ubiquitin, 2 µM ATP, and 4 µg of purified UBE3A. The reaction was allowed to proceed at room temperature for the indicated times. Reactions were stopped by adding 2x Laemmli buffer and boiling for 5 min. UBE3A ubiquitination was also assayed in HEK293T cells treated with 30 µM MG-132 for 1 h. The cells were then lysed in RIPA buffer containing 1% SDS and 30 µM MG-132. Cell lysates were boiled for 20 min and clarified by centrifugation at 15,000×g at 4 °C. The resulting supernatant was diluted 1:10 (v/v) in an immunoprecipitation buffer (20 mM HEPES pH 7.4, 50 mM KCL, 1% Triton X-100). For UBE3A immunoprecipitation, an anti-Myc-conjugated affinity gel (Sigma #A740) was used at 4 °C for 1 h. The final complex was washed three times with wash buffer (immunoprecipitation buffer containing 125 mM NaCl), resuspended in sample buffer, and subject to SDS-PAGE and immunoblot analysis.

Protein modeling with Rosetta

To model ubiquitin bound to the exosite of UBE3A, we used the Rosetta Relax application through the Rosetta scripts interface47,57. To build a starting structure we aligned UBE3A (PDB ID: 1C42) to NEDD4 in the NEDD4 ubiquitin co-complex (PDB ID: 4B8N)58, which has a complete ubiquitin tail. We used this starting structure with the relax application in Rosetta to generate 30,000 models of ubiquitin docked to UBE3A. To calculate the RMSD of the ubiquitin tail, we used ProFit with residues 71–76 of ubiquitin. The same protocol was used with WT UBE3A, and the QS88e and QS88m mutations which were generated using the Rosetta faab application46.

Sequence alignments of UBE3A from various species were performed using amino acid sequences with the following accession numbers: mouse (NP_001007319.1), chicken (NP_001072258.1), fly (NP.648452.1), frog (NP.001029134.1), and fish (NP.001029134.2). Sequence alignments of UBE3A with human NEDD4 subfamily enzymes were performed using amino acid sequences from the following accession numbers: NEDD4 (NP_006145.2), NEDD4-2 (NP_001138439.1), HUWE1 (NP.115584.3), HECW1 (NP_055867.3), HECW2 (NP_001335697.1), SMURF1 (NP_001029776.1), SMURF2 (NP.001003652.1), and SMURF3 (NP.001257383.1), and ITCH (NP_003111272.1). PDB IDs for additional structural comparisons of HECT domains used in this study are as follows: NEDD4-2 (3JVZ), HUWE1 (3H1D), WWP1 (6Y1J), and ITCH (STUG).

Luciferase assays

Luciferase assays were performed in 96-well plates for all experiments. HEK293T cells were plated at a density of 10,000/well. Cells were transiently transfected with 10 ng of plK-TK-Replica, 30 ng of BAR-pGL3, and 30 ng of the indicated constructs using the FuGENE (Promega) transfection reagent according to the manufacturer’s instructions. Forty-eight hours after transfection, reporter gene expression was assessed using the Dual-Luciferase reporter assay system (Promega) and measured on a Synergy HTX Multi-Mode Reader (BioTek) using Gen5 software v3.08. Luciferase activity was normalized against Renilla activity, and UBE3A missense variant signals were normalized to WT UBE3A.

Protein expression and purification

For protein expression, pET-45b(+) UBE3A constructs were transformed into chloramphenicol-resistant Rosetta™ BL21DE3 competent E. coli cells (Sigma-Aldrich #70954). The next day, a colony was picked and transferred into 1 mL of growth media (10% tryptone, 15% yeast extract, 5% NaCl, and 5% K2HPO4). Just before inoculation, the following was added: 1 mM MgSO4, 10 mL/L trace metals (pre-mixed in deionized water and stored at RT: 0.3/µL CoSO4 7 H2O, 1.5/µL MnCl2 4 H2O, 0.22/µL CuSO4 5 H2O, 0.3/µL H3BO3, 0.25/µL Na2MoO4 2 H2O, 0.5/µL ZnCl2, 0.93/µL NaEDTA + 2 H2O, and 6.17/µL FeCl3), 0.5% glycerol, 100 µg/mL ampicillin, and 30 µg/mL chloramphenicol). Cultures were allowed to shake at 225 RPM at 37 °C for 2−5 h. Afterwards, 1 mL of culture was transferred into 50 mL of growth media and incubated overnight at 225 RPM at 25 °C. The next morning, 500 mL of induction media (inoculation media + 0.5% glycerol) was inoculated to an OD600 of 0.4. The induction culture was shaken at 175 RPM at 37 °C until an O.D. of 0.7 was reached (~2 h). Five hundred microliters of 1 M IPTG was then added to induce UBE3A protein expression and the culture was allowed to shake at 175 RPM at 25 °C for 6 h. Cells were pelleted by centrifugation at 1425 g at 4 °C for 15 min. Protein pellets were resuspended in 25 mL buffers: (25 mM HEPES pH 7.4, 10 mM NaCl, 1% EDTA-free Pierce® protease inhibitor mini tablets (ThermoFisher #A32955), 10 mM imidazole and lysed by sonication. After centrifugation at 13,000×g for 15 min at 4 °C, the resulting lysate was incubated with His Pur® Ni-NTA Resin beads (ThermoFisher #88221) at 4 °C for 60 min. The beads were washed with wash buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM imidazole, 20% glycerol), 20 µg/mL Rosetta® Blue and 500 mM imidazole. UBE3A was eluted with 500 mM imidazole. UBE3A was further purified through size-exclusion chromatography and buffer exchange with 7 K MWCO Zeba® Spin desalting columns (Thermo Scientific #70004) according to the manufacturer’s instructions (final buffer: 25 mM Tris 7.5, 150 mM NaCl, 10% glycerol).
Coated ferromagnetic beads were diluted approximately 2000-fold, to reduce the probability of bead aggregation. Beads were placed into channels on the coated slides and imaged. PSuperM slide containing functionalized substrate was placed in a Helmholtz-inspired apparatus. A clockwise field was actuated at 1 Hz for 5 s, turned off for 5 s, then a counter-clockwise field was actuated at 1 Hz for 5 s, then turned off for 5 s. This actuation cycle was repeated 18 times. Video captured from the CMOS camera was converted into an avi file using ESoft Media Converter software. Images were introduced between His-GST and the HECT domain to excise the affinity tag. The UBE3A Q588E mutant was made by site-directed mutagenesis. Both wild type and UBE3A Q588E were transformed into E. coli BL21 codon plus RIL (Aglent) cells, and expression was induced by adding 0.6 mM IPTG at 16 °C for 18 h. UBE3A was purified on an affinity gravity column followed by TEV protease digestion and size exclusion purification on an AKTA FPLC (GE healthcare). The purify of the fractions was confirmed by SDS-gel electrophoresis, combined, and concentrated to ~10 mg/mL for biophysical assays. N-terminal fluorescein-labeled ubiquitin (Anaspec) was prepared as described previously. Mixtures of 1 μM fluorescein-labeled ubiquitin and varied concentrations of UBE3A were prepared in a 384-well plate. Milli-fluorescence polarization (mP) values were detected by a Tecan Infinite M1000Pro plate reader at 30 °C. Iterative nonlinear curve fitting using a one-site specific binding model was applied in ProFit to obtain binding affinities (Kd).

**Fluorescence polarimetry.** The HECT domain from WT UBE3A was cloned into pShiFduet-1 (Novagen) with an Octa-histidine-GST double affinity tag fused to the N-terminal of the TEV protease digestion sequence introduced between His-GST and the HECT domain to excise the affinity tag. The UBE3A Q588E mutant was made by site-directed mutagenesis. Both wild type and UBE3A Q588E were transformed into E. coli BL21 codon plus RIL (Aglent) cells, and expression was induced by adding 0.6 mM IPTG at 16 °C for 18 h. UBE3A was purified on an affinity gravity column followed by TEV protease digestion and size exclusion purification on an AKTA FPLC (GE healthcare). The purity of the fractions was confirmed by SDS-gel electrophoresis, combined, and concentrated to ~10 mg/mL for biophysical assays. N-terminal fluorescein-labeled ubiquitin (Anaspec) was prepared as described previously. Mixtures of 1 μM fluorescein-labeled ubiquitin and varied concentrations of UBE3A were prepared in a 384-well plate. Milli-fluorescence polarization (mP) values were detected by a Tecan Infinite M1000Pro plate reader at 30 °C. Iterative nonlinear curve fitting using a one-site specific binding model was applied in ProFit to obtain binding affinities (Kd).

**Generation of UBE3A Q588E mice.** All animal procedures were performed in accordance with guidelines set by the Washington University Institutional Animal Care and Use Committee. The mice were maintained on a standard 12:12 h light/dark cycle, where food and water were provided ad libitum. Each generation was crossed to wildtype C57BL/6 mice from Jackson labs. An analogous Q585E mutation in mouse UBE3A (Q585E, NP_001029134.1) was created by designing single guide RNAs (sgRNAs) to create a C<G substitution where ~200 bp isopropanol precipitation. PCR was performed by amplifying a 407 bp fragment with PCR purified kit (Qiagen) and submitted for Sanger sequencing (Genewiz). PCR products were digested with NcoI and XbaI and inserted into pSuperM. PCR products were ligated into pSuperM to ensure homology. The gRNAs were in vitro transcribed using Ambion mMessageMachine kit (Ambion). mRNA of mouse UBE3A was created by designing single guide RNAs (sgRNAs) to create a C<G substitution on the paternal copy of UBE3A. Both wild type and UBE3A Q588E were induced by adding 0.6 mM IPTG at 16 °C for 18 h. UBE3A was purified on an affinity gravity column followed by TEV protease digestion and size exclusion purification on an AKTA FPLC (GE healthcare). The purity of the fractions was confirmed by SDS-gel electrophoresis, combined, and concentrated to ~10 mg/mL for biophysical assays. N-terminal fluorescein-labeled ubiquitin (Anaspec) was prepared as described previously. Mixtures of 1 μM fluorescein-labeled ubiquitin and varied concentrations of UBE3A were prepared in a 384-well plate. Milli-fluorescence polarization (mP) values were detected by a Tecan Infinite M1000Pro plate reader at 30 °C. Iterative nonlinear curve fitting using a one-site specific binding model was applied in ProFit to obtain binding affinities (Kd).
Figure 5a. One-sample t-test (two-tailed) with Benjamini–Hochberg multiple comparisons correction (false discovery rate \( p < 0.05 \)). 

Figure 5b. Tukey’s multiple comparison test detected a significant interaction between WT UBE3A \( \times Q588R \): \( q = 8.580, p < 0.001 \), and between WT UBE3A \( \times Q588R \): \( q = 7.004, p < 0.001 \).

Figure 5c, d. Pearson Chi-square test, exact significance (two-sided). \( p < 0.05 \), \( \chi^2 < 0.005 \), *** \( p < 0.005 \).

Figure 5e, f. Mann–Whitney U-test, exact significance (two-tailed). \( p < 0.05 \), *** \( p < 0.005 \), *** *** \( p < 0.0005 \).

Figure 6h, k. Mann–Whitney U-test, exact significance (two-tailed). \( p < 0.05 \), *** \( p < 0.005 \), *** *** \( p < 0.0005 \).

Figure 6l. Repeated measures ANOVA. \( p < 0.05 \). Supplementary Fig. 1b. One-sample t-test (two-tailed) with Bonferroni multiple comparisons correction (critical value \( p < 0.05 \)). Transform enzyme protein levels were normalized to WT UBE3A and tested for deviance from a theoretical mean abundance level of 100. \( p < 0.05 \). Supplementary Fig. 2b. One-sample t-test (two-tailed) with Bonferroni multiple comparisons correction (critical value \( p < 0.05 \)). RING1B protein levels for each UBE3A mutant-transfected sample was normalized to WT UBE3A and tested for deviance from a theoretical mean abundance level of 100. \( p < 0.05 \). Supplementary Fig. 3d. One-sample t-test (two-tailed) with Benjamini–Hochberg multiple comparisons correction (false discovery rate \( p < 0.05 \)). \( p < 0.05 \), *** *** *** *** \( p < 0.0005 \).

All behavioral statistical analyses were performed in SPSS (v.27). ANOVA assumption of normality was assessed by using the Shapiro–Wilk test. For all reported behavioral tests which were found to violate the assumptions of ANOVA, non-parametric tests were performed. Chi-square goodness of fit tests was used to assess associations between categorical variables. For tests performed across multiple ages, the linear mixed model was used to account for repeated measures.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Protein structures used in this study can be found in the Protein Data Bank (PDB) under the following accession numbers, 6U19, 4GZ2, 1CGZ, 4BNB, 3TJVZ, 3HIH, and 3WUG. Missense variants were identified in the ClinVar database unless otherwise noted, and information was obtained without restrictions on data access. ClinVar accession numbers for variants are listed in Supplementary Data 1. Data supporting the findings in this manuscript are available within the article and in the Source Data file. Source data are provided with this paper.

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Author contributions

K.P.W., X.G., J.Z. K.-S.K. and J.J.Y. performed experiments, and acquired and analyzed data. Y.-C.L. and K.-P.W. performed florescence polarimetry. J.P.S. and J.S.H. performed the METRIS assay and J.S.H. performed Rosetta modeling. S.E.M. assisted with the design and analysis of animal behavior experiments, J.G., S.P. and M.S. provided detailed patient information. The manuscript was written by K.P.W. and J.J.Y. All authors provided critical editing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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