

Mutations in *MAST1* cause mega-corpus-callosum syndrome with cerebellar hypoplasia and cortical malformations.

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Summary

Corpus callosum malformations are associated with a broad range of neurodevelopmental diseases. We report that *de novo* mutations in *MAST1* cause mega-corpus-callosum syndrome with cerebellar hypoplasia and cortical malformations (MCC-CH-CM) in the absence of megalencephaly. We show that MAST1 is a microtubule associated protein that is predominantly expressed in post-mitotic neurons, and is present in both dendritic and axonal compartments. We further show that *Mast1* null animals are phenotypically normal, whereas the deletion of a single amino acid (Leu278del) recapitulates the distinct neurological phenotype observed in patients. In animals harboring *Mast1* microdeletions we find that the PI3K/AKT3/mTOR pathway is unperturbed, whereas Mast2 and Mast3 levels are diminished, indicative of a dominant negative mode of action. Finally, we report that *de novo* MAST1 substitutions are present in patients with autism and microcephaly, raising the prospect that mutations in this gene give rise to a spectrum of neurodevelopmental diseases.

Introduction

The bilateral integration of sensory, motor and cognitive inputs is mediated by the corpus callosum, the largest white matter tract of the brain (Paul et al., 2007). A broad array of neurodevelopmental disorders is known to be associated with malformations of this structure. For example, corpus callosum agenesis has been reported in patients with microcephaly, lissencephaly and polymicrogyria, and has been described in patients with autism (Parrini et al., 2016). Mouse and human genetic studies have provided insight into the molecular machinery that is required for the development of this important anatomical feature (Edwards et al., 2014). It has been shown that the midline crossing of post-mitotic neurons requires transcription factors such as *Satb2* (Britanova et al., 2008), cell adhesion molecules such as L1-CAM (Demyanenko et al., 1999), guidance molecules such as *Netrin1* and the semaphorins (Niquille et al., 2009; Serafini et al., 1996), and cytoskeletal proteins including *Map1b* and the β -tubulin *Tubb3* (Meixner et al., 2000; Tischfield et al., 2010). Collectively these molecules specify the fate of neurons destined to traverse the cerebral hemispheres, guiding their leading process to the correct destination.

While thinning of the corpus callosum is relatively common, in rare instances patients present with a thickening of this myelinated structure (Edwards et al., 2014; Marsh et al., 2017). To date, this phenotype has been reported in patients with neurofibromatosis and megalencephaly-polymicrogyria-mega-corporus-callosum syndrome (DiMario et al., 1999; Gohlich-Ratmann et al., 1998). These diseases are associated with a generalised enlargement in brain size, driven by activation of the PI3K/AKT3/mTOR pathway attributable to mutations in *PIK3R2* and *NF1* (Johannessen et al., 2005; Nguyen et al., 2016; Terrone et al., 2016). As mutations in these genes do not account for all cases, it is apparent that our understanding of the molecular pathology that underlies mega corpus callosum syndromes is incomplete (Hengst et al., 2010). Here, we present a cohort of patients with an enlarged corpus callosum in the absence of megalencephaly, harboring mutations in the uncharacterized microtubule associated protein *MAST1*.

Results

Identification of *MAST1* Mutations

As part of an ongoing endeavor to identify genetic variants associated with structural brain malformations, we undertook whole exome sequencing on 7 patient-parent trios in which the affected individual presented with a striking enlargement of the corpus callosum. This led to the identification of *de novo* mutations in the gene *MAST1* (*microtubule associated serine threonine kinase 1*) in 6 of the 7 affected individuals (Figure 1A-T). Each of these patients presented with a hyperplastic corpus callosum (particularly over the genu and mid-body), cerebellar hypoplasia, ventricular dilation, and impaired motor and verbal performance (Table 1). Four patients presented with gyral simplification (P2, P3, P5, P6), three with dysplastic longitudinal gyri (P1, P5, P6) (Pierson et al., 2008), and one (P4) with periventricular nodules of white matter. We refer to this syndrome as mega-corpus-callosum with cerebellar-hypoplasia and cortical malformations (MCC-CH-CM).

Through the Genematcher platform, four additional patients with *de novo* mutations in *MAST1* were identified. These individuals presented with either microcephaly accompanied by motor deficits (P7, P8) or autism spectrum disorder (P9, P10) (Figure S1A-C,D,E, Table S1) (Gilissen et al., 2014; Sobreira et al., 2015). In each case the mutation was verified by Sanger sequencing, was unreported in publicly available genome databases (e.g. dbSNP, 1000 genome, ExAC, see Table S2), and was in a highly conserved residue (Figure S1F-L) that was predicted to be deleterious when mutated (Table 1, Table S1, CADD score) (Kircher et al., 2014; Lek et al., 2016). We did not identify any unreported variants in our patient cohort with the exception of a silent mutation in *TUBGCP5* (T457T) in P2 and a *de novo* mutation in NIMA related kinase 1 (NEK1) in P8 (Table 1 and Table S1). It should be noted that mutations in NEK1 have been previously associated with amyotrophic lateral sclerosis, cilial dysfunction and skeletal diseases; however, patient P8 did not present with symptoms consistent with these disorders (Kenna et al., 2016; Thiel et al., 2011). Three of the MCC-CH-CM associated variants in *MAST1* were single amino acid deletions positioned in the hydrophobic core of a four-helix bundle in the domain of unknown function DUF1908 (P1: p.Glu194del; P2: p.Lys276del; P3: p.Leu278del) (Figure S1M) while the remainder (P4-P6: p.Gly517Ser) harbored a recurrent missense mutation in the kinase domain of the protein (Figure 1U-V). These microdeletions were not found in control individuals in the

ExAC genome browser; however, a single microdeletion between the kinase and PDZ domains (Glu697del) has been reported (See Table S2).

MAST1 is a microtubule associated protein

Little is known of the function of the MAST family of proteins (MAST1-4). MAST2 was initially cloned from mouse testes, and was shown to function as a kinase and to interact with microtubules via other microtubule associated proteins (MAPs) (Walden and Cowan, 1993). To ascertain whether MAST1 associates with the microtubule cytoskeleton, we performed coupled *in vitro* murine (m)Mast1-driven transcription and translation (TnT) reactions in rabbit reticulocyte lysate containing ³⁵S-methionine (mMast 1 shares 94% sequence identity with human MAST1). The reaction products were then assayed for their ability to bind to microtubules in the absence or presence of MAPs. We found that mMast1 associates with taxol-stabilized microtubules in a MAP-dependent manner (Figure 1W,X; n=3; $p<0.05$). Next, we asked whether the *MAST1* mutations found in our patient cohort influence microtubule binding. To do this, we expressed each Mast1 mutation as a radiolabeled polypeptide via TnT, and repeated the microtubule binding assay. While translation efficiency was similar for all variants, we found that the K276 deletion significantly enhanced Mast1 binding to microtubules (Figure 1Y,Z; n=9; WT vs K276del $P<0.05$). Taken together, these data show that Mast1 binds to microtubules in a MAP-dependent manner, and that mutations can result in perturbation of this interaction.

MAST1 is Expressed in Post-Mitotic Neurons

To gain insight into the role of MAST1 in neurodevelopment, we analyzed its expression in the human and mouse brain during early developmental stages (Figure 2A and 2B). To this end we extracted mRNA from the developing murine brain (E10.5, E12.5, E14.5, E16.5, E18.5, P0 and P6), generated cDNA, and performed qPCR analysis. We observed that Mast1 expression begins at E12.5, peaks at E16.5, and decreases postnatally (Figure 2B). Consistent with this result, qPCR analysis of human fetal brain cDNA showed moderate expression of MAST1 at gestation week 13 and 22 (Figure 2A). To determine which cells express Mast1, we performed immunohistochemistry on the murine cortex at E12.5, E14.5, E16.5 and P0 (n=3). We used a commercially available Mast1 antibody, having validated its specificity employing a Mast1

knockout animal (Figure S2G-L, Figure S3B-D). We observed staining in the developing cortical plate and intermediate zone at these time points (Figure 2C-F). Further analysis at E14.5 and E16.5 showed that Mast1 is located in the cytoplasm in Tuj positive post-mitotic neurons, but is largely absent from Sox2 positive progenitors and Tbr2 positive intermediate progenitors (Figure 2S-AP) (n=3). This expression pattern is mirrored in human cerebral organoids that were cultured for 64 days, where we again observed co-localisation with post mitotic markers (Figure S2A-F) (n=10 organoids) (Lancaster et al., 2013). Immunostaining on P0 mouse brain sections showed that Mast1 is present in Tuj positive corpus callosal fibers that cross the midline (n=3 animals) (Figure S2G-L). To gain further insight into the subcellular localisation of Mast1, we cultured primary P0 cortical neurons and P7 cerebellar granule neurons and performed immunostaining. We again validated the specificity of our Mast1 antibody for cell culture experiments employing a Mast1 KO animal (Figure S2S-T). We performed double staining with antisera specific for the axonal marker Tau or the dendritic marker Map2 (Kosik and Finch, 1987). We observed Mast1 staining throughout the soma, as well as in dendritic and axonal compartments in cortical neurons (Figure 2G-R, n=5) and in cerebellar granule neurons (Figure S2M-R, n=3). This staining had a punctate appearance, suggesting that Mast1 may associate with vesicular structures that are trafficked along the microtubule cytoskeleton. qPCR analysis of Mast1 expression in the adult mouse showed that its expression persists in all brain regions (albeit at much lower levels), and that it is present in the testes, liver and spleen at very low levels (n=3) (Figure S2U). These results are consistent with expression data that is available through online databases (e.g. Allen brain atlas, GTEx) (Consortium, 2013; Morris et al., 2010). Taken together, these data show that Mast1 is predominantly expressed in post-mitotic neurons in the developing nervous system.

An Enlarged Corpus Callosum in a *Leu278Del Mast1* Mouse Model

To investigate the effect of *Mast1* mutations *in vivo*, we exploited the power of the CRISPR-Cas9 genome editing system to generate a *Mast1* knockout (KO) mouse and a *Mast1* *Leu278del* (*L278del*) mouse employing *guide RNAs* targeted to exon 8 (Figure S3A) (Wang et al., 2013). We decided to recapitulate the mutation identified in patient P3 because of the striking callosal, cortical and cerebellar phenotypes. As homozygous *L278del/del* animals die shortly after birth, we focused our initial analysis on homozygous *Mast1* KO and heterozygous *L278del/+* mice,

which are viable. We analyzed the thickness of the genu of the corpus callosum on Nissl stained coronal sections. While *Mast1* KO animals were indistinguishable from wild type littermate controls (Figure S3E-G; n=5; +/+ v KO/KO; P>0.5), *L278del*/+ animals presented with a significantly thicker corpus callosum in comparison to wild-type littermate controls (Figure 3A-E; n=5; +/+ v *L278del*/+; P<0.001). High resolution volumetric magnetic resonance imaging (MRI) analysis confirmed an increased corpus callosum volume in *L278del*/+ animals (Figure 3H; n=3; +/+ v *L278del*/+; P<0.05), and revealed that the thickening was most prominent in the regions encompassing the genu and the mid-body (Figure 3F-G). Analysis of animals aged P10 again revealed a thicker corpus callosum in *L278del*/+ mice (Supplementary Figure 3K-M, n=5; +/+ v *L278del*/+; P<0.05). While off-target mutations in Cas9-modified mice are rare (Iyer et al., 2015), we confirmed that this phenotype was not due to a linked mutation by undertaking whole exome sequencing for our *L278del*/+ mouse line. We identified no other non-synonymous mutations on chromosome 8 in this line (Table S3). Moreover, we generated a second independent *L278del* mouse line, and once again observed an enlarged corpus callosum (Figure S3H-J; +/+ v *Leu278del*/+_Line#7; n=3; P<0.005).

We then asked whether the increase in the thickness of the corpus callosum of the *L278del*/+ mice was due to an increase in the thickness of myelinated axons. In light of our MRI studies, we focused our analysis on the mid body of the corpus callosum where we observed the most pronounced phenotype in *L278del*/+ mutants. The corpus callosum of adult animals was microdissected, samples stained to enable clear visualisation of myelinated neurons crossing the midline, and images captured at high resolution (8900x) with transmission electron microscopy (Figure 3I-J) (Sturrock, 1980; West et al., 2015). We observed no significant difference in either myelin thickness (Figure 3K; n=5; n>500 axons per animal; +/+ v *L278del*/+; P>0.1) or axonal caliber (Figure 3L; n=5; n>1500 axons per animal; +/+ v *L278del*/+; P>0.1) when comparing littermate controls with *L278del*/+ animals. An analysis of the distribution of axon calibers in *L278del*/+ animals again revealed no significant difference in comparison to littermate controls (n= 5; +/+ v *L278del*/+; P>0.1 for all bins) (Figure S3K). Reflecting this, an assessment of the G-ratio (axonal diameter/total fiber diameter), revealed no significant difference between genotypes (n=5, n>300 fibers animal, +/+ vs *L278del*/+; P>0.1) (Guy et al., 1989).

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217 Given these results, we asked whether more myelinated axons might cross the midline in
 218 *L278del/+* animals. To assess this, TEM images were acquired at a magnification of 710x along
 219 the dorso-ventral axis of the corpus callosum. These images (10-12 per animal) were tiled,
 220 resulting in a single image that encompassed the entire cross section of the corpus callosum. A
 221 box 30µm wide was overlaid onto the structure, and the total number of myelinated axons counted
 222 manually (Figure 3M-N). Statistical analysis revealed a significant increase in the number of
 223 myelinated axons within that box in *L278del/+* animals in comparison to littermate controls (n=5
 224 animals per genotype, n=3 images per animal, *+/+ v L278del/+*; *P*<0.005). Finally, we counted
 225 the number of Olig2 positive oligodendrocytes and GFAP positive astrocytes in a 200µm wide
 226 box that was overlaid onto coronal sections of the corpus callosum (Figure S3O-R). This
 227 revealed that in *Leu278del/+* animals there are more oligodendrocytes and astrocytes in
 228 comparison to wild-type controls (n=5; *+/+ v L278del/+*; Olig2: *P*<0.05; GFAP: *P*<0.05) (Figure
 229 S3S,V). There is, however, no significant difference in the density of these cell types when
 230 comparing genotypes (n=5; *+/+ v L278del/+*; Olig2: *P*>0.1; GFAP: *P*>0.05) (Figure S3T,W).
 231 Taken together these data show that our *L278del/+* mouse model recapitulates the enlarged
 232 corpus callosum observed in affected patients, which we attribute to an increase in the number
 233 of myelinated axons crossing the midline.

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235 **Cortical and cerebellar hypoplasia in the *L278del Mast1* Mouse Model**

236 We extended our analysis of the *L278del* and *KO* mouse lines to the cortex and cerebellum, which
 237 are consistently affected in our *MAST1* patient cohort. MRI volumetric analysis of adult animals
 238 revealed an overall reduction in cortical volume in *L278del* heterozygotes (Figure 4A-C; n=3; *+/+ v L278del/+*; *P*<0.05). We confirmed this finding by undertaking Nissl and NeuN staining, where
 239 we observed a significant reduction in cortical thickness in *L278del* animals that was most
 241 pronounced in caudal regions (Nissl: Figure 4D-F; n=5; *+/+ v L278del/+*; *P*<0.001; NeuN: Figure
 242 S4N-Q; n=5; *+/+ v L278del/+*; *P*<0.0001). This was most marked for Er81 positive layer V neurons
 243 (Figure 4J-L; n=5; *+/+ v L278del/+*; *P*<0.005). We did not observe a significant difference in the
 244 thickness of Foxp2 positive layer VI neurons (Figure S4J-M; n=5; *+/+ v L278del/+*; *P*>0.1) (Ferland
 245 et al., 2003) or Cux1 positive granule cells (Figure 4G-I; n=5; *+/+ v L278del/+*; *P*>0.5) (Cubelos

et al., 2015). Analysis of the cortex at P10 by Nissl staining again revealed that it was thinner in *L278del/+* mutants (Supplementary Figure 4Y-AA, n=5; *+/+* v *L278del/+*; $P<0.05$). Consistent with our previous findings, adult *Mast1* KO animals appeared to be phenotypically normal, without morphological or layering defects in the adult KO cortex (Figure S4A-Q).

MRI volumetric analysis of the cerebellum revealed a severe reduction in *L278del* heterozygotes (Figure 5A-C; n=3; *+/+* v *L278del/+*; $P=0.0005$). Nissl staining as well as NeuN and calbindin immunohistochemistry showed that the foliation and lamination of the cerebellum was unaffected in *L278del* heterozygotes; however, there was a significant reduction in the thickness of the granule cell layer and the molecular cell layer (Figure 5D-I). We calculated the density of granule cells as well as the linear density of Purkinje cells, allowing us to estimate the total number of these cell types in mid sagittal sections. While the density of both cell types was similar in *Leu278del* mutants in comparison to littermate controls (Figure S5A-B), we observed a significant reduction in the total number of granule cells per section (Figure 5J; n=5; *+/+* v *L278del/+*; $P<0.05$). We also observed a reduction in the total number of Purkinje cells per section, but this reduction was not statistically significant (Figure 5K; n=5; *+/+* v *L278del/+*; $P>0.5$). Volumetric analysis of the colliculi, putamen, thalamus and olfactory bulbs revealed no significant difference between wild-type littermates and *L278del* heterozygotes (Figure S5; n=3; *+/+* v *L278del/+*; $P>0.5$ for all regions). There was a reduction in the volume of the hippocampus in *L278del* heterozygotes, but this difference was not statistically significant (Figure S5; n=3; *+/+* v *L278del/+*; $P=0.34$).

Volumetric analysis of the colliculi, putamen, thalamus and olfactory bulbs revealed no significant difference between wild-type littermates and *L278del* heterozygotes (Figure S5; n=3; *+/+* v *L278del/+*; $P>0.5$ for all regions). There was a reduction in the volume of the hippocampus in *L278del* heterozygotes, but this difference was not statistically significant (Figure S5; n=3; *+/+* v *L278del/+*; $P=0.34$).

Neuronal apoptosis in the *L278Del Mast1* Mouse Model

To determine if the smaller cortex and cerebellum in our *L278del* mutant mice is a result of neuronal cell death during development, we performed an activated caspase 3 staining. We

detected a dose-dependent increase in the number of apoptotic cells in the P0 cortex of wild type versus heterozygous and homozygous *L278del* mice (Figure 4P-U,Y, AB; n=5; +/+ vs *L278del*/+ $P<0.005$; +/+ vs *L278del*/del $P<0.0001$; *L278del*/+ vs *L278del*/del $P=0.0001$). Consistent with our anatomical analysis of adult animals, we found that apoptosis was more severe in the caudal regions of the P0 cortex in the *L278del*/+ and *L278del*/del animals (Supplementary Figure 4X, n=5; rostral vs caudal: *L278del*/+ $P<0.001$; *L278del*/del $P<0.0001$). At P10 and in 8 week old adult mice, apoptosis in *L278del*/+ mice was comparable to wildtype controls (Supplementary Figure 4AB-AE, n=5; +/+ vs. *L278del*/+; P10, $P>0.1$; Adult: $P>0.1$). An analysis of activated caspase 3 staining in the P0 cerebellum revealed a large increase in *L278del*/+ and *L278del*/del animals in comparison to wildtypes (Figure 5L,M-R) (n=3; +/+ vs *L278del*/+ $P<0.01$; +/+ vs *L278del*/del $P=0.0001$; *L278del*/+ vs *L278del*/del $P<0.005$). To ascertain whether this cell death is associated with intrinsic suicide pathways, we stained P0 cortical sections with sera that targets activated Caspase 9, an upstream regulator of caspase 3 that is associated with mitochondrial mediated apoptosis (Hyman and Yuan, 2012). We observed a dose-dependent increase in the number of activated caspase 9 positive cells in the cortex of heterozygous and homozygous *L278del* mice (Figure 4V-Y,Z; n=5; +/+ vs. *L278del*/+ $P<0.05$; +/+ vs. *L278del*/del $P<0.0001$; *L278del*/+ vs. *L278del*/del $P<0.01$). Intriguingly, this was not associated with the upregulation of the tumour suppressor p53 (Figure S4R-W), which has been associated with a range of stressful cellular events including DNA damage, defects in cell cycle progression, and oxidative and nutritional stress (Szybinska and Lesniak, 2017). Taken together, these data show that the *L278del* mutation results in a hypoplastic cortex and cerebellum, a phenotype that is associated with neuronal apoptosis mediated by activated caspases 3 and 9, but not p53 upregulation.

The *L278* Microdeletion Alters *Mast1/2/3* Protein Levels but not the PI3K/AKT3/mTOR Pathway

Next, we exploited these mouse models to investigate the underlying molecular impairment associated with *MAST1* mutations. Western blot analysis of P0 brain lysates showed a complete absence of Mast1 protein in *KO* animals (n=6) with an unexpected dose dependent reduction in Mast1 levels in the *L278del* line 9 (Figure 6A, n=4, Mast1: +/+ vs *L278del*/+ $P<0.0001$; +/+ vs *L278del*/del $P<0.0001$). This absence of protein in the Mast 1KO animals correlates with a

dramatic reduction in mRNA levels, but this is not the case in *L278del* mice where *Mast1* transcript levels are statistically similar in *+/+*, *L278del/+*, and *L278del/L278del* animals (Figure S6A, E). We assessed whether the reduction in *Mast1* protein levels would influence the levels of other MAST family members. Relying on western blot analysis, we observed a striking reduction in the levels of *Mast2* and *Mast3* in our *L278del* animals (Figure 6A-B; *n*=4; *Mast2*: *+/+* vs *L278del/+* *P*<0.0001; *+/+* vs *L278del/L278del* *P*<0.0001; *Mast3*: *+/+* vs *L278del/+* *P*<0.0001; *+/+* vs *L278del/L278del* *P*<0.0001). This was not attributable to changes in the mRNA levels of *Mast2* and *Mast3*, which were similar in *+/+*, *L278del/+*, and *L278del/L278del* animals (Figure S6B-C). In contrast, in our *Mast1* KO animals we observed a significant increase in the levels of *Mast2* and *Mast3* (*Mast2*: *+/+* vs KO *P*<0.0001; *Mast3*: *+/+* vs KO *P*<0.001), again with no change in the mRNA levels (Figure S6E-F). These data imply that the *L278del* mutation in *Mast1* acts by a dominant negative mode of action, whereas a form of post-transcriptional compensation occurs in the *Mast1* KO line.

Finally, as previous studies have implicated activation of the PI3K/AKT3/mTOR pathway in corpus callosum hyperplasia associated with megalencephaly, we assessed the phosphorylation state of AKT and rpS6 in our *L278del* mice (Figure 6B) (Broix et al., 2016; Poduri et al., 2012; Riviere et al., 2012; Terrone et al., 2016). We observed no difference in the levels of p-AKT_{S473} and p-rpS6_{S240/244} when comparing heterozygous and homozygous *L278del* brain lysates with wild type controls (Figure 6C,D; *n*=5 animals; *+/+* vs *L278del/+* AKT *P*>0.5; *+/+* vs *L278del/L278del* AKT *P*>0.5; *L278del/+* vs *L278del/L278del* AKT *P*>0.5; *+/+* vs *L278del/+* rpS6 *P*>0.5; *+/+* vs *L278del/L278del* rpS6 *P*>0.5; *L278del/+* vs *L278del/L278del* rpS6 *P*>0.5).

Discussion

Here we report that *de novo* mutations in *MAST1* cause MCC-CH-CM, a disease characterised by a striking enlargement of the corpus callosum, cerebellar hypoplasia and cortical malformations. For patients with this constellation of phenotypes, mutations in *MAST1* appear to be the primary genetic cause, as we observed *MAST1* variants in 6 of the 7 patients that we studied. We have further shown that *MAST1* is expressed predominantly in post-mitotic neurons in the developing nervous system, and that it is present in the soma as well as dendritic and

axonal compartments. We demonstrate that a *L278del* mouse line recapitulates the enlarged corpus callosum and cerebellar hypoplasia observed in patients, while the *Mast1* KO line lacks any morphological defects. We report extensive cortical and cerebellar apoptosis in our *L278del* mice that is mediated by activated caspases 3 and 9, but not by p53 upregulation. Our results show that unlike other syndromes associated with an enlarged corpus callosum, activation of the PI3K/AKT3/mTOR pathway does not appear to be the underlying pathogenic driver (Terrone et al., 2016).

What molecular mechanism underlies MCC-CH-CM? We have shown that MAST1 binds to microtubules in a MAP-dependent manner, but this binding is only altered in the case of the K276del mutation. It therefore seems unlikely that alterations in microtubule affinity cause the spectrum of phenotypes we observe in patients with MMC-CH-CM. Analysis of our Leu278del mouse model has shown a dramatic reduction in the levels of Mast 2 and 3. This result suggests that pathogenic microdeletions in Mast1 act by a dominant negative mechanism, and that the Mast proteins (like other AGC kinases) may physically interact (Leroux et al., 2017). Notably, three of the deletions detected in patients were located in the domain of unknown function (DUF1608) which adopts a 4-helix bundle structure in solution (PDB 2M9X). Since the helices pack together to form a hydrophobic core, it is expected that the Glu194del, Lys276del, and Leu278del microdeletions would disrupt this structure by changing the register of the alpha helices. It is therefore conceivable that these MAST1 microdeletions exert a dominant negative effect by titrating out functional endogenous MAST proteins and/or targeting them for degradation. The consequence of the reduction in Mast1/2/3 levels is likely to be complex, influencing the phosphorylation of multiple targets. Little is known of the targets of the MAST family; however, Andrade and colleagues have recently shown that in mature dopaminergic neurons in the striatum, MAST3 phosphorylates ARPP-16 at Ser46, which in turn inhibits the serine-threonine phosphatase PP2A (Andrade et al., 2017). Mast2 has been reported to interact with β 2-syntrophin, but its targets are otherwise undefined (Lumeng et al., 1999). Future experiments that define and validate the targets and binding partners of the MAST family in the developing brain will provide valuable insight on this front.

Why do our *L278del* mice have a thicker corpus callosum? We report that our *Leu278del* mice present with an enlarged corpus callosum that is most severe in the midbody and genu, and manifests despite an increase in cortical apoptosis. Electron microscopic analysis has revealed that this phenotype is not attributable to either an increase in the caliber of myelinated neurons, or the thickness of the myelin sheath itself, but is associated with an increase in the number of axons that project to the contralateral hemisphere. Such a phenotype may result from: (1) an increase in the branching of callosal fibers; (2) the formation of multiple axons originating from the soma of projection neurons; or (3) a change in the fate of differentiating neurons during embryogenesis, resulting in an increase in the number of callosal projection neurons at the expense of sub-cortical projection neurons (Baranek et al., 2012; Britanova et al., 2008).

Finally, we report the presence of *de novo* *MAST1* substitutions in patients with severe autism and microcephaly. Notably, a *de novo* P500L mutation in *MAST1* has also been reported in a patient presenting with cerebral palsy (McMichael et al., 2015). This suggests that mutations in *MAST1* give rise to a spectrum of neurodevelopmental diseases. Unlike those mutations that we describe in patients with MCC-CH-CM, these variants are all substitutions, which might account for the different clinical phenotypes. In light of these findings it is conceivable that mutations in *MAST2* and *MAST3*, both of which are expressed in the developing and adult brain, might also result in neurological disease (Garland et al., 2008). In conclusion, we have defined the genetic lesion that causes MCC-CH-CM, and revealed the importance of the *MAST* family in global brain development.

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Author Contributions

Conceptualisation: R.T.; D.A.K.; Methodology: R.T.; Validation: R.T.; M.F.M.; Formal Analysis: R.T.; A.W.W.; I.L.; M.C.S. L.L.; Visualisation: R.T.; I.L.; M.C.S; Software: S.L.; Investigation: R.T.; I.L.; A.W.W.; T.V.D.; J.W.; B.W.B.; M.C.S.; T.G.; M.B.; G.T.; N.B.B.; A.R.P.; A.P.; G.T.; G.V.; E.D.G.; N.B.P.; A.D.; N.V.; E.F.; U.K.; T.A.L.; S.V.; L.A.; S.M.H.; K.A.A.; W.B.D.; G.M.; Writing original draft: R.T.; D.A.K; Writing reviewing and editing: all authors; Funding acquisition: W.B.D.; J.C.T.; A.R.; J.A.B.; L.B.; G.M.C.; T.M.P.; F.B.; T.A.L.; J.C.; N.J.C.; D.A.K.; Supervision: J.C.T.; A.R.; J.A.B.; L.B.; G.M.C.; T.M.P.; F.B.; J.C.; N.J.C.; D.A.K.

Declaration of Interests

The authors declare no competing interests.

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Figure Legends

554 **Figure 1. Patients with *MAST1* mutations.** Selected magnetic resonance images from patients
555 P1 (**A-D**), P2 (**E-H**), P3 (**I,J**), P4 (**K,L**), P5 (**M-P**) and P6 (**Q-T**) in the midline sagittal plane (**A, E,**
556 **I, K, M**), parasagittal plane (**Q**), and axial planes through the brainstem (**F, N**), lateral ventricles
557 (**B, C, G, J, L, O, R, S, T**), and high convexities (**D, H, P**). All patients have a cortical malformation
558 or dysgyria characterized by diffuse undersulcation, shallow sulci (arrowheads in **B, C, G, J, L,**
559 **O, R, S, T** point to selected more obvious areas), and in the more severely affected mildly thick
560 cortex (**J, G**; thus consistent with mild lissencephaly). While diffused, the cortical malformation
561 appears most severe in the posterior frontal and perisylvian regions. The lateral ventricles are
562 mildly to moderately enlarged, and the corpus callosum is abnormally thick (arrows in **A, E, I, K,**
563 **M, Q**), accompanied by mildly thick white matter. The brainstem - especially the pons - is mildly
564 (**E**) or moderately (**A, I, K, M, Q**) small, and in at least one child a prominent ventral midline cleft
565 of the pons is seen (arrow in **N**). Available axial images through the high convexity of the cerebral
566 hemispheres showed a very dysplastic, longitudinally oriented gyral pattern (long arrows in **D, H,**
567 **P**). (**U**) Schematic representation of the *MAST1* genomic locus shows the position of the
568 mutations identified in patients P1-P6. (**V**) The *MAST1* protein consists of a domain of unknown
569 function (DUF1908, shown in red), a kinase domain (shown in yellow) and a PDZ domain (shown
570 in blue). The amino acid boundaries of each of the domains are shown. (**W-Z**) Autoradiograph
571 showing the results of the microtubule binding assay with Mast1. Murine Mast1 was radiolabeled
572 (³⁵S) by *in vitro* transcription and translation in rabbit reticulocyte lysate, before incubation with a
573 porcine microtubule extract in the presence or absence of microtubule associated proteins

(MAPs) (W,X). Following microtubule polymerization in the presence of Taxol, pelleted microtubules were analyzed by polyacrylamide gel electrophoresis (PAGE) and the ratio of pelleted radiolabeled-mMast1 to tubulin levels determined. This experiment revealed a decrease of binding of Mast1 to microtubules in the absence of MAPs (X; n=3 technical replicates; two-tailed unpaired t-test; $t_4=3.265$, $P<0.05$). Patient mutations were introduced into mMast1, radiolabeled by *in vitro* transcription and translation (TnT), and microtubule binding assessed (Y,Z). As each mutant peptide was radiolabeled, the ratio of microtubule bound Mast1 (in the pelleted fraction), to total radiolabelled Mast1 was determined. Comparison of pelleted wild-type Mast1 to the K276del mutation shows a significant alteration in microtubule binding, and a similar trend for the L278del. (Z; n=6-9 repeated experiments; one-way ANOVA with Dunnett's multiple comparison; WT vs K276del $P<0.05$). * shows $P<0.05$; ** shows $P<0.01$; *** shows $P<0.001$; **** shows $P<0.0001$. Error bars show mean \pm the standard error of the mean.

Figure 2. Mast1 expression in human and mouse embryonic brain. (A) qPCR analysis reveals that *MAST1* mRNA is expressed in the human developing fetal brain at gestational week (GW) 13 and the fetal frontal lobe at GW22. (B) qPCR analysis performed on mouse brain cDNA libraries from E10.5 to P6 show that Mast1 expression peaks at E16.5 in mice (n=3 animals per timepoint). (C-F) Immunohistochemistry employing a validated Mast1 antibody indicates staining in the post mitotic cortical plate and intermediate zone from E12.5 to P0 in mice. (G-R) Cultured P0 cortical neurons at 5-DIV staining with the axonal marker Tau (G-L) and dendritic marker Map2 (M-R) showing that Mast1 (I,L,O,R) is present in both axonal and dendritic compartments. Dashed boxes in G-I and M-O and expanded in J-L and P-R, respectively. (S-AP) Immunohistochemistry employing the progenitor marker Pax6 (S-U, AE-AG), intermediate progenitor marker Tbr2 (W-Y, AI-AK), and post mitotic marker Tuj (AA-AC, AM-AO) on E14.5 (S-AD) and E16.5 (AE-AP) murine sections reveal that Mast1 expression is restricted to post-mitotic neurons at these time points (PP: preplate; CP: cortical plate; MZ: marginal zone; IZ: intermediate zone; VZ: ventricular zone).

Figure 3. L278del mice have an enlarged corpus callosum associated with an increase in the number of callosal axons. (A-D) Nissl-stained sections of 8-week old adult brains highlighting the thicker corpus callosum in *L278del/+* animals compared to wild type littermates

(black boxes in **A,B** expanded in **C,D**). Quantification of the thickness at the septum (**E**) reveals a significantly thicker corpus callosum in *L278del/+* animals (n=5 animals per genotype; unpaired t-test; $+/+ \text{ v } L278del/+$; $t_8=6.217$, $P<0.001$). (**F-G**) MRI reconstructions of the corpus callosum in wild-type controls (**F**) and *L278del/+* mice (**G**). The heat map reflects the thickness of the corpus callosum (blue: thinner region, red: thicker region). Note that in *L278del/+* animals the genu and mid body region are most affected. (**H**) MRI volumetric quantification of the corpus callosum shows that this structure is significantly larger in *L278del/+* animals compared to wild type littermates (n=3 animals per genotype; two-tailed unpaired t-test; $+/+ \text{ v } L278del/+$; $t_4=4.233$, $P<0.05$). (**I-J**) Electron microscopy images showing cross-sections of the corpus callosum at the midbody (region depicted with boxes in F and G). Myelinated axons can be clearly seen. (**K**) Quantification of myelin thickness reveals no significant difference between *L278del/+* animals and wild type littermates (n=5 animals per genotype, >500 myelinated axons per animal; two-tailed unpaired t-test; $t_8=1.001$, $P>0.1$). (**L**) Quantification of axonal caliber reveals no significant difference between *L278del/+* animals and wild type littermates (n=5 animals per genotype, >1500 myelinated axons per animal; $+/+ \text{ v } L278del/+$; two-tailed unpaired t-test; $t_8=1.786$, $P>0.1$). (**M-N**) Assessment of the total number of myelinated axons within a 30 μ m wide box extending along the ventro-dorsal axis of the corpus callosum. (**O**) Quantification reveals a significant increase in axonal count in the *L278del/+* animals in comparison to wild-type littermates (n=5 animals per genotype, 3 images analyzed per animal; two-tailed unpaired t-test; $t_8=4.095$, $P<0.01$). * shows $P<0.05$; ** shows $P<0.01$; *** shows $P<0.001$; **** shows $P<0.0001$. Error bars show mean \pm the standard error of the mean.

Figure 4. *L278del* mice have a reduction in cortical volume associated with an increase in neuronal apoptosis. (**A,B**) MRI reconstructions of the cortex in adult animals reveals a reduction in cortical volume in *L278del/+* mice in comparison to littermate controls. (**C**) Quantification of the MRI cortical volume (n=3 animals per genotype; two-tailed unpaired t-test; $+/+ \text{ vs } L278del/+$; $t_4=2.902$, $P<0.05$). (**D-E**) Nissl stained sections of the adult somatosensory cortex reveals a reduction in cortical thickness in *L278del/+* adult mice in comparison to littermate controls. (**F**) Quantification of cortical thickness (n=5 animals per genotype; two-tailed unpaired t-test; $+/+ \text{ vs } L278del/+$; $t_8=5.417$, $P<0.001$). (**G-H**) Labeling of Cux1-positive layer II-III and (**J-K**) Er81-positive

layer 5 neurons in wild type and *L278del/+* adult old mice. **(I)** Quantification reveals no significant difference in Cux1 layer thickness when comparing genotypes, but a significant reduction in the size of the Er81-positive layer **(L)** (n=5 animals per genotype; 2-way repeated measures ANOVA with a Bonferroni test for multiple comparisons; *+/+* vs *L278del/+*; Cux1 layer $P>0.5$; Er81 layer $P<0.01$). **(M-O)** Representative Nissl stained and **(P-U)** activated caspase-3 stained P0 cortical sections of littermate controls **(M,P,S)**, *L278del/+* **(N,Q,T)**, and *L278del/del* **(O,R,U)** animals. The white boxes in **P,Q,R** are expanded in **S,T,U**. The number of Caspase3-positive apoptotic cells increases in a dose dependent manner in heterozygous and homozygous *L278del* animals in comparison to wild type siblings. **(V-X)** P0 cortical sections stained with sera against activated caspase-9 again reveals a dose- dependent increase in staining in *L278del/+* and *L278del/del* animals in comparison to wild type siblings. **(Y)** Quantitation of capase-3 staining and **(Z)** caspase-9 staining (n=5 animals per genotype; 2-way repeated measures ANOVA with a Bonferroni test for multiple comparison; Caspase-3: *+/+* vs. *L278del/+* $P<0.01$; '*+/+* vs. *L278del/del* $P<0.0001$; *L278del/+* vs. *L278del/del* $P<0.001$; Caspase-9: *+/+* vs. *L278del/+* $P<0.05$; '*+/+* vs. *L278del/del* $P<0.0001$; *L278del/+* vs. *L278del/del* $P<0.01$). * shows $P<0.05$; ** shows $P<0.01$; *** shows $P<0.001$; **** shows $P<0.0001$. Error bars show mean \pm the standard error of the mean.

Figure 5. *Leu278del* mice have a hypoplastic cerebellum. **(A-B)** MRI reconstructions of the cerebellum from wild type littermates **(A)** and *Leu278del/+* **(B)** adult animals. **(C)** Quantification reveals a significant reduction in cerebellar volume (mm^3) in *Leu278del/+* animals (n=3 animals per genotype; two-tailed unpaired t-test; *+/+* vs. *L278del/+*; $t_4=10.16$, $P<0.001$). **(D-G)** Nissl stained sagittal sections of 8-week old cerebellum confirms the reduction in cerebellar size and indicates that lamination within the cerebellum is normal in *Leu278del/+* animals. **(H-I)** Immunostaining employing the neuronal marker NeuN (shown in red) and the Purkinje cell marker Calbindin (shown in green) revealed a reduction in the thickness of the granule cell layer and molecular layer in *Leu278del/+* animals. **(J-K)** Estimates of the total number of granule cells **(J)** and Purkinje cells **(K)** per midsagittal section in *Leu278del/+* animals and littermate controls (n=5 animals per genotype; two-way ANOVA with a Bonferroni correction; *+/+* vs. *L278del/+*; granule cell counts: $P<0.05$; Purkinje cell counts: $P>0.5$). **(L-Q)** Immunohistochemistry employing sera for

activated Caspase3 reveals an increase in apoptosis in the *L278del/+* and *L278del/del* animals in the developing cerebellum at P0. The white boxes in **L,N,P** are expanded in **M,O,Q**, respectively. **(L)** Quantification of capase3 staining (n=3 animals per genotype; one-way ANOVA with Tukey multiple comparison; *+/+* vs. *L278del/+* $P<0.01$; *+/+* vs. *L278del/del* $P<0.001$; *L278del/+* vs. *L278del/del* $P<0.01$). * shows $P<0.05$; ** shows $P<0.01$; *** shows $P<0.001$; **** shows $P<0.0001$. Error bars show mean \pm the standard error of the mean.

Figure 6. The *L278del* mutation influences Mast1/2/3 protein levels, but does not activate the PI3K/AKT3/mTOR pathway. (A-B) Western blot analysis of Mast1, Mast2 and Mast3 on P0 cortical lysates from *L278del* animals. Quantification reveals a dramatic reduction of Mast1, Mast2 and Mast3 protein levels in *Leu278del* heterozygotes and homozygotes in comparison to littermate controls (n=4 animals per genotype; 2-way repeated measures ANOVA with a Bonferroni correction; Mast1: *+/+* vs *L278del/+* $P<0.0001$; *+/+* vs *L278del/L278del* $P<0.0001$; Mast2: *+/+* vs *L278del/+* $P<0.0001$; *+/+* vs *L278del/L278del* $P<0.0001$; Mast3: *+/+* vs *L278del/+* $P<0.0001$; *+/+* vs *L278del/L278del* $P<0.0001$). **(C-D)** Western blot analysis of Mast1, Mast2 and Mast3 on brain lysates from Mast1 KO animals. While Mast1 is absent, there is a significant increase in the levels of Mast2 and Mast3 when compared to littermate controls (n=6 animals per genotype; 2-way repeated measures ANOVA with a Bonferroni test for multiple comparison; Mast1: *+/+* vs KO $P<0.0001$; Mast2: *+/+* vs KO $P<0.0001$; Mast3: *+/+* vs KO $P<0.001$). **(E-F)** Levels of phosphorylated AKT and ribosomal S6 proteins, indicators of activation of PI3K/AKT/mTOR pathway, are not significantly different in wild type and *L278del* P0 cortex (n=5 animals per genotype; 2-way repeated measures ANOVA with a Bonferroni test for multiple comparison; *+/+* vs *L278del/+* AKT $P>0.5$; *+/+* vs *L278del/L278del* AKT $P>0.5$; *L278del/+* vs *L278del/L278del* AKT $P>0.5$; *+/+* vs *L278del/+* rpS6 $P>0.5$; *+/+* vs *L278del/L278del* rpS6 $P>0.5$; *L278del/+* vs *L278del/L278del* rpS6 $P>0.5$). * shows $P<0.05$; ** shows $P<0.01$; *** shows $P<0.001$; **** shows $P<0.0001$. Error bars show mean \pm the standard error of the mean.

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STAR Methods

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. David Keays (david.keays@imp.ac.at).

Experimental Model and Subject Details

Animals

Mice were maintained on a 12:12 light dark cycle and food was available ad libitum. Mice of ages E10.5, E12.5, E14.5, E16.5, P0, P6, 8-10 weeks were used in this study. Animals were not subject to randomization and allocated to experimental groups according to their genotypes as littermates regardless of the sex. For experiments with mice of ages E10.5, E12.5, E14.5, E16.5, P0, P6 the sex was not determined. For generating CRISPR mouse lines, female BL6/CBA F1 mice were used, whereas all backcrosses were performed using C57/BL6 mice to the 7th generation. All procedures were carried out according to legal requirements and covered by an approved license (M58/006093/2011/14).

Human studies

Informed consent was obtained from all patients included in this study. Details of each patient and the condition is provided in Table 1 and Supplementary Table 1. This study was conducted within approved ethical frameworks of University of Oxford (08/MRE09/55); Stanford University Institutional Review Board (28362); University of Alabama at Birmingham (X130201001); and at the Seattle Children's Research Institute (IRB protocol #13291); and by the Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen (NL36191.091.11).

Cell Lines, primary cultures, microbe strains

For generating cerebral organoids, feeder free H9 human ES cells were obtained from Wicell (WA09), having a normal karyotype, female sex, and no contaminants. These cells were maintained using the commercially available mTESR media (Stem cell technologies #05850) at

721 37°C with 5% CO₂ levels. These cells were not authenticated. Primary cell cultures were generated
722 from P0 and P7 cortex or cerebellum. The sex of these pups was not determined. The primary
723 neurons were cultured in Neurobasal media with B27, Penstrep and L-glutamine supplements at
724 37°C with 5% CO₂ levels. The Escherichia coli DH5alpha strain was used for cloning purposes.

725

726 **Method Details**

727 **Exome Sequencing**

728 Patients with structural brain phenotypes and their parents were recruited in accordance with
729 existing ethical frameworks and internal review boards. DNA from blood was extracted using
730 standard methods and subject to exome sequencing. We exploited capture arrays (such as the
731 Agilent Human All exon 50Mb array) to capture exonic genomic DNA, which was then subject to
732 next generation paired end sequencing (Illumina). Bioinformatic filtering employed platforms such
733 as the Genome Analysis Toolkit (GATK), and excluded variants with a population frequency greater
734 than 1%. For this purpose, we relied on available genomic databases including dbSNP and the
735 1000 Genome server. Sanger sequencing was employed to confirm any putative de novo variants.
736 This study was conducted within an approved ethical framework (08/MRE09/55). For mouse
737 exome sequencing genomic DNA was extracted from the tails of BL6J, CBA, and Leu278del mice
738 and then subjected to Illumina library preparation and captured on the Agilent SureSelect mouse
739 (51Mb) array. The samples were sequenced on an Illumina HiSeq2500 with 100-125 nucleotides
740 paired-end reads followed by data quality analysis using FASTQC. Reads were mapped to the
741 mouse genome (GRCm38, mm10) using BWA (Li and Durbin, 2010). The BAM files were used as
742 an input to UnifiedGenotyper module from the GATK-lite toolkit (v2.3). (McKenna et al., 2010).
743 Variants were called and dbSNP variants were used as known sites, according to GATK Best
744 Practices recommendations (DePristo et al., 2011). Finally, variants were annotated by SnpEff v4.1
745 (Cingolani et al., 2012). Sanger sequencing was employed to confirm putative de novo variants on
746 chromosome 8, potentially linked to the Leu278del mutation (Table S2).

747

748 **qPCR**

749 The following tissues were dissected from C57/BL6 mice: embryonic brains at stages E10.5, E12.5,
750 E14.5, E16.5, E18.5 (undetermined sex); brains from early postnatal mice at P0, P6 (undetermined

sex); the brain regions (cortex, cerebellum, hippocampus, striatum, midbrain, colliculi, hypothalamus, brain stem, spinal cord, olfactory bulbs) and organs (liver, spleen, heart, muscle, testis, kidney, lung) from male adult littermates (n=3). To ascertain **Mast1, Mast2 and Mast3**, mRNA levels in Leu278del **and KO** animals, cortices from littermate homozygote, heterozygote and wild type P0s were obtained (undetermined sex) (n=4). Tissue samples were snap frozen before total RNA extraction and cDNA synthesis (SuperScript III First-Strand Synthesis System, Invitrogen, 18080-051). We used SYBR green on a Bio-Rad Cyclor together with intro spanning primers to amplify murine Mast1 (mMAST1_qPCR_F/GCAAGGTGTACAGCAGTATGG, mMAST1_qPCR_R/TGGGTCCCGCTTGCTG). For qPCR on human fetal tissue (hMAST1_qPCR_F/GGTGCATCTGGAGGAACAG, hMAST1_qPCR_R/GATGGTATCGAAGTCATTCTCCC) we performed technical triplicates on two different cDNA libraries (Biochain; C1244051, C1244035) again using intro spanning primers. In addition, we amplified three control genes (Pgk1, Tfr, and Hprt). For each control gene technical triplicates were performed, and the geometric mean of the Ct values for the three control genes calculated. This geometric mean was then used to obtain the delta Ct value by subtracting it from the average (of triplicate runs) Ct value for Mast1. The relative mRNA levels were then obtained by taking into account the efficiency of the qPCR primers (see Braun et al., 2010).

768

769 **Generation of CRISPR Mice**

770 Female BL6/CBA F1 mice (3-4 weeks old) were superovulated according to standard protocols. 771 Zygotes were isolated from donor females at the day of the coagulation plug (=E0.5). Surrounding 772 cumulus cells were removed by incubation of the cumulus-zygote-complexes in hyaluronidase 773 solution (at ~0.3mg/ml). The injection mix (50ng/μl Cas9 mRNA + 50ng/μl guide RNA + 200ng/μl 774 Leu278del-ssODN repair template) was injected into the cytoplasm of zygotes. Injected zygotes 775 were incubated for at least 15 minutes in a CO₂ incubator at 37°C. Zygotes that survived 776 microinjection were transferred the same day into the oviducts of pseudopregnant recipient females 777 (=0.5 days post coitum). Resultant pups were genotyped by PCR amplification of chromosome 8 778 followed by sequencing using primers GGAGAGGGTCTTACTTGCTT and 779 TCTTCTGGGTTGAATTCCTA, and backcrossed to C57/BL6.

780

781 **MRI Analysis**

782 MRI analysis was performed as described previously (Breuss et al., 2016). Briefly, eight-week old
783 littermates were perfused with 0.9% NaCl and 4% PFA supplemented with 10% ProHance Solution
784 (Bracco Imaging Group, 4002750). Images were then acquired with a 15.2 T Biospec horizontal
785 bore scanner (Bruker BioSpin, Ettlingen, Germany). Brain regions of interest were manually
786 segmented, blinded to the genotype, using Amira 5.6 (Visualization Science Group), relying on a
787 mouse brain atlas, and the volumes obtained.

788

789 **Electron Microscopy Studies**

790 Mice aged 8-9 weeks (n=5) were sacrificed and brain tissue prepared for electron microscopy
791 (Korogod et al., 2015). Briefly, animals were perfused employing a constant flow of 7 mL/min with
792 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Following
793 dissection of the brain, 80-µm thick coronal slices were prepared on a vibratome (Leica VT 1000S).
794 Matching slices were selected from the medial region of each brain (Bregma -0.10), the corpus
795 callosum microdissected, and then post fixed for 40 min in 1% osmium tetroxide in cacodylate
796 buffer (0.1 M, pH 7.4). Samples were then washed twice for 5 min in ddH2O and stained with 1%
797 uranyl acetate for 40 min before being dehydrated in a graded acetone series and embedded in
798 Epon resin. Ultrathin sections were prepared (70nm), mounted on 50 mesh grids and post-stained
799 with uranyl acetate (10mins, 2% in water) and with Reynolds' lead citrate (5 min). To assess
800 differences in myelin thickness and axon, internal diameter images were captured at 8900x using
801 a FEI Morgagni 268D (FEI Company) 100 kV transmission electron microscope operating at 80 kV.
802 A customized script written in Definiens Architect XD (Version: 2.7.0; Build 60765X64) was
803 employed to ascertain axonal surface area and myelin thickness determined using ImageJ. To
804 ascertain the number of axons crossing the midline, images were acquired at 710x along the dorsa-
805 ventral axis. These images (approx 10-12) per animal were then tiled employing iTEM, resulting in
806 a single image. A box measuring 30µm along the x axis was then overlaid onto the structure, and
807 the total number of myelinated axons counted manually. Three stitched images were prepared per
808 animal and all analysis was performed blind to genotype. To determine the G-ratio of axons
809 crossing the midline we measured the total diameter of fibers (>300 per animal) and the axonal

diameter (without myelin) on high resolution EM images. The G-ratio was then calculated (axon diameter/fiber diameter) (Guy et al., 1989).

812

813 **Generation of organoids**

814 Human cerebral organoids were generated from H9 human ES cells (Wicell, WA09) as previously
815 described (Lancaster and Knoblich, 2014)_ENREF_14. 64 days-old organoids were then
816 embedded in Neg-50 Medium (Richard-Allan Scientific) and sliced into 20 µm sections in a cryostat.
817 The slides were dried overnight before storing at -20°C and stained as described below.

818

819 **Nissl Staining and Immunohistochemistry**

820 Brains were removed at E12.5, E14.5, E16.5, E18.5, P0 and drop-fixed in 4% PFA overnight
821 followed by dehydration in 30% sucrose. Brains were embedded in Neg-50 Medium (Richard-Allan
822 Scientific) and sliced into 12 µm sections in a cryostat. Adults brains were recovered from the
823 animals after perfusion with 0.9% NaCl and 4% PFA. These brains were post-fixed, dehydrated
824 and sliced using a sledge microtome into 40 µm sections. For Nissl staining, sections were washed
825 in PBS, and bathed in Cresyl Violet for 3 min [0.25% Cresyl Violet acetate (Sigma, C5042)
826 dissolved in distilled water with ten drops of glacial acetic acid per 100 ml of solution]. After briefly
827 washing in PBS, slides were dehydrated in an alcohol series (30%, 70%, 96% and 100% ethanol,
828 2 min each), xylol (twice, 2 min each), mounted with DPX mountant (Fluka, 44581) and left
829 overnight at room temperature. Images were acquired using the transmitted Mirax slide scanner
830 (Zeiss).

831

832 Immunofluorescence experiments were performed as previously described (Breuss et al., 2016;
833 Breuss et al., 2015). Where necessary antigen retrieval was performed on sections by slow heating
834 of the slides in antigen retrieval buffer (Vector, H-3301) up to 90°C, followed by gradual cooling at
835 room temperature for 20 minutes. Primary antibody in 0.1%-0.3% Triton-X100/PBS with 4% donkey
836 serum as the blocking agent, was incubated overnight at 4°C. The following concentrations of
837 primary antibodies were used: 1:300 Mast1 (Santa Cruz, sc-55851), 1:100 Mast1 (Santa Cruz, sc-
838 373845) 1:300 cleaved-Caspase3 (Cell Signaling, #9661), 1:300 cleaved-Caspase9 (Cell
839 Signaling #9509), 1:300 Pax6 (Covance, PRB-278P), 1:300 Tbr2 (Abcam, ab23345), 1:1000 Tuj

(Covance, MMS-435P), 1:300 NeuN (Millipore, MAB377), 1:100 Cux1 (Santa Cruz, sc-6327), 1:1000 Er81 (Abcam, ab36788), 1:300 FoxP2 (Abcam, ab16046), 1:250 Calbindin (Millipore, AB1778), 1:1000 Olig2 (Millipore, AB9610), GFAP (Dako, Z0334), 1:300 Map2 (Abcam, ab24640), 1:300 Tau (Abcam, ab61493), 1:1000 p53 (Leica, P53-CM5P). The next day, sections were washed in PBS and a species-specific secondary antibody (Molecular Probes, A-10037, A-11057, A-10042, A-21206; 1:500) was applied for 1 h in blocking solution at 4°C, followed by Hoechst 33342 staining (1:2000 in PBS) for 5 min. Subsequently the slides were mounted with Fluorescent Mounting Medium (Dako, S302380) and dried overnight at 4°C before imaging. Images were taken using a laser scanning confocal microscope (LSM 780 Zeiss).

849

850 Quantification of Histological Data

851 To quantify the thickness of the corpus callosum in Nissl-stained sections, an average of three
852 measurements at the septum in the genu region of matched sections was taken and expressed in
853 μm . To determine the cortical thickness in of Nissl sections, as well as the fluorescently stained
854 cortical layers, the average of three closely placed measurements in the somatosensory region of
855 matched cortical regions (Bregma co-ordinates -1.82mm) was taken and expressed in μm .
856 Caspase3 and Caspase9 positive puncta were counted in matched sections of the cortex and
857 cerebellum in P0 animals. To quantify the number of oligodendrocytes and astrocytes within the
858 corpus callosum, coronal sections from 8 week old animals were stained with Olig2 and GFAP. A
859 box measuring 200 μm wide was placed over the septum of the corpus callosum extending from
860 the ventral to ventral edges, and positively stained cells were manually counted. To estimate the
861 number of Purkinje and granule cells in the cerebellum, free-floating, 40 μm , sagittal midline
862 cerebellar sections were stained with anti-sera for Calbindin and NeuN, followed by counterstaining
863 with DAPI (n=2 sections per animal). Folium III was imaged and all Purkinje cells in this folium were
864 counted allowing determination of the Purkinje cell linear density (cells/mm of Purkinje cell layer).
865 The total number of Purkinje cell cells per section was then estimated by measuring the entire
866 length of the Purkinje cell layer. To estimate the number of granule cells per section 4 images per
867 animal were captured within the granule cell layer of folium III, and the density of granule cells/ mm^2
868 of granule layer (GL) was determined. The total number of cells per section was then estimated by

869 measuring the entire surface of the GL and multiplying it by the average density for that animal. All
870 quantifications were done using ImageJ software and performed blinded to the genotype.

871

872 **Immunoblotting**

873 Brains were snap frozen in liquid nitrogen and stored at -80°C. Protein lysates were prepared from
874 frozen tissue samples, homogenized in chilled lysis buffer (20 mM TrisHCl pH 7.5, 100 mM NaCl,
875 10% glycerol, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Pierce
876 #88668). Using a Tungsten Carbide Bead the tissue was lysed in a Qiagen Tissue Lyser (2 x 1min,
877 20Hz), incubated at 4°C for 1h, and then centrifuged twice for 20 min. Protein concentration of the
878 collected supernatant was measured using the Pierce BCA Protein Assay Kit (#23225). Protein
879 lysates were run on NuPAGE protein gels, blotted onto nitrocellulose membranes and blocked in
880 5% skimmed milk/TBST. Primary antibodies [1:2000 GAPDH (Millipore, MAB374); 1:1000 Mast1
881 (Proteintech, 13305-1-AP); 1:300 Mast2 (Santa Cruz, sc-377198); 1:500 Mast3 (Novus; NBP1-
882 82993); 1:1000 pan-Akt (Cell Signaling Technology #9272); 1:1000 Phospho-Akt (Ser473) (Cell
883 Signaling Technology #4060); 1:500 S6 Ribosomal Protein (Cell Signaling Technology #5548);
884 1:1000 Phospho-S6 Ribosomal Protein (Ser240/244) (Cell Signaling Technology #2215)] were
885 incubated overnight in blocking solution at 4°C and the corresponding HRP conjugated secondary
886 antibody (abcam) was added for 1 hour at room temperature. The signal was detected on
887 Amersham Hyperfilm™ ECL film using the ECL™ Western Blotting Detection Reagents kit (GE
888 healthcare, RPN2106). Western blots were quantified using ImageJ.

889

890 **Primary neuron culture**

891 Cortical neurons were prepared from brains of C57/BL6 or Mast1 KO P0 mice while P7 mice were
892 used for cerebellar neuronal cultures. Briefly, the brain region of interest was isolated, and the
893 meninges carefully removed. The tissue was treated with 0.25% trypsin solution in a 37°C water
894 bath for 15 mins, followed by three washes in HBSS (20mM HEPES, 2mM CaCl₂, 5.4mM KCl,
895 1mM MgCl₂, 1mM NaPi, 5.6mM glucose, pH7.3). The trypsinised tissue was triturated in plating
896 media (DMEM, 10% horse serum, 200mM L-glutamin, 100mM sodium pyruvate), first using a
897 P1000 pipette and then a P200 pipette. The cells were passed through a 70µm strainer and the
898 resulting cell suspension counted. Neurons plated at $\sim 1 \times 10^5$ cells per well in a 24-well plate

899 containing poly-L-lysine coated coverslips. After 3-4 hours the medium was changed to Neurobasal
900 containing B27, L-glutamine and PenStrep supplements. After 4-5 days in culture the coverslips
901 were fixed in 4% PFA and stained with anti-Map2, anti-Tau and anti-Mast1 antibodies.

902

903 **In vitro transcription and translation of Mast1 and microtubule-binding assay**

904 These experiments were performed as previously described (Keays et al., 2007; Walden and
905 Cowan, 1993). Briefly, the full-length murine Mast cDNA was cloned into the pcDNA 3.1+ vector
906 (Invitrogen) driven by the T7 promoter. The different patient mutations screened were introduced
907 by site-directed mutagenesis using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit
908 (Agilent #210518) kit. Plasmids (20 ng/μL final concentration) were used to drive expression in
909 rabbit reticulocyte lysate (TnT[®] T7 Coupled Reticulocyte Lysate System, Promega) containing 35S-
910 methionine (specific activity: 1000 Ci/mMol; Perkin Elmer). Reactions were incubated at 30°C for
911 90 min. Prior to co-polymerization reactions, the TnT reaction product was cleared by centrifugation
912 at 60,000g for 10 min at 4°C. MAP rich porcine brain tubulin (Cytoskeleton Inc. #ML116) was
913 depolymerized in tubulin buffer (0.1 M PIPES buffer pH 6.9, 1 mM MgCl₂ and 0.5 mM EGTA) by
914 incubation on ice. Co-polymerization reactions were set up by adding the ³⁵S-labeled Mast1 to MAP
915 rich tubulin (1mg/ml) in tubulin buffer containing 1 mM GTP, 2 mM AMP-PNP and 20 μM taxol. To
916 investigate Mast1 binding to microtubules in either the presence or absence of MAPs, the labeled
917 Mast1 reaction was distributed equally between MAP rich and 99% pure porcine brain tubulin
918 (Cytoskeleton Inc. #T240). All co-polymerization reactions were incubated at 30 °C for 30 min to
919 promote microtubule polymerization and binding of associated proteins. Reaction products were
920 then loaded onto cushions (0.20 mL) containing 1 M sucrose in tubulin buffer, 0.5 mM GTP, 1 mM
921 AMP-PNP and 10 μM taxol and centrifuged at 60,000g for 15 min at 30°C. All centrifugations were
922 done in a Beckman TL-100.3 rotor. Proteins in the pellet were solubilized in SDS loading dye and
923 separated in a 7% SDS-PAGE. Radioactivity content of the microtubule pellets obtained was
924 determined following an overnight exposure to a high-sensitive film (KODAK BioMax MR film).

925 **Quantification and Statistical Analyses**

926 All statistical analysis was executed using GraphPad Prism software package (v7.0c). Numbers
927 (n) of animals or replica for experiments are indicated in the text and the statistical test used

928 indicated in individual figure legends. Statistical tests used in this study include the Student's T-
929 test, one-way ANOVA with Dunnett's or Tukey's multiple comparisons test, and two-way repeated
930 measures ANOVA with Bonferroni's multiple comparisons test. Samples and animals were not
931 subject to randomization, but were assigned to experimental groups based on their genotype.
932 Shapiro-Wilk or Kolmogorov-Smirnov tests were applied to assess whether assumptions of
933 normality were met, and corrected for multiple comparisons were necessary. A summary of the
934 statistical tests applied for each experiment, the n numbers, and the results obtained are shown in
935 Table S4.

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