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A microglia clonal inflammatory disorder in Alzheimer's Disease

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Abstract

Summary

Somatic genetic heterogeneity resulting from post-zygotic DNA mutations is widespread in human tissues and can cause diseases, however few studies have investigated its role in neurodegenerative processes such as Alzheimer's Disease (AD). Here we report the selective enrichment of microglia clones carrying pathogenic variants, that are not present in neuronal, glia/stromal cells, or blood, from patients with AD in comparison to age-matched controls. Notably, microglia-specific AD-associated variants preferentially target the MAPK pathway, including recurrent CBL ring-domain mutations. These variants activate ERK and drive a microglia transcriptional program characterized by a strong neuro-inflammatory response, both *in vitro* and in patients. Although the natural history of AD-associated microglial clones is difficult to establish in human, microglial expression of a MAPK pathway activating variant was previously shown to cause neurodegeneration in mice, suggesting that AD-associated neuroinflammatory microglial clones may contribute to the neurodegenerative process in patients.

One-Sentence Summary

A subset of Alzheimer Disease patients carry mutant microglia somatic clones which promote neuro-inflammation.

eLife assessment

This **fundamental** study enhances our understanding of how somatic variants in microglia might influence the onset and progression of neurodegenerative diseases such as Alzheimer's. The evidence supporting the conclusions is **compelling**, with the authors employing a multi-faceted approach to identify an enrichment of potentially pathogenic somatic mutations in Alzheimer's disease microglia. This research will be of significant interest to those investigating somatic mutations, Alzheimer's disease, microglial biology and cell signalling pathways.

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Introduction

Neurodegenerative diseases are a frequent cause of progressive dementia. Alzheimer's disease (AD) is diagnosed in ~90% of cases, with an estimated prevalence of ~10% in the population over 65 years of age [1,2]. The role of germline genetic variation in neurodegenerative diseases and AD has been studied intensely. Although autosomal dominant forms of AD due to rare germline variants with high penetrance account only for an estimated ~1% of cases [3–8], a number of common variants were also shown to contribute to disease risk. Carriers of one germline copy of the epsilon4 (E4) allele of the apolipoprotein E gene (APOE4), present in ~15 to 20% of population, have a three-fold higher risk of AD, while two copies (~2 to 3 % of population) increase the risk by ~10-fold [9–12]. Genome-wide association studies (GWAS) have identified an additional ~50 common germline variants that more moderately increase the risk of AD, including TREM2, CD33, and MS4A6A variants [13–15]. Interestingly, the APOE4 allele is responsible for an increased inflammatory and neurotoxic response of microglia and astrocytes in the brain of carriers [16–18], and it was noted that the majority of the other germline AD-risk variants are located within or near genes expressed in microglia [15] and in particular at microglia-specific enhancers [19]. These data, together with transcriptional studies [20–22] support the hypothesis that genetic variation in microglia may contribute to the pathogenesis of neurodegeneration and AD.

Somatic genetic heterogeneity (mosaicism), resulting from post-zygotic DNA mutations, is widespread in human tissues, and a cause of tumoral, developmental, and immune diseases [23–26]. Aditionally, a role of somatic variants in neuropsychiatric disorders is also suspected [27]. Mosaicism has been documented in the brain tissue of AD patients in several deep-sequencing studies [28–30], showing that the enrichment of putative pathogenic somatic mutations in the PI3K-AKT, MAPK, and AMPK pathway do occur in the brain of patients in comparison to controls [30]. However these studies performed in whole brain tissue lacked cellular resolution and mechanistic insights, and the role of somatic mutants in neurodegenerative diseases remains poorly understood [23]. Somatic variants that activate the PI3K-AKT-mTOR or MAPK pathways in neural progenitors are a cause of cortical dysplasia and epilepsy [31–34] and developmental brain malformations [35], while somatic variants that activate the MAPK pathway in brain endothelial cells are associated with arteriovenous malformations [36]. Interestingly, we reported that expression of a somatic variant activating the MAPK pathway in microglia causes neurodegeneration in mice [37], but the presence and contribution of microglial somatic clones in neurodegenerative diseases and AD remains unknown.

Here, we investigated the presence and nature of somatic variants in brain cells from control and AD patients. In an attempt to examine all brain cells at the same resolution, nuclei from neurons, glia cells and microglia, which only represent ~5% of brain cells, were pre-sorted. Human

microglia are reported to develop in embryo and renew by local proliferation within the brain [38–40]. However, bone marrow-derived myeloid cells can enter the brain, in particular during pathological processes, and may not be distinguishable from resident microglia by transcriptomics alone [41]. In order to distinguish somatic variants carried by resident microglia from the one carried by myeloid cells of peripheral origin, we analyzed matched peripheral blood from control and patients, to 'barcode' somatic mutants shared between microglia and blood. Finally, in order to achieve high sensitivity in the detection of variants that confer a proliferative or activation advantage (pathogenic mutations) and support the emergence or pathogenicity of mosaic clones [42], and/or that have been previously associated with neurological diseases, we initially performed a targeted deep-sequencing of a panel of 716 genes covering somatic variants reported in clonal proliferative disorders and genes associated with neurodegenerative diseases diseases.

We found that microglia from AD patients were enriched for pathogenic variants in comparison to age-matched controls. Furthemore, we found that these microglia-specific AD-associated variants preferentially target the MAPK pathway, including recurrent CBL ring-domain mutations. In addition, we showed that these variants drive a microglia transcriptional program characterized by a strong neuro-inflammatory response previously associated with neurotoxicity, including the production of IL1 and TNF, both in *in vitro* microglia models and in patients. The natural history of the AD-associated microglia clonal inflammatory disorder we describe here is difficult to establish. Specifically, we do not know whether it contributes to the onset of the neuro-inflammatory process at an early stage of the disease, or if microglia carrying pathogenic mutations preferentially expand later during the course of the disease in response to tissue inflammation. Under both hypotheses however, the presence of neuro-inflammatory microglial clones may contribute to the neurodegenerative process in a subset of AD patients. This report reveals a previously unrecognized presence of AD-associated microglia harboring pathogenic somatic variants in humans and provides mechanistic insight for neurodegenerative diseases by delineating cell-type specific variant recurrence.

Results

Clonal diversity among brain cells and blood from controls and AD patients

We examined post-mortem frozen brain samples and matching blood from 45 patients with intermediate-onset sporadic AD and 44 control individuals who died of other causes, including 27 donors age and sex-matched donors with the AD cohort (**Fig. 1A**; Supplementary Fig. 1A and Supplementary Table 1). APOE risk allele frequency for patients and controls was comparable to published series [10–12] (Supplementary Fig. 1A), and analysis of germline mutations did not identify deleterious variants in the 140 genes associated with neurological diseases. Myeloid/microglia, neurons, and glia/stromal cells were purified by flow cytometry using antibody against PU.1 and NeuN [43] (**Fig. 1B**, Supplementary Fig. 1B and 1C). Single nuclei (sn)RNAseq was performed on PU.1$^+$ nuclei from one control and 3 AD patients to evaluate microglia enrichment following PU.1$^+$ purification, and a cell-type annotation analysis indicated that ~94% of PU.1$^+$ nuclei correspond to microglia (**Fig. 1C** and Supplementary Fig. 1D-1H). Cortex samples were obtained from all donors but hippocampus samples were mostly obtained from AD patients (**Fig. 1A**; Supplementary Table 1). A total of 744 DNA samples from blood, PU.1$^+$ nuclei, NeuN$^+$ nuclei, and Double Negative nuclei (glia/stromal cells) from patients and controls (**Fig. 1A**) were submitted to targeted hybridization/capture and deep-DNA targeted sequencing (TDS, **Fig. 1D**, see Methods), at mean coverage of ~1,100x (Supplementary Fig. 1I), for a panel of 716 genes (3.43 Mb, referred to below as BRAIN-PACT) which included genes reported to carry somatic variants in clonal proliferative disorders (n=576 genes)[44,45] or that have been reported to be associated with neurodegenerativediseases (n=140) [46–54] (Supplementary Table 2, see Methods).

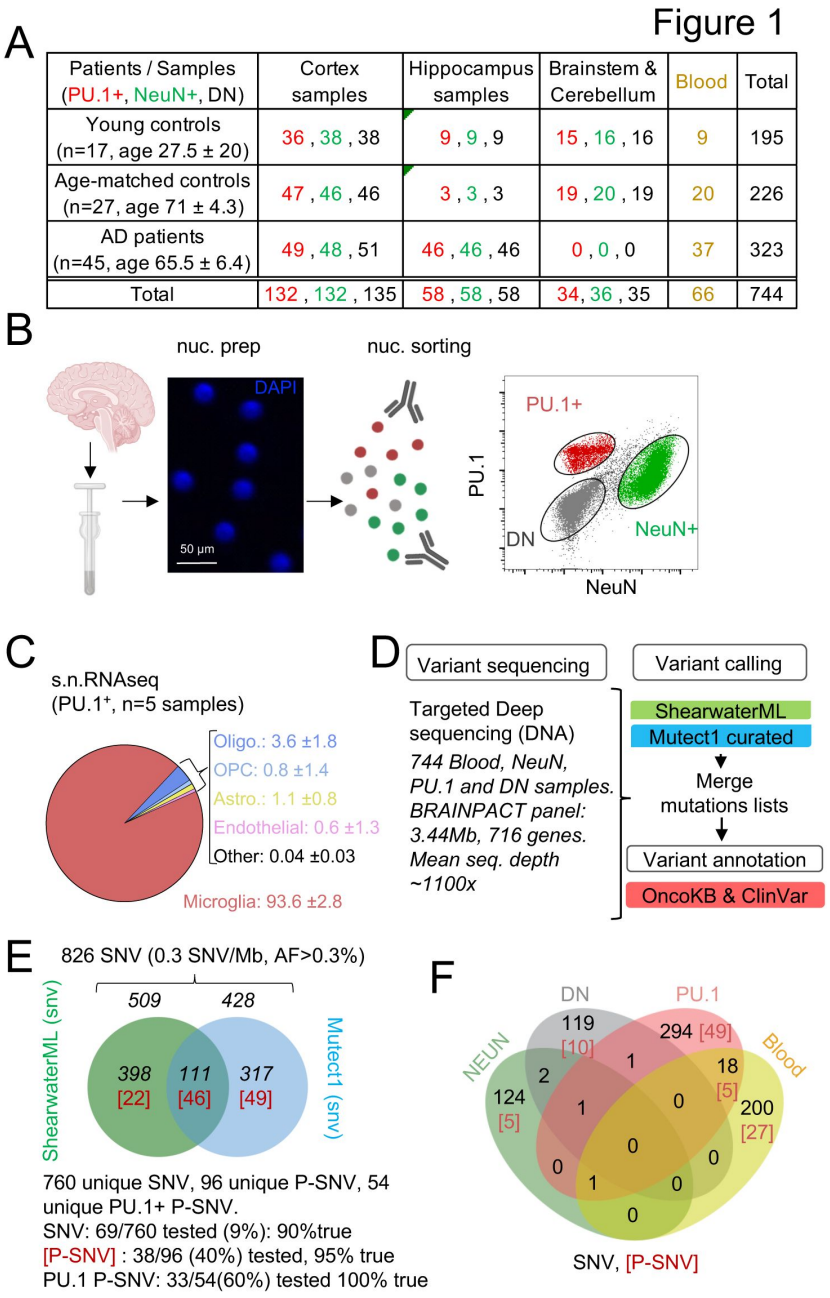


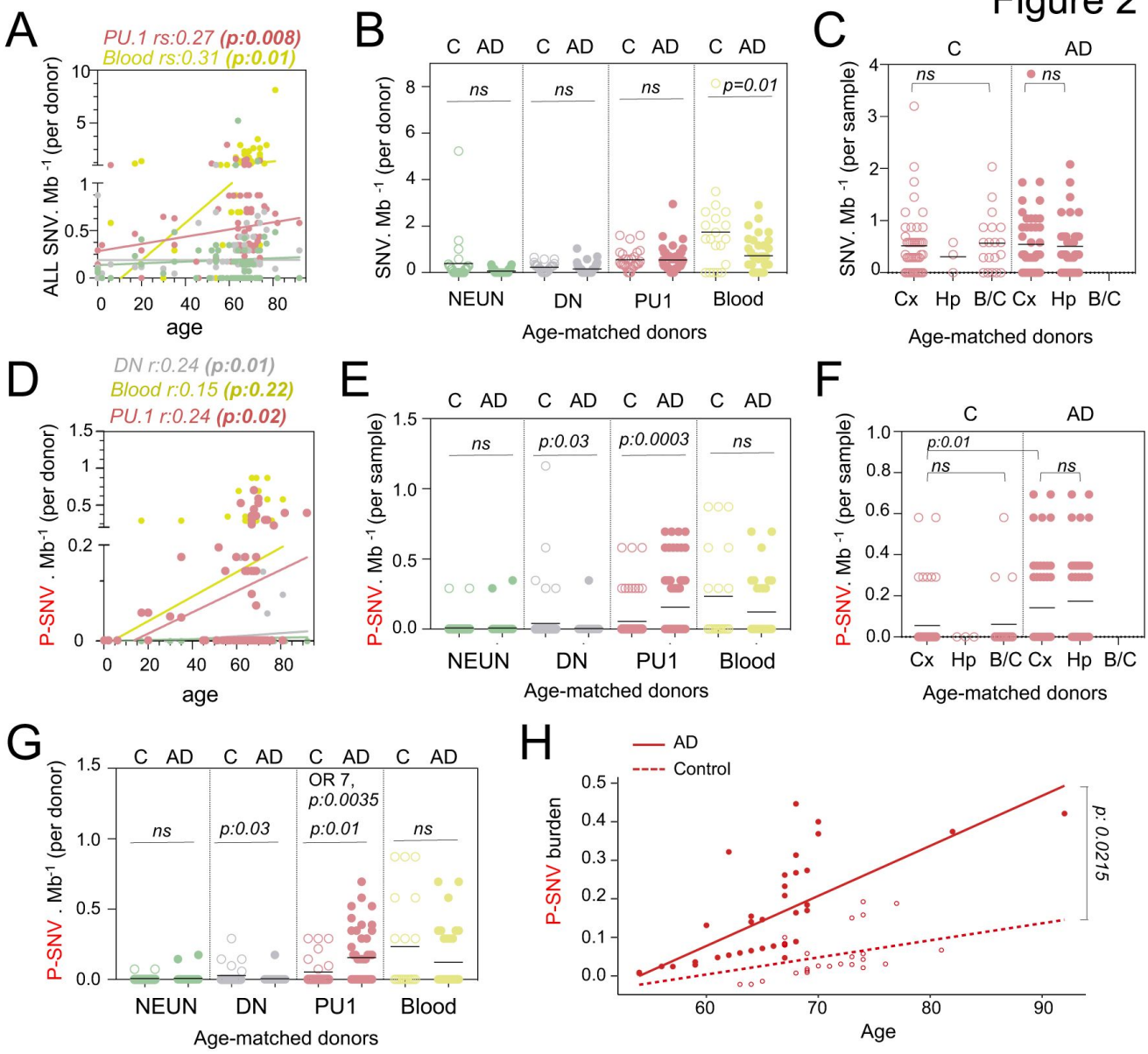
Fig. 1

Detection of mutations in brain cell types and blood

(A) Table with patient and sample information. **(B)** Schematic represents the isolation and labelling of nuclei from post-mortem frozen brain samples from controls and Alzheimer's disease patients with DAPI and antibodies against PU.1^{+} (myeloid/microglia) and NeuN^{+} (neurons).Representative flow cytometry dot-plot of nuclei separation. Double negative nuclei are labeled 'DN'. **(C)** Percentage of cell types obtained in sorted PU.1^{+} nuclei determined by single-nuclei RNAseq in 5 brain samples from 4 individuals. **(D)** Schematic represents the sequencing strategy. Two algorithms (ShearwaterML and Mutect1) were used for variant calling. After annotation, pathogeneity was determined using OncoKb and ClinVar. **(E)** Venn diagram represents the number of variants and overlap between the ShearwaterML and Mutect1. Numbers in red indicate pathogenic variants (P-SNV). Validation of variants was performed by droplet digital (dd)PCR on pre-amplified DNA when available. **(F)** Venn diagrams represent the repartition per cell type of the 826 single-nucleotide variations (SNVs) identified in NeuN^{+}: Neurons, PU.1^{+}: microglia, DN: glia, and matching blood. [Numbers] in red indicate pathogenic variants P-SNV

After QC and filtering of germline variants, variant calling using ShearwaterML and a curated Mutect1 analysis identified 826 somatic synonymous and non-synonymous single-nucleotide-variations (SNVs), at an allelic frequency > 0.3% (mean 1.3%) in the 744 samples, corresponding to an overall variant burden of 0.3 mut/Mb (**Fig. 1E**). Sixty-six/826 SNV were present in more than one sample (Supplementary Table 3). Droplet digital-PCR performed on pre-amplification DNA for ~10% of the 760 unique SNV was positive in 90% of cases (**Fig.1E**; Supplementary Table 3). After annotation using the OncoKB [55] and ClinVar [56] databases for disease-associated or causative variants (**Fig. 2D,F**; Supplementary Table 3), 96 unique SNV were classified as Pathogenic (P)-SNV. 40% of these P-SNV were tested by droplet digital-PCR and confirmed in 95% of cases (**Fig. 1E** and Supplementary Table 3). Positive and negative results in matching brain samples from individual donors were confirmed in 100% of samples at a mean depth of ~5000x (range 648-23.000x) (Supplementary Table 3). A venn-diagram analysis of SNVs detected in $PU.1^+$, $NeuN^+$, DN, and blood samples indicated that most (>90%) SNV and P-SNV were cell-type or tissue specific, with ~ 5% of SNV and ~ 8% of P-SNV shared between the blood and brain of individual donors (**Fig. 1F**; Supplementary Table 3). These data indicate that targeted deep-sequencing of purified nuclei allows to detect clonal mosaic variants with high sensitivity and specificity. In addition, 'bar-coding' of clonal variants across tissues suggests that infiltrating myeloid cells of peripheral origin account for ~5% of microglia somatic diversity, and therefore that blood clones have a detectable but minor contribution to microglia, consistent with its local maintainance and proliferation [38,39].

Figure 2


A
PU.1 rs:0.27 (p:0.008)
Blood rs:0.31 (p:0.01)
ALL SNV. Mb -1 (per donor)
age
B
C AD C AD C AD C AD
ns ns ns p=0.01
SNV. Mb -1 (per donor)
NEUN DN PU1 Blood
Age-matched donors
C
C AD
ns ns
SNV. Mb -1 (per sample)
Cx Hp B/C Cx Hp B/C
Age-matched donors
D
DN r:0.24 (p:0.01)
Blood r:0.15 (p:0.22)
PU.1 r:0.24 (p:0.02)
P-SNV . Mb -1 (per donor)
age
E
C AD C AD C AD C AD
ns p:0.03 p:0.0003 ns
P-SNV . Mb -1 (per sample)
NEUN DN PU1 Blood
Age-matched donors
F
C AD
p:0.01
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P-SNV. Mb -1 (per sample)
Cx Hp B/C Cx Hp B/C
Age-matched donors
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OR 7,
p:0.0035
ns p:0.03 p:0.01 ns
P-SNV . Mb-1 (per donor)
NEUN DN PU1 Blood
Age-matched donors
H
AD
Control
P-SNV burden
p: 0.0215
Age

Fig. 2

Pathogenic variants are enriched in microglia from AD patients.

(A) Correlation plot represents the mean number of variants per cell type and donor (n=89) (Y axis), as a function of age (X axis). Each dot represents mean value for a donor. Statistics: fitted lines, the correlation coefficients (rs) and associated *p values* were obtained by linear regression (Spearman's correlation). **(B)** Number of SNV per Mb and cell types per donor, of age-matched controls (n=27) and AD patients (n=45). Each dot represents mean value for a donor. Statistics: *p-values* are calculated with unpaired two-tailed Mann-Whitney U. Note: non-parametric tests were used when data did not follow a normal distribution (D'Agostino-Pearson normality test). **(C)** Number of SNV per Mb in PU.1$^+$ samples across brain regions, of age-matched controls (n=27) and AD patients (n=45). Each dot represents a sample. Statistics: *p-values* are calculated with Kruskal–Wallis, multiple comparisons. Note: non-parametric tests were used when data did not follow a normal distribution (D'Agostino-Pearson normality test). **(D)** Correlation plot represents the mean number of pathogenic variants (P-SNV) as determined by ClinVar and/or OncoKB, per cell type and donor (n=89) (Y axis), as a function of age (X axis). Each dot represents mean value for a donor. Statistics: fitted lines, the correlation coefficients (rs) and associated *p values* were obtained by linear regression (Spearman's correlation). **(E)** Number of P-SNV per Mb and cell types per sample, of age-matched controls (n=27) and AD patients (n=45). Each dot represents a sample. Statistics: *p-values* are calculated with unpaired two-tailed Mann-Whitney U. Note: non-parametric tests were used when data did not follow a normal distribution (D'Agostino-Pearson normality test). **(F)** Number of P-SNV per Mb in PU.1 samples across brain regions, of age-matched controls (n=27) and AD patients (n=45). Each dot represents a sample. Statistics: *p-values* for comparison within each group (controls and patients) are calculated with Kruskal–Wallis test and Dunn's test for multiple comparisons. *p-values* for the comparison of P-SNV between the cortex of C and the cortex of AD (0.01) was calculated with unpaired two-tailed Mann-Whitney U. Note: non-parametric tests were used when data did not follow a normal distribution (D'Agostino-Pearson normality test). **(G)** Number of P-SNV per Mb and and cell types per donor for age-matched controls (n=27) and AD patients (n=45). Each dot represents mean value for a donor. Statistics: *p-values* are calculated with unpaired two-tailed Mann-Whitney U test. Odds ratio (95% CI, 2.049 to 29.02) and *p values* for the association between AD and the presence of pathogenic variants are calculated by multivariate logistic regression, with age and sex as covariates. **(H)** P-SNV burden as a function of age and disease status (age-matched controls and AD). Linear lines represent trend lines from mixed-effects linear regression that incorporates individual donor as a random effect (blue, control: P=0.0025, R^2=0.13; red, NDD: P=9.1x10^-16, R^2=0.50 by Pearson's correlation). The model's total explanatory power is substantial (conditional R^2=0.48). Both age and AD are associated with a significant increase in SNV burden in this model (P<1x10^-4 and P=1x10^-4, respectively, by likelihood ratio test). Anatomical regions of the brain specimen and originating brain banks were not incorporated because the models incorporating those parameters did not significantly improve the overall model fitting by likelihood ratio test (see Methods). Graph depicts SNV burden corrected by the mixed-effects model (See Fig S2D for observed P-SNV burden)

Somatic clonal diversity of the different cell types, as evaluated by the SNV/megabase burden was higher in blood (1 mut/Mb) and PU.1$^+$ nuclei (0.5 mut/Mb) than for DN and neurons (0.18 mut/Mb) (Supplementary Fig. 2A). The SNV/mb burden of blood and PU.1$^+$ nuclei increased as a function of age (**Fig. 2A** and Supplementary Table 3) as previously reported for proliferating cells [24,57–59]. Interestingly, the SNV/mb burden of blood cells from age-matched controls was higher than for AD patients (**Fig. 2B**). In contrast, there was no difference in SNV/mb burden between PU.1$^+$, NEUN$^+$ and DN samples from AD patients and age-matched controls (**Fig. 2B**), and between PU.1$^+$ nuclei from the cortex, hippocampus, and brainstem/cerebellum samples (**Fig. 2C**). These data altogether indicate that the clonal diversity of microglia and blood both increase with age, and that the clonal diversity of blood cells is lower in AD than in age-matched controls who died of other causes including cancer and cardiovascular diseases (see Methods). This is consistent with recent studies showing that clonal hematopoiesis is associated with a higher risk of several diseases related to ageing such as cardiovascular diseases, but inversely associated with the risk of AD [60,61].

Microglia clones carrying pathogenic variants are enriched in AD patients

In contrast to the global SNV burden, increased P-SNV burden was correlated not only with age (**Fig. 2D**), but also with the disease status (AD) (**Fig. 2E-H**). Within the control group, the SNV and P-SNV burden was higher in the blood of controls treated for cancer (Supplementary Fig. 2B). The P-SNV burden per Mb was selectively and highly enriched in PU.1^{+} samples from AD patients in comparison to age-matched controls (p=0.0003, **Fig. 2E**). Analysis of PU1^{+} P-SNV/Mb burden per brain region indicated that the P-SNV/Mb burden was similar between brain regions within each group (**Fig. 2F** and Supplementary Fig. 2C), and therefore attributable to AD status rather than sampling bias. Analysis of mutational load per donor confirmed that microglial clones carrying P-SNV were enriched in the brain of AD patients in comparison to age-matched controls (**Fig. 2G**). Despite the relatively modest cohort size, a logistic regression analysis confirmed the association between the presence of P-SNVs in PU.1^{+} nuclei and AD after adjusting for sex and age (*OR= 7; p=0.0035,* **Fig. 2G** and Supplementary Fig. 2D). A mixed-effects linear regression model analysis also showed an excess of P-SNVs in AD independently of the effect of age (p = 0.0215) (**Fig. 2H** and Supplementary Fig. 2E).

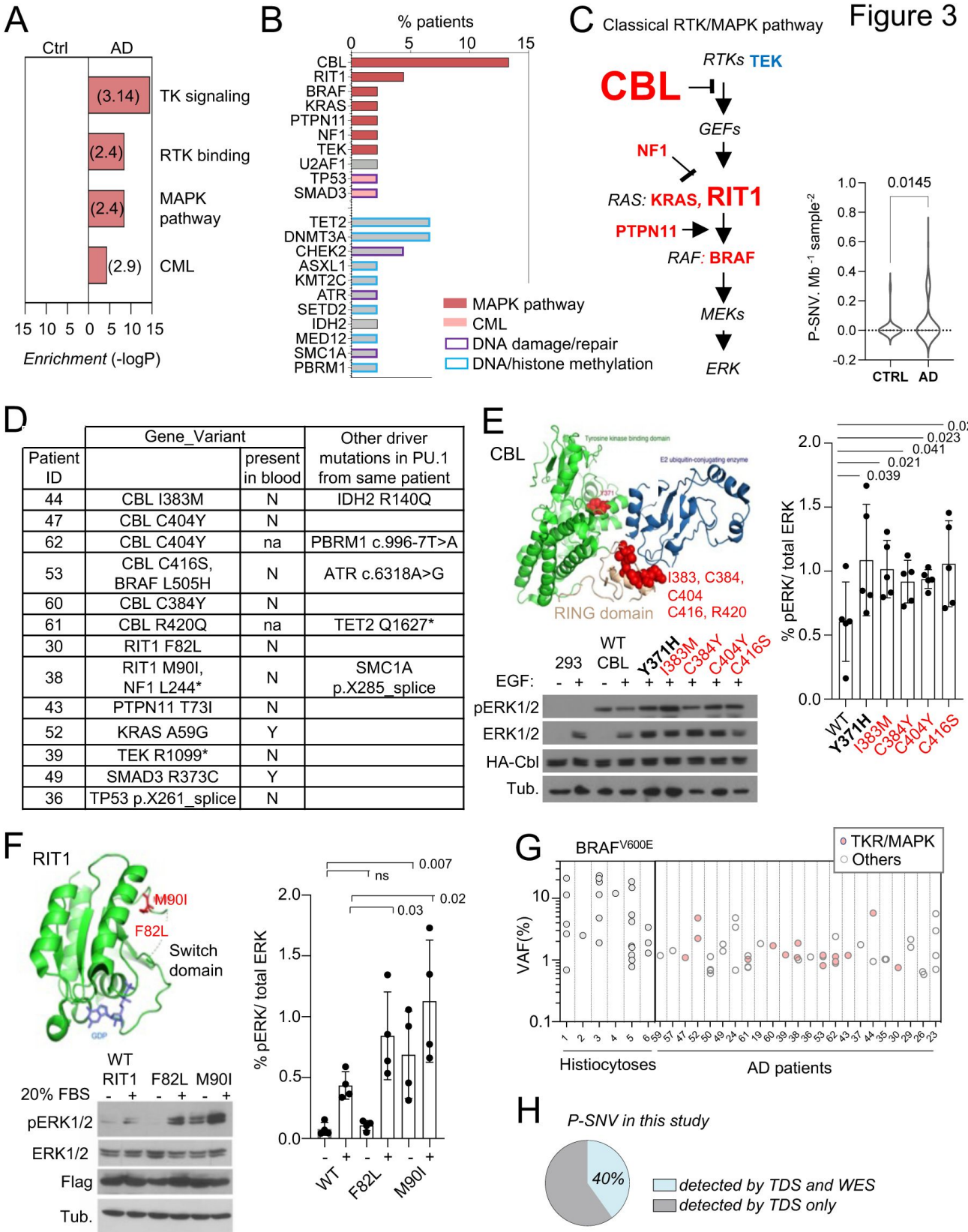
In addition, genes targeted by P-SNV were all expressed in microglia (Supplementary Fig. 2F and Supplementary Table 3) and the analysis of P-SNV/Mb mutational load restricted to genes that are not expressed in microglia did not show an enrichment of candidate pathogenic variants in AD patients (Supplementary Fig. 2G and Supplementary Table 3). Altogether, these results show an association between microglia clones carrying P-SNV and AD in this series.

AD patients carry microglial clones with MAP-Kinase pathway variants including recurrent CBL variants

Pathways analysis of genes carrying P-SNV in microglia from AD patients, against the background of the 716 genes sequenced, showed that the most significant pathways enriched were the receptor tyrosine kinase /MAP-Kinase pathways (Reactome, GO, and canonical pathways, **Fig. 3A** and Supplementary Table 4), corresponding to pathogenic/oncogenic variants in 6 of the 15 genes of the classical MAPK pathway [62] (CBL, BRAF, RIT1, NF1, PTPN11, KRAS), TEK, and the KEGG Chronic Myeloid Leukemia (CML) pathway, which includes the former plus SMAD5 and TP53 (**Fig. 3B**, 3C and Supplementary Fig. 3). Mutational load for MAPK genes was significantly higher in AD patients in comparison to age-matched control (**Fig. 3C**). Other enriched pathways, albeit less significant, included genes involved in DNA repair and chromatin binding/ methyltransferase activity (**Fig. 3B**; Supplementary Table 4). No pathway was enriched in age matched controls. Of note we did not observe microglia P-SNVs within genes reported to be associated with neurological disorders (Supplementary Table 2) in patients (Supplementary Table 3). P-SNV targeting genes of the classical RTK/ MAPK pathway (**Fig. 3C**) were detected in the PU.1^{+} samples from ~25% of the AD patients tested (p=0.0145 vs age-matched controls, **Fig. 3D** and Supplementary Fig. 3). Strikingly, half of these patients (6 patients, 13% of AD patients in this series) carried reccurent P-SNV in the RING domain of CBL [63–73] (**Fig. 3B**-**3E**). Two additional patients presented with P-SNV in the Switch II domain of RIT1 [74] (**Fig. 3B**-**3F**). Microglia from the 3 other patients carried activating KRAS (p.A59G), PTPN11 (p.T73I) and TEK (p.R1099*) oncogenic variants previously described in cancer and sporadic venous malformations [75–77] (**Fig. 3B** and **3D**). In addition, a 12th patient carried a gain of function (GOF) U2AF1 (p.S34F) variant [78], which is not a 'classical MAPK gene' but activates the MAPK pathway in myeloid malignancies [79] (**Fig. 3B** and Supplementary Fig. 3). Two patients carried 2 different MAPK activating variants: microglia from 1 patient carried an activating BRAF (p.L505H) variant [80] in addition to loss of function (LOF) variant CBL (p.C416S), and another patient carried the NF1 (p.L2442*) LOF variant [81,82] in addition to the activating RIT1 (p.M90I) variant (**Fig. 3D** and Supplementary Fig. 3). Five patients also carried additional P-SNV targeting genes involved in DNA repair with tumor

suppressor function [83,84], including the loss of function variants in ATR (c.6318A>G) [85] and SMC1A (p.X285_splice) (**Fig. 3D** and Supplementary Fig. 3), and in DNA/histone methylation including TET2 (p.Q1627*) [63,86], IDH2 (p.R140Q) [87], and PBRM1 (c.996-7T>A) [88]) (**Fig. 3D** and Supplementary Fig. 3). Finally, two patients carried oncogenic variants in genes from the KEGG Chronic Myeloid Leukemia (CML) pathway, SMAD3 (p.R373C) [89] and TP53 (pX261_splice) [90] (**Fig. 3D** and Supplementary Fig. 3). The detection of multiple oncogenic variants in the same patients is reminiscent of the features observed in myeloproliferative disorders described outside the brain [73,86].

Figure 3



D

Patient ID	Gene_Variant	present in blood	Other driver mutations in PU.1 from same patient
44	CBL I383M	N	IDH2 R140Q
47	CBL C404Y	N	
62	CBL C404Y	na	PBRM1 c.996-7T>A
53	CBL C416S, BRAF L505H	N	ATR c.6318A>G
60	CBL C384Y	N	
61	CBL R420Q	na	TET2 Q1627*
30	RIT1 F82L	N	
38	RIT1 M90I, NF1 L244*	N	SMC1A p.X285_splice
43	PTPN11 T73I	N	
52	KRAS A59G	Y	
39	TEK R1099*	N	
49	SMAD3 R373C	Y	
36	TP53 p.X261_splice	N	

Fig. 3

Somatic microglial clones with multiple and recurrent CBL and MAP-Kinase pathway activating variants.

(A) Pathway enrichment analysis for the genes target of P-SNVs using the panel of 716 genes as background set. Graph shows the most enriched pathways by: Reactome Gene Sets, GO Molecular Functions, Canonical Pathways and KEGG Pathway (see complete list in Supplementary Table 4). **(B)** Bar plot indicates the genes carrying P-SNV (y-axis) and the % of AD patients carrying P-SNV for each gene (x-axis). Genes are color-coded by pathway. **(C)** Representation of the classical MAPK pathway, the 6 genes mutated in AD patients are labeled in red, TEK is labeled in blue, and larger font size indicate reccurence of variants in a given gene. Violin plot shows distribution of P-SNV in genes from classical MAPK pathway per Mb sequenced and per sample in patients and controls, *p-value*: unpaired two-tailed Mann-Whitney U test. **(D)** Summary Table showing patients carrying P-SNV in the classical RTK/MAPK pathway and CML associated genes (see Supplementary Table 3) and indicating the detection of variants in blood, and their association with other variants in microglia. **(E)** Recurrent variants in the ring-like domain of CBL are indicated in red on the diagram structure of gene, above representative Western blot from cell lysates from HEK293T cells expressing WT, positive control (Y371H), or CBL variants alleles found in patients, and stimulated with EGF or control, probed with antibodies against Phospho-p44/42 MAPK (Erk 1/2, Thr202/Tyr204), total p44/42 MAPK (Erk1/2), HA-tag and tubulin (BOTTOM). Histogram (RIGHT) represents quantification of the increase of the Phospho-ERK1/2 /total ERK1/2 ratio in western blots in n=5 independent experiments, statistics: unpaired one-tailed t-test. **(F)** RIT1 M90I and F82L are represented on the 3D structure of the gene (pdb code: 4klz, F82 is within a segment whose structure was not resolved) and representative western blot from HEK293T cells expressing Flag-RIT1 (WT and mutants) and treated -/+ 20% FBS before harvesting. Lysates were probed with antibodies against Phospho-p44/42 MAPK (Erk 1/2, Thr202/Tyr204), total p44/42 MAPK (Erk1/2, (MAPK)), Flag, and tubulin. Histogram (RIGHT) represents quantification of the increase of the Phospho-ERK1/2 /total ERK1/2 ratio in western blots in n=4 independent experiments, statistics: unpaired one-tailed t-test. **(G)** Variant allelic frequency (VAF, %) for the $BRAF^{V600E}$ allele in $PU.1^{+}$ nuclei from brain samples from histiocytosis patients (each dot represents a sample) and for P-SNVs in in PU.1+ nuclei from brain of AD patients (each dot represent a variant). Note: non-parametric tests were used when data did not follow a normal distribution (D'Agostino-Pearson normality test). **(H)** Percentage of P-SNVs detected by targeted deep sequencing (TDS) which were also detected by Whole-Exome-Sequencing (WES).

Recurrent CBL and RIT1 variants activate the MAPK pathway

CBL is an E3 ubiquitin-protein ligase that negatively regulates RTK signaling via MAPK [91]. CBL somatic and germ-line LOF variants such as R420Q have been previously associated with tumoral diseases including clonal myeloproliferative disorders [63–73] and RASopathies [92] respectively. We confirmed that CBL RING-domain variants found in AD patients increased MAPK phosphorylation in response to EGF upon expression of HA-tagged WT or mutant alleles in HEK293T cells (**Fig. 3E** and Supplementary Fig. 4A). RIT1 is a RAS GTPase, and somatic or germ-line GOF variants such as RIT1 F82L and RIT1 M90I, also enhance MAPK signaling in malignancies [74] and RASopathies [93,94] respectively. As in the case of CBL variants, the 2 RIT1 variants found in AD patients increased MAPK phosphorylation in response to FBS in HEK293T cells expressing these mutant alleles (**Fig. 3F**, Supplementary Fig. 4B and 4C). These data altogether indicate that a subset of AD patients (12/45, ~ 27% of this series) present with microglial clones carrying one or several oncogenic variants that activate the RTK/MAPK pathway, and are characterized by recurrent oncogenic variants in CBL and RIT1.

Allelic frequency of the patients' MAPK activating variants

The allelic frequencies at which MAPK activating variants are detected in brain samples from AD patients range from ~1-6% in microglia (**Fig. 3G**), which correspond to mutant clones representing 2 to 12% of all microglia in these samples, assuming heterozygosity. This range of

allelic frequency is frequently observed for the MAPK activating BRAF^{V600E} variant in microglia isolated from brain samples of 6 patients diagnosed with *BRAF*$^{V600E+}$ histiocytosis, a rare clonal myeloid disorder associated with neurodegeneration [37,95–98] (**Fig. 3G** and Supplementary Table 5). These data suggested that the size of the mutant microglial clones in AD patients was compatible with a role in a neuro-inflammatory/neurodegeneration process.

Other variants found in microglia from AD patients

Pathogenic variants that did not involve the MAPK pathway included LOF variants in the DNA repair gene CHEK2 including CHEK2 c.319+1G>A [99] and CHEK2 R346H (Supplementary Fig. 3 and 4D), Mediator Complex gene MED12 [100], Histone methyltransferases SETD2 [101] and KMT2C/MLL3, the DNA methyltransferase DNMT3A [86,102], DNA demethylating enzymes TET2 and the Polycomb proteins ASXL1 [86]. Of note, TET2, DNMT3 and KMT2C variants when present, were frequently detectable in the patients' matching blood at low allelic frequency (Supplementary Fig. 3). TET2, DNMT3 and KMT2C are frequently mutated in clonal hematopoiesis [58,59], suggesting that in contrast to other variants, the presence of TET2, DNMT3 and KMT2C/MLL3 in the brain of patients may reflect the entry of blood clones in the brain.

In half of the AD patients, no microglia pathogenic variants were identified. Targeted deep sequencing (TDS) cannot identify variants located outside of the BRAIN-PACT panel, such as other potential additional variants that would activate the MAPK pathway. Therefore, we performed whole exome sequencing (WES) of PU.1^{+} nuclei at an average depth ~400x, in selected samples from 48 donors, including samples from most of the patients negative for pathogenic variants by TDS (n=17 out of 22), a selection of patients with variants identified by TDS (n=16 out of 23), and 15 controls, followed by a curated Mutect analysis. Only 6/15 (40%) of the pathogenic SNVs previously identified by TDS and confirmed by ddPCR were detectable by WES in these samples (**Fig. 3H**), indicating a lower sensitivity of WES. Nevertheless, after annotation by 4 modeling predictors (Polyphen, SIFT, CADD/MSC and FATHMM-XF [103–108] additional SNVs predicted to be deleterious with high confidence were identified in 8/22 patients without pathogenic variants identified by TDS (Supplementary Fig. 3 and Supplementary Table 6). Interestingly, 4 of the predicted deleterious variants identified by WES targeted genes that regulate the MAPK pathway (ARHGAP9, ARHGEF26, CHD8, and DIXDC1 (Supplementary Fig. 3 and SupplementaryTable 6).

The patients' MAPK activating variants increases ERK phosphorylation, proliferation, inflammatory and mTOR pathways in murine microglia and macrophages

CBL variants increased ERK phosphorylation upon lentiviral transduction in BV2 murine microglial cells [109,110] (Supplementary Fig. 4E). However as this line was immortalized by v-Raf, which might interfere with the study of the MAPK pathway, we also stably expressed WT and variant CBL, RIT1, KRAS, PTPN11 alleles in SV-U19–5 transformed mouse 'MAC' lines [111,112] (see Methods and Supplementary Fig. 5A,B). MAC lines expressing CBL, RIT1, KRAS and PTPN11 variants presented with increased ERK phosphorylation and/or increased proliferation in comparison to their WT controls, as measured by Western immunoblotting and EdU incorporation (**Fig. 4A** and Supplementary Fig. 5A,B). In addition, Hallmark and KEGG pathway analysis of RNAseq data from control and mutant lines showed increased RAS, TNF, IL6 and JAK STAT signaling, complement, inflammatory responses, and mTOR pathway activation signatures in mutants (**Fig. 4B** and Supplementary Table 7). These data indicated that microglia variants from patient's activate murine microglial cells and growth factor-dependent macrophages with proliferative and inflammatory responses *in vitro*. However, overexpression of mutant alleles in mouse cell lines does not necessarily recapitulate or predict the effects of a heterozygous genetic variant in physiological conditions. Thus, we investigated the role of CBL^{C404Y} allele in heterozygous human primary microglia-like cells.

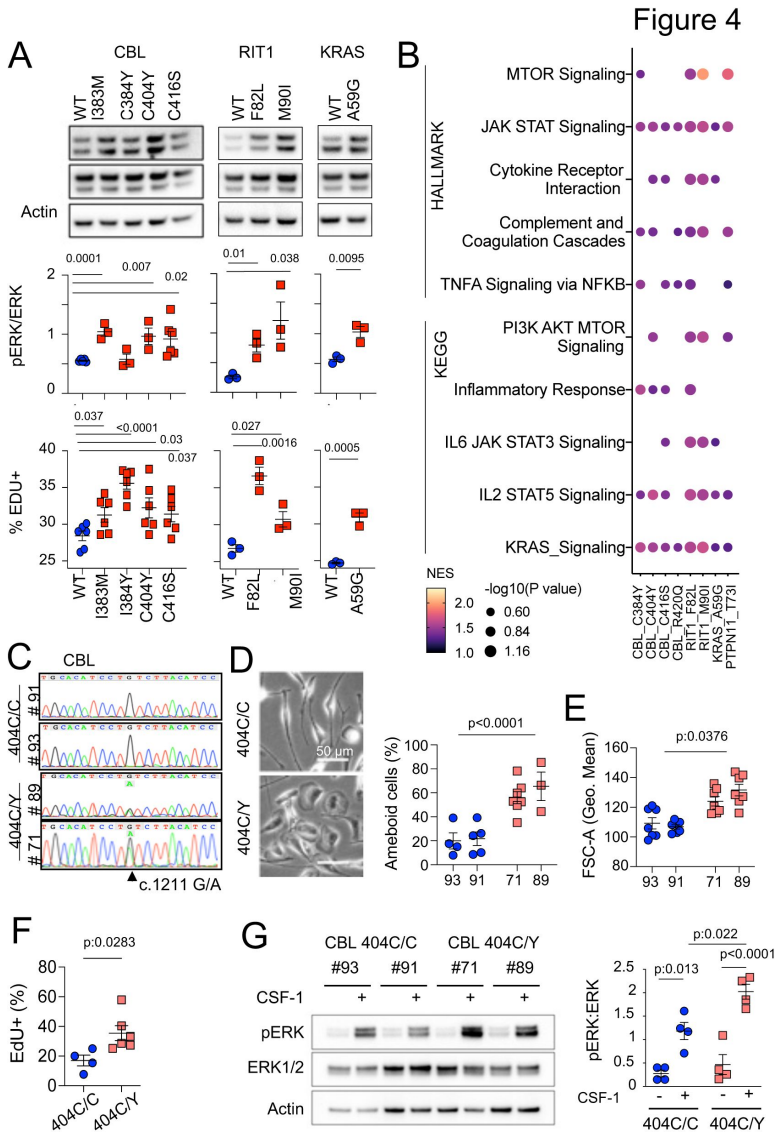


Fig. 4

MAPK pathway activating variants in mouse macrophages and human iPSC-derived microglia-like cells.

(A) Representative western-blot analysis (Top panels) and quantification (Middle panels) of phospho-and total-ERK in lysates from a murine CSF-1 dependent macrophage cell line expressing CBL^{WT}, CBL^{I383M}, CBL^{C384Y}, CBL^{C404Y}, CBL^{C416S} (n=3-6), and $RIT1^{WT}$, $RIT1^{F82L}$ and $RIT1^{M90I}$ (n=3), $KRAS^{WT}$ and $KRAS^{A59G}$ (n=3). Bottom panels depicts flow cytometry analysis of EdU incorporation in the same lines. Statistics, Unpaired t-test. **(B)** HALLMARK and KEGG pathways (FDR / adj.p value <0.25, selected from Supplementary Table 7) enriched in gene set enrichment analysis (GSEA) of RNAseq from mutant CSF-1 dependent macrophages lines CBL^{I383M}, CBL^{C384Y}, CBL^{C404Y}, CBL^{C416S}, CBL^{R420Q}, $RIT1^{F82L}$ $RIT1^{M90I}$, $KRAS^{A59G}$, and $PTPN11^{T73I}$ (n=3-6) in comparison with their wt controls. NES: normalized enrichment score. **(C)** Sanger sequencing of 2 independent hiPSC clones (#93 and #91) of $CBL^{404C/Y}$ heterozygous mutant carrying the c.1211G/A transition on one allele and 2 independent isogenic control $CBL^{404C/C}$ clones (#71 and #89) all obtained by prime editing. **(D)** Photomicrographs in $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells.**(E)** Quantification of leading edge and lateral lamellipodia in $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells. n=3-7, statistics: p-value are obtained by nested one-way ANOVA. **(F)** Flow cytometry analysis of cell size for the same lines (n>3) statistics: p-value are obtained with nested one-way ANOVA,). **(G)** Flow cytometry analysis of EdU incorporation in $CBL^{404C/C}$ and $CBL^{404C/Y}$ microglia-like cells after a 2 hours EdU pulse. n=3, unpaired t-test). **(H)** Western-blot analysis (left) and quantification (right) of phospho- and total-ERK proteins in lysates from $CBL^{404C/C}$ and $CBL^{404C/Y}$ microglia-like cells starved of CSF-1 for 4 h and stimulated with CSF-1 (5 min, 100 ng/mL) (n=4), statistics: pvalue are obtained with two-way ANOVA.

Heterozygosity for a CBL variant allele activates human microglia-like cells

We used prime editing [113] of human induced pluripotent stem cells (hiPSCs, see Methods) to generate isogenic hiPSCs clones heterozygous for the patients' variants (**Fig. 3C** and Supplementary Fig. 5). We focused our analysis on $CBL^{404C/Y}$ mutant lines because CBL was mutated in 6 patients and 2 of them carried the same *CBL c.1211G>A* p.C404Y variant (**Fig. 3D**). Microglia-like cells were differentiated from two independent hiPSC-derived $CBL^{404C/Y}$ lines and their isogenic $CBL^{404C/C}$ controls (**Fig. 4C** and Supplementary Fig. 5C). $CBL^{404C/Y}$ and isogenic $CBL^{404C/C}$ microglia-like cells expressed similar amount of CBL total mRNA and protein, and $CBL^{404C/Y}$ cells expressed wt and mutant mRNA in similar amounts, as expected assuming bi-allelic expression of CBL (Supplementary Fig. 5D-5F). $CBL^{404C/Y}$ cells presented with a phenotype comparable to isogenic $CBL^{404C/C}$ microglia-like cells for expression of IBA1, CSF1R, NGFR, EGFR, CD11b, MRC1, CD36, CD11c, Tim4, CD45, and MHC Class II (Supplementary Fig. 5G). Their viability was also comparable to control (Supplementary Fig. 5H). However, $CBL^{404C/Y}$ cells were larger and presented with more lamellipodia, resulting in an amoeboid morphology less frequently observed in isogenic controls (**Fig. 4D**,**4E**), and their proliferation rate was slightly increased, as measured by EdU incorporation (**Fig. 4F**). Moreover $CBL^{404C/Y}$ cells cultured in CSF1-supplemented medium also presented with a higher basal pERK level than control when restimulated with CSF1 (Supplementary Fig. 5I), and ERK phosphorylation after stimulation of starved microglia-like cells with CSF-1 was increased by ~2 fold in comparison to isogenic WT (**Fig. 4G**). Altogether, these results showed that heterozygosity for a CBL^{C404Y} allele is sufficient to activate human microglia-like cells increasing their proliferation and ERK activation.

Heterozygosity for a CBL^{C404Y} allele drives a microglial neuroinflammatory /AD associated signature

Gene Set Enrichment Analyses (GSEA) of RNAseq comparing $CBL^{404C/Y}$ and isogenic $CBL^{404C/C}$ microglia-like cells showed upregulation of Glycolysis, Oxidative Phosphorylation, and mTORC1 signatures, indicating increased metabolism and energy consumption by the mutant cells (**Fig. 5A** and Supplementary Table 8). In addition, as observed in MAC lines, $CBL^{404C/Y}$ cells upregulated complement, TNF, and JAK STAT signaling and inflammatory signatures (**Fig. 5A**; Supplementary Table 8) [114]. Increased production of TNF, IL-6, IFN-ψ, IL-1β, C3 and complement Factor H (CFH) by $CBL^{404C/Y}$ cells was confirmed by ELISA (**Fig. 5B**). In addition, $CBL^{404C/Y}$ microglia-like cells also presented with signatures from the KEGG database associated with neurodegenerative disorders (**Fig. 5A**; Supplementary Table 8), and for the recently published human microglia AD scRNAseq signature, obtained by analysis of 24 sporadic AD patients and 24 controls [21] (**Fig. 5C**). These data indicated that heterozygosity for the CBL^{C404Y} allele is sufficient to drive expression of a neuroinflammatory /AD signature in a human microglia-like cell type, characterized by increased metabolism and the production of neurotoxic cytokines known to interfere with normal brain homeostasis.

The MAPK variant neuroinflammatory microglial signature is detectable in patients

Analysis of the snRNAseq data from 5 samples of purified microglia nuclei from 4 donors (control, AD without and with pathogenic variants (**Fig.1C**, Supplementary Fig. 1D-H and Supplementary Table 9) using unsupervised Louvain clustering and GSEA showed that microglia samples from patients carrying variants were enriched for the signatures observed in the MAC lines and $CBL^{404C/Y}$ cells (**Fig. 5D,E** and Supplementary Fig. 6). In particular microglia cluster 2 and 2B, were most enriched for the inflammatory, TNF, mTOR and oxidative phosphorylation and

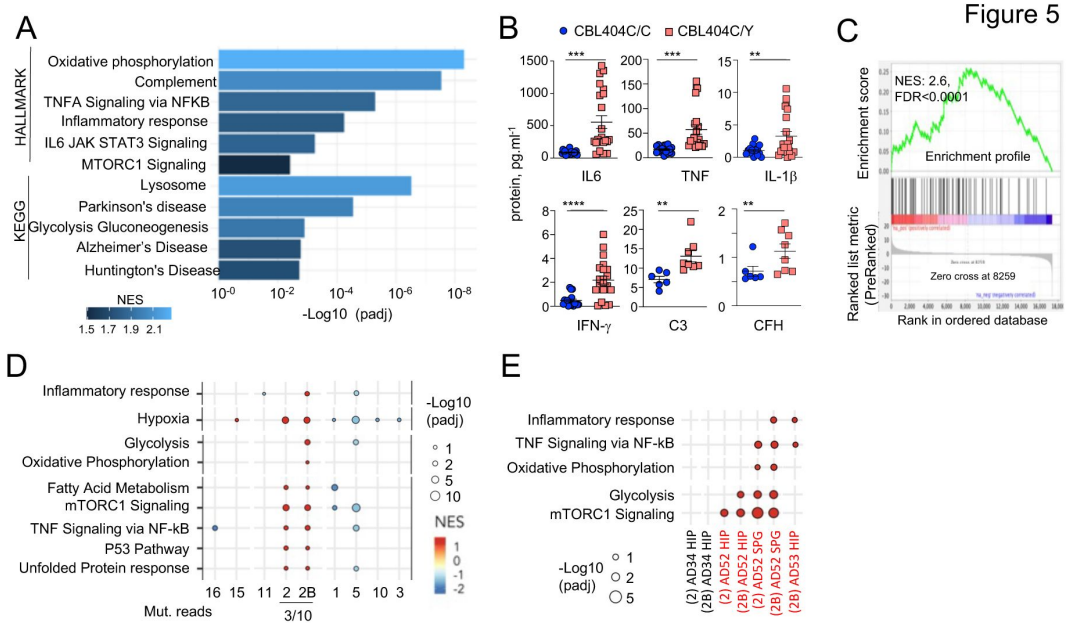


Fig. 5

CBL$^{404C/Y}$ microglia signature.

(A) HALLMARK and KEGG pathways (FDR /adj.p value <0.25, selected from Supplementary Table 8) enriched in gene set enrichment analysis (GSEA) of RNAseq from from CBL$^{404C/Y}$ iPSC-derived macrophages and isogenic controls NES, normalized enrichment score. **(B)** ELISA for pro-inflammatory cytokines (n=3) and complement proteins (n=2) in the supernatant from CBL$^{404C/Y}$ iPSC-derived microglial like cells and isogenic controls. Statistics: *p-value* are obtained by nonparametric Mann-Whitney U test,* *0.05, ** 0.01, *** 0.001, **** 0.0001*. **(C)** GSEA analysis for enrichment of the human AD-microglia snRNAseq signature (MIC1) [21] in differentially expressed genes between CBL$^{404Y/C}$ microglial like cells and isogenic controls. **(D)** Dot plot represents the GSEA analysis of HALLMARK and KEGG pathways enriched in snRNAseq microglia clusters (samples from all donors). Genes are pre-ranked per cluster using differential expression analysis with SCANPY and the Wilcoxon rank-sum method. Statistical analyses were performed using the fgseaMultilevel function in fgsea R package for HALLMARK and KEGG pathways. Selected gene-sets with p-value < 0.05 and adjusted p-value < 0.25 are visualized using ggpubr and ggplot2 R package (gene sets/pathways are selected from Supplementary Fig. 6B, Supplementary Table 9). **(E)** Dot plot represents the GSEA analysis (as in (E)) of HALLMARK and KEGG pathways enriched in cluster 2 / 2B and deconvoluted by donor samples (selected from Supplementary Fig. 6A).

glycolysis signatures in patients carrying variants (AD52, AD53) but not the controls (C11, AD34) (**Fig. 5D,E** and Supplementary Fig. 6). Despite the small size of the mutant clones and the low sensitivity of scRNAseq to detect rare allelic variants, KRAS A59G variant reads were detected in cluster 2/2B from patient AD52.

Altogether, the above results support the hypothesis that patients' microglial clones carrying pathogenic mutations are associated with a metabolic and neuroinflammatory signature that includes the production of neurotoxic cytokines *in vitro* and *in vivo*.

Discussion

We report here that microglia from a cohort of 45 AD patients with intermediate-onset sporadic AD (mean age 65 y.o) is enriched for clones carrying pathogenic/oncogenic variants in genes associated with clonal proliferative disorders (Supplementary Table 2) in comparison to 44 controls. Of note we did not observed microglia P-SNVs within genes reported to be associated with neurological disorders in the patients.

These pathogenic variants are absent from blood, glia or neurons in most cases. They are found predominantly in the MAPK pathway and include reccurent variants (CBL RING domain variants in 6 patients), which promote microglial proliferation, activation, and expression of a neuroinflammatory/neurodegereration-associated transcriptional programme *in vitro* and *in vivo*, and the production of neurotoxic cytokines IL1b, TNF, and IFNg [115–118]. Heterozygous expression of pathogenic CBL variant in human microglia-like cells was sufficient to drive a transcriptional program that associates with increased metabolic activity and a neurotoxic inflammatory response, also observed in microglia from patients with MAPK-activating variants.

The association between AD and MAPK pathway variants is consistent with a previous study where WES performed on unseparated brain tissue from AD patients showed that putative pathogenic somatic variants were enriched for the MAPK pathway, despite the lower sensitivity of the approach and the lack of cellular specificity [30]. The pathogenic role of the somatic pathogenic variants in the MAPK pathway associated with the microglia of AD patients is supported by several lines of evidence. We show here that they promote a neuroinflammatory/neurodegereration-associated transcriptional programme in microglia like cells. In addition, somatic variants that activate the MAPK pathway in tissue macrophages cause a clonal proliferative and inflammatory disease called Histiocytosis, strongly associated with neurodegeneration [37,95–97], and introduction in mouse microglia of the variant allele most frequently associated with histiocytosis ($BRAF^{V600E}$) causes neurodegeneration in mice [37]. The allelic frequencies of pathogenic variants found in AD patients is lower than values classically observed in solid tumors or leukemia, but within the range of the clonal frequency of pathogenic T cells observed in auto-immune diseases [119], and we found that they were in the range of the allelic frequencies observed for the $BRAF^{V600E}$ variant in microglia in the brain of Histiocytosis patients. Moreover, the RAS/MAPK signaling pathway is involved in microglia proliferation, activation and inflammatory response [120–122], neuronal death, neurodegeneration, and AD pathogenesis [15,19,37,123], and its activation has been proposed to be an early event in the pathophysiology of AD in human [124]. Neuroinflammation is an early event in AD pathogenesis, increasingly considered as critical in pathogenesis initiation and progression [16,125,126]. This is underscored by the observation that the main known genetic risk factor for sporadic AD is the APOE4 allele, responsible for an increased inflammatory response in the brain of APOE4 carriers [16]. In this regard, the contributing role of MAPK activating variants could be comparable to that of the APOE4 allele, and we noted that the allelic frequency of APOE4 allele is lower in patients with pathogenic variants (16/46 alleles, 34%) than patients without detected variant (23/44 alleles, 53%) although the difference did not reach significance in this series.

Variants targeting the DNA-repair and DNA/histone methylation pathways are also enriched among AD patients, sometimes associated in the same patients, albeit their functional significance was not investigated here. Of note however, germline variants of the DNA-repair transcription factor TP53, and DNA damage sensors ATR and CHEK2 were shown to promote accelerated neurodegeneration in human [83,84].

Microglia variants are frequently absent from blood, and our DNA sequencing barcoding approach does not support a model where blood cells massively infiltrate the brain or replace the microglia pool in patients from our series, but instead consistent with the local maintenance and proliferation of microglia [38,39]. In addition, our results are consistent with a recent study showing that clonal hematopoiesis was inversely associated with the risk of AD[61].

The association of microglia clones carrying pathogenic variants with AD in a subset of patients is not a consequence of an overall increase in microglia mutational load (SNV) in AD. Together with evidence that pathogenic variants drive neuroinflammation, these data suggest that these clones could contribute to AD pathogenesis, together with other genetic and environmental factors. Lewy bodies, amyloid angiopathy, tauopathy, or alpha synucleinopathy, were equally distributed among AD patients with or without microglia clones carrying MAPK activating variants. The natural history of the microglial clones is difficult to study in human. It is possible that microglial clones with proliferative and activation advantage and a neuroinflammatory and neurotoxic profile may be present at the onset and contribute to the early stages of the disease. Alternatively it is also possible that the microglial clones carrying the pathogenic mutations appear or are selected later during the course of the disease in the inflammatory milieu of the AD brain. In the latter case, pathogenic microglial clones may contribute to disease progression, *i.e.* neuroinflammation and neurodegeneration.

Competing interests

FG has been a paid consultant (no equity) to Third Rock Ventures from 2018 to 2020. Sequencing costs and analysis in this study were covered in part by a SRA between Third Rock venture and MSKCC. This work led to patents PCT/US2022/037893/WO2023004054A1 *'Methods and compositions for the treatment of alzheimer's disease'* by MSKCC and PCT/US2018/047964 'Kinase mutation-associated neurodegenerative disorders by MSKCC'.

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Authors contribution

RV and FG designed the study and wrote the draft of the manuscript. RV designed, performed and supervised the collection of brain samples, brain nuclei separation and preparation of samples for DNA and snRNA sequencing with help from AA, OA and AB. DNA and bulk and snRNAseq sequencing were performed in MSKCC genomics core under supervision of AV and NS. DNA sequencing data were analyzed, validated, and interpreated RV and SF, with support from NS, BB, MO, JLC, and FG. Transfected/transduced lell lines were generated by BPC, SYH, WTM (HEK cells) and RV (BV-2 lines) and LW (MAC lines). hiPSCs clones heterozygous for the patients' variants and isogenic controls were generated and validated with the SKI Stem Cell Research Core by TZ, LW and TL. Biochemical analysis of HEK and BV2 lines was performed by BPC, SYH, WTM. Protocols for culture and analysis of hiPSC-derived cells were designed by TL. Biochemical and phenotypic analysis and RNA preparation for MAC lines hiPSC-derived cells was performed by LW. Analysis of bulk RNAseq data was performed by SF (hiPSC derived cells) and NS (Mac lines). Analysis of snRNAseq was performed by YH, LW, and OE. The Netherland Brain Bank, and MSKCC LWP (CAI-D and RK) provided patients and control brain and blood samples. RMR, RC, and OAW contributed to the discussion of the results and edited the manuscript. FG supervised all aspect of the work. RV and FG prepared the initial and revised manuscripts. All authors contributed to the manuscript.

Methods

Tissue samples

The study was conducted according to the Declaration of Helsinki. Human tissues were obtained with patient-informed consent and used under approval by the Institutional Review Boards from Memorial Sloan Kettering Cancer Center (IRB protocols #X19-027). Snap-frozen human brain and matched blood were provided by the Netherlands Brain Bank (NBB), the Human Brain Collection Core (HBCC, NIH), Hospital Sant Joan de Déu and the Rapid Autopsy Program (MSKCC, IRB #15-021). Samples were neuropathologically evaluated and classified by the collaborating institutions as Alzheimer's disease (AD) [1–5] or non-dementia controls. The mean age of AD patients is 65 years old (55.5% female, 44.5% male). The mean age of all controls is 54 years old (60% female, 40% male), and the mean age of AD age-matched controls was 70 years old (60% female , 40% male). The overall mean of the post-mortem delay interval was 9.8 hours. Patients did not present with germline pathogenic PSEN1/2/3 or APP AD's associated variants. For additional information on donor's brain regions, sex, age, cause of death, Apoe status, Braak status see Supplementary Table S1. To avoid possible contamination of sequencing data with mutations associated with donor's tumoral disease in the group of non-dementia controls, we refrained from selecting cases with blood malignancies or with brain tumors. Samples from histiocytosis patients were collected under GENE HISTIO study (approved by CNIL and CPP Ile-de France) from Pitié-Salpêtrière Hospital and Hospital Trousseau and from Memorial Sloan Kettering Cancer Center.

Nuclei isolation from frozen brain samples, FACS-sorting and DNA extraction

All samples were handled and processed under Air Clean PCR Workstation. An average of 400 mg of frozen brain tissues were homogenized with a sterile Dounce tissue grinder using a sterile non-ionic surfactant-based buffer to isolate cell nuclei ('homogenization buffer': 250 mM Sucrose, 25 mM KCL, 5 mM MgCl2, 10 mM Tris buffer pH 8.0, 0.1% (v/v) Triton X-100, 3 µM DAPI, Nuclease Free Water). Homogenate was filtered in a 40-µm cell strainer and centrifuged 800g 8 min 4°C. To clean-up the homogenate, we performed a iodixanol density gradient centrifugation as follow: pellet was

gently mixed 1:1 with iodixanol medium at 50% (50% Iodixanol, 250 mM Sucrose, 150 mM KCL, 30 mM MgCl2, 60 mM Tris buffer pH 8.0, Nuclease Free Water) and homogenization buffer. This solution layered to a new tube containing equal volume of iodixanol medium at 29% and centrifuged 13.500g for 20 min at 4°C. Nuclei pellet was gently resuspended in 200 µl of FACS buffer (0.5% BSA, 2mM EDTA) and incubated on ice for 10 min. After centrifugation 800g 5 min 4°C, sample was incubated with anti-NeuN (neuronal marker, 1:500, Anti-NeuN-PE, clone A60 Milli-Mark™) for 40 min. After centrifugation 800g 5 min 4°C, sample was washed with 1X Permeabilization buffer (Foxp3 / Transcription Factor Staining Buffer Set, eBioscience™) and centrifuged 1300g for 5 min, without breaks to improve nuclei recovery. Staining with anti-Pu.1 antibody in 1X Permeabilization buffer (myeloid marker 1:50, Pu.1-AlexaFluor 647, 9G7 Cell Signaling) was performed for 40 min. After a wash with FACS buffer samples were prepared for FACS. Nuclei were FACS-sorted in a BD FACS Aria with a 100-µm nozzle and a sheath pressure 20 psi, operating at ~1000 events per second. Nuclei were sorted into 1.5 ml certified RNAse, DNAse DNA, ATP and Endotoxins tubes containing 100µl of sterile PBS. For detailes on sorted samples see Supplementary Table S1 . Sorting purity was >95%. Sorting strategy is depicted in Supplementary Fig 1. Of note, the Double-negative gate is restricted to prevent cross-contamination between cell types. Nuclei suspensions were centrifuged 20 min at 6000g and processed immediately for gDNA extraction with QIAamp DNA Micro Kit (Qiagen) following manufacture instructions. DNA from whole-blood samples was extracted with QIAamp DNA Micro Kit (Qiagen) following manufacture instructions. Flow cytometry data was collected using DiVa 8.0.1 Software. Subsequent analysis was performed with FlowJo_10.6.2. For sorting strategy, see Supplementary Fig 1.

DNA library preparation and sequencing

DNA samples were submitted to the Integrated Genomics Operation (IGO) at MSKCC for quality and quantity analysis, library preparation and sequencing. DNA quality mas measured with Tapestation 2200. All samples had a DNA Integrity Number (DIN) >6. After PicoGreen quantification, ~200ng of genomic DNA were used for library construction using the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) with 8 cycles of PCR. After sample barcoding, 2.5ng-1µg of each library were pooled and captured by hybridization with baits specific to either the HEME-PACT (Integrated Mutation Profiling of Actionable Cancer Targets related to Hematological Malignancies) assay, designed to capture all protein-coding exons and select introns of 576 (2.88Mb) commonly implicated oncogenes, tumor suppressor genes [6] and/or HEME/BRAIN-PACT (716 genes, 3.44 Mb, Supplementary Table S2) an expanded panel that included additional custom targets related to neurological diseases including, Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS) and others (tableS1) [7–15]. To simplify, in the manuscript the combined panel is referred to as 'BRAIN-PACT'. In Supplementary Table S3, 'Heme-only' or 'Brain-only' is indicated in the cases for which only one or the other panels were used. Capture pools were sequenced on the HiSeq 4000, using the HiSeq 3000/4000 SBS Kit (Illumina) for PE100 reads. Samples were sequenced to a mean depth of coverage of 1106x (Control samples: 1071x, AD samples 1100x). For detailed information on the sample quality control checks used to avoid potential sample and/or barcode mix-ups and contamination from external DNA, see [6].

Mutation data analysis

The data processing pipeline for detecting variants in Illumina HiSeq data is as follows. First the FASTQ files are processed to remove any adapter sequences at the end of the reads using cutadapt (v1.6). The files are then mapped using the BWA mapper (bwa mem v0.7.12). After mapping the SAM files are sorted and read group tags are added using the PICARD tools. After sorting in coordinate order the BAM's are processed with PICARD MarkDuplicates. The marked BAM files are then processed using the GATK toolkit (v 3.2) according to best practices for tumor normal pairs. They are first realigned using ABRA (v 0.92) and then the base quality values are recalibrated with the BaseQRecalibrator. Somatic variants are then called in the processed BAMs using MuTect (v1.1.7) for SNV and ShearwaterML[16–18].

muTect (v1.1.7)

to identify somatic variants and eliminate germline variants, we run the pipeline as follow: PU.1, DN and Blood samples against matching-NeuN samples, and NeuN samples against matching-PU.1. In addition, we ran all samples against a Frozen-Pool of 10 random genomes. We selected Single Nucleotide Variations (SNVs) [Missense, Nonsense, Splice Site, Splice Regions] that were supported by at least 4 or more mutant reads and with coverage of 50x or more. Fill-out file for each project (~27 samples per sequencing pool), were used to exclude by manual curation, variants with high background noise. This resulted in 428 variants (Missense, Nonsense, Splice_site, Splice_Region).

ShearwaterML

was used to look for low allelic frequency somatic mutations as it has been shown to efficiently call variants present in a small fraction of cells with true positives being ~90%. Briefly, the basis of this algorithm is that is uses a collection of deep-sequenced samples to learn for each site a base-specific error model, by fitting a beta-binomial distribution to each site combining the error rates across all normal samples both the mean error rate at the site and the variation across samples, and comparing the observed variant rate in the sample of interest against this background model using a likelihood-ratio test. For detailed description of this algorithm please refer to [16,17]. In our data set, for each cell type (NeuN, DN, PU.1) we used as "normal" a combination of the other cell types, i.e PU.1 vs NeuN+DN, DN vs NeuN+PU.1, NEUN vs PU.1+DN, Blood vs NeuN+DN. Since all samples were processed and sequenced using the same protocol, we expect the background error to be even across samples. More than 400 samples were used as background leading to an average background coverage >400.000x. Resulting variants for each cell type were filtered out as germline if they were present in more than 20% of all reads across samples. Additionally, variants with coverage of less than 50x and more than 35% variant allelic frequency (VAF) were removed from downstream analysis. P-values were corrected for multiple testing using Benjamini & Hochberg's False Discovery Rate (FDR) [19] and a q-value of cutoff of 0.01 was used to call somatic variants. Variants were required to have a least one supporting read in each strand. Somatic variants within 10bp of an indel were filtered out as they typically reflect mapping errors. We selected Single Nucleotide Variations (SNVs) [Intronic, Intergenic, Missense, Nonsense, Splice Site, Splice Regions] that were supported by at least 4 or more mutant reads and annotated them using VEP. Finally, to reduce the risk of SNP contamination, we excluded variants with a MAF (minor allelic frequency) cutoff of 0.01 using the gnomeAD database. This resulted in 509 SNVs.

We compared the final mutant calls from Muetct1 and ShearwaterML and found that 30% of the events (111 variants) that were called by MuTect1 were also called by ShearwaterML. Overall a total of 826 variants (Supplementary Table S3) were found, with a mean coverage at the mutant site of 668.3X (10% percentile: 276X, 90% percentile: 1181X) and a mean of 29.1 mutant reads (10% percentile: 4, 90% percentile: 52), with 84% of mutated supported by at least 5 mutant reads (Supplementary Table S3). The median allelic frequency was ~1.34% (Supplementary Table S3). Negative results for matching brain negative samples were confirmed in 100% of samples at a mean depth of ~5000x (range 648-23.000x) (Supplementary Table S3), confirming nuclei sorting purity of >95% for $PU.1^{+}$, DN, and $NEUN^{+}$ populations.

Validation of variants by droplet-digital-PCR (ddPCR)

We performed validation of ~11% of unique variants (69/760) by droplet-digital PCR (ddPCR) on pre-amplified DNA or on libraries (in the cases where DNA was not sufficient). Around 15% (15/69) of the variants analyzed by ddPCR were called by ShearwaterML, ~44% (34/69) were called by Mutect1 and 40% (24/69) by both ShearwaterML+ Mutect1. Altogether we confirmed 62/69 of variants tested (~90%). In addition, 61 assays (from variants detected in PU.1+ nuclei) were tested in paired cell types isolated from the same brain region). Assays were also run in matching blood when available. The mean depth of ddPCR was ~5000x and mutant counts of 3 or more were

considered positive. VAF obtained by ddPCR correlated with original VAF by sequencing (R2 0.93, p<0.0001). For **KRAS_G12D:** Bio-Rad validated assay (Unique Assay ID: dHsaMDV2510596) and **MTOR_Arg1616His_c.4847G>A:** Bio-Rad validated assay (Unique Assay ID: dHsaMDV2510596) were used. The remaining assays were designed and ordered through Bio-Rad. For setting-up the right conditions for newly designed assays, cycling conditions were tested to ensure optimal annealing/extension temperature as well as optimal separation of positive from empty droplets. All reactions were performed on a QX200 ddPCR system (Bio-Rad catalog # 1864001). When possible, each sample was evaluated in technical duplicates or quartets. Reactions contained 10ng gDNA, primers and probes, and digital PCR Supermix for probes (no dUTP). Reactions were partitioned into a median of ~31,000 droplets per well using the QX200 droplet generator. Emulsified PCRs were run on a 96-well thermal cycler using cycling conditions identified during the optimization step (95°C 10'; 40-50 cycles of 94°C 30' and 52-56°C 1'; 98°C 10'; 4°C hold). Plates were read and analyzed with the QuantaSoft sotware to assess the number of droplets positive for mutant DNA, wild-type DNA, both, or neither. ddPCR results are listed in Supplementary Table S3.

Classification of variants

To classify somatic variants according to their pathogenicity we did as follow: Variants were classified as 'pathogenic (P-SNV)' if reported as 'pathogenic/likely pathogenic' by ClinVar[20] and/or 'oncogenic/predicted oncogenic/likely oncogenic' by OncoKb[21]. (Supplementary Table S3). These two databases report pathogenicity in cancer and other diseases, based on supporting evidence from curated literature (see corresponding citations in Supplementary Table S3). We considered classical-MAPK-pathway genes those reported to be mutated in RASopathies: *BRAF, CBL, KRAS, MAP2K1, NF1, PTPN11, SOS1, RIT1, SHOC2, NRAS, RAF1, RASA1, HRAS, MAP2K2,SPRED1* [22,23]. (Supplementary Table S3).

Quantification of mutational load and statistics

We defined mutational load or mutational burden as the number of synonymous and non-synonymous somatic single-nucleotide-variant (SNV) per megabase of genome examined [24]. Overall, a total of 826 single-nucleotide-variants (SNV) were detected resulting in 0.3 mutations/Mb sequenced. As detailed in the manuscript, the mutational load varies considerably across cell types and patients. To quantify mutational load we took into consideration the panel used for sequencing each sample: HEME-PACT (2.88 Mb) or the extended panel BRAIN-PACT (3.44 Mb) (see Supplementary Table S2). Therefore, the number of mutations was normalized by the number of Mb sequenced for that specific sample. In the cases where we calculated mutational load per patient, we averaged the mutational load of each sample from that patient for a given cell type [(i.e if for one patient, 2 PU.1 samples were sequenced, one from hippocampus and one from superior parietal cortex (with BRAIN-PACT) then the mutational load for PU.1 for that patient is the mean of the mutational load of the 2 PU.1 samples analyzed). For the quantification of 'pathogenic' variants, the same analysis is performed, quantifying only variants that are reported as pathogenic by ClinVar and/or OncoKb. Statistical significance was analyzed with GraphPad Prism (v9) and R (3.6.3). Non-parametric tests were used when data did not follow a normal distribution (Normality test: D'Agostino-Pearson and Shapiro-Wilk test). For normally distributed data, unpaired t-test was used to compare two groups and one-way, nested one-way or two-way analyses of variance (ANOVA) were used for comparing more than two groups, as indicated in the Figure legends. For data that did not have a normal distribution, the tests performed were unpaired two-tailed Mann-Whitney U test and Kruskal–Wallis test and Dunn's test for multiple comparisons. Pearson and Spearman were used for correlation analysis. In **Fig. 2G**, we used multivariate logistic regression analysis to test if there was an association between Alzheimer's disease and the presence of pathogenic variants in $PU.1^{+}$ nuclei. We used Alzheimer's disease as a dependent variable, and age, sex, and the presence of pathogenic variant/s (Yes/No) as co-variates. In all the statistical tests, significance was considered at $P < 0.05$. For Venn Diagram plots, we used [25].

Mixed-effects modelling of somatic P-SNV burden

To evaluate the correlation between P-SNV burden and disease status (non-dementia controls and AD) after adjusting for other factors such as individual donor and age, we performed linear mixed-effects regression modeling using the nlme package in R. In the subsequent analysis, we also tested another framework implemented in the lme4 package and confirmed similar findings. We estimated the linear mixed model via maximum likelihood method with nlminb optimizer. The model included individual donor as a random effect. We also tested if inclusion of other co-variates (i.e., sex, anatomical location of the brain, and source biobank of brain samples) as random effects inproved the overall model fitting via likelihood ratio test. However, none of these co-variates improved the model fitting (P>0.99). Thus, we used a relatively simple model that incorporated disease status and age as fixed effects and donor as random effect. The total explanatory power of the final model is substantial (conditional R^2=0.48). To assess the significance of age or disease status in predicting P-SNV burden, we constructed another model that does not incorporate the variable as fixed effect, and compared the two models via likelihood ratio test. The variable was considered significantly associated with P-SNV burden when the P value was below 0.05 and the AIC increased after removing the variable.

Pathway enrichment analysis of genes target of variants

was performed using Metascape [26] and the following ontology sources: KEGG Pathway [27,28], GO Molecular function [29,30], Reactome Gene Sets [29,30] and Canonical Pathways [31]. The list of 716 genes from the targeted panel were used as the enrichment background. Terms with a p-value< 0.05, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are shown. p-values are calculated based on the cumulative hypergeometric distribution [32].

Expression of target genes in microglia

To evaluate the expression levels of the genes identified in this study as target of somatic variants, we consulted a publicly available database (*https://www.proteinatlas.org/*), and also plotted their expression as determined by RNAseq in 2 studies (Galatro et al. GSE99074 [33], and Gosselin et al. [34]) (Table S3 and Figure S2). For data from Galatro et al. (GSE99074) [33], normalized gene expression data and associated clinical information of isolated human microglia (N = 39) and whole brain (N = 16) from healthy controls were downloaded from GEO. For data from Gosselin et al. [34], raw gene expression data and associated clinical information of isolated microglia (N = 3) and whole brain (N = 1) from healthy controls were extracted from the original dataset. Raw counts were normalized using the DESeq2 package in R [35].”

Nuclei isolation from frozen brain samples for sn-RNAseq

For sn-RNAseq studies we only selected samples with a RIN score in whole tissue of 6 or more. All samples were handled and processed under Air Clean PCR Workstation. About 250-400 mg of frozen brain tissues were homogenized with a sterile Dounce tissue grinder using a sterile homogenization buffer to isolate cell nuclei (250 mM Sucrose, 25 mM KCL, 5 mM MgCl2, 10 mM Tris buffer pH 8.0, 0.1% (v/v) Triton X-100, 3 µM DAPI, Nuclease Free Water and 20 U/ml of Superase-In RNase inhibitor and 40 U/ml RNasin ribonuclease inhibitor). Homogenate was filtered in a 40-µm cell strainer and centrifuged 800g 8 min 4°C. To clean-up the homogenate, we performed a iodixanol density gradient centrifugation as follow: pellet was gently mixed 1:1 with iodixanol medium at 50% (50% Iodixanol, 250 mM Sucrose, 150 mM KCL, 30 mM MgCl2, 60 mM Tris buffer pH 8.0, Nuclease Free Water) and homogenization buffer. This solution layered to a new tube containing equal volume of iodixanol medium at 29% and centrifuged 13.500g for 20 min at 4°C. Nuclei pellet was resuspended in FACS buffer with RNAse inhibitors (0.5% BSA, 2mM EDTA, Superase-In RNase inhibitor and 40 U/ml RNasin ribonuclease inhibitor) and centrifuged 800g 5 min, 4 °C. Nuclei pellet was fixed with 90% ice-cold methanol and incubated for 10 min on

ice, followed by a centrifugation at 1300g (without brakes, which improves with nuclei recovery after fixation). The pellet was resuspended in permeabilization buffer (6% BSA, Superase-In RNase inhibitor 20 U/mL, RNasin ribonuclease inhibitor 40 U/mL and 0.05% Triton) followed by a centrifugation at 1300g. Sample was incubated with anti-Pu.1 antibody (microglia marker 1:50, Pu.1-AlexaFluor 647, 9G7 Cell Signaling) in permeabilization buffer. After a wash with FACS buffer sample were ready for sorting. Nuclei are FACS-sorted in a BD FACS Aria with a 100-µm nozzle and a sheath pressure 20 psi, operating at ~1000 events per second. Nuclei were sorted into 1.5 ml certified RNAse, DNAse DNA, ATP and Endotoxins tubes containing 100µl of sterile PBS. For each population we sorted >10^5 nuclei into FACS buffer.

Sn-RNAseq library preparation and sequencing

The single-nuclei RNA-Seq of FACS-sorted nuclei suspensions was performed on Chromium instrument (10X genomics) following the user guide manual (Reagent Kit 3' v3.1). Each sample, containing approximately 10,000 nuclei at a final dilution of ~1,000 cells/µl was loaded onto the cartridge following the manual. The individual transcriptomes of encapsulated cells were barcoded during RT step and resulting cDNA purified with DynaBeads followed by amplification per manual guidelines. Next, PCR-amplified product was fragmented, A-tailed, purified with 1.2X SPRI beads, ligated to the sequencing adapters and indexed by PCR. The indexed DNA libraries were double-size purified (0.6–0.8X) with SPRI beads and sequenced on Illumina NovaSeq S4 platform (R1 – 26 cycles, i7 – 8 cycles, R2 – 70 cycles or higher). Sequencing depth was ~200 million reads per sample on average. FASQ files were processed using SEQC pipeline [36] for quality control, mapping to GRCH38 reference genome, and log2 transformation of the data with the default SEQC parameters to obtain the gene-cell count matrix.

Sn-RNAseq analysis

Seurat v4.0.3 with default parameters was used to perform sctransform (SCT) normalization, integration and Uniform Manifold Approximation and Projection (UMAP) for dimensionality reduction. The FindClusters function was used for cell clustering. To improve clustering, all samples were analyzed in an integrated analysis, based on canonical correlation analysis (CCA). Cell types were annotated using the top 500 DEGs of each cell type in a human cortex database. Data can be accessed at *https://weillcornellmed.shinyapps.io/Human_brain/*. The removal of doublets using DoubletFinder and cells with high mitochondrial content (>10% mitochondrial RNA) yielded between 6,437 and 9,241 nuclei per patient and sample. Microglia represented 94 ± 3% of total cells. Unique Molecular Identifiers (UMIs) per nucleus and gene count per nucleus were comparable between donors. Integrated_snn at resolution 0.2 outlined 16 microglia clusters. Except for cluster 13 consisting at 97% of cells from the healthy control Control 11_AG, all donors and samples were represented in every cluster. One cluster contained few cells (0.84% of total microglia, for an average of 6.20 ± 1.60% for other clusters) and was marked by a low number of cluster-enriched genes and was excluded from further analyses. For pathway enrichment analysis, genes were pre-ranked using differential expression analysis in SCANPY [37] with Wilcoxon rank-sum method. Statistical analysis were performed using the fgseaMultilevel function in fgsea R package [38] for HALLMARK and KEGG pathways. Gene sets with p-value < 0.05 and adjusted p-value < 0.25 were selected and visualized using ggpubr and ggplot2 [39] R package. For the variant analysis of AD52_HIP harboring a $KRAS^{A59G}$ (c.176C>G) clone, Integrative Genomics Viewer (IGV) software was used to display sequencing reads at KRAS c.176C (exon 3; GRCh38 chr12:25,227,348). Cells within each cluster were identified based on the 16-digit barcodes from SEQC-aligned reads. Barcodes were converted to the 10X Genomics format and used to sample reads from each cluster within the original BAM file. BAM subsets for each cluster were read with IGV and reads with identical UMIs were filtered out to account for amplification bias.

Whole-Exome-Sequencing and analysis

Remaining libraries from a selected group of PU.1 and NEUN samples sequenced with BRAIN-PACT (see above) were sequenced by Whole-Exome-Sequencing (WES). Matching NEUN samples were sequenced to extract the germline variants. Around 100 ng of library were captured by hybridization using the xGen Exome Research Panel v2.0 (IDT) according to the manufacturer's protocol. PCR amplification of the post-capture libraries was carried out for 12 cycles. Samples were run on a NovaSeq 6000 in a PE100 run, using the NovaSeq 6000 S4 Reagent Kit (200 Cycles) (Illumina). Samples were covered to an average of 419X. The data processing pipeline for detecting variants in Novaseq data is as follows. First the FASTQ files are processed to remove any adapter sequences at the end of the reads using cutadapt (v1.6). The files are then mapped using the BWA mapper (bwa mem v0.7.12). After mapping the SAM files are sorted and read group tags are added using the PICARD tools. After sorting in coordinate order the BAM's are processed with PICARD MarkDuplicates. The marked BAM files are then processed using the GATK toolkit (v 3.2) according to the best practices for tumor normal pairs. They are first realigned using ABRA (v 0.92) and then the base quality values are recalibrated with the BaseQRecalibrator. Somatic variants are then called in the processed BAMs using muTect (v1.1.7) for SNV and the Haplotype caller from GATK with a custom post-processing script to call somatic indels. The full pipeline is available here *https://github.com/soccin/BIC-variants_pipeline* and the post processing code is at *https://github.com/soccin/Variant-PostProcess*. We selected Single Nucleotide Variants (SNVs) [Missense, Nonsense, Splice Site, Splice Regions] that were supported by at least 8 or more mutant reads, variant allelic frequency above 5% and with coverage of 50x. Annotation was performed using VEP. Finally, to reduce the risk of SNP contamination, we excluded variants with a MAF (minor allelic frequency) cutoff of 0.01 using the genomeAD database. Variants were classified as 'candidate pathogenic' when SNV is predicted to affect the protein as determined by PolyPhen-2 (possibly and probably damaging) and SIFT (deleterious) and CADD-MSC (high) and FATHMM-XF (Functional Analysis through Hidden Markov Models (pathogenic) [40] (Supplementary Table S5).

Cell lines

HEK293T cell culture and transfection

HEK 293T cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Sigma) and 1000 IU/ml penicillin, 1000 IU/ml streptomycin.

BV2 microglial cell line

BV2 murine microglial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) High Glucose medium (Gibco), Glutamax (Gibco), sodium pyruvate, 1% non-essential amino acids (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS, EMD Millipore). For MAPK activation experiments, cells were treated with M-CSF1 100 ng/ml for 5 min.

MAC cell lines

Mouse primary CSF-1 dependent macrophages immortalized with the SV-U19-5 retrovirus [41] were a gift of Dr. E. R. Stanley (Albert Einstein College of Medicine, Bronx, NY). They were cultured in RPMI 1640 medium with Glutamax (Gibco), 10 % heat-inactivated fetal bovine serum (FBS, EMD Millipore) and 100 ng/mL recombinant CSF-1 (gift from Dr. E. R. Stanley). Growth medium was renewed every second day. When confluency reached 80%, cells were passaged by cell scraping and plated at 5 X 10^4 cells/ cm^2 in tissue culture treated plates. For signaling pathway analyses, cell proliferation assays or collection for RNA sequencing, cells were plated one day prior at 5 X 10^4

cells/ cm^2 in tissue culture treated plates. For signaling pathway analyses, cell proliferation assays or collection for RNA sequencing, cells were plated one day prior at 5 X 10^4 cells/ cm^2 in medium containing 10 ng/mL CSF-1 for lines expressing wild-type (WT) and mutant CBL, RIT1 and KRAS proteins and 100 ng/mL CSF-1 for lines expressing WT and mutant PTPN11 proteins. Cells were grown at 37°C and 5% CO2.

Human induced pluripotent stem cell (hiPSC) culture

Human induced Pluripotent Stem Cell (hiPSC) lines were derived from peripheral blood mononuclear cells (PBMCs) of a healthy donor. Written informed consent was obtained according to the Helsinki convention. The study was approved by the Institutional Review Board of St Thomas' Hospital; Guy's hospital; the King's College London University; the Memorial Sloan Kettering Cancer Center and by the Tri-institutional (MSKCC, Weill-Cornell, Rockefeller University) Embryonic Stem Cell Research Oversight (ESCRO) Committee. hiPSC were derived using Sendai viral vectors (ThermoFisher Scientific; A16517). Newly derived hiPSC clones were maintained in culture for 10 passages (2-3 months) to remove any traces of Sendai viral particles. Over 90% of hiPSCs in the derived lines expressed high levels of the pluripotency markers NANOG and OCT4 by flow cytometry. The C12 hiPSC WT line was engineered to carry a CBL p.C404Y, c.1211G>A heterozygous variant at the endogenous CBL locus. HiPSCs of passage 25-35 were cultured on confluent irradiated CF1 mouse embryo fibroblasts (MEFs, Gibco) in hiPSC medium consisting of knock-out DMEM (Invitrogen), 10% knock-out-Serum Replacement (Invitrogen), 2 mM L-glutamine (Gibco), 100 U/mL penicillin-streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (R&D). hiPSC medium was supplement with 10 ng/mL bFGF (PeproTech) and changed every second day. Two days before culture with hiPSCs, MEFs were plated at 20,000 cells/cm^2 in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS, EMD Millipore), 100 U/mL penicillin-streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol (R&D Systems) on 150 mm tissue culture plates coated with 0.1% gelatin (Sigma). hiPSCs were passaged weekly with 250 U/mL collagenase type IV (ThermoFisher Scientific) at a 1:4 to 1:6 ratio onto MEF cells in hiPSC medium supplemented with 10 μM Rock inhibitor (Y-27632 dihydrochloride, Sigma). Cells were maintained at 37°C and 5% CO2 and they were routinely tested for mycoplasma and periodically assessed for genomic integrity by karyotyping. Microglia-like cells were obtained from hiPSCs using an embryoid body (EB)-based protocol as previously described [42]. Briefly, hiPSC were loosened with 250 U/mL collagenase type IV (ThermoFisher Scientific) and lifted with cell scraping. For EBs formation, hiPSC colonies were transferred to suspension plates on an orbital shaker in hiPSC medium supplemented with 10 μM Rock inhibitor (Y-27632 dihydrochloride, Sigma). After 6 days, EBs were transferred to 6-wells tissue culture treated plates in STEMdiff APEL 2 medium (Stem Cell Technology) with 5% Protein Free Hybridoma Media (Gibco), 100 U/mL penicillin-streptomycin (Invitrogen), 25 ng/mL IL-3 (Peprotech) and 50 ng/mL CSF-1 (Peprotech).

Microglia-like cells were harvested every week from the supernatant of EBs cultures. Collected microglia-like cells were used immediately for signaling pathway analyses or plated for 6-7 days in RPMI 1640 medium with Glutamax supplement (Gibco), 10 % heat-inactivated fetal bovine serum (FBS, EMD Millipore) and 100 ng/mL human recombinant CSF-1 (Peprotech) in tissue culture plates for cytology, flow cytometry, RNA sequencing and supernatant analyses of cytokines release. Microglia like cells differentiation was monitored by May-Grunwald Giemsa staining and flow cytometry analyses of myeloid markers.

Plasmids used in *in-vitro* studies (HEK293, BV2, and MAC lines)

The expression vectors for Flag-tagged CHK2 kinase and RIT1 were from Sino Biological and Origene, respectively. The vector encoding pcDNA3-HA-tagged c-Cbl was a kind gift from Dr. Nicholas Carpino (Stony Brook). $RIT1^{M90I,}$ $RIT1^{F82L}$, CBL^{I383M}, CBL^{C404Y}, CBL^{C416S}, CBL^{C384Y}, CBL^{Y371H} were generated by site-directed mutagenesis using the QuikChange Kit (Agilent). pHAGE_puro was a gift from Christopher Vakoc (Addgene plasmid # 118692; *http://n2t.net/addgene:*

118692; RRID:Addgene_118692) [43]. pHAGE-KRAS was a gift from Gordon Mills & Kenneth Scott (Addgene plasmid # 116755; *http://n2t.net/addgene:116755*; RRID: Addgene_116755) [44]. pHAGE-PTPN11 was a gift from Gordon Mills & Kenneth Scott (Addgene plasmid # 116782; *http://n2t.net/addgene:116782*; RRID: Addgene_116782) [44]. pHAGE-PTPN11-T73I was a gift from Gordon Mills & Kenneth Scott (Addgene plasmid # 116647; *http://n2t.net/addgene:116647*; RRID: Addgene_116647) [44]. pDONR223_KRAS_p.A59G was a gift from Jesse Boehm & William Hahn & David Root (Addgene plasmid # 81662 ; *http://n2t.net/addgene:81662* ; RRID:Addgene_81662) [45], Phage-CBL, Phage-CBL^{I383M}, Phage-CBL^{C404Y}, Phage-CBL^{C416S}, Phage-RIT1, Phage-RIT1^{M90I} and Phage-RIT1^{F82L} and Phage-KRAS^{A59G} were generated by Azenta Life Sciences via a PCR cloning approach. pHAGE-CBL^{C384Y} plasmid was generated at Azenta Life Science by targeted mutagenesis of pHAGE-CBL.

Generation of mutant lines

HEK293 were transfected 24 hours after plating with 2.5 µL of Mirus Transit LT1 per µg of DNA. Cells were harvested and lysed 48 hrs after transfection using a buffer containing 25 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 200 µM PMSF, and 0.2 mM Na_3VO_4. For EGF stimulation, the media was replaced 24 hours after transfection with DMEM containing 1% FBS and antibiotics. After a further 24 hours in this starvation media, the cells were stimulated with 50ng/ml EGF for 5 minutes at 37°C. **Lentiviral production and transduction of BV2 and MAC cell lines**. For BV2 cell line, cells were transduced for 24 hours without the presence of Vpx VLPs and selected with 2.5 µg/mL puromycin (Fisher Scientific). For 'MAC' lines, Vpx-containing virus-like particles (Vpx VLPs) were produced by transfection of HEK293T cells with 4.8 ug VSV-g plasmid and 31.2 ug pSIV3/Vpx plasmid, a gift from Dr. M. Menager (Imagine Institute, Paris, France) using TransIT-293 Transfection Reagent (Mirus Bio, Fisher Scientific). Forty-eight hours after transfection, the supernatant containing Vpx VLPs was collected and used immediately for lentiviral transduction of macrophages. Viral supernatants were obtained by transfection of HEK293T cells using X-tremeGENE HP DNA Transfection Reagent (Sigma). Packaging vectors used were psPAX2 (gift from Didier Trono Addgene plasmid # 12260; *http://n2t.net/addgene:12260*; RRID: Addgene_12260) and pMD2.G (gift from Didier Trono, Addgene plasmid # 12259; *http://n2t.net/addgene:12259*; RRID:Addgene_12259). Cells were transduced for 24 h in presence of Vpx VLPs. Transduced macrophages were selected with 5 µg/mL puromycin (Fisher Scientific).

Generation of the CBL$^{+/C404Y}$ and isogenic WT hiPSC lines

The CBL^{C404Y} (c.1211 G>A) variant was inserted at the endogenous locus in the C12 WT hiPSC using Cytidine base editing (CBE) with CBE enzyme BE3-FNLS [46]. Briefly, the sgRNA for CBE was designed to target the non-coding strand and introduce the position 6 "C-to-T" conversion, to create the G-to-A conversion on the coding strand. The sgRNA target sequence was cloned into the pSPgRNA (Addgene plasmid # 47108) [47] to make the gene targeting construct. To introduce the CBL C404Y variants, the WT hiPSC (C12) were dissociated using Accutase (Innovative Cell Technologies) and electroporated (1 x10^6 cells per reaction) with 4 µg sgRNA-construct plasmid and 4 µg CBE enzyme coding vector BE3-FNLS (Addgene plasmid # 112671) [46] using Lonza 4D-Nucleofector and the Nucleofector solution (Lonza V4XP-3034) following our previously reported protocol [48]. The cells were then seeded, and 4 days later, the hiPSC were dissociated into single cells by Accutase and re-plated at a low density (4 per well in 96-well plates) to get the single-cell clones. Ten days later, individual colonies were picked, expanded and analyzed by PCR and DNA sequencing to identify the clones carried the desired CBL^{C404Y} heterozygous variant and the isogenic WT control clones. The sgRNA target, PCR and sequencing primers are listed below.

	sgRNA target	PCR-Forward primer	PCR-Reverse primer (used for sequencing)
CBL^{C404Y}	TAAGACAGGATGTGCACATG	TGGGCTCCACATTCCAACTA	GCCCTGACCTTCTGATTCCT

Western Blotting

For HEK293 cells, lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with the appropriate antibodies. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and Western blotting substrate (Thermo) were used for detection. For anti-Cdc42 immunoprecipitation experiments, cell lysates (1 mg total protein) were incubated overnight with 1 µg of anti-Cdc42 antibody (Santa Cruz) and 25 µL of protein A agarose (Roche) at 4°C. Anti-Flag immunoprecipitations were done with anti-Flag M2 affinity resin (Sigma). The beads were washed three times with lysis buffer, then eluted with SDS-PAGE buffer and resolved by SDS-PAGE. The proteins were transferred to PVDF membrane for Western blot analysis. Antibodies used are Phospho-p44/42 MAPK (pErk 1/2) (Thr202/Tyr204) is from Cell Signaling #4370, total p44/42 MAPK (Erk1/2) is from Cell Signaling #9102, HA tag from Millipore # 05-904, Flag antibody is from Sigma (#A8592), pCHEK2 (T383) antibody is from Abcam, #ab59408, Cdc42 antibody is from Santa Cruz (#sc87) and Anti-γ-Tubulin antibody (Sigma T6557). **For Immunoprecipitation Kinase assay in HEK293T cells,** cell lysates (1 mg protein) were incubated overnight with 30 µL of anti-Flag M2 affinity resin on a rotator at 4°C, then washed three times with Tris-buffered saline (TBS). A portion of each sample was eluted with SDS-PAGE sample buffer and analyzed by anti-Flag Western blotting. The remaining sample was used for a radioactive kinase assay. The immunoprecipitated proteins were incubated with 25 µL of reaction buffer (30 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mg/mL BSA, 400 µM ATP), 650 µM CHKtide peptide (KKKVRSGLYRSPSMPENLNRPR, SignalChem), and 50 – 100 cpm/pmol of [γ^{32}-P] ATP at 30°C for 15 minutes. The reactions were quenched using 45 µL of 10% trichloroacetic acid. The samples were centrifuged and 30 µL of the reaction was spotted onto Whatman P81 cellulose phosphate paper. After washing with 0.5% phosphoric acid, incorporation of radioactive phosphate into the peptide was measured by scintillation counting. **For MAC lines and hiPSC-derived cells,** cell lysates obtained with RIPA buffer + 1:1000 Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific) were sonicated 3 times for 30sec at 4°C (Bioruptor, Diagenode). Protein quantification of supernatant was done with Precision Red Advanced Protein Assay (Cytoskeleton). Proteins were boiled for 5 min at 95°C in NuPAGE LDS sample buffer (Invitrogen) and separated in NuPAGE 4%–12% Bis-Tris Protein Gel (Invitrogen) in NuPAGE MES SDS Running Buffer (Invitrogen). Electrophoretic transfer to a nitrocellulose membrane (ThermoFisher Scientific) was done in NuPAGE Transfer Buffer (Invitrogen). Blocking was performed for 60 min in TBS-T + 5% nonfat milk (Cell Signaling) and incubated with primary antibodies at 4°C: rabbit anti-p44/42 MAPK (ERK1/2) (Cell Signaling; 1:1000); rabbit anti-P-p44/42 MAPK (Cell Signaling, 1:1000); rabbit anti-c-CBL (Cell signaling, 1:1000); rabbit anti-RIT1 (Abcam, 1:1000); mouse anti-KRAS (clone 3B10-2F2, Sigma, 1 µg/mL); mouse anti-Actin (clone MAB1501, Sigma, 1:10,000). Primary antibodies were detected using the secondary anti-rabbit IgG HRP-linked (Cell Signaling, 1:1000) or the anti-mouse IgG HRP-linked (Cell Signaling, 1:1000) were used to detect primary antibodies, with SuperSignal™ West Femto Chemiluminescent Substrate (ThermoFischer Scientific) using a ChemiDoc MP Imaging System (Bio-Rad). pERK/ERK ratios were measured with ImageJ software.

DNA/ RNA isolation, dd-PCR and RTqPCR in MAC lines and hiPSC-derived cells

Genomic DNA was extracted using QIAamp DNA Micro Kit (50) (Qiagen), following the manufacturer's instructions. Total RNA was extracted using RNeasy Mini kit (Qiagen), following the manufacturer's instructions. cDNA was generated by reverse transcription using Invitrogen SuperScript IV Reverse Transcriptase (Invitrogen) with oligo(dT) primers. The TaqMan gene expression assays used were c-CBL FAM (Hs01011446_m1), CBLb FAM (Hs00180288_m1) and GAPDH VIC (Hs02786624_g1) (ThermoFisher Scientific). RT-qPCR was performed using Applied Biosystems TaqMan Fast Advanced Master Mix (ThermoFisher Scientific) and a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific). The results were normalized to GAPDH. For droplet PCR analyses, assays specific for the detection of I383M, C384Y, C404Y and C416S in CBL and F82L and M90I in RIT1, A59G in KRAS and corresponding WT sequences (listed below) were obtained from Bio-Rad. Cycling conditions were tested to ensure optimal annealing/extension temperature as well as optimal separation of positive from empty droplets. Optimization was done with a known positive control. After PicoGreen quantification, 2.6-9 ng gDNA or cDNA were combined with locus-specific primers, FAM- and HEX-labeled probes, HaeIII, and digital PCR Supermix for probes (no dUTP). All reactions were performed on a QX200 ddPCR system (Bio-Rad catalog # 1864001) and each sample was evaluated in technical duplicates. Reactions were partitioned into a median of ~19,000 droplets per well using the QX200 droplet generator. Emulsified PCRs were run on a 96-well thermal cycler using cycling conditions identified during the optimization step (95°C 10'; 40 cycles of 94°C 30' and 52-55°C 1'; 98°C 10'; 4°C hold). Plates were read and analyzed with the QuantaSoft software to assess the number of droplets positive for mutant or wild-type DNA.

Assay Name	Assay ID
CBL_I383M	dHsaMDS675699482
CBL_C384Y	dHsaMDS386449640
CBL_C404Y	dHsaMDS437459772
CBL_mRNA_C404Y	dMDS334857054
CBL_C416S	dHsaMDS613275900
RIT1_ F82L	dMDS959028273
RIT1_M90I	dHsaMDS133045056
KRAS_A59G	dHsaMDS581417660

Flow cytometry analyses

for surface antigens CSF1-R, CD11b, MRC1, α5β3, CD11c, Tim4, HLA-DR, CD45, CD14, NGFR, EGFR, CD36 and SIRPα were performed using PE-conjugated anti-CD115 (CSF1-R) (clone 9-4D2, BD Pharmingen), PE/Cy7-conjugated anti-CD11b (clone ICRF44, Biolegend), Alexa Fluor 488-conjugated anti-CD206 (MRC1) (clone 19.2, ThermoFisher Scientific), PE-conjugated anti-integrin α5β3 (clone 23C6, R&D systems), PE/Cy5-conjugated anti-CD11c (Clone B-ly6, BD Pharmigen), APC-conjugated anti-Tim4 (Clone 9F4, BioLegend), PE/Cy7-conjugated anti-HLA-DR (clone G46-6, BD Pharmigen), BV650-conjugated anti-CD45 (clone HI30, BD Horizon), APC/Cy7-conjugated anti-CD14 (clone M5E2, Biolegend), PE-conjugated anti-NGFR (clone ME20.4, eBioscience), Alexa Fluor 647-conjugated anti-EGFR (clone EGFR.1, BD Pharmigen), APC/Cy7-conjugated anti-CD36 (clone 5-271, BioLegend) and APC-conjugated anti-CD172a (SIRPα) (Clone: 15 414, ThermoFisher Scientific) antibodies. Iba1

expression was detected following fixation and permeabilization of macrophages using BD Cytofix/Cytoperm solution (BD Pharmingen). Cells were marked with Zombie Violet Viability (Biolegend). After incubation with FcR Blocking Reagent (Miltenyi Biotec), cells were stained with Alexa Fluor 555-conjugated anti-Iba1 antibody (clone E4O4W, Cell Signaling). Flow cytometry was performed using a BD Biosciences LSR Fortessa flow cytometer with Diva software. Data were analyzed using FlowJo (BD Biosciences LLC).

Cell proliferation analyses

For hiPSC-derived cells, cell suspension was filtered through a 100 µm nylon mesh (Corning) and marked with Zombie Violet Viability (Biolegend). After incubation with FcR Blocking Reagent (Miltenyi Biotec), surface receptors were labelled with PE/Cy7-conjugated anti-CD11b (clone ICRF44, Biolegend), Alexa Fluor 488-conjugated anti-CD206 (MRC1) (clone 19.2, ThermoFisher Scientific), BV650-conjugated anti-CD45 (clone HI30, BD Horizon), APC/Cy7-conjugated anti-CD14 (clone M5E2, Biolegend) prior to EdU detection. For proliferation studies in the mouse macrophage cell lines, macrophages were incubated with 10 µM EdU (ThermoFischer Scientific) for 2 hours at 37°C and collected by cell scraping and marked with Zombie Violet Viability (Biolegend) prior to EdU detection. EdU detection was performed using the Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (ThermoFischer Scientific), following manufacturer's instructions. hiPSC-derived macrophages were analyzed using a BD Biosciences Aria III cell sorter and macrophages were identified as $CD11b^{+}CD45^{+}CD14^{+}MRC1^{+}$. The macrophage cell lines were analyzed using a BD Biosciences LSR Fortessa flow cytometer. Data were analyzed using FlowJo 10.6 (BD Biosciences LLC).

Enzyme-linked immunosorbent assay

Supernatants of iPSC-derived microglia-like cells were analyzed for human inflammatory cytokines IL-6, TNFα, IL-1β, IFNψ and for the complement C3 and complement Factor H by Enzyme-linked immunosorbent assay (ELISA) at Eve Technologies (Calgary, AB).

Bulk RNA sequencing (RNAseq)

Three biological replicates were processed for each condition/cell line. In view of RNA sequencing, phase separation in cells lysed in 1 mL TRIzol Reagent (ThermoFisher Scientific) was induced with 200 µL chloroform and RNA was extracted from the aqueous phase using the miRNeasy Mini Kit (Qiagen) on the QIAcube Connect (Qiagen) according to the manufacturer's protocol with 350 µL input, or using the MagMAX mirVana Total RNA Isolation Kit (ThermoFisher Scientific) on the KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific) according to the manufacturer's protocol with 350 µL input. Samples were eluted in 30 µL RNase-free water. After RiboGreen quantification and quality control by Agilent BioAnalyzer, 231-500 ng of total RNA with RIN values of 9.4-10 underwent polyA selection and TruSeq library preparation according to instructions provided by Illumina (TruSeq Stranded mRNA LT Kit, Illumina), with 8 cycles of PCR. Samples were barcoded and run on a NovaSeq 6000 in a PE100 run, using the NovaSeq 6000 S4 Reagent Kit (200 Cycles) (Illumina). An average of 90 million paired reads was generated per sample. Ribosomal reads represented 0-1.6% of the total reads generated and the percent of mRNA bases averaged 79%.

Bulk RNAseq analysis

FastQ files of 2x100bp paired-end reads were quality checked using FastQC (*https://www.bioinformatics.babraham.ac.uk/projects/fastqc/*, 2012). Samples with high quality reads (Phred score >= 30) were aligned to the Mus musculus genome (GRCm38.80) for the MAC lines or Homo sapiens (assembly GRCh38.p14) for the IPSCs lines using STAR aligner. For the MAC lines, we computed the expression count matrix from the mapped reads using HTSeq (*www-huber.embl.de/users/anders/HTSeq*) and one of several possible gene model databases. The raw

count matrix generated by HTSeq are then be processed using the R/Bioconductor package DESeq (www-huber.embl.de/users/anders/DESeq) which is used to both normalize the full dataset and analyze differential expression between sample groups. For the ISPCs line dataset, gene quantification was performed using feature counts from the Subread package in R. Gene expression levels were normalized and log2 transformed using the Trimmed Mean of M-values (TMM) method and differential expression analysis was performed using the edgeR package in R. For hiPSC derived cells, gene-set enrichment analysis (GSEA) (Hallmark, KEGG, GO, REACTOME) were performed using the fgsea package in R on a pre-ranked list (formula: sign(ogFC) * -log10(PValue)) on all expressed genes in the dataset. For the MAC cell lines dataset, GSEA was performed using gsea4.3.2 for KEGG and HALLMARK canonical pathways in MSigDB v 7.5.1. Significant genesets were selected based on an FDR <= 0.25. For lists of differentially expressed genes, genes were selected with controlled False Positive Rate (B&H method) at 5% (FDR <= 0.05). Genes were considered upregulated/downregulated for log2 fold change> 1.5 or <-1.5.

Statistical analysis

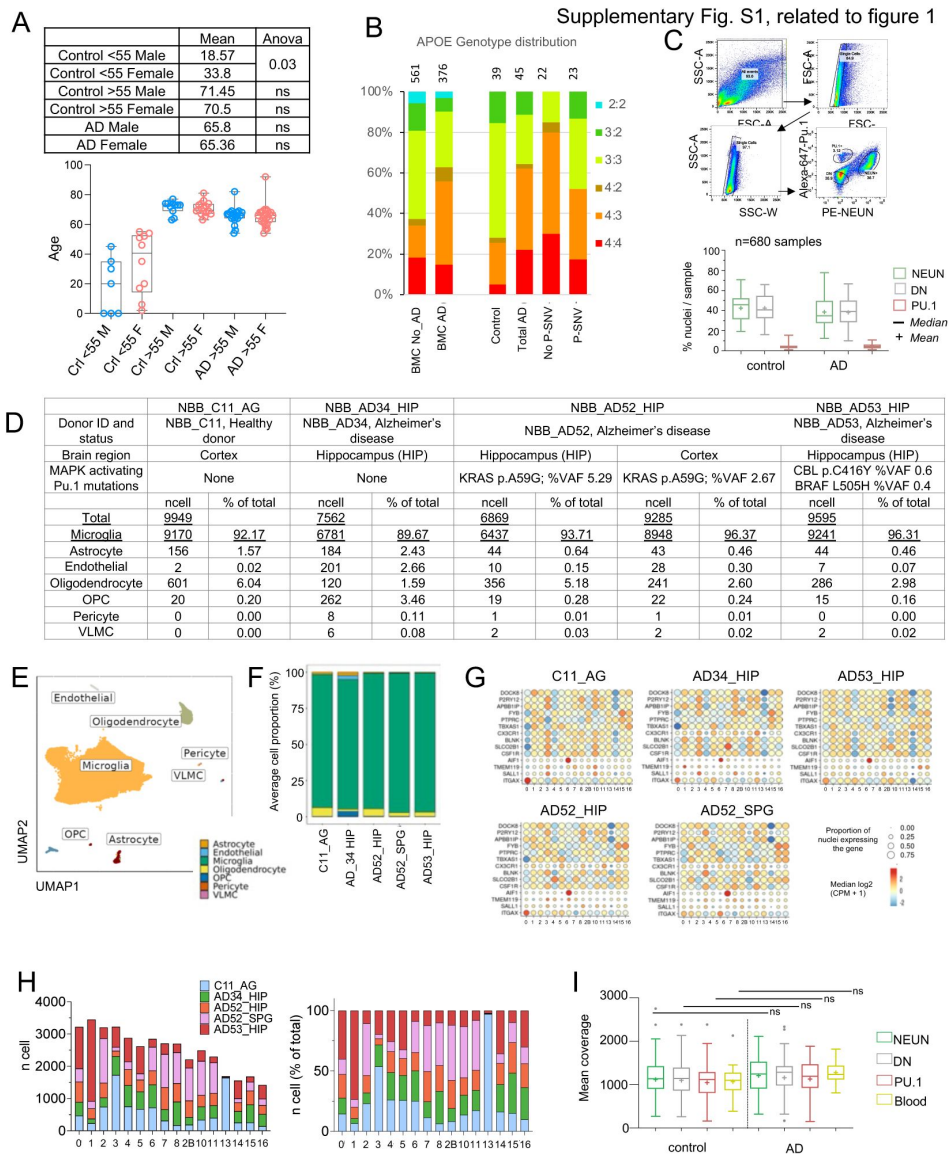
Statistical methods are detailed in the corresponding sections above (Quantification of mutational load and statistics, Bulk RNAseq analysis, Sn-RNAseq analysis) and in the Fig. legends. P values of 0.05 and adj. P values (FDR) of 0.25 are considered significant unless otherwise specified.

Data availability

DNA sequencing data processed for selection of somatic variants are available for all patients and samples in Supplementary Table S3. Raw DNA sequencing data (FASTQ files) from targeted-deep sequencing are deposited in dbGaP under project accession number phs002213.v1.p1, for samples where patient-informed consent for public deposition of DNA sequencing data was obtained. Bulk RNAseq raw data are deposited in GEO (number pending). Sn-RNAseq raw data are deposited in GEO (number pending) and as an interactive analysis web tool accessible at *https://weillcornellmed.shinyapps.io/Human_brain/*.

Code availability

All code used in this study has been previously published as referenced in the method section above.

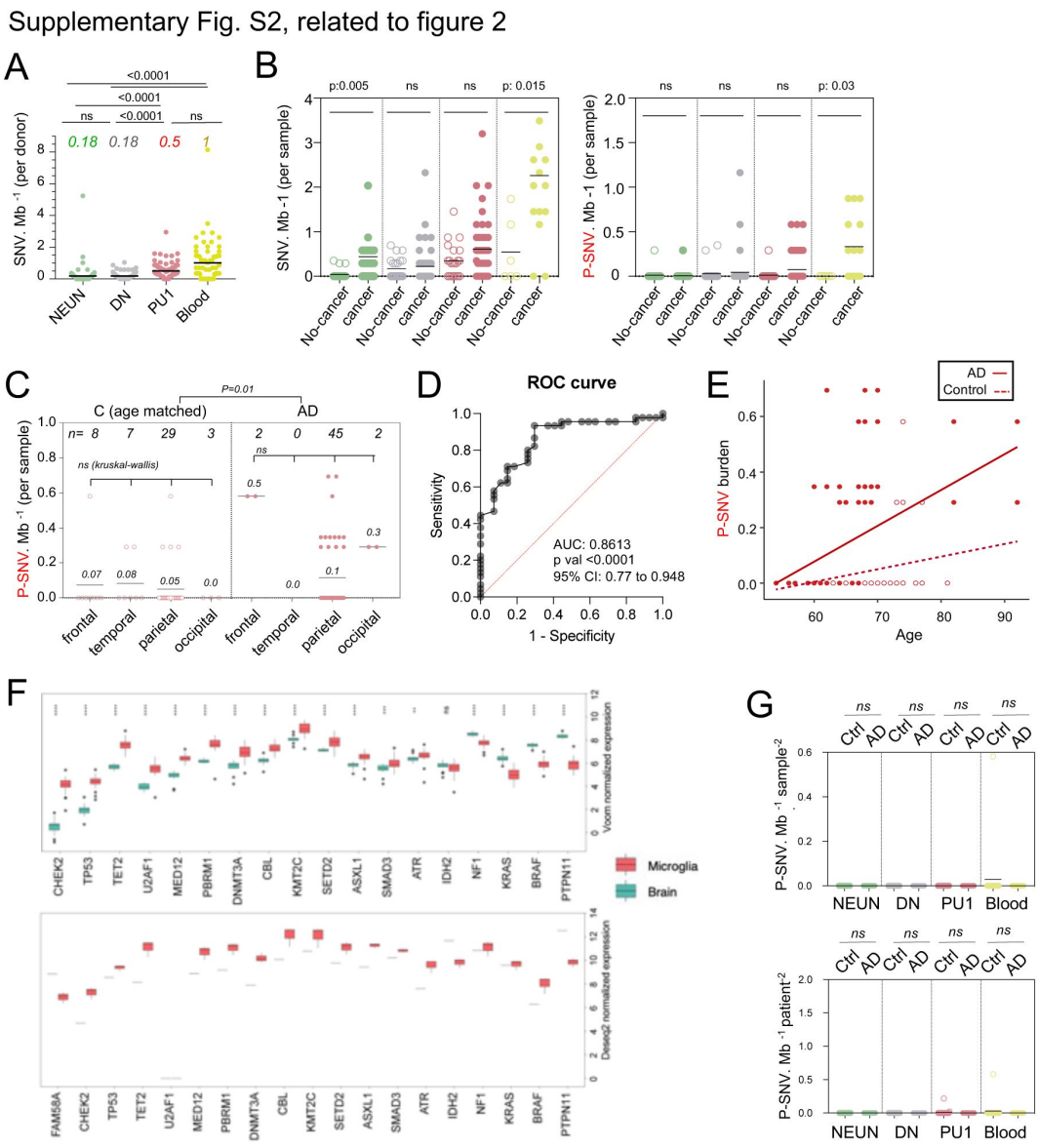


D	NBB_C11_AG		NBB_AD34_HIP		NBB_AD52_HIP				NBB_AD53_HIP	
Donor ID and status	NBB_C11, Healthy donor		NBB_AD34, Alzheimer's disease		NBB_AD52, Alzheimer's disease				NBB_AD53, Alzheimer's disease	
Brain region	Cortex		Hippocampus (HIP)		Hippocampus (HIP)		Cortex		Hippocampus (HIP)	
MAPK activating Pu.1 mutations	None		None		KRAS p.A59G; %VAF 5.29		KRAS p.A59G; %VAF 2.67		CBL p.C416Y %VAF 0.6 BRAF L505H %VAF 0.4	
	ncell	% of total	ncell	% of total	ncell	% of total	ncell	% of total	ncell	% of total
Total	9949		7562		6869		9285		9595	
Microglia	9170	92.17	6781	89.67	6437	93.71	8948	96.37	9241	96.31
Astrocyte	156	1.57	184	2.43	44	0.64	43	0.46	44	0.46
Endothelial	2	0.02	201	2.66	10	0.15	28	0.30	7	0.07
Oligodendrocyte	601	6.04	120	1.59	356	5.18	241	2.60	286	2.98
OPC	20	0.20	262	3.46	19	0.28	22	0.24	15	0.16
Pericyte	0	0.00	8	0.11	1	0.01	1	0.01	0	0.00
VLMC	0	0.00	6	0.08	2	0.03	2	0.02	2	0.02

Supplementary Figure 1.

Quality control for DNA analysis and snRNAseq.

(A) patients characterisitic: age and sex distribution of young controls, age-matched controls and AD patients, Statistics: 2 way Anova. **(B)** Distribution of APOE genotype in a historical cohort of controls and AD patients [49] (Left) and the present series (Right) of Control, AD and AD without and with pathogenic (P-SNV) microglia variants. Numbers on top of the bars show patient number in each group. **(C)** Sorting strategy to separate PU.1^{+}, NEUN^{+} and DN nuclei from post-mortem brain samples. Boxplot represents relative frequencies, median, mean, 25-75th quartiles (boxes) and minimum/maximum (whiskers) of nuclei for each cell type in controls (n=63 brain samples) and AD patients (n=99 brain samples). **(D)** SnRNA-seq analysis of Facs-sorted PU.1+ nuclei from 4 donors. Table indicate donor characteristics, number of nuclei analyzed after quality control (see methods) and cell types as determined by unsupervised clustering of normalized and integrated gene expression of nuclei from 5 PU.1^{+} samples. **(E)** UMAP representation of cell types from (C). **(E)** Cell proportion plot of the 5 PU.1^{+} samples from (C). **(F)** Boxplot showing the coverage of targeted DNA deep sequencing per cell type in AD and control samples. Box plots show median (+ mean) and 25th and 75th percentiles; whiskers extend to the largest and smallest values. Dots show outliers. **(G)** Expresion of microglia markers by sn-RNAseq across samples and clusters. **(H)** Number (TOP) and proportiton (BOTTOM) of cells from each sample, per-cluster. **(I)** Boxplot showing the coverage of targeted DNA deep sequencing per cell type in AD and control samples. Box plots show median (+ mean) and 25th and 75th percentiles; whiskers extend to the largest and smallest values. Dots show outliers.

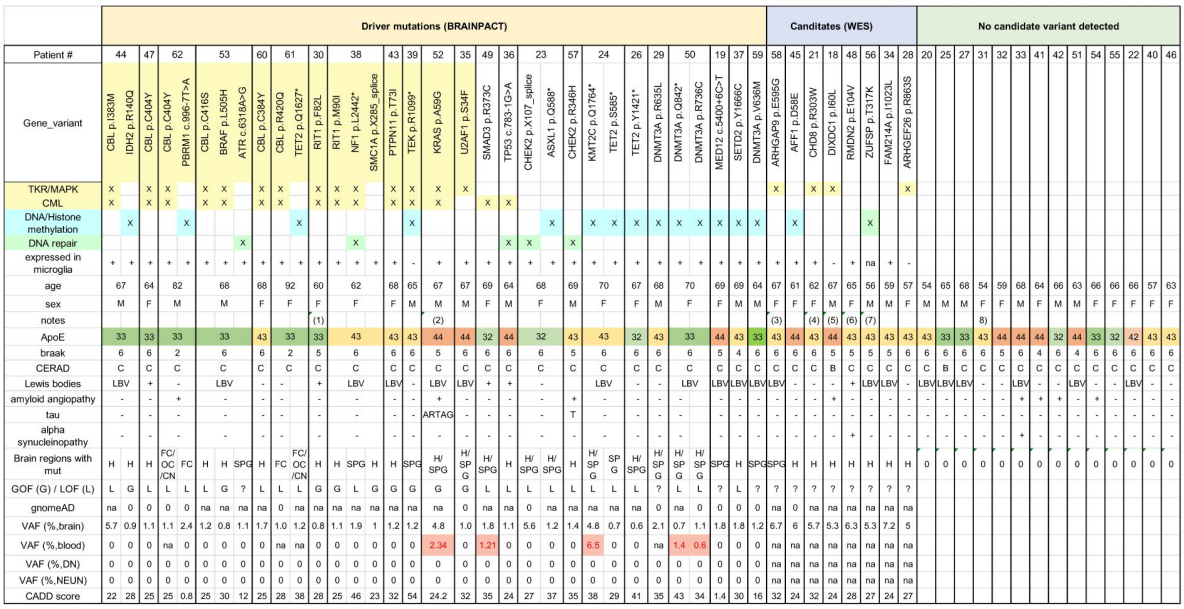


Supplementary Figure 2.

Analysis of pathogenic variants.

(A) Number of SNV per Mb, per donor, and cell types. Each dot represents the mean of a donor. NeuN n=226, DN n=229, PU.1 n=225, Blood n=66). Values (color, *italics)* indicate the mean number of variants /Mb per cell type. Statistics: *p-value* are calculated by Kruskal–Wallis test and Dunn's test for multiple comparisons. **(B)** Number of SNV (Left) and P-SNV (right) per Mb in controls (age-matched with the patients) with or without cancer per sample and cell types. Each dot represents a sample. Statistics: *p-values* within each group are calculated with Kruskal–Wallis, multiple comparisons. **(C)** Number of SNV per Mb in PU.1 samples across cortical samples, of age-matched controls (n=27) and AD patients (n=45). Each dot represents a sample. Statistics: *p-values* within each group are calculated with Kruskal–Wallis, multiple comparisons. **(D)** Receiver operating characteristic (ROC) curve showing the accuracy of the multivariate logistic regression model in predicting the association of AD and the presence or not of pathogenic variants in PU.1^{+} nuclei. Note: non-parametric tests were used as data did not follow a normal distribution (D'Agostino-Pearson normality test). **(E)** Observed SNV burden for **Fig. 2H**. **(F)** Expression of pathogenic genes in microglia and whole brain tissue, reported in [33] (TOP, sorted microglia n= 39 and whole brain n=16) and [34] (BOTTOM, sorted microglia n= 3 and whole brain n=1. **(G)** Graph depicts mean number of pathogenic variants in a group of control genes not expressed by the brain or by microglia (see Supplementary Table 3), per Mb, and samples (LEFT) and donor (RIGHT), in NEUN, DN, PU.1 nuclei and matching blood from all controls and AD patients. Each dot represents the mean for each donor. Statistics: *p-values* are calculated with unpaired two-tailed Mann-Whitney U test comparing AD to controls.

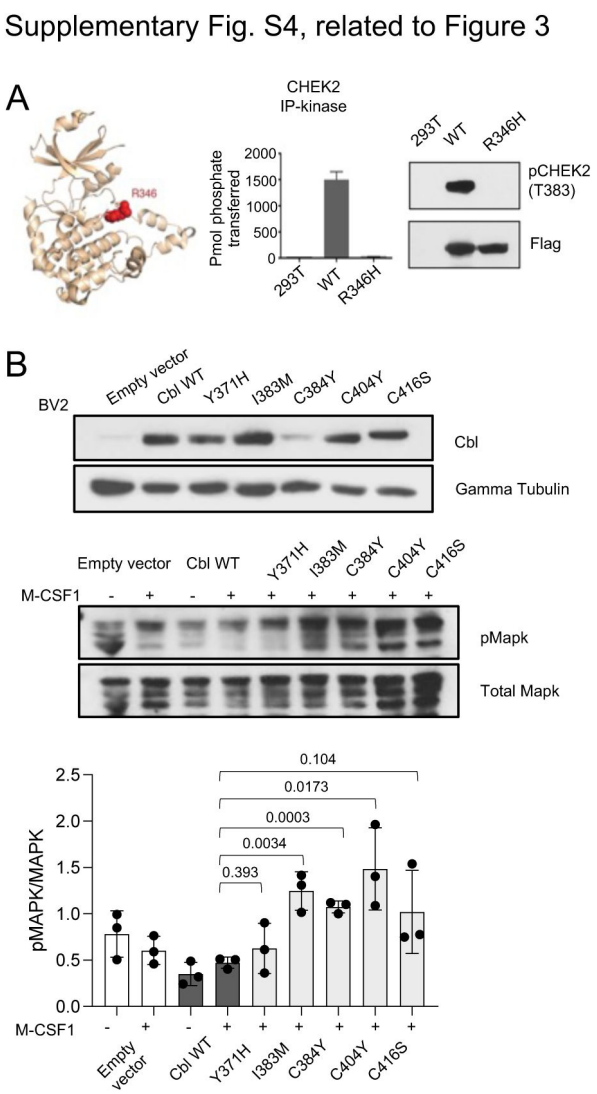
Supplementary Fig. S3, related to Figure 2 and 3



Supplementary Figure 3.

Summary of AD patients characteristics and pathogenic variants.

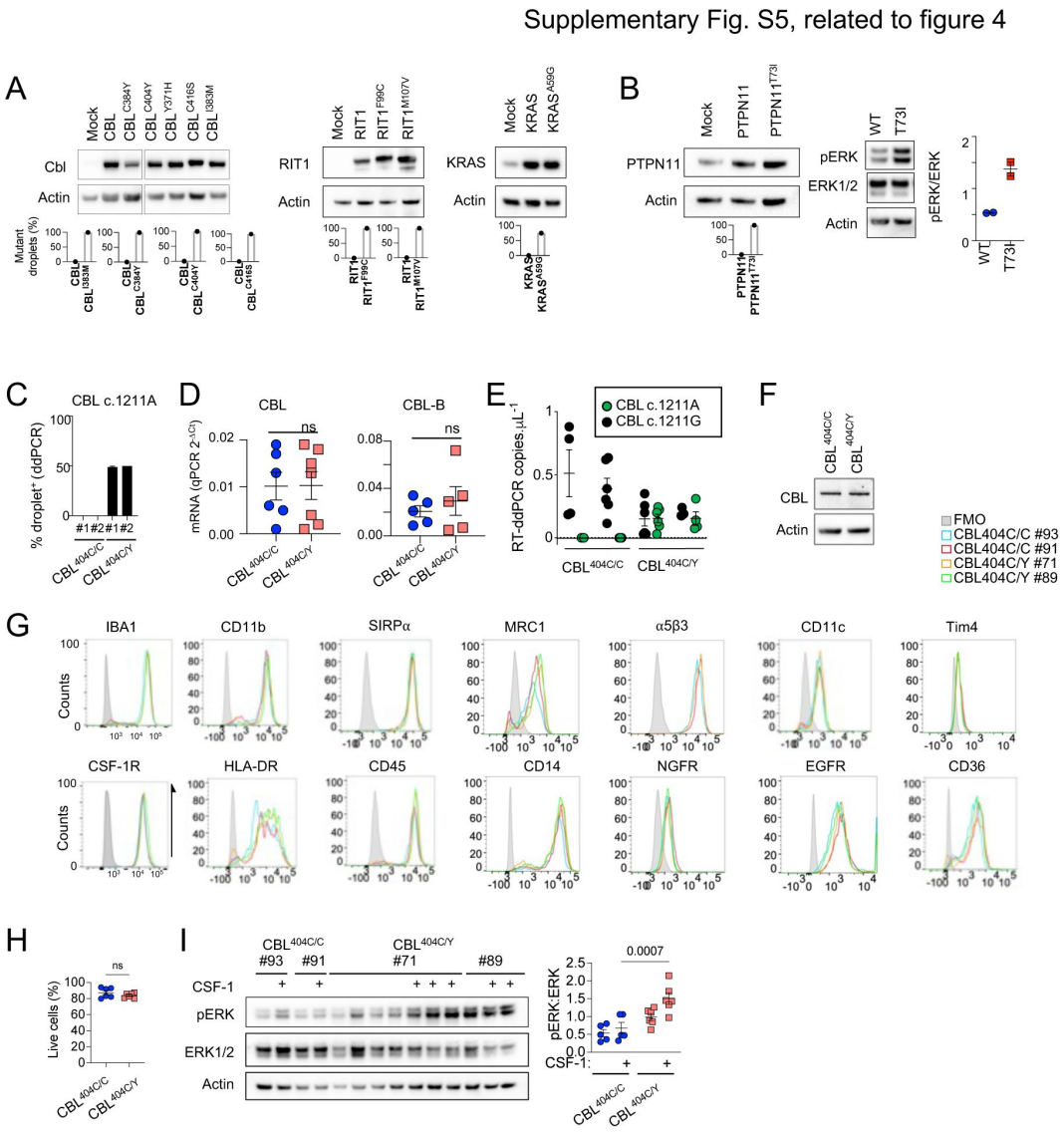
Table shows for all AD patients studied, the detection of pathogenic variants by TDS, candidates identified by WES, categories of gene functions (MAPK pathway, DNA repair, DNA/Histone methylation), expression in microglia, and patient information (age/sex/Apoe genotype/braak status/CERAD score/presence of lewis bodies/presence of amyloid angiopathy). #Brain regions: number of brain regions where variant was detected. GOF (G, Gain of Function) / LOF (L, Loss of Function) as reported in bibliography (see manuscript for references). gnomeAD shows the minor allele frequency of each variant in the population. VAF: variant allelic frequency (%) by BRAIN-PACT in brain cell types and matching-blood when available. CADD score (Combined Annotation Dependent Depletion) of each variant. Notes: (1) Trisomy 21, Down syndrome. (2) familial history of AD, no variant in AD associated genes. (3) MAPK docking protein. (4) cooperative interaction with ELK1 on chromatin. (5) inhibits JNK activation, murine KO has a neurological phenotype[50] (6) microtubule binding, involved in b-amyloid aggregation. (7) DNA repair gene. (8) Mosaic trisomy 21.



Supplementary Figure 4.

Functional analysis of variants in HEK293 and BV2 cell lines

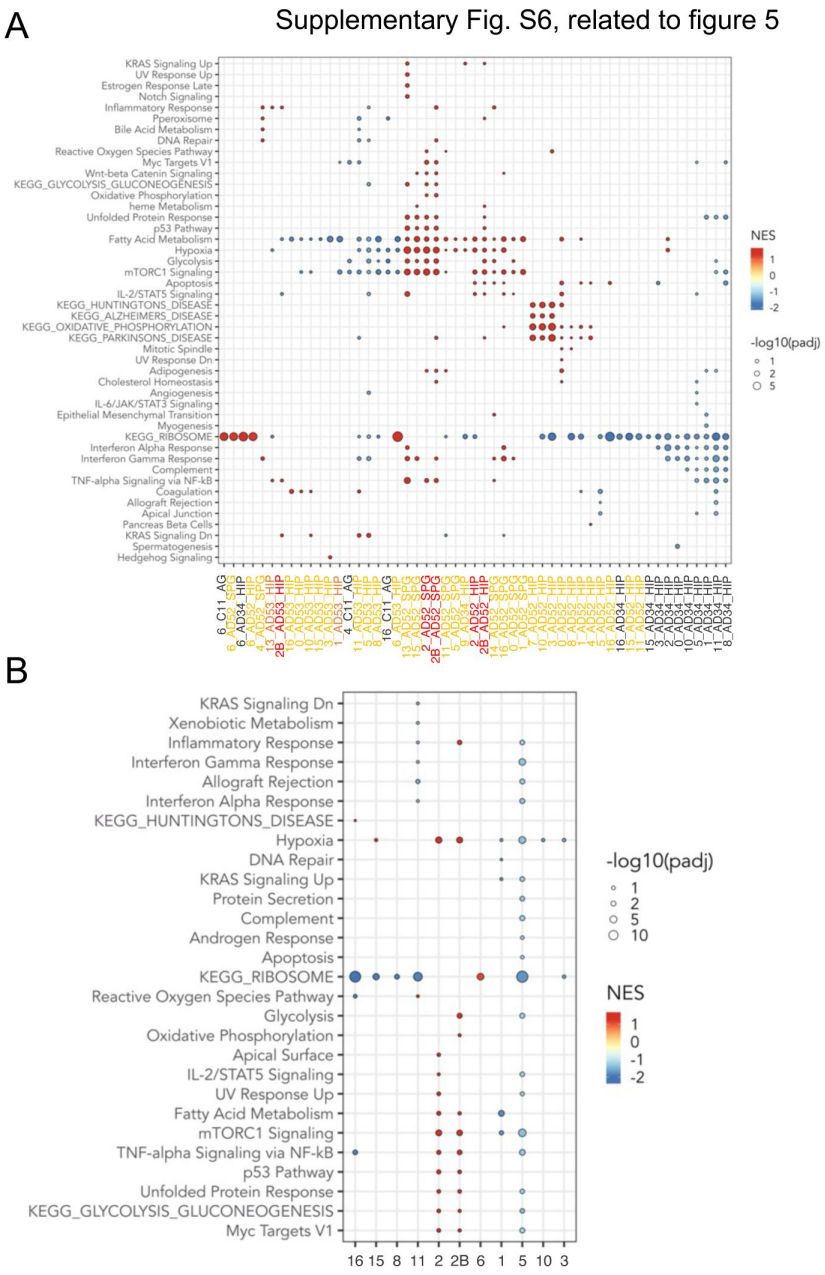
(A) Quantification of Western blot from cell lysates from HEK293T cells expressing WT of mutant CBL alleles and stimulated with EGF or control were probed with antibodies against Phospho-p44/42 MAPK (Erk 1/2, Thr202/Tyr204, (pMAPK)), total MAPK (p44/42 MAPK, Erk1/2, (MAPK)), and HA-tag (BOTTOM). n= 4 independent experiments. Statistic: Student t-test. **(B)** HEK293T cells expressing Flag-RIT1 (WT and mutants) were treated -/+ 20% FBS before harvesting and Lysates were probed with antibodies against Phospho-p44/42 MAPK (Erk 1/2, Thr202/Tyr204, (pMAPK)), total MAPK (p44/42 MAPK, Erk1/2, (MAPK)), and Flag. n= 5 independent experiments. Statistic: Student t-test. **(C)** Flag-tagged RIT1 constructs were expressed in HEK293T cells. Lysates were used in pulldown reactions with immobilized GST-PAK1-CRIB domain and in immunoprecipitation reactions with Cdc42 antibody. Bound RIT1 was measured by anti-Flag Western blotting. Lysates were also analyzed by anti-Flag and anti-MAPK Western blotting. **(D)** CHEK2 R346H is a loss-of-function mutant. The R346H variant is located within the catalytic loop of the protein kinase domain and shown in red on the 3D structure of CHEK2 kinase domain (pdb code: 2cn5) (LEFT). CHEK2 R346 Lysates from HEK293T cells expressing Flag-WT or CHEK2 R346 were probed with antibodies that recognizes the auto phosphorylated and activated form of CHEK2 and Flag (MIDDLE). Flag-tagged WT and R346H CHK2 were expressed in HEK293T cells, proteins were isolated by immunoaffinity capture using anti-Flag resin. CHK2 activity was measured with [^{32}P]-labeled ATP and a synthetic CHEK2 substrate peptide. Wild-type CHEK2 showed robust activity, while the R346H mutant was inactive (RIGHT). **(E)** Western-blot analysis of CBL expression (TOP), pMAPK and total MAPK (MIDDLE) and respective quantification (BOTTOM) in BV2 cell lines transduced with empty vector, CBL^{WT}, CBL^{Y371H}, CBL^{I383M}, CBL^{C384Y}, CBL^{C404Y} and CBL^{C416S}. For MIDDLE panel, cells were treated with M-CSF1 100 ng/ml for 5 min. Statistics: *p-values* are calculated with t-test. n=3.



Supplementary Figure 5.

Analysis of mouse and human microglia-like cells.

(A) Western-blot analysis of CBL, RIT1, and KRAS expression in lysates from a growth factor-dependent macrophage cell line expressing CBL^{WT}, CBL^{I383M}, CBL^{C384Y}, CBL^{C404Y}, CBL^{C416S}, CBL^{R420Q}, $RIT1^{WT}$, $RIT1^{F99C}$, $RIT1^{M107V}$, $KRAS^{WT}$ and $KRAS^{A59G}$ alleles (TOP), and ddPCR analysis of wt and mutant alleles in DNA from the same cell lines (BOTTOM). (**B**) Western-blot analysis of PTPN11 expression and phospho- and total-ERK in lysates from growth factor-dependent macrophage cell line expressing $PTPN11^{WT}$ or $PTPN11^{T73I}$ alleles, and ddPCR analysis of wt and variant alleles in DNA from the same lines. **(C)** Genomic DNA ddPCR of 2 independent hiPSC clones (#1 and #2) of $CBL^{404C/Y}$ heterozygous mutant carrying the c.1211G/A transition on one allele and 2 independent isogenic control $CBL^{404C/C}$ clones all obtained by prime editing. **(D)** CBL and CBL-B mRNA expression assessed by Taqman assay in $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells. Unpaired t-test. **(E)** RT-ddPCR of CBL reference allele (CBL c.1211A) and CBL variant CBL c.1211G transcripts in $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived macrophages. n=4-6 independent experiments. **(F)** Western-blot analysis of CBL expression in lysates from $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells. **(G)** Representative flow cytometry analysis of the expression of surface receptors and Iba1 in $CBL^{404C/C}$ and $CBL^{404C/Y}$ cells (n=3) **(H)** Viability of $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells estimated by flow cytometry analysis after DAPI staining. Unpaired t-test. n=6. **(I)** Western-blot analysis and quantification of phospho- and total-ERK proteins in lysates from $CBL^{404C/C}$ and $CBL^{404C/Y}$ iPSC-derived microglia-like cells untreated or re-stimulated with CSF-1 cells (5 min, 100 ng/mL). (Two-way ANOVA, n=6-7).



Supplementary Figure 6.

snRNAseq analysis of microglia.

(A) Dot plot represents the significant pathways by GSEA analysis of HALLMARK and KEGG pathways of snRNAseq analysis of microglia, by samples and clusters. Genes from all samples are pre-ranked per cluster using differential expression analysis with SCANPY [37] and the Wilcoxon rank-sum method. Statistical analysis were performed using the fgseaMultilevel function in fgsea R package [38]f or HALLMARK and KEGG pathways. Only HALLMARK and KEGG gene sets with p-value < 0.05 and adjusted p-value < 0.25 are visualized, using ggpubr and ggplot2 [39] R package. **(B)** Dot plot represents the same GSEA analysis of HALLMARK and KEGG pathways enriched in snRNAseq microglia clusters as in A, but samples from all donors are grouped by microglia clusters.

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Reviewer #1 (Public review):

In the revised manuscript Vicario et al. provide new insights on a potential contribution of somatic mutations within the microglia population of the CNS that accelerates microglia activation and disease-associated gene signatures in Alzheimer's disease. Here they especially identified an "enrichment" of pathological SNVs in microglia, but not the peripheral blood, that are associated with clonal proliferative disorders and neurological diseases in a subset of patients with AD. They identified P-SNVs in microglia of AD patients located within the ring domain of CBL, a negative regulator of MAPK signaling. They further provide mechanistic insights how these variants result in MAPK over-activation and subsequently in a pro-inflammatory phenotype in human microglia-like cells in vitro.

Overall, this study provides novel evidence from an AD patient cohort pointing to a potential contribution of microglia-specific somatic mutations to disease onset and/or progression in at least a subset of patients with Alzheimer's disease.

The work within this study is highly relevant and will open new study lines to explore somatic mutations within the microglia compartment and neurodegenerative diseases.

Strengths:

As outlined above, the study identified P-SNVs in microglia of AD patients associated with clonal proliferative disorders, but also give an in depth analysis in re-occurring P-SNVs located within the ring domain of CBL, a negative regulator of MAPK signaling. They further provide mechanistic insights how these variants result in MAPK over-activation and subsequently in a pro-inflammatory phenotype in HEK cells, BV2 cells, MAC cells and human microglia-like cells in vitro. The over-activation of the cells in vitro is convincing.

Great care was taken to identify the limitations of the possible conclusions and to make careful conclusions. For example, they highlight that the pathway proposed to be affected may be an explanation for a subset of AD patients, and emphasize that it is yet unclear whether this accumulation of pathological SNVs is a cause or consequence of disease progression

The study supports an enrichment of P-SNVs in several genes associated clonal proliferative disorders in microglia and nicely separates this from SNVs associated with clonal hematopoiesis in the peripheral blood found in AD patients and controls.

The authors further acknowledged that several age matched control patients were diagnosed with cancer or tumor-associated diseases and carefully dissected the occurring SNVs in these patients are not associated with the P-SNVs identified in the microglial compartment of the AD cohort.

Weaknesses:

The revised study is overall convincing and has improved in the revised version, but some points especially regarding the clear connection of the seen somatic variants in microglia with a potential role in disease progression remain unanswered.

A potential connection between P-SNVs in microglia and disease pathology and symptoms was not further explored by the authors but might be in future work.

Taken this into account, maybe the title is a bit overstated and could be tuned down.

https://doi.org/10.7554/eLife.96519.2.sa2

Reviewer #2 (Public review):

Summary:

In this study, Vicaro et al. aimed to quantify and characterize mosaic mutations in human sporadic Alzheimer's disease (AD) brain samples. They focused on three broad classes of brain cells, neurons that express the marker NeuN, microglia that express the marker PU.1, and double-negative cells that presumably comprise all other brain cell types, including astrocytes, oligodendrocytes, oligodendrocyte progenitor cells, and endothelial cells. The authors find an enrichment of potentially pathogenic somatic mutations in AD microglia compared to controls, with MAPK pathway genes being particularly enriched for somatic mutations in those cells. The authors report a striking enrichment for mutations in the gene CBL and use in vitro functional assays to show that these mutations indeed induce MAPK pathway activation.

The current state of the AD and somatic mutation fields puts this work into context. First, AD is a devastating disease whose prevalence is only increasing as the population of the U.S. is aging, necessitating the investigation of novel features of AD to identify new therapeutic opportunities. Second, microglia have recently come into focus as important players in AD pathogenesis. Many AD risk genes are selectively expressed in microglia, and microglia from AD brain samples show a distinct transcriptional profile indicating an inflammatory phenotype. The authors' previous work shows that a genetic mouse model of mosaic BRAF activation in macrophages (including microglia) displays a neurodegenerative phenotype similar to AD (Mass et al., 2017, doi:10.1038/nature23672). Third, new technological developments have allowed for identifying mosaic mutations present in only a small fraction of or even single cells. Together, these data form a rationale for studying mosaic mutations in microglia in AD. In light of the authors' findings regarding MAPK pathway gene somatic mutations, it is also important to note that MAPK has previously been implicated in AD neuroinflammation in the literature.

Strengths:

The study demonstrated several strengths. Firstly, the authors used two methods to identify mosaic mutations: 1) deep (~1,100x) DNA sequencing of a targeted panel of >700 genes they hypothesized might, if mutated somatically, play a role in AD, and 2) deep (400x) whole-exome sequencing (WES) to identify clonal mosaics outside of those genes. A second strength is the agreement between these experiments, where WES found many variants identified in the panel experiment, and both experiments revealed somatic mutations in MAPK pathway genes. Third, the authors demonstrated in several in vitro systems that many mutations they identified in MAPK genes activate MAPK signaling. Finally, the authors showed that in some human brain samples, single-cell gene expression analysis revealed that cells bearing a mosaic MAPK pathway mutation displayed dysregulated inflammatory signaling and dysregulation in other pathways. This single-cell analysis was in agreement with their in vitro analyses.

Weaknesses:

The study also showed some weaknesses. The sample size (45 AD donors and 44 controls) is small, reflected in the relatively modest effect sizes and p-values observed. This weakness is partially ameliorated by the authors' extensive molecular and functional validation of mutation candidates. Secondly, as the authors point out, this study cannot conclude whether microglial mosaic mutations cause AD or are an effect of AD. Future studies may shed more light on this important question.

Conclusions and Impact:

Considering the study's aims, strengths, and weaknesses, I conclude that the authors achieved their goal of characterizing the role of mosaic mutations in human AD. Their data strongly suggest that mosaic MAPK mutations in microglia are associated with AD. The impacts of this study remain to be seen, but they could include attempts to target CBL or other mutated genes in the treatment of AD. This work also suggests a similar approach to identifying potentially causative somatic mutations in other neurodegenerative diseases.

https://doi.org/10.7554/eLife.96519.2.sa1

Author response:

The following is the authors' response to the original reviews.

We edited the manuscript for clarity, added information described in new figure panels (below) and corrected typos.

In figure 1 we corrected a typo.

In figure 2, panel 2H, and Figure S2E, we included a new statistical analysis (mixed effect linear regression) to compare mutational burden in controls and AD patients.

In figure 3, and Figure S4B, we revised the western blots panels in Panel 3E,F, to improve presentation of controls and quantification.

we corrected typos.

In figure 5 we removed a panel (former 5D) which did not add useful information.

In Figure S1A we included information about sex and age from the control and patients analyzed. In Figure S2B, we added an analysis of the mutational burden in controls, distinguishing controls with and without cancer.

We modified Table S1 for completeness of information for all samples analyzed.

> ***Reviewer #1:***
>
> *Weaknesses:*
>
> *Even though the study is overall very convincing, several points could help to connect the seen somatic variants in microglia more with a potential role in disease progression. The connection of P-SNVs in the genes chosen from neurological disorders was not further highlighted by the authors.*

All P-SNVs are reported in Table S3.

We observed only two P-SNVs within genes associated to neurological disorders (brain panel in Table S2). - SQSTM1 (p.P392L) was identified in blood but not in brain from the patient AD48A.

- OPTN was identified (p.Q467P) in PU.1 from control 25.

To highlight this point, we modified the first paragraph of the discussion as follow:

"We report here that microglia from a cohort of 45 AD patients with intermediate-onset sporadic AD (mean age 65 y.o) is enriched for clones carrying pathogenic/oncogenic variants in genes associated with clonal proliferative disorders (Supplementary Table 2) in comparison to 44 controls. Of note we did not observe microglia P-SNVs within genes

reported to be associated with neurological disorders (Supplementary Table 2) in patients, and one such variant was identified in a control (Supplementary Table 3) “.

> *The authors show in snRNA-seq data that a disease-associated microglia state seems to be enriched in patients with somatic variants in the CBL ring domain, however, this analysis could be deepened. For example, how this knowledge may translate to patient benefits when the relevant cell populations appear concentrated in a single patient sample (Figure 5; AD52) is unclear; increasing the analyzed patient pool for Figure 5 and showcasing the presence of this microglia state of interest in a few more patients with driving mutations for CBL or other MAPK pathway associated mutations would lend their hypotheses further credibility.*

We acknowledge this limitation, but we respectfully submit that the analysis was performed in 2 patients. AD 53 also show a MAPK-associated inflammatory signature in the microglia clusters associated with mutations.

We performed the analysis on all FACS-purified PU.1+ nuclei samples that passed QC for single nuclei RNAseq. It should be noted that this analysis is extremely difficult with current technologies because microglia nuclei need to be fixed for PU.1 staining and FACS purification and the clones are small (~1% of microglia).

> *A potential connection between P-SNVs in microglia and disease pathology and symptoms was not further explored by the authors.*

At the population level, Braak/CERAD scores, the presence of Lewy bodies, amyloid angiopathy, tauopathy, or alpha synucleinopathy were not different between AD patients with or without pathogenic microglial clones (Figure S3 and Table S1). Of note, we studied here a homogenous population of AD patients.

At the tissue level, the roles of mutant microglia in plaques for example is being investigated, but we do not have results to present at this time.

> *A recent preprint (Huang et al., 2024) connected the occurrence of somatic variants in genes associated with clonal hematopoiesis in microglia in a large cohort of AD patients, this study is not further discussed or compared to the data in this manuscript.*

This pre-print supports the high frequency of detection of oncogenic variants associated with clonal proliferative disorders, they hypothesize that the mutations may be associated with microglia, but they only check a few mutations in purified microglia. Most of the study is performed in whole brain tissue. It does not really bring new information as compared to other study we cite in the introduction (and to our manuscript).

> ***Reviewer #2 (Recommendations For The Authors):***
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> *Suggestions for improved or additional experiments, data, or analyses:*
>
> *The authors can demonstrate that identified pathological SNVs from their AD cohort also lead to the activation of human microglia-like cells in vitro, but do not provide any data from histological examination of the patient cohort (e.g. accumulation at the plaque site, microglia distribution, and cell number). The study could be further supported by providing a histological examination of patients with and without P-SNVs to identify if microglia response to pathology, microglia accumulation, or phagocytic capacity are altered in these patients.*

We performed IBA1 staining in brain samples from control and from AD patients, with or without microglial clones and microglia density was not different between patient with and without mutations. In addition, histological reports from the brain bank (Braak/CERAD scores, Lewis bodies, amyloid angiopathy, tauopathy, or alpha synucleinopathy did not suggest differences between patient with and without mutations (Figure S3). These results are preliminary and further investigations are ongoing.

> *It would have been interesting to see if for example, transgenic AD mice with an introduced somatic mutation in microglia show an altered disease progression with alterations in amyloid pathology or cognition.*

We agree with the reviewer. We performed an in vivo study with mice expressing a 5xFAD transgene, an inducible microglia Cx3cr1CreERt2 BrafLSL-V600E transgene, or both, and performed survival, behavioral (Y-Maze and Novel Object Recognition), and histological analyses for β-Amyloid, p-Tau and Iba1 staining.

Microgliosis was increased in the group with the 2 transgenes, however the phenotype associated with the expression of a BrafV600E allele in microglia (Mass et al Nature 2017) was strongly dominant over the phenotype of 5xFAD mice, which did not allow us to conclude on survival and behavioral analyses.

Other studies with different transgenes are in progress but we have no results yet to include in this revised manuscript.

> *To connect the somatic mutations in microglia better to a potential contribution in neurodegeneration or neurotoxicity, the authors could provide further details on how to demonstrate if human microglia-like cells respond differentially to amyloid or induce neurotoxicity in a co-culture or slice culture model.*

These studies are undertaken in the laboratory, but unfortunately, we have no results as yet to include in this revised manuscript.

> *The number of samples analyzed for hippocampi, especially in the age-matched controls might be underpowered.*

Unfortunately, despite our best efforts, we were not able to analyze more hippocampus from control individuals. To control for bias in sampling as well as to other potential bias in our analysis, we investigated the statistical analysis of the cohorts for inclusion of age as a criterion (age matched controls), inclusion of a random effect structure, and possible confounding factor such as sex, brain bank site, and samples' anatomical location (see revised Methods and revised Fig. 2C, F, and H, and S2B).

We first tested whether the inclusion of age is appropriate in a fixed-effects linear regression using a generalized linear model (GLM) with gaussian distribution. Compared to the baseline model, the model with age had significantly low AIC (from -66.6 to -71.9, P = 0.0067 by chi-square test). Therefore, the inclusion of age as a fixed effect is appropriate. We next tested multiple structures of mixed-effects linear modeling. We used donors as random effects, while utilizing age, disease status (neurotypical control vs. AD), or both as fixed effects. Fitting was performed using the lme function implemented in the nlme package with the maximum likelihood (ML) method. The incorporation of age and disease status significantly improved overall model fitting. Both age and AD are associated with a significant increase in SNV burden in this model (P<1x10^-4 and P=1x10^-4, respectively, by likelihood ratio test). The model's total explanatory power is substantial (conditional R^2=0.48). We also asked if the addition of potential confounding factors to the model is justified. Three factors were

tested via the two above-mentioned methods: sex, brain bank site, and the anatomical location of the samples. In all cases, the AIC increased, and the P values by likelihood ratio tests were higher than 0.99. Therefore, from a statistical standpoint, the inclusion of these potential confounding factors does not seem to improve overall model fitting.

> *Minor corrections to the text and figures:*
>
> *The authors made a great effort to analyze various samples from one individual donor. One can get a bit confused by the sentence that "an average of 2.5 brains samples were analyzed for each donor". Maybe the authors could highlight more in the first paragraph of the results section and in Figure 1A, that there are multiple samples ("technical replicates") from one individual patient across different brain regions used.*

We removed the '2.5' sentence and rewrote the paragraph for clarity. Samples information's are now displayed in Table S1.

> *In the method section is a part included "Expression of target genes in microglia", it was very hard to allocate where these data from public data sets were actually used and for which analysis. Maybe the authors could clarify this again.*

AU response: we apologize and corrected the paragraph in the methods (page 6) as follow: " Expression of target genes in microglia. To evaluate the expression levels of the genes identified in this study as target of somatic variants, we consulted a publicly available database (https://www.proteinatlas.org/), and also plotted their expression as determined by RNAseq in 2 studies (Galatro et al. GSE99074 33, and Gosselin et al. 34) (Table S3 and Figure S2). For data from Galatro et al. (GSE99074) 33, normalized gene expression data and associated clinical information of isolated human microglia (N = 39) and whole brain (N = 16) from healthy controls were downloaded from GEO. For data from Gosselin et al. 34, raw gene expression data and associated clinical information of isolated microglia (N = 3) and whole brain (N = 1) from healthy controls were extracted from the original dataset. Raw counts were normalized using the DESeq2 package in R 35."

> *Table S3 is very informative, but also very complex. The reader could maybe benefit a lot from this table if it can be structured a bit easier especially when it comes to identifying P-SNVs and in which tissue sample they were found and if this was the same patient. The sorting function on top of the columns helps, but the color coding is a bit unclear.*

Despite our best efforts we agree that the table, which contain all sequencing data for all samples, is complex. The color coding (red) only highlights the presence of pathogenic mutation.

> ***Reviewer #3 (Recommendations For The Authors):***
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> *This is a well-done study of an important problem. I present the following minor critiques:*
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> *At the bottom of Page 4 and into the top of Page 5, the authors state that 66 of the 826 variants identified in their panel sequencing experiment were found in multiple donors. Then the authors proceed to analyze the remaining 760 variants. It seems that the authors concluded that these multi-donor mosaics were artifacts, which is why they were excluded from further analysis. I think this is a reasonable assumption, but it should be stated explicitly so it is clear to the reader. Complicating this assumption, however, the authors later state that one of their CBL variants was found in two donors, and it is treated as a true mosaic. The authors should make it clear whether recurrent variants were filtered out of any given analysis. It remains possible that all recurrent variants are*

> *true mosaics that occurred in multiple donors. The authors should do a bit more to characterize these recurrent variants. Are they observed in the human population using a database like gnomAD, which, together with their recurrence, would strongly suggest they are germline variants? Are they in MAPK genes, or otherwise relevant to the study?*

We apologize for the confusion. Our original intent for the ddPCR validation of variants (Figure 1E) was to count only 1 'unique' variant for variants found for example in 1 brain sample and in the blood from the same patient, or in 2 brain regions from one patient, in order to avoid the criticism of overinflating our validation rate. This was notably the case for TET2 and DNMT3 variants. For example, validation of a TET2 variant found in 2 different brain areas and blood of the same donor is counted as 1 and not 3. We did not eliminate these variants from the analysis as they passed the criteria for somatic variants as presented in Methods.

In contrast, when a specific variant was found and validated in two different donors, we counted it as 2.

The characterization of variants included multiple parameters and databases, including for example AF and gnomAD, as indicated in Methods and reported in Table S3.

All ddPCR results can be found at the end of Table S3.

> *Figure 2B labels age-matched controls as "C", but Figure 2C labels age-matched controls as AM-C. Labels should be consistent throughout the manuscript.*

We corrected this in the revised version.

> *It is not clear if the "p:0.02" label in Figure 2F is referring to AM-C Cx vs. AD-Cx or AM-C vs. AD. Please clarify.*

We apologize for the confusion, and we corrected the legend. The calculated p value is for the comparison between Cortex from Controls (age-matched) and the Cortex from AD.

> *On Page 7, the authors state, "The allelic frequencies at which MAPK activating variants are detected in brain samples from AD patients range from ~1-6% of microglia (Fig. 3G), which correspond to clones representing 2 to 12% of mutant microglia in these samples, assuming heterozygosity." I understand what the authors mean here but I think it's a bit confusingly stated. I suggest something like "The allelic frequencies at which MAPK activating variants are detected in brain samples from AD patients range from ~1-6% in microglia (Figure 3G), which correspond to mutant clones representing 2 to 12% of all microglia in these samples, assuming heterozygosity."*

We thank the reviewer for this suggestion and re-wrote that sentence.

> *Is there any evidence that the transcriptional regulators mutated in AD microglia (MED12, SETD2, MLL3, DNMT3A, ASXL1, etc.) are involved in regulating MAPK genes? This would tie these mutations into the broader conclusions of the paper.*

This is a very interesting question, and indeed published studies indicate that some of the transcriptional /epigenetic regulators regulate expression of MAPK genes. However, in the absence of experimental evidence in microglia and patients, the argument may be too speculative to be included.

> *Do the authors have any thoughts as to whether germline variants in CBL are linked to AD? If not, why do they think germline mutations in CBL are not relevant to AD?*

This is also a very interesting question. As indicated in our manuscript, germline mutations in CBL (and other member of the classical MAPK genes, see Figure 3C) cause early onset (pediatric) and severe developmental diseases known as RASopathies, characterized by multiple developmental defects, and associated with frequent neurological and cognitive deficits.

It is possible that some other (and more frequent?) germline variants may be associated with a late-onset brain restricted phenotype, but we did not find germline pSNV in our patients. GWAS studies may be more appropriate to test this hypothesis.

> *Do any donors show multiple variants? I don't think this is addressed in the text.*

We do find donors with multiple variants (see Figure 3D and Figure S3), however at this stage, we did not perform single nuclei genotyping to investigate whether they are part of the same clone.

> *Figure S3 appears to be upside down.*

This was corrected

> *Figure 5C should have some kind of label telling the reader what gene set is being depicted.*

We added this information above the panel (it was in the corresponding legend).

> *At the top of Page 12, Lewy bodies are written as Lewis bodies.*

This was corrected

> *Many control donors died of cancer (Table S1). Is there any information on which, if any, chemotherapeutics or radiation these patients received? Might this impact the somatic mutation burden? The authors should compare controls with and without cancer or with and without cancer treatments to rule this out.*

As suggested by the reviewer, we analyzed the mutational load of age-matched controls with and without cancer (revised Figure S2B). As expected, we saw an increase in the mutational load in controls with cancer, particularly in their blood. This information was added in the result section.

This is most likely associated with the treatments received as well as possible cancer clones.

> *The formatting for Table S3 is odd. Multiple different fonts are used (this is also seen in Table S5). Column Q has no column ID. The word "panel" is spelled "pannel." The word "expressed" is spelled "expressd" in one of the worksheet labels. Columns BG-BN in the ALL-SNV worksheet are blank but seemingly part of the table.*

We fixed this error in Table S3.

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