Integrated multi-omics analysis reveals common and distinct dysregulated pathways for genetic subtypes of Frontotemporal Dementia

Kevin Menden[1,*] (KM); Margherita Francescatto[1] (MF); Tenzin Nyima[1] (TN); Cornelis Blauwendraat[1,2] (CB); Ashutosh Dhingra[1] (AD); Melissa Castillo-Lizardo[1] (MC); Noémia Fernandes[1] (NF); Lalit Kaurani[4] (LK); Deborah Kronenberg-Versteeg[1,3] (DKV); Burcu Atarsu[1,3] (BA); Eldem Sadikoglou[1] (ES); Barbara Borroni[6] (BB); Salvador Rodriguez-Nieto[1] (SRN); Javier Simon-Sanchez[1,3] (JSS); Andre Fischer[4] (AF); David Wesley Craig[7] (DWC); Manuela Neumann[1] (MN); Stefan Bonn[1,5] (SB); Patrizia Rizzu[1] (PR); Peter Heutink[1,*] (PH).

Affiliations

1 German Center for Neurodegenerative Diseases, Tübingen, Germany

2 National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

3 Hertie Institute for Clinical Brain Research, Tübingen, Germany

4 German Center for Neurodegenerative Diseases, Göttingen, Germany

5 Institute of Medical Systems Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

6 Neurology Unit, Department of Clinical and Experimental Sciences, University of Brescia, Italy

7 University of Southern California, California, USA

* corresponding authors

Peter Heutink: peter.heutink@dzne.de

Kevin Menden: kevin.menden@qbic.uni-tuebingen.de

Abstract

Understanding the molecular mechanisms underlying frontotemporal dementia (FTD) is essential for the development of successful therapies. Here we integrated transcriptomic and epigenomic analyses of postmortem human brains of FTD patients with mutations in MAPT, GRN and C9orf72 and detected common and distinct dysregulated cellular pathways between patient groups. Our results highlight that excitatory neurons are the most vulnerable neuronal cell type and that vascular aberrations are a common hallmark in FTD. Via integration of multi-omics data, we detected several transcription factors and pathways which regulate the strong neuroinflammation observed in FTD-GRN. Small RNA-seq data and verification experiments in cellular models identified up-regulated miRNAs that inhibit cellular trafficking pathways in FTD and lead to microglial activation. These findings shed light on novel mechanistic and pathophysiological hallmarks of FTD. The data represent the

1st phase of a multi-omics, multi-model data resource for FTD research which allows in-depth molecular research into disease mechanisms that will further mechanistic FTD research.

Introduction

Frontotemporal Dementia (FTD) is a devastating pre-senile dementia characterized by progressive deterioration of the frontal and anterior temporal lobes [1]. The most common symptoms include severe changes in social and personal behaviour as well as a general blunting of emotions. Clinically, genetically, and pathologically there is considerable overlap with other neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Progressive Supranuclear Palsy (PSP) and Cortical Basal Degeneration (CBD) [2]. Research into FTD has made major advances over the past decades. Up to 40% of cases [3] have a positive family history and up to 60% of familial cases can be explained by mutations in the genes Microtubule Associated Protein Tau (MAPT), Granulin (GRN) and C9orf72 [4] which has been key to the progress in our understanding of its molecular basis. Several other disease-causing genes have been identified that account for a much smaller fraction of cases [5]. Mutations in MAPT lead to accumulation of the Tau protein in neurofibrillary tangles in the brain of patients while mutations in GRN and C9orf72 lead to the accumulation of TDP-43 [6], as well as dipeptide repeat proteins (DPRs) and RNA foci in the case of C9orf72 [7].

As of today, no therapy exists that halts or slows the neurodegenerative process of FTD and in order to develop successful therapies there is an urgent need to determine whether a common target and therapy can be identified that can be exploited for all patients, or whether the distinct genetic, clinical and pathological subgroups need tailored treatments. Therefore, the development of remedies relies heavily on a better understanding of the molecular and cellular pathways that drive FTD pathogenesis in all FTD subtypes.

Although our knowledge of FTD pathogenesis using molecular and cellular biology approaches has significantly advanced during recent years, a deep mechanistic understanding of the pathological pathways requires simultaneous profiling of multiple regulatory mechanisms. As neurodegenerative diseases develop over time, it is furthermore important to examine temporal changes. While post-mortem human brain tissue represents the end-stage of disease, well-defined rodent models can be used to address the temporal component. Lastly, experimental validation of derived hypotheses can be achieved in cellular systems, such as neurons derived from induced pluripotent stem cells (iPSCs) as well as in rodent models.

The Risk and modifying factors in Frontotemporal Dementia (RiMod-FTD) consortium[8] generates a multi-model and multi-omics data resource with the focus on mutations in the three most common causal genes: MAPT, GRN and C9orf72. The data resource will consist of multi-omics datasets from multiple post-mortem human brain regions, and matching iPSC derived neurons and brain tissue of transgenic mouse models at different time points.

Here, we report on data derived from the post-mortem human brain RNA-seq, CAGE-seq, smRNA-seq and methylation datasets from RiMod-FTD. We identified dysregulation of overlapping pathways in all disease groups that indicates converging disease mechanisms manifesting during disease progression. Using deconvolution analysis, we have identified changes in cellular composition that are either common or distinct to genetic subgroups. Through integration of smRNA-seq and CAGE-seq data, we could furthermore highlight potential regulatory molecules that might play important roles in FTD pathogenesis, within the identified functional gene modules and pathways.

Results

Multi-omics Data Resource for Frontotemporal Dementia

We have analysed data from brain tissue from diseased patients carrying mutations in the MAPT (n=17), GRN (n=11) or C9orf72 (n=17) genes and non-demented controls (n=16). The average age of FTD groups was lower than that of healthy controls (Table S1). We obtained tissue from up to 7 regions for each brain. The temporal and frontal lobes are the most affected areas in FTD, but we also obtained material from the occipital lobe, hippocampus, caudate, putamen and cerebellum for verification experiments. We performed CAGE-seq, smRNA-seq and quantitative proteomics (Miedema et al., manuscript in preparation) on tissue from frontal and temporal lobes, and generated methylation and RNA-seq data for frontal lobe tissue. Additional data types such as ATAC-seq, transgenic mouse models and iPS derived data are planned for future releases. The resulting, comprehensive multi-omics data resource enables the study of disease mechanisms in FTD subtypes to greater detail than single genomics experiments (Fig. 1).

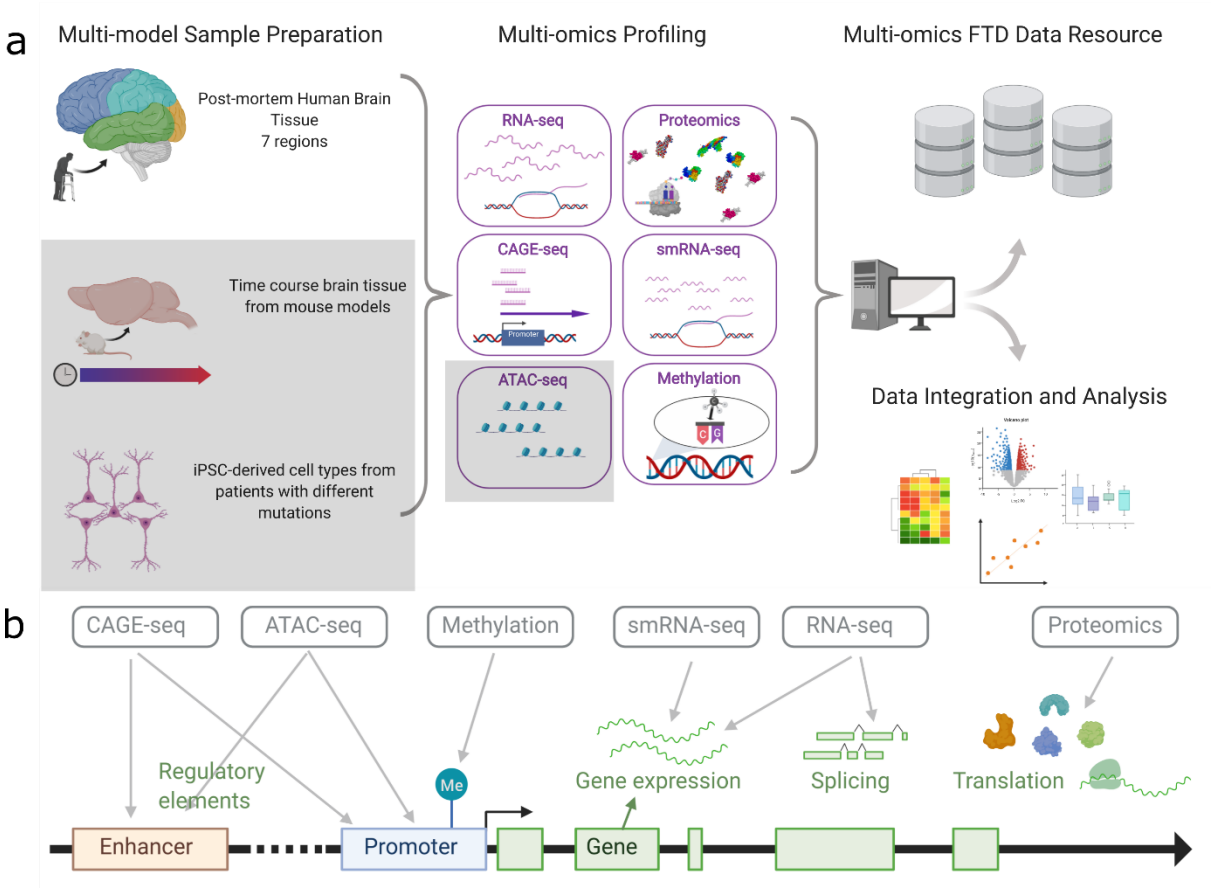

Figure 1: Graphical overview of the RiMod-FTD project. a In phase 1, Human Post-mortem brain tissue samples from multiple regions of patients with mutations in GRN, MAPT and C9orf72 have been collected and used for multi-omics data generation. Grey shading indicates datasets that will be completed in future phases. The datasets have been integrated and analysed and published to be accessible as FTD resource. In subsequent phases additional datatypes will be added (i.e. ATAC-seq) and the resource will be extended with data from matching mouse models and iPSC derived celltypes. **b** The multi-omics approach allows to profile multiple regulatory features of gene expression, including enhancer- and promoter-based regulation, epigenetic regulation, alternative splicing, post-transcriptional regulation (miRNAs) and regulation of translation (proteomics).

In the current study, we have integrated RNA-seq, CAGE-seq, smRNA-seq and methylation data from the Gyrus Frontalis Medialis (GFM) (Table S2), as this brain region is strongly affected in FTD.

Differential gene expression analysis and cellular deconvolution of the GFM in FTD.

To identify general gene expression patterns in the GFM of patients with FTD, we performed differential gene expression (DGE) and principal component analysis (PCA) using RNA-seq data. The PCA indicates considerable heterogeneity between samples, as can be expected from post-mortem human brain tissue (Fig. 2a). However, a difference between FTD cases and control samples is clearly visible. Differentially expressed genes (DEGs) were calculated for all disease groups (FTD-MAPT, FTD-GRN, FTD-C9orf72) compared to controls while controlling for gender and pH-value (see Methods). We observed the largest number of DEGs (adj. P-value < 0.05) for FTD-GRN, followed by FTD-MAPT and FTD-C9orf72 (Fig. 2b). DGE of smRNA-seq data yielded 78, 21 and 39 differentially expressed miRNAs in FTD-MAPT, FTD-GRN and FTD-C9orf72, respectively (Fig. 2c).

Due to the neurodegenerative nature of FTD, it is likely that there exists a systematic difference in cell composition between cases and controls which can affect DGE analysis due to differences in gene expression between cell types - a problem which has often been overlooked in tissue expression studies. Here, we account for this problem by applying a conservative filtering approach and removing DEGs that are associated with changing cellular composition (see Methods). All further analyses were based on the filtered set of DEGs, unless otherwise specified. Note that this method could only be applied to the total

RNA-seq dataset because similar cell type specificity data (here, single-cell RNA-seq data) was not available for other data types.

Activation of extracellular matrix (ECM) associated pathways and circulatory system development.

We next performed pathway enrichment analysis with DEGs from the RNA-seq data using go:Profiler[9] to identify the most affected cellular pathways. Down-regulated genes are strongly enriched for mitochondrial and oxidative phosphorylation pathways in both FTD-GRN and FTD-MAPT (Fig. 2d, Fig. S1), indicating a dysfunctional energy metabolism - a well-known hallmark of many neurodegenerative diseases[10]. Neuronal system pathways are enriched among down-regulated genes for both groups as well. This might be explained by dysfunctional neurons that have not yet undergone apoptosis or by a general impairment of neuronal function caused by the disease. Other significantly down-regulated pathways include ubiquitin-dependent protein metabolism and vesicle-mediated transport (FTD-GRN). In all three groups, up-regulated genes are enriched for extracellular matrix (ECM) associated pathways and circulatory system development (Fig. S1). Genes involved in Hippo-signalling are enriched in FTD-GRN and FTD-MAPT (Fig. 2d), and immune system related genes are enriched in FTD-GRN. ECM dysregulation, in particular, has been implicated with several neurodegenerative diseases. For instance, studies in mouse models showed that tau pathology can lead to ECM reorganization and that reducing ECM proteins could reverse memory deficits in an AD model[11,12]. While the role of the ECM in FTD remains unknown, our results suggest a prominent involvement in end-stage FTD.

We also specifically examined the DEGs with the largest fold-changes in the RNA-seq data because large expression fold-changes often signify strong dysregulation. These results

support the importance of ECM in FTD as for all disease groups, multiple matrix metalloproteinase enzymes (MMPs) are among the DEGs with the largest LFCs (Fig. S2, Fig. S3A). Elevated RNA levels of MMP genes have been reported for many neurodegenerative diseases, and MMPs target a wide range of ECM [13]indicating their importance in neurodegenerative mechanisms[14]. Protein interaction networks of up-regulated genes in FTD-MAPT and FTD-GRN show the central importance of MMPs in these networks (Fig. S3 B & C).

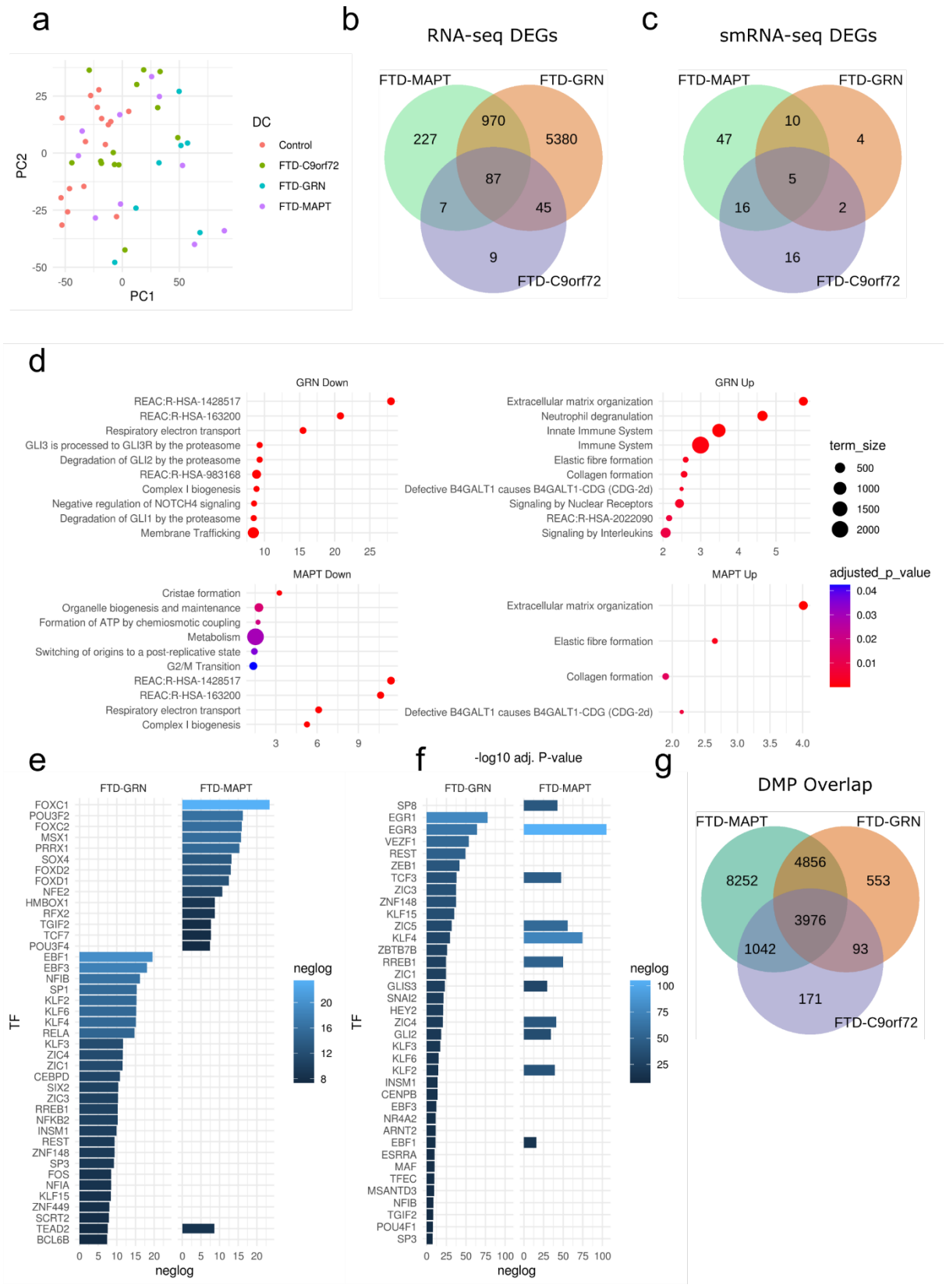

Figure 2: **Gene- and Pathway-level transcriptional changes in FTD. a** Principal component analysis of variance stabilized RNA-seq expression values, coloured by group. **b**

Overlap between RNA-seq DEGs from different disease groups. **c** Overlap between smRNA-seq DEGs from different disease groups. **d** Enriched Reactome pathways in RNA-seq up- and down-regulated DEGs. Shown are the ten most significant pathways per group; the x-axis signifies the negative log10 P-value. Colour corresponds to adjusted P-value and node size corresponds to the number of genes in a pathway. **e,f** Best candidates for active and inactive TFs in FTD-GRN and FTD-MAPT, respectively. The x-axis signifies the negative log10 P-value. **g** Overlap of DMPs in different disease groups.

Regulatory mechanisms associated with differential expression

To better understand relevant regulatory mechanisms leading to these gene expression changes, we generated a set of candidate driver transcription factors (TFs) using the GFM CAGE-seq data. CAGE-seq cluster counts, when assigned to the closest gene, correlate well with RNA-seq expression data (average sample-wise correlation coefficient: 0.6, Fig. S4). We used the CAGE-seq data to predict candidate driver TFs for up- and down-regulated genes (see Methods for details). TEAD2, a TF central to the Hippo signalling pathway, is the only predicted active TF common to FTD-GRN and FTD-MAPT (Fig. 2e), while there is greater overlap among inactive TFs (here: inactive TF = has down-regulated targets, Fig. 2f). Moreover, we performed miRNA-target gene mapping to evaluate potential regulatory roles of miRNAs. Expression values of miRNAs were correlated with their predicted targets using matching samples from the RNA-seq data. Only miRNA-target pairs with considerable negative correlation were retained (see Methods).

DNA methylation is another important regulatory mechanism that can affect gene expression. We generated Illumina Infinium EPIC methylation data from the GFM and considered the most variable CpG sites (28,173) corrected for possible confounding effects

using surrogate variable analysis (SVA) to perform differential methylation analysis (see Methods). We detected 18,126, 9,478 and 5,282 significantly differentially methylated positions (DMPs) for FTD-MAPT, FTD-GRN, and FTD-C9orf72, respectively (Fig. 2g). The C9orf72 repeat expansion is known to be associated with hypermethylation[15] and we confirmed in our data that a CpG site located at the 5'-end of the C9orf72 gene, only 14 bp away from the repeat expansion, is hypermethylated (log fold-change: 0.6, Fig. S4A). Pathway enrichment analysis of genes in proximity to DMPs yielded enrichment of genes involved in nervous system development for hypermethylated CpG sites. Genes close to hypomethylated sites were enriched for system development and vasculature development (Fig. S5C). As hypermethylation of CpG sites at promoter regions is associated with decreased expression, this indicates epigenetically controlled expression inhibition of genes important for neuronal function, or remnants of cell composition effects that could not be entirely alleviated by SVA (see Methods). Performing biological age prediction using the methylation data resulted in underestimated age predictions for all groups, albeit to a lesser extent for FTD groups, which indicates accelerated aging in FTD (Fig. S5B).

Vulnerability of excitatory neurons and enrichment of endothelial cells

To identify vulnerable cell types and disease-related cell composition changes, we inspected the results from the RNA-seq deconvolution analysis (Methods) with respect to genetic FTD subtypes. As expected, fractions of neuronal cells are systematically lower in all FTD groups compared to controls (Fig. S6). Consequently, virtually all other cell types show increasing percentages. We therefore calculated the percentage-wise change for each cell type and assessed statistical significance (see Methods). Strongest neuronal loss was observed in FTD-GRN, followed by FTD-MAPT and FTD-C9orf72 (Fig. 3a, Table S3), which agrees with studies that have shown that the frontal lobe is most strongly affected in FTD-GRN[16–18]. Moreover, neuronal loss can be primarily attributed to loss of excitatory neurons, while

fractions of inhibitory are not significantly different to controls (Table S3). Our results confirm findings from recent studies that found excitatory neurons to be especially vulnerable to tau pathology[19] and we specifically detected an important role of glutamatergic neurotransmission in FTD [20,21]. Closer examination of the KEGG pathway 'glutamatergic synapse' suggests that AMPA receptors are mainly affected, while we could not see signs of dysregulation for NMDA receptors (Fig. S7 A-C). Analysis of candidate regulator TFs highlighted the TF Early Growth Response 3 (EGR3), targets of which are enriched for glutamatergic synapse genes (Fig. S7D), indicating involvement in excitatory neuronal function.

To validate our computational deconvolution, we considered the fractions of excitatory neurons as a proxy of neurodegeneration and correlated them with manually determined degeneration scores by a neuropathologist (Fig. 3b, Methods). Indeed, excitatory neuron fractions show strong negative correlation with pathology scores (Pearson's correlation coefficient = -0.78, P-value = 2.8e-07), thereby providing experimental confirmation of our computational predictions.

The strongest growth in percentage compared to the baseline is observed for endothelial cells in FTD-MAPT and FTD-C9orf72 disease groups, but not FTD-GRN, where microglial cells show the strongest increase. Circulatory system development is among the most significantly up-regulated biological processes in all three disease groups (Fig. 3 c & d). The role of the circulatory system in FTD is relatively unexplored. However, Bennet et al. recently found increased vasculature growth in mouse models of FTD-MAPT with a P301L mutation[22]. Interestingly, endothelial enrichment in FTD-MAPT is particularly strong in patients with a P301L mutation (Fig. 3a). Another recent study observed a particular microvascular structure with increased frequency in brains of patients with frontotemporal

254 lobar degeneration (FTLD)[23] and Park et al. have shown that soluble tau can interfere with
255 nitric oxide production and thus lead to reduced vasodilation of blood vessels, ultimately
256 leading to insufficient blood supply [24].

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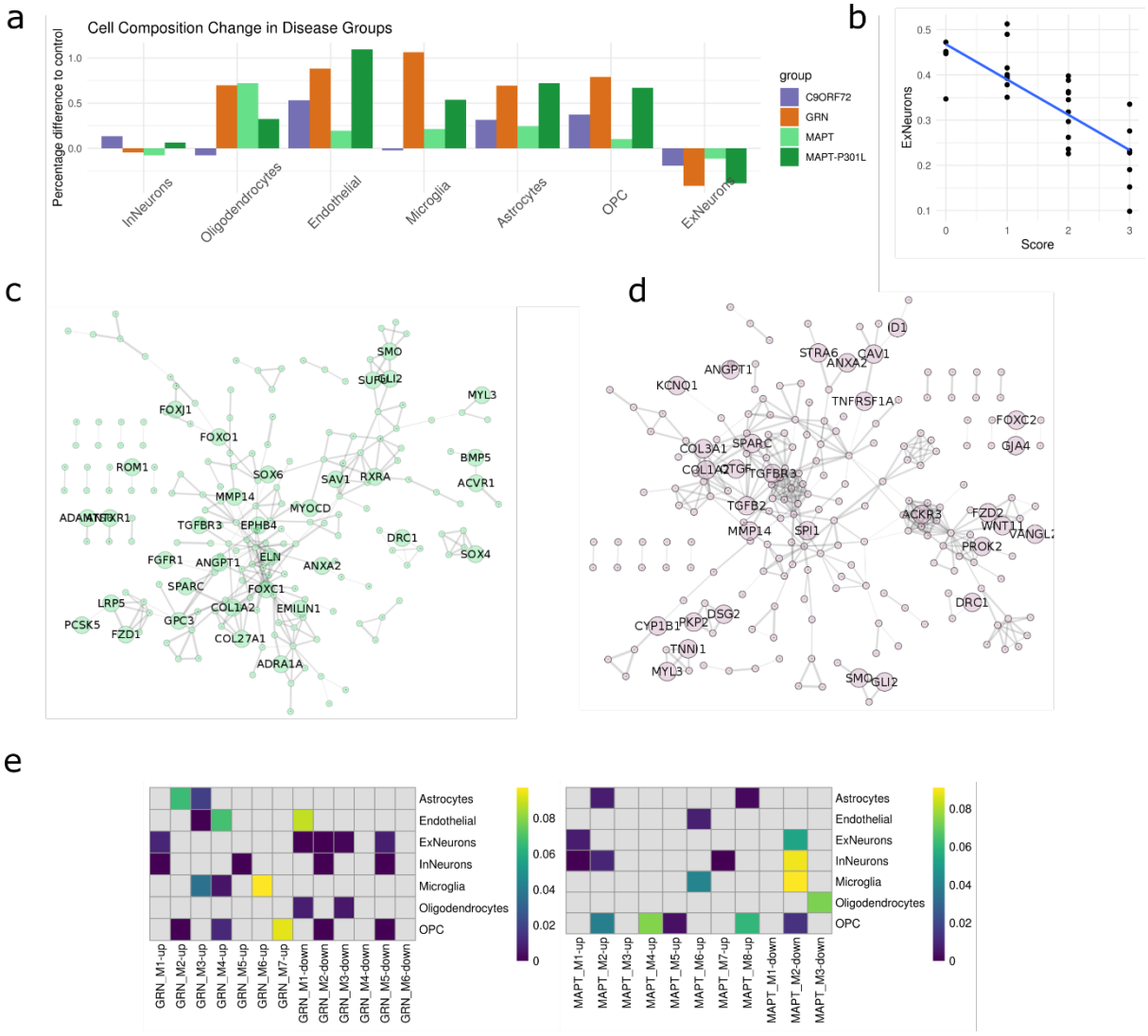

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259 **Figure 3: Cell composition changes in FTD. a** Percentage change of averaged fractions
260 per group compared to the average of the control group. Genetic subtypes are indicated with
261 different colours. **b** Regression of excitatory neuron fractions (y-axis) against neuropathology
262 scores (x-axis). **c**, **d** PPI networks of genes up-regulated in FTD-MAPT and FTD-GRN (log-
263 fold-change > 1), respectively. Genes involved in the biological process “circulatory system
264 development” are labelled. **e** Heatmap of EWCE analysis results for HumanBase modules of

FTD-GRN and FTD-MAPT. Different modules are lined up on the x-axis, different cell types on the y-axis. Tile colour signifies the EWCE P-value. Tiles with P-values above 0.1 are marked grey.

To better understand transcriptional changes and regulatory mechanisms, we performed tissue-specific functional module detection with HumanBase[25] and assessed cell type specificity of modules using EWCE[26]. Both for FTD-MAPT and FTD-GRN, most modules show specificity for a few cell types (Fig. 3e). Up-regulated modules in both groups are significantly enriched for endothelial genes (P-value < 0.1). Genes within these modules have been associated with blood vessel development (FTD-MAPT M6-up) and endothelial cell growth (FTD-GRN M4-up) by HumanBase (Fig. 3 c & d), further supporting a distinct involvement of endothelial genes in these FTD subtypes.

Increased Inflammatory response in FTD-GRN

In patients with FTD-GRN, microglial fractions show an even larger relative increase than endothelial cells, indicating increased microglial activity. The protein encoded by GRN is well known for its importance to lysosomal function, is highly expressed in microglia and well-known for having important functions in the immune system [27,28]. Microglia are also slightly enriched in FTD-MAPT (P-value = 0.037) but not in FTD-C9orf72 (P-value = 0.475).

Here, we have observed a prominent increase in microglial cell fractions and up-regulation of immune system pathways in FTD-GRN, a feature of GRN deficiency that has been frequently shown in mouse models[28–30]. We therefore wanted to further characterize potential underlying regulatory mechanisms. First, we examined FTD-GRN modules for enrichment of immune system-related terms. Indeed, several up-regulated modules are

enriched for genes related to the immune system, while we could not find enrichment among down-regulated modules. The module FTD-GRN M1-up contains genes important for neutrophil migration and response to interleukins (Fig. 4 a & d). Both modules M3-up and M4-up contain genes relevant to NF-kappa-B (NFkB) signalling, as well as genes involved in tumour necrosis factor (TNF) production (Fig. 4 b & c, respectively). Finally, the module M6-up is enriched for genes involved in T cell activation. Modules M3-up, M4-up and M6-up are furthermore enriched for microglial-specific genes (Fig. 3e). Interestingly, several necroptosis-related genes are up-regulated (M1-up: TLR3, TLR8, RIPK3; M4-up: RIPK2), suggesting this pathway as a potential driver of neuronal death. While we did not detect prominent signals for neuroinflammation in FTD-MAPT, the FTD-MAPT module M3-up contains several genes involved in T cell and TNF signalling (EZR, RAB29, CARD8, HIPK1). However, neuroinflammation is much less prominent in FTD-MAPT and FTD-C9orf72 compared to FTD-GRN.

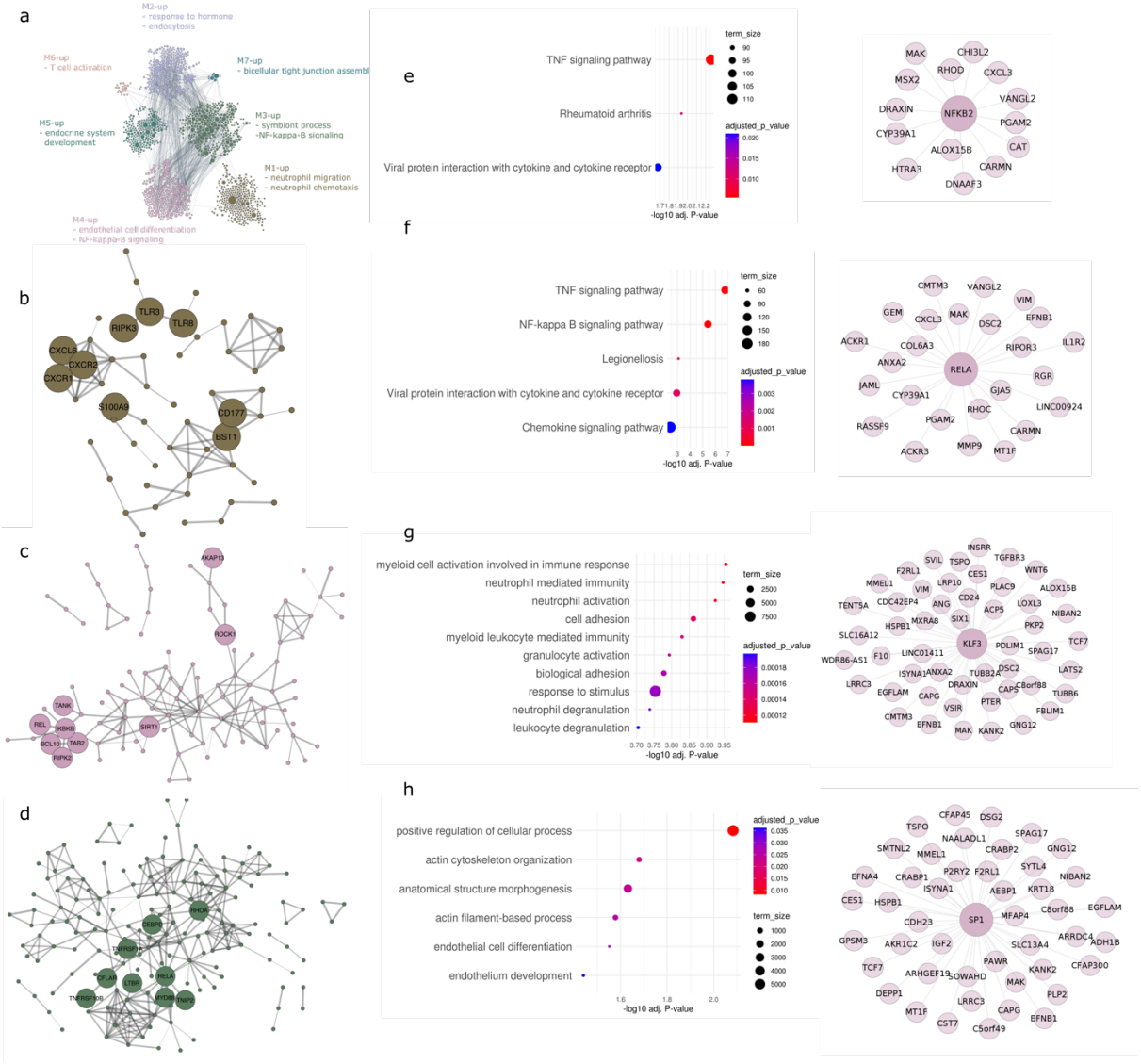

Figure 4: **Neuroinflammation in FTD-GRN. a** Up-regulated HumanBase modules in FTD-GRN with most significant terms. **b** Protein-protein interaction (PPI) network (made with String-DB) of FTD-GRN M1 up-module. Genes involved in necroptosis, interleukin response and neutrophil migration are indicated **c** PPI network of FTD-GRN M4-up module. Genes involved in NFkB signalling are indicated. **d** PPI network of FTD-GRN M3-up module. Genes involved in NFkB signalling and CEPBD are indicated. **e** and **f** KEGG pathway enrichment of predicted targets of TFs NFKB2 and RELA, respectively. **g** and **h** GO:BP pathway enrichment of predicted targets of TFs KLF3 and SP1, respectively.

Inspection of our candidate regulator TFs indicated the TFs Nuclear Factor Kappa B Subunit 2 (NFKB2) and RELA which together form the NFkB signalling complex, as potential drivers in FTD-GRN (Fig. 2e). Enrichment analysis of predicted NFKB2 and RELA targets in FTD-GRN indeed revealed TNF signalling and NFkB signalling as the most significantly enriched KEGG pathways (Fig. 4 e & f). Furthermore, enrichment analysis indicated targets of the TFs SP1 and KLF3 as highly enriched among genes in the FTD-GRN M3-up module. Predicted KLF3 targets are enriched for immune system genes (Fig. 4g). SP1 target genes do not show a strong enrichment but have roles in actin cytoskeleton organization and endothelial cell differentiation, among others (Fig. 4h). We also investigated predicted targets of down-regulated miRNAs and genes proximal to hypomethylated CpG sites for involvement in the immune system in FTD-GRN but could not detect any significant immune system-relevant enrichment suggesting they do not play a major role in regulating the immune response.

To closer examine which parts of the NFkB and TNF signalling pathways are affected in FTD-GRN and in FTD in general, we inspected fold-changes of genes from the corresponding KEGG pathways. Interestingly, the pro-inflammatory cytokine Interleukin 1 Beta (IL1B) is down-regulated in all disease groups, although only significantly in FTD-MAPT (Fig. S9A). Similarly, the inflammatory cytokine Interleukin 6 (IL6) has negative fold-changes in all disease groups. Downstream effector genes with positive fold-changes include multiple chemokines, Interleukin 18 Receptor 1 (IL18R1) and several metalloproteinases.

GRN deficiency in mouse models leads to overactivation of microglia[28] and GRN deficiency leads to NFkB overactivation in microglia in a GRN loss-of-function mouse model[29]. Hyperactivation of TNF by NFkB signalling has been linked to obsessive-compulsive

behaviour (OCB) and inhibition of NFkB was sufficient to rescue the behavioural symptoms linking these TFs to the OCB observed in patients.

Dysfunctional energy metabolism and cellular trafficking in FTD

Among the most significantly down-regulated pathways in FTD-GRN and FTD-MAPT are several pathways involved in energy metabolism and oxidative phosphorylation (Fig. 2d, Fig. S1). Inspection revealed the modules FTD-GRN M1-down and FTD-MAPT M1-down as being most significantly associated with the term NADH dehydrogenase complex assembly (Fig. 5 d & e, Tables S4 and S5). Further inspection of the FTD-MAPT and FTD-GRN M1-up modules revealed that they contain several NADH:Ubiquinone Oxidoreductase Subunit genes (Fig. 5 b & c), which are necessary for functional oxidative phosphorylation and hence energy production. The FTD-GRN module is moreover enriched for genes involved in intracellular transport and autophagy. The FTD-GRN M1-down module contains several genes associated with FTD or ALS: Superoxide Dismutase 1 (SOD1), Dynactin Subunit 1 (DCTN1), PTEN Induced Kinase 1 (PINK1), Huntingtin (HTT), and CHCHD10. All these genes show lower expression values in every genetic subgroup, although they do not reach significant levels in all groups (Fig. 5a).

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357 -

358 **Figure 5: Impaired oxidative phosphorylation and cellular trafficking in FTD. a**

359 Expression levels (variance stabilized with DESeq2) of the genes CHCHD10, PINK1, SOD1,

360 DCTN1 and HTT in different groups. **b** STRING-DB PPI of FTD-MAPT M1-down module.

361 Genes involved in oxidative phosphorylation are labelled. **c** PPI of FTD-GRN M1-down

362 module. Genes involved in NADH dehydrogenase complex assembly and mitophagy are
363 labelled, as well as CHCHD10. **d, e** Most significant results from pathway enrichment
364 analysis with g:Profiler (GO biological process) for the FTD-GRN M1-down module and the
365 FTD-MAPT M1-down module, respectively. Node colour corresponds to adjusted P-value
366 and node size to term size. **f** Most significant results from enrichment analysis (Reactome) of
367 targets of up-regulated miRNAs in all disease groups. **g, h** Heatmaps of intersection-over-
368 union scores between predicted miRNA targets and down-regulated modules in FTD-GRN
369 and FTD-MAPT, respectively. **i** Top enrichment results of g:Profiler (GO biological process)
370 for the FTD-GRN M3-down module. Node colour corresponds to adjusted P-value and node
371 size to term size. **j** PPI network (String-DB) of FTD-GRN M3-down module. Predicted targets
372 of up-regulated miRNAs are labelled. **k** PPI network of predicted targets of up-regulated
373 miRNAs in FTD-MAPT.

374

375 Cellular transport is thought to play a key role in FTD pathogenesis as impaired trafficking
376 can affect protein and mitochondria homeostasis. Here, we show that mitochondrial function
377 is strongly impaired in end-stage FTD and that transport pathways are tightly connected to
378 this pathology. We thus looked for potential regulatory mechanisms driving the pathological
379 changes. Enrichment analysis of targets of up-regulated miRNAs in all disease groups
380 revealed cellular localization as the most significantly enriched biological process (GO:BP)
381 and membrane trafficking as the most significant Reactome pathway (Fig. 5f). Up-regulated
382 miRNAs in FTD therefore seem to primarily target cellular transport pathways and might play
383 important roles in dysfunctional transportation.

384

385 To detect modules and genes predominantly targeted by up-regulated miRNAs, we
386 calculated the intersection-over-union (IoU) of up-regulated miRNA targets with down-
387 regulated modules for FTD-GRN and FTD-MAPT (Fig. 5 g & h). The FTD-GRN M3-down

388 module is most strongly targeted by miRNAs and contains genes involved in metabolic
389 processes and cellular localization (Fig. 5i, Table S4). Five miRNAs have putative target
390 genes in this module: hsa-miR-150-5p, hsa-miR-142-3p, hsa-miR-193a-3p, hsa-miR-148a-
391 3p and hsa-miR-363-3p, which are all significantly up-regulated in FTD-MAPT as well,
392 except hsa-miR-363-3p. We generated networks of the above-mentioned candidate miRNAs
393 combined with a PPI network of the FTD-GRN M3-down module (Fig. 5j) and a PPI network
394 of all predicted targets in FTD-MAPT (Fig. 5k), as we could not detect a similar module in
395 FTD-MAPT. In total, we observed 31 common putative miRNA targets in both networks.

396

397 Next, we selected hsa-miR-193a-3p, hsa-miR-150-5p and hsa-miR-19b-3p for further
398 characterization in iPSC models (Fig. 6a). The first two miRNAs are DE in all three disease
399 groups and have many targets among module genes (Fig. 5 j & k). The miRNA hsa-miR-
400 19b-3p is up-regulated in all disease groups, although it does not reach significance after
401 (FTD-MAPT and FTD-C9orf72) or before (FTD-GRN) multiple testing correction.
402 Nevertheless, down-regulated genes were predicted to be enriched for targets of hsa-miR-
403 19b-3p by g:Profiler, the miRNA is known to inhibit autophagy [31] and it is highly expressed in
404 neurons. We performed RNA-seq on iPSC-derived neurons and microglia (Methods) that
405 were transfected with miRNA mimics and inhibitors for the three selected miRNAs. Here, we
406 focused on the mimic experiments, as the mimics should in theory reproduce the effects of
407 miRNA overexpression. Inhibition and mimicking of miR-150-5p in neurons had only minor
408 effects in neurons, while in microglia, the miR-150-5p mimic had strong effects, leading to
409 237 down-regulated and 236 up-regulated DEGs, enriched for cellular transport and immune
410 system pathways of the latter and nervous system development of the former (Fig. 6b).
411 Inhibition of miR-150-5p had even stronger effects (3221 DEGs), indicating an important
412 function of this miRNA in microglia. Transfection of miR-193a-3p mimic and inhibitor only
413 resulted in significant expression changes in microglia, where the mimic had strong effects
414 with 1756 down-regulated and 1474 up-regulated genes. Up-regulated genes were enriched

of mitochondrial functions like oxidative phosphorylation, while down-regulated genes were enriched for localization and vesicle-mediated transport pathways (Fig. 6c).

In neurons, the miR-19b-3p mimic resulted in 89 down- and 137 up-regulated DEGs (inhibitor: 8 down-regulated, 31 up-regulated). Genes down-regulated by the mimic and up-regulated in the inhibitor experiment are involved in neuronal system pathways, enriched for miR-19b-3p targets and share 17 common genes, thus providing evidence for these genes to be regulated by miR-19b-3p. In microglia, stronger effects of the miR-19b-3p mimic compared to the inhibitor were observed (1518 compared to 608 DEGs). Genes down-regulated by the miR-19b-3p mimic were enriched for catabolic processes, autophagy and vesicle-mediated transport, up-regulated genes were enriched for cell cycle and immune system related genes (Fig. 6d). These results provide strong evidence that hsa-miR-19b-3p and hsa-miR-193a-3p indeed regulate cellular trafficking pathways. Furthermore, hsa-miR-150-5p is important for microglia function and up-regulation could lead to immune system activation.

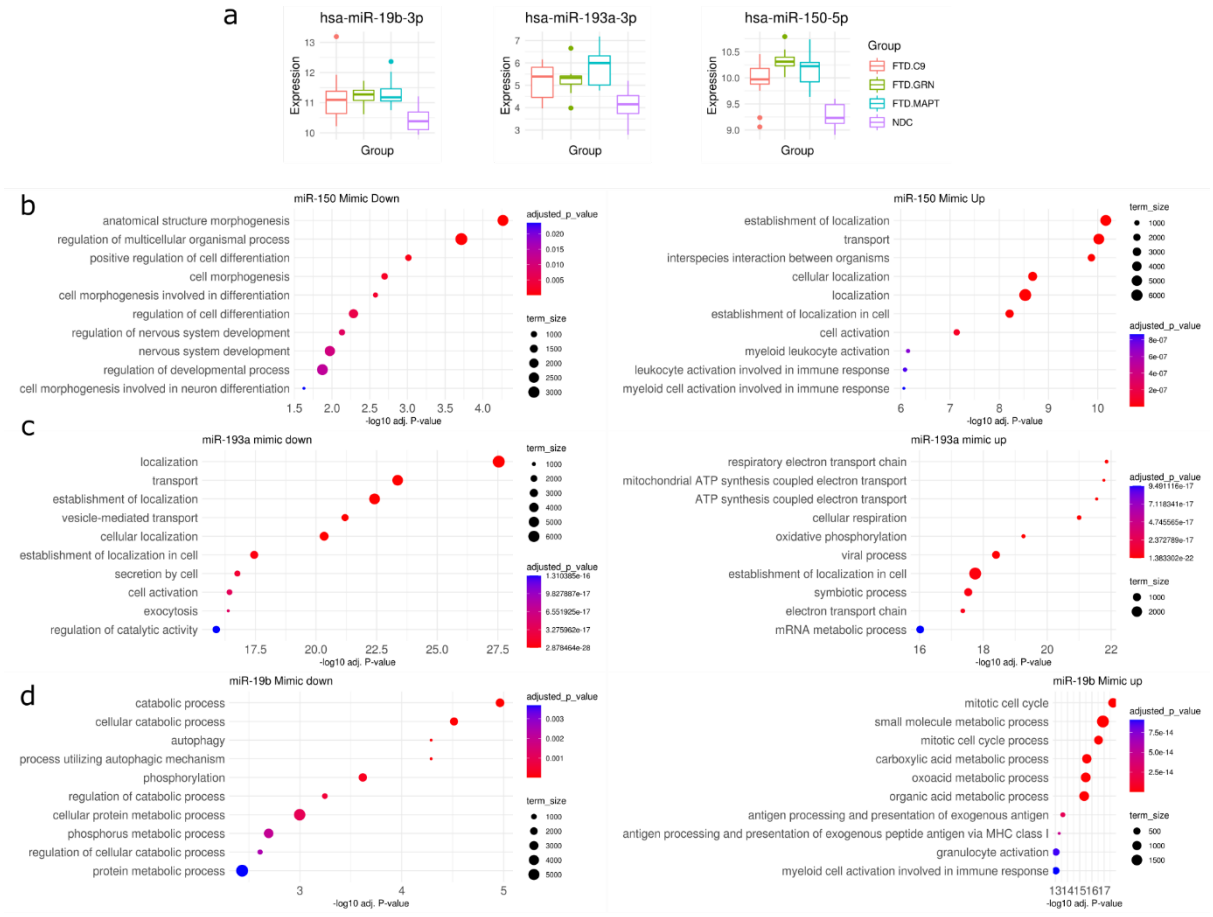

Figure 6: Effects of miRNA mimic and inhibitor experiments in iPSC-derived microglia. **a** Boxplots of normalized expression values for the selected miRNAs. **b**, **c**, **d** The top ten most significantly enriched biological processes of up- and down-regulated genes after transfection with mimics for miR-150-5p, miR-193a-3p and miR-19b-3p, respectively. Node size corresponds to the number of genes in the biological process term and node colour corresponds to the P-value adjusted for multiple testing.

Discussion

Here, we present the data from the 1st phase of the RiMod-FTD project, a multi-omics, multi-model data resource for FTD research that aims to understand the role of distinct genetic

risk factors in the disease. Generated by the RiMod-FTD consortium over several years, the resource depicts a valuable tool for FTD researchers that will help to accelerate scientific progress towards a better understanding of relevant disease mechanisms in FTD. Additional multi-omics data from iPSC derived cell types, transgenic mouse models and other brain regions will be added over time.

By studying post-mortem tissue of the GFM we detected the largest transcriptional dysregulation and greatest neuronal loss in FTD caused by mutations in GRN, agreeing with previous findings of a more pronounced frontal lobe atrophy in FTD-GRN compared to other subtypes[32]. Our deconvolution analysis indicates that excitatory neurons are the most affected cell type in all genetic subtypes of FTD, which was confirmed in the RiMod-FTD proteomics data (Mediema et al., manuscript in preparation) as well. Recently, evidence from multiple studies has accumulated pointing toward a strong involvement of glutamatergic synapses in FTD[33]. While it has been previously reported that densities of both ionotropic glutamate receptors, AMPA and NMDA receptors, are reduced in post-mortem brain tissue of FTD patients[21], we see evidence that AMPA receptors are particularly affected. Intriguingly, a recent single-nucleus RNA-seq study in a GRN mouse model could show that hyperactivation of microglia leads to selective loss of excitatory neurons[34], which confirms our hypothesis that excitatory neurons are especially vulnerable in FTD.

Strong neuroinflammation is a distinct feature of FTD-GRN, which is confirmed in our data and by increased microglial cell numbers in this FTD sub-type, in line with recent findings showing increased microglial burden in FTD-GRN[35,36]. Using an integrative approach, we identified TFs: NFKB2, RELA, KLF3 and SP1 as key inflammatory drivers, leading to activation of the NFkB- and TNF-signalling pathways.

We found indicators of activated necroptosis, suggesting this pathway as potential cause for cell death. The necroptosis cell death pathway is deregulated in several neurodegenerative

disorders[37], and a recent study has shown that TBK1, a genetic cause of ALS and FTD (here down-regulated in FTD-GRN), is an endogenous inhibitor of RIPK1, an upstream regulator of RIPK3[38]. The authors showed that embryonic lethality of TBK1-knockout mice is dependent on RIPK1 activity, suggesting that the necroptosis pathway is indeed an important player in FTD pathogenesis. In a recent review, Molnár and colleagues have discussed several available drugs that could potentially regulate necroptosis[39], highlighting the potential of this pathway as a drug target for developing therapies for FTD.

Our pathway enrichment and deconvolution analyses pointed toward increased blood vessel abundance and growth in FTD brains compared to controls, which is consistent with the results from the RiMod-FTD proteomics data (Mediema et al., manuscript in preparation). It is generally not known how and if the vasculature system is involved in FTD pathogenesis, although recent studies have observed abnormalities in a mouse model of tau pathology and post-mortem human brains[22,23]. To our knowledge, angiogenesis as a pathological feature in several genetic FTD subtypes has not been reported before and therefore depicts an important subject for FTD research.

In all three disease groups, we have observed prominent up-regulation of ECM pathways and MMP enzymes, suggesting MMPs as important regulators in FTD pathogenesis. While it has been increasingly recognized that MMPs are important regulators in many neurodegenerative diseases [40,41], the role of MMPs in FTD pathogenesis has not been investigated in depth. In mouse models of ALS, inhibition of the MMPs MMP2 and MMP9 could indeed prolong survival and reduce symptoms[42,43]. Moreover, TIMP3, which is up-regulated in our data, was found to be partly responsible for neuronal apoptosis in an ALS model [44], which points towards TIMP3 as a potential apoptosis mechanism in FTD. MMPs are furthermore tightly involved in the inflammatory response, and can activate the tumour

necrosis factor (TNF) gene[45]. Inflammatory cytokines, hypoxia and reactive oxygen species can lead to the activation of MMPs[40,46] and MMPs can digest the ECM, stimulate increased production of growth factors and thereby promote the growth of blood vessels, providing a potential causal link to the prominent enrichment of endothelial cells[46]. Given their important biological functions and their involvement in all genetic FTD subgroups, it will be important to further investigate how MMPs contribute to FTD and whether they can be exploited as drug targets, as MMP inhibition in model system has shown promising results[47,48].

Impaired cellular trafficking mechanisms is very likely a key feature of FTD pathogenesis and it has been shown multiple times that FTD-causal mutations lead to trafficking deficits [49–51]. However, it is not always clear which mechanisms continue to dysfunctional transport mechanisms. Here, using multi-omics data and validation experiments, we show that elevated expression of several miRNAs contributes to the inhibition of genes important for cellular transport. Additional studies are necessary to further validate this hypothesis, which directly suggests several miRNAs as putative drug targets.

While our study is reasonably powered, increasing the sample size for individual groups such as FTD-C9orf72 would further increase the power of our analysis and help to better define which pathways are truly distinct to certain subtypes. It will therefore be an objective for future iterations of the RiMod-FTD resource to include larger numbers of samples.

To conclude, we present here an integrated multi-omics analysis on data from Phase 1 of the RiMod-FTD project and developed new hypotheses on FTD disease mechanisms. The data presented here highlights several regulator molecules important for FTD pathogenesis and their consequences such as vascular abnormalities and thereby we show the value of an integrated multi-omics data analysis for hypothesis generation and testing. The RiMod-

FTD data will be freely accessible to the scientific community through the European Genome-phenome Archive (**EGA**) and a dedicated RiMod-FTD web application (https://www.rimod-ftd.org) thus enabling scientists to derive new mechanisms and hypotheses from the data.

Methods

Donor samples employed in this study

Post mortem human brains

Tissues were obtained under a Material Transfer Agreement from the Netherlands Brain Bank, and additional samples were provided by the Queen Square Brain Bank of Neurological Disorders and MRC, King College London. Demographic details about human brain samples are summarized in Table S1.

GFM and GTM tissue from each subject was divided into three pieces for transcriptomic, proteomic and epigenetic experiments in a dry-ice bath using precooled scalpels and plasticware.

hIPS-derived NGN2 neurons and miRNA mimics and inhibitors transfection

smNPC were derived from hiPSc cells (Cell line id: GM23280 obtained from the Coriell Institute) using the protocol described by Reinhardt et al[52]. The differentiation protocol from

544 smNPC to neurons involves over-expression of Neurogenin-2 (NGN2) using a modified
545 version of the NGN2 lentiviral inducible vector system (single vector
546 pLV_TRET_hNgn2_UBC_BSD_T2A_rtTA3). The detailed description about protocol,
547 reagents and media composition is available in Dhingra et al.[53].

548 Briefly, stable NGN2 smNPC are grown for six days in expansion medium N2B27
549 supplemented with CHIR99021 (CHIR) 3 µM, Purmorphamine (PMA) 0.5 µM and L-ascorbic
550 acid 2-phosphate magnesium (AA) 64 mg/l. For differentiation, cells are plated (80,000
551 cells/cm2) onto Poly L-orithine and laminin coated plates in N2B27 medium supplemented
552 with doxycycline (dox) at 2.5 µg/mL, and 2 µM DAPT. On day 4 of differentiation,
553 transfection was performed in n=3 replicate plates using lipofectamine RNAiMax
554 (ThermoFisher Scientific) with a final concentration of miRNA mimic and inhibitors (miR-19b-
555 3p and miR-1505p mimics and inhibitors from Qiagen and miR-193a-3p mimic and inhibitor
556 from ThermoFisher Scientific) in the range of 5 to 10 nM as per the manufactures' guidelines
557 along with their corresponding controls. Next day (day 5 of differentiation), the complete
558 media was changed with N2B27 media supplemented with dox, 10 ng/mL brain-derived
559 neurotrophic factor (BDNF), 10 ng/mL glial cell-derived neurotrophic factor (GDNF), 10
560 ng/mL neurotrophic factor 3 (NT-3), 1 µg/mL Laminin, and 10 µM DAPT. Thereafter, half
561 media was changed on day 8 of differentiation. On day 11, cells were gently washed with
562 PBS and processed for RNA isolation.

563

564 *hIPS-derived microglia and miRNA mimics and inhibitors transfection*

565 hiPSCs were differentiated as previously described (van Wilgenburg et al[54]). In brief, 3 x
566 10^6 iPSCs were seeded into an Aggrewell 800 well (STEMCELL Technologies) to form
567 embryoid bodies (EBs), in mTeSR1 and fed daily with medium plus 50ng/ml BMP4 (Miltenyi
568 Biotec), 50ng/ml VEGF (Miltenyi Biotec), and 20ng/ml SCF (R&D Systems). Four-day EBs
569 were then differentiated in 6-well plates (15 EBs/well) in X-VIVO15 (Lonza) supplemented

with 100ng/ml M-CSF (Miltenyi Biotec), 25ng/ml IL-3 (Miltenyi Biotec), 2mM Glutamax (Invitrogen Life Technologies), and 0.055mM beta-mercaptoethanol (Thermo Fisher Scientific), with fresh medium added weekly. Microglial precursors emerging in the supernatant after approximately 1 month were collected and isolated through a 40um cell strainer and plated in N2B27 media supplemented with 100 ng/ml M-CSF, 25 ng/ml interleukin 34 (IL-34) for differentiation. Thereafter, the media is reshred every 2 days supplemented with 100 ng/ml M-CSF, and 25 ng/ml IL-34. The cells were cultured for additional 6 days with media refresh every 2 days. On day 7 of maturation, transfection was performed in n=3 replicate plates using lipofectamine RNAiMax with a final concentration of miRNA mimics and inhibitors in the range of 5 to 10 nM as per the manufactures' guidelines along with their corresponding controls (miR-19b-3p and miR-1505p mimics and inhibitors from Qiagen and miR-193a-3p mimic and inhibitor from ThermoFisher Scientific). Next day complete media was refreshed. On day 11, cells were gently washed with PBS and processed for RNA isolation.

Genetic analysis

Genomic DNA was isolated from 50 mg of GFM frozen brain tissue by using the Qiamp DNA mini kit (Qiagen) following the manufacturer protocol. DNA concentration and purity were assessed by nanodrop measurement. DNA integrity was evaluated by loading 100 nanogram per sample on a 0,8% agarose gel and comparing size distribution to a size standard.

Presence of C9orf72-HRE in postmortem brain tissues and hIPS cells was confirmed by primed repeat PCR according to established protocols. Reported mutations for MAPT and GRN were verified by sanger sequencing.

Transcriptomic procedures

596 *RNA isolation from human brain tissue*

597 Total RNA for CAGE-seq and RNAseq was isolated from ±100mg of frozen brain tissue with
598 TRIzol reagent (Thermo Fischer Scientific) according to the manufacturer recommendation,
599 followed by purification with the RNeasy mini columns (Qiagen) after DNAse treatment.

600

601 Total RNA for smallRNA-seq was isolated from frozen tissue using the TRIzol reagent
602 (ThermoFischer Scientific). After isopropanol precipitation and 80% ethanol rinsing RNA
603 pellet was resuspended in RNAse free water and up to 10 micrograms of RNA was
604 incubated with 2U of Ambion DNAse I (ThermoFischer) at 37°C for 20 minutes. DNA-free
605 RNA samples were then further purified by phenol-chloroform-isoamyl-alchol extraction
606 followed by ethanol precipitation.

607

608 *RNA isolation from smNPC-derived neurons and microglia*

609 Total RNA was isolated from NGN2 driven neurons and microglia cells after transfection with
610 miRNA mimics and inhibitors. Briefly at day 11 of transfection cells were carefully rinsed with
611 PBS and lysed in Qiazol buffer (Qiagen). Further DNAse treatment and purification were
612 carried out with the miRNeasy micro kit (Qiagen) according to the manufacturer protocol.

613

614 **RNA QC**

615 For each RNA sample, RNA concentration (A_{260}) and purity ($A_{260/280}$ and $A2_{60/230}$) were
616 determined by Nanodrop measurement and RNA integrity (RIN) was assessed on a
617 Bioanalyser 2100 system and/or Tape station 41200 (Agilent Technologies Inc.)

618

619 *CAGE-seq libraries*

CAGE-seq libraries were prepared from 5 micrograms of RNA from frozen brain tissues according to a published protocol[55]. Libraries were sequenced on a HiSeq 2000 and/or HiSeq2500 on a 1x50 bp single read (SR) flow cell (Illumina) at an average of 20M reads/sample.

RNAseq libraries

Total RNAseq libraries were prepared from 1 microgram of total RNA from frozen brain tissue using the TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina) according to the protocol specifications. RNAseq libraries were sequenced on a Hiseq2500 and HISeq4000 on a 2x100 bp paired end (PE) flow cell (Illumina) at an average of 100M PE/sample.

smallRNAseq libraries

Small RNA-seq libraries were prepared from 1 microgram of total RNA from NPC-derived neurons and 300 nanograms of microglia after miRNA mimics and inhibitors transfection, using the mRNA TrueSeq Stranded kit (Illumina). mRNAseq libraries were sequenced on a NextGen550 on a 75 cycles flow cell (Illumina). Small RNAseq libraries from frozen tissue were prepared starting from 2 micrograms of total RNA using the Nextflex Small RNA-seq kit v3 (Bioo Scientific) and the NEBNext Small RNA library prep set for Illumina (New England Biolabs). Libraries were sequenced on a NextSeq550 on a 75 cycles flow cell.

Methylation assay

643 To assess the methylation status of over 850000 CpG sites in promoter, gene body and
644 enhancer regions we have used the MethylationEPIC bead chip arrays (Illumina).

645 Bisulfite conversion of genomic DNA, genome amplification, hybridization to the beadchips,
646 washing, staining and scanning procedure was performed by Atlas Biolabs (Atlas Biolabs,
647 Berlin, Germany). Cases and controls DNAs were distributed randomly across each array.

648

649 **HumanBase Module Analysis**

650 Functional gene modules were generated using the HumanBase tool at:
651 https://hb.flatironinstitute.org/. We divided DEGs into up- and down-regulated genes as we
652 were looking for active and repressed modules in FTD. Modules were downloaded for further
653 analysis. Cell type enrichment analysis was performed for genes of each modules using
654 EWCE[56] as described further down.

655 **RNA-seq processing and analysis**

656 Raw FastQ files were processed using the RNA-seq pipeline from nf-core (nf-core/rnaseq
657 v1.3) [57], with trimming enabled. Gene quantification was subsequently done using Salmon
658 (v0.14.1)[58] on the trimmed FastQ files. Alignment and mapping were performed against the
659 human genome hg38. DESeq2 (v.1.26.0)[59] was used to perform differential expression
660 analysis. We corrected for the covariates gender and PH-value. Genes were considered
661 differentially expressed when having a Benjamini-Hochberg corrected P-value below 0.05.

662 **Cell type deconvolution and filtering**

663 We performed cell type deconvolution on the RNA-seq data using Scaden[60]. For training we
664 used the human brain training dataset used in the Scaden publication. Each ensembl model
665 was trained for 5000 steps. To filter differentially expressed genes for false positives caused
666 by cell composition bias, we first calculated the correlation of gene expression with cell type
667 fraction. Then, we calculated a cell type specificity score as defined in Skene et al. [56] for

each gene available in the scRNA-seq dataset from Darmanis et al. [61]. We filtered out all genes that had a specificity score of at least 0.5 and a positive correlation of at least 0.4 with the cell type fractions of the most specific cell type. False positive DEGs that are caused by systematic increase or decrease of a specific cell type will show high correlation with the cell type fractions and can thus be identified and removed from the analysis. A specificity score of 0.5 means that half of the total gene expression for a certain gene can be attributed to a single cell type, assuming a uniform cell type composition. The cut-offs for specificity score and correlation were chosen based on an informed decision. Relative changes in cell type composition were quantified by first calculating the average fractions of a cell type for all groups and then calculating the percentual change of cell fractions compared to the average control fractions. This allows to detect relative changes in cell type compositions. Statistical significance between cell type fractions of groups was assessed using a t-test in the R language.

Cell type enrichment analysis

We performed cell type enrichment analysis of genesets using the EWCE R package[56]. Cell type specificity of genes was calculated from the single-cell RNA-seq cortex dataset of Darmanis and colleagues[61]. EWCE analysis was done following instructions from https://github.com/NathanSkene/EWCE.

CAGE-seq processing and analysis

Sequencing adapters and barcodes in CAGE-seq FastQ files were trimmed using Skewer (v.0.1.126)[62]. Sequencing artefacts were removed using TagDust (v1.0)[63]. Processed reads were then aligned against the human genome hg38 using STAR (v.2.4.1)[64]. CAGE detected TSS (CTSS) files were created using CAGEr (v1.10.0)[65]. With CAGEr, we removed the first G nucleotide if it was a mismatch. CTSS were clustered using the 'distclu' method with a maximum distance of 20 bp. For exact commands used we refer to the reader to the scripts used in this pipeline: https://github.com/dznetubingen/cageseq-pipeline-mf.

Transcription factor activity analysis

To identify candidate regulatory transcription factors, we first performed differential expression analysis with all CAGE-seq clusters (see RNA-seq analysis). Then, we extracted the sequence 600 bp up-stream and 300 bp downstream around all detected clusters. We used Homer[66] to look for significant TFBS enrichment in the regions around up- and down-regulated clusters (similar to [67]). TFBS motifs were downloaded from the JASPAR database[68]. When calculating enrichment, we considered all extracted regions that are not part of the set of interest as background. The complete pipeline can be found at https://github.com/KevinMenden/tf-activity. We selected all TFs with significant enrichment (p-value <= 0.001) for either up-regulated or down-regulated CAGE clusters as candidate regulators. We considered genes as potential targets of a TF if a TFBS could be found in their promoter region. As an additional filter, we selected only TFs with evidence for differential expression in the RNA-seq data (adj. P-value < 0.05, not filtered for cell composition).

smRNA-seq processing and analysis

After removing sequencing adapters, all FastQ files were uploaded to OASIS2 [69] for analysis. Subsequent differential expression analysis was performed on the counts yielded from OASIS2, using DESeq2 and correcting for gender and PH-value, as was done for the RNA-seq data. Additionally, we added a batch variable to the design matrix to correct for the two different batches of this dataset. For the target prediction analysis, we first downloaded all targets from mirBase[70]. Then, we correlated the expression of miRNAs with their predicted targets using matching samples from the RNA-seq data. We removed all predicted targets with a correlation above -0.4, thus only considering miRNA-target pairings with high negative correlation.

Methylation data processing and analysis

The Infinium MethylationEPIC BeadChip data was analyzed using the minfi R package[71]. We

721 removed all sites with a detection P-value above 0.01, on sex chromosomes and with single
722 nucleotide polymorphisms (SNPs). Data normalization was done using stratified quantile
723 normalization. Sites with a standard deviation below 0.1 were considered uninformative and
724 filtered out, to increase detection power. Surrogate variable analysis[72] was performed to
725 determine confounding factors. Differential methylation analysis was done using the limma
726 package[73] and controlling for the detected surrogate variables. Sites with a Benjamini-
727 Hochberg [74] adjusted P-value below 0.05 were considered differentially methylated.

728 **Age prediction**

729 We predicted the biological age of donors using the methylation data and the Wenda
730 algorithm[75]. Training data was kindly provided by the authors of Wenda. We subsetted the
731 data for CpG sites found in our data (11,729) sites and performed the prediction as
732 described at https://github.com/PfeiferLabTue/wenda.

733

734 **Analysis of mRNA-seq data from cellular models**

735 This section describes the analysis of mRNA-seq data generated for the miRNA mimic and
736 inhibitor experiments. FastQ files were mapped and gene counts quantified using Salmon
737 and differential expression analysis was performed with DESeq2 (see post-mortem brain
738 RNA-seq analysis). DEGs were examined for pathway enrichment using go:Profiler.

739

740 **Assessment of degeneration**

741 For assessment of neurodegeneration, H&E stained paraffin sections of the frontal and
742 temporal cortex were graded as absent (0), mild (1), moderate (2) and severe (3) based on
743 the presence of spongiosis, neuronal loss and gliosis.

744

Data Availability

All data used in this study and published as phase 1 of the RiMod-FTD resource have been deposited at the European Phenome-genome Archive (EGA) under accession number EGAS00001004895.

Code Availability

The code used for generating the analysis results is made freely available in the GitHub repository https://github.com/dznetubingen/rimod-ftd-paper.

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Acknowledgements

Post-mortem brain tissue was obtained from the Dutch Brain Bank, Netherlands Institute for Neuroscience, Amsterdam, and from the London Neurodegenerative Disease Brain Bank, King's College London, London, UK. The London Neurodegenerative Disease Brain Bank is part of the Brains for Dementia Research Initiative.

Author contributions

The project was initiated and designed by PH. Small RNA-seq experiments were performed by AF, LK, PR and NF. RNA-seq and CAGE-seq experiments were performed by CB, PR, NF, MC. iPSC-derived neuron and microglia experiments were performed by AD and DKV.

Analysis of CAGE-seq data was done by MF, TN and KM. JSS, BA and KM analysed the RNA-seq data. KM analysed the smRNA-seq and methylation data. PH, SB, PR planned and interpreted all performed analyses. KM, PH, PR and SB wrote the manuscript.

Funding

This work was funded in part by the EU Joint Programme - Neurodegenerative Disease Research (JPND) project: Risk and Modifying factors for FTD (RiMod-FTD), NOMIS Foundation and BMBF IDSN. DKV is in receipt of an Alexander von Humboldt Research Fellowship.

RiMod-FTD Partners

Prof. Dr. P. Heutink: Kevin Menden; Margherita Francescatto; Tenzin Nyima; Cornelis Blauwendraat; Burcu Atarsu; Javier Simon Sanchez; Stefan Bonn; Patrizia Rizzu;

German Center for Neurodegenerative Diseases (DZNE)-Tübingen

Otfried Müller Strasse 23

967 72076 Tübingen

968 Germany

969 Tel.: +49 (0)7071 9254 050

970 E-Mail: peter.heutink@dzne.de

971

972 Prof. Dr. J. Hardy:

973 Reta Lila Weston Research Laboratories, Dept. of

974 Molecular Neuroscience, UCL Institute of

975 Neurology, London WC1N 3BG, England.

976 Tel: +44 (0) 207 679 4297.

977 E-Mail: j.hardy@UCL.AC.UK

978

979 Prof. Dr. A. Brice

980 Research Centre of the Brain and Spine Institute

981 (CRicm UMR_S975), Pitié-Salpêtrière Hospital, 47

982 boulevard de l'Hôpital, 75013 Paris, France

983 Tel: +33 1 572746 82

984 E-Mail: alexis.brice@upmc.fr

985

986 Prof. Dr. J.C. van Swieten; Fenne Riemslagh

987 Prof. Dr. A.B. Smit: Pim van Nierop; Suzanne Miedema

988 Dept. Neurology, ErasmusMC, 's-Gravendijkwal 230

989 3015 CE Rotterdam, Netherlands.

990 Tel: +31 107033274

991 E-Mail: j.c.vanswieten@erasmusmc.nl

992

993 Prof. Dr. P. Longone/

994 Prof. Dr. E. Buratti/

995 Prof. Dr. A. Alberici

996 Molecular Neurobiology Unit

997 Santa Lucia Foundation

998 Via del Fosso di Fiorano 64

999 00179 Roma Italia

1000 Tel. +39-06501703151

1001 E-Mail: p.longone@hsnatalucia.it

1002

1003 Prof. Dr. P. van Damme

1004 Laboratory for Neurobiology (Vesalius Research

1005 Center) O&N IV Herestraat 49 - bus 912.

1006 3000 Leuven, Belgium

1007 Tel. +32 16 3 73181

1008 E-Mail: philip.vandamme@vib-kuleuven.be

1009

1010 Dr. A. Sandelin

1011 Department of Biology, Copenhagen University,

1012 Ole Maaoles vej 5,

1013 DK2200 Copenhagen, Denmark.

1014 Tel +45 35321285/ Email: albin@binf.ku.dk

1015

1016 Prof. Dr. A. Fischer

1017 Dept. of Psychiatry and Psychotherapy, University

1018 of Göttingen, German Center for Neurodegenerative

1019 Diseases (DZNE) Göttingen, Grisebach Str. 5,

1020 37077 Göttingen, Germany.

1021 Tel: +49 551 30178.

1022 E-Mail: afische2@gwdg.de

1023

1024 Prof. Dr. S. Lichtenthaler

1025 Technical University Munich (TUM) and German

1026 Center for Neurodegenerative Diseases (DZNE),

1027 Max-Lebsche Platz 30, 81377 Munich, Germany.

1028 Tel: +49 89 7095 8405

1029 E-Mail: stefanlichtethaler@dzne.de

Prof. Dr. E. Mandelkow + Prof. Dr. E. Mandelkow + Marta Anglada

German Center for Neurodegenerative Diseases

(DZNE), Ludwig-Erhard-Allee 2 53175 Bonn,

Germany.

Tel: +49 (0) 228 / 43302-630

E-Mail: eckhard.mandelkow@dzne.de

Competing Interests

The authors declare no competing interests.