Non-Coding Genetic Analysis Implicates Interleukin 18 Receptor Accessory Protein 3'UTR in Amyotrophic Lateral Sclerosis

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Abstract:

The non-coding genome is substantially larger than the protein-coding genome but is largely unexplored by genetic association studies. Here, we performed region-based burden analysis of >25,000 variants in untranslated regions of 6,139 amyotrophic lateral sclerosis (ALS) whole-genomes and 70,403 non-ALS controls. We identified Interleukin-18 Receptor Accessory Protein (IL18RAP) 3'UTR variants significantly enriched in non-ALS genomes, replicated in an independent cohort, and associated with a five-fold reduced risk of developing ALS. Variant IL18RAP 3'UTR reduces mRNA stability and the binding of RNA-binding proteins. Variant IL18RAP 3'UTR further dampens neurotoxicity of human iPSC-derived C9orf72-ALS microglia that depends on NF-κB signaling. Therefore, the variant IL18RAP 3'UTR provides survival advantage for motor neurons co-cultured with C9-ALS microglia. The study reveals direct genetic evidence and therapeutic targets for neuro-inflammation, and emphasizes the importance of non-coding genetic association studies.

One Sentence Summary: Non-coding genetic variants in IL-18 receptor 3'UTR decrease ALS risk by modifying IL-18-NF-κB signaling in microglia.

[Main Text:]

Introduction

Genomic sequencing technologies facilitate the identification of variants in open reading frames (ORFs). Although allelic variants in non-coding regions are expected to be numerous [1, 2] they are largely overlooked.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative syndrome, primarily affecting the human motor neuron system with a strong genetic predisposing component [3, 4]. Thus far, mutations in approximately 25 protein-coding genes have been associated with ALS [3, 5-7]. Hexanucleotide repeat expansion in an intronic sequence of the *C9orf72* gene is the most common genetic cause of ALS [8-10] and enrichment of variants was recently discovered in the CAV1 enhancer [11]. However, non-coding nucleotide variants in ALS have yet to be systematically explored.

Burden analysis is a genetics approach that is based on the rationale that different rare variants in the same gene may have a cumulative contribution [12]. Therefore, burden analysis allows the identification of genes containing an excess of rare and presumably functional variation in cases relative to controls. Although de novo mutations in non-coding regions were recently shown in family-based autism studies [13], variants in non-coding regions are not routinely included in rare-variant burden association studies. The application of burden analysis to non-coding regulatory variation is constrained by the availability of whole-genome sequencing (WGS) data, and the ability to recognize functional variants in non-coding regulatory regions, which is currently far less effective than for protein-coding genes.

MicroRNAs (miRNAs) are endogenous posttranscriptional repressors that silence mRNA expression through sequence complementarity. miRNA primarily act on 3' untranslated regions (3'UTRs) [14], which are non-coding parts of messenger RNAs (mRNAs) and often regulate degradation and translation [15]. miRNA dysregulation has been implicated in ALS pathogenesis, and ALS-associated RNA-binding proteins, TARDBP/TDP-43 and FUS, regulate miRNA biogenesis [16-27].

Microglia are the resident immune cells of the central nervous system and are the primary mediators of neuroinflammation in neurodegeneration [28, 29]. In ALS, microglia induce motor neuron death via the classical nuclear factor-κappa B (NF-κB) pathway [29-35]. One suggested mechanism for microglia-induced cytotoxicity is based on detection of extracellular TDP-43 aggregates and triggering of IL-1beta and Interleukin 18 (IL-18; also known as: IGIF/IL1F4/IL-1g) signaling [31]. Accordingly, IL-18 levels are elevated in ALS patient tissues and biofluids [36-39], supporting a role for IL-18 signaling in the disease's neuroinflammatory milieu [40]. IL-18 also induces NF-κB signaling by binding and dimerising the two IL-18 receptor subunits, IL18RAP (also known as: AcPL/CD218b/IL-

30 18R-Beta) and IL18R1 [31, 40-48]. In turn, NF-κB contributes to microglial inflammation [49, 50], microglial-mediated motor
31 neuron death [51] and to disease progression [52, 53].

32 Here, we identified rare variants in miRNAs and 3'UTR of mRNAs, and performed collapsed genetic analysis [54] to
33 test if these regulatory RNAs are associated with ALS. We discovered an enrichment of rare variants in the IL18RAP
34 3'UTR and provide experimental evidence for their relevance to human ALS. Therefore, non-coding variant
35 analysis reveals a genetic and mechanistic link for the IL-18 pathway in ALS and encourages systematic exploration
36 of non-coding regions to uncover genetic mechanisms of disease.

37 **Results**

38 To test whether genetic variations in non-coding regulatory regions are associated with ALS, we analyzed regions
39 of interest in WGS data from the Project MinE ALS sequencing consortium [55] (Supplementary Fig. 1A,B and
40 Supplementary Tables 1,2). The discovery cohort consisted of 3,955 ALS patients and 1,819 age- and sex-matched
41 controls, for a total of 5,774 whole-genomes from the Netherlands, Belgium, Ireland, Spain, United Kingdom,
42 United States, and Turkey (Project MinE Datafreeze 1). We performed a region-based burden test, in which rare
43 genetic variants with minor allele frequencies (MAF) ≤0.01 are binned together, to weight their contribution to
44 disease, in 295 non-coding 3'UTRs of candidate genes, linked to sporadic ALS via GWAS [56] or genes encoding RNA-
45 binding proteins (Supplementary Table 3). In addition we tested all autosomal human-pre-miRNA genes (1,750
46 pre-miRNAs; miRBase v20 [57]).

47 As a positive control, we also performed an association analysis of rare variants in the open reading frames of
48 these 295 genes. For the proteins, we called variants that are predicted to cause frameshifting, alternative splicing,
49 an abnormal stop codon, or a deleterious non-synonymous amino acid substitution that were detected in ≥ 3 of 7
50 independent dbNSFP prediction algorithms [58] (Fig. 1A and Supplementary Table 3). In total, 30,721 rare qualifying
51 variants were identified (Supplementary Table 4). Optimized Sequence Kernel Association Test (SKAT-O) [59]
52 identified a significant excess of deleterious minor alleles in the ALS genes *NEK1* (127 cases; 19 controls [3.21%;
53 1.04%]: $P = 8x10^{-7}$; $P_{corrected} = 2.3x10^{-4}$), comparable with a reported prevalence of 3% [60], and in *SOD1* (36 cases
54 [0.91%]; 0 controls: $P = 2.6x10^{-4}$; $P_{corrected} = 3.73x10^{-2}$) [61], which is below the reported 2% prevalence [5, 62] (Fig. 1B,
55 Supplementary Fig. 2A and Supplementary Table 5). Other known ALS genes did not reach statistical significance
56 (Supplementary Table 3), consistent with reported statistical power limitations of Project MinE WGS data in
57 assessing the burden of rare variants [63]. Our analysis did not consider the *C9orf72* hexanucleotide (GGGGCC)
58 repeat expansion region.

59 The burden of rare variants did not identify a disease association for any of the autosomal pre-miRNAs in the
60 human genome, nor for any of the predicted genetic networks based on variants aggregated over specific mature
61 miRNAs and their cognate downstream 3'UTR targets. This may be because the small size of miRNA genes makes
62 genetic aggregation studies particularly challenging (Supplementary Fig. 2B,C).

63 When we tested the burden of variants in 3'UTRs, the strongest association found was for the 3'UTR of IL18RAP
64 (Fig. 1B, Supplementary Fig. 2D and Supplementary Table 5). This association was higher than expected at random
65 ($P = 1.93x10^{-5}$, $P_{corrected} = 5.41x10^{-3}$) and from the association gained for all protein-coding ALS genes in this cohort,

with the exception of *NEK1*. Notably, the signal was more prevalent in controls [12/1819, 0.66%] relative to ALS patients [6/3955, 0.15%], indicating that these variants might act as protective variants against ALS.

IL18RAP 3'UTR also ranked as the top hit by three other algorithms – the Sequence Kernel Association Test (SKAT, $P = 1.77x10^{-5}$; permutated P-value < 10^{-4}), the Combined Multivariate and Collapsing (CMC, $P = 8.78x10^{-4}$) or Variable Threshold (VT) with permutation analysis (permutated P-value = $1.75x10^{-3}$, suggesting that the association does not depend on a particular statistical genetics method (Supplementary Fig. 3A-C). Furthermore, when we tested the burden of variants in miRNA recognition elements (MREs) in 3'UTRs (variants that are potentially either abrogating conserved miRNA binding sites or creating new miRNA binding sites in 3'UTRs), the strongest association was also gained for the 3'UTR of IL18RAP (SKAT-O P-value = $3.42x10^{-5}$, Supplementary Fig. 3D, see Methods). A diagram of variants in IL18RAP 3'UTR is presented in Supplementary Fig. 3E and a description of IL18RAP 3'UTR variants in Supplementary Table 6. The top 10 principal components (PCs) of common variant-based ancestry information and sex were included as covariates in the SKAT-O, SKAT, CMC, and VT analyses.

In addition, genome-wide analysis of all known human 3'UTRs (16,809 3'UTRs from RefSeq [64]) identified IL18RAP 3'UTR as the most significant 3'UTR associated with ALS in the Project MinE cohort (Fig. 1C).

Finally, we tested if different functional genetic classes were enriched overall for ALS risk/protection variants by testing the burden of rare variants in all genes pooled together. SKAT-O signal for open reading frames of 295 proteins, the 3'UTR of the same 295 genes, all autosomal pre-miRNA genes [miRBase v20; [57]] or networks composed of all miRNA genes and their cognate set of downstream targets (TargetScan) were all not significant (P-values of 0.024, 0.59, 0.08, 0.58, respectively). Therefore, results from these burden tests do not implicate any of the functional class of genomic elements in ALS risk.

Because the number of ALS genomes was ~2.17-fold larger than the number of controls, the data depict a 4.35-fold enrichment in the abundance of variants in controls over cases. IL18RAP 3'UTR potentially-protective variants reduced the disease odds ratio by five-fold (OR = 0.23; Fig. 2A), and was consistent across independent population strata (Fig. 2B), whereas *NEK1* and *SOD1* increased the disease odds ratio (OR = 3.14, 33.89, respectively; Fig. 2A).

To determine if the rare IL18RAP 3'UTR variants are depleted in another ALS cohort, we performed independent replication studies. Similar results for rare IL18RAP 3'UTR variants were reproduced in the New York Genome Center (NYGC) ALS Consortium cohort (2,184 ALS genomes), which was studied against: (i) 263 non-neurological controls from the NYGC; (ii) 62,784 non-ALS genomes from NHLBI's Trans-Omics for Precision Medicine (TOPMed); and (iii) 5,537 non-ALS genomes from gnomAD. This replication effort yielded a joint analysis P-value = $9.58x10^{-4}$ ($\chi2$ with Yate's correction; OR=0.32; 95% CI: 0.16 – 0.64; Fig. 2C and Supplementary Table 7). Combining this

96 cohort with our discovery cohort from Project MinE, yielded a superior joint P-value < 1.00×10^{-5} ($\chi 2$ with Yate's
97 correction; OR=0.20; 95% CI: 0.12 – 0.34; Fig. 2C). A meta-analysis of Project MinE datafreeze 1 and 2 [7], which
98 consisted of 5,185 ALS patients and 2,262 age- and sex-matched controls, reproduced the initial signal (P-value =
99 7.6×10^{-4}).

100 Together, IL18RAP 3'UTR sequence variants are associated with a lower risk of suffering from ALS, which is
101 approximately one-fifth of the general population, although it did not reach conventional exome-wide multiplicity-
102 adjusted significance threshold ($\alpha \approx 2.6 \times 10^{-6}$, ref. [12]) in our study.

103 To investigate the source of the signal in the IL18RAP 3'UTR in a posthoc analysis, we divided the 11 variant
104 nucleotides into two synthetic sets, of either nine singleton variants (9 variants / 3 controls / 6 patients) or two
105 variants that were identified solely in controls (2 variants / 9 controls / 0 patients). While the signal of the nine
106 singleton variants was not statistically significant, analysis of the two control variants, which were identified in
107 multiple samples, derived an improved significance compared to the original signal (SKAT-O P-value = 4.36×10^{-6}).
108 Thus, these two rare variants (V1, Chr2:103068691 C>T; V3, Chr2:103068718 G>A) are likely central in generating
109 the genetic association signal in IL18RAP 3'UTR.

110 Because of the enrichment of V1 and V3 at the proximal (5') side of the IL18RAP 3'UTR, we tested if restricting
111 burden analysis to the 5' end of the 3'UTR, might boost the association signal. However, the P-values gained from
112 the 3'UTRs proximal quadrant were comparable to that of the full 3'UTRs in the cohort of 295 3'UTRs (Wilcoxon
113 matched-pairs P-value > 0.05, Cohen's d effect size = 0.1, Supplementary Fig. 4A,B), suggesting that the apparent
114 spatial distribution of variants in the case of IL18RAP 3'UTR is a particular case rather than part of a global pattern.

115 To determine the functional impact of the IL18RAP 3'UTR variants we analyzed IL18RAP expression in
116 lymphoblastoid cell lines (LCLs) from the UK MNDA DNA Bank [65] that were derived from twelve different
117 individuals: 4 healthy individuals (without ALS), carrying the canonical IL18RAP 3'UTR sequence (Control;
118 Canonical IL18RAP 3'UTR); 4 sporadic ALS patients, carrying the canonical IL18RAP 3'UTR sequence (sALS;
119 Canonical IL18RAP 3'UTR); two healthy individuals, carrying a variant form of IL18RAP 3'UTR (Control;
120 VariantIL18RAP 3'UTR) and two sporadic ALS patients carrying a variant form of IL18RAP 3'UTR (sALS;
121 VariantIL18RAP 3'UTR; see Supplementary Table 8 for list of variants).

122 ALS-derived LCLs carrying the canonical IL18RAP 3'UTR sequence expressed higher levels of IL18RAP (Fig. 3A,B).
123 In addition, LCLs from both healthy and ALS individuals harboring the IL18RAP 3'UTR variant significantly down-
124 regulated IL18RAP mRNA and protein expression (Fig. 3A,B and Data File S1). Phosphorylation of the nuclear factor
125 kappa-light-chain-enhancer of activated B cells (p-NF-κB), an established intracellular effector downstream of IL-

126 18 signaling, was similarly higher in the ALS LCLs with canonical IL18RAP 3'UTR and also significantly reduced in
127 control and ALS LCLs harboring IL18RAP variants (Fig. 3C,D and Data File S1). Consistent results were obtained
128 with C9orf72 hexanucleotide expansion ALS LCLs (Supplementary Fig. 5 and Data File S2). Accordingly, variants of
129 IL18RAP 3'UTR reduced NF-κB activity, relative to the canonical 3'UTR in an NF-κB reporter assay in U2OS cells
130 (Supplementary Fig. 6). Therefore, variant forms of IL18RAP 3'UTR correlate with reduced expression of the
131 endogenous IL18RAP and reduced NF-κB signaling.

132 To further establish the functional relevance of the IL18RAP 3'UTR variants, we edited the genome of human-
133 induced pluripotent cells (iPSCs) donated by ALS patients with C9orf72 repeat expansion ([66] NINDS/Coriell Code:
134 ND10689, ND12099, see Supplementary Table 8) to include two point mutations that recapitulate the most
135 prevalent variants (Chr2:103068691 C>T (V1) and Chr2:103068718 G>A (V3)) in the IL18RAP 3'UTR sequence (Fig.
136 4A). The resulting isogenic pair lines all carry C9orf72 repeat expansion and vary by only the presence of the
137 canonical or a variant IL18RAP 3'UTR.

138 We explored the receptive cell type involved in IL-18 receptor signaling by profiling dissociated mouse brain cells,
139 namely, neurons, microglia, and astrocytes. Fluorescence cytometric gating on CD11b+ and CD45+ and IL18RAP
140 (CD218b) revealed that IL18RAP is mainly expressed on microglia cells (Supplementary Fig. 7A-C). Although IL-18
141 and IL18RAP expression increases in ALS motor neurons (Supplementary Fig. 8A-C), our observations are
142 consistent with the accepted notion that the role of IL-18 and other cytokines in disease heavily rests on a chronic
143 inflammatory state established particularly by microglia [67].

144 Therefore, we next differentiated the isogenic IL18RAP 3'UTR lines into human microglia following the protocol
145 of Haenseler et al. [68] (Fig. 4A). iPSC-derived microglia differentiation was validated by immunofluorescence
146 staining of the microglial-specific marker, TMEM119 (Supplementary Fig. 9). In differentiated human microglia,
147 we detected a ~5-6 fold downregulation in the levels of the variant IL18RAP protein, as well as in the levels of the
148 IL18RAP mRNA, relative to the canonical sequence of the isogenic line (Fig. 4B,C and Data File S3). Therefore, the
149 variants at the 3'UTR regulate IL18RAP mRNA and protein expression and provide a conceivable explanation for
150 the variant function in human C9-ALS microglia. Next, we investigated the molecular mechanism that controls the
151 IL18RAP mRNA levels by performing an mRNA stability assay in human microglia. We measured an mRNA
152 degradation rate that is twice as fast with the rare 3'UTR variants, relative to the canonical sequence, after
153 inhibition of mRNA transcription by actinomycin D (Fig. 4D). Thus, the mechanism for reduced IL18RAP mRNA
154 levels is associated with destabilization of IL18RAP mRNA via variants in the 3'UTR.

155 We sought the potential trans-acting factors that might differentially bind to the canonical and variant 3'UTRs. To
156 this end, we performed RNA-pulldown assays and mass spectrometry on *in vitro* transcribed canonical and variant
157 forms of the IL18RAP 3'UTRs, V1 and V3 (Fig. 5A diagram of exp. design). Mass spectrometry after pull-down
158 identified 552 proteins with good confidence (passed all QC filters, found in 50% of the repeats in at least one
159 experimental group, and were represented by at least 2 unique peptides, Supplementary Table 9), that were
160 enriched in comparison to the negative control. Principal component analysis demonstrated a clear separation of
161 proteomes bound by the canonical and variant IL18RAP 3'UTRs (Fig. 5B and Supplementary Table 9). Gene set
162 enrichment analysis (GSEA) revealed a reduction in the association of double-stranded RNA (dsRNA) binding
163 proteins, to V1 IL18RAP 3'UTR, relative to the canonical 3'UTR (ELAVL1/Hur; PRKRA, EIF2AK2/PKR; ADAR; ADARB1;
164 ILF2; ILF3; DHX9; DHX58; DDX58, Fig. 5C,D,E and Supplementary Table 10). These dsRNA binding proteins were
165 reported in other contexts to play roles in controlling the stability of mRNA [69-75], consistent with the observed
166 changes to IL18RAP mRNA stability. A similar analysis of the V3 variant was unproductive (Supplementary Fig.
167 10A).

168 In accordance, RNA Fold analysis predicted that the canonical 3'UTR sequence consists of a more stable dsRNA
169 structure than the V1 variant sequence (minimum free energy (MFE) of canonical and variant IL18RAP 3'UTR, -
170 39.9 kcal/mol and -27.8 kcal/mol, respectively) (Fig. 5F and Supplementary Fig. 10B). In light of these results, we
171 propose that variant-dependent changes to the secondary structure of IL18RAP 3'UTR attenuate the binding of
172 one or more of the dsRNA proteins and may be involved in controlling the stability of IL18RAP mRNA.

173 To study the potential protective impact of IL18RAP 3'UTR mutations, we performed survival analyses in a
174 coculture system of human iPSC-derived isogenic IL18RAP 3'UTR microglia (on a *C9orf72 repeat expansion*
175 *background)* with human iPSC-derived lower motor neurons (i^3LMNs; healthy, non-ALS, [76]). Time-lapse microscopy
176 was used to quantify motor neuron survival after microglia activation with a cocktail of LPS and the cytokine IL-18
177 (experimental design, Fig. 6A). Motor neuron survival was significantly improved in the presence of microglia
178 harboring the IL18RAP 3'UTR variants relative to microglia harboring the canonical IL18RAP 3'UTR (two
179 independent isogenic pairs, based on independent patient C9orf72 lines, n=3 independent differentiation
180 procedures from different passages per line, with 3-8 co-culture wells per passage; Fig. 6B-D, Supplementary
181 movie and Data File S4). Based on these studies, we conclude that rare variants of IL18RAP 3'UTR increase *C9orf72*
182 microglia-dependent motor neuron survival and hence convey a protective property.

183 To determine whether the mutant IL18RAP 3'UTR is also protective in human patients with ALS, we tested the
184 association between age of diagnosis and age of death in ALS patients harboring canonical or variants of the
185 IL18RAP 3'UTR. Of 4216 patients for whom data on the age of diagnosis was available (Project MinE and NYGC

186 cohorts), 8 harbored IL18RAP 3'UTR variants. Of 4263 patients for whom the age of death was available, 9
187 harbored IL18RAP 3'UTR variants. IL18RAP 3'UTR variants are expected to be depleted in ALS genomes,
188 nonetheless, in those extremely rare patients harboring IL18RAP 3'UTR variants, these were associated with an
189 older age of death and an older age of diagnosis. On average, the age of death was higher by 6.1 years after the
190 average for patients with canonical Il18RAP 3'UTR (Permutation P-value = 0.02, Cohen's d effect size = 0.65; Fig.
191 6E and Supplementary Table 11), and the age of diagnosis was higher by 6.2 years after the average for patients
192 with canonical IL18RAP 3'UTR (Permutation P-value = 0.05, Cohen's d effect size = 0.62; Fig.6F and Supplementary
193 Table 11). Thus, variants in IL18RAP 3'UTR are protective against ALS in a tissue culture model and correlate with
194 survival advantage for patients suffering from the disease.

195 To study the role of NF-κB signaling in our system, we analyzed NF-κB phosphorylation and the impact on the
196 transcriptome after microglia activation (Fig. 7A). Western blot analysis revealed reduced levels of phospho-NF-
197 κB in variant IL18RAP 3'UTR relative to isogenic control (Fig. 7B and Data File S5). Reduced phosphorylation is
198 associated with decreased nuclear localization and transcriptional activity of NF-κB [77-80]. In parallel, we conducted
199 a next-generation sequencing study (Supplementary Table 12, Gene Expression Omnibus accession number:
200 GSE186757) of the differentially expressed transcriptomes in microglia harboring variant vs. canonical IL18RAP
201 3'UTR. Over-representation analysis (ORA) of differentially expressed genes (DEGs) revealed downregulation of
202 the NF-κB signaling pathway in microglia harboring the variant IL18RAP 3'UTR (KEGG Pathway enrichment results:
203 Ratio = 3.77, FDR P-value = 7.34×10^{-6}; Gene Ontology Biological Process enrichment results: Ratio = 3.48, FDR P-
204 value = 3.70×10^{-12}, Fig. 7C,D and Supplementary Table 13). In addition, an unsupervised study of NF-κB pathway
205 mRNAs (GO:0007249) demonstrated broad downregulation of pathway-associated mRNAs in microglia with the
206 variant IL18RAP 3'UTR, relative to the isogenic control (Fig. 7E). Therefore, microglia's NF-κB transcriptomic
207 signature depends on signaling via the IL-18 receptor and is attenuated by protective IL18RAP 3'UTR variants.

208 To test a plausible neurotoxic role for NF-κB downstream of the IL-18 receptor in this system, we next performed
209 a co-culture survival assay with or without IKK16, a selective IκB kinase (IKK) inhibitor that inhibits NF-κB signaling
210 [81]. In human microglia with the canonical IL18RAP 3'UTR, IKK16 significantly ameliorated motor neuron toxicity,
211 relative to control (carrier alone, Fig. 7F). However, in human microglia with the protective variant IL18RAP 3'UTR,
212 inhibition of NF-κB had no effect (two independent isogenic pairs, based on independent patient C9orf72 lines
213 with 3-8 co-culture wells per line, Fig. 7F). This suggests that NF-κB neurotoxic function resides epistatically
214 downstream of IL18RAP in human microglia. Together, rare variants in IL18RAP 3'UTR diminish NF-κB signaling,
215 thus increasing *C9orf72* microglia-dependent motor neuron survival.

216

217 **Discussion**

218 Data from the Project MinE and NYGC ALS consortia provide unprecedented opportunities for investigating the
219 role of the non-coding genome in ALS. By performing rare variant aggregation analysis in regulatory non-coding
220 regions, we demonstrate that variants in the 3'UTR of IL18RAP are enriched in non-ALS genomes, indicating that
221 these are relatively depleted in ALS. IL18RAP 3'UTR variants reduced the chance of developing ALS five-fold, and
222 delayed onset and therefore age of death in people with ALS.
223 These protective variants recall other protective variants that have been reported in the past in protein-coding
224 regions in Alzheimer's disease [82-85] and implicated in ALS as well [86, 87]. In addition, deleterious variants were
225 suggested in VEGF promoter/5'UTR and within CAV1/CAV2 enhancers [11, 88]. However, the 3'UTR of IL18RAP is a
226 protective non-coding allele associated with a neurodegenerative disease.
227
228 Elevated levels of the cytokine IL-18 were reported in tissues and biofluids of ALS patients [36-38]. Accordingly, we
229 reveal the upregulation of endogenous IL18RAP in sporadic and C9orf72 lymphoblastoid cells. In addition, we
230 demonstrate the downregulation of IL18RAP expression in lymphoblastoid cells harboring variant versions of the
231 IL18RAP 3'UTR.
232
233 We elucidated the regulatory changes affected by the IL18RAP 3'UTR variants by showing destabilization of the
234 IL18RAP mRNA and downregulation of IL18RAP mRNA levels. Sequence analysis suggests that at least one variant
235 (V1) potentially reduced the propensity of the 3'UTR to form a double-stranded secondary structure. Accordingly,
236 unbiased proteomics demonstrated that the 3'UTR harboring the variant fails to bind dsRNA binding proteins that
237 are known to stabilize mRNAs. Together, this supports a mechanism for reduced IL18RAP signaling involving
238 changes to mRNA stability and differential binding of stabilizing RNA-binding proteins.
239
240 Neuro-inflammation is prevalent in neurodegeneration, including in ALS [89], and is often characterized by the
241 activation of microglia [29-35]. The cytokine, IL-18, is part of this neuro-inflammatory milieu, promoting receptor
242 subunit (IL18RAP, IL18R1) dimerization on the membrane of cells [40], and activating intracellular signaling cascades,
243 including NF-κB.
244
245 By CRISPR editing of two independent C9orf72 lines, from one female and one male patient, we created isogonic
246 IL18RAP 3'UTR cell lines (canonical or harboring V1 and V3 variants), at the endogenous gene locus. By
247 differentiating these lines to human microglia, we demonstrated that variants downregulated IL18RAP mRNA and

248 protein expression. In addition, tracking of human (wild-type) motor neuron survival, in co-culture with microglia,
249 over 21 days, demonstrated the neuroprotective effect of microglia carrying the variant IL18RAP 3'UTR.
250
251 Finally, we demonstrate that variant IL18RAP 3'UTR attenuates NF-κB signaling in lymphoblastoid cells and in
252 microglia. Unbiased next-generation sequencing of microglia RNA demonstrated broad transcriptomic changes,
253 typical of reduced NF-κB signaling. In addition, inhibition of NF-κB was able to ameliorate motor neuron death
254 when co-cultured with microglia harboring the canonical IL18RAP 3'UTR. However, inhibition of NF-κB was not
255 further protective if microglia with variant IL18RAP 3'UTR were present, suggesting an epistatic relationship,
256 whereby IL18RAP is upstream of NF-κB in this system. We conclude that IL18RAP acts in microglia and controls
257 the cytotoxicity conveyed to motor neurons, at least in human C9orf72 types of disease.
258
259 The discovery of functional, disease-modifying IL18RAP 3'UTR variants underscores the need to explore the role
260 of additional non-coding genomic regions in ALS. One limitation of our study is that IL18RAP 3'UTR signal did not
261 reach the conventional exome-wide multiplicity-adjusted significance threshold ($\alpha \approx 2.6 \times 10^{-6}$, ref. [12]). However,
262 IL18RAP 3'UTR signal is comparable to that of protein-coding ALS-causing genes, such as *SOD1* and *NEK1.*
263 Furthermore, the key findings were reproduced in a genome-wide study of all human 3'UTRs and in an
264 independent replication study. Limitations in the statistical power might have prevented the discovery of other
265 non-coding variants and may be overcome with larger ALS and control cohorts, which are not currently available.
266 Additionally, we have focused our tissue culture studies on human C9orf72 microglia. Therefore, the involvement
267 of IL18RAP 3'UTR in other ALS-associated genetic backgrounds remains to be experimentally explored, as is the
268 relevance to other neurodegenerative diseases. Finally, the mechanism underlying IL18RAP dose sensitivity is not
269 fully understood. While we provide evidence that variant IL18RAP 3'UTR endows neuroprotection via dampening
270 of microglia-dependent neurotoxicity, additional studies should explore the degree to which other cell types, such
271 as motor neurons and astroglia, are involved.
272
273 In summary, we have identified the IL18RAP 3'UTR as a non-coding genetic disease modifier by burden analysis of
274 WGS data using ALS case-control cohorts. We show that IL-18 signaling modifies ALS susceptibility and
275 progression, delineating a neuro-protective pathway and identifying potential therapeutic targets for ALS.
276 Whereas the 3'UTR of IL18RAP is a protective non-coding allele associated with a neurodegenerative disease, the
277 increasing wealth of WGS data in Project MinE, NYGC and elsewhere, indicates that the exploration of non-coding
278 regulatory genomic regions should reveal further disease-relevant genetic mechanisms.

279 **Methods**

280

281 **Human genetic cohorts**

282 All participants contributed DNA after signing informed consent at the submitting sites. Human materials were
283 studied under approval of the Weizmann Institute of Science Institutional Review Board (Weizmann IRB: 1039-1).

284 Discovery cohort: Project MinE ALS sequencing consortium Datafreeze 1 includes 3,955 ALS patients and 1,819
285 age- and sex-matched controls, free of any neurodegenerative disease, for a total of 5,774 quality control (QC)
286 passing whole-genomes, from the Netherlands, Belgium, Ireland, Spain, United Kingdom, United States, and
287 Turkey. Rare variant association in cases versus controls was evaluated for regions of interest, when we could
288 identify ≥2 variants per region, by SKAT-O, SKAT, CMC, and VT in RVTESTS environment [90], with sex and the top 10
289 principal components (PCs) as covariates. To construct the PCs of the population structure, an independent set of
290 ~450,000 SNPs was sampled from WGS, (MAF ≥0.5%) followed by LD-pruning. Rare genetic variants were included
291 based on minor allele frequencies (MAF) ≤0.01 within the controls in the current data set.

292 Replication cohorts: Utilized for testing rare variant alleles (MAF < 0.01) in human IL18RAP 3'UTR (GRCh37/hg19
293 chr2:103068641-103069025 or GRCh38 chr2:102452181-102452565) from Project MinE datafreeze 2: ~1300
294 European heritage ALS genomes without middle eastern (Turkish and Israelis) genomes. The New York Genome
295 Center (NYGC) ALS Consortium (2,184 ALS Spectrum MND and 263 non-neurological control genomes from
296 European/Americas ancestries), NHLBI's Trans-Omics for Precision Medicine (TOPMed; 62,784 non-ALS genomes)
297 and gnomAD (5,537 non-ALS genomes; Europeans, non-Finnish, non-TOPMed). Joint analysis in replication cohort
298 was performed by Chi-square test with Yate's correction. Meta-analysis was not possible because TOPMed and
299 gnomAD covariate information is not available.

300 **Quality control procedures in Project MinE genomics**

301 Sample selection, data merging, and sample- and variant level quality control procedures for Project MinE ALS
302 sequencing consortium genomes are described in full previously [63]. Briefly, 6,579 Project MinE ALS sequencing
303 consortium whole genomes were sequenced on Illumina HiSeq2000 or HiSeqX platforms. Reads were aligned to
304 human genome build hg19 and sequence variants called with Isaac Genome Alignment Software and variant caller
305 [91]. Individual genomic variant call format files (GVCFs) were merged with 'agg' tool: a utility for
306 aggregating Illumina-style GVCFs. Following completion of the raw data merge, multiple QC filtering steps were
307 performed: (i) setting genotypes with GQ<10 to missing; (ii) removing low-quality sites (QUAL< 30 and QUAL< 20

308 for SNPs and indels, respectively); (iii) removing sites with missingness > 10%. (iv) Samples excluded if deviated
309 from mean by more than 6SD for total numbers of SNPs, singletons and indels, Ti/Tv ratio, het/hom-non-ref ratio,
310 and inbreeding (by cohort). (v) missingness > 5%, (vi) genotyping-sequence concordance (made possible by
311 genotyping data generated on the Illumina Omni 2.5M SNP array for all samples; 96% concordance), (vii) depth of
312 coverage, (viii) a gender check (to identify mismatches), (ix) relatedness (drop samples with >100 relatedness
313 pairs). (x) Related individuals were further excluded until no pair of samples had a kinship coefficient > 0.05. (xi)
314 missing phenotype information. Following QC, 312 samples with expended/inconsistent *C9orf72* status were
315 omitted from further analysis. A total of 5,774 samples (3,955 ALS patients and 1,819 healthy controls) passed all
316 QC and were included in downstream analysis. Per-nucleotide site QC was performed on QC-passing samples only,
317 for Biallelic sites: variants were excluded from analysis based on depth (total DP < 10,000 or > 226,000),
318 missingness > 5%, passing rate in the whole dataset < 70%, sites out of Hardy–Weinberg equilibrium (HWE; by
319 cohort, controls only, $p < 1x10^{-6}$) and sites with extreme differential missingness between cases and control
320 samples (Overall and by cohort, $p < 1x10^{-6}$). Non-autosomal chromosomes and multiallelic variants were excluded
321 from analysis.

322 **Selection of regions of interest**

323 Discontinuous regions of interest approximating in total ~5Mb, include coding sequences and 3' untranslated
324 regions (3'UTRs) of 295 genes (Supplementary Table 3) encoding for proteins that were: (i) previously reported to
325 be associated with ALS, (ii) RNA-binding proteins including miRNA biogenesis or activity factors [UCSC gene
326 annotation; [92]]. In addition to (iii) all 1,750 autosomal human pre-miRNA genes [miRBase v20; [57]]. In addition,
327 genome-wide analysis of all known human 3'UTRs (RefSeq [64]). Variants in regions of interest were extracted from
328 Project MinE ALS sequencing consortium genomes using vcftools [93] according to BED file containing genomic
329 coordinates of interest (hg19) ±300 bp that ensures covering splice junctions and sequence (Supplementary Table
330 14).

331 **Annotation and burden analysis**

332 After quality control and extraction of regions of interest, we performed functional annotation of all variants.
333 Indels were left-aligned and normalized using bcftools and multiallelic sites were removed. For variant annotation
334 we developed a pipeline that calculates the impact of genetic variation in coding regions as well as in 3'UTR and
335 miRNA regions, using ANNOVAR [94]. The frequency of the variants in the general population was assessed by
336 screening the 1000 Genomes Project, the Exome Aggregation Consortium (ExAC), and NHLBI Exome Sequencing
337 Project (ESP). For protein-coding ORFs, association analysis of deleterious rare variants was performed, i.e.,
338 frameshift variants, deviation from canonical splice variant, stop gain/loss variants, or a non-synonymous
339 substitution, as predicted by at least three prediction programs (SIFT, Polyphen2 HVAR, LRT, MutationTaster,
340 MutationAssessor, FATHMM, MetaLR) in dbNSFP environment [v2.0; [58]].

341 Non-coding sequence burden analysis included (i) 3'UTRs, (ii) variants in miRNA recognition elements (MREs) in
342 3'UTRs (Supplementary Table 3): Variants that impaired conserved-miRNA binding sites in 3'UTRs (predicted loss
343 of function) were called by TargetScan [v7.0; [95]]. Newly created miRNA binding sites in 3'UTRs (predicted gain of
344 function) were called by textual comparison of all possible mutated seeds around a variant to all known miRNA
345 seed sequences in the genome, (iii) all human pre-miRNAs (mirBase v20 [57]) and (iv) miRNAs:target gene networks:
346 mature miRNA sequences (mirBase v20 [57]) and cognate targets within the 3'UTRs (Supplementary Table 3). Variant
347 annotation scripts are available at GitHub: https://github.com/TsviyaOlender/Non-coding-Variants-in-ALS-genes-

348 **Mammalian Cell Cultures**

349 Lymphoblastoid cell lines (LCLs) from the UK MNDA DNA Bank [65] were originally derived from sixteen different
350 individuals: 4 healthy individuals (without ALS), carrying the canonical IL18RAP 3'UTR sequence (Control;
351 Canonical IL18RAP 3'UTR); 4 sporadic ALS patients, carrying the canonical IL18RAP 3'UTR sequence (sALS;
352 Canonical IL18RAP 3'UTR); two healthy individuals, carrying a variant form of IL18RAP 3'UTR (Control; Variant
353 IL18RAP 3'UTR); two sporadic ALS patients carrying a variant form of IL18RAP 3'UTR (sALS; Variant IL18RAP 3'UTR)
354 and 4 C9orf72 ALS patients, carrying the canonical IL18RAP 3'UTR sequence (C9orf72; Canonical IL18RAP 3'UTR)
355 (Cell lines listed in Supplementary Table 8; Weizmann IRB: 537-1). LCLs were cultured in RPMI-1640 (Gibco,
356 21875091) with 20% inactivated fetal bovine serum (FBS, Biological Industries, 04-001-1A), 1% L-glutamine and
357 1% penicillin-streptomycin (Biological Industries, 03-0311B) at 37°C, 5% CO2. Human Bone Osteosarcoma
358 Epithelial Cells (U2OS), were maintained in Dulbecco's Modified Eagle Medium (DMEM, Biological Industries, 01-
359 050-1A) supplemented with 10% FBS, 1% penicillin-streptomycin at 37°C, 5% CO2. Human iPSCs were cultured on
360 Matrigel (Corning, 354277) coated plated in mTeSR1 medium (Stemcell technologies, 85850) according to the

361 manufacturer's instructions. Briefly, cells were passaged at 70–90% confluent with StemPro accutase (Gibco,
362 A11105-01) and seeded in mTeSR1 medium supplemented with 10 nM Y-27632 dihydrochloride (Tocris, 1254).
363 Cells were refreshed with mTeSR1 medium every 24 hours until passaged.

364 **Isolation and Culture of Rat Cortical Astrocytes**

365 All experiments were performed in accordance with relevant guidelines and regulations of the Institutional Animal
366 Care and Use Committee at Weizmann Institute of Science (IACUC 09491120-1). Primary cortical astrocytes were
367 isolated and cultured as previously described [96] with several modifications. Briefly, the cerebral cortex of postnatal
368 day 1 (P1) Sprague-Dawley rat pups was dissected and placed in DMEM/F12 containing 0.5% trypsin (biological
369 industries, 03-046-5B). After 30 min incubation at 37 °C water bath, the cortical tissues were mechanically
370 dissociated with pipette into single cells and were seeded on poly-D-lysine (Sigma Aldrich, 7405) coated T75
371 culture flask in Astrocytes medium (DMEM/F12 (Gibco, 31330) supplemented with 10% FBS, 50U/mL Pen-strep
372 and 2Mm Glutamax (Gibco, 35050-038)). The confluent cultures were shaken for 4 hours at 200 rpm to remove
373 microglial cells. Each T75 flask was trypsinized and splited into three new T75 flasks. After 7-8 days the confluent
374 flasks were trypsinized and were frozen (in 90% FBS, 10% DMSO) until further use.

375 **I^3LMNs neuronal differentiation and *Syn::GFP+* transduction**

376 Differentiation of hiPSCs into lower motor neurons (i^3LMNs, iPSCs containing Doxycycline induced human NGN2,
377 ISL1, and LHX3 (hNIL)) was performed as described previously [76]. Briefly, iPSCs were seeded on day 0 into mTeSR1
378 medium supplemented with 10 nM Y-27632 dihydrochloride. Few hours after seeding, cells were transduced with
379 *Syn::GFP* lentivirus (pHR-hSyn-EGFP, Addgene #114215). 24 hrs. after seeding the cells medium was replaced with
380 differentiation medium (DMEM/F12 (Gibco, 31330-038) with 1× MEM non-essential amino acids (Gibco, 11140-
381 035), 2mM GlutaMAX (Gibco, 35050-038), 1× N-2 supplement (Gibco, 17502-048), 2 µg/mL Doxycycline (Sigma
382 Aldrich, D9891-1G.) and 10 nM Y-27632 dihydrochloride). On day 3, cells were split using accutase, counted and
383 re-seeded on poly-D-Lysine coated dishes containing Rat astrocytes in neuronal medium (B27 Electrophysiology
384 medium (Gibco, A14137-01) supplemented with 1× MEM non-essential amino acids, 2mM GlutaMAX, 1× N-2
385 supplement, and 1 µg/mL mouse laminin (Gibco, 23017-015)). Twice a week half of the media was removed, and
386 an equal volume of fresh media was added.

387

388

389 **Generation of IL18RAP 3'UTR rare variant hiPSCs lines**

390 iPSCs were generated by the Ichida lab from human lymphocytes from ALS patients obtained from the National
391 Institute of Neurological Disorders and Stroke (NINDS) Biorepository at the Coriell Institute for Medical Research.
392 lymphocytes were reprogrammed into iPSCs as previously described [66]. The NINDS Biorepository requires
393 informed consent from patients.

394 Human iPSC lines were maintained on irradiated MEFs in hESCs medium [DMEM/F12 (Sigma-Aldrich, D6421)
395 supplemented with 20% KO Serum Replacement (Gibco, 10828-028,), 1% GlutaMax (Gibco, 35050038), 1% MEM-
396 NEAA (Biological Industries, 01-040-1A), 0.1mM 2-Mercaptoethanol (31350-010, Gibco), 10ng/ml hFGF
397 (PeproTech, 100-18B)] and passaged twice a week with Collagenase IV (Worthington, LS004188).

398 CRISPR guides were chosen using several design tools, including: the MIT CRISPR design tool [97] and sgRNA
399 Designer, Rule set 2 [98], in the Benchling implementations (www.benchling.com), SSC [99], and sgRNAscorer [100], in
400 their websites.

401 Prior to CRISPR procedure iPSCs were passaged once in feeder-free condition [LDEV Free GelTrex matrix (Gibco,
402 A1413202), mTESR1 medium (StemCell Technologies, 85850)], dislodged as single cells using StemPro Accutase
403 (Gibco, A11105-01), washed twice with Opti-MEM (Gibco, 31985-047) and counted. 90ul cells suspension
404 containing 1M cells was mixed with 10 uL DNA mix: 4 ug pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene #48139),
405 0.4 ug gRNA encoding plasmid (pKLV-U6gRNA(BbsI)-PGKzeo2ABFP, derived from pKLV-U6gRNA(BbsI)-
406 PGKpuro2ABFP (Addgene)), 1 ug (8 pmol) ssODN repair template (Supplementary Table 15) (IDT, 400 bases
407 Megamer DNA Oligonucleotide) and 2.6 ug carrier plasmid DNA. CRISPR reaction components were introduced to
408 iPSCs by single round electroporation using Nepa21 system (NEPA GENE). 100 uL cells and DNA suspension was
409 transferred to Nepa Electroporation Cuvette 2 mm gap (Nepa Gene, EC-002). Electroporation conditions: 150 V
410 Poring pulse; 5 ms Pulse length; 20 V Transfer pulse; 50 ms Pulse length. Electroporated cells were transferred to
411 two GelTrex coated 100 mm dishes (1K and 10K) in mTeSR medium supplemented with 10 uM ROCK inhibitor
412 (PeproTech, 1293823) and placed into CO2 incubator for 2 days. 48h past electroporation cells were treated with
413 0.5 ug/mL Puromycin (Sigma-Aldrich) for 2 consecutive days. Survived cells were maintained until clones
414 development. Single clones were picked and transferred to 96 well plates. Matured clones were genotyped at the
415 first passage. Additionally, the top five predicted off-target sites for the guide RNA were sequenced
416 (Supplementary Table 16). Selected clones containing desired mutations were expanded, cryopreserved, and used
417 for the downstream experiments.

418 **Differentiation and culturing of human iPSC-derived microglia**

419 hiPSCs were differentiated to microglia-like cells as previously described [68]. Briefly, to form embryoid bodies (EBs),
420 iPSCs were seeded into 96 well suspension plates in mTeSR1 media supplemented with 50 ng/mL rhBMP4
421 (Peprotech, 314-BP), 50 ng/mL VEGF (Peprotech, 100-20), 20 ng/mL SCF (Peprotech, 300-07) and 10 nM Y-27632
422 dihydrochloride. Everyday half of the medium was removed, and an equal volume of fresh media was added. After
423 four days 12 EBs were transferred into each well of 6 well plate in X-VIVO 15 (Lonza, BE02-060Q) containing 100
424 ng/mL M-CSF (Peprotech, 300-25), 25 ng/mL IL-3 (Peprotech, 200-03), 2 mM Glutamax, 55 uM 2-mercaptoethanol
425 (Gibco, 31350-10) and 100 U/mL penicillin/streptomycin (Biological Industries, 03-031-1B). iPSC derived
426 progenitor microglia (ipMG) were collected weekly from the supernatant and were co-cultured with iPSC derived
427 neurons in 96 well plates (Greiner, 655090) in neuronal medium containing 10 ng/mL IL-34 (Peprotech,200-34).
428 EB medium was refreshed weekly.

429 **i^3LMNs survival assay**

430 Survival assay was conducted by monitoring eGFP signal of day 5 i^3LMNs co-cultured with two independent
431 CRISPR-edited isogenic iPSC-derived microglia (harboring canonical or variant IL18RAP 3'UTR), with C9orf72
432 genetic background. Cells were monitored for over 20 days using Incucyte® Live-Cell Analysis System (Sartorius).
433 Daily longitudinal microscopic tracking was performed following Lipopolysaccharide (LPS, 100 ng/mL) and IL-18
434 treatment (100 ng/mL). i^3LMNs survival assay was performed using three individual replicates for each line, with
435 3-8 co-culture wells per condition. Twice a week half of the media was removed, and an equal volume of fresh
436 media containing LPS and IL-18 was added.

437 **Cloning**

438 Full IL18RAP coding sequence (CDS) and 3'UTR sequence (2223bp) in pMX vector was purchased from GeneArt
439 (Invitrogen, Supplementary Table 15) and subcloned with V5 epitope into pcDNA3. Different mutants, including:
440 WT IL18RAP CDS + mutant 3'UTR (V1 or V3), and a dominant-negative coding mutant E210A-Y212A-Y214A CDS +
441 WT 3'UTR (3CDS) [41] created by Transfer-PCR mutagenesis[101]. Next, WT and mutants full IL18RAP were subcloned
442 into pUltra vector (a gift from Malcolm Moore, Addgene plasmid #24130, for which mCherry was replaced with
443 EGFP), downstream of the human Ubiquitin C promoter and EGFP-P2A. Cloning procedures were done via
444 restriction-free cloning [102]. List of primers used for cloning and Transfer-PCR mutagenesis described in
445 Supplementary Table 16.

446

447 **Transfection**

448 Transfection to U2OS cells at 1.9 cm^2 corning plates was performed at 70–80% confluence, 24 hrs. post-plating in
449 antibiotic-free media, using Lipofectamine 2000, 0.5 µL per well (Thermo Fisher Scientific, Cat# 11668027). Each
450 well was considered as a single replicate. For NF-κB reporter assay, U2OS cells were induced with/without
451 recombinant IL-18 (5 ng/mL) 72 hrs. post-transfection with full coding sequence of IL18RAP coding region + 3'UTRs
452 (pUltra vector 500 ng / 1.9 cm^2 plate), luc2P/NF-κB-RE (pGL4.32 100 ng) luciferase and Renilla luciferase (hRluc 10
453 ng). Following 6 hrs. cells were harvested for Dual-Luciferase® Reporter Assay (E1960) and luminescence was
454 quantified using Veritas™ Microplate Luminometer.

455 **RNA extraction, cDNA synthesis, and quantitative real-time PCR.**

456 Total RNA from LCLs was extracted using Direct-Zol RNA MiniPrep (Zymo Research ,R2052) according to
457 manufacturer instructions. Total RNA from ipMGs was extracted using miRNeasy micro Kit (QIAGEN, 217084)
458 according to manufacturer instructions. Total RNA was reverse transcribed using High Capacity cDNA Reverse
459 Transcription Kit (applied biosystem, 4368814) according to manufacturer instructions, except for the mRNA
460 stability assay, where equal volume of RNA (and not equal amounts of RNA) from each sample was used to
461 generate cDNA. Quantitative Real-time PCR was performed using TaqMan Universal PCR master Mix (applied
462 biosystem, 4304437) or KAPA SYBR FAST (Roche, KK4605). Primers and TaqMan probes are shown in
463 Supplementary Table 16.

464 **Bulk MARS-Seq**

465 200,000 ipMGs harboring variant or canonical IL18RAP 3'UTR (n=4) were treated with 100 ng/mL LPS + 100 ng/mL
466 IL-18 for 6 hrs. in ipMG media (Advanced DMEM (Gibco, 12491-015) containing 1× N-2 supplement (Gibco, 17502-
467 048), 2mM GlutaMAX (Gibco, 35050-038), 55 uM 2-mercaptoethanol (Gibco, 31350-10), 50 U/mL
468 penicillin/streptomycin (Biological Industries, 03-031-1B) and 100 ng/mL IL-34 (Peprotech, 200-34). Following 6
469 hrs. RNA was extracted as described above and a bulk adaptation of the MARS-Seq protocol (Jaitin et al., Science
470 2014; Keren-Shaul et al., Nature Protocols, 2019) was used to generate 3' RNA-Seq libraries for expression
471 profiling. Briefly, 50 ng of input RNA from each sample was barcoded during reverse transcription and pooled.
472 Following Agencourt Ampure XP beads cleanup (Beckman Coulter), the pooled samples underwent second strand
473 synthesis and were linearly amplified by T7 in-vitro transcription. The resulting RNA was fragmented and
474 converted into a sequencing-ready library by tagging the samples with Illumina sequences during ligation, RT, and
475 PCR. Libraries were quantified by Qubit and TapeStation as well as by qPCR for GAPDH gene as previously

476 described (Jaitin et al., Science 2014; Keren-Shaul et al., Nature Protocols, 2019). Sequencing was done on a
477 NovaSeq 6000 system, SP Reagent Kit, 100 cycles (Illumina; paired-end sequencing).

478 Analysis of the MARS-seq was done using the UTAP pipeline ([103]; the Weizmann Institute Bioinformatics Unit) to
479 map the reads to the human genome and to calculate Unique Molecule Identifier (UMI) counts per gene. Reads
480 were trimmed from their adapter using cutadapt (parameters: -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
481 -a "A(10)" –times 2 -u 3 -u −3 -q 20 -m 25) and mapped to hg38 genome (STAR v2.4.2a). The pipeline removes UMI
482 redundancy and quantifies the 3' of RefSeq annotated genes (1,000 bases upstream and 100 bases downstream
483 of the 3' end). Genes having a minimum of five reads in at least one sample were considered for further analysis.
484 Differentially expressed (DE) gene detection and count normalization analysis were performed by DESeq2. P-
485 values in the UTAP results were adjusted for multiple testing using the Benjamini and Hochberg procedure.
486 Thresholds for significant DE genes: padj < 0.01, |log2FoldChange| >= 0.585, baseMean > 20. This assay was done
487 with critical advice from Dr. Hadas Keren-Shaul from the Genomics Sandbox unit at the Life Science Core Facility
488 of Weizmann Institute of Science.

489 **Cell lysis and Western blot**

490 LCLs were washed in PBSx1, centrifuged at 800 × *g* for 5 min at 4°C, pelleted, and lysed in ice-cold RIPA buffer
491 (Supplementary Table 17) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche, 4693116001) and
492 PhosSTOP™ (Roche, 4906837001). The lysates were cleared by centrifugation at 15,000 × g for 10 min at 4°C.
493 Protein concentrations quantified with Protein Assay Dye Reagent (Bio-Rad, 500-0006), resolved at 30-50µg of
494 total protein/well by 8-10% polyacrylamide / SDS gel electrophoresis at 100-120 V for 70 min. After gel
495 electrophoresis proteins were transferred to nitrocellulose membrane (Whatmann, 10401383) at 250 mA for 70
496 min. Membranes were stained with Ponceau (Sigma, P7170), blocked for 1 hour at RT with 3% Bovine albumin
497 fraction V (MPBio 160069) or 5% milk protein in PBST (PBS containing 0.05% TWEEN-20), and then incubated with
498 primary antibodies (see Supplementary Table 18) O.N. at 4°C with rocking in antibody-Solution [5% albumin, 0.02%
499 sodium azide, 5 drops of phenol red in 0.05% PBST]. Following primary antibody incubation, membranes were
500 washed 3 times for 5 min at RT with 0.05% PBST then incubated for 1 hour at RT with horseradish peroxidase
501 (HRP)-conjugated species-specific secondary antibodies, washed 3 x 5 min in 0.05% PBST at RT, and visualized
502 using EZ-ECL Chemiluminescence (Biological Industries, 20500-120) by ImageQuant™ LAS 4000 (GE Healthcare Life
503 Sciences). Densitometric analysis was performed using ImageJ (NIH).

504

505 **In-Vitro Transcription of biotinylated IL18RAP 3'UTR**

506 To identify the potential trans-acting factors that might differentially bind to the canonical and variant 3'UTRs an
507 RNA-pulldown and mass spectrometry assay was performed on *in vitro* transcribed canonical and variant forms
508 of the IL18RAP 3'UTRs, V1 and V3. Briefly, The canonical, V1 and V3 biotinylated-IL18RAP 3'UTR sequences
509 (384nt), and the negative control (ultrapure water only), were produced by using in vitro transcription HiScribe™
510 T7 ARCA Kit (NEB, E2060S) following the manufacturer instructions. Briefly, 300 ng of purchased DNA template
511 (50 ng/uL) (Twist, Supplementary Table 15) was incubated with unlabeled ATP/GTP/CTP and 5% biotin-labelled
512 UTP, at 37°C for 3 hrs. Next, DNase treatment was performed by incubating the reactions at 37°C for 30 min and
513 was followed by incubation at 65°C for 10 min to terminate the reaction. The RNA products were purified by an
514 RNA cleanup purification kit (Zymo Research, R1015). The concentrations of the purified RNA samples were
515 measured by nanodrop and the expected length was analyzed by TapeStation.

516 **Pull Down of IL18RAP 3'UTR RNA-associated proteins**

517 LCL cell pellets were suspended and lysed in RIPA buffer followed by centrifugation at 15,000xg for 10 min at 4°C.
518 The concentrations of the cleared supernatants were measured by Bradford assay. 1 mg lysate per sample was
519 incubated with Pierce streptavidin magnetic beads (Thermo Scientific, 88817) for 30 min at 4°C in rotation, to pre-
520 clear the lysates from endogenous biotinylated-proteins. To bind IVT products (WT, V1, V3 and negative control;
521 n=6 repeats/group) to the beads, new prepared binding Pierce streptavidin magnetic beads were incubated by
522 rotation with equal amounts of IVT products for 30 min at 4°C (100 uL beads/10 pmol RNA product). After 30 min,
523 the tubes of incubated IVT products with beads were washed three times, and then the cleared lysate was added
524 equally to each tube and incubated for 30 min at 4°C. In the next step, the samples were washed three times by
525 magnetizing the beads and resuspended by vortex with a high salt buffer. The bound beads were magnetized and
526 suspended in 20 ul RNase-free PBSx1 for on-bead digestion procedure.

527 **Liquid Chromatography and Mass Spectrometry**

528 The resulting peptides were analyzed using nanoflow liquid chromatography (nanoAcquity) coupled to high
529 resolution, high mass accuracy mass spectrometry (Q-Exactive HF). Each sample was analyzed on the instrument
530 separately in a random order in discovery mode.

531

532

533 **Raw proteomic data processing**

534 Raw MS data were processed using MaxQuant version 1.6.6.0 (Cox and Mann, 2008). Database search was
535 performed with the Andromeda search engine (Cox and Mann, 2011; Cox et al., 2011) using the human Uniprot
536 database, appended with common lab protein contaminants. Forward/decoy approach was used to determine
537 the false discovery rate and filter the data with a threshold of 1% false discovery rate (FDR) for both the peptide-
538 spectrum matches and the protein levels. The label-free quantification (LFQ) algorithm in MaxQuant (Cox et al.,
539 2014) was used to compare between experimental samples. Additional settings included the following
540 modifications: Fixed modification- cysteine carbamidomethylation. Variable modifications- methionine oxidation,
541 asparagine and glutamine deamidation, and protein N-terminal acetylation.

542 **Proteomics statistical analysis**

543 ProteinGroups output table was imported from MaxQuant to Perseus v.1.6.2.3 environment (Tyanova et al.,
544 2016). Quality control excluded reverse proteins, proteins identified only based on a modified peptide, and
545 contaminants. Non-specific streptavidin-bead binders were excluded by the following procedure: LFQ Intensity
546 values were log2 transformed, and two outlier samples were excluded from further analysis. Missing values were
547 imputed by creating an artificial normal distribution with a downshift of 1.8 standard deviations and a width of
548 0.4 of the original ratio distributions. Student's t-test with S0 = 0.1 was performed with FDR P-value ≤ 0.05
549 between the experimental groups (Canonical, V1 and V3) and the negative control group, which was defined as a
550 single control group. Proteins that passed all QC filters were separated for each of the experimental groups and
551 compared to the negative control samples (ultrapure water). The statistically significant-associated proteins were
552 filtered to retain only proteins that were found in 50% of the repeats in at least one experimental group and were
553 represented by at least one unique peptide. The enriched proteins were subjected to student's t test between
554 every two groups (canonical vs. V1 and canonical vs. V3), with S0 = 0.1, FDR P-value ≤ 0.05 and fold-change
555 threshold >2.

556 **Processing of Mouse Brain Samples for Flow Cytometry**

557 Wild-type C57BL/6 mice were euthanized with CO2 and perfused with PBS through the left ventricle of the heart.
558 Dissected mouse cortex was cut into smaller pieces using scissors and digested in 0.5 mg/mL Collagenase IV
559 (Worthington Biochemical), 10 µg Deoxyribonuclease (Sigma-Aldrich), 10% HI-FBS, RPMI1640 (Gibco) at 37°C for
560 30 minutes with continuous agitation. Digested samples were gently triturated for 1 minute and the enzymatic
561 reaction was stopped by adding 1 mM EDTA in PBS. The homogenate was filtered through a 100 µm cell strainer
562 and centrifuged at 400 x g for 8 minutes at 4°C to pellet the cells and myelin. This was followed by myelin removal

563 step by gradient centrifugation with 30% Percoll (Sigma-Aldrich) in PBS (700 x g for 20 minutes at 21oC; without
564 brakes during deceleration). After myelin (the top white layer) separation, the middle transparent layer was
565 collected, washed in PBS, and centrifuged at 400 x g for 8 minutes at 4°C to pellet the cells.

566 Cells pellets were incubated with Mouse Fc block (BD Biosciences 553142), Fixable Viability Stain 620 (BD
567 Biosciences 564996) and the following antibody mixture in PBS at 4°C for 30 minutes: BV421 Rat Anti-CD11b (BD
568 Biosciences 562605), BV510 Hamster Anti-Mouse TCR β Chain (BD Biosciences 563221), BV711 Rat Anti-Mouse
569 Ly-6G (BD Biosciences 563979), APC-Cy7 Rat Anti-Mouse CD45 (BD Biosciences 557659), and Polyclonal Goat IgG
570 Anti-Mouse IL-18Rβ (R&D Systems AF199). Samples were then washed with PBS and incubated with Alexa Fluor
571 647 Donkey Anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody (Invitrogen A-21447) in PBS at 4°C for 30
572 minutes. Surface-stained samples were washed with PBS and fixed and permeabilized with BD
573 Fixation/Permeabilization solution (BD Biosciences 554714) at 4°C for 30 minutes, followed by intracellular
574 staining with Alexa Fluor 488 Anti-NeuN Antibody (EMD Millipore MAB377X) and eFluor 570 Anti-GFAP
575 (eBioscience 41-9892-82) in BD Perm/Wash Buffer (BD Biosciences 554714) at 4°C for 30 minutes. Cells were
576 washed with BD Perm/Wash Buffer and resuspended in PBS for analysis with a FACSymphony (BD Biosciences).
577 Data were collected as FCS files and analyzed with FlowJo v10 software (BD Biosciences). Antibody specificity was
578 assessed using relevant isotype control antibodies and fluorescence minus one. Compensation was adjusted using
579 single-stained samples.

580 The expression of IL-18RAP (IL-18Rβ) was expressed as Mean Fluorescence Intensity (MFI) or % frequency after
581 gating for the following cell types: immune cells (CD45^{hi}), microglia (MG: CD45^{int} CD11^{hi}), neurons (CD45^{-} CD11b^{-}
582 NeuN^{+}), and astrocytes (CD45^{-} CD11b^{-} GFAP^{+}). Animal procedures were approved by the Walter and Eliza Hall
583 Institute Animal Ethics Committee (Ethics application: 2020.017).

584

585 **Statistical analysis**

586 Statistics performed with Prism Origin (GraphPad). Shapiro-Wilk test was used to assess normality of the data.
587 Pairwise comparisons passing normality test were analyzed with Student's *t*-test, whereas the Mann-Whitney test
588 was used for pairwise comparison of nonparametric data. Multiple group comparisons were analyzed using
589 ANOVA with post hoc tests. For age of diagnosis and age of death a Permutation Test was used (a Monte-Carlo
590 simulation test on the t-test between ALS patients harboring canonical or variants of the IL18RAP 3'UTR).
591 Statistical P-values <0.05 were considered significant. Data are shown as scatter dot plot with mean and SEM, box
592 plots, or as noted in the text.

593 **Supplementary Materials**

594 Fig. S1. Study design.

595 Fig. S2. Region-based rare-variant association analyses.

596 Fig. S3. 3'UTR-based rare-variant association analysis, using different algorithms, and illustration of rare variants
597 identified in the IL18RAP 3'UTR.

598 Fig. S4. Restricting burden analysis to the proximal part of 3'UTRs does not improve the association signal.

599 Fig. S5. IL18RAP and p-NF-κB expression is elevated in lymphoblastoid cells from patients with the C9orf72
600 repeat expansion.

601 Fig. S6. IL18RAP 3'UTR variant attenuates IL-18 - NF-κB signaling in U2OS cells.

602 Fig. S7. IL18RAP is mainly expressed on mouse microglia cells.

603 Fig. S8. Evaluation of IL18RAP and IL-18 mRNA expression in motor neurons of patients with ALS.

604 Fig. S9. iPSC-derived microglia express the microglial-specific marker, TMEM119.

605 Fig. S10. Differentially bound RNA binding proteins to variant 3'UTR (V3) relative to canonical 3'UTR.

606 Table S1. Total number of samples before and after quality control procedures, stratified by country.

607 Table S2. Samples quality control procedures.

608 Table S3. Candidate genes list.

609 Table S4. Number of rare genetic variants identified.

610 Table S5. Detailed description of variants in protein-coding sequences of *NEK1* and *SOD1* and the IL18RAP 3'UTR,
611 in Project MinE discovery cohort.

612 Table S6. Identified IL18RAP 3'UTR variants in Project MinE discovery cohort.

613 Table S7. Identified IL18RAP 3'UTR variants in discovery and replication cohorts.

614 Table S8. Control and patient human cell lines information.

615 Table S9. Proteomics data from IL18RAP 3'UTR pull-down experiments.

616 Table S10. Gene set enrichment analysis of differentially bound proteins in canonical vs. V1 IL18RAP 3'UTR.

617 Table S11. Phenotypic and variant information for patients with protective 3'UTR variants in Project MinE and
618 NYGC cohorts.

619 Table S12. MARS-seq data for mRNAs measured in isogenic ipMG harboring variant vs. canonical IL18RAP 3'UTR.

620 Table S13. KEGG Pathway and Gene Ontology (GO) Biological Process Enrichment Results.

621 Table S14. BED file containing genomic coordinates of regions of interest.

622 Table S15. IL18RAP oligonucleotides.

623 Table S16. List of primers.

624 Table S17. Medium formulation.

625 Table S18. Materials and Antibodies.

626 Data File S1. Source data for IL18RAP and p-NF-κB western blot studies in LCLs (Figure 3D).

627 Data File S2. Source data for IL18RAP and p-NF-κB western blot studies in Control vs. C9orf72 LCLs (Supplementary
628 figure 5D).

629 Data File S3. Source data for IL18RAP western blot studies in isogenic microglia (Figure 4B).

630 Data File S4. Source data for motor neuron survival assays (Figure 6B,C).

631 Data File S5. Source data for pNF-κB western blot studies in isogenic microglia, following microglia activation
632 (Figure 7B).

633 Project MinE ALS Sequencing Consortium PI List

634 NYGC ALS Consortium PI List

635 Supplementary movie. Motor neuron survival was significantly improved in the presence of microglia harboring
636 variant IL18RAP 3’UTR relative to canonical IL18RAP 3’UTR.

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698 in the genetic association analysis of rare non-coding variants in human patients with ALS; S.-T.H. and J.K.I.
699 provided stem cells and initial data; S.B.-D., E.A., G.B. and H.M.-K. were involved in the design, generation and
700 validation of CRISPR-edited IL18RAP isogenic iPSCs; H.M.-K. and Y.M.D. performed the pull down experiments of

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702 derived microglia differentiation and culturing protocols, performed motor neuron survival experiments and
703 interpreted data; A.Si., N.R. and C.E. performed molecular biology studies in LCLs and U2OS cell lines, including
704 reporter assays, qPCR and protein quantification by western blots; E.Y. performed Bulk MARS-Seq experiment; C.-
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708 **Competing interests:** J.K.I. is a co-founder of AcuraStem Incorporated. J.K.I. declares that he is bound by
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714 Consortium, the Genome Aggregation Database (gnomAD), and NHLBI's Trans-Omics for Precision Medicine
715 (TOPMed). Gene Expression Omnibus accession number: GSE186757. All Other data used for this manuscript are
716 available in the manuscript. **Code availability:** Variant annotation scripts are available at GitHub:
717 https://github.com/TsviyaOlender/Non-coding-Variants-in-ALS-genes-.

FIGURES

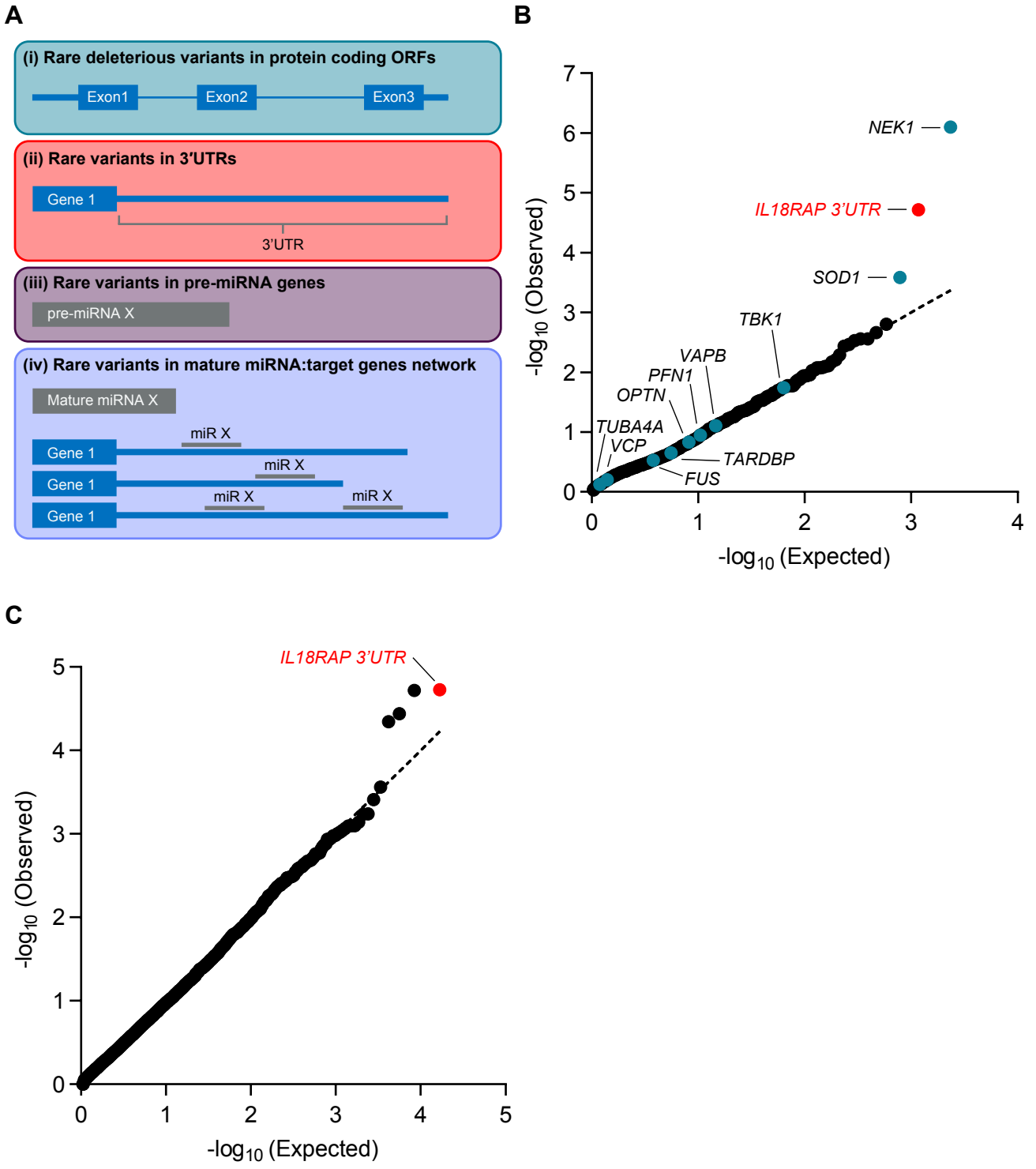


Fig.1 - Eitan et al. (Hornstein)

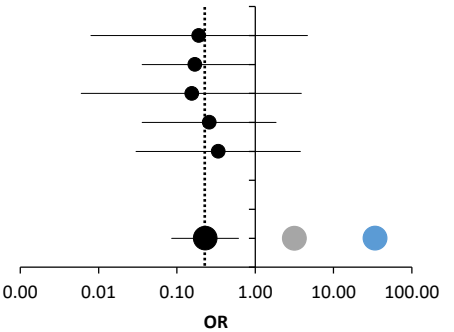
720 **Fig. 1. Region-based rare-variant association analysis reveals association of IL18RAP 3'UTR with ALS. (A)** Diagram
721 of study design. Collapsed region-based rare-variant (MAF ≤0.01) association analysis was performed on: (i) 295
722 candidate protein-coding genes (Supplementary Table 3), encoding for ALS-relevant proteins or proteins
723 associated with miRNA biogenesis/activity. Variants were included if predicted to cause frameshifting, alternative
724 splicing, an abnormal stop codon, or a deleterious non-synonymous amino acid substitution, in ≥ 3 of 7
725 independent dbNSFP prediction algorithms; (ii) variants in 3'-untranslated regions (3'UTRs) of the 295 genes
726 (Supplementary Table 3); (iii) all known autosomal pre-miRNA genes in the human genome; and (iv) predicted
727 networks, comprised of aggregated variants detected in a specific mature miRNA sequence and its cognate down-
728 stream 3'UTR targets. **(B)** QQ plot of obtained and expected P-values for the burden of rare variants (log scale),
729 gained by collapsed region-based association analysis of all genomic regions described in (A). Data were obtained
730 from 3,955 ALS cases and 1,819 controls (Project MinE). Features positioned on the diagonal line represent results
731 obtained under the null hypothesis. Open-reading frames of 10 known ALS genes (blue). IL18RAP 3'UTR (red).
732 Genomic inflation $\lambda = 1.2$. **(C)** QQ plot of obtained and expected P-values for the burden of rare variants (log scale),
733 gained by collapsed region-based association analysis for all known human 3'UTRs (RefSeq). The IL18RAP 3'UTR
734 (red) is the most significant 3'UTR associated with ALS. P-values, calculated with Optimized Sequence Kernel
735 Association Test, SKAT-O (genomic inflation $\lambda = 0.97$).

A

Gene	Region	ALS (3955)	Control (1819)	OR	OR 95% CI	P	$P_{corrected}$
NEK1	Coding	127	19	3.14	1.93-5.11	8.00×10^{-7}	2.30×10^{-4}
SOD1	Coding	36	0	33.89	2.08-552.47	2.60×10^{-4}	3.73×10^{-2}
IL18RAP	3'UTR	*6*	*12*	0.23	0.09-0.61	1.93×10^{-5}	5.41×10^{-3}

B

Cohort	Cases	Control	OR	OR 95% CI
Ireland	0/239	1/136	0.19	0.008-4.662
Netherlands	2/1633	7/1004	0.17	0.036-0.842
Turkey	0/142	1/67	0.16	0.006-3.870
UnitedKingdom	2/1043	2/272	0.26	0.036-1.850
USA	2/398	1/68	0.34	0.030-3.784
Belgium	0/295	0/172	-	-
Spain	0/205	0/100	-	-
Total	6/3955	12/1819	0.23	0.086-0.611



C

Cohort	Cases	Control	OR	95% CI	P	
					χ2	SKAT-O
Discovery: Project MinE	6/3955	12/1819	0.23	0.086-0.611	3.00×10^{-3}	1.93×10^{-5}
Replication: NYGC, TOPMed & gnomAD	8/2184	786/68584	0.32	0.158-0.637	9.58×10^{-4}	-
Joint analysis: Discovery & replication	14/6139	798/70403	0.20	0.118-0.338	$< 1.00 \times 10^{-5}$	-

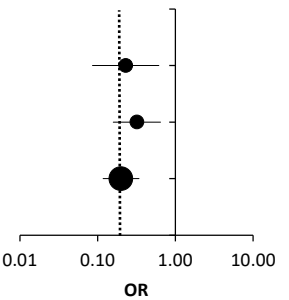
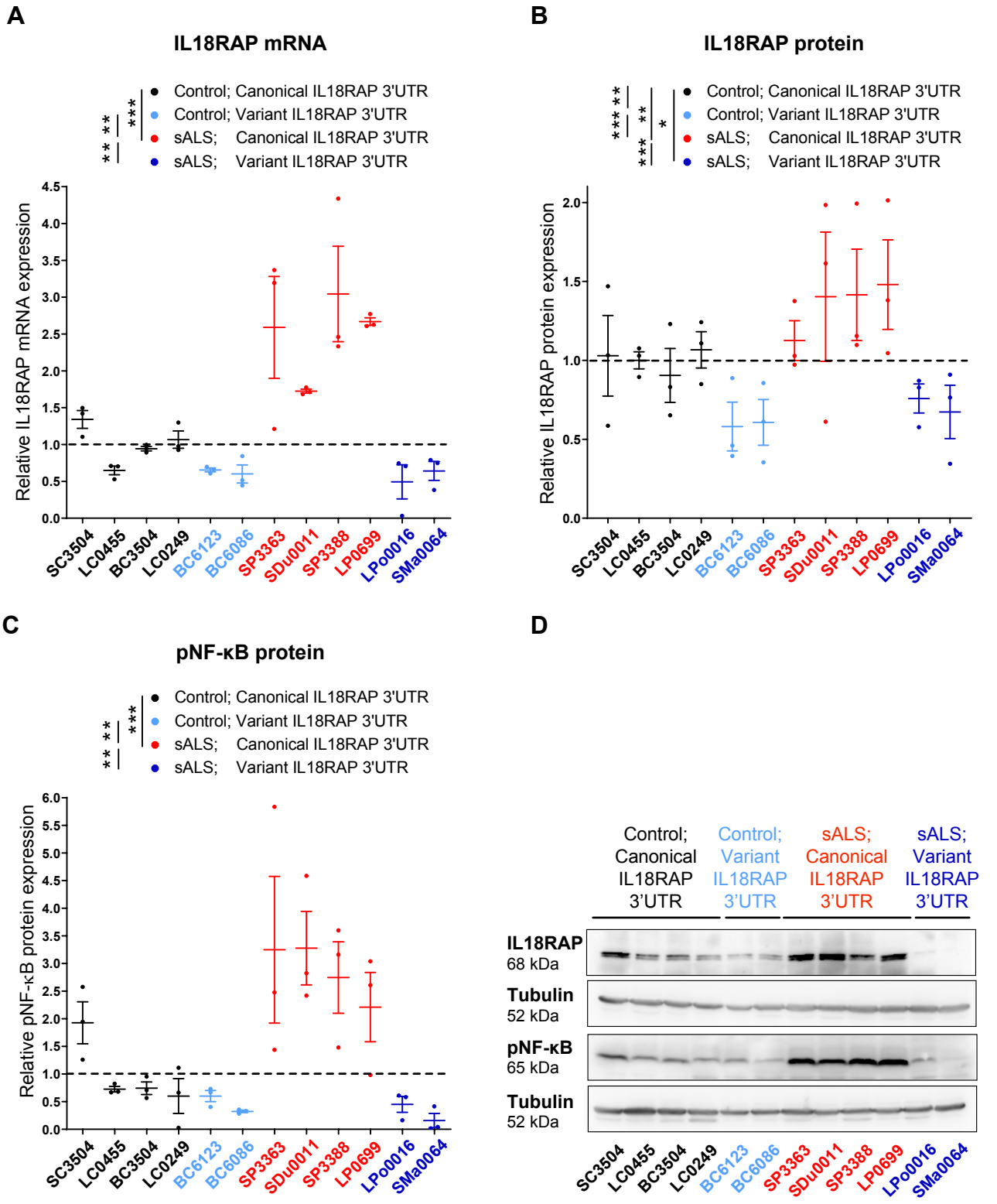


Fig.2 - Eitan et al. (Hornstein)

736 **Fig 2. Odds of ALS are reduced with rare variants in the IL18RAP 3'UTR. (A)** Odds ratio (OR) estimates with 95%
737 confidence intervals (CI) for NEK1 (coding), SOD1 (coding) and IL18RAP (3'UTR). P-values corrected for false
738 discovery rate (FDR). **(B)** Stratification of data pertaining to IL18RAP 3'UTR in seven geographically-based sporadic
739 ALS sub-cohorts and forest plot (OR on log scale with whiskers for 95% CI). *NEK1* (grey) and *SOD1* (blue) signals
740 are from combined data of all cohorts. Vertical dotted line denotes OR=0.23. **(C)** Stratification of IL18RAP 3'UTR
741 variants data across discovery and replication cohorts and joint analysis thereof; Forest plot (OR on log scale with
742 whiskers for 95% CI). Vertical dotted line denotes OR=0.2. P-values, calculated with SKAT-O or Chi-squared test
743 with Yate's correction.

Lymphoblastoid cell lines



744 **Fig. 3. IL18RAP 3'UTR variant correlates with attenuated IL-18 - NF-κB signaling in human lymphoblastoid cells.**
745 **(A)** IL18RAP mRNA expression (qPCR normalized to IPO8 mRNA levels) and **(B)** IL18RAP or **(C)** p-NF-κB protein
746 expression (Western blots, normalized to Tubulin). Scatter dot plot with mean and SEM. **(D)** Representative blots
747 processed with anti-IL18RAP, anti p-NF-κB and anti-Tubulin antibodies. Extracts from twelve different human
748 lymphoblastoid cell lines (listed in Supplementary Table 8): Four lines of healthy individuals (without ALS), carrying
749 the canonical IL18RAP 3'UTR sequence (Control; Canonical IL18RAP 3'UTR, black); Four sporadic ALS patients,
750 carrying the canonical IL18RAP 3'UTR sequence (sALS; Canonical IL18RAP 3'UTR, red); Two healthy individuals,
751 carrying a variant form of IL18RAP 3'UTR (Control; Variant IL18RAP 3'UTR, light blue) and two sporadic ALS
752 patients carrying a variant form of IL18RAP 3'UTR (sALS; Variant IL18RAP 3'UTR, navy blue). One-way ANOVA
753 followed by Newman-Keuls multiple comparisons test, was conducted based on the mean value of three
754 independent passages for each of the twelve human lymphoblastoid cell lines. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

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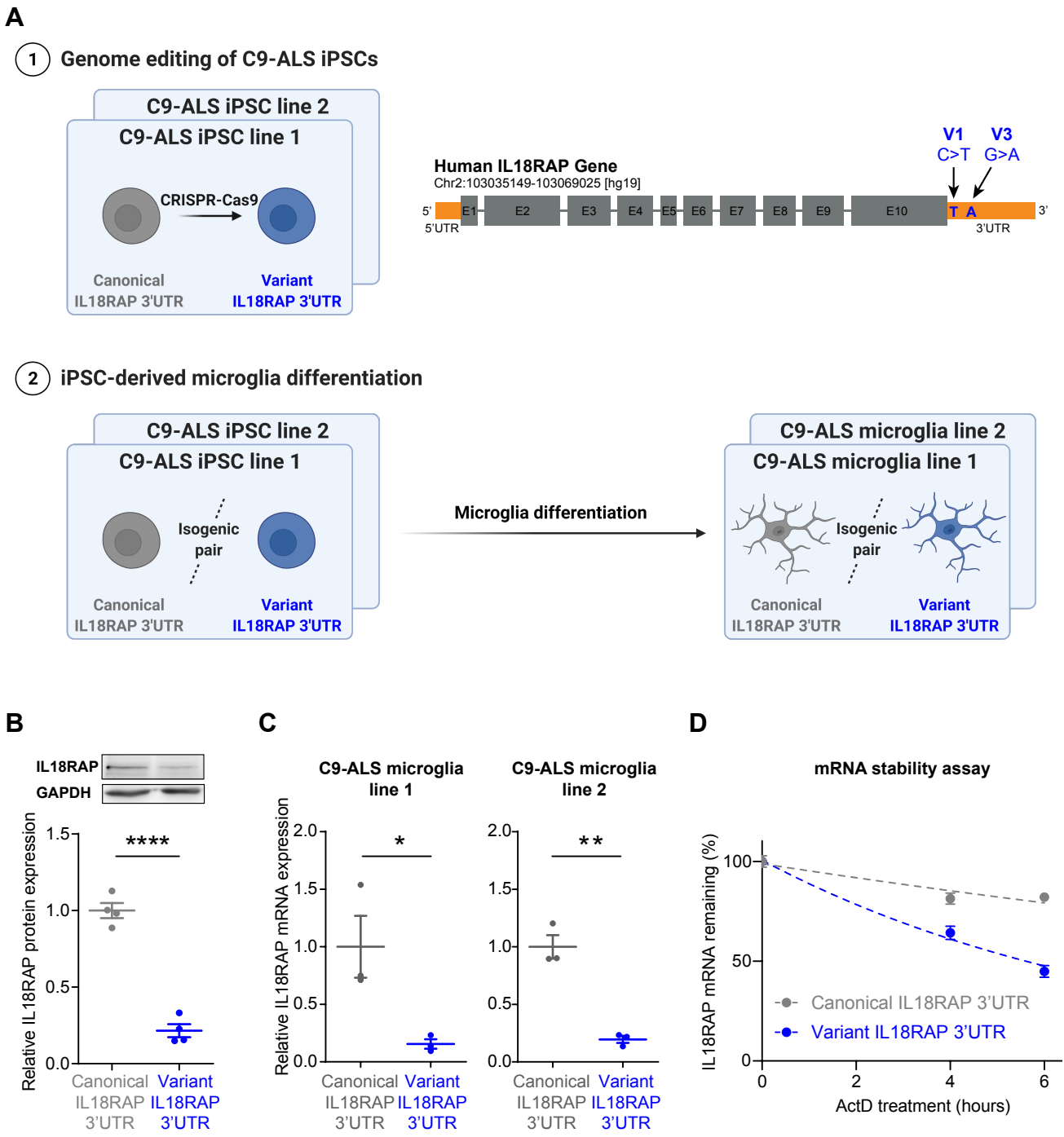
A
1 Genome editing of C9-ALS iPSCs
C9-ALS iPSC line 2
C9-ALS iPSC line 1
CRISPR-Cas9
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
Human IL18RAP Gene
Chr2:103035149-103069025 [hg19]
V1 C>T
V3 G>A
5' 5'UTR E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 T A 3'UTR 3'
2 iPSC-derived microglia differentiation
C9-ALS iPSC line 2
C9-ALS iPSC line 1
Isogenic pair
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
Microglia differentiation
C9-ALS microglia line 2
C9-ALS microglia line 1
Isogenic pair
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
B
IL18RAP
GAPDH
Relative IL18RAP protein expression
1.5
1.0
0.5
0.0
****
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
C
C9-ALS microglia line 1
C9-ALS microglia line 2
Relative IL18RAP mRNA expression
2.0
1.5
1.0
0.5
0.0
*
**
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
D
mRNA stability assay
IL18RAP mRNA remaining (%)
100
50
0
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
0
2
4
6
ActD treatment (hours)

768 **Fig. 4. IL18RAP 3'UTR variant destabilizes IL18RAP mRNA in CRISPR-edited isogenic iPSC-derived microglia, with**
769 **C9orf72 genetic background. (A)** Diagram of experimental design. (1) Genome editing with CRISPR Cas9 of point
770 mutations that recapitulate the most prevalent variants (Chr2:103068691 C>T (V1) and Chr2:103068718 G>A (V3))
771 in the IL18RAP 3'UTR sequence in human induced pluripotent stem cells (iPSCs) donated by ALS patients with a
772 C9orf72 repeat expansion ([66] NINDS/Coriell Code: ND10689, ND12099, Supplementary Table 8). The two
773 independent isogenic pairs of cell lines both carry the C9orf72 repeat expansion and vary only by the presence of
774 the canonical or a variant IL18RAP 3'UTR. (2) The four IL18RAP 3'UTR lines (two isogenic pairs) were differentiated
775 into human microglia [68]. Dot plots of IL18RAP **(B)** protein levels (by Western blot analysis, normalized to GAPDH,
776 N=3, Data File S3) and **(C)** mRNA (by qPCR, normalized to IPO8 mRNA N=3) in differentiated human microglia. **(D)**
777 IL18RAP mRNA degradation rate studied in human isogenic microglia at 0, 4 and 6 hrs after introduction of a
778 transcriptional block with actinomycin D (7.5 µg/mL, Sigma-Aldrich A9415) (by qPCR, normalized to average of
779 IPO8 and GAPDH mRNA expression, n = 4 independent wells per time point with two technical duplicates). Variant
780 3'UTR destabilizes the IL18RAP mRNA relative to the canonical sequence. Scatter dot plot with mean and SEM.
781 Two sided t-test P- values * <0.05, ** <0.01, **** <0.0001.

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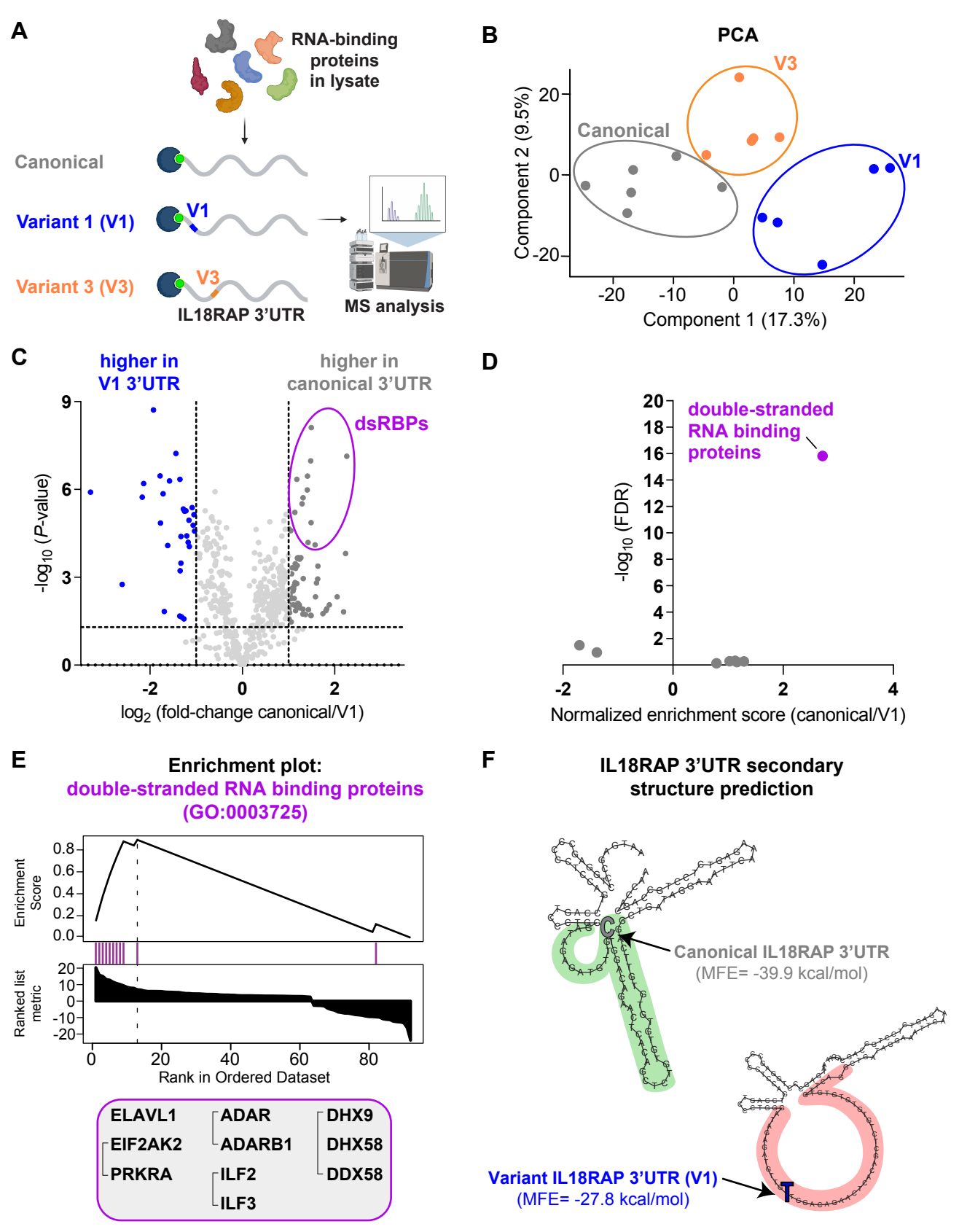
A
RNA-binding proteins in lysate
Canonical
Variant 1 (V1)
V1
Variant 3 (V3)
V3
IL18RAP 3'UTR
MS analysis
B
PCA
V3
Canonical
V1
Component 2 (9.5%)
Component 1 (17.3%)
C
higher in V1 3'UTR
higher in canonical 3'UTR
dsRBPs
-log10 (P-value)
log2 (fold-change canonical/V1)
D
double-stranded RNA binding proteins
-log10 (FDR)
Normalized enrichment score (canonical/V1)
E
Enrichment plot:
double-stranded RNA binding proteins
(GO:0003725)
Enrichment Score
Ranked list metric
Rank in Ordered Dataset
ELAVL1
EIF2AK2
PRKRA
ADAR
ADARB1
ILF2
ILF3
DHX9
DHX58
DDX58
F
IL18RAP 3'UTR secondary structure prediction
Canonical IL18RAP 3'UTR
(MFE= -39.9 kcal/mol)
Variant IL18RAP 3'UTR (V1)
(MFE= -27.8 kcal/mol)

793 **Fig. 5. Reduced association of double-stranded RNA binding proteins to variant IL18RAP 3'UTR. (A)** Diagram of
794 mass spectrometry of RNA binding proteins pulled-down by IL18RAP 3'UTR sequences (canonical, V1 and V3). **(B)**
795 Principal-component analysis (PCA) of IL18RAP 3'UTR-associated proteomes pulled down by the canonical (grey,
796 N=6 experimental repeats), V3 (orange, N=5), and V1 (blue, N=5) biotin-tagged, in-vitro transcribed oligos. **(C)**
797 Volcano plot of protein abundance associated with the canonical relative to variant (V1) IL18RAP 3'UTR (x-axis
798 log2 scale), analyzed by MS. Y-axis depicts P-values (−log10 scale). Proteins significantly enriched in association
799 with canonical/variant 3'UTR are colored (grey/blue). Double-stranded RNA-binding proteins (dsRBPs) are
800 demarcated by a purple oval. Features above the horizontal dashed line demarcate proteins with adjusted $p <$
801 0.05, in student's t-test with FDR correction to multiple hypotheses. Vertical dashed lines are of 2 or ½ fold change.
802 A non-significant data point of KIF13B (P-value = 0.08) is not shown for clarity of the illustration (Supplementary
803 Table 9) **(D)** Volcano plot of normalized enrichment score of the Gene Ontology (GO) molecular function gene sets
804 from GSEA analysis of differentially expressed proteins (canonical vs. V1 IL18RAP 3'UTR). Reduced association of
805 double-stranded RNA binding proteins (GO:0003725; purple) with V1 IL18RAP 3'UTR, relative to the canonical
806 3'UTR. All gene sets are described in Supplementary Table 10. **(E)** Profile of GSEA enrichment score and positions
807 of the 10 double-stranded RNA binding proteins (purple) within all differentially expressed proteins, ranked from
808 most enriched in canonical 3'UTR to most depleted protein (Supplementary Table 10; WebGestalt [104]). **(F)**
809 Prediction of 3'UTR secondary structure by RNA Fold [105] suggests a more stable dsRNA structure of canonical 3'UTR
810 (green), with lower minimum free energy (MFE) than that of the sequence harboring a V1 variant (red).

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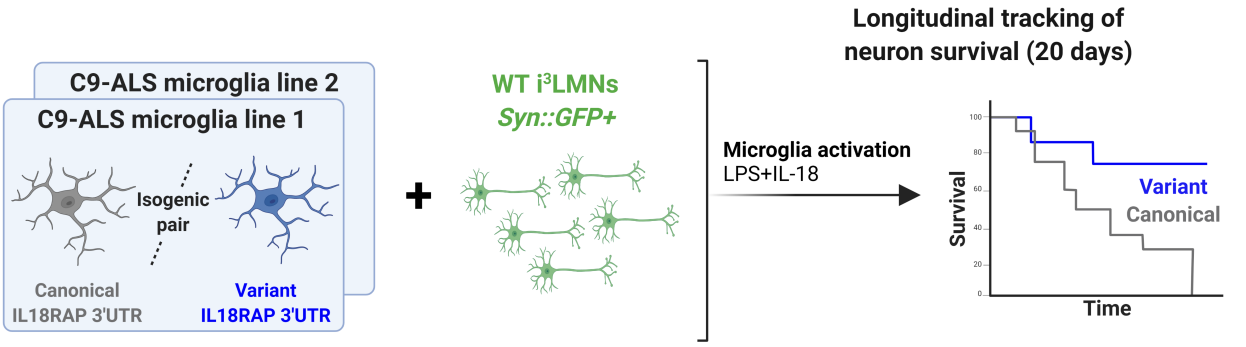
A Co-culture of isogenic IL18RAP 3'UTR C9-ALS microglia and WT iMNs



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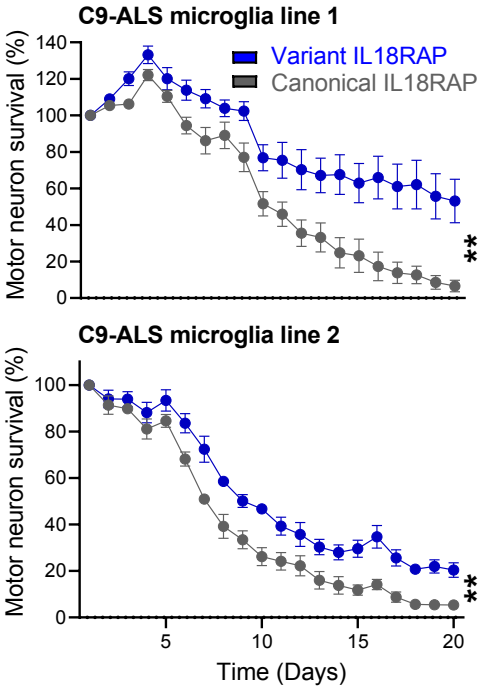

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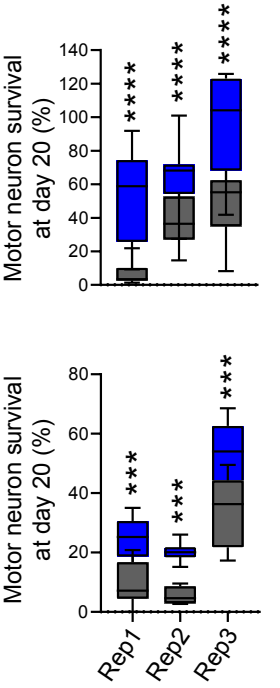

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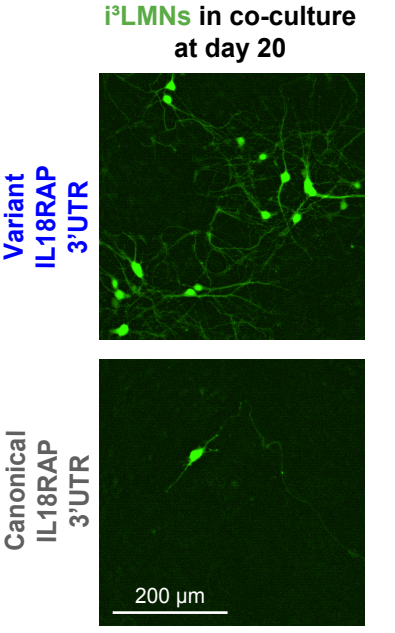

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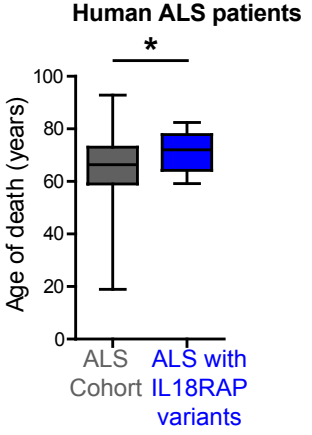

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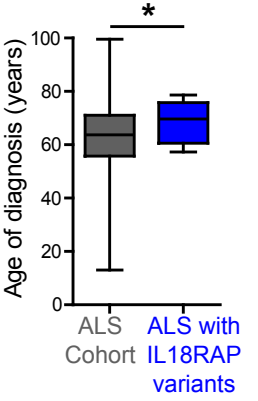

820 **Fig. 6. Variant IL18RAP 3'UTR are protective in human microglia and in patients with ALS. (A)** Diagram of
821 experimental design. Co-culture of human iPSC-derived transcription factor-induced motor neurons (i^3LMNs) that
822 express GFP driven by the synapsin (Syn) promoter (healthy, non-ALS, [76]) and human iPSC-derived isogenic
823 IL18RAP 3'UTR microglia (on a *C9orf72 repeat expansion background)*. Time-lapse microscopic analyses of i^3LMNs
824 survival, after microglia activation with a cocktail of LPS and the cytokine IL-18. **(B,C)** i^3LMNs survival over 20 days
825 in the presence of microglia harboring variant (blue) or canonical (grey) IL18RAP 3'UTR (two independent isogenic
826 pairs, based on independent patient C9orf72 lines, n=3 independent differentiation procedures from different
827 passages per line, with 3-8 co-culture wells per passage). **(B)** Survival plot of i^3LMNs in a representative experiment
828 for each isogenic pair (Two-way ANOVA) and **(C)** Box plot depicting the percentage of i^3LMNs survival on day 20
829 of co-culture, median, upper and lower quartiles of all experiments. Two independent isogenic pairs, based on
830 independent patient C9orf72 lines, n=3 independent differentiation procedures from different passages per line,
831 with 3-8 co-culture wells per passage. Two-way ANOVA followed by Tukey's multiple comparison test. **(D)**
832 Representative micrographs of fluorescent i^3LMNs after 20 days of culture with C9-ALS microglia. **(E)**. Association
833 of age of death (9 patients with protective 3'UTR variants /4263 patients with available phenotypic data in Project
834 MinE and NYGC cohorts, or **(F)** age of diagnosis (8/4216 patients). IL18RAP variant is associated with delayed age
835 of death (+6.1 years, Permutation P-value = 0.02, Cohen's d effect size = 0.65) and age of diagnosis (+6.2 years,
836 Permutation P-value = 0.05, Cohen's d effect size = 0.62), relative to the mean age of all Project MinE and NYGC
837 ALS patients. Box plots depicting median, upper and lower quartiles, and extreme points. * P<0.05, ** P<0.01,
838 *** P<0.001, **** P<0.0001.

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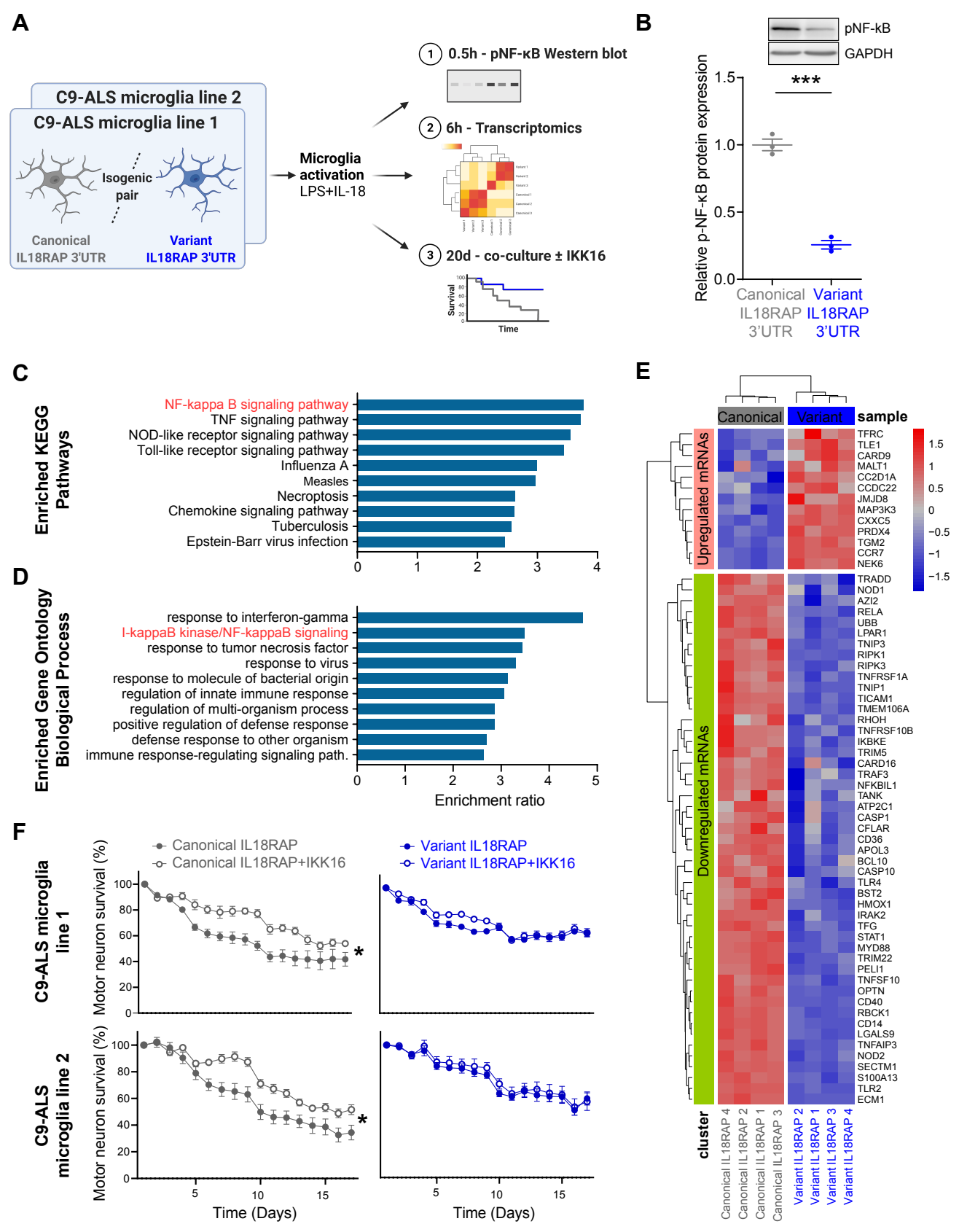
A
C9-ALS microglia line 2
C9-ALS microglia line 1
Isogenic pair
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
Microglia activation LPS+IL-18
1 0.5h - pNF-κB Western blot
2 6h - Transcriptomics
3 20d - co-culture ± IKK16
Survival
Time
B
pNF-kB
GAPDH
***
Relative p-NF-κB protein expression
1.5
1.0
0.5
0.0
Canonical IL18RAP 3'UTR
Variant IL18RAP 3'UTR
C
Enriched KEGG Pathways
NF-kappa B signaling pathway
TNF signaling pathway
NOD-like receptor signaling pathway
Toll-like receptor signaling pathway
Influenza A
Measles
Necroptosis
Chemokine signaling pathway
Tuberculosis
Epstein-Barr virus infection
0 1 2 3 4
D
Enriched Gene Ontology Biological Process
response to interferon-gamma
I-kappaB kinase/NF-kappaB signaling
response to tumor necrosis factor
response to virus
response to molecule of bacterial origin
regulation of innate immune response
regulation of multi-organism process
positive regulation of defense response
defense response to other organism
immune response-regulating signaling path.
0 1 2 3 4 5
Enrichment ratio
E
Canonical
Variant
sample
Upregulated mRNAs
Downregulated mRNAs
TFRC
TLE1
CARD9
MALT1
CC2D1A
CCDC22
JMJD8
MAP3K3
CXXC5
PRDX4
TGM2
CCR7
NEK6
TRADD
NOD1
AZI2
RELA
UBB
LPAR1
TNIP3
RIPK1
RIPK3
TNFRSF1A
TNIP1
TICAM1
TMEM106A
RHOH
TNFRSF10B
IKBKE
TRIM5
CARD16
TRAF3
NFKBIL1
TANK
ATP2C1
CASP1
CFLAR
CD36
APOL3
BCL10
CASP10
TLR4
BST2
HMOX1
IRAK2
TFG
STAT1
MYD88
TRIM22
PELI1
TNFSF10
OPTN
CD40
RBCK1
CD14
LGALS9
TNFAIP3
NOD2
SECTM1
S100A13
TLR2
ECM1
1.5
1
0.5
0
−0.5
−1
−1.5
cluster
Canonical IL18RAP 4
Canonical IL18RAP 2
Canonical IL18RAP 1
Canonical IL18RAP 3
Variant IL18RAP 2
Variant IL18RAP 1
Variant IL18RAP 3
Variant IL18RAP 4
F
C9-ALS microglia line 1
C9-ALS microglia line 2
Canonical IL18RAP
Canonical IL18RAP+IKK16
Variant IL18RAP
Variant IL18RAP+IKK16
Motor neuron survival (%)
100
80
60
40
20
0
*
5
10
15
Time (Days)

848 **Fig. 7. Variant IL18RAP 3'UTR dampens neurotoxic NF-κB signaling in human microglia. (A)** Diagram of
849 experimental design. Four IL18RAP 3'UTR lines (two isogenic pairs) were differentiated into human microglia [68]
850 and analyzed for phosphorylated NF-κB protein levels, transcriptomics, and neuronal survival in co-culture
851 with/without IKK16, following activation with a cocktail of LPS and the cytokine IL-18, for 0.5h, 6h and 20 days,
852 respectively. **(B)** Western blot analysis revealed reduced levels of phosphorylated NF-κB in variant IL18RAP 3'UTR
853 relative to isogenic control. Scatter dot plot with mean and SEM (Two sided t-test P-value *** <0.001, N=3, Data
854 File S5). mRNA extracted from human microglia was subjected to a next generation sequencing study with
855 downstream bioinformatics studies **(C-E)**. Over-representation analysis (ORA) within **(C)** KEGG Pathways and **(D)**
856 Gene Ontology biological processes, of the differentially expressed transcriptome in microglia harboring variant
857 vs. canonical IL18RAP 3'UTR. Bar graph depicting the Ratio of enrichment for significantly enriched pathways (FDR
858 ≤ 0.05) are shown (Supplementary Table 13; WebGestalt [104]). **(E)** Unsupervised study of the NF-κB transcriptomic
859 signature (GO:0007249 pathway-associated mRNAs) in microglia with the variant relative to the isogenic canonical
860 IL18RAP 3'UTR. **(F)** Time-lapse microscopic analyses of co-cultured human i^3LMNs (healthy, non-ALS, [76]) with
861 human iPSC-derived isogenic IL18RAP 3'UTR microglia (on a *C9orf72 repeat expansion background)*, activated with
862 a cocktail of LPS and the cytokine IL-18, without (carrier alone, DMSO), or with IKK16 (200nM), a selective IκB
863 kinase (IKK) inhibitor that inhibits NF-κB signaling [81]. IKK16 significantly ameliorates motor neuron death, relative
864 to control only in the context of canonical IL18RAP 3'UTR, but did not further contribute to rescue in human
865 microglia with the protective variant IL18RAP 3'UTR (two independent isogenic pairs, based on independent
866 patient C9orf72 lines with 3-8 co-culture wells per line). Two-way ANOVA P-value * <0.05.

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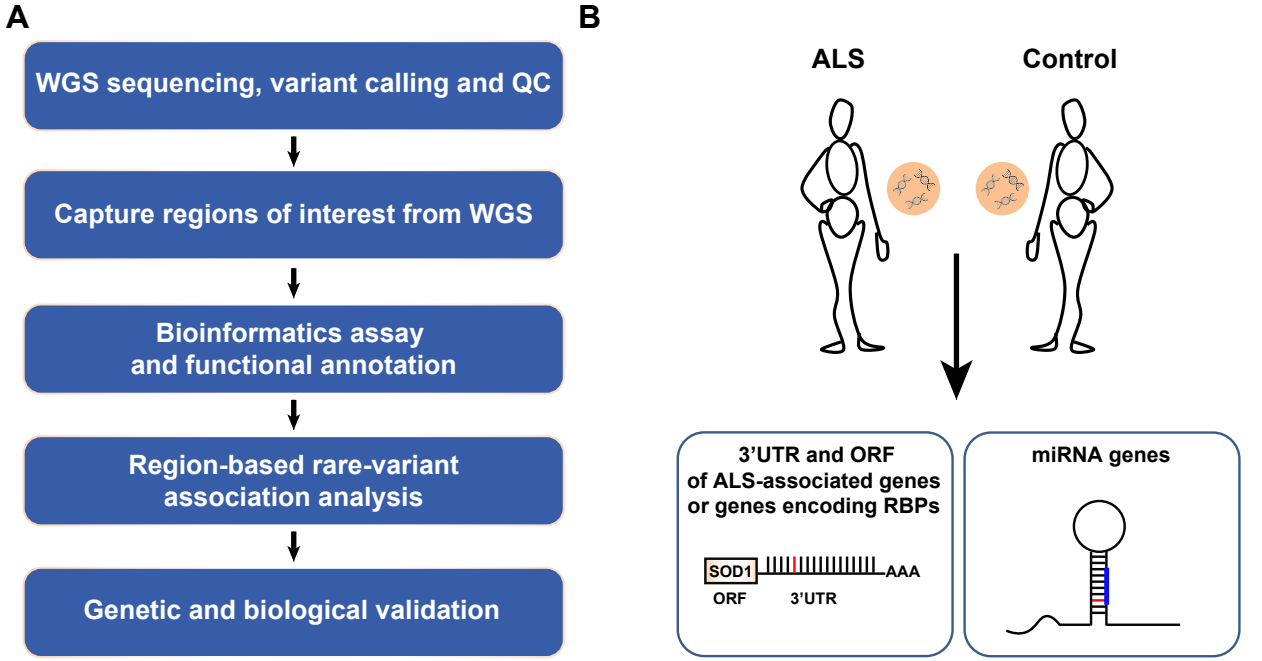

Supplementary Fig.1 - Eitan et al. (Hornstein)

871 **Supplementary Fig. 1. Study design. (A)** Flow chart of approach for discovery of region-based rare-variants in
872 non-coding genomic regions via association studies and **(B)** diagram depicting regions of interest comprising of
873 1,750 autosomal human pre-miRNA genes, 295 open reading frames encoding for proteins of interest, and 295
874 3'UTRs.

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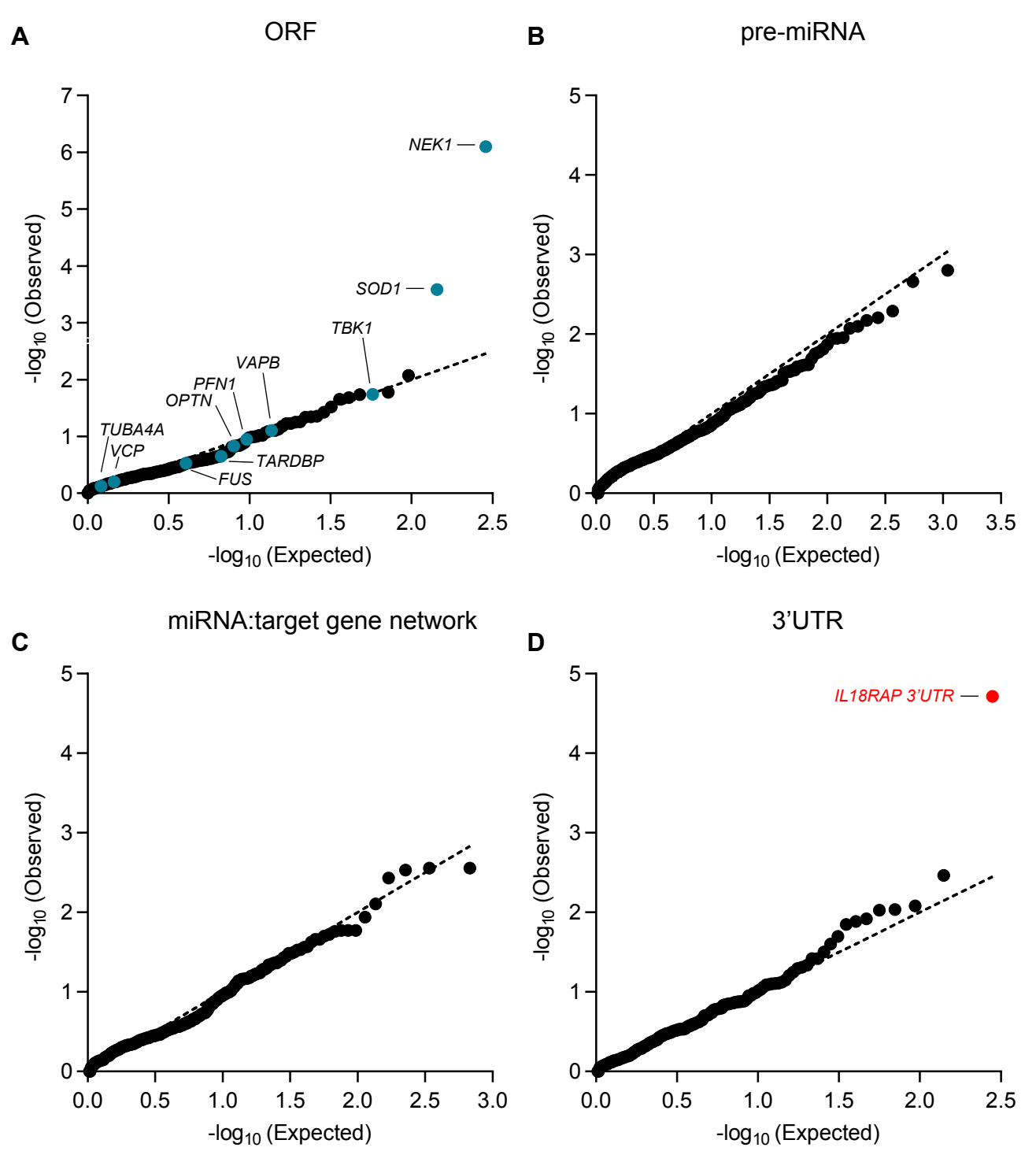
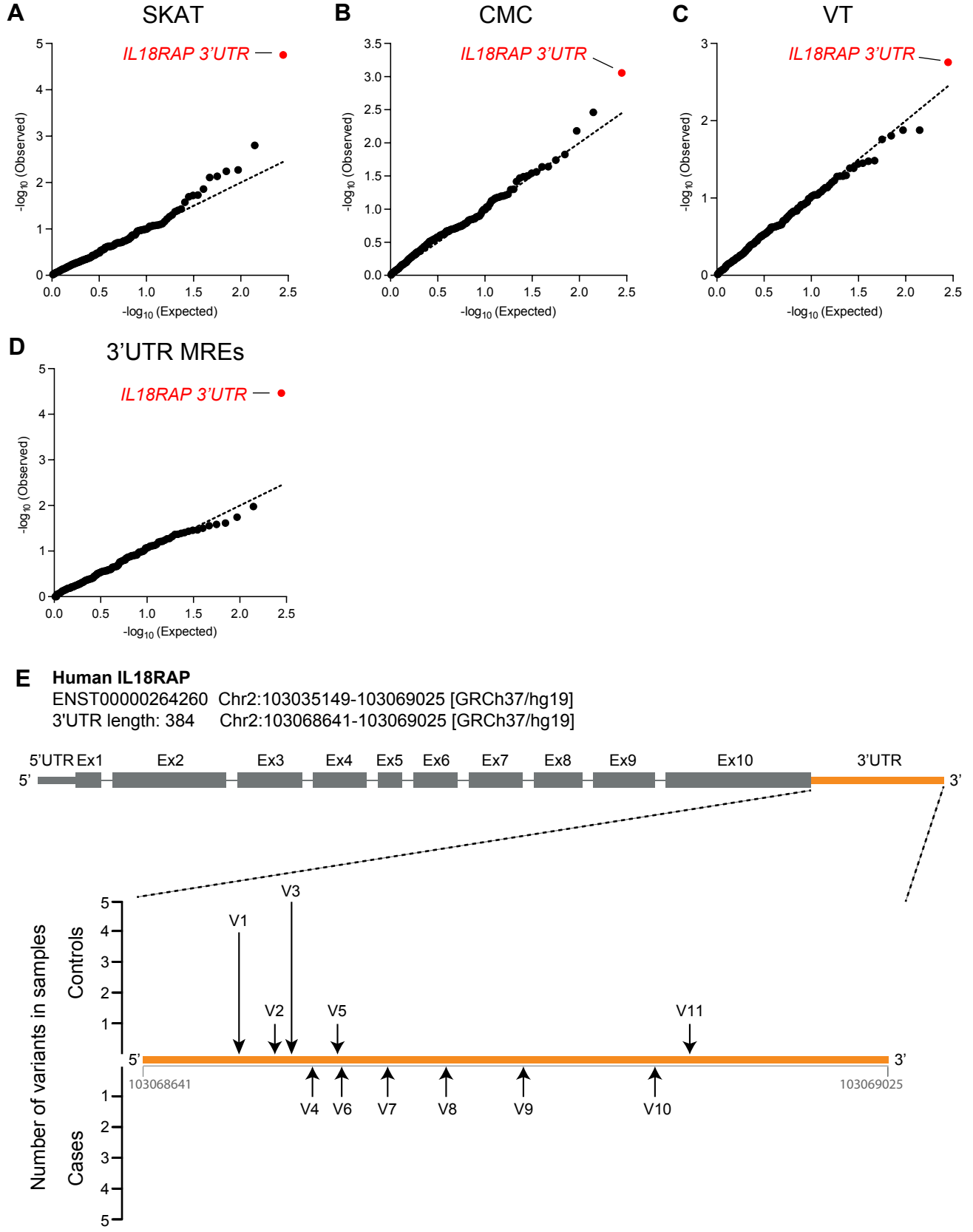


Supplementary Fig.2 - Eitan et al. (Hornstein)

877 **Supplementary Fig. 2. Region-based rare-variant association analyses. (A-D)** QQ plot of obtained and expected
878 P-values for the burden of rare-variants (log scale) gained by collapsed region-based association analysis of
879 different genomic regions, comprised of (A) 295 candidate protein-coding regions listed in Supplementary Table
880 3. These ORFs encode for ALS-relevant proteins or proteins that are associated with miRNA biogenesis or activity.
881 Variants were depicted if predicted to cause frameshifting, alternative splicing, abnormal stop codon or a
882 deleterious non-synonymous amino acid substitution, in ≥ 3 of 7 independent dbNSFP prediction algorithms
883 (genomic inflation $\lambda = 0.96$), (B) All known pre-miRNA genes in the human genome (genomic inflation $\lambda = 1.31$),
884 (C) predicted networks, comprised of aggregated variants detected on a specific mature miRNA sequence and its
885 cognate downstream 3'UTR targets (genomic inflation $\lambda = 1.16$), and (D) variants in 3'UTRs of the same 295 genes
886 listed in Supplementary Table 3 (genomic inflation $\lambda = 1.08$). Data was obtained from 3,955 ALS cases and 1,819
887 controls (Project MinE). Features positioned on the diagonal line represent results obtained under the null
888 hypothesis. Open-reading frames of 10 known ALS genes (blue). IL18RAP 3'UTR (red). P-values, calculated with
889 SKAT-O.


A
SKAT
IL18RAP 3'UTR
-log10 (Observed)
-log10 (Expected)
B
CMC
IL18RAP 3'UTR
-log10 (Observed)
-log10 (Expected)
C
VT
IL18RAP 3'UTR
-log10 (Observed)
-log10 (Expected)
D
3'UTR MREs
IL18RAP 3'UTR
-log10 (Observed)
-log10 (Expected)
E
Human IL18RAP
ENST00000264260 Chr2:103035149-103069025 [GRCh37/hg19]
3'UTR length: 384 Chr2:103068641-103069025 [GRCh37/hg19]
5'UTR Ex1 Ex2 Ex3 Ex4 Ex5 Ex6 Ex7 Ex8 Ex9 Ex10 3'UTR
5'
3'
Number of variants in samples
Controls
Cases
V1
V2
V3
V4
V5
V6
V7
V8
V9
V10
V11
103068641
103069025

890 **Supplementary Fig. 3. 3'UTR-based rare-variant association analysis, using different algorithms, and illustration**
891 **of rare variants identified in the IL18RAP 3'UTR. (A-D)** QQ plot of obtained and expected P-values for the burden
892 of rare variants (log scale) gained by collapsed region-based association analysis of genomic regions comprised of
893 295 3'UTRs listed in Supplementary Table 3, in the Project MinE cohort (3,955 ALS cases and 1,819 non-ALS
894 controls). Features positioned on the diagonal line represent results obtained under the null hypothesis. IL18RAP
895 3'UTR (red) is the most significant 3'UTR associated with ALS using different algorithms: **(A)** Sequence Kernel
896 Association Test, SKAT (genomic inflation $\lambda = 1.02$), **(B)** Combined Multivariate and Collapsing, CMC (genomic
897 inflation $\lambda = 1.34$), **(C)** Variable Threshold with permutation analysis, VT (genomic inflation $\lambda = 1.03$). **(D)** IL18RAP
898 3'UTR also ranked as the top hit when aggregating variants abrogating or gaining miRNA recognition elements
899 (MREs) in 3'UTRs (genomic inflation $\lambda = 1.04$). **(E)** Schematic of the IL18RAP transcript and 3'UTR (5' to 3') showing
900 the number of control (upper) or ALS (lower) samples in which variants (black arrow) were identified in the Project
901 MinE discovery cohort (Supplementary Table 6).

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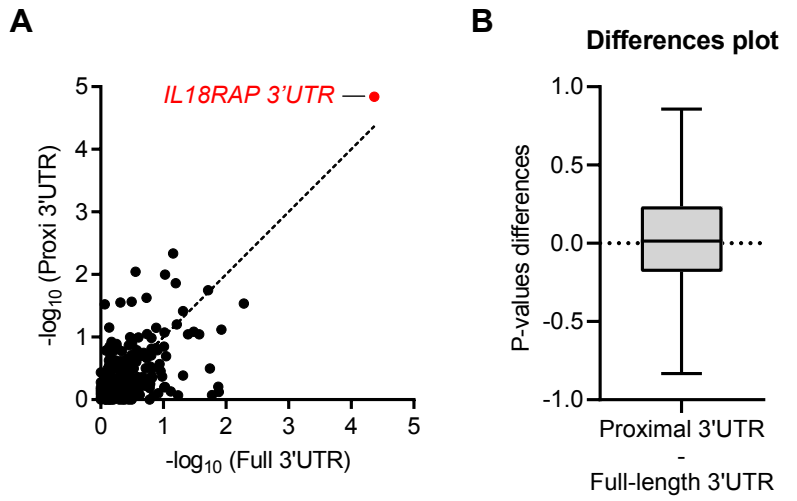
A
IL18RAP 3'UTR
-log10 (Proxi 3'UTR)
-log10 (Full 3'UTR)
0
1
2
3
4
5
B
Differences plot
P-values differences
1.0
0.5
0.0
-0.5
-1.0
Proximal 3'UTR
-
Full-length 3'UTR

915 **Supplementary Fig. 4. Restricting burden analysis to the proximal part of 3'UTRs does not improve the**
916 **association signal. (A)** Scatter plot with SKAT-O P-values (log scale) calculated for the burden of rare variants
917 gained by collapsed region-based association analysis of the full 3'UTRs on the x-axis versus the 3'UTRs proximal
918 quadrant on the y-axis, for the 295 3'UTRs listed in Supplementary Table 3, in the Project MinE cohort (3,955 ALS
919 cases and 1,819 non-ALS controls) (Pearson correlation coefficient (r=0.61) and P-value ****<0.0001). The 45-
920 degree diagonal line represents a perfect correlation of r=1. IL18RAP 3'UTR (red). **(B)** A Difference plot showing
921 the difference between the two P-value measurements (3'UTRs proximal quadrant minus the full 3'UTRs). The
922 bias (difference between means) is only 0.03. Overall the P-values gained from the 3'UTRs proximal quadrant were
923 comparable to that of the full 3'UTRs in the cohort of 295 3'UTRs. Box plots depict median, upper and lower
924 quartiles, and extreme points (Wilcoxon matched-pairs P-value > 0.05, Cohen's d effect size = 0.1). Hence, the
925 apparent spatial distribution of variants in IL18RAP 3'UTR seems to be a particular case, rather than part of a
926 global pattern.

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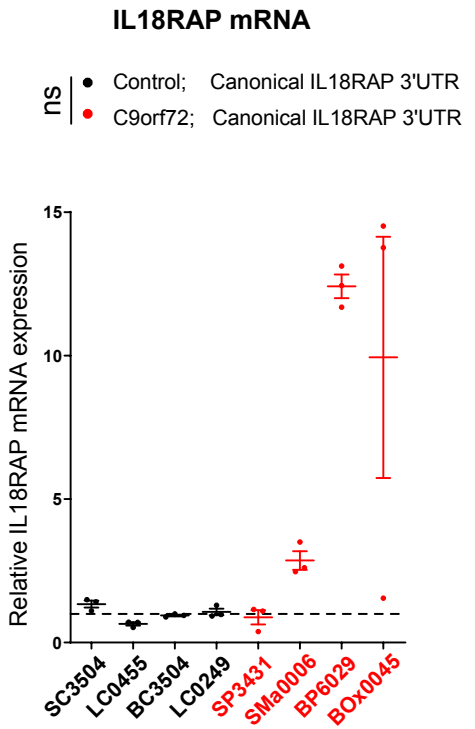
Lymphoblastoid cell lines

A



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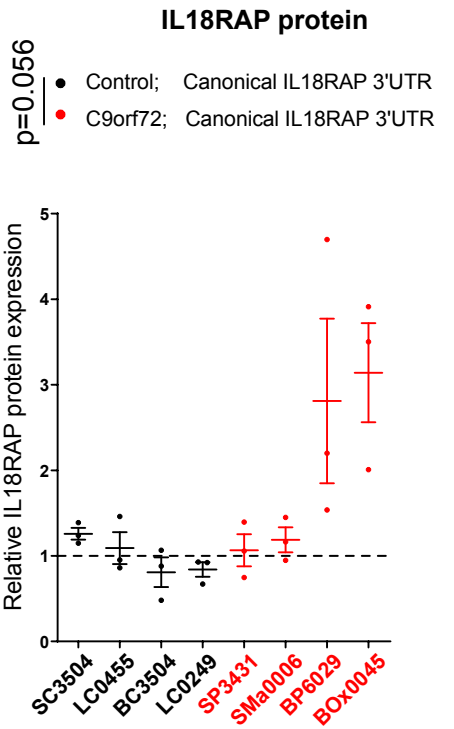

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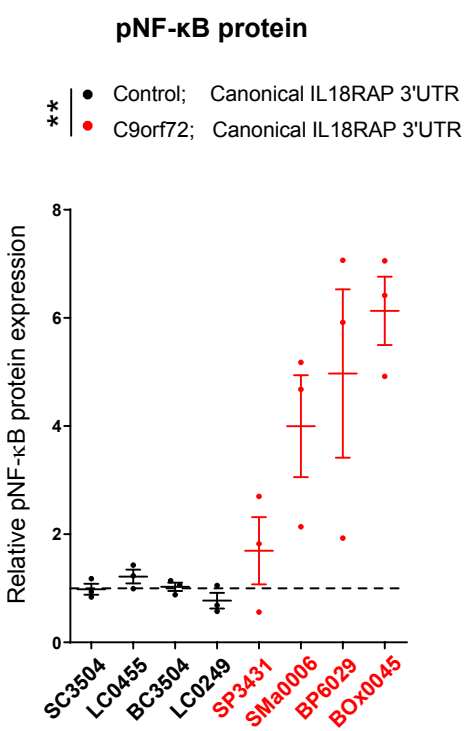
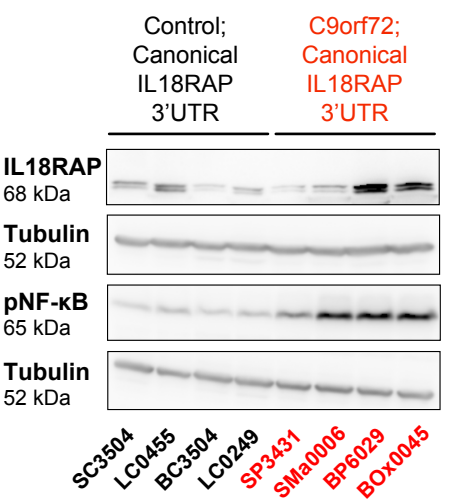


D



940 **Supplementary Fig. 5. IL18RAP and p-NF-κB expression is elevated in lymphoblastoid cells from patients with**
941 **the C9orf72 repeat expansion. (A)** IL18RAP mRNA expression (qPCR normalized to IPO8 mRNA levels) and **(B)**
942 IL18RAP or **(C)** p-NF-κB protein expression (Western blots, normalized to Tubulin). Extracts from eight different
943 human lymphoblastoid cell lines (listed in Supplementary Table 8): Four lines of healthy individuals (without ALS)
944 carrying the canonical IL18RAP 3'UTR sequence (Control; Canonical IL18RAP 3'UTR, black) and four C9orf72 ALS
945 patients carrying the canonical IL18RAP 3'UTR sequence (C9orf72; Canonical IL18RAP 3'UTR, red). **(D)**
946 Representative blots processed with anti-IL18RAP, anti p-NF-κB and anti-Tubulin antibodies. Mann-Whitney test
947 (A) or one-sided student's t-test with Welch's correction on log-transformed data (B,C), was conducted based on
948 the mean value of three independent passages for each of the eight human lymphoblastoid cell lines. Scatter dot
949 plot with mean and SEM. ** $P<0.01$.

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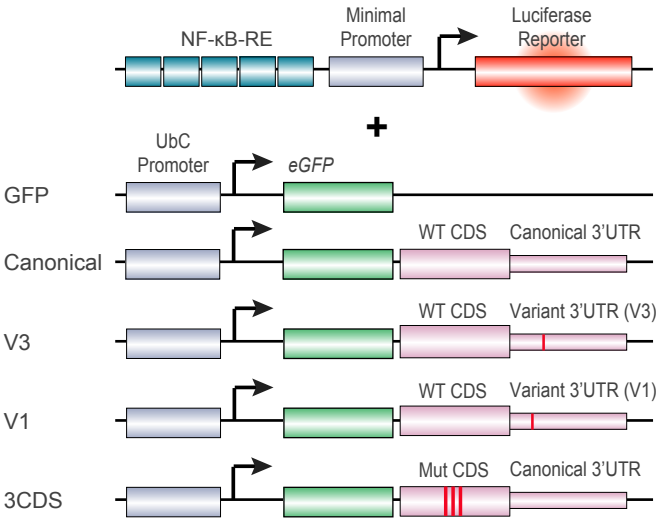
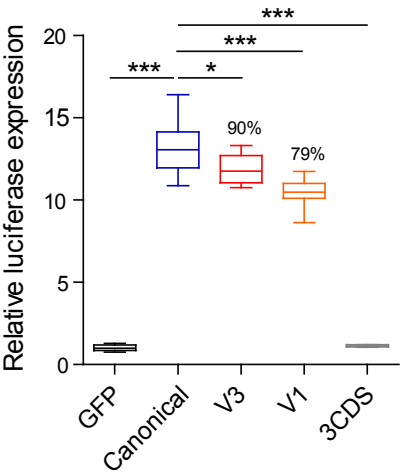
A Biosensor for NF-κB pathway activity



B U2OS cells



964 **Supplementary Fig. 6. IL18RAP 3'UTR variant attenuates IL-18 - NF-κB signaling in U2OS cells.** Diagram **(A)** and
965 quantification **(B)** of NF-κB reporter assays in human U2OS cell line. To determine the ability of the IL18RAP
966 variants V3 and V1 to induce NF-κB activity, U2OS cells were co-transfected with different IL18RAP coding region
967 (CDS) and 3'UTR constructs (GFP, Canonical, V3, V1, n=9; 3CDS, *n*=4), along with an NF-κB activity reporter that
968 drives luciferase (Luc2P) transcription via five copies of the NF-κB response element. NF-κB signaling was induced
969 by adding human recombinant IL-18 to the medium. Variants V3 and V1 of the IL18RAP 3'UTR reduced NF-κB
970 activity by ~10% and ~21%, respectively, relative to the WT IL18RAP 3'UTR. GFP vector and a dominant-negative
971 coding mutant E210A-Y212A-Y214A CDS + WT 3'UTR (3CDS) [41], served as controls. Luciferase expression was
972 normalized to transfected U2OS cells that were not induced with human recombinant IL-18. One-way ANOVA
973 followed by Dunnett's multiple comparison test was performed on square root-transformed data. Box plots depict
974 median, upper and lower quartiles, and extreme points. * P<0.05; *** P<0.001. The experiment was repeated
975 independently three times with similar results.

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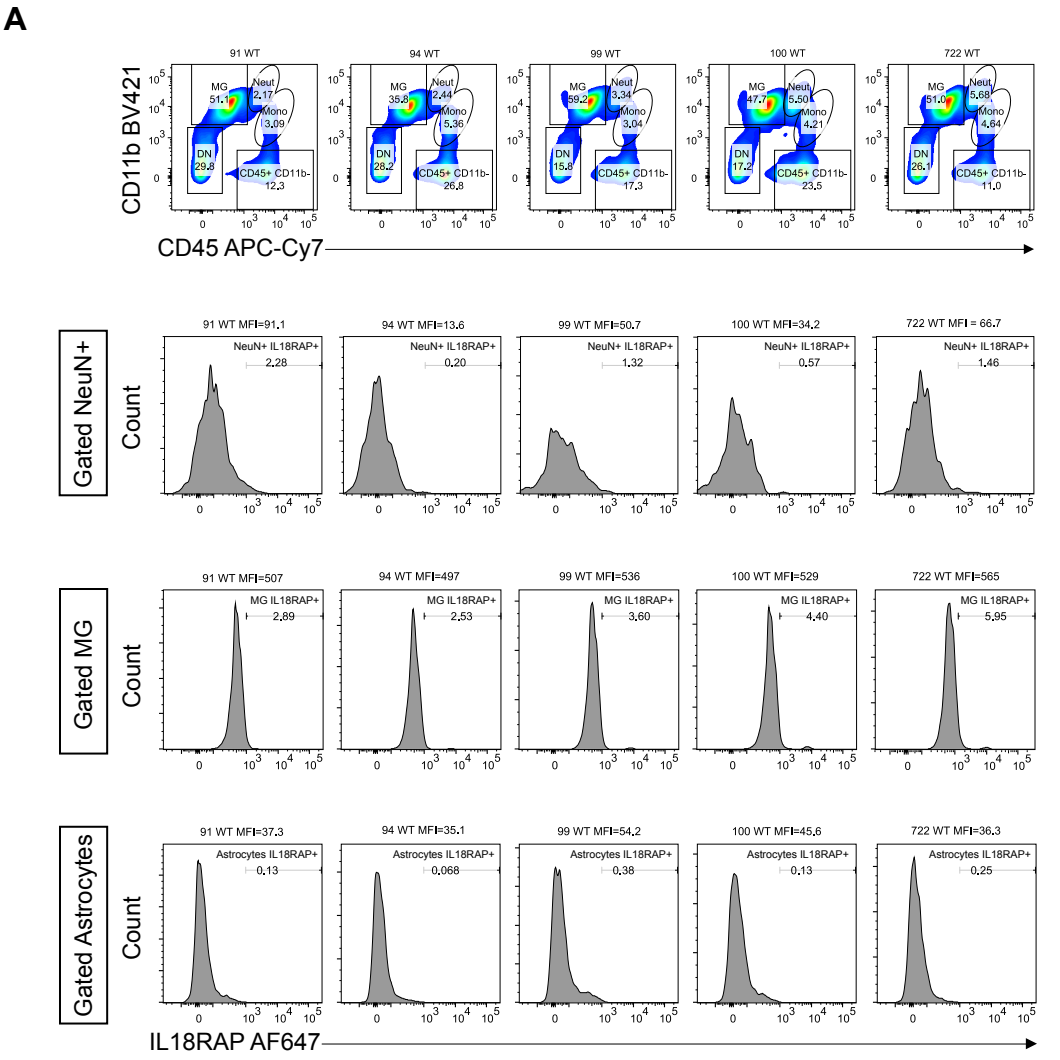
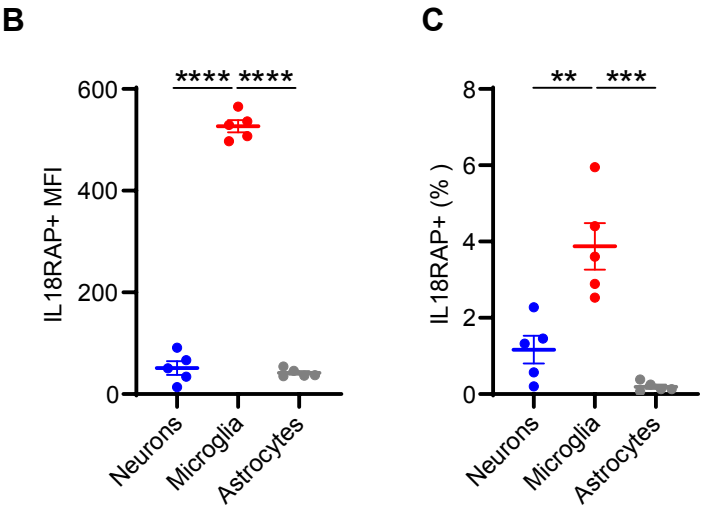

A
91 WT
94 WT
99 WT
100 WT
722 WT
CD11b BV421
CD45 APC-Cy7
MG 51.1
Neut 2.17
Mono 3.09
DN 29.8
CD45+ CD11b- 12.3
MG 35.8
Neut 2.44
Mono 5.36
DN 28.2
CD45+ CD11b- 26.8
MG 59.2
Neut 3.34
Mono 3.04
DN 15.8
CD45+ CD11b- 17.3
MG 47.7
Neut 5.50
Mono 4.21
DN 17.2
CD45+ CD11b- 23.5
MG 51.0
Neut 5.68
Mono 4.64
DN 26.1
CD45+ CD11b- 11.0
Gated NeuN+
Count
91 WT MFI=91.1
NeuN+ IL18RAP+ 2.28
94 WT MFI=13.6
NeuN+ IL18RAP+ 0.20
99 WT MFI=50.7
NeuN+ IL18RAP+ 1.32
100 WT MFI=34.2
NeuN+ IL18RAP+ 0.57
722 WT MFI = 66.7
NeuN+ IL18RAP+ 1.46
Gated MG
Count
91 WT MFI=507
MG IL18RAP+ 2.89
94 WT MFI=497
MG IL18RAP+ 2.53
99 WT MFI=536
MG IL18RAP+ 3.60
100 WT MFI=529
MG IL18RAP+ 4.40
722 WT MFI=565
MG IL18RAP+ 5.95
Gated Astrocytes
Count
91 WT MFI=37.3
Astrocytes IL18RAP+ 0.13
94 WT MFI=35.1
Astrocytes IL18RAP+ 0.068
99 WT MFI=54.2
Astrocytes IL18RAP+ 0.38
100 WT MFI=45.6
Astrocytes IL18RAP+ 0.13
722 WT MFI=36.3
Astrocytes IL18RAP+ 0.25
IL18RAP AF647


B
IL18RAP+ MFI
600
400
200
0
**** ****
Neurons
Microglia
Astrocytes
C
IL18RAP+ (%)
8
6
4
2
0
** ***
Neurons
Microglia
Astrocytes

989 **Supplementary Fig. 7. IL18RAP is mainly expressed on mouse microglia cells.** (A-C) Flow cytometry was used to
990 characterize IL18RAP expression levels in dissociated wild-type mouse cortex cells. The expression of IL-18RAP (IL-
991 18Rβ) was expressed as Mean Fluorescence Intensity (MFI) and % frequency after gating for the following cell
992 types: immune cells (CD45hi), microglia (MG: CD45int CD11hi), neurons (CD45- CD11b- NeuN+), and astrocytes
993 (CD45- CD11b- GFAP+). FACS analysis reveals that IL18RAP is mainly expressed on microglia cells. A scatter dot
994 plot with mean and SEM values for the median fluorescence intensity (MFI) and percentage of IL18RAP+ cells is
995 shown. One-way ANOVA followed by Tukey's multiple comparison test. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

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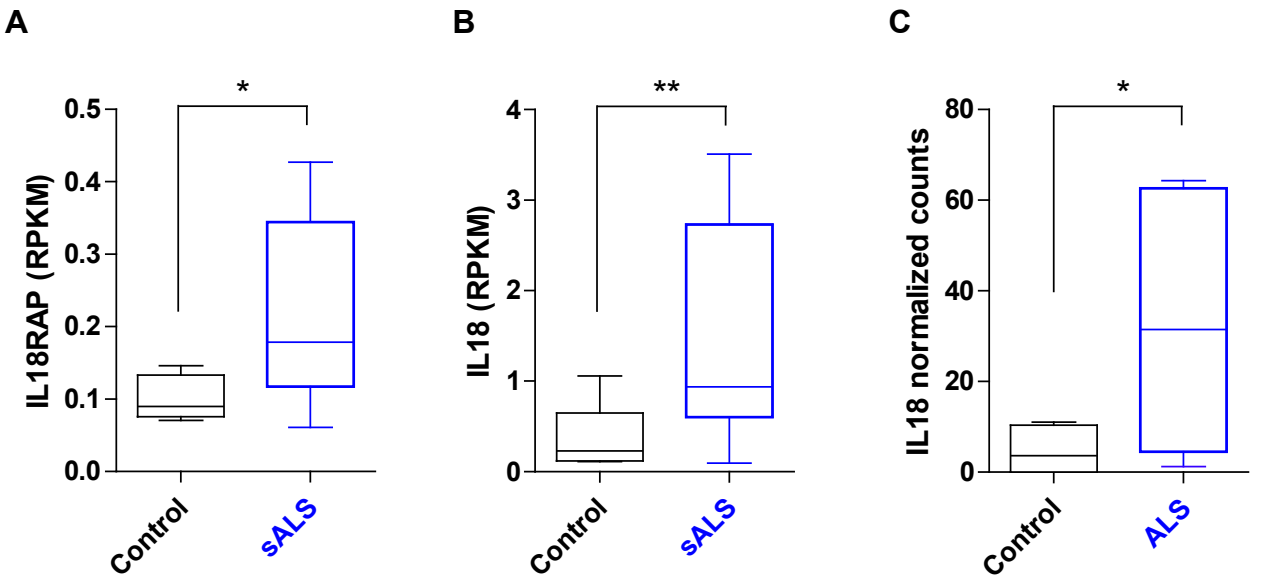
A
B
C
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**
*
IL18RAP (RPKM)
IL18 (RPKM)
IL18 normalized counts
0.5
0.4
0.3
0.2
0.1
0.0
4
3
2
1
0
80
60
40
20
0
Control
sALS
Control
sALS
Control
ALS

1013 **Supplementary Fig. 8. Evaluation of IL18RAP and IL-18 mRNA expression in motor neurons of patients with ALS.**
1014 **(A-B)** mRNA expression of IL18RAP **(A)** and IL-18 **(B)**, as reads per kilobase million (RPKM), from NGS study of laser
1015 capture microdissection–enriched surviving motor neurons from lumbar spinal cords of patients with sALS with
1016 rostral onset and caudal progression (n = 12) and non-neurodegeneration controls (n = 9; [106] GSE76220). Two-
1017 sided Student's t test with Welch's correction on log-transformed data. **(C)** IL-18 mRNA expression, as log2-
1018 normalized counts, from NGS study of induced ALS motor neurons (n = 4 different donors in duplicates) or non-
1019 neurodegeneration controls (n=3 different donors in duplicates; [107] DESeq analysis). Box plots depict median,
1020 upper and lower quartiles, and extreme points. *P < 0.05; **P < 0.01.

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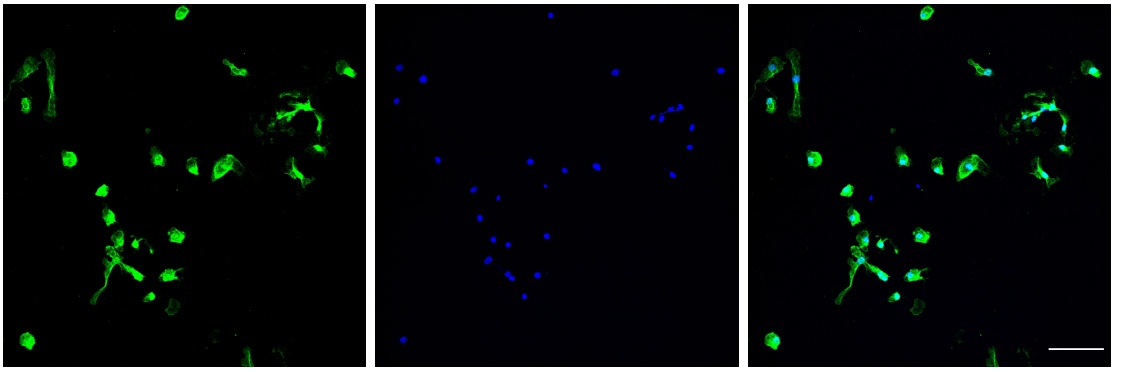
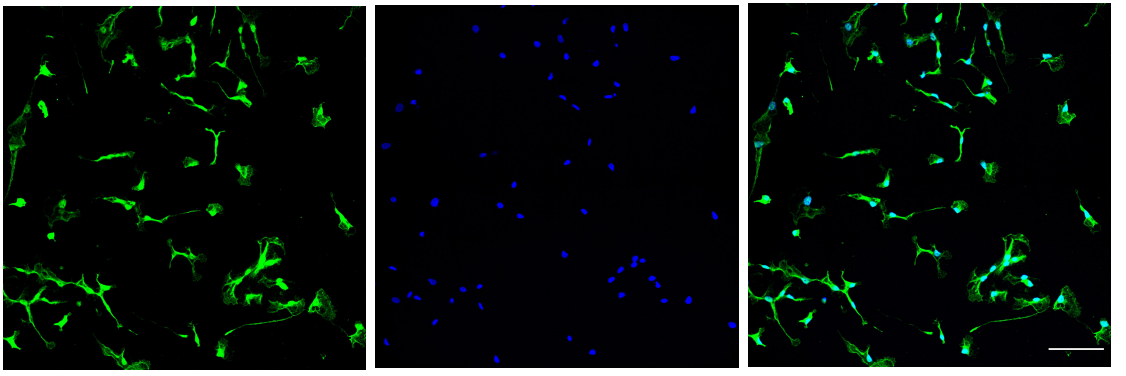
C9-ALS microglia line 1



C9-ALS microglia line 2



1037 **Supplementary Fig. 9. iPSC-derived microglia express the microglial-specific marker, TMEM119.**
1038 Immunofluorescence staining of TMEM119 (green) and DAPI (blue), in two different C9orf72 iPSC-derived
1039 progenitor microglia lines. Lens, ×20; scale bar, 100 µm.

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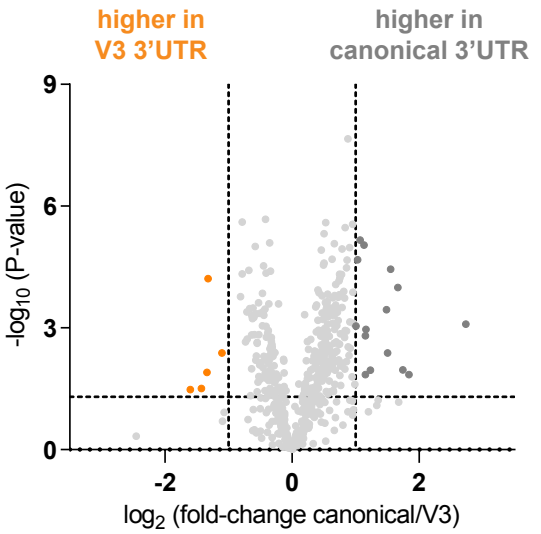
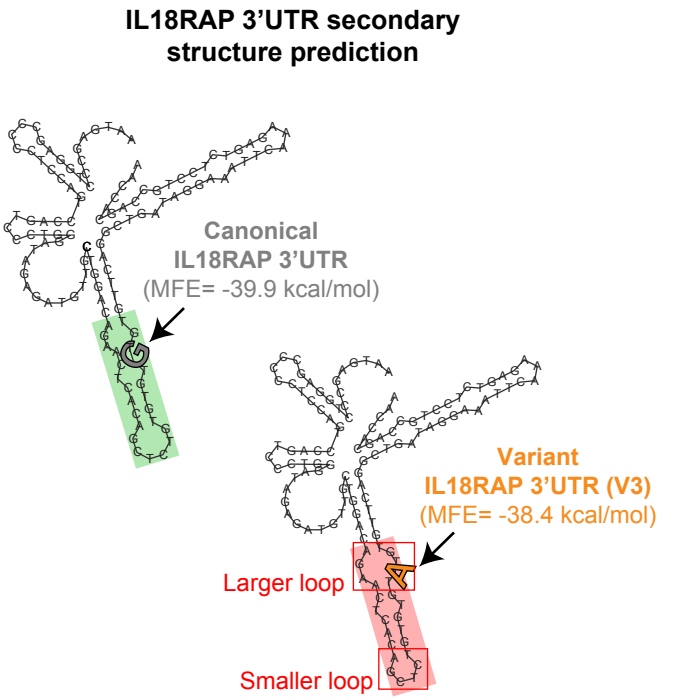
A


higher in
V3 3'UTR
higher in
canonical 3'UTR
9
6
3
0
-log10 (P-value)
-2
0
2
log2 (fold-change canonical/V3)

B


IL18RAP 3'UTR secondary
structure prediction
Canonical
IL18RAP 3'UTR
(MFE= -39.9 kcal/mol)
Variant
IL18RAP 3'UTR (V3)
(MFE= -38.4 kcal/mol)
Larger loop
Smaller loop

1059 **Supplementary Fig. 10. Differentially bound RNA binding proteins to variant 3'UTR (V3) relative to canonical**
1060 **3'UTR. (A)** Volcano plot of protein abundance associated with the canonical relative to variant (V3) IL18RAP 3'UTR
1061 (x-axis log2 scale), analyzed by MS. Y-axis depicts P-values (−log10 scale). Proteins significantly enriched in
1062 association with canonical/variant 3'UTR are colored (grey/orange). Features above the horizontal dashed line
1063 demarcate proteins with adjusted $p < 0.05$, in student's t-test with FDR correction to multiple hypotheses. Vertical
1064 dashed lines are of 2 or ½ fold change (Supplementary Table 9). **(B)** Prediction of 3'UTR secondary structure by
1065 RNA Fold [105], suggests a minor change to the structure of the sequence harboring a V3 variant (red), relative to
1066 the canonical 3'UTR (green).

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1083 **References**

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