

Molecular basis of neurodegeneration in a mouse model of *Polr3*-related disease

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Abstract

Pathogenic variants in subunits of RNA polymerase (Pol) III cause a spectrum of *Polr3*-related neurodegenerative diseases including 4H leukodystrophy. Disease onset occurs from infancy to early adulthood and is associated with a variable range and severity of neurological and non-neurological features. The molecular basis of *Polr3*-related disease pathogenesis is unknown. We developed a postnatal whole-body mouse model expressing pathogenic *Polr3a* mutations to examine the molecular mechanisms by which reduced Pol III transcription results primarily in central nervous system phenotypes. *Polr3a* mutant mice exhibit behavioral deficits, cerebral pathology and exocrine pancreatic atrophy. Transcriptome and immunohistochemistry analyses of cerebra during disease progression show a reduction in most Pol III transcripts, induction of innate immune and integrated stress responses and cell type-specific gene expression changes reflecting neuron and oligodendrocyte loss and microglial activation. Earlier in the disease when integrated stress and innate immune responses are minimally induced, mature tRNA sequencing revealed a global reduction in tRNA levels and an altered tRNA profile but no changes in other Pol III transcripts. Thus, changes in the size and/or composition of the tRNA pool have a causal role in disease initiation. Our findings reveal different tissue- and brain region-specific sensitivities to a defect in Pol III transcription.

eLife Assessment

This study provides **important** insights into the mechanistic basis of neurological manifestations of RNA polymerase III-related disease by creating a mutant mouse to dissect transcriptional changes. The data provide **compelling** evidence for disease progression initiated by a global reduction in tRNA levels leading to integrated stress and innate immune responses and neuronal loss. The work will be of interest to those engaged in the study of chromosome biology, developmental biology and neurodegeneration.

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Introduction

Biallelic pathogenic mutations in multiple subunits of RNA polymerase (Pol) III are causally associated with a spectrum of neurodegenerative diseases (Lata et al. 2021 [↗](#); Watt et al. 2023 [↗](#)). These *Polr3*-related disorders include a prevalent form of leukodystrophy with hypomyelination, hypodontia and hypogonadotropic hypogonadism (4H leukodystrophy) as distinguishing features along with cerebellar atrophy, myopia and short stature (Wolf et al. 2014 [↗](#)). Patients with *Polr3*-related leukodystrophy typically present in early childhood with motor deficits and developmental delay. The disease is inherited in an autosomal recessive manner, is progressive and exhibits wide ranging phenotypic severity from premature death during infancy to mild forms that have been diagnosed in adults (Watt et al. 2023 [↗](#)). The neurologic and non-neurologic manifestations of *Polr3*-related disorders are variably penetrant. Notably, initial findings of hypomyelination, suggesting oligodendrocyte involvement, are not universal as some patients have normal or near normal myelination but distinct MRI and neuropathological changes that suggest a largely neuronal phenotype (Perrier et al. 2022 [↗](#)). Other atypical forms of *Polr3*-related disease have also been reported (Lata et al. 2021 [↗](#); Watt et al. 2023 [↗](#)). The bases for these different disease presentations are unclear. It is also unclear why mutations in Pol III, a ubiquitously-expressed, essential enzyme responsible for the synthesis of critical non-coding RNAs central to protein synthesis, pre-mRNA splicing, secretion and other processes, result in phenotypes primarily of the central nervous system.

Phenotypic variability in *Polr3*-related disease is likely to reflect differences in the abundance of Pol III, its residual activity and how these changes affect the Pol III transcriptome at sensitive periods during embryonic and postnatal development. These properties are difficult to assess given the structural complexity of the 17 subunit Pol III enzyme and the fact that most patients carry compound heterozygous mutations that are likely to differentially affect enzyme function (Wolf et al. 2014 [↗](#)). Genetic modifiers of *Polr3*-related phenotypes are also likely given the observed phenotypic variation between unrelated patients with the same *Polr3* mutation (Bernard et al. 2011 [↗](#); Perrier et al. 2020 [↗](#)). Few studies have examined the effect of pathogenic *Polr3* mutations on the levels of Pol III-derived transcripts in mammalian cells. Collectively, experiments with patient-derived fibroblasts and CRISPR-Cas9 engineered cell lines show that precursor tRNA levels are generally lower but only one of four mature tRNAs tested in two different studies were reduced, suggesting that high tRNA stability can compensate for defects in synthesis (Dorboz et al. 2018 [↗](#); Choquet et al. 2019 [↗](#)). 7SL RNA levels were uniformly lower in agreement with a separate analysis of Pol III-derived transcripts in patient blood (Azmanov et al. 2016 [↗](#)). Other Pol III-transcribed RNAs (5S RNA) showed discordant results or, based on one report, U6, Y, RPPH1, RMRP and 7SK RNAs were unchanged (Choquet et al. 2019 [↗](#)). The extent to which the preceding findings reflect altered RNA levels in the brain is unknown.

In previous work we showed that brain-specific expression of pathogenic *Polr3a* mutations (W671R/G672E) in the mouse Olig2 lineage resulted in impaired growth and developmental delay, deficits in cognitive, sensory, and fine sensorimotor function, and hypomyelination in multiple regions of the cerebrum and spinal cord (Merheb et al. 2021 [↗](#)). We report here a new model of *Polr3*-related disease in which expression of a Pol III enzyme bearing the *Polr3a* mutation is broadly induced postnatally in adolescent mice. Overt pathology was noted only in the exocrine pancreas and cerebrum despite similarly efficient recombination in multiple tissues. Analysis of the Pol III transcriptome reveals a decrease in pre-tRNA and mature tRNA populations and few if any changes among other Pol III transcripts across multiple tissues. Analysis of the Pol II transcriptome reveals activation of the integrated stress response in cerebra but not in other surveyed tissues. The molecular and cellular changes that underlie cerebral neurodegeneration in *Polr3a* mutant mice, which include induction of an innate immune response and activation of microglia, indicate different brain region-specific sensitivities to defective Pol III activity.

Results

A postnatal whole-body *Polr3a* mutant mouse

Patients with *Polr3*-related leukodystrophy express mutant forms of Pol III in all their cells. We sought to generate mice with a similarly broad cellular distribution of a Pol III enzyme bearing the *Polr3a* W671R/G672E mutation. To circumvent the embryonic lethality of the mutation, we employed a ubiquitously-expressed tamoxifen-inducible CAGGCre-ERTM transgene to knockin the *Polr3a* mutant allele postnatally in diverse tissues (Hayashi and McMahon 2002 [↗](#)). Five tamoxifen injections were administered every other day starting at postnatal day 28 (P28, **Fig. 1A,B** [↗](#)). Recombination frequencies determined by flow cytometry or fluorescence microscopy using a dual tdTomato-EGFP reporter were ~60% in liver, ~70% in cerebrum, ~80% in cerebellum and kidney and ~90% in heart (Supplementary Fig. S1). Tamoxifen-induced knockin mice (denoted as *Polr3a*-tamKI mice) have significantly lower body weights than tamoxifen-injected WT controls. Body weight differences appear in both sexes at P34 and become progressively larger as *Polr3a*-tamKI mice lose weight while the control mice gain weight over time (**Fig. 1C** [↗](#)). These differences are due in part to the smaller body size of *Polr3a*-tamKI mice post-injection (**Fig. 1D** [↗](#)), suggesting a growth defect. Notably, a growth defect was reported previously for *Polr3a*-Olig2KI mice (formerly *Polr3a*-cKI mice, Merheb et al. 2021 [↗](#)), consistent with the reduced stature of many patients with Pol III-related leukodystrophy (Watt et al. 2023 [↗](#)). However, in contrast to the current model, *Polr3a*-Olig2KI mice gain weight, albeit more slowly than controls, from adolescence to adulthood despite their smaller size (Merheb et al. 2021 [↗](#)). Thus, the loss of body weight in *Polr3a*-tamKI mice demonstrates a more severe failure to thrive phenotype, in line with the widespread expression of the *Polr3a* mutation.

Impaired myelination and exocrine pancreatic atrophy

We conducted a broad histopathological analysis of the effect of the *Polr3a* mutation in adolescent (P42) and adult (P90) mice. Gross morphology was assessed by Hematoxylin and Eosin (H&E) staining and in the CNS, myelin and neurons were examined using Luxol Fast Blue (LFB) and Nissl, respectively. LFB staining of coronal sections of the cerebrum revealed impaired myelin deposition in *Polr3a*-tamKI cortex, hippocampus and thalamus with qualitatively greater differences in the cortex at P90 (**Fig. 1E** [↗](#)). In contrast, LFB- and Nissl-stained sagittal sections of the cerebellum showed no differences in myelin or granular layer neurons, respectively, and no difference in Purkinje cell density (Supplemental Fig. S2A,B). The absence of a myelination defect in the cerebellum was previously noted in *Polr3a*-Olig2KI mice (Merheb et al. 2021 [↗](#)). Non-CNS tissues (>20 examined) were histologically normal except for the pancreas. *Polr3a*-tamKI mice exhibit pronounced and severe exocrine pancreatic atrophy at P42 and P90, respectively (**Fig. 1F** [↗](#)). This condition is characterized by insufficient production of digestive enzymes (Kleeff et al. 2017 [↗](#)) and correlates with the lower body weight of *Polr3a*-tamKI mice, the marked reduction in subcutaneous and visceral adipose tissue at P90, and the poor health of the mice at this age. In contrast to the paucity of body fat at P90, echoMRI measurements at P42 showed that body fat as a percentage of total body weight was comparable to WT (Supplemental Fig. S2C). To assess whether the exocrine pancreatic defect might extend to the endocrine pancreas, glucose homeostasis was examined in adolescent mice. Blood glucose concentrations increased upon feeding, as expected, and were not significantly different between *Polr3a*-tamKI and WT mice in either the fasted or the refed state (Supplemental Fig. S2D). Similarly, a glucose tolerance test showed comparable responses (Supplemental Fig. S2E). These data suggest that endocrine pancreatic functions controlling glucose homeostasis are not significantly impaired at P42 by the *Polr3a* mutation.

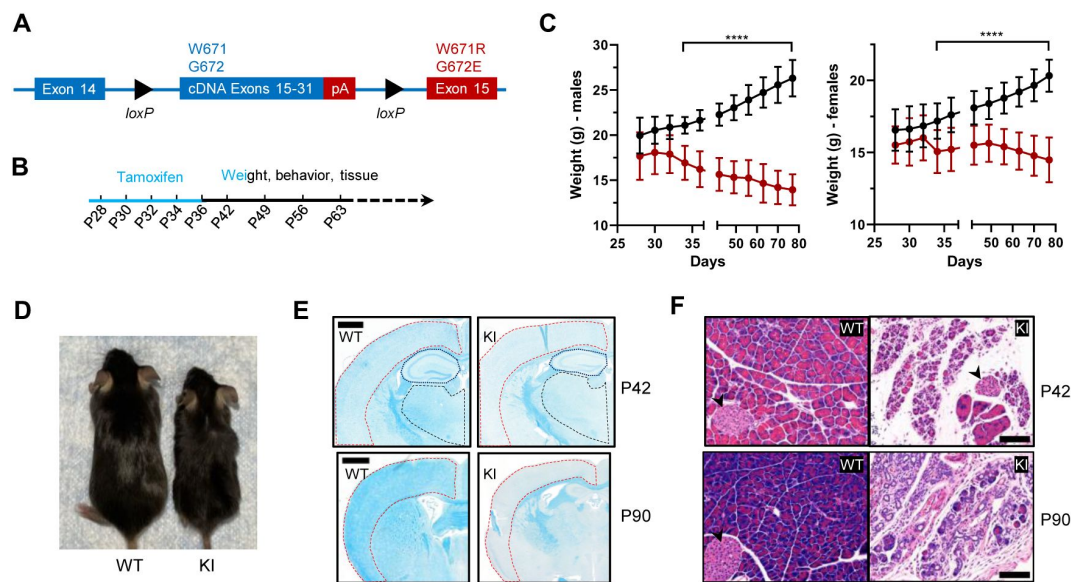


Figure 1.

Body weight, length and histological characteristics of *Polr3a*-tamKI mice.

(A) Schematic of the modified *Polr3a* locus showing floxed WT sequences encoding exons 15-31 with adjacent sequences for termination and polyadenylation (pA) and *Polr3a* exon15 containing the leukodystrophy mutations.

(B) Tamoxifen injections and experimental timeline.

(C) Lower body weights of *Polr3a*-tamKI (red) versus WT (black) mice (males n=18/group, nested t test $p < 0.0001$ ****, females n= 20/group, nested t test $p < 0.0001$ ****). Data show mean $\pm$ SD.

(D) Body length differences at P56. Images are representative.

(E) LFB-staining of WT and KI brain sections at P42 and P90. The cerebral cortex, hippocampus and thalamus are marked by red, blue and black lines, respectively. Scale bar, 1000 μ m.

(F) H&E-stained WT and KI pancreata at P42 and P90 shows a dramatic loss of acinar cells. Pancreatic islets are marked with a black arrowhead. Scale bar, 100 μ m.

Polr3a-tamKI mice exhibit multiple behavioral changes

A behavioral spectrometer with video tracking and pattern-recognition software was used to quantify ~20 home cage-like behaviors in a longitudinal study of behavior. Testing began at P42 and was continued at weekly intervals (**Fig. 1B**). No differences were observed in anxiety-like behavior, which was assessed using metrics that measure the natural tendency of mice to avoid open spaces (Supplemental Figs. S3A-C). However, overall locomotor activity was decreased in Polr3a-tamKI mice as indicated by the reduced track length at P42, P49, P56 and P63 (**Fig. 2A**). Polr3a-tamKI mice also spent less time running and more time standing still at all time points compared to WT mice, consistent with a locomotor deficit (**Fig. 2B,C**). Risk assessment was measured by orientation behavior according to three discrete metrics that can be summed to provide an overall evaluation of this behavior (Brodtkin et al. 2014). Individual orientation behaviors were significantly lower in Polr3a-tamKI mice at all time points compared to WT mice, except for Orient-shuffle at P42 where there was a trend towards lower performance in the knockin mice (Supplemental Fig. S3D-F). The sum of these behaviors shows that Polr3a-tamKI mice spend less time assessing risk at all time points (**Fig. 2D**). Exploratory behavior was investigated via three rearing motions performed when the mice are standing on their hind legs. Polr3a-tamKI mice spent less time performing these motions at most timepoints and a tendency towards diminished performance at other times (Supplemental Fig. S3G-I). When all three rearing metrics were combined, Polr3a-tamKI mice showed lower levels of exploration compared to WT mice at all timepoints although the data did not reach significance at P49 (**Fig. 2E**). Grooming behavior was evaluated by measuring the time each animal spent attending to a specific region of their body (**Fig. 2F**; Supplemental Fig. S3J,K). The Polr3a-tamKI mice spent less time grooming their legs and back compared to WT mice starting at P49 and progressing to P63. Head grooming was also reduced at P63. In summary, the findings reveal behavioral phenotypes in Polr3a-tamKI mice at the earliest time point in the study, two weeks after introduction of the mutation, consistent with neurobehavioral deficits (Brodtkin et al. 2014). As none of the affected behaviors were apparent in Polr3a-Olig2KI mice (Merheb et al. 2021), the data further demonstrate that the mutation has a more severe impact on animal behavior when its expression is induced postnatally in many cell types than when it is induced during embryonic development only in the Olig2 lineage.

A reduction in Pol III transcripts in the cerebra of adult Polr3a-tamKI mice is accompanied by induction of innate immune and integrated stress responses

To investigate the molecular changes to Pol III transcript levels caused by the *Polr3a* mutation and any secondary effects on the Pol II transcriptome, we initially focused on the cerebra of adult mice at P75. Precursor tRNA levels reflect Pol III transcription activity since these nascent RNAs are rapidly processed into mature sized tRNA by the removal of 5' leader, 3' trailer and, in some instances, intronic sequences (Berg and Brandl 2021). The levels of four precursor tRNAs, determined either by northern blotting for intron sequences or by RT-qPCR with precursor-specific primers, were reduced ~three-fold in Polr3a-tamKI cerebra (**Fig. 3A-C**). Mature tRNAs and other Pol III transcripts have relatively long half-lives and thus their levels reflect steady state abundance rather than synthesis (Nwagwu and Nana 1980). Nonetheless, northern blotting of four mature tRNAs showed that all were lower in Polr3a-tamKI cerebra with two of them reduced to ~60% of the WT (**Fig. 3A,B**).

Despite the defect in precursor tRNA synthesis and the reduction in mature tRNAs, the Pol III transcriptome was not universally affected (**Fig. 3A-C**). Transcripts from genes with a tRNA-like Type II promoter (e.g. 7SL, BC1 and vault RNA genes) showed a decrease in abundance to between 45% and 60% of the WT level (**Fig. 3C**). Transcripts from genes with a Type III promoter (e.g. U6,

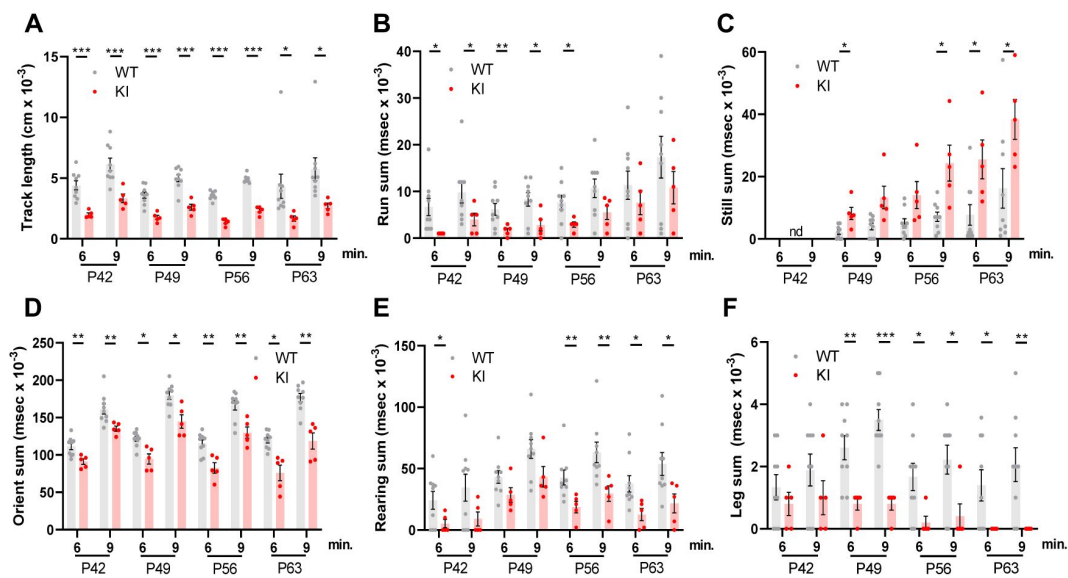


Figure 2.

Behavioral studies of WT and Polr3a-tamKI mice.

(A-C) Locomotor activity is reported as the total distance traveled (Track length) and the total time during which the mice are running (Run sum) or standing still (Still sum).

(D) Risk assessment (Orient sum) is the sum of three orientation behaviors (see Supplemental Fig. S3).

(E) Exploratory behavior (Rear sum) is the sum of three rearing behaviors (see Supplemental Fig. S3).

(F) Leg grooming. Data were collected at weekly intervals beginning at P42 (WT n= 9 and KI n= 5). Mice were tested for nine minutes with recording in three intervals of three minutes each. Values show the mean $\pm$ SEM at six- and nine-minute timepoints. Multiple t tests and one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

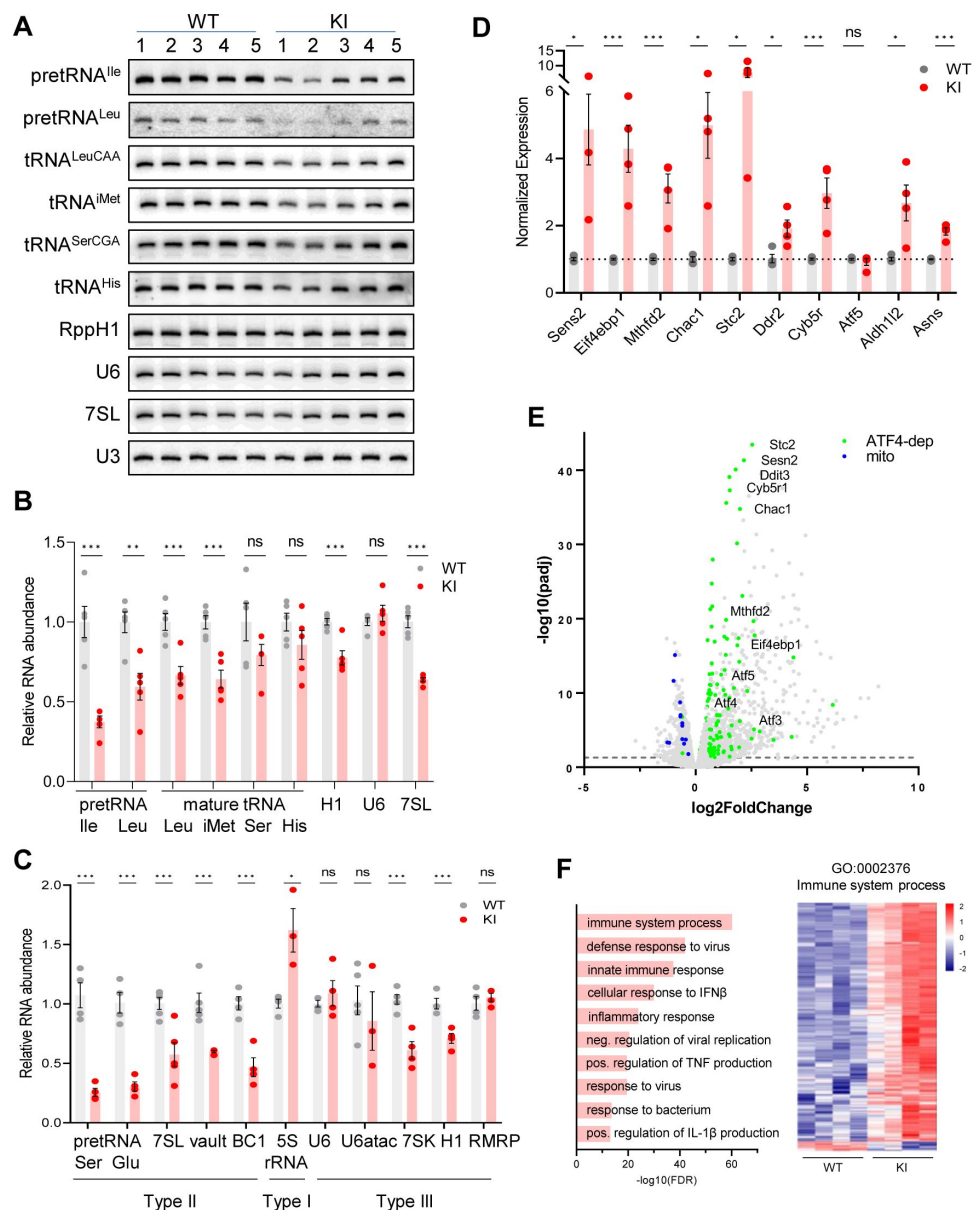


Figure 3.

Transcriptome analysis of Polr3a-tamKI cerebra at P75.

(A) Northern blot of Pol III transcripts. Precursor tRNAs (Ile-TAT, Leu-CAA), mature tRNAs (Leu-CAA, iMet, Ser-CGA, His), RppH1, U6 and 7SL RNAs and the loading control U3 snRNA were detected by hybridization using transcript-specific oligonucleotide probes. All blots represent sequential probing of a single gel. The cropped images frame the relevant regions.

(B) Quantitation of Pol III transcripts in panel A (mean ± SEM, n= 5 biological replicates).

(C) RT-qPCR of Pol III transcript abundance (mean ± SEM, n= 3-6 biological replicates).

(D) RT-qPCR of selected ATF4-regulated ISR genes (mean ± SEM, n= 3-5 biological replicates).

(E) Global gene expression quantified by RNA-seq. The volcano plot shows gene expression changes (KI/WT, n= 4 biological replicates). Differentially expressed (DE) ATF4-regulated genes (green) (p-adj < 0.05, log2FC > |0.58|) and mitochondrially-encoded transcripts (blue). ATF4-regulated genes assayed in panel D and Ddit3/Chop are labeled.

(F) Top ten GO bioprocesses among up-regulated DE genes are shown with a heatmap of Z-scores for DE genes annotated to the GO:0002376 immune system process. For all graphs: WT, gray bars and circles; KI, red bars and circles. ns, not significant; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.005.

RMRP, RppH1 and 7SK genes), were variably decreased: RppH1 and 7SK RNAs were decreased to 70% and 56%, respectively, while U6, U6atac and RMRP RNAs were unaffected. The level of 5S rRNA, the only RNA produced using a Type I promoter, was elevated (**Fig. 3C** [↗](#)).

Mouse models of neurodegeneration with underlying defects in the translation machinery induce an adaptive response to cellular stress known as the integrated stress response (ISR) (Ishimura et al. 2016 [↗](#); Abbink et al. 2019 [↗](#); Terrey et al. 2020 [↗](#); Spaulding et al. 2021 [↗](#)). Canonical activation of the ISR is initiated by stress-activated protein kinases which phosphorylate eIF2 α to globally attenuate bulk mRNA translation initiation, increase translation of uORF-containing mRNAs including transcription factors such as ATF4 and induce a stress response transcription program (Costa-Mattioli and Walter 2020 [↗](#)). We measured the expression of 10 ATF4-regulated ISR target genes to evaluate whether the ISR had been activated in Polr3a-tamKI cerebra. A two to six-fold upregulation was measured for seven of these genes, two others were induced at lower levels and one was unaffected (**Fig. 3D** [↗](#)). These results point to altered translation in Polr3a-tamKI cerebra.

Bulk RNAseq was used to broadly profile the transcriptional changes in Polr3a-tamKI cerebra at P75. Differentially expressed (DE) genes (p adj. <0.05, log2FoldChange >|0.58|) were biased towards upregulation; 83% of DE genes were increased in the *Polr3a* mutant (**Fig. 3E** [↗](#); Supplemental Table S1). We compared our DE genes with a list of 774 ISR genes that show ATF4-dependent induction following tunicamycin treatment of mouse embryo fibroblasts (Torrence et al. 2021 [↗](#)) and found 192 genes upregulated in Polr3a-tamKI cerebra (**Fig. 3E** [↗](#), annotated in green, Supplemental Fig. S4A; Supplemental Table S1). Among these were key ISR regulators, such as uORF-containing transcription factors (ATF3, ATF5 and CHOP, aka Ddit3), pro-apoptotic targets of CHOP (Chac1 and Bbc3), and negative ISR regulators (PPP1r15a, aka GADD34 and TRIB3) that function to antagonize the response (**Fig. 3E** [↗](#)). Interestingly, expression of the transcription factor ATF6 and its canonical ERAD targets (Pdia4, Pdia6, EDEM1, GRP78 aka Hspa5, GRP94 aka Hsp90b1, XBP1 and calnexin) were not affected suggesting that ISR induction in the *Polr3a* mutant does not involve an ER stress-mediated Unfolded Protein Response (UPR, Supplemental Table S1) (Hillary and FitzGerald 2018 [↗](#)). This was confirmed by showing that the relative levels of inactive unspliced and active spliced forms of XBP1, a critical transcription factor for the UPR, were unchanged (Supplemental Fig. S4B).

Metascape pathway enrichment of upregulated DE genes identified activation of the immune system and related processes as major functional categories (**Fig. 3F** [↗](#); Supplemental Table S1). These data point to activation of microglia (see below) and the upregulation of signaling pathways that promote inflammation and cell death by multiple mechanisms (Butovsky and Weiner 2018 [↗](#)). For example, pyroptosis is indicated by the upregulation of caspases 1 and 4, the inflammasome sensor NLRP3 and its adapter protein, PYCARD, interleukin-1 β and the plasma membrane pore-forming protein, Gasdermin D, among others (Supplemental Table S1). The most enriched functional categories among genes down-regulated in Polr3a-tamKI cerebra were cholesterol biosynthesis and electron transport (Supplemental Table S1). All mitochondrially-encoded transcripts were also down-regulated in the *Polr3a* mutant (**Fig. 3E** [↗](#), annotated in blue). In contrast, nuclear-encoded mitochondrial genes were mostly unaffected although 24 were found in the upregulated gene set (Supplemental Table S1; Supplemental Fig. S4C). These observations suggested a loss of mitochondrial DNA. Indeed, qPCR analysis showed that the ratio of two mitochondrial genes (16S rRNA and ND1) to a nuclear gene (HK2) was substantially reduced in Polr3a-tamKI cerebra (Supplemental Fig. S4D). Thus, the loss of mitochondrial genomes underlies the reduction in mitochondrially-encoded RNA detected by RNAseq. *Hmgcr* and *Sqle* were among numerous sterol biosynthetic genes down-regulated in Polr3a-tamKI cerebra (Supplemental Fig. S4E; Supplemental Table S1). These enzymes function in rate-limiting steps in *de novo* cholesterol synthesis, suggesting dysfunctional cholesterol production. Cholesterol synthesis in the brain occurs predominantly in astrocytes and oligodendrocytes, accounts for almost one quarter of total body cholesterol and cannot be compensated by the periphery since cholesterol uptake is blocked by the blood-brain barrier (Saher and Stumpf 2015 [↗](#)). Astrocytes synthesize cholesterol for export

to neurons while oligodendrocytes (OLs) synthesize 70-80% of brain cholesterol that resides in myelin (Barber and Raben 2019 [↗](#); Li et al. 2022 [↗](#)). Impairment of cholesterol biosynthesis in either cell type can result in cognitive and behavioral phenotypes (Li et al. 2022 [↗](#)) and thus may contribute to the behavioral defects noted in Polr3a-tamKI mice.

Changes in cell-type specific gene expression reflect the loss of neurons and oligodendrocytes and activation of microglia

We queried the Polr3a-tamKI DE genes against consensus sets of mouse brain cell type gene expression signatures for OLs, neurons, astrocytes, microglia and endothelial cells (McKenzie et al. 2018 [↗](#)). Twenty-one percent of Polr3a-tamKI DE genes ($p\text{-adj} < 0.05$) could be assigned to one of these five cell types with microglia and neurons constituting the bulk of the cell type-specific genes (Supplemental Table S2). OL and neuronal gene expression was predominantly down-regulated suggesting a reduction in these cell populations while microglia were predominantly elevated (Fig. 4A-C [↗](#); Supplemental Table S2). Immunohistochemistry confirmed that these changes in cell type gene expression signatures correlated with changes in cell-specific protein markers (Fig. 4D-H [↗](#)). Myelination in the mouse begins at birth and is largely complete by weaning although it continues with decreasing frequency through adulthood (Hammelrath et al. 2016 [↗](#); Nishiyama et al. 2021 [↗](#)). Thus, under our treatment regimen, substantial myelination has occurred by P28 when the *Polr3a* mutation is introduced. This, together with the long half-life of myelin (Barnes-Velez et al. 2023 [↗](#)) contributes to a high background level of myelin. Accordingly, the mean fluorescence intensity of myelin basic protein (MBP) staining in the corpus callosum of adult Polr3a-tamKI mice was not significantly diminished relative to WT. However, the density of MBP staining was reduced as was the number of OLs staining positive with the CC1 antibody (Fig. 4D,E [↗](#)). In the cerebral cortex, the mean fluorescence intensity of NeuN staining and the number of NeuN positive neuronal nuclei was lower in Polr3a-tamKI mice (Fig. 4F [↗](#)) consistent with the decrease in neuron-specific gene expression. Additionally, we observed reactive microglia in Polr3a-tamKI cerebra, as evidenced by the increased cell density and altered morphology (enlarged cell bodies and shorter, thicker processes) of Iba1-stained cells in the cortex and particularly in the striatum (Fig. 4G,H [↗](#)). Together, these data indicate that behavioral deficits, induction of the ISR, loss of mitochondrial genomes, an elevated immune response and neurodegeneration are causally linked to the loss of Pol III transcription in Polr3a-tamKI cerebra.

To assess whether the changes observed in Pol III and Pol II transcripts in P75 cerebra occurred in other tissues, we examined cerebellum, heart, kidney and liver from the same animals. The levels of three precursor tRNAs in the cerebellum, heart, kidney were reduced ~three-fold, comparable to the changes seen in cerebra (Supplemental Fig. S5A). However, in the liver, where the efficiency of CAGGCre-ERTM recombination is lower (Supplemental Fig. S1C; Hayashi and McMahon 2002 [↗](#)), only one of the three precursor tRNAs was reduced (Supplemental Fig. S5A). The levels of two mature tRNAs were variably affected, being unchanged in liver and cerebella, trending lower in heart and reduced in kidney to ~70% of WT (Supplemental Fig. S5A). Among other Pol III transcripts, tissues other than cerebra showed few changes: 7SL and RMRP RNA levels were reduced in cerebella but otherwise no reductions were detected (Supplemental Fig. S5B). Similarly, there was little indication of an ISR response in the tested tissues other than cerebra (Supplemental Fig. S5C). These data imply that the diminished activity of Pol III in these tissues is above the threshold necessary for ISR induction.

A reduction in Pol III transcription and mature tRNA levels precedes induction of innate immune and integrated stress responses

To separate causal changes in Pol III activity from secondary stress responses that could down-regulate Pol III transcription, we profiled both the Pol II and Pol III transcriptomes of WT and Polr3a-tamKI cerebra at P42, two weeks after induction of recombination. Similar to the P75 time

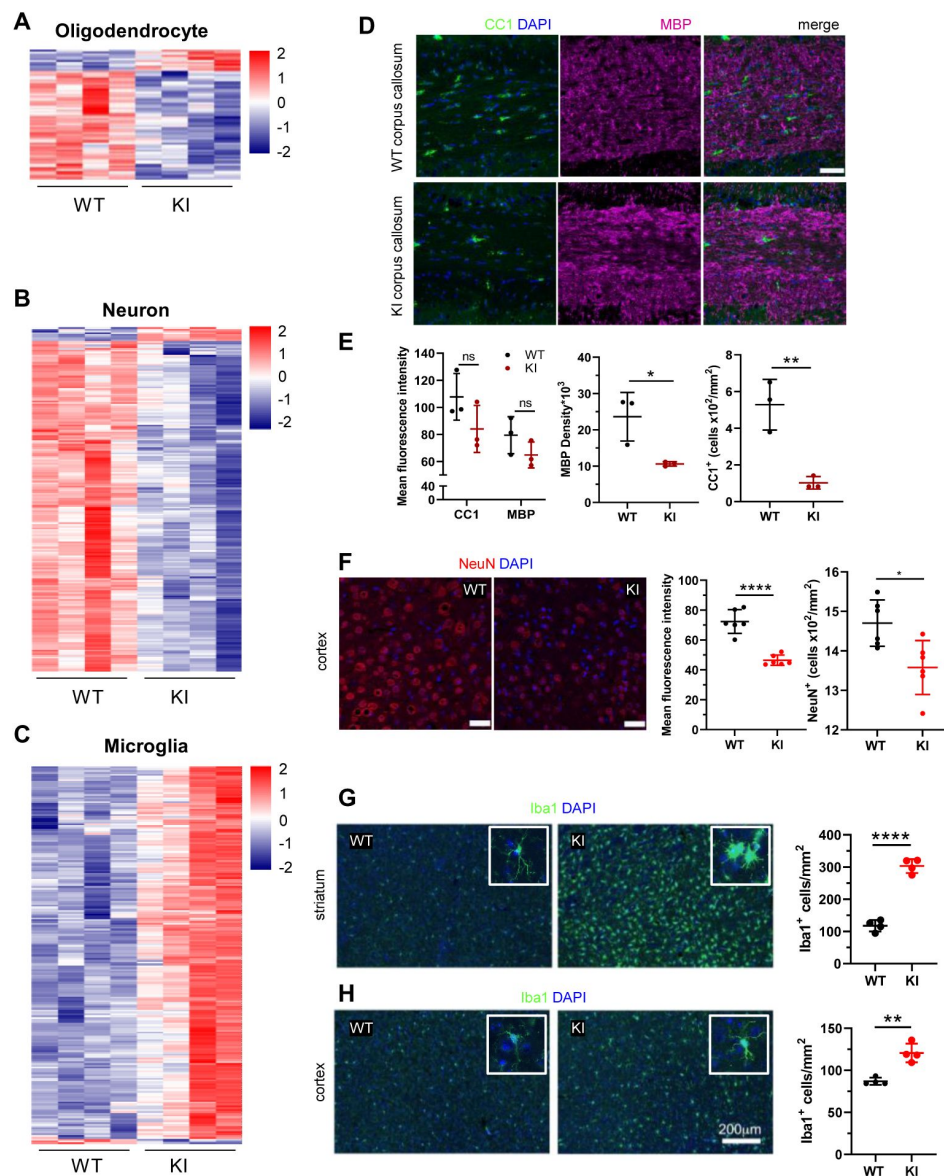


Figure 4.

Cell type-specific changes in Polr3a-tamKI cerebra at P75.

(A-C) Oligodendrocyte (A), neuron (B), and microglia (C) cell type-specific DE genes. Heatmaps show Z-scores of DE genes identified in the top 500 mouse cell type-specific genes defined in McKenzie et al. 2018.

(D-E) Immunostaining of oligodendrocytes at the midline of the corpus callosum. Scale bar, 40 μ m. Mean fluorescence intensities of MBP and CC1, MBP staining density and CC1 cell counts represent mean $\pm$ SD, $n=3$.

(F) NeuN immunostaining of neurons in the motor cortex. Scale bar, 40 μ m. Mean fluorescence intensities and cell counts represent mean $\pm$ SD of bilaterally symmetric regions ($n=3$).

(G-H) Iba1 staining of microglia in the striatum (G) and cerebral cortex (H). Insets show larger magnifications of individual microglia. Note the striking shortening of processes and enlargement of the cell body in the striatum. Scale bar, 200 μ m. Cell counts represent mean $\pm$ SD of bilaterally symmetric regions, ($n=2$). For all graphs: WT, black; KI, red; ns, not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p < 0.0001$.

point, *Polr3a*-tamKI pre-tRNA levels were reduced ~three-fold and three out of four mature tRNAs were reduced to ~60% of the WT level (compare **Fig. 5A-C** with **Fig. 3A-C**). No Pol III transcripts other than tRNAs were decreased at P42 (**Fig. 5C**). Thus, decreased production of precursor and mature tRNA is the earliest detectable Pol III defect in *Polr3a*-tamKI cerebra.

Analysis of the ATF4-regulated ISR gene panel revealed that fewer genes were induced in *Polr3a*-tamKI cerebra at P42 and the response was less robust than at P75 (compare Supplemental Fig. S6A and **Fig. 3D**). *Sesn2*, *Mthfd2*, *Chac1*, *Ald1l2* and *Asns* were up-regulated two-fold or less indicating partial activation of the ISR. Bulk RNAseq showed fewer significant DE genes at P42 (181 genes) with 68% up-regulated and minimal overlap with the P75 DE gene set (**Fig. 5D**; Supplemental Fig. S6B; Supplemental Table S3). Although no GO terms were enriched, the P42 DE gene set contained a small number of ISR genes (20 genes), the majority of which are ATF4-regulated and showed increased expression (Supplemental Fig. S6B; Supplemental Table S3). Similarly, a small number of up-regulated genes were associated with the immune response GO bioprocess (21 genes at P42 versus 582 genes at P75, Supplemental Table S3). Additionally, the expression of mitochondrially-encoded genes was unaffected at P42, as was mitochondrial genome content (Supplemental Fig. S6C; Supplemental Table S3). Consistent with these data at P42 and in contrast to the findings at P75, *Iba1*-staining did not reveal any increase in the density of microglia in the cerebral cortex or the striatum (Supplemental Fig. S6F,G). Together these data indicate a progression of stress and immune response programs with time in *Polr3a*-tamKI cerebra.

The *Polr3a* mutation reduces total cytoplasmic tRNA levels and alters the tRNA profile

The effect of the *Polr3a* mutation on the tRNAome in P42 cerebra was assessed by QuantM-tRNA-seq (Pinkard et al. 2020). This method exploits the conserved 3'-CCA end of mature tRNAs using a splint-ligation approach to prepare DNA libraries for sequencing. Prior to sample workup, a synthetic tRNA spike-in based on *E. coli* tRNA^{Gln} was added for normalization of tRNA reads to total input RNA. Since mitochondrially-encoded mRNAs and mitochondrial DNA content were comparable between WT and *Polr3a*-tamKI cerebra at P42 (Supplemental Fig. S6C; Supplemental Table S3), mitochondrially-encoded tRNAs were also used for normalization. Total nuclear-encoded tRNA reads normalized with either or both standards were similar and significantly lower in *Polr3a*-tamKI cerebra suggesting an ~25% reduction in total tRNA abundance (**Fig. 6A**; Supplemental Fig. S6D,E). Differential expression (DE) analysis indicated that the majority of tRNA isodecoders (i.e. tRNAs that share the same anticodon but have different body sequences) were significantly lower in the *Polr3a* mutant (58%, 108/187 tRNAs with $p\text{-adj} < 0.05$, Supplemental Table S5). DE was observed across the entire read depth which spans 5 orders of magnitude and included tRNAs whose sequences could be uniquely mapped as well as redundantly-encoded tRNAs (**Fig. 6B**). Compared to most elongator tRNAs, the three tRNA^{Met} isodecoders were less dramatically affected, reduced to 86-88% of the WT level. The level of tRNA^{Arg}-TCT-4-1 (aka *Tr20*), an abundant neuron-enriched tRNA that is mutated in the C57BL/6J background and causes synthetic phenotypes when combined with ribosome rescue factor mutants (Ishimura et al. 2014; Terrey et al. 2020), was unchanged in the *Polr3a* mutant. Thus, the cerebral phenotypes of *Polr3a*-tamKI mice are unlikely to result from synthetic effects of the tRNA^{Arg}-TCT-4-1 and *Polr3a* mutations.

Grouping tRNA isodecoders into their isoacceptor families illustrates that most members of each family were reduced in the *Polr3a* mutant (**Fig. 6C**). Indeed, when tRNA read counts were combined into their respective decoder (i.e. anticodon) groups, all decoders were reduced in the *Polr3a* mutant, and for 30 decoders (60%), the reductions were significant (**Fig. 6F**; Supplemental Table S5). To assess how these changes affect the composition of the tRNA population (i.e. the tRNA profile), each decoder was plotted as a percentage of the total tRNA population for the *Polr3a* mutant versus WT (**Fig. 6E**). This revealed changes in the relative

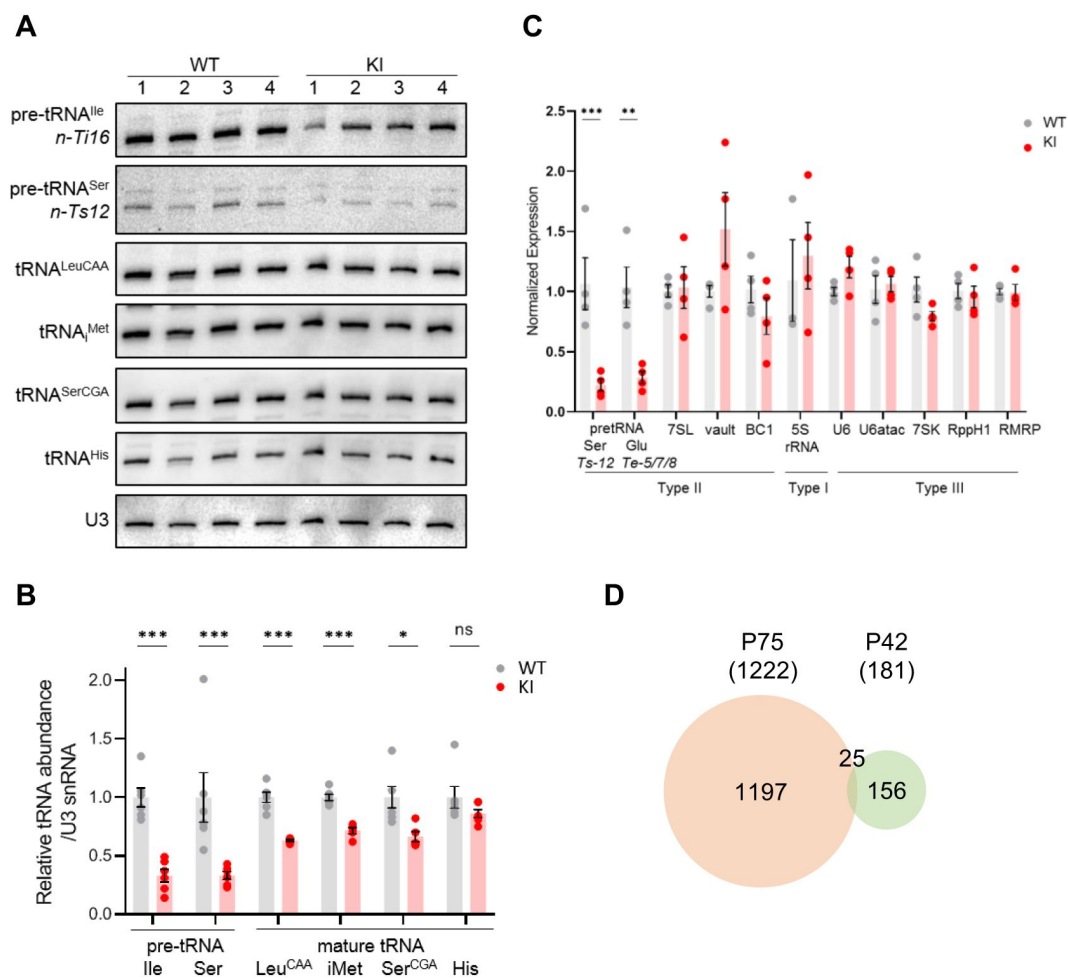


Figure 5.

Transcriptome Analysis of Polr3a-tamKI cerebra at P42.

(A) Northern blots of Pol III transcripts. Precursor tRNAs (Ile-TAT, Ser-CGA), mature tRNAs (Leu-CAA, iMet, Ser-CGA, His) and U3 snRNA were detected as in [Fig. 3A](#).

(B) Quantitation of Pol III transcripts in panel A. Mean $\pm$ SEM, $n = 5$ biological replicates.

(C) RT-qPCR of Pol III transcript abundance. Mean $\pm$ SEM, $n = 3-5$ biological replicates.

(D) Venn diagram of the overlap between P75 and P42 DE genes ($p\text{-adj} < 0.05$, $\log_2\text{FC} > |0.58|$).

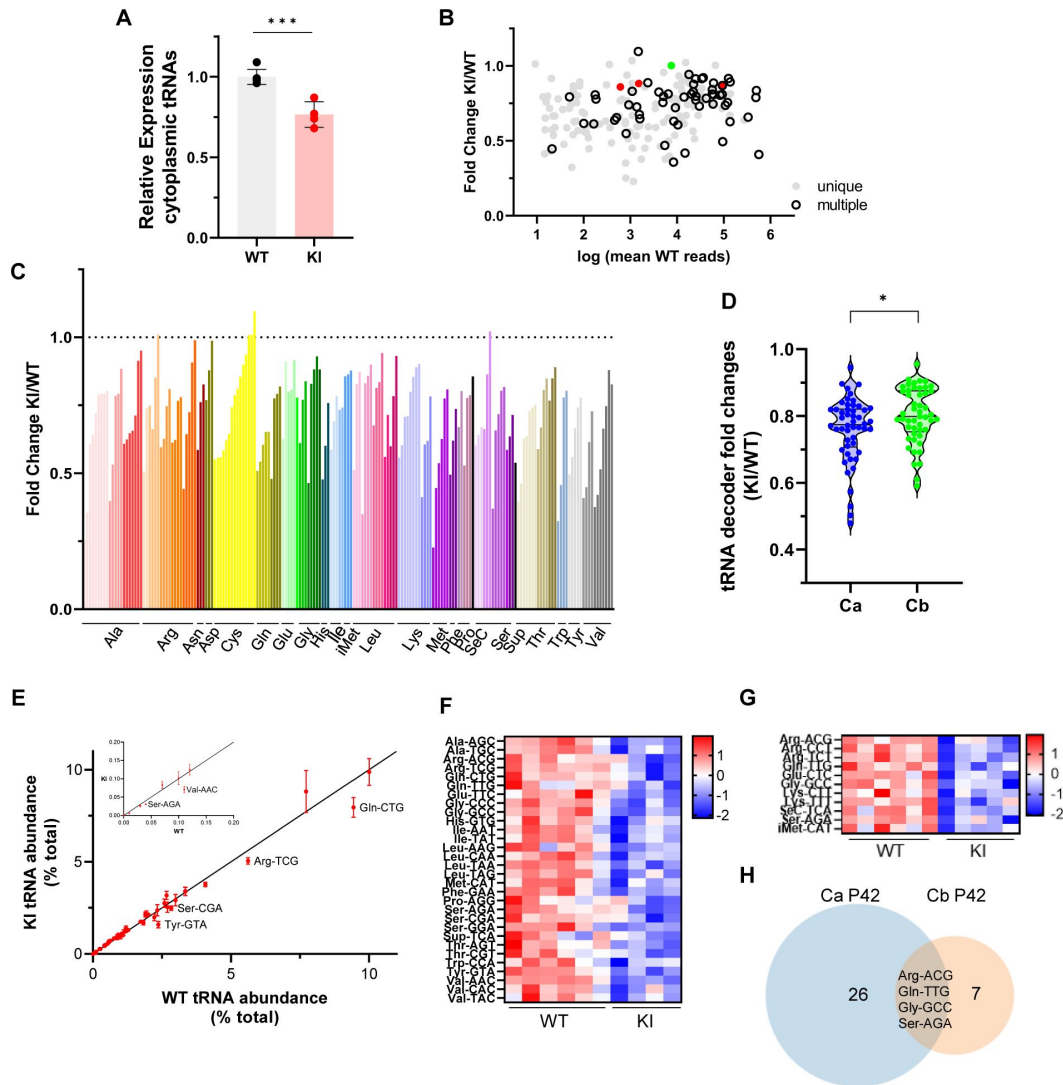


Figure 6.

Cytosolic tRNA abundance in Polr3a-tamKI cerebra at P42.

- (A) Total cytoplasmic tRNA reads in WT and KI cerebra, normalized to the sum of spike-in and mitochondrially-encoded tRNA reads are expressed relative to the mean WT value. Mean $\pm$ SD, KI $n = 4$, WT $n = 6$ biological replicates, $p = 0.0003$.
- (B) tRNA fold change (KI/WT) is plotted against Log mean WT reads. Symbols show tRNAs encoded by unique loci (gray), identical tRNAs encoded by multiple loci (hollow black), iMet tRNAs (red) and tRNA^{Arg-TCT-4-1} (green).
- (C) tRNA fold change (KI/WT) for all decoder families. Individual tRNAs are ordered from most to least fold change and grouped by codon recognition (tRNA decoder family). The amino acid for each tRNA decoder family is indicated.
- (D) Violin plots of tRNA decoder fold changes (KI/WT) for cerebra (Ca) and cerebella (Cb) at P42. tRNA reads were summed for each tRNA decoder family and normalized to spike-in and mitochondrial tRNA reads, $p = 0.0250$. For Ca, $n = 4$ (KI) and $n = 6$ (WT) biological replicates and for Cb, $n = 5$ (KI) and $n = 6$ (WT) biological replicates.
- (E) The cytoplasmic tRNA profile for KI cerebra is plotted against the WT profile. tRNA decoder reads are expressed as a percentage of their respective total cytoplasmic decoder pool, mean $\pm$ SEM. tRNA decoders that are significantly lower in the KI compared to WT ($p \leq 0.05$) fall below the regression line and are labeled. The inset shows tRNA decoders representing $<0.2\%$ of the total tRNA pool.
- (F) DE tRNA decoders in cerebra (Ca).
- (G) DE tRNA decoders in cerebella (Cb). Heatmaps represent Z scores of normalized read counts for significant DE genes ($p\text{-adj} < 0.05$).
- (H) Venn diagram of the overlap between DE tRNA decoders in Ca and Cb ($p\text{-adj} < 0.05$).

proportions of some tRNAs. For example, tRNA^{Val}(AAC) and tRNA^{Tyr}(GTA) were proportionally less abundant than tRNA^{His}(GTG), and seven other tRNAs, respectively. These changes coupled with the overall decrease in tRNA abundance have the potential to change the kinetics of tRNA binding to the ribosome (decoding speed) as well as the kinetic competition with near-cognate tRNAs for ribosome binding. The most severely affected decoders were tRNA^{Val}(AAC) (to 48% of the WT level), tRNA^{Tyr}(GTA) (to 50%), tRNA^{Sup}(TCA) (to 53%), tRNA^{Ser}(AGA) (to 63%) and tRNA^{Gln}(CTG) (to 64%). In mouse, tRNA^{Ser}(AGA) and tRNA^{Val}(AAC) decode 41% and 42% of their respective codons (accounting for 3rd base wobble), tRNA^{Gln}(CTG) decodes 75% of glutamine codons and tRNA^{Tyr}(GTA) decodes 100% of tyrosine codons. These findings suggest that the decrease in tRNA decoders is likely to negatively affect the tRNA decoding potential at the earliest stage of disease pathogenesis.

All tRNA isodecoder families are reduced in *Polr3a-tamKI* cerebellum but few decoders are affected

An initial analysis of the Pol III transcriptome in *Polr3a-tamKI* cerebella at P42 revealed comparable findings to the cerebra with decreases in several precursor and mature tRNAs and no effects on other Pol III transcripts (compare Supplemental Fig. S7A,B with **Fig. 5B,C**). QuantM-tRNA-seq indicated that total tRNA abundance was reduced in cerebella by ~23% in the *Polr3a* mutant (Supplemental Fig. S7D), similar to cerebra, and the expression of tRNA isodecoders was broadly decreased (Supplemental Fig. S7E,F). However, a comparative DE analysis showed that fewer tRNAs (30 versus 108) and fewer tRNA decoders (11 versus 30) achieved statistical significance in *Polr3a-tamKI* cerebella compared to cerebra (**Fig. 6G,H**; Supplemental Table S6) and the distribution of fold change values spanned a narrower range and was shifted towards smaller differences relative to WT (**Fig. 6D**). In addition, the tRNA profile was minimally altered in cerebella (Supplemental Fig. S7G). These differential effects on the tRNA population in cerebella versus cerebra are consistent with bulk RNA-seq data from cerebella which showed few significant DE genes (13 genes, $p\text{-adj} < 0.05$, $\log_2\text{Fold Change} > |0.58|$, Supplemental Table S4) and limited overlap with cerebral DE genes (Supplemental Fig. S7C). The minimal effect of the *Polr3a* mutation on the Pol II transcriptome of cerebella at P42 and the absence of ISR induction at P75 suggests that any reduction in tRNA decoding potential is insufficient to cause significant pathology in this tissue.

Discussion

Patients with *Polr3*-related disease express *Polr3* mutations in all cells during development and throughout postnatal life yet exhibit predominantly CNS phenotypes. To gain insight into this neural sensitivity, we introduced a pathogenic *Polr3a* mutation into mice using a ubiquitously-expressed tamoxifen-inducible Cre recombinase and examined the effects of reduced Pol III transcription in various tissues in a largely post-developmental state. The paucity of gross phenotypes identified by histopathology across many *Polr3a* mutant tissues reflects that cell-specific requirements for a minimum threshold of Pol III transcription have mostly been met. At the molecular level, our analysis of heart, kidney, cerebella and cerebra showed that Pol III transcription, as reported by pre-tRNA levels, was substantially reduced at P75, six weeks after induction of the mutation. These changes were paralleled by smaller decreases in mature tRNAs (Supplemental Fig. S5A) suggesting that tRNA half-lives are either much longer than expected in WT mice (Nwagwu and Nana 1980) and/or that tRNA turnover is decreased in the *Polr3a* mutant. Surprisingly, the levels of non-tRNA Pol III transcripts were not decreased in heart or kidney and only 7SL and RMRP RNAs were lower in cerebella at P75. In contrast, reductions among non-tRNA transcripts were widespread in cerebra at the same age although two RNAs remained unaffected (Supplemental Fig. S5B). Given the levels of recombination in these tissues (which were higher in cerebella, kidney and heart than in cerebra, Supplemental Fig. S1C), the results point to a particularly high sensitivity of cerebra to the *Polr3a* mutation and a remarkable

insensitivity of many non-tRNA transcripts in cerebella, kidney and heart to the reduction in Pol III activity. This insensitivity of non-tRNA transcripts may result from tissue-specific differences in the recruitment of the mutant Pol III to its genes, differences in the stability or nuclease accessibility of the RNA in specific ribonucleoprotein complexes, the ability of some Pol III promoters to be transcribed by RNA polymerase II (James Faresse et al. 2012 [↗](#); Dergai et al. 2018 [↗](#); Gao et al. 2018 [↗](#)) or other effects that remain to be defined.

The higher sensitivity of cerebra versus cerebella, heart and kidney to the *Polr3a* mutation was apparent at both P42 and P75. In cerebella, only 13 DE genes were identified at P42 whereas in cerebra the number of DE genes was more than an order of magnitude higher and included a nascent ATF4-dependent ISR that was absent in cerebella at this age. Moreover, while the induction of ISR genes in cerebra increased at P75, there was no appreciable ISR induction in cerebella, heart and kidney. Together, these data suggest different thresholds of sensitivity to defects in Pol III transcription in different tissues and/or cell populations. Indeed, within the cerebra, the more robust activation of microglia in the striatum versus the cortex suggests significant differences in sensitivity to the *Polr3a* mutation between these regions. Thus, different thresholds of Pol III activity may be needed to support the functions of these regions, e.g. the continuing maturation of cortical thickness and myelination that occurs from P28 into adulthood (Hammelrath et al. 2016 [↗](#); Nishiyama et al. 2021 [↗](#)) versus the functions other cells such as medium spiny neurons that comprise ~95% of the neurons in the striatum (Tepper and Bolam 2004 [↗](#)).

In contrast to the cerebra, the apparent insensitivity of the cerebella to the *Polr3a* mutation may reflect that neurogenesis and synapse formation is largely complete by P21, before introduction of the mutation (Zeiss 2021 [↗](#)). However, it remains possible that more subtle phenotypes such as dendritic pruning, which continues in the cerebellum through two months of age, may be affected (Leto et al. 2016 [↗](#)). Of note, our developmental oligodendrocyte lineage model (*Polr3a*-Olig2KI mice), which showed reduced growth and neurological deficits along with hypomyelination in the cerebra and spinal cord (Merheb et al. 2021 [↗](#)), also did not show cerebellar phenotypes. Together, these observations suggest that oligodendrocyte lineage cells in the cerebellum have a different threshold for minimal Pol III activity through development and maturation than in other regions of the brain.

The sensitivity of the mouse exocrine pancreas to the *Polr3a* mutation was unexpected given the absence of reports on acinar dysfunction associated with *Polr3*-related disease but is consistent with the knowledge that a *Polr3b* mutation disrupts development of the exocrine pancreas and intestine in zebrafish (Yee et al. 2007 [↗](#)). Thus, our finding may inform undiagnosed digestive issues in the patient population. The unique codon usage of mouse and human pancreatic acinar cells, which differs from most other cell types and is skewed by the codon composition of highly expressed secreted proteins may underlie this phenotype (Gao et al. 2022 [↗](#)). Alternatively, the *Polr3a* pancreatic phenotype may reflect unique differences in the development of rodent versus human pancreata: The increase in the size of mouse pancreas from birth to adulthood results largely from an increase (~19-fold) in the volume of acinar cells (Anzi et al. 2018 [↗](#)). This hypertrophy of murine acinar cells is attributed to their higher biosynthetic rate and tetraploid genome content. In contrast, postnatal growth of human pancreas involves an increase in cell number, with acinar cells maintaining a constant volume (Anzi et al. 2018 [↗](#)).

Pol III dysfunction and the reduction in the cerebral tRNA population at P42 coincides with behavioral deficits and precedes substantial downstream alterations in the Pol II transcriptome, which include induction of an innate immune response (IR) and an ISR, and indicators of neurodegeneration (i.e., activation of cell death pathways and loss of mitochondrial DNA). These findings suggest a causal role for the lower tRNA abundance and/or altered tRNA profile in disease progression. Whether the decrease in the abundance of other Pol III transcripts in the cerebrum at

later times contributes to disease progression is an open question. These changes may represent secondary effects of IR/ISR signaling or cell death stressors and may be occurring in cells that have already committed to a pathway of programmed cell death.

Recent studies have shown that decreased levels of a single cytoplasmic tRNA isoacceptor or deletion/mutation of a single nuclear-encoded tRNA gene is sufficient to alter translation and cause neuronal dysfunction: Dominant mutations in the glycyl-tRNA synthetase gene associated with Charcot-Marie-Tooth disease CMT2D, cause sequestration of tRNA^{Gly}, neuropathy, impair neuronal translation and induce the ISR (Spaulding et al. 2021 [↗](#); Zuko et al. 2021 [↗](#)). Mutation or deletion of the tRNA^{Arg}-TCT-4-1 isodecoder expressed in neurons, reduced the total tRNA^{Arg} pool, caused ribosome pausing at AGA codons, ISR induction, defects in neuronal function and sensitization to loss of ribosome quality control proteins (Ishimura et al. 2014 [↗](#); Kapur et al. 2020 [↗](#); Terrey et al. 2020 [↗](#)). Global deletion of the tRNA^{Phe}1-1 isoacceptor is sufficient to cause transcription and proteomic changes, neurological deficits, increased ribosome stalling, and altered protein expression (Hughes et al. 2023 [↗](#)). In the cerebra of *Polr3a*-tamKI mice, the induction of an ATF4-dependent ISR at P42 and its enhancement at P75 is a clear indicator that translation kinetics have been impacted by the global decrease in tRNA abundance, which extends across most decoders and changes the tRNA profile. Accordingly, the *Polr3a* mutation is predicted to compromise translation efficiency and change the frequency and sites of ribosome pausing and/or stalling. These events can trigger ISR induction via protein misfolding or unresolved ribosome collisions (Costa-Mattioli and Walter 2020 [↗](#); Park et al. 2021 [↗](#)). We propose that in the context of *Polr3*-related disease, chronic ISR activation ultimately fails to restore cellular homeostasis, and thus leads to neurodegeneration through amplification of the IR and activation of cell death pathways (Costa-Mattioli and Walter 2020 [↗](#)).

Materials and Methods

Mouse husbandry

All experiments involving mice were performed using protocols approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine (AECOM). Mice were housed in a barrier facility at 22°C on a 12-hour light/dark cycle with constant access to food (PicoLab® Mouse Diet 20) and water. C57BL/6J-*Polr3a*^{tm1Iwil} mice were described previously (Merheb et al. 2021 [↗](#)) and were bred to B6.Cg-Tg(CAG-cre/Esr1*5Amc/J) mice (CAGGCre-ERTM, #004682) and to B6.129(Cg)-Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Lo/J} mice (tdTomato-EGFP, #007676). Whole-body inducible *Polr3a* mutant mice and Cre-minus WT controls were obtained by breeding male mice heterozygous for both the floxed *Polr3a* allele and Cre recombinase to female mice homozygous for the floxed *Polr3a* allele. The breeding of mice to assess recombination used females homozygous for the tdTomato-EGFP reporter. Experimental animals (knockin mutants and WT controls) were treated with tamoxifen (6 mg/40 g body weight, five injections i.p.) every other day starting at P28. Unless otherwise indicated, all experiments were performed with male and female mice and the data were pooled for analysis.

Recombination efficiency

Single cell suspensions of dissected cerebrum and cerebellum were prepared using an adult brain dissociation kit (Miltenyi). Flow analysis was performed on a LSRII-U flow cytometer (BD Biosciences) with single color controls to establish gating prior to sample analysis (Merheb et al. 2021 [↗](#)). For liver, heart and kidney, fixed, sectioned tissue was mounted with ProLong diamond antifade with DAPI (Invitrogen) and imaged using a 3D Histech p250 high-capacity slide scanner. Manual cell counts were performed in ImageJ (five sections/tissue, >150 cells/section).

Purkinje Cell Counts

Images of Nissl-stained sagittal sections of cerebellum were viewed using CaseViewer software for manual counting of Purkinje cells in lobes III, IV/V, VI/VII and VIII. The open polygon tool was used to measure the length of the Purkinje layer for calculating Purkinje cell density.

Histology

Necropsy and staining of tissues with H&E, LFB and Nissl was performed in the Histopathology and Comparative Pathology Facility at AECOM. Images were acquired using a 3D Hitech p250 high-capacity slide scanner.

Mouse behavior

Behavioral studies were conducted during the light cycle using a Behavioral Spectrometer (BiObserve) equipped with video-tracking, infrared beams and a vibration-sensitive floor to record motion in the X, Y and Z planes and to measure the frequency and magnitude of vibrations in a 40 cm² arena. Locomotor activity was tracked and stereotyped behaviors were measured algorithmically using Viewer Software (BiObserve) (Brodtkin et al. 2014 [DOI](#)). Specifically, locomotor classes of behavior include Run, Trot, Walk and Still (not moving) metrics. Locomotor activity - open field-like behavior is defined by Track Length (total distance travelled in the arena). Exploratory classes of behavior include Rearing and Orientation behaviors. Rearing movements include Rear Climb (e.g. trying to climb walls), Rear Bob (moving body in an up/down vertical motion) and Rear Sniff (head movement in a nodding arc) activities. Orientation movements include Orient Shuffle (repositioning of all 4 feet and facing a different direction), Orient Creep (repositioning 1-3 steps more or less in the same direction) and Orient Sniff (moving less than a body length with no head movement) motions. Grooming behaviors are defined by Scratches (involving the hind legs) and grooming of specific body parts (Genital, Tummy, Back, Leg, Head, Face, Nose, and Paw). Anxiety-like behavior is defined by Center visits and Center duration which score the number and duration of visits to a 15 cm² central area of the arena and Center track (total distance travelled in the central area). Individual mice were placed in the instrument for a total of 9 minutes and recorded in 3 minute intervals (3 recordings in total). Behaviors are reported after 6 minutes (2 recordings) and 9 minutes (all 3 recordings). Approximately, equal numbers of mice of both genders (5 WT females, 4 WT males and 3 KI females, 2 KI males) were examined at weekly intervals on postnatal days 42, 49, 56 and 63. The data for each genotype at each age were combined for analysis. Significance was calculated by multiple t tests and one-way ANOVA in Graphpad Prism.

Immunohistochemistry

Mice at P63-P66 were transcardially perfused with 4% paraformaldehyde (PFA), brains were removed and postfixed overnight in 4% PFA at 4°C before storage in 25% sucrose. Brains were embedded in OCT medium, cut into 10 µm thick matched sections and stored at -20°C. Sections were post-fixed in 4% PFA for 10 min. at room temperature and rinsed in PBS 3x 5 min before permeabilization and blocking in 1% Triton X-100, 10% goat serum, 10% BSA, PBS for 2 hours at room temperature prior to staining. Slides were incubated overnight at 4°C with primary antibodies, MBP (Abcam Cat# ab7349, RRID:AB_305869; 1:100), CC1 (Millipore Cat# OP80, RRID:AB_2057371; 1:20) or NeuN (Abcam Cat# ab104224, RRID:AB_10711040; 1:100) in 0.1% Triton X-100, 1% goat serum, 10% BSA, PBS and then washed with PBS 3x 5 min. Secondary antibodies (1:1000) were conjugated to Alexa-Fluor 488 (Invitrogen A28175), Alexa-Fluor 568 (Invitrogen A21144) or Alexa Fluor 633 (Invitrogen A21094). For Iba1 staining, antigen retrieval (0.025% Tween-20 in 10 mM citrate buffer pH 6.0) and non-specific blocking (10% donkey serum) was performed before incubation overnight with primary antibody (Wako Chemicals RRID:AB_839504; 1:250) and detection with IgG conjugated to Alexa 488 (Invitrogen A11008). After washing in PBS,

slides were mounted with ProLong diamond antifade with DAPI (Invitrogen). Images were captured using a 3D Histech P250 High Capacity Slide Scanner and Z-stack images were acquired using a Leica SP8 inverted DMI8 confocal microscope (40X N.A.1.3).

Immunofluorescence and quantitation

LIF micrographs were analyzed using Volocity software. Composite images were generated and then split into individual channels. The threshold for the green, red and magenta channels was set uniformly across all sections according to signal intensity. Mean fluorescent intensity and area values were reported directly from Volocity software. CC1 stained cells were counted at the midline of the corpus callosum and NeuN stained cells were counted in the motor cortex. Colocalization of CC1 and MBP signals was interrogated in the merged image throughout the Z-stack and confirmed in the individual split channels. Quantification of Iba1 staining was performed manually in areas covering $\sim 1.3 \text{ mm}^2$ of the cortex and striatum.

Nucleic acid preparation for RNA and DNA analysis

Tissues (50 to 100 mg, freeze-clamped and flash frozen in liquid nitrogen) were homogenized into TRIzol Reagent. RNA was reprecipitated before quantification. DNA recovered using the TRIzol DNA isolation protocol was used in mitochondrial genome content assays (Quiros et al. 2017 [\[1\]](#)). Total RNA (2 μg) was used for cDNA synthesis with SuperScript III (SSIII, Invitrogen). qPCR followed the Lightcycler 480 SYBR Green I Master mix product manual for 384 multiwell plates (Roche). All primers are listed in Supplemental Table S7. Each sample was run in triplicate with 4-6 biological replicates. $\Delta\Delta\text{Ct}$ values were calculated using two reference genes (GAPDH, β -actin and/or γ -tubulin) (Taylor et al. 2019 [\[2\]](#)).

Northern blotting

Total RNA (5 μg) was resolved by denaturing polyacrylamide electrophoresis before electrophoretic transfer to Nytran N or SPC membranes (Cytiva), and hybridization to [^{32}P]-end-labeled oligonucleotide probes at 37°C (Bonhoure et al. 2015 [\[3\]](#)). Transcripts detected by phosphorimaging were quantified using ImageQuant software, normalized to U3 snRNA, and expressed as a fraction of the mean WT value. Oligonucleotide probes are listed in Supplemental Table S7.

tRNA-sequencing and differential expression analysis

Total RNA was mixed with a synthetic spike-in tRNA based on *E. coli* tRNA^{Gln} (Supplemental Table S7). The spike-in tRNA was denatured and refolded (80°C 2 min with a 0.1°C/s ramp to 20°C in 0.5 mM EDTA), and then added to 2.5 μg of total RNA from P42 cerebra (0.375 ng spike-in) or P42 cerebella (0.750 ng spike-in) prior to tRNA library preparation. The RNA mixture was deacylated (37°C for 45 minutes in 20 mM Tris-HCl pH 9.0, RNaseOUT (1 $\mu\text{g}/\mu\text{L}$, Invitrogen) and then 3'-end dephosphorylated and 5'-end phosphorylated using T4 polynucleotide kinase (NEB). tRNA library preparation used SuperScript™ IV following the QuantM-tRNA-seq protocol (Pinkard et al. 2020 [\[4\]](#)). Library multiplexing, sequencing (Illumina NextSeq500, single-end 150 bp reads) and mapping was performed by the Center for Epigenomics/Computational Genomics Core at AECOM. Library reads were trimmed and mapped, as specified previously (Pinkard et al. 2020 [\[4\]](#)), to a custom look-up table containing the high confidence set of nuclear-encoded cytosolic tRNAs (gtRNAdb grcm38/mm10), mitochondrially-encoded tRNAs (RNACentral) and the spike-in tRNA sequence. tRNA reads > 10 were used for further analyses. Decoder families were created by summing reads for all tRNAs with the same anticodon sequence. tRNAseq raw read counts for tRNAs and tRNA decoder families were normalized to the mitochondrial tRNA and spike-in tRNA read counts using the estimateSizeFactors control gene function in DESeq2 (Love et al. 2014 [\[5\]](#)). Differential expression and significance were determined using the likelihood ratio test (LRT) in DESeq2. Data visualization and plotting was performed in Prism (v9, GraphPad).

RNAseq

Poly(A) selected mRNA libraries were generated from total RNA (RINs > 8.9, Agilent Bioanalyzer), sequenced (Illumina PE150) and processed by Novogene. Differential expression and significance was determined using DESeq2 in R studio for all read counts >10 using default settings and the Wald test. DE was defined as adjusted p-value < 0.05. Downstream data visualization and plotting were performed using heatmap in R and Prism (v9, GraphPad).

Statistical analysis

Statistical analyses were performed using Prism (v9 GraphPad) or Excel. Two-tailed unpaired Student's t tests were used for experiments with two conditions. Behavioral testing used multiple unpaired t tests and one-way ANOVAs. In molecular analyses, all samples represent biological replicates (no samples were pooled). Significance was determined at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean $\pm$ standard deviation (SD) or $\pm$ standard error (SEM) as indicated.

Data availability

RNA-seq and QuantM-tRNA-seq data are available on the Gene Expression Omnibus (GEO) under accession number GSE246162.

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Editors

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Senior Editor

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Joint Public Review:

Summary:

The authors present an intriguing investigation into the pathogenesis of Pol III variants associated with neurodegeneration. They established an inducible mouse model to overcome developmental lethality, administering 5 doses of tamoxifen to initiate the knock-in of the mutant allele. Subsequent behavioral assessments and histological analyses revealed potential neurological deficits. Robust analyses of the tRNA transcriptome, conducted via northern blotting and RNA sequencing, suggested a selective deleterious effect of the variant on the cerebrum, in contrast to the cerebellum and non-cerebral tissues. Through this work, the authors identified molecular changes caused by Pol III mutations, particularly in the tRNA transcriptome, and demonstrated its relative progression and selectivity in brain tissue. Overall, this study provides valuable insights into the neurological manifestations of certain genetic disorders and sheds light on transcripts/products that are constitutively expressed in various tissues.

Strengths:

The authors utilize an innovative mouse model to constitutively knock in the gene, enhancing the study's robustness. Behavioral data collection using a spectrometer reduces experimenter bias and effectively complements the neurological disorder manifestations. Transcriptome analyses are extensive and informative, covering various tissue types and identifying stress response elements and mitochondrial transcriptome patterns. Additionally, metabolic studies involving pancreatic activity and glucose consumption were conducted to eliminate potential glucose dysfunction, strengthening the histological analyses.

Comments on revised version from expert Editor #1:

The authors in the revised manuscript have effectively responded to all of the comments and suggestions raised by both reviewers. Overall, I find the revised version to be an important contribution to the field and the strength of evidence supporting the work's claims to be compelling.

Comments on revised version from expert Editor #2:

The authors have responded constructively to all the comments in the first round of reviews and clarified many issues in the manuscript. The current report represents a significant advance.

Comments on revised version from Reviewer #2:

The authors should include their clarifications of all concern raised by reviewer #2 (mentioned in the previous weaknesses) in the main text. They should consider including point #2 to point #10 in the main text (discussion section). The should highlight limitations of this study in discussion.

Also, they should clearly state that deciphering brain area specific behavioural deficits is beyond the scope of the manuscript with appropriate justification mentioned in the rebuttal letter.

I still do not agree with the author to state that "brain region-specific sensitivities to a defect in Pol III transcription". The changes are global and also not restricted to brain. Authors may consider restating this sentence. It is obvious that transcription defects related to tRNA production will lead to alteration in whole body physiology.

<https://doi.org/10.7554/eLife.95314.2.sa1>

Author response:

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

Reviewer #1 (Public Review):

Summary:

Moir, Merheb et al. present an intriguing investigation into the pathogenesis of Pol III variants associated with neurodegeneration. They established an inducible mouse model to overcome developmental lethality, administering 5 doses of tamoxifen to initiate the knock-in of the mutant allele. Subsequent behavioral assessments and histological analyses revealed potential neurological deficits. Robust analyses of the tRNA transcriptome, conducted via northern blotting and RNA sequencing, suggested a selective deleterious effect of the variant on the cerebrum, in contrast to the cerebellum and non-cerebral tissues. Through this work, the authors identified molecular changes caused by Pol III mutations, particularly in the tRNA transcriptome, and demonstrated its relative progression and selectivity in brain tissue. Overall, this study provides valuable insights into the neurological manifestations of certain genetic disorders and sheds light on transcripts/products that are constitutively expressed in various tissues.

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The authors utilize an innovative mouse model to constitutively knock in the gene, enhancing the study's robustness. Behavioral data collection using a spectrometer reduces experimenter bias and effectively complements the neurological disorder manifestations. Transcriptome analyses are extensive and informative, covering various tissue types and identifying stress response elements and mitochondrial transcriptome patterns. Additionally, metabolic studies involving pancreatic activity and glucose consumption were conducted to eliminate potential glucose dysfunction, strengthening the histological analyses.

Weaknesses:

The study could have explored identifying the extent of changes in the tRNA transcriptome among different cell types in the cerebrum. Although the authors attempted to show the temporal progression of tRNA transcriptome changes between P42 and P75 mice, the causal link was not established. A subsequent rescue experiment in the future could address this gap.

Nonetheless, the claims and conclusions are supported by the presented data.

We thank Reviewer 1 for their thoughtful review and commentary. We appreciate the reviewer's finding that our "claims and conclusions are supported by the presented data."

We note that our findings on the temporal progression of transcriptional changes between P42 and P75 apply to both the Pol II and Pol III transcriptomes. Importantly, in the case of Pol III, only precursor and mature tRNAs are affected at P42 whereas at P75, numerous other Pol III transcripts are also changed. We therefore attribute the changes in tRNA as being causal in disease initiation since this is the earliest direct consequence of the *Polr3a* mutation.

To expand on the evidence demonstrating the progressive nature of *Polr3*-related disease in our mouse model, the revised manuscript includes new immunofluorescence data showing no change in microglial cell density in the cerebral cortex or the striatum at an early stage in the disease (Supplementary Fig. S6F, G). This is in striking contrast to the findings at later times (P75) where the number of microglia increased significantly in the *Polr3a* mutant and exhibit an activated morphology (Fig. 4G,H).

We agree with the reviewer that it will be interesting in the future to assess the impact of the *Polr3a* mutation in different neural cell types and to explore opportunities for suppressing disease phenotypes.

Reviewer #2 (Public Review):

Summary:

The study "Molecular basis of neurodegeneration in a mouse model of Polr3 related disease" by Moir et.al. showed that how RNA Pol III mutation affects production, maturation and transport of tRNAs. Furthermore, their study suggested that RNA pol III mutation leads to behavioural deficits that are commonly observed in neurodegeneration. Although, this study used a mouse model to establish theses aspects, the study seems to lack a clear direction and mechanism as to how the altered level of tRNA affects locomotor behaviour. They should have used conditional mouse to delete the gene in specific brain area to test their hypothesis. Otherwise, this study shows a more generalized developmental effect rather than specific function of altered tRNA level. This is very evident from their bulk RNA sequencing study. This study provides some discrete information rather than a coherent story. My enthusiasm for publication of this article in eLife is dampened considering following reasons mentioned in the weakness.

Reviewer 2's summary contains two misstatements:

Moir et.al. showed that how RNA Pol III mutation affects production, maturation and transport of tRNAs.

Our experiments document the effect of a neurodegenerative disease-causing mutation in RNA polymerase III on the Pol III transcriptome with a particular focus on the tRNA^{ome} (i.e. the mature tRNA population). Experiments on the maturation and transport of tRNA were not performed as there was no indication that these processes might be negatively impacted at the earliest time point (P42). Additional comments about tRNA maturation and export are provided under points 8 and 9 (see below).

The study seems to lack a clear direction and mechanism as to how the altered level of tRNA affects locomotor behaviour.

This comment misstates the purpose of our study while overlooking the important results. As stated in the abstract, our goal was to develop “a postnatal whole-body mouse model expressing pathogenic *Polr3a* mutations to examine the molecular mechanisms by which reduced Pol III transcription results primarily in central nervous system phenotypes.”

Accordingly, our work provides the first molecular analysis of RNA polymerase III transcription in an animal model of *Polr3*-related disease. The novelty and importance of the findings, as stated in the abstract, include the discovery that a global reduction in tRNA levels (and not other Pol III transcripts) at an early stage in the disease precedes the frank induction of integrated stress and innate immune responses, activation of microglia and neuronal loss at later times. These later events readily account for the observed neurobehavioral deficits that collectively include risk assessment, locomotor, exploratory and grooming behaviors.

Strengths:

The study created a mouse model to investigate role of RNA PolIII transcription. Furthermore, the study provided RNA seq analysis of the mutant mice and highlighted expression specific transcripts affected by the RNA PolIII mutation.

Weaknesses:

(1) The abstract is not clearly written. It is hard to interpret what is the objective of the study and why they are important to investigate. For example: "The molecular basis of disease pathogenesis is unknown." Which disease? 4H leukodystrophy? All neurodegenerative disease?

We have modified the abstract to more clearly frame the objective of the study and its importance as reflected in the title "Molecular basis of neurodegeneration in a mouse model of *Polr3*-related disease". We hope the reviewer will agree that the fourth sentence of the abstract, unchanged from the initial submission, clearly outlines the objective of the study.

(2) How cerebral pathology and exocrine pancreatic atrophy are related? How altered tRNA level connects these two axes?

It is not known how cerebral pathology and exocrine pancreatic atrophy are related beyond their shared Pol III dysfunction in our mouse model of *Polr3*-related disease. We anticipate that altered tRNA levels connect these two axes. Indeed, the pancreas and the brain are both known to be highly sensitive to perturbations affecting translation (Costa-Mattioli and Walter, 2020 Science doi: 10.1126/science.aat5314). Changes to the tRNA population in the cerebrum and cerebellum of *Polr3a* mutant mice were extensively documented in the manuscript (e.g. Figs. 3, 5 and 6). We also found reduced tRNA levels in the pancreas of the mutant mice but did not report these findings due to the absence of a stable reference transcript in total RNA from the atrophied pancreatic tissue, even at the earliest time point examined (P42).

(3) Authors mentioned that previously observed reduction mature tRNA level also recapitulated in their study. Why this study is novel then?

Our study reports the novel finding that a pathogenic *Polr3a* mutation causes a global reduction in the steady state levels of mature tRNAs, i.e. the levels of all tRNA decoders were reduced with the vast majority these reaching statistical significance (Fig. 6D and 6F). In the introduction we refer to several studies that examined the effect of pathogenic *Polr3* mutations on the levels of Pol III-derived transcripts. We noted that these studies examined only a small number of Pol III transcripts in CRISPR-Cas9 engineered cell lines, patient-derived fibroblasts and patient blood. Thus, no study until now has tested for or reported a global defect in the abundance of mature tRNAs in any model of *Polr3*-related disease. Moreover, no previous study of *Polr3*-related disease has analyzed Pol III transcript levels in the brain or in any other tissue.

(4) It is very intuitive that deficit in Pol III transcription would severely affect protein synthesis in all brain areas as well as other organs. Hence, growth defect observed in Polr3a mutant mice is not very specific rather a general phenomenon.

While we agree with the simple assumption that a “deficit in Pol III transcription likely would affect protein synthesis in all brain areas as well as other organs”, this turned out not to be the case. In fact, a novel finding of our study is that not all *Polr3a* mutant tissues show a translation stress response despite reduced Pol III transcription and reduced mature tRNA levels. This implies that in some tissues the reduction in tRNA levels caused by the *Polr3a* mutation is not sufficient to affect protein synthesis, at least to a point where the Integrated Stress Response is induced. The underlying basis for the growth deficit has not been defined in this work. However, we noted in the discussion that a growth defect was previously seen in mice where expression of the *Polr3a* mutation was restricted to the Olig2 lineage. In the present postnatal whole-body inducible model, we anticipate that the diminished growth of the mice results from a combination of hormonal and nutritional deficits caused by cerebral and pancreatic dysfunction.

(5) Authors observed specific myelination defect in cortex and hippocampus but not in cerebellum. This is an interesting observation. It is important to find the link between tRNA removal and myelin depletion in hippocampus or cortex? Why is myelination not affected in cerebellum?

We agree that the specific myelin defect observed in the cortex and hippocampus, but not the cerebellum, is an interesting observation. Pol III dysfunction in this model and reduced tRNA levels are common to both cerebra and cerebella, yet the pathological consequences differ between these regions. While we do not know why this is the case, the cells that oligodendrocytes support in these regions are functionally different. We suggest in the discussion that subtle defects in oligodendrocyte function in the cerebellum may be uncovered using more sensitive or specific assays than the ones we have employed to date. In addition, consistent with our findings in other tissues where Pol III transcription and tRNA levels are reduced but phenotypes are lacking, we suggest that oligodendrocytes in the cerebellum may have a different minimum threshold for Pol III activity than in other regions of the brain.

(6) How was the locomotor activity measured? The detailed description is missing. Also, locomotion is primarily cerebellum dependent. There is no change in term of growth rate and myelination in cerebellar neurons. I do not understand why locomotor activity was measured.

We used a behavioral spectrometer with video tracking and pattern-recognition software to quantify ~20 home cage-like behaviors, including locomotor activity, as part of our phenotypic characterization of the mice. This experimenter-unbiased approach reported several metrics of locomotion, specifically, total Track length (the total distance traveled in the instrument), Center Track length and the time spent running (Run Sum) and standing still (Still Sum) in a longitudinal study (Figs. 2A-C and Supplemental Fig. S3A-C). The Materials and Methods section on mouse behavior has been amended to provide a detailed description of these experiments.

locomotion is primarily cerebellum dependen_t

While we agree that the cerebellum plays a critical role in balance and locomotion, regions of the cerebrum that are affected in our mice, including the primary motor cortex and the basal ganglia (Fig. 4), also have important roles in locomotor activity and control.

(7) The correlation with behavioural changes and RNA seq data is missing. There a number of transcripts are affected and mostly very general factors for cellular metabolism. Most of them are RNA Pol II transcribed. How a Pol III mutation influences RNA Pol II driven transcription? I did not find differential expression of any specific transcripts associated with behavioural changes. What is the motivation for transcriptomics analysis? None of these transcripts are very specific for myelination. It is rather a general cellular metabolism effect that indirectly influences myelination.

The differentially expressed mRNAs identified in our RNAseq analysis at P75 reflect both direct and secondary consequences of dysfunctional Pol III transcription on Pol II transcription. These effects can be achieved by multiple mechanisms. Induction of the Integrated Stress Response (ISR) due to insufficient tRNA can be considered a direct consequence of diminished Pol III transcription on Pol II transcription. An example of a secondary response is the activation of microglia and the innate immune response (which is known to accompany prolonged activation of the ISR), and the loss of neurons and oligodendrocytes. These changes are documented in Figs. 3 and 4. Importantly, loss of neurons, activated microglia and reduced oligodendrocyte numbers are each readily reconciled with changes in behavior.

None of these transcripts are very specific for myelination

The RNAseq data at P75 indicates only a modest reduction in oligodendrocyte-specific gene expression (as defined by single-cell RNAseq studies of purified cell populations, Mackenzie et al., 2018 Sci. Rep. doi: 10.1038/s41598-018-27293-5). Despite this, some oligodendrocytespecific transcripts with well-known roles in myelination were down-regulated in the *Polr3a* mutant (e.g. Plp1, Mog and Mobp). In addition, steroid synthesis pathway transcripts involved in the production of cholesterol, an abundant and essential component of myelin, were also downregulated (Supplementary Fig. S4E).

(8) What genes identified by transcriptomics analysis regulates maturation of tRNA? Authors should at least perform RNAi study to identify possible factor and analyze their importance in maturation of tRNA.

Of the many proteins involved in the maturation of tRNA (Phizicky and Hopper, 2023 RNA doi: 10.1261/rna.079620.123), RNAseq analysis at P75 identified only amino-acyl tRNA synthetases as being differentially-expressed (fold change >1.5, p adj. < 0.05, Table S1). These genes are canonical indicators of the ATF4-dependent Integrated Stress Response and their upregulation is widely interpreted as an attempt to restore efficient translation. In addition, our analysis of Pol III transcripts at P75 identified a reduction in the level of RppH1 (Fig. 3C), the RNA component of RNase P, which removes the 5' leader of precursor tRNAs. However, at P42, there was no effect on RppH1 abundance, or the expression of amino-acyl tRNA synthetase genes (Fig. 5C and Table S3). Thus, an RNAi study to identify and analyze a possible factor involved in the maturation of tRNA is neither warranted nor relevant to the current body of work.

(9) What factors are influencing tRNA transport to cytoplasm? It may be possible that Polr3a mutation affect cytoplasmic transport of tRNA. Authors should study this aspect using an imaging experiment.

Our analysis of tRNA populations in this study employed total cellular RNA and thus reflect the abundance of mature tRNA from all cellular compartments. We have not assessed whether the reduction in tRNA abundance caused by the *Polr3a* mutation alters the dynamics of tRNA transport from the nucleus to the cytoplasm. However, we consider it highly unlikely

that the *Polr3a* mutation would have a significant effect on cytoplasmic transport of tRNA. Imaging experiments along these lines are beyond the scope of the current study.

(10) Does alteration of cytoplasmic level of tRNA affects translation? Author should perform translation assay using bio-orthogonal amino acid (AHA) labelling.

It is not known whether the reduced tRNA levels affect translation globally in the *Polr3a* mutant, but we predict that this may not be the case. Since tissues (heart and kidney) and brain regions (cerebrum and cerebellum) that share a decrease in tRNA abundance do not share activation of the Integrated Stress Response (a reporter of aberrant translation), we anticipate that effects on translation may be limited to specific regions or cell populations and to specific mRNAs within these cells. The current study provides the foundation for further work to address these questions.

Reviewer #1 (Recommendations For The Authors):

Below are a few comments, mostly regarding typographical errors, presentation, and clarity, that we believe would enhance this manuscript:

On the heatmaps generated, it would be ideal to place "WT" before "KI," with "WT" on the left. This will maintain consistency with the rest of the manuscript, where "WT" conditions precede "KI" conditions, as observed in the bar graphs and dot plots.

All heatmaps have been remade with WT on the left and KI on the right to maintain consistency throughout the manuscript.

Authors mentioned in several instances (Discussion Pg 19 Line 2, for instance) the analysis of changes in the "Pol II transcriptome." Is this a typographical error?

The reference to the Pol II transcriptome is not a typographical error (Discussion Pg 19 Line2). Here and elsewhere in the manuscript, we are distinguishing between changes to the Pol III transcriptome and the timing of subsequent changes to the Pol II transcriptome. The text has been edited to clarify this relationship in several places.

(1) Introduction, Page 4, last paragraph.

Analysis of the Pol III transcriptome reveals a common decrease in pre-tRNA and mature tRNA populations and few if any changes among other Pol III transcripts across multiple tissues. Analysis of the Pol II transcriptome reveals activation of the integrated stress response in cerebra but not in other surveyed tissues.

(2) Results, page 8, 2nd paragraph

To investigate the molecular changes to Pol III transcript levels caused by the *Polr3a* mutation and any secondary effects on the Pol II transcriptome, we initially focused on the cerebra of adult mice at P75.

(3) Discussion, Page 19, second paragraph

Pol III dysfunction and the reduction in the cerebral tRNA population at P42 coincides with behavioral deficits and precedes substantial downstream alterations in the Pol II transcriptome, which include induction of an innate immune response (IR) and an ISR, and indicators of neurodegeneration (i.e., activation of cell death pathways and loss of mitochondrial DNA). These findings suggest a causal role for the lower tRNA abundance and/or altered tRNA profile in disease progression.

In supplementary figure 1, authors validated the expression of their systems using flow cytometry and observed a high level of recombination frequency in different tissue types. Can the flow cytometry data distinguish between cell types within the cerebrum (neurons/microglia/astrocytes)?

The flow cytometry experiments reported in Supplementary Fig. S1 used a dual tdTomato-EGFP reporter to assess recombination. The cerebral and cerebellar samples were gated on fluorescence from endogenous expression of tdTomato (red), EGFP (green) and DAPI (blue) staining. In principle, flow cytometry could be used to distinguish between cell types within the cerebrum (neurons/microglia/astrocytes). However, this would require (i) an antibody to a cell surface marker on the cell type of interest and (ii) a fluorescent probe conjugated to the primary antibody or a fluorescent secondary antibody that is spectrally well resolved from the emission spectra of tdTomato, eGFP and DAPI.

Results section 1: Is there any particular reason why P28 was chosen as the commencement of tamoxifen injection?

P28 was chosen so that any effect of the *Polr3a* mutation on development and differentiation would be limited in the tissues we examined.

Fig 1C: The number of asterisks does not match between the graph and the figure legend.

Fig. 1C has been corrected to match the number of asterisks in the graph and figure legend.

Results section 3:

This section seemed a little brief, especially when compared to the depth of the succeeding sections. Authors can state in greater detail which behaviors were quantified. In S3A-C, my understanding is that the animals were placed in an open-field test. This procedure can be briefly mentioned in the methods, as well as in the main manuscript text.

In the legends of S3, a bracket is missing for "(D-F)" on line 5. Additionally, the alignment of legends for each bar graph could be consistent for all graphs except under the condition of spatial constraint.

Detailed methods pertaining to the measurement and calculation of home cage-like behaviors reported by the behavioral spectrometer have been added to the Methods section on Mouse Behavior.

In the Results, Figs. S3A-C show anxiety-like behaviors which measure the number and duration of visits and the distance traveled in a 15 cm² central area of the arena. Figs. 2A-C show locomotor behaviors including Tracklength, Run sum and Still sum. The open field-like behavior is reported as total Tracklength in the behavioral spectrometer, i.e. the total distance travelled in the arena. This is now more clearly described in the main manuscript and the Methods section. "overall locomotor activity was decreased in *Polr3a*-tamKI mice as indicated by the reduced track length at P42, P49, P56 and P63 (Fig. 2A)."

The legend of S3, now has the missing bracket "(D-F)" on line 5.

The legends within each bar graph are now consistent and aligned as much as spatial constraints allow.

Results section 4:

Similar to our earlier questions for S1, is it possible to distinguish samples derived from different cell types (neurons/glia)? In figure 4, this is mainly done post-hoc, based on the known gene expression. Maybe the authors could discuss this small limitation? In Fig S4C, the color contrast for the heatmap legend needs to be corrected.

It is not possible to accurately distinguish different neural cell sub-types, such as different types of neurons, or different types of oligodendrocytes in bulk RNAseq. Hence, we have reported only high confidence correlations based on known gene expression signatures (Fig. 4). We discuss only the data for which we can draw confident conclusions. The heatmap and legend in Fig. S4C has been amended.

Results section 5:

In figure S5A, the alignment of asterisk significance markers could be adjusted.

Asterisks have been realigned in Fig. S5A

Reviewer #2 (Recommendations For The Authors):

Methods Section should include detailed procedure.

A detailed description of the methods pertaining to the measurement and calculation of behaviors using the behavioral spectrometer has been added to the Methods section.

Statistical tests should have detailed information

Statistical tests are detailed in the Methods section “Statistical Analysis”. Additional details pertaining to calculations of behavioral data have been added to the “Mouse behavior” section of the Methods.

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