Iron Responsive Element (IRE)-mediated responses to iron dyshomeostasis in Alzheimer's disease

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Abstract

Background: Iron trafficking and accumulation has been associated with Alzheimer's disease (AD) pathogenesis. However, the role of iron dyshomeostasis in early disease stages is uncertain. Currently, gene expression changes indicative of iron dyshomeostasis are not well characterized, making it difficult to explore these in existing datasets.

Objective: We identified sets of genes predicted to contain Iron Responsive Elements (IREs) and used these to explore iron dyshomeostasis-associated gene expression responses in cultured cell, human, mouse, and zebrafish datasets.

Results: IRE gene sets were sufficiently sensitive to distinguish not only between iron overload and deficiency in cultured cells, but also between AD and other pathological brain conditions in different brain regions. Notably, we see changes in IRE transcript abundance as amongst the earliest observable changes in zebrafish familial AD (fAD)-like brains, preceding other AD-typical pathologies such as inflammatory changes. Unexpectedly, while some IREs in the 3' untranslated regions of transcripts show significantly increased stability under iron deficiency in line with current assumptions, many such transcripts instead show decreased stability, indicating that this is not a generalizable paradigm.

Conclusion: Our results reveal IRE gene expression changes as early markers of the pathogenic process in fAD and are consistent with iron dyshomeostasis as an important driver of this disease. IRE gene expression changes can also distinguish AD from other neuropathologies. Our work demonstrates how differences in the stability of IRE-containing transcripts can be used to explore and compare iron dyshomeostasis-associated gene expression responses in different species, tissues, and conditions.

Keywords

Iron responsive element, iron homeostasis, Alzheimer's disease, gene expression, Computational Biology, Transcriptomics, familial Alzheimer's disease

Introduction

The pathological processes underlying Alzheimer's Disease (AD) commence decades before symptoms become evident [1,2]. Since the early days of AD research, iron trafficking and accumulation has been observed to be altered in AD [3–7]. However, it is still unclear whether iron dyshomeostasis represents a late symptom or an early pathological driver of AD. Iron homeostasis is closely linked to many critical biological processes including cellular respiration, hypoxia, and immune responses, all of which are disrupted in AD. Consistent with this, discoveries over the past decade have shown that disruptions to iron homeostasis can drive feedback loops that worsen AD pathology [8,9]. However, evidence for iron dyshomeostasis as an early driver of the disease is only just emerging. While iron accumulation associated with amyloid plaque formation has been observed in various transgenic mouse models of AD [10–13], the age at which iron dyshomeostasis first occurs is uncertain [14].

Currently, gene expression patterns representing responses to iron dyshomeostasis are not well-characterized. Cellular responses to iron dyshomeostasis are complex and involve several systems and layers of regulation. The stability of the transcription factor HIF1 α , (a component of HIF1, a master regulator of responses to hypoxia) is regulated by an iron-dependent mechanism so that transcriptional responses to iron deficiency can resemble hypoxia responses [15]. However, cellular iron homeostasis is also regulated at the post-transcriptional level by the IRP/IRE system [16–18]. In this system, altered levels of available

ferrous iron (Fe^{2+}) cause Iron Responsive Proteins (IRP1 or IRP2) to change conformation or stability respectively [19,20]. This alters their ability to bind *cis*-regulatory Iron Responsive Element (IRE) stem-loop motifs in the 3' or 5' untranslated regions (UTRs) of genes encoding products related to iron metabolism. Only a few IRE-containing genes have been characterized in detail, including transferrin receptor 1 (*TFR1*; 3' UTR IRE), divalent metal transporter 1 (*DMT1* or *SLC11A2*; 3' UTR IRE), ferritin heavy chain and ferritin light chain (*FTH1* and *FTL*; both 5' UTR IRE), and ferroportin (*SLC40A1*; 5' UTR IRE) [18]. In general, these previously-characterized IRE-containing genes suggest that IRPs binding to 3' IREs tend to stabilize transcripts to increase protein translation, while binding to 5' IREs suppresses translation [17]. It is important to note that while the ancestral ferritin IRE is present even in lower metazoans (including sponges), IRE elements in other genes have likely more recently arisen through convergent evolution [21]. Perhaps because of this, some heterogeneity in regulatory mechanisms and effects for IRE genes has been reported. For example, *SLC11A2* is stabilized by IRP1 binding to a greater extent than by IRP2 binding (as its 3' IRE has a greater affinity for IRP1) [22]. Similarly, while *CDC42BPA* and *TFRC* both possess 3' IREs, *CDC42BPA* is stabilized to a greater extent than *TFRC* under iron deficiency [23]. Currently however, global gene expression changes mediated by genes in the IRP/IRE system have not been well-defined, and the overall expression patterns of IRE-containing genes have not been explored in the context of AD. In addition, it is unclear how expression of these genes might differ between AD and other neurodegenerative diseases, or how AD risk factors such as aging and hypoxia might contribute.

In this study, we utilized the SIREs (Searching for Iron Responsive Elements) tool [24] to predict and identify sets of IRE-containing genes in human, mouse, and zebrafish. We then applied these gene sets to explore overall IRP/IRE-mediated iron dyshomeostasis-associated responses in datasets involving: (1) a cultured cell line subjected to iron overload

and deficiency treatments, (2) a cohort of AD patients, healthy controls, and two other pathological conditions affecting the brain, (3) 5XFAD mice used to model the amyloid and tau pathology seen in AD, and (4) a zebrafish knock-in model possessing a familial AD (fAD)-like mutation.

Our IRE gene sets displayed significant enrichment in AD, the 5XFAD mouse model, and an fAD-like zebrafish model, demonstrating for the first time the early and extensive involvement of IRP/IRE-mediated iron dyshomeostasis-associated gene expression responses in the context of AD. IRE gene sets were sufficiently sensitive to distinguish not only between iron overload and deficiency in a cultured cell line dataset, but also between AD and other pathological conditions affecting the brain (pathological aging and progressive supranuclear palsy), implying that the dysregulation of IRE-containing genes in AD may differ from other conditions. Changes in IRE gene expression were already evident in young adult brains of animal models of AD (3-month-old 5XFAD mice, 6-month-old fAD-like zebrafish). Overall, our observations do not support the current assumption that IRP binding to 3' IREs generally stabilizes transcripts as we observed both increases and decreases in abundance of transcripts with either 3' or 5' IREs under most conditions.

Results

Defining sets of genes containing Iron Responsive Elements (IREs)

We utilized the SIREs (Searching for Iron Responsive Elements) tool, to define species-specific IRE gene sets by searching for IRE and IRE-like motifs in the 3' and 5' untranslated regions (UTRs) of the reference transcriptomes of human (hg38), mouse (mm10), and zebrafish (z11). *SIREs* assigns a quality-score to all detected IREs, with "high-quality"

scores corresponding to canonical IREs and “medium-quality” or “low-quality” scores reflecting deviations from the canonical IRE (alternative nucleotide composition in the apical loop, one bulge at the 3’ or one mismatch in the upper stem) that would still produce an IRE-like motif with potential to be functional [24–28]. **Figure 1** summarizes the IRP/IRE interaction effects on transcripts and gives examples of canonical and non-canonical IREs.

We defined four gene sets for each species as follows: **HQ 3’ IREs** (high-quality predicted 3’ IRE genes), **HQ 5’ IREs** (high-quality predicted 5’ IRE genes), **all 3’ IREs** (including all low, medium, and high-quality predicted 3’ IRE genes), and **all 5’ IREs** (including all low, medium, and high-quality predicted 5’ IRE genes). The size of these gene sets for each species and the overlap present is shown in **Figure 2** and the gene sets are provided in **Supplementary Table 1**. Overall, searching through human UTRs uncovered the largest number of predicted IRE genes, followed by mouse, and then zebrafish. Overlap between homologous genes from the IRE gene sets of different species was generally poor. The largest sets of genes shared between species were consistently found between human and mouse, which is reflective of their closer evolutionary divergence.

While not many high-quality IRE genes were identified across all three species, the few we identified are consistent with known and well-characterized IREs in the literature [29,30]. For example, the single shared **HQ 3’ IRE** gene between the three species was *TFRC* (transferrin receptor), while the shared **HQ 5’ IRE** genes between the three species included *FTH1* (ferritin heavy chain 1) and *ALAS2* (5'-aminolevulinate synthase 2). We note that the scoring of genes for the **HQ 3’ IRE** and **HQ 5’ IRE** sets is considered stringent (i.e. only canonical IREs) and that other human genes with well-characterized IREs experimentally shown to bind IRPs are recovered in the **All 3’ IRE** and **All 5’ IRE** sets across mouse and zebrafish as well. These genes include *CDC14A* (3’ IRE; [31]), *CDC42BPA* (3’ IRE; [23,32],

SLC40A1 (5' IRE; [33]), and *FTL* (5' IRE; [34]). In addition, the 3' IRE of *SLC11A2* (also known as *DMT1*) [22] which is only found in mammals was also recovered in the mouse **All 3' IREs** set (but not for zebrafish). The expression of these previously characterized IRE genes across the human, mouse, and zebrafish datasets analyzed in this current work is described in **Supplementary Text 1** and discussed further in the analyses we later describe.

IRE gene sets are over-represented within up-regulated AD genes, but overall not well-represented in existing gene sets

We explored the biological relevance of the predicted IRE gene sets described above by testing whether genes within them were over-represented in existing gene sets from the Molecular Signatures Database (MSigDB; available at <https://www.gsea-msigdb.org/gsea/msigdb>). MSigDB represents one of the largest publicly-available collection of gene sets collated from existing studies and other biological databases [35]. We limited our analysis to gene sets from the following collections: Hallmark (non-redundant sets of ~200 genes each representing various biological activities), C2 (gene sets from databases including KEGG and Reactome and published studies), C3 (gene sets containing genes with motif elements), and C5 (gene sets based on gene ontology terms) (see **Methods**). We performed over-representation analysis for the predicted IRE gene sets (**all 3' IREs** and **all 5' IREs**) separately for each species (human, mouse, zebrafish), and used Wilkinson's meta-analytic approach to determine gene sets that were significantly over-represented across the three species' IRE gene sets. Our results indicated that 1,148 of 10,427 tested gene sets displayed over-representation of IRE gene sets across all three species (Bonferroni-adjusted Wilkinson's p -value < 0.05) (**Supplementary Table 2**). Remarkably, the "Blalock Alzheimer's Disease Up" gene set from the C2 collection was significantly over-represented in both the sets of **all 3' IREs** (Bonferroni-adjusted Wilkinson's p -value = 3.9×10^{-14}) and **all 5' IREs** (Bonferroni-adjusted Wilkinson's p -value = 2.1×10^{-26}) in the meta-analysis,

and was also the gene set with the most significant over-representation of the human ***all 3'*** ***IREs*** set (Bonferroni-adjusted Fisher's exact test p -value = $7.8e-58$) (**Supplementary Table 2**). This supports that disturbance of iron homeostasis particularly distinguishes Alzheimer's disease from other disease conditions and pathways represented within the C2 collection. In addition, the top 15 MSigDB gene sets showing the most significant over-representation generally differed between species. However, in all cases, a large proportion of IRE genes from the predicted IRE gene sets were not contained within any of these top-ranked MSigDB gene sets or within the Heme Metabolism gene set belonging to the Hallmark collection (**Figure 3**). This demonstrates that the predicted IRE genes we defined are not fully captured by existing gene sets and so may be uniquely useful for investigating gene expression changes during the IRE-IRP response to iron dyshomeostasis.

Involvement of transcription factor regulation within IRE gene sets

To be confident that IRE gene sets accurately capture information about the IRP/IRE-mediated responses, we needed to investigate possible co-ordinate regulation by other factors. As a starting point, we examined whether known binding motifs for transcription factors were significantly over-represented in the promoter regions of genes within each IRE gene set (***all 3' IREs***, ***all 5' IREs***, ***HQ 3' IREs***, and ***HQ 5' IREs***) for each species. We detected significant over-representation of several transcription factors including the Klf14 motif in the zebrafish ***all 3' IREs*** set (FDR-adjusted p -value = 0.049), the E2F motif in the human ***all 5' IREs*** set (FDR-adjusted p -value = 0.049) and the Bach1 (FDR-adjusted p -value = 0.012), Nrf2 (FDR-adjusted p -value = 0.013), and NF-E2 (FDR-adjusted p -value = 0.019) motifs in the zebrafish ***HQ 5' IREs*** set (**Supplementary Table 3**). This suggests that the following results should be interpreted with the knowledge that expression of subsets of genes in some of the IRE gene sets may be influenced by factors other than iron ions.

Gene set enrichment testing approach

Our predicted IRE gene sets can be used in any standard gene set enrichment testing approach to detect potential changes in iron homeostasis between conditions. Our workflow, which we later apply on human, mouse, and zebrafish datasets, is shown in **Figure 4**. Due to variability in the results produced by different gene set enrichment testing methods, we were inspired by the EGSEA framework [36] to combine the results from different methods. Based on an initial analysis using EGSEA, we chose *fry/mroast* [37,38], *camera* [39], and *fgsea* [40,41] as the representative methods to use. (See **Supplementary Figure 2** for a principal component analysis of results from different gene set enrichment analysis approaches.) A summary of the different characteristics of the three methods is shown in **Table 1**. The raw enrichment *p*-values from these approaches can be combined to obtain an overall enrichment *p*-value for each gene set. In accordance with EGSEA default parameters, we used Wilkinson's method to combine raw *p*-values, and then applied adjustment for multiple testing on all combined *p*-values. Along with performing this gene set enrichment testing on our IRE gene sets, we also recommend using the same method to test for enrichment for the MSigDB Hallmark gene sets as the diverse biological activities they represent help to provide context for interpreting the IRE enrichment results. To explore further the results of IRE gene set enrichment analysis, we use UpSet plots to display the overlap between sets of "leading-edge" genes in the *all 3' IREs* and *all 5' IREs* gene sets. The leading-edge genes can be interpreted as the core (often biologically important) genes of a gene set that account for the significant enrichment as calculated by GSEA [41].

Differences in IRE gene set enrichment during iron deficiency and iron overload in a cultured cell line

We first tested how our enrichment approach illuminated the effects of iron overload and deficiency in a cultured cell line microarray dataset from Caco-2 cells (GEO accession:

GSE3573). As only one cell type contributed to this dataset, interpretation of the IRE enrichment results is simplified by not having to consider the differing iron requirements of different cell types. This allowed us to focus on whether the iron dyshomeostasis treatments (iron overload and iron deficiency) could be detected and distinguished in terms of their IRP/IRE system-driven transcript abundance response. Because the dataset was from a microarray experiment, we performed only the differential gene expression and gene set enrichment testing portions of our workflow. The results of Principal Component Analysis and differential gene expression analysis are provided in **Supplementary Figure 3**. We note that this particular dataset was chosen despite not being derived from a central nervous system cell type as maintaining iron homeostasis is fundamental to all cell types (regardless of their tissue of origin). In addition, this particular dataset included iron deficiency and iron overload treatments in the same batch and with sufficient sample number, and no other cultured cell line gene expression dataset was publicly available at the time that fulfilled these criteria.

In general, we found iron deficiency and iron overload treatments resulted in different gene expression responses. In terms of classic IRE genes such as *TFRC*, *FTL*, and *FTH1*, iron deficiency is associated with increased expression of *TFRC* (consistent with increased transcript stabilization of this 3' IRE gene) and decreased expression of the 5' IRE genes *FTL* and *FTH1*, while these changes are in the opposite direction during iron overload (see **Supplementary Text 1** and **Supplementary Figure 1A**). Differential gene expression across all detected genes in the dataset additionally revealed that iron deficiency was associated with 96 differentially expressed genes of which 10 possessed predicted IREs while iron overload was associated with 212 differentially expressed genes (FDR-adjusted p -value from *limma* < 0.05) of which 33 possessed predicted IREs (**Supplementary Figure 3B**). There were 17 differentially expressed genes in common between iron deficiency and

iron overload treatments, and all moved in opposite directions according to the treatment (i.e. increased abundance under iron deficiency and decreased abundance under iron overload, or vice versa). These clear differences between iron deficiency and iron overload were reflected in gene set enrichment analyses using the MSigDB Hallmark gene sets, where the gene sets involved and the proportions of gene transcripts with increased or decreased abundance differed (**Figure 5A**). As expected, IRE gene sets also showed significant enrichment under iron deficiency and overload conditions (**Figure 5B**) (Bonferroni adjusted p -value < 0.05).

We expected that iron deficiency would result in increased expression of genes with 3' IREs under the IRP/IRE paradigm similar to the *TFR* gene as seen in **Supplementary Text 1**. However, both the 3' and 5' IRE gene sets displayed mixed patterns of increased and decreased expression under both the iron deficiency and iron overload treatments (**Figure 5B**). This indicates it would be difficult to distinguish between these conditions based purely on overall increases or decreases in the expression of IRE gene sets. Despite this, we see that the iron deficiency and iron overload treatments can be distinguished by their “leading-edge” genes (those genes contributing most to the enrichment signal for the predicted IRE gene sets (*all 3' IREs* and *all 5' IREs*) (**Figure 5C**). This supports that gene set enrichment using predicted IRE gene sets may be sufficiently sensitive to detect whether iron dyshomeostasis is present and potentially more informative than individual IRE genes in distinguishing between different IRP-IRE system-mediated gene expression responses in iron deficiency and iron overload treatments in this cell line.

A distinct iron homeostasis response in human AD patients compared to other neuropathologies

Given that IRE gene sets could distinguish between iron overload and deficiency in a cultured cell line, we next tested our gene sets on a more complex dataset including cerebellum and temporal cortex tissue samples from post-mortem human brains. The brains originated from either healthy controls or patients afflicted with one of three conditions: Alzheimer's disease (AD); pathological aging (PA), a condition involving amyloid pathology but no significant dementia symptoms; or progressive supranuclear palsy (PSP), a tauopathy without amyloid pathology [42]. An important characteristic of the dataset is that both cerebellum and temporal cortex tissue samples were available from each patient. A summary of the 236 patients whose data we included in this analysis is shown in **Table 2** and the results of differential gene expression analysis and IRE gene set enrichment analysis are shown in **Table 3** and **Figure 6A**. The expression of classic IRE genes including *TFRC*, *FTL*, and *FTH1* in this dataset is shown in **Supplementary Text 1** and **Supplementary Figure 1B-C**. In our analyses, we focus mainly on comparing conditions within each tissue rather than between tissues. This is because we found significant differences in the AD vs. control comparison in the temporal cortex compared to the cerebellum (see **Supplementary Figure 4**).

Overall, our IRE enrichment analyses indicate significant enrichment of IRE gene sets in all pathological conditions (AD, PA or PSP) compared to healthy controls within the cerebellum and temporal cortex (**Figure 6A**). In all pathological conditions, 3' and 5' IRE gene transcripts show overall mixed patterns of abundance (e.g. increased and decreased). Further examination of the leading-edge genes from these IRE gene sets gives more insight regarding potential differences and similarities between AD, PA, and PSP (**Figure 6B**). Overall, AD, PSP, and PA appear to involve distinct yet partially overlapping IRE gene

expression responses in a tissue-specific manner. Within the temporal cortex, there are 3' IRE leading-edge genes and 178 5' IRE leading-edge genes exclusively present in the “AD vs. control” comparison. These are greater numbers than for the other two conditions. Interestingly, AD and PA share only relatively small numbers of leading-edge genes despite the fact that many regard PA as a prodrome of AD [43]. Interestingly, in the cerebellum, while AD and PSP share many 3' and 5' IRE leading-edge genes, PA is associated with a large number of unique leading-edge 3' and 5' IRE genes, further emphasizing its difference from AD and also PSP. These observations are supported when considering expression of previously characterized IRE genes individually (see **Supplementary Text 1** and **Supplementary Figure 1B-C**). The results in this Supplementary Text suggest that in particular, some 3' IRE genes (e.g. *TFRC*, *CAV3*, *CDC14A*, *CDC42BPA*, *SERTAD2*, *TRAM1*) show increased expression in AD (relative to controls) whereas PA and PSP do not. Overall, our results suggest that IRP-IRE system-mediated gene expression changes may be sufficiently sensitive to discern and identify potential differences in the way iron homeostasis is regulated in AD compared to PA and PSP.

Age-dependent disruption of IRE-driven iron homeostasis in the 5XFAD mouse model

Alzheimer’s disease is thought to develop over decades [44–46]. However, detailed molecular studies of AD brains can only be based on post-mortem tissues. To reveal the early molecular changes that initiate the progression to AD we must study the brains of animal disease models. Given that the IRE gene sets appear to work well in human datasets, we then tested our mouse IRE gene sets on an RNA-seq dataset derived from brain cortex tissue of the 5XFAD transgenic mouse AD model (GEO: GSE140286). The 5XFAD mouse is one of the most common systems used to model the amyloid beta histopathology of AD brains and brain gene expression patterns in this model were

previously shown in a concordance analysis to resemble those of human AD brains more than four other mouse models (3xTg, CK-p25, J20, and Tg2576) [47]. 5XFAD mice possess two transgenes that include a total of five different mutations, each of which individually causes fAD in humans. In this dataset, the mice analyzed were either 3, 6 or 12 months of age. We acknowledge that the small sample size of this particular dataset means that results should be interpreted tentatively (see Discussion).

Using gene set enrichment testing methods as before, we observed significant enrichment of the *all 3' IREs* and *all 5' IREs* gene sets in several comparisons. These included 5XFAD vs. wild type comparisons and wild type aging comparisons (Bonferroni-adjusted enrichment p -value < 0.05) (**Figure 7**). Notably, even the youngest age group of 5XFAD mutant mice (3 months) displayed significant enrichment of genes containing 3' or 5' IREs compared to age-matched wild types. This is consistent with an enrichment of immune-related Hallmark gene sets that we observed in this age group (see **Figure 7B**) and with a previous transcriptome analysis suggesting immune activation in 5XFAD mice as early as 2-4 months [48]. UpSet plots of overlapping leading-edge genes suggest that the IRE responses due to aging and due to the 5XFAD transgenes may involve partially overlapping gene expression changes (**Figure 8**). In addition, the UpSet plots reveal subsets containing large numbers of IRE-containing genes which uniquely contribute to enrichment of IRE gene sets in only one comparison (e.g. 256 3' IRE genes only contribute to enrichment in the “5xFAD vs WT at 6 months” comparison). Notably, there are 126 shared 3' IRE genes contributing to enrichment in the “5xFAD vs WT at 6 months” and “5xFAD vs WT at 3 months” comparisons, but no genes shared between these comparisons and the “5xFAD vs WT at 12 months” comparison. These 126 genes were found to show over-representation of Gene Ontology terms including “Hsp70 protein binding” and “nuclear import signal receptor” (FDR-adj. p -value < 0.05) (**Supplementary Table 4**), suggesting that subsets of leading-edge genes

may have biological relevance, and that these may give clues into the biologically distinct age-dependent iron dyshomeostasis responses caused by the 5XFAD transgenes. Although beyond the scope of our current analysis, further investigation into these subsets of genes may further help to define differences in iron dyshomeostasis responses under different biological conditions of interest.

Similarities in IRE-driven iron homeostasis responses during hypoxia and a familial AD-like mutation in a zebrafish model

Concerns have been raised over the relevance of transgenic mouse models in modelling the early stages of AD (reviewed by [49]). Meanwhile, knock-in models of familial AD (fAD) (that possess a single, heterozygous mutation in an endogenous gene like the human version of the disease) only exhibit subtle pathological changes with little to no visible amyloid or tau pathology present [50]. We make an important distinction between these fAD-like models that resemble the genetic state of human fAD as closely as possible and other knock-in models that invoke genetic states which have never been observed in human fAD (e.g. models possessing homozygous mutations that have only been observed in a heterozygous state in humans and/or several fAD-causative mutations simultaneously as described by Saito et al. [51] or Serneels et al. [52]). Despite the comparatively more subtle pathology seen in fAD-like knock-in models, because they more closely mimic the genetic state of fAD, it would be expected that gene expression analysis of knock-in models may reveal informative early molecular changes that are relevant to early fAD states in human brains. We had access to whole-brain RNA-seq data from a knock-in zebrafish model of fAD possessing a single fAD-like mutation in its endogenous *psen1* gene (*psen1*^{Q96_K97del/+}) (henceforth referred to as the “fAD-mutation-like” model in this paper). Previous analysis of a subset of this dataset involving young adults (6-month-old brains) revealed gene expression changes related to energy metabolism and mitochondrial function, especially ATP synthesis

and other ATP-dependent functions including lysosomal acidification [53]. (In contrast, the 3-month-old young adult 5XFAD mouse brain dataset is dominated by immune/inflammation responses, **Figure 7B**). Considering the critical role of iron homeostasis in energy metabolism, we decided to revisit this zebrafish dataset. We performed IRE gene set enrichment on the entire dataset in order to include exploration of the effects of aging and acute hypoxia (two important risk factors for sporadic late onset AD) and to analyze how these effects interact with the fAD-like *psen1*^{Q96_K97del/+} mutant genotype. The experimental design and the results of the differential gene expression analyses and gene set enrichment tests are summarized in **Figure 9**.

We first turned our attention to the comparison between young adult (6-month-old) *psen1*^{Q96_K97del/+} mutant zebrafish and their wild-type siblings. At this age, gene expression changes in the mutant fish likely represent early stresses driving the development of fAD in humans. Gene set enrichment tests in this comparison identify alteration of energy metabolism-related Hallmark gene sets (e.g. OXIDATIVE PHOSPHORYLATION, GLYCOLYSIS) (**Figure 9B**) which is consistent with a previous analysis of gene ontology terms with this dataset [53]. In addition, we see enrichment of other gene sets including FATTY ACID METABOLISM, PI3K AKT MTOR SIGNALLING, MTORC1 SIGNALLING, and HEME METABOLISM. All of these gene sets which show enrichment in 6-month-old *psen1*^{Q96_K97del/+} mutants relative to wild-type siblings are also enriched in 24-month-old *psen1*^{Q96_K97del/+} mutants relative to their wild-type siblings (**Figure 9B**). This supports that the biological activities represented in these gene sets are amongst the earliest altered in this particular fAD mutation model.

In Lumsden et al. [14] we predicted that fAD mutations in the major locus *PSEN1* would cause an early cellular deficiency of ferrous iron due to the observation of insufficient

acidification of the endolysosomal pathway in in-vitro *PSEN1* mutation studies [54,55]. In Newman et al. [53] we saw that GO analysis of 6 month old *psen1^{Q96_K97del/+}* zebrafish brain supported that lysosomal acidification was affected. Therefore, we decided to apply our IRE enrichment analysis to test for evidence of iron dyshomeostasis in these fish. The enrichment of the ***all 3' IREs*** set in 6-month-old *psen1^{Q96_K97del/+}* zebrafish brains supports that iron dyshomeostasis is an important stress in the early stages of fAD (**Figure 10A**).

While almost all pairwise comparisons in the zebrafish dataset show significant enrichment of at least one IRE gene set (**Figure 10A**), the expression of IRE genes appears to differ in terms of the proportions of IRE-containing transcripts which show increased versus decreased abundance (**Figure 10B**). In addition, the Principal Component Analysis plot of expression of the ***all 3' IREs*** set over all samples shown in **Figure 10C** suggests that different conditions appear to have distinct expression patterns of these genes. Across the first principal component, different age groups (6- and 24-month-old brains) differ in their expression of predicted 3' IRE genes, while the second principal component appears to separate *psen1^{Q96_K97del/+}* mutants from their wild-type siblings. This separation between *psen1^{Q96_K97del/+}* mutants and wild type siblings is even more pronounced when both are exposed to hypoxia.

To gain more insight into the similarities and differences between the IRE responses in the *psen1^{Q96_K97del/+}* vs. wild type comparison, we plotted UpSet plots of overlapping leading-edge genes (**Figure 10D**). These plots suggest that the IRE responses during hypoxia, aging, and due to this fAD-like mutation are mostly distinct from each other with unique leading-edge genes. However, similarities in the IRE response between different conditions are suggested by the existence of some shared leading-edge genes. For example, for the set of ***all 3' IREs***, the “6-month-old *psen1^{Q96_K97del/+}* vs. wild type” comparison shares 19

leading-edge genes with the “24-month-old *psen1*^{Q96_K97del/+} vs. wild type” comparison. As an initial exploration of the biological relevance of these genes, we submitted them to the STRINGR tool. This indicated that the proteins products of these genes were significantly associated with each other (e.g. in terms of text-mining from PubMed articles, experimentally determined interactions, and co-expression). These proteins were significantly over-represented in the sets “MAPK pathway”, “AP-1 transcription factor”, “Jun-like transcription factor”, and “signaling by TGF-beta family members” (FDR-adjusted over-representation p-value < 0.05; **Supplementary Figure 5**). The AP-1 and MAPK pathways have previously been shown to be stimulated by iron depletion [56,57]. Therefore, mechanistically, it is possible that the IRE-IRP response to iron dyshomeostasis in the fAD-like zebrafish mutant might involve these pathways, although this requires confirmation *in vivo*.

The fact that aging, hypoxia, and a fAD-like mutation all cause changes in the abundance of IRE-containing transcripts in zebrafish raised concerns regarding the specificity of such changes. Therefore, as a negative control, we examined changes in IRE transcript abundance in a brain transcriptome dataset derived from zebrafish heterozygous for a *psen1* mutation now thought not to be fAD-like, *psen1*^{K97fs} [58]. In this dataset, *psen1*^{K97fs/+} zebrafish are compared to their wild type siblings at 6 and 24 months. We tested this dataset for enrichment using our zebrafish 3’ and 5’ IRE gene sets but found no significant enrichment of any of our predicted IRE gene sets in *psen1*^{K97fs/+} vs. wild type comparisons at any age (**Table 4; Supplementary Figure 6**). Reassuringly, we still observed significant enrichment of both 3’ and 5’ IRE gene sets during wild type aging (24-month-old wild types vs. 6-month-old wild types), consistent with the equivalent comparison in the *psen1*^{Q96_K97del/+} dataset. These results support that IRE-containing transcript abundance changes are sufficiently sensitive to reflect differences in iron homeostasis between different mutation models.

Simultaneous stabilization of some 3' IRE transcripts and destabilization of others

In the cultured cell line dataset analyzed above, we noticed that even a straightforward iron deficiency treatment resulted in the simultaneous increase and decrease in expression of 3' IRE-containing genes. These findings are difficult to reconcile with the current paradigm that stabilization of 3' IRE-containing genes occurs under iron deficiency and suggest that the current model of the IRP/IRE system may be incomplete or insufficient for describing the regulation of non-canonical IREs. Given that many predicted 3' IRE genes with non-canonical IREs (e.g. in the *all 3' IREs* set) displayed enrichment and different gene expression patterns in the fAD-like zebrafish dataset, we decided to explore further the stability changes of these genes by comparing the expression of spliced and unspliced transcripts for each gene (**Supplementary Text 2**). We found that transcripts of some predicted 3' IRE genes were significantly increased in stability while others were significantly decreased in stability (**Supplementary Figure 7; Supplementary Table 5**).

Discussion

In this study we identified comprehensive sets of genes predicted to contain IREs in human, mouse, and zebrafish. Surprisingly, IRE gene sets were generally not well-represented in existing gene sets, and because of this, we believe they represent a novel resource for researchers interested in exploring iron dyshomeostasis gene expression responses in detail. Notably, our finding that IRE genes were most significantly over-represented in an existing gene set previously shown to be up-regulated in AD brains (the “Blalock Alzheimer’s Disease Up” gene set from MSigDB) supports the unique importance of IRE genes in AD. Furthermore, IRE gene sets displayed significant enrichment in postmortem brains from a human AD cohort, 5XFAD mouse model, a zebrafish model of a fAD-like mutation. Taken

together, our results demonstrate for the first time the involvement of a coordinated IRP/IRE-mediated gene expression response associated with AD.

Overall, IRE gene sets were sensitive enough to capture several interesting phenomena. The relatively tightly controlled conditions in the mouse and zebrafish model datasets revealed a strong age-dependent effect on the transcript abundances of these predicted IRE-containing genes. Notably, 3' IRE gene expression changes were amongst the earliest changes observable in the zebrafish fAD-like mutation model alongside changes in energy metabolism. These 3' IRE gene expression changes preceded other signals of pathological change in the transcriptome (such as altered expression of inflammatory response pathways) commonly associated with AD. Our findings raise the question of how early IRE gene expression changes and iron dyshomeostasis more broadly may start to occur in fAD brains. In a recent analysis of transcriptome data from pools of 7-day-old larvae heterozygous for the fAD-like mutation *psen1*^{Q96_K97del} (compared to wild type half-siblings), we have observed over-representation of the GO term “iron ion transport” without significant changes in 3' IRE or 5' IRE transcript abundance [59]. This supports that the 3' IRE transcript abundance signal in 6-month-old brains of this mutant line reflects changes in ferrous iron abundance that develop with age in response to an underlying defect in cellular iron regulation.

In relation to our findings associated with the 5XFAD mouse model, we acknowledge that the low sample number is a limitation in this analysis and constrains our interpretation of results from this particular model. Unfortunately, to our knowledge, no other publicly-available 5XFAD mouse dataset was available containing (1) both young and aged adults in the same batch (where young is defined as ~2-4 months old mice as this is when the earliest signs of pathology are observed for this model [60,61]), (2) 5XFAD and WT genotype mice

in the same batch, (3) information about biological sex for each sample (so that sex-specific effects could be accounted for in the statistical model for differential gene expression), and (4) separation of groups by biological factors (e.g. age, genotype) rather than unidentifiable batch effects when inspected with Principal Component Analysis. Our original rationale for analyzing the 5XFAD mouse model came from results from an independent concordance analysis from various mouse models and human AD [47]. In that study, it was found that 5XFAD mice brain transcriptomes showed greatest (although still fairly poor) concordance to human AD compared to other mouse models including the 3xTg model. However, we also acknowledge the limitations associated with only analyzing the 5XFAD mouse model here, and note that it would be useful to analyze other mouse models of AD in the future to compare the involvement of IRE genes across different models. Likewise, we anticipate that further analysis of fAD-like mutation models in general will help to extend our knowledge of the early molecular changes contributing to the development of AD.

Notably, both classic IRE genes (e.g. *TFRC*, *FTL*, and *FTH1*) or IRE gene sets were sufficiently sensitive to distinguish not only between iron overload and deficiency in a cultured cell line dataset, but also between AD and other neuropathologies (i.e. PA and PSP). This suggests that the dysregulation of IRE-containing genes and iron homeostasis in general in AD may differ from other neuropathological conditions. The differences in both classic IRE genes and IRE gene set expression between AD and PA are of particular interest. Considering that presence of amyloid pathology is required to diagnose individuals with AD [62,63] and sufficient for preclinical AD [2], it will be interesting to explore further the biological relevance and impact of these IRE gene expression differences using other techniques in future work to gauge whether PA is a prodrome of, or a separate pathological condition to, AD.

One of our original intentions for IRE gene set analysis of the human AD and animal model datasets was to gain insight on whether these brains were in a state of iron deficiency or overload at the gene expression-level. Unfortunately, it became clear that interpretation of IRE gene expression changes was more complex than expected, both because of the use of bulk brain tissue samples for the human and animal model datasets (which obscured differences between cell type and tissue) and because the regulation of IRE-containing genes we observed did not follow the expected paradigm. To address the former issue, in future work we would like to expand the use of IRE gene sets to single cell RNA-seq (scRNA-seq) datasets so that the IRE gene expression patterns of different cell types can be deconvoluted. Recently, a large-scale scRNA-seq study of AD brains indicated highly cell-type specific changes related to inflammation during early stages of disease progression within the prefrontal cortex [64], highlighting the importance of studying iron homeostasis responses at the single-cell level in the context of AD. The failure of 3' IRE genes to follow the expected expression paradigm is a limitation that we did not foresee when beginning this work. Findings indicating that many predicted 3' IRE genes were able to show both increased and decreased expression under iron deficiency in a cultured cell line (suggesting they are not stabilized as would be predicted by the IRE paradigm) and the decreased stability of many 3' IRE transcripts in the fAD-mutation-like zebrafish model are interesting and challenge the simplistic paradigm of stabilization of 3' IRE-containing transcripts under ferrous iron deficiency. Previous research holds that under iron deficiency, 3' IRE-containing genes such *TFRC* are stabilized and hence increased in expression [20] in order to increase ferrous iron availability. However, our findings reveal that this same principle may not apply to other 3' IRE-containing genes, including those with canonical IREs (e.g. *SLC11A2* in the cultured cell line), and genes with IRE-like elements deviating from the canonical IRE sequence. Many of these IRE-like elements are likely to have some functionality [26–28], and their expression changes in our analyses indicate that they are

associated with AD and able to distinguish AD from other neuropathological conditions (i.e. PSP and PA). Detailed characterization of these genes and their biological significance is beyond the scope of this current work. We expect our findings to provide a starting point for further research involving IRE genes and their regulation beyond the classic paradigm.

It is important to acknowledge that at least some part of the complexity seen with IRE gene expression may be due to other activities known to influence expression of IRE genes, including oxidative stress [65–67]), oxidative phosphorylation [68], and cell cycle control [69,70]. In addition, several transcription factor motifs potentially associated with other biological pathways were also found in our analysis to be over-represented in IRE gene sets. These biological activities may be expected to show some alteration in AD, other neurodegenerative diseases, and during normal aging, which is likely to explain at least some of the IRE gene set enrichment observed in the datasets analyzed, and potentially some of the expression changes that are otherwise difficult to explain (e.g. decreased expression of 3' IRE genes). Changes in expression patterns in predicted IRE genes which we observed in these datasets must be carefully interpreted as not only reflecting iron dyshomeostasis responses, but also co-occurring changes in other linked processes. The overlap between gene expression responses to iron dyshomeostasis and other processes was particularly evident in our analysis of the Caco-2 cultured cell line dataset where even straightforward iron overload and iron deficiency treatments resulted in significant enrichment in gene sets related to the reactive oxygen species pathway and hypoxia. We expect that future work deconvoluting IRE expression patterns at the cellular level will be required to determine to what extent IRE expression changes are influenced by cell / tissue type compared to other linked biological activities.

Most previous work has assumed that accumulation of iron in the brain with age (a phenomenon observed broadly across animal phyla [71,72]) is indicative of cellular iron overload driving oxidative stress [73]. Indeed, iron accumulation and oxidative stress apparently contribute to the insolubility of amyloid plaques and neurofibrillary tangles in AD brains [74]. However, a recent publication by Yambire et al. [75] showed that disturbed lysosomal function leading to increased lysosomal pH causes a deficiency of functional ferrous iron (Fe^{2+}) while non-functional ferric iron (Fe^{3+}) accumulated in lysosomes. The deleterious effects of this on the brains of mice (defective mitochondrial biogenesis and function and stimulation of inflammatory responses) could be alleviated by increasing the levels of iron in their diet. The observations of Nixon and colleagues that acidification of the endolysosomal pathway is affected both by fAD mutations in *PSEN1* [54] and excessive dosage of the amyloid beta A4 precursor protein (*AβPP*) gene [76], together with our observations from our fAD-mutation-like *psen1*^{Q96_K97del/+} mutant zebrafish both in this study and in ref. [53] (where gene expression changes associated with lysosome and mitochondria for this model were first described), support the possibility that AD brains may suffer a ferrous iron deficiency in a background of ferric iron overload. This is discussed further below.

Although the evidence from human AD brains is more tentative at this stage, our findings of increased expression of *TFRC* expression and other well-characterized 3' IRE genes in the temporal cortex tissue of human AD brains is consistent with a cellular ferrous iron deficiency, although more research is required to confirm this at the single-cell level. Intriguingly, the greatest genetic risk factor for late onset AD, the ε4 allele of the gene *APOE*, appears to increase lysosomal pH [77] but ε4's increased risk of AD is alleviated in individuals who possess the *HFE* 282Y allele that predisposes to the iron overload disease hemochromatosis [78]. Given that many other risk loci for sporadic late onset AD also affect

endolysosomal pathway function (reviewed in [79]) it is reasonable to suggest that disturbed iron homeostasis may afflict brains with this disease.

The proposal that fAD mutations drive a ferrous iron deficiency that contributes to AD pathology is not necessarily in disagreement with the experimental data recently reported in Tsatsanis et al. [80] regarding an association between amyloidogenic processing of A β PP and impaired export of iron from cells. In that study, the authors extended investigation of the idea that the protein product of the fAD locus *A β PP* stabilizes the iron exporting protein ferroportin, providing a connection between *A β PP* and iron homeostasis in AD. The idea that A β PP interacts with ferroportin has been the subject of some contention [81,82]. Tsatsanis et al. observed that inhibition of the amyloidogenic cleavage of A β PP by the enzyme β -secretase in mouse N2a neuroblastoma cells is associated with stabilization of ferroportin on the cell surface and a reduced, intracellular labile iron pool. However, these authors did not consider the evidence that the ferroxidase hephaestin, (well established as complexing with, and stabilizing, ferroportin to export iron [83–85]), is likely also cleaved by β -secretase (see Figure 7 in [86]). It is probable that reduced β -secretase activity increases the hephaestin available to stabilize ferroportin.

We have previously noted that all the fAD mutations of A β PP (including gene duplications) are expected to have the singular quality of increasing the abundance of the amyloidogenic β -CTF / C99 fragment of A β PP (see Figure 2 in Lumsden et al. [14]). Recently Jiang et al. [76] showed that increased β -CTF reduces lysosomal acidification. According to the results of Yambire et al. cited above, this would be expected to reduce cellular ferrous iron levels (while causing accumulation of ferric iron in lysosomes). This idea is consistent with the existence of an IRE in the 5' UTR of A β PP transcripts that inhibits A β PP translation (and

hence β -CTF production) when cytosolic ferrous iron levels are low [87]. Thus, A β PP may influence cellular ferrous iron levels by modulating lysosomal pH to regulate importation of ferrous iron into the cytosol, rather than by modulating ferroportin stability to regulate iron export from the cytosol.

Overall, our results demonstrate for the first time the involvement of a coordinated IRP/IRE-mediated gene expression response in the context of AD. By searching entire human, mouse, and zebrafish transcriptomes for all genes containing potential IREs, we formed comprehensive gene sets applicable to the analysis of any human, mouse, or zebrafish gene expression dataset. For the first time, our work suggests the existence of gene expression changes associated with the IRP/IRE system in the young, pre-pathology brains of *PSEN1* fAD mutation carriers, and is consistent with the idea of iron dyshomeostasis as an early contributor to the disease process. It also reinforces the critical importance of IRE genes in general in the development of the late onset, sporadic form of AD. More broadly, our approach highlights how changes in the stability and abundance of IRE-containing transcripts can be used to give insight into iron dyshomeostasis-associated gene expression responses in different species, tissues, and conditions.

Methods

Defining IRE gene sets for human, mouse, and zebrafish

We extracted all 3' and 5' UTR sequences from the human, mouse and zebrafish genome assemblies using the Bioconductor packages BSgenome.Hsapiens.UCSC.hg38, BSgenome.Mmusculus.UCSC.mm10 and BSgenome.Drerio.UCSC.danRer11, and gene definitions from the Ensembl 94 release. Each set of UTR sequences was then submitted input into the SIREs web server (v.2.0 [24]). The SIREs algorithm assigns quality scores to predicted IREs taking into account whether the sequence is canonical, whether it contains any mismatches and bulges, and the free energy of the secondary structure. Canonical sequences are tagged by SIREs as being high-quality, while IRE-like sequences are tagged as low or medium quality. Given that High-quality IRE predictions miss the majority of true IREs, this enables a more comprehensive sampling of IRE motifs. For human, mouse, and zebrafish, we separately defined the following four gene sets: ***HQ 3' IREs*** and ***HQ 5' IREs*** (representing genes with high-quality predicted IREs), along with ***all 3' IREs*** and ***all 5' IREs***, which included genes containing any predicted IRE in the respective UTR. Comparisons between gene sets were performed using the UpSetR package (v.1.4.0 [88]) with gene ID mappings between species obtained by BioMart [89].

Over-representation of IRE gene sets in existing MSigDB gene sets

We downloaded the following gene set collections from MSigDB (v.6.0 [35]): Hallmark, C2 (gene sets from online pathway databases and biomedical literature, including KEGG and the REACTOME databases), C3 (motif gene sets based on regulatory targets), and C5 (gene sets defined by Gene Ontology terms). We excluded the following collections from analysis: C1 (specific to human chromosomes and cytogenetic bands, while our analysis involves different species), C4 (computationally-defined cancer-focused gene sets), C6

(oncogenic signatures) and C7 (immunologic signatures). C4, C6, and C7 were not included as the level of detail in the gene sets in these specific collections is more domain-specific rather than broad-level. We used Fisher's exact test to determine whether any IRE gene set was significantly over-represented in each MSigDB gene set. Gene sets were defined as having significant enrichment for IRE gene sets if the FDR-adjusted p -value from Fisher's exact test was below 0.05. UpSet plots were produced using the UpSetR package (v.1.4.0 [88]) while network representations were produced in Gephi (v.0.9.3 [90]). To produce network visualizations, we first exported node and edge tables from R. The nodes table contained the following gene sets: top 15 gene sets (ranked by Fisher's exact test p -value), Hallmark Heme Metabolism gene set, *all 3' IREs*, *all 5' IREs*, and all genes contained within these gene sets. The edges table contained gene – gene set edges which indicated the gene set(s) that genes belonged to. To create the network plots in Gephi, we used "Force Atlas 2" as the initial layout algorithm, followed by the "Yifan Hu" [91] layout algorithm to improve visual separation between distinct groupings of genes.

Over-representation analysis of transcription factor motifs in IRE gene promoters

Defining promoter regions as being 1500 bp upstream and 200 bp downstream of the transcription start site for each gene, we used the findMotifs.pl script from HOMER (v.4.11 [92,93]) to search for known transcription factor binding site (TFBS) motifs in the promoters of each IRE gene set. The HOMER Motif database contains 363 vertebrate transcription factor binding motifs based on analysis of high-quality public ChIP-seq datasets (<http://homer.ucsd.edu/homer/motif/HomerMotifDB/homerResults.html>). We considered TFBS motifs as being significantly enriched in a gene set if the FDR-adjusted p -value (q -value provided in HOMER output) was less than 0.05.

Gene set enrichment testing

We performed all gene set enrichment tests in R v3.6.1 [94] using *fry* [37,38], *camera* [39], and *fgsea* [40,41]. For *fry*, and *camera*, we used model fits obtained using *limma* [95,96], whilst for *fgsea*, a ranked list was obtained using *moderated t*-statistics taken from *limma*. All genes were used in gene set enrichment tests (i.e. not just DE genes). We combined the raw *p*-values from *fry*, *camera*, and *fgsea* using Wilkinson's method [97] with default parameters, followed by FDR-adjustment. When performing gene set enrichment testing on the MSigDB Hallmark gene sets, we applied FDR-adjustment to combined *p*-values and defined significant enrichment as gene sets having an adjusted *p*-value < 0.05. When performing gene set enrichment on the four IRE gene sets (***all 3' IREs***, ***all 5' IREs***, ***HQ 3' IREs***, ***HQ 5' IREs***), we applied Bonferroni-adjustment to combined *p*-values to further protect against Type I errors and defined significant enrichment as gene sets having an adjusted *p*-value < 0.05. Depending on the species in the dataset being analyzed, we used the respective IRE gene sets defined for human, mouse, or zebrafish.

Analysis of the Caco-2 cultured cell line dataset

We downloaded processed microarray data from GEO (accession number: GSE3573). This study investigated gene expression responses to iron treatments, including iron deficiency (cells in iron-free medium vs. cells cultivated in ferric ammonium nitrate, and iron overload (cells cultivated in DMEM-FBS medium with hemin vs. cells in DMEM-FBS medium) [98]. We performed differential gene expression analysis using the "lmFit" and eBayes" functions in *limma* [95]. Genes were defined as differentially expressed when their FDR-adjusted *p*-value < 0.05.

Analysis of the Mayo Clinic RNA-seq dataset

No experiments of human subjects were performed in this study as all human data was accessed from public databases and was anonymous. We downloaded processed CPM count data from Synapse (<https://www.synapse.org/#!Synapse:syn5550404>). We matched cerebellum and temporal cortex samples by their patient ID, and only retained genes which were present across all samples and patients for which there were both cerebellum and temporal cortex samples (n=236 patients with measurements for cerebellum and temporal cortex, 472 samples in total). We performed analysis using *limma* [95,96] and determined differentially expressed genes between conditions. In addition, we used the “duplicateCorrelation” function in *limma*, setting the “block” parameter to the patient ID. Genes were considered differentially expressed if their FDR-adjusted *p*-value < 0.05.

Zebrafish husbandry and animal ethics

All zebrafish work was conducted under the auspices of the Animal Ethics Committee (permit numbers S-2017-089 and S-2017-073) and the Institutional Biosafety Committee of the University of Adelaide. Tübingen strain zebrafish were maintained in a recirculated water system.

fAD-like *psen1*^{Q96_K97del/+} zebrafish

The isolation of the *psen1*^{Q96_K97del} mutation has previously been described [53]. Mutations were only analyzed in the heterozygous state in this study.

Acute hypoxia treatment of zebrafish

psen1^{Q96_K97del/+} mutants and their wild-type siblings were treated in low oxygen levels by placing zebrafish in oxygen-depleted water for 3 hours (oxygen concentration of 6.6 ± 0.2 mg/L in normoxia and 0.6 ± 0.2 mg/L in hypoxia).

Whole brain removal from adult zebrafish

After normoxia or hypoxia treatment adult fish were euthanized by sudden immersion in an ice water slurry for at least ~30 seconds before decapitation and removal of the entire brain for immediate RNA or protein extraction. All fish brains were removed during late morning/noon to minimize any influence of circadian rhythms.

RNA extraction from whole brain

Total RNA was isolated from heterozygous mutant and WT siblings using the *miRvana* miRNA isolation kit (Thermo Fisher). RNA isolation was performed according to the manufacturer's protocol. First a brain was lysed in a denaturing lysis solution. The lysate was then extracted once with acid-phenol:chloroform leaving a semi-pure RNA sample. The sample was then purified further over a glass-fiber filter to yield total RNA. Total RNA was DNase treated using the DNA-free™ Kit from Ambion, Life Technologies according to the manufacturer's instructions. Total RNA was then sent to the Genomics Facility at the South Australian Health and Medical Research Institute (Adelaide, Australia) to assess RNA quality and for subsequent RNA sequencing (*using poly-A enriched RNA-seq technology, and estimated gene expression from the resulting single-end 75 bp reads using the reference GRCz11 zebrafish assembly transcriptome*)

786

787 Pre-processing of the fAD-like *psen1*^{Q96_K97del/+} zebrafish dataset:

788 RNA-seq libraries contained single-end 75bp Illumina NextSeq reads. We performed quality
789 trimming with *AdapterRemoval* using default parameters, followed by quality assessment
790 with FastQC and ngsReports. Trimmed reads were pseudo-aligned to the reference
791 zebrafish transcriptome using *Kallisto* (v.0.45) [99] and transcript descriptions from Ensembl
792 release 94. The “catchKallisto” function from *edgeR* [100] was used to import and
793 summarise counts from transcript-level to gene-level, with all subsequent analyses
794 performed at the gene-level.

795

796 Differential gene expression analysis for fAD-like zebrafish dataset

797 For differential gene expression analysis, we retained all genes with expression of at least 1
798 cpm in 4 or more samples, and used voomWithQualityWeights to downweight lower quality
799 samples [96]. Contrasts were defined to include all relevant pairwise comparisons between
800 conditions, and genes were considered as differentially expressed using an FDR-adjusted *p*-
801 value < 0.05. The *limma* model used to create the design matrix for this dataset included
802 biological sex as a variable (~0 + biologicalSex + group, where biologicalSex is either male
803 or female and group refers to the combination of age, hypoxia treatment, and genotype of
804 the samples) to minimize any sex-specific effects in the gene expression analysis. More
805 details regarding the modest effects of biological sex in this dataset are provided in

806 **Supplementary Text 3.**

807

808 Estimation of spliced and unspliced gene expression in fAD-like zebrafish dataset

809 For spliced transcripts in Ensembl release 94, we additionally defined unspliced genes
810 including intronic regions. Unspliced transcripts were appended to the end of the reference

transcriptome and used to build a new Kallisto [99] index. Estimated counts for spliced transcripts and unspliced genes were imported into R using the “catchKallisto” function from *edgeR* [100].

Gene set enrichment tests for non-fAD-like (K97fs/+) zebrafish dataset

Please refer to ref. [53] for details on RNA-seq data processing and analysis of the non-fAD-like dataset. In the current work, we used the gene expression counts matrix with *limma*. The voom, design, and contrasts objects produced as part of the *limma* analysis were used for gene set enrichment analysis with the zebrafish IRE gene sets we defined as well as the MSigDB Hallmark gene sets. Significantly enriched Hallmark gene sets had FDR-adjusted p -value < 0.05 while IRE gene sets were considered significantly enriched if the Bonferroni-adjusted p -value < 0.05 .

Gene set enrichment tests for 7-day-old Q96 K97del/+ dataset

Please refer to ref. [59] (GEO accession: GSE148631) for details on RNA-seq data processing and analysis. In the current work, we used the gene expression counts matrix with *limma* to perform gene set enrichment analysis. IRE gene sets we defined as well as the MSigDB Hallmark gene sets. Significantly enriched Hallmark gene sets had FDR-adjusted p -value < 0.05 while IRE gene sets were considered significantly enriched if the Bonferroni-adjusted p -value < 0.05 .

Differential transcript stability analysis

The estimated spliced and unspliced transcript count estimates from *kallisto* [99] were imported into R using the catchKallisto function from *edgeR* [100]. We used *limma* [95] to determine the logFC of spliced transcripts and unspliced transcripts for each comparison. To

test for whether there was a significant difference in the logFC of the spliced and unspliced transcripts, we used Welch's *t*-test with the *s2.prior* values from *limma* as the variances of the spliced and unspliced transcripts. We defined the null (no stabilization of transcript) and alternate (stabilization of transcript) hypotheses for each gene as follows, where *s* and *u* refer to the spliced and unspliced versions of a particular gene:

$$H_0: \log FC_s = \log FC_u$$

$$H_a: \log FC_s \neq \log FC_u$$

We defined genes with FDR-adjusted *p*-values < 0.05 as having differential stability.

Checks that observed gene expression differences are not artifacts of differences in proportions of cell types

The Mayo Clinic RNA-seq study, 5XFAD mice, and fAD-like zebrafish datasets are bulk RNA-seq datasets. To confirm that any gene expression changes were likely due to altered transcriptional programs rather than changes in cell type proportions, we compared expression of marker genes for four common neural cell types (astrocytes, neurons, oligodendrocytes, microglia) in conditions within each dataset. The marker genes for astrocytes, neurons, and oligodendrocytes were obtained from MSigDB gene sets from [101] which were based on studies in mice. The marker genes for microglia were derived from [102] which was based on studies in human and mouse. All gene IDs were converted to human, mouse, or zebrafish Ensembl IDs using BioMart [89] for each dataset. Please refer to **Supplementary Text 4** and **Supplementary Figures 8-10** for details.

Declarations

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Conflict of Interest / Disclosure Statement

The authors have no conflict of interest to report.

Ethics approval and consent to participate

No experiments of human subjects were performed in this study as all human data was accessed from public databases and was anonymous. All zebrafish work was conducted under the auspices of the Animal Ethics Committee (permit numbers S-2017-089 and S-2017-073) and the Institutional Biosafety Committee of the University of Adelaide.

Consent for publication

All authors give consent for publication of the manuscript.

887 Datasets and Data Articles

888 The fAD-like zebrafish dataset supporting the conclusions of this article is available in the
889 GEO repository with accession number GSE149149.

890

891 Authors' contributions

892 NH performed the bioinformatics analysis and drafted the manuscript. MN generated the
893 mutant zebrafish, isolated brain RNA and protein, drafted the description of this and edited
894 the manuscript. SP directly supervised NH, guided the analysis, and edited the manuscript.
895 ML conceived the project, supervised all aspects of it, and edited the manuscript. All authors
896 read and approved the manuscript.

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Tables

Table 1. Summary of gene set testing approaches used in our analyses.

Method	Hypothesis	Focused / battery approach	Accounts for inter- gene correlation	R implementation
Fry / Roast	Self-contained null hypothesis (genes within a set have no association with experimental condition)	Focused (each gene set is tested on its own terms, and multiple testing adjustment is applied afterwards)	Yes	fry() and mroast() functions in the <i>limma</i> Bioconductor package [103,104]
Camera	Competitive null hypothesis (genes within a set do not have a stronger association with experimental condition compared to a random gene set)	Focused (each gene set is tested on its own terms, and multiple testing adjustment is applied afterwards)	Yes	camera() function in the <i>limma</i> Bioconductor package [105]
fgsea (fast implementation of GSEA in R)	Self-contained null hypothesis (genes within a set have no association with experimental condition)	Battery (gene sets are pitted against each other to determine which ones are more significantly enriched)	No	fgsea() function in the <i>fgsea</i> Bioconductor package [40,106]

Table 2. Samples analyzed from Mayo Clinic RNA-seq study.

Group	Total number	% Female	Mean age $\pm$ s.d (years)	Diagnosis
Alzheimer's disease (AD)	134	56.7	82.6 $\pm$ 7.3	Braak Stage $\geq$ 4, diagnosis according to NINCDS-ADRDA criteria
Control	130	46.2	82.9 $\pm$ 8.3	Braak Stage $\leq$ 3, No or sparse CERAD neuritic and cortical plaque density, no diagnoses for any neurodegenerative disease
Pathological Aging (PA)	44	54.5	84.5 $\pm$ 4.3	Braak Stage $\leq$ 3, CERAD neuritic and cortical plaque density of 2 or more, no diagnoses for any neurodegenerative disease or mild cognitive impairment
Progressive supranuclear palsy (PSP)	164	40.2	73.8 $\pm$ 6.5	Braak Stage $\leq$ 3, no or sparse CERAD neuritic and cortical plaque density

Table 3. Differential gene expression and IRE gene set enrichment results from Mayo Clinic RNA-seq study. Asterisks (*) indicate significant enrichment (Bonferroni-adjusted Wilkinson's *p*-value from *fry*, *camera*, and *fgsea* < 0.05).

	Comparison	No. of DE genes (FDR <i>p</i> -value < 0.05 and abs(logFC) > 0.5)	All 3' IREs enrichment <i>p</i> -value	All 5' IREs enrichment <i>p</i> -value	HQ 3' IREs enrichment <i>p</i> -value	HQ 5' IREs enrichment <i>p</i> -value
Effect of Alzheimer's disease	AD vs. control (cerebellum)	Down: 1,422 Up: 1,308	0.0109*	0.128	0.770	0.770
	AD vs. control (temporal cortex)	Down: 1,650, Up: 1,929	9.03E-15*	2.44E-14*	4.50E-15*	8.95E-08*
Effect of pathological aging (amyloid pathology, no dementia symptoms)	PA vs. control (cerebellum)	Down: 254, Up: 463	0.000116*	0.00139*	0.0201*	0.770
	PA vs. control (temporal cortex)	Down: 466, Up: 512	0.000521*	0.0461*	0.000065*	0.0000682*
Effect of progressive supranuclear palsy (PSP)	PSP vs. control (cerebellum)	Down: 2,550, Up: 1,080	1.81E-22*	3.45E-22*	1.43E-12*	0.0399*
	PSP vs. control (temporal cortex)	Down: 1,271, Up: 622	3.58E-11*	5.17E-13*	1.39E-06*	0.0399*
Differences between Alzheimer's disease and pathological aging	AD vs. PA (cerebellum)	Down: 1,728, Up: 1,006	0.000116*	0.00139*	0.0201*	0.770
	AD vs. PA (temporal cortex)	Down: 2,633, Up: 2,174	0.000521*	0.0461*	0.000065*	0.0000682*
Differences between Alzheimer's disease and progressive supranuclear palsy	AD vs. PSP (cerebellum)	Down: 108, Up: 659	0.0019*	0.00184*	0.158	0.158
	AD vs. PSP (temporal cortex)	Down: 2,052, Up: 2,354	9.12E-22*	2.10E-22*	5.35E-16*	0.040*
Differences between cerebellum and temporal cortex tissue	Cerebellum vs. temporal cortex in controls	Down: 10,925, Up: 9,302	0.00E+00*	0.00E+00*	2.82E-269*	1.60E-81*
	Cerebellum vs. temporal cortex in AD	Down: 11,966, Up: 9,950	0.00E+00*	0.00E+00*	7.71E-302*	9.35E-92*
	Cerebellum vs. temporal cortex in PA	Down: 10,200, Up: 8,737	1.87E-153*	5.88E-160*	1.22E-89*	1.31E-88*
	Cerebellum vs. temporal cortex in PSP	Down: 11,552, Up: 9,576	0.00E+00*	0.00E+00*	0.00E+00*	4.74E-104*

Table 4. Enrichment of Iron Responsive Element (IRE) gene sets in fAD-like zebrafish dataset. Raw *p*-values from *fry*, *camera* and *fgsea* were combined with Wilkinson's method, with combined *p*-values then Bonferroni-adjusted for multiple testing. The same process was repeated for the K97fs/+ dataset, which involves an independent family of fish (†).

	Comparison	All 3' IREs enrichment <i>p</i> - value	All 5' IREs enrichment <i>p</i> - value	HQ 3' IREs enrichment <i>p</i> - value	HQ 5' IREs enrichment <i>p</i> - value
Effect of <i>psen1</i> mutation	6-month-old Q96_K97del/+ vs. 6- month-old +/+ all under normoxia	0.0109	0.128	0.770	0.770
	6-month-old Q96_K97del/+ vs. 6- month-old +/+ all under hypoxia	9.03E-15	2.44E-14	4.50E-15	8.95E-08
	24-month-old Q96_K97del/+ vs. 24- month-old +/+ all under normoxia	0.000116	0.00139	0.0201	0.770
	24-month-old Q96_K97del/+ vs. 24- month-old +/+ all under hypoxia	0.000521	0.0461	0.000065	0.0000682
	6-month-old K97fs/+ vs. 6-month-old +/+ all under normoxia †	0.199	0.289	0.0197	0.289
	24-month-old K97fs/+ vs. 24-month-old +/+ all under normoxia †	0.116	0.115	0.00000118	0.979
	6-month-old +/+ under hypoxia vs. 6-month-old +/+ under normoxia	1.10E-18	1.10E-18	8.19E-13	1.27E-08
Effect of hypoxia	24-month-old +/+ under hypoxia vs. 24-month-old +/+ under normoxia	3.67E-15	7.98E-11	2.94E-07	3.31E-05
	6-month-old Q96_K97del/+ under hypoxia vs. 6-month-old Q96_K97del/+ under normoxia	1.13E-24	9.88E-22	2.28E-13	2.86e- 3
	24-month-old Q96_K97del/+ under hypoxia vs. 24-month-old Q96_K97del/+ under normoxia	0.000521	0.015	0.128	0.770
	24-month-old +/+ vs. 6- month-old +/+ all under normoxia	6.80E-35	4.82E-27	3.41E-18	1.26E-21
	24-month-old +/+ vs. 6- month-old +/+ all under hypoxia	1.55E-35	6.84E-31	2.36E-20	7.40E-23
Effect of aging	24-month-old Q96_K97del/+ vs. 6- month-old Q96_K97del/+ all under normoxia	1.85E-27	1.03E-21	8.09E-17	1.07E-17
	24-month-old Q96_K97del/+ vs. 6- month-old Q96_K97del/+ all under hypoxia	3.77E-30	1.59E-23	2.11E-19	2.46E-16
	24-month-old +/+ vs. 6- month-old +/+ all under normoxia (K97fs family) †	1.69E-10	3.96E-08	5.95E-10	4.59E-06
	24-month-old K97fs/+ vs. 6-month-old K97fs/+ all under normoxia †	0.199	0.289	0.0197	0.289

Figure Legends

Figure 1. Iron Responsive Element (IRE) background. A. Model of altered transcript

stability due to the IRP/IRE system under iron dyshomeostasis. When cellular iron levels (through FeS) are low, Iron Responsive Proteins (IRP1 or IRP2) bind IREs in the 5' or 3' untranslated regions (UTRs) of genes involved in iron homeostasis. In general, genes with IREs in their 3' UTRs will be stabilised and hence increased in expression while genes with IREs in their 5' UTR will have repressed translation from IRP binding. However, when cellular iron levels are high, IRP1 undergoes a conformation change and is now unable to bind IREs. IRP1 now acts as a cytosolic aconitase (ACO1). IRP2 is degraded in high iron conditions and is also unable to bind IREs. Without IRPs binding to IREs, genes with 5' IREs will have increased translation, while those with 3' IREs will have decreased mRNA stability, causing their expression to be decreased. **B. Consensus IRE secondary structure and**

examples of high-quality and IRE-like motifs predicted by SIREs. IRE-like motifs with non-canonical structure are able to be detected by SIREs if they have up to one mismatch pair in the upper stem (e.g. *Hao1*) or 1 unpaired bulge nucleotide on the 3' strand of the upper stem (e.g. *EPAS1*). For more details on the prediction of non-canonical IRE motifs, please refer to Figure 1 of Campillos et al. [24].

Figure 2. Overlap between predicted IRE gene sets for human, mouse, and zebrafish.

The number of genes in the gene set for each species is shown at the bottom-left bars of each UpSet plot, while genes with shared homologs across species are indicated in the main plot region. IRE genes in mouse and zebrafish gene sets were excluded from this plot if they did not have a human homolog.

Figure 3. MSigDB gene sets showing over-representation of predicted 3' and 5' IRE

genes in human, mouse, and zebrafish. The top 15 MSigDB gene sets ranked by Fisher's

exact test p -value (testing for over-representation of the *all 3' IREs* and/or *all 5' IREs* sets) are shown for each species. In the network plots, the top 15 MSigDB gene sets are shown as large nodes, with genes represented as small nodes. Edges connecting genes to gene sets indicate the gene set(s) that a gene belongs to. Overall, the *all 3' IREs* and *all 5' IREs* gene sets have a large proportion of genes which are not included in any of the top ranked MSigDB gene sets for each species.

Figure 4. IRE-containing gene expression analysis workflow. The section including identification of candidate 3' IRE genes stabilized under iron deficiency was only applied to the fAD-like zebrafish dataset due to unavailable raw RNA-seq reads for the other datasets needed to identify expression of unspliced genes.

Figure 5. Analysis of Caco-2 cultured cell line microarray dataset. A. Gene set enrichment testing results for human predicted IRE gene sets for the iron overload and iron deficiency treatments. Dots indicate if a gene set was considered significantly enriched (Bonferroni-adjusted p -value < 0.05) in the iron overload (on left) or iron deficiency (on right) treatment. **B. Gene set enrichment testing results for MSigDB Hallmark gene sets in the iron overload and iron deficiency treatments.** Dots indicate if a gene set was considered significantly enriched (FDR-adjusted p -value < 0.05) in the iron overload (on left) or iron deficiency (on right) treatments. **C. UpSet plots showing overlap between iron overload and iron deficiency treatments in GSEA leading-edge genes for the *All 3' IREs* and *All 5' IREs* gene sets.** The bars to the lower-left indicate the number of the leading-edge genes for iron overload and iron deficiency treatments, while the bars in the main plot region indicate the number of leading-edge genes which are unique or shared between the treatments.

Figure 6. Analysis of Mayo Clinic RNA-seq dataset with human IRE gene sets. A. Enrichment analysis results of IRE gene sets in comparisons of AD (Alzheimer's Disease), PA (Pathological Ageing) and PSP (Progressive Supranuclear Palsy) vs. control. The proportions of IRE genes within each IRE gene set with increased, decreased, or unchanged expression are indicated as coloured bars. The dots indicate that the IRE gene set was significantly enriched (Bonferroni-adjusted enrichment p -value < 0.05). **B. UpSet plots showing overlap between leading-edge IRE genes in comparisons of AD (Alzheimer's Disease), PA (Pathological Aging), and PSP (Progressive Supranuclear Palsy) vs. control.** The bars to the lower-left region of each UpSet plot indicate the number of leading-edge IRE genes for each comparison, while bars in the main plot region indicate the number of leading-edge IRE genes which are unique or shared between comparisons.

Figure 7. Analysis of 5XFAD mouse dataset. A. Experimental design and results of IRE gene set enrichment analysis. The gene sets *all 3' IREs* and *all 5' IREs* derived from searching for IRE sequences in the UTRs of genes in the reference mouse genome mm10 are represented here as "3' IRE" and "5' IRE" respectively. Asterisks (*) indicate that the gene set was significantly enriched in a particular comparison (Bonferroni-adjusted p -value < 0.05). **B. Proportions of genes in IRE and MSigDB Hallmark gene sets which are increased ($t > 2$) or decreased ($t < -2$) in expression in all "5XFAD vs. wild type" comparisons.** A dot next to a bar indicates that the gene set was significantly enriched (FDR-adjusted p -value < 0.05).

Figure 8. UpSet plots showing the overlap in GSEA leading-edge genes between all comparisons in the 5XFAD mouse datasets for the gene sets all 3' IREs and all 5' IREs. Numbers of genes for each intersection are shown above intersection bars.

Figure 9. Differential gene expression and gene set enrichment analysis in the fAD-like zebrafish dataset. A. Results of differential gene expression analysis. Genes which were significantly increased or decreased in expression are indicated in boxes. These differentially expressed genes have FDR-adjusted p -value < 0.05 . **B. Gene set enrichment with MSigDB Hallmark gene sets.** The comparisons between Q96_ K97del/+ fAD-like mutants and their wild-type siblings are shown for the 6-month-old (young adult) and 24-month-old (infertile adult) age groups. Dots indicate gene sets which are significantly enriched (FDR-adjusted p -value < 0.05).

Figure 10. Iron Responsive Element (IRE)-containing gene expression in the fAD-like zebrafish dataset. A. Results of gene set enrichment testing using predicted IRE gene sets. We represent the gene sets all 3' IREs and all 5' IREs derived from searching for IRE and IRE-like sequences from z11 reference zebrafish gene UTRs as "3' IRE" and "5' IRE" in the panel. **B. Proportions of predicted IRE genes which are increased ($t > 2$) or decreased ($t < -2$) in expression for each pairwise comparison in the dataset. C. Principal component analysis of all genes in the sets all 3' IREs and all 5' IREs for all samples.** Circles on the 3' IRE plot show that different conditions generally have distinct expression of genes in the *all 3' IREs set* but not in the *all 5' IREs set*. **D. UpSet plot showing overlap in leading-edge genes for the *all 3' IREs* gene set for select comparisons.**

Supplementary Information

Supplementary Text

- **Supplementary Text 1.** Expression of previously characterized 3' and 5' IRE genes in the gene expression datasets analyzed in this paper
- **Supplementary Text 2.** Identifying predicted 3' IRE genes with altered stability.
- **Supplementary Text 3.** Effects of biological sex on gene expression in the fAD-mutation-like zebrafish dataset.
- **Supplementary Text 4.** Neural cell type proportions in the gene expression datasets analyzed in this paper.

Supplementary Tables

- **Supplementary Table 1.** Iron Responsive Element (IRE) gene sets defined from human, mouse, and zebrafish transcriptomes. The untranslated regions (UTR) of all known genes were searched for IRE and IRE-like motifs. Four gene sets are included for each species: ***all 3' IREs*** (all genes with predicted IRE in their 3' UTR), ***all 5' IREs*** (all genes with predicted IRE in their 5' UTR), ***HQ 3' IREs*** (genes with high-quality predicted IRE in 3' UTR), and ***HQ 5' IREs*** (genes with high-quality predicted IRE in 5' UTR). Genes are provided as ENSEMBL gene identifiers.
- **Supplementary Table 2.** Molecular Signature Database (MSigDB) gene sets with significant over-representation of IRE gene sets in human, mouse, and zebrafish. Gene sets were defined to have significant over-representation of IRE gene sets if the FDR-adjusted Fisher's exact test p -value was < 0.05 .
- **Supplementary Table 3.** Promoter motif over-representation analysis results for all IRE gene sets in human, mouse, and zebrafish. Promoter regions were defined as being 1500 bp upstream and 500 bp downstream of genes. See Methods for details. We define significant over-representation as FDR-adjusted enrichment p -value < 0.05 .
- **Supplementary Table 4.** Gene ontology (GO) analysis results for leading edge genes shared between the "5XFAD vs. WT (3-months-old)" and "5XFAD vs. WT (6-months-old)" comparisons. Significant over-representation of GO terms was determined as GO terms having an FDR-adjusted p -value < 0.05 .

- **Supplementary Table 5.** Altered stability of transcripts in the fAD-mutation-like zebrafish dataset. See Methods and Supplementary Text 2 for details on how stability was defined and calculated. Genes which contain a 3' IRE (belonging to one of the 3' IRE gene sets) or 5' IRE (belonging to one of the 5' IRE gene sets) are indicated with the "has3ire" or "has5ire" columns.

Supplementary Figures

- **Supplementary Figure 1.** Expression of previously characterized IRE genes in datasets analyzed. **A.** Caco-2 cell line dataset. **B.** Mayo Clinic RNA-seq (Human) dataset – Temporal Cortex tissue. **C.** Mayo Clinic RNA-seq (Human) dataset – Cerebellum tissue. **D.** 5XFAD mouse dataset. **E.** fAD-mutation-like zebrafish dataset.
- **Supplementary Figure 2.** Principal Component Analysis of results from different gene set testing methods.
- **Supplementary Figure 3.** Analysis of cultured Caco-2 cell line dataset. **A.** Principal Component Analysis plot of gene expression in the cultured Caco-2 cell line dataset. **B.** Volcano plots indicating differential gene expression due to iron overload (DMEM-FBS Medium + Hemin vs. DMEM-FBS Medium) and iron deficiency (Iron-free medium vs. Iron-free medium + FAC) treatments in the Caco-2 cell line dataset.
- **Supplementary Figure 4.** Gene set enrichment testing results for the AD vs. control comparison in cerebellum and temporal cortex.
- **Supplementary Figure 5.** STRINGR protein-protein interaction network plot between the 19 shared leading-edge genes between the *psen1*^{Q96_K97del/+} and *psen1*^{+/+} comparisons at 6 and 24-months-old.
- **Supplementary Figure 6.** Principal Component Analysis plots showing lack of clear association between IRE gene expression and the *psen1*^{K97fs/+} mutant genotype.
- **Supplementary Figure 7.** Predicted 3' IRE containing transcripts with significant differences in stability between conditions in the fAD-like zebrafish dataset.
- **Supplementary Figure 8.** Age-dependent expression of neural marker genes (microglia, astrocyte, neuron, oligodendrocyte) in the Mayo Clinic RNA-seq dataset.
- **Supplementary Figure 9.** Age-dependent expression of neural marker genes (microglia, astrocyte, neuron, oligodendrocyte) in 5XFAD mouse dataset.
- **Supplementary Figure 10.** Age- and hypoxia- dependent expression of neural marker genes (microglia, astrocyte, neuron, oligodendrocyte) in fAD-like zebrafish dataset.

Figure 1. Iron Responsive Element (IRE) background. A. Model of altered transcript stability due to the IRP/IRE system under iron dyshomeostasis. When cellular iron levels (through FeS) are low, Iron Responsive Proteins (IRP1 or IRP2) bind IREs in the 5' or 3' untranslated regions (UTRs) of genes involved in iron homeostasis. In general, genes with IREs in their 3' UTRs will be stabilised and hence increased in expression while genes with IREs in their 5' UTR will have repressed translation from IRP binding. However, when cellular iron levels are high, IRP1 undergoes a conformation change and is now unable to bind IREs. IRP1 now acts as a cytosolic aconitase (ACO1). IRP2 is degraded in high iron conditions and is also unable to bind IREs. Without IRPs binding to IREs, genes with 5' IREs will have increased translation, while those with 3' IREs will have decreased mRNA stability, causing their expression to be decreased. **B. Consensus IRE secondary structure and examples of high-quality and IRE-like motifs predicted by SIREs.** IRE-like motifs with non-canonical structure are able to be detected by SIREs if they have up to one mismatch pair in the upper stem (e.g. Hao1) or 1 unpaired bulge nucleotide on the 3' strand of the upper stem (e.g. EPAS1). For more details on the prediction of non-canonical IRE motifs, please refer to Figure 1 of Campillos et al. [24].

All predicted 3' IRE genes

All predicted 5' IRE genes

High-quality predicted 3' IRE genes

High-quality predicted 5' IRE genes

Figure 2. Overlap between predicted IRE gene sets for human, mouse, and zebrafish. The number of genes in the gene set for each species is shown at the bottom-left bars of each UpSet plot, while genes with shared homologs across species are indicated in the main plot region. IRE genes in mouse and zebrafish gene sets were excluded from this plot if they did not have a human homolog.

Human

Mouse

Zebrafish

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