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The zebrafish equivalent of Alzheimer's disease-associated PRESENILIN isoform PS2V regulates inflammatory and other responses to hypoxic stress

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Abstract

Dominant mutations in the *PRESENILIN* genes *PSEN1* and *PSEN2* cause familial Alzheimer's disease (fAD) that usually shows onset before 65 years of age. In contrast, genetic variation at the *PSEN1* and *PSEN2* loci does not appear to contribute to risk for the sporadic, late onset form of the disease (sAD), leading to doubts that these genes play a role in the majority of AD cases. However, a truncated isoform of *PSEN2*, PS2V, is upregulated in sAD brains and is induced by hypoxia and high cholesterol intake. PS2V can increase γ-secretase activity and suppress the unfolded protein response (UPR) but detailed analysis of its function has been hindered by lack of a suitable, genetically manipulable animal model since mice and rats lack this PRESENILIN isoform. We recently showed that zebrafish possess an isoform, PS1IV, that is cognate to human PS2V. Using an antisense morpholino oligonucleotide we can block specifically the induction of PS1IV that normally occurs under hypoxia. Here, we exploit this ability to identify the gene regulatory networks that are modulated by PS1IV. When PS1IV is absent under hypoxia-like conditions we observe changes in expression of genes controlling inflammation (particularly sAD-associated *IL1B* and *CCR5*), vascular development, the UPR, protein synthesis, calcium homestasis, catecholamine biosynthesis, TOR signaling and cell proliferation. Our results imply an important role for PS2V in sAD as a component of a pathological mechanism that includes hypoxia / oxidative stress and support investigation of *PS2V*'s role in other diseases, including schizophrenia, when these are implicated in the pathology.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with several pathological characteristics, including severe neuronal loss, glial proliferation, extracellular deposition of senile plaques composed of amyloid-β, and deposition of intracellular neurofibrillary tangles (reviewed in [1, 2]). The overwhelming majority of AD cases are sporadic (sAD), with more than 15 million people affected worldwide. However, the cause(s) of sAD is poorly understood and therapeutic approaches are not yet well developed.

The *PRESENILIN1* (*PSEN1*) and *PRESENILIN2* (*PSEN2*) genes are associated with the relatively rare, dominantly inherited form of AD, familial AD (fAD, reviewed in [3]). These genes encode transmembrane proteins embedded in the membranes of the endoplasmic reticulum (ER), Golgi, plasma membrane, endosomes and lysosomes [4]. Intriguingly, although the PRESENILIN proteins provide the γ-secretase enzyme activity that is critical for production of amyloid-β peptide (Aβ, regarded by many as central to pathological processes causing AD [5]) no genetic variation at the *PSEN1* or *PSEN2* loci has yet been discovered to contribute to the risk for developing sAD [6]. This, combined with the failure of γ-secretase inhibitors as AD-therapeutics and other observations inconsistent with the idea that Aβ is a pathogenic agent, has encouraged a search for other changes in *PRESENILIN* gene function that may contribute to AD pathogenesis (reviewed in [7]).

One PRESENILIN-related difference observed between normal and sAD brains is expression of a truncated isoform of PSEN2 protein, PS2V [8]. PS2V is produced by alternative splicing of *PSEN2* transcripts that is induced specifically by hypoxia (see below). PS2V protein accumulates in intracellular inclusion bodies termed PS2V bodies. These are observed in pyramidal cells of the cerebral cortex and in the hippocampus of sporadic AD patients during early stages [9]. *In vitro* experiments indicate that PS2V protein impairs the unfolded protein response thereby sensitizing cells to various ER stresses and significantly stimulating the production of Aβ_{40} and Aβ_{42} peptides [9]. PS2V also changes the conformation of the protein tau, which is a major component of neurofibrillary tangles [10]. Interestingly, PS2V was seen to be elevated in brains from people with schizophrenia to an even greater extent than

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in sporadic AD brains (although the sample size was small [11]). This suggests that PS2V may play an important role in the pathogenesis of AD and other diseases in which hypoxia and oxidative stress are implicated.

Using human neuoblastoma SK-N-SH cells, Sato et al. showed that the exclusion of exon 5 from *PSEN2* transcripts to form PS2V mRNA is induced by hypoxia but not a number of other cellular stressors [9]. The oxidative stress produced by hypoxia induces expression of the protein HIGH MOBILITY GROUP AT-HOOK 1 isoform a (HMGA1a) in neurons and this binds to a sequence in exon 5 of *PSEN2* transcripts. This interferes with spliceosome complex function causing exon 4 to be ligated to exon 6 and generating a frameshift that leads to a premature termination codon in exon 6 sequence [8]. Remarkably, although PS2V lacks critical amino acid residues required for γ-secretase catalysis, it nevertheless can increase γ-secretase activity and production of Aβ peptide from the Aβ Precursor Protein (AβPP). To do this PS2V probably requires the presence of full-length PSEN protein [12]. Interestingly, the pathogenic K115Efx10 mutation of *PSEN2* (causing fAD) truncates the open reading frame in a very similar position to the frameshift that generates PS2V [13, 14] and our previous analyses have shown that a peptide generated from K115Efx10 transcripts would have similar effects to PS2V on γ-secretase activity and the UPR [14]. This strongly supports that PS2V plays a role in development of sAD brain pathology. We recently documented that PS2V is not a uniquely human isoform but is widely conserved among most mammals although not in mice or rats [15]. Indeed, we showed that zebrafish possess a PS2V-cognate isoform, PS1IV, regulated by a conserved mechanism (i.e. involving a zebrafish orthologue of HMGA1a) but generated from the fish's *PSEN1* orthologous gene (*psen1*) rather than its *PSEN2* orthologue (*psen2*). Thus, human PS2V and zebrafish PS1IV appear to share a common origin in an hypoxia-induced isoform of the ancestral *PSEN* gene that duplicated to form the contemporary vertebrate *PSEN1* and *PSEN2* genes [14]. Like human PS2V, zebrafish PS1V can stimulate γ-secretase activity and suppress the unfolded protein response [14]. However, aside from these activities, we have no idea how this PRESENILIN isoform modulates cellular responses to hypoxia and oxidative stress.

We recently demonstrated an ability to inhibit, specifically, the induction by hypoxia of the PS1IV isoform in zebrafish embryos. When embryos are placed in a sodium azide solution to mimic hypoxia, expression of Hmga1a is induced [20] but binding of this protein specifically to nascent *psen1* transcripts can be inhibited by the presence of a morpholino antisense oligonucleotide that binds competitively to the Hmga1a binding site. This prevents the alternative splicing event that forms PS1IV mRNA under hypoxia while nevertheless allowing formation of translatable mRNA encoding full-length Psen1 protein [14]. Since so little is known regarding the function of PS1IV or its cognate human isoform PS2V, we exploited this ability to investigate changes in the zebrafish transcriptome caused by absence of PS1IV during hypoxia. We used a comprehensive microarray detecting zebrafish mRNAs to observe transcriptome-level changes in gene expression. These data were then subjected to various forms of genetic network analysis to reveal greater biological meaning from the observed expression changes. The results provide evidence supporting modulation by PS1IV of numerous processes that, in humans, are implicated in AD pathology and also in other diseases. In particular, PS1IV is required for upregulation of the IL1B gene that is a recognized AD risk locus and is central to neuroinflammation. Our results support that PS2V plays an important role in a pathological process in sAD possibly involving chronic hypoxia.

Materials and Methods

Ethics statement

All animal experiments including experiments on genetically modified organisms were conducted under the auspices of the University of Adelaide's Animal Ethics Committee and Institutional Biosafety Committee.

Zebrafish husbandry, morpholino injection and exposure to NaN$_3$

Danio rerio were bred and maintained at 28 °C on a 14 h light/10 h dark cycle. Embryos were collected from natural mating from Tubigen strain (Tu), grown in embryo medium (E3) and staged [16]. MoHmga1aBindBlock morpholino was synthesized by GeneTools LLC (Corvallis, OR, USA) and injected at 0.5mM (diluted to this concentration with MoCont as previously described [17]) at the one-cell stage.

Sodium azide, NaN$_3$, induces has previously been used for mimicry of hypoxia [18, 19] including in zebrafish [20, 21]. Exposure of embryos to NaN$_3$ (Sigma-Aldrich CHEMIE Gmbh, Steinheim, Germany) was performed at 100μM from 6 hours post fertilization (hpf) until embryos reached the developmental stage equivalent to that attained under normoxia at 48 hpf at 28.5 °C. 30 embryos were used for each treatment and replicate.

Oligonucleotides

The sequences of the microRNA primers and qPCR primers used in this study are given in Supplemental Data 1. The sequence of morpholino oligonucleotide MoHmga1aBindBlock was 5'-CTTGTAGAGCACCACCAGCACCAGG-3'.

Microarray experiment, experimental design, and statistical analysis

For microarray analysis, total RNA was extracted from batches of injected embryos using the RNeasy Mini Kit according to the manufacturer's specifications (Qiagen GmbH, Hilden, Germany). For each sample, the RNA concentration was determined using a NanoVue™ UV–vis spectrophotometer (GE Healthcare Life Sciences, Fairfield, USA). RNA integrity and quality were then estimated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the RNA integrity number (RIN) index was calculated for each sample. Only RNAs with a RIN number >7.0 were processed further.

To evaluate genome-wide changes in gene transcription related to the presence or absence of PS1IV under mimicry of hypoxia, we performed hybridisation analysis using the Zebrafish Gene 1.0 ST Array (Affymetrix Inc. Santa Clara, CA). Briefly, 300ng of total RNA derived from embryos was converted to amplified sense strand cDNA using the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA). The resulting sense cDNAs were fragmented and Biotin end labelled using the Affymetrix Genechip WT Terminal Labeling Kit prior to hybridisation to the arrays at 45 °C for 16 hours. Finally, the signal intensity of the chip was scanned using a GeneChipR Scanner 3000TG and analysed using Expression Console software (www. Affymetrix.com) as described previously . Cell files were imported and intensities adjusted by RMA background correction and quantile normalization [22-24]. Each treatment had 4 biological replicates. The cell files which did not pass quality control were removed based on their quality assessments obtained by Expression Console reports.

The final list of genes significantly (p≤0.05) over-expressed or under-expressed as a result of blocking PS1IV formation under hypoxia was generated using the following formula:

Impact of PS1IV splicing blockage under hypoxia = [Comparison of (HMGA1aBindBlock) vs (Control Morpholino) under chemical mimicry of Hypoxia] – [Comparison of (HMGA1aBindBlock) vs (Control Morpholino) under Normoxia] – [Comparison of (Control Morpholino) vs (Uninjected) under chemical mimicry of Hypoxia] – [Comparison of (Control Morpholino) vs Uninjected under Normoxia].

A Bayesian t-test using the FlexArray package (McGill University, Canada) was employed to find significantly over-expressed and under-expressed genes (p≤0.05) for each of the dual comparisons in the above formula as previously described. Removal of genes with significant alteration in the control comparisons increases the reliability of discovery of genes influenced specifically by PS1IV expression.

Gene ontology analysis was carried out using the "comparative GO" web application [25, 26].

Statistics-based subnetwork discovery and regulatory network analysis after loss of PS1IV under mimicry of hypoxia

To understand the consequences of loss of PS1IV under hypoxia, we used the integrative approach of "gene set enrichment" (based on Fisher's exact test) [27] with union selected subnetworks [28]). This reveals significantly altered subnetworks as well as underlying regulatory mechanisms controlling alteration of gene expression patterns in an integrative network.

Subnetwork discovery

For determination of statistically altered subnetworks after loss of PS1IV splicing under mimicry of hypoxia, the following steps were performed:

1- An enriched mammalian database of gene/protein/small RNA interactions, ResNet, was implemented in the Pathway Studio 10 package (Elsevier). ResNet is a database of biological relations, ontologies and subnetworks, compiled by Ariadne (Elsevier) for mammalian research (20, 21). One of the major advantages of this database is inclusion of information on the subcellular localization of gene products which provides the opportunity to evaluate the contribution of different cell compartments to biological networks.

A range of interaction sets are deposited in the ResNet database such as regulation, expression, promoter binding, molecular transport, protein modification, binding, molecular synthesis, chemical reaction, direct regulation, miRNA effect, protein complex, and small molecule function. Furthermore, we updated the network database Pathway Studio 10 using Medscan language programming via text mining [29] with the keywords of names of over-expressed and under-expressed genes after *PS1IV* loss of splicing, Alzheimer's disease, and hypoxia to enrich the deposited networks in our reference database. MedScan is an NLP (Natural Language Processing) application for reading scientific literature and extracting protein-centric biological relations [29].

2- Using as input the set of genes showing significantly altered genes expression after loss of PS1IV, different possible subnetworks to which these genes may contribute were called from the updated database of the Pathway Studio package.

3- The gene set enrichment concept was used to distinguish and select important subnetworks. Gene set enrichment measures the enrichment of different subnetworks by an imported list of genes/miRNAs and highlights the statistically enriched subnetworks at p≤0.05, based on various statistical tests and particularly Fisher's exact test [27]. As an example, if a particular subnetwork has 15 genes and 14 out of 15 genes of this subnetwork are present in the list of those displaying altered expression after loss of PS1IV, this subnetwork has a high probability of existence according to Fisher's exact test.

Integrative regulatory network construction

For construction of an integrative regulatory network, various algorithms such as "*add neighbours* (with varying ranges of directly and indirectly interacting neighbours – "expansions" of 1, 2 or 3)", "*add direct interaction*", "*add shortest path*", "*add common targets*", "*add common regulators*", and "*union selected subnetworks*" [28, 30-33] were compared.

The *add neighbours* algorithm adds a new interacting element (direct or indirect interaction) to an imported gene entry if that new element is in within defined interacting range. Here, expansions of 1, 2, and 3 were tested. *Add direct interaction* only adds direct interactions to the imported gene list and produces the smallest networks. The next algorithm was the *add shortest path* algorithm. Two elements in the network may interact with each other via many different routes. The *add shortest path* algorithm selects the shortest route as that is the most probable to occur. The *add common targets* algorithm searches for interactions that are affecting a shared target. *Add common regulators* looks for common regulators of imported genes such as transcription factors. *Union selected subnetworks* is a reliable algorithm which attempts to join together statistically significant subnetworks (from the previous stage of subnetwork discovery) and finds the underlying relationship between subnetworks based on the shared elements. Due to the ability of transcription factors (TFs), ligands, and microRNAs, to regulate multiple systems, it can be expected that these play central roles in joining various subnetworks. In fact, *union selected subnetworks* can be seen as a crosstalk discovery tool.

Topological analysis of regulatory networks

Topological analyses of the regulatory networks of over-expressed, under-expressed as well as the combined set of over- and under-expressed genes (constructed using union selected subnetworks) were performed using the cyto-Hubba package [34] in Cytoscape software [35]. Centrality indices including Degree, clustering coefficient, Edge Percolated Component (EPC), Maximum Neighbourhood Component (MNC), Density of Maximum Neighbourhood Component (DMNC), Maximal Clique Centrality (MCC) and centralities based on shortest paths, such as Bottleneck (BN), EcCentricity, Closeness, Radiality, Betweenness, and Stress were used to identify the highest-ranked genes (hubs) in the structure of the regulatory networks using the cyto-Hubba plugin [34, 36]. For details regarding the employed indices, please see the cyto-Hubba URL: http://hub.iis.sinica.edu.tw/cytoHubba/supplementary/index.htm. The genes which received the highest scores in the above mentioned indices were selected as the highest-ranked genes (hubs) in the regulatory networks. The 10 highest-ranked genes according to Bottleneck score were visualised

in Cytoscape for the regulatory networks of over-expressed and under-expressed genes, and for the combined set of over- and under-expressed genes.

Validation of network nodes by quantitative real-time PCR (qPCR)

The relative standard curve method for quantification was used to determine the expression of experimental samples compared to a basis sample. For experimental samples, target quantity was determined from the standard curve and then compared to the basis sample to determine fold changes in expression. Gene specific primers were designed for amplification of target cDNA and the cDNA from the ubiquitously expressed control gene *eef1a1l1*. The reaction mixture consisted of 50ng/μl of cDNA, 18 μM of forward and reverse primers and *Power* SYBR green master mix PCR solution (Applied Biosystems Foster City, CA, USA).

To generate the standard curve, cDNA was serially diluted (100 ng, 50 ng, 25 ng, 12.5 ng). Each sample and standard curve reaction was performed in triplicate for the control gene and experimental genes. Amplification conditions were 2 min at 50 °C followed by 10 min at 95 °C and then 40–45 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) using 96 well plates. Cycle thresholds obtained from each triplicate were averaged and normalized against the expression of *eef1a1l1*. Each experimental sample was then compared to the basis sample to determine fold changes of expression. For each gene, 3 biological replicates and 3 technical replicates were used in qPCR.

Validation of predicted miRNAs by quantitative real-time PCR (qPCR)

MicroRNA PCRs were performed on replicated biological samples using TaqMan microRNA assays (Life Technologies). Briefly, a multiplex cDNA reaction was performed on 10ng of RNA using a TaqMan MicroRNA Reverse Transcription Kit together with specific TaqMan microRNA assay primers (Life Technologies). Real-time PCR was performed in triplicate with a 1:3 dilution of cDNA using specific TaqMan MicroRNA assays and TaqMan

Universal Mastermix (Life Technologies) on a Rotorgene 6000 series PCR machine (Corbett Research). The relative

expression of microRNA was calculated using the comparative Ct method with U6 as the endogenous reference. A

list of the primers used for the miRNA qPCR assay is given in Supplemental Data 1.

Results

In the work described below, we used microarray analysis to assess changes in the expression of genes when PS1IV expression is blocked during hypoxia-like conditions in zebrafish embryos. However, since numerous human genes have "co-orthologues" in zebrafish (due to a whole genome duplication that occurred early in the teleost evolutionary lineage [37], and since the GO and Pathway Studio databases contain predominantly mammalian genetic information, we refer to genes using their human orthologue names. Raw microarray expression data gene designations are given in Supplemental Data 2 and 3.

Genes with altered expression after failure to form PS1IV under hypoxia

PS2V is a truncated isoform of PSEN2 that shows elevated expression in the brains of people with sporadic AD. However, the cellular role of PS2V cannot be investigated using mouse or rat models since the rapid evolution of some of their AD-related genes has resulted in loss of their ability to induce this isoform [15]. In contrast, zebrafish produces a PS2V-cognate isoform named PS1IV [37]. We can block induction of PS1IV in embryos subjected to hypoxia by injection of the morpholino oligonucleotide Hmga1aBindBlock [14]. Since so little is known about PS2V function, we exploited this phenomenon to investigate the effects on cellular function of loss of PS1IV under hypoxia using gene network analysis of relative expression data from microarrays.

Zebrafish embryos were injected at the 1-cell stage with MoHmga1aBindBlock or the negative control morpholino MoCont. They were then incubated in 100 μM sodium azide, NaN_3 to mimic the effects of hypoxia (a treatment hereafter referred to simply as "hypoxia") from 36 hpf to 48 hpf before extraction of mRNA and analysis of relative gene expression on the Affymetrix Zebrafish Gene 1.0 ST Array. A number of control microarray comparisons were also made to identify genes for which expression is affected by the act of injection or by the non-specific effects of the presence of morpholino oligonucleotides (Figure 1). Any genes for which expression was significantly altered by injection or non-specific morpholino effects were excluded from subsequent analyses (see below).

Bayesian t-tests produced lists of genes with significantly increased or decreased transcript levels ("expression") in our microarray comparisons. We describe these genes below as either "over-" or "under-expressed" in response to loss of PS1IV under hypoxia. The final list of genes with significant (p≤0.05) over- or under-expression was generated after removal of any genes showing significantly altered expression in the controls (see Figure1, Table 1, Supplemental Data 2 and Supplemental Data 3).

Among the genes showing over-expression under hypoxia without PS1IV (i.e. genes that would normally be repressed by the formation of PS1IV) were numerous genes involved in modulation of immune responses (*HRH2*, *IL17RA*, *CD22*, *ACY1*, *IRF4*, *XCL2* etc.), in regulation of TOR signalling (*RICTOR*, *PKD1*), in regulation of blood pressure and formation of vascular structures (e.g. *AGTR1* and *ANGPTL2*), and in cell proliferation (*PIM3*, *ACY1*, *OLFM4*) (Supplemental Data 2). This set of genes also included two genes known to be involved in neurological conditions, *GAK* (associated with Parkinsons Disease) and *CAMTA1* (associated with cerebellar ataxia, nonprogressive, with mental retardation (Supplemental Data 2). Two non-coding RNAs were also seen in the list of genes over-expressed in the absence of PS1IV, si:dkey-167i21.2 and si:ch73-110p20.1.

A greater number of genes were identified as showing significant under-expression under hypoxia without PS1IV (i.e. genes that are normally dependent on PS1IV for stable or increased expression under hypoxia). These included genes encoding transcription factors or proteins involved in chromatin remodelling (e.g. *NKX3-2*, *SRCAP*, *GBX2*, *TCF25*, *SMARCA4*, *POU4F2*), genes involved in ER function (*CALR*), intracellular signalling (*OCRL*, *AIDA*, *PLEKHA4*, *PLCB1*, *MAPKAPK5*) and a considerable number of ribosomal proteins (see Table 1).

An overview of our genetic network analysis of transcriptome changes occurring due to failure to form the PS1IV isoform under hypoxia

Gene regulatory network analysis offers a more comprehensive level of understanding of biological mechanisms compared to analyses of individual (separate) gene expression changes.

Within cells, the transcripts and proteins encoded by genes can have many functionally interacting partners. "Subnetworks" are sets of genes with biologically important interactive relationships. The Pathway Studio 10 software package (based on the Fisher Exact Test) accepts a set of genes with significantly altered expression and evaluates the genes with respect to their contribution to all the possible subnetworks in its database. It then ranks these subnetworks in terms of statistical significance to create a list of "statistically significant subnetworks". We used the Pathway Studio 10 software package to examine the sets of genes showing significantly increased or decreased transcript levels when formation of the PS1V isoform is blocked during hypoxia. Subnetworks with a statistical significance of $p \leq 0.05$ were then ranked.

Once ranked lists of statistically significant subnetworks had been determined, we then used the algorithm "*union selected subnetworks*" to attempt to join different subnetworks into larger aggregate networks (integrative networks). Networks were produced based on the set of genes with significantly increased transcript levels, the set of genes with significantly decreased transcript levels, and the set of all genes with either significantly increased or decreased transcript levels (see later). The *union selected subnetworks* algorithm looks for elements shared between subnetworks that could act as links between them. This procedure is valuable as a test of the veracity of the existence of the subnetworks identified within our experimental system since subnetworks that show greater numbers of shared elements are less likely to be spurious.

A number of other methods for network analysis were also applied to our sets of genes that are "over"- and "under"-expressed in the absence of PS1IV. These other methods are summarised in the Materials and Methods section. In general, these analyses confirmed the major features of the networks produced by *union selected subnetworks* analysis. Of all the methods used, "*union selected subnetworks*" proved to be the most efficient network prediction algorithm for combining the statistically significant subnetworks and revealing their regulatory components

(transcription factors, microRNAs, and ligands). The veracity of the networks predicted by *union selected subnetworks* was confirmed by qPCR analysis of predicted changes of transcript levels for a number of genes identified as nodal within the networks (see the section "Validation of network nodes (hubs) by RT-qPCR" below and Figure 2). In the description that follows we have focused on the *union selected subnetworks* analysis since it is the method producing the most statistically rigorous output. (The *union selected subnetworks* algorithm has also been recently used in the discovery of transcriptomics-based regulatory networks of biological phenomena such as apoptosis and stem cell differentiation [31, 32, 38]).

Significant subnetworks formed by genes over-expressed after failure to form PS1IV under hypoxia

Genes over-expressed after failure to form PS1IV under hypoxia were examined for their contribution to formation of statistically significant subnetworks ($p \leq 0.05$) based on the "gene set enrichment" approach [27] using the Pathway Studio 10 software package. The identified significant subnetworks and their corresponding *p*-values are presented in Table 2. The genetic relationships underlying each subnetwork and citations upon which these relationships are based are presented in Supplemental Data 4. The subnetworks are: *neighbours of GABARAP* (Gamma-Aminobutyric Acid Receptor-Associated Protein), *PDLIM5* (PDZ And LIM Domain 5), *MIR20A* (microRNA 20a), *LPS* (lipopolysaccharides), *BTK* (Bruton A gamma globulinemia), and *NOTCH2* (notch2). These subnetworks and their interactions (relationships) are visualised in Figure 3. The gene *GABARAP* encodes a ligand-gated chloride channel that mediates inhibitory neurotransmission and interacts with the cytoskeleton. GABARAP protein has a direct regulatory effect on the angiotensin receptor AGTR1 that regulates blood pressure and vascular development. GABARAP also mediates membrane expression of AGTR1 (Figure 3A) [39]. Interestingly (and with relevance to AD), GABARAP is also involved in apoptosis and autophagy [40-45]. As presented in Figure 3B, *PDLIM5* may be involved in the observed over-expression of *AGTR1* and another gene, *PKD1*, after loss of PS1IV [46, 47]. *PDLIM5* has been implicated in bipolar disorder, depression, and schizophrenia via regulation of dendritic spine morphogenesis in neurons [48, 49]. This is especially interesting when one considers that the human homologue of PS1IV, PS2V, is greatly increased in the brains of some cases of schizophrenia [11]. It has recently been suggested that *PDLIM5*

plays an important role in heart development and cardiomyocyte expansion [50]. Since mutations in both human *PSEN1* and *PSEN2* can cause Dilated Cardiomyopathy (DC) this suggests that the *PRESENILIN* genes together with *PDLIM5*, *PKD* and *AGTR1* may form a genetic network involved in cardiovascular disease.

One subnetwork is formed by *NOTCH2* together with *FOXE3*. *NOTCH* genes encode transmembrane receptors that signal directly to the nucleus. When NOTCH receptors are cleaved by PSEN proteins within γ-secretase complexes, their intracellular domains move into the nucleus to act as transcription factors [51]. NOTCH receptors have important roles in maintenance of undifferentiated stem cell states, particularly under hypoxic conditions [52, 53]. During zebrafish embryo development, the gene *foxe3* is expressed mainly in the developing lens of eyes that are relatively much larger than in humans. The *foxe3* gene also shows restricted expression in the brain and in the hatching gland [51], a structure not found in mammals. This reminds us that our microarray analysis involves all the cells of a developing zebrafish at 48 hpf and not only those with relevance to the brain. This means that the networks we subsequently identify should not be interpreted as representing single overall regulatory networks within a single cell type. Some of the subnetworks will exist only within particular cell types and some apparent linkages between putative subnetworks may be artefactual.

A subnetwork illustrating the ability of microRNAs to coordinate regulation of multiple gene transcripts is formed by the apparent inhibitory effect of the microRNA *MIR20A* on *PKD1*, *AGTR1*, and *CAMTA1* (Figure 3C). Since these 3 genes are over-expressed in the absence of PS1IV, we would predict *MIR20A* to be downregulated in this circumstance. *MIR20A* expression is not measured by the Affymetrix Zebrafish Gene 1.0 ST Array but subsequent qPCR analysis of its expression under normoxia and mimicry of hypoxia in the presence or absence of PS1IV supports that PS1IV may be important for maintaining levels of *MIR20A* expression under hypoxia (Figure 4). *MIR20A* belongs to the miR-17 microRNA precursor family that includes *miR-20a/b*, *miR-93*, and *miR-106a/b*. These microRNAs negatively regulate gene expression through complementarity to the 3' UTR of specific target messenger RNAs (miRBase, http://www.mirbase.org/).

From our analysis, another microRNA, *MIR1-1*, is implicated in a putative subnetwork regulating *NOTCH2* and *GAK* (Figure 3F). Decreased NOTCH signaling has been suggested to underlie the development of AD pathology [54] so the downregulation of *NOTCH2* by PS1IV under hypoxia may be significant. Decreased expression of CYCLIN G-ASSOCIATED KINASE, GAK has been shown to inhibit NOTCH signaling and to increase neural cell death in zebrafish [55]. GAK has also been associated with Parkinson Disease (PD) in genome wide association studies (GWAS) [56], consistent with its interaction with the PD-associated protein α–synuclein [57]. Previously, we observed increased expression of *cyclin G2* gene expression when zebrafish *psen1* or *psen2* activity was suppressed in embryos in a morpholino-based study [58]. We have also argued that insufficient delivery of oxygen to the brain may play an important role in AD pathogenesis [59] and, in this light, it is interesting that both the MIR1-1- and MIR20A-coordinated subnetworks described here involve genes important for vascular function and development (e.g. see [60] and [61]). The analysis summarised in Figure 5 also illustrates that both these subnetworks are potentially linked through the transcription factor ESTROGEN RECEPTOR 2, ESR2. The *ESR2* gene shows homology to the *Hairy/enhancer of split* (*HES*)-related genes that are typically directly activated by NOTCH signaling. Also, the estrogen molecule estradiol has been shown to up-regulate *Agtr1* in rat heart [62] .

Finally, lipopolysaccharides are known to have positive regulatory effects on all the receptors seen to be over-expressed after loss of PS1IV (*AGTR1, HRH2, IL17RA, CD22,* and *NOTCH2*) [63-65]. Together these form a statistically significant subnetwork (Figure 3D). Lipopolysaccharides commonly induce inflammatory responses [66] while hypoxia (that induces PS2V and PS1IV formation) is also pro-inflammatory. The fact that PS1IV upregulates expression of the pro-inflammatory cytokine IL1B and the chemokine receptor CCR5 (see later) suggests that the truncated *PRESENILIN* transcript isoforms induced by hypoxia act as important modulators of inflammatory responses.

Prediction of a regulatory network underlying genes over-expressed after failure to form PS1IV

Transcription factors and microRNAs commonly establish the crosstalk and coordination between different regulatory subnetworks since any one transcription factor or microRNA usually has the ability to regulate multiple gene transcripts. To detect potentially coordinated subnetworks, genes significantly over-expressed after failure to form PS1IV under hypoxia were subjected to various network construction algorithms including "*add interacting neighbours* (interacting range of 1, 2, and 3, as mentioned in Material and Methods)", "*add direct interaction*", "*add shortest path*", "*add common targets*", "*add common regulators*", and "*union selected subnetworks*".

Figure 5 shows the network generated when the *union selected subnetworks* algorithm was applied to the list of statistically significant subnetworks given in Table 2. Significant features of this network are described below. Also, Supplemental Data 5 demonstrates the underlying relationships of the network.

The transcription factor encoded by *ATF2* (also known as *CREBP1*) regulates *PKD1* and *ANGPTL2* within this network. Loss of *ATF2* has been shown to affect mouse *Psen1* expression and to cause cranial motoneuron degeneration during brain development [67]. The ATF2 protein is involved in regulation of cellular responses to numerous stresses [68]. The role of *MIR20A* as a key subnetwork coordinator controlling expression of *CAMTA1, AGTR1* and *PKD1* is evident (see below and Figure 5). The *CAMTA1* and *AGTR1* genes are described above but *PKD1* encodes a protein that has been observed in astrocytes and has been shown to activate the JAK-STAT signaling pathway [69]. Another microRNA suggested to coordinate a subnetwork, *MIR1-1* (see above) regulates NOTCH2 and MRC2 receptor expression and expression of GAK in the Golgi apparatus. In the network analysis, the number of interactions for any gene product can be used as an index of that gene's regulatory importance. The structure of our network of genes over-expressed after failure to form PS1IV highlights the products of *PKD1, AGTR1, ANGPTL2*, and *NOTCH2* as central regulatory elements since these each show large numbers of interactions.

The results of an alternative strategy for network assembly – the *shortest path* method – are presented in Supplemental Data 6 and emphasise the importance of the genes *CAMTA1* and *AGTR1* as coordinating regulatory

nodes. Calmodulin has a considerable number of interactions within that network. Noticeably, *CAMTA1* expression is under the control of many microRNAs, such as mir20A and mir34A emphasing both its functional importance and the selective pressure for its tight regulatory control. This is not unexpected when one considers the important role that Ca^{2+} signaling and the PRESENILIN proteins play in interactions between the ER and mitochondria [70, 71]. *CAMTA1* encodes a Ca^{2+}-regulated transcription factor and changes in *CAMTA1* expression have been associated with early-onset nonprogressive cerebellar ataxia and mild mental retardation [72-74]. The relationships within this network are presented in Supplemental Data 7.

Decreased expression of *MIR20A* after loss of PS1IV under hypoxia

In network analysis, a "seed" is defined as the central gene (node) in a subnetwork if it has the highest number of interactions with other subnetwork members. As seen in Figures 3 and 5, *MIR20A* is the seed of an interesting subnetwork which negatively modulates expression of important genes that are over-expressed after failure to form PS1IV. These genes include *PKD1*, *AGTR1*, and *CAMTA1*. We have analysed the expression of *MIR20A* in the presence and absence of PS1IV under hypoxia in zebrafish (Figure 4). Interestingly, the expression of *MIR20A* appears to be decreased after loss of PS1IV (by 1.6 fold on average although p is only 0.1). As microRNAs bind to the mRNA of target genes to decrease their expression it is expected that down–regulation of *MIR20A* will result in up-regulation of its targets in the network, i.e. *PKD1*, *AGTR1*, and *CAMTA1*.

It should be noted that changes in the expression levels of some microRNAs can cause disproportionately greater changes in the expression of their target mRNAs. This is because microRNAs can function at much lower concentrations than mRNAs and need not be translated before binding to mRNAs to rapidly affect gene expression. Therefore, typically a fold change of 1.2 – 1.5 is used as a lower threshold when identifying microRNA s with altered expression [75, 76].

Significant subnetworks formed by genes under-expressed after failure to form PS1IV under hypoxia

When formation of PS1IV is suppressed during hypoxia, a number of genes show decreased expression. These genes require PS1IV for normal or increased expression during chemical mimicry of hypoxia and are listed in Table 1. We used these genes to construct subnetworks using the Pathway Studio 10 software (as described above for those genes with increased expression in the absence of PS1IV).

Significant subnetworks generated using the under-expressed genes are shown in Table 3 and Figure 6. Many of the subnetworks are related to apoptosis and immune response, including genes such as *CASP5, NLRP12, IL1B, TRIM22* and *CCR2*. The underlying relationships of these subnetworks are presented in Supplemental Data 8. It is apparent that *IL1B* is shared in many subnetworks. The regulatory subnetwork with the greatest significance (smallest p-value) is controlled by the transcription factor *MEF2D* (myocyte enhancer factor 2D) (Figure 6C). *MEF2D* is a stress-induced gene involved in cardiac muscle development as well as neuronal differentiation and survival. *MEF2D* plays diverse roles in cell growth, survival and apoptosis via the p38 MAPK signaling pathway. It has been suggested that *MEF2D* is a critical protein in the regulation of neuronal apoptosis and autophagy [77-80]. Another important subnetwork coordinates around Ca^{2+} ion concentration (Figure 6D) again emphasizing the relationship between *PRESENILIN* gene function and this ion.

Prediction of a regulatory network coordinating genes under-expressed after failure to form PS1IV

A regulatory network for under-expressed genes was constructed based on the *union selected subnetworks* algorithm as discussed above. This network is presented in Figure 7 and its underlying relationships are given in Supplemental Data 9. A variety of cellular organelles and structures respond to modulation by PS1IV including the mitochondrion, endoplasmic reticulum, Golgi apparatus, nucleolus, and plasma membrane emphasing the functional significance of this PRESENILIN isoform (Figure 7).

The following nodes are central in the generation of the regulatory network of under-expressed genes: (1) *IL1B*, (2) *CCR5*, (3) *TH*, (4) *CALR*, (5) *SMARCA4* and (6) *MEF2*. According to the Gene Ontology classification of *CCR5* and based on our ComparativeGO database [25, 26], this gene, *CCR5* is involved in aging and promotion of apoptosis. *CCR5* has previously been suggested to have a modulatory effect on the activated microglia seen in AD brains [81] and its expression is elevated in peripheral blood mononuclear cells [82] and in other cells of the immune system in AD patients [83]. In rats, *CCR5* is expressed by microglia in the developing brain and predominantly in the endothelial cells of the adult brain [84]. It is highly up-regulated in the brain by hypoxia/ischaemia supporting that hypoxia is an important element of AD pathology [84]. However, some researchers have reported that hypoxia can inhibit upregulation of *CCR5* in mouse macrophages [85].

As can be seen in Figure 7, *TH* and *IL1B* represent intensive nodes in the network. *TH* encodes the enzyme *TYROSINE HYDROXYLASE* that is rate limiting in the catecholamine biosynthetic pathway required for production of dopamine. Interestingly, hypoxia is known to regulate TH activity in selected areas of the brain [86]. It has recently been documented that disruption of *TH* activity in humans causes Segawa syndrome, an inherited form of infantile Parkinsonism [87, 88]. TH has also been seen to modulate the release of inflammatory cytokines so the observed coupling of the TH and IL1B nodes (below) in our network is not unexpected. Also intriguing is the existence of another gene associated with Parkinson disease as a node in our network, *LRRK2*. Mutations in *LRRK2* are one of the most common causes of familial Parkinson disease. The protein product of *LRRK2* binds to the product of the *TUBULIN, BETA* gene, *TUBB,* that is the most under-expressed gene when PS1IV is absent under hypoxia. LRRK2 has also been seen to stimulate protein expression by acting on Argonaut proteins to repress microRNA action [89].

Cytokines are signaling compounds and major mediators of immune responses which are involved in stress responses, cell differentiation, and apoptosis. The IL1B protein is a member of the interleukin 1 cytokine family which is proteolytically processed to its active form by CASPASE 1 (CASP1/ICE). The importance of IL1B in sporadic AD is demonstrated by identification of polymorphisms at this gene locus associated with increased AD risk [90]. *IL1B* mRNA is highly significantly under-expressed in the absence of PS1IV and this gene forms a major node in our

genetic network. Thus PS1IV contributes significantly to induction of *IL1B* expression under hypoxia. In our network, *IL1B* communicates with *CCR5* the product of which has important roles in mediation of neuroinflammatory responses to excitotoxicity (see above and [91]). Our predicted network also shows the transcription factor encoded by *STAT4* as increasing the expression of *CCR5* and *IL1B*. STAT4 protein is well recognized as playing a stimulatory role in inflammation including in the brain [92].

The family of *MEF* transcription factors figures prominently in our network of under-expressed genes. These transcription factors control neuronal survival, apoptosis, and autophagy [79, 80]. We also see involvement of the gene encoding CREATINE KINASE, MITOCHONDRIAL 1B, (*CKMT1B*), a protein of the inner membrane of mitochondria involved in lipid transfer between membranes. The gene *CALR*, that encodes a stress response protein binding Ca^{2+} ions in the ER and that is also found in the nucleus possibly acting as a transcription factor [93] is an important node in this network. Interestingly, in the ER, CALR binds to PROGRANULIN, (a protein implicated in frontotemporal dementia), where it forms a complex together with protein disulfide isomerases [94]. The mitochondrial associated membranes (MAM) are foci for oxidative protein folding (disulphide bond formation) and are a major subcellular location for PRESENILIN protein expression [95]. Changes in PRESENILIN expression affect the lipid constitution of the MAM that is a site of oxygen use and that produces high levels of reactive oxygen species (ROS). ROS induces expression of PS2V via upregulation of HMGA1a to alter *PSEN2* transcript splicing [20]). Numerous mutations in *PSEN1* are linked to frontotemporal dementia (usually in association with AD). Thus, our network analysis supports involvement of the PSEN proteins in frontotemporal dementia via regulatory interactions with PROGRANULIN in the MAM under hypoxic conditions.

SMARCA4 is another node in the network (Figure 7). *SMARCA4* encodes a calcium signaling related protein with helicase and ATPase activities that can regulate the expression of genes by altering their chromatin structure [96]. Interestingly, significant down regulation of *SMARCA4* has been reported in severe AD [97].

MAPKAPK5, encoding a protein of the serine/threonine kinase family, governs another node in the network. This kinase is expressed in response to cellular stress and proinflammatory cytokines [98, 99]. As a tumor suppressor protein, the major functions of *MAPKAPK5* are activation of apoptosis and mediation of senescence [100]. Interestingly, *MAPKAPK5* is also involved in amyloidosis and yellow fever diseases [101]. This supports the idea that PS2V might be important for the accumulation of amyloid in AD (e.g. through its suppression of the UPR, [9, 14]).

In our network of under-expressed genes, the microRNAs *MIR137* and *MIR26A1* potentially negatively regulate the nodal gene, *PHOSPHOLIPASE C, BETA-1*, (*PLCB1*), that encodes a key protein regulating signal transduction pathways involving IP$_3$ and diacylglycerol. Deletions of *PLCB1* have been observed in the brains of people with schizophrenia [102] which may be significant since PS2V has also been observed to be greatly elevated in schizophrenia brains [11]. This supports that neural hypoxia may be an important environmental influence in schizophrenia [103] via PS2V.

Finally, a considerable number of genes encoding ribosomal proteins are under-expressed in the absence of PS1IV, for example RPL9, RPS15A, RPL28, RPL26 and RPS19. The stimulation of expression of these proteins by PS1IV under hypoxia appears consistent with the action of this truncated PRESENILIN protein and its cognate PS2V isoform to repress the UPR [104]. The UPR usually acts to suppress protein synthesis when cellular stresses (e.g. lack of oxygen for oxidative protein folding) lead to increased levels of unfolded proteins [104]. As mentioned above, the stimulation of LRRK expression by PS1IV is also consistent with its opposition to the UPR since this should increase protein synthesis. Notably, among the genes normally suppressed by PS1IV expression, the gene with most significantly altered expression is *HSPA8* encoding the chaperone HEAT-SHOCK 70-KD PROTEIN 8. Upregulation of chaperone protein expression is an important element of the UPR. However, the action of PS1IV on other UPR actors is less easy to explain. PS1V stimulates *CALR* expression (see above), the product of which is involved in the UPR and is normally upregulated by ER stress [105]. The CALRETICULIN protein can form complexes with the Ca^{2+} pump ATPase, Ca(2+)-TRANSPORTING, FAST-TWITCH 1 (ATP2A1, also known as SERCA) and disulphide isomerases required for oxidative protein folding [106]. PRESENILIN1 holoprotein binds ATP2A1 and is, itself,

upregulated by unfolded protein stress [107]. Indeed, the PRESENILIN1 holoprotein has been observed to act a chaperone [108, 109].

Topological analysis of regulatory networks

To identify those genes central to the structure of the regulatory networks, Bottleneck, DMNC, MNC and MCC centrality indices were calculated. The results are presented in Supplemental Data 10 and the genes are ranked based on overall scores of importance in various indices. Also, the 10 highest-ranked genes according to Bottleneck are presented in Figure 8.

AGTR1, *NOTCH2*, LPS, are *PKD1* are central hubs in the network structure of genes over-expressed after failure to form PS1IV under hypoxia (Supplemental Data 10). MIR20A received a high rank in network topological indices (Supplemental Data 10, Figure 8)

The Inflammatory response genes *IL1B* and *CCR5* are central in the regulatory network of genes under-expressed after failure to form PS1IV under mimicry of hypoxia (Supplemental Data 10). Also, STAT4, a transcription factor involved in stimulation of brain inflammation [92], forms a hub in this network (Supplemental Data 10, Figure 8).

The raw expression value within different biological replicates of the treatments in this study is presented in Supplemental Data 11. Low standard deviations within different biological treatments show that gene expression is consistent across the biological replicates of each treatment.

In the regulatory network constructed from the combined set of over- and under-expressed genes after loss of PS1IV, the highest ranked genes (hubs) were: *IL1B*, *TH*, *LPS*, *CCR5*, *AGTR1*, *HSPA8*, *PKD1*, *CALR*, and *MAPKAPK5*. Ca^{2+} was also identified as a hub. Overall, the topological analyses support the importance of these genes/factors in the function exercised by PS1IV.

A global regulatory network of both over- and under-expressed genes after failure to induce PS1IV during hypoxia

The genes observed to be over- or under-expressed in our study were detected after a only one form of experimental manipulation, i.e. blockage of PS1IV expression under hypoxia. Therefore, we can expect both the over- and under-expressed genes to show regulatory interactions within a global network. To examine this, the combined set of over- and under-expressed genes was subjected to subnetwork discovery using the "gene set enrichment" approach followed by integrative network construction using the *union selected subnetworks* algorithm. Supplemental Data 12 shows the subnetworks enriched by this global gene set. The network underlying these genes regulated by PS1IV is presented in Supplemental Data 13 and its constructing relationships in Supplemental Data 14.

The combined, global network does not reveal very significant new nodes but does highlight the intensity of the nodal roles played by *IL1B*, *PLCB1, NOTCH2, AGTR1, SMARCA4*, *CALR*, *PKD1* and *HSPA8*. The regulation by PS1IV of processes in the ER, mitochondrion, nucleus and on the plasma membrane is evident. The contribution of the ER and mitochondrial dysfunction to induction and progression of AD has been discussed recently [108, 110, 111]. Our results support that PS2V may play a significant role in this.

Validation of network nodes (hubs) by RT-qPCR

Finally, to confirm the validity of our constructed networks, RT-qPCR was performed for a subset of those genes located at network nodes (Figure 2). The results showed high correspondence between the microarray and RT-qPCR results. *CAMTA1* showed more than a 2-fold increase in mRNA level in both microarray and qPCR analyses. On the other hand, *IL1B* was down regulated by more than 2-fold in both analyses. Both methods of analysis showed *PKD1* to be significantly over-expressed after inhibition of PS1IV formation under hypoxia while *MAPKAPK5* and *SMARCA4* were significantly under-expressed.

Several factors have inhibited analysis of the correspondence between changes in mRNA expression and consequent protein expression changes. There is a dearth of commercially available antibodies that detect zebrafish proteins and proteomic analyses of zebrafish embryos can be problematic due to interference from the high levels of yolk vitellogenins present at these stages [112]. Hopefully, the results of our analyses will direct future proteomic experimentation in cultured human cells that will support our findings.

Literature mining-based network analysis of under-expressed genes after failure to form PS1IV highlights PS1IV's involvement in cancer and cardiomyopathy

During our construction of various networks, one was of particular interest. By using the *shortest path* algorithm to analyse genes under-expressed after failure to form PS1IV and by incorporating disease terms (to extract relationships between these genes and different diseases from the scientific literature, see Material and Methods) we identified a network that emphasizes the pathological importance of changes in expression of *IL1B*, *HSPA8*, *TH* and *CALR* (see Supplemental Data 15 and Supplemental Data 16). This suggests that PS1IV's human homologue, PS2V, may play roles in the progression of cancer and in preeclampsia. Changes in the expression of the under-expressed genes *TH*, *IL1B*, *PLCB1* and *AMPD1* are also associated with heart failure. This is interesting since mutations in both human *PSEN1* and *PSEN2* exist that are thought to cause dilated cardiomyopathy [113].

Discussion

Despite their importance as fAD loci, genome-wide association studies have failed to identify the PRESENILIN genes as risk loci in the predominant, late onset form of AD, sAD. This has raised concern that the pathological mechanisms underlying these two forms of AD may differ. However, a particular isoform of PSEN2 upregulated by hypoxia [8] and high cholesterol [15], PS2V, shows increased expression in sAD brains. Therefore it is important to understand the role this isoform plays in gene regulation. Investigation of PS2V function in the genetically manipulable rodent models is thwarted by the accelerated evolution of the fAD genes in these animals and their consequent loss of ability to express PS2V [114]. However, the ability to express this isoform in response to hypoxia has been retained by zebrafish and the molecular mechanism controlling induction of zebrafish PS1IV and human PS2V under hypoxia is conserved [14].We have also previously observed that the pathological function of the *PRESENILIN* genes cannot be correctly understood in physiologically abnormal systems [14]. Our ability to inhibit specifically the induction of the endogenous PS1IV isoform under hypoxia-like conditions in developing zebrafish embryos enables us to examine its function in an otherwise normal cellular state (in contrast to the abnormal cell types and growth conditions commonly involved in studies using tissue culture).

Aside from the abilities of PS2V / PS1IV to suppress the UPR and stimulate γ-secretase activity, nothing is known regarding the function of this isoform, why this function has been conserved over nearly half a billion years since the tetrapod/teleost divergence, or why this isoform was lost from the rodent family *Muridae* but not other rodents or mammals [14]. Disconcertingly, our results show that this isoform regulates expression of a number of genes and cellular systems associated with a variety of neurodegenerative conditions including AD (see below), Parkinson disease (e.g. *LRRK2*, *TH* and *GAK*), schizophrenia (e.g. *PLCB1*, *PDLIM5*), early-onset nonprogressive cerebellar ataxia (*CAMTA1*) and others. This reinforces concerns that mouse models of Alzheimer's disease and other neurological diseases do not accurately represent the human conditions [115].

Our microarray analysis has shown that loss of PS1IV function in zebrafish embryos under hypoxia-like conditions significantly alters the expression of a number of genes implicated in AD pathology in humans. We have listed a number of these genes in this paper but a greater depth of analysis is required in order to understand the changes in cell biology that these gene expression changes represent. Gene regulatory network analysis can provide greater insight into function than simply identifying genes with greatest and/or most significantly changed expression. This is because small changes in the expression of genes at nodal positions in networks can have greater systemic effects on cell biology than large changes in the expression of non-nodal genes. One example of this is the *TUBB* gene that shows the most statistically significant and greatest fold change in expression of any gene in our analysis but that is not very enlightening in terms of understanding the functional consequences of PS1IV loss. (Interestingly, however, problems with tubulin polymerization into microtubules have been observed in AD brains [116] and, of course, AD pathology is associated with abnormal aggregation of MICROTUBULE-ASSOCIATED PROTEIN TAU, MAPT into neurofibrillary tangles. In contrast, it was significant to see that PS1IV is required to maintain or increase the expression of the nodal gene *CALR* under hypoxia since the CALR protein acts as a chaperone in the maturation of glycoproteins including AβPP [117] and has also been observed to be down-regulated in neurons in AD brains [118]. This supports that expression of PS2V in AD brain is an attempt by neural cells to cope with the stresses that are driving the development of AD pathology. CALR protein is known to exist in complexes with the protein PGRN implicated in frontotemporal dementia (FTD) and has also been shown to bind the ATP2A1 protein that binds PSEN1 [119]. This supports a putative functional link between the major genetic locus mutated in familial AD (*PSEN1*) and the second most commonly mutated gene in familial FTD (*PGRN*). Interestingly, there are mutations in *PSEN1* that are thought to cause FTD rather than FAD and these have been observed to differ in their effects on subcellular calcium transport [120, 121]. This emphasizes the importance of Ca^{2+} in PRESENILIN function. Since the PRESENILINs and ATP2A1 are all MAM-resident proteins, the data above imply that PGRN will also show localization in the MAM. Of course, since PGRN possesses a very high number of disulphide bonds [122], the oxidative protein folding that occurs in the MAM must be central to PGRN function. Reassuringly, the involvement of

CALR, a protein important for quality control of protein folding and for signal transduction [122], showed that our microarray and network analyses are consistent with the known roles of PS1IV and PS2V in modulation of the UPR.

Other observations from our network analysis further support that the human equivalent of PS1IV, PS2V, plays a role in AD pathology. Somewhat unexpectedly, we saw that PS1IV is important for the upregulation of *1L1B* and *CCR5* that, in humans, have been strongly implicated in the inflammation characterising AD brains. The hypoxia that upregulates PS1IV can also drive inflammatory, innate immune responses (e.g. [123]), possibly by increasing the amount of incorrectly folded proteins in a cell [124]. In our recent paper describing PS1IV we argued that such truncated PSEN isoforms function to inhibit the over-activation of the UPR under hypoxia that can lead to cell death [14]. By inhibiting the UPR, PS1IV may permit higher levels of mis-folded proteins under hypoxia and thus stimulate inflammatory responses including *IL1B* expression. We note also that the NLRP3 inflammasome that can promote cleavage of pro-IL1β to form active IL1β protein is apparently localised to the MAM [125]. Recently, activation of the innate immune response was shown to be suppressed in the brains of mice lacking expression of the fAD gene AβPP [126]. In terms of AD, the AβPP protein is the most important substrate for the γ-secretase activity of the PSEN proteins and both PS2V and PS1IV can act to increase γ-secretase activity in cells. Thus, another molecular subnetwork connecting PS2V/PS1IV to increased *IL1B* expression in sAD may be via the Aβ and/or AICD production from AβPP that would increase with increased γ-secretase activity.

Upregulation of PS2V mRNA expression [8, 11] and accumulation of PS2V protein [127] in the human brain are markers of sAD. Since hypoxia is important for induction of PS2V via oxidative stress, this supports that insufficient brain oxygenation may be a fundamental driver of sAD pathology. This is consistent with evidence that serum markers of hypoxia can distinguish people who progress from mild cognitive impairment (MCI) to AD versus from those who do not [128]. Also, hypoxia has been seen to upregulate production of the AD-associated peptide Aβ in cultured human neuroblastoma cells and to greatly raise Aβ in serum [129]. Thus, in humans, PS2V may form a facilitating link between hypoxia and a number of pathological processes in AD brains. It is possible that blockage of

PS2V action in AD brains might inhibit some of these pathological processes such as the inflammation provoked by IL1B expression. It might relieve suppression of the UPR that normally acts to restrict generation of inappropriately folded, aggregation-prone proteins. On the other hand, PS2V suppression might increase the cell death that results from over-stimulation of the UPR [14] while decreasing expression of CALR with negative consequences for protein folding (see above) . Understanding the multifaceted actions in sAD of the human PS2V isoform will require using the data we have obtained from zebrafish to generate hypotheses that can be tested in human cellular systems such as in neurons derived from induced pluripotent stem cells (iPSCs)[130] .

Our analyses in this paper show that extending examination of the consequences of gene/isoform dysregulation from simplistic observation of statistically significant changes in gene expression to construction of statistically significant theoretical gene interaction networks enhances our understanding of the normal functions of dysregulated genes and protein isoforms. Thus, gene network analysis can contribute greatly to our understanding of the pathological mechanisms underlying complex disorders such as AD. The fact that, despite publication of over 80,000 scientific papers on AD (excluding reviews), there is no firm consensus on either its underpinning molecular mechanisms or disease treatment reinforces the need to implement more advanced analytical strategies such as machine learning (data mining), network analysis, promoter architecture analysis, promoter-binding based network analysis, and gene ontology illustration to reveal the cellular changes and stresses underlying AD pathology [26, 128, 131-134] .

Acknowledgments

Conflict of Interest Statement

The authors declare that they have is no conflict of interest.

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Table 1 A. The most significantly over-expressed genes after blockage of PS1IV formation during mimicry of hypoxia. The complete list is presented at Supplementary 2. It should be noted that these genes are normally repressed by the formation of PS1IV.

Gene name	Affymetrix transcript ID	Fold change	P-value	Regulation	Description
ACY1	13124285	1.43802	0.003253	UP	aminoacylase 1
AGTR1	13148727	1.212083	0.032714	UP	angiotensin II receptor, type 1
ANGPTL2	13001248	2.020158	3.07E-07	UP	angiopoietin-like 2
CAMTA1	12976155	2.474301	2.73E-07	UP	calmodulin binding transcription activator 1
CD22	12959348	1.338294	0.019724	UP	CD22 molecule
EBP	13139214	1.367905	0.039918	UP	emopamil binding protein (sterol isomerase)
ECM2	13119638	1.493006	0.000693	UP	extracellular matrix protein 2, female organ and adipocyte specific
FOXE3	13244659	1.310028	0.00264	UP	forkhead box E3
GAK	13201928	1.232148	0.006471	UP	cyclin G associated kinase
HLA-DPB1	13254612	1.670167	0.002895	UP	major histocompatibility complex, class II, DP beta 1
HRH2	13007297	1.716072	9.85E-05	UP	histamine receptor H2
HSPA4	13242974	3.854714	1.09E-11	UP	heat shock 70kDa protein 4
HTR3B	13110168	1.303078	0.00088	UP	5-hydroxytryptamine (serotonin) receptor 3B
IL17RA	13189932	1.474021	0.00115	UP	interleukin 17 receptor A

Gene name	Affymetrix transcript ID	Fold change	P-value	Regulation	Description
MRC2	13225339	1.228197	0.047657	UP	mannose receptor, C type 2
MSL1	13174217	1.402262	0.018025	UP	male-specific lethal 1 homolog (Drosophila)
MUM1 (IRF4)	13090651	1.666235	0.001652	UP	melanoma associated antigen (mutated) 1 (INTERFERON REGULATORY FACTOR 4)
NOTCH2	13244612	1.4356	0.039308	UP	notch 2
NRN1L	13237032	1.389511	0.050836	UP	neuritin 1-like
OLFM4	13170496	1.39979	0.000831	UP	olfactomedin 4
PIM3	13256027	2.796259	0.000201	UP	pim-3oncogene
PKD1	13145022	1.766053	0.000564	UP	polycystic kidney disease 1 (autosomal dominant)
POLR3G	13198080	1.355577	0.000653	UP	polymerase (RNA) III (DNA directed) polypeptide G (32kD)
PTPRH	13144075	1.579611	0.003511	UP	protein tyrosine phosphatase, receptor type, H
RBM5	13217068	1.644646	0.001741	UP	RNA binding motif protein 5
RICTOR	13192653	1.834237	8.6E-05	UP	RPTOR independent companion of MTOR, complex 2

Table 2 B. The most significantly under-expressed genes after blockage of the formation of PS1IV during mimicry of hypoxia. The complete list is presented in Supplemental Data 3. It should be noted that these genes are normally up-regulated by the formation of PS1IV.

Gene name	Affymetrix transcript ID	Fold change*	P-value	Regulation	Description
ACTC1B	13043120	-1.795448	0.0019	DOWN	actin, alpha, cardiac muscle 1b

AIDA	13099479	-2.237709	1.0207E-08	DOWN	axin interactor, dorsalization associated
AKAP1B	13021077	-1.537301	0.02472395	DOWN	A kinase (PRKA) anchor protein 1b
AMPD1	13243554	-2.080487	0.0008468	DOWN	adenosine monophosphate deaminase 1
CALR	13125424	-2.135172	0.00020882	DOWN	calreticulin
CETN2	13013035	-1.694809	1.9335E-05	DOWN	centrin, EF-hand protein, 2
CKMT1A	13161691	-2.35532	8.4446E-10	DOWN	creatine kinase, mitochondrial 1A
EEF1B2	13212503	-1.911308	0.02344456	DOWN	eukaryotic translation elongation factor 1 beta 2
GBX2	13212750	-2.016745	1.2832E-06	DOWN	gastrulation brain homeobox 2
GORASP1	13148147	-1.235676	0.0491116	DOWN	golgi reassembly stacking protein 1, 65kDa
GRAMD1B	13065585	-1.134146	0.02169604	DOWN	GRAM domain containing 1B
GZMM	13122283	-1.07633	0.04767155	DOWN	granzyme M (lymphocyte met-ase 1)
HSDL2	12961615	-2.07654	4.9238E-08	DOWN	hydroxysteroid dehydrogenase like 2
HSPA8	12969404	-1.667337	0.01602544	DOWN	heat shock 70kDa protein 8
IL1B	12971431	-2.867022	0.00750858	DOWN	interleukin 1, beta
LSMD1	13208954	-1.911134	2.0139E-08	DOWN	LSM domain containing 1
MAPKAPK5	13210281	-1.601199	7.2304E-06	DOWN	mitogen-activated protein kinase-activated protein kinase 5
MRPS12	13116129	-1.303939	0.00596112	DOWN	mitochondrial ribosomal protein S12
NLRP12	12948631	-1.280818	0.00166266	DOWN	NLR family, pyrin domain containing 12
OCRL	13016081	-1.528282	0.00261043	DOWN	oculocerebrorenal syndrome of Lowe

PLCB1	13106935	-1.357149	0.00755506	DOWN	phospholipase C, beta 1 (phosphoinositide-specific)
PLEKHA4	13147690	-1.13717	0.01677694	DOWN	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4
POP7	13227367	-1.851494	1.6801E-06	DOWN	processing of precursor 7, ribonuclease P/MRP subunit (S. cerevisiae)
POU4F2	12948390	-2.494429	3.6522E-12	DOWN	POU class 4 homeobox 2
RAB3C	13251770	-1.803195	5.4026E-05	DOWN	RAB3C, member RAS oncogene family
RGS8	13214775	-1.479107	0.00691868	DOWN	regulator of G-protein signaling 8
RPL26	13012615	-1.393831	0.00272945	DOWN	ribosomal protein L26
RPL28	13035086	-1.393911	0.00271368	DOWN	ribosomal protein L28
RPL7A	13196034	-1.576352	1.1759E-05	DOWN	ribosomal protein L7a
Rpl9	12946795	-2.255041	2.09E-06	DOWN	ribosomal protein L9
RPL9	12946795	-2.255041	2.09E-06	DOWN	ribosomal protein L9
RPS15A	13167130	-1.785676	0.04753847	DOWN	ribosomal protein S15a
RPS19	13031783	-1.093388	0.05029653	DOWN	ribosomal protein S19
Rps20	13240815	-1.429581	0.01435088	DOWN	ribosomal protein S20
RRM2	13103533	-1.70361	0.00079271	DOWN	ribonucleotide reductase M2
SGCG	13018621	-1.739535	0.00064507	DOWN	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)
SHISA7	13073348	-1.505375	0.01521283	DOWN	shisa homolog 7 (Xenopus laevis)
SLC25A5	13010725	-1.568371	0.01842738	DOWN	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5

SMARCA4	13205694	-1.442988	0.0126954	DOWN	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4
SRCAP	12985971	-2.4494	6.6558E-07	DOWN	Snf2-related CREBBP activator protein
SSU72	13119040	-1.988331	8.3227E-05	DOWN	SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)
SYN2	13137535	-2.097409	9.4208E-07	DOWN	synapsin II
TCF25	13059029	-1.827271	4.2207E-06	DOWN	transcription factor 25 (basic helix-loop-helix)
TRIAP1	12960177	-1.455814	9.1656E-05	DOWN	TP53 regulated inhibitor of apoptosis 1
TRIB2	13098805	-1.65455	4.836E-07	DOWN	tribbles homolog 2 (Drosophila)
TTC26	13218326	-1.583521	0.00509751	DOWN	tetratricopeptide repeat domain 26
TUBB	13030037	-4.01288	2.33E-15	DOWN	tubulin, beta
UBAC1	13198301	-1.918137	3.9775E-06	DOWN	UBA domain containing 1

*Negative sign means under-expression.

Table 2. Significant subnetworks (p ≤0.05) formed by over-expressed genes after blockage of PS1IV formation under mimicry of hypoxia. It should be noted that these subnetworks are normally repressed by the formation of PS1IV.

Name	Total number of Neighbours[1]	Overlap[2]	Percent Overlap[3]	Gene Set Seed[4]	Overlapping Entities[5]	p-value[6]
Neighbours of PDLIM5	67	2	2	PDLIM5	AGTR1,PKD1	0.00175519
Neighbours of MIR20A	264	3	1	MIR20A	AGTR1,PKD1,CAMTA1	0.00177078
Neighbours of LPS	2721	8	0	LPS	HRH2,AGTR1,NOTCH2,IL17RA,CD22,PIM3,ANGPTL2,NLRC3	0.00235013
Neighbours of BTK	298	3	1	BTK	PKD1,NOTCH2,CD22	0.00249463
Neighbours of ADRBK2	124	2	1	ADRBK2	HRH2,AGTR1	0.0057829
Neighbours of NOTCH2	184	2	1	NOTCH2	NOTCH2,FOXE3	0.0122797
Neighbours of MIR1-1	582	3	0	MIR1-1	NOTCH2,MRC2,GAK	0.0156391
Neighbours of DLL1	210	2	0	DLL1	NOTCH2,FOXE3	0.0157536
Neighbours of CHRM3	214	2	0	CHRM3	HRH2,PKD1	0.0163214
Neighbours of CCKBR	224	2	0	CCKBR	HRH2,PKD1	0.0177791
Neighbours of TCR	1762	5	0	TCR	AGTR1,PKD1,NOTCH2,IL17RA,NLRC3	0.0201721
Neighbours of GRK	245	2	0	GRK	HRH2,AGTR1	0.0210128
Neighbours of CD19	246	2	0	CD19	PKD1,CD22	0.0211725
Neighbours of p70RSK	253	2	0	p70RSK	RICTOR,IL17RA	0.0223048
Neighbours of HRH3	266	2	0	HRH3	HRH2,AGTR1	0.024473
Neighbours of MIR221	286	2	0	MIR221	ANGPTL2,HSPA4	0.0279705
Neighbours of radiation	1312	4	0	radiation	AGTR1,PKD1,OLFM4,HSPA4	0.0300081
Neighbours of MIR146A	317	2	0	MIR146A	AGTR1,NOTCH2	0.0337632
Neighbours of ATF2	324	2	0	ATF2	PKD1,ANGPTL2	0.0351313
Neighbours of NFATC1	344	2	0	NFATC1	CD22,ANGPTL2	0.0391576
Neighbours of ESR2	875	3	0	ESR2	AGTR1,NOTCH2,CD22	0.0445307
Neighbours of hypoxia	2205	5	0	hypoxia	AGTR1,RICTOR,NOTCH2,ANGPTL2,POLR3G	0.0467763
Neighbours of ARRB1	386	2	0	ARRB1	AGTR1,PKD1	0.0481522
Neighbours of cortisol	904	3	0	cortisol	HRH2,AGTR1,NOTCH2	0.0482578
Neighbours of Na+	915	3	0	Na+	HRH2,AGTR1,CD22	0.0497112
Neighbours of PRKD1	393	2	0	PRKD1	AGTR1,PKD1	0.049719

[1]Total number of genes in pathway. [2]Number of overlapping genes between sample and pathway. [3]Percentage of overlap between sample and pathway. [4]Central gene in pathway. [5]Shared members between pathway and sample. [6]Statistical significance based on "gene set enrichment" approach and Fisher's exact test.

Table 3. Significant subnetworks (p ≤0.05) formed by genes under-expressed after blockage of PS1IV formation under mimicry of hypoxia. It should be noted that these subnetworks are normally up-regulated by the formation of PS1IV.

Name	Total number of Neighbours[1]	Overlap[2]	Percent Overlap[3]	Gene Set Seed[4]	Overlapping Entities[5]	p-value[6]
Neighbours of MEF2D	76	3	3	MEF2D	CALR,TH,SMARCA4	0
Neighbours of RPL28	15	2	12	RPL28	RPS19,RPL28	0
Neighbours of mitogen-activated protein kinase kinase	534	5	0	mitogen-activated protein kinase kinase	IL1B,CCR5,HSPA8,CALR,TH	0.001
Neighbours of TRIM22	32	2	6	TRIM22	IL1B,CCR5	0.001
Neighbours of MAPKAPK2	316	4	1	MAPKAPK2	IL1B,HSPA8,TH,MAPKAPK5	0.001
Neighbours of RAN	146	3	2	RAN	IL1B,CCR5,HSPA8	0.001
Neighbours of MECOM	156	3	1	MECOM	CALR,TH,SMARCA4	0.002
Neighbours of Ca2+	3235	12	0	Ca2+	IL1B,CCR5,HSPA8,CALR,PLCB1,TH,RPS19,MAPKAPK5,CETN2, SLC25A5,SYN2,RAB3C	0.002
Neighbours of mitogen	610	5	0	mitogen	IL1B,CCR5,HSPA8,TH,SLC25A5	0.002
Neighbours of casein kinase II	909	6	0	casein kinase II	IL1B,HSPA8,CALR,TH,EEF1B2,UBAC1	0.002
Neighbours of MEF2A	381	4	1	MEF2A	HSPA8,CALR,TH,SGCG	0.002
Neighbours of RNA polymerase II	642	5	0	RNA polymerase II	IL1B,CCR5,HSPA8,CALR,TH	0.002
Neighbours of CASP5	50	2	3	CASP5	IL1B,NLRP12	0.003
Neighbours of RPS6KA5	190	3	1	RPS6KA5	TH,SMARCA4,RPL26	0.003
Neighbours of MAPKAPK3	52	2	3	MAPKAPK3	TH,MAPKAPK5	0.003
Neighbours of HOXA2	55	2	3	HOXA2	NKX3-2,GBX2	0.003
Neighbours of glyceraldehyde	55	2	3	glyceraldehyde	HSPA8,PLCB1	0.003
Neighbours of HAND2	200	3	1	HAND2	IL1B,TH,NKX3-2	0.003
Neighbours of MAPKAPK5	57	2	3	MAPKAPK5	TH,MAPKAPK5	0.003
Neighbours of KDM6A	57	2	3	KDM6A	IL1B,SMARCA4	0.003
Neighbours of NLRP12	57	2	3	NLRP12	IL1B,NLRP12	0.003
Neighbours of glycerol	427	4	0	glycerol	IL1B,CCR5,HSPA8,POP7	0.003
Neighbours of endo-RNase	59	2	3	endo-RNase	IL1B,CALR	0.003
Neighbours of LRRK2	208	3	1	LRRK2	IL1B,TH,TUBB	0.003
Neighbours of E2	209	3	1	E2	HSPA8,TH,CETN2	0.004
Neighbours of ZFP42	60	2	3	ZFP42	CCR5,SMARCA4	0.004
Neighbours of RCVRN	62	2	3	RCVRN	HSPA8,TH	0.004
Neighbours of galactosidase	62	2	3	galactosidase	IL1B,TH	0.004
Neighbours of STAT4	215	3	1	STAT4	IL1B,CCR5,SMARCA4	0.004
Neighbours of dehydroascorbate	66	2	2	dehydroascorbate	IL1B,TH	0.004
Neighbours of RPS19	67	2	2	RPS19	IL1B,RPS19	0.004
Neighbours of diterpenoids	69	2	2	diterpenoids	IL1B,MAPKAPK5	0.005
Neighbours of CCL16	70	2	2	CCL16	IL1B,CCR5	0.005
Neighbours of FRK	70	2	2	FRK	IL1B,TH	0.005
Neighbours of MAPK1	2726	10	0	MAPK1	IL1B,CCR5,PLCB1,TH,SMARCA4,MAPKAPK5,POU4F2,GORASP1,RPL26,GBX2	0.005

Neighbours of LAG3	72	2	2	LAG3	IL1B,CCR5	0.005
Neighbours of SNCA	479	4	0	SNCA	IL1B,CALR,PLCB1,TH	0.005
Neighbours of POLR2A	73	2	2	POLR2A	SSU72,RPL28	0.005
Neighbours of butanoic acid	780	5	0	butanoic acid	IL1B,CCR5,HSPA8,TH,SLC25A5	0.005
Neighbours of MAPK11	244	3	1	MAPK11	CALR,TH,MAPKAPK5	0.005
Neighbours of melanin	76	2	2	melanin	IL1B,TH	0.006
Neighbours of MIR125B1	495	4	0	MIR125B1	CCR5,TRIB2,TRIAP1,SYN2	0.006
Neighbours of mitogen-activated protein kinase	2810	10	0	mitogen-activated protein kinase	IL1B,CCR5,HSPA8,CALR,PLCB1,TH,MAPKAPK5,POU4F2,GORASP1,SYN2	0.006
Neighbours of LILRB4	80	2	2	LILRB4	IL1B,NLRC3	0.006
Neighbours of pathogen	261	3	1	pathogen	IL1B,CCR5,HSPA8	0.007
Neighbours of 5-hydroxytryptophan	83	2	2	5-hydroxytryptophan	IL1B,TH	0.007

[1]Total number of genes in the subnetwork. [2]Number of overlapping genes between the sample and subnetwork. [3]Percentage of overlap between the sample and subnetwork. [4]Central gene in the subnetwork. [5]Shared members between the subnetwork and sample. [6]Statistical significance based on "gene set enrichment" approach and Fisher's exact test.
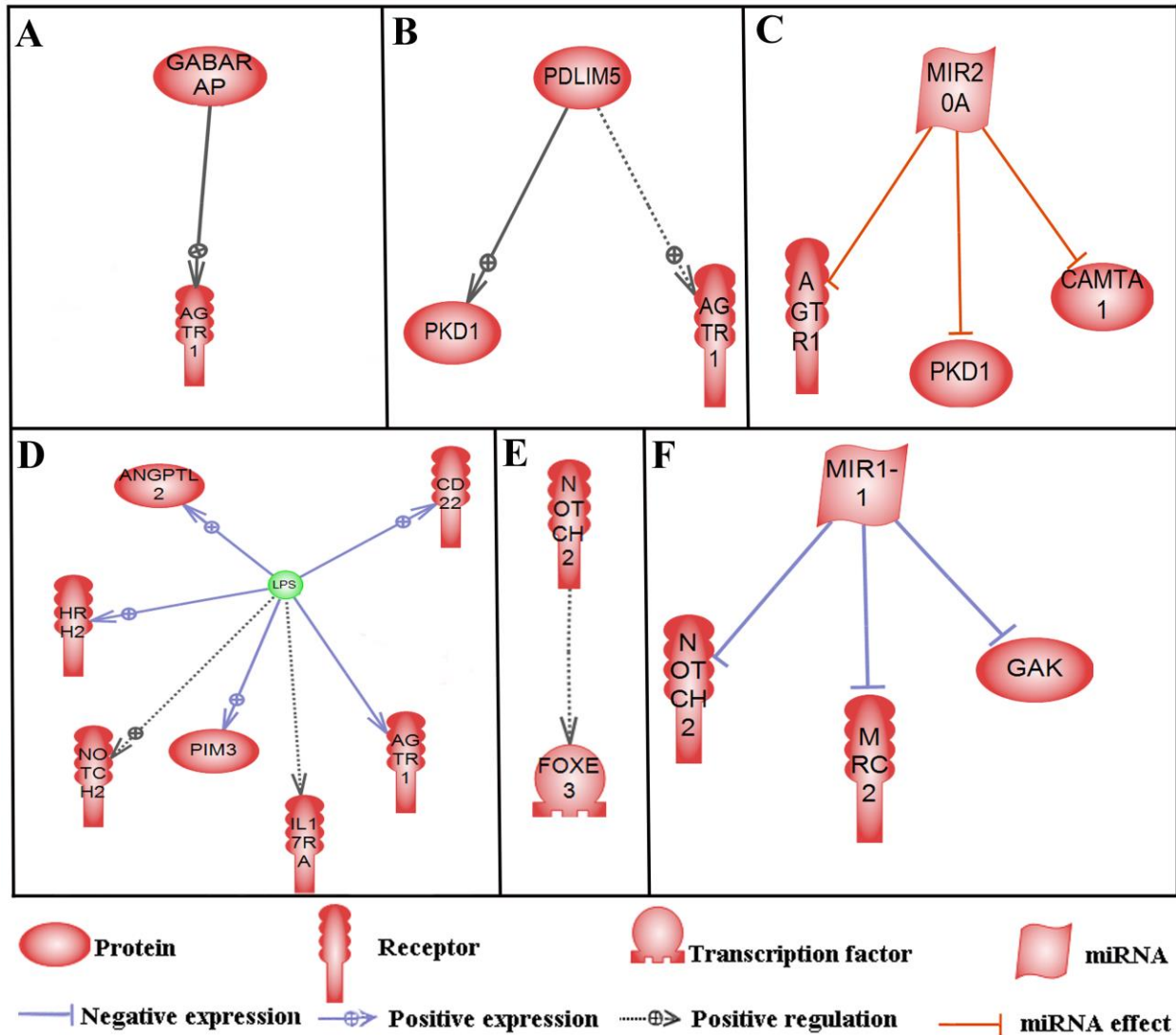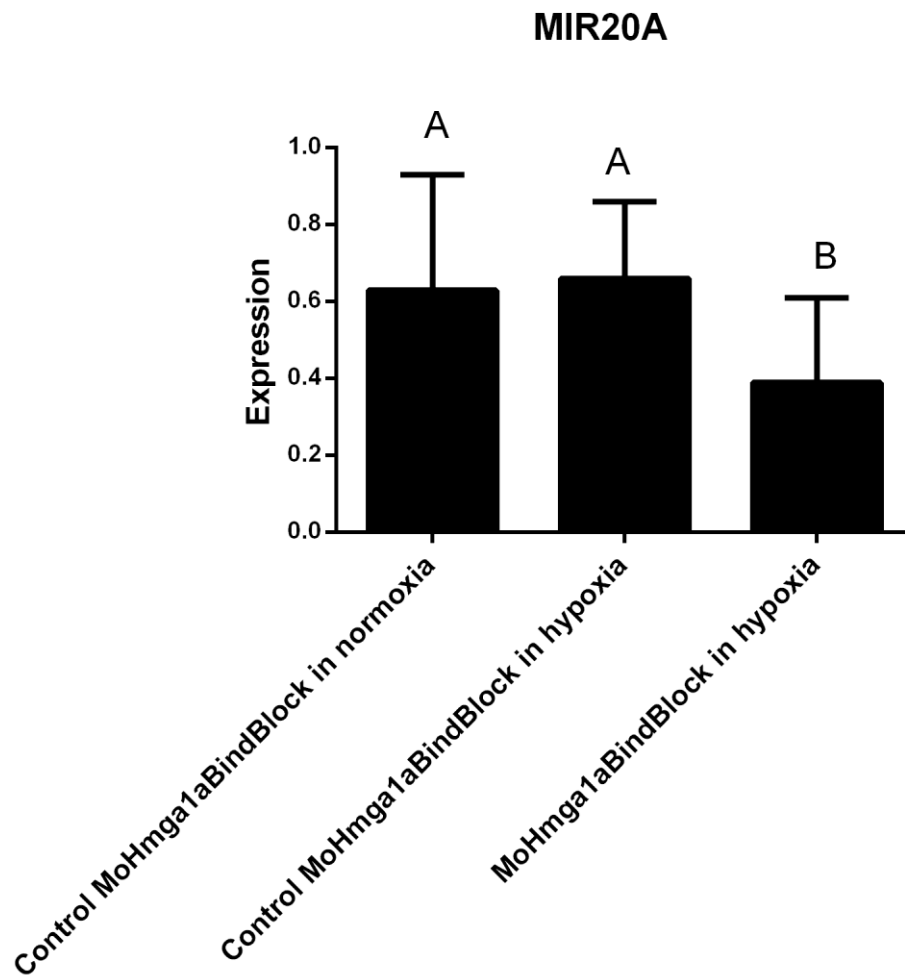
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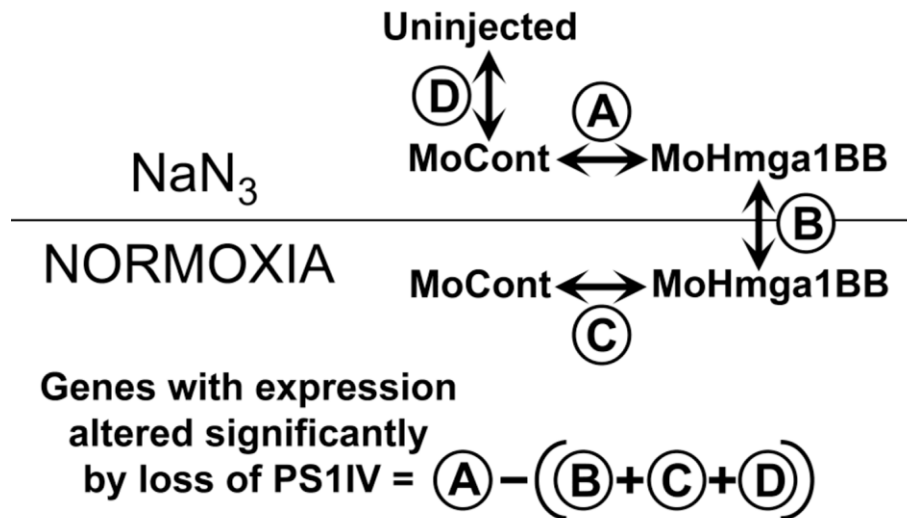
Figure 1. Microarray comparisons to detect statistically significant changes in gene expression after morpholino-inhibition of the induction of PS1IV that occurs under mimicry of hypoxia using NaN_3. "MoCont" and "MoHmga1BB" represent embryos injected with the negative control and PS1IV-inhibiting morpholinos respectively. "Uninjected" represents uninjected embryos. Comparison A is of primary interest but genes for which expression was significantly altered by the act of injection itself (D), by PS1IV-independent differential effects of MoCont and MoHmga1BB injection (C), and by cellular responses to hypoxia (B) were subtracted from the set of genes seen in A before gene regulatory network analysis was performed.
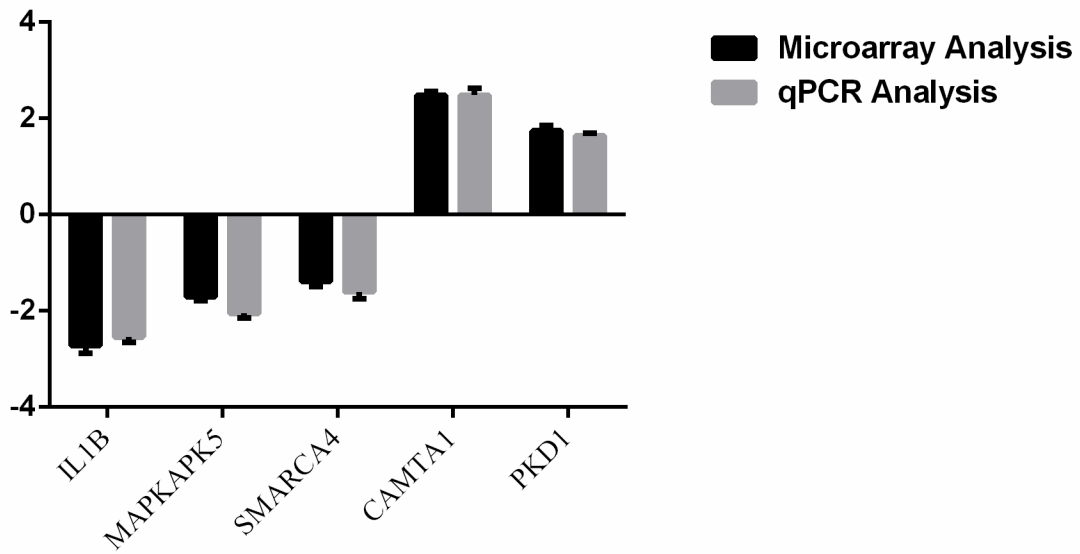
Figure 2. Comparison of the results of microarray and qPCR analyses for validation of nodal genes in the networks identified after failure to form PS1IV under mimicry of hypoxia. *CAMTA1* and *PKD1* are found to be over-expressed by both methods. *IL1B*, *MAPKAPK5* and *SMARCA4* are found to be under-expressed by both methods. Error bars represent standard errors of the means.

Figure 3. Schematic representation of some of the subnetworks for genes over-expressed after failure to form PS1IV under mimicry of hypoxia. (A) *Neighbours of GABARAP* (Gamma-Aminobutyric Acid Receptor-Associated Protein), (B) *Neighbours of PDLIM5* (PDZ And LIM Domain 5), (C) *Neighbours of MIR20A* (microRNA 20a), (D) *Neighbours of LPS* (lipopolysaccharides).

MIR20A

Figure 4. qPCR analysis supports upregulation of miR-20A by the presence of PS1IV under hypoxia. This is indicated by decreased expression of miR-20A in embryos injected with the Hmga1aBindBlock morpholino. Values are normalised against those for Hmga1aBindBlock in normoxia. The letters represent the significant difference at P<0.1 calculated using a Bayesian t-test. The microRNA expression is slightly different in MoHmga1aBindBlock verses the controls at P<0.1
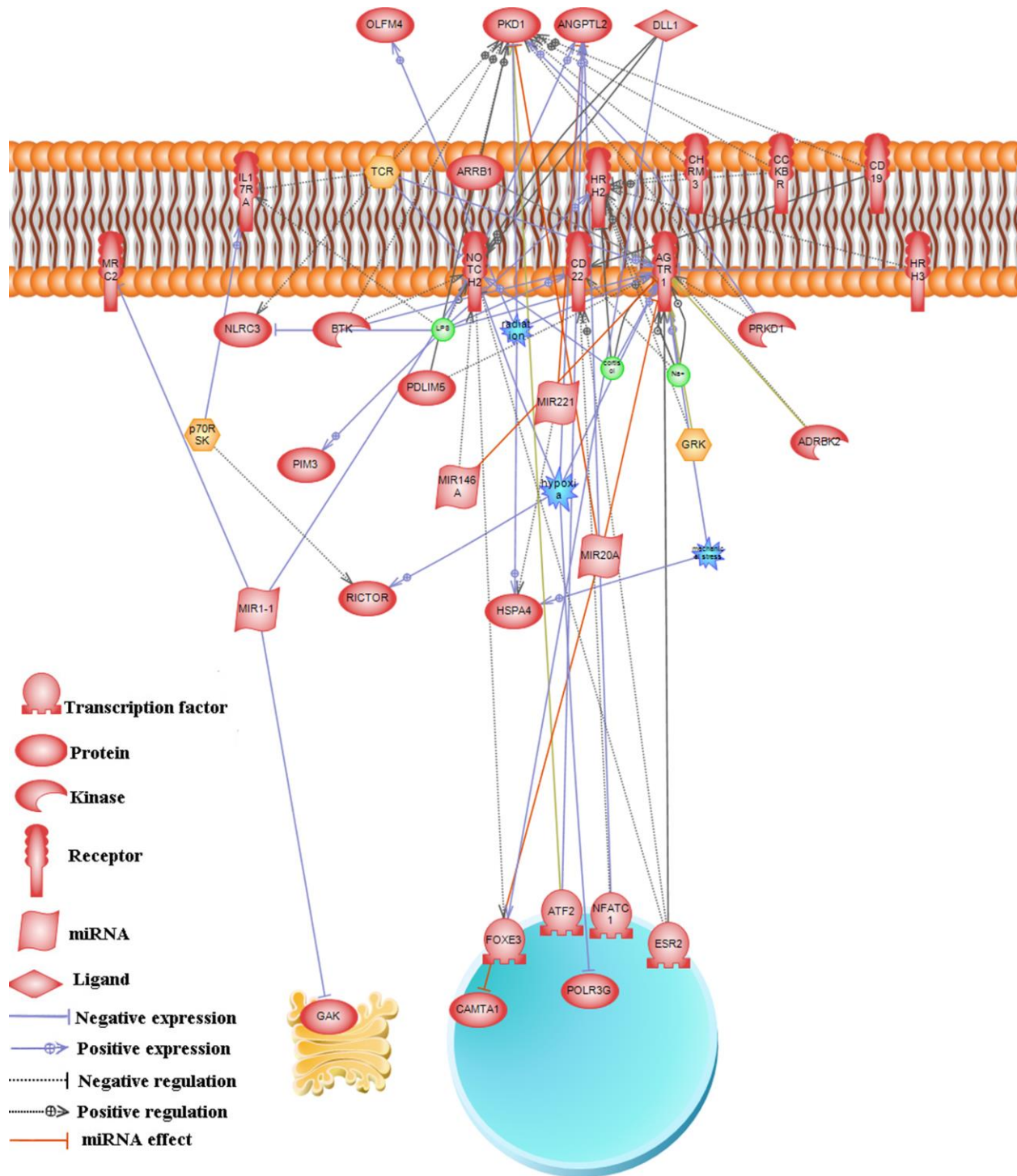
Figure 5. Regulatory network for genes over-expressed after failure to form PS1IV under mimicry of hypoxia. This network was predicted using the *union selected subnetworks* algorithm. Yellow, green, and blue structure represent small molecules, Gene Ontology (Biological process), and treatments (such as hypoxia, radiation, etc), respectively.
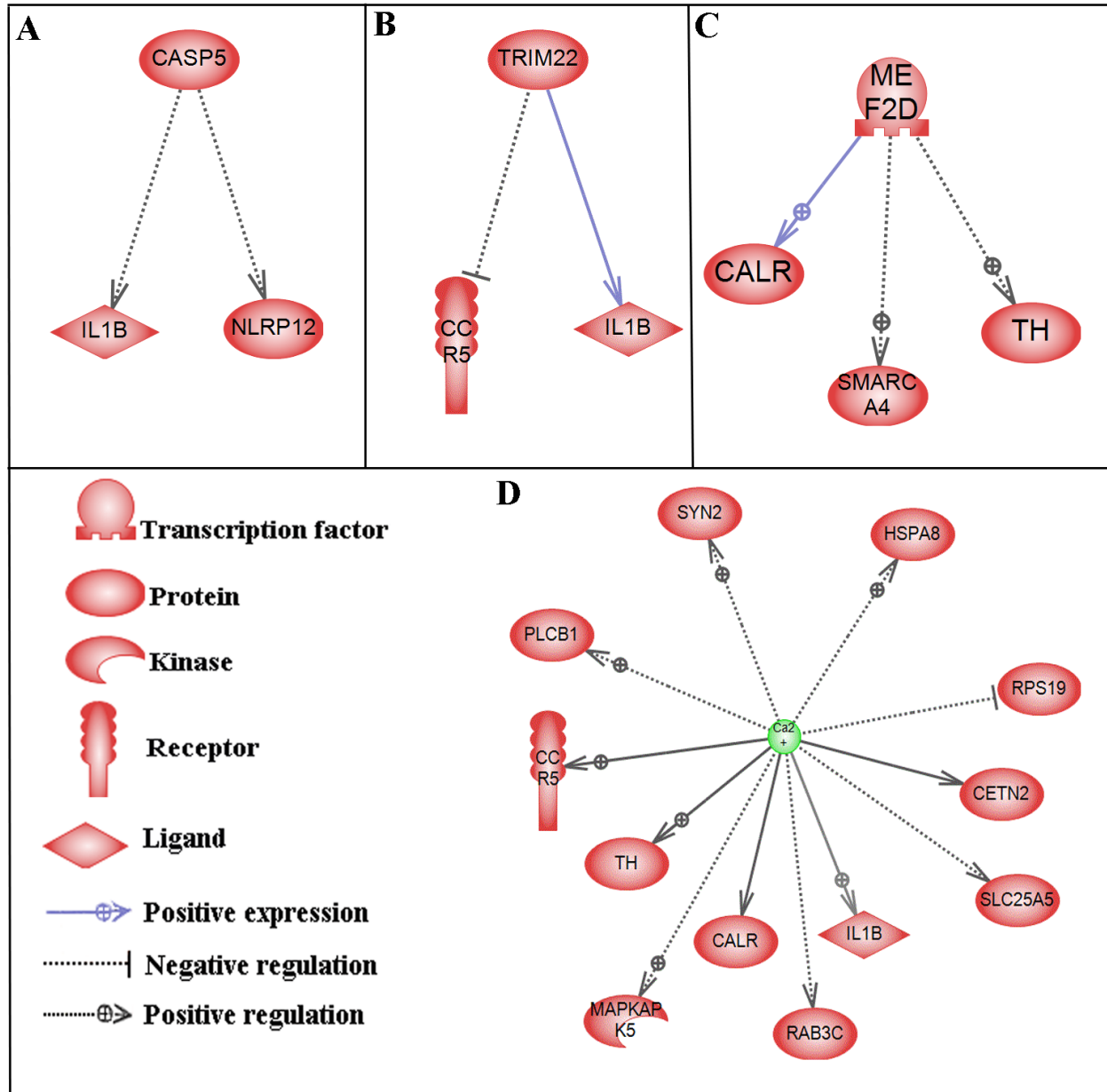
Figure 6. Significant pathways generated by under-expressed genes after failure to form PS1IV under mimicry of hypoxia. (A) *Neighbours of CASP5.* (B) *Neighbours of TRIM22.* (C) *Neighbours of MEF2D.* (D) *Neighbours of Ca2+.*
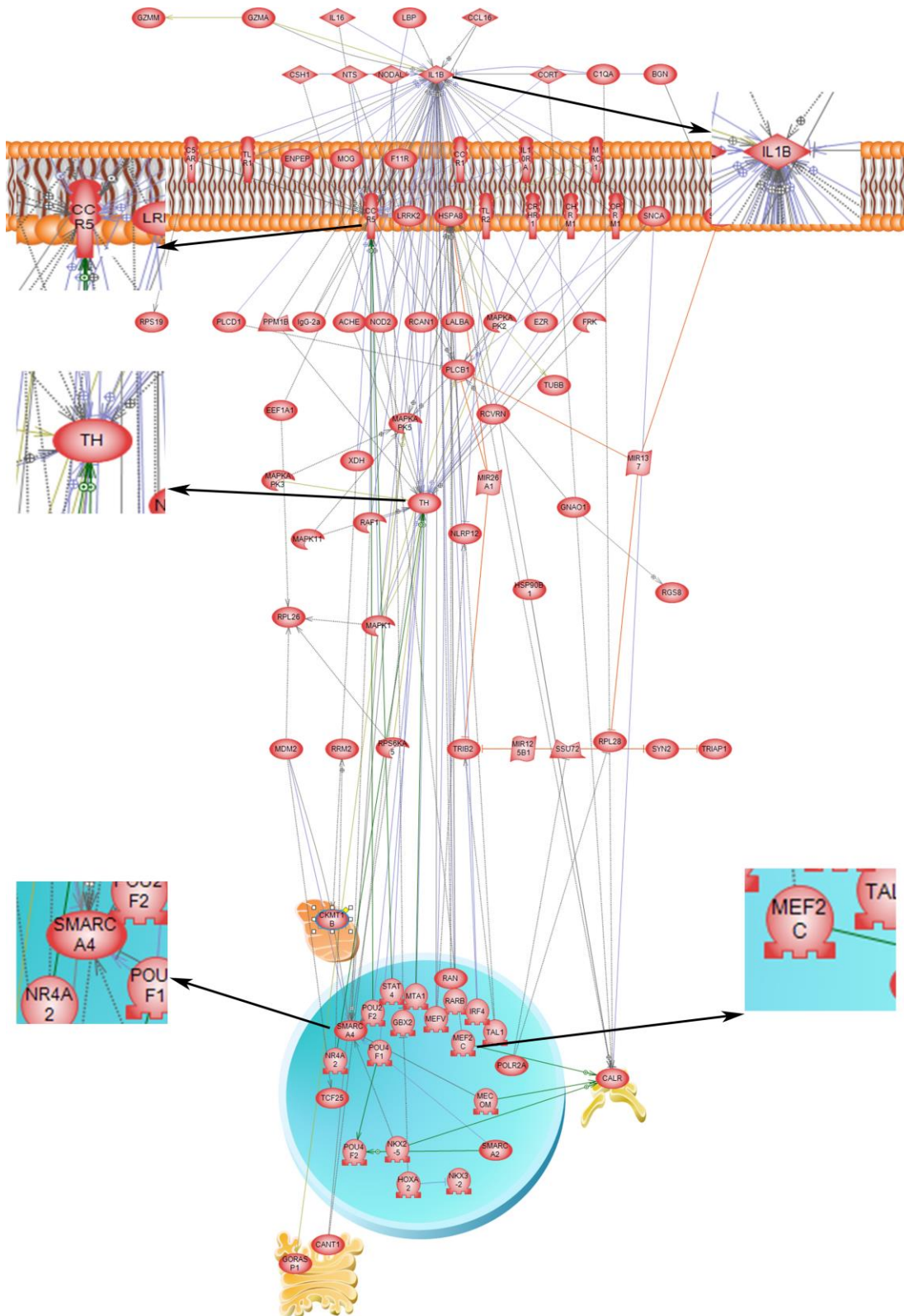
Figure 7. Regulatory network for genes under-expressed genes after failure to form PS1IV under mimicry of hypoxia. The network was constructed using the *union selected subnetworks* algorithm. The central/important genes in the network including *IL1B*, *CCR5*, *TH*, *CALR*, *SMARCA4* and *MEF2* are highlighted.
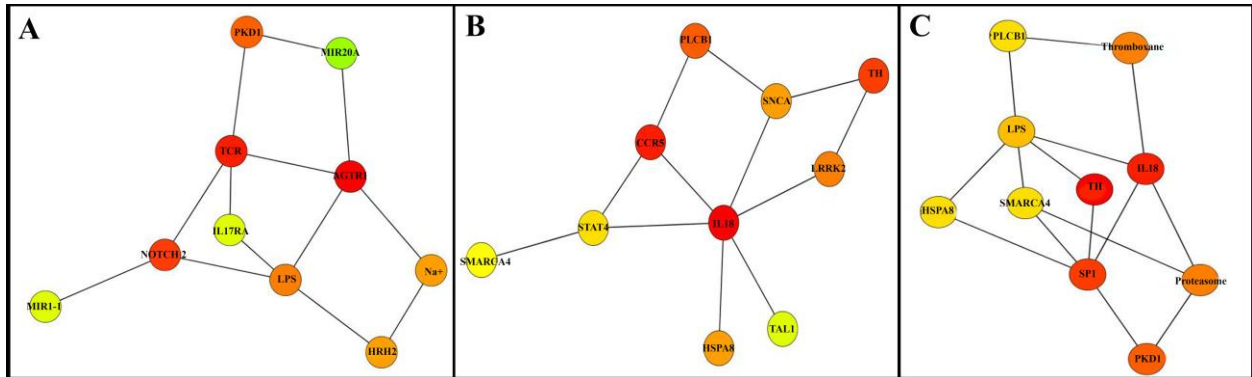
Figure 8. The 10 highest ranked genes (hubs) in the regulatory networks of genes (A) over-expressed, (B) under-expressed, and (C) the combined set of these genes after failure to form PS1IV under mimicry of hypoxia according to the Bottleneck (BN) centrality index.

Supplementary files

The supplementary files are shared in this link (59 MB): https://drive.google.com/folderview?id=0B2Npj-saFbgeSG5GLUx4cENfZXM&usp=sharing

Supplemental Data 1. Primers used for qPCR.

Supplemental Data 2. List of all over-expressed genes after failure to form PS1IV under mimicry of hypoxia

Supplemental Data 3. List of all under-expressed genes after failure to form PS1IV under mimicry of hypoxia

Supplemental Data 4. Relationships underlying significant subnetworks of the over-expressed genes

Supplemental Data 5. Relationships underlying the regulatory network of over-expressed genes after failure to form PS1IV under mimicry of hypoxia. This network was predicted based on the *union selected subnetworks* algorithm.

Supplemental Data 6. *Shortest path* network using genes over-expressed after failure to form PS1IV under mimicry of hypoxia.

Supplemental Data 7. Relationships underlying the *shortest path* network using genes over-expressed after failure to form PS1IV under mimicry of hypoxia.

Supplemental Data 8. Relationships underlying significant subnetworks of under-expressed genes

Supplemental Data 9. Relationships underpinning the regulatory network for genes under-expressed after failure to form PS1IV under mimicry of hypoxia. The network was constructed using the *union selected subnetworks* algorithm.

Supplemental Data 10. Topological analysis of the regulatory networks of genes under-expressed, over-expressed, and the combined set of these genes after failure to form PS1IV under mimicry of hypoxia.

Supplemental Data 11. Comparison of the gene relative expression values within different biological replicates of the treatments *HMGA1aBindBlock in chemical Hypoxia*, *Control Morpholino in chemical Hypoxia*, *HMGA1aBindBlock in Normoxia*, *Control Morpholino in Normoxia*, *Control Morpholino in chemical Hypoxia*, *Uninjected in chemical Hypoxia*, *Control Morpholino*, *Uninjected in Normoxia*

Supplemental Data 12. Subnetworks enriched by over- and under-expressed genes after failure to form PS1IV under mimicry of hypoxia

Supplemental Data 13. Regulatory network underlying over- and under-expressed genes after failure to form PS1IV under mimicry of hypoxia. The network was constructed using the *union selected subnetworks* algorithm.

Supplemental Data 14. Relationships underlying the regulatory network of over- and under-expressed genes after failure to form PS1IV under mimicry of hypoxia. The network was constructed using the *union selected subnetworks* algorithm.

Supplemental Data 15. Network of interaction between under-expressed genes (after failure to form PS1IV under mimicry of hypoxia) with other diseases.

Supplemental Data 16. Relationships underlying the network of interaction between under-expressed genes (after failure to form PS1IV under mimicry of hypoxia) with other diseases

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