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Global transcriptome dysregulation in second trimester fetuses with *FMR1* expansions

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Abstract

Objective—We tested the hypothesis that *FMR1* expansions would result in global gene dysregulation as early as the second trimester of human fetal development.

Method—Using cell-free fetal RNA obtained from amniotic fluid supernatant and expression microarrays we compared RNA levels in samples from fetuses with premutation or full mutation allele expansions to control samples.

Results—We found clear signals of differential gene expression relating to a variety of cellular functions, including ubiquitination, mitochondrial function and neuronal/synaptic architecture, among others. Additionally, among the genes showing differential gene expression, we saw links to related diseases of intellectual disability and motor function. Finally, within the unique molecular phenotypes established for each mutation set, we saw clear signatures of mitochondrial dysfunction and disrupted neurological function. Patterns of differential gene expression were very different in male and female fetuses with premutation alleles.

Conclusion—These results support a model for which genetic misregulation during fetal development may set the stage for late clinical manifestations of *FMR1*-related disorders.

INTRODUCTION

FMR1 is an X-linked gene encoding the fragile-X mental retardation protein (FMRP). Mice demonstrate ubiquitous *FMR1* expression by embryonic day ten[1]. In both mice and humans, expression is markedly higher in the brain and gonads than other tissues by adulthood[1]. FMRP accomplishes diverse cellular functions, including directing mRNA localization and

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translational regulation for hundreds of proteins, including many involved in synaptic plasticity and motor function[2–5].

The 5' untranslated region of *FMR1* contains a triplet repeat CGG of variable length, with a normal range being <45 repeats. The full mutation allele (≥ 200 repeats, frequency 1:5,000 males /1:8,000 females[6–7]) results in gene hypermethylation and inhibition of *FMR1* expression[8–9]. Fragile-X syndrome (FXS), characterized by seizures, anxiety, obsessive-compulsion, and language delay,[10] is the most common genetic basis for intellectual disability (ID) and constitutes a leading monogenic cause of autism (1–2%)[11]. Many individuals with FXS are also mosaic for smaller, premutation length alleles; mosaicism among males with a FXS diagnosis is estimated at 19–41%[12–13].

Expansions in the premutation range (55–200 repeats, frequency 1:209 females /1:430 males[14]) can result in increased *FMR1* RNA expression. Twenty percent of female carriers of the premutation allele will develop fragile-X associated primary ovarian insufficiency (FXPOI), defined as reduced ovarian function, and in the extreme case, premature ovarian failure, a cessation of menses prior to age 40[15–18]. By the age of 60, many men (40%) and women (16%) with premutation alleles will develop fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder characterized by tremors, cognitive decline, gait ataxia and autonomic dysfunction, with neurological signatures of white matter disease and the loss of axons and myelin (reviewed[19–21]). FXTAS has also been demonstrated to co-occur with dementia and Parkinson's disease in males and with Alzheimer's disease in females[19]. Postmortem exams of human cortex and cerebellum tissues reveal that the premutation allele is associated with ubiquitin-positive intranuclear inclusions in neurons and astrocytes[22–24]. Although the likelihood of developing FXTAS decreases for lower range premutation alleles, an elevated risk for development of parkinsonian disorders remains, perhaps as a result of the mitochondrial dysfunction resulting from elevated *FMR1* transcription[25]. Additional medical conditions associated with the premutation length allele include migraine, fibromyalgia, neuropathy, psychiatric disorders, hypothyroidism, hypertension, and immune-mediated disorders among others (reviewed[26]).

Historically, the premutation allele was thought to be related to only adult-onset disorders, but recent studies offer compelling evidence that clinical symptoms may manifest throughout the lifetime (reviewed[20–21,26]). Premutation mouse models have shown altered neuronal migration and differentiation in the embryonic neocortex[27]. Cultured neurons from neonatal premutation mice exhibit oxidative stress and mitochondrial dysfunction as well as elevated expression of stress proteins and shorter dendritic length with reduced branching, likely as a result of asynchronized calcium oscillations[28–30]. Human infants with the premutation allele have demonstrable deficits in visual motion processing[31]. Carriers of the premutation allele may present in childhood with anxiety, attention-deficit hyperactivity disorder and autism spectrum disorders[32–34]. In light of these observations, a recent review by Hagerman and Hagerman[26] proposed that human carriers of the premutation allele are subject to lifelong disruptions in calcium regulation and mitochondrial function as a result of a background state of cellular dysfunction established early in development.

While the disorders associated with each allelic expansion have distinct clinical features and underlying molecular mechanisms, areas of overlap blur these distinctions. As such, the phrase “fragile X-associated disorder (FXD)” captures the continuity and breadth of clinical involvement resulting from all expansions.

To our knowledge, there are three studies showing that fetuses with the full mutation allele lose *FMR1* expression in the first trimester[35–37]. Cell-free fetal RNA (cffRNA) in amniotic fluid supernatant (AFS) from fetuses with expanded *FMR1* alleles offers the opportunity to examine transcription in a diverse array of fetal tissues. This approach has been used in prior studies of genetic conditions using AFS cffRNA[38–42]. Given that an *FMR1* expansion allele is known to have molecular effects on somatic organs of the adult in addition to nervous system tissues[43], we used AFS cffRNA to examine transcription in different tissue types from the developing fetus[44].

METHODS

Ethical Approval/Samples

The procedures employed in this study were reviewed and approved by the Tufts Medical Center Institutional Review Board (Tufts Medical Center protocol #5582), and are in accordance with the guidelines set forth by this journal. Anonymized amniotic fluid supernatant (AFS) samples (n=40) were received from Integrated Genetics/LabCorp (Westborough, MA) and the New York State Institute for Basic Research in Developmental Disabilities (Staten Island, NY).

Genotyping

Fetal sex and *FMR1* triplet repeat number were determined per routine clinical protocols for all samples, including control fetuses, by collaborating CLIA-certified diagnostic laboratories, using both Southern blot and PCR assays. Genotypes were subsequently grouped according to allele-size, corresponding to either normal (repeat size <45), premutation (55–200) or full mutation (>200) size. Samples with intermediate (repeat size 45–54) alleles were excluded from the analysis.

Expression Microarray Preparation and Data Analysis

Full details available in Supplementary Methods. Cell-free fetal RNA was extracted from residual amniotic fluid supernatant as described elsewhere[45]. Raw microarray CEL files, along with normalized values are publicly available at NCBI’s Gene Expression Omnibus[46] using the GEO Series accession number GSE83556. Normalization of microarrays from all *FMR1* allele groups was performed as a batch; methods for this and for identification of differential gene expression are described elsewhere[38].

In Silico Functional Analyses

Full details available in Supplementary Methods. As is described elsewhere[44], tissue-specific gene expression was assessed using BioGPS Gene Expression Atlas, http://biogps.org. Candidate genes of interest from male premutation cases were manually curated for disease-specific associations using NCBI’s Online Mendelian Inheritance in Man

(OMIM, http://omim.org/) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed, accessed 5/27/15). A Gene Set Enrichment Analysis[47] with fetus-specific functional annotation, DFLAT[48], was also undertaken (pre-ranked based on paired *t*-scores, FDR q ≤0.25). The advantage of GSEA with DFLAT over the previously used Ingenuity Pathway Analysis software is that DFLAT most accurately reflects the current understanding of fetal biology, including the rapid physiologic cellular proliferation in fetal tissues that may falsely present a signature of cancer when relying solely on adult annotations[49].

RESULTS

FMR1 Expansions and Differential Gene Expression

The premutation male analysis data set contained 12 pairs of gestational age (GA)- and sex-matched fetuses with *FMR1* CGG repeats in the normal or premutation length (Table 1, Table S1). Forty-five (45) probes representing 36 genes were significantly differentially expressed, with fold-change magnitude ≥2, a BH p-value ≤0.05, and direction of differential gene expression (DGE) consistent among at least 10 pairs (Table S2).

The premutation female analysis data set contained 6 pairs of GA-and sex-matched fetuses with *FMR1* CGG repeats in the normal or premutation length (Tables 1, 2 and S1). Due to the smaller sample size, a fold-change magnitude ≥2.5 was used to increase the specificity of results. With a BH p-value ≤ 0.05, and direction of differential gene expression (DGE) consistent among at least 5 pairs, 243 probes (174 unique genes) showed statistically significant differential expression (Table S3).

For both female and male premutation samples, differentially expressed genes were found throughout the genome (Figure 1, Figure S1). The increased numbers of samples in the male premutation set allowed greater stringency in the identification of candidate genes, resulting in fewer differentially expressed genes as compared to the female premutation data.

As proof of principle for our ability to report on multiple fetal tissues, Affymetrix probe sets showing statistically significant DGE in either male or female premutation samples were queried for tissue-specific expression using the publicly available BioGPS database. Probe sets corresponding to 13 genes were shown to be tissue-specific (Table 3). The tissues contributing cffRNA to the AFS are not limited to this list, but the presence of transcripts expressed in a tissue-specific manner illustrates that gene expression in these diverse sets of fetal tissues, including those of the central and peripheral nervous systems, was successfully detected by this experiment.

Due to the relative rarity of amniotic fluid supernatant samples from full mutation fetuses, only four pairs of GA- and sex-matched fetuses with either normal or full mutation length *FMR1* alleles were available for this study (Table 1, Table S1). Although the sample size was too small to provide statistically significant conclusions, preliminary analyses can, in broad strokes, outline the altered terrain of fetal gene expression. There were 1,610 probes representing 1,257 genes that showed DGE, with fold-change magnitude ≥ 2.5, a BHp-value ≤ 0.05, and direction of differential regulation consistent among all four pairs (Table S4).

There were some commonalities in the gene families and general gene functions among the three sets of genes showing DGE, yet the majority of genes were unique to one sample set or another. Indeed, no single gene was found to be differentially regulated in both the male and female premutation data sets (Table S5). These unique signatures thus suggest specific molecular phenotypes associated with each *FMR1* allele size group.

Trends in Gene Function and Human Disease

The GSEA analysis revealed an enrichment in gene ontologies related to mitochondrial function, the immune system, the cell cycle, regulation of mRNA and proteins, neurological development, hormone signaling, phosphorylation and ubiquitination, and cellular energy metabolism, among others. The female premutation dataset demonstrated four enriched terms, including RNA splicing, translation initiation and nuclear import of proteins bearing a nuclear localization signal. Analysis of the full mutation data set revealed enrichment for a diversity of terms, the most frequent of which relate to lipid biosynthesis and energy metabolism (Figure 2, Table S6).

Cross-referencing the lists of both male and female premutation results with OMIM and NCBI PubMed databases revealed that many genes had previously known associations with other diseases. Genes of particular interest, given clinical implications in neurological diseases, include: *CHRNE, GRIA4, HPCA, RMST, SLC6A6, TAF1*, and *UBE3A* in the premutation male; and *CP, FGFR2, GRIK2, GRIN2A, L1CAM, NRXN1, NTRK2, SIX3, SNAP25*, and *SNCA* in the premutation female (Figure 3, Table S7).

DISCUSSION

To our knowledge, this is the first study of differential gene expression resulting from the expanded *FMR1* allele in ongoing human pregnancies. This increases knowledge of the effects of *FMR1* expansion alleles on human fetal development and suggests areas for further investigation of the pathophysiology of related adult-onset diseases. A major strength of this work is its rigorous design, made all the more notable in light of the technical difficulties associated with studying a living fetus. Prior studies of mammalian development and *FMR1* gene expression relied on samples from animal studies, aborted human fetal tissues, induced pluripotent stem cells (iPSC), or cells cultured from amniocytes or chorionic villi, the latter three being limited to mitotically active cells and possibly affected by the culturing process[27,35–37,50].

Biological variation among our samples was minimized through careful matching of sex and GA (± 7 days), both of which are known to affect fetal gene expression[51]. Rigorous statistical corrections were applied to limit the effects of multiple hypothesis testing in the identification of differential gene expression and altered developmental pathways. In order to minimize technical variation, all samples were processed by the same person using the same techniques. By reporting only genes for which DGE was observed in ≥10 of the 12 pairs for premutation males and ≥5 of the 6 pairs for premutation females, we are focusing only on the most consistent signals. This requirement unavoidably leads to possible under-reporting of some of the gene expression changes associated with various expansions in

FMR1, particularly given the heterogeneity of clinical outcomes and the tissue-specific somaticisms that have been reported for some *FMR1* expansions[52–52].

While *FMR1* is an X-linked gene, differentially expressed genes are found genome-wide, in line with our understanding of the complex and interrelated nature of expression regulation and the pleiotropic phenotypes associated with *FMR1* mutations (Figure 1, Figure S1). The results for male and female premutation length *FMR1* alleles are distinct from signatures for prior diseases we have studied, with only five probe sets reported in both[42] (Table S8). Most of the genes reported here have not previously been studied in the context of FXD, with several notable exceptions (Table S9). Some other genes of interest from prior FXD studies do show *suggested* differential gene expression, defined as a relaxation of the fold-change magnitude threshold to ≥ 1.5 (Table S9). Further study and critical analyses are necessary to determine the validity of these latter *suggested* differences in gene expression.

There are multiple possible explanations for the fact that *FMR1* does not show consistent differential gene expression in our studies. It may be that at this stage of development, changes in gene expression levels for *FMR1* are negligible, but the physical presence of the premutation RNA molecule itself disrupts the regulation of gene expression of other genes. This possibility is in line with the toxic-gain-of function model, in which the expanded CGG repeat of the *FMR1* RNA molecule itself binds directly to and sequesters one more cellular proteins, removing the proteins from normal cellular functioning (reviewed[26]). Another possibility is that at this stage of development, the effect on gene expression falls below detection level in our study, while still affecting transcriptional regulation of other genes. Finally, some of the cffRNA may have originated from undifferentiated fetal stem cells, in which *FMR1* expression levels may not yet differ among allele types[54].

DGE signals within each study set were enriched for certain molecular and physiological functions. Many of these functional terms were enriched regardless of whether the biological function was defined narrowly or broadly (Table S6), reflecting both the strength and the breadth of these findings. Selections chosen based on clinical significance are discussed below.

Neuro- and Neuromuscular Development and Calcium

Among the genes found misregulated in male premutation carriers are several with well-established links to ID (Table S7). *UBE3A* normally drives ubiquitination, marking proteins for degradation by the 26S proteasome – a process regulating synaptic development[55]. Loss of function of the maternally inherited *UBE3A* allele is the basis for Angelman syndrome, a genetic disease causing developmental delay, speech and cognitive impairments, tremors and seizures[56]. Changes in *UBE3A* expression have previously been linked to premutation length *FMR1* alleles[57]. *ABHD11, LAT2* and *NCF1*, which are DGE in the female premutation samples, and *ADCY10*, which is downregulated in the male premutation samples, are all located on 7q11, in a region deleted in Williams syndrome. Individuals with this genetic disorder exhibit abnormalities in cardiovascular, ophthalmologic, renal, connective and dental tissues, and variable neurological and cognitive abilities[58–60]. Given the overlapping clinical features between Williams syndrome and some juveniles or adults with *FMR1* premutation mutations, it may be that affected cellular pathways are affected in

both cases. Another example is *UPB1*, which was differentially expressed in female premutation samples. Mutations in *UPB1* have been linked to Beta-ureidopropionase deficiency in a handful of patients, with effects that range from severe neurologic involvement with mental retardation and seizures to no discernable effects on neurologic development[61].

In addition to genes implicated in ID, DGE was observed for several genes known to be important in neurodevelopment or neurodegeneration, including those modulating the concentration of intracellular Ca^{2+}, which regulates dendritic growth and retraction. In the premutation male, DGE was seen for *SLC6A6*, which encodes a neurotransmitter transporter for the GABAA agonist taurine, modulating both neuronal excitability and calcium flux. These data provide the first evidence of Ca^{2+} signaling misregulation in the developing fetus of the premutation carrier, and lend support to the hypothesis that adult-onset consequences of the premutation allele could be traced to early fetal development. *PCDH11X*, which was differentially expressed in the female premutation set, belongs to a family of protocadherin genes that is especially prevalent in the central nervous system, and performs calcium-dependent cell adhesion and cell recognition functions. *PCDH11X* has been implicated in late-onset Alzheimer's disease[62]. Premutation males also showed DGE of *CHRNE*, mutations of which have been shown to result in slow-channel congenital myasthenic syndrome, a postsynaptic neuromuscular junction disorder resulting in early-onset and progressive muscle weakness[63]. A similar phenotype is observed for mutations in *SNAP25*, which showed evidence of DGE in premutation females. *SNAP25* encodes an acetyl choline receptor and has a role in calcium-triggered neuronal exocytosis. Similarly, psychomotor developmental delays have been seen in a family with a mutation in the gene *CAD*[64]. The DGE of *CHRNE, SNAP25*, and *CAD* suggests a possible link to the adult-onset neuromuscular clinical phenotype associated with the premutation allele.

Among the female premutation results were other differentially-expressed genes with important roles in synaptic functions, including mediation of synaptic transmission and synaptogenesis and dentritic spine morphology: *GRIK2, GRIN2A, L1CAM* and *SRCIN1* (Table S7). Additional differentially regulated genes include: *GRIA4*, mutations in which result in absence epilepsy due to increased duration of synaptic responses in a mouse model[65]; *NRXN1*, mutations in which are known to alter synaptic function, resulting in epilepsy, ID and autism in human subjects[66–68]; *SNCA* proteins are thought to integrate presynaptic signaling, and aggregations of these proteins are hallmarks of neurodegenerative diseases, including Alzheimer's disease[69]. Overexpression of *SNCA* is also linked to mitochondrial fragmentation in cell lines[70].

Mitochondria

Mitochondria have several key cellular roles, including phospholipid biosynthesis and inter-organelle trafficking, glycosphingolipid anabolism, metabolism of ceramide and cholesterol, and intracellular Ca^{2+} homeostasis in mammalian cells[71–73]. The male premutation GSEA results show enrichment for pathways related to cell-death, cholesterol metabolism, phospholipid trafficking, carbohydrate metabolism, regulation of oxygen species and ion

transport, organelle localization and transport, all of which suggest disrupted mitochondrial function (Table S6).

Mitochondrial disorders in the context of *FMR1* expansions have previously been explored. Tasha *et al.*[74] found FMRP to be physically associated with the heavy membrane of mitochondria in HeLa cells, suggesting a functional interaction. Ross-Inta *et al.*[75] found evidence for disruption of ATP synthesis as well as evidence of low rates of NAD- and FAD-linked oxygen uptake in cultured fibroblasts from male premutation carriers. They propose that these mitochondrial deficits, found regardless of whether or not the premutation carriers had presented with FXTAS, predispose individuals to associated neurodegenerative disorders, such as Parkinson's disease. A study of the Drosophila homolog of *FMR1, dfmr1* suggests that another function of *dfmr1* is to negatively regulate mitochondrial numbers and transport[76]. Cultured neurons from newborn premutation knock-in mice possess fewer mitochondria, and those mitochondria suffer from reduced mobility[28]. Hagerman[21] posited that the neurodegeneration seen in FXS can be traced to the failure of mitochondria to meet the particularly high energy needs of neurons as compared to other cell types. Similarly, Hagerman and Hagerman[26] suggest that this mitochondrial insufficiency reduces dendritic growth of premutation mouse neurons, resulting in long-term neurological dysfunction.

Differential expression of genes related to mitochondrial functioning was also found among the female premutation samples. These included: *ACAD10*, which has a role in mitochondrial fatty acid beta-oxidation; *NDUFA10*, which is involved in the transfer of electrons from NADH to the respiratory chain and is related to Leigh syndrome; and *REXO2*, which is necessary for mitochondrial protein synthesis and DNA repair.

We cannot know which fetuses among the male and female premutation carriers might go on to develop associated disorders such as FXTAS or FXPOI. However, the presence of these molecular signals suggests that a background state of cellular dysregulation far predates the clinical onset of symptoms. Indeed, Hagerman and Hagerman[26] review several neuroimaging studies showing that CNS changes predate FXTAS, and propose that the transition from genetic predisposition to clinical manifestation may rely on a combination of additional mutations, or a variety of environmental insults encountered throughout life.

Preliminary Analyses from Full Expansion Alleles

With four full mutation samples and their matched controls, the limited size of the full mutation sample set makes the resulting data appropriate only for preliminary interpretation. The most interesting of these preliminary results relates to the consistent differential gene expression of genes previously studied in the context of neurological diseases, including FXD (Table S9). Due to the nature of genomic work, some of these findings may be spurious; however, they are worthy of preliminary consideration, particularly in light of the unique nature of these data. Among the results of interest is differential expression of *NLGN4*, mutations in which are recognized as a monogenic source of autism and X-linked mental retardation[77–78]. Genes previously studied in context of FXS full mutation include *NRNX1* and *UBE3A*, discussed in detail above, as well as: *DSCAM, MAPK3, NCS1, PRKCD* and *PRKCE* (Table S9). Cvetkovska *et al.*[79] found that Drosophila FMRP null mutants showed overexpression of *DSCAM* and synaptic targeting errors, suggesting a role

for *DSCAM* in FXS-associated ID. Similarly, a Drosophila knockout model of *dfmr1* showed decreased expression of the Drosophila homolog of *NCS1 (frequenin)* in the fourth day of life[80], a finding mirrored in our full mutation samples. Gene expression downregulation was previously seen for *MAPK3, PRKCD* and *PRKCE* in fibroblasts from a mouse knockout mode[81].

Molecular signatures for mitochondrial dysfunction are also observed in our full mutation samples, including consistent differential expression of *NRF1*, which encodes a transcription factor acting on nuclear genes encoding mitochondrial proteins. Similarly, the GO terms most enriched for the full mutation analysis included those related to lipid biosynthesis and transport, carbohydrate metabolism and nucleic acid metabolism, known functions of mitochondria.

CONCLUSIONS

Using a discovery-driven approach, we have revealed a rich landscape of altered gene expression throughout a variety of fetal tissues, with prominent signatures of mitochondrial and neurological dysfunction. Our work shows that the *FMR1* expansions affect gene regulation as early as the second trimester, a developmental period previously unexplored in the context of FXD. Our results suggest that developmental aberrations beginning during this critical period of development may set the stage for later clinical manifestations. Future research might focus on prenatal use of preventative therapies, including antioxidants to combat oxidative stress or dietary therapies used for mitochondrial diseases so as to lower the lifetime risk of FXD.

Finally, further studies are needed to elucidate the relationship between transcription of the expanded allele and the molecular phenotypes we have reported. Indeed, the relationship between FMR1 transcription and FXTAS-associated mitochondrial disorders is by no means clear[26]. Future studies with larger sample sets and prospective tracking of life-time environmental risk and disease incidence would allow for a richer understanding of the complex relationship between the premutation allele and FXD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AUTHORS' ROLES

SLN, and PMO, and ME procured the samples and edited the manuscript. SLN provided expertise in *FMR1* analysis and medical genetics. DWB and LMZ designed the experiments and drafted the manuscript. HCW and LMZ performed the GSEA analysis. LMZ performed all other laboratory and computational experiments and data analyses.

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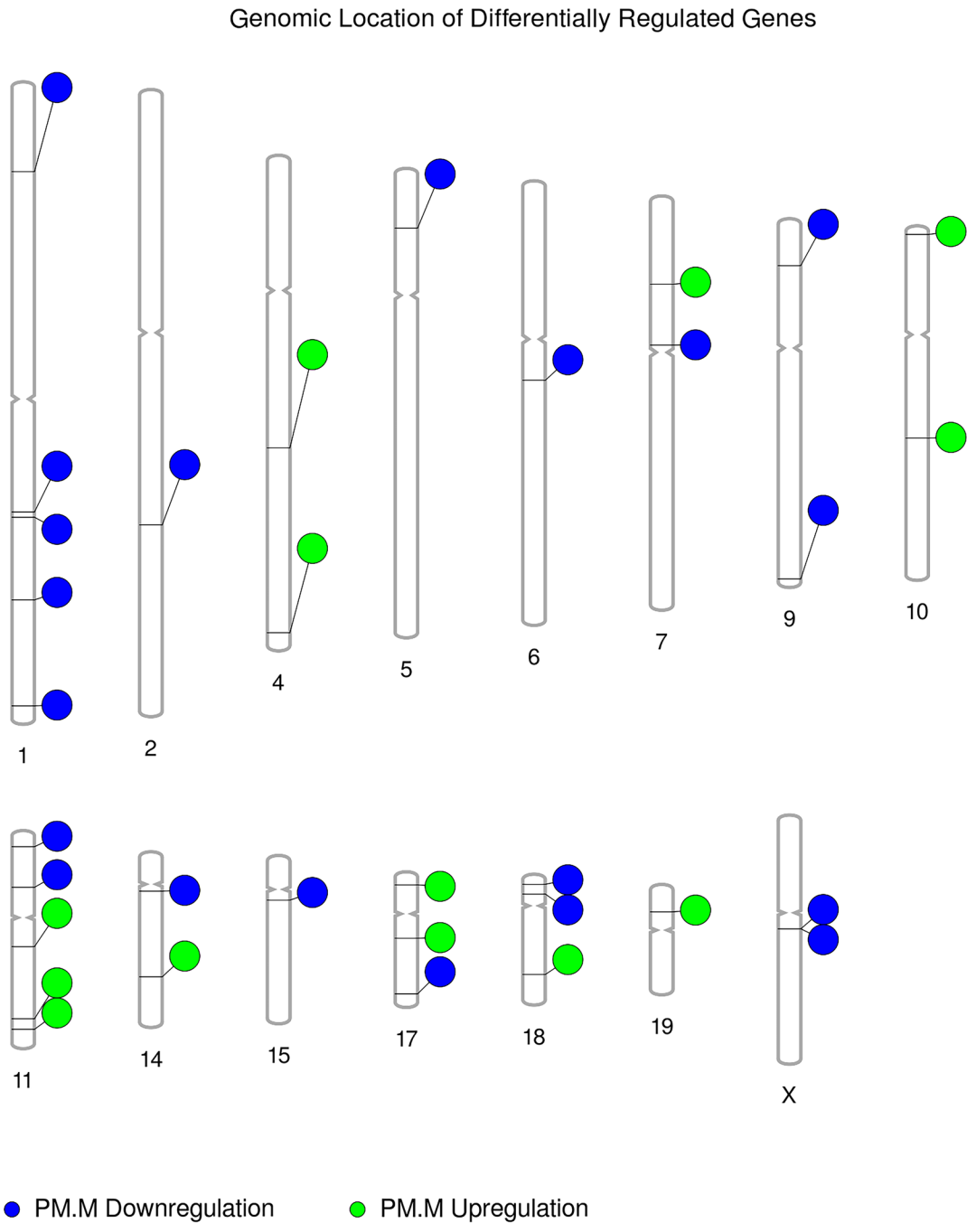
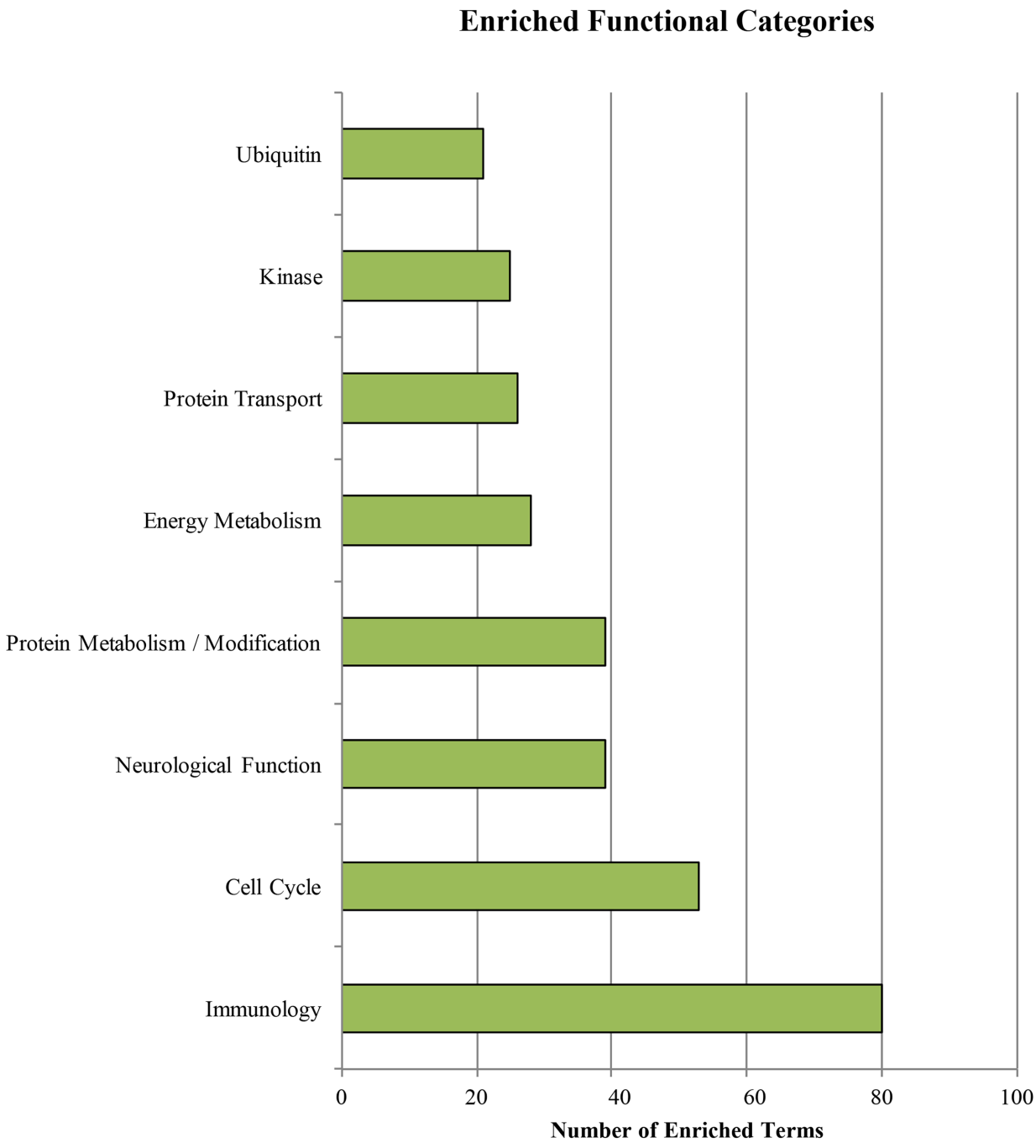
WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Expansions in the fragile X mental retardation 1 gene (*FMR1*) have been studied extensively in the context of their juvenile and adult-onset diseases.
- To date, however, little is known about the effects of these genetic changes early in human development.

WHAT DOES THIS STUDY ADD?

- This study presents for the first time an analysis of gene expression in developing fetuses with expanded *FMR1* alleles (both premutation and full mutation)
- In fetuses with premutation alleles, differential expression was observed in many genes, including those involved in ubiquitination, mitochondrial function and neuronal/synaptic architecture.
- Male and female fetuses with premutations had very different transcription signatures.





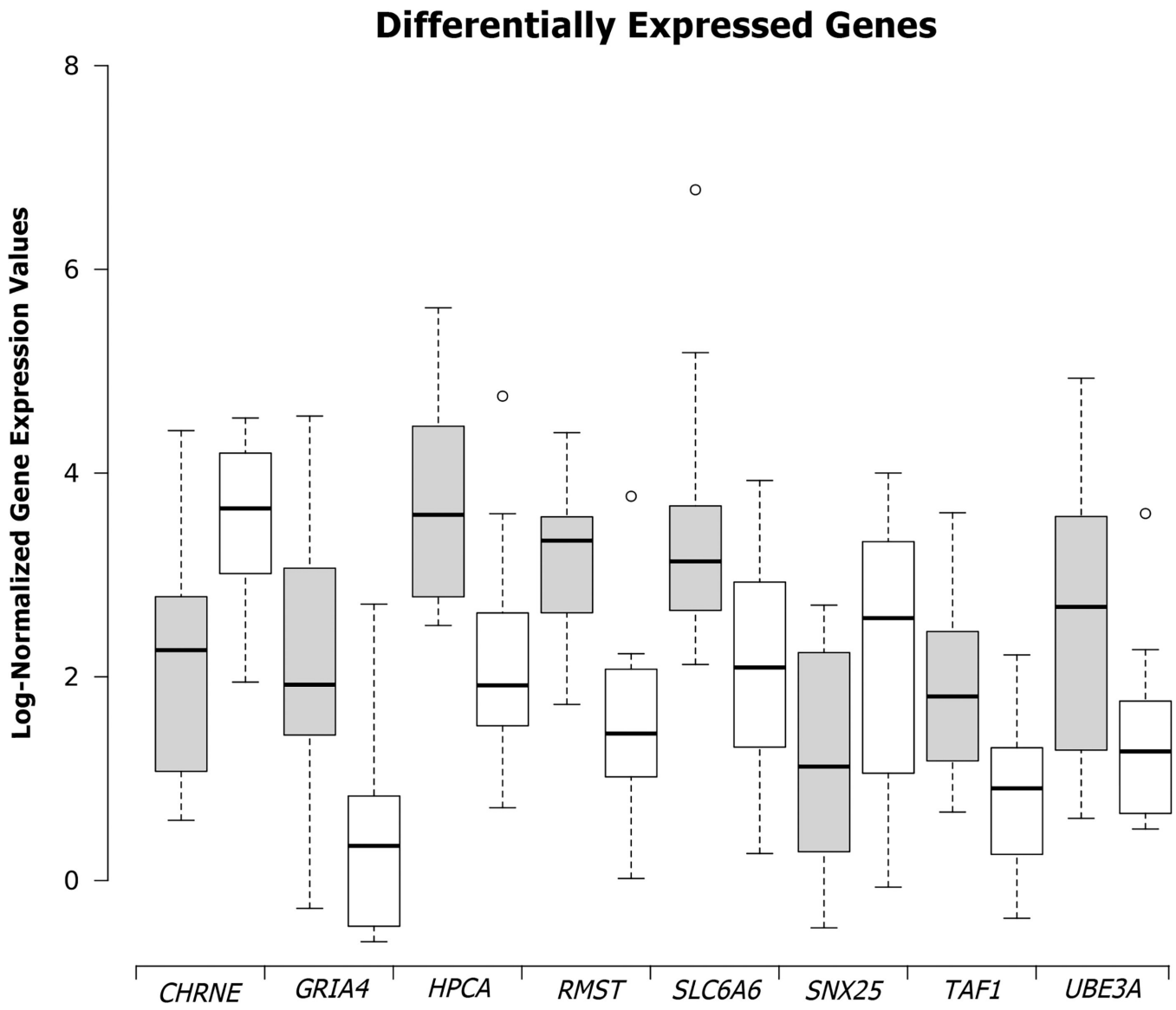


Table 1

Amniotic Fluid Samples Used in Study

Sample	GA	Number of Repeats	Diagnosis	Fetal Sex
Nl-1	15+5	29	Normal	M
Nl-2	15+6	31	Normal	M
Nl-3	16+2	24	Normal	M
Nl-4	16+2	36	Normal	M
Nl-5	16+3	24	Normal	M
Nl-6	16+4	32	Normal	M
Nl-7	16+6	29	Normal	M
Nl-8	17+3	20	Normal	M
Nl-9	17+5	25	Normal	M
Nl-10	18+0	43	Normal	M
Nl-11	20+2	30	Normal	M
Nl-12	20+3	30	Normal	M
Pm-1	15+0	67	Premut	M
Pm-2	16+3	55	Premut	M
Pm-3	16+4	56	Premut	M
Pm-4	16+5	66	Premut	M
Pm-5	16+5	66	Premut	M
Pm-6	16+5	61	Premut	M
Pm-7	16+6	99	Premut	M
Pm-8	17+2	62	Premut	M
Pm-9	17+5	57	Premut	M
Pm-10	19+0	64	Premut	M
Pm-11	19+4	60	Premut	M
Pm-12	20+2	144, 165	Premut	M
Nl-13	16+6	27, 29	Normal	F
Nl-14	17+1	21, 30	Normal	F
Nl-15	17+3	29, 30	Normal	F
Nl-16	17+4	29, 30	Normal	F
Nl-17	19+2	29, 30	Normal	F
Nl-18	19+5	30, 43	Normal	F
Pm-13	16+6	23, 55	Premut	F
Pm-14	17+0	37, 56	Premut	F
Pm-15	17+6	29, 64	Premut	F
Pm-16	17+6	29, 64	Premut	F
Pm-17	19+0	23, 83	Premut	F
Pm-18	19+0	23, 83	Premut	F
Fm-1	16+4	30, >200	Full	F
Fm-2	17+5	30, >200	Full	F
Fm-3	16+6	155, 171, >200	Full	M

Sample	GA	Number of Repeats	Diagnosis	Fetal Sex
Fm-4	20+0	>200	Full	M

GA, weeks+days gestational age; Nl, Normal; Pm, Premutation; Fm, Full mutation

Table 2

Analysis groups comprising this study.

Characteristics				Criteria for Differential Gene Expression			
Analysis Group	**Samples (Nl)**	**Samples (Pm or Fm)**	**GA Range**	**Fold-Change Thres-hold**	**Concor-dant Direct-ion**	**BH-p Thres-hold**	**Number of Different-ially Expressed Genes**
Pm Male	12	12	15–20	2	10/12	0.05	36
Pm Female	6	6	16–20	2.5	5/6	0.05	174
Fm (2M, 2F)	4	4	16–20	2.5	4/4	0.05	1, 257

GA, weeks gestational age; Nl, Normal; Pm, Premutation; Fm, Full mutation

Table 3

Probe sets known to have tissue-specific expression in humans. These probe sets showed significantly different gene expression in premutation males or females compared to their matched controls.

Gene Symbol	Probe Set	Tissue	DGE	Magnitude Fold-Change
SNAP25	202508_s_at	Amygdala	Downregulation FP	2.60
TMEFF1	205122_at	Amygdala	Upregulation FP	2.56
HPCA	205454_at	Caudate Nucleus	Downregulation MP	2.96
NCF1	204961_s_at	CD19+ B Cells (Neg. Sel.)	Downregulation FP	3.43
AL928768.3	215118_s_at	CD19+ B Cells (Neg. Sel.)	Upregulation FP	2.85
IRF5	205469_s_at	CD33+ Myeloid Cells	Upregulation FP	3.14
CD3D	213539_at	CD4+ T Cells	Downregulation FP	4.72
L1CAM	204584_at	Fetal Brain	Upregulation FP	3.87
UPB1	220507_s_at	Liver	Downregulation FP	4.28
SFTPD	214199_at	Lung	Upregulation MP	2.11
ADAM12	213790_at	Placenta	Upregulation FP	2.91
AKR1B10	206561_s_at	Small Intestine	Downregulation FP	3.26
ARHGAP25	38149_at	Whole Blood	Upregulation FP	2.53

DGE, Differential Gene Expression; MP, Male Premutation; FP, Female Premutation.