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A Multidimensional Investigation of 3q29 Deletion Syndrome

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A Multidimensional Investigation of 3q29 Deletion Syndrome

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Abstract

A Multidimensional Investigation of 3q29 Deletion Syndrome
By Rebecca Marie Pollak

The reciprocal 3q29 deletion and duplication syndromes (3q29del and 3q29dup) are rare genomic disorders characterized by a 1.6 Mb recurrent copy number variant. 3q29del is associated with a range of phenotypes including reduced birthweight, failure to thrive, developmental delay (DD), intellectual disability (ID), autism spectrum disorder (ASD), and schizophrenia (SZ). The phenotypic spectrum of 3q29dup is not well-described, but case reports have identified phenotypes including obesity, seizures, DD, and ID. Developing a clearer understanding of the phenotypes associated with 3q29del and 3q29dup, and the biological mechanisms underlying those phenotypes, is critical for the development of effective therapeutic strategies. With data from the online 3q29 registry (https://3q29deletion.org) and the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model of the 3q29 deletion, we sought to develop an improved phenotypic description of 3q29del and 3q29dup, and to interrogate the role of metabolic function in the development of 3q29 deletion-associated phenotypes. Self-report data from the 3q29 registry revealed a significantly increased rate of ASD diagnosis in our 3q29del study population, and a high rate of social disability irrespective of reported ASD diagnostic status. We also defined a spectrum of ASD-associated phenotypes that is distinct from the profile observed in idiopathic ASD, with 3q29del individuals showing relatively well-preserved social motivation. We next evaluated the phenotypic spectrum of 3q29dup, and found that feeding problems, learning problems, and seizures are all prevalent among 3q29dup study subjects. Additionally, we found a high rate of reported ASD diagnosis, demonstrating that the 3q29 duplication may confer a previously unrecognized ASD liability. Finally, we used the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model to interrogate metabolic dysregulation caused by the 3q29 deletion. We found that the 3q29 deletion has a substantial and sex-specific impact on fat metabolism. Taken together, these data highlight the broad phenotypic spectrum of 3q29del and 3q29dup and provide inroads toward mechanistic understanding of these complex disorders.

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CHAPTER 1. Understanding the phenotypic spectrum of copy number variant disorders and their underlying biological mechanisms

1.1 Copy number variants are a class of human genetic mutations

Copy number variants (CNVs) are rare human genetic mutations characterized by deletions or duplications of genetic material (1-4). CNVs can be *de novo* or inherited. An inherited CNV is transmitted from parent to child, whereas a *de novo* CNV arises from a spontaneous genomic event. CNVs are further classified as novel or recurrent; recurrent means that a CNV with the same or similar breakpoints is observed in multiple unrelated individuals. The genomic architecture surrounding many recurrent CNV loci provides clues to their formation; many recurrent loci are flanked by segmental duplications, which are composed of low copy repeats (LCRs). These regions of segmental duplications can misalign during DNA replication, resulting in the formation of deletions or duplications (1, 3-5). Deletions and duplications of the same CNV locus are referred to as reciprocal CNVs. For a given reciprocal CNV interval, the number of copies in the general population ranges from one to three: an individual with a deletion carries one copy of the interval, an individual with a duplication carries three copies, and an individual without a CNV carries two copies.

1.2 CNV disorders contribute to human morbidity

In addition to classifying CNVs as *de novo* or inherited and as novel or recurrent, CNVs are also defined as benign or pathogenic. On average, pathogenic CNVs are larger and/or more gene-rich than benign CNVs (1, 6). Pathogenic CNVs are the cause of CNV disorders, which can cause a wide range of physical, neurodevelopmental, and neuropsychiatric phenotypes. However, while many CNVs confer increased liability for complex phenotypes such as autism spectrum disorder (ASD), intellectual disability (ID), and schizophrenia (SZ) (7-12), there is still variation in the phenotypic presentation of CNV disorders within affected populations (13). This is referred to as phenotypic heterogeneity: not all individuals with a given CNV disorder are equally impacted, resulting in differing degrees of disability for individuals with the same genetic diagnosis.

1.3 CNVs can be leveraged to gain insight into human phenotypes

In the general population, the prevalence of ID, ASD, and SZ is 1.14%, 1.47%, and 0.5-1%, respectively (14-16). However, while these complex disorders affect a significant percentage of the

population, the biological mechanisms underlying their development are neither simple nor well-understood. A major roadblock to dissecting these mechanisms is the genetic and phenotypic heterogeneity within the affected populations. Studies have identified common and rare genetic variants associated with these disorders (7-11, 17-27). Common variants have a small effect on liability to develop the disorder, while some rare variants have a strong effect (28). Each individual with a given disorder has a combination of genetic risk factors that combine to produce the phenotype of interest, and this combination of genetic factors varies between affected individuals. Further, the phenotypic heterogeneity within complex disorders is substantial. Complex disorders such as ASD and SZ are defined by a variety of features, not all of which are present in all affected individuals. This combination of genetic and phenotypic heterogeneity makes it difficult to detect meaningful biological signals from heterogeneous patient populations.

CNVs that underlie a complex disorder can be effectively leveraged against the problem of heterogeneity. By definition, individuals with a given CNV disorder share the same genetic lesion, minimizing the effect of genetic heterogeneity within the study population. Further, many reciprocal CNVs are associated with increased risk for ID, ASD, and/or SZ, resulting in a significant enrichment for these phenotypes within the patient population. Ascertaining a study cohort based on a genetic diagnosis rather than a neurodevelopmental or neuropsychiatric diagnosis will reduce heterogeneity and increase statistical power to detect meaningful patterns in data. This is referred to as a genetics-first strategy; the use of this strategy in the study of ASD was pioneered by the Simons Foundation (29). By performing detailed phenotyping on a discrete group of patients defined by a similar genetic lesion with large effect size on the phenotype of interest, a genetics-first strategy allows for a nuanced understanding of subtypes of complex disorders defined by a specific genetic etiology (30-32). In turn, this more detailed description will help to guide clinical care for individuals with the CNV disorder in question. Further, the understanding gleaned from genetics-first study may be generalizable to the broader population of individuals with complex etiologies of disorders such as ASD or SZ, ultimately improving the current understanding of these complex phenotypes and potentially improving therapeutic strategies.

1.4 Methods to study CNVs

1.4.1 The study of CNVs in human populations

In order to understand CNV disorder phenotypes, studies of affected individuals are critical. Research studies with human subjects can take several different forms. Studies using direct observation of participants are a common way to collect phenotype data and to perform assessments on affected individuals. However, the feasibility of direct observation to study rare phenomena, including CNV disorders, is limited by the ability to gather a large enough cohort of affected individuals to gain meaningful insight into the disorder of interest. In these instances, remote phenotyping is an invaluable tool to gather information on a large cohort of affected individuals scattered across a country or around the globe. Online patient registries are one method of performing remote data collection; a registry permits an affected individual or their parent/guardian to complete questionnaires and surveys. Researchers can then securely download the de-identified data for analysis, so participant privacy and confidentiality is maintained. Data from direct observation studies, including case reports on individuals with rare disorders, can be used to inform the structure of registries and remote data collection tools, including the creation of customized surveys to gather information about specific phenotypes of interest. In turn, registries can be used to support direct observation studies via building a database of affected individuals that can be invited to participate in more extensive phenotyping studies and other in-person research projects.

While human subject research is invaluable to our understanding of complex disorders, it is not without limitations. Remote phenotyping tools such as registries rely on self-report data, the reliability of which has been called into question in some studies (33). Further, both remote phenotyping and direct observation assess participants at one point in time. Longitudinal studies are informative but require years of follow-up before results are available. These studies are also expensive, logistically demanding, and subject to participant attrition. Finally, studies with human subjects are observational, rather than experimental. Human subject research is critical to developing a phenotypic understanding of CNV

disorders, but experimental approaches are often necessary to reveal biological changes underlying these phenotypes.

1.4.2 Rodent models can be used to study CNVs

Rodent models, including rat and mouse models, are an experimental system that can be used to study the underlying biology of CNV disorders. Rodent models can be subjected to experimental manipulations including genetic, dietary, pharmacological, and surgical interventions that are too invasive to be studies in human subjects. The shorter lifespan of rodent models compared to humans also makes them an excellent model system for longitudinal research, which can provide insight into phenotypes that may change depending on the developmental stage of the organism. In the study of CNV disorders, mouse models have proved particularly valuable in understanding both behavioral and physical alterations that may contribute to phenotypes in human patients. Mouse models have been created for several CNV loci, including the 1q21.1 deletion, 3q29 deletion, 7q11.23 deletion, 16p11.2 deletion, 16p11.2 duplication, and 22q11.2 deletion (34-43). These models show some phenotypic similarities to human patients, including behavioral phenotypes and growth abnormalities (34-38, 40-43). Further, several of these mouse models have been used to define cellular phenotypes, including transcriptional dysfunction, changes in neurotransmitter function, and brain structural alterations compared to wild type littermates (34, 36, 38-41).

There are important phenotypic similarities between human patients and mouse models of CNVs; however, not all phenotypes observed in mouse models are consistent with those in human patients. For example, the effect of the reciprocal 16p11.2 CNV on growth in mice is opposite its effect in humans; in humans, the 16p11.2 deletion is associated with obesity, while the 16p11.2 duplication is associated with an extreme underweight phenotype (44). In mice, the orthologous 16p11.2 deletion is associated with an underweight phenotype, while the orthologous 16p11.2 duplication is associated with an overweight phenotype (38). There are also important differences in brain structure and function between mice and humans, which can limit the direct translation of findings in murine models to human studies (45). Additionally, mouse models inherently model symptoms or features of complex disorders such as ASD

and SZ rather than modeling the actual disorder, further complicating the translation of findings from mice to humans (45-47).

While limitations exist for both human subject research and research using mouse models, these strategies can be combined to form an effective multidimensional research strategy. Studies with human participants enable researchers to build a detailed phenotypic description of the disorder. Detailed phenotyping information can inform patient care and can help guide experimental design for more in-depth studies using rodent models. The results from studies with rodent models, in turn, can be used to start building more targeted therapeutic strategies. Ultimately, the goal of a multidimensional research strategy incorporating data from human subjects and rodent models is to build a more complete understanding of CNV disorders, consequently improving treatment strategies and long-term outcomes.

1.5 The 3q29 CNV locus is poorly understood

The 3q29 reciprocal CNV locus is a 1.6 Mb interval on chromosome 3 (hg19, chr3:195725000-197350000) (48-50). The interval contains 21 distinct protein-coding genes, as well as five antisense transcripts, three long noncoding RNAs, and one microRNA (Figure 1-1). The locus is flanked by LCRs, which mediate the formation of deletions or duplications of the interval. The 3q29 deletion and duplication are relatively recent additions to the CNV literature (49-52), and the phenotypic presentation of affected individuals has not been well-described.

The 3q29 deletion is a rare (~1:30,000), typically *de novo* genomic event (48-50, 53-55). Phenotypes associated with 3q29 deletion syndrome (3q29del) present across the lifespan and have been described in case reports (49, 50, 56-60) and in a prior study of individuals in the online 3q29 registry (3q29deletion.org) by our team (53). At birth, individuals with 3q29del have significantly reduced birthweight compared to typically developing peers, after correcting for sex and gestational age (53). Additionally, approximately 25% of individuals with 3q29del are born with a congenital heart defect (53, 56). A large majority of individuals with 3q29del experience medical challenges in the first year of life, most commonly feeding problems and failure to thrive (49, 53). Developmental delay, mild to moderate ID, and ASD are commonly reported (49, 50, 53, 56-59); however, individuals without cognitive

impairment have been identified (60, 61). Studies with a large sample size have shown that the 3q29 deletion is associated with a 19-fold increased risk for ASD (17, 19), supporting the high rate of reported ASD in the 3q29del patient population (49, 50, 53, 56-59). Other medical phenotypes presenting during childhood include recurrent ear infections, feeding problems beyond the first year of life, and dental abnormalities (49, 50, 53, 56, 59, 60). Significant neuropsychiatric liability has also been associated with 3q29del. Multiple studies have shown that the 3q29 deletion confers a 20 to 40-fold increased risk for SZ (7, 9, 10, 62, 63), and data suggests that attention deficit/hyperactivity disorder, bipolar disorder, and anxiety disorders may also be associated with 3q29del (49, 50, 53, 56-60).

3q29 duplication syndrome (3q29dup) is a rare disorder, with prevalence estimates ranging from 1:75,000 to 1:8,000 (54, 64-67). The 3q29 duplication has been observed as both a de novo and an inherited event (50, 68, 69). The phenotypic spectrum of 3q29dup has been poorly described and case reports have identified individuals with varying duplication sizes, further obscuring the true phenotypic spectrum of the duplication (50). Case reports have identified some features that are consistent with those reported in 3q29del, including developmental delay, ID, dental phenotypes, and cardiac abnormalities (50, 52, 68, 70-73). Other phenotypes include ocular abnormalities, obesity, and seizures (50, 52, 68-76). Little is known about neurodevelopmental and neuropsychiatric phenotypes of 3q29dup beyond developmental delay and ID, with just one case report describing an individual with behavioral similarities to ASD (75).

The current understanding of 3q29del and 3q29dup suggests that metabolic dysregulation may be associated with the 3q29 CNV locus. Of the 21 protein-coding genes in the 3q29 interval, four (*TFRC*, *PCYT1A*, *SENP5*, and *BDH1*) have direct links to metabolism. The other genes in the interval are not known to be directly involved in metabolism, but functional roles for these genes may emerge with improved annotation. 3q29del is associated with significantly reduced birthweight and a high proportion of feeding problems and failure to thrive compared to the general population (53). 3q29dup is associated with obesity (50, 52, 68-73), suggesting a potential mirror effect of the 3q29 locus on metabolism, similar to what has been reported for the 16p11.2 locus (44). Robust weight deficits have been identified in both

mouse models of the 3q29 deletion (35, 36), further supporting a possible unidentified metabolic disruption associated with the 3q29 deletion.

1.6 Research aims

The detailed phenotypic spectrum of 3q29del and 3q29dup have not been well-described. Further, the mechanisms contributing to these phenotypes is unknown. The objectives of this dissertation were to more clearly define the phenotypes associated with 3q29del and 3q29dup, and to interrogate biological mechanisms related to metabolism in 3q29del. These goals were accomplished through three aims:

I. Define the spectrum of ASD-associated phenotypes in 3q29del

II. Create a comprehensive description of the phenotypic spectrum of 3q29dup

III. Investigate the role of metabolic function in 3q29del-associated phenotypes

The first aim of this dissertation was to define the spectrum of ASD-associated phenotypes in a cohort of individuals with 3q29del. Previous work found that the 3q29 deletion is associated with a 19-fold increased risk for ASD (17, 19), and a prior study by our group found increased rates of reported ASD diagnosis in a cohort of individuals with 3q29del (53). However, the specific ASD features present in individuals with 3q29del were not defined. I leveraged the online 3q29 registry (3q29deletion.org) (53) to collect data using standardized ASD questionnaires, which allowed us to systematically assess functional domains relevant to ASD (77). These data are discussed in Chapter 2.

The second aim of this project was to define the phenotypic spectrum of 3q29dup. Previous studies had examined small cohorts of individuals (n=1-19), resulting in an incomplete phenotypic description of the syndrome (50, 52, 68-73). I evaluated self-reported phenotypes from the largest cohort of individuals with 3q29dup assembled to date, using a combination of custom and standardized questionnaires deployed through the 3q29 registry (3q29deletion.org) (53, 78). These data are discussed in Chapter 3.

The final aim of this dissertation was to investigate the role of metabolic function in 3q29del, using the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model of the 3q29 deletion created by members of our team (35). This

mouse model is powered to dissect the underlying biology of the 3q29 deletion, due to the synteny of the interval in humans and mice (35). A prior study by our group identified reduced birthweight in individuals with 3q29del (53); this weight deficit is robustly recapitulated in both mouse models of the 3q29 deletion (35, 36). Additionally, individuals with 3q29del report a high prevalence of feeding problems (53). These data, together with the fact that four genes in the 3q29 interval have direct links to metabolism (*Bdh1*, *Senp5*, *Pcyt1a*, and *Tfrc*), led to the hypothesis that there may be a previously unidentified metabolic disturbance in 3q29del. These data are discussed in Chapter 4.

The ultimate goal of this dissertation was to improve the current understanding of 3q29del and 3q29dup. I first articulated the phenotypic spectrum in the largest patient cohorts to date by systematically ascertaining features of 3q29del and 3q29dup. These data, in combination with previously published studies, provided the foundation for the hypotheses investigated in aim 3; namely, metabolic disruption associated with the 3q29 deletion, and the role of sex in modifying metabolic phenotypes. Refining our understanding of 3q29del and 3q29dup is the first step in translating research findings into improved therapeutic strategies for patients, ultimately improving long-term outcomes and quality of life for individuals with 3q29del and 3q29dup. Further, cultivating a better understanding of 3q29del and 3q29dup may provide a scaffold for the investigation of complex disorders like ASD and SZ.

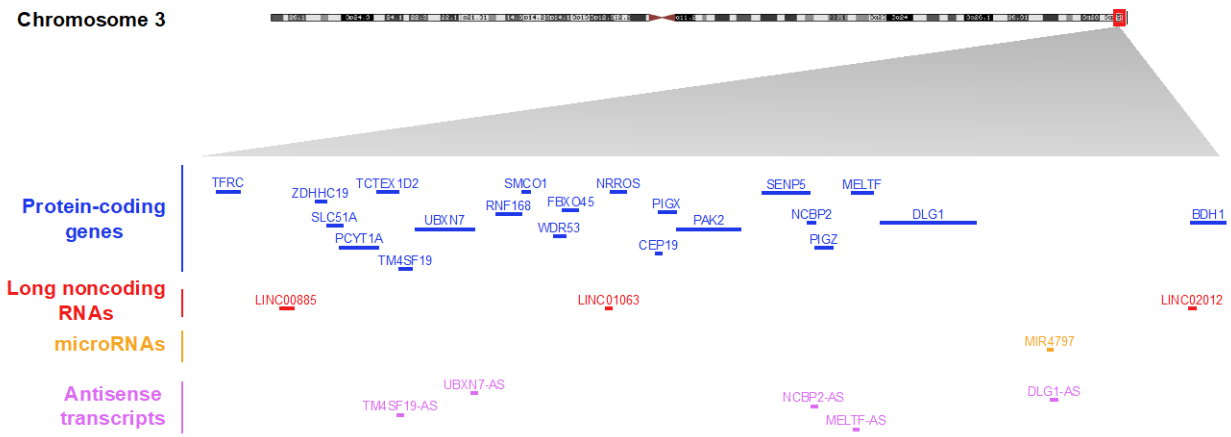

Figure 1-1. Structure of the 3q29 reciprocal CNV locus. The 3q29 CNV locus is highlighted on the chromosome map in red. The 21 protein-coding genes in the interval are indicated in blue, the three long noncoding RNAs are indicated in red, the microRNA is indicated in orange, and the five antisense transcripts are indicated in purple.

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Chapter 2. Neuropsychiatric phenotypes and a distinct constellation of ASD features in 3q29 deletion syndrome: Results from the 3q29 registry

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BACKGROUND

3q29 deletion syndrome (3q29Del) is a rare (~1 in 30,000) (1, 2) genomic disorder characterized by a 1.6 Mb typically *de novo* deletion on chromosome 3 (3-5). The interval contains 21 distinct protein-coding genes, 3 antisense transcripts, 1 long noncoding RNA, and 1 microRNA. Our understanding of the syndrome phenotype continues to evolve. Initial reports found developmental delay/intellectual disability universal among 3q29 deletion carriers, though some case reports have since identified individuals without cognitive impairment (6). The 3q29 deletion is associated with a 20-40-fold increased risk for schizophrenia (SZ), with multiple replication studies supporting this association (7-11). Case reports also indicate other neuropsychiatric phenotypes may exist, including attention deficit/hyperactivity disorder (ADHD) and bipolar disorder (3, 4, 12-16). Previous work by our team examining self-report data from 44 individuals with 3q29Del revealed a high prevalence (~20%) of generalized anxiety disorder (5). Further, case reports have long suggested an association with autism spectrum disorder (ASD), and studies with large sample sizes indicate that the 3q29 deletion may confer a 19-fold increased risk for ASD ($p = 0.001$) (17, 18).

The range of neuropsychiatric manifestations in 3q29Del is consistent with other genomic disorders. For example, the 22q11.2 deletion has a well-known association with schizophrenia but is also associated with intellectual disability (ID), ASD, anxiety, mood disorders, and ADHD (19, 20). A similar constellation of phenotypes, including ASD, ADHD, ID, SZ, and anxiety, has been identified in 16p11.2 deletion and duplication syndromes (21, 22), 7q11.23 duplication syndrome (23), and 1q21.1 deletion syndrome (24). Thus, risk for multiple neuropsychiatric phenotypes appears to be a feature common to many genomic disorders, including 3q29 deletion syndrome.

The present study aims to improve the current understanding of 3q29 deletion-associated neuropsychiatric and neurodevelopmental phenotypes, and ASD in particular, by examining data from comprehensive, standardized questionnaires in the largest cohort of individuals with 3q29Del ever assembled. Developing a clearer and more comprehensive picture of 3q29 deletion-associated phenotypes will aid in management of the syndrome for both families and clinicians, which may in turn improve

long-term outcomes. Additionally, a careful description of the phenotypic spectrum of 3q29Del provides a basis for cross-disorder comparison between genomic disorders, which may ultimately create inroads for identifying common mechanisms underlying 3q29Del and similar CNV disorders.

METHODS AND MATERIALS

Sample

Individuals with 3q29Del were ascertained through the internet-based 3q29 deletion registry (https://3q29deletion.patientcrossroads.org) as previously reported (5). Briefly, information about the registry was emailed to health care providers, medical geneticists, genetic counselors, and support organizations; the registry is also advertised via Google AdWords, where specific keywords were chosen to target the registry website in internet searches. Participant recruitment, informed consent and assent, and data collection are all performed through the registry website. Data were securely downloaded and de-identified for analysis. After data cleaning of the electronic records (removing spam accounts, duplicate records, and related individuals), 93 3q29Del registrants (58.1% male) were included in the present study, ranging in age from 0.1-41.0 years (mean = 10.0 ± 8.6 years). Clinical diagnosis of 3q29 deletion syndrome was confirmed in 58% of our study subjects via review of clinical genetics reports and/or medical records. To confirm that adaptation of standardized questionnaires to an online format did not skew results, 64 typically developing controls (51.6% male) were included, ranging in age from 1.0-41.0 years (mean = 9.9 ± 7.2 years). Controls were recruited via emails sent to intramural CDC and Emory listservs and invited to fill out surveys in an identical fashion to cases. Controls reporting a clinical diagnosis of any neurodevelopmental disorder were excluded (n = 1). Description of the study sample can be found in Table 2-1. This study was approved by Emory University's Institutional Review Board (IRB00064133).

Questionnaires

Upon registration, the participant or his/her parent completed a custom medical and demographic questionnaire. This questionnaire includes questions on the sex, birthdate, race, and ethnicity of the

participant, as well as a detailed medical history, including developmental milestones and prior clinical diagnoses of any neuropsychiatric or neurodevelopmental disorders (5).

Four standardized questionnaires were used to assess ASD-related symptomology and general behavioral problems in the participants. The Social Responsiveness Scale (SRS; preschool, school-age, and adult forms; n = 48 3q29Del, 56 controls) is a 65-item, 4 point Likert-scaled questionnaire designed to assess ASD-related symptoms along a normative continuum (25). The Social Communication Questionnaire (SCQ, n = 33 3q29Del, 46 controls) is a 40-item, yes/no questionnaire designed to assess ASD-related symptoms keyed to DSM criteria (26). The Autism Spectrum Screening Questionnaire (ASSQ, n = 24 3q29Del, 35 controls) is a 27-item, yes/somewhat/no questionnaire designed to assess ASD-related symptoms in high-functioning individuals with no to mild ID (27). The Child Behavior Checklist (CBCL) and Adult Behavior Checklist (ABCL) are 100-, 113-, or 126-item (CBCL preschool, CBCL school-age, and ABCL, respectively; n = 48 3q29Del, 57 controls), 3 point Likert-scaled questionnaires designed to assess behavioral or developmental problems (28, 29). Data from the CBCL and ABCL were pooled for analysis. All standardized questionnaires were adapted for the online 3q29 deletion registry and were completed by the participant or parent/guardian of the participant upon registration. Some participants were not eligible to complete the standardized questionnaires because the proband was too young. Demographic characteristics of the respondents for each questionnaire can be found in Table S2-1, demonstrating that the average age and sex distribution of participants who completed the medical and demographic questionnaire was not different from the average age and sex distribution of participants who completed each standardized form.

Analysis

Data from standardized questionnaires were imported into R (30) and were recoded and scored according to the publisher's guidelines. Features of interest from the medical history questionnaire (heart defects, age at walking, ASD diagnosis, global developmental delay/mental retardation (GDD/MR) diagnosis) were recoded for analysis as follows: heart defects, yes/no; age at walking, binned as normal (≤18 months), delayed (19-24 months), and extremely delayed (>24 months); ASD diagnosis, yes/no;

GDD/MR diagnosis yes (reported diagnosis of global developmental delay and/or mental retardation)/no. To compare responses between 3q29Del cases and controls, linear models and logistic regression models were implemented using the stats R package (30). To perform case-only analysis within 3q29Del cases, linear models and logistic regression models were implemented using the stats R package (30) and cumulative link proportional-odds models were implemented using the ordinal R package (31). All statistical models included age, race, and sex as covariates. To compare rates of self-reported diagnoses and demographic parameters between 3q29Del cases and controls, Fisher's exact test was implemented using the stats R package (30). To compare rates of self-reported diagnoses in 3q29Del cases to population prevalence values, one-sample proportion tests with Yates' continuity correction were implemented using the stats R package (30). To compare sex distribution between 3q29Del participants and controls, Pearson's chi square test was implemented using the stats R package (30). To compare age distribution in 3q29Del participants and controls, two sample t-test was implemented using the stats R package (30). To compare scores in 3q29Del participants to mean values for children with idiopathic ASD, one sample t-test was implemented using the stats R package (30). Odds ratios and p values were calculated using the fmsb R package (32). Figures were generated using the plotly and ggplot2 R packages (33, 34).

Sensitivity Analysis

The questionnaires for 90 participants with 3q29Del (96.8%) were completed by a parent or guardian ("parent-registered"), while 3 participants with 3q29Del (3.2%) completed all questionnaires themselves ("self-registered"). All control participants were parent-registered. To assess whether responses from the self-registered 3q29Del participants were influencing the results, self-registrants were removed and the data were re-analyzed. Self-registrants were not found to have a significant effect on the analyses (Tables S2 and S3). All results include both parent- and self-registrants.

RESULTS

Self-report of neuropsychiatric diagnosis in 3q29Del

Self-report of neuropsychiatric diagnoses in our 3q29Del study subjects (Table 2-2) revealed a higher prevalence of neuropsychiatric disorder diagnoses compared to controls, including anxiety (28.0%), and compared to general population frequencies, including ASD (29.0%, Figure 2-2A) and GDD/MR (59.1%) (Table 2-2), confirming prior work by our group (5). Reported rates of conduct disorder (1.1% vs. 3.5%) and oppositional defiant disorder (3.2% vs. 3.5%) were similar to those observed in the general population. While a small proportion of participants reported diagnoses of bipolar/manic depression (4.3%), depression (6.5%), and schizophrenia (4.3%), we focused on ASD due to the young age (mean = 10.0 years) of our study population, since many study participants have not reached the age of risk for schizophrenia and other adult-onset disorders. Despite this young age, the self-reported rate of SZ diagnoses in our adult study subjects (age > 18 years, n = 13) was 15-30 times higher than expected (15.4% compared to an expected 0.5-1% in the general population; n = 2) (35-39) and the frequency of bipolar disorder was ~1.8 times higher than expected (40). A summary of neuropsychiatric diagnoses can be found in Table 2-2.

SRS, SCQ, ASSQ, and CBCL/ABCL scores

In 3q29 deletion study subjects, the mean SRS score was in the moderate range (T-score = 71.8), the mean ASSQ score was in the clinical range (mean = 22.2), and the mean CBCL/ABCL score was in the borderline range (T-score = 62.5). The mean SCQ score in 3q29 deletion carriers was at the extremely high end of the normal range (mean = 13.9, clinical cutoff = 15) and elevated as compared to controls (mean = 3.5). Mean scores for typically developing controls were all in the normal range (SRS T-score = 45.9, ASSQ mean = 2.2, CBCL/ABCL T-score = 41.8, SCQ mean = 3.5) (Figure 2-1). Participants with 3q29Del scored significantly higher than typically developing controls on all four scales ($p < 3.0E-12$, Table S2-4).

Standardized scores stratified by ASD diagnosis

Next, we examined the relationship between SRS scores and reported ASD diagnosis, to determine whether the score inflation we observed in our study population as a whole was largely due to the increased prevalence of ASD. As expected, we observed that individuals with 3q29Del and an ASD

diagnosis scored significantly higher than both controls and individuals with 3q29Del without an ASD diagnosis (3q29Del with ASD n = 17, T-score = 82.41; 3q29Del without ASD n = 31, T-score = 65.90; control n = 56, T-score = 45.90; $p < 3.0E-13$; Figure 2-2B). We were interested to observe that individuals with 3q29Del without an ASD diagnosis also scored significantly higher than controls (3q29Del without ASD n = 31, T-score = 65.90; control n = 56, T-score = 45.90; $p = 2.16E-13$; Figure 2-2B), indicating that increased SRS scores in individuals with 3q29Del are not driven by ASD diagnostic status alone (Table S2-5). Similar features were observed in the contribution of ASD diagnosis status to SCQ scores (Figure S2-1, Table S2-6).

Standardized scores stratified by sex

Both males and females with 3q29Del reported a significantly increased frequency of ASD diagnoses, with a substantially greater burden for ASD on females with 3q29Del. Males with 3q29Del are at 16-fold increased risk for ASD as compared to the general population (37.0% vs. 2.34%, OR = 24.6, $p = 6.06E-09$) and females are at 34-fold risk compared to the general population (17.9% vs. 0.52%, OR = 41.8, $p = 4.78E-05$) (Figure 2-2A) (41), resulting in a male:female ratio in our study population of 2:1, as compared to the general population ratio of 4:1. Taken together, this indicates that the 3q29 deletion elevates the risk for ASD in females more substantially than in males.

Based on the sex differences in ASD risk for individuals with 3q29Del, we also examined possible sex differences in scores. We found that both males and females with 3q29Del scored significantly higher than controls (3q29Del male n = 26, T-score = 74.31; control male n = 30, T-score = 45.80; $p = 7.70E-11$; 3q29Del female n = 22, T-score = 68.73; control female n = 26, T-score = 46.04; $p = 7.42E-09$); while 3q29Del males have higher scores than females, the differences are not statistically significant (3q29Del male n = 26, T-score = 74.31; 3q29Del female n = 22, T-score = 68.73; $p > 0.05$; Figure 2-2C). After stratifying our study population further by sex and ASD diagnosis status, we determined that both male and female 3q29Del participants without an ASD diagnosis had significantly higher scores than controls (3q29Del male without ASD n = 14, T-score = 66.29; control male n = 30, T-score = 45.80; $p = 1.20E-06$; 3q29Del female without ASD n = 17, T-score = 65.69; control female n =

26, T-score = 46.04; p = 5.04E-07; Figure 2-2D). Taken together, this suggests that increased SRS scores in individuals with 3q29Del are not driven by sex alone or by sex and ASD diagnosis status in combination (Table S2-5); rather, the presence of the deletion itself confers a greater risk for social disability. Furthermore, these data show an enrichment for female ASD in our study population, based on the reduction in male bias and the highly similar scores between males and females with 3q29Del, irrespective of ASD diagnosis status. Similar features were observed in the contribution of sex to SCQ scores (Figure S2-1, Table S2-6).

ASD presentation of 3q29Del

While total scores on the SRS, SCQ, ASSQ, and CBCL/ABCL can give an indication of the overall level of impairment of individuals, sub-scores can reveal nuanced deficits in specific behavioral domains. To this end, we analyzed all SRS sub-scales (Social Awareness, Social Cognition, Social Communication, Social Motivation, Restricted Interests and Repetitive Behaviors, and Social Communication and Interaction) to better understand the extent of social disability in our study population; our goal was to determine whether our observed total score inflation was due to a specific severe deficit in a few domains, or if individuals with 3q29Del showed high scores across all sub-scales. The mean score for the Restricted Interests and Repetitive Behaviors sub-scale was in the severe range (T-score = 77.3). Mean scores for Social Awareness (T-score = 67.3), Social Cognition (T-score = 69.1), Social Communication (T-score = 69.7), and Social Communication and Interaction (T-score = 69.5) were all in the moderate range. Notably, the mean score for Social Motivation was in the mild range (T-score = 62.1, Figure 2-3A, Table 2-3). This sub-score profile is strikingly different from that reported in studies of idiopathic ASD, where children tend to score equally high on all sub-scales (3q29Del Social Motivation T-score = 62.1, idiopathic ASD Social Motivation T-score = 78.4, p = 7.66E-11) (42). This atypical behavioral profile is supported by clinical data; direct assessment of individuals with 3q29Del by clinicians affiliated with the Emory 3q29 Project (http://genome.emory.edu/3q29/, (43)) show less impaired social motivation as compared to children with idiopathic ASD.

ASD presentation stratified by sex

To determine whether this unusual SRS sub-score profile was influenced by sex, we examined profiles of male and female 3q29 deletion carriers separately. We found that the shape of the profiles were identical, with males scoring on average 5 points higher than females on every sub-scale (n = 26 male, 22 female; $p > 0.05$; Figure 2-3B; Table 2-3), demonstrating that the social disability in 3q29Del is not qualitatively different between males and females.

ASD presentation stratified by ASD diagnosis

We then stratified our study subjects according to reported ASD diagnosis status and examined subscale scores separately for 3q29Del individuals reporting a diagnosis of ASD and those not reporting a diagnosis of ASD. We observed that the shape of the profile is shared between 3q29Del individuals reporting a diagnosis of ASD and those not reporting a diagnosis of ASD, with individuals reporting a diagnosis of ASD scoring on average 10-15 points higher on every sub-scale (Figure 2-3C). As expected, 3q29Del participants with ASD scored significantly higher on all sub-scales than 3q29Del participants without ASD (n = 17 with ASD, 31 without ASD; $p < 0.005$; Table 2-3); however, 3q29Del participants without ASD still scored significantly higher than controls on all sub-scales (n = 31 without ASD, 56 control; $p < 5.0E-05$; Table 2-3).

Additional neuropsychiatric phenotypes in 3q29Del

To further assess behavioral features associated with the 3q29 deletion, we examined the DSM-oriented Attention Deficit/Hyperactivity Problems, Anxiety Problems, and Depressive Problems sub-scales from the CBCL and ABCL. These DSM-oriented sub-scales align with neuropsychiatric diagnoses reported by individuals with 3q29Del (5). Individuals with 3q29Del scored significantly higher than typically developing controls on all three scales (3q29Del Attention Deficit/Hyperactivity Problems T-score = 61.0, control Attention Deficit/Hyperactivity Problems T-score = 51.3, 3q29Del Anxiety Problems T-score = 60.9, control Anxiety Problems T-score = 52.9, 3q29Del Depressive Problems T-score = 62.7, control Depressive Problems T-score = 52.3, all $p < 0.001$, Figure 2-3D, Table S2-7), supporting previous reports of increased risk for neuropsychiatric phenotypes associated with the 3q29 deletion (5).

Confounding due to heart defects and/or ID-related phenotypes

A previous study of 3q29Del by our group showed that approximately 25% of individuals with 3q29Del reported a congenital heart defect (5). Early hypoxic insult due to a heart defect has been hypothesized to contribute to later neuropsychiatric and neurodevelopmental outcomes (44-49). To determine if the high frequency of heart defects in our study population was driving adverse neurodevelopmental outcomes within 3q29Del cases, we implemented generalized linear and cumulative link models to assess the relationship between congenital heart defects and clinical ASD diagnosis, GDD/MR diagnosis, and age at walking, which has been reported to be a suitable proxy for ID in the absence of available IQ and adaptive behavior measures (50) . Congenital heart defects were not associated with self-reported ASD or GDD/MR diagnoses or age at walking ($p > 0.05$, Table S2-8). Individuals with 3q29Del are also commonly diagnosed with mild to moderate ID (5). To ask whether ASD phenotypes or ASD features were disproportionately overrepresented in individuals with more pronounced ID-related phenotypes and/or heart defects, we stratified the data according to these phenotypes. Within our 3q29Del study population, congenital heart defects were associated with significantly increased scores on the SCQ and CBCL/ABCL ($p < 0.05$); however, reported GDD/MR diagnosis and age at walking were not significantly associated with scores on the SRS, SCQ, ASSQ, or CBCL/ABCL ($p > 0.05$, Table S2-9). These data indicate that ID-related phenotypes were not driving the increased scores in our study population.

DISCUSSION

Previous studies have found enrichment of the 3q29 deletion in large samples ascertained based on clinical ASD diagnosis (17, 18). We have approached the association of 3q29Del with ASD from a different angle; by ascertaining subjects with 3q29Del and investigating the prevalence of reported ASD diagnosis and ASD-related phenotypes, the current study complements the existing literature, providing additional evidence for the 3q29 deletion as a genetic risk factor for ASD. Notably, the male:female ratio of self-reported ASD diagnosis in our study population is 2:1. This is a reduction from the 4:1 male bias observed in idiopathic ASD in the general population. A substantial reduction in male bias in ASD

prevalence has been observed in studies of other CNVs and single-gene mutations; a recent study has shown that as the severity of a mutation increases, the sex ratio in ASD prevalence approaches 1:1 (51). Taken together, this suggests that the 3q29 deletion is approaching the severe end of the spectrum of ASD-associated mutations.

We have shown that compared to typically developing children, our 3q29Del sample is significantly enriched for ASD features and other behavioral problems, irrespective of a clinical ASD diagnosis. This finding is particularly concerning; while individuals with 3q29Del who have an ASD diagnosis tend to score higher on symptomology scales overall, 3q29Del individuals without an ASD diagnosis still score significantly higher than typically developing children. This indicates several possible explanations: a) an enrichment for ASD features or social disability that falls short of diagnostic criteria, b) possible undiagnosed ASD in our study population, or c) non-specificity of the SRS, and potentially SCQ, for phenotypes other than ASD, such as anxiety. The possibility of undiagnosed ASD in our study population is aligned with anecdotal reports from parents of our study participants, where they have reported concerns about atypical social development that do not appear to have been addressed using gold-standard ASD evaluations. Based on the elevated symptomology scores in our study population, the substantially increased risk for ASD associated with the 3q29 deletion, and the apparent severity of the 3q29 deletion, our data suggest that gold-standard ASD evaluations should be the recommended standard of care for individuals diagnosed with 3q29Del. If implemented, this practice would enable patients to gain access to early interventions, treatments, and therapeutic programs that are known to improve later outcomes.

Based on the SRS sub-scales, participants with 3q29Del display a strikingly different behavioral profile as compared to a study of children with idiopathic ASD (42). Male and female 3q29Del individuals show substantially less impaired social motivation in the context of an otherwise typical ASD profile, with the most severe deficits in the Restricted Interests and Repetitive Behaviors domain. This profile is also observed when dividing scores for 3q29Del participants based on reported ASD diagnosis. This qualitative difference from idiopathic ASD may serve as an inroad to therapeutic interventions in

3q29Del, as well as an investigative inroad to a distinct subtype of ASD. Because social motivation appears to be relatively well-preserved in 3q29Del, this suggests that therapies such as cognitive-behavioral therapy to teach social skills and effective strategies for social interaction may be particularly successful in this patient population.

Some facets of the difference in ASD features between 3q29Del and idiopathic ASD are recapitulated by the scores on the Withdrawn sub-scale of the CBCL and ABCL. Previous studies utilizing the CBCL in idiopathic ASD have found that mean scores for participants with ASD are in the borderline range, with over 50% of subjects scoring in the borderline or clinical range (52, 53). While 3q29Del participants generally, as well as males and females separately, score significantly higher than controls, their mean score is still in the normal range (Figure S2-2A and B). However, 60% of 3q29Del participants reporting an ASD diagnosis score in the borderline or clinical range (Figure S2-2C, Table S2-10), which is in line with what is expected based on studies of idiopathic ASD (52, 53). This is in conflict with the relatively well-preserved social motivation in 3q29Del individuals with ASD identified in our analysis of the SRS sub-scales and suggests that a more refined analysis is merited to identify the true degree of social disability in this population.

We tested the hypothesis that the score inflation observed in our 3q29Del study subjects may be due to the high prevalence of developmental delay or congenital heart defects (5). Our available data do not support this hypothesis, and instead reveal that social disability is equally distributed in our study population. Lack of direct measures of intellectual disability, and errors or missing data in self-report measures, may obscure this relationship; however, numerous studies of the relationship between ID and ASD in genomic disorders suggests that when the population is stratified by the presence of a specific genetic variant, the association between these two phenotypes diminishes. A large study of several genetic disorders showed that the prediction of genetic diagnosis based on ADI-R scores was not confounded by IQ (54); a study of 7q11.23 duplication found that IQ was not significantly associated with ASD status (55); and multiple studies of 22q11.2 deletion have shown that IQ is not significantly associated with SRS score, ASD severity, and ASD status (56-58). A question ripe for future investigation is the potential role

for microcephaly in the ASD-related phenotypes observed in 3q29Del. Microcephaly, ASD, and ID are associated with the 16p11.2 duplication (21); microcephaly has been shown to be associated with ASD and ID in probands with pathogenic CNVs (59); and children with "complex autism", defined as ASD with microcephaly and/or dysmorphology, have significantly lower cognitive function than children with "essential autism" (60). Reports have shown a high prevalence of microcephaly in 3q29Del (3, 4, 12); however, this question was not probed in the current study due to the high rate (>50%) of 3q29Del participants responding "Unsure" to the medical history questionnaire regarding their child's head circumference at birth, rendering this data unreliable. Ongoing studies with direct evaluation of study subjects (43) will address these questions.

While this study is the most comprehensive study of behavioral phenotypes in 3q29Del to date, it is not without limitations. All of the data used in the present study were collected from questionnaires completed by the parents and guardians of individuals with 3q29Del, which introduces several potential sources of bias. Some studies have questioned the validity and reliability of parent-report data (61); however, a recent study in Williams syndrome patients has shown that parents are more accurate in predicting their child's social behaviors than the child themselves (62). The responses to the medical and demographic questionnaire are more likely to include error due to the fact that the data is retrospective. By limiting our study to only a few key points in the medical history (heart defects, age at walking, and ID/ASD diagnosis) we aimed to reduce recall errors; however, we only had proxies for ID, rather than direct evaluation of cognitive ability. Further, the sample sizes for our stratified analyses were small, rendering them underpowered; while the differences between males and females were not statistically significant, males do score higher than females on all measures. Studies with larger sample size will be better able to assess the importance of and estimate the true effect size of any difference between males and females. Additionally, there is likely ascertainment bias within our sample. First, our sample of 93 individuals with 3q29Del is 87.1% white, indicating that we are not adequately reaching minority populations. Second, parents that register their children and complete in-depth questionnaires are likely to be highly motivated, possibly because their children experience significant morbidity – a potential

indication that we are sampling from the extreme of the phenotypic distribution of 3q29Del. Thus, scores on the standardized questionnaires, as well as rates of heart defects and clinical neuropsychiatric diagnoses, may be higher in our study sample than in the general 3q29Del population. Additionally, the odds ratios calculated for the increased risk for ASD associated with the 3q29 deletion may also be overestimated, due to the combined effects of self-report data and ascertainment bias; however, if this increased risk is replicated using gold-standard diagnostic measures, it could provide valuable insight into possible sex-specific effects of the deletion. Finally, the lack of observed association between congenital heart defects and neurodevelopmental outcomes may be obscured by the high rate of patent ductus arteriosus in 3q29 deletion syndrome (5), which is a relatively mild heart defect; however, the low number of participants with different types of heart defects rendered analyses to assess their associations with neurodevelopment underpowered (Table S2-11). Ongoing studies by the Emory 3q29 Project (http://genome.emory.edu/3q29/), including direct in-person patient evaluations (43) aim to address some of the weaknesses of the present work by performing comprehensive gold-standard evaluations by expert clinicians.

While direct in-person evaluations are the ideal method to corroborate the findings of this study, the low population frequency of the 3q29 deletion and geographic dispersal of our study population (Figure S2-3) renders this approach infeasible for a large number of study subjects. However, a small number of 3q29 deletion study subjects have been directly assessed as part of the Emory 3q29 Project (http://genome.emory.edu/3q29/). We confirm high concordance between registry-leveraged data and gold-standard direct evaluation, as all participants qualifying for an ASD diagnosis based on gold-standard evaluation have clinically significant scores on the SRS and all participants reporting an ASD diagnosis qualified for an ASD diagnosis after gold-standard assessment by the Emory 3q29 Project team (Table S2-12). Notably, one participant that did not report a prior diagnosis of ASD received an ASD diagnosis after assessment by our team, supporting our hypothesis that ASD may be underdiagnosed in the 3q29Del population. Five additional participants with a clinically significant SRS score did not qualify for an ASD diagnosis, suggesting that the SRS is not selectively identifying children with ASD in

participants with 3q29Del, possibly due to the high rates of reported anxiety in our study population. However, this comparison does suggest that our analysis, though based on self-report data, reveals valid conclusions about behavioral phenotypes in 3q29 deletion syndrome. For genetic syndromes with low population frequencies, data collection through remote means such as online patient registries remains a valuable phenotyping tool.

While the current understanding of the 3q29 deletion is still evolving, there are more well-understood CNV disorders that can be used as a comparison point to determine whether the social disability phenotypes described in this study are distinct to 3q29Del. These include Williams Syndrome (WS, or the 7q11.23 deletion), the reciprocal 7q11.23 duplication, 16p11.2 deletion and duplication, Smith-Magenis Syndrome (SMS), and 22q11.2 deletion. WS is typically associated with hyper-sociability (63), and patients with WS show more problems with social cognition than with pro-social behaviors (64), similar to what we have observed in our population of individuals with 3q29Del. However, the prevalence of restricted interests and repetitive behaviors appears to be lower in WS as compared to 3q29Del (64), and the mean SRS sub-scale Social Motivation score indicates enhanced social motivation in WS as compared to 3q29Del (WS mean T-score = 55.24, 3q29Del mean T-score = 62.1, $p = 0.0005$) (65). Studies of the reciprocal 7q11.23 duplication showed that parent-reported ASD symptomology via standardized questionnaires was higher than ASD features as assessed by gold-standard instruments; that some probands had been diagnosed with ASD based on delayed speech and social anxiety but did not qualify for ASD via gold-standard measures; that substantially more males than females qualified for an ASD diagnosis; and that 7q11.23 duplication probands were indistinguishable from children with idiopathic ASD on measures of ASD severity and diagnosis status (55, 66, 67). This is qualitatively different from our 3q29Del population; all of the participants with a prior ASD diagnosis who were later assessed by the Emory 3q29 Project team had their diagnosis confirmed using gold-standard measures (Table S2-12), the male:female ratio in our sample is 2:1, and we see significant differences between 3q29Del cases and idiopathic ASD (42) on the SRS Social Motivation sub-scale.

Similar to 7q11.23 duplication, ASD probands with 16p11.2 deletion or duplication were indistinguishable from idiopathic ASD probands (67); probands with 16p11.2 deletion also have a significantly higher mean SRS score as compared to 3q29Del (16p11.2 mean T-score = 77.8, 3q29Del mean T-score = 71.8, p = 0.003) (22), and males with 16p11.2 deletion are at increased risk for ASD compared to females and are overrepresented when cases are ascertained based on neurodevelopmental disorders (68, 69), indicating a different sex-based ASD risk as compared to 3q29Del. A study of 16p11.2 duplication probands found that scores on the SRS Social Motivation sub-scale were not significantly different from controls and that ASD cases had specific impairments in social cognition and communication (70); 3q29Del cases score significantly higher than controls on the SRS Social Motivation sub-scale, and do not have substantially higher scores on the Social Cognition or Social Communication sub-scales relative to the other SRS sub-scales.

A recent study of SMS showed that female probands scored higher than males on SRS sub-scales and the sex ratio of ASD was reversed, with more females than males qualifying for a diagnosis (71), which we do not observe in our 3q29Del study population. Finally, studies of 22q11.2 deletion show some similarities with 3q29Del, including SRS total scores that are not significantly different, high levels of ASD features in the absence of ASD diagnosis, and a male:female ASD ratio of approximately 1:1 (19, 57, 58, 72); however, 22q11.2 deletion probands have a significantly lower mean ASSQ score as compared to 3q29Del (22q11.2 mean = 11, 3q29Del mean = 22.2, p = 0.00004), and 3q29Del cases have significantly higher scores on several CBCL/ABCL sub-scales (Table S2-13) (73, 74). Taken together, this evidence suggests that while the ASD features in 3q29Del reported in this study share some characteristics with other CNV disorders, the complete constellation of symptoms is discrete from previously described genomic syndromes.

There are significant strengths of this study as compared to previous studies of 3q29Del. First, this is the largest cohort of individuals with 3q29Del ever assembled. This is a critical step in capturing the true phenotypic spectrum associated with the 3q29 deletion. Our use of standardized questionnaires allowed for comparison between ASD features present in 3q29Del and those reported in idiopathic ASD

and ASD in other CNV disorders. Additionally, our online patient registry allows for remote data collection, which has enabled us to expand our sample size. This study has shown that high-quality, comprehensive medical history and symptomology data can be collected through an online patient registry, effectively reducing the patient-ascertainment burden associated with studying rare disorders. Taken together, these attributes make the present study an excellent complement to previously published case reports on individuals with 3q29Del; by capturing a larger patient base with systematic assessments, we are able to more accurately measure the presence of a variety of neuropsychiatric and neurodevelopmental phenotypes associated with the 3q29 deletion. The findings reported here indicate that comprehensive neuropsychiatric and neurodevelopmental assessments with gold standard tools are merited for individuals diagnosed with 3q29Del, and that such assessments should be the standard of care for this patient population.

CONCLUSIONS

The present study confirms previous reports of phenotypes in 3q29Del, as well as expanding the spectrum of behavioral phenotypes associated with the deletion. We found that individuals with 3q29Del report a significantly higher prevalence of ASD diagnosis than the general population, and significantly elevated scores on the SRS, SCQ, ASSQ, and CBCL/ABCL irrespective of ASD diagnosis indicate significant social disability overall in our study population. Further, 3q29Del participants showed a distinct profile of ASD-related phenotypes on the SRS sub-scales, marked by less impaired scores on the Social Motivation sub-scale and extremely high scores on the Restricted Interests and Repetitive Behaviors sub-scale. This score profile is consistent between 3q29Del males and females and between 3q29Del participants with and without ASD, suggesting that it may be a hallmark behavioral feature of the syndrome and providing a potential therapeutic inroad for the treatment of individuals with 3q29Del. Finally, we identify a high degree of social disability in female 3q29Del participants; the 3q29 deletion elevates the risk ASD in females (OR=41.8, p=4.78E-05) more substantially than in males (OR=24.6, p=6.06E-09). These results demonstrate that there is a benefit to studying rare CNVs such as 3q29Del;

studying a single genomic variant with large effect allows us to control for genetic etiology and unmask the mechanisms underlying the development of neuropsychiatric and neurodevelopmental disorders.

Table 2-1: Characteristics of study participants with 3q29Del and controls.

	3q29 Deletion Syndrome	Control	P value
Age, years (mean ± SD)	10.0 ± 8.6	9.9 ± 7.2	0.945
Sex (n, %)			0.521
Male	54 (58.1%)	33 (51.6%)	
Female	39 (41.9%)	31 (48.4%)	
Race (n, %)			0.0003
White	81 (87.1%)	41 (64.1%)	
Black/African American	2 (2.2%)	12 (18.8%)	
Other	10 (10.8%)	9 (14.1%)	
Blank	0 (0%)	2 (3.1%)	
Heart Defect (n, %)			2.37E-07
Yes	27 (29.0%)	2 (3.1%)	
No	54 (58.1%)	61 (95.3%)	
Blank	12 (12.9%)	1 (1.6%)	
Age at Walking (n, %)			2.16E-09
Normal	42 (45.7%)	60 (93.8%)	
Delayed	23 (25.0%)	1 (1.6%)	
Extremely Delayed	12 (13.0%)	1 (1.6%)	
Unsure	10 (10.9%)	2 (3.1%)	
Not applicable	5 (5.4%)	0 (0%)	

Demographic data collected from the custom Medical & Demographic Questionnaire completed by participants upon enrollment in the online 3q29 Registry. P values were calculated with Student's t-test (age), Fisher's exact test (race, heart defect, age at walking), or Pearson's chi square test (sex).

Table 2-2: Self-reported neuropsychiatric diagnoses.

		3q29 Deletion Syndrome			Control			P value; 3q29Del vs. Control
		Total	Male	Female	Total	Male	Female	
GDD/MR (n, %)								<2.20E-16
	Yes	55 (59.1%)	31 (57.4%)	24 (61.5%)	1.14%*	1.48%*	0.90%*	
	No	38 (40.9%)	23 (42.6%)	15 (38.5%)	64 (100%)	33 (100%)	31 (100%)	
ASD (n, %)								<2.20E-16
	Yes	27 (29.0%)	20 (37.0%)	7 (17.9%)	1.47%*	2.34%*	0.52%*	
	No	66 (71.0%)	34 (63.0%)	32 (82.1%)	64 (100%)	33 (100%)	31 (100%)	
Anxiety (n, %)								0.001
	Yes	26 (28.0%)	15 (27.8%)	11 (28.2%)	4 (6.2%)	2 (6.1%)	2 (6.5%)	
	No	67 (72.0%)	39 (72.2%)	28 (71.8%)	60 (93.8%)	31 (93.9%)	29 (93.5%)	
Bipolar/Manic Depression (n. %)								0.146
	Yes	4 (4.3%)	2 (3.7%)	2 (5.1%)	0 (0%)	0 (0%)	0 (0%)	
	No	89 (95.7%)	52 (96.3%)	37 (94.9%)	64 (100%)	33 (100%)	31 (100%)	
Conduct Disorder (n, %)								1.00
	Yes	1 (1.1%)	1 (1.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	No	92 (98.9%)	53 (98.1%)	39 (100%)	64 (100%)	33 (100%)	31 (100%)	
Depression (n, %)								1.00
	Yes	6 (6.5%)	2 (3.7%)	4 (10.3%)	4 (6.2%)	4 (12.1%)	0 (0%)	
	No	87 (93.5%)	52 (96.3%)	35 (89.7%)	60 (93.8%)	29 (87.9%)	31 (100%)	
Oppositional Defiant Disorder (n, %)								0.271
	Yes	3 (3.2%)	2 (3.7%)	1 (2.6%)	0 (0%)	0 (0%)	0 (0%)	
	No	90 (96.8%)	52 (96.3%)	38 (97.4%)	64 (100%)	33 (100%)	31 (100%)	

Panic Attacks (n, %)							0.045
	Yes	12 (12.9%)	9 (16.7%)	4 (10.3%)	2 (3.2%)	0 (0%)	2 (6.5%)
	No	81 (87.1%)	45 (83.3%)	35 (89.7%)	62 (96.8%)	33 (100%)	29 (93.5%)
Schizophrenia (n, %)							0.146
	Yes	4 (4.3%)	1 (1.9%)	3 (7.7%)	0 (0%)	0 (0%)	0 (0%)
	No	89 (95.7%)	53 (98.1%)	36 (92.3%)	64 (100%)	33 (100%)	31 (100%)

Characteristics of self-reported neuropsychiatric diagnoses in study participants with 3q29Del and controls. Asterisks indicate where 3q29Del was compared to general population prevalence values (41, 75). P values were calculated with one-sample proportion test with Yates' continuity correction when comparing to population prevalence and Fisher's exact test when comparing to controls.

Table 2-3: SRS sub-scale score comparison stratified by genotype, ASD status, and sex.

	Social Awareness		Social Cognition		Social Communication		Social Motivation		RRB		SCI	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Genotype												
Control	47.04 ± 8.88	-	45.27 ± 7.64	-	45.88 ± 8.14	-	46.13 ± 7.66	-	47.66 ± 8.51	-	45.50 ± 7.74	-
3q29Del	67.33 ± 13.28	1.45E-13	69.06 ± 15.51	1.20E-15	69.69 ± 13.92	<2.00E-16	62.10 ± 13.52	1.62E-10	77.31 ± 14.25	<2.00E-16	69.52 ± 14.63	<2.00E-16
Sex												
Male control	45.97 ± 9.15	-	45.23 ± 7.42	-	45.73 ± 6.34	-	46.57 ± 6.58	-	47.63 ± 5.59	-	45.37 ± 6.60	-
Male 3q29Del	69.92 ± 13.92	7.61E-09	71.08 ± 17.65	1.07E-08	72.12 ± 15.47	1.74E-10	63.92 ± 15.23	3.39E-06	79.92 ± 14.82	2.84E-13	72.00 ± 16.34	7.12E-10
Female control	48.27 ± 8.56	-	45.31 ± 8.03	-	46.04 ± 9.95	-	45.62 ± 8.85	-	47.69 ± 11.08	-	45.65 ± 9.01	-
Female 3q29Del	64.27 ± 12.08	4.52E-06	66.68 ± 12.51	1.29E-08	66.82 ± 11.52	2.99E-08	59.95 ± 11.14	1.36E-05	74.23 ± 13.22	1.30E-09	66.59 ± 12.02	1.87E-08
ASD Status												
Control	47.04 ± 8.88	-	45.27 ± 7.64	-	45.88 ± 8.14	-	46.13 ± 7.66	-	47.66 ± 8.51	-	45.50 ± 7.74	-
No ASD diagnosis 3q29Del	62.61 ± 13.20	5.17E-09	64.10 ± 15.80	5.77E-11	64.61 ± 13.54	1.20E-12	58.00 ± 13.15	1.42E-06	70.58 ± 11.50	<2.00E-16	64.13 ± 14.39	5.19E-12
ASD diagnosis 3q29Del	75.94 ± 8.33	2.43E-15	78.12 ± 10.18	<2.00E-16	78.94 ± 9.18	<2.00E-16	69.59 ± 10.99	5.12E-12	89.59 ± 10.04	<2.00E-16	79.35 ± 9.02	<2.00E-16

Comparison of mean scores on the SRS sub-scales between study participants with 3q29Del and controls. 3q29Del participants were stratified by ASD status and sex for further analysis. P values were calculated using simple linear regression, adjusting for age, race, and sex.

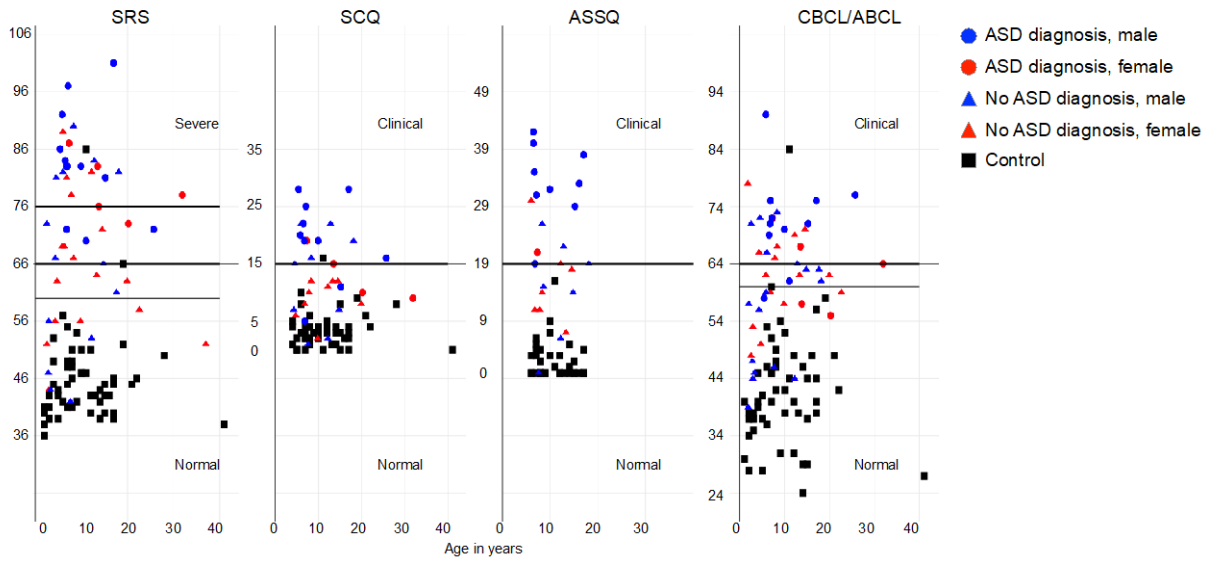

Figure 2-1. Score distribution for 3q29Del and controls on the SRS, SCQ, ASSQ, and CBCL/ABCL. Total scores on the SRS (n=48 3q29Del, 56 control), SCQ (n=33 3q29Del, 46 control), ASSQ (n=24 3q29Del, 35 control), and CBCL/ABCL (n=48 3q29Del, 57 control) for registry participants. Self-reported diagnosis of ASD is denoted by shape (circle/ASD, triangle/no ASD), and sex of participant is denoted by color (red/female, blue/male). Controls are shown in black.

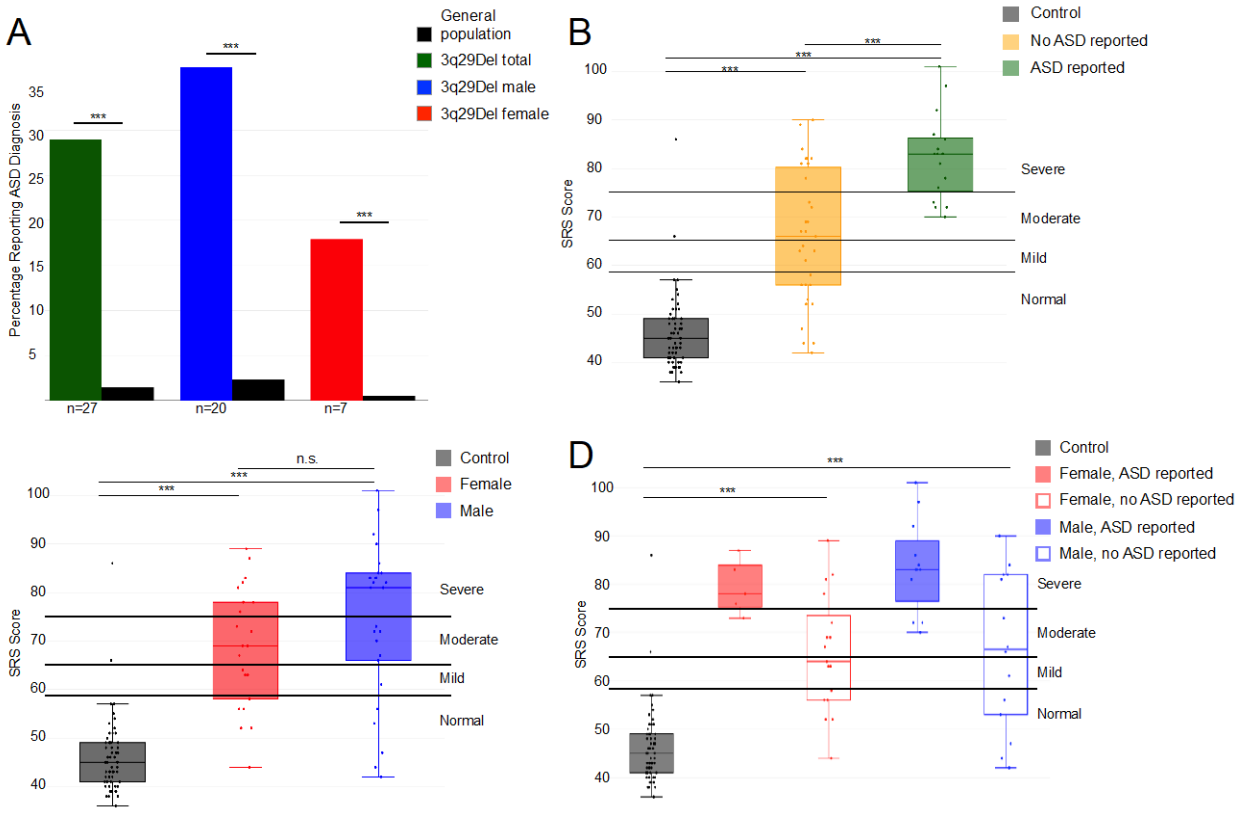

Figure 2-2. Comparison of ASD prevalence and SRS scores between 3q29Del and controls. A) Proportion of participants with 3q29Del self-reporting a diagnosis of ASD (not all respondents completed symptom questionnaires); 27 cases report an ASD diagnosis (green), comprised of 20 males (blue) and 7 females (red). Compared to general population frequencies (black), cases report significantly higher incidence of ASD. **B)** SRS scores split by control (n=59), 3q29Del not reporting an ASD diagnosis (n=31), and 3q29Del reporting an ASD diagnosis (n=17), showing a significant association between self-reported diagnostic status and SRS score. **C)** SRS scores split by sex, with control (n=59), 3q29Del female (n=22), and 3q29Del male (n=26), showing a lack of sex bias in scores for 3q29Del participants. **D)** SRS scores split by sex and self-reported diagnostic status, with control (n=59), 3q29Del female reporting ASD (n=5), 3q29Del female not reporting ASD (n=17), 3q29Del male reporting ASD (n=12), and 3q29Del male not reporting ASD (n=14), showing inflated scores for 3q29Del participants irrespective of sex or diagnostic status. ***, $p<0.001$

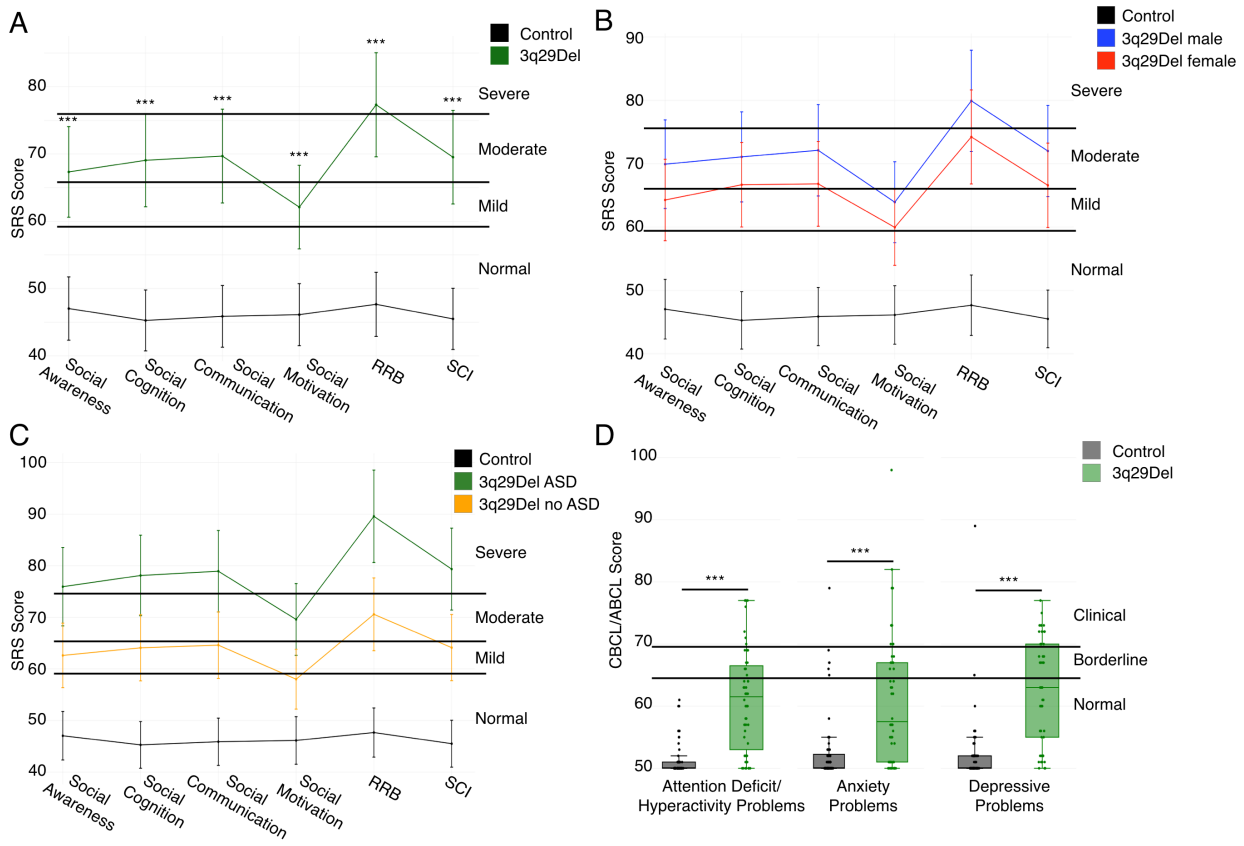

Figure 2-3. Comparison of SRS sub-scales and CBCL/ABCL DSM-oriented sub-scales between 3q29Del and controls. A) Profile of individuals with 3q29Del (n=48) and controls (n=59) across SRS sub-scales, showing moderate to severe impairment of 3q29Del participants in all domains except Social Motivation (RRB, Restricted Interests and Repetitive Behaviors; SCI, Social Communication and Interaction). **B)** Profile of 3q29Del males (n=26) and females (n=22) and controls (n=59) across SRS sub-scales, showing that 3q29Del males and females both score significantly higher than controls and that there are no significant differences in score between males and females. **C)** Profile of 3q29Del participants reporting an ASD diagnosis (n=17) and participants not reporting an ASD diagnosis (n=31) and controls (n=59) across SRS sub-scales, showing that 3q29Del participants score significantly higher than controls irrespective of ASD status, with 3q29Del participants reporting an ASD diagnosis scoring significantly higher than those not reporting an ASD diagnosis. **D)** Profile of 3q29Del participants (n=48)

and controls (n=57) across 3 DSM-oriented sub-scales from the CBCL and ABCL, showing significantly increased pathology in 3q29Del participants in all 3 domains. ***, $p<0.001$

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Supplemental information

Table S2-1: Questionnaire demographics. Characteristics of study participants with 3q29Del and controls completing each questionnaire utilized in the present study.

	Medical and Demographic Questionnaire		Social Responsiveness Scale (SRS)		Social Communication Questionnaire (SCQ)		Autism Spectrum Screening Questionnaire (ASSQ)		Achenbach Behavior Checklists (CBCL/ABCL)	
	3q29Del	Control	3q29Del	Control	3q29Del	Control	3q29Del	Control	3q29Del	Control
Age, years (mean ± SD)	10.0 ± 8.6	9.9 ± 7.2	10.7 ± 7.6	10.4 ± 7.3	11.2 ± 6.5	11.8 ± 6.9	10.4 ± 3.9	10.9 ± 3.7	9.8 ± 6.8	9.5 ± 7.0
Sex (%, n)										
Male	58.1% (54)	51.6% (33)	54.2% (26)	53.6% (30)	57.6% (19)	52.2% (24)	66.7% (16)	48.6% (17)	58.3% (28)	49.1% (28)
Female	41.9% (39)	48.4% (31)	45.8% (22)	46.4% (26)	42.4% (14)	47.8% (22)	33.3% (8)	51.4% (18)	41.7% (20)	50.9% (29)

Sensitivity analysis testing for effect of self-registrants

To examine the effect of self-registrants on all analyses, we stratified participants based on registration status. Of 93 total 3q29Del cases, 3 were self-registered (3.2%) and 90 were parent-registered (96.8%). Of 64 total controls, 0 were self-registered (0%) and 64 were parent-registered (100%). Self-registrants in the study population were considered to have a significant effect on the results if the estimates for any analysis were changed by 10% or more, or if the conclusions from any analyses changed. All analyses were conducted identically when comparing the complete data and the stratified data, as outlined in Methods.

Table S2-2: Sensitivity analysis for self-reposted neuropsychiatric diagnoses. Comparison of self-reported diagnoses in the full 3q29Del dataset versus the reduced dataset with self-registrants removed. Asterisks indicate where 3q29Del was compared to general population prevalence values (41, 75). P values were calculated with one-sample proportion test with Yates' continuity correction when comparing to population prevalence and Fisher's exact test when comparing to controls.

		3q29Del Prevalence	P value; 3q29Del vs. Control
GDD/MR*			
	Full dataset	55 (59.1%)	<2.2E-16
	Reduced dataset	55 (61.1%)	<2.2E-16
ASD*			
	Full dataset	27 (29.0%)	<2.2E-16
	Reduced dataset	26 (28.9%)	<2.2E-16
Anxiety			
	Full dataset	26 (28.0%)	0.0007
	Reduced dataset	25 (27.8%)	0.0007
Bipolar/Manic Depression			
	Full dataset	4 (4.3%)	0.146
	Reduced dataset	3 (3.3%)	0.267
Conduct Disorder			
	Full dataset	1 (1.1%)	1.00
	Reduced dataset	1 (1.1%)	1.00
Depression			
	Full dataset	6 (6.5%)	1.00
	Reduced dataset	5 (5.6%)	1.00
Oppositional Defiant Disorder			
	Full dataset	3 (3.2%)	0.271
	Reduced dataset	3 (3.3%)	0.267
Panic Attacks			
	Full dataset	12 (12.9%)	0.045
	Reduced dataset	12 (13.3%)	0.044
Schizophrenia			
	Full dataset	4 (4.3%)	0.144
	Reduced dataset	4 (4.4%)	0.142

Table S2-3: Sensitivity analysis for symptomology questionnaire scores. Comparison of estimates and p values for the contribution of the 3q29 deletion versus controls to scores on the SRS, SCQ, ASSQ, and CBCL/ABCL for the full dataset and the reduced dataset with self-registrants removed. P values were calculated using simple linear regression, adjusting for age, race, and sex.

		Estimate	P value
SRS			
	Full dataset	26.38	<2.00E-16
	Reduced dataset	26.35	<2.00E-16
SCQ			
	Full dataset	10.71	2.40E-13
	Reduced dataset	10.80	3.44E-13
ASSQ			
	Full dataset	19.61	2.19E-12
	Reduced dataset	19.61	2.19E-12
CBCL/ABCL			
	Full dataset	20.58	4.30E-16
	Reduced dataset	20.62	7.55E-16

Self-registrants did not have a significant effect on the conclusions from this study; none of the estimates changed by more than 10%, and the conclusion from each analysis was consistent for the full and reduced datasets.

Table S2-4: Symptomology questionnaire score comparison. Comparison of mean scores on each symptom questionnaire between study participants with 3q29Del and controls. P values were calculated using simple linear regression, adjusting for age, race, and sex.

Scale	3q29Del (mean ± SD)	Control (mean ± SD)	P value
SRS	71.8 ± 14.6	45.9 ± 8.0	<2.00E-16
SCQ	13.9 ± 7.4	3.5 ± 3.1	2.40E-13
ASSQ	22.2 ± 11.4	2.2 ± 3.4	2.19E-12
CBCL/ABCL	62.5 ± 10.4	41.8 ± 9.8	4.30E-16

Table S2-5: SRS score comparison stratified by ASD status and sex. Comparison of mean scores on the SRS between study participants with 3q29Del stratified by ASD status and sex to controls (mean ± SD = 45.9 ± 8.0). P values were calculated using simple linear regression, adjusting for age.

	Mean ± SD	P value
Sex		
Male control	45.80 ± 6.38	-
Male 3q29Del	74.31 ± 15.97	7.70E-11
Female control	46.04 ± 9.62	-
Female 3q29Del	68.73 ± 12.40	7.42E-09
ASD Status		
Control	45.91 ± 7.97	-
No ASD diagnosis 3q29Del	65.90 ± 13.87	2.16E-13
ASD diagnosis 3q29Del	82.41 ± 8.70	<2.00E-16
ASD Status and Sex		
Male control	45.80 ± 6.38	-
Male 3q29Del, no ASD diagnosis	66.29 ± 16.17	4.33E-07
Male 3q29Del, ASD diagnosis	83.67 ± 9.63	1.13E-13
Female control	46.04 ± 9.62	-
Female 3q29Del, no ASD diagnosis	65.59 ± 12.17	1.60E-07
Female 3q29Del, ASD diagnosis	79.40 ± 5.59	3.07E-08

Table S2-6: SCQ score comparison stratified by ASD status and sex. Comparison of mean scores on the SCQ between study participants with 3q29Del stratified by ASD status and sex to controls (mean ± SD = 3.5 ± 3.1). P values were calculated using simple linear regression, adjusting for age.

		Mean ± SD	P value
Sex			
	Male control	3.38 ± 2.14	-
	Male 3q29Del	16.00 ± 8.33	7.64E-08
	Female control	3.68 ± 3.97	-
	Female 3q29Del	11.00 ± 4.76	4.44E-07
ASD Status			
	Control	3.52 ± 3.12	-
	No ASD diagnosis 3q29Del	11.16 ± 6.53	2.17E-08
	ASD diagnosis 3q29Del	17.57 ± 7.05	3.78E-14
ASD Status and Sex			
	Male control	3.38 ± 2.14	-
	Male 3q29Del, no ASD diagnosis	12.33 ± 8.25	1.67E-04
	Male 3q29Del, ASD diagnosis	19.30 ± 7.27	1.19E-08
	Female control	3.68 ± 3.97	-
	Female 3q29Del, no ASD diagnosis	10.10 ± 4.72	1.65E-05
	Female 3q29Del, ASD diagnosis	13.25 ± 4.65	6.71E-06

Table S2-7: CBCL/ABCL DSM-oriented sub-scale score comparison. Comparison of mean scores on the CBCL/ABCL DSM-oriented attention deficit/hyperactivity problems, anxiety problems, and depressive problems sub-scales between 3q29Del participants and controls. P values were calculated using simple linear regression, adjusting for age, race, and sex.

DSM-oriented sub-scale	3q29Del (mean ± SD)	Control (mean ± SD)	P value
Attention deficit/hyperactivity Problems	60.96 ± 8.17	51.30 ± 2.63	4.70E-13
Anxiety Problems	60.94 ± 10.67	52.93 ± 5.95	1.74E-05
Depressive Problems	62.65 ± 8.41	52.28 ± 5.69	1.65E-11

Table S2-8: Contribution of congenital heart defects to phenotypes of interest. Examination of the relationship between congenital heart defects and self-reported ASD and GDD/MR diagnoses and age at walking within 3q29Del cases. P values were calculated using logistic (ASD, GDD/MR) and ordinal (age at walking) regressions.

Outcome	Covariate	Estimate	P value
ASD			
	Heart defect	0.02	0.967
	Sex	0.79	0.152
	Age	0.03	0.318
	Race	1.25	0.259
GDD/MR			
	Heart defect	-0.52	0.278
	Sex	-0.005	0.992
	Age	-0.0006	0.984
	Race	0.46	0.513
Age at walking			
	Heart defect	0.25	0.648
	Sex	0.22	0.641
	Age	-0.04	0.291
	Race	-0.24	0.733

Table S2-9: Test for confounding factors contributing to symptomology questionnaire scores.

Possible confounding factors for the increased symptom questionnaire scores observed in 3q29Del participants. With the exception of the presence of heart defects being significantly associated with the SCQ and CBCL/ABCL scores, no other confounders were significantly associated with scores. P values were calculated using simple linear models; separate models were run for each predictor-scale pair, with the exception of the age at walking variable (comparing both "delayed" and "extremely delayed" to "normal" within the same model). All models were run controlling for age, race, and sex

Scale	Predictor	Estimate	P value
SRS			
	Heart defect	6.66	0.200
	GDD/MR	6.72	0.135
	Age at walking (Delayed)	3.66	0.504
	Age at walking (Extremely delayed)	6.83	0.373
SCQ			
	Heart defect	6.93	0.039
	GDD/MR	0.61	0.835
	Age at walking (Delayed)	-0.56	0.867
	Age at walking (Extremely delayed)	5.44	0.230
ASSQ			
	Heart defect	7.46	0.253
	GDD/MR	4.31	0.487
	Age at walking (Delayed)	-3.52	0.602
	Age at walking (Extremely delayed)	-2.28	0.799
CBCL/ABCL			
	Heart defect	7.15	0.043
	GDD/MR	2.91	0.376
	Age at walking (Delayed)	1.85	0.644
	Age at walking (Extremely delayed)	2.13	0.682

Table S2-10: CBCL/ABCL Withdrawn sub-scale score comparison. Comparison of mean scores on the CBCL/ABCL Withdrawn sub-scale between 3q29Del participants and controls (mean ± SD = 52.3 ± 5.8). 3q29Del participants were stratified by ASD status and sex for further analysis. P values were calculated using simple linear regression, adjusting for age, race, and sex.

		Mean ± SD	P value
Genotype			
	Control	52.30 ± 5.80	-
	3q29Del	62.35 ± 8.84	3.11E-10
Sex			
	Male control	52.00 ± 3.74	-
	Male 3q29Del	63.04 ± 10.35	1.24E-05
	Female control	52.59 ± 7.33	-
	Female 3q29Del	61.40 ± 6.27	1.02E-05
ASD Status			
	Control	52.30 ± 5.80	-
	No ASD diagnosis	60.56 ± 7.98	4.39E-07
	ASD diagnosis	65.94 ± 9.62	3.77E-09

Table S2-11: Heart defects present in study sample. Types of heart defects reported by both 3q29Del and control study participants. 27 total 3q29Del participants reported heart defects: one participant reported atrial septal defect and mitral valve regurgitation; one participant reported hypoplastic left heart syndrome, interrupted aortic arch/ventricular septal defect, and single ventricle anomalies; two participants reported atrial septal defect and pulmonary valvar stenosis; one participant reported atrial septal defect, pulmonary atresia, and tricuspid valve regurgitation; one participant reported atrial septal defect and pulmonary atresia; one participant reported pulmonary valvar stenosis and ventricular septal defect; one participant reported atrial septal defect and ventricular septal defect; and one participant reported atrial septal defect, patent ductus arteriosus, pulmonary valvar stenosis, pulmonary valve regurgitation, and tricuspid valve regurgitation.

Type of defect	3q29Del	Control
Aortic valvar stenosis	2	0
Atrial septal defect	7	0
Atrioventricular septal defect (or atrioventricular canal defect)	1	0
Hypoplastic left heart syndrome	1	0
Interrupted aortic arch/Ventricular septal defect	1	0
Mitral valve regurgitation	1	0
Patent ductus arteriosus	8	0
Pulmonary atresia	3	0
Pulmonary valvar stenosis	5	0
Pulmonary valve regurgitation	1	0
Single ventricle anomalies	1	0
Tricuspid valve regurgitation	2	0
Ventricular septal defect	2	0
Unsure/Not indicated	6	2

Table S2-12: Comparison of 3q29 registry-leveraged and gold-standard phenotyping measures. SRS total scores and category (Normal, Mild, Moderate, Severe) for 16 (56.25% male) study participants with 3q29Del, with parent-reported ASD diagnosis in the Medical & Demographic Questionnaire via the online 3q29 registry, and ASD diagnosis as determined by gold-standard direct evaluation by members of the Emory 3q29 Project team. Note that all parent-reported ASD diagnoses are supported by direct, in-person phenotyping, with one participant (3558) qualifying for a new ASD diagnosis after gold-standard phenotyping.

Subject ID	Sex	Age (years)	SRS total score	SRS category	Parent-reported ASD diagnosis	Gold-standard ASD diagnosis
3557	Female	17.5	83	Severe	Yes	Yes
3563	Female	21.17	73	Moderate	Yes	Yes
3548	Male	14	70	Moderate	Yes	Yes
3558	Male	10.83	90	Severe	No	Yes
3678	Male	16.08	81	Severe	Yes	Yes
3600	Female	10.5	56	Normal	No	No
3607	Female	8.67	63	Mild	No	No
3627	Female	6.08	78	Severe	No	No
3658	Female	27.33	54	Normal	No	No
3797	Female	14.92	50	Normal	No	No
3540	Male	7.67	69	Moderate	No	No
3575	Male	15.83	84	Severe	No	No
3582	Male	18.17	66	Moderate	No	No
3590	Male	7.42	56	Normal	No	No
3625	Male	18.67	82	Severe	No	No
3647	Male	9	42	Normal	No	No

Table S2-13: Comparison of CBCL/ABCL sub-scale scores between 3q29Del and 22q11.2 deletion. Comparison of the mean scores for 3q29Del on the CBCL/ABCL Withdrawn, Somatic Complaints, Anxious/Depressed, Social Problems, and Thought Problems sub-scales to those reported in a sample of 22q11.2 deletion probands [71]. The Social Problems sub-scale is only included on the school-age CBCL; the Thought Problems sub-scale is included on the school-age CBCL and ABCL. 3q29Del sample size for each sub-scale is indicated in parentheses.

CBCL/ABCL sub-scale	3q29Del mean (n)	22q11.2 deletion mean	P value
Withdrawn	62.4 (48)	58.2	0.0010
Somatic Complaints	62.0 (48)	57.4	0.0002
Anxious/Depressed	60.3 (48)	57.2	0.0157
Social Problems	65.5 (26)	62.4	0.0053
Thought Problems	67.9 (31)	61.1	0.0004

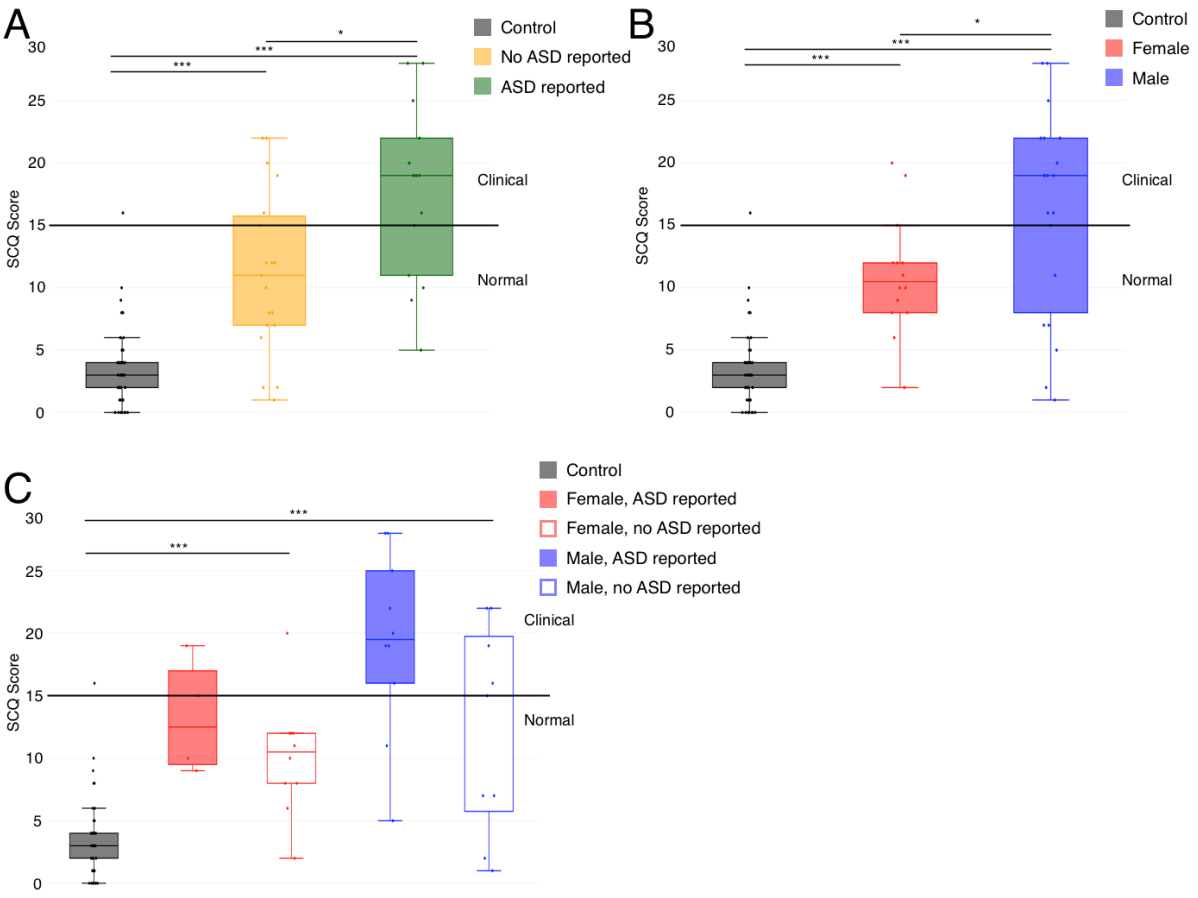

Figure S2-1. Comparison of SCQ scores between 3q29Del and controls, stratified by ASD status and sex. A) SCQ scores split by control (n=46), 3q29Del not reporting an ASD diagnosis (n=19), and 3q29Del reporting an ASD diagnosis (n=14), showing a significant association between self-reported diagnostic status and SRS score. **B)** SCQ scores split by sex, with control (n=46), 3q29Del female (n=14), and 3q29Del male (n=19), showing a lack of sex bias in scores for 3q29Del participants. **C)** SCQ scores split by sex and self-reported diagnostic status, with control (n=46), 3q29Del female reporting ASD (n=4), 3q29Del female not reporting ASD (n=10), 3q29Del male reporting ASD (n=10), and 3q29Del male not reporting ASD (n=9), showing inflated scores for 3q29Del participants irrespective of sex or diagnostic status. *, $p < 0.05$; ***, $p < 0.001$

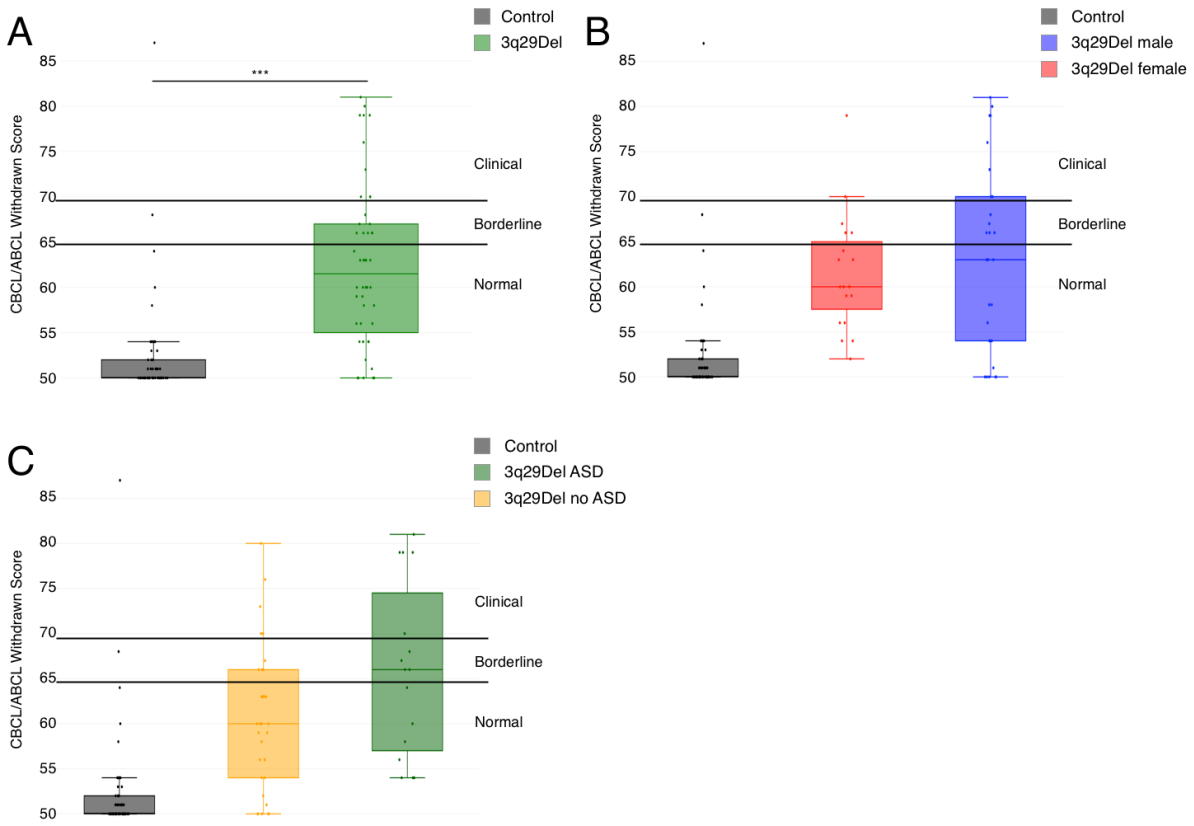

Figure S2-2. Comparison of CBCL/ABCL Withdrawn sub-scale scores between 3q29Del and controls, stratified by ASD status and sex. A) Profile of 3q29Del participants (n=48) and controls (n=57) on the Withdrawn sub-scale from the CBCL and ABCL, showing a significantly higher score in 3q29Del participants, with a mean score for both groups in the normal range. **B)** Profile of 3q29Del males (n=28) and females (n=20) and controls (n=57) on the Withdrawn sub-scale from the CBCL and ABCL, showing that scores are not significantly different between males and females. **C)** Profile of 3q29Del participants reporting an ASD diagnosis (n=16) and not reporting an ASD diagnosis (n=32) and controls (n=57) on the Withdrawn sub-scale from the CBCL and ABCL, showing that scores are not significantly different for 3q29Del participants based on self-reported ASD status. ***, $p < 0.001$

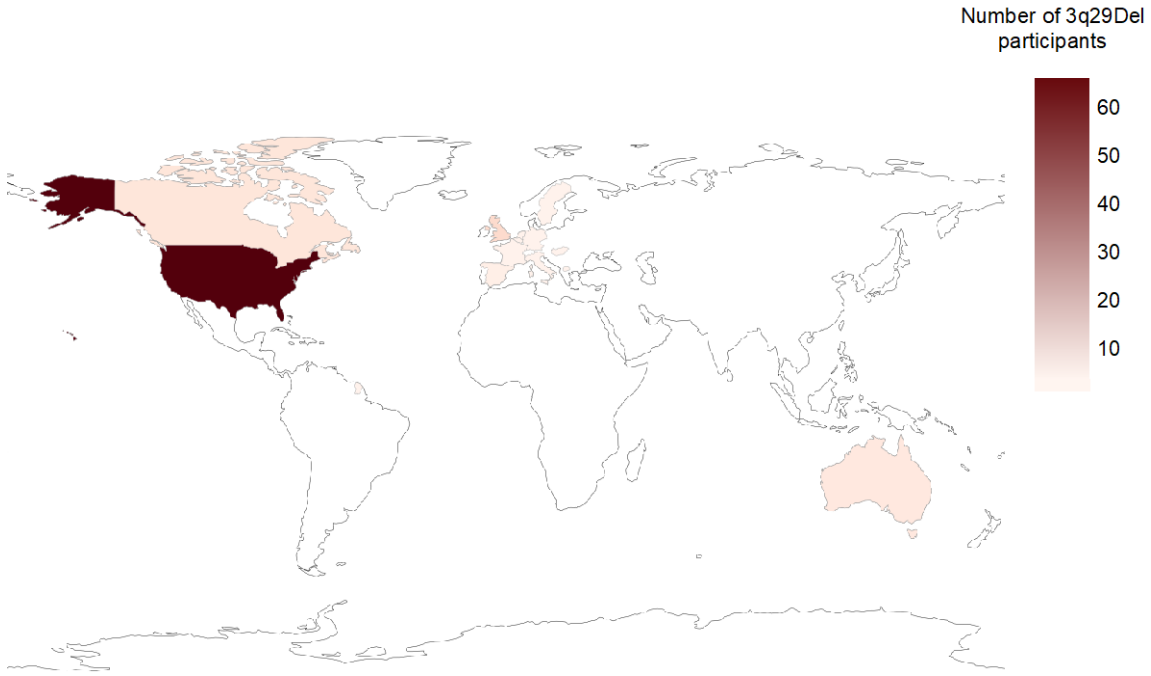

Figure S2-3. Geographic distribution of participants with 3q29Del. Geographic distribution of participants with 3q29Del (n=93). White indicates countries not represented in the present study sample.

Chapter 3. New phenotypes associated with 3q29 duplication syndrome: Results from the 3q29 registry

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INTRODUCTION

3q29 duplication syndrome (3q29dup) is a genomic disorder caused by duplication of a 1.6 Mb interval on human chromosome 3 (GRCh38 chr3: 195,998,000 – 197,623,000). The reciprocal deletion of this interval causes 3q29 deletion syndrome (3q29del). While the breakpoints of the duplication and reciprocal deletion can vary due to the presence of low copy repeats flanking the interval, the canonical interval contains 21 distinct protein-coding genes, 3 antisense transcripts, 3 long noncoding RNAs, and 1 microRNA (1, 2). 3q29dup has been observed as both an inherited and *de novo* event (2-4). The estimated prevalence of 3q29dup from population-based studies ranges from ~1:75,000 to ~1:8,000 (5-9). Studies of individuals from clinical cohorts referred for microarray testing indicate a prevalence of ~1:2,000, suggesting that, with larger sample size, the 3q29 duplication may be more common in cohorts ascertained for neurodevelopmental and neuropsychiatric phenotypes than in unselected populations (2, 7, 9-13). However, the phenotype of 3q29dup is not fully understood; the purpose of the present study is to assess 3q29 duplication-associated phenotypes in a standardized manner.

Case reports of 3q29dup report a range of associated characteristics, including developmental delay, speech delay, intellectual disability (ID), ocular and cardiac anomalies, microcephaly, dental anomalies, obesity, and seizures (2-4, 14-20). Additionally, some case reports have described a disruptive behavioral profile (21) and behavioral similarities to autism spectrum disorder (ASD) (16), and one case report identifies an individual with spina bifida (22). These case reports are based on extremely small samples, commonly of one individual or of related individuals, highlighting the need for additional phenotypic data on 3q29dup collected from larger patient populations. In the largest case series published to date, which reports on 19 individuals with 3q29dup by Ballif et al (2), only five individuals had the canonical 1.6 Mb duplication; the other 14 cases had duplications of sizes varying from 200 kb to 2.4 Mb. Additionally, only seven cases had clinical information (2); based on these factors, it is unclear whether the observed phenotypic heterogeneity is an accurate reflection of heterogeneity in 3q29dup, or if it is largely attributable to the varying duplication sizes within the described cases. For the three subjects with the canonical 1.6 Mb duplication and clinical information, the only common feature was mild/moderate

intellectual disability (2). Based on the genomic heterogeneity at the 3q29 locus in these cases, there is limited ability to draw conclusions based on this study alone. Taken together, the existing case report literature of 3q29dup is not robust enough to appreciate the full range of syndromic phenotypes associated with the canonical 3q29 duplication.

Because the 3q29 duplication can be inherited from apparently unaffected parents, case reports published to date may reflect the extreme end of the phenotypic distribution associated with 3q29dup. Additionally, carriers of 3q29dup can appear phenotypically normal; these factors combined have resulted in a lack of consensus about the clinical significance of the duplication. For example, in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), of 19 submission entries for the 1.6 Mb 3q29 duplication, the variant is classified as pathogenic 13 times (68%) with the remaining entries classified as "Uncertain significance" (n = 5) or with "conflicting data from submitters" (n = 1). This means that genetic testing labs identifying the identical variant may classify it differently, with some labs identifying the 3q29 duplication as pathogenic while others fail to do so, which is confusing for families and clinicians alike – especially in cases where a parent transmitting the duplication is seemingly unaffected. To bridge this knowledge gap and better characterize the syndrome, we have created an internet-based registry for individuals with 3q29dup and 3q29del (https://3q29.com) and implemented standardized instruments for systematic ascertainment of self-reported phenotypes. Here we present results from 31 individuals, the largest cohort of individuals with 3q29dup ever described. We also compare phenotypes to 117 individuals with 3q29del, to identify shared or divergent phenotypes that may be present across the syndromes.

Developing a clearer understanding of the phenotypic spectrum of 3q29dup is crucial for clinicians, caregivers, and the probands themselves, so that evidence-based interventions can be synthesized. Furthermore, these data may provide insight into the potential molecular mechanism and gene dosage effects that have a role in the 3q29 CNVs. Additionally, it will be of clinical utility to determine whether the high rates of neuropsychiatric diagnoses and social disability in 3q29del (23, 24)

are shared or distinct from 3q29dup phenotypes, as this will provide guidance for developing clinical standards of care for this understudied population.

METHODS

Sample

Individuals with 3q29dup were ascertained through the internet-based 3q29 registry (https://3q29.com) as previously reported (23). Briefly, at launch in 2013, information about the registry was emailed to health care providers, medical geneticists, genetic counselors, and support organizations; the registry is currently advertised via Google AdWords, where specific keywords were chosen to target the registry website in internet searches. Participant recruitment, informed consent and assent, and data collection are all performed through the registry website. In April 2019, a data freeze was implemented and existing records were securely downloaded and de-identified for analysis. After data cleaning (removing spam accounts, duplicate records, and individuals with additional significant genetic diagnoses), 31 3q29dup registrants (48.4% male) were included in the present study, ranging in age from 0.3-52.2 years (mean = 10.0 +/- 10.8 years). 117 individuals with 3q29del (55.6% male) were also obtained through the 3q29 registry, ranging in age from 0.1-41.0 years (mean = 9.4 +/- 8.0 years). Clinical diagnosis of 3q29dup or 3q29del was confirmed via review of clinical genetics reports and/or medical records. Data from typically developing controls (n = 64, 51.6% male) ranging in age from 1.0-41.0 years (mean = 10.5 +/- 7.2 years) were obtained as a comparison group (24). Description of the study sample can be found in Table 3-1. This study was approved by Emory University's Institutional Review Board (IRB00064133).

Questionnaires

Upon registration, the participant or his/her parent or caregiver completed a custom medical and demographic questionnaire. This questionnaire includes questions on the sex, birthdate, race, and ethnicity of the participant, as well as a detailed medical history covering seven domains of physical and mental development: birth history, development, ear/nose/throat, gastrointestinal, renal, oral/dental, and seizures/psychiatric (23).

In addition to medical phenotypes, two standardized questionnaires were used to assess ASD-related symptomology and general behavioral problems in the participants. The Social Responsiveness Scale (SRS; preschool, school-age, and adult forms; n = 15 3q29dup, 67 3q29del, 56 controls) is a 65-item, 4 point Likert-scaled questionnaire designed to assess ASD-related symptoms along a normative continuum (25). The Child Behavior Checklist (CBCL) and Adult Behavior Checklist (ABCL) are 100-, 113-, or 126-item (CBCL preschool, CBCL school-age, and ABCL, respectively; n = 15 3q29dup, 64 3q29del, 57 controls), 3 point Likert-scaled questionnaires designed to assess behavioral or developmental problems (26, 27). Data from the CBCL and ABCL were pooled for analysis. All standardized questionnaires were adapted for the online 3q29 registry and were completed by the participant or parent/guardian of the participant upon registration. Some participants were not eligible to complete the standardized questionnaires because the proband was too young. Demographic characteristics of the respondents for each questionnaire can be found in Table S3-1, demonstrating that the average age and sex distribution of participants who completed the medical and demographic questionnaire was not different from the average age and sex distribution of participants who completed each standardized form.

Analysis

Diagnoses and health problems from the medical history questionnaire were recoded for analysis as yes/no binary variables; global developmental delay/intellectual disability (GDD/ID) diagnosis was recoded as yes (reported diagnosis of global developmental delay and/or intellectual disability)/no. Birthweight is coded in 1 lb increments in the online 3q29 registry (https://3q29.com); the midpoint of the interval was assumed as the birth weight of the participant for analysis. Developmental milestones are coded in “bins” of time in the registry; for analysis, it was assumed that the milestone was reached at the midpoint of the selected interval. For milestones marked as “more than 10 years”, it was assumed the participant achieved the milestone at the midpoint between 10 years and their age at registration. For participants who had not yet reached a developmental milestone, their data were treated as censored observations, where time in the study is recorded consistent with age at the time of entry into the registry.

To compare birthweight between 3q29dup cases, 3q29del cases, and controls, linear regression and goodness-of-fit analyses were implemented using the stats R package (28), controlling for sex, gestational age, and race. To compare reported diagnoses between 3q29dup cases and 3q29del cases, Fisher's exact test and chi-squared tests were implemented using the stats R package (28). To compare length of time spent in the hospital between 3q29dup cases and controls, two sample t-test was implemented using the stats R package (28). To compare rates of self-reported seizures and psychiatric diagnoses in 3q29dup cases to population prevalence values, one-sample proportion tests with Yates' continuity correction were implemented using the stats R package (28). Data from standardized questionnaires were imported into R (28) and were recoded and scored according to the publisher's guidelines. To compare standardized questionnaire scores between 3q29dup cases, 3q29del cases, and controls, linear regression was implemented using the stats R package (28), controlling for age, race, and sex. To compare scores in participants with 3q29dup to mean values reported for children with idiopathic ASD (29), one sample t-test was implemented using the stats R package (28). Kaplan-Meyer time-to-event analysis for developmental milestones was implemented using the survival R package (30). Figures were generated using the plotly, ggplot2, and VennDiagram R packages (31-33).

RESULTS

Birth weight

The average birth weight for 31 participants with 3q29dup is 6.50 lbs (2948.35 g), with an average gestational age of 38.1 weeks, as compared to an average birth weight of 7.6 lbs (3447.30 g) and average gestational age of 39.2 weeks in our 64 typically developing controls (Figure 3-1). Gestational age was significantly reduced in 3q29dup cases relative to controls (3q29dup mean = 38.1, control mean = 39.2, $p = 0.04$). After adjusting for gestational age, sex, and race, participants with 3q29dup weigh significantly less than controls ($p = 0.005$), with an effect size of -0.74, indicating that participants with 3q29dup, on average, weigh 0.74 lbs (11.84 oz, 335.66 g) less than control participants at birth. Additionally, goodness-of-fit analysis shows that including genotype (3q29dup vs. control) in the model fits the data significantly better than only including gestational age, sex, and race ($p = 0.005$, Table S3-2).

Due to the fact that the racial makeup is not matched between participants with 3q29dup and controls, we restricted the analysis to those participants that self-identify as white. We find that the magnitude of the effect size for the 3q29 duplication increases slightly, to -0.83 ($p = 0.001$), indicating that within self-identified white registrants, babies with 3q29dup weigh 0.83 lbs (13.28 oz, 376.48 g) less at birth than typically developing controls.

Problems in the first year of life

Participants with 3q29dup spent longer in the hospital immediately after birth, with an average stay of 9.8 days (+/- 14.5 days) as compared to an average of 3.8 days (+/- 6.4 days) for typically developing controls ($p = 0.037$). Consistent with this longer hospital stay, 80.6% of participants with 3q29dup ($n = 25$) reported significant health problems in the first year of life, as compared to 39.1% of controls ($n = 25$). Some of these problems include: feeding problems (54.8%, $n = 17$); failure to gain weight (41.9%, $n = 13$); hypotonia (38.7%, $n = 12$); and respiratory distress (29.0%, $n = 9$). More data on problems reported in the first year of life can be found in Figure 3-2 and Table S3-3.

Delay of developmental milestones

In the 3q29 registry, data are collected on social-emotional, communication, gross motor, and fine motor developmental milestones (23). We used survival analysis to estimate the average time-to-event for developmental milestones for participants with 3q29dup and typically developing controls. One representative milestone was selected for each category; time-to-event curves for participants with 3q29dup and controls are shown in Figure 3-3. For each milestone shown, participants with 3q29dup achieved that milestone on average 10 to 25 months later than typically developing controls ($p < 0.005$); however, the majority of participants do eventually achieve each milestone. A full account of all milestones investigated is available in Table S3-4. Interestingly, while social-emotional, gross motor, and fine motor milestones on average are delayed by a similar amount (10 months, 15 months, and 16 months, respectively), communication milestones are more substantially delayed in participants with 3q29dup, with an average delay of 25 months.

Learning disabilities

One of the most common phenotypes reported in prior studies of 3q29dup is mild to moderate ID (2, 17, 18, 20), with one case report of a child with severe ID (19). Further, developmental delay, speech delay, and learning disabilities have been reported in individuals with 3q29dup (3, 20, 21), suggesting that neurodevelopmental and learning disabilities are common to individuals with 3q29dup. Indeed, in our study population 71.0% (n = 22) of participants report at least one diagnosed learning problem, as compared to 4.7% (n = 3) of controls. Commonly reported early learning problems include expressive language delay (54.8%, n = 17), global developmental delay (41.9%, n = 13), and receptive language delay (29.0%, n = 9); common school-age learning problems include learning disability in math (33.3%, n = 7), and learning disability in reading (33.3%, n = 7). A full account of learning disabilities reported by 3q29dup and control participants can be found in Figure 3-4 and Tables S5A and S5B.

Gastrointestinal phenotypes

While gastrointestinal problems have not been previously reported in individuals with 3q29dup, we find that 54.8% (n = 17) of participants with 3q29dup report at least one gastrointestinal problem (Table S3-6), including feeding problems beyond the first year of life (38.7%, n = 12) and chronic constipation (35.5%, n = 11).

Seizures and neuropsychiatric phenotypes

Seizures: 25.8% (n = 8) of participants with 3q29dup reported seizures, consistent with prior reports of individuals with 3q29dup (15, 16, 19, 20) and significantly elevated relative to the general population (general population prevalence = 1.2%, $p < 2.20E-16$) (34).

Neuropsychiatric diagnosis: 32.3% (n = 10) of participants with 3q29dup reported a diagnosis of anxiety disorder, significantly higher than the general population lifetime prevalence (5.7% vs. 32.3%, $p = 1.05E-09$). 38.7% (n = 12) of our participants with 3q29dup report a clinical diagnosis of ASD, a rate substantially higher than both that reported for 3q29dup in the literature to date and that reported for the general population, with an estimated 26-fold increased risk for ASD for individuals with 3q29dup. Subsets of participants with 3q29dup also report conduct disorder (6.5%, n = 2), depression (16.1%, n = 5), oppositional defiant disorder (6.5%, n = 2), and panic attacks (6.5%, n = 2) (Table S3-7). Additionally,

there is only partial overlap between participants with 3q29dup reporting global developmental delay, ASD, seizures, and anxiety, indicating that these inflated rates are not due to a subset of severely affected participants, but rather are due to increased risks for these disorders in 3q29dup (Figure 3-5).

SRS social disability phenotypes and ASD features

While developmental delay and behavioral similarities to ASD have been identified in 3q29dup (3, 16, 20), social disability phenotypes have not been quantitatively described. Using standardized self-report tools, we find that participants with 3q29dup have significantly higher total SRS scores than typically developing controls (3q29dup mean T-score = 79.1, control mean T-score = 45.9, p = 1.09E-15), indicating that 3q29dup cases have significantly more social disability than typically developing controls. We observe this increased burden of social disability across sexes and ASD status within our participants with 3q29dup; individuals with 3q29dup score significantly higher than controls irrespective of sex (3q29dup female mean T-score = 77.1, control female mean T-score = 46.0, p = 2.22E-07; 3q29dup male mean T-score = 82.0, control male mean T-score = 45.8, p = 1.83E-07) (Figure 3-6A) and ASD status (3q29dup with ASD mean T-score = 87.2, control mean T-score = 45.9, p = 3.3E-12; 3q29dup without ASD mean T-score = 73.7, control mean T-score = 45.9, p = 2.9E-09) (Figure 3-6B) (Table S3-8).

SRS sub-scale profile in 3q29dup

While the SRS total score can give an indication of the overall degree of social impairment for an individual, the SRS sub-scales can provide more detail about specific domains of social functioning that may be compromised. We analyzed all SRS sub-scales (Social Awareness, Social Cognition, Social Communication, Social Motivation, Restricted Interests and Repetitive Behaviors, and Social Communication and Interaction) to determine whether the inflated SRS total scores we observed are attributable to substantial deficits in all domains or functioning, or if individuals with 3q29dup show specific impairments in a few domains. Mean scores for Social Communication (T-score = 77.3), Restricted Interests and Repetitive Behaviors (T-score = 81.5), and Social Communication and Interaction (T-score = 77.3) were in the severe range, while mean scores for Social Awareness (T-score = 72.5), Social Cognition (T-score = 74.5), and Social Motivation (T-score = 71.1) were in the moderate range

(Figure 3-6C, Table 3-2). Notably, participants with 3q29dup have significantly lower Social Motivation scores than those reported in cases of idiopathic ASD (3q29dup Social Motivation T-score = 71.1, idiopathic ASD T-score = 78.4, $p = 0.040$) (29), indicating a preservation of social motivation relative to other domains assessed by the SRS. Participants with 3q29dup scored significantly higher than typically developing controls on all sub-scales ($p < 4.0E-11$) (Table 3-2).

SRS sub-scale profile stratified by sex

We find that males and females with 3q29dup do not score significantly differently from each other on any sub-scale ($p > 0.05$); however, both males and females with 3q29dup score significantly higher than controls ($p < 0.0005$, Table 3-2), similar to our previous finding in 3q29del (24). We note that females with 3q29dup score approximately 2 points higher than males on the Social Awareness sub-scale, while males with 3q29dup score slightly higher than females on all other sub-scales (Figure 3-6D, Figure S3-1); however, a larger sample size is needed to determine the biological relevance of any sex-specific differences.

SRS sub-scale profile stratified by ASD diagnosis

Similar to 3q29del (24), we find that the shape of the SRS sub-score profile is shared between participants with 3q29dup with and without ASD, with participants with 3q29dup reporting an ASD diagnosis scoring on average 10 to 15 points higher on every sub-scale than participants with 3q29dup not reporting an ASD diagnosis (Figure 3-6E). Consistent with participants with 3q29dup having higher total SRS scores than controls irrespective of ASD status, participants with 3q29dup also score in the moderate or severe range, and significantly higher than controls, on all SRS sub-scales irrespective of ASD status ($p < 2.0E-06$, Table 3-2).

CBCL/ABCL behavioral phenotypes

CBCL/ABCL Withdrawn sub-scale

To further investigate social disability phenotypes in 3q29dup, we used the Withdrawn sub-scale of the CBCL and ABCL. Previous studies have shown that individuals with idiopathic ASD, on average, score in the borderline range on this sub-scale, and the majority of individuals score in the borderline or

critical range (35, 36). Here, we find that participants with 3q29dup score significantly higher than controls (3q29dup mean T-score = 66.2, control mean T-score = 52.4; p = 6.3E-08; Table 3-3). The average score for participants with 3q29dup overall, and males and females separately, is in the borderline range (Figure 3-7A and B, Table 3-3). This supports the SRS data and suggests a previously unidentified social disability phenotype in 3q29dup. Over 50% of participants with 3q29dup score in the borderline or clinical range, with similar proportions observed in participants reporting a diagnosis of ASD and those not reporting a diagnosis of ASD (Figure 3-7C, Table 3-3).

CBCL/ABCL DSM-oriented sub-scales

To assess additional behavioral features of 3q29dup, we examined the DSM-oriented Attention Deficit/Hyperactivity Problems, Anxiety Problems, and Depressive Problems sub-scales from the CBCL and ABCL. Participants with 3q29dup score significantly higher than controls on every sub-scale (3q29dup Attention Deficit/Hyperactivity Problems T-score = 63.0, control Attention Deficit/Hyperactivity Problems T-score = 51.3; 3q29dup Anxiety Problems T-score = 64.4, control Anxiety Problems T-score = 53.2; 3q29dup Depressive Problems T-score = 65.8, control Depressive Problems T-score = 52.3; all $p < 0.0005$) (Figure 3-7D). These data suggest additional neuropsychiatric phenotypes are associated with the 3q29 duplication.

Other phenotypes

13 (42%) participants reported ear problems (Table S3-9), 21 (68%) participants reported dental problems (Table S3-10), two participants (6%) reported heart defects, six participants (19%) reported genitourinary phenotypes, one participant (3%) reported renal phenotypes, and one participant (3%) reported cleft palate (Supplemental Information).

Comparison of 3q29dup and 3q29del

Medical phenotypes: To determine whether there is evidence for divergent phenotypes associated with 3q29dup and 3q29del, we compared overall rates of reported problems in the first year of life, heart defects, learning problems, GDD/ID, ear problems, gastrointestinal problems, genitourinary problems, renal problems, dental problems, seizures, and psychiatric diagnoses between participants with 3q29dup

and 3q29del (Table 3-4). Congenital heart defects are reported at a significantly higher rate by participants with 3q29del as compared to participants with 3q29dup (24.8% vs. 6.5%, p = 0.047). Although not statistically significant, participants with 3q29dup reported seizures at a rate substantially greater than participants with 3q29del (25.8% vs. 15.4%, p = 0.276). Rates of all other reported problems were remarkably similar between participants with 3q29dup and 3q29del, suggesting that the reciprocal 3q29 CNVs may have similar effects on organ systems.

Psychiatric phenotypes and social disability: ASD and anxiety disorder are present at a similar frequency in both 3q29dup and 3q29del (38.7% in 3q29dup vs 29.1%% in 3q29del, p = 0.416; 32.3% in 3q29dup vs 28.2% in 3q29del, p = 0.826) (Table S3-7). The 3q29 deletion is established as an ASD-risk variant (24, 37) but it is not known whether social disability phenotypes are similarly present in the 3q29 duplication. Using the SRS, we find that 3q29 Dup participants score similarly to participants with 3q29del (3q29dup mean T-score = 79.1, 3q29del mean T-score = 72.9, p = 0.107) (Figure 3-8A), indicating a similar burden of social disability shared between 3q29dup and 3q29del. The distribution of SRS sub-scores between 3q29 Dup and 3q29 Del is qualitatively similar (Figure 3-8B). However, participants with 3q29dup score significantly higher on Social Motivation than participants with 3q29del (3q29dup Social Motivation T-score = 71.1, 3q29del Social Motivation T-score = 63.5, p = 0.043), indicating that cases with 3q29dup have an intermediate social motivation phenotype, with significantly more impairment than that observed in 3q29del, but significantly less impairment that that reported in idiopathic ASD (29). For the CBCL/ABCL Withdrawn sub-scale, we have previously reported that individuals with 3q29del have mean scores in the normal range on this sub-scale, and that over 50% of individuals with 3q29del reporting a diagnosis of ASD score in the borderline or clinical range (24). Here, we find that participants with 3q29dup and 3q29del both score significantly higher than controls (3q29dup mean T-score = 66.2, control mean T-score = 52.4, p = 6.33E-08; 3q29del mean T-score = 63.2, control mean T-score = 52.4, p = 1.6E-09) and that they do not score significantly differently from each other (p = 0.318). However, the average score for participants with 3q29dup overall, and males and females separately, is in the borderline range (Figure 3-7A and B, Table 3-3), whereas the mean score for participants with 3q29del is in the

normal range, suggesting a more substantial, and previously unidentified, social disability phenotype in 3q29dup as compared to 3q29del.

To determine whether 3q29dup shares some behavioral features with 3q29del, we examined the DSM-oriented Attention Deficit/Hyperactivity Problems, Anxiety Problems, and Depressive Problems sub-scales from the CBCL and ABCL. Both participants with 3q29dup and 3q29del score significantly higher than controls on every sub-scale (3q29dup Attention Deficit/Hyperactivity Problems T-score = 63.0, 3q29del Attention Deficit/Hyperactivity Problems T-score = 61.7, control Attention Deficit/Hyperactivity Problems T-score = 51.3; 3q29dup Anxiety Problems T-score = 64.4, 3q29del Anxiety Problems T-score = 61.4, control Anxiety Problems T-score = 53.2; 3q29dup Depressive Problems T-score = 65.8, 3q29del Depressive Problems T-score = 63.1, control Depressive Problems T-score = 52.3; all $p < 0.0005$), and participants with 3q29dup and 3q29del do not score significantly differently from each other (all $p > 0.282$, Figure 3-8C), suggesting that participants with 3q29dup and 3q29del have shared liability for these neuropsychiatric phenotypes previously associated with 3q29del (23).

DISCUSSION

This study is the first to report on phenotypes associated with 3q29dup using a systematic, standardized approach. We find a high prevalence of problems in the first year of life, including feeding problems, failure to gain weight, hypotonia, and respiratory distress, suggesting that individuals with 3q29 duplication require extra clinical attention during infancy. We also find that seizures, frequently described in case reports of 3q29 duplication syndrome (15, 16, 19, 20), are reported in 25% of our study subjects, thus individuals with 3q29 duplication syndrome should be evaluated by a pediatric neurologist. We find feeding problems and chronic constipation are reliably manifest, such that a pediatric gastroenterologist should administer an evaluation. Our data also suggest that ASD and social disability phenotypes are enriched in 3q29 duplication syndrome, and individuals with the 3q29 duplication should therefore be evaluated for ASD using gold-standard clinical measures.

We have found that the 3q29 duplication registry participants report a substantial reduction in birthweight (0.74 lbs, 11.84 oz, 335.66 g), similar to that previously reported for the reciprocal 3q29 deletion (13.9 oz, 394 g) (23). In 3q29del, unpublished data from human subjects assessed by the Emory 3q29 Project (http://genome.emory.edu/3q29/) (38) show that the weight deficit in 3q29del persists into adolescence and data from two independent 3q29 mouse models document diminished weight as a robust feature in these animal models (39, 40). However, in an apparent paradox, the 3q29 duplication is associated with obesity in childhood through adulthood (2-4, 17, 19), thus 3q29 duplication carriers weigh less at birth but may exhibit accelerated weight gain at an unknown developmental timepoint. Although the 3q29 registry does not collect data on current weight and height for study participants, these data suggest a compelling future direction for longitudinal data collection on weight and height. These findings also support a complex dose-response relationship between 3q29 interval genes and metabolic phenotypes.

Prior to this study, a significant link between 3q29dup and ASD and related behavioral phenotypes had not been established. One case study reported behavioral similarities to ASD (16); however, 3q29dup cases have not been identified to be significantly enriched in cohort studies of ASD (37, 41). It is possible that the population frequency of the 3q29 duplication is low, such that current studies of ASD are underpowered to find association with ASD. As larger cohorts become available, an association between ASD and 3q29dup may become apparent. It is also possible that with existing genomics technologies (such as exome sequencing), duplications are challenging to identify in a research setting and are susceptible to high false-negative rates. Improved analysis methods or new technologies (42) may rectify this problem. It is also possible that our study suffers from ascertainment bias, where individuals with ASD are referred for genetic testing and are coincidentally found to have a 3q29 duplication. However, our own data partially contradict this possibility. In figures 6 and 7, we show that substantial social disability is present even among individuals without a diagnosis of ASD, as assessed by both the SRS and the CBCL. Our data suggest the 3q29 duplication is a susceptibility locus for ASD, and that gold-standard ASD evaluations should be standard of care for individuals with 3q29dup.

We found that the rate of reported ASD diagnoses in our 3q29dup study population is similar to that reported in 3q29del (38.7% vs. 29.1%, p = 0.416). Because this is the first study to use standardized, quantitative measures to assess dimensions of social disability in 3q29dup, we are able to evaluate nuances of social behavior in our study sample. Our findings suggest that the degree of social disability in this population has been underappreciated in the literature. Further, we found a profile of ASD features on the SRS that is similar to the profile we had previously identified in 3q29del (24), suggesting that the 3q29 CNVs harbor similar risk for social disability. Furthermore, in both disorders social disability is present irrespective of ASD diagnosis status. Previous work by our group found that individuals with 3q29del have relatively well-preserved social motivation as assessed by the SRS Social Motivation sub-scale (24); here, we report that individuals with 3q29dup appear to have an intermediate social motivation phenotype, with Social Motivation sub-scale scores significantly higher (more impaired) than 3q29del, but significantly lower (less impaired) than those reported in a study of idiopathic ASD (29). Additionally, we found that participants with 3q29dup, on average, scored in the borderline range for the Withdrawn sub-scale of the CBCL and ABCL, which further supports a previously unappreciated degree of social disability in the 3q29dup population. Taken together, this indicates that, similar to 3q29del, individuals diagnosed with 3q29dup should receive gold-standard ASD evaluations as a standard of care.

Familial cases of 3q29dup have been reported in the literature (2-4); the proportion of 3q29 duplication cases that are inherited is unknown. Anecdotal data from our registry families and prior reports in the literature imply that there are several occurrences where the 3q29 duplication can be inherited from a mildly affected parent. A quantitative estimate of the proportion of 3q29 duplications that are inherited is challenging to obtain because parental genetic testing may not be covered by health insurance, and out of pocket expense for a microarray test may be a significant barrier to testing for many families. Indeed, while most clinical genetics reports for individuals in our study include a recommendation for parental testing and genetic counseling, many of our parents do not follow through in part because they perceive there will be high costs, a high bureaucratic and administrative burden, and hostile interactions with insurance companies for genetic testing to be considered a reimbursable expense.

However, knowledge of a parent's carrier status is critical for genetic counseling, and for informing future reproductive choices. In light of our results about the disability that can be associated with the 3q29 duplication, parent genetic testing and genetic counseling should be standard of care for all families of an individual with 3q29dup and should be covered by health insurance to ensure equal access.

While we identified several similarities between 3q29dup and 3q29del, including the effect of the 3q29 CNV on birthweight, SRS scores, and CBCL/ABCL scores, there are also features where 3q29dup and 3q29del diverge. We found that there is a significantly higher rate of congenital heart defects in 3q29del as compared to 3q29dup (24.8% vs. 6.5%, $p = 0.047$), which is in line with previously published studies of 3q29del and 3q29dup. We also found a higher rate of participants with 3q29dup reporting seizures as compared to participants with 3q29del (25.8% vs. 15.4%, $p = 0.276$); while this difference is not statistically significant, it lends additional support to the association between 3q29dup and seizure phenotypes previously described in case reports (15, 16, 19, 20). The registry asks only about the presence or absence of seizures; collecting more data about seizure phenotypes associated with the 3q29 duplication is an important future direction for this work. While the overall rate of individuals reporting at least one psychiatric diagnosis was similar between 3q29dup and 3q29del (58.1% vs. 48.7%, $p = 0.500$), the rate of each psychiatric disorder varies between 3q29dup and 3q29del (Table S3-7). As ongoing efforts to articulate the molecular mechanism and downstream targets impacted by the 3q29 duplication and 3q29 deletion bear fruit, it will be productive to compare convergent and divergent downstream pathways with the concordant and discordant phenotypic spectra of these reciprocal disorders.

Although there are significant strengths to this study, it is not without limitations. While this is the largest cohort of individuals with 3q29dup reported on to date, the small sample size restricts our ability to draw definitive conclusions due to relatively low statistical power. We found substantial differences in some health domains, most notably seizures, that did not reach statistical significance due to our sample size. Studies with larger sample size will be better able to assess the importance of these differences between 3q29dup and 3q29del. Additionally, all of the data used in this study were collected using parent-report measures, which introduces the potential of recall bias due to the data being

retrospective. However, a previous study by our group using the same measures as the present study found high concordance between parent-reported diagnoses and direct assessment (24). Lastly, there are two potential sources of ascertainment bias in our study. First, our sample of participants with 3q29dup is overwhelmingly white, suggesting that we are not effectively reaching minority populations with our recruitment efforts. Second, we note that parents that register their children and complete time-consuming questionnaires are likely to be highly motivated, potentially because their children are severely affected. If our study sample is taken from the extreme end of 3q29dup phenotypes, scores on the SRS and CBCL/ABCL and reported health problems and diagnoses are likely to be inflated as compared to the true prevalence in the 3q29dup population. Direct assessment of individuals with 3q29dup by the Emory 3q29 Project (http://genome.emory.edu/3q29/) (38) aim to address some of the weakness of this work by performing comprehensive gold-standard evaluations by expert clinicians.

There are significant strengths of this study, most notably that we have reported on the largest cohort of individuals with 3q29dup to date. Further, we have systematically ascertained phenotypes from our study population and from a population of individuals with 3q29del, and we were able to compare phenotypes between the two groups. Although we had a relatively small sample size, we were able to identify significant differences between participants with 3q29dup and 3q29del in the frequency of congenital heart defects, and we were able to find suggestive evidence of other phenotypic divergences between the reciprocal CNVs. We were also able to identify areas of phenotypic concordance between 3q29dup and 3q29del; if these similarities are borne out by studies with larger sample size, it could provide meaningful insight into the molecular mechanisms driving phenotype development, including neuropsychiatric and neurodevelopmental phenotypes. Finally, we found a substantial degree of social disability in our 3q29dup population that has not been previously reported in the literature, which suggests that gold-standard ASD evaluations should be standard of care for individuals with 3q29dup. Taken together, this study serves as a valuable complement to previously published case studies of 3q29dup; by systematically ascertaining phenotypes and comparing them to 3q29del and typically developing controls, we are able to add to the body of knowledge regarding the 3q29dup phenotype and

find relationships between 3q29dup and 3q29del, similar to those identified in other reciprocal CNV disorders. These data will assist clinicians, caregivers, and probands in developing comprehensive treatment plans to improve long-term outcomes for individuals with 3q29dup.

Table 3-1: Characteristics of study participants with 3q29dup, 3q29del, and controls.

	3q29dup	3q29del	Control
Age, years (mean ± SD)	10.0 ± 10.8	9.4 ± 8.0	10.5 ± 7.2
Sex (n, %)			
Male	15 (48.4%)	65 (55.6%)	33 (51.6%)
Female	16 (51.6%)	52 (44.4%)	31 (48.4%)
Race (n, %)			
White	27 (87.1%)	103 (88.0%)	41 (64.1%)
Black/African American	0 (0%)	1 (0.9%)	13 (20.3%)
Other	4 (12.9%)	11 (9.4%)	8 (12.5%)
Blank	0 (0%)	2 (1.7%)	2 (3.1%)

Demographic data collected from the custom Medical & Demographic Questionnaire completed by participants upon enrollment in the online 3q29 Registry.

Table 3-2: SRS sub-score comparison stratified by genotype, sex, and ASD status.

	Social Awareness		Social Cognition		Social Communication		Social Motivation		RRB		SCI	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Genotype												
Control	47.04 ± 8.88	-	45.27 ± 7.64	-	45.88 ± 8.14	-	46.13 ± 7.66	-	47.66 ± 8.51	-	45.50 ± 7.74	-
3q29dup	72.53 ± 16.69	1.43E-10	74.53 ± 18.45	4.27E-13	77.27 ± 17.65	3.37E-14	71.13 ± 14.89	3.61E-12	81.47 ± 20.28	1.48E-13	77.27 ± 18.43	3.24E-14
Sex												
Male control	45.97 ± 9.15	-	45.23 ± 7.42	-	45.73 ± 6.34	-	46.57 ± 6.58	-	47.63 ± 5.59	-	45.37 ± 6.60	-
Male 3q29dup	71.83 ± 23.66	0.0003	78.67 ± 22.41	1.04E-06	78.83 ± 21.64	3.27E-07	73.33 ± 13.35	3.72E-07	85.83 ± 23.81	6.41E-08	79.67 ± 22.13	3.38E-07
Female control	48.27 ± 8.56	-	45.31 ± 8.03	-	46.04 ± 9.95	-	45.62 ± 8.85	-	47.69 ± 11.08	-	45.65 ± 9.01	-
Female 3q29dup	73.00 ± 11.72	1.47E-07	71.78 ± 16.15	6.24E-07	76.22 ± 15.80	1.82E-07	69.67 ± 16.45	6.74E-06	78.56 ± 18.47	9.13E-07	75.67 ± 16.76	1.71E-07
ASD status												
Control	47.04 ± 8.88	-	45.27 ± 7.64	-	45.88 ± 8.14	-	46.13 ± 7.66	-	47.66 ± 8.51	-	45.50 ± 7.74	-
No ASD diagnosis 3q29dup	68.22 ± 17.09	1.74E-06	70.33 ± 19.66	1.50E-08	72.00 ± 18.73	3.56E-09	67.11 ± 16.83	1.00E-07	75.56 ± 21.37	1.29E-08	72.00 ± 19.72	3.31E-09
ASD diagnosis 3q29dup	79.00 ± 15.15	5.86E-09	80.83 ± 15.99	1.24E-10	85.17 ± 13.64	2.85E-12	77.17 ± 9.77	3.80E-10	90.33 ± 16.23	7.50E-12	85.17 ± 14.27	3.15E-12

Comparison of mean scores on the SRS sub-scales between study participants with 3q29dup and controls. Participants with 3q29dup were stratified by sex and ASD status for further analysis. P values were calculated using simple linear regression, adjusting for age, race, and sex. RRB, Restricted Interests and Repetitive Behaviors; SCI, Social Communication and Interaction.

Table 3-3: CBCL/ABCL Withdrawn sub-score comparison stratified by genotype, sex, and ASD status.

	Mean ± SD	P value
Genotype		
Control	52.37 ± 5.85	-
3q29dup	66.20 ± 12.76	6.33E-08
Sex		
Male control	52.14 ± 3.89	-
Male 3q29dup	65.00 ± 9.96	5.56E-05
Female control	52.59 ± 7.33	-
Female 3q29dup	67.00 ± 14.87	0.0004
ASD status		
Control	52.37 ± 5.85	-
No ASD diagnosis 3q29dup	64.88 ± 13.91	4.42E-05
ASD diagnosis 3q29dup	67.71 ± 12.20	6.06E-06

Comparison of mean scores on the CBCL/ABCL Withdrawn sub-scale between study participants with 3q29dup and controls. Participants with 3q29dup were stratified by sex and ASD status for further analysis. P values were calculated using simple linear regression, adjusting for age, race, and sex.

Table 3-4: Comparison of reported medical and neuropsychiatric phenotypes between 3q29dup (n = 31) and 3q29del (n = 117) participants.

Category	3q29dup (%, n)	3q29del (%, n)	P value
Problems in the first year of life	80.6% (25)	82.1% (96)	1.000
Heart defects	6.5% (2)	24.8% (29)	0.047
Learning problems (excluding GDD/ID)	71.0% (22)	78.6% (92)	0.500
GDD/ID	45.2% (14)	55.6% (65)	0.400
Ear problems	41.9% (13)	35.9% (42)	0.682
Gastrointestinal problems	54.8% (17)	67.5% (79)	0.270
Genitourinary problems	19.4% (6)	17.9% (21)	0.800
Renal problems	3.2% (1)	6.0% (7)	1.000
Dental problems	67.7% (21)	62.4% (73)	0.734
Seizures	25.8% (8)	15.4% (18)	0.276
Psychiatric diagnoses (including ASD)	58.1% (18)	48.7% (57)	0.500

Comparison of medical and neuropsychiatric diagnosis categories collected from the custom Medical & Demographic Questionnaire completed by participants upon enrollment in the online 3q29 Registry. P values were calculated using chi-squared test or Fisher's exact test.

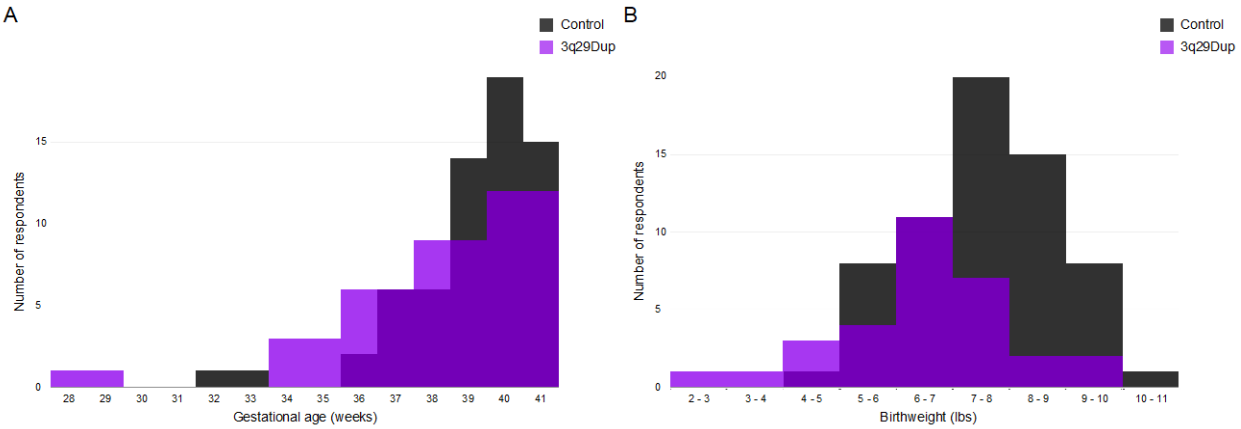

Figure 3-1. Gestational age and birthweight distributions for 3q29dup and controls. A) Gestational age distribution for participants with 3q29dup (n = 31) and typically developing controls (n = 64). **B)** Birthweight distribution for participants with 3q29dup (n = 31) and typically developing controls (n = 64).

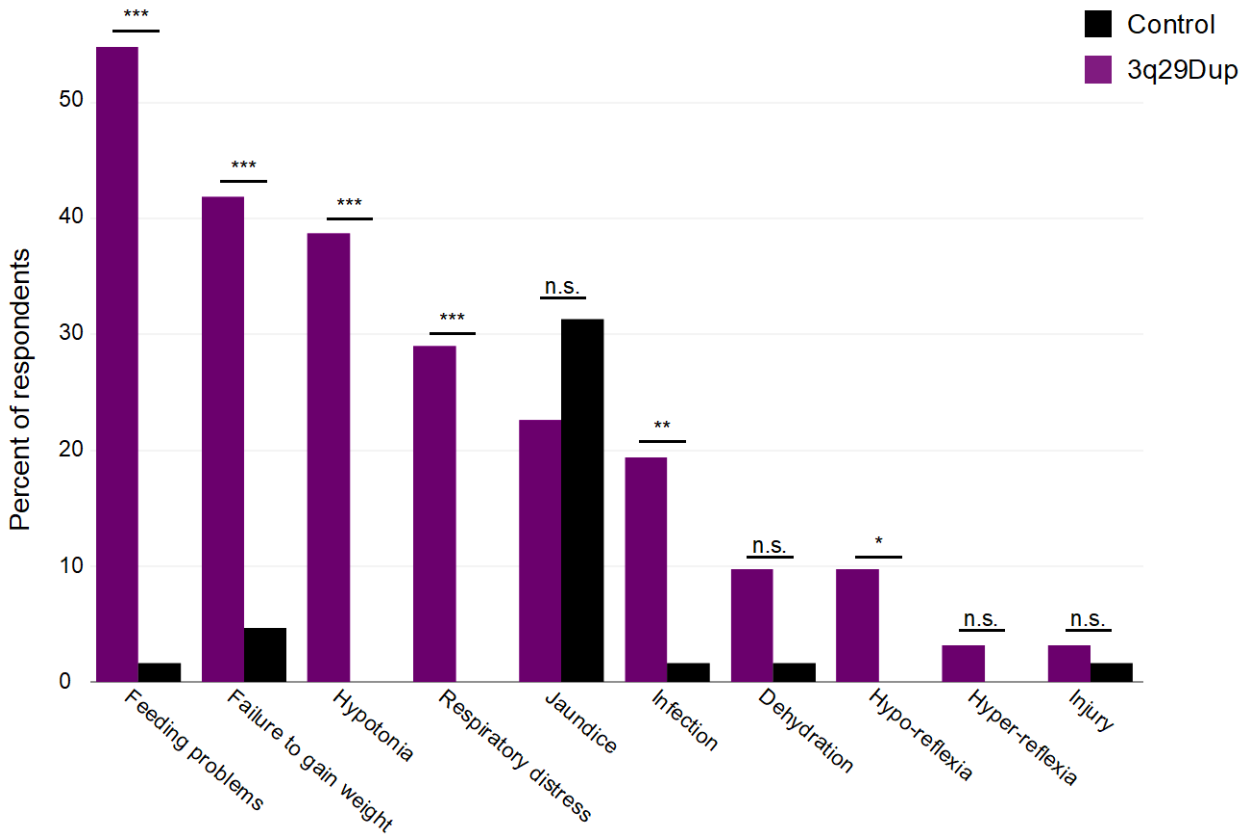

Figure 3-2. Reported problems in the first year of life by participants with 3q29dup and controls. Rate of problems in the first year of life reported by participants with 3q29dup (n = 31) and typically developing controls (n = 64), showing that participants with 3q29dup report substantially more problems in the postnatal period. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant

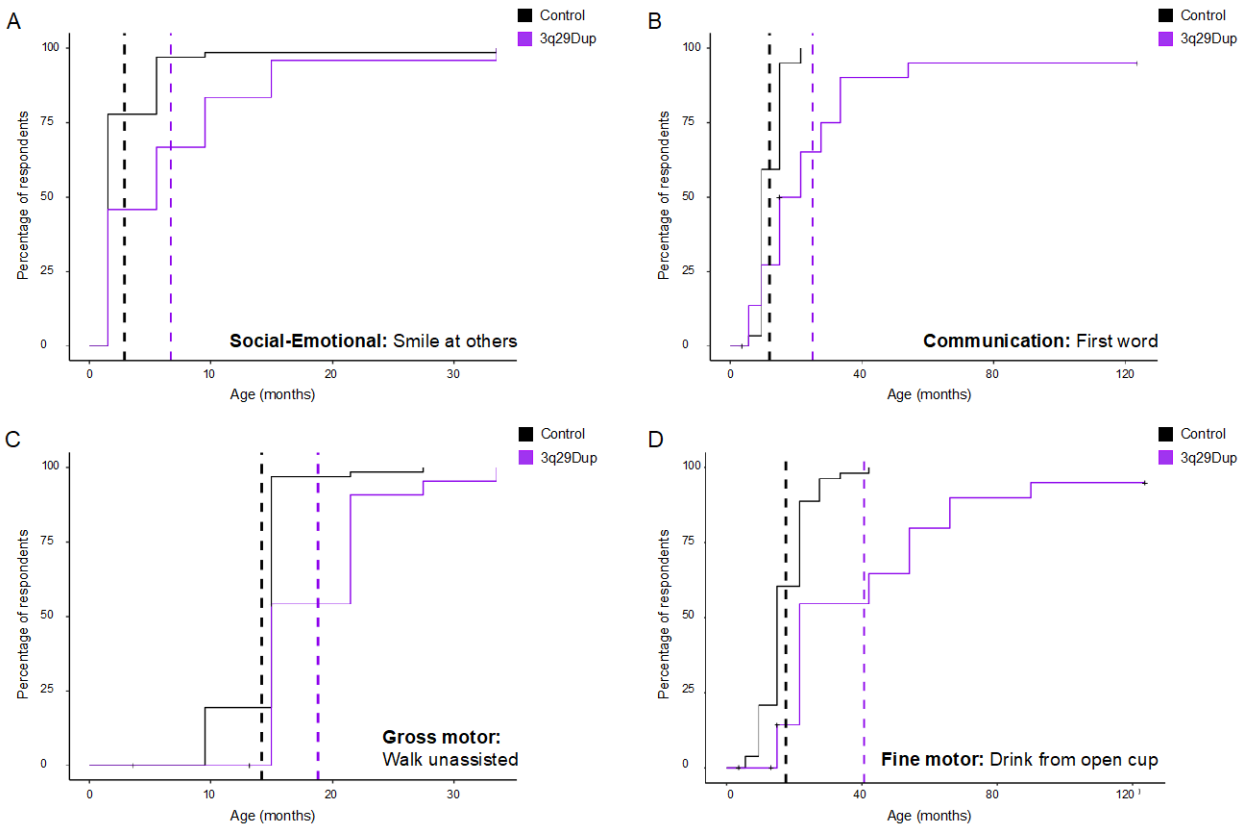

Figure 3-3. Comparison of developmental milestone achievement between participants with 3q29dup and controls. A) Kaplan-Meier time-to-event analysis of the representative social-emotional milestone, smile at others, showing that participants with 3q29dup (n = 24) on average achieve this milestone 3.79 months later than typically developing controls (n = 63). **B)** Kaplan-Meier time-to-event analysis of the representative communication milestone, first verbal word, showing that participants with 3q29dup (n = 23) on average achieve this milestone 13.4 months later than typically developing controls (n = 59). **C)** Kaplan-Meier time-to-event analysis of the representative gross motor milestone, walk unassisted, showing that participants with 3q29dup (n = 26) on average achieve this milestone 4.56 months later than typically developing controls (n = 62). **D)** Kaplan-Meier time-to-event analysis of the representative fine motor milestone, hold and drink from open cup, showing that participants with 3q29dup (n = 23) on average achieve this milestone 23.29 months later than typically developing controls (n = 53).

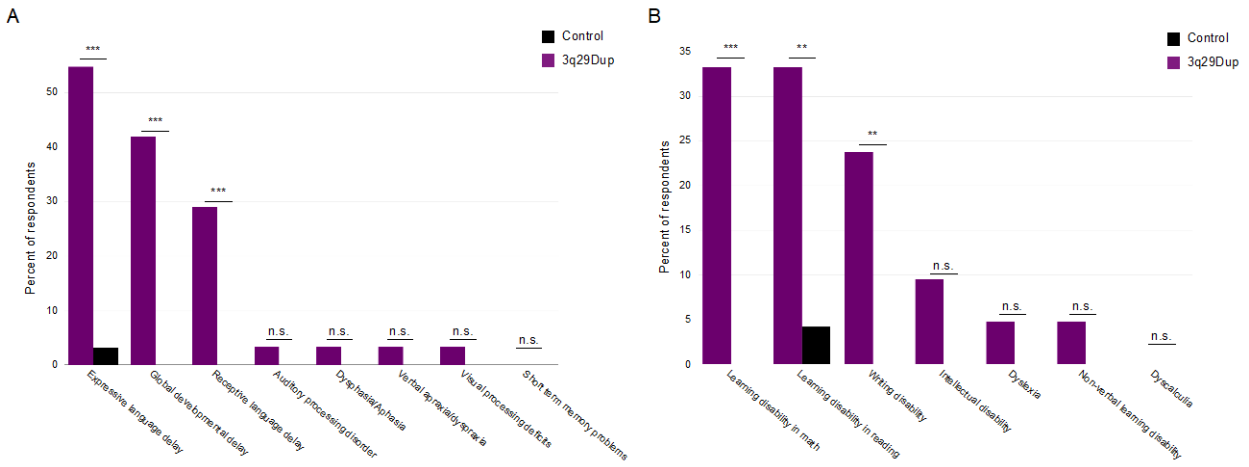

Figure 3-4. Reported learning problems by participants with 3q29dup and controls. A) Rate of early learning problems reported by participants with 3q29dup (n = 31) and typically developing controls (n = 64), showing that participants with 3q29dup report substantially more early learning problems. **B)** Rate of school-age learning problems reported by participants with 3q29dup (n = 21) and typically developing controls (n = 48) over 5 years of age, showing that participants with 3q29dup report substantially more school-age learning problems. P values were calculated using Fisher's exact test. ***, $p < 0.001$; **, $p < 0.01$; n.s., not significant

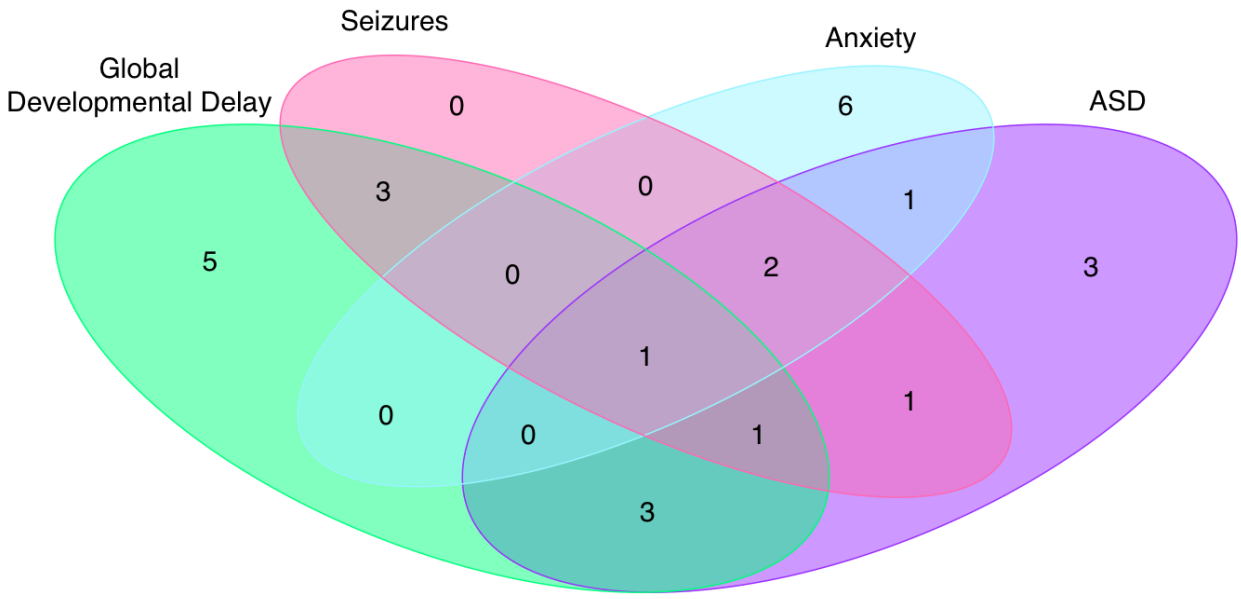

Figure 3-5. Overlap between reported global developmental delay, seizures, anxiety, and ASD among participants with 3q29dup. Venn diagram showing the overlap between reported global developmental delay, seizures, anxiety, and ASD within our 3q29dup study population, demonstrating that these diagnoses are distributed through the population rather than clustered in a small group of severely affected participants.

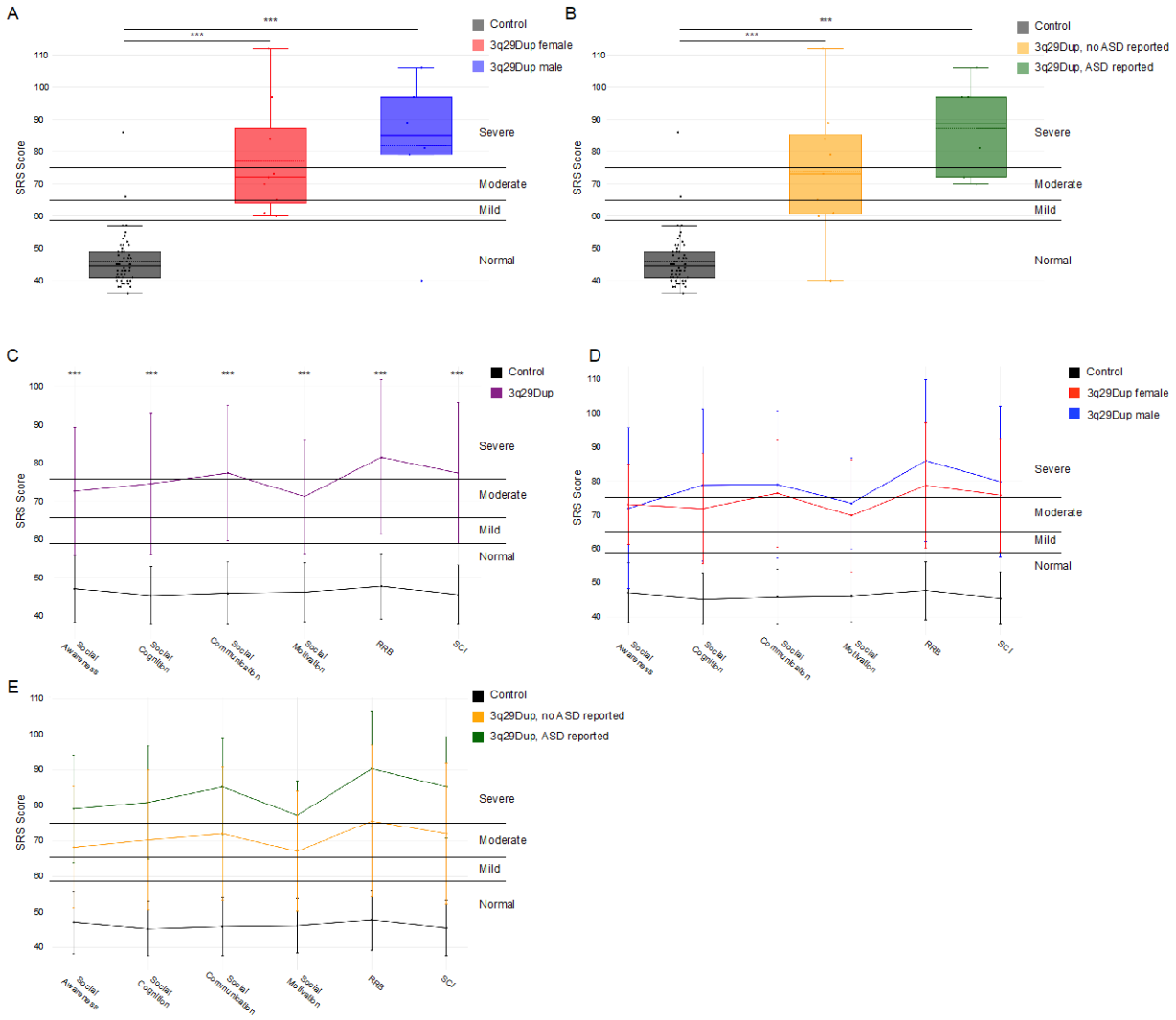

Figure 3-6. Comparison of SRS total scores and sub-scores between 3q29dup and controls. A) SRS total scores split by controls (n = 56), female with 3q29dup (n = 9), and males with 3q29dup (n = 6), showing that participants with 3q29dup score significantly higher than controls irrespective of sex. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **B)** SRS total scores split by controls (n = 56), 3q29dup not reporting an ASD diagnosis (n = 9), and 3q29dup reporting an ASD diagnosis (n = 6), showing that participants with 3q29dup score significantly higher than typically developing controls irrespective of self-reported ASD diagnosis status. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **C)** Profile of individuals with 3q29dup (n = 15) and controls (n = 56) across SRS sub-scales, showing moderate to

severe impairment of participants with 3q29dup in all domains (RRB, Restricted Interests and Repetitive Behaviors; SCI, Social Communication and Interaction). **D)** Profile of females with 3q29dup (n = 9), males with 3q29dup (n = 6), and controls (n = 56) across SRS sub-scales, showing that males and females with 3q29dup both score significantly higher than controls and that the overall shape of the profile is consistent between males and females with 3q29dup. **E)** Profile of participants with 3q29dup reporting an ASD diagnosis (n = 6), participants with 3q29dup not reporting an ASD diagnosis (n = 9), and controls (n = 56) across SRS sub-scales, showing that participants with 3q29dup score significantly higher than controls irrespective of ASD status. ***, $p < 0.001$

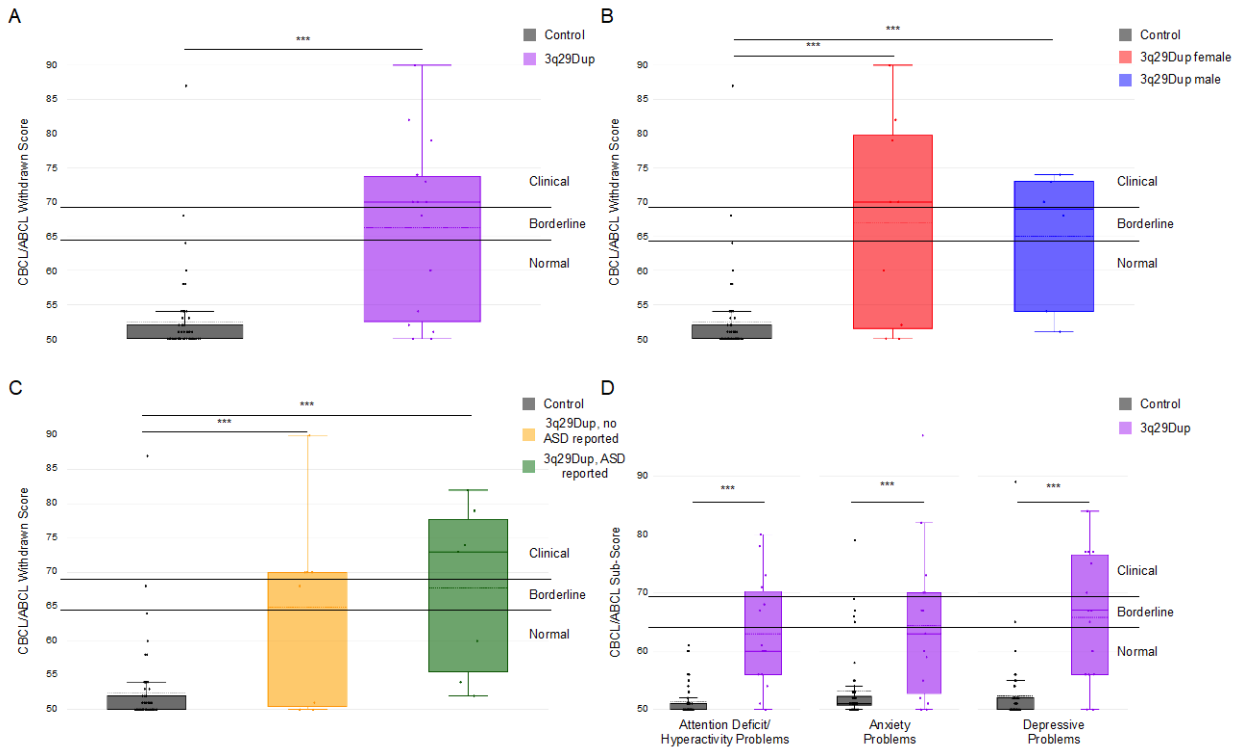

Figure 3-7. Comparison of CBCL/ABCL Withdrawn and DSM-oriented sub-scales between 3q29dup and controls. A) Profile of participants with 3q29dup (n = 15) and controls (n = 57) on the Withdrawn sub-scale from the CBCL and ABCL, showing a significantly higher score in participants with 3q29dup, with a mean score in the borderline range. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **B)** Profile of males with 3q29dup (n = 6), females with 3q29dup (n = 9), and controls (n = 57) on the Withdrawn sub-scale of the CBCL and ABCL, showing that both males and females score significantly higher than typically developing controls. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **C)** Profile of participants with 3q29dup reporting an ASD diagnosis (n = 7) and not reporting an ASD diagnosis (n = 8) and controls (n = 57) on the Withdrawn sub-scale of the CBCL and ABCL, showing that participants with 3q29dup score significantly higher than typically developing controls irrespective of ASD status. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **D)** Profile of participants with 3q29dup (n = 15) and controls (n = 57) across 3 DSM-oriented sub-scales from the CBCL and ABCL, showing significantly increased pathology in participants with

3q29dup across all domains. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. ***, $p < 0.001$

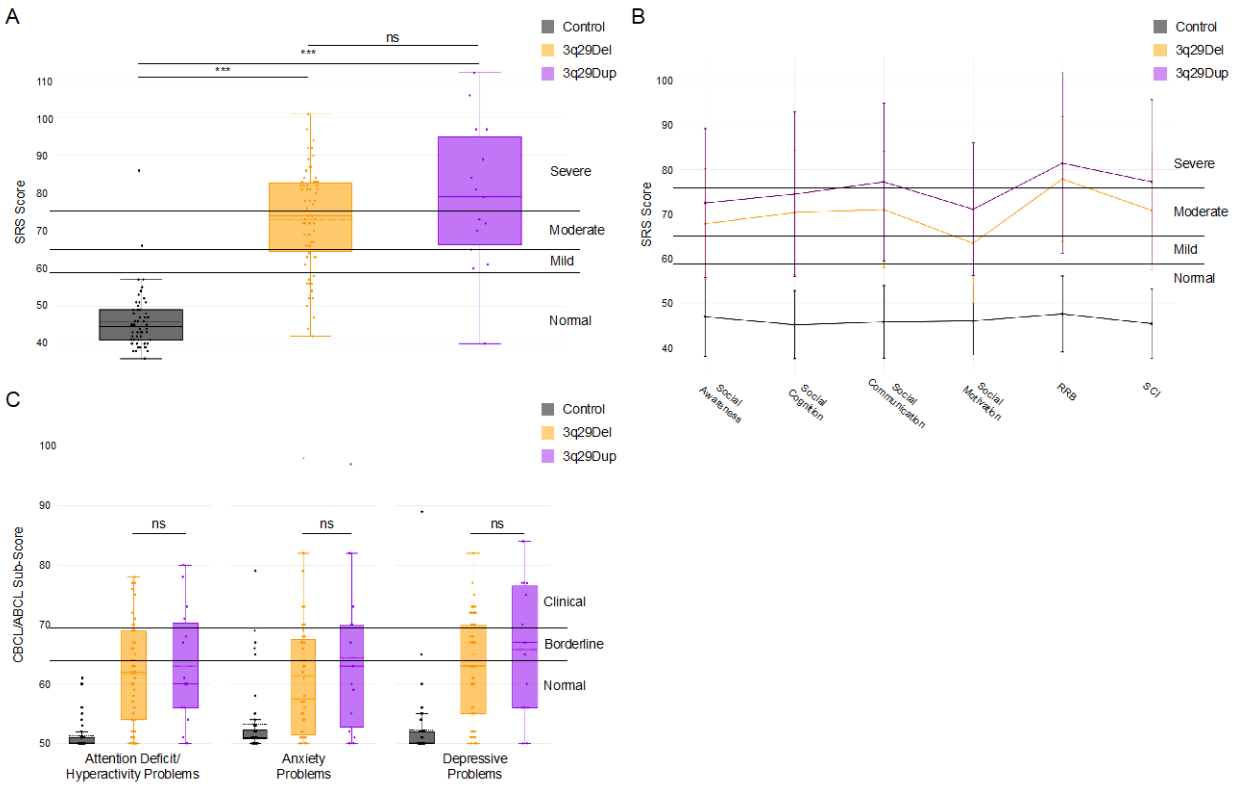

Figure 3-8. Comparison of SRS and CBCL/ABCL scores between 3q29dup and 3q29del. A) Total scores on the SRS for participants with 3q29dup (n = 15), participants with 3q29del (n = 67), and controls (n = 56), showing that participants with 3q29dup and 3q29del do not score significantly differently. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **B)** SRS sub-scale profile for participants with 3q29dup (n = 15), participants with 3q29del (n = 67), and controls (n = 56), showing that the shape of the SRS sub-scale profile is conserved between participants with 3q29dup and 3q29del (RRB, Restricted Interests and Repetitive Behaviors; SCI, Social Communication and Interaction). **C)** Profile of participants with 3q29dup (n = 15), participants with 3q29del (n = 64), and controls (n = 57) across 3 DSM-oriented sub-scales from the CBCL and ABCL, showing that participants with 3q29dup and 3q29del have similar levels of pathology in all 3 domains. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. ***, $p < 0.001$; n.s., not significant

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Supplemental information

Table S3-1: Questionnaire demographics. Characteristics of study participants with 3q29dup, 3q29del, and controls completing each questionnaire utilized in present study.

	Medical and Demographic Questionnaire			Social Responsiveness Scale (SRS)			Achenbach Behavior Checklists (CBCL/ABCL)		
	3q29dup	3q29del	Control	3q29dup	3q29del	Control	3q29dup	3q29del	Control
Age, years (mean +/- SD)	10.0 +/- 10.8	9.4 +/- 8.0	10.5 +/- 7.2	11.5 +/- 9.3	10.4 +/- 7.3	10.9 +/- 7.2	9.1 +/- 6.2	9.7 +/- 6.7	10.0 +/- 7.0
Sex (%, n)									
Male	48.4% (15)	55.6% (65)	51.6% (33)	40.0% (6)	55.2% (37)	53.6% (30)	40% (6)	56.3% (36)	49.1% (28)
Female	51.6% (16)	44.4% (52)	48.4% (31)	60.0% (9)	44.8% (30)	46.4% (26)	60.0% (9)	43.8% (28)	50.9% (29)

Table S3-2: Goodness-of fit analysis. Goodness-of-fit analysis for testing the effect of genotype on birthweight, showing that a model including genotype fits the data significantly better than one without. Y is birthweight in pounds.

Model: $Y \sim \beta0 + \beta1(\text{Sex}) + \beta2(\text{Gestational age in weeks}) + \beta3(\text{Race}) + \beta4(\text{Duplication})$

Coefficient	Estimate	Std. error	t value	p value
Sex	0.46	0.23	1.99	0.050
Gestational age	0.43	0.05	7.90	8.29E-12
Race (Black)	-0.93	1.11	-0.84	0.405
Race (Other)	0.03	1.12	0.02	0.981
Race (White)	-0.27	1.08	-0.25	0.802
Duplication	-0.74	0.25	-2.90	0.005

Goodness of fit:
Model 1: $Y \sim \beta0 + \beta1(\text{Sex}) + \beta2(\text{Gestational age in weeks}) + \beta3(\text{Race}) + \beta4(\text{Duplication})$
Model 2: $Y \sim \beta0 + \beta1(\text{Sex}) + \beta2(\text{Gestational age in weeks}) + \beta3(\text{Race})$

Model 1 vs. Model 2:

F	p value
8.39	0.005

Table S3-3: Problems in the first year of life. Proportion of participants with 3q29dup (n = 31) and typically developing controls (n = 64) reporting problems in the first year of life.

Condition	3q29dup (%, n)	Control (%, n)
Dehydration	9.7% (3)	1.6% (1)
Failure to gain weight	41.9% (13)	4.7% (3)
Feeding problems	54.8% (17)	1.6% (1)
Hyper-reflexia (overactive reflexes)	3.2% (1)	0% (0)
Hypo-reflexia (underactive reflexes)	9.7% (3)	0% (0)
Hypotonia (low muscle tone)	38.7% (12)	0% (0)
Infection	19.4% (6)	1.6% (1)
Injury	3.2% (1)	1.6% (1)
Jaundice	22.6% (7)	31.3% (20)
Respiratory distress	29.0% (9)	0% (0)
Unsure	6.5% (2)	0% (0)

Table S3-4: Kaplan-Meier time-to-event analysis for developmental milestones. Time-to-event analysis for developmental milestones across the social-emotional, communication, gross motor, and fine motor domains for participants with 3q29dup and typically developing controls.

Developmental Milestone	**3q29dup**					**Control**				
	N observations	Mean time to milestone (months)	Median time to milestone (months)	Range		N observations	Mean time to milestone (months)	Median time to milestone (months)	Range	
				Lower	Upper				Lower	Upper
Social-Emotional										
Smile at others	24	6.69	5.50	1.50	33.50	63	2.90	1.50	1.50	33.50
Play peek-a-boo	22	14.78	9.50	5.50	33.50	51	6.72	5.50	1.50	15.00
Initiate social interaction by smiling, moving arms, and/or vocalizing	21	9.45	5.50	1.50	33.50	56	3.91	1.50	1.50	15.00
Cling to caregivers/familiar adults in presence of stranger	19	19.15	9.50	1.50	33.50	60	9.56	9.50	1.50	42.00
Smile when praised, repeats action for more praise	20	26.17	15.00	5.50	78.00	52	10.35	9.50	1.50	33.50
Respond affectionately to caregivers (gives hug/kiss when asked)	21	22.47	15.00	5.50	90.00	59	12.45	9.50	1.50	42.00
Social smile	17	29.92	15.00	1.50	102.00	57	7.41	5.50	1.50	15.00
Communication										

Single syllable utterances (e.g. ma, da)	23	18.73	9.50	5.50	33.50	59	7.98	5.50	1.50	15.00
First verbal word (for example: go)	23	25.33	18.25	5.50	54.00	59	11.93	9.50	5.50	21.50
Verbal two word sentences	25	33.26	27.50	5.50	66.00	56	16.04	15.00	9.50	27.50
Recognizes written letters/numbers	25	66.29	66.00	15.00	102.00	53	29.09	27.50	9.50	54.00
Phonics-style reading (sounds out words)	25	98.16	90.00	33.50	148.80	57	55.28	54.00	9.50	78.00
Read whole words (words as an individual unit)	25	104.64	102.00	54.00	149.00	59	66.30	66.00	9.50	90.00
Write/type using a keyboard	24	105.66	102.00	54.00	149.00	59	87.79	78.00	54.00	165.00
Gross motor										
Hold head up on his/her own	22	8.29	5.50	1.50	54.00	59	3.33	1.50	1.50	9.50
Roll over back to stomach	21	10.75	5.50	1.50	78.00	59	4.42	5.50	1.50	9.50
Sit when placed	22	10.43	9.50	5.50	21.50	61	6.44	5.50	5.50	15.00
Crawl on hands and knees	21	13.64	15.00	5.50	27.50	62	8.62	9.50	5.50	21.50
Walk unassisted	26	18.80	15.00	15.00	33.50	62	14.24	15.00	9.50	27.50
Climb stairs standing up without help	23	41.30	27.50	15.00	66.00	53	19.58	21.50	5.50	33.50
Descend stairs without help	23	47.92	33.50	15.00	66.00	55	23.01	21.50	15.00	54.00
Jump with both feet	21	55.45	54.00	15.00	78.00	49	24.41	21.50	15.00	54.00
Pedal tricycle	22	64.25	54.00	21.50	114.00	51	32.50	27.50	21.50	54.00

Fine motor										
Look at, reach, and grasp objects placed at a distance	20	11.50	5.50	1.50	33.50	52	6.49	5.50	1.50	42.00
Transfer object between hands	22	14.21	15.00	5.50	33.50	49	7.68	5.50	1.50	21.50
Isolate index finger to point	18	40.43	21.50	9.50	78.00	41	10.22	9.50	5.50	15.00
Clap hands	22	18.38	15.00	5.50	42.00	56	9.04	9.50	5.50	15.00
Deliberately release object to a container	17	24.76	21.50	5.50	90.00	45	10.79	9.50	5.50	33.50
Hit two objects together	19	19.94	15.00	5.50	78.00	51	9.85	9.50	5.50	21.50
Acquired pincer grasp	14	35.09	21.50	5.50	90.00	45	11.34	9.50	5.50	21.50
Hold and drink from open cup unassisted	23	40.64	21.50	15.00	90.00	53	17.35	15.00	5.50	42.00
Turn knobs	21	41.60	42.00	15.00	102.00	46	21.64	21.50	9.50	33.50
Stack and balance blocks	23	36.98	27.50	15.00	90.00	49	18.15	15.00	9.50	42.00

Table S3-5A: Early learning problems. Proportion of participants with 3q29dup (n = 31) and typically developing controls (n = 64) reporting early learning problems.

Condition	3q29dup (%, n)	Control (%, n)
Auditory processing disorder	3.2% (1)	0% (0)
Dysphasia/Aphasia	3.2% (1)	0% (0)
Global developmental delay	41.9% (13)	0% (0)
Language (receptive) delay (problems understanding language)	29.0% (9)	0% (0)
Short term memory problems	0% (0)	0% (0)
Speech (expressive) delay (problems getting words out)	54.8% (17)	3.1% (2)
Verbal apraxia/dyspraxia	3.2% (1)	0% (0)
Visual processing deficits	3.2% (1)	0% (0)
Unsure	0% (0)	1.6% (1)

Table S3-5B: School-age learning problems. Proportion of participants with 3q29dup (> 5 years old; n = 21) and typically developing controls (> 5 years old; n = 48) reporting school-age learning problems.

Condition	3q29dup (%, n)	Control (%, n)
Dyscalculia	0% (0)	0% (0)
Dyslexia	4.8% (1)	0% (0)
Learning disability in math	33.3% (7)	0% (0)
Learning disability in reading	33.3% (7)	4.2% (2)
Intellectual disability (mild, moderate, severe)	9.5% (2)	0% (0)
Non-verbal learning disability	4.8% (1)	0% (0)
Writing disability	23.8% (5)	0% (0)
Unsure	0% (0)	2.1% (1)

Table S3-6: Gastrointestinal problems. Proportion of participants with 3q29dup (n = 31) and typically developing controls (n = 64) reporting gastrointestinal problems.

Condition	3q29dup (%, n)	Control (%, n)
Anterior displaced anus	0% (0)	0% (0)
Autistic enterocolitis	0% (0)	0% (0)
Barrett's esophagus	0% (0)	0% (0)
Chronic constipation	35.5% (11)	7.8% (5)
Chronic diarrhea	6.5% (2)	4.7% (3)
Diaphragmatic hernia	0% (0)	0% (0)
Dysphagia (difficulty swallowing)	16.1% (5)	0% (0)
Feeding problems	38.7% (12)	1.6% (1)
Gastroesophageal reflux (GERD)	12.9% (4)	6.3% (4)
Hiatial hernia	0% (0)	0% (0)
Inflammatory bowel disease (Crohn's disease, Ulcerative colitis)	3.2% (1)	0% (0)
Intestinal malrotation	0% (0)	0% (0)
Irritable bowel syndrome (IBS)	0% (0)	0% (0)
Peptic ulcers	0% (0)	0% (0)
Pyloric stenosis	0% (0)	0% (0)
Silent Reflux	9.7% (3)	3.1% (2)
Unsure	16.1% (5)	0% (0)

Table S3-7: Psychiatric diagnoses. Proportion of participants with 3q29dup (n = 31), participants with 3q29del (n = 117), and typically developing controls (n = 64) reporting psychiatric diagnoses. P values comparing reported diagnoses between 3q29dup and 3q29del were calculated using chi-square test or Fisher's exact test.

Diagnosis	3q29dup (%, n)	3q29del (%, n)	Control (%, n)	P value
ASD	38.7% (12)	29.1% (34)	0% (0)	0.416
Addiction	0% (0)	2.6% (3)	6.3% (4)	1.00
Anxiety disorder	32.3% (10)	28.2% (33)	0% (0)	0.826
Bipolar/manic depression	0% (0)	5.1% (6)	0% (0)	0.344
Conduct disorder	6.5% (2)	3.4% (4)	6.3% (4)	0.606
Depression	16.1% (5)	6.0% (7)	0% (0)	0.130
Oppositional defiant disorder	6.5% (2)	4.3% (5)	3.1% (2)	0.637
Panic attacks	6.5% (2)	12.0% (14)	0% (0)	0.525
Schizophrenia	0% (0)	3.4% (4)	0% (0)	0.580

Table S3-8: SRS score comparison stratified by ASD status and sex. Comparison of mean scores on the SRS between study participants with 3q29dup stratified by ASD status and sex to controls (mean +/- SD = 45.91 +/- 7.97). P values were calculated using simple linear regression, adjusting for age and race.

	Mean +/- SD	P value
Sex		
Male control	45.80 +/- 6.38	-
Male 3q29dup	82.00 +/- 22.91	1.83E-07
Female control	46.04 +/- 9.62	-
Female 3q29dup	77.11 +/- 17.51	2.22E-07
ASD status		
Control	45.91 +/- 7.97	-
No ASD diagnosis 3q29dup	73.67 +/- 20.60	2.93E-09
ASD diagnosis 3q29dup	87.17 +/- 14.91	3.29E-12

Table S3-9: Ear problems. Proportion of participants with 3q29dup (n = 31) and typically developing controls (n = 64) reporting ear problems.

Condition	3q29dup (%, n)	Control (%, n)
Dizziness and/or vertigo	9.7% (3)	0% (0)
Ear pain	16.1% (5)	7.8% (5)
Meniere's disease	0% (0)	0% (0)
Recurrent ear infections	35.5% (11)	20.3% (13)
Tinnitus (ringing in the ear)	3.2% (1)	0% (0)
Unsure	9.7% (3)	0% (0)

Table S3-10: Dental problems. Proportion of participants with 3q29dup (n = 31) and typically developing controls (n = 64) reporting dental problems.

Condition	3q29dup (%, n)	Control (%, n)
Cone-shaped teeth	9.7% (3)	0% (0)
Crowded teeth	6.5% (2)	3.1% (2)
Enamel hypoplasia	6.5% (2)	1.6% (1)
Extra teeth	0% (0)	1.6% (1)
High number of cavities	25.8% (8)	6.3% (4)
Large gap between two front teeth on top	22.6% (7)	6.3% (4)
Large teeth	3.2% (1)	0% (0)
Malocclusion	3.2% (1)	1.6% (1)
Missing teeth	3.2% (1)	1.6% (1)
Small teeth	16.1% (5)	3.1% (2)
Tooth in palate (roof of mouth)	0% (0)	1.6% (1)
Tooth/teeth extraction (pulled)	19.4% (6)	6.3% (4)
Weak/soft tooth enamel	12.9% (4)	1.6% (1)
Widely spaced teeth	6.5% (2)	1.6% (1)
Unsure	16.1% (5)	0% (0)

Additional Phenotypes

Heart Defects: Two participants with 3q29dup reported heart defects (6%); one reported atrial septal defect (3.2%) and one reported tricuspid atresia (3.2%).

Genitourinary Problems: Six participants with 3q29dup reported genitourinary problems (19.4%); one reported micropenis (3.2%); one reported recurrent UTIs, bladder diverticulum, bladder exstrophy, and difficulty emptying bladder (3.2%); and four reported recurrent UTIs (12.9%).

Renal Problems: One participant with 3q29dup reported kidney reflux as a renal problem (3.2%).

Clefting: One participant with 3q29dup reported cleft palate (3.2%).

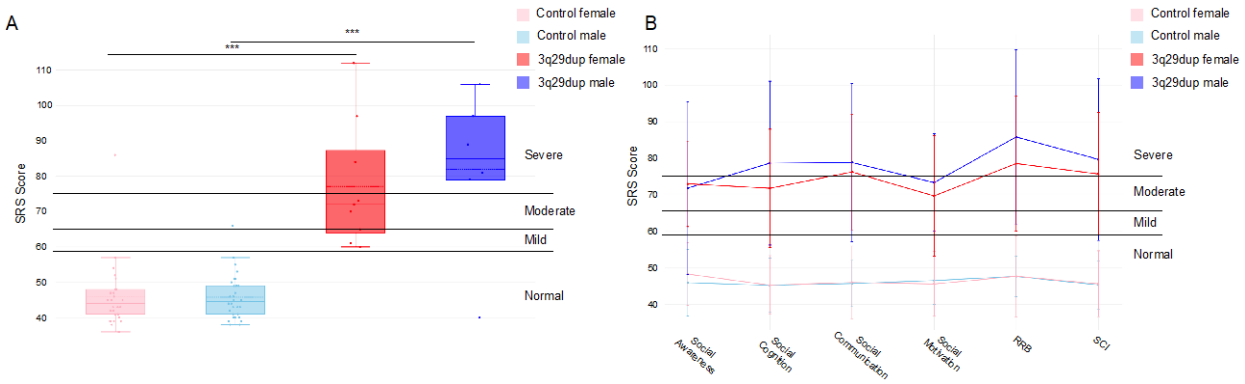

Figure S3-1. Comparison of SRS scores between participants with 3q29dup and controls, stratified by sex. A) SRS total scores split by control females (n = 26), control males (n = 30), 3q29dup females (n = 9), and 3q29dup males (n = 6), showing the distribution of scores split by sex and genotype. For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. **B)** Profiles of control females (n = 26) control males (n = 30), 3q29dup females (n = 9), and 3q29dup males (n = 6) across SRS sub-scales, showing the distribution of sub-scale scores and shape of the profile divided by sex and genotype (RRB, Restricted Interests and Repetitive Behaviors; SCI, Social Communication and Interaction). For each box plot, the dashed line indicates the mean value, while the solid line indicates the median. ***, $p < 0.001$

Chapter 4. Metabolic effects of the schizophrenia-associated 3q29 deletion are highly sex-specific and uncoupled from behavioral phenotypes

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INTRODUCTION

3q29 deletion syndrome (3q29del) is a rare (~1 in 30,000) (1, 2) genomic disorder characterized by a typically *de novo* 1.6 Mb deletion on chromosome 3 (hg19, chr3:195725000-197350000) (3-5). The 3q29 deletion is associated with neurodevelopmental and neuropsychiatric phenotypes, including mild to moderate intellectual disability (ID) (3, 4, 6), a 19-fold increased risk for autism spectrum disorder (ASD) (7-9), and a 20-40-fold increased risk for schizophrenia (SZ) (10-14). Two independently generated mouse models of the 3q29 deletion show behavioral manifestations, including social interaction and cognitive deficits and altered acoustic startle reflex, sensorimotor gating, and amphetamine-induced locomotion (15, 16). The range of neurodevelopmental and neuropsychiatric manifestations in 3q29del is consistent with that observed in other CNV disorders, including 22q11.2 deletion syndrome (17, 18), 16p11.2 deletion and duplication syndromes (19, 20), 7q11.23 duplication syndrome (21), and 1q21.1 deletion syndrome (22). These data demonstrate that risk for multiple neuropsychiatric phenotypes is a feature common to many genomic disorders.

There is growing evidence that metabolic alterations can contribute to neurodevelopmental and neuropsychiatric diseases. Many inborn errors of metabolism have neurodevelopmental and neuropsychiatric manifestations, including Wilson's disease, cerebrotendinous xanthomatosis, Niemann-Pick disease type C, phenylketonuria, and classical galactosemia (23-34). Additional molecular studies of the 22q11.2 deletion have highlighted the role of oxidative stress and mitochondrial dysfunction as major contributors to synaptic phenotypes (35-37). Mitochondrial function, oxidative stress, and small molecule dysregulation have also been implicated in the pathogenesis of idiopathic ASD, bipolar disorder, major depression, and SZ (38-60), highlighting some etiological similarities between syndromic and idiopathic cases of neuropsychiatric disorders. In light of this evidence linking metabolic function and neuropsychiatric outcomes, it is noteworthy that four of the 21 genes in the 3q29 deletion interval (the SUMO-specific protease SENP5, the fatty acid catabolism protein BDH1, the transferrin receptor TFRC, and the phosphatidylcholine biosynthesis protein PCYT1A) are directly involved in metabolism. Individuals with 3q29del report significantly reduced birthweight and a high proportion of feeding

problems and failure to thrive compared to the general population (5), and robust weight deficits have been observed in both mouse models of the 3q29 deletion (15, 16). Additionally, our team previously identified sex differences in the degree of the 3q29 deletion-associated weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, with female animals more substantially affected than males (15). These data inspired the hypothesis that metabolic disruption may contribute to 3q29 deletion syndrome phenotypes, and further suggest that there may be sex-specific effects of the 3q29 deletion on metabolic phenotypes.

Although there are established links between recurrent CNV disorders, metabolism, and neurodevelopmental and neuropsychiatric liability, metabolic function has not been interrogated in the context of the 3q29 deletion. Existing evidence of birthweight deficits in humans with 3q29del, weight deficits in 3q29 deletion mouse models, and the metabolic genes contained in the 3q29 interval motivated our investigation of a possible unidentified metabolic disturbance associated with the 3q29 deletion (Figure 4-1). Furthermore, to determine whether metabolic disruption and adverse behavioral outcomes arise from a shared mechanism, we interrogated the relationship between metabolic function and behavioral phenotypes in our mouse model. Finally, we have explicitly considered sex as a modifier of 3q29 deletion metabolic biology, based on our previously reported finding of sex differences in the severity of the 3q29 deletion-associated weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice (15) (Figure 4-1). The new results described here have important implications for our mechanistic understanding of phenotype development in 3q29del and help to further elucidate the relationship between metabolism and neurodevelopmental and neuropsychiatric disease risk.

RESULTS

Reduced respiratory exchange ratio and energy expenditure, but not reduced energy consumption, in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice

To investigate whether the 3q29 deletion-associated weight deficit is attributable to increased energy expenditure or decreased energy consumption, we performed 5 days of indirect calorimetry on male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice and WT littermates using CLAMS/Metabolic Cages (Columbus Instruments). Energy expenditure was similar between WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males (Figure 4-2A); in

females, energy expenditure was *reduced* in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals relative to WT (Figure 4-2B). These data show that the 3q29 deletion-associated weight deficit is not due to increased energy expenditure; rather, B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice burn *fewer* calories than their WT littermates. To understand whether the 3q29 deletion influences the calorie source that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice use, we evaluated the respiratory exchange ratio (RER). If an animal is predominantly using lipids as an energy source, the RER will approach 0.7, whereas if an animal is predominantly utilizing carbohydrates, the RER will approach 1 (61). Both male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice demonstrated reduced RER relative to WT (Figure 4-2C-D), indicating that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals preferentially use dietary lipids as an energy source, whereas WT animals use more carbohydrates.

To determine whether the weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice is simply due to decreased caloric intake, we evaluated food consumption. There was no significant difference in food consumption (Figure S4-1A) between male or female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals after controlling for the reduced weight of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals. There were only minor differences in locomotor activity, with male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice *less* active than WT littermates, and no differences between female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice (Figure S4-1C-D). These data show that the weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice is not attributable to a decrease in energy consumption nor an increase in energy expenditure. Further, the reduced RER in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice indicates that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals are preferentially using lipids as an energy source rather than carbohydrates. Together, these data support our hypothesis of altered metabolic function associated with the 3q29 deletion.

Untargeted metabolomics reveals small molecule alterations in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice that are highly sex-dependent

To identify metabolic pathway differences between B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice and WT littermates, we performed untargeted metabolomic profiling on liver samples (62). Males and females were analyzed separately. We compared all nominally significant metabolic features between the male and female datasets and found that only 22 features were shared (Figure 4-3A, full details in Supplement), highlighting the sex-dependent effect of the 3q29 deletion on the metabolic environment. Using the top

250 ranked metabolic features, we were able to cluster WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples with 84.2%±0.2% accuracy for males (p=0.003) and 76.9%±0% accuracy for females (p=0.006, Figure 4-3B-E), indicating a strong effect of the 3q29 deletion on the metabolic environment. We also created random forest classifiers using the top 250 ranked features; our male classifier attained an AUC of 0.909±0.002 (p=0.001) and a prediction accuracy of 84.2%±0.2% (p=0.003, Figure 4-3D), which translates to a 90.9% chance that the classifier will assign an unknown male sample to the correct genotype, and that 84.2% of input samples were correctly classified as WT or B6.Del16$^{+/Bdh1\text{-}Tfrc}$. Our female classifier attained an AUC of 0.905±0.001 (p=0) and a prediction accuracy of 76.9%±0% (p=0.006, Figure 4-3E), which translates to a 90.5% chance that the classifier will assign an unknown female sample to the correct genotype, and that 76.9% of input samples were correctly classified as WT or B6.Del16$^{+/Bdh1\text{-}Tfrc}$. These data show that there is a substantial effect of the 3q29 deletion on the metabolic environment, and this effect is highly sex dependent.

Consistent with the significant reduction in RER in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, pathway enrichment analysis of altered features using Mummichog (63) revealed that pathways related to fat metabolism were identified in both the male and female datasets, including bile acid biosynthesis and the carnitine shuttle in males; and glycerophospholipid metabolism, arachidonic acid metabolism, phosphatidylinositol metabolism, and ganglio-series glycosphingolipid biosynthesis in females (Figure 4-3F-G). In addition to the lack of individual feature overlap, there was also no pathway-level overlap between the sexes, again highlighting the sex-dependent effects of the 3q29 deletion on metabolism. Despite the lack of pathway overlap, the pathways identified were generally related to fat metabolism, supporting our finding of reduced RER in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice and further demonstrating that these processes may be altered in both male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals.

A high-fat diet attenuates the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ weight deficit and affects RER in a sex-specific manner

From the RER data, we determined that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals preferentially use fat as an energy source; untargeted metabolomics data confirmed there are differences in fat metabolism pathways in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on a standard diet (STD). As the STD contains only 13.4% of total calories from fat; we hypothesized that increased availability of fat calories would cause B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals to gain weight and remedy the 3q29 deletion-associated weight deficit. To test this hypothesis, we implemented a high-fat diet (HFD) challenge from postnatal day 21 to euthanasia (16-20 weeks), using a commercially available diet (TD.88137, Teklad Custom Diets, Envigo) containing 42% of total calories from fat. We found that the weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice was partially ameliorated in female mice but was largely unchanged in male mice (Figure 4-4A-B). After HFD treatment, male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD weighed on average 1.72 g less than WT littermates ($p=0.015$, Figure 4-4A), compared to 1.61 g less than WT littermates on the STD (15). Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD weighed on average 1.66 g less than WT littermates ($p=0.004$, Figure 4-4B), versus 2.24 g less than WT littermates on the STD (15). When these effect sizes were considered relative to total body weight, the effect of the HFD intervention became clearer. Male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD were 4.33% smaller than WT littermates at 16 weeks, compared to 5.16% smaller on the STD. Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD were 5.16% smaller than WT littermates at 16 weeks, compared to 10.29% smaller on the STD at the same timepoint. These data demonstrate a sex-specific effect of the 3q29 deletion on the response to the HFD, further supporting the differential impact of the 3q29 deletion on metabolism in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice.

To further investigate the metabolic consequences of the HFD intervention, we performed 5 days of indirect calorimetry in the HFD-treated male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ and WT mice. There were no differences in RER between male WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, indicating that male WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals have similar energy source utilization on the HFD (Figure 4-4C). By contrast, female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD had reduced RER peaks compared to WT animals, consistent with the STD data (Figure 4-4D). Even after HFD treatment, metabolism in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice was still shifted toward preferentially utilizing lipids rather than carbohydrates, whereas male

B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice had comparable macronutrient utilization to WT animals after the HFD intervention. These data suggest that the HFD rescued the shift in macronutrient utilization in males, but females may have a pronounced need for dietary lipids that was not fully satisfied by the HFD intervention. Consistent with the STD results, there were no differences between male or female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice in food or water consumption (Figure S4-1E-F) or activity (Figure S4-1G-H). There was no difference in energy expenditure in male HFD animals (Figure S4-1I) and a slight difference in energy expenditure in female HFD animals (Figure S4-1J). Together, these data provide further support to the conclusion that male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice are differentially impacted by 3q29 deletion-associated metabolic deficits.

Widespread changes in the global metabolic environment of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice after HFD treatment

We found that the HFD intervention altered RER in male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice and partially ameliorated the 3q29 deletion-associated weight deficit in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. Based on these data, we hypothesized that the HFD intervention may also affect changes in the global metabolic environment of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals. To test this hypothesis, we performed untargeted metabolomic profiling of liver samples from HFD-treated animals (62). Males and females were analyzed separately. Similar to the results from our metabolomics analysis of STD-treated animals, comparison of all nominally significant metabolic features between the male and female datasets revealed only 7 shared features between sexes (Figure 4-5A, full details in Supplement), highlighting a substantial sex-dependent effect of the 3q29 deletion on the metabolic environment after HFD treatment. Using the top 250 ranked metabolites, WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples clustered with 100%±0% accuracy in males (p=0) and 95%±0% accuracy in females (p=0, Figure 4-5B-E). These data suggest that even after the HFD intervention, pronounced metabolic differences exist between the WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. Additionally, random forest classifiers using the top 250 ranked features achieved excellent classification (AUC=1±0 in males and 1±0.001 in females) and high prediction accuracy (100%±0% in males and

95%±0% in females, Figure 4-5D-E) in both sexes. Pathway enrichment analysis of altered features using Mummichog (63) identified pathways with diverse functions in both datasets, including pyrimidine metabolism and aspartate and asparagine metabolism in males, and vitamin B6 metabolism and propanoate metabolism in females (Figure 4-5F-G). Pathways related to fat metabolism were identified in the female dataset, including *de novo* fatty acid biosynthesis and ganglio-series glycosphingolipid biosynthesis, as they were in the STD pathway analysis. This result is concordant with our finding of persistent RER shifts in female, but not male, B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. At the small molecule level, the HFD treatment did not restore fat metabolism functions in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice to WT levels, supporting our hypothesis that the HFD intervention did not fully satisfy the increased metabolic demand for fat in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. As seen in the STD result and the lack of individual feature overlap in the HFD datasets, there was no pathway-level overlap between the sexes, further demonstrating that a robust sex effect of the 3q29 deletion on the metabolic environment remains after HFD treatment.

To test for changes in metabolism resulting from the HFD intervention, we performed a direct comparison between the metabolomics results from the STD and HFD cohorts, stratified by sex. We compared the statistically significant high-confidence annotated metabolic features in each dataset. In the male datasets, there were 61 significant annotated features in the STD cohort, and 56 significant annotated features in the HFD cohort, and only four of those features were identified in both datasets (Figure 4-5H, full details in Supplement). The comparison between the female STD and HFD cohorts yielded similar results; there were 79 significant annotated features in the female STD cohort and 51 significant annotated features in the female HFD cohort, with only three features identified in both datasets (Figure 4-5I, full details in Supplement). Further, we found that these major shifts in the metabolic environment were recapitulated at the pathway level; ganglio-series glycosphingolipid biosynthesis was the only metabolic pathway identified in both female cohorts, and pyrimidine metabolism was identified in the female STD cohort and the male HFD cohort (Figure 4-5G). Together, these data demonstrate that that the HFD intervention resulted in major shifts in the global metabolic environment of both male and female animals, but response to the HFD was highly sex-specific. Further,

the finding that ganglio-series glycosphingolipid biosynthesis was identified in both female cohorts suggests that the HFD intervention did not fully restore fat metabolism in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice to WT levels. Finally, although body weight of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD approached that of WT littermates, the substantial genotype differences in the global metabolic environment show that the underlying metabolism of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice still did not recapitulate that of WT animals.

HFD treatment does not affect B6.Del16$^{+/Bdh1\text{-}Tfrc}$ brain size or behavioral phenotypes

Reduced brain size has been described in both mouse models of the 3q29 deletion (15, 16). Additionally, prior work by our team identified an increased brain:body weight ratio in female, but not male, B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice compared to WT littermates (15). An increase in the brain:body weight ratio has been observed in human and animal models of starvation, lending support to the hypothesis that the brain is metabolically privileged (64-67). Because female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed metabolic improvement after HFD treatment, we hypothesized that the brain:body weight ratio in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD would be reduced to WT levels. We found that, consistent with prior reports, brain weight was reduced in both male (p=3E-6) and female (p=0.04) B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice relative to WT littermates (Figure 4-6A). Additionally, we found that the brain:body weight ratio in male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice was identical to that in WT animals (p=1); however, the brain:body weight ratio in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice was increased relative to WT littermates (p=0.04, Figure 4-6B). These data show that while the HFD intervention resulted in metabolic changes in male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, and partially ameliorated the weight deficit in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, these positive effects did not extend to the brain:body weight ratio, indicating that early neurodevelopmental processes may not have been impacted by the HFD.

The 3q29 deletion has a well-established association with neurodevelopmental and neuropsychiatric phenotypes (3, 4, 6-8, 10-14); behavioral deficits have also been identified in two independent mouse models of the 3q29 deletion (15, 16). To understand the effect of the HFD intervention on behavioral phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, we performed a pilot study using a battery of assays designed to test learning and memory, acoustic startle, sensorimotor gating, and

amphetamine sensitivity. We replicated several phenotypes previously identified by our group, including spatial learning and memory deficits, an elevated acoustic startle response, sensorimotor gating deficits, and attenuated amphetamine-induced locomotion (15). We observed some sex differences between our findings and previously published results (15); however, the present study focused on diet and was sufficiently powered for metabolic analyses but not for subtle behavioral phenotypes. There was a significant main effect of the diet intervention ($p<0.05$) in the Morris water maze (MWM), acoustic startle, fear conditioning, and amphetamine-induced locomotor activity for both male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals. However, the effect of the diet was shared across genotypes; the HFD intervention did not differentially impact behavior based on genotype (full details in Supplement). Together, these data demonstrate that the HFD did not introduce any appreciable changes to behavioral phenotypes of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, suggesting that 3q29 deletion-related metabolic and behavioral phenotypes may arise from uncoupled, independent mechanisms.

DISCUSSION

This is the first study to examine metabolic function associated with the 3q29 deletion in a comprehensive manner. Previous work by our group characterized a persistent, sex-dependent weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice (15), and a study of an independently generated mouse model of the 3q29 deletion also identified a reduced weight phenotype in males, but did not examine female animals (16). In the current study we expanded upon this work by exploring mechanisms that may lead to this reduced weight phenotype, incorporating both male and female animals in our study design to explicitly assess the effect of sex on metabolic phenotypes (Figure 4-1). We identified pervasive sex effects of the 3q29 deletion on metabolic function, including differential effects on the metabolic environment and the response to HFD. After HFD treatment, female animals showed a change in weight, and male and female animals showed contrasting changes in the metabolic profile. The HFD intervention did not rescue behavioral phenotypes in male or female animals, suggesting that metabolic and behavioral phenotypes in the context of the 3q29 deletion may arise from independent mechanisms. This study is a step toward unraveling the biology underlying the development of diverse phenotypic outcomes in 3q29 deletion

syndrome. Furthermore, the substantial sex effects identified here show it is imperative to evaluate sex as a biological variable in metabolic studies.

In light of our data, in particular the RER shifts in STD-treated animals and the response to the HFD, we conclude that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice preferentially use lipids as an energy source. This preference for lipids was more pronounced in female animals and was partially corrected by the HFD. The HFD intervention also affected substantial changes in the global metabolic environment as assessed via untargeted metabolomics; the small molecule profile was substantially altered after HFD treatment in both males and females. Consistent with the lack of overlap in the small molecule profile after the HFD intervention, the altered metabolic pathways in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice had minimal overlap between the STD and HFD datasets. Notably, the ganglio-series glycosphingolipid biosynthesis pathway was altered in both STD- and HFD-treated female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice; this supports our conclusion that the HFD did not fully rescue fat metabolism deficits in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females and further emphasizes the persistent sex-dependent effects of the 3q29 deletion.

The striking lack of overlap in male and female metabolic effects of the 3q29 deletion was unexpected. We sought to integrate our data with the larger metabolomics literature, but our initial literature search suggested that most studies did not include sex-stratified analysis of mouse metabolomics data. Our literature search also indicated that female mice were often excluded from the study design. To test this hypothesis, we conducted a formal meta-analysis of the literature. We searched for the terms “mouse metabolomics” and identified 2,601 possible studies (full details in Supplement). We selected 500 of these studies at random and classified them according to whether both sexes were included, and whether sex-stratified analysis was conducted. Of the 500 publications we evaluated, only 44 (8.8%) included both sexes, and only 17 (3.4%) analyzed the data separately by sex. Of these 17 studies, 9 (52.9%) reported substantial sex-dependent differences in the metabolome, indicating there may be widespread but unappreciated differences between male and female mouse metabolomics data. Our conclusion that the metabolomic differences due to the 3q29 deletion are highly-sex dependent is consistent with the larger, albeit limited, scope of the literature. This literature meta-analysis highlights a

pronounced knowledge gap in the field of metabolomics research, as there may be substantial, unappreciated sex-dependent metabolic differences in mouse models. These results have profound implications for the design of future metabolic studies; it is imperative that males and females be included and analyzed separately to rigorously assess the role of sex as a biological variable.

There are well-established links between sex and metabolism. Males and females have different patterns of fat deposition, and differences in fat metabolism have been identified in both humans and rodents (68-73). Studies in rodents have revealed that the sex chromosome complement affects fat metabolism; methods such as the four core genotypes model (74) have helped to disentangle the effects of sex hormones and sex chromosomes on fat metabolism (75-78). These findings are supported at the level of gene expression. A large proportion of liver-expressed genes in humans show sex-biased expression, and the complement of sex-biased genes are enriched for fat metabolism functions (79). Sex hormones, specifically estrogen, also appear to have a role in sex-dependent differences in fat metabolism; oral estrogen therapy in postmenopausal women leads to well-documented changes in fat metabolism (80, 81), and endogenous levels of sex hormones also impact fat metabolism and fat distribution (68, 72, 73, 76, 82, 83). Data from animal models have revealed pervasive roles for estrogen and estrogen-related signaling in metabolic processes including fat metabolism and storage (73). An independent study by our group using transcriptome network analysis identified a co-expression module significantly enriched for estrogen-dependent signaling (p=5.08E-06) that contained the 3q29 deletion interval gene *PAK2* (84). Together with the existing literature on sex differences in fat metabolism and our finding that male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice are differentially affected by 3q29 deletion-associated metabolic phenotypes, this finding suggests that sex is an important consideration in defining the biological mechanisms underscoring phenotypes in 3q29del.

The links between the 3q29 deletion and metabolic function (5, 15, 16) are not unique in the broader context of recurrent CNV disorders. Weight changes and failure to thrive are associated with many recurrent CNVs, including the 22q11.2, 16p11.2, 17p11.2, and 1q21.1 loci (85-98). Evidence has shown that pediatric feeding disorders and nutrient deficiencies can exacerbate existing

neurodevelopmental and cognitive deficits (99-101). In this context, addressing feeding disorders and metabolic concerns in individuals with CNV disorders should be a priority, to minimize the adverse effects of poor nutrition on long-term outcomes. In the present study, we found that a HFD treatment improved metabolic phenotypes but did not affect behavioral phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice, suggesting that a lipid-rich dietary intervention in humans with 3q29del may improve weight phenotypes and nutritional status without exacerbating behavioral phenotypes.

The effects of recurrent CNVs on growth-related phenotypes have been relatively well-described; however, the current understanding of the biological mechanisms leading to these phenotypes is lacking. Recent molecular studies have started to elucidate these mechanisms for 22q11.2 deletion syndrome; mitochondrial dysfunction has been identified as a key contributor to neuronal and synaptic defects associated with the deletion (35-37, 102). Additionally, a recent study of a mouse model of the 16p11.2 deletion and duplication revealed opposite effects of the CNV on metabolic function (90). However, these studies largely focused on targeted metabolic measurements, and failed to address sex as a potential mediator of metabolic phenotypes. The incorporation of untargeted approaches into studies of CNV disorders, and the rigorous interrogation of the role of sex in CNV-associated phenotypes, may expedite our understanding of the biological mechanisms at play in these complex disorders.

While there are established links between neurodevelopmental and neuropsychiatric disorders and metabolic function, we found that the HFD intervention improved metabolic phenotypes but did not affect behavioral phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. There are several possible explanations for this outcome. First, the HFD intervention was implemented starting at postnatal day 21. It is possible this was too late to impact the neurodevelopmental processes that contribute to 3q29 deletion phenotypes. In future experiments, the HFD could be applied to pregnant or nursing dams, potentially exposing 3q29 deletion pups to abundant lipid sources earlier in development (103). It is also possible that the behavioral assays we used and/or the sample size we evaluated could only detect large effects on behavior; the HFD may have caused subtle behavioral improvements that we were unable to detect with the behavioral battery we performed. Additionally, the HFD intervention only targeted fat metabolism, while other metabolic

alterations in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice would not have been improved by the HFD. Our observation that the weight deficit was only partially ameliorated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice supports this hypothesis, and suggests that the underlying biology of the 3q29 deletion involves multiple metabolic processes. Data from the present study suggest that metabolism and neurodevelopment may be unlinked in the context of the 3q29 deletion and may be influenced by separate sets of genes within the deletion interval.

The present study was the first to identify metabolic deficits in the context of the 3q29 deletion. Furthermore, we found pervasive sex-specific effects of the 3q29 deletion; these findings are supported by transcriptome network analysis by our team that identified a module enriched for estrogen-related signaling (84). These results have important implications, both for the 3q29 deletion specifically and for metabolic and mechanistic studies more generally. Our findings suggest that metabolic and behavioral phenotypes may arise from independent mechanisms in the context of the 3q29 deletion, and that these mechanisms may be sex specific. This study underscores a critical need for metabolic and mechanistic experiments to include samples from both male and female subjects, and to analyze the data in a sex-specific manner. Due to the substantial, well-documented metabolic, medical, and neurodevelopmental and neuropsychiatric differences between males and females (68, 104-109), it is not surprising that by analyzing only one sex, or by pooling data from males and females, important metabolic insights may be obscured. Additionally, mechanistic studies in complex disorders that combine data from males and females may miss important sex dependent differences in mechanism, which could delay advancements in available therapeutics. Together, our data highlight sex dependent differences in metabolic function in a mouse model of the 3q29 deletion, adding to our current understanding of 3q29del and creating a framework for future mechanistic studies of complex disorders.

STAR METHODS

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jennifer Mulle (jmulle@emory.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The datasets and code supporting the current study are available from the corresponding author upon reasonable request.

Experimental Model and Subject Details

Animals, Husbandry, and Diets

All studies were performed on male and female C57BL/6N- Del16$^{+/Bdh1\text{-}Tfrc}$ (B6.Del16$^{+/Bdh1\text{-}Tfrc}$) mice and wild type (WT) littermates (15). All animals were maintained on a C57BL/6N background sourced from Charles River Laboratories. Mice were group housed (maximum of 5 animals per cage) during the entire experiment, except for a five-day separation during indirect calorimetry when a subset of mice were singly housed. Mice were on a 12-hour light/dark cycle and were given food and water *ad libitum*. Starting at postnatal day 21, mice were fed either a standard diet (STD, LabDiet 5001) low in fat (13.4% energy from fat) or a high-fat diet (HFD, Teklad TD.88137, 42.0% energy from fat) for the remainder of their lives. Body weight was monitored weekly from 1-16 weeks of age. Indirect calorimetry and behavioral assays were performed on mice between 16-20 weeks of age. At the conclusion of indirect calorimetry, mice were euthanized, and tissues were collected for metabolomics analysis. Mice were not fasted prior to euthanasia and tissue collection. All animal protocols were performed under the approved guidelines of the Emory University Institutional Animal Care and Use Committee. Both males and females were used in all experiments, and the data were analyzed separately. Number of animals used in experiments is indicated in figure legends.

Method Details

Indirect Calorimetry

Mouse metabolic rate was assessed by indirect calorimetry for 5 days in Oxymax chambers using the Comprehensive Lab Animal Monitoring System (Oxymax CLAMS-HC, Columbus Instruments). Mice were singly housed with *ad libitum* access to water and food and were maintained at 20-22°C under a 12/12 hr light/dark cycle (light period 07:00-19:00). A mass-sensitive flow meter was used to maintain a constant airflow of 0.6 L/min. n=8-12 mice/group; age=16-18 weeks

Metabolomics

Untargeted metabolomics analysis on mouse liver tissue was performed as previously described (62). Four blind replicate samples were included in the STD analysis (Figure S4-2A-H) and two blind replicate samples were included in the HFD analysis (Figure S4-2I-L) to ensure data quality. All samples were analyzed in triplicate, and the feature intensities were median summarized. Metabolite profiles were analyzed using the xmsPANDA R package (110). Data from males and females were analyzed separately. Features missing from more than 50% of all samples, or more than 80% of samples from one group, were removed from the dataset. Feature selection was performed using partial least squares regression with a cutoff of VIP score>1.5 and linear regression with a cutoff of p value<0.05. Pathway enrichment analysis was performed using mummichog 2.3.3 (63). n=8-12 mice/group; age=17-19 weeks

Behavior Tests

Morris Water Maze

The Morris water maze (MWM) was conducted to test for deficits in spatial learning and memory. Briefly, the MWM was conducted in a circular tank (52 inches in diameter) filled with water, made opaque with white paint, at 23°C. A hidden circular platform (30 cm in diameter) in the northwestern quadrant of the tank was present 1 cm below the surface of the water. The tank was surrounded by white walls on the north and east sides and white curtains on the west and south sides, all containing external cues for spatial reference. Mice were trained to find the hidden platform over 5 days by being released

into the tank from each quadrant (north, south, east, and west) in a randomized order each day. Each trial lasted a maximum of 60 s; if a mouse did not find the platform in that time, it was guided to the platform and allowed to rest on the platform for 10 s. On the sixth day, the platform was removed from the tank and a probe trial was conducted, in which the mouse was placed in the tank at the south start point and allowed to swim for 60 s. An automated tracking system (TopScan, CleverSys) was used during training to record the latency and distance to the platform and swim speed and was used during the probe trial to record the duration and distance the mice spent in each quadrant of the maze. n=5-13 mice/group; age=16-20 weeks

Acoustic Startle and *Prepulse Inhibition*

To test for deficits in the acoustic startle response and sensorimotor gating, acoustic startle and prepulse inhibition (PPI) were performed. To test the acoustic startle response, mice were subjected to a series of increasing startle tons (75, 80, 85, 90, 95, 100, 115, and 120 db) for 40 ms each, and the response of the animal was measured by the SR-LAB startle response system (San Diego Instruments) accelerometer. A startle curve was constructed to ensure the animal was responding to the increasing stimulus. On the second day, PPI was evaluated. The mice were exposed to 6 blocks of startle conditions with each block consisting of 12 trials, so that each trial was presented to the animal 6 times. The 12 trials were randomly ordered in each block, and the animal's response to the stimulus was measured after each trial. The 12 trials consisted of the following conditions: background (68 db) for 20 ms; startle (120 db) for 40 ms; prepulses 1-5 (PP1=70 db, PP2=74 db, PP3=78 db, PP4=82 db, PP5=86 db) for 20 ms each; and the prepulse.startle combinations (PP1.startle, PP2.startle, PP3.startle, PP4.startle, and PP5.startle), where each prepulse tone was followed by the 120 db startle tone. In the prepulse.startle trials, the mouse was exposed to the prepulse for 20 ms and the startle for 40 ms with a 100 ms gap between the two tones. Each trial was averaged over the 6 blocks, and percent PPI was calculated as:

$$\%PPI = \left(\frac{startle\ response - prepulse.startle\ response}{startle\ response}\right) \times 100$$

n=8-13 mice/group; age=16-20 weeks

Fear Conditioning

To test for deficits in associative learning and memory, we performed a 3-day fear conditioning paradigm. Training and testing were performed in a chamber (H10-11M-TC, Coulbourn Instruments) equipped with a house light, a ceiling-mounted camera, and a speaker. On days 1 and 2, the chamber was equipped with an electric grid shock floor (H10-11M-TC-SF, Coulbourn Instruments); on day 3, the chamber was equipped with a non-shock wire mesh floor (H10-11M-TC-NSF, Coulbourn Instruments). On day 1, the animals were subjected to a 7 min training trial consisting of a 3 min acclimation period followed by three tone-shock pairings during which a tone was played for 20 s immediately followed by a 1 s, 0.5 mA foot shock. On day 2, the animals were placed back in the same chamber as day 1 and were left for 7 min without presentation of the tone or foot shock to test contextual memory. On day 3, the animals were placed in a different chamber, and the chamber floor was replaced with the non-shock wire mesh floor. The animals were in the chamber for 7 min, and the shock-associated tone was played for the last 320 s of the trial to test cued memory. Freezing behavior was automatically recorded using FreezeFrame (Coulbourn Instruments) during each trial. n=9-13 mice/group; age=16-20 weeks

Amphetamine-Induced Locomotor Activity

To evaluate amphetamine sensitivity, amphetamine-induced locomotor activity was measured. The assay was performed in a locomotor chamber (San Diego Instruments) consisting of a plexiglass cage (48x25x22 cm) containing corncob bedding. The locomotor chamber was placed inside an apparatus that projected an 8x4 grid of infrared beams, with beams placed 5 cm apart. When a mouse crossed two consecutive beams, it was considered one ambulation. After a 2 hr acclimation period, mice were given an intraperitoneal injection of either saline or 7.5 mg/kg D-amphetamine and post-injection ambulations were recorded for 2 hr with the accompanying Photobeam Activity System software (San Diego Instruments). Treatments were spread over 2 weeks and were randomized, so that not all of the mice received the same injection in a given week. n=9-13 mice/group; age=16-20 weeks

Meta-analysis

A PubMed search using the keywords “mouse metabolomics” was conducted on July 7, 2020. Papers were filtered to only include publicly available studies published in English between 2015 and 2020, resulting in a list of 2601 papers. 500 papers were randomly selected using a random number generator for analysis. If a study did not use metabolomics or if a study did not use metabolomics performed on primary mouse tissue or cultured mouse cells, or if the study was not a primary research paper, it was excluded and replaced with another randomly selected study from the list of 2601 papers. Papers were reviewed and coded as one of the following to indicate how sex as a biological variable was addressed: “males only”, “females only”, “sex not specified”, “both sexes, not stratified”, or “stratified”.

Quantification and Statistical Analysis

Males and females were analyzed separately in all analyses. All data is represented as mean □standard error of the mean (SEM), and sample size is included in the figure legend. Values of $p<0.05$ were considered statistically significant. WT was set as the reference genotype and the STD was set as the reference diet for all analyses. All plots were created using the plotly R package (111) unless otherwise specified. All analyses performed in R utilized R 3.5.3 (112). All analyses performed in Prism used Prism 8.3.1 (GraphPad). Specific details for each analysis are as follows:

Growth Curves

Growth curve data were analyzed in R (112) using the geepack package to implement generalized estimating equations (GEE) that regressed weight measurements on genotype and age while accounting for within-subject correlation of measurements resulting from multiple time points of data collection (113-115). Age was dichotomized to “early” (1-3 weeks of age) and “late” (4-16 weeks of age) to coincide with time of weaning. All analyses were repeated after applying an inverse normal transformation to the weight data to better satisfy modeling assumptions. Results using the raw and transformed data led to identical conclusions, so the results from the analysis of the raw weight data were presented for ease of interpretation. Using the GEE framework, we performed four distinct sets of analyses. We first compared weight measurements between B6.Del16$^{+/Bdh1\text{-}Tfrc}$ and WT mice on the STD. We then compared weight measurements between an independent set of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ and WT mice

on the HFD. To test for sex-specific differences in effect size in each diet condition, we pooled the male and female data from that diet treatment and fit an additional GEE model that regressed weight measurements on genotype, age, sex, and a genotype-by-sex interaction term. The interaction term was tested to determine whether the effect size of the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ genotype significantly differed by sex. Finally, to test for the effect of the HFD intervention on effect size within each sex, we pooled the STD and HFD data from each sex and fit a new GEE model that regressed weight measurements on genotype, age, diet, and a genotype-by-diet interaction term. The interaction term was tested to determine whether the effect size of the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ genotype significantly differed between diet treatments.

Indirect Calorimetry

All data were analyzed in R (112). The data were filtered to exclude observations with a respiratory exchange ratio (RER) less than 0.650 or greater than 1.05, because they are outside the dynamic range of the measurement. Observations with a negative value for cumulative food or water consumption were excluded, because they indicate periods where the animal had climbed onto the sensor. The final dataset was trimmed to remove intervals at the beginning and end of the experiment that did not have observations for every subject. Ambulations were analyzed with a simple linear model implemented through the stats R package regressing ambulations on genotype and age (112). Ambulations were averaged over all observations in the light and dark cycles for each animal, and light and dark cycle data were analyzed separately. For food and water consumption, the final value for cumulative consumption was used, and was divided by the animal's body weight to find the g food consumed per g body weight. The relative food and water consumption data were analyzed with simple linear models implemented through the stats R package regressing relative food or water consumption on genotype and age (112). For food consumption, a mediation analysis was performed using the R package mediation to determine if body weight mediated the relationship between genotype and total food consumption (116). For energy expenditure and RER, the data were subsetted to only the peaks and troughs in the RER curve; the peak and trough were identified via manual inspection, and one interval to either side of the peak or trough was included, for a total of 3 data points per interval and 7 total intervals (the day 1 and day 5 light cycles

were excluded because the entire cycle was not captured). Each interval was analyzed with a GEE implemented through the geepack R package that regressed either energy expenditure or RER on genotype and interval while accounting for within-subject correlation of measurements resulting from multiple time points of data collection (113-115).

Metabolomics

All data were analyzed in R (112). Median summarized ComBat batch-corrected data was used for all analyses (117). To determine the similarity between blind replicate samples, correlation tests were performed using the stats R package (112). Data filtering and feature selection with partial least squares regression and linear regression were performed using the xmsPANDA R package (110). Stepwise feature selection was performed, where the data were first filtered based on a VIP score>1.5, and then filtered again based on a p value<0.05. All features with a p value<0.05 also had a VIP score>1.5, so the p values from linear regression were used for pathway enrichment analysis. Pathway enrichment analysis was performed using mummichog 2.3.3 with a p value<0.05 cutoff (63). For hierarchical clustering, the linear regression results from the HILIC and C18 columns were pooled, and the top 250 ranked features across the two datasets based on VIP score and p value were used as input. Hierarchical clustering was implemented via the xmsPANDA R package using Spearman correlation (110). For predictive modeling, we used the R package randomForest (118) and the top 250 ranked metabolic features to train a random forest with 25,001 trees to predict genotype. Trees were grown to the maximum size possible; by default, 15 features were considered as candidates at each split, and splitter importance was calculated as the mean decrease in Gini impurity. To account for unequal sample sizes in the STD data, we used weights equal to the inverse of the sample size for each genotype. The receiver operating characteristic (ROC) curves and the area under the ROC curves (AUC) were generated using the R package ROCR (119). P values for the AUC and prediction accuracy were calculated by permuting genotype 10,000 times. Venn diagrams were constructed to compare male and female datasets within a diet condition, as well as to compare STD and HFD datasets within sex, using the VennDiagram R package (120). Male-female comparisons within a diet condition were performed on the top 250 ranked features. STD-HFD

comparisons within sex were performed on statistically significant high-confidence annotated features, defined as an xMSannotator confidence level of 2 or 3 (121).

Behavior Tests

MWM

Animals that did not swim during the probe trial were removed from all analyses. Training data (swim distance, latency to platform, and swim speed) from the STD and HFD cohorts were analyzed separately in Prism (GraphPad) using two-way repeated measures ANOVA followed by multiple comparisons with Sidak's correction when a significant genotype effect or interaction was observed. Unpaired t-tests were implemented using the stats R package to separately analyze probe trial data (proportion of time the animals spent in the quadrant of the maze that formerly contained the platform) from the STD and HFD cohorts (112). To test for the effect of the diet intervention in the training phase, linear mixed-effects models were implemented using the lme4 R package (122). When analyzing the training phase data, we fit models with genotype as the predictor and diet and day as covariates, with subject ID as a random effect. We started with a model including up to a three-way interaction between genotype, diet, and day. To identify the most parsimonious model, we performed backward elimination via likelihood ratio tests implemented using the lmtest R package (123) and removed any higher-order interaction terms that were not significant and refit the model. We performed this process with three-way interactions followed by two-way interactions if the three-way interaction was not significant. The final models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. P values were calculated using Satterthwaite's method via the lmerTest R package (124). To test for the effect of the diet intervention in the probe trial, simple linear models regressing the proportion of time spent in the platform quadrant on genotype, diet, and a genotype-by-diet interaction were implemented using the stats R package (112).

Acoustic Startle

Startle response to 70 db was excluded from the dataset for all animals. The inverse normal transformation was applied to transform the data to an approximately normal distribution. Proper transformation of the data was confirmed with the Shapiro-Wilk normality test implemented using the stats R package (112). Linear mixed-effects models were implemented using the lme4 R package (122). When analyzing the data for each diet separately, all models included genotype, decibel level, and weight as fixed effects and subject ID as a random effect. The models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. To test for the effect of the diet intervention, we fit models with genotype as the predictor and diet, decibel level, and weight as covariates, with subject ID as a random effect. We started with a model including up to a four-way interaction between genotype, diet, decibel, and weight. To identify the most parsimonious model, we performed backward elimination via likelihood ratio tests implemented using the lmtest R package (123) and removed any higher-order interaction terms that were not significant and refit the model. We performed this process with four-way interactions, followed by three-way interactions if the four-way interaction was not significant, and followed by two-way interactions if the three-way interactions were not significant. The final models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. P values were calculated using Satterthwaite's method via the lmerTest R package (124).

PPI

Only the prepulse.startle trials were used to calculate % PPI, as shown in the equation above. The response to the PP1.startle condition (70 db prepulse) was excluded from analysis. Data from the STD and HFD cohorts were analyzed separately in Prism (GraphPad) using 2-way repeated measures ANOVA followed by multiple comparisons with Sidak's correction when a significant genotype effect or interaction was observed. To test for the effect of the diet intervention, linear mixed-effects models were implemented using the lme4 R package (122). We fit models with genotype as the predictor and diet and prepulse decibel as covariates, with subject ID as a random effect. We started with a model including up

to a three-way interaction between genotype, diet, and prepulse decibel. To identify the most parsimonious model, we performed backward elimination via likelihood ratio tests implemented using the lmtest R package (123) and removed any higher-order interaction terms that were not significant and refit the model. We performed this process with three-way interactions followed by two-way interactions if the three-way interaction was not significant. The final models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. P values were calculated using Satterthwaite's method via the lmerTest R package (124).

Fear Conditioning

Data from each day of the task were analyzed separately. Data from the STD and HFD cohorts were analyzed separately in Prism (GraphPad) using 2-way repeated measures ANOVA followed by multiple comparisons with Sidak's correction when a significant genotype effect or interaction was observed. To test for the effect of the diet intervention, linear mixed-effects models were implemented using the lme4 R package (122). All models included genotype, diet, and a genotype-by-diet interaction as fixed effects and subject ID and time as random effects. The models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. P values were calculated using Satterthwaite's method via the lmerTest R package (124).

Amphetamine-Induced Locomotor Activity

Because the ambulation data were not normally distributed, the inverse normal function was used to transform the data to an approximately normal distribution. Proper transformation of the data as confirmed with the Shapiro-Wilk normality test implemented using the stats R package (112) Linear mixed-effects models were implemented using the lme4 R package (122). Saline was set as the reference treatment for all analyses. When analyzing the data for each diet separately, all models included genotype, treatment, and a genotype-by-treatment interaction as fixed effects and subject ID and timepoint as random effects. The models were fit with both maximum likelihood estimation and restricted maximum

likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. To test for the effect of the diet intervention, as well as for differences in the effect of the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ genotype after the HFD intervention, we fit models with genotype as the predictor and diet and treatment as covariates, with subject ID and time as random effects. We started with a model including up to a three-way interaction between genotype, diet, and treatment. To identify the most parsimonious model, we performed backward elimination via likelihood ratio tests implemented using the lmtest R package (123) and removed any higher-order interaction terms that were not significant and refit the model. We performed this process with three-way interactions followed by two-way interactions if the three-way interaction was not significant. The final models were fit with both maximum likelihood estimation and restricted maximum likelihood estimation; the fits were comparable, so the results from the models fitted with maximum likelihood are presented. P values were calculated using Satterthwaite's method via the lmerTest R package (124).

Brain Weight

Data were analyzed in R (112). To calculate the brain:body weight ratio, the brain weight was divided by the body weight of the animal at euthanasia. Brain weight and the brain:body weight ratio were analyzed by unpaired t-test using the stats R package (112).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Mouse: Del16$^{+/Bdh1\text{-}Tfrc}$: C57BL/6N-Del16$^{+/Bdh1\text{-}Tfrc}$	Rutkowski et al., 2019	N/A
Software and Algorithms		
R 3.5.3	R Foundation for Statistical Computing	https://cran.r-project.org
R 3.6.2	R Foundation for Statistical Computing	https://cran.r-project.org
Mummichog 2.3.3	Li et al., 2013	http://mummichog.org
Prism 8.3.1	GraphPad	http://www.graphpad.com/scientific-software/prism; RRID: SCR_015807
Microsoft Excel (version 16.37)	Microsoft Corp.	https://www.microsoft.com/en-gb/; RRID: SCR_016137
TopScan	CleverSys	http://cleversysinc.com/CleverSysInc/?csi_products=topscan-suite; RRID: SCR_017141
FreezeFrame	Coulbourn Instruments	https://www.coulbourn.com/category_s/277.htm
Other		
Laboratory Rodent Diet	LabDiet	Cat#5001
Teklad Rodent Diet	ENVIGO	Cat#88137
Oxymax CLAMS-HC	Columbus Instruments	http://www.colinst.com/docs/CLAMS-HC-CF-WC-2018.pdf
SR-LAB startle response system	San Diego Instruments	https://sandiegoinstruments.com/product/sr-lab-startle-response/
Mouse test cage	Coulbourn Instruments	Cat#H10-11M-TC
Shock floor for mouse test cage	Coulbourn Instruments	Cat#H10-11M-TC-SF
Non-shock floor for mouse test cage	Coulbourn Instruments	Cat#H10-11M-TC-NSF
Photobeam Activity System - Home Cage	San Diego Instruments	https://sandiegoinstruments.com/wp-content/uploads/2018/08/PAS-Home-Cage-DataSheet.pdf

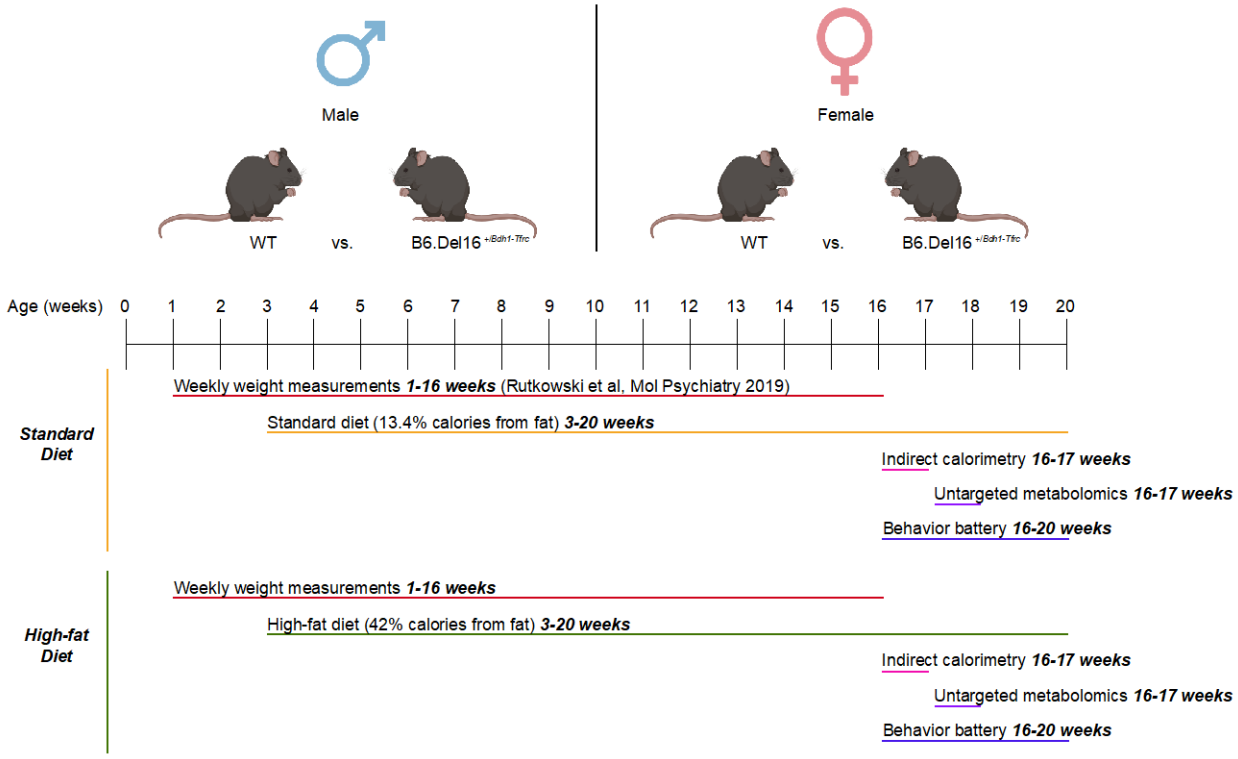

Figure 4-1. Experimental approach to interrogating the effect of the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ genotype on metabolism and the effect of sex on 3q29 deletion-associated metabolic phenotypes.

All experiments were performed on male and female animals, and the sexes were analyzed separately. Animals were fed either standard diet chow (STD) or high-fat diet chow (HFD) from week3 to week 20. Animals were weighed weekly from week 1 to week 16; STD weights were previously published by our group (15). At week 16, a subset of STD- and HFD-treated animals was subjected to indirect calorimetry to assess feeding behavior and metabolic function. At the conclusion of indirect calorimetry, liver tissue was collected for untargeted metabolomics analysis. From weeks 16-20, another subset of STD- and HFD-treated animals was subjected to a behavioral battery.

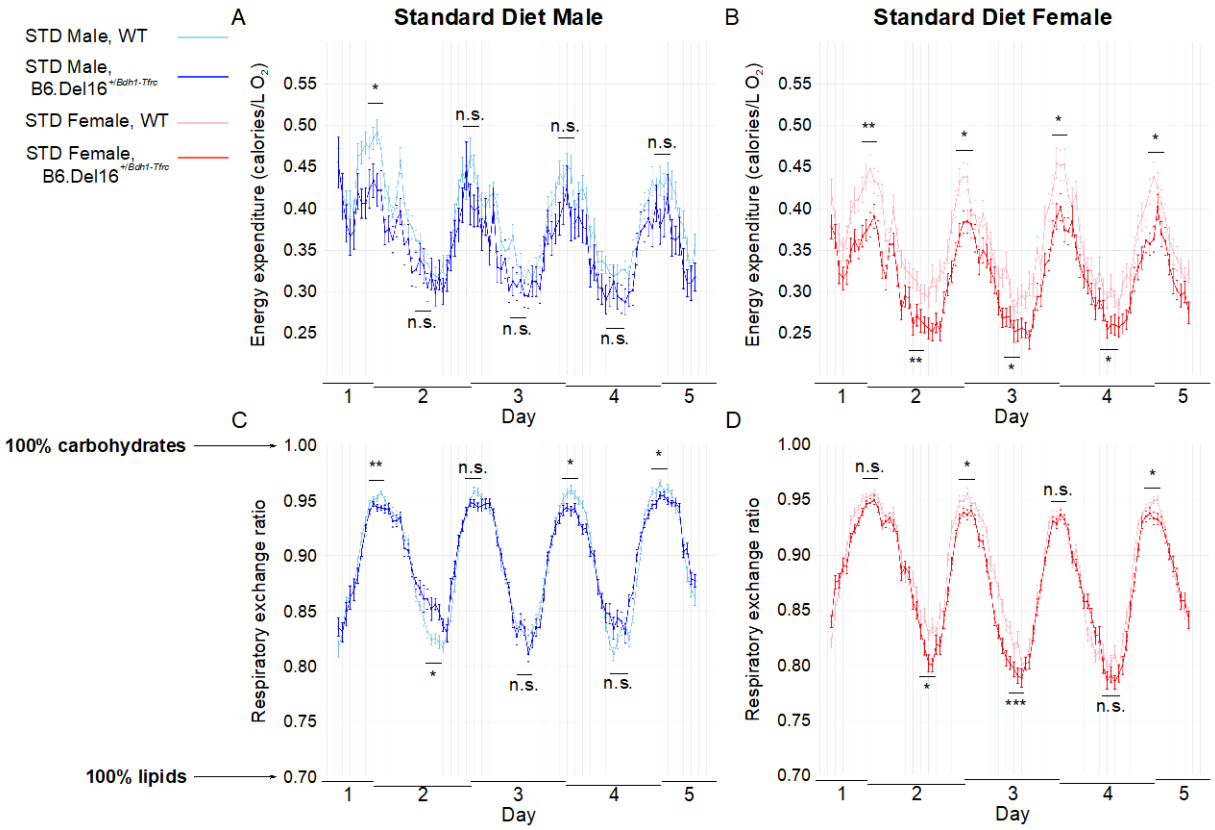

Figure 4-2. Reduced energy expenditure and respiratory exchange ratio in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice.

A and B) Energy expenditure for A) male (n=11 WT, 8 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and B) female (n=14 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) mice on the STD over 5 days in CLAMS/Metabolic Cages.

C and D) RER curves for C) male and D) female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD over 5 days in CLAMS/Metabolic Cages.

Data are represented as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001

Statistical analysis was performed using generalized linear models.

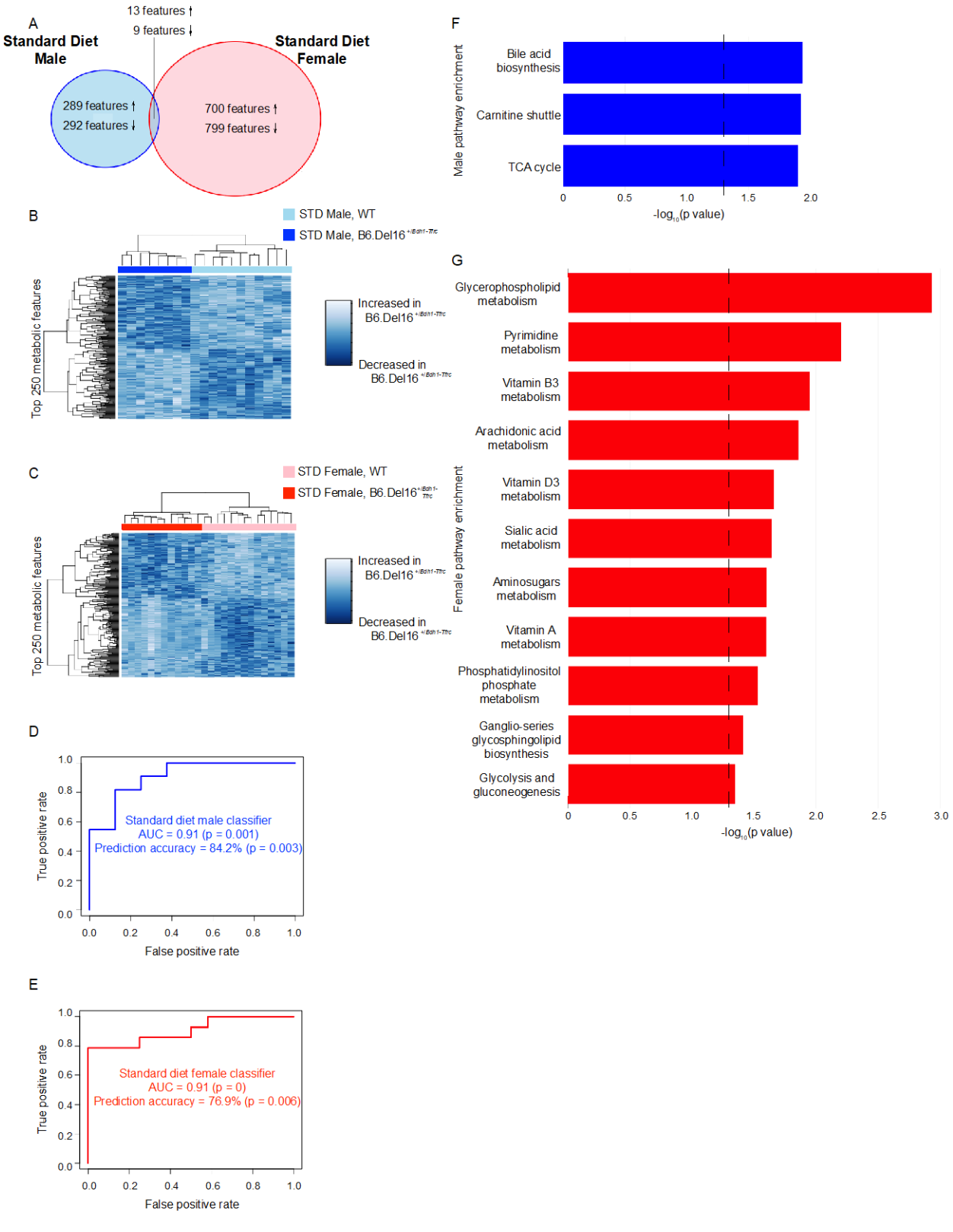
A
Standard Diet Male
Standard Diet Female
13 features ↑
9 features ↓
289 features ↑
292 features ↓
700 features ↑
799 features ↓
B
STD Male, WT
STD Male, B6.Del16+/Bsh1-Tfrc
Top 250 metabolic features
Increased in B6.Del16+/Bsh1-Tfrc
Decreased in B6.Del16+/Bsh1-Tfrc
C
STD Female, WT
STD Female, B6.Del16+/Bsh1-Tfrc
Top 250 metabolic features
Increased in B6.Del16+/Bsh1-Tfrc
Decreased in B6.Del16+/Bsh1-Tfrc
D
True positive rate
Standard diet male classifier
AUC = 0.91 (p = 0.001)
Prediction accuracy = 84.2% (p = 0.003)
False positive rate
E
True positive rate
Standard diet female classifier
AUC = 0.91 (p = 0)
Prediction accuracy = 76.9% (p = 0.006)
False positive rate
F
Male pathway enrichment
Bile acid biosynthesis
Carnitine shuttle
TCA cycle
-log10(p value)
G
Female pathway enrichment
Glycerophospholipid metabolism
Pyrimidine metabolism
Vitamin B3 metabolism
Arachidonic acid metabolism
Vitamin D3 metabolism
Sialic acid metabolism
Aminosugars metabolism
Vitamin A metabolism
Phosphatidylinositol phosphate metabolism
Ganglio-series glycosphingolipid biosynthesis
Glycolysis and gluconeogenesis
-log10(p value)

Figure 4-3. Untargeted metabolomics reveals small molecule alterations in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice that are highly sex-dependent.

A) Comparison of all nominally significant metabolomic features between the male and female datasets. Up arrows indicate metabolites significantly upregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples, down arrows indicate metabolites significantly downregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples. Also refer to Supplement.

B and C) Hierarchical clustering of B) male (n=11 WT, 8 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and C) female (n=14 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) samples using the top 250 ranked metabolomic features.

D and E) ROC curves for random forest predictors generated using the top 250 ranked metabolomic features in D) male and E) female datasets.

F and G) Altered pathways in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice identified via pathway enrichment analysis of F) male and G) female datasets. Dashed line denotes statistical significance.

Statistical significance of ROC curves (D and E) was assessed using 10,000 permutations of the data.

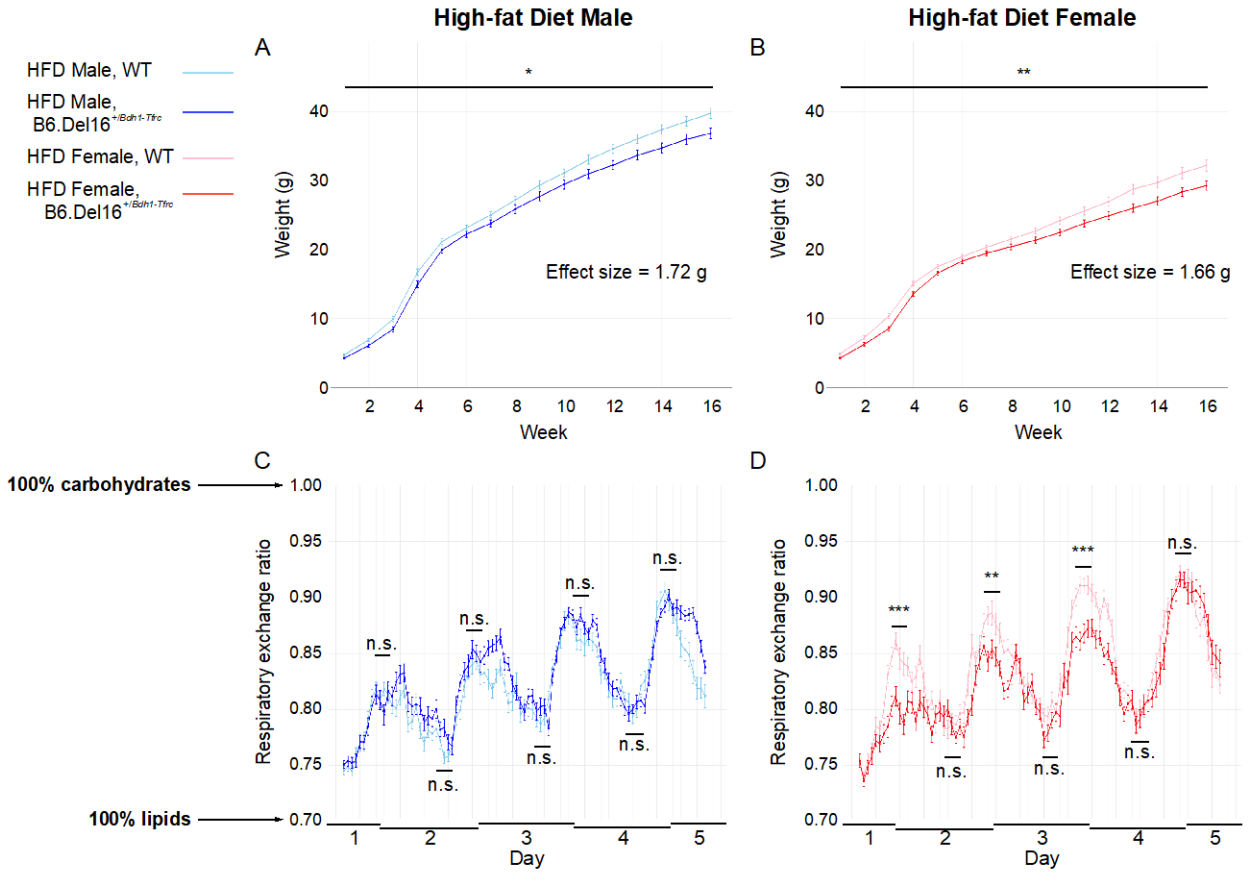

Figure 4-4. A high-fat diet reduces the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ weight deficit and affects RER in a sex-specific manner.

A and B) 16-week growth curves for HFD-treated A) male (n=50 WT, 30 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and B) female (n=42 WT, 32 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) mice.

C) Raw effect size of the 3q29 deletion on weight in HFD-treated male and female mice.

D) Effect size of the 3q29 deletion on weight relative to average WT body weight at week 16 in HFD-treated male and female mice.

E and F) RER curves for E) male (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and F) female (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) mice on the HFD over 5 days in CLAMS/Metabolic Cages.

Data are represented as mean ± SEM. n.s., $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Statistical analysis of growth curves (A-D) was performed using generalized estimating equations.

Statistical analysis of RER (E-F) was performed using generalized linear models.

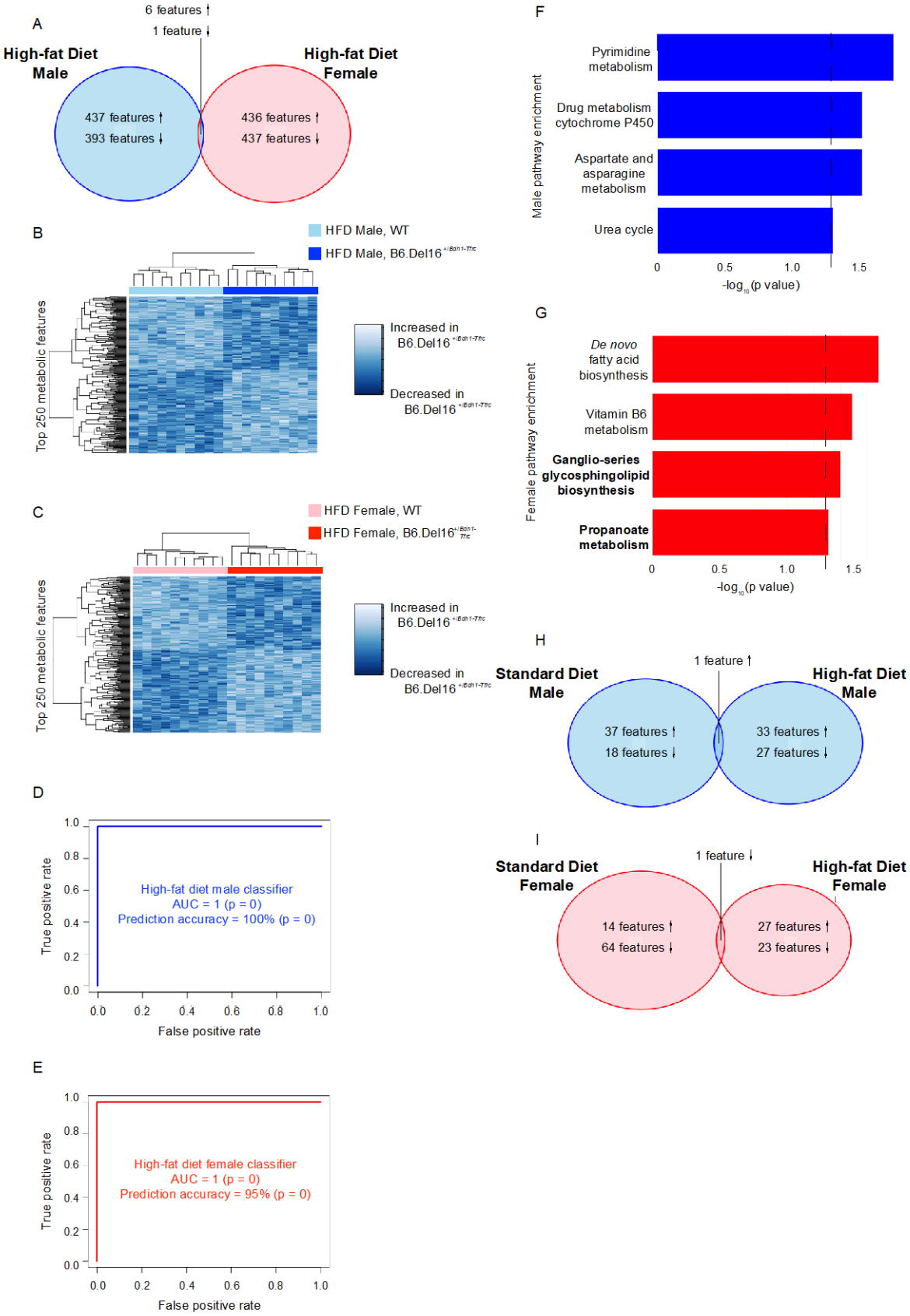
A
6 features ↑
1 feature ↓
High-fat Diet Male
High-fat Diet Female
437 features ↑
393 features ↓
436 features ↑
437 features ↓
B
HFD Male, WT
HFD Male, B6.Del16 +/Bdh1-Tfrc
Top 250 metabolic features
Increased in B6.Del16 +/Bdh1-Tfrc
Decreased in B6.Del16 +/Bdh1-Tfrc
C
HFD Female, WT
HFD Female, B6.Del16 +/Bdh1-Tfrc
Top 250 metabolic features
Increased in B6.Del16 +/Bdh1-Tfrc
Decreased in B6.Del16 +/Bdh1-Tfrc
D
True positive rate
False positive rate
High-fat diet male classifier
AUC = 1 (p = 0)
Prediction accuracy = 100% (p = 0)
E
True positive rate
False positive rate
High-fat diet female classifier
AUC = 1 (p = 0)
Prediction accuracy = 95% (p = 0)
F
Male pathway enrichment
Pyrimidine metabolism
Drug metabolism cytochrome P450
Aspartate and asparagine metabolism
Urea cycle
-log10(p value)
G
Female pathway enrichment
De novo fatty acid biosynthesis
Vitamin B6 metabolism
Ganglio-series glycosphingolipid biosynthesis
Propanoate metabolism
-log10(p value)
H
Standard Diet Male
High-fat Diet Male
1 feature ↑
37 features ↑
18 features ↓
33 features ↑
27 features ↓
I
Standard Diet Female
High-fat Diet Female
1 feature ↓
14 features ↑
64 features ↓
27 features ↑
23 features ↓

Figure 4-5. Widespread changes in the global metabolic environment of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice after HFD treatment.

A) Comparison of all nominally significant metabolomic features between the HFD-treated male and female datasets. Up arrows indicate metabolites significantly upregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples, down arrows indicate metabolites significantly downregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples. Also refer to Supplement.

B and C) Hierarchical clustering of HFD-treated B) male (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and C) female (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) samples using the top 250 ranked metabolomic features.

D and E) ROC curves for random forest predictors generated using the top 250 ranked metabolomic features in HFD-treated D) male and E) female datasets.

F and G) Altered pathways in HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice identified via pathway enrichment analysis of F) male and G) female datasets. Dashed line denotes statistical significance. Bold text denotes pathways that were identified in both the STD and HFD experiments.

H and I) Comparison of nominally significant annotated features between STD-treated and HFD-treated H) male and I) female datasets. Up arrows indicate metabolites significantly upregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples, down arrows indicate metabolites significantly downregulated in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ samples. Also refer to Supplement.

Statistical significance of ROC curves (D and E) was assessed using 10,000 permutations of the data.

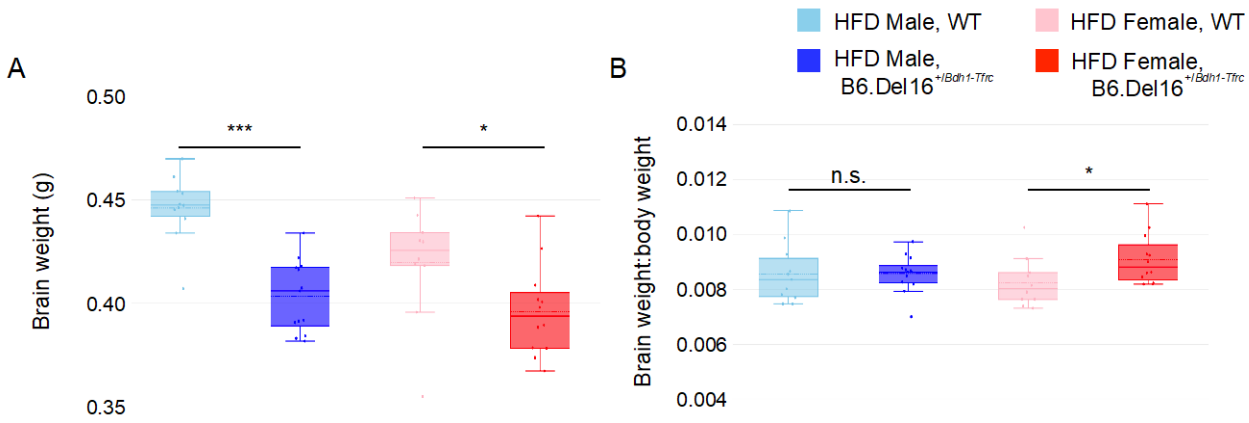

Figure 4-6. HFD treatment does not affect B6.Del16$^{+/Bdh1\text{-}Tfrc}$ brain size.

A) Brain weight in HFD-treated male (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) and female (n=10 WT, 10 B6.Del16$^{+/Bdh1\text{-}Tfrc}$) mice.

B) Brain weight:body weight ratio in HFD-treated male and female mice.

For each box plot, the dashed line indicates the mean value, and the solid line indicates the median. n.s., $p>0.05$; *, $p<0.05$; ***, $p<0.001$

Statistical analysis was performed using unpaired t-tests.

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Supplemental information

Energy and water consumption are not reduced in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD

We performed 5 days of indirect calorimetry on male and female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice using CLAMS/Metabolic Cages (Columbus Instruments). To determine whether the weight deficit in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice is simply due to decreased calorie intake, we evaluated food consumption. There was no significant difference in food consumption (Figure S4-1A) between male or female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals after controlling for the reduced weight of B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals; mediation analysis showed that including weight in the model fully accounted for the relationship between genotype and food consumption. Additionally, there were no differences between male or female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice in water consumption (Figure S4-1B).

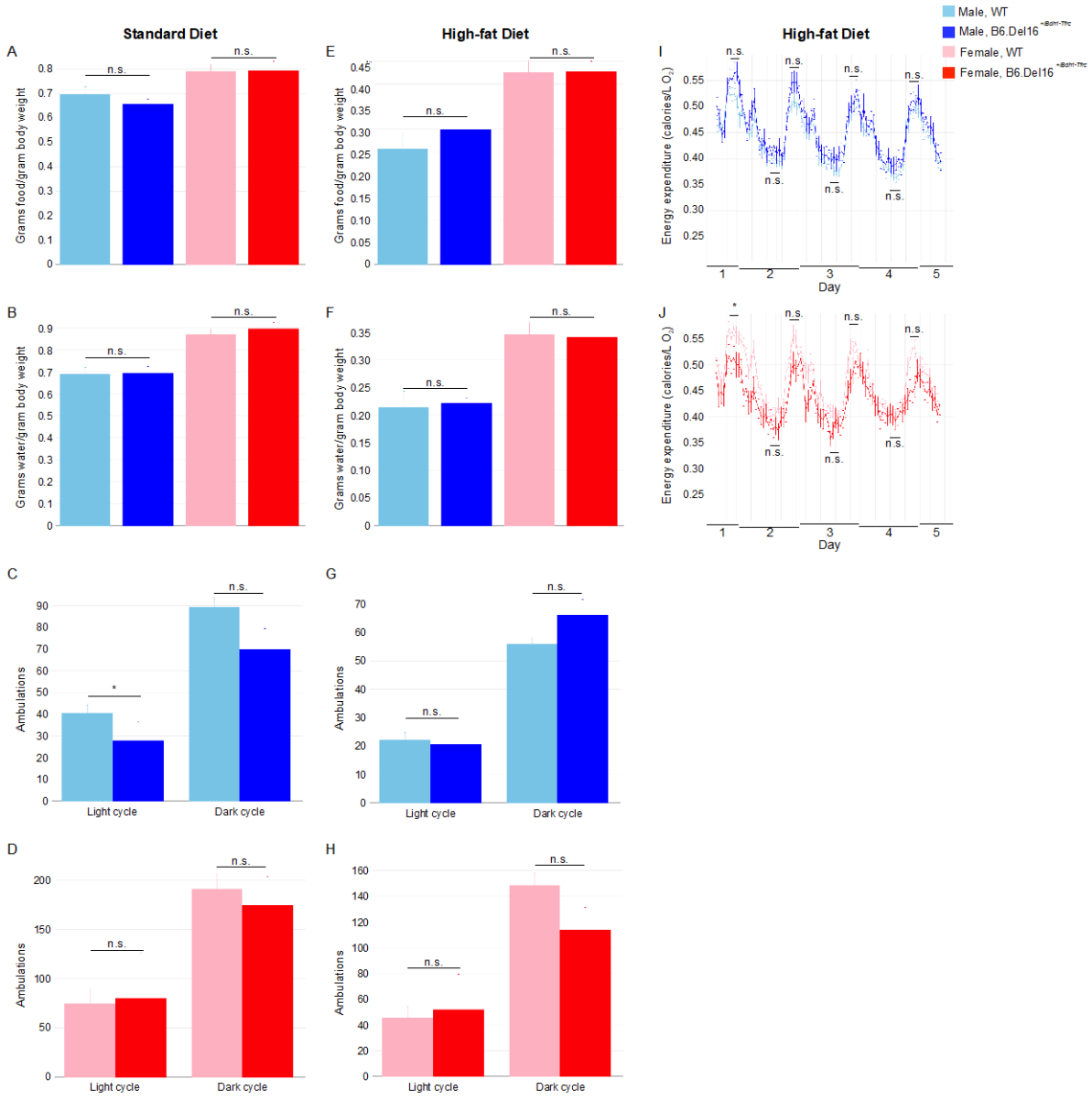

Figure S4-1. Indirect calorimetry of WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD and HFD. Related to Figures 4-2 and 4-4.

A) Total food consumption for male and female WT (n= 11 male, 14 female) and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ (n= 8 male, 12 female) mice on the STD over 5 days in CLAMS/Metabolic Cages.

B) Total water consumption for male and female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD over 5 days in CLAMS/Metabolic Cages.

C and D) Average ambulations during the light and dark cycles for C) male and D) female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD over 5 days in CLAMS/Metabolic Cages.

E) Total food consumption for male and female WT (n= 10 male, 10 female) and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ (n= 10 male, 10 female) mice on the HFD over 5 days in CLAMS/Metabolic Cages.

F) Total water consumption for male and female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD over 5 days in CLAMS/Metabolic Cages.

G and H) Average ambulations during the light and dark cycles for G) male and H) female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD over 5 days in CLAMS/Metabolic Cages.

I and J) Energy expenditure for I) male and J) female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD over 5 days in CLAMS/Metabolic Cages.

Data are represented as mean ± SEM. n.s., $p>0.05$; *, $p<0.05$

Statistical analysis of food consumption, water consumption, and ambulations (A-H) was performed using simple linear regression. Statistical analyses of energy expenditure (I and J) were performed using generalized linear models.

Sex-specific and diet-specific small molecule changes identified via untargeted metabolomics

We performed untargeted metabolomics of liver samples from WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD and HFD. To understand the effect of sex on the metabolic environment of animals on the STD, we compared all nominally significant features between males and females. Only 22 features were identified in both datasets (Figure 4-3A). Of those 22 features, 13 were enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals and 9 were depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals. An additional 17 features were identified in both datasets but were discordant, so they were not counted as common features. Of the 17 discordant features, 9 were enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females, while the other 8 were depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females. When we compared all nominally significant features between samples from HFD-treated males and females, a similar pattern emerged, with only 7 features identified in both datasets (Figure 4-5A). Of those 7 features, 6 were enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals and one was depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals. An additional 18 features were identified in both datasets but were discordant, so they were not counted as common features. Of the 18 discordant features, 7 were enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females, while the other 11 were depleted in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females.

To understand the effect of the HFD on the metabolic environment, we compared the statistically significant high-confidence annotated features between STD-treated and HFD-treated males, and between STD-treated and HFD-treated females. One feature was enriched in samples from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals in both male datasets (Figure 4-5H). Three additional features were identified in both datasets but were discordant, so they were not counted as common features. Of the three discordant features, two were enriched in samples from STD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but depleted in samples from HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males, and one was depleted in samples from STD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males but enriched in samples from HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ males. One feature was depleted in samples

from B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals in both female datasets (Figure 4-5G). An additional two features were identified in both datasets but were discordant, so they were not counted as common features. Both discordant features were depleted in samples from STD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females and enriched in samples from HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ females.

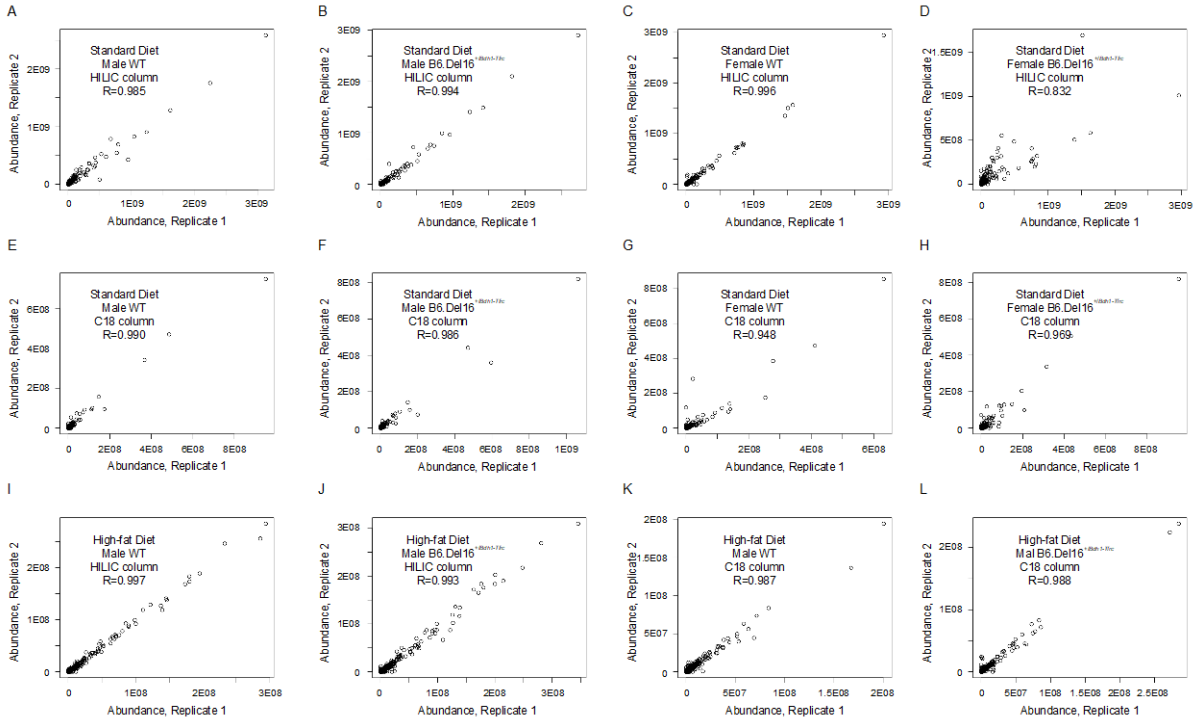

Figure S4-2. Correlation between replicate samples from untargeted metabolomics.

A, B, C, D, E, F, G, and H) Correlation between blind replicate samples for liver metabolomics of STD-treated animals using A, B, C, D) the HILIC column and E, F, G, H) the C18 column.

I, J, K, and L) Correlation between blind replicate samples for liver metabolomics of HFD-treated animals using I, J) the HILIC column and K, L) the C18 column.

Behavioral phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice are not impacted by HFD treatment

Spatial learning and memory

In the Morris water maze (MWM), we found no differences between male WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD in swimming distance ($p>0.05$), latency ($p>0.05$), or swim speed ($p>0.05$) during the training portion (Figure S4-3A-C). Male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed increased latency ($p=0.002$) and swam a greater distance ($p=0.002$) to reach the hidden platform compared to WT littermates, but did not show any difference in swim speed ($p>0.05$) during the training portion (Figure S4-3G-I). When the data from STD- and HFD-treated males were directly compared, we observed a significant main effect of genotype on latency and swim distance ($p<0.05$), where male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice took longer to reach the platform and swam a farther distance compared to WT littermates, and a significant main effect of diet on swim distance and swim speed ($p<0.05$), where males on the HFD swam a shorter distance and swam more slowly than males on the STD (Figure S4-3A-C, G-I).

Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed increased swimming distance ($p=0.005$), but no differences in latency ($p>0.05$) or swim speed ($p>0.05$) compared to WT littermates in the training portion of the MWM (Figure S4-3D-F). Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed increased latency ($p=0.025$), but no differences in distance ($p>0.05$) or swim speed ($p>0.05$) compared to WT littermates in the training portion of the MWM (Figure S4-3J-L). When data from STD- and HFD-treated females were directly compared, we observed a significant main effect of genotype on latency and swim distance ($p<0.05$), where female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice took longer to reach the platform and swam a farther distance compared to WT littermates, and a significant main effect of diet on latency, where females on the HFD took longer to reach the platform than females on the STD (Figure S4-3D-F, J-L).

In the probe trial of the MWM, there was no difference in the percentage of time in the quadrant that formerly contained the platform between male or female WT and B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD (Figure S4-3M-N). There was no difference in the percentage of time in the quadrant that formerly contained the platform between male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ and WT mice on the HFD ($p>0.05$); however,

female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD spent significantly less time in the platform quadrant compared to WT littermates (p=0.022, Figure S4-3M-N). When the data from STD- and HFD-treated males were directly compared, we found a significant main effect of diet, where males on the HFD spent more time in the quadrant that formerly contained the platform than males on the STD (p=0.02). When the data from STD- and HFD-treated females were directly compared, there were no main effects of genotype or diet (p>0.05, Figure S4-3M-N).

Contextual learning and memory

In the fear conditioning assay, male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed no significant differences in freezing percentage in the training phase, but showed decreased freezing compared to WT littermates in both the context (p=0.01) and cue (p=0.004) phases (Figure S4-3O-Q). Likewise, male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed no significant differences in freezing percentage in the training phase, but showed decreased freezing compared to WT littermates in both the context (p=0.001) and cue (p=0.006) phases (Figure S4-3U-W). When the data from STD- and HFD-treated males were directly compared, we found no main effects of genotype or diet in the training phase (p>0.05, Figure S4-3O, U). In the context phase, there was a significant main effect of genotype, where male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed decreased freezing compared to WT littermates (p=0.002, Figure S4-3P, V). In the tone phase, there was a significant main effect of genotype, where male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed decreased freezing compared to WT littermates (p=0.001), and a significant main effect of diet, where male animals on the HFD showed increased freezing relative to males on the STD (p=0.008, Figure S4-3Q, W).

Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD had similar freezing percentages to WT littermates during all phases of the fear conditioning assay (p>0.05, Figure S4-3R-T). Likewise, female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD had similar freezing percentages to WT littermates during all phases of the task (p>0.05, Figure S4-3X-Z). When the data from STD- and HFD-treated females were directly compared, we found no main effects of genotype or diet in the training phase (p>0.05, Figure S4-3R, X). In the context phase there were no main effects of genotype or diet (p>0.05, Figure S4-3S, Y). In the tone

phase there was a significant main effect of diet, where females on the HFD showed increased freezing relative to females on the STD (p=0.007, Figure S4-3T, Z).

Acoustic startle response and sensorimotor gating

Male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed an increased acoustic startle response compared to WT littermates (p<0.05, FigureS4A, C). Body weight was not significantly associated with startle response for male or female animals on the STD (p>0.05), indicating that the reduced weight phenotype in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ animals on the STD did not affect the measured startle response. Male and female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed an increase in acoustic startle response compared to WT littermates (p<0.05, Figure S4-4B, D). For male animals on the HFD, body weight was not associated with startle response (p>0.05). Weight was significantly associated with startle response in female animals on the HFD (p=0.001), suggesting that the decreased weight in female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD may have impacted the measured startle response. When the data from STD- and HFD-treated males were directly compared, we found a significant main effect of diet, where males on the HFD showed an increased acoustic startle response compared to males on the STD (p=0.02, Figure S4-4A-B). When the data from STD- and HFD-treated females were directly compared, we found a significant main effect of diet, where females on the HFD showed a decreased acoustic startle response compared to females on the STD (p=0.03), and a significant main effect of genotype, where female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed a decreased acoustic startle response compared to WT littermates (p=0.02, Figure S4-4C-D).

Male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed reduced prepulse inhibition (PPI) compared to WT littermates (p=0.02, Figure S4-4E), indicating a mild impairment in sensorimotor gating. Female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed similar PPI to WT littermates at all prepulse levels (p>0.05, Figure S4-4G). Likewise, male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed significantly reduced PPI compared to WT littermates (p=0.003, Figure S4-4F), while female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed similar PPI to WT littermates (p>0.05, Figure S4-4H). When the data from STD- and HFD-treated males were directly compared, we found a significant main effect of genotype, where male

B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed significantly reduced PPI compared to WT littermates (p=1.4E-6, Figure S4-4E-F). When the data from STD- and HFD-treated females were directly compared, there were no significant effects of genotype or diet (p>0.05, Figure S4-4G-H).

Amphetamine sensitivity

In the amphetamine-induced locomotor activity task, there were no differences in ambulatory activity following saline administration between male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD and WT littermates (p>0.05). Likewise, there were no differences in ambulatory activity following saline administration between male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD and WT littermates (p>0.05). After administration of 7.5 mg/kg amphetamine, male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed similar levels of amphetamine-induced locomotion relative to WT littermates (p>0.05, Figure S4-4I), whereas male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed significantly attenuated amphetamine-induced locomotion relative to WT littermates (p=0.015, Figure S4-4J). When the data from STD- and HFD-treated males were directly compared, we found a significant main effect of diet, where males on the HFD showed reduced activity compared to males on the STD (p=0.004), a significant genotype-by-treatment interaction, where male B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed reduced activity after amphetamine administration compared to WT littermates (p=0.002), and a significant diet-by-treatment interaction, where males on the HFD showed reduced activity after amphetamine administration compared to males on the STD (p=2.26E-13, Figure S4-4I-J).

There were no differences in ambulatory activity following saline administration between female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD and WT littermates (p>0.05). Likewise, there were no differences in ambulatory activity following saline administration between female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD and WT littermates (p>0.05). After administration of 7.5 mg/kg amphetamine, female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the STD showed significantly attenuated amphetamine-induced locomotion relative to WT littermates (p=9.32E-6, Figure S4-4K), whereas female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice on the HFD showed similar levels of amphetamine-induced locomotion relative to WT littermates (p>0.05, Figure S4-4L). When the data from STD- and HFD-treated females were directly compared, we found a significant main

effect of diet, where females on the HFD showed reduced activity compared to males on the STD (p=0.04), a significant genotype-by-treatment interaction, where female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed reduced activity after amphetamine administration compared to WT littermates (p=2.74E-5), a significant diet-by-treatment interaction, where females on the HFD showed reduced activity after amphetamine administration compared to females on the STD (p=6.43E-13, Figure S4-4K-L).

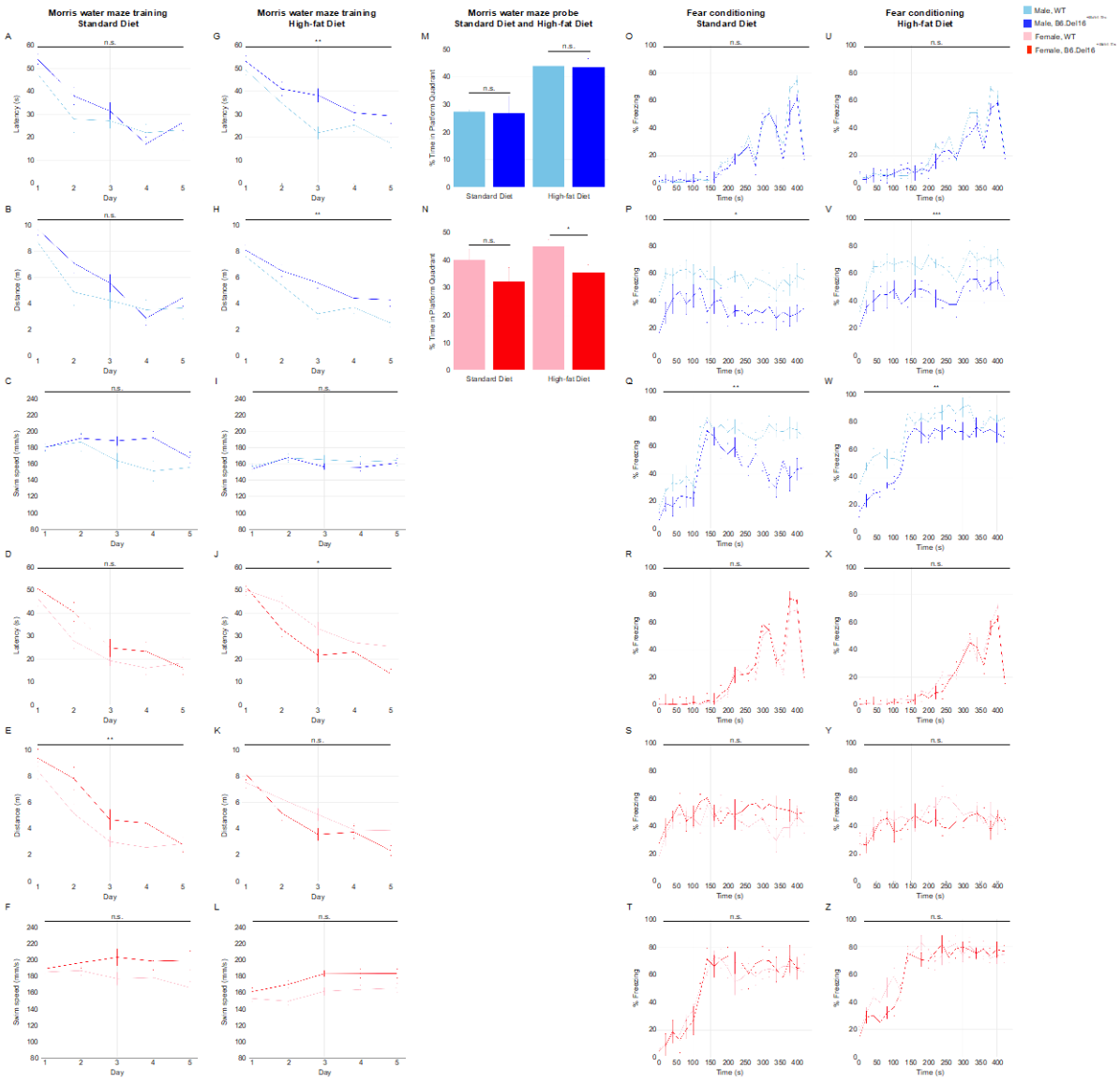

Figure S4-3. Learning phenotypes in STD- and HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice.

A, B, and C) MWM training A) latency to the hidden platform, B) swim distance, and C) swim speed in STD-treated males (n=5 WT, 8 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

D, E, and F) MWM training D) latency to the hidden platform, E) swim distance, and F) swim speed in STD-treated females (n=8 WT, 7 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

G, H, and I) MWM training G) latency to the hidden platform, H) swim distance, and I) swim speed in HFD-treated males (n=12 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

J, K, and L) MWM training J) latency to the hidden platform, K) swim distance, and L) swim speed in HFD-treated females (n=11 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

M and N) Percentage of time spent in the quadrant that formerly contained the platform in the probe trial of the MWM in STD- and HFD-treated M) males and N) females.

O, P, and Q) Percent freezing behavior during the fear conditioning O) training phase, P) context test, and Q) tone test in STD-treated males (n=9 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

R, S, and T) Percent freezing behavior during the fear conditioning R) training phase, S) context test, and T) tone test in STD-treated females (n=9 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

U, V, and W) Percent freezing behavior during the fear conditioning U) training phase, V) context test, and W) tone test in HFD-treated males (n=12 WT, 13 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

X, Y, and Z) Percent freezing behavior during the fear conditioning X) training phase, Y) context test, and Z) tone test in STD-treated males (n=12 WT, 13 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

Data are represented as mean ± SEM. n.s., $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Statistical analysis of MWM training phase and fear conditioning (A-L, O-Z) was performed using two-way repeated measures ANOVA. Statistical analysis of MWM probe trial (M and N) was performed using unpaired t-test.

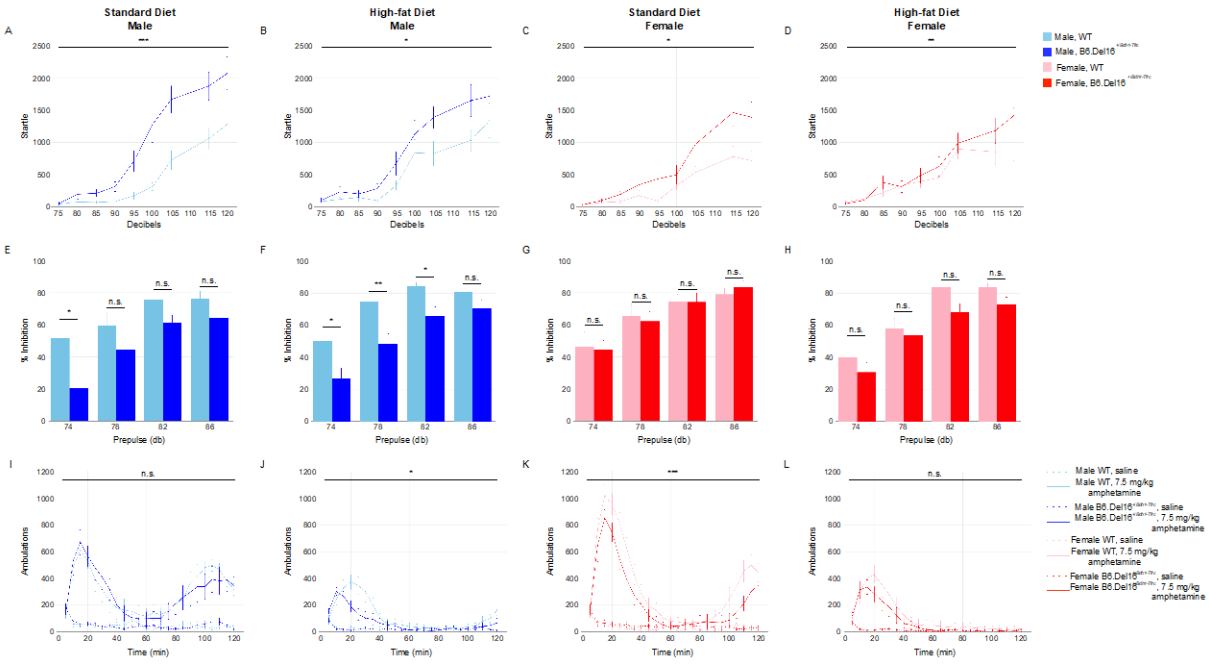

Figure S4-4. Acoustic startle, prepulse inhibition, and amphetamine-induced locomotion phenotypes in STD- and HFD-treated B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice.

A, B, C, and D) Acoustic startle response in A) STD-treated males (n=9 WT, 8 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), B) HFD-treated males (n=12 WT, 13 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), C) STD-treated females (n=10 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), and D) HFD-treated females (n=11 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

E, F, G, and H) Prepulse inhibition in E) STD-treated males (n=9 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), F) HFD-treated males (n=12 WT, 13 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), G) STD-treated females (n=10 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), and H) HFD-treated females (n=11 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

I, J, K, and L) Amphetamine-induced locomotor activity in I) STD-treated males (n=9 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), J) HFD-treated males (n=11 WT, 13 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), K) STD-treated females (n=10 WT, 9 B6.Del16$^{+/Bdh1\text{-}Tfrc}$), and L) HFD-treated females (n=9 WT, 12 B6.Del16$^{+/Bdh1\text{-}Tfrc}$).

Data are represented as mean ± SEM. n.s., $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Statistical analysis of acoustic startle and amphetamine-induced locomotor activity (A-D, I-L) was performed using linear mixed models. Statistical analysis of prepulse inhibition (E-H) was performed using two-way repeated measures ANOVA.

Meta-analysis of metabolomics literature

We performed a meta-analysis of existing literature to test the hypothesis that sex is not commonly addressed as a biological variable in mouse metabolomics studies. A PubMed search for the keywords “mouse metabolomics” yielded 2,601 studies published since 2015. We randomly selected 500 studies for further analysis using a random number generator. We classified the studies based on how sex was addressed as a biological variable; studies were coded as “males only”, “females only”, “sex not specified”, “both sexes, not stratified”, or “stratified”. Of the 500 randomly selected studies, only 44 (8.8%) studied both sexes, and only 17 (3.4%) performed a stratified analysis. 69 studies used only female samples (13.8%), 248 studies used only male samples (49.6%), and 139 studies did not specify the sex of the samples (27.8%).

Chapter 5. Discussion and future directions

5.1 Summary

The 3q29 interval is a new frontier for understanding neurodevelopmental and neuropsychiatric phenotypes. In this work, I performed a multidimensional investigation of 3q29 deletion syndrome (3q29del) and the reciprocal 3q29 duplication syndrome (3q29dup). Previous studies have shown that 3q29del is associated with a wide variety of neurodevelopmental, neuropsychiatric, and medical phenotypes (1-16). However, the nuances of social disability and ASD phenotypes in individuals with 3q29del have not been previously described. Additionally, the phenotypic spectrum of 3q29dup has not been systematically defined. Further, our current understanding of 3q29del and 3q29dup is based largely on case reports, which focus on severely affected individuals and are not generalizable to the larger patient population. Finally, although a majority of individuals with 3q29del experience reduced birthweight, significant feeding problems, and failure to thrive (4), the possibility of metabolic alterations underlying these phenotypes have not been investigated. The possibility of an unidentified metabolic disturbance is supported by the robust weight deficits observed in both mouse models of the 3q29 deletion (17, 18). These gaps in the knowledge of 3q29del and 3q29dup can be a barrier to care; without a clear understanding of the phenotypic consequences of 3q29del and 3q29dup, there is little information to guide medical providers in caring for affected individuals.

In chapter two, I used data from the online 3q29 registry (3q29deletion.org) to explore features of ASD present in the largest cohort of individuals with 3q29del assembled to date. I found that the rate of reported ASD diagnosis in our study population was substantially elevated compared to the general population (29% in 3q29del versus 1.47% in the general population), and that the 3q29 deletion confers a greater influence on risk for ASD in females (OR=41.8) than in males (OR=24.6). I also found that individuals with 3q29del show substantial social vulnerability independent of their reported ASD diagnosis status. Further, I found that the presentation of ASD-related phenotypes in individuals with 3q29del was distinct from individuals with idiopathic ASD. The ASD profile in 3q29del was characterized by severe deficits in restrictive interests and repetitive behaviors, but relatively well-preserved social motivation. Together, these data demonstrate a previously unappreciated degree of social

vulnerability independent of ASD status in 3q29del and highlight features that differentiate 3q29del-associated ASD from idiopathic ASD.

While the broad phenotypic spectrum of 3q29del has been relatively well-described, little is known about the range of phenotypes associated with 3q29dup. In chapter three, I analyzed data from the online 3q29 registry (3q29deletion.org) to develop a clearer understanding of the phenotypic spectrum of 3q29dup. I collected data on the largest existing cohort of individuals with 3q29dup using a customized medical questionnaire and standardized ASD symptom scales. I found enrichment for a variety of phenotypes in our 3q29dup study population, including reduced birthweight, feeding problems, developmental delay, ASD, and anxiety. I also identified a previously unappreciated degree of social vulnerability in individuals with 3q29dup; individuals with 3q29dup reported a significantly increased prevalence of ASD diagnosis compared to the general population. Further, individuals with 3q29dup showed a high degree of social vulnerability and ASD-related phenotypes independent of their reported ASD diagnostic status. These data show that the 3q29 duplication is associated with a wide range of phenotypes comparable to that observed in individuals with 3q29del, and that the 3q29 duplication may be a previously unrecognized ASD risk locus.

In addition to systematically describing the phenotypic spectrum of 3q29dup in chapter three, I also performed a direct comparison between 3q29del and 3q29dup. Some phenotypes were consistent between 3q29del and 3q29dup, including reduced birthweight, feeding problems, developmental delay, ASD, and anxiety. 3q29del and 3q29dup diverged in other phenotypic domains, including congenital heart defects and seizures. The prevalence of congenital heart defects was significantly lower in individuals with 3q29dup as compared to individuals with 3q29del, and the prevalence of seizures was increased in individuals with 3q29dup as compared to individuals with 3q29del. I also found qualitative differences between 3q29del and 3q29dup in some ASD-related phenotypes. Individuals with 3q29dup had significantly greater social motivation deficits compared to individuals with 3q29del, suggesting that individuals with 3q29dup have a more substantial social disability phenotype than individuals with 3q29del.

The 3q29 deletion is associated with reduced birthweight, feeding problems, and failure to thrive in human patients (4) and robust weight deficits in mouse models (17, 18); I hypothesized the 3q29 deletion results in a previously unidentified metabolic disturbance. In chapter four, I investigated metabolic function, and the role of sex as a modifier of metabolic function, associated with the 3q29 deletion using the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model. I found that the 3q29 deletion has substantial, sex-dependent effects on fat metabolism. Further, I found that the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ response to a high-fat diet (HFD) intervention was also sex-specific; these data showed that female B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice have a more pronounced need for dietary lipids that was only partially corrected by the HFD. I also tested the effect of the HFD intervention on behavioral phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice and found that the HFD intervention does not affect behavioral phenotypes, indicating that metabolic and behavioral phenotypes in 3q29del may be uncoupled. These data underscore the importance of evaluating sex as a biological variable in metabolic studies and serve as the first step in understanding the biological mechanisms underlying phenotypes in 3q29del.

This work builds upon the existing understanding of 3q29del and 3q29dup. Most importantly, these data provide guidance for the clinical management of individuals with 3q29del and 3q29dup. Based on my data in chapters two and three, I proposed that gold-standard ASD evaluation should be standard of care for all individuals diagnosed with 3q29del or 3q29dup. These evaluations are of critical importance, as they are the first step in accessing care that can improve long-term outcomes for affected individuals. In chapter three I also identified additional areas of concern for individuals with 3q29dup and their caregivers that had not been previously acknowledged in the literature, including a substantial burden of failure to thrive and a high proportion of feeding problems. The enrichment for feeding problems in both 3q29del and 3q29dup populations is a particular area of concern, because feeding problems and subsequent nutritional deficiencies can exacerbate existing neurodevelopmental and medical challenges (19-21). In chapter four, I found that B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice showed some metabolic improvement after a HFD intervention, and that the HFD did not result in behavioral change. These data suggest that a HFD intervention in individuals with 3q29del may improve metabolic phenotypes without exacerbating

behavioral manifestations. Together, this work identified areas of improvement in the clinical management of individuals with 3q29del and 3q29dup, and highlighted areas of future therapeutic exploration. Combining these strategies may serve as an effective way to improve long-term outcomes and quality of life for individuals with 3q29del and 3q29dup.

5.2 Limitations

In chapter two, I described the spectrum of ASD-related phenotypes in 3q29del using data from the 3q29 registry (3q29deletion.org). While this is the most comprehensive description of social disability phenotypes in 3q29del to date, there are limitations to consider. This study relied entirely on registry-solicited data, which is subject to ascertainment bias. In order to be included in the study, participants had to have joined the registry and completed at least one questionnaire. Individuals who performed these tasks are likely to be highly motivated to participate in research, possibly because their child is severely affected. This could skew the data, as I may have sampled from the extreme end of the phenotypic distribution. Additionally, because the registry is web-based, internet access is a precondition of participation. This may have limited the participation of historically underserved communities; the study sample was 87.1% white, which supports this conclusion and suggests the registry has not adequately reached minority populations. Finally, the data in this study was collected using standardized questionnaires rather than gold-standard diagnostic instruments; however, I was able to compare the registry-collected data to data from in-person evaluations for a subset of participants and found high concordance, suggesting that registry-based data collection is a viable tool for studying rare disorders.

In chapter three I also used data from the 3q29 registry (3q29deletion.org); therefore, the study has many of the same limitations as chapter two. In addition to the limitations outlined above, there are two additional limitations to the study in chapter three. First, the 3q29 registry was originally conceived to study 3q29del; as such, the medical and demographic questionnaire used to gather information about medical, neurodevelopmental, and neuropsychiatric diagnoses was customized to focus on phenotypic domains highlighted in case reports of 3q29del (4). I identified several areas of phenotypic divergence between 3q29del and 3q29dup, including congenital heart defects and seizures; however, there may be

additional phenotypes present in 3q29dup that were not assessed in the registry. Additionally, I was not able to compare the results from the standardized ASD questionnaires to gold-standard evaluations for any participants. However, the high concordance between the standardized questionnaires and gold-standard evaluations in chapter two suggests the standardized questionnaires accurately capture ASD-relevant phenotypes.

In chapter four, I studied metabolic function using the B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model of the 3q29 deletion. The use of a mouse model rather than patient-derived samples introduces some inherent limitations. While the study design was a necessity given the invasive nature of the study, I was unable from these experiments alone to determine whether humans with 3q29del share the same metabolic alterations. An additional limitation is that the behavioral experiments were underpowered, so I was unable to detect small changes in behavioral phenotypes resulting from the HFD. Finally, the HFD intervention was implemented at postnatal day 21, so it did not impact early neurodevelopment. However, the findings outlined in chapter four provide a strong foundation for future metabolic studies of the 3q29 deletion and identify areas of metabolic function that should be investigated in greater detail.

5.3 Future directions

There are several areas of expansion to continue building the phenotypic understanding of 3q29del and 3q29dup. In-depth evaluations of individuals with 3q29del and 3q29dup using gold-standard instruments will help shape the clinical standard of care recommendations made in chapters two and three. This work is an ongoing part of the Emory 3q29 Project (https://genome.emory.edu/3q29) (22) and will continue to refine the current understanding of 3q29del and 3q29dup. Evaluating phenotypes in larger cohorts of affected individuals will illuminate core phenotypes associated with the 3q29 interval, which is key to moving toward improved therapeutic strategies.

In addition to guiding clinical care of individuals with 3q29del and 3q29dup, more refined phenotyping data will help inform future molecular studies of the 3q29 interval. A goal of the Emory 3q29 Project is to dissect biological mechanisms underlying 3q29del- and 3q29dup-associated phenotypes. The work presented in chapter 4 investigated metabolism, specifically fat metabolism, in the

B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mouse model; however, other metabolic alterations in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice were not evaluated. The data in chapter 4 supports additional metabolic alterations associated with the 3q29 deletion, based on the partial correction of metabolic phenotypes by the HFD intervention. Additionally, we were unable to directly link 3q29 interval genes to specific metabolic phenotypes using currently available techniques. Techniques currently under development, including metabolic flux analysis, are a natural extension of the metabolomics data presented in chapter 4 and will help refine our understanding of 3q29 deletion-associated metabolic changes. Further, the majority of 3q29 interval genes are not known to be involved in metabolism; future experiments are needed to determine how haploinsufficiency of these genes contributes to 3q29 deletion-associated phenotypes. Finally, the work in chapter 4 highlighted the role of sex as a mediator of metabolic phenotypes in B6.Del16$^{+/Bdh1\text{-}Tfrc}$ mice. This finding needs further investigation, as sex has not previously been considered as a modifier of 3q29 deletion and duplication phenotypes.

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