## EXTENDED EXPERIMENTAL PROCEDURES

### Patient Assessment

Approval to initiate patient re-contact from the anonymized cohorts for comprehensive phenotypic workup was obtained through the institutional review boards (IRBs) for each of the patient cohorts (University of Washington IRB protocol HSD#42744). For all participants, characterization consisted of review of medical and research records and consultation with the treating clinician, if appropriate. Review of records and consultation focused on clarification of the physical phenotype as reported by clinical geneticist following physical exam, psychiatric diagnosis and diagnostic assessment process and measures, cognitive and adaptive functioning and assessment processes and measures used, medical comorbidities, and identification of gastrointestinal (GI), sleep, and other behavioral concerns based on parent and physician reports. Following the informed consent process, the participants completed a comprehensive, standardized assessment battery that targeted diagnostic clarification; identification of GI, sleep, and other behavioral concerns based on parent and physician report; cognitive, adaptive, language, and motor assessment; and physical exam. The diagnostic battery consisted of clinical interview with caregiver and patient, gold-standard autism spectrum disorder (ASD) diagnostic tools (Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994); Autism Diagnostic Observation Schedule (ADOS; Lord et al., 1999)), and application of Diagnostic and Statistical Manual – 5$^{th}$ Edition criteria using expert clinical judgment (American Psychiatric Association, 2013) by a licensed psychologist with research reliability on the ADOS and ADI and expertise in ASD. The cognitive and adaptive battery included the Differential Ability Scales-2$^{nd}$ Edition (DAS-2; Elliott, 2007), Mullen Scales of Early Learning (Mullen, 1995) for younger/lower functioning children, and the Vineland Adaptive Behavior Scales, 2$^{nd}$ Edition (VABS-2; Sparrow et al., 2005). Autism was dimensionally assessed using the Social Responsiveness Scale (SRS; Constantino and Gruber, 2005) and behavioral/emotional problems were assessed using the Child Behavior Checklist (Achenbach and Rescorla, 2001). Language was evaluated using the Peabody Picture Vocabulary Test-4$^{th}$ Edition (PPVT-4; Dunn and Dunn, 2007) and the Nonword Repetition subtest of the Comprehensive Test of Phonological Processing (CTOPP; Wagner et al., 1999). Fine motor skills were assessed using the Purdue Pegboard (Tiffin, 1968). The physical exam was conducted by a behavioral pediatrician with expertise in clinical genetics. Standardized *z* scores were derived from the head circumference measurements using established norms (Roche et al., 1987). For two study participants, longitudinal examination of head circumference was achieved through retrospective medical records review. Raw occipital frontal head circumference values were culled from medical records from all-well baby and pediatric care visits. For one patient, 11 measurements between birth and 5 years of age were obtained and, for the other, 19 measurements between birth and 6 years of age were obtained.

### Detailed Case Reports

#### *Summary*

In our case series of 15 individuals with *CHD8* disruptive mutations, we identified several characteristics common to most of the cohort, including a diagnosis of ASD; macrocephaly; a facial phenotype marked by prominent forehead, wide-set eyes, and pointed chin; as well as increased rates of GI complaints and marked sleep dysfunction. Our findings suggest that *CHD8* disruptions represent a specific pathway in the development of ASD and define a distinct ASD subtype.

Macrocephaly was observed in 73% of the case series through analysis of occipitofrontal circumference (OFC) measurements. Importantly, these OFC measurements reflect the pronounced supraorbital brow ridges that were reported in many of the patients for whom physical exams had been completed. This suggests that the observed increase in head circumference may be reflecting particular overgrowth of the underlying frontal cortex in early brain development. While increased height was also observed in this case series with some regularity (43% with height *z* scores $\geq$ +2.0), there was not 1:1 correspondence to those with macrocephaly. Furthermore, per recommendations by Chaste et al. (2013) who performed a rigorous assessment of head circumference in ASD, a mean score for mother and father head circumference was calculated for individuals in the SSC to account for the familial impact of head size when considering head circumference in offspring. After controlling for parental mean head circumference, age at evaluation, and height, individuals with *CHD8* in the SSC had significantly larger heads compared to individuals without a *CHD8* event, $F(1,2639) = 4.79$, $p = 0.029$, Cohen's $d = 3.10$ (large effect size based on estimated marginal means).

Although 67% of the cohort presented with significant sleep problems, high rates of overall sleep problems are reported in ASD with rates ranging from 44% to 83% reported in the literature (Clements et al., 1986; Hoshino et al., 1984; Patzold et al., 1998; Polimeni et al., 2005; Richdale and Prior, 1995; Wiggs and Stores, 1996). When only difficulties specific to falling asleep are examined in ASD, consistent rates of 53% (Gail Williams et al., 2004) and 51% (Krakowiak et al., 2008) have been reported. These rates are reflected in this cohort. When comparing the children with *CHD8* mutations in the SSC cohort to children with ASD in the SSC without a *CHD8* event, 67% (versus 60% in overall SSC) had significant difficulty with sleep onset (defined as difficulty going to bed, difficulty falling asleep, or requiring a parent to lay down with child prior to sleeping). These results were not significant (Fisher's exact test, $p = 0.747$). Notably, for two of the patients in this case series, the difficulties with falling asleep were so pronounced that the children would reportedly remain awake for days or sleep very little over a period of days. However, a comparison to the general SSC cohort was unavailable for this particular question. Additionally, 67% of the cohort (versus 40% in overall SSC) had excessive nighttime awakenings (defined as frequent/prolonged awakenings after falling asleep, sleepwalking or frequent nightmares); this result was also non-significant (Fisher's exact test, $p = 0.169$). Larger samples with more specific inquiry into the precise nature of the sleep disturbances will be required.

Regression, medical and behavioral concerns were examined in the case series. Regression was observed in 6 of the 15 cases in this series. This rate roughly mirrors the 32% rate of regression reported in a recent meta-analytic review of regression research in ASD from 1980 to 2010 (Barger et al., 2013).

As clarified in the patient descriptions (see below), the two parents with *CHD8* events identified through cascade testing showed a varied behavioral presentation. The father of patient Nij07-06646 demonstrated similar behavioral challenges related to autism (although not formally evaluated), had a large head circumference, and recurrent sleeping and GI difficulties. In contrast, the father of patient T102.03, with a paternally inherited duplication, showed no behavioral atypicalities, did not have macrocephaly or similar facial features, and did not have any reported GI or sleep disturbances.

Five of the 15 patients (33%) in this case series reported a C-section birth. This rate is higher than reported in the general population of 22.9% across the United States in 2000, although not significantly higher than the rate observed in ASD (29.9%; Brimacombe et al., 2007). While C-section rates are consistently increasing, rates based on 2000 data are more relevant given the age of the reported cases. It is plausible that this high rate of C-sections reflects birth complications related to the increased head size observed in patients with *CHD8* mutations.

Similarly, induction and augmentation of labor is noted in 6 of the 15 patients (40%) with available birth history information. Although highly variable and increasing, average ranges of labor induction and labor augmentation in the general population in 2001 were reported to be 20.5% and 17.5%, respectively (Simpson and Atterbury, 2003). Of note, compared to non-ASD births, higher percentages of induced or augmented births are reported for ASD births after controlling for fetal distress and other birth complications, with 32% of boys and 29% of girls with ASD having had induction or augmentation during their delivery (Gregory et al., 2013).

***Patient SSC 12714.p1***

Patient is a 4-year-old Caucasian male. He has above average head circumference (*4 years* HC = 53 cm, $z$ = 1.0) and is tall with a normal BMI (height = 116.5 cm, $z$ = 2.4; weight = 21.5 kg, $z$ = 1.5). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria). Cognitive abilities fall in the borderline range (Verbal IQ = 75, Nonverbal IQ = 78, Full Scale IQ = 74 by DAS-2) and adaptive scores in the low average range (Adaptive Composite = 80). Patient currently speaks in sentences. He first used single words at 30 months and first phrases at 42 months. Problems were first noted in his development at 12 months of age. A possible loss of language skills during development is noted. Patient was diagnosed with chronic constipation at 1.5 years, which resolved at 2.5 years. Patient also has a history of chronic loose stool beginning in infancy. No sleep abnormalities are noted. Elevated internalizing behaviors are endorsed (social withdrawal, and somatic complaints) with no comorbid diagnoses. Impairment in receptive language is noted (PPVT-4 Standard Score = 71). Average overall motor skills are reported by parent on the VABS (Motor Standard Score = 91), but significant challenges with fine motor coordination were noted upon assessment (Purdue Pegboard Dominant Hand T scores = 31, Nondominant Hand T score = 15, and Both Hands T score = 18). Parent report about his social responsiveness on the SRS suggests severely impacted social communication, social cognition, and autistic mannerisms, and mild-to-moderate impairment in social motivation and social awareness. Patient was born vaginally at 38 weeks gestation following a labor augmented by Pitocin. Hyperbilirubinemia was present at birth but no treatment was prescribed.

***Patient SSC 13986.p1***

Patient is a 5-year-old Caucasian male. Large head circumference is noted (*5 years* HC = 55 cm, $z$ = 2.0). Patient is tall with a normal BMI (height = 123.5 cm, $z$ = 2.1, weight = 24.7 kg, $z$ = 0.7). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and intellectual disability (confirmed with cognitive and adaptive testing). Respiratory problems (retraction in lungs) were diagnosed at 12 months and cerebral palsy was diagnosed at 18 months. Imaging results were normal at 18 months and EEG results were normal at 24 months. Patient's cognitive and adaptive abilities fall in the extremely low range (Verbal IQ = 25, Nonverbal IQ = 38, Full Scale IQ = 32, by Mullen; and Adaptive Composite = 57). Problems were first noted in his development at 9 months of age, and he remains nonverbal at age 5. There are no reported GI disturbances, but patient has reported allergies to gluten and casein. Patient has sleep difficulties characterized as frequent night-time awakenings. He also has significant affective and attention problems, and below average motor skills, per parent report on the VABS (Motor Standard Score = 72). Parent report about his social responsiveness on the SRS suggests severely impacted social communication, social cognition, and social awareness, and mild-to-moderate impairment in social motivation and autistic mannerisms. Patient was born vaginally at 42 weeks gestation following lab augmentation with Pitocin due to failure to progress. Patient's mother had a previous 2$^{nd}$ trimester miscarriage. Mother experienced an upper respiratory infection during pregnancy (trimester unknown) with the patient.

***Patient SSC 11654.p1***

The patient is a 12-year-old Caucasian female. Facial features include hypertelorism, broad forehead with prominent supraorbital ridges, down slanted palpebral fissures, broad nose, and pointed chin, (See Figure 2a). Patient has a large head circumference (*12 years* HC = 58.2 cm, $z$ = 3.1; *10 years* HC = 56.3 cm, $z$ = 2.9; *9 years* HC = 56.0 cm, $z$ = 2.4; *8 years* HC = 55.2, $z$ = 2.3). Patient is tall with a normal BMI (*12 years*: height = 166 cm, $z$ = 1.5; weight = 57.7 kg, $z$ = 1.3; *10 years*: height = 147.5 cm, $z$ = 1.7; weight = 40.4 kg, $z$ = 1.2; *9 years*: height = 144 cm, $z$ = 1.7; weight = 37.4 kg, $z$ = 1.2; *8 years*: height = 140.5 cm, $z$ = 2.4, weight = 34.5 kg, $z$ = 1.5). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and intellectual disability (confirmed with cognitive and adaptive testing). Patient meets strict criteria for ASD with significant social communication impairment (impaired eye contact, proxemics, use of language, limited gestures, no peer relationships, no emotional/affective reciprocity, reduced sharing, showing, giving, and reduced joint attention and social orienting) and frequently recurring,

significantly impairing repetitive behaviors (e.g., repetitive motor movements) and stereotypic use of language. Patient's cognitive and adaptive abilities fall in the extremely low range (Verbal IQ = 37, Nonverbal IQ = 47, Full Scale IQ = 43, by DAS-2; and Adaptive Composite = 59). She first used single words at 9 months, but did not develop phrase speech until 48 months. Patient currently uses limited phrase speech. Abnormalities were first noted in her development at 8 months of age and her parents report loss of skills in the first 2 years of life. While she has no diagnosed seizures, subclinical seizure symptoms have been reported although imaging and EEG at age 5 were clinically normal. Parents report patient complains of commonly recurring headaches. Precocious puberty is reported. Patient has significant history of constipation and periodic leakage. This has ameliorated somewhat over time, but persists. She also has a history of significant sleep problems. Parents report that patient has significant problems with falling asleep and will remain awake without any apparent fatigue for multiple days. Sleep problems were present from early development. Patient has significant attention problems, substantial impairment in receptive language (PPVT-4 Standard Score = 45), intact phonological short-term memory skills (CTOPP Scaled Score = 8), and extreme difficulties in fine motor coordination (Purdue Pegboard T scores all < 0). Mother's report about patient's social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, social awareness, and autistic mannerisms. Patient was born via C-section due to large head size at 40 weeks gestation following a labor induced by Pitocin. The pregnancy was unremarkable.

***Patient SSC 13844.p1***

Patient is an 8-year-old Caucasian male. Large head circumference is noted (*8 years* HC = 56.5 cm, $z = 2.6$). Patient is tall with a normal BMI (height = 132.7 cm, $z = 1.5$, weight = 29.9 kg, $z = 0.1$). He was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and intellectual disability (confirmed with cognitive and adaptive testing). Respiratory problems were diagnosed at 11 months and kidney problems were diagnosed at 9 months. Patient's cognitive and adaptive abilities fall in the extremely low range (Verbal IQ = 20, Nonverbal IQ = 34, Full Scale IQ = 27, by DAS-2 and Mullen; and Adaptive Composite = 59). He first used single words at 20 months, but has not developed phrase speech. He currently speaks in fewer than 5 single words. Abnormalities were first noted in his development at 12 months of age and parents report a possible loss of language skills during early development. Patient has a significant history of constipation with bloating and abdominal pain as well as allergies to gluten and casein. There are no reported sleep disturbances. Patient is reported to be withdrawn. He also has significantly impaired receptive language (PPVT-4 Standard Score = 52) and extreme difficulties with fine motor coordination (Purdue Pegboard T scores all < 0). Parent report about patient's social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, social awareness, and autistic mannerisms. Patient was born vaginally at 37 weeks gestational age. Patient received oxygen supplementation at birth.

***Patient SSC 14016.p1***

Patient is an 8-year-old Caucasian male. Facial features include broad forehead with prominent supraorbital ridges, hypertelorism, down slanted palpebral fissures and pointed chin (See Figure 2b). Patient has a large head circumference measurements (*5 years* HC = 55.4 cm, $z = 2.3$; *18 months* HC = 50.8 cm, $z = 1.9$; *15 months* HC = 49.9 cm, $z = 1.2$; *13 months* HC = 52.1 cm, $z = 3.9$; *12 months* HC = 49.2 cm, $z = 1.8$; *9 months* HC = 48.3 cm, $z = 2.1$, *6 months* HC = 47.0 cm, $z = 2.6$; *4 months* HC = 45.4 cm, $z = 1.4$; *2 months* HC = 41.9 cm, $z = 1.2$; *1 month* HC = 39.4, $z = 1.4$; *birth* HC = 35.9, $z = 0.8$). Patient is average height and build (*8 years*: height = 137 cm, $z = 1.2$, weight = 25.0 kg, $z = -0.4$; *6 years*: height = 123.2 cm, $z = 1.5$, weight = 23.2 kg, $z = 0.7$; *4 years*: height = 104.1 cm, $z = 0.4$, weight = 16.1 kg, $z = -0.1$; *2 years*: height = 87.6 cm, $z = 0.0$, weight = 12.9 kg, $z = 0.1$; *1 year*: height = 80 cm, $z = 1.3$, weight = 9.0, $z = -1.4$). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria). He shows significant autism-related impairments in social communication (social reciprocity, no peer relationships, limited showing, sharing, and giving, foundation social skills such as joint attention and social orienting are intact though), and restricted interests. Patient's cognitive abilities fall in the low average range (Verbal IQ = 85, Nonverbal IQ = 86, Full Scale IQ = 84 by DAS-2) while his adaptive abilities fall in the significantly impaired range (Adaptive Composite = 69). Patient is verbally fluent, but is still very quiet, rarely initiates spoken language, and is significantly anxious around unfamiliar people. He first used single words at 36 months and first phrases at 42 months. Abnormalities were first noted in his development at 2 months of age. Patient has been diagnosed with hypotonia and as excessively clumsy/uncoordinated. His early history is also significant for febrile seizures, which have resolved. EEG and imaging have been conducted and results have been normal. Patient has a history of intermittent unusual stool, diarrhea, and constipation during early childhood that have remediated somewhat, but not completely. Patient has significant sleep problems, specifically with falling asleep. Parents report that patient will remain awake for up to three days before falling sleep. Melatonin and anti-anxiety medication have had limited efficacy in managing sleep problems. Patient has significant problems with attention, anxiety, aggressive behavior, and affect. He also has below average receptive language (PPVT-4 Standard Score = 74), below average fine motor coordination (Purdue Pegboard Dominant Hand T scores = 27, Nondominant Hand T score = 29, and Both Hands T score = 31), and below average phonological short-term memory skills (CTOPP Scaled Score = 6). Parent report about his social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, and autistic mannerisms, and mild-to-moderate impairment in social awareness. Patient was born vaginally at 38 weeks gestation following a labor induced by Pitocin due to failure to progress. Mother reports having a viral illness during pregnancy with him. The mother had two (one before and one after the patient's pregnancy) miscarriages, without any ascertained cause.

***Patient SSC 12991.p1***

Patient is a 16-year-old Caucasian male. Facial features include high forehead, hypertelorism, large ears, and fleshy earlobes. (See Figure 2c). Other physical findings include minimal arch and flat feet. Large head circumference is noted (*16 years*

HC = 59.0 cm, $z$ = 1.7; *12.5 years* HC = 58.0 cm, z = 2.3) and overweight BMI is recorded (*16 years* height = 170 cm, $z = -0.7$, weight = 80.3 kg, $z$ = 1.2; *12 years* height = 158, $z$ = 0.6, weight = 57.7, $z$ = 1.3). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and mild intellectual disability (confirmed with cognitive and adaptive testing). He shows significant autism-related impairments in social communication (unusual prosody, stereotyped social response, few peer relationships, decreased eye contact with intact gesture use and facial expressions), repetitive/scripted speech, repetitive play, and motor mannerisms. He first used single words at 15 months and first phrases at 36 months. Patient is now verbally fluent. Abnormalities were first noted in his development at 24 months of age. Patient has a history of ADHD with antidepressant and stimulant medication use. Seizures and abnormal EEG and MRI were noted starting at age 12. Patient is currently taking antiepileptic medication. Patient is reportedly excessively clumsy/uncoordinated. Patient's cognitive and adaptive abilities fall in the extremely low range (Verbal IQ = 44, Nonverbal IQ = 67, Full Scale IQ = 59, by DAS-2; Adaptive Composite = 63). Patient has chronic diarrhea and excessive gas. He has had significant difficulty falling asleep since 5 years of age and has frequent night-time awakenings. Mother reports that he sleeps for 4 hr per night. Patient had hernia surgery at 10 months of age. Patient has significant problems with anxiety and depressive symptoms, substantial impairment in receptive language (PPVT-4 Standard Score = 47), and below average to significantly impaired fine motor coordination skills (Purdue Pegboard Dominant Hand T scores = 33, Nondominant Hand T score = 0, and Both Hands T score = 33). He has intact phonological short-term memory skills (CTOPP Scaled Score = 8). Mother's report about patient's social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, social awareness, and autistic mannerisms. Patient was born via C-section at 39 weeks due to failure to progress following labor augmentation with Pitocin. Mother took oral fertility medication.

***Patient SSC 12752.p1***

Patient is a 4-year-old Caucasian female. Large head circumference (*4 years* HC = 53.5 cm, $z$ = 2.4) is noted and an underweight BMI is recorded. Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria). Patient's cognitive abilities fall in the normative range (Verbal IQ = 90, Nonverbal IQ = 93, Full Scale IQ = 91 by DAS-2) while her adaptive skills are significantly impaired (Adaptive Composite = 59). She first used single words at 15 months and developed phrase speech at 42 months. Abnormalities were first noted in her development at 36 months of age. Patient has been diagnosed with chronic constipation, ongoing since 3.5 months of age with intermittent episodes of abnormal stool. Patient has significant sleep disturbances, including difficulty going to bed, disordered breathing, being excessively tired with frequent naps, and a history of sleep walking. Parent report indicates attention and affective problems as well as emotional reactivity. Extremely low motor skills are reported on the VABS (Motor Standard Score = 40) and patient has below average receptive language (PPVT-4 Standard Score = 84). Parent report about patient's social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, social awareness, and autistic mannerisms. Patient was born via C-section due to breech position at 38 weeks gestational age.

***Patient SSC 14233.p1***

Patient is a 16-year-old Caucasian male. Large head circumference is noted (*16 years* HC = 61 cm, $z$ = 3.0) and a normal BMI is recorded (height = 182.9 cm, $z$ = 1.1, weight = 77.3 kg, $z$ = 0.7). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and intellectual disability (confirmed with cognitive and adaptive testing). Patient's cognitive and adaptive abilities fall in the extremely low range (Verbal IQ < 30, Nonverbal IQ < 30, Full Scale IQ < 30, by DAS-2 and Mullen; Adaptive Composite = 39). He first used single words at 7 months, but has not developed phrase speech. He currently speaks in single words. Abnormalities were first noted in his development at 21 months of age and parents report a regression in language skills. Both imaging and EEG testing yielded normal findings. Patient also has inflammatory bowel disease and has had hernia surgery. The patient has a history of sleep problems including problems with falling asleep, nighttime awakenings, difficulty waking in the morning, and a generally irregular bedtime. Patient also has a history of elevated attention problems. Extreme difficulties with fine motor coordination are also noted (Purdue Pegboard T scores all < 5). Parent report about his social responsiveness on the SRS suggests severely impacted social communication, and mild-to-moderate impairment in social cognition, social awareness, social motivation and autistic mannerisms. Patient was born vaginally at 41 weeks gestation without induction. During pregnancy with the patient, his mother was diagnosed with anemia during second and third trimesters and took iron supplements. She had a viral illness during the second trimester and reports having significant edema during the pregnancy.

***Patient SSC 14406.p1***

Patient is a 13-year-old Caucasian male. Large head circumference is noted (*13 year* HC = 57 cm, $z$ = 1.6) and an overweight BMI is recorded (height = 168.5 cm, $z$ = 1.1, weight = 77.5 kg, $z$ = 2.3). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria). Patient's cognitive abilities fall in the normative range (Verbal IQ = 84, Nonverbal IQ = 98, Full Scale IQ = 92 by DAS-2) while his adaptive skills are significantly impaired (Adaptive Composite = 66). He first used single words at 15 months and currently speaks in sentences. Abnormalities were first noted in his development at 18 months of age and a mild hearing impairment necessitated accommodation at school. There are no reported GI disturbances. Patient has a history of significant sleep problems including difficulties falling asleep and frequent awakenings at night. Parents report social, attention, and affective problems. He also has below average fine motor coordination (Purdue Pegboard Dominant Hand T scores = 29, Nondominant Hand T score = 33, and Both Hands T score = 25), and below average phonological short-term memory skills (CTOPP Scaled Score = 6). Parent report about his social responsiveness on the SRS suggests severely impacted social communication, social cognition, social motivation, social awareness, and autistic mannerisms. Patient was born at 38 weeks

gestation following labor induction through multiple methods (Pitocin, Prostaglandins, and Amniotomy) for unspecified reasons. Mother had herpes 1 virus during first, second, and third trimesters of pregnancy; UTI during 2nd trimester; and gestational diabetes during pregnancy. No medications were taken by mother during pregnancy.

***Patient Nijmegen DNA07-06646***

The patient is a 17-year-old Caucasian male. Facial features include broad forehead with prominent supraorbital ridges, down slanted palpebral fissures, full nasal tip with broad alae nasi, high palate, and simple ears (See Figure 2d). Other physical findings include long extremities, and flat feet. At the age of 17 years, patient's head circumference was 58.4 cm ($z$ = 1) (previous records 55.5 cm ($z$ = 2) at 14 years; 44 cm ($z$ = 0) at 6 months; 37.5 cm ($z$ = 0) at 1 month). He was tall (197.7 cm; $z$ = 2.2) (previous records 172.2 ($z$ = 3) at 14 years; 115 cm ($z$ = 2.5) at 13 years; 94.5 cm ($z$ = 2) at 3 years; 79 cm ($z$ = 1) at 1 year) and had a normal BMI (86.5 kg; $z$ = 0.5) (previous records ~100 kg ($z$ = 2) at the age of 16 years; 21.5 kg ($z$ = 1) at 13 years). He was diagnosed with an autism spectrum disorder (confirmed by Dutch autism-related screener called the AVZ-R (score 14) and clinical judgment using DSM-IV criteria at the age of 6 years and 9 years) and reading disability. Patient's cognitive abilities fall in the borderline range (Full Scale IQ = 76 as measured by NIO at age 11 years; WISC-R at age 8 years FSIQ 82, VIQ 75, PIQ 95). He showed significant autism related impairments in social interaction, adherence to routines, stereotypic behavioral patterns and fascinations, although these had improved significantly by the age of 17 years. The patient reports some problems with falling asleep and variable stools, changing from diarrhea to constipation. He was born by caesarian section at 38 weeks of gestation because of solutio placentae.

The patient's father was reported to have similar autistic like features as his son, although this was never assessed formally. He followed normal secondary education. He had a history of diarrhea, problems falling asleep and he was said to have had a large head circumference. His father was also tall (height = 198 cm, $z$ = 2). He was diagnosed with a cT2N1 rectum carcinoma at the age of 42 years for which he was treated by radiotherapy and rectum amputation. At the age of 46 years he developed a melanoma on his trunk, which was excised. At the age of 46 years metastases of the rectum cancer were identified in his lungs and mediastinum and he underwent chemotherapy subsequently. At the age of 49 years also metastases of the melanoma to his lymph nodes were noted. He died of the complications of the metastases, including brain metastasis, at the age of 49 years.

***Patient Troina2037***

Patient is a 41-year-old Caucasian female. Facial features include flat lateral profile, coarse features, prominent supraorbital ridges with hypertelorism and down slanted palpebral fissures, large ears with fleshy upturned lobes, and full fleshy lips (See Figure 2f). Other notable features include large, wide hands with some stiffness in the hyperextension of the interphalangeal joints. The feet are large and flat. At the age of 41 years, large head circumference was recorded (z > 2.0). Height at 41 years 165.1 cm (z = 0.3) while weight was 83.7 kg (z = 2.0; BMI = 31). A large uterine leyomioma appeared at abdominal ultrasound and surgical removal was suggested. Cardiological evaluation and electrocardiogram and EEG, performed at 36 years, were normal although skull CT showed widened cranial vault. Patient was diagnosed with intellectual disability (moderate severity; confirmed with cognitive and adaptive testing) and although psychotic disorder was suspected, this was ruled out by clinician diagnosis. Poor attention skills and rare repetitive behaviors have been noted. All developmental milestones were delayed. Gastropathy has been reported. There are no reported sleep disturbances. Patient's cognitive and adaptive abilities fall in the extremely low range (Full Scale IQ = 40 by Raven's Colored Progressive Matrices; Adaptive Composite = 60). She was born vaginally, at term following an uneventful pregnancy.

***Patient Troina2659***

Patient is a 13-year-old Caucasian male. Facial features include prominent supraorbital ridges, down slanted palpebral fissures, and pointed chin (See Figure 2e). Additional physical findings include posterior plagiocephaly, winged scapulae, joint hyperlaxity, muscular hypotonia, and intermittent exotropia on the left eye. Patient has a large head circumference ($z$ = 2.0), is tall (height z = 2.0), and has a normal BMI (weight $z$ = 0.7). Patient was diagnosed with autism spectrum disorder (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria) and intellectual disability (confirmed with cognitive and adaptive testing). EEG was normal although MRI showed a delayed myelinic maturation on the temporal poles. Normal developmental milestones were achieved, followed by a striking regression at 2.5 years of age in communicative language proficiency, social withdrawal, poor eye contact, reduced response to name, inadequate social gestures, and the appearance of stereotyped movements and behaviors, with echolalia. Starting in his first year, patient has struggled with consistent constipation with stooling every 2-3 days that continues today. There are no reported sleep disturbances. Patient's cognitive and adaptive abilities fall in the extremely low range (Full Scale IQ = 46 by Leiter-R; Adaptive Composite = 36). Pregnancy was characterized by vaginal bleeding in first trimester, treated with progestinic and cortisonic up to the fifth month, and was supplemented with iron and folate. The mother had four (three before and one after the patient's pregnancy) spontaneous abortions, without any ascertained cause. Delivery was at term by cesarean section for breech presentation.

***Patient APP 109580-100***

Patient is a 6-year-old Caucasian male. Facial features include midface hypoplasia and hypertelorism. Large head circumference ($z$ = 3.2) is noted and a high average BMI is recorded (height $z$ = 2.0, weight $z$ = 1.3). Patient was diagnosed with autism spectrum disorder by clinician diagnosis and global developmental delay (confirmed with cognitive and adaptive testing). Attention problems are also noted. Parents also report mild GI impairment, reporting that constipation occurs often and pain on stooling occurs at times. Sleep problems are also noted, and melatonin is prescribed to manage sleep difficulties. Developmental quotient and adaptive skills fall in the extremely low range (Verbal ability = 27, Nonverbal ability = 41, Overall ability = 34, by Mullen; Adaptive Composite = 62). The patient is nonverbal and parents report a history of regression.

### *Patient Nijmegen DNA-010878*

The patient is an 11-year-old Caucasian male. Facial features include a square face with full cheeks, medially extending eyebrows, periorbital fullness with deep-set eyes, and bilateral epicanthic folds. Other physical findings were obesity, an increased lumbar lordosis, short hands and fingers with shortening of 4$^{th}$ metacarpals, small feet with partial cutaneous syndactyly of 2$^{nd}$ and 3$^{rd}$ toes and shortening of 3$^{rd}$ and 4$^{th}$ metatarsals. Patient's head circumference was 56.5 cm ($z$ = 1.5) at 11 years of age and height 160 cm ($z$ = 1) (previous records 101.5 cm ($z$ = 1.0) at age 3 years; 62 cm ($z$ = −0.8) at 4 months) and his weight 66 kg ($z$ = 2.5) (previous records 18.2 kg ($z$ = 1.1) at 3 years; 6.1 kg ($z$ = −1) at 4 months). He was diagnosed with ADHD by clinical judgment at age 6 years, as he had hyperactivity, concentration problems and destructive behavior. Sleeping problems and constipation have not been reported. Patient's cognitive abilities fall in the borderline range (Full Scale IQ = 77, Verbal IQ = 68, Nonverbal IQ = 79 as measured by WISC-RN at age 7 years). He was born by an uncomplicated delivery.

### *Patient Nijmegen DNA023486*

The patient is a 15 years old Caucasian female. Facial features included brachycephaly, prominent supraorbital ridges, mild hypertelorism with down slanted palpebral fissures, divergent strabismus, long eyelashes, large, fleshy ears and pointed chin. Other physical findings included large halluces, sandal gaps of the feet and two café-au-lait spots. At the age of 5 years and 6 months, patient's head circumference was 54.2 cm ($z$ = 2) and her height was 126 cm ($z$ = 2) and at the age of 15 years 57.5 cm (90$^{th}$ centile) and 174 cm (85$^{th}$ centile) respectively.

She was diagnosed with autism spectrum disorder and intellectual disability. She started walking and said her first words at the age of 18 months. IQ testing at the age of three years indicated Verbal IQ scores of 88 and Nonverbal IQ scores of 69, while at four years these were 66 and 55, respectively. At the age of 13 years and 8 months her Full scale IQ score tested by WPPSI-R was 50, with a Verbal score of 41 and a Nonverbal IQ score of 57. She ''lived in her own world'' and made poor eye contact. She talked to herself often and showed echolalia and had a characteristic tic (continuous movement of the hands in a stereotypic manner). Periods of unresponsiveness and eye movements were reported, but EEG registrations showed isolated centrotemporal spike-wave complexes, but no indication for epilepsy. In addition, she had problems falling asleep for which was treated with melatonin and had constipation. She was formally diagnosed with autism and an attention deficit disorder (ADD). She was born vaginally after an uncomplicated pregnancy and delivery at 43 weeks of gestation with a birth weight of 3980 g ($z$ = 1). Her Apgar scores were 9 and 10 after 1 and 5 min, respectively. She had feeding problems in the neonatal period.

The patient's mother was reported to have autistic like features, albeit significantly milder than her daughter, although this was never assessed formally. She completed secondary education, although she needed additional support. As a child she had difficulties with falling asleep but no gastrointestinal problems were noted. As an adult she had difficulties maintaining a job and appropriate interactions with her colleagues. She had a head circumference of 57.5 cm (90$^{th}$ centile) and a height of 178 cm (85$^{th}$ centile). Facially she resembled her daughter (not specified further).

She carried the same CHD8 mutation as her affected daughter. Testing of the grandmother did not reveal the CHD8 mutation in her. The grandfather was deceased and therefore not available for testing.

## Additional Patients Identified with Events with Limited Impact to *CHD8*

### *Patient Leuven 445853*

The patient is a 7-year-old Caucasian male. No atypical facial features are noted nor are any additional physical features reported. Patient's head circumference at 7.5 years was 51 cm ($z$ = −1.0); height was 127 cm ($z$ = 0.3); weight was 27 kg ($z$ = 0.6). He was diagnosed with ASD (confirmed with ADOS and clinical judgment). Sleeping problems and GI problems have not been reported. Patient's cognitive abilities fall in the normal range (as measured by SON-R IQ) although there is a significant history of language delay (first speech after 2 years of age) and mild motor delay. He was born via C-section due to breech presentation following an uneventful pregnancy and preceding a normal perinatal period.

### *Patient T102.03*

Patient is a 10-year-old Caucasian female with an inherited CNV [copy number variant]. Facial features include a broad forehead, an upswept lateral left eyebrow and a broad nasal base (See Figure 1b). Additional findings include a mild shortening of the left 4th metacarpal. According to her mother, she has a curved spine, which causes her stomach to protrude. Additionally, her mother reports that her fontanelles remained open for an extended period of time (not closed at 2 year well-child visit). Large head circumference ($z$ = 2.0) has been noted since 1 year of age and normal BMI is recorded (height $z$ = 0.3, weight $z$ = 1.0). Patient is suspected of having an autism spectrum disorder by professionals and is undergoing a formal assessment. Patient reportedly achieved her developmental milestones on time, but has shown delays in social interactions (e.g., impaired peer interactions, reduced responsiveness to her name or social situations, difficulties understanding abstract language) and autism-related behaviors (e.g., interests of unusual intensity, sensory sensitivities including sound). She is also reportedly very distractible. She has speech articulation problems. Patient is reportedly ambidextrous, excessively clumsy/uncoordinated, and has a history of croup and a urinary tract infection at age 6. Impetigo also occurred as a young child. There are no reported GI disturbances. She has difficulties falling asleep, although once asleep will sleep through the night without problems. Patient was born at 39 weeks gestation via planned C-section due to mother's previous birth trauma (postpartum hemorrhaging). Patient had difficulty breathing at birth, and was admitted to the equivalent of a NICU for 2 days to regulate her breathing. Patient's mother had a previous ectopic pregnancy and was not expected to conceive again. The patient's father, also a carrier of the CNV, is a 51-year-old Caucasian male who does not have any notable facial features nor any

psychiatric diagnoses. He was diagnosed with asthma as an adult, but has no reported GI disturbances. He has difficulty breathing at night, related to his diagnosis of asthma and reports waking once nightly as a result.

### Generation of Deletions in *chd8* by CRISPR/Cas9 System

Plasmids and Generation of *chd8* Guide RNAs. The *nls-Cas9-nls* gene sequence inserted into pCS2 vector and the pT7-gRNA were generated by the W. Chen lab (Jao et al., 2013) and obtained from Addgene, Cambridge, MA (Addgene plasmid numbers 47929 and 46759, respectively). Three guide RNAs (gRNA) were designed to target the exon 2 of *chd8* utilizing the CRISPR design tool (http://crispr.mit.edu/). Oligonucleotides were annealed and cloned into the pT7-gRNA vector digested by BsmBI. Oligonucleotides inserted into pT7-gRNA were as follow: *chd8*-gRNA1, 5′- TAGGGACCCCTCTTAGGCCCGGCA-3′, 5′-AAACTGCCGGGCCT AAGAGGGGTC-3′; *chd8*-gRNA2, 5′-TAGGCTAGCGAGGGTGACTTTCCC-3′, 5′-AAACGGGAAAGTCACCCTCGCTAG-3′; *chd8*-gRNA3, 5′-TAGGACTGCCATGCCGGGCCTAAG-3′, 5′-AAACCTTAGGCCCGGCATGGCAGT-3′. The data presented herein have been obtained with *chd8*-gRNA2. Oligonucleotides targeting zebrafish *tyrosinase* (*tyr*) were also annealed and cloned into pT7-gRNA. The *tyr*-gRNA was used as a positive control and was previously described (Jao et al., 2013). For making g-RNAs, the template DNA was linearized by BamHI and gRNA was generated by in vitro transcription using MEGAshortscript T7 kit (Invitrogen). For making *nls-Cas9-nls* RNA, the template DNA was linearized by NotI and RNA was synthesized using mMessage mMachine SP6 kit (Invitrogen).

To test the efficiency of each of the three gRNAs, a mix of gRNA (100 ng) and *nls-Cas9-nls* capped-RNA (150 ng) was injected directly into ~100 one-cell-stage embryo clutches. Each injection cocktail had one *chd8* guide and the *tyr* guide positive control. We first scored qualitatively for albinism (evidence of pigmentation) to ask whether the CRISPR experiment worked. We saw 60%–70% of embryos were affected. About a third of these were completely white, the rest had some patchy pigment sites that on a cylindrical structure are readily quantifiable. We phenotyped all batches for guides 1, 2, and 3 and reproduced, quantitatively, the head size increase and gut neuronal defect only with gRNA2. Guide 1 and 3 clutches were indistinguishable from dye-injected controls and represent *de facto* controls. The experiment was repeated with guides 1 and 2. We saw precisely the same result both at the genotype and phenotype level indicating that the findings were not the result of potential off-target interaction of the *tyr* and *chd8* guide RNAs. To validate genetic editing mediated by our gRNA2 *chd8*-CRISPR/Cas9 system in the injected embryos (founders, F0), total genomic DNA was prepared from 30 randomly selected individuals and a short stretch of DNA (Exon 2 of zebrafish *chd8*) flanking the target site was PCR amplified from the genomic DNA with the following primers: Forward 5′- CAGCAGACTC AGCAAATCACT-3′, Reverse 5′- CGCAGGAGATGTCGCATTTA-3′. A T7 endonuclease I (T7EI) assay was then performed as previously described (Jao et al., 2013). Founders were phenotyped for head size measurements and enteric neuron counts at 4.3 dpf and 6 dpf, respectively. Furthermore, we amplified a larger fragment (920 base pairs) of DNA flanking the putative gRNA2 cut site from the genomic DNA of 36 control fish and 31 *chd8*-CRISPR fish independently using the following primers: Forward 5′- GCAGGGTCAG ACTCAAGTGC-3′, Reverse 5′- GCAGCTCCACCTTGTGAGG-3′. PCR products were quantified, equimolar concentrations were pooled and SMRT (single-molecule, real-time) sequencing libraries were generated for the control and *chd8*-CRISPR guide 2 pools. Each library was sequenced using the PACBIO RS II with P4/C2 chemistry run with 120 min movies (Pacific Biosciences). Consensus sequences were generated for 100-110 molecules from each of two pools and compared to the target zebrafish reference sequence.

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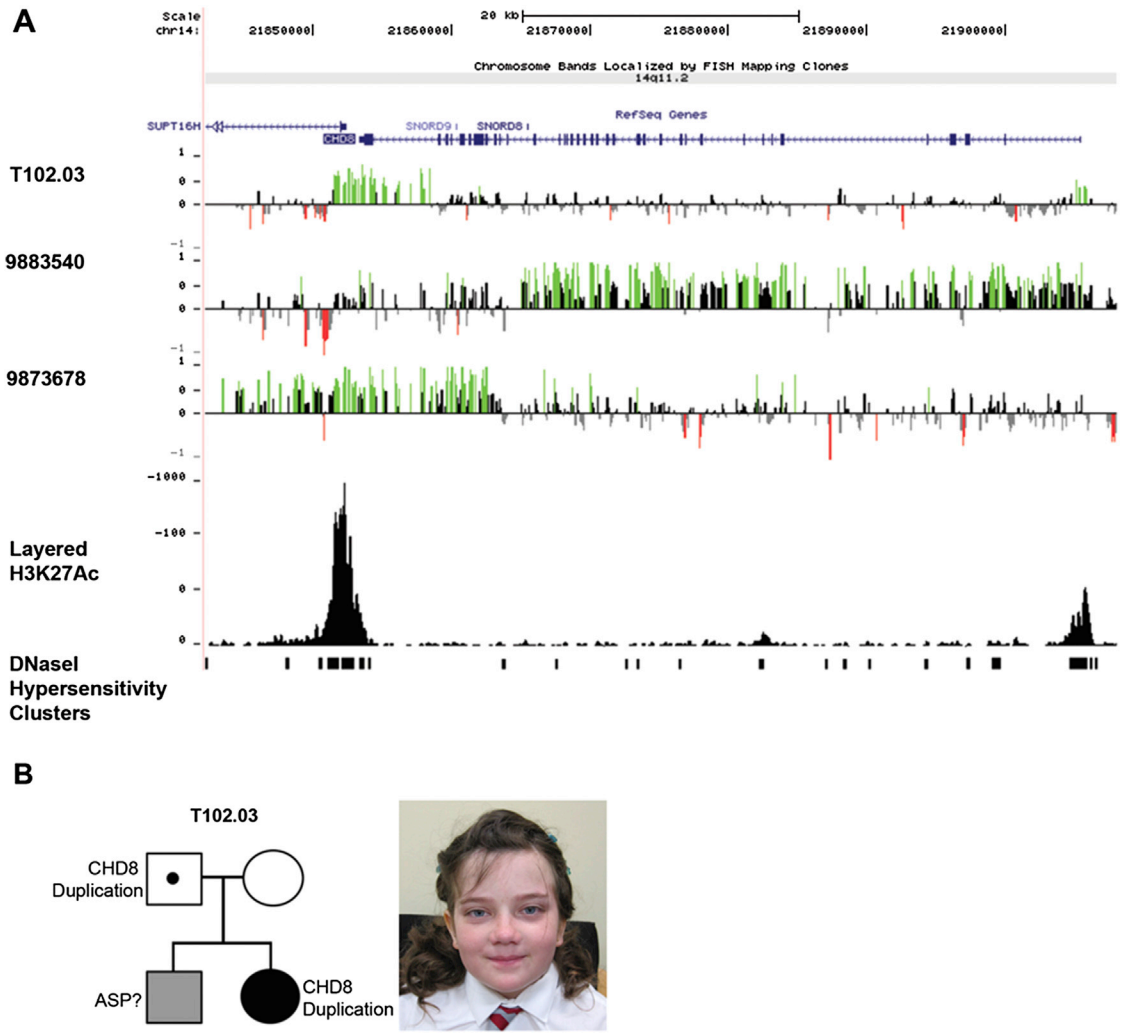


**Figure S1. Focal *CHD8* CNVs and Inherited *CHD8* Cases, Related to Figure 1**

(A) Array CGH of three probands with *CHD8* gene-disruptive CNVs. Also shown are predicted regulatory elements based on H3K27 acetylation marks and DNaseI hypersensitivity sites reported by the ENCODE project.

(B) Pedigree showing one family with inherited CNV event in *CHD8* transmitted from the father. Individuals shown in gray have reported characteristics of ASD but have not been diagnosed by a clinician. Image of Patient T102.03 is shown. ASP = Asperger syndrome.

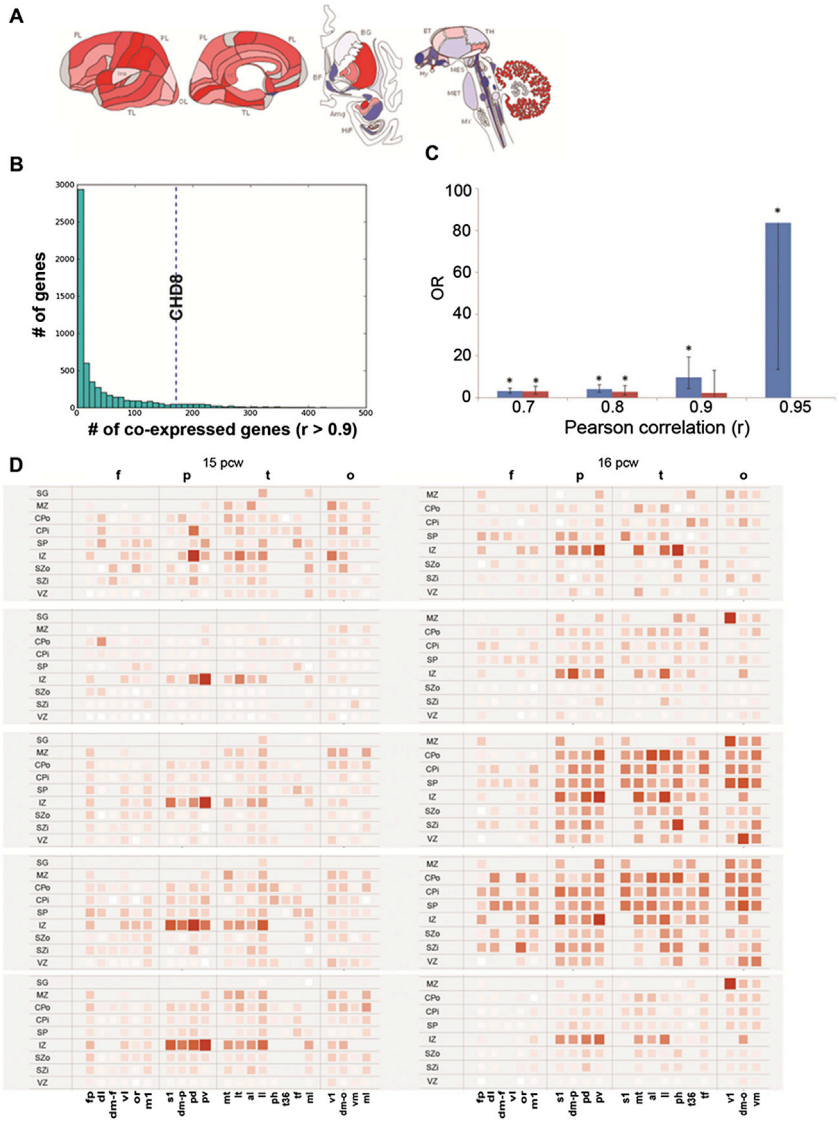


**Figure S2. Expression Patterns of *CHD8*, Related to Figure 3**

(A) Anatomical heatmap of *CHD8* expression in adult human (left two panels) and adult mouse (right two panels) brains.

(B) The number of genes co-expressed with *CHD8* during human brain development (blue dashed line) in comparison to the number of genes co-expressed with each of the other genes from the BrainSpan Atlas (histogram). Co-expression was defined as Pearson correlation coefficient r > 0.9 (left figure) or 0.7 (right figure) between pairs of gene-expression profiles, calculated for human brain RNaseq data. Only genes that are co-expressed with at least one other gene are shown.

(C) Enrichment of genes reported as carrying de novo disruptive mutations in ASD probands (blue) or unaffected siblings or controls (red) in the set of genes that co-express with *CHD8*. Odds ratios (y axis) were calculated using different thresholds to define co-expression (x axis). Data sources include four ASD studies (Iossifov et al., 2012; Neale et al., 2012; O'Roak et al., 2012a; Sanders et al., 2012) for the ASD probands analyses. For the unaffected siblings and controls analyses the data include these same studies and additional two intellectual disability studies (Gulsuner et al., 2013; Rauch et al., 2012).

(D) Heatmap showing localized expression of *NAV1*, *MLL*, *ARID1A*, *RPRD2*, and *ZNF462* (top to bottom) at 15 pcw (left) and 16 pcw (right) in the intermediate zone (IZ). Red indicates increased expression and white indicates no expression. f = frontal; p = parietal; t = temporal; o = occipital; SG = supragenicular nucleus of the thalamus; MZ = marginal zone; CPo = cortical plate; CPi = cortical plate; SP = subplate zone; SZo = subventricular zone; SZi = subventricular zone; VZ = ventricular zone; a1 = primary auditory cortex; dl = dorsolateral prefrontal cortex; dm-f = dorsomedial frontal cortex; dm-o = dorsomedial extrastriate cortex; dm-p = dorsomedial parietal cortex (area 7 m); fp = frontal polar cortex; il = inferolateral temporal cortex; lt = lateral temporal-occipital cortex; m1 = posterior frontal cortex (motor cortex); mi-t = midinferior temporal cortex; ml = midlateral extrastriate cortex; mt = medial temporal-occipital cortex; or = orbital frontal cortex; pd = posterosuperior (dorsal) parietal cortex; ph = posterior parahippocampal cortex; pv = posteroinferior (ventral) parietal cortex; s1 = primary somatosensory cortex; sl = superolateral temporal cortex; t36 = (rostral) midinferior temporal cortex (area 36); tf = caudal midinferior temporal cortex (area TF); tp = temporal polar cortex; v1 = primary visual cortex; vl = ventrolateral prefrontal cortex; vm = ventromedial extrastriate cortex.

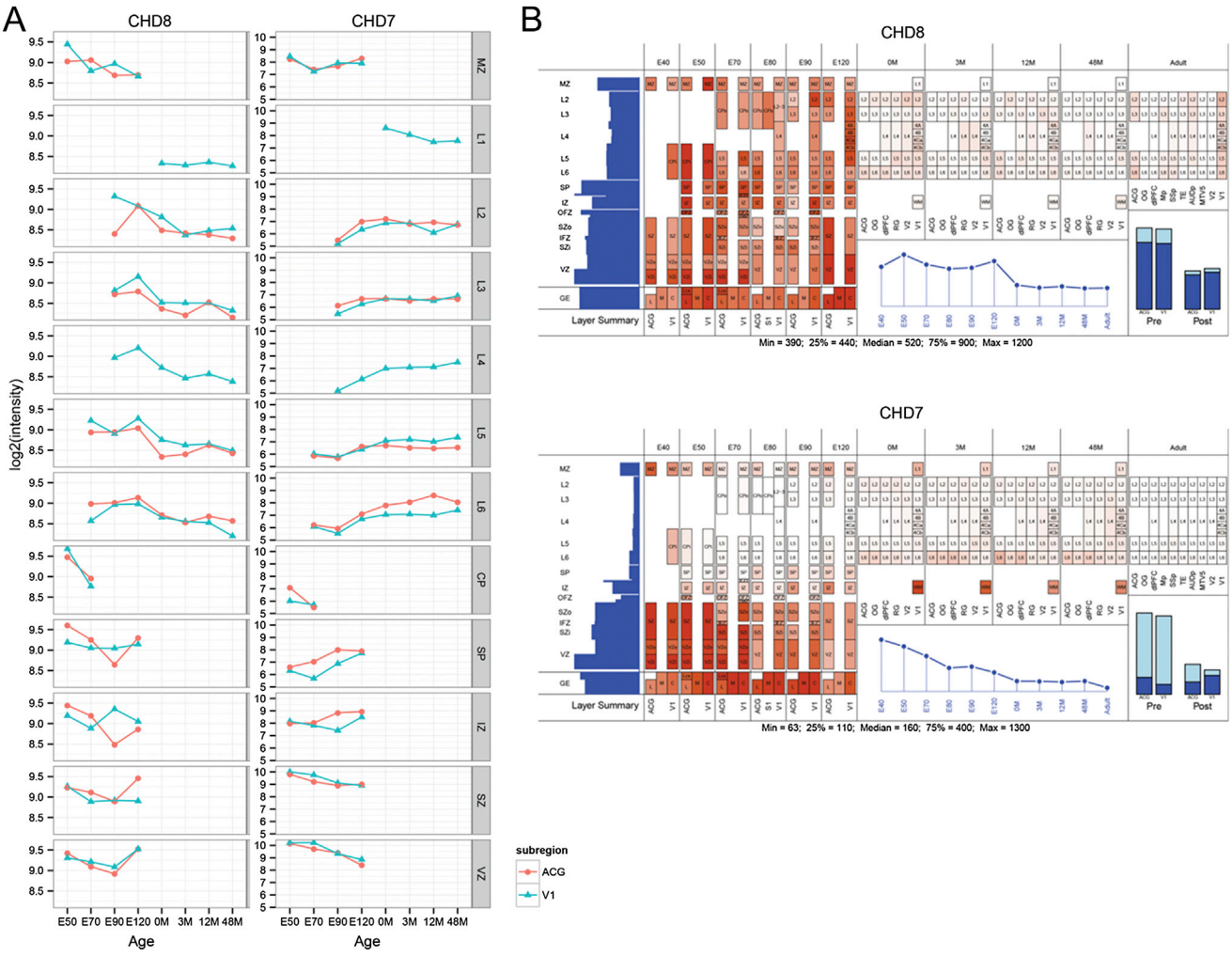


**Figure S3. Expression Patterns of *chd8* in Rhesus Macaque Brain, Related to Figure 3**

(A) Graphs showing the expression of *Chd8* (left) and *Chd7* (right) by Affymetrix array in Rhesus macaque brain over the course of development from embryonic day 50 (E50) to 48 months (48M) after birth in two tissues, the anterior cingulate gyrus (ACG, red) and the visual cortex (V1, blue). VZ = ventricular zone; SZ = subventricular zone; IZ = intermediate zone; SP = subplate zone; CP = cortical plate; L6 = layer 6; L5 = layer 5; L4 = layer 4; L3 = layer 3; L2 = layer 2; L1 = layer 1; MZ = marginal zone.

(B) Each box represents samples (n = 4) taken from the labeled cortical region and layer and age. Darker box colors correspond to greater mean expression level in that sample with the color range defined by the quantiles of expression intensity listed at the bottom. Column labels indicate the 11 developmental time points that were assessed, including 6 prenatal (40 – 120 post-conceptional days) and 5 postnatal (0 – 48 months after birth and ~8.5 year old adults). Row labels indicate the cortical layer and are ordered from apical to basal layers at the top. VZ = ventricular zone; SZ = subventricular zone; IZ = intermediate zone; SP = subplate zone; CP = cortical plate; L6 = layer 6; L5 = layer 5; L4 = layer 4; L3 = layer 3; L2 = layer 2; L1 = layer 1; MZ = marginal zone. Columns within ages are labeled near the bottom of the figure and indicate the cortical region sampled including primary visual (V1) and somatosensory cortex (S1) and anterior cingulate gyrus (ACG) during prenatal development. More cortical regions were sampled after birth (OG = orbital gyrus; dlPFC = dorsolateral prefrontal cortex; RG = rectus gyrus) and are ordered from left to right in a rostral to caudal order. The bar plot on the left shows mean expression by layer across all ages, and the bar plot in the lower right hand corner shows mean (total bar height) and standard deviation (light blue bar height) of expression across all layers in ACG and V1 in prenatal and postnatal cortex. The line plot shows the mean expression during development across all samples.

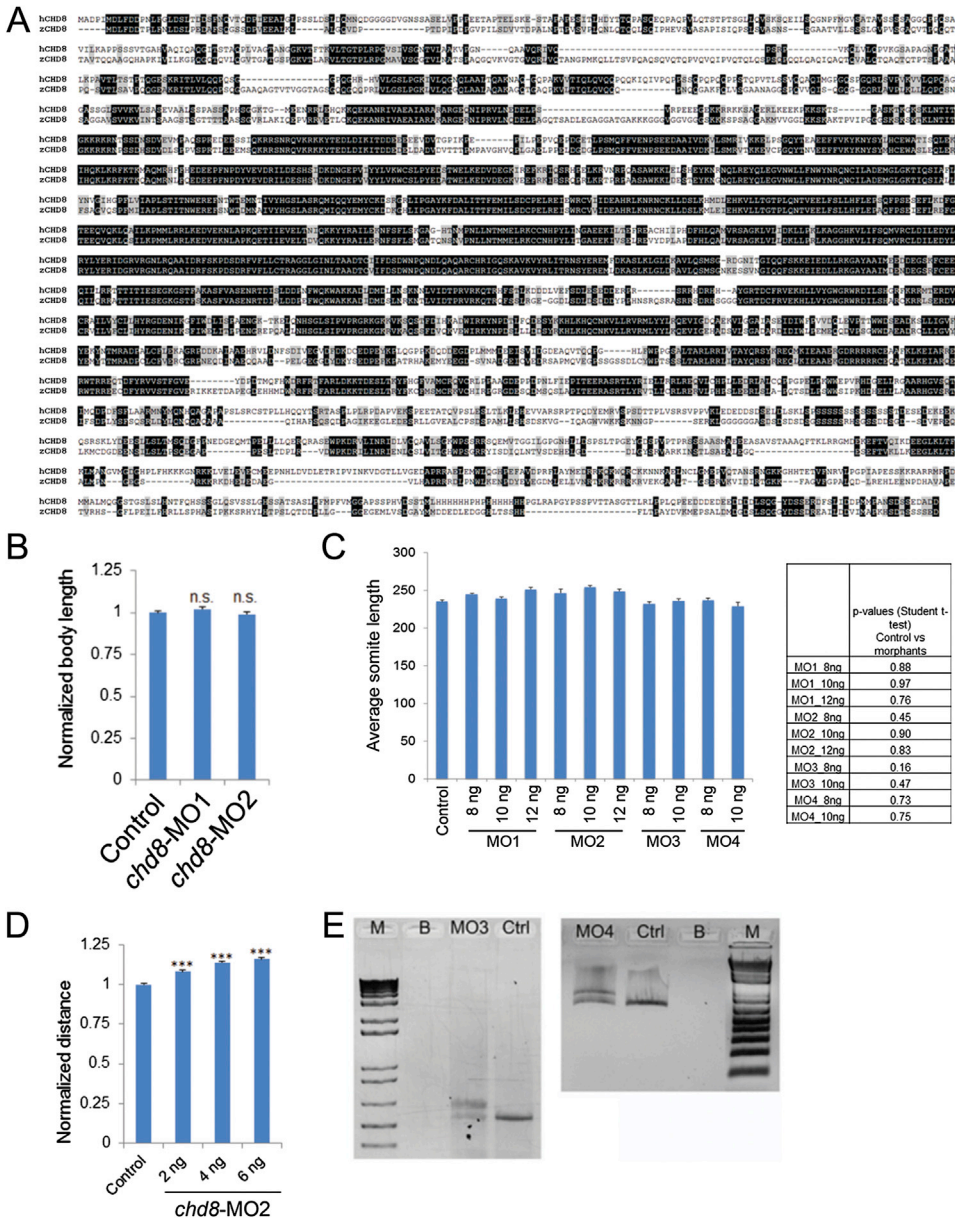


**Figure S4. *chd8* MOs Efficiently Disrupt the Zebrafish Endogenous Message, Related to Figures 4 and 5**

(A) Amino acid residues conserved between human *CHD8* and the zebrafish ortholog are shadowed in black (same) and gray (similar). The overall sequence identity is 62% and sequenced similarity is 72%.

(B) 4.3 days after MO1 or MO2 injection, the total body length of *chd8* morphants and controls were measured showing no significant (n.s.) difference. Data are represented as mean ± SEM.

(C) The average length (μm) between five consecutive chevrons for n = 20 embryos injected with *chd8*-MO1, MO2, MO3, MO4, or controls were measured blind to injection cocktail. The table on the right shows no significant difference in somite length between *chd8* morphants and controls. Data are represented as mean ± SEM.

(D) 4.3 days after MO2 injection, the interorbital distances were measured. Increased dosage of MO2 injection caused further enlargement of the eye distance. Data are represented as mean ± SEM. ***$p < 0.0001$

(E) Two morpholinos (MO3 and MO4) were independently designed to target two exon/intron junctions of *chd8*. It was predicted that MO injection would lead to inclusion of the adjacent intron into the mature message. To validate the morpholino effects on *chd8* message, 10 ng (MO3 and MO4) were injected into zebrafish embryos. Total mRNA was extracted at 24 hr postfertilization (hpf) followed by reverse transcription and PCR using primers flanking the targeted junctions showing abnormal splicing of the *chd8* message. M, 1 kbp plus ladder; B, PCR blank; MO3, *chd8* MO3-injected; MO4, *chd8* MO4-injected; Ctrl, sham-injected.

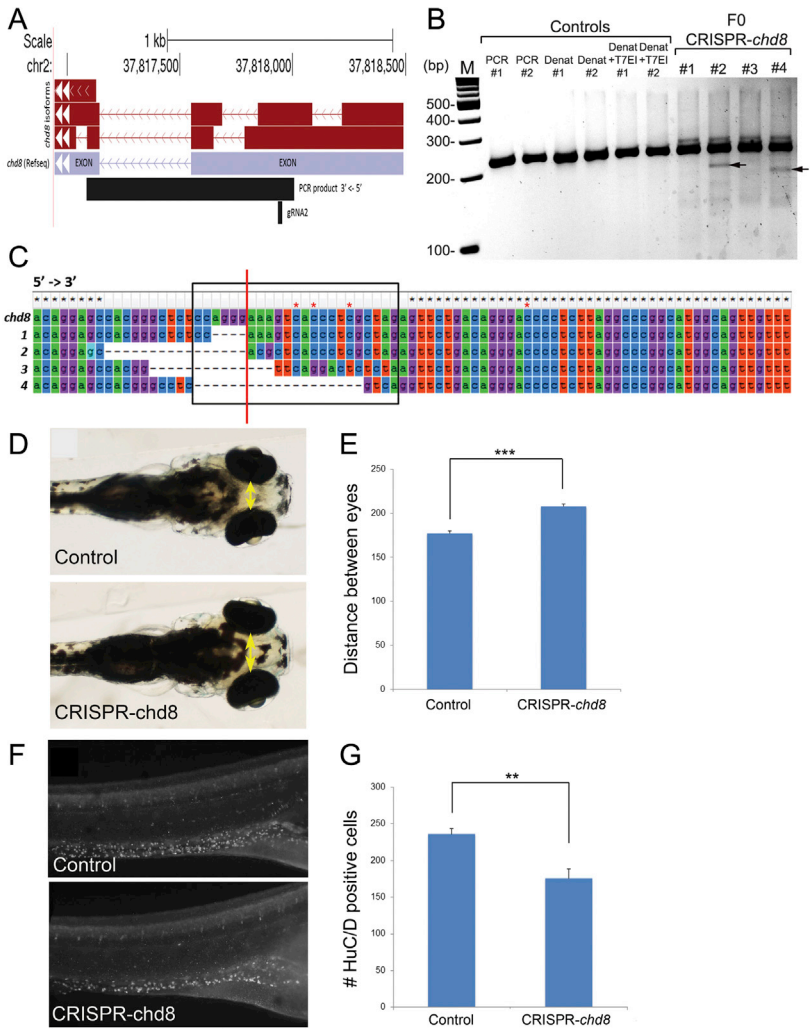


**Figure S5. *Chd8*-CRISPR Zebrafish Embryos Phenocopy *chd8* Morphant Phenotypes, Related to Figures 4 and 5**

(A) Location of the guide RNA (gRNA) 2 used for zebrafish *chd8*-CRISPR experiments and PCR product for sequencing experiments in the context of *chd8* gene structure.

(B) At 5 dpf, a total of 26 founders and 30 controls were randomly selected and were subjected to T7 endonuclease I (T7EI) assay. Representative gel picture shows two controls and four founders subjected to T7EI assay. T7EI fragments are noted with black arrows for the positive founders #2 and #4. Lanes from left to right: M: 100 bp DNA ladder; PCR #1: PCR of exon2 of *chd8* for control 1; PCR #2: PCR of exon2 of *chd8* for control 2; DENAT #1: PCR of exon2 of *chd8* after denaturation/reannealing for control 1; DENAT #2: PCR of exon2 of *chd8* after denaturation/reannealing for control 2; DENAT+T7EI #1: PCR of exon2 of *chd8* after denaturation/reannealing, and after T7EI digest for control 1; DENAT+T7EI #2: PCR of exon2 of *chd8* after denaturation/reannealing, and after T7EI digest for control 2; F0 #1, #2, #3, #4: random selection of four embryos injected with *chd8*-gRNA2. We noted the presence of a T7EI fragment for a total of 13 out 26 founders subjected to T7EI assay, indicating that 50% of the founders have indels of exon 2 of *chd8*. No T7EI fragment was detected in the 30 controls tested.

(C) Multiple sequence alignment of reference sequence (top) to four *chd8*-CRISPR variants generated from SMRT sequencing of the 920 base pair amplified product (Figure S5A). The black box indicates the sequence targeted by the guide RNA (gRNA2), and the red line marks the putative CRISPR cut site based on the location of the PAM recognition motif (i.e., CCA). Black stars indicated conserved residues, and red stars indicate positions of single base pair insertions present in the sequencing data.

(D) 4.3 days after *chd8*-gRNA2 and *nls-Cas9-nls* co-injection, the distance between the convex tips of the eyes were measured (interorbital distance, yellow arrows).

(E) Bar graph represents the distance between the eyes (μm) for controls and embryos injected with *chd8*-gRNA/Cas9. A Student's t test was performed and the corresponding p value is denoted on the bar graph. ***$p < 0.0001$.

(F) 6 days after *chd8*-gRNA2 and *nls-Cas9-nls* co-injection, the embryos were stained with HuC/D antibody, imaged and the number of enteric neurons was quantified utilizing ImageJ.

(G) Quantification of the number of HuC/D positive cells in the GI tract in controls and embryos injected with *chd8*-gRNA/Cas9. A Student's t test was performed and the corresponding p value is denoted on the bar graph. **$p < 0.001$.

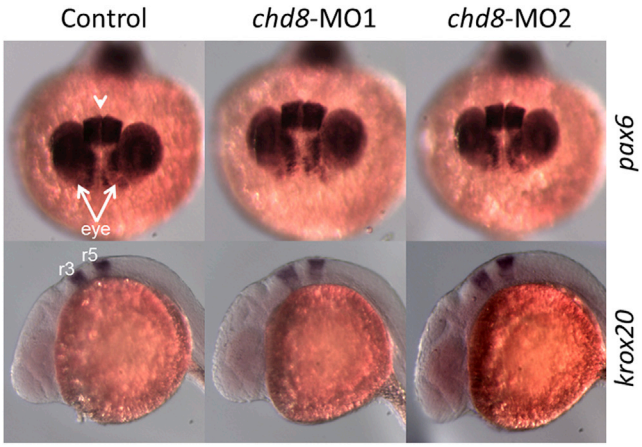


**Figure S6. Additional Marker Analyses in *chd8* Morphants, Related to Figure 4**

Embryos were injected with 4 ng of *chd8*-MO1 or 4 ng *chd8*-MO2. Paired-box 6 (*pax6*) is a marker of the eye region and the telencephalon. Expression of *pax6* at 24 hr stage, top view. Arrows point at the eye region and arrowhead points at the telencephalon region. No significant change is observed for *pax6* expression (top panels). Expression of *krox20* at 24 hr stage, lateral view. Rhombomere 3 (r3) and 5 (r5) of the hindbrain is labeled. No significant change is observed (bottom panels).