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Haploinsufficiency of *KDM6A* is associated with severe psychomotor retardation, global growth restriction, seizures and cleft palate

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Abstract

We describe a female subject (DGAP100) with a 46,X,t(X;5)(p11.3;q35.3)inv(5)(q35.3q35.1)dn, severe psychomotor retardation with hypotonia, global postnatal growth restriction, microcephaly, globally reduced cerebral volume, seizures, facial dysmorphism and cleft palate. Fluorescence in situ hybridization and whole-genome sequencing demonstrated that the X chromosome breakpoint disrupts *KDM6A* in the second intron. No genes were directly disrupted on chromosome 5. *KDM6A* is a histone 3 lysine 27 demethylase and a histone 3 lysine 4 methyl-transferase. Expression of *KDM6A* is significantly reduced in DGAP100 lymphoblastoid cells compared to control samples. We identified nine additional cases with neurodevelopmental delay and various other features consistent with the DGAP100 phenotype with copy number variation encompassing *KDM6A* from microarray databases. We evaluated haploinsufficiency of *kdm6a* in a zebrafish model. *kdm6a* is expressed in the pharyngeal arches and ethmoid plate of the developing zebrafish, while a *kdm6a* morpholino knockdown exhibited craniofacial defects. We conclude *KDM6A* dosage regulation is associated with severe and diverse structural defects and developmental abnormalities.

Introduction

Histone methylation is an epigenetic mechanism that plays an important role in regulation of global and tissue-specific gene expression at precise developmental stages (Berger 2007; Kouzarides 2007). Different histone lysine (K) methylation states (mono-, di-, tri-; me1, me2, me3) are associated with gene transcriptional activation or repression depending on the location of the lysine residue. For example, histone H3 lysine 4 (H3K4) di- and tri-methylation are associated with gene activation, while H3K27 di- and tri-methylation are associated with gene repression (Bernstein et al. 2002; Martin and Zhang 2005; Santos-Rosa

et al. 2002; Schuettengruber et al. 2007; Sims Iii et al. 2003). Methylation sites are often found at promoters, insulators, enhancers and transcribed regions (Barski et al. 2007). Histone methylation is dynamic and regulated by specific methyltransferases and demethylases (Wysocka et al. 2005).

Lysine-specific demethylase 6A (*KDM6A*; MIM 300128), previously designated as *UTX*, is both a histone lysine methyltransferase and demethylase (Lee et al. 2007). It also plays a role in general chromatin remodeling (Miller et al. 2010). *KDM6A* interacts with mixed-lineage leukemia complex 2 (*MLL2*), in a conserved SET-1 like complex that trimethylates H3K4 (Issaeva et al. 2007), while also complexing with *MLL3* and *MLL4* although the function of these interactions is unknown (Lee et al. 2007). *KDM6A* contains six tetratricopeptide repeats (TPR) in the N-terminal portion of the protein, a JmjC domain and a novel zinc-binding domain, conserved in the *KDM6* family, in the C-terminus. The JmjC domain is required for its demethylase activity. The proximity of the zinc-binding domain, which binds H3 residues 17–21, and the JmjC domain confers *KDM6A*-specific demethylase activity to H3K27me3/me2/me1 (Agger et al. 2007; Hong et al. 2007; Kim and Song 2011; Lan et al. 2007; Lee et al. 2007; Sengoku and Yokoyama 2011).

KDM6A predominantly targets genes involved in developmental and cellular processes including cell adhesion, cytoskeletal organization, chromosome organization and biogenesis, transcriptional regulation, RNA splicing and the cell cycle. For example, in fibroblasts, *KDM6A* occupies 1,945 promoters, with most transcriptionally active genes marked with H3K4me2. In contrast, the target genes of H3K27me3 are inactive and enriched for involvement in development, such as *HOX* genes, which have previously demonstrated *KDM6A* regulation and activation during developmental stages (Agger et al. 2007; Lan et al. 2007; Lee et al. 2007; Wang et al. 2010).

It is proposed that *KDM6A* plays a role at specific loci throughout development (Agger et al. 2007; Hong et al. 2007; Lee et al. 2007). *kdm6a1* is important for *hox* gene expression and posterior development in zebrafish, while *Kdm6a* is essential for proper cardiac and neural tube development in mice (Lan et al. 2007; Lee et al. 2012; Shpargel et al. 2012). *Drosophila dUTX* mutants demonstrate rough eyes, dysmorphic wings and abnormal sex combs (Herz et al. 2010). During embryogenesis *KDM6A* is involved in endoderm differentiation, the regulation of muscle-specific genes, as well as in activation of beta-globin in differentiated erythroid cells (Chaturvedi et al. 2009; Jiang et al. 2012; Seenundun et al. 2010). *KDM6A* has also been shown to play a role in the reestablishment of pluripotency in somatic cells and germ cell development (Mansour et al. 2012).

Six individuals with Kabuki syndrome were recently identified with either point mutations or microdeletions encompassing portions of *KDM6A* (Lederer et al. 2012; Miyake et al. 2013). Kabuki syndrome is characterized by moderate intellectual disability, postnatal growth retardation, dysmorphic facial features (long palpebral fissures, with eversion of the lateral third of lower eyelids, high arched eyebrows, long lashes, broad and depressed nasal tip, large ears), clinodactyly, and recurrent otitis media in infancy. Additional anomalies can include skeletal and dental abnormalities, cleft palate, and persistent fetal finger pads (Kuroki et al. 1981; Niikawa et al. 1981). The frequency of *KDM6A* mutations in Kabuki syndrome remains to be determined. Presently, mutations in *MLL2* are recognized as the most common cause of Kabuki syndrome in 56–72 % of patients (Hannibal et al. 2011; Li et al. 2011; Micalé et al. 2011; Ng et al. 2010; Paulussen et al. 2011).

We present a female subject with a complex chromosomal rearrangement that includes a balanced translocation between the X chromosome and an inverted region of chromosome 5. Although the phenotype of this individual overlaps with clinical features of Kabuki

syndrome, her clinical findings exhibit greater morbidity. Using fluorescence in situ hybridization (FISH) and DNA sequencing, *KDM6A* was found disrupted in one of the translocation breakpoints; molecular genetic analyses were performed to explore further the role of *KDM6A* in development.

Methods

Subject recruitment

Informed consent, medical records and blood samples were obtained through an Institutional Review Board approved protocol of Partners HealthCare System titled “Developmental Genome Anatomy Project”, and the subject received the study number DGAP100.

Generation of lymphoblast cell lines

A peripheral blood specimen was used for Epstein–Barr virus transformation as previously described (Bernacki et al. 2003) at Massachusetts General Hospital Genomics Core Facility in the Center for Human Genetic Research (Boston, MA).

Cytogenetic studies and array comparative genomic hybridization

GTG-banded karyotyping was performed in the clinical work-up of DGAP100. For FISH, lymphoblast cells were harvested and metaphase spreads were prepared by standard protocols as previously described (Ney et al. 1993). Fosmid (<http://www.bacpac.chori.org/>) and RP11 BAC clones were selected using the University of California Santa Cruz (UCSC) Genome Browser, GRCh37/hg19 assembly (<http://www.genome.ucsc.edu>). DNA extracted from clones was labeled with either SpectrumOrange or SpectrumGreen conjugated dUTP using a nick translation kit, according to the manufacturer’s protocol (Abbott Molecular, Des Plaines, IL). Labeled pairs of probes were hybridized overnight to metaphase chromosome preparations. After 4× SSC/0.3 % Tween, 2× SSC/0.1 % Tween, and phosphate-buffered detergent washes, chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Abbott Molecular) and analyzed with a Zeiss Axioplan2 microscope (Thornwood, NY) with epi-fluorescence and Applied Imaging CytoVision software (Santa Clara, CA). An array comparative genomic hybridization (aCGH) experiment to assess genomic copy number in DNA extracted from peripheral blood lymphocytes was performed using an Agilent 1M array at the Cytogenetics Core of Dana Farber Harvard Cancer Center (Boston, MA).

Custom bar coded jumping library and sequencing

DNA extracted from peripheral blood was sequenced using a customized whole-genome large insert jumping library for Illumina HiSeq2000, as previously described (Chiang et al. 2012; Talkowski et al. 2011a, b) and analyzed with publicly available resources including BWA (Li and Durbin 2009), SAMtools (Li and Durbin 2009), and a C++ program, BamStat (<http://www.mappingtools.chgr.org>). All genome coordinates are listed as the GRCh37/hg19 assembly.

X-inactivation study

Lymphoblast cells were synchronized for 15 h with 0.3 mg/ml thymidine (Sigma, Natick, MA), then washed three times in Hank’s solution without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Invitrogen, Carlsbad, CA) and resuspended in complete RPMI medium (Invitrogen). 5-bromo-2′-deoxyuridine (BrdU, Sigma) was added to the culture to a final concentration of 30 $\mu\text{g}/\text{ml}$ for 5.5 h prior to harvest. Slides were fixed in 70 % ethanol for 30 min, treated with 0.07 N NaOH for 45 s and washed with phosphate-buffered saline (PBS, Invitrogen). BrdU incorporation was detected with BrdU-AlexaFluor488-conjugated mouse monoclonal antibodies (Invitrogen)

diluted to 1:35 in a 0.5 % Tween-20/PBS solution. The anti-BrdU AlexaFluor488 solution was added to the slide and incubated for 30 min and then washed with PBS. Chromosomes were stained with DAPI.

RNA expression analysis of lymphoblastoid cells

RNA was extracted from lymphoblastoid cell lines of DGAP100 and two sex-matched controls (an 8-year-old female, GM03299, and a 16-year-old female, GM07533, obtained from the NIGMS Human Genetic Cell Repository, Coriell Cell Repositories, Camden, NJ) using the Qiagen RNeasy Mini Kit (Valencia, CA). Concentration and purity of RNA were assessed using a Thermo Scientific NanoDrop 1000 (Waltham, MA). First-strand cDNA synthesis using Superscript III First Strand Synthesis System for RT-PCR (Invitrogen) was prepared with 1 µg of total RNA. For quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), primers were designed for use with probes from the Roche Universal Probe Library for genes of interest and for *GAPDH* (MIM 138400) as an internal control. All samples of cDNA were assayed in triplicate in 10 µl reaction volumes. Two control samples were analyzed for each replicate expression assay to ensure expression data were consistent. Quanta PerfeCta 2X qPCR Master Mix (Quanta Biosciences, Gaithersburg, MD), primers, probe and deionized water were mixed together in a fixed ratio, and 8 µl added to each well with 2 µl of ~50 ng/µl cDNA. PCR conditions were the standard conditions of the CFX Real Time PCR Detection System (Bio-Rad, Hercules, CA). The cycle threshold (CT) was determined during the geometric phase of the PCR amplification plots, as recommended by the manufacturer. Relative differences in transcript levels were quantified using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Acquired data were analyzed using Bio RadCFX Manager (Bio-Rad) and Microsoft Excel.

Evaluation of copy number variants in independent subjects

A copy number variant (CNV) burden analysis was performed as previously described (Talkowski et al. 2012). CNV data were compiled and analyzed from 33,573 clinical genome-wide microarray cases from the molecular diagnostic laboratories of Signature Genomic (SG) Laboratories and Boston Children's Hospital (BCH). 19,556 cases were identified with a neurodevelopmental disorder (NDD), while the remaining 14,017 cases had no clinical indication of NDD. Controls were obtained from the International Schizophrenia Consortium (ISC; $n = 7,878$) (Ferreira et al. 2008; ISC 2008; O'Donovan et al. 2008; Purcell et al. 2009; Smith et al. 2009; Sullivan et al. 2008) and from the recently published cohort analysis of Cooper et al. (2011) that did not overlap with ISC controls ($n = 6,113$ after filtering WTCCC controls). Of these controls, only 6,239 were analyzed for CNVs on the X chromosome, and this reduced comparison group was used for analyses.

Zebrafish lines and maintenance

All embryos and fish were raised and cared for essentially as described (Westerfield 1993), using established protocols as per the Subcommittee on Research Animal Care, Massachusetts General Hospital.

Cloning of the zebrafish *kdm6a* ortholog

KDM6A orthologous candidate genes were identified in the zebrafish genome using the human *KDM6A* mRNA sequence and protein sequences, respectively (NCBI Reference Sequence: NM_021140.2, NP_066963.2), in the NCBI BLAST tool. The zebrafish gene with the greatest sequence homology is annotated as *kdm6a* (NCBI Reference Sequence: XM_692654.5 and XP_697746.2). The full-length 4.4 kb fragment was PCR amplified from 48 h post-fertilization (hpf) embryo cDNA using forward primer, 5'-CCGAGGATCCATGAAATCGTGCGGAGTGTC GG-3' and reverse primer 5'-

CCGCTCGAGGCAACCCATTTTCTTTGTGA-3'. The amplified fragment was cloned into pGEM T-Easy Vector System (Promega, Madison, WI), and the resulting clone was verified by sequencing. This clone was used as a template to synthesize DIG-labeled antisense riboprobe for whole-mount RNA in situ hybridization. Full-length *kdm6a* cDNA was subcloned into a pCS7 expression vector for capped mRNA synthesis.

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization was performed as described (Thisse and Thisse 2008) with digoxigenin-labeled antisense RNA probes generated using forward primer 5'-CCGAGGATCCATGAAATCGTGCGGAGTGTCTGG-3' and reverse primer 5'-CGCGGATCCAATTAACCCTCACTAAAGGGACAACACACCGATTGAGCAC-3', respectively, using T3 polymerase. Sense RNA probes were generated using forward primer 5'-TAATACGACTCACTATAGGGATGAAATCGTGCGGAGT-3' and reverse primer 5'-ACAACACACCGATTGAGCAC, respectively, using T7 polymerase. Stained embryos/larvae were imaged using a Nikon AZ100 microscope and processed with NIS-Elements software, using the maximum intensity projection feature applied to z-stacks for greater depth of clarity.

Cartilage staining

Alcian blue staining was performed to analyze the craniofacial skeleton essentially as described (Walker and Kimmel 2007) and images were obtained on a Nikon 80i compound microscope.

Morpholino knockdown

Translation-blocking *kdm6a* and *kdm6a-like* morpholinos (MO) were designed by Gene Tools (Philomath, OR) with the following nucleotide sequence: 5'-CCACCGACACTCCGCACGATTTTCAT-3', *kdm6a-like* 5'-AGCTCCGAGCGTCCAAAAGCCACAA-3'. Knockdown was performed injecting a 2 ng dose (1 nl) of morpholino into one-cell stage embryos, and the resulting phenotype was assessed at day 5 post-fertilization. We screened 24 hpf embryos for toxicity, determined by neural necrosis, which was below 10 %, and such embryos were excluded from analysis. Double morpholino knockdown was performed co-injecting smaller doses of *kdm6a* and *kdm6a-like* morpholinos in the same embryo. A total of 1 ng (1 nl of a 500 μ M stock solution) of *kdm6a* and 1 ng (1 nl of a 250 μ M stock solution) of *kdm6a-like* were injected, respectively. Capped full-length *kdm6a* mRNA was synthesized from pCS7:*kdm6a* plasmid using Sp6 mMessage mMachine kit (Ambion, Foster City, CA) and stored at -80 °C. This stock solution was diluted to 150 ng/ μ l for rescue injections, and 2 ng *kdm6a* MO (1 nl) was co-injected with 150 pg (1 nl) *kdm6a* mRNA into one-cell stage embryos.

Results

Clinical history for DGAP100

DGAP100 is a 19-year-old female with a karyotypic interpretation of an apparently balanced translocation that we determined to represent a complex chromosomal rearrangement including a balanced inversion, 46,X,t(X;5)(p11.3;q35.3)inv(5)(q35.3q35.1)dn. She is nonverbal and has abnormal clinical findings of facial dysmorphism, large and slightly posteriorly rotated ears, repaired cleft palate, shovel-like teeth with caries, medial notches and enamel dysplasia, gingival hyperplasia, severe myopia, short stature, microcephaly, mild pectus excavatum, clinodactyly, puffy fingers with deep-set and hyperconvex nails, short metatarsals, neuromuscular scoliosis, cerebral atrophy with mild ventriculomegaly, hypotonia, history of seizures, and global developmental and severe psychomotor delay with

intellectual disability (Fig. 1). Facial dysmorphic features of DGAP100 include a flattened profile with a short columella, long and downslanting palpebral fissures with long eyelashes, and peaked, arched eyebrows. She also exhibits a thin upper lip with downturned corners of the mouth. After repair for a congenital high arched cleft palate performed at 9 months of age, she still has a poorly molded, short palate.

Family history

A three-generation pedigree of DGAP100 does not demonstrate similar abnormalities in any family members.

Birth

DGAP100 was born after a 38-week uncomplicated pregnancy by Cesarean section for failure to progress. Birth parameters included a weight of 3,275 g (80th percentile), length of 48 cm (25th percentile), and head circumference of 33 cm (10th percentile). She demonstrated poor suck-swallow and arousal responses, metabolic acidosis, and hypoglycemia. GTG-banded karyotyping was performed at 5 days of life revealing an apparently balanced translocation of Xp11.23 and 5q35. A brain MRI performed at 7 days after birth was normal.

General

DGAP100 demonstrated growth retardation following birth; her height and weight deteriorated to the third percentile by 3 months. Failure to thrive was observed throughout infancy and DGAP100 remained in the third percentile for weight and height at 5 years and 11 months of age. By 8 years and 4 months, her weight dropped below the third percentile to four standard deviations below the mean with no progression in height. She has remained proportionately short at seven standard deviations below the mean. Head circumference centile has decreased steadily since birth and is currently 3.5 standard deviations below the mean.

Skin

At 5 years and 11 months, she had an eczematoïd rash on her cheeks and thighs which still persists at 18 years of age. Her hands and feet become cyanotic and cool periodically. At 8 years of age, she had mild hypertrichosis and a 4 mm nevus surrounded by a lighter color halo, both on her back.

Ears, nose, throat, and eyes

At 5 years and 11 months DGAP100 passed an audiogram after a history of otitis media, otorrhea and hearing impairment. At 8 years of age, a cycloplegic retinoscopy revealed severe myopia (right eye: spherical -10.00 diopters, cylinder +4.50 diopters, axis 110°; left eye: spherical -9.50 diopters, cylinder +4.50 diopters, axis 75°).

Respiratory and cardiovascular

There were no abnormal respiratory findings except nasal congestion. Cardiovascular exam at 5 months identified a grade II systolic ejection murmur with regular rate and rhythm, which was assessed to be benign. Cardiovascular system evaluation was unremarkable at her later visits.

Gastrointestinal

DGAP100 had feeding difficulties starting from birth and became gastrostomy tube dependent for feeding.

Endocrine and metabolism

At 5 months she was admitted to the hospital due to failure to thrive, with chronic diarrhea since birth, dehydration, and chronic hypoglycemia. Chronic metabolic acidosis was determined to be secondary to chronic diarrhea. Testing for urine organic acids, reducing substances, and serum amino acids was normal, ruling out galactosemia and disorders of amino or organic acids.

Musculoskeletal

In addition to the described anomalies of the hands and fingers, mild pectus excavatum was noted at 8 years, and there was no scoliosis at the time. She was also treated with ankle orthotics for tight heel cords. At 8 years 3 months her bone age correlated to that of a female of 6 years 10 months according to Greulich and Pyle standards, identified by generalized demineralization and coarsening of the trabeculae in the osseous structures. By 16 years of age she developed neuromuscular scoliosis and subsequently underwent lumbar-sacral spinal fusion surgery at 17.5 years.

Neurologic

DGAP100 has abnormal neurologic findings with severe global development delay since birth. At 9 days of life she had an abnormal neurological exam with decreased tone in lower extremities and hyperreflexia and hypertonicity in upper extremities. At 3 months, she had 3+ deep tendon reflexes at the knees without clonus. At 5 months an upper gastrointestinal series showed swallowing incoordination and tracheal aspiration; poor sucking and swallowing motions were observed throughout infancy. CT scan of the brain performed at 10 months revealed prominent lateral and third ventricles suggesting mild ventriculomegaly while the electroencephalogram (EEG) was normal for her age. At 5 years and 11 months the EEG reported the waking record as mildly to moderately abnormal because of background slowing. She was nonverbal and non-ambulatory except for rolling. Her facies moved symmetrically, all of her movements were slow, she tended to shake her legs, and she had brisk reflexes without spasticity. Of note, she was capable of demonstrating mirror hand movements and could walk ten steps with a walker at 8 years and 4 months of age. DGAP100 was diagnosed with intractable epilepsy at age 14. The longest seizure-free time period is 5–6 months since diagnosis. Her most recent MRI, at 16 years of age, revealed global cerebral volume loss and possible mucocoeles of the sphenoid sinus. Now at 19 years of age, DGAP100 is still nonverbal with severe psychomotor retardation.

Medications

She is currently on antiepileptic, pain, and anti-inflammatory medications as well as vitamin D and calcium supplements. She takes her medicine through her gastrostomy tube. Of note DGAP100 is allergic to sulfa drugs, Vancomycin, Ibuprofen and had Stevens–Johnson reaction to Trileptal.

Molecular and cytogenetic analyses of DGAP100

Karyotypic analysis in the newborn period was reported as 46,X,t(X;5)(p11.23;q35)dn. aCGH results were interpreted as normal, and all copy number variants (CNVs) detected are present in the Database of Genomic Variants (DGV; <http://www.projects.tcag.ca/variation/>).

FISH analysis identified two split clones: g248p80280c4 and g248p84552g9 (Fig. 2d), narrowing the X chromosome breakpoint to within a 33 kb region disrupting *KDM6A* in the second intron. Because *KDM6A* escapes X inactivation (Greenfield et al. 1998), an X-inactivation/late replication study was performed. The der(X) was determined to be active

and the normal X chromosome homolog inactive (Fig. 3b). Long range PCR was attempted to determine the breakpoint sequence, but was unsuccessful.

Whole-genome sequencing revealed loss of four bases at the chr 5 translocation breakpoint (chr 5:176906708-176906713) with inverted segments at both translocation breakpoints, suggesting the translocation was nested within an inverted genomic region. Consistent with this observation, an inversion between chr 5:170261000~ and chr 5:178185000~ was identified by the jumping library sequence data. The breakpoints of the inversion could not be delineated precisely to nucleotide resolution due to the presence of flanking segmental duplications, suggesting a mechanism of formation that includes non-allelic homologous recombination (NAHR). No genes or mRNA coding regions were disrupted by any of the three chromosome 5 breakpoints. The X chromosome breakpoint was found at chr X: 44759733 (Fig. 3a), with loss of one base, disrupting the second intron of *KDM6A* and confirming the FISH mapping. The translocation breakpoints were Sanger sequenced for validation. *KDM6A* was the only gene directly disrupted as detected by sequence analyses. The next-gen cytogenetic karyotype for DGAP100 is designated as 46,X,der(X) (5qter->178185000~::170261000~>176906708::44759735->Xqter),der(5)(5pter->170261000~::178185000~>176906713::44759733->Xpter)dn[GRCh37/hg19] (Fig. 2a).

Quantitative RT-PCR was performed to investigate expression of *KDM6A*. Relative differences in transcript levels were quantified and showed that *KDM6A* exhibited at least ~60 % reduction of mRNA expression compared to control samples (Fig. 3c), suggesting that the DGAP100 phenotype was caused by haploinsufficiency of *KDM6A*. In addition, the chromosome 5q35.3 translocation breakpoint is 6 kb upstream of *DBN1* (MIM 126660) and 3.7 kb downstream of *PDLIM7* (MIM 605903). Both *DBN1* and *PDLIM7* demonstrated expression reduced by ~38 and ~50 %, respectively, in DGAP100 lymphoblast cell lines compared to control cell lines (data not shown).

Evaluation of copy number variants in independent subjects

To strengthen our hypothesis of haploinsufficiency of *KDM6A* in the DGAP100 phenotype, we screened the microarray databases of SG and BCH (NDD and non-NDD cohorts), as well as DGV and DECIPHER (<http://www.decipher.sanger.ac.uk/>). Six individuals with a variety of neurodevelopmental abnormalities contained CNVs encompassing *KDM6A*, two of whom were classified as autism spectrum disorder (Fig. 3a). No CNVs were observed in controls, suggesting a statistically significant increase in CNV burden among cases with NDD ($p = 0.04$). To substantiate that these findings were not peculiar to technical differences in array technology between the clinical diagnostic NDD cohort and the control microarray studies, we compared the NDD cases to those analyzed on the same diagnostic arrays but without an indication of a neurodevelopmental disorder. No *KDM6A* disruptions were detected among the non-NDD cohort, replicating the initial observation ($p = 0.04$; case burden compared to combined control and non-NDD samples, $p = 0.005$). Three additional cases were identified in DECIPHER with two deletions and one duplication including *KDM6A*, while disruptions of exon 1 were noted in DGV. Five of the cases were female, two were male and the gender was unknown for the remaining two cases. Eight cases were diagnosed with intellectual disability, developmental delay or autism spectrum disorder. Two individuals demonstrated short stature, four suffered from seizures and three patients had dysmorphic features. Despite having a duplication, SG9651 also presented with wide-set and downslanting eyes, a short nose, clinodactyly, constipation, and most noticeably only had a vocabulary of 10 words at 4 years of age. DECIPHER130 had hypoglycemia and DECIPHER4479 was diagnosed with cleft palate, scoliosis, and eczema. Of note, *KDM6A* was the only gene within the minimal region of overlap in CNVs from all subjects, but was not the only gene involved in all subjects. It is thus possible that other loci may be contributory to the phenotypes in some individuals (Table 1).

***kdm6a* expression in zebrafish**

Zebrafish have two *KDM6A* homologs: *kdm6a* (ENSDARG 00000061759) and *kdm6a-like* (ENSDARG00000059794). Protein sequence alignments show that *kdm6a* (75 % sequence similarity) and not *kdm6a-like* (64 % sequence similarity) is the ortholog of *KDM6A*. The full-length *kdm6a* zebrafish homolog was cloned through RT-PCR and its spatiotemporal expression examined across different time points during embryogenesis. *kdm6a* demonstrated weak expression at early somite stages but robust expression thereafter. Expression of *kdm6a* was initially detected at 24 h post-fertilization (hpf) in the brain, otic placode and developing pharyngeal arches 1 and 2 (Fig. 4a asterisk, arrow and arrowheads, respectively). At 48 hpf, expression continued in the brain and in the first and second developing pharyngeal arches (Fig. 4b asterisk and arrowheads, respectively). By 72 hpf, *kdm6a* expression in the brain decreased but continued in the first and second pharyngeal arches as well as in the developing third pharyngeal arch (Fig. 4d 1, 2, 3). At 96 hpf, expression was found in the ethmoid plate (Fig. 4e, arrowhead) and pharyngeal arches 2–7 (Fig. 4f 2, 3, 4, 5, 6, 7). The WISH using sense probe showed no staining as expected.

Craniofacial defects in *kdm6a* morpholino knockdown

To uncover the potential role of *KDM6A* in development, *kdm6a* in zebrafish was targeted by morpholino knockdown. Surviving embryos were followed until 96 hpf, a time point when all craniofacial structures should be fully formed, and the craniofacial cartilage was stained using Alcian blue. Knockdown of *kdm6a* resulted in embryos with a smaller lower jaw (Fig. 5e, k), hypoplastic Meckel's cartilage, palatoquadrate, ceratohyals, diminutive ceratobranchials (Fig. 5h, k), and the ethmoid plate failed to extend along its axis (Fig. 5n, q). To verify the knockdown phenotype, specificity rescue experiments were performed injecting 150 pg/nl of wild-type *kdm6a* capped mRNA, resulting in an almost complete rescue of the phenotype in the majority of the cases (Fig. 5c, f, i, l, o, r). We further analyzed the phenotype with the stable transgenic line *sox10::kaede*, where the reporter protein is restricted to the neural crest lineage by the *sox10* promoter (Dougherty et al. 2012). We were able to assess *kdm6a* regulation of morphogenetic events by following migration of cranial neural crest cells (CNCC) in vivo across embryonic developmental stages. Although the mandible and the ceratohyals phenotype appeared similar to the Alcian blue-stained morphants, the ceratobranchials were present but hypoplastic. CNCC can migrate to the pharyngeal arches 3–7, but the chondrocytes failed to terminally differentiate.

Given the high sequence similarity of *kdm6a* and *kdm6a-like* homologs with human *KDM6A* and a possible overlap in function, we performed a double morpholino knockdown co-injecting smaller doses of *kdm6a* and *kdm6a-like* morpholinos in the same embryo, resulting in early death, thereby suggesting an early requirement during embryogenesis.

Discussion

We performed molecular genetic analyses for a 19-year-old female with multiple dysmorphic features and neurodevelopmental findings who has a balanced translocation that resulted in disruption of three non-genic regions on chromosome 5 and *KDM6A* on chromosome X. Analyses of gene expression revealed haploinsufficiency of *KDM6A*, and this gene was found to be the only locus within the minimal region of overlap from analyses of genomic alterations in nine independent individuals with similar phenotypic features to DGAP100.

The chromosomal rearrangement was complex with the chromosome 5 breakpoint nested within an apparent NAHR-mediated inversion. None of the chromosome 5 breakpoints directly disrupted genes, though dysregulation of nearby genes by positional effects might

be contributing to the phenotype of DGAP100 and warrant consideration. The chromosome 5q35.3 translocation breakpoint lies in a region associated with Sotos syndrome (critical region: chr5:175130402-177456545). Sotos syndrome is characterized by excessive growth, macrocephaly, dysmorphic facial features, prominent jaw, mental retardation, and advanced bone age (Cole and Hughes 1994; Sotos et al. 1964; Tatton-Brown et al. 2005). Mutations in or haploinsufficiency of *NSD1* (MIM606681; chr5:176560080-176727214) are the etiologies in a large proportion of individuals with Sotos syndrome (Höglund et al. 2003; Kurotaki et al. 2002). No statistically significant correlation between microdeletion size and phenotype has been reported (Tatton-Brown et al. 2005), although some larger microdeletion cases had more severe mental retardation (Kurotaki et al. 2003). In one reported case, a duplication downstream of *NSD1* was speculated to cause reduced expression of *NSD1*, resulting in a Sotos phenotype (Kasnauskiene et al. 2011). The chromosome 5q35.3 translocation breakpoint of DGAP100 (chr5:176906708–176906713) is near *DBN1* and *PDLIM7*. These genes are within the critical microdeletion region, but have no known role in Sotos syndrome. In addition, deletions of *DBN1* are present in three apparently healthy individuals in the DGV database. The DECIPHER database displays a predicted haploinsufficiency score, which considers genes with scores in the top 10 % as candidates contributing to the phenotype (Corpas et al. 2001; Huang et al. 2010). Haploinsufficiency scores for *PDLIM7* and *DBN1* are 78 and 43 %, respectively, suggesting neither locus is predicted to be a major contributing factor. Because DGAP100's phenotype does not correlate with that of Sotos syndrome, reduced expression of *PDLIM7* and *DBN1* by qRT-PCR most likely are non-contributory in her phenotype.

The 5q35.1 inversion breakpoint is ~28 kb upstream of *RANBP17* (MIM 606141) and ~20 kb downstream of *GABRP* (MIM 602729), while the 5q35.3 inversion breakpoint is ~27 kb upstream of *ZNF354A* (MIM 602444) and ~7 kb downstream of *AACSP1* (non-coding RNA). *AACSP1* and *GABRP* expression were not measured because they are not expressed in lymphoblast cells, and *RANBP17* and *ZNF354A* showed low expression that could not be quantified. In DECIPHER, *ZNF354A* received a haploinsufficiency score of 43 %, *GABRP* was 71 % and *RANBP17* was 53 %, none of which are predicted to be pathogenic. Multiple CNVs are found in the DGV database spanning *RANBP17*. In addition, when analyzing the potential chromatin structure in the breakpoint regions in seven human cell lines using the UCSC Genome Browser, "Integrated Regulation from ENCODE" track (GRCH37/hg19), there were no peaks indicating H3K4ME1, H3K4ME3 or H3K27Ac, which are indicative of promoters or other regulatory elements, at either inversion breakpoint. This suggests that no regulatory elements were disrupted or separated from the target gene by either the 5q35.1 or 5q35.3 breakpoints that would affect gene expression, although the qRT-PCR experiments are indicative of the contrary and could reflect separation of regulatory elements at a greater distance from the breakpoints.

Functional and expression data from the zebrafish suggest that disruption of *KDM6A* represents a likely pathogenic defect in DGAP100. Translation-blocking morpholino disruption of *kdm6a* resulted in aberrant craniofacial development, while the body plan extended normally. It is not surprising that the phenotype included a significantly smaller lower jaw with hypoplastic Meckel's cartilage, palatoquadrates and ceratohyals and diminutive ceratobranchials 1–5 (pharyngeal arches 3–7), as *kdm6a* is specifically expressed in the pharyngeal arches. Meckel's cartilage and palatoquadrates are derived from the first pharyngeal arch. In zebrafish, Meckel's cartilage forms the mandible and the palatoquadrates articulate with the ethmoid plate, which is analogous to the amniote palate. The ceratohyals originate from the second pharyngeal arch and form part of the hyoid bone (Schilling and Kimmel 1997). We also reproduced the phenotype of a previously published *kdm6a-like* morpholino knockdown zebrafish that resulted in abnormal posterior development. The knockdown did not have the typical chevron shape, exhibited a shortened

caudal trunk, degeneration of the posterior notochord (somites 13–23), and slight dorsal curvature of the tail (Lan et al. 2007). Because both zebrafish homologs have high sequence similarity with human *KDM6A* mRNA, it is possible that duplication and subsequent specialization of *KDM6A* in zebrafish generated homologs with overlapping functions. Both *kdm6a* and *kdm6al* have a tetratricopeptide repeat-containing domain and a JmjC domain. Furthering this hypothesis, our *kdm6a* and *kdm6al* double morpholino knockdown model was embryonic lethal prior to gastrulation, suggesting requirement of *kdm6a* in early embryogenesis, consistent with observations of embryonic lethality in *Drosophila* and mouse knockouts (Herz et al. 2010; Lee et al. 2012).

The craniofacial defects observed in the *kdm6a* zebrafish knockdown suggest the cleft palate and dysmorphic facial features seen in DGAP100 are attributable to *KDM6A* haploinsufficiency. *kdm6a* was expressed in the pharyngeal arches, and the cartilage structures that developed from them were abnormal in the morpholino knockdown. In human development, neural crest from the first pharyngeal arch gives rise to the lower jaw (mandibular prominence/Meckel's cartilage) and part of the upper jaw (maxillary prominence/palatopterygoquadrate bar). Meckel's cartilage also forms the malleus, sphenomandibular ligament and the anterior ligament of the malleus, while the maxillary cartilage gives rise to the incus and alisphenoid bones. The first pharyngeal arch also contributes muscles for mastication and other bones of the lower face. Cartilage from the second and third pharyngeal arches forms the hyoid bone, which functions in both swallowing and vocalization. The second arch also forms the stapes of the middle ear, the styloid process of the temporal bone and the stylohyoid ligament. The fourth and sixth pharyngeal arches both contribute to formation of the larynx. If the human embryonic expression patterns parallel those seen in zebrafish, it is possible that DGAP100 has abnormal middle or inner ear structures that correlate with her recurrent otitis media history; abnormal development of the hyoid bone and larynx could potentially participate in poor coordination of the suck-swallow response as an infant and explain why she continues to be non-verbal at age 18. It is also of interest that *kdm6a* is expressed in the ethmoid plate/palate and disrupted function results in a short ethmoid plate, because DGAP100 was born with cleft palate.

In mice, *Kdm6a* has enriched expression in the anterior region of the neural tube, ventricular zone of the caudal neural tube, neural crest cells and somites at E8.5; expression is enriched in the heart, myotome of the somites, limb buds, branchial arches, isthmus, cortex and eyes at E11.5 (Lee et al. 2012). Previous studies demonstrated that *Kdm6a* is expressed at significantly higher levels in the brain and other tissues of female mice compared to male mice across developmental time points. Although *Kdm6a* escapes inactivation, it was shown that the inactive X chromosome contributes less *Kdm6a* transcript than the active X chromosome in mice (Xu et al. 2002; Xu et al. 2008). Furthermore, *Kdm6a*^{-/-} female mice did not survive past E10.5, while heterozygous mice were viable and fertile. Female knockout mice exhibited severe cardiac malformations, anterior neural tube closure defects, and were significantly growth retarded compared to wild-type littermates, while female heterozygous mice displayed normal cardiac morphogenesis (Lee et al. 2012). Variation in phenotype severity was observed in the female knockout embryos (Shpargel et al. 2012). Most male knockout mice also demonstrated embryonic lethality and severe developmental delay, but some lived until adulthood (Lee et al. 2012). Also of note, some heterozygous mice have eye phenotypes and malocclusion (S–K Lee, unpublished data).

The mouse knockout model demonstrates abnormal neurologic, cardiac, eye and dental development as well as enriched expression in tissues where DGAP100 has an abnormal phenotype, such as the myotome, cortex, limb buds and eyes. This supports an interpretation

that reduction of *KDM6A* expression is responsible for the phenotypic findings of DGAP100.

DGAP100 has overlapping phenotypes with those described in six patients with Kabuki syndrome including hypoglycemia, feeding difficulties in infancy, developmental delay, intellectual disability, hypotonia, arched eyebrows, long palpebral fissures and eyelashes, short columella and mild ear abnormalities (Lederer et al. 2012; Miyake et al. 2013). Interestingly, in two female Kabuki patients described with microdeletions, the microdeletion is located on the inactive X chromosome (Lederer et al. 2012), while DGAP100's derivative X chromosome, containing the disrupted copy of *KDM6A*, is the active X chromosome. Because it has been previously shown in mice that the active and inactive X chromosomes contribute disproportionate amounts of transcript, and the fact that DGAP100 had a greater than 50 % reduction in *KDM6A* expression, DGAP100 could potentially have a more significant reduction in expression, leading to a more severe phenotype than that observed in the microdeletion cases. In addition, positional effects of the chromosome 5 disruptions should be considered as potential phenotypic contributors implicated by expression analyses of the nearby genes.

We also identified nine patients with deletions or duplications including *KDM6A* who were not previously diagnosed with Kabuki syndrome, in addition to DGAP100. Seven of these subjects had duplications, some deemed pathogenic by the diagnostic laboratory. These patients displayed variable phenotypes with some overlapping clinical manifestations typically found in Kabuki syndrome such as cleft palate, abnormal dentition, hypoglycemia, developmental delay, intellectual disability, seizures, hypotonia and ophthalmologic problems (myopia, retinal coloboma). Differences in the severity of the phenotypes might be attributed to the sizes of the CNVs, as well as the involvement of other potential pathogenic genes (Table 1). Interestingly, there were also phenotypic distinctions as none of the ten patients we describe presented with congenital heart defects, another common occurrence in Kabuki syndrome, nor did all of the subjects present with the classic facial features.

We conclude that *KDM6A* is critical in early development and that abnormal *KDM6A* dosage has variable manifestations in individuals, although tight regulation of its expression appears to be crucial for normal development. The impact of dosage effects and the specific mechanisms by which *KDM6A* orchestrates its role in development are likely complex and warrant a supplemental approach to achieve a deeper understanding.

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Fig. 1.

a Photograph of DGAP100 at 19 years of age with deformities of the extremities. **b, c** Note the flattened profile with full cheeks, short columella, down slanting and long palpebral fissures with long eyelashes, peaked/arched eyebrows, large and posteriorly rotated ears, and thin upper lip. **d–f** In addition she has puffy fingers with deep-set and hyperconvex nails and short metatarsals

Fig. 2.

a Ideogram and GTG-banded normal chromosomes X and 5 and der(X) and der(5) chromosomes identified in DGAP100 karyotype: 46,X,t(X;5)(p11.3;q35.3)inv(5)(q35.3q35.1)dn. The *black lines* and *asterisks* identify the chromosome inversion locations in the ideogram. **b–d** FISH images confirm the breakpoint locations. **b** RP11-962J7 spans the chromosome 5 translocation breakpoint and is found on chr 5, der(5) and der(X). **c** Three signals are also demonstrated for RP11-867J19, which spans the 5q35.3 inversion breakpoint. **d** G248P80280C4 spans the X chromosome breakpoint and hybridizes to chr X, der(X) and der(5)

Fig. 3.

a DGAP100 breakpoint location and sequence with a loss of four bases from chromosome 5 and a loss of one base from the X chromosome, and nine cases with deletions (*red*) or duplications (*blue*) encompassing *KDM6A* that appear to confer risk for an overlapping phenotype to DGAP100 identified in the Signature Genomics, Boston Children's Hospital and DECIPHER databases. BCH5562 duplication is specific to *KDM6A*. Bases in *blue text* refer to the X chromosome breakpoint while those in *red* identify the chromosome 5 breakpoint. The base in *black text* indicates a one base pair addition on the der(X) and bases in *purple* represent homology found on the X chromosome and chromosome 5 at the breakpoint. **b** An X-inactivation study shows the normal X chromosome is inactive as identified by anti-BrdU Alexafluor488 staining. The *arrow* identifies the inactive X chromosome. **c** qRT-PCR expression of *KDM6A* in age- and sex-matched controls and DGAP100 lymphoblastoid cell lines. *KDM6A* is approximately 60 % reduced in DGAP100 compared to two controls (color figure online)

Fig. 4.

WISH analysis of zebrafish *kdm6a* expression comparing antisense probe (a–f) to sense probe control (a'–f'). Images of whole-mount embryos across development starting at 24 hpf (a, a'), 48 hpf (b, b'), 72 hpf lateral and ventral views, respectively (c, c', d, d'), and 96 hpf lateral and ventral views respectively (e, e', f, f'). Expression was detected initially in the brain, otic placode and developing pharyngeal arches at 24 hpf, with a progressively decreasing expression in the brain and the ear, while the expression in the pharyngeal arches and their derived structures increased until 96 hpf. The WISH using sense probe showed no staining as expected (a'–f')

Fig. 5.

Morpholino knockdown of *kdm6a* disrupts craniofacial development. Images of live larvae (a–c), lateral views of Alcian blue-stained larvae (d–f) with their respective ventral views (g–i), flat mount of the lower jaw (j–l) and ethmoid plate dissections (m–r) with a magnified view of the dissected ethmoid plate (p–r). *kdm6a* morpholino knockdown (b, e, h, k, n, q) resulted in a shortened ethmoid plate (n) with hypoplastic Meckel's cartilage, palatoquadrates and ceratohyals (k). Injection of wild-type *kdm6a* mRNA partially rescued the knockdown phenotype (c, f, i, l, o, r). Scale bars 100 μm. Graph representing number of injected embryos on the x axis and type of injection on the y axis with the wild-type phenotype is represented in dark purple and the morphant phenotype in light purple. MM mismatch morpholino, MO morpholino, M Meckel's cartilage, PQ palatoquadrate cartilage, CH ceratohyals, CB ceratobranchials 1–5 (color figure online)

Table 1

Patients with CNVs encompassing *KDM6A*

Patient	Age in years gender	Aberration	Location	Clinical description	Other potential phenotype contributing genes in addition to <i>KDM6A</i> ^a
DECIPHER130	2F	3.72 Mb loss, de novo	X:42660069–46378186	Short stature, low birthweight (<3rd centile), hypoglycemia	N/A
DECIPHER4479	F	3.52 Mb loss, de novo	X:41666611–45185654	Short stature, asthenic habitus, microcephaly, cleft palate, coloboma of retina/choroid, intellectual disability, scoliosis, seizures, spasticity, abnormality of the skin (eczema)	<i>CASK</i>
DECIPHER254319	M	6.03 Mb gain, inherited from normal parent	X:38965125–44995301	Mental retardation/developmental delay, generalized obesity	<i>DDX3X</i> <i>CASK</i>
SG57940	4F	7.9 Mb gain, pathogenic	X:40088624–47947626	Developmental delay, dysmorphic features	<i>DDX3X</i> <i>CASK</i> <i>ARAF</i> <i>ELK1</i>
SG9651	4F	7.6 Mb gain, de novo, pathogenic	X:41290770–48859176	Moderate global developmental delay (especially speech; only 10 word vocabulary), constipation, dysmorphic features: wide-set and downslanting eyes, epicanthal folds and bushy eyebrows, low set ears over folded helices, short nose with anteverted nares and a prominent columella, long upper lip and pointy chin, bilateral fifth finger clinodactyly but normal palmar creases, very flat feet	<i>CASK</i> <i>ARAF</i> <i>ELK1</i> <i>WAS</i> <i>PIM2</i>
SG55459	15F	6.4 Mb gain, pathogenic	X:43113035–49496089	Developmental delay, dysmorphic features, seizure disorder, macrocephaly	<i>ARAF</i> <i>ELK1</i> <i>WAS</i> <i>PIM2</i>
SG44724	M	7.2 Mb gain	X:39976597–47216121	Encephalopathy unspecified, complex partial epilepsy, intractable pervasive developmental disorder	<i>DDX3X</i> <i>CASK</i>
BCH5561	N/A	713 Kb gain	X:44350469–45063265	ASD, epilepsy/seizures	N/A
BCH5562	N/A	210 Kb gain	X:44706657–44917073	ASD	N/A

^aHaploinsufficiency score in top 10 % to be a gene candidate for the phenotype by DECIPHER