Received: 30 June 2023 | Revised: 4 September 2023 | Accepted: 14 September 2023

DOI: 10.1002/bdr2.2253

REVIEW ARTICLE

Birth Defects Research | Society for Birth Defects Research & Prevention | WILEY

SETting up the genome: KMT2D and KDM6A genomic function in the Kabuki syndrome craniofacial developmental disorder

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Funding information
National Institute of Dental and Craniofacial Research

[Correction added after first online publication on 21 October 2023. Author name 'Gabrielle A. Quickstad' has been changed to 'Gabrielle Quickstad'.]

Abstract

Background: Kabuki syndrome is a congenital developmental disorder that is characterized by distinctive facial gestalt and skeletal abnormalities. Although rare, the disorder shares clinical features with several related craniofacial syndromes that manifest from mutations in chromatin-modifying enzymes. Collectively, these clinical studies underscore the crucial, concerted functions of chromatin factors in shaping developmental genome structure and driving cellular transcriptional states. Kabuki syndrome predominantly results from mutations in KMT2D, a histone H3 lysine 4 methylase, or KDM6A, a histone H3 lysine 27 demethylase.

Aims: In this review, we summarize the research efforts to model Kabuki syndrome in vivo to understand the cellular and molecular mechanisms that lead to the craniofacial and skeletal pathogenesis that defines the disorder.

Discussion: As several studies have indicated the importance of KMT2D and KDM6A function through catalytic-independent mechanisms, we highlight noncanonical roles for these enzymes as recruitment centers for alternative chromatin and transcriptional machinery.

KEYWORDS

craniofacial and skeletal disorder, histone methylase, Kabuki syndrome, KMT2D, MLL4

1 | KABUKI SYNDROME IS A HETEROGENEOUS DEVELOPMENTAL DISORDER OF CHROMATIN REGULATORS

Kabuki syndrome was first clinically characterized by Norio Niikawa in 1981 in pediatric patients featuring craniofacial, growth, and cognitive abnormalities (Niikawa et al., 1981). Due to ocular components such as elongated palpebral fissures (length of eye-opening) and out turned lower eyelids, the authors proposed calling the syndrome "Kabuki" with reference to the makeup worn by Kabuki actors. The prevalence is estimated at 1 in 32,000 individuals. Over the past 50 years, clinical and basic research have made tremendous progress in advancing our understanding of the pathogenesis of this disorder. Kabuki syndrome patients develop a myriad of multi-organ deficiencies that manifest in a variety of clinical features. In craniofacial tissues, Kabuki patients exhibit facial hypoplasia that produces a broad, depressed nasal tip

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that can be accompanied with cleft or high-arched palate, high-arched eyebrows, elongated palpebral fissures, lower eyelid eversion, prominent ears, and micrognathia (see Figure 1a for craniofacial frequencies; Adam et al., 2019; Wessels et al., 2002; Porntaveetus et al., 2018). Craniofacial dysmorphism is accompanied by a variety of dental abnormalities including excessive caries, enamel hypoplasia, altered tooth shape, size, and agenesis (Porntaveetus et al., 2018; Teixeira et al., 2009). Irregularities in skeletal development produce short stature with reduced postnatal growth, scoliosis, hip dysplasia, joint laxity, hypermobility, and patellar dislocation (see Figure 1b for skeletal frequencies with regards to ossification origins; Adam & Hudgins, 2005; Barry et al., 2022; Bogershausen et al., 2016; Schrander-Stumpel et al., 2005; Wessels et al., 2002). Outside of craniofacial and skeletal features, alterations in other organ systems result in intellectual deficiencies, dermatoglyphic abnormalities, developmental delay, otitis media and recurrent infections, and a variety of congenital heart problems including atrial and ventricular septal defects as well as aortic coarctation (Barry et al., 2022; Bögershausen & Wollnik, 2013).

Kabuki syndrome mutations were first identified in KMT2D (also termed MLL4), a histone H3 lysine 4 (H3K4) methylase (Ng et al., 2010). However, the disorder is genetically heterogenous with causative heterozygous mutations in *KMT2D* (39%–74% of patients; Banka et al., 2015; Bogershausen et al., 2016; Cocciadiferro et al., 2018; Hannibal et al., 2011; Makrythanasis et al., 2013; Micale et al., 2011; Miyake et al., 2013; Ng et al., 2010), mutations in *KDM6A* (3%–6% of patients; Banka et al., 2015; Bogershausen et al., 2016; Cocciadiferro et al., 2018; Lederer et al., 2012; Miyake et al., 2013), and infrequent mutations in a variety of other transcriptional and chromatin regulatory proteins (Lintas & Persico, 2018). KDM6A (also termed UTX) is an X-linked gene with a Y-chromosome homolog termed UTY. Kabuki syndrome can result from female heterozygous or male hemizygous *KDM6A* mutations. KDM6A

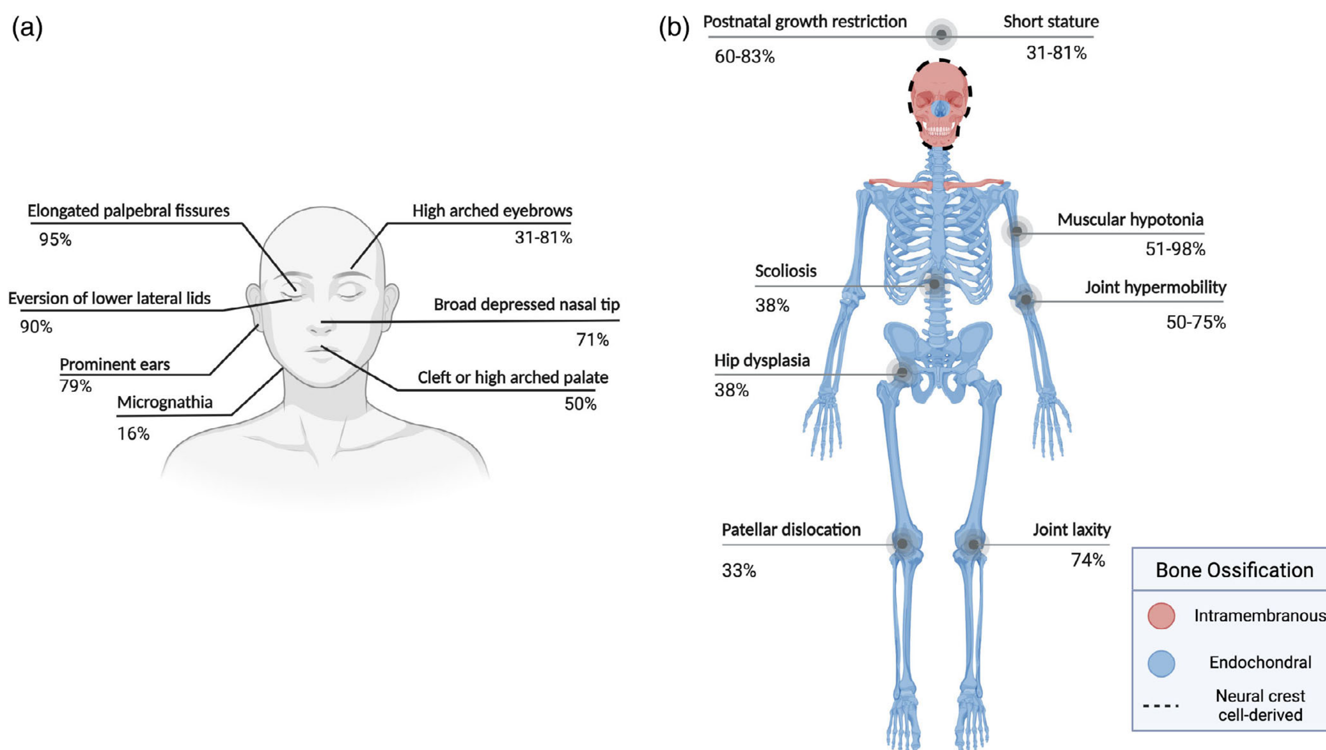


FIGURE 1 Craniofacial and skeletal features in Kabuki syndrome patients. (a) Frequencies of common craniofacial features found in Kabuki syndrome patients. Phenotypic frequencies in parts (a) and (b) were compiled from the following References: (Adam & Hudgins, 2005; Barry et al., 2022; Schrander-Stumpel et al., 2005; Wessels et al., 2002). (b) Illustration demonstrating frequencies of common skeletal features present in Kabuki syndrome patients with reference to mode of bone formation and cellular origin. Ossification is the process by which new bone tissue is formed during skeletal development, or during remodeling and repair of bones. There are two primary types of ossification: endochondral ossification, where bone forms from a cartilaginous precursor, and intramembranous ossification, where bone forms directly from sheets of connective tissue. Endochondral ossification occurs in most of the skeleton, including long bones and the cranial base (blue). Intramembranous ossification (red) occurs primarily in skull bones, facial bones, clavicles, and some irregular bones such as the scapula and ossicles of the ear. All anterior craniofacial bone and cartilage are derived from neural crest stem cells (dashed area). Figure created with BioRender.com.

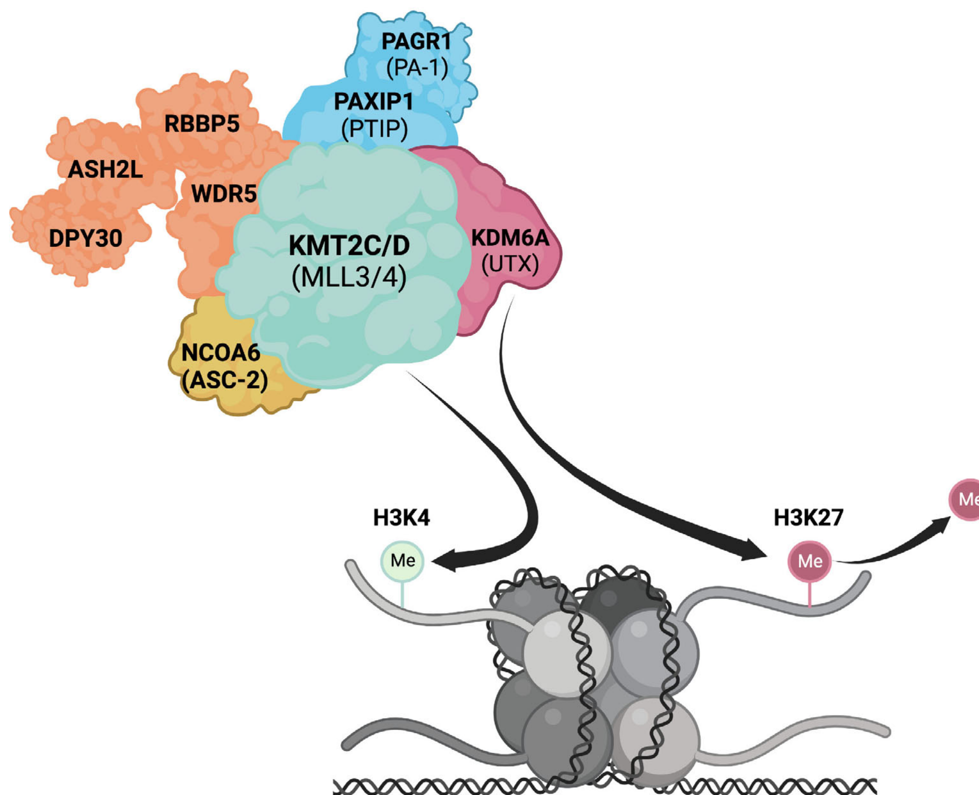


FIGURE 2 The KMT2D and KDM6A COMPASS complex regulates histone methylation. The mixed-lineage leukemia (MLL) complexes, also known as COMPASS (complex of proteins associated with Set1), are a family of multi-subunit proteins responsible for histone 3 lysine 4 (H3K4) mono-, di-, or trimethylation and gene regulation in mammals. KMT2C (MLL3) and KMT2D (MLL4) are histone methyltransferases that play crucial roles in gene activation by mono- and di-methylation of H3K4. WDR5, RBBP5, ASH2L, and DPY30 are subunits common to all complexes in the MLL family. PAGR1 (PA-1), PAXIP1 (PTIP), and KDM6A (UTX) are unique to KMT2C/D COMPASS complexes. KDM6A is responsible for demethylating histone 3 lysine 27 (H3K27), removing methylation marking areas of gene repression. Figure created with BioRender.com.

functions as a histone H3 lysine 27 (H3K27) demethylase and it is hypothesized that removal of this repressive histone modification may be coordinated with KMT2D deposition of activating H3K4 methylation (Figure 2).

2 | KMT2D IS REQUIRED FOR COMPASS COMPLEX INTEGRITY AND KDM6A STABILIZATION

KMT2D and KDM6A associate together in a protein complex (Cho et al., 2007) termed COMPASS, an acronym for complex of proteins associated with SET1 (see Figure 2; Miller et al., 2001). Although a variety of H3K4 methylases can comprise the catalytic core of COMPASS complexes (SET1-2 or KMT2A-D; Shilatifard, 2012), KDM6A is only found in those containing KMT2C or KMT2D (Cho et al., 2007). Other components of the KMT2D and KMT2C COMPASS complexes include WDR5, RBBP5, ASH2L, and DPY30, the WRAD portion of the complex that regulates KMT2D methylase activity (Zhang et al., 2015). KMT2C mutations have not been described in Kabuki syndrome but can result in a distinct intellectual disability syndrome with craniofacial dysmorphism (Koemans et al., 2017). More peripheral components of the COMPASS complex include NCOA6 as well as PTIP and PA1 which are not thought to be essential for KMT2D catalytic activity (Cho et al., 2007).

As the central component of the COMPASS complex, KMT2D is required to maintain the integrity of the associated components. In the absence of KMT2D, PTIP, and PA1 lose biochemical association with the WRAD components of the COMPASS complex. Moreover, KDM6A protein levels are reduced when KMT2D is lost in several cell types (embryonic tissue, embryonic stem (ES) cells, mesenchymal stem cells, adipocytes) from various organismal models such as Human, Mouse, and *Drosophila* (Dorighi et al., 2017; Fasciani et al., 2020; Herz et al., 2012; Lee et al., 2013; Rickels et al., 2020; Xie et al., 2023). The *trr Drosophila* ortholog of KMT2C/D binds to KDM6A (UTX) through a domain termed the UTX stabilization domain (USD; Rickels et al., 2020).

When fused to the KMT2D high-mobility group DNA binding motif, the USD was capable of restoring KDM6A binding to chromatin in KMT2D null HCT116 cells with partial restoration of gene expression (Rickels et al., 2020).

3 | GERMLINE ANIMAL MODELS OF KABUKI SYNDROME

Diverse animal models have been developed to unravel mechanisms behind Kabuki syndrome pathogenesis. In several mouse models for COMPASS complex function, *Kmt2d*, *Kmt2c*, or *Kdm6a* mutations are established in the germline and propagated throughout all embryonic and adult tissues. Homozygous deletion of *Kmt2d* throughout the entire organism is lethal before embryonic mid-gestation with observed growth retardation and developmental delay (Ashokkumar et al., 2020; Lee et al., 2013; Shpargel et al., 2020). *Kmt2d* heterozygous deletion throughout all mouse tissues is haploinsufficient in producing facial hypoplasia, postnatal growth retardation with reduced bone length and thickness, reduced hippocampal neurogenesis and memory, mild aorta narrowing, and can exhibit exencephaly (Ang et al., 2016; Ashokkumar et al., 2020; Bjornsson et al., 2014; Carosso et al., 2019; Fahrner et al., 2019). Mouse homozygous mutation of *Kmt2c* exhibits partial perinatal lethality with surviving mice experiencing reductions in postnatal growth (Ashokkumar et al., 2020; Lee et al., 2006). *Kdm6a* mouse deletion results in female-specific embryonic lethality during a similar developmental period as *Kmt2d* with developmental delay, deficiencies in mesoderm differentiation, and cardiac abnormalities (Lee et al., 2012; Shpargel et al., 2012; Wang et al., 2012; Welstead et al., 2012). The X-chromosomal mutation of *Kdm6a* features sexual dimorphism due to the male presence of the *Uty* Y-chromosome homolog that retains redundant function (Shpargel et al., 2012). Male mice carrying *Kdm6a* mutations experience partial penetrance of perinatal lethality and postnatal growth deficiency (Shpargel et al., 2012; Welstead et al., 2012).

4 | KMT2D AND KDM6A MECHANISMS IN NONMAMMALIAN CRANIAL AND SKELETAL DEVELOPMENT

Modeling KMT2D or KDM6A function using nonmammalian model organisms has proved instrumental in understanding broader cellular and molecular mechanisms relevant to Kabuki syndrome cranial and skeletal dysplasia. Zebrafish morpholinos inhibiting expression of *Kmt2d* or *Kdm6a* orthologs in early development produced craniofacial hypoplasia, loss of branchial arches, absence or reduced dimensions of cranial cartilage, and convergent-extension developmental growth deficits (Bögershausen et al., 2015; van Laarhoven et al., 2015). In a similar fashion, *Kmt2d* homozygous deletion throughout all zebrafish tissues resulted in microcephaly, shortened body axis, underdeveloped neurocranium, altered palate structure, and absence or reduction in cartilaginous jaw structures (de Serrano et al., 2019). Chondrocyte organization in the jaw was altered in *Kmt2d* morphant zebrafish with a lack of columnar orientation and polarization (Bögershausen et al., 2015). These phenotypes were dependent on GTPase signaling as morpholino knockdown of *Rap1* enhanced *Kmt2d*-dependent phenotypes, and exogenous RAP1A expression ameliorated developmental defects. KMT2D inhibition in *Xenopus* through early embryonic morpholino knockdown resulted in craniofacial and cartilaginous deformities (Schwenty-Lara et al., 2019). KMT2D loss of function led to deficiencies in neural crest cell (NCC) specification and migration. KMT2D loss impacted *Xenopus Sema3F* expression, a transmembrane regulator of NCC migration.

5 | MOUSE NEURAL CREST-SPECIFIC MUTATION OF KMT2D AND KDM6A TO MODEL FACIAL DEVELOPMENT

Cranial NCCs are a multipotent stem cell population that is specified in the dorsal neural tube. After migration, these stem cells differentiate into osteoblast and chondrocyte lineages that will develop anterior facial bone and cartilage (Figure 1b; Dupin et al., 2006; Trainor, 2005; Santagati & Rijli, 2003; Jiang et al., 2002; McBratney-Owen et al., 2008). Mouse NCC-specific homozygous deletion of *Kmt2d* models the craniofacial features of Kabuki syndrome as these mice develop hypoplasia of anterior facial bones, depressed snouts, and die at birth with cleft palate (Shpargel et al., 2020). *Kmt2d* heterozygous NCC-specific deletion was viable with mild facial phenotypes and altered postnatal growth. Mouse *Kdm6a* NCC-specific deletion also recapitulated many features of human Kabuki syndrome including craniofacial dysmorphism, postnatal growth retardation, and cardiac abnormalities (Shpargel et al., 2017).

At the cellular level, KMT2D and KDM6A loss of function in NCCs had a significant impact on differentiation of cell types relevant to mammalian bone and cartilage development. Deletion of KMT2D or KDM6A did not alter NCC specification or migration as these cells populated anterior facial regions (Shpargel et al., 2017,

2020). However, KMT2D or KDM6A loss of function did affect distribution of NCC-differentiated osteoblasts in ossification center primordia that form frontal bones (Shpargel et al., 2017, 2020). KMT2D NCC deletion also impaired expression of extracellular matrix components within osteoblasts of the developing palatal shelf (Shpargel et al., 2020). KDM6A deletion downstream of osteoblast specification produced a loss of skeletal bone volume and mineralization that resulted from alterations in osteocyte differentiation from osteoblasts (Xia et al., 2022). Collectively, these studies highlight the importance of Kabuki-causative histone modifiers in multiple stages of osteoblast differentiation.

6 | KMT2D AND KDM6A REGULATION OF CHONDROCYTE DIFFERENTIATION AND SKELETAL DEVELOPMENT

Unlike most facial bones that form directly by osteoblast-dependent intramembranous ossification, the cranial base is a support structure that forms by endochondral ossification whereby NCC-derived chondrocytes in cartilage enter a terminal hypertrophic differentiation stage, forming a scaffold for bone formation (Figure 1b; McBratney-Owen et al., 2008; Szabo-Rogers et al., 2010). KMT2D deletion prevented terminal hypertrophic differentiation of NCC-derived chondrocytes in the cranial base which led to deficiencies in endochondral bone formation (Shpargel et al., 2020). Alternative mouse models have implicated more extensive roles for KMT2D and KDM6A during endochondral ossification of skeletal development (Figure 1b). The postnatal growth deficits of KMT2D heterozygous gene trap mice resulted from an expansion of long bone growth plates due to precocious, unregulated chondrocyte differentiation to restrict bone formation (Fahrner et al., 2019). KMT2D and KDM6A loss in mesenchymal stem cells impaired chondrocyte or osteoblast differentiation (Fasciani et al., 2020; Hemming et al., 2014). These broader functions in stem cell to chondrocyte transitions may underly the skeletal and joint phenotypes prevalent in Kabuki syndrome (Figure 1b).

7 | CANONICAL GENOMIC FUNCTIONS OF KMT2D AND KDM6A: ESTABLISHING ENHANCER CHROMATIN

At the genome level, tri-methylation of H3K4 is commonly found at active promoters, however, mono-methylation of H3K4 is enriched at enhancers (Heintzman et al., 2007). These enhancers can be subclassified as poised with co-enriched methylation of H3K27 or active with accompanying acetylation of H3K27 (Calo & Wysocka, 2013; Creyghton et al., 2010; Rada-Iglesias et al., 2011). KMT2D and KMT2C proteins (KMT2C/D) contain carboxy-terminal SET domains that catalyze methylation of H3K4 in vitro (Goo et al., 2003). Although H3K4 can be mono, di, or tri-methylated in the genome with correlations to transcriptional activation (Ruthenburg et al., 2007), KMT2D demonstrates the highest catalytic activity in producing H3K4me1 and weak activity in generating H3K4me2 (Zhang et al., 2015). KMT2C/D redundantly regulates H3K4me1 and H3K4me2 in vivo as global levels of these histone modifications drop with mutations of both enzymes in mammalian cells (Hu et al., 2013; Lee et al., 2013; Wang et al., 2016), similar to mutation of *Kmt2d* orthologous genes in *Xenopus* or *Drosophila* (Herz et al., 2012; Schwenty-Lara et al., 2019). Accordingly, KMT2C/D has been observed to function at enhancers during stem cell differentiation (Wang et al., 2016). Mutation of both redundant methylases in mouse ES cells were dispensable in maintaining stem cell properties and gene expression, however, upon embryoid body differentiation, KMT2D binding shifted to novel developmental enhancers where KMT2C/D were required for histone acetylase recruitment, H3K27 acetylation, gene induction, and proper embryoid body formation (Wang et al., 2016).

8 | KMT2D MOLECULAR FUNCTION IN CRANIOFACIAL AND SKELETAL DEVELOPMENT

KMT2D mechanisms regulating chondrocyte differentiation have been explored through gene expression analysis and histone methylation profiling at candidate promoters. Through these approaches, KMT2D was discovered to regulate H3K4me3 at the *Shox2* promoter and region surrounding the transcription start site (TSS; Fahrner et al., 2019). Upon differentiation to chondrocytes, KMT2D mutant mouse ATDC5 teratocarcinoma cells reduced H3K4me3 surrounding the *Shox2* TSS, failed to properly activate *Shox2* expression, and enhanced SOX9-mediated chondrocyte differentiation due to a loss of SHOX2 dependent repression. KMT2D regulated RAP1B expression by regulating promoter H3K4me3 in zebrafish and Kabuki patient fibroblasts with alterations in MEK/ERK signaling and hyperactivation of RAS/MAPK pathways (Bögershausen et al., 2015). Craniofacial phenotypes in KMT2D mutants were suppressed by a RAS/MAPK inhibitor (Tsai et al., 2018). The

implications of KMT2D function in these instances of promoter H3K4me3 are unclear as KMT2D lacks tri-methylation activity in vitro (Zhang et al., 2015). Further studies will be required to characterize KMT2D genome-wide binding during osteoblast or chondrocyte differentiation to identify direct genomic functions in regulating craniofacial or skeletal development.

9 | KDM6A MOLECULAR FUNCTION IN CRANIOFACIAL DEVELOPMENT IS CATALYTIC INDEPENDENT

In several cell culture osteochondral models, KDM6A regulated differentiation through control of H3K27 methylation at candidate genomic sites. KDM6A promoted chondrogenic differentiation of periodontal ligament stem cells by demethylation of the SOX9 promoter (Wang et al., 2018). KDM6A regulated MSC osteogenic differentiation by regulating H3K27 demethylation at promoters for RUNX2, Osteopontin, and Osteocalcin (Hemming et al., 2014). Mesenchymal KDM6A knockout inhibited cranial suture development and restricted calvaria osteoblast differentiation by regulating H3K27me3 levels on osteogenic promoters (Pribadi et al., 2023). This compilation of studies demonstrates that KDM6A loss can lead to elevated H3K27 methylation in specific genomic instances, however, the direct requirements of H3K27 demethylation on differentiation and gene expression were not assayed.

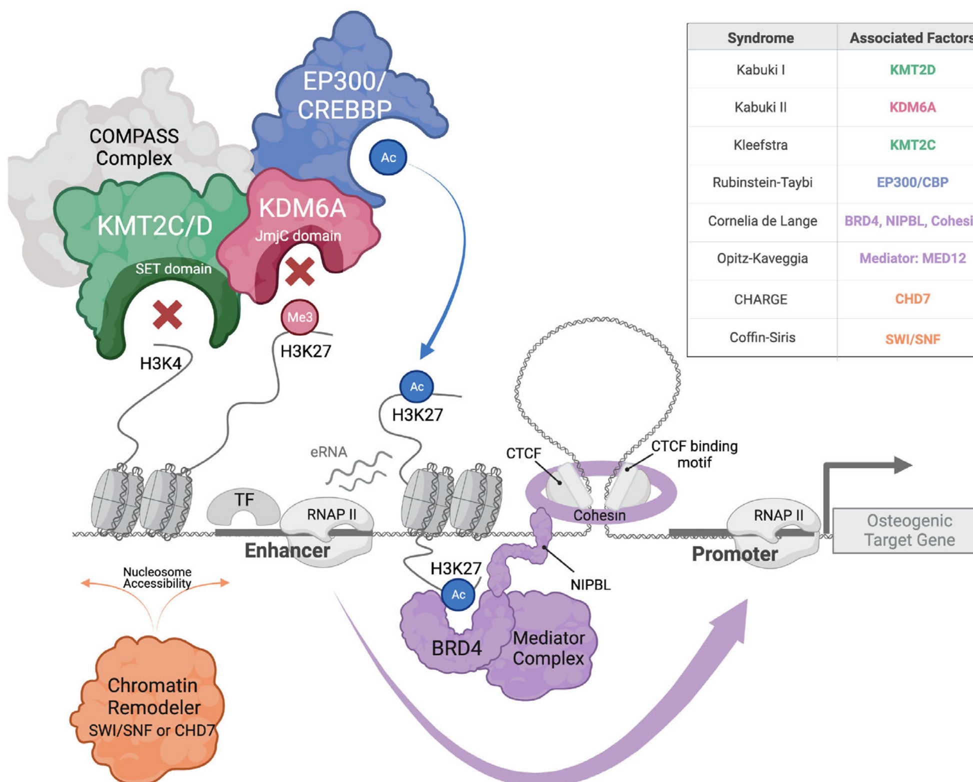
During in vivo NCC differentiation, KDM6A does not require demethylation activity (Shpargel et al., 2017). KDM6A is required for appropriate NCC osteoblast differentiation (Shpargel et al., 2020) and frontal bone development (Shpargel et al., 2017). KDM6A bound and regulated gene expression of Notch and Wnt signaling factors important for NCC and craniofacial development (Shpargel et al., 2017). However, when KDM6A genome binding was assessed in differentiating embryonic NCCs, KDM6A-dependent gene expression did not correlate well with H3K27me3 increases in mutant cells (Shpargel et al., 2017). KDM6A-dependent craniofacial phenotypes were mild in male NCC knockout animals due to redundancy with the catalytically inactive Y-chromosomal UTY homolog. Moreover, homozygous demethylase-dead point mutation of KDM6A throughout all mouse female tissues fully supported normal facial development (Shpargel et al., 2017). KDM6A catalytic activity is also not required in other developmental decisions such as mesoderm differentiation (Wang et al., 2012) or in other organismal models as the *Caenorhabditis elegans* ortholog is required for embryonic development independent of demethylase activity (Vandamme et al., 2012).

10 | EVIDENCE FOR CATALYTIC INDEPENDENT FUNCTION OF KMT2C AND KMT2D IN STEM CELL BIOLOGY

The connections between KMT2D enhancer H3K4 mono-methylation and regulation of osteochondral gene expression are unknown; however, more recent evidence in other instances of stem cell differentiation indicates that KMT2D may not function via histone methylation. In mouse ES cell culture, homozygous point mutations in the KMT2D and KMT2C SET domains rendered the proteins incapable of catalyzing H3K4 methylation (Dorighi et al., 2017). Catalytic inactivation led to loss of enhancer H3K4me1, but KMT2C/D target genes largely maintained appropriate expression. Mice carrying germline homozygous catalytic-dead mutations in *Kmt2c* were viable in contrast to perinatal lethality in *Kmt2c* null mice (Xie et al., 2023). Homozygous catalytic-dead mutations in *Kmt2d* produced perinatal lethality whereas *Kmt2d* null mutations are early embryonic lethal (Xie et al., 2023). These results indicate that KMT2C and KMT2D have non-enzymatic functions during development. Early embryonic tissues did not require KMT2C/D catalytic activity as methylase-dead ES cells could differentiate to all three germ layers and lineage-specific enhancers could become active with H3K27 acetylation in the absence of methylation. A conditional approach with *Sox2-Cre* to selectively eliminate catalytic function in embryonic tissues revealed that embryos could develop normally to mid-gestation in the absence of KMT2C/D methylation (Xie et al., 2023). Similarly, a catalytic-deficient transgene of *trr*, the *Drosophila* KMT2C/D ortholog, was capable of rescuing embryonic lethality of null mutations (Rickels et al., 2017). It is currently unknown if KMT2D methylation activity is essential for osteochondral differentiation or skeletal development.

11 | A MODEL FOR COMPASS ENZYMATIC-INDEPENDENT CRANIOSKELETAL DEVELOPMENT

In both cell culture and differentiating embryoid bodies, KMT2D can retain ability to bind chromatin in the absence of H3K4 methylase activity (Rickels et al., 2020; Xie et al., 2023). KMT2D encodes for tandem plant homeodomain (PHD) regions that bind to histones methylated



Syndrome	Associated Factors
Kabuki I	KMT2D
Kabuki II	KDM6A
Kleefstra	KMT2C
Rubinstein-Taybi	EP300/CBP
Cornelia de Lange	BRD4, NIPBL, Cohesin
Opitz-Kaveggia	Mediator: MED12
CHARGE	CHD7
Coffin-Siris	SWI/SNF

FIGURE 3 A model for COMPASS enzymatic-independent cranioskeletal development. An inactivating point mutation in the SET domain of KMT2C/D (MLL3/4) results in loss of methylase activity. The resulting decrease in histone H3K4 methylation, a mark associated with gene activation, can result in minor effects on transcription and H3K27 acetylation, suggesting alternative roles for KMT2C/D in enhancer activation and gene regulation. Point mutations of the KDM6A Jumonji C (JmjC) domain that inactivate histone H3K27 demethylation can support normal mouse development. In the absence of KMT2C/D or KDM6A catalytic activity, COMPASS complex enhancer relevant functions may compensate for the loss of enzymatic activity. Blue: KMT2C/D and KDM6A can regulate EP300/CREBBP binding and enhancer recruitment for H3K27 acetylation. Orange: The COMPASS complex is co-recruited to enhancers with SWI/SNF remodelers and can associate with CHD7. KMT2C/D specify enhancer RNA polymerase II occupancy, impact lineage transcription factor binding, and influence eRNA production. Purple: KMT2C/D presence is required to promote BRD4 reading of H3K27ac, association with the Mediator complex, and proper localization of cohesin/CTCF-mediated DNA looping to facilitate enhancer-promoter interactions. Depicted in the table, mutations in these histone- and chromatin-modifying proteins are responsible for a variety of cranioskeletal development disorders (KMT2D: Kabuki syndrome type 1, KDM6A: Kabuki syndrome type 2; KMT2C: Kleefstra syndrome; EP300/CREBBP: Rubinstein-Taybi syndrome; SWI/SNF: Coffin-Siris syndrome; CDH7: CHARGE syndrome; BRD4, NIPBL, and cohesin: Cornelia de Lange syndrome; MED12 in Mediator complex: Opitz-Kaveggia (FG) syndrome). Figure created with BioRender.com.

at H4R3 (Dhar et al., 2012) or acetylated H4K16 (Zhang et al., 2019). Thus, KMT2D and the COMPASS complex may have a prominent scaffolding function in the recruitment of alternative histone modification, chromatin regulatory, or transcription factors (Figure 3). Notably, many chromatin complexes that have been tied to COMPASS co-regulation at enhancers are mutated in craniofacial disorders (table in Figure 3). Although patients diagnosed with specific disorders have distinct facial gestalt and unique syndromic features, they share common elements such as hypoplasia and dysmorphic facial structures in combination with short stature and growth deficits. Similarity between these disorders can even result in a blending of phenotypic presentation whereby KMT2D mutations have been ascribed to alternative syndromes (Sakata et al., 2017; Schulz et al., 2014; Verhagen et al., 2014). We hypothesize that KMT2D and KDM6A catalytic-independent function in Kabuki syndrome craniofacial and skeletal development involves enhancer co-regulation with these related factors. Although the coordinated functional mechanisms between Kabuki causative factors and other enhancer chromatin disorders have not been extensively studied in cranioskeletal development, in the remainder of this review we highlight known connections gleaned from other developmental systems.

12 | CATALYTIC INDEPENDENT FUNCTION OF KMT2D/AND KDM6A: ENHANCER ACETYLATION

Mutation of CREB-binding protein (CREBBP) and EP300 histone acetylases (Figure 3: blue) manifest in the Rubinstein-Taybi craniofacial disorder with overlapping clinical features to Kabuki syndrome (Petrij et al., 1995; Roelfsema et al., 2005). Genomic binding assays indicate that predominant KMT2D and KDM6A localization is at active enhancers featuring H3K27 acetylation (Hu et al., 2013; Lee et al., 2013; Wang et al., 2016; Wang et al., 2017). In *Drosophila*, KDM6A can bind directly to the CREBBP that acetylates H3K27 to modulate levels of histone acetylation in the genome (Tie et al., 2012). During differentiation, KMT2C/D mutant ES cells demonstrated a loss of enhancer H3K27ac due to a failure to recruit EP300 (Wang et al., 2016). Loss of acetylation is likely due to direct binding between KDM6A and EP300 (Wang et al., 2017). Protein complexes containing both KMT2D and KDM6A enhanced EP300 acetylase activity on chromatin using in vitro assays and KDM6A knockout significantly decreased H3K27 acetylation levels at genomic target enhancers (Wang et al., 2017). In germinal center B-cells, CREBBP binds to and acetylates KMT2D to regulate methylase activity (Vlasevska et al., 2023). Histone deacetylase inhibition improved neurological phenotypes in KMT2D heterozygous mice, further illustrating the role of acetylation in Kabuki-like pathogenesis (Bjornsson et al., 2014).

Although there is an abundance of experimental evidence supporting KMT2D and KDM6A involvement in establishing active enhancer acetylation, there are caveats suggesting that these Kabuki factors are not absolutely required for enhancer acetylation. As ES cells shift to a formative pluripotent state, KMT2C/D are required for instances of de novo H3K4 methylation and H3K27 acetylation, however, acetylation can occur independently of KMT2C/D and did not correlate with transcriptional activation (Boileau et al., 2023). In fact, H3K27 acetylation may be dispensable for enhancer regulation. The variant histone H3.3 is commonly located at enhancers (Chen et al., 2013), and when H3.3 lysine 27 is mutated to arginine, acetylation is lost at mouse ES cell enhancers, yet enhancer accessibility and gene expression are largely unaffected (Zhang et al., 2020). ES cell pan-H3K27R mutations in all histone H3 variants demonstrated that H3K27 acetylation is not required for de novo gene activation during differentiation and that RNA polymerase and Mediator complexes can bind genes and enhancers in the absence of H3K27 acetylation (Sankar et al., 2022). Thus, due to conflicting data, the connections between KMT2D or KDM6A, enhancer acetylation, and transcription remain unclear and will require further exploration, particularly in osteochondral lineages relevant to cranial or skeletal development.

13 | CATALYTIC INDEPENDENT FUNCTION OF KMT2D/KDM6A: CHROMATIN ACCESSIBILITY

Various chromatin remodeling components that regulate DNA accessibility are mutated in craniofacial disorders (Figure 3: orange). Components of the SWI/SNF remodeler are mutated in the Coffin-Siris craniofacial disorder (Santen et al., 2012; Tsurusaki et al., 2012). KMT2D mutations have also been described in patients featuring the CHARGE craniofacial syndrome (Sakata et al., 2017; Schulz et al., 2014; Verhagen et al., 2014) that typically carry alterations to the CHD7 chromatin remodeler (Vissers et al., 2004). KMT2D and KDM6A can influence chromatin accessibility to regulate enhancer function. During adipogenic differentiation, KMT2D and KDM6A associate with SWI/SNF chromatin remodeling complexes (Park et al., 2021). Mutation of KMT2D or SMARCB1 (SWI/SNF complex member) demonstrated reciprocal roles in co-recruitment to enhancers to regulate chromatin accessibility. KDM6A bound to SMARCA4 (BRG1 in SWI/SNF complex) to regulate associations with T-box transcription factors during T-cell differentiation (Miller et al., 2010). Similarly, CHD7 can physically associate with KMT2D, KDM6A, and the COMPASS complex (Schulz et al., 2014; Ufartes et al., 2021). SWI/SNF and CHD7 remodeling complexes coordinate to regulate neural crest enhancer activity and craniofacial development, however, the role of enhancer priming by the COMPASS complex and coordination with remodeling complexes is unclear within these lineages (Bajpai et al., 2010; Chandler & Magnuson, 2016; Sanosaka et al., 2022; Sperry et al., 2014).

14 | CATALYTIC INDEPENDENT FUNCTION OF KMT2D/KDM6A: ENHANCER-PROMOTER CONTACTS

Chromatin-associated factors play an essential function in bridging enhancer to promoter contacts to drive transcription (Figure 3: purple). Factors that facilitate enhancer to promoter crosstalk are mutated in craniofacial disorders (Krantz et al., 2004; Mannini et al., 2013; Olley et al., 2018; Risheg et al., 2007; Schwartz et al., 2007; Vulto-van Silfhout et al., 2013) including BRD4, NIPBL, or cohesin subunits (Cornelia de Lange syndrome) as well as MED12 of the Mediator complex

(Opitz-Kaveggia or FG, Lujan-Fryns, and Ohdo syndromes). KMT2C/D were essential for enhancer associations with promoter elements in ES cells and during neural progenitor differentiation to regulate gene expression (Yan et al., 2018). Upon loss of KMT2C/D, these enhancers lacked cohesin, a protein complex forming a ring involved in DNA loop extrusion and long-range chromatin contacts (Davidson & Peters, 2021). KMT2D-dependent enhancer to promoter communication and cohesin loading required KMT2D catalytic methylase activity and the presence of H3K4me1 (Yan et al., 2018). Other chromatin factors critical for linking enhancer activity to promoters bind downstream of KMT2D function. During adipogenesis, KMT2D is required for enhancer recruitment of BRD4, a tandem bromodomain encoding protein that binds to acetylated histones (Lee et al., 2017). BRD4 interacts with components of the Mediator complex (Jang et al., 2005) and the cohesin-loading protein NIPBL (Olley et al., 2018). BRD4 recruits Mediator to KMT2D bound enhancers (Lee et al., 2017). In turn, Mediator can communicate to promoters with the aid of cohesin (Pherson et al., 2019). Both NIPBL and BRD4 are required within NCCs for mouse craniofacial development, however, their genomic mechanisms with regard to enhancer regulation in mammalian cranial lineages and cooperation with KMT2D or KDM6A are unknown (Linares-Saldana et al., 2021; Smith et al., 2014).

15 | CATALYTIC INDEPENDENT FUNCTIONS OF KMT2D/KDM6A: REGULATION OF TRANSCRIPTION

KMT2D and KMT2C may function directly in regulating active transcription. In KMT2C/D mutant ES cells, target enhancers have a reduction in RNA polymerase binding and eRNA transcription (Dorighi et al., 2017). Although promoter RNA Pol II loading was unaffected, KMT2C/D loss resulted in increased promoter pausing that was not dependent on methylase catalytic activity. During muscle cell differentiation, KDM6A is recruited by SPT6, a histone chaperone that associates with and regulates RNA Pol II elongation (Wang et al., 2013). KMT2C/D binding to enhancers is a prerequisite for lineage-determining transcription factors in adipocyte differentiation (Lai et al., 2017; Lee et al., 2017). KDM6A is also known to be associated with transcription factors in cardiac development (Lee et al., 2012; Shpargel et al., 2012). KMT2D binds to enhancers with the MEF2 transcription factor to coactivate muscle myofiber gene expression programs (Liu et al., 2020). Similar to a growing list of chromatin regulators, KMT2D can stimulate nuclear biomolecular condensate formation through a prion-like domain that facilitates liquid–liquid phase separation (Fasciani et al., 2020). These nuclear condensates may provide a mechanism to compartmentalize active transcribing regions in the nucleus. This property of KMT2D may also impact nuclear mechanical stress and cellular properties as deficiencies in KMT2D mutant chondrocyte differentiation were alleviated by inhibition of ATR, a checkpoint that responds to nuclear mechanical stress (Fasciani et al., 2020). In summary, KMT2D and KDM6A have been implicated in transcriptional activation by regulating transcription factor activity, RNA polymerase extension, and nuclear compartmentalization of active genes.

16 | NEXT STAGES FOR RESEARCH ON CHROMATIN REGULATION IN CRANIOFACIAL DISORDERS

Chromatin-modifying enzymes perform multifaceted functions in the pathogenesis of craniofacial and skeletal developmental syndromes. These disorders feature overlapping clinical characteristics that can culminate in misclassification of causative genes within related disorders. Therefore, substantial chromatin regulatory complexes may cooperate and co-function within cellular lineages critical for craniofacial and skeletal development. The haploinsufficient nature of these syndromes indicates that these cellular origins are particularly sensitive to dosage of chromatin factors. Cellular differentiation events are regulated by drastic alterations in gene expression, and as such, chromatin structure may provide vital roles in fine-tuning transcriptional output to control developmental decisions. Current research has demonstrated the essential functions of Kabuki-dependent KMT2D and KDM6A histone-modifying enzymes in regulating osteochondral cellular lineages critical for facial and skeletal development. Although several organismal and tissue-specific models have concentrated on cellular pathogenesis that promotes Kabuki-like phenotypes, the lack of genomic exploration in this field of research has limited the understanding of broader KMT2D and KDM6A mechanisms. Further complicating mechanistic interpretation is the fact that catalytic function is not required for KDM6A and may only partially contribute to KMT2D function. A multitude of diverse genomic mechanisms are established for KMT2D and KDM6A in other cell types to facilitate enhancer histone modifications, chromatin accessibility, recruitment of transcription factors and RNA polymerase, and regulation of enhancer-promoter associations. These functions, catalytic requirements, and coordination can vary depending

on cell type. As chromatin states can be altered by therapeutic inhibition of regulatory proteins, a more comprehensive genomic inquiry in cell types relevant to osteochondral development will create a foundation to understand the mechanisms underlying craniofacial and skeletal disorders, identify potential routes of treatment, and, by extension, have important implications for our understanding of Kabuki syndrome pathogenesis.

FUNDING INFORMATION

This work was financially supported by an NIH R01 (R01DE030530, KS) award from the NIDCR.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Shpargel, K. B., & Quickstad, G. (2023). SETting up the genome: KMT2D and KDM6A genomic function in the Kabuki syndrome craniofacial developmental disorder. *Birth Defects Research*, *115*(20), 1885–1898. https://doi.org/10.1002/bdr2.2253