UTX-guided neural crest function underlies craniofacial features of Kabuki syndrome

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Kabuki syndrome, a congenital craniofacial disorder, manifests from mutations in an X-linked histone H3 lysine 27 demethylase (UTX/KDM6A) or a H3 lysine 4 methylase (KMT2D). However, the cellular and molecular etiology of histone-modifying enzymes in craniofacial disorders is unknown. We now establish Kabuki syndrome as a neurocristopathy, whereby the majority of clinical features are modeled in mice carrying neural crest (NC) deletion of UTX, including craniofacial dysmorphism, cardiac defects, and postnatal growth retardation. Female UTX NC knockout (FKO) demonstrates enhanced phenotypic severity over males (MKOs), due to partial redundancy with UTX, a Y-chromosome demethylase-dead homolog. Thus, NC cells may require demethylase-independent UTX activity. Consistently, Kabuki causative point mutations upstream of the JmjC domain do not disrupt UTX demethylation. We have isolated primary NC cells at a phenocritical postmitogenic timepoint in both FKO and MKO mice, and genome-wide expression and histone profiling have revealed UTX molecular function in establishing appropriate chromatin structure to regulate crucial NC stem-cell signaling pathways. However, the majority of UTX-regulated genes do not experience aberrations in H3K27me3 or H3K4me3, implicating alternative roles for UTX in transcriptional control. These findings are substantiated through demethylase-dead knockin mutation of UTX, which supports appropriate facial development.

UTX | KDM6A | Kabuki syndrome | histone demethylation | neural crest

Neurocristopathies are defined as disorders originating from deficiencies in any aspect of neural crest (NC) biology including specification, migration, and differentiation (1). NC cells comprise a multipotent stem-cell lineage that is specified along the dorsal axis of the vertebrate neural tube. Upon specification, NC cells migrate ventrally and are responsible for diverse differentiation events, depending on rostral–caudal positioning upon reaching their embryonic destination (2–4). Cranial NC cells can differentiate into sensory ganglia, preosteoblasts, and chondrocytes to pattern and form all anterior facial bone and cartilage. Cardiac NC cells differentiate into smooth muscle and are required for outflow tract septation and patterning of the aortic arch arteries. Trunk NC cells develop into melanocytes and dorsal root ganglia, while vagal and sacral NC cells form the enteric nervous system. Thus, neurocristopathies will compromise common sets of organ systems featuring craniofacial, cardiac, and neurological dysfunction. Classical neurocristopathies such as Treacher Collins syndrome result from transcriptional deficiencies, while more recently, neurocristopathies have been described from mutations in chromatin remodeling machinery (5–7).

Kabuki syndrome is a human disorder defined largely by facial characteristics. Affected individuals present midfacial hypoplasia with a broad depressed nasal tip, elongated palpebral fissures with partial eyelid eversion, and large prominent abnormal earlobes (8, 9). Accompanying partially penetrant features include cleft or high arched palate, postnatal growth retardation, and cognitive disability. The disorder can also include several forms of congenital cardiac abnormalities, including ventricular septal closure defects, patent ductus arteriosus, and aortic coarctation. Kabuki syndrome results in the majority of cases (60–70%) from mutations in KMT2D (MLL2, also referred to as MLL4), which encodes a histone H3 lysine 4 (H3K4) methylase that creates an active chromatin environment for enhancing genetic transcription (10–12). A smaller percentage of Kabuki individuals (6–14%) carry mutations in UTX (KDM6A), a histone H3 lysine 27 (H3K27) demethylase that removes repressive chromatin modifications (10, 11, 13). Active H3K4 trimethylation (H3K4me3) can co-occur in promoter regions together with repressive H3K27 trimethylation (H3K27me3) in the form of bivalent chromatin (14). Genes regulated by bivalent chromatin have been identified in developmental stem cells and are hypothesized to be poised for rapid gene induction in cellular differentiation events. Similarly, UTX and KMT2D coexist in a protein complex (15) that may be designed to resolve bivalent chromatin through coordinated removal of H3K27me3 and enhancement of H3K4me3. Given the spectrum of phenotypes in Kabuki syndrome resembling a neurocristopathy and the phenotype of Kabuki causative UTX and KMT2D mutations, NC cells represent a relevant model system to study the coordinated functions of these histone-modifying enzymes.

Kabuki causative mutations in UTX have been identified in both female and male patients (13, 16). This is surprising, given UTX’s location on the X chromosome. Kabuki syndrome results from hypomorphic female heterozygous mutation and null male hemizygous mutation of UTX. Although male patients lack UTX activity, there may be compensation by UTY, a Y-chromosome homolog of UTX. Given that UTY has lost enzymatic activity

Significance

Several chromatin-modifying enzymes are mutated in human craniofacial disorders. These factors function genome-wide to regulate accessibility and expression of extensive gene sets. Therefore, understanding chromatin-modifier function requires identification of responsible cellular origins and genomic characterization of phenotypes in primary cells. We now combine reporter flow cytometry with low cell number genomics to identify neural crest stem-cell factors as molecular targets of UTX in Kabuki syndrome. UTX demethylates histones to regulate some gene expression, but many UTX-bound regions are subject to novel mechanisms of transcriptional regulation. This study identifies UTX cellular and molecular targets in craniofacial development and this methodology is broadly adaptable to study genome distribution of other chromatin factors in neural crest disorders.

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UTX-mediated H3K27me3 demethylation may not be essential for function in craniofacial development. We have developed a NC mouse model to examine UTX molecular function in craniofacial pathogenesis. Our genetic studies indicate that UTX has both demethylase-dependent and -independent roles in NC development. Epigenomic analysis of primary NC cells supports this observation as UTX regulates H3K27me3 demethylation at a subset of gene loci, while other transcriptional targets experience unaltered histone methylation. We find that the majority of Kabuki mutations disrupt UTX demethylation; however, two point mutations that reside outside of the JmjC demethylase domain inhibit alternative UTX functions. Because demethylase-dead knockin (KI) mutation of UTX facilitates normal craniofacial development, we conclude that UTX functions mainly through demethylase-independent transcriptional control of stem-cell signaling pathways to influence NC cell viability.

Results

Mouse UTX NC Knockout Models Several Features of Human Kabuki Syndrome. We have previously contrasted male and female mouse Utx mutant phenotypes to elucidate molecular function based on the presence of the demethylase-dead Y-chromosome homolog, UTy (17). If male Utx mutation elicits a phenotype, then UTy cannot fully compensate for UTX loss, signifying demethylase dependency. In contrast, if Utx homogous females demonstrate enhanced phenotypic severity over Utx hemizygous males, then UTy is providing demethylase-independent compensation for loss of UTX. To model Kabuki syndrome in the mouse, we drove NC-specific deletion of a conditional Utx allele with a Wnt1-Cre transgene (20). This Utx allele has a floxed (fl) third exon, and Cre-mediated deletion produces a nonsense frameshift null for UTX protein (17). While Utx<sup>fl/fl</sup> Wnt1-Cre males (hereafter abbreviated as MKO for male NC knockout) are viable based on expected Mendelian genotype frequencies, Utx<sup>fl/fl</sup> Wnt1-Cre females (abbreviated FKO for female NC knockout) demonstrate significant postnatal lethality as ~40% die before weaning (Fig. 1L). Both MKO and FKO mice experience significant reductions in weight starting at postnatal day 3 (P3) that persist throughout adulthood with FKO mice more severely afflicted (Fig. 1B).

Anterior facial lineages are derived from cranial NC, and MKO mice exhibited frontonasal hypoplasia with a depressed snout, an increased facial angle producing a more prominent forehead, and reduced palpebral fissures (Fig. 1C). FKO mice displayed enhanced MKO characteristics, including greater facial depression, a broader dome-shaped forehead, severe palpebral fissure reduction, and rounded low-set earlobes (Fig. 1C). Partially penetrant cleft palate was specific to FKO embryos (Fig. 1D), which is a likely cause for lower than expected FKO recovery between P1 and P5 (Fig. 1L). Furthermore, FKO mice also exhibited patent ductus arteriosus congenital heart defects (Fig. 1E) without abnormalities in ventricular septal closure (Fig. S1L).

We observed a similar spectrum of phenotypes when UTX knockout is driven by another NC Cre line derived by insertion into the Pax3 locus (21). The dysmorphic facial phenotypes were also observed when NC knockout was driven by Pax3-Cre (Fig. S1B). Utx<sup>fl/fl</sup> Pax3-Cre males had enhanced frontonasal depression, and Utx<sup>fl/fl</sup> Pax3-Cre females could not be recovered at weaning, due to deficiencies in lung inflation at birth (Fig. S1 C and D). These contrasts to Wnt1-Cre–driven phenotypes may be due to leakiness of Pax3-Cre in intercostal skeletal muscle and/or hypomorphic loss of Pax3 in combination with Utx knockout (21, 22).

In summary, NC-specific knockout of Utx establishes a mouse model for Kabuki syndrome that exhibits many features of the human disorder (Fig. 1F). MKO mice have a Kabuki-like manifestation of mild craniofacial features and postnatal growth retardation, indicative of demethylase-dependent UTX function. FKO mice have more severe growth, craniofacial, and cardiac abnormalities. Thus, UTy performs some demethylase-independent UCX compensation in NC development. UTy only partially compensates for loss of UTX in mouse NC, creating an allelic phenotypic gradient. Heterozygous Utx females having one copy of Utx are asymptomatic for facial phenotypes, MKO having one copy of UtY manifest mild phenotypes, and FKO completely lacking both homologs are more severely affected. Similar dosage sensitivity exists in Kabuki patients. Utx<sup>−/−</sup> males have more severe developmental delay and learning disabilities compared with Utx<sup>−/+</sup> females (11, 16, 23). Therefore, UTX has some function, but a single copy of UTX<sup>−/−</sup> is not as functional as a single copy of UTX<sup>−/+</sup>.

NC Cells Require UTX for Appropriate Anterior Cranial Bone Structure. To detail the skeletal structure that may underlie MKO and FKO facial dysmorphism, we performed alizarin red (bone) and alcian blue (cartilage) staining on P10 skull preparations. Gross bone composition was normal; however, FKO mice exhibited dramatic shortening of nasal and frontal bones (Fig. 2A). Measurement of bone lengths verified significant shortening of FKO frontal and nasal structures, while MKO mice experienced mild reductions that failed to reach statistical significance (Fig. 2B). Posterior cranial structures such as parietal bones that are derived from non-NC origins were unaffected in MKO and FKO mice (Fig. 2B). Mutant mice displayed no deficiencies in cartilage distribution, but exhibited a persistence of mandibular cartilage (Fig. 2A). Skeletal abnormalities manifested in embryonic development as similar craniofacial phenotypes were present at late embryonic stages (Fig. 2C). Quantification of E18.5 facial angle revealed significant alterations in both MKO and FKO embryos (Fig. 2C). Alizarin red stained transverse sections of E18.5 cranial regions revealed decreased frontal bone thickness in FKO embryos (Fig. 2D). Therefore, the primary defects in MKO and FKO anterior cranial structure occur earlier in embryonic NC development.

UTX NC Loss of Function Results in Postmigratory Embryonic Neural Crest Deficiencies. We traced embryonic NC cells utilizing a Cre-activated Tomato reporter inserted into the Rosa locus (24). Cranial NC cells
complete migration to ventral facial regions by E9 (25). They continue to proliferate and expand before differentiation to facial lineages at E12 (26). Compared with Tomato reporter fluorescence driven by Wnt1-Cre in WT embryos, FKO NC fluorescence was normal subsequent to migration completion and across proliferative phases (E11.5), but was diminished at differentiation onset (E13.5, Fig. 3A). E13.5 sectioning and Tunel assay of anterior facial regions indicated elevated FKO NC cell death, particularly in the future frontonasal suture and nasal septum regions (Fig. 3B).

Cranial NC cells were isolated by dissection of frontal facial regions rostral from the maxilla and anterior to the otic vestibule (dashed lines in Fig. 3A). This tissue was dissociated and flow sorted for live (based on DAPI exclusion) cranial NC (based on Tomato fluorescence, Fig. 3C). Based on relative sorted cell percentages, FKO NC cell numbers were normal at E12.5, but were significantly reduced by E13.5 (Fig. 3D). Nuclear staining of isolated cranial NC for KI67 and flow cytometry revealed no differences in cellular proliferation (Fig. S2 A and B); however, surface staining for annexin V (recognizing plasma membrane phosphatidylserine and early apoptosis) demonstrated a greater percentage of FKO Tomato-positive cells exhibiting high annexin staining (Fig. 3E and F). Flow cytometry revealed UTX protein levels were elevated in Tomato-positive WT NC relative to Tomato-negative non-NC cells, and verified that UTX was lost in FKO NC (Fig. 3G and H).

We examined UTX function in the O9-1 self-renewing female primary cranial NC cell line that was obtained by similar sorting methodology at E8.5 (27). The O9-1 cranial NC line identity was verified by RT-PCR for NC stem-cell markers (Fig. S2C). UTX was knocked out by lentiviral infection of the NC line with Cas9 and Utx guide RNAs followed by puromycin selection for transduction. UTX protein was lost after 3 d of selection for UTX knockout (FKO) but was unaffected by a nontargeting control guide RNA (CTL) (Fig. 3J). Knockout of UTX in this cell line resulted in a significant reduction in cranial NC cells across the onset of UTX protein depletion (Fig. 3J). In summary, UTX is required for postmigratory cranial NC viability.

UTX Molecular Analysis of Cranial NC Cells. Isolated primary cranial NC cells were subjected to molecular analysis at E12.5, before onset of FKO NC loss. We performed mRNA-sequencing (RNA-seq) on biological replicates of flow-sorted WT, MKO, and FKO NC cells. These expression data were correlated with histone profiles of both H3K27me3 and H3K4me3 ChIP-seq on pooled biological replicates of WT and FKO. The UTX antibody specifically immunoprecipitated UTX (Fig. S2D), but was not as efficient for ChIP analysis and required greater quantities of cells. Therefore, UTX ChIP-seq was performed on the O9-1 self-renewing primary cranial NC cell line (27). To confirm the validity of expression and ChIP-seq data, we examined WT enrichment at the Hoxb genomic region. As Hox gene expression is repressed in cranial NC cells, the Hoxb cluster has high enrichment of H3K27me3 without RNA expression (Fig. 4A). This bipartite pattern of H3K27me3 enrichment is similar to what has been observed at Hoxb in undifferentiated mouse ES cells (28). Proximal to Hoxb is a set of genes, including Atp5g1, that is highly expressed by RNA-seq, have strong peaks of H3K4me3 at genic transcription start sites (TSSs), and lack H3K27me3 enrichment (Fig. 4A). Similarly, Twist1, a highly expressed gene essential for NC viability and differentiation (29), has high RNA-seq signal and H3K4me3 accumulation, but...
Demethylase-Dependent and -Independent Roles for UTX in Cranial NC Gene Regulation. To develop an overview of the relationship between UTX binding and histone methylation, we examined the overlap of UTX, H3K27me3, and H3K4me3 in cranial NC cells on a genomic level. UTX, H3K27me3, and H3K4me3 peaks of enrichment were identified by the MACS peak calling algorithm (version 2) (31). Due to the use of male and female samples in our analysis, all genes and peaks on the X and Y chromosomes were excluded from analysis. MACS identified 21,656 UTX peaks, 38,134 H3K4me3 peaks, and 17,867 H3K27me3 peaks of enrichment. UTX peaks overlapped more frequently with H3K4me3 peaks than H3K27me3 peaks, possibly due to the sheer number of H3K4me3 peaks (Fig. 4B). UTX did frequently overlap with H3K27me3 (17% of UTX peaks), and most of these sites had a corresponding peak of H3K4me3 (92%, 3,377 total peaks, Fig. 4B).

UTX is an H3K27me3 demethylase utilized to remove repressive H3K27me3 in gene activation events. Therefore, we expected direct UTX targets to experience elevated H3K27me3 in FKO NC cells relative to WT at sites of UTX binding. EdgeR compared read counts at prominent peaks (with cpm >1) to identify those with significantly [false discovery rate (FDR) < 0.05] elevated FKO H3K27me3. Overall, 15% of all H3K27me3 peaks were significantly increased in FKO NC (2,705 peaks). We assessed the frequency with which elevated FKO H3K27me3 co-occurred with WT UTX binding as evidence of direct UTX-mediated demethylation. A total of 23% of all UTX-occupied H3K27me3 peaks were elevated in FKO NC cells, compared with only 13% of all UTX-unoccupied H3K27me3 peaks (Fig. 4C); thus UTX binding significantly correlates with H3K27me3 demethylation. A profile across all UTX peaks demonstrated overlap with H3K4me3 enrichment and H3K27me3 depletion (Fig. 4D). Only a subset of these UTX peaks fit criteria for demethylation (UTX peaks that overlap with elevated FKO K3K27m3) and display particularly high H3K27me3 (even in WT samples) with an enrichment spike at the UTX peak center (Fig. 4E).

Based on gene annotation data, UTX and H3K4me3 peaks were found at more genic regions than H3K27me3 (Fig. S2F). UTX was bound more frequently to promoters and TSSs, while H3K27me3 had more intergenic localization. To characterize NC UTX transcriptional targets, EdgeR identified RNA-seq transcripts that were significantly (FDR < 0.05) reduced in FKO cells relative to WT. A profile of all UTX-bound TSSs reveals extensive H3K4me3 enrichment (Fig. S2F, Left). UTX binding spans these TSSs with little H3K27me3 accumulation. However, UTX-bound transcriptional targets with expression significantly reduced in FKO NC cells demonstrate elevated H3K27me3 as a potential regulatory feature (Fig. S2F, Right).

RNA-seq analysis identified a high confidence set of 508 potential demethylase-dependent genes (expression reduced in both MKO and FKO NC relative to WT, FDR < 0.05, Fig. 4F). An additional set of 84 FKO-specific reductions (relative to WT levels) comprise potential demethylase-independent genes (Fig. 4F). We used this total set of 592 FKO-affected genes to identify direct expression relative to WT or 84 FKO specific genes (592 total). (G) The total set of 592 genes with decreased (dec.) FKO expression were overlaid with UTX-bound genes (UTX ChIP-seq peak within 10 kb of gene body) to identify direct UTX gene targets. (H) A total of 279 direct UTX targets were classified as having peaks of H3K27me3 (K27), H3K4me3 (K4), K4/K27, or UTX only within 10 kb of the gene body. (I) A total of 279 direct UTX targets were classified as experiencing enhanced FKO H3K27me3 (FKO-K27up), reduced FKO H3K4me3 (FKO-K4dn), both alterations, or bound by UTX with no alterations within 10 kb of the gene body.

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UTX-bound targets (Fig. 4G). Notably, of 592 genes with altered NC body) to direct UTX binding. UTX-bound transcriptional targets frequently (80%) had peaks of H3K27me3 that coincided with genomic H3K4me3 enrichment (Fig. 4H). However, only 40% of UTX direct transcriptional targets experienced a demethylation phenotype (FKO-K27up, 106 total genes, Fig. 4I). An equally large set of UTX direct targets of activation (110 genes) had no significant alterations of H3K27me3 or H3K4me3 distribution upon UTX loss of function (Fig. 4J); therefore, UTX has both histone demethylase-dependent and –independent roles in transcriptional regulation of NC target genes.

The reason for only partial function relative to UTX may occur because NC requires some form of demethylation for establishing the appropriate cellular transcriptome. Alternatively, UTY may have additional deficiencies in other functions such as altered protein associations or chromatin binding properties that render the protein hypomorphic compared with UTX. To specifically assay NC demethylation requirements, we utilized a demethylation-dead knockin point mutation of Utx (KI allele) (allele schematic in Fig. 6A) (41, 42). Mutation of UTX H11146 and E11148 to alanine will disrupt JmJ-C-mediated iron cofactor binding and histone demethylation. These point mutations have been validated to eliminate histone demethylation through both in vitro assays with purified KI complexes (41) and in vivo overexpression assays (17). The KI mutant line has deficiencies in muscle regeneration and fails to demethylate several UTX targets of muscle differentiation from primary myoblasts (42). KI mutations were verified by sequencing of PCR and RT-PCR products (Fig. 6B). Both heterozygous+/KI and homozygous KI/KI females had normal facial characteristics lacking any previously observed MKO and FKO phenotypic appearance (Fig. 6C). Compared with WT mice, KI/KI homozygotes had normal body weight and facial angular measurements at early postnatal timepoints (Fig. 6D and E).

One caveat in contrasting KI/KI phenotypes to NC-specific Utx deletion is that KI/KI homozygotes have biallelic demethylase-deficient UTX, while MKO hemizygotes have monoallelic demethylase-dead WT UTY. For this reason, comparison of KI+-/KI– and FKO over a Utx null allele (–, gene trap, UtxGTT) (17). The monoallelic KI (K/–) female mice displayed mild snout depression phenotypes similar to MKO (Fig. 6F). In contrast, Utx+/– heterozygous female mice lacked facial phenotypes but did exhibit mild postnatal growth retardation (Fig. 6F and S3). Therefore, UTX can support craniofacial development through demethylase-independent mechanisms; however, under hypomorphic conditions, UTX-mediated demethylation may be required for appropriate NC cellular gene activation.

**Kabuki Syndrome Can Manifest Through Demethylase-Independent UTX Mutation.** To investigate the molecular requirements of UTX that give rise to human Kabuki syndrome, we assessed the spectrum of mutations identified in Kabuki patients (10, 11, 13, 16, 23, 43–46). Kabuki causative UTX protein coding mutations vary in extent from complete Utx deletion to single amino acid point substitutions (Fig. 7A). Despite no obvious correlations in patient sex or domain-specific mutation, one commonality is the prevalence of nonsense mutations through frameshift or the introduction of stop codons. As the JmJC demethylation domain is downstream in a carboxyl-terminal (C terminal) region, the majority of these mutations will eliminate UTX-dependent demethylation activity (15 of 23 mutations). A smaller set of point mutations is predominantly located in proximity to the JmJC domain (Fig. 7A, Top); however, the molecular consequence of these mutations is unknown.

Demethylation activity can be assayed by transiently transfecting HEK293T cells to overexpress WT Flag-UTX and dramatically reduce H3K27me3 levels by immunofluorescence relative to untransfected cells (17, 19, 47). By introducing Kabuki causative point mutations into Flag-UTX, we assayed the effect that these mutations have on demethylation activity (Fig. 7B). We scored cells expressing moderate to high levels of Flag-UTX protein for any observable reduction in H3K27me3 immunofluorescence over untransfected cells in the same field. These assays were performed with the UTX C terminus (C-ter), encoding the JmJC and surrounding domains identified as essential for proper structure and function (48). Most point mutations or small deletions (D980V, S1025G, ΔL1119, R1255W, W1239*, and R1351*) eliminated UTX demethylation activity as no cells experienced H3K27me3 reduction (Fig. 7B and C). Only two Kabuki causative point mutations, K188R (assayed in full-length UTX) and N910S, maintained enzymatic activity (Fig. 7B and C) with 100% of medium-high cells exhibiting loss of

**Full Dosage of Demethylase-Dead UTX Can Support Normal Craniofacial Development.** Genetic analysis of NC UTX versus UTY function has revealed that UTY can partially compensate for loss of UTX. Therefore, UTY can partially compensate for loss of UTX.
H3K27me3. Most of these constructs exhibited overall expression levels similar to WT (Fig. S4 A and B).

To quantify demethylation activity, these transfected cells were subjected to flow cytometry. Cells were gated based on untransfected (Flag negative) or transfected (Flag positive) signal (Fig. 7D). Cells expressing WT full-length UTX and K188R mutation exhibited a significant, mild reduction in H3K27me3 levels compared with untransfected cells (Fig. 7 E and F). Expression of the WT C-ter UTX construct was much more efficient in demethylating H3K27me3 (Fig. 7 G–I). The demethylation-inactivating S1025G mutation exhibited H3K27me3 levels similar and even higher than untransfected cells (Fig. 7 H and I). Both WT C-ter UTX and N910S mutation dramatically reduced the profile of H3K27me3 (Fig. 7H) resulting in highly significant demethylation (Fig. 7J). We conclude that K188R and N910S mutations have demethylation activities similar to WT and disrupt alternative UTX functions in Kabuki patients.

As the K188R mutation resides in the tetratricopeptide (TPR) protein association domain, we reasoned that K188R might disrupt UTX protein association with the KMT2D H3K4 methylation complex. The TPR domain is required for this association, as its removal in Flag-UTX (ATPR) reduces immunoprecipitation of RBBP5, a KMT2D complex protein (Fig. S4C). The TPR domain alone is sufficient for RBBP5 association; however, K188R does not appreciably affect KMT2D complex interaction.

To test the function of demethylase-independent Kabuki causative point mutations, we attempted to rescue NC cellular deficits caused by UTX knockout (Fig. 3). CRISPR lentiviral UTX knockout of the O9-1 NC cell line was performed in combination with additional exogenous UTX lentivirus carrying a synonymous substitution in the guide targeting sequence. Expression of WT UTX from this exogenous lentivirus partially restored knocked out protein levels (Fig. 7J) and deficits in cellular numbers (Fig. 7K). Expression of exogenous UTX K188R failed to rescue UTX knockout cellular phenotypes; however, N910S and S1025G appeared similar to WT. We also examined regulated expression of one UTX target, Notch1. A Notch1-Luciferase reporter construct was utilized containing a 6-kb promoter region upstream of the Notch1 TSS. Upon transfection into HEK293 cells, this reporter was activated by cotransfection with WT UTX (Fig. 7L). Only S1025G mutation reduced Notch1 promoter activation relative to WT UTX (Fig. 7L). Notch1 activation was also examined through O9-1 osteoprogenitor differentiation following UTX knockout/lentiviral rescue (Fig. 7M). UTX N910S and S1025G mutation failed to restore Notch1 activation to WT levels (Fig. 7M). Overall, elimination of UTX demethylation activity is a common feature of Kabuki syndrome, but the disorder can manifest through disruption of alternative UTX functions, which have varying effects on NC cellular culture properties.

Discussion

Chromatin-modifying enzymes are increasingly prevalent in biological research pertaining to neural crest cellular function. These enzymes that catalyze histone posttranslational modifications or nucleosomal remodeling have been mutated in several distinct craniofacial human syndromes with overlapping clinical features, including histone methylases (KMT2D: Kabuki syndrome), histone demethylases (UTX: Kabuki syndrome), chromatin remodelers (CHD7: CHARGE syndrome and SWI/SNF: Coffin-Siris syndrome), and histone acetylases (CBP: Rubinstein-Taybi syndrome).
syndrome) (12, 13, 40, 49–51). Many of these craniofacial factors form protein associations (17, 52, 53) and may function not only within the confines of given enzymatic activity, but may play enzymatic-independent roles in the recruitment of alternative chromatin machinery within a common neural crest cellular precursor. BRG1, ARID1A, and CHD7 chromatin remodeling enzymes all produce NC cell intrinsic functions in migration and differentiation based on tissue-specific knockout studies (6, 54, 55). In fact BRG1 and CHD7 cooperate in activation of NC enhancer activation. UTX and UTY can also physically associate with KMT2D and CBP, which function in enhancer activation (69). We found UTX at a large percentage of distal elements (such as H1146–E1148) and KI/KI catalytic UTX mutants also misexpress CHD7, which is frequently mutated in CHARGE syndrome and can have overlapping clinical features to Kabuki syndrome (40, 61).

Pathway analysis identified the strongest transcriptional deficiencies in Notch and Wnt signaling pathways. These pathways experience moderate reductions in gene expression of several pathway members. Embryos with complete knockout of NC Wnt signaling fail to form any anterior facial structures; however, genetic modulation by hypomorphic mutations in multiple components (Wnt1 and Wnt3a) can produce similar facial hypoplasial phenotypes to UTNC mutation (62, 63). Similar facial characteristics can also be derived from combinatorial hypomorphic mutation of Notch signaling (Notch1/Dlll) (64). The FKO and MKO phenotypes may be due to a combination of genes and pathways identified in our study, and future research will uncover the contribution of various pathways to M KO and FKO pathogenesis.

M KO mice with a single copy of demethylase-dead UT Y or KI/− mice with a single copy of demethylase-dead UTX both develop mild craniofacial phenotypes. However, KI/KI catalytic UTX mutation has normal facial development. Therefore, UTX demethylation events (such as Notch1) can be overcome under normal U TX dosage (K I/K I) by alternative UTX functions. UTX physically associates with KMT2D and CBP, which function in enhancer activation through H3K27 acetylation (53, 65–68). UTX has previously been localized to both promoters and enhancers in mouse mammary tissue (57) and has been correlated with ES cell enhancer activation (69). We found UTX at a large percentage of distal elements (Fig. 5B and Fig. 5E) and UTX may regulate NC enhancer activation through demethylase-independent acetyl transfer recruitment. Future research will be required to determine the extent of U TX and KMT2D overlap at NC enhancers and identify roles in NC enhancer activation. UTX and UT Y can also physically associate with SWI/SNF chromatin remodeling machinery as well as lineage-specific transcription factors (17, 52, 53, 70). Now that we have established a model system capable of assessing NC genomics, we can begin to examine genomic overlap and coregulation potential of alternative demethylase-independent UTX regulatory factors.

Materials and Methods

Mice. All mouse experimental procedures were approved by the University of North Carolina institutional Animal Care and Use Committee. All mice were maintained on outbred genetic backgrounds. The Utxa and UtxG77 alleles are described (17, 28). The Wnt1-Cre transgene, Pax3-Cre knockout, and Tomato reporter were imported from The Jackson Laboratory (20, 21, 24). Utx demethylase-dead knockin mice were developed as described (41, 42).
Constructs, Transfections, Immunofluorescence, Western Blotting, and Antibodies.

Transfection of HEK293T was accomplished with Lipofectamine 3000 as directed (Invitrogen). Fixation, extraction, and immunofluorescence were performed as described (17). Immunofluorescence antibodies include anti-Flag (Sigma, F3165; 1:500) and anti-H3K27me3 (Cell Signaling, 9733S; 1:500). Tunel was performed with the Roche cell death detection kit as directed (11684795910). Cells were imaged with Zeiss axiovision software. Image stacks were deconvolved and Z projected. For Western blotting, nuclear lysates were prepared according to Invitrogen’s nuclear extraction protocol. Immunoprecipitations were carried out with 10 μL antobody and 50 μL protein A/G beads (Santa Cruz, sc-2003) in buffer A as described, using 500 μg of lysate (15). Immunoprecipitation reactions were boiled off beads and run with 10% input.

Fig. 7. Demethylase-independent Kabuki causative UTX mutations. (A) Schematic of UTX protein with Kabuki causative mutations. Pink, female mutations; blue, male; black, undescribed sex; and green, familial male and female. Point mutations are listed Above the protein schematic and nonsense mutations are listed Below. Underlining means tested for demethylation. (B) Demethylation assay whereby HEK293 cells overexpressing Flag-UTX K188R lose H3K27me3 relative to untransfected cells. Flag-UTX S1025G fails to demethylate H3K27me3. (C) Summary of demethylation assay as percentage of scored cells with observable H3K27me3 demethylation (n = 50). (D) Full-length UTX-Flag transfected cells were subject to flow cytometry for Flag and H3K27me3. Cells were gated as transfected (Flag-Pos, positive) or untransfected (Flag-Neg, negative). (E) Histogram of H3K27me3 levels for untransfected or Flag-Pos WT UTX or K188R. (F) Full-length WT UTX and K188R transfected cells had significant reduction in H3K27me3 (Un, untransfected; t test *P < 0.05, 50,000 cells scored, n = 2). (G) C-terminal WT UTX-Flag (amino acids 880–1,401) transfected cells were subject to flow cytometry for Flag and H3K27me3. (H) Histogram of H3K27me3 levels for C-terminal UTX transfections. (I) C-terminal WT UTX and N910S transfected cells had significant reduction in H3K27me3 (*t test *P < 0.02, 50,000 cells scored, n = 2). (J) O9-1 CRISPR UTX knockout (FKO) rescued with exogenous lentivirus for WT UTX or indicated point mutations. Nln, nucleolin control. (K) CellTiter-Glo numbers for rescue treatments (J), plotted as fold increase across D2–D5 of selection. (t test green *P < 0.03 for WT over FKO, t test red *P < 0.01 for K188R relative to WT, n = 3). (L) Notch1–Luciferase was transfected alone into HEK293 (Un) or cotransfected with indicated full-length UTX constructs (t test green *P < 0.02 for WT over Un, t test red *P < 0.01 for S1025G vs. WT, n = 3). (M) O9-1 NC were undifferentiated (D0) or differentiated to osteoprogenitors for 2 d with UTX FKO CRISPR or rescued with indicated WT or UTX mutant lentivirus (t test *P < 0.05 relative to WT, n = 3).
on a 6% SDS/PAGE gel. Western blotting was performed as described (17) with anti-UTX (Cell Signaling, 33510S; 1:4,000), anti-RBBP5 (Bethyl Labs, A300-109A; 1:5,000), and anti-nucleolin (Bethyl Labs, A300-711A; 1:5,000).

Cell Culture and Constructs. HEK293T were maintained in DMEM supplemented with glutamine, penicillin-streptomycin, and 10% FBS. Flag-human UTX was described (17). The Flag-human UTX C-terminal construct containing residues 880–1,401 (GenBank: NP_066963.2) was described (17). Site-directed mutagenesis was performed via QuikChange Lightning (Agilent) as directed to produce point mutations or small deletions. All mutations were introduced into the UTX C-terminal construct for demethylase assay except for UTX K188R, which was mutated in the full-length construct. To assay demethylase activity, constructs were transfected into HEK293T and processed for immunofluorescence or flow cytometry 2 d later. The O9-1 cranial NC cell line was generated, cultured, and differentiated as described (27). Luciferase assays were performed following transfection of a 6-kb Notch1 promoter-Luciferase construct (71) as described (17).

Histology. Embryonic fixation and sectioning was performed as described (17). Alizarin red and alcin blue staining was performed as described (72).

Primary NC Isolation. Anterior facial regions from E12.5 WT (male), MKO, or FKO embryos carrying the Tomato reporter were isolated by forceps dissection of facial regions rostral from the maxilla and anterior to the otic vesicle. This tissue was dissociated in HBSS containing 0.25% trypsin and 0.7 mg/mL DNase I for 8 min at 37 °C followed by pipetting and neutralization with 12% FBS as described (73, 74). Cells were washed on ice and resuspended in RPMI with 10% FBS, 2% BSA, and 100 ng/mL DAPI. Cells were filtered through a 70-μm cell strainer and sorted on a Beckman Coulter MoFlo XDP (University of North Carolina [UNC] Flow Cytometry Core) based on Tomato fluorescence and DAPI exclusion into either TRizol-LS (Thermo Fisher) for RNA preparation or RPMI 10% FBS for ChIP. Cells for ChIP were washed in PBS and the cell pellet was frozen. Cellular staining and flow cytometry were performed as described (56) with anti-UTX (Cell Signaling, 33510S; 1:300), anti-Ki67 (BioLegend, 350507; 1:30), and annexin V (BioLegend, 640919).

RNA-seq and ChiP-seq Library Preparation. RNA was isolated with TRizol-LS as directed (Thermo Fisher) from two biological replicates of 2 × 10^6 WT, MKO, or FKO E12.5 sorted cranial NC cells and mitochondrial DNA was isolated for cDNA synthesis, tranglutimation of UTX, and KDM6A mutations. UTX 1:5,000), and anti-nucleolin (Bethyl Labs, A300-711A; 1:5,000). Western blotting was performed as described (17) with anti-UTX (Cell Signaling, 33510S; 1:300), anti-Ki67 (BioLegend, 350507; 1:30), and annexin V (BioLegend, 640919).

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In Situ Hybridization. A Notch1 in situ hybridization probe was amplified, corresponding to a previously utilized region of Notch1 (88). Whole mount in situ hybridization was performed as described (6, 89).

Primers. All genotyping, mutagenesis, and qPCR primers are available on request.

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