Delineation of a Human Mendelian Disorder of the DNA Demethylation Machinery: *TET3* Deficiency

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Germline pathogenic variants in chromatin-modifying enzymes are a common cause of pediatric developmental disorders. These enzymes catalyze reactions that regulate epigenetic inheritance via histone post-translational modifications and DNA methylation. Cytosine methylation (5-methylcytosine [5mC]) of DNA is the quintessential epigenetic mark, yet no human Mendelian disorder of DNA demethylation has yet been delineated. Here, we describe in detail a Mendelian disorder caused by the disruption of DNA demethylation. TET3 is a methylcytosine dioxygenase that initiates DNA demethylation during early zygote formation, embryogenesis, and neuronal differentiation and is intolerant to haploinsufficiency in mice and humans. We identify and characterize 11 cases of human *TET3* deficiency in eight families with the common phenotypic features of intellectual disability and/or global developmental delay; hypotonia; autistic traits; movement disorders; growth abnormalities; and facial dysmorphism. Mono-allelic frameshift and nonsense variants in *TET3* occur throughout the coding region. Mono-allelic and bi-allelic missense variants localize to conserved residues; all but one such variant occur within the catalytic domain, and most display hypomorphic function in an assay of catalytic activity. *TET3* deficiency and other Mendelian disorders of the epigenetic machinery show substantial phenotypic overlap, including features of intellectual disability and abnormal growth, underscoring shared disease mechanisms.

Post-translational modifications of histone tails and DNA methylation play essential roles in development by regulating chromatin structure and gene expression. Inherited conditions that disrupt these processes—chromatin-modifying disorders or Mendelian disorders of the epigenetic machinery—account for a substantial percentage of neurodevelopmental and growth abnormalities in children.[1,2] Most known disorders in this class are caused by pathogenic variants in either histone-modifying enzymes or chromatin remodelers. Far fewer have been linked to deficiencies in the DNA methylation machinery.[3–5] The latter include disorders caused by defects in DNA methylation "writers," or DNA methyltransferases (DNMTs). For example, immunodeficiency-centromeric instability-facial anomalies syndrome 1 (ICF syndrome) results from bi-allelic variants in *DNMT3B* (MIM: 242860). Tatton-Brown-Rahman syndrome results from mono-allelic variants in *DNMT3A (*MIM: 615879). Defects in "reader" proteins that bind to DNA methylation lead to disorders including Rett syndrome, which is caused by variants in *MECP2* (MIM: 312750).[3–5] No Mendelian disorder has been consistently linked to the multi-step and tightly regulated process that removes DNA methylation.

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The roles of DNMTs and proteins such as MECP2 in "writing" and "reading" methyl marks on DNA have been known for decades, whereas the existence of enzymes that can actively reverse or "erase" DNA methylation was discovered more recently.[6,7] The ten-eleven translocase (TET) family of enzymes consists of three methylcytosine dioxygenases (TET1, TET2, and TET3) that initiate DNA demethylation through a series of sequential oxidation reactions that convert 5-methyl cytosine (5mC) first to 5-hydroxymethylcytosine (5hmC), which is then converted to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are removed either passively by replication-dependent dilution or actively by thymidine DNA glycosylase.[8] The process ultimately results in the loss of the methylated base and its replacement with an unmethylated cytosine,[6,7,9,10] effectively leading to DNA demethylation.

In addition to being an intermediate in the active removal of 5mC, 5hmC is suggested to have an independent role in gene regulation, although the exact nature of this role remains unclear. Notably, the amounts of 5hmC differ globally based on cell lineage and are particularly enriched in mammalian brains.[8,11] 5fC and 5caC are less well understood and might have unique functions as well.[8] *Tet3* is highly expressed in oocytes, zygotes, and neurons, and ablation of *Tet3* in mice leads to embryonic lethality.[8] TET3 plays an important role in rapidly demethylating the paternal genome after fertilization, producing genome-wide increases in the oxidized 5mC intermediates 5hmC, 5fC, and 5caC.[12–16] Importantly, *Tet3* haploinsufficiency causes neonatal sublethality or sub-Mendelian ratios in mice.[17–19] Furthermore, inhibition or depletion of *Tet3* in mouse differentiated neurons can impact synaptic function.[20–22] In humans, *TET3* is highly intolerant to loss-of-function alleles in control databases,[23] and homozygous missense variants in *TET3* were recently implicated as a possible cause for autosomal-recessive intellectual disability in a single consanguineous family (further described here as family 3).[24] Together, these findings illustrate the important role of TET3 in early embryonic development and neuronal function.

Here, we provide a detailed description of a cohort of individuals with a Mendelian disorder that is due to disruption of the DNA demethylation machinery, namely the TET3 enzyme. Although inheritance patterns vary and include both autosomal-dominant and autosomal-recessive forms, all affected individuals have in common a deficiency in TET3 function. This is either due to one or more missense variants within the highly conserved catalytic domain, most of which have been functionally validated to result in decreased TET3 activity, or due to a single frameshift or nonsense variant. The phenotype is remarkably similar among affected individuals and is consistent with the broader group of Mendelian disorders of the epigenetic machinery; such disorders often show global developmental delay and/or intellectual disability and other neurological manifestations, growth abnormalities, and characteristic craniofacial features.[1,25,26]

Individual 1-II-1 presented with developmental delay, generalized overgrowth including macrocephaly, and some facial features reminiscent of Sotos syndrome (Table 1; Figure 1A). Targeted testing for Sotos syndrome (MIM: 117550; *NSD1*) and the related Malan syndrome (MIM: 614753; *NFIX*), as well as methylation testing for Beckwith-Wiedemann syndrome (MIM: 130650) and array comparative genomic hybridization (CGH), failed to detect a pathogenic alteration. We performed research-based trio exome sequencing and identified bi-allelic rare variants in *TET3* (GenBank: NM_001287491.1; c.2254C>T [p.Arg752Cys] and c.3265G>A [p.Val1089Met]). Through collaborations with other institutions and Genematcher,[27] we subsequently identified an additional ten affected individuals in seven unrelated families with overlapping phenotypes and rare *TET3* variants predicted to negatively impact catalytic function (Table 1; Figure 1B).

To delineate the phenotypic spectrum associated with variants in *TET3*, we collected detailed clinical information on all affected individuals, who ranged in age from 11 months to 56 years at the time of assessment (Table 1). We observed striking phenotypic overlap among affected individuals (Table 1). All had global developmental delay and/or intellectual disability, and the vast majority (9/11) had hypotonia and/or joint hypermobility. Other commonly observed findings were autistic features including difficulty with social interactions (6/11), growth abnormalities (8/11), movement disorders (5/11), and overlapping common facial characteristics (Figure 1A). The developmental delay/intellectual disability ranged from mild to severe and included gross motor delay with or without speech delay in almost all cases (Table 1). Seizures and/or electroencephalogram (EEG) abnormalities occurred in 4/11 individuals; other movement disorders were also noted and included tics, dystonia, extensor posturing, and myoclonic jerks (Table 1). Brain magnetic resonance imaging (MRI) demonstrated non-specific abnormalities, such as periventricular white-matter changes and increased extra-axial spaces, including ventriculomegaly, in two individuals (Table 1). Postnatal growth abnormalities, most often involving head size, were identified in 7/11 affected individuals, three of whom (1-II-1, 4-II-1, and 5-II-1) had true macrocephaly (OFC $\geq$ 2 SD above the mean), one of whom (6-II-1) had borderline and relative macrocephaly, and two of whom (3-II-1 and 3-II-3) were siblings from a consanguineous family and had microcephaly (Table 1). In individual 1-II-1, macrocephaly was accompanied by tall stature (height $\geq$ 2 SD above the mean), and in individual 3-II-1, microcephaly was accompanied by short stature (height $\leq$ 2 SD below the mean; Table 1). Three individuals (3-II-1, 3-II-3, and 6-II-1) were born small for their respective gestational ages, suggesting a potential effect on prenatal growth; however, two of these were siblings from the same consanguineous family, and we cannot rule out other genetic causes, maternal factors, exposures, or poor prenatal care without additional information. The other individual born small for his gestational

Table 1. Clinical Characteristics of Individuals Harboring *TET3* Variants

Individual	1-II-1	2-II-1	3-II-1 [24]	3-II-2 [24]	3-II-3 [24]	4-II-1	5-II-1	6-II-1	7-II-1	7-I-1	8-II-1
Variant Characteristics											
Variant cDNA[a]	c.2254C>T (paternal); c.3265G>A (maternal)	c.3215T>G (paternal); c.3226G>A (maternal)	c.2722G>T (maternal; paternal)	c.2722G>T (maternal; paternal)	c.2722G>T (maternal; paternal)	c.2552C>T	c.5083C>T[d]	c.5030C>T[d]	c.4977_4983del[d]	c.4977_4983del[d]	c.1215delA
Amino acid changes[b]	p.Arg752Cys; p.Val1089Met	p.Phe1072Cys; p.Ala1076Thr	p.Val908Leu	p.Val908Leu	p.Val908Leu	p.Thr851Met	p.Gln1695*[d]	p.Pro1677Leu[d]	p.His1660Profs*52[d]	p.His1660Profs*52[d]	p.Trp406Glyfs*135
Genomic coordinates[c]	chr2: 74275298C>T; chr2: 74320791G>A	chr2: 74320741T>G; chr2: 74320752G>A	chr2: 74314999G>T	chr2: 74314999G>T	chr2: 74314999G>T	chr2: 74300733C>T	chr2: 74328998C>T	chr2: 74328945C>T	chr2: 74328892_ 74328898del	chr2: 74328892_ 74328898del	chr2: 74274259_ 74274259delA
CADD score	23.6; 29.1	28.2; 25.9	27	27	27	26.2	44	25.7	NA	NA	NA
gnomAD alleles	29 (0 homozygotes); 0	0; 0	0	0	0	0	0	0	0	0	0
Inheritance	AR	AR	AR	AR	AR	AD *de novo*	AD *de novo*	AD *de novo*	AD inherited	AD	AD *de novo*
Demographics of Affected Individuals											
Ethnic origin	Caucasian	Caucasian	Asian	Asian	Asian	Caucasian	West Indies (father); Morocco (mother)	Estonian (mother); Finnish (father)	White British	White British	Ashkenazi Jewish
Sex	female	female	male	female	female	male	male	male	male	male	male
Age at evaluation	7 years	3 years 3 months	21 years	24 years	27 years	11 months	7 years 6 months	18 months	11 years	56 years	9 years 11 months
Gestation	Term	41 weeks	Term	Term	Term	40 weeks	Term	35 weeks	Term	NA	40 weeks, 3 days
Growth											
Birth weight in g (SD)	3,370 (−0.07)	3,230 (−0.57)	1,360 (−4.7)	NA	2,270 (−2.44)	3,865 (+0.66)	4,300 (+1.49)	1,475 (−2.37)	3,170 (−0.73)	NA	3,685 (+0.21)
Birth length in cm (SD)	48.3 (−0.92)	53.98 (+1.26)	NA	NA	NA	48.26 (−1.23)	53 (+0.68)	42 (−1.56)	NA	NA	57.2 (+2.38)
Birth OFC in cm (SD)	NA	NA	NA	NA	NA	35.56 (+0.52)	NA	31 (−0.66)	NA	NA	34.9 (+0.04)
Weight at evaluation in kg (SD)	41.6 (+2.61)	13.7 (−0.37)	38 (−5.16)	NA	47 (−1.52)	9.35 (−0.06)	27.7 (+0.79)	8.9 (−1.85; −3.0 Estonian chart)	38.55 (+0.36)	126 (>+2.0)	24.3 (−1.76)
Length/height at evaluation in cm (SD)	135.3 (+2.25)	100.3 (+1.13)	159 (−2.48)	NA	165 (+0.26)	75.5 (+0.41)	120 (−0.88)	82 (−0.10)	134.3 (−1.34)	185 (+1.15)	131 (−1.12)
OFC at evaluation in cm (SD)	57.9 (>+2.00)	47 (−1.04)	53 (−2.00)	NA	51 (<-2.00)	48.3 cm (+2.00)	55 (>+2.00)	50 (+1.98)	52.5 (−0.67)	56.5 (+0.67)	52 (−0.7)

(*Continued on next page*)

Table 1. ***Continued***

Individual	1-II-1	2-II-1	3-II-1 [24]	3-II-2 [24]	3-II-3 [24]	4-II-1	5-II-1	6-II-1	7-II-1	7-I-1	8-II-1
Neurodevelopment											
Intellectual disability (degree)	Y (mild)	NA	Y (moderate)	Y (moderate)	Y (moderate)	NA	NA	NA	Y (moderate)	Y (mild to moderate)	N
Global developmental delay	Y	Y (severe)	Y	Y	Y	Y	Y	Y (severe)	Y	Y	Y
Gross motor delay	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y
Fine motor delay	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	N
Speech delay	Y	Y	Y	Y	Y	Y	NA	Y	Y	NA	Y
Behavior											
Autistic features/ASD	Y (obsessive-compulsive tendencies)	NA	NA	NA	NA	Y	NA	Y (poor eye contact)	Y (routine-oriented, obsessions)	N	Y
Difficult/delayed social interactions	Y	NA	NA	NA	NA	Y	NA	Y	Y	Y	Y
Other behavioral concerns	anxiety, ADHD	high pain tolerance	NA	NA	NA	NA	NA	N	ADHD	anxiety, depression	anxiety, ADHD
Neurological Findings											
Seizures (type)	Y (? absence spells)	Y	N	N	N	N	Y (febrile partial and myoclonic)	Y (infantile spasms)	N	N	N
EEG	abnormal focus on right	abnormal	NA	NA	NA	normal	bioccipital bi-phasic spikes; then centro-temporal spikes with continuous spikes + waves during sleep	epileptic activity	N	N	NA
Other abnormal movements	tic disorder	extensor posturing	N	N	N	myoclonic movements and periodic limb movement in sleep	dysmetria	dystonias	N	N	N
Hypotonia	Y	Y	Y	Y	Y	Y	Y	Y (central)	N	N	N
Hypertonia	N	N	N	N	N	NA	N	Y (peripheral)	N	N	N
Brain MRI	increased extra-axial spaces, mild ventriculomegaly	normal	NA	NA	NA	normal	normal at 2.5 years and 5.5 years	periventricular leukomalacia; increased extra-axial spaces	N	N	NA

(*Continued on next page*)

Table 1. ***Continued***

Individual	1-II-1	2-II-1	3-II-1 [24]	3-II-2 [24]	3-II-3 [24]	4-II-1	5-II-1	6-II-1	7-II-1	7-I-1	8-II-1
Ophthalmological findings	No	nystagmus	N	N	N	NA	N	nystagmus; strabismus; ptosis; lacrimal duct stenosis	N	N	N
Cardiovascular anomalies	cardiomegaly, valve abnormality, abnormal EKG	N	NA	NA	NA	NA	N	VSD, aortic valve insufficiency	N	N	N
Musculoskeletal findings	advanced bone age; pes planus	scoliosis	NA	NA	NA	N	N	hip dysplasia	joint hypermobility	NA	N
GI manifestations	infantile feeding difficulties	feeding difficulties/ G-tube, constipation	NA	NA	NA	feeding difficulties, GER, delayed gastric emptying	NA	feeding difficulties	N	N	N
Craniofacial dysmorphisms											
Brachycephaly	N	Y	NA	NA	NA	Y	N	N	Y	Y	N
Tall or broad forehead	Y	Y	Y	NA	Y	NA	Y	Y	N	N	N
Long face	Y	N	Y	NA	Y	NA	Y	Y	N	N	N
Protruding ears	N	Y	Y	NA	NA	NA	Y	Y	N	N	N
Short nose/long philtrum	Y	Y	N	NA	N	NA	N	Y	Y	N	N
Hypotonic face/open mouth	Y	Y	Y	NA	NA	NA	N	Y	N	N	N
Highly arched palate	Y	N	NA	NA	NA	Y	N	Y	N	N	N

Abbreviations are as follows: CADD, combined annotation dependent depletion; AR, autosomal recessive; AD, autosomal dominant;SD, standard deviation from the mean; OFC, occipitofrontal circumference (head circumference); Y, yes, indicates presence of feature; N, no, indicates absence of feature; NA, not available; ADHD, attention deficit hyperactivity disorder; VSD, ventricular septal defect; GER, gastresophageal reflux; and G-tube, gastrostomy tube.

[a]All cDNA variants based on GenBank: NM_001287491.1.

[b]All amino acid changes based on GenBank: NP_001274420.1.

[c]Genomic coordinates based on GRCh37/hg19.

[d]Denotes variants in last exon. Birth-growth parameters plotted with the growth calculator from Olsen *et al.*, 2010 (see Web Resources); growth parameters for children 0–2 were plotted on WHO 0–2y growth charts; height and weight of older children/adults were plotted on CDC 2–20y growth charts; head circumferences/OFCs of children 2–5 plotted on 2–5 WHO growth charts and children and adults over 5 plotted on Nellhaus chart. If SD is significantly different on local growth chart, both are indicated.

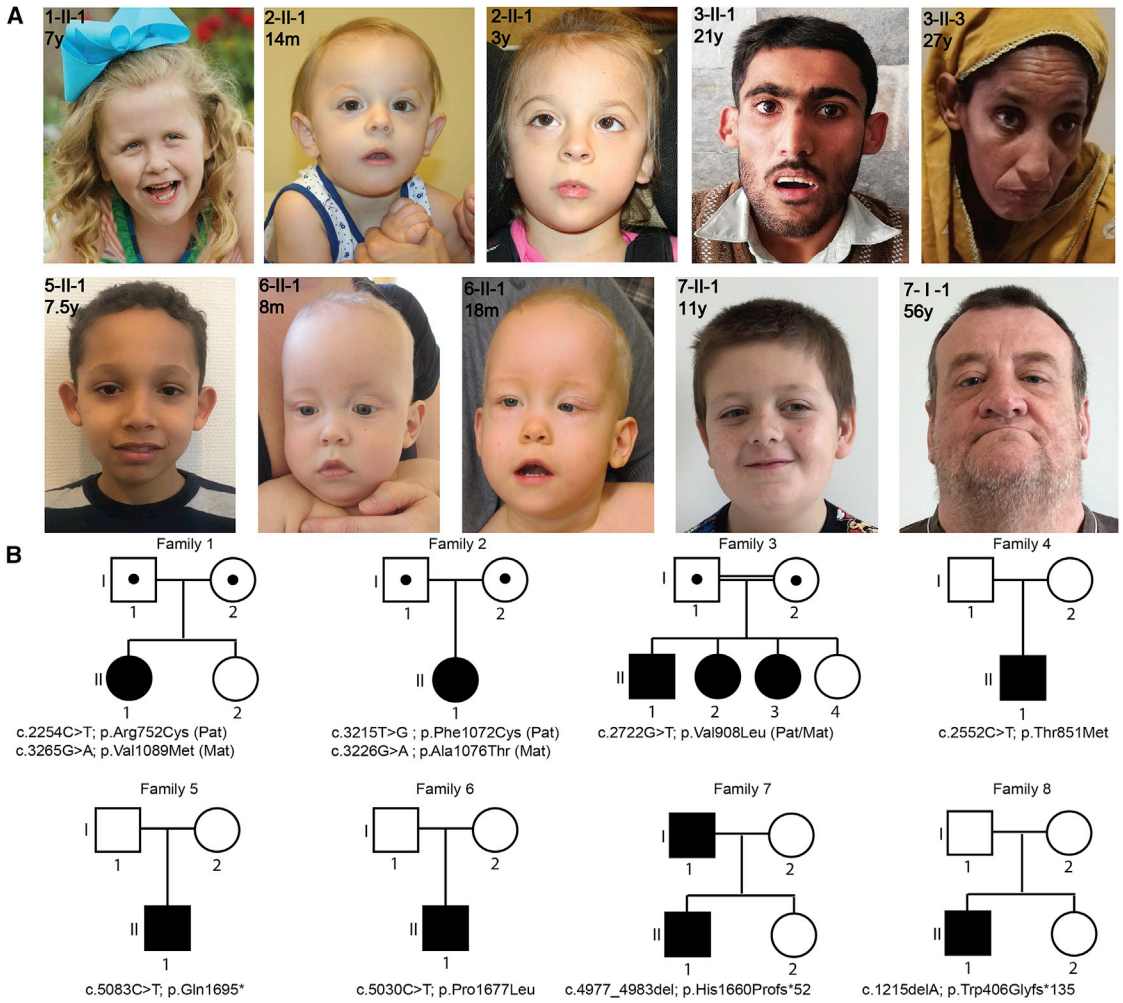
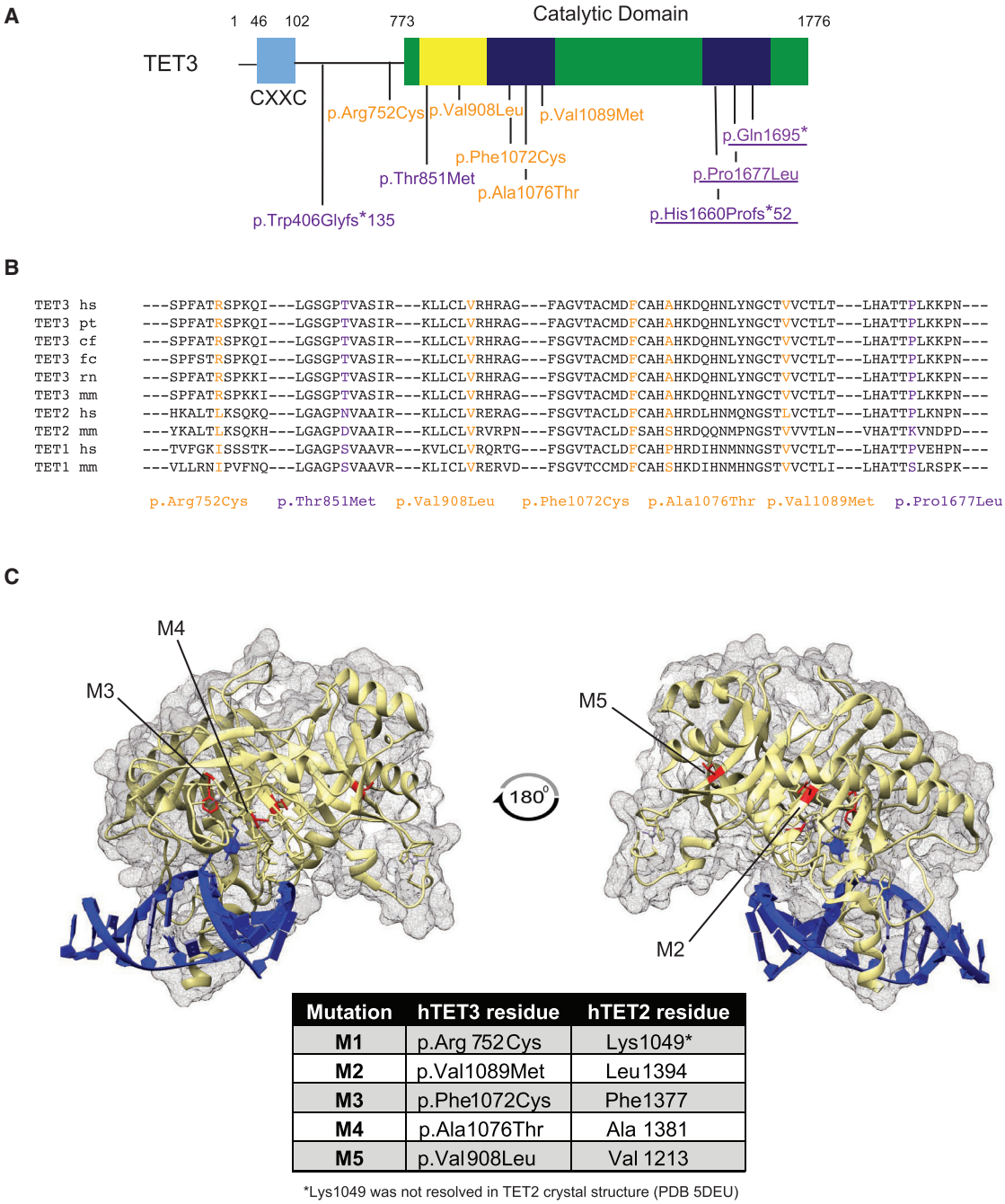


Figure 1. Craniofacial Features and Inheritance in Individuals with *TET3* Deficiency
(A) Images showing craniofacial characteristics of a subset of affected individuals.
(B) Pedigrees illustrating inheritance patterns in each family; specific variants are listed. Abbreviations are as follows: Pat, paternal; Mat, maternal; y, years; and m, months.

age (6-II-1) continued to exhibit poor weight gain but developed borderline macrocephaly by 18 months of age (Table 1). Distinctive craniofacial features common to these affected individuals include a tall and/or broad forehead (6/11) and long face (5/11; Figure 1A). Less commonly noted features were brachycephaly (4/11); a short nose and long philtrum, particularly in younger individuals (4/11); hypotonic facies with open mouth appearance (4/11); protruding ears (4/11); and a highly arched palate (3/11). A few individuals had feeding difficulties (4/11) and eye findings including nystagmus (2/11). Detailed case reports for all subjects are included in the Supplemental Information.

Five affected individuals in three distinct families had bi-allelic variants, consistent with autosomal-recessive inheritance (Table 1; Figure 1B). Two of these individuals were compound heterozygotes, whereas the other three were siblings from a consanguineous family and were homozygous for the same variant (Table 1; Figure 1B).[24] In all three of the families showing an autosomal-recessive inheritance pattern, at least one parent appeared mildly affected. Both parents in family 2 had mild learning difficulties requiring individualized educational plans (IEPs) in school; in addition, the father had attention deficit hyperactivity disorder (ADHD), and the mother had a history of seizures requiring medication in childhood. The mother in family 3—the most severely affected carrier parent—had severe anxiety, problems with short-term memory, and borderline psychosis, whereas the parental phenotypes in family 1 appear milder. In family 1, the father and unaffected sister had specific and similar mild childhood learning disabilities, and the mother had occasional depression, significant anxiety, and possible ADHD, the latter two of which were also confirmed in her affected daughter. All parents were able to live independently and/or hold jobs.

In terms of specific variants, all five of the individuals who were from three distinct lineages and showed autosomal-recessive inheritance (Figure 1B; Table 1) harbored either rare or novel missense changes at conserved residues within or adjacent to the catalytic domain of TET3 (Figures



Mutation	hTET3 residue	hTET2 residue
M1	p.Arg 752 Cys	Lys1049*
M2	p.Val1089Met	Leu1394
M3	p.Phe1072Cys	Phe1377
M4	p.Ala1076Thr	Ala 1381
M5	p.Val 908Leu	Val 1213

*Lys1049 was not resolved in TET2 crystal structure (PDB 5DEU)

Figure 2. ***TET3*** **Variants and Predicted Functional Consequences**
(A) Schematic depiction of TET3 showing domain structure with the catalytic dioxygenase domain in green (aa, amino acids 773–1776) and specific subdomains indicated as follows: the Cys-rich insert in yellow (aa 825–1012) and the double-stranded β helix domain in dark blue (DSBH; aa 1012–1159; aa 1636–1719). The DSBH domain is split in two by a low-complexity insert. The N-terminal CXXC DNA binding domain is shown in light blue (aa 46–102). Specific variants are annotated in orange for recessive alleles and purple for dominant alleles, and underlined variants occur within the last exon.
(B) Alignment of missense variants in *TET3* across multiple species, including hs, *Homo sapiens*; pt, *Pan troglodytes*; *cf.*, *Canis familiaris*; fc, *Felis catus*; rn, *Rattus norvegicus*; and mm, *Mus musculus*, and among TET enzymes.
(C) Crystal structure of TET2 (PDB: 5DEU)[28] bound to DNA; *TET3* mutations M2, M3, M4, and M5 are highlighted.

2A and 2B), which consists of a dioxygenase domain separated by a spacer and a cystine-rich domain (Figure 2A). Specifically, individual 1-II-1 had a paternally inherited c.2254C>T (p.Arg752Cys) variant just upstream of the catalytic domain and a maternally inherited c.3265G>A (p.Val1089Met) variant within the dioxygenase domain; individual 2-II-1 had a paternally inherited c.3215T>G (p.Phe1072Cys) variant and a maternally inherited c.3226G>A (p.Ala1076Thr) variant, both of which were in the dioxygenase domain (Figure 2A). Individuals

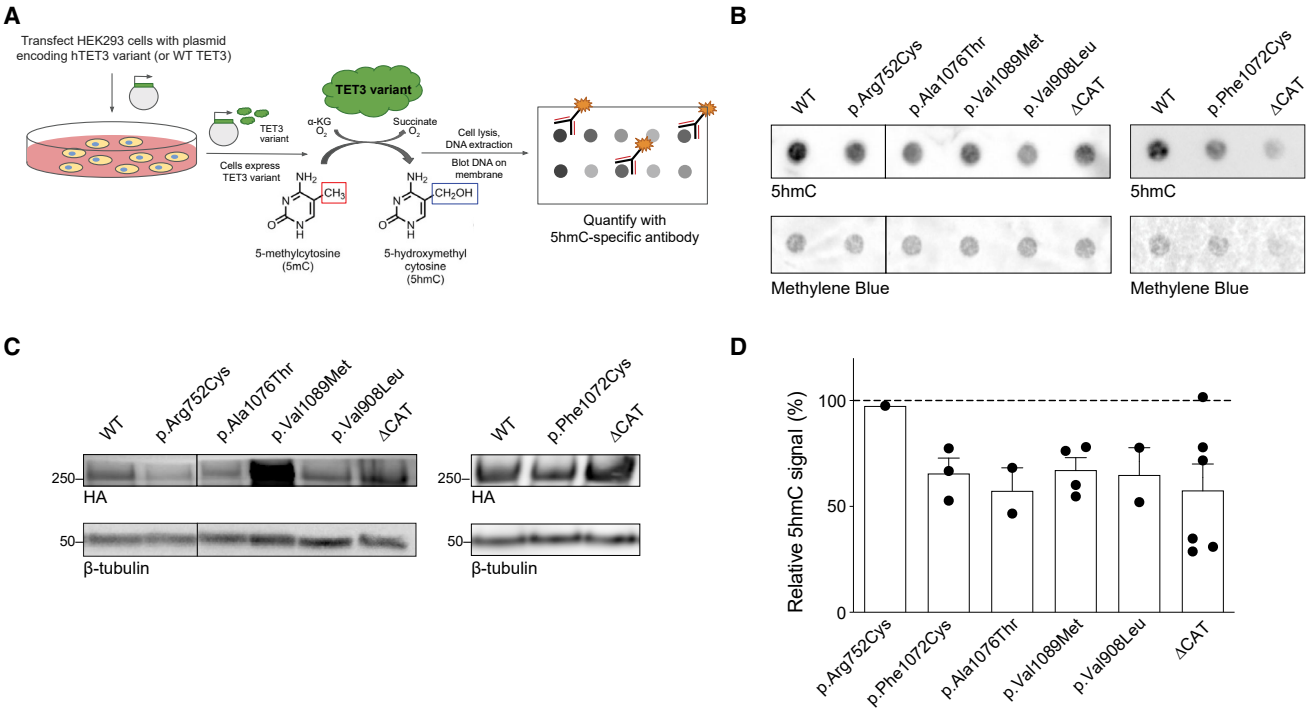


Figure 3. Cells Overexpressing *TET3* Variants Have Decreased Amounts of 5hmC
(A) Schematic depiction outlining the enzymatic activity assay for measuring TET3 catalytic activity.
(B) Representative dot blot showing 5hmC amounts in HEK293 cells overexpressing wild-type or mutant HA-tagged *TET3* constructs.
(C) Representative western blots showing wild-type and mutant TET3 protein expression in HEK293 cells. β-tubulin was used as a loading control.
(D) Quantification of the 5hmC signal relative to TET3 wild-type transfections. The dotted line indicates the wild-type signal. Error bars represent the standard error of the mean. Abbreviations are as follows: WT, wild type; and ΔCAT, catalytically inactive control (p.His1077Tyr/Asp1079Ala, also known as HxD[29]).

3-II-1, 3-II-2, and 3-II-3 were siblings from a consanguineous family and shared the c.2722G>T (p.Val908Leu) homozygous variant[24] within the cystine-rich domain (Figure 2A). Because the crystal structure of TET3's catalytic domain has not been reported, we mapped the autosomal-recessive variants in *TET3* to the published crystal structure of the well-conserved TET2 catalytic domain bound to DNA,[28] and all but one (p.Arg752Cys) could be visualized (Figure 2C). p.Val1089Met and p.Phe1072Cys are in close proximity to TET2's Asn1387 residue, which forms a hydrogen bond with the cytosine base of 5hmC to stabilize binding. Ala1076 is found adjacent to TET2's Thr1393, which participates in hydrogen bonding with the N4 exocyclic amino group of cytosine.[28]

In addition, we identified six individuals in five families with rare mono-allelic *TET3* variants suggestive of autosomal-dominant inheritance (Table 1; Figure 1B). One variant was inherited and the rest occurred *de novo*. In family 7, a similarly affected father and son both harbor the same frameshift variant, c.4977_4983del (p.His1660Profs*52), in the catalytic domain (Figure 2A), consistent with autosomal-dominant inheritance. Individual 5-II-1 had a *de novo* nonsense variant, c.5083C>T (p.Gln1695*), which is also located within the dioxygenase domain, and both this and the inherited variant occur in the last exon (Figure 2A). Individual 8-II-1 had a *de novo* frameshift variant, c.1215delA (p.Trp406Glyfs*135), upstream of the catalytic domain (Figure 2A), and individuals 4-II-1 and 6-II-1 harbored *de novo* missense variants, namely c.2552C>T (p.Thr851Met) and c.5030C>T (p.Pro1677Leu); the former was located in the cystine-rich domain and the latter within the dioxygenase domain (Figure 2A). Strikingly, in both autosomal-recessive and autosomal-dominant cases, all missense variants (except for p.Arg752Cys) were located within the catalytic domain (Figure 2A); moreover, all occurred at residues highly conserved across species, and many occurred at positions also conserved among human and sometimes mouse TET enzymes (Figure 2B).

In the first step of DNA demethylation, 5mC is converted to 5hmC by TET enzymes.[8] To analyze the effect of individual variants on TET3 catalytic activity, we measured 5hmC production by using a cell-culture system whereby recessively inherited TET3 variants (p.Arg752Cys, p.Val1089Met, p.Phe1072Cys, p.Ala1076Thr, and p.Val908Leu) were overexpressed in HEK293 cells and total amounts of 5hmC were measured with a dot blot assay (Figure 3A). We compared the activity of TET3 variants to that of a known catalytically inactive mutant (ΔCAT; p.His1077Tyr/Asp1079Ala, also known as HxD[29]) and to full-length wild-type TET3 (Figures 3B–3D). All but one of the affected individual-derived variants that we tested demonstrated a defect in converting 5mC to 5hmC (Figures 3B and 3D). The exception was

p.Arg752Cys, which is outside the catalytic domain and not conserved among TET enzymes (Figures 2A and 2B). Of note, both variants in family 2 (p.Phe1072Cys and p.Ala1076Thr) were located in close proximity to those disrupted in the well-described ΔCAT/HxD[29] mutant, and so it is not surprising that they lead to reduced catalytic activity (Figures 3B and 3D). The observed defects were consistent across biological replicates, despite fluctuations in the levels of TET3 variant expression (Figure S1). For quantification, we normalized the amounts of 5hmC from cells expressing TET3 mutants to those measured in cells transfected with the wild-type TET3 construct to obtain a relative 5hmC signal (Figure 3D). These results showing decreased cellular amounts of 5hmC suggest that the vast majority of the missense variants identified in affected individuals have hypomorphic function. The observation that affected individuals with nonsense and frameshift variants have phenotypes similar to those with hypomorphic missense variants further supports the hypothesis that decreased TET3 catalytic activity causes disease.

TET3, like most genes encoding components of the epigenetic machinery, is highly dosage sensitive in both model organisms and humans.[1,17,23] *TET3* has a pLI (probability of being intolerant to loss of function) score of 1 (observed/expected = 0.02), suggesting nearly complete intolerance to loss-of-function variation.[23] On the basis of this high pLI score and its high degree of coexpression across diverse tissues, *TET3* was recently predicted bioinformatically to be a candidate epigenetic-machinery gene that would cause disease when mutated.[30] Consistent with dosage sensitivity, most Mendelian disorders of the epigenetic machinery are autosomal dominant and result from haploinsufficiency. In line with these observations, we identified affected individuals with mono-allelic missense or loss-of-function (nonsense and frameshift) variants in *TET3*. However, we also report on individuals who have overlapping phenotypes and carry bi-allelic hypomorphic missense variants, most with mildly decreased catalytic activity according to our overexpression assay. Because of limitations in the ability of our *in vitro* assay to measure small perturbations in TET3 activity in the presence of wild-type alleles, we did not test the mono-allelic variants for comparison. We expect that in both cases—presumed mono-allelic loss of function variants and bi-allelic hypomorphic variants—there will be a similar reduction in total enzymatic activity causing a conserved disease mechanism across inheritance types, though an alternative and more sensitive assay is required to test this hypothesis. Interestingly, nonsense and frameshift variants were only identified in the heterozygous state, suggesting that some residual TET3 activity is required for viability. Conversely, perhaps if TET3 activity falls below a certain threshold, developmental phenotypes result regardless of whether reduced TET3 activity is caused by missense or loss-of-function heterozygous alleles or by bi-allelic hypomorphic alleles.

Within our cohort, we identified affected individuals with autosomal-recessive inheritance and mildly affected carrier parents, which is consistent with a causal relationship between perturbations of TET3 activity and disease manifestations and suggests an inverse correlation between residual TET3 activity and phenotypic severity. Similar examples of complex inheritance involving both mono-allelic and bi-allelic variants in another component of the epigenetic machinery, *KDM5B,* have been reported recently.[31,32] An alternative hypothesis for differential modes of inheritance other than absolute activity levels could be that mono-allelic mutations have activating or dominant-negative effects, whereas bi-allelic mutations lead to loss of function; other examples of this exist in human disease.[33,34] We cannot rule this out here because the nonsense and frameshift variants identified in families 5 and 7, respectively, are located in the last exon and might escape nonsense-mediated mRNA decay, raising the possibility of a dominant-negative mutation mechanism and warranting further mechanistic studies to better correlate genotype and phenotype.

Along these lines, despite the evidence for multiple modes of inheritance, we cannot discount the possibility that individuals with *de novo* variants have additional non-coding sequence variants *in trans,* given that exome sequencing, not genome sequencing, was performed. Similarly, we have not ruled out *in trans* epigenetic alterations, such as DNA methylation. Moreover individuals can have additional variants that contribute to their phenotype, as is the case for individual 8-II-1, with a paternally inherited 16p11.2 duplication, and individual 2-II-1, with a maternally inherited 16q22.1q22.2 duplication. Further studies, including genome sequencing and methylation analysis, could shed light on the molecular mechanisms involved. However, evidence from family 7, with two sequential affected generations, supports autosomal-dominant inheritance, particularly when this evidence is considered along with that from the four *de novo* variants. Together, our observations strongly support two distinct modes of inheritance for *TET3*-deficiency syndrome.

Another explanation for the observed mono-allelic and bi-allelic variants is sex-specific differences. All probands with mono-allelic variants are male, whereas all but one of the individuals with bi-allelic variants are female. Although it remains true that most (if not all) of the carrier mothers appear to have mild phenotypic manifestations, these were not sufficient to bring them to medical attention. It therefore remains possible that males are more susceptible to *TET3* deficiency and only require a single mono-allelic variant to express the full phenotype, whereas females require bi-allelic variants. Certainly, these sex-specific findings might be due to chance given the small total number of individuals; therefore, identification of additional affected individuals and further investigation into the mechanisms associated with specific mutations is required before we can fully delineate the mode of pathogenesis.

TET3-deficient individuals have significant phenotypic overlap with the broader group of Mendelian disorders of

the epigenetic machinery; these disorders are characterized by global developmental delay and/or intellectual disability and other neurobehavioral findings, as well as growth abnormalities, including growth retardation or overgrowth.[1,25,26] Tatton-Brown *et al*. previously showed that variants in epigenetic-machinery genes account for approximately 45% of overgrowth co-occurring with intellectual disability and that many of the same genes are somatically mutated in cancer.[25] More recently, mono-allelic germ-line mutations in *TET2* were suggested to cause a cancer-predisposition syndrome in a single family and mild speech delay in a single individual from a distinct lineage.[35] The most common overgrowth and intellectual-disability disorder identified by Tatton-Brown *et al*. is Sotos syndrome, which results from variants in *NSD1*.[25] Our data suggest *TET3* deficiency might fall into this group of overgrowth and intellectual-disability disorders, given that all individuals with *TET3* deficiency have intellectual disability or global developmental delay, and a subset have overgrowth. Among individuals displaying both phenotypes, most also exhibit facial features reminiscent of Sotos syndrome, including a long face and tall forehead. phenotypic overlap is particularly intriguing in the context of known biochemical interactions between NSD proteins and TET3.[36] Further support comes from observations that TET3 also binds other epigenetic factors encoded by genes responsible for overgrowth and intellectual disability disorders. Such genes include *SETD2*, responsible for Luscan-Lumisch syndrome (MIM: 616831); *HIST1H1E,* responsible for Rahman syndrome (MIM: 617537); and *SUZ12,* which causes a recently described Weaver-like syndrome.[25,36–38] The subset of *TET3*-deficient individuals who exhibited overgrowth (macrocephaly) in addition to intellectual disability is mostly confined to those with mono-allelic variants (affected individuals from families 4, 5, and 6), which is consistent with the established inheritance pattern of overgrowth and intellectual-disability disorders.[25] The exception is individual 1-II-1, who had overgrowth consisting of macrocephaly and tall stature but who inherited bi-allelic variants *in trans* from carrier parents, which we initially thought indicated autosomal recessive inheritance. However, her p.Arg752Cys variant was the only tested variant that did not show decreased TET3 activity in our enzymatic assay, suggesting the possibility that the other p.Val1089Met variant is mostly, if not solely, responsible for the disease phenotype or that a defect in converting 5mC to 5hmC is not required for pathogenicity and that other disease mechanisms exist. In support of the former, her mother, who carried the confirmed p.Val1089Met hypomorphic variant, exhibited potentially relevant features, including significant anxiety and possible ADHD, although she was of average height and her head circumference measurement was unavailable. Further supporting this, the father and unaffected non-carrier sister had specific and similar mild childhood learning disabilities, which are most likely unrelated to the proband's phenotype. It therefore remains possible that the phenotype of individual 1-II-1 more closely resembles that of others with autosomal-dominant inheritance. The pathogenicity of the p.Arg752Cys variant and the inheritance pattern in family 1 are therefore still open questions. Future studies of individual mutation mechanisms will shed light on disease pathogenesis.

In addition, genome-wide DNA methylation profiling might provide insight into disease mechanisms and could help define molecular subgroups. *TET3* deficiency directly impacts the DNA demethylation system, and like many other Mendelian disorders of the epigenetic machinery, this disorder might have a specific genome-wide DNA methylation signature.[39–41] In related disorders, specific signatures have been shown to differentiate affected individuals from unaffected controls,[39–41] as well as affected males with the X-linked disorder Claes-Jensen syndrome (MIM: 300534) from female carriers of the same variants and both of these groups from unaffected non-carriers.[42] Moreover, two distinct and specific DNA methylation signatures, which correlate with gene variant position, have been described in a related chromatin-modifying disorder, Helsmoortel-van der Aa syndrome (MIM: 615873).[43] Studies that attempt to identify a specific DNA methylation signature for *TET3* deficiency are ongoing and might help to resolve some of the outstanding questions.

Together, our clinical observations and biochemical studies define a neurodevelopmental disorder due to reduction in TET3 catalytic activity. TET3 plays a key role in actively reversing DNA methylation during development. Individuals with TET3 deficiency display phenotypic features that overlap with those of other Mendelian disorders of the epigenetic machinery; namely, such features include developmental delay and intellectual disability, other neurobehavioral manifestations, and growth abnormalities. By describing in detail a deficiency in the DNA demethylation pathway, our work defines a distinct biochemical category of epigenetic-machinery disorders and expands our knowledge of this important group of diseases. Given the central role of DNA methylation in epigenetic inheritance, this disorder provides important initial insights into the dynamic regulation of DNA methylation in humans. Further characterization of *TET3*-deficient individuals, their specific variants, and their resulting molecular perturbations will lead to a deeper understanding of the role of DNA methylation and demethylation in human development and disease.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.12.007.

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Declaration of Interests

R.J.L. is a clinical laboratory director in molecular genetics at the Greenwood Genetic Center, and the Greenwood Genetic Center receives fee income from clinical laboratory testing. A.T. and K.M. are employees of GeneDx.

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Web Resources

CADD, https://cadd.gs.washington.edu/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
DDD study, https://www.ddduk.org/
DECIPHER, http://decipher.sanger.ac.uk/
gnomAD Browser, https://gnomad.broadinstitute.org/
Olsen et al. 2010 growth calculator, https://peditools.org/olsen2010/
OMIM, https://www.omim.org/
PyMOL, https://pymol.org/2
RCSB Protein Data Bank, http://www.rcsb.org

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