Homozygous Nonsense Mutations in *TWIST2* Cause Setleis Syndrome

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The focal facial dermal dysplasias (FFDDs) are a group of inherited developmental disorders in which the characteristic diagnostic feature is bitemporal scar-like lesions that resemble forceps marks. To date, the genetic defects underlying these ectodermal dysplasias have not been determined. To identify the gene defect causing autosomal-recessive Setleis syndrome (type III FFDD), homozygosity mapping was performed with genomic DNAs from five affected individuals and 26 members of the consanguineous Puerto Rican (PR) family originally described by Setleis and colleagues. Microsatellites D2S1397 and D2S2968 were homozygous in all affected individuals, mapping the disease locus to 2q37.3. Haplotype analyses of additional markers in the PR family and a consanguineous Arab family further limited the disease locus to ~3 Mb between D2S2949 and D2S2253. Of the 29 candidate genes in this region, the bHLH transcription factor, *TWIST2,* was initially sequenced on the basis of its known involvement in murine facial development. Homozygous *TWIST2* nonsense mutations, c.324C>T and c.486C>T, were identified in the affected members of the Arab and PR families, respectively. Characterization of the expressed mutant proteins, p.Q65X and p.Q119X, by electrophoretic mobility shift assays and immunoblot analyses indicated that they were truncated and unstable. Notably, Setleis syndrome patients and *Twist2* knockout mice have similar facial features, indicating the gene's conserved role in mammalian development. Although human *TWIST2* and *TWIST1* encode highly homologous bHLH transcription factors, the finding that *TWIST2* recessive mutations cause an FFDD and dominant *TWIST1* mutations cause Saethre-Chotzen craniocynostosis suggests that they function independently in skin and bone development.

The focal facial dermal dysplasias (FFDDs) are a group of inherited syndromes characterized by distinctive bitemporal scar-like depressions resembling forceps marks.[1] Three subtypes have been delineated on the basis of their clinical features: type I FFDD, or Brauer syndrome (MIM 136500), is inherited as an autosomal-dominant trait, and most affected patients have only the characteristic bitemporal lesions; type II FFDD (MIM 227260) is the autosomal-recessive form of Brauer[2] syndrome without additional features;[1] and type III FFDD, or Setleis syndrome (MIM 227260), first described in patients from consanguineous Puerto Rican (PR) families,[3] is characterized by bilateral temporal marks and additional facial features, including an aged-leonine appearance, absent eyelashes on both lids or multiple rows on the upper lids, absent Meibomian glands, slanted eyebrows, chin clefting, and other nonfacial manifestations[1,3–6] (Figure 1A). The disease is panethnic, having been described in White, Hispanic, Asian, and American Indian patients from North America, Europe, Japan, the Middle East, and Samoa.[3,7–10] The mode of inheritance of Setleis syndrome has been variably reported as autosomal dominant (e.g., [11,12]), autosomal dominant with variable expressivity and decreased penetrance in families in which a parent had minimal to mild facial dysmorphia or in sporadic cases in which neither parent had manifestations (e.g., [7,13–16]), and autosomal recessive (e.g., [1,3,5,17]). These reports suggest that Setleis syndrome is genetically heterogeneous, perhaps reflecting the interactive nature of the underlying gene defects.

Histologically, the bitemporal lesion is a mesodermal dysplasia with near absence of subcutaneous fat and with skeletal muscle almost contiguous with the epidermis,[6] suggesting insufficient migration of neural crest cells into the frontonasal process and the first branchial arch.[4] To date, the genetic bases of the FFDDs have not been identified. Here, we report that homozygous *TWIST2* (MIM 607556) nonsense mutations, identified by positional cloning, cause Setleis syndrome. TWIST2 is a member of the bHLH transcription factor family first described in mice (*Dermo1*),[18] whose function has been characterized in knockout (KO) mice.[19]

For identification of the Setleis syndrome locus, a genome scan was performed on five affected individuals and 26 family members from the original consanguineous family from the San Sebastian and Lares regions of Puerto Rico described by Setleis et al.[3] (Figure 2). Informed consent was provided by the subjects and/or their parents, and each subject was examined and provided blood samples. Several affected individuals provided skin biopsies and were photographed. Genomic DNAs were isolated

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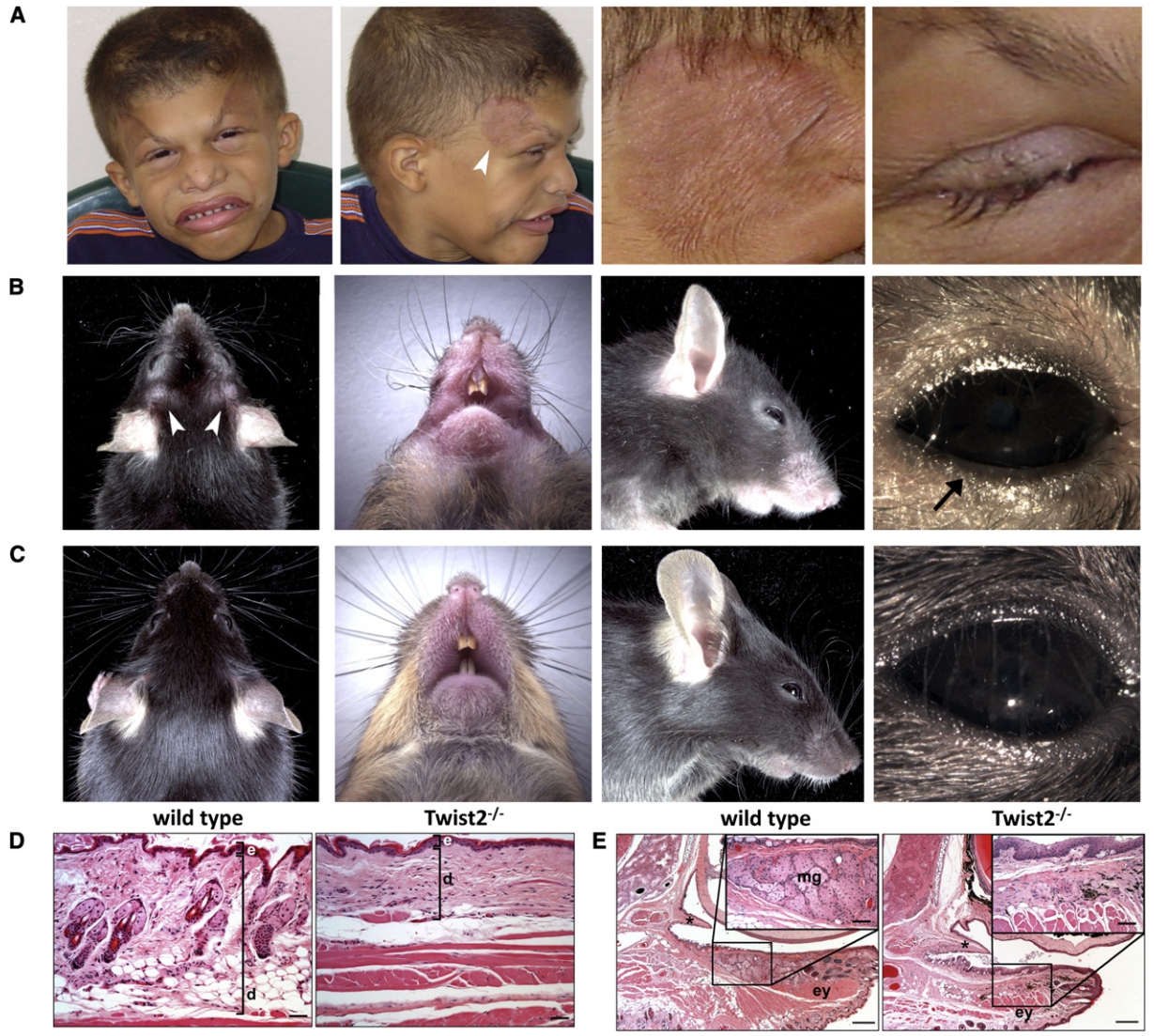


Figure 1. Similarity of Facial Characteristics of Setleis Syndrome Patients and Wild-Type and *Twist2* Knockout Mice
(A) A 5-year-old affected PR male. Note leonine facial appearance, bitemporal lesions, and upper eyelash abnormalities.
(B) *Twist2* knockout (KO) mice. Note similar facial and eye abnormalities, including bitemporal lesions (white arrowheads), narrow snout, pointed chin, and sparse or absent eyelashes (black arrow).
(C) Wild-type 129/C57 mice. All mouse comparisons were performed on littermate controls.
(D and E) Hematoxylin- and eosin-stained skin sections of alopecic areas (D) and palpebral margins (E) at nictitating membrane (*) from *Twist2* KO and wild-type mice. Insets: Note absence of Meibomian glands, and decreased eyelash follicles. e = epidermis; d = dermis; mg = Meibomian gland; ey = eyelid. Scale bars represent 200 μm in (E). Insets represent 40 μm in (D) and (E).

with the Puregene Isolation Kit according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN, USA). Cultured fibroblasts were established from skin biopsies by standard procedures. Peripheral blood and/or cultured fibroblasts also were obtained from patients and members of four unrelated families with FFDD or Setleis syndrome phenotypes. These included an Arab family[8] and three United States families of Hispanic, German Italian, and Cherokee Indian ancestry.

Linkage analysis was performed by genotyping 501 microsatellite markers at a 10 cM density (Genome-Wide Screening Set, version 9, Single Chromosome Set, Invitrogen Life Technologies, Carlsbad, CA, USA) in genomic DNAs from the five affected members of the PR family as previously described.[20] Microsatellite assays were analyzed on a ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with GeneScan Analysis software (version 3.1.2) and Genotyper software (version 2.5) (Perkin-Elmer-Cetus, Norwalk, CT, USA). Homozygous markers in all five affected individuals were then genotyped in all available family members for determination of their segregation and for haplotype analyses. Additional markers (D2S2344, D2S206, D2S1279, D2S2348, D2S2973, D2S1397, D2S2968, D2S2285, D2S2253, D2S125, D2S395, and D2S140) were genotyped, and the critical region was further narrowed by haplotype analyses of other putative FFDD families, including two informative affected first cousins from a consanguineous Arab family

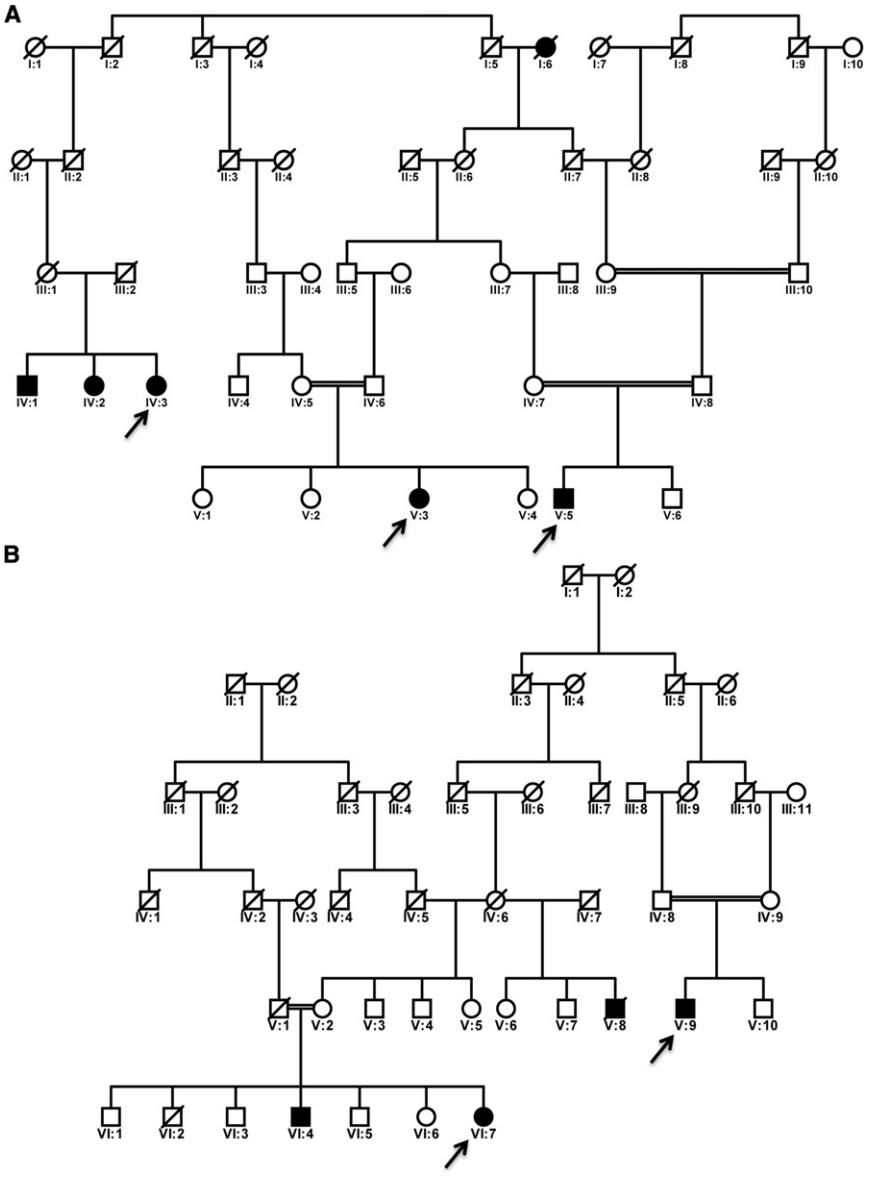


Figure 2. Partial Pedigrees of a Large Setleis Syndrome Family from Puerto Rico
Arrow indicates the five affected individuals who were genotyped.

from the United Arab Emirates.[8] Linkage calculations were made with the SimWalk2 program[21] and were confirmed with the Linkmap program.[22]

Only microsatellites D2S1397 and D2S2968 were homozygous in all affected individuals from the PR family, mapping the disease locus to 2q37.3 (Figure 3A). Saturation of the region with additional markers further defined the locus to 17 cM (~7 MB) between D2S206 and D2S2253 (LOD score Z = 5.22 at D2S1397). These markers were analyzed in three unrelated Setleis syndrome families. Only the consanguineous Arab patients[8] were homozygous for the region, and their haplotypes further narrowed the centromeric boundary, reducing the critical region to 8 cM (~3 MB) between D2S2949 and D2S2253 (Figures 4A–4D), which localized the Setleis syndrome critical region between 238,061,150 and 241,439,288 bp on the chromosome 2 physical map. Inspection of the 29 genes in this region suggested *TWIST2* as an initial candidate for sequencing on the basis of the spatial and temporal dermal facial expression pattern of the murine ortholog during embryogenesis.[18] The promoter, exonic, adjacent intronic, and 3′ untranslated regions of the *TWIST2* gene were amplified via PCR from genomic DNAs of these patients and sequenced on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems).

Sequencing of *TWIST2* revealed homozygous nonsense mutations in the PR (c.486C>T [p.Q119X]) and Arab (c.324C>T [p.Q65X]) patients (Figure 3C), which segregated in respective family members in an autosomal-recessive pattern with full penetrance. Both nonsense mutations predicted early truncation of the TWIST2 transcription factor; the c.486C>T mutation truncated the C-terminal region after the bHLH motif, and the c.324C>T mutation eliminated the bHLH region and the C terminus (Figure 3D). Sequencing of *TWIST2* in five other unrelated familial and/or sporadic Setleis syndrome and FFDD patients did not identify disease-causing mutations, emphasizing the genetic heterogeneity of the bilateral temporal lesions that characterize the Setleis syndrome and FFDD phenotype.

For characterization of the mRNA levels and truncated proteins expressed by these mutations, Myc-tagged constructs of wild-type and mutated (c.324C>T and c.486C>T) *TWIST2* cDNAs were prepared in the pCINEO vector and used for transient transfection of HeLa cells. Quantitative PCR revealed approximately 3-fold to 4-fold decreased amounts of the c.324C>T and c.486C>T fibroblast mRNAs compared to wild-type levels (not shown). The expressed *TWIST2* c.324C>T and c.486C>T mutant proteins in HeLa cells were unstable, consistent with the decreased amounts of the p.Q119X protein and even less of the p.Q65X mutant protein, detected by immunoblot analysis (Figure 3E). The instability of both mutant transcripts and their truncated proteins is consistent with nonsense-mediated decay and might explain why the patients with different mutations had similar phenotypes.

Twist2 KO mice on a 129 genetic background develop cachexia and die within days after birth as a result of

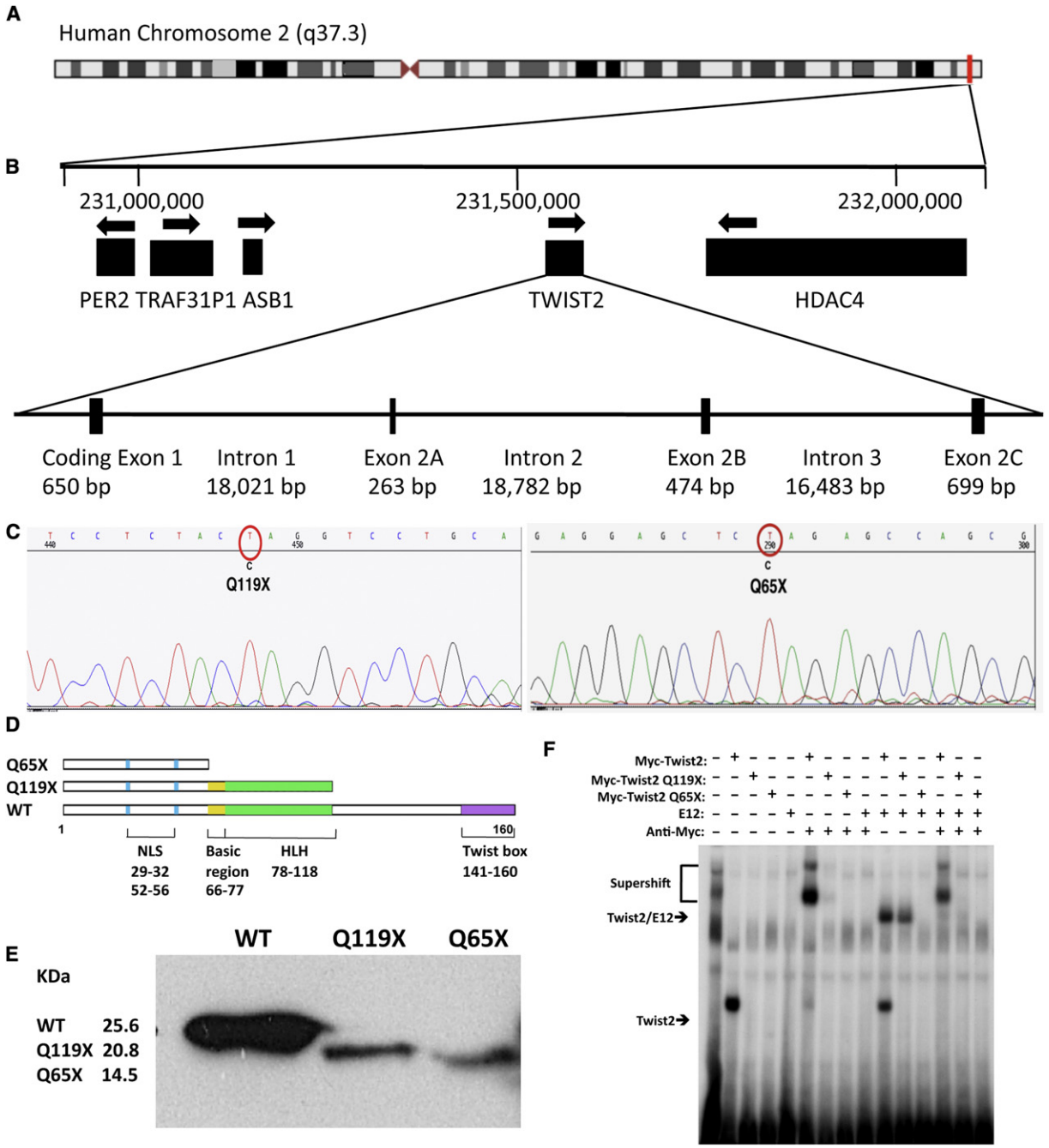


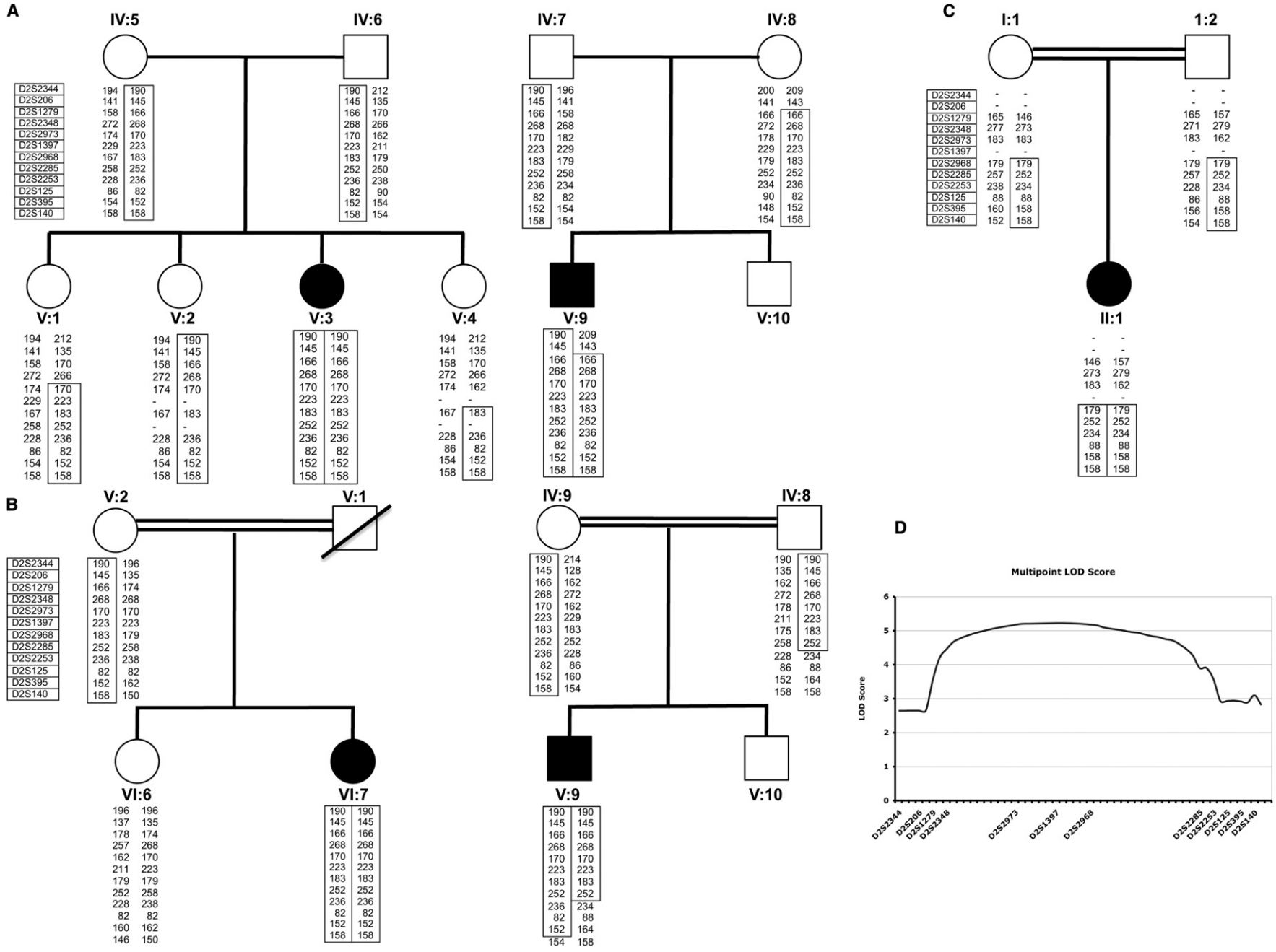
Figure 3. Mapping the Setleis Syndrome Gene

(A–C) Chromosome 2 cytogenetic map (A), critical region genes and *TWIST2* gene structure[29] (B), and electropherograms of c.324C>T and c.486C>T mutations (C). Sequencing was performed on an ABI Prism 3730*xl* Capillary Array Sequencer with the use of ABI Prism BigDye Terminator Ready Reaction Mix (Perkin-Elmer-Cetus). Electropherograms were inspected with ABI Prism Sequencing Analysis software (version 3.4.1) and were aligned to the reference sequence with the BLAST engine.

(D and E) Cartoon depicting 160 amino acid TWIST2 protein domains; NLS, nuclear localization signal (D), and immunoblot analysis of Myc-tagged normal and mutated (p.Q119X and p.Q65X) TWIST2 proteins expressed in HeLa cells (E). HeLa cells were transfected with Lipofectamine 2000 reagent (Invitrogen). Equal amounts of total cell extracts from transfected cells were loaded for immunoblot analysis, and anti-Myc antibody (Sigma-Aldrich) was used to detect the tagged TWIST2 proteins according to manufacturer's instructions.

(F) EMSA of TNF-α promoter E box oligonucleotide and recombinant Myc-tagged TWIST2 proteins. EMSA experiments were done with in vitro translated proteins with the use of the TnT T7 Quick Coupled Transcription/Translation System (Promega) as previously described.[19]

elevated proinflammatory cytokines.[19] However, *Twist2* KO mice on a 129/C57 mixed background have a milder phenotype, some attaining adulthood and living a normal life span.[19] Analysis of the 129/C57 KO mice revealed a facial phenotype similar to that of Setleis syndrome patients, including thin skin and sparse hair with bilateral


A
IV:5
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B
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V:10
C
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D2S2344
D2S206
D2S1279
D2S2348
D2S2973
D2S1397
D2S2968
D2S2285
D2S2253
D2S125
D2S395
D2S140

Table 1. Phenotypic Similarities of Setleis Syndrome Patients and *Twist2* Knockout Mice

Setleis Syndrome Patients	Knockout Mice
Bitemporal lesions resembling forceps marks	Alopecic areas between ears and eyes
Absent lower eyelashes	Absent lower eyelashes
Multiple rows of eyelashes in upper eyelid	Multiple rows of eyelashes in upper eyelid
Sparse hair	Sparse hair
Abnormal pattern of hair growth	Abnormal pattern of hair growth
Bulbous nasal tip	Narrowing of the snout
Absent Meibomian glands	Absent Meibomian glands
Protruding soft tissue in chin, median ridge of the chin	Pointed chin

pronounced alopecic areas between the ears and the eyes resembling the bitemporal forceps marks in humans. They also had absent lower eyelashes, narrow snouts, protruding chins, short anterior-posterior head diameters, and low-set dysmorphic ears (Figures 1B and 1C, Table 1).

Histologically, the bilateral alopecic areas in the KO mice were similar to the defects in Setleis syndrome patients,[4] including the hypoplastic dermis and absence of epidermal appendages and subcutaneous fat (Figure 1D). In mice, *Twist2* is expressed in the dermis, not the epidermis,[18] and the alopecic areas are located in the region with predominantly mesoderm and not neural-crest-derived dermis.[23] The KO mice also have absent/hypoplastic Meibomian glands (Figure 1E), as seen in Setleis syndrome patients.[5] Development of eyelids in mammals is governed by bidirectional signaling between surface epithelium and underlying neural-crest-derived mesenchyme.[24] Mice with *Wnt1*-Cre-mediated deletion of *Twist2* do not show an eyelid phenotype or bilateral head skin lesions (data not shown), indicating that unlike *Twist1*, *Twist2* does not function in the cranial neural crest. We suggest that *Twist2* is involved in cranial dermal mesenchyme development, while indirectly regulating overlying epidermis and induction of its appendages. Furthermore, murine *Twist2* and human *TWIST2* may regulate mesenchymal signal(s) for migration and/or differentiation of neural crest cells involved in Meibomian gland and eyelash development. Thus, *Twist2* KO mice provide an excellent model for the investigation of its role in facial development.

The murine Twist2 and human TWIST2 proteins are 100% identical, whereas their TWIST1 (MIM 601622) proteins have 92% identity. Human TWIST1 and TWIST2 proteins are 98% identical in the ~100 residues in the bHLH region and C terminus (Figure 5). The main difference between these two human proteins lies in the N terminus, where TWIST2 lacks two glycine-rich regions found in TWIST1. Throughout their coding regions, the identity and similarity are 65% and 71%, respectively. In mice, *Twist1* is essential for embryonic viability and cranial neural tube formation.[25] In humans, *TWIST1* haploinsufficiency causes Saethre-Chotzen syndrome (MIM 101400).[26]

The bHLH proteins of the TWIST subfamily form heterodimers with ubiquitous E proteins, such as E12, as well as homodimers. For determining the DNA-binding properties of the TWIST2 mutated proteins, EMSA studies were conducted with the wild-type and mutant proteins p.Q65X and p.Q119X, as previously described.[27] Relative positions of TWIST homodimers, TWIST2/E12 heterodimers, and supershifted complexes are shown in Figure 3F. A ^{32}P-labeled E box containing oligonucleotide derived from the proximal region of the human TNF-α promoter was used as a probe (5′-GGGCCGACTACCGCTTCCTCC AGATGAGCTCATGGGTTT-3′). This probe is part of an evolutionarily conserved DNA region upstream of the TNF-α transcription start site that has been shown previously to bind both mouse Twist1 and Twist2 proteins.[19]

The gel mobility shift assays revealed that the human TWIST2 protein bound to the DNA probes containing a conserved E box, both as homodimers and as heterodimers (Figure 3F). However, neither mutant protein was able to bind a DNA probe as a homodimer. Mutant protein p.Q119X, but not p.Q65X, bound DNA as a heterodimer, albeit at reduced levels in comparison to full-length TWIST2 (Figure 3F). The outcome of this binding is unclear, because the C terminus contains an important inhibitory domain that can repress target gene transcription.[19,27] Therefore, the p.Q119X mutant protein could cause aberrant expression of target genes, given that its *trans*-activating effects presumably differ from those of the wild-type protein. The p.Q65X protein likely represents a true loss-of-function mutant. Notably, the mouse phenotypes of the *Twist1* null mutant and the mutant with C-terminal mutations ("Charlie Chaplin" strain) differ significantly.[28] We speculate that dominant-negative mutations in *TWIST2* or partner proteins could cause autosomal-dominant inheritance of FFDD.

In summary, Setleis syndrome results from *TWIST2* homozygous nonsense mutations that affect TWIST2 protein stability and DNA binding, providing the first genetic basis for an FFDD syndrome. The similar facial dysmorphia in *Twist2* KO mice demonstrates the conservation of TWIST2 function in mammalian facial dermal development. Thus, *Twist2* KO mice represent an appropriate animal model for the study of the molecular mechanism(s) leading to the FFDD syndromes. Our results suggest that TWIST2 binding partners or other dermal-development transcription factors may be defective in other Setleis syndrome patients or FFDD syndromes.

Figure 4. Genetic Analysis of Chromosome 2 for the Puerto Rican and United Arab Emirates Setleis Syndrome Families
(A and B) Chromosome 2 haplotypes of PR family members.
(C) Haplotypes for the family from the United Arab Emirates.
(D) Plot of the LOD scores for chromosome 2q37.3.

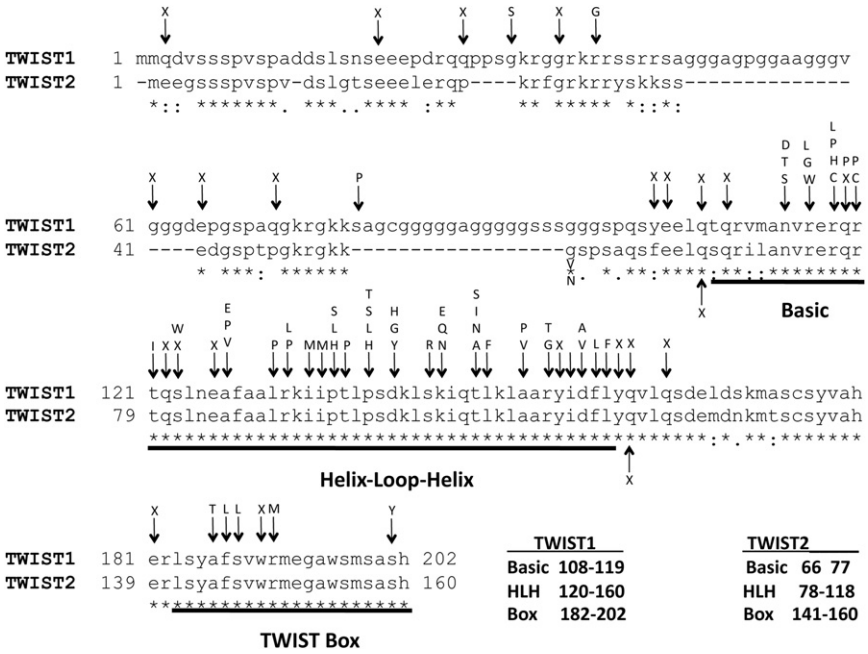

Figure 5. Alignment of the Human TWIST1 and TWIST2 Amino Acid Sequences

The functional domains and residues are indicated where disease-causing *TWIST1 or TWIST2* nonsense (X) or missense (arrows with substituted amino acids) mutations occurred in Saethre-Chotzen syndrome and Setleis syndrome, respectively. Solid bars indicate functional domain regions. Amino acid identity (*), conserved substitutions (:), and semiconserved substitutions (·) are indicated below the TWIST2 residues.

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Web Resources

The URL for data presented herein is as follows:

BLAST, http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html

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