**Supplementary Table S1.** Sequences of Primers used in this study for deletion of conserved regions in the TWIST2 coding sequence, amplification of the human *CHRDL1* gene upstream region and of the *TCF3* gene coding region for production of an expression construct. The primers used to amplify the human *CHRDL1* gene sequences for reporter gene assays contained additional nucleotides to add KpnI and XhoI sites at the ends of the PCR product for directional cloning into the pGL4.14 vector. Sequences of the oligonucleotides used for competition reactions in electrophoretic mobility shift assays are also included, these oligonucleotides were used as double stranded molecules. E-box and SP1 elements (normal or altered) are shown in italics. E-box bases changed are underlined.

Name of Primers/Oligo-Nucleotide	Sequence	Size	Application
Twist2ΔA	FW: 5'-CGATGAGGAGGGCGTGGACAGCCTGG-3' RV: 5'-CCAGGCTGTCCACGCCCTCCTCATCG-3'	26 bases	Deletion Mutagenesis of first sub-motif SSSPVSP
Twist2ΔB	FW: 5'-CAGCCTGGGCACCCTCGAGAGGCAGC-3' RV 5'-GCTGCCTCTCGAGGGTGCCCAGGCTG-3'	26 bases	Deletion Mutagenesis of second sub-motif SEEE
CH3KLuc-FW	5'-AC<u>GGTACC</u>GGAAGGGAAAAGATGGGTGT-3' Includes KpnI site (underlined) for cloning	28 bases	PCR forward primer used for Preparation of *-3KCHRDL1-pGL4 Luc* construct
CH3KLuc-RV	5'-A<u>CTCGAG</u>TGCTCACTAACCTGGGCACT-3' Includes XhoI site (underlined) for cloning	27 bases	PCR reverse primer used for Preparation of *-3KCHRDL1-pGL4 Luc* construct
TCF3FW	5'-ATGAACCAGCCGCAGAGGATGG-3'	22 bases	PCR forward primer used for preparation of TCF3 expression construct in pCRII-TOPO vector
TCF3RV	5'-CGGAGGCATACCTTTCACAT-3'	20 bases	PCR reverse primer used for preparation of TCF3 expression construct in pCRII-TOPO vector
SP1 oligo	5'- ATTCGATCG*GGGCGG*GGCGAGC-3' 3'-TAAGCTAGC*CCCGCC*CCGCTCG-5'	22 base pairs	Non-specific competitor
CH2600TWI	5'-TATATACACAGG*CAAATG*AGTGCATATAAA-3' 3'-ATATATGTGTCC*GTTTAC*TCACGTATATTT-5'	30 base pairs	EMSA probe

CH2700EA	5'-TGGTGGTGGT*CACATG*AATATA*CACATG*CGATAAAATT-3' 3'-ACCACCACCA*GTGTAC*TTATAT*GTGTAC*GCTATTTTAA-5'	38 base pairs	Specific competitor
CH2700EMAW	5'-TGGTGGTGGT*C<u>G</u>CAC<u>G</u>*AATATA*CACATG*CGATAAAATT-3' 3'-ACCACCACCA*G<u>C</u>GTG<u>C</u>*TTATAT*GTGTAC*GCTATTTTAA-5'	38 base pairs	Competitor
CH2700EWAM	5'-TGGTGGTGGT*CACATG*AATATA*C<u>G</u>CAC<u>G</u>*CGATAAAATT-3' 3'-ACCACCACCA*GTGTAC*TTATAT*G<u>C</u>GTG<u>C</u>*CGCTATTTTAA-5'	38 base pairs	Competitor
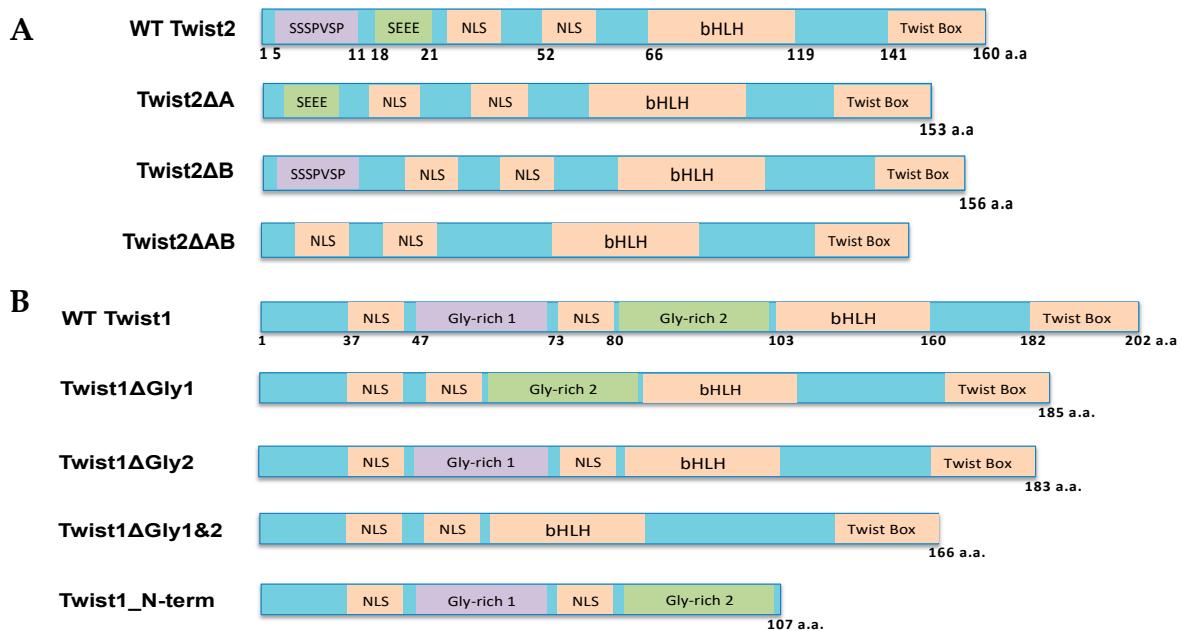
Supplementary Figure S1. Diagram of the TWIST2 and TWIST1 N-terminal mutant proteins produced by deletion mutagenesis. A. We generated mutant TWIST2 expression constructs where we removed two conserved sub-motifs [43], which were predicted to be associated with protein binding, the first sub-motif SSSPVSP (construct TWIST2ΔA), the second sub-motif SEEE (construct TWIST2ΔB), or both sub-motifs (construct TWIST2ΔAB). B. Diagram of TWIST1 glycine-rich deletion mutant proteins and the N-Terminal construct used for EMSAs, which were generated by gene synthesis. NLS = nuclear localization signal; bHLH = basic helix loop helix; TWIST box = protein domain which interacts with the Runx2 DNA binding domain to inhibit its function [9].

A

Macaque
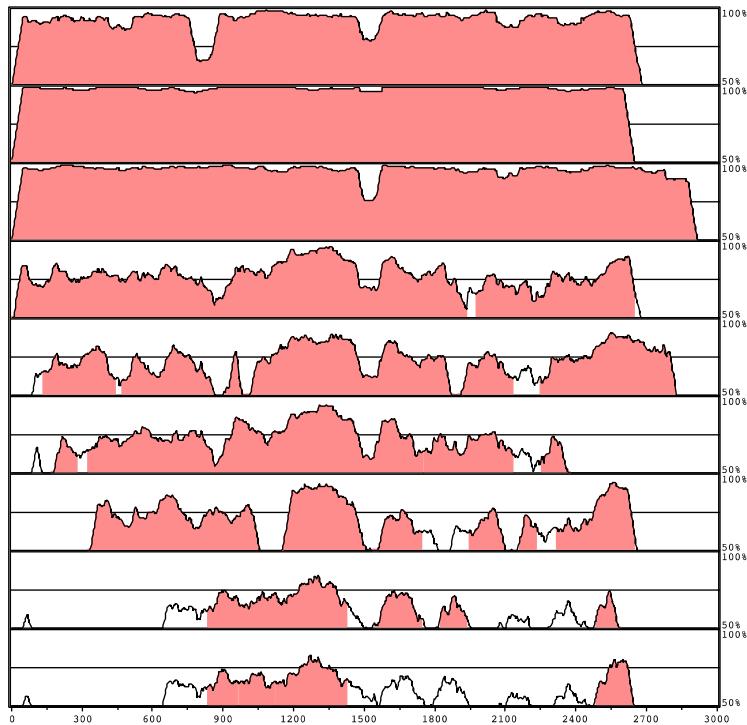
Chimpanzee
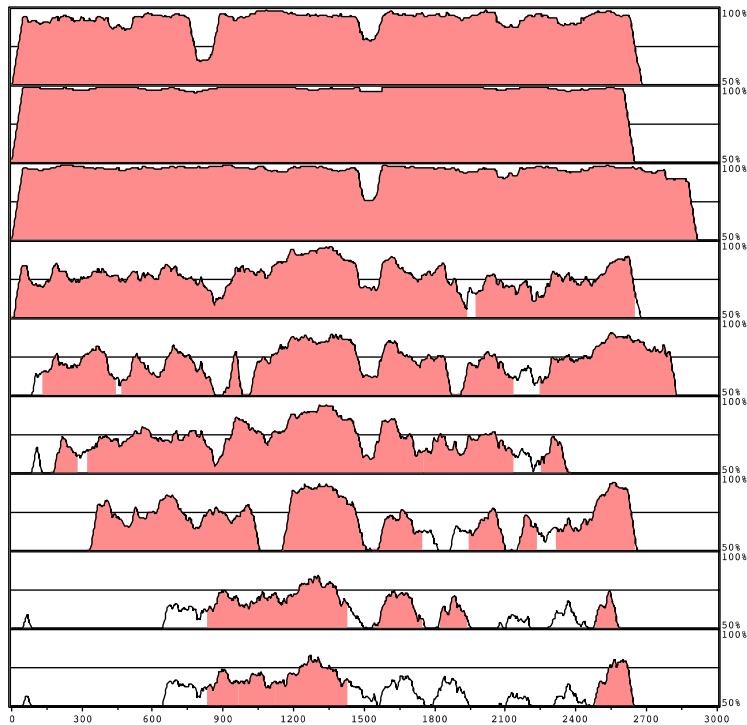
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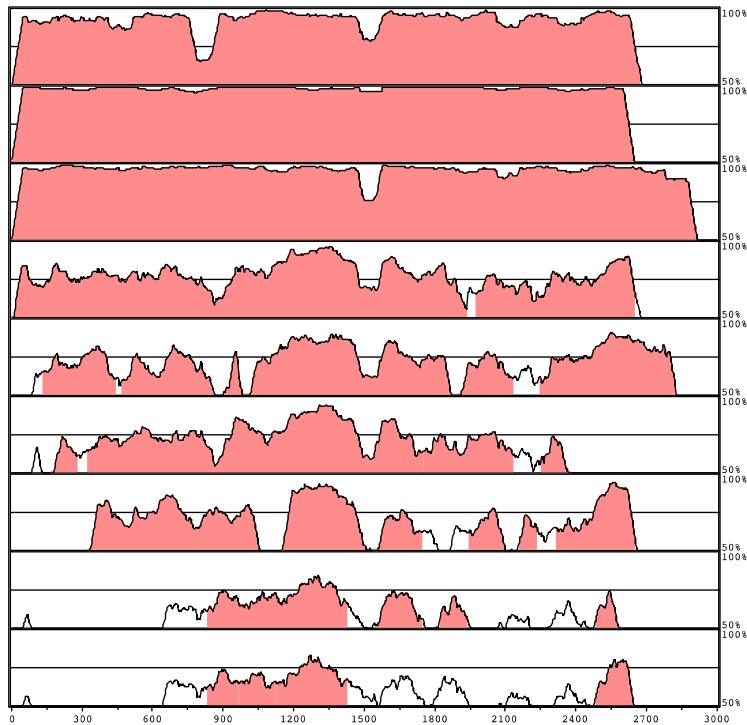
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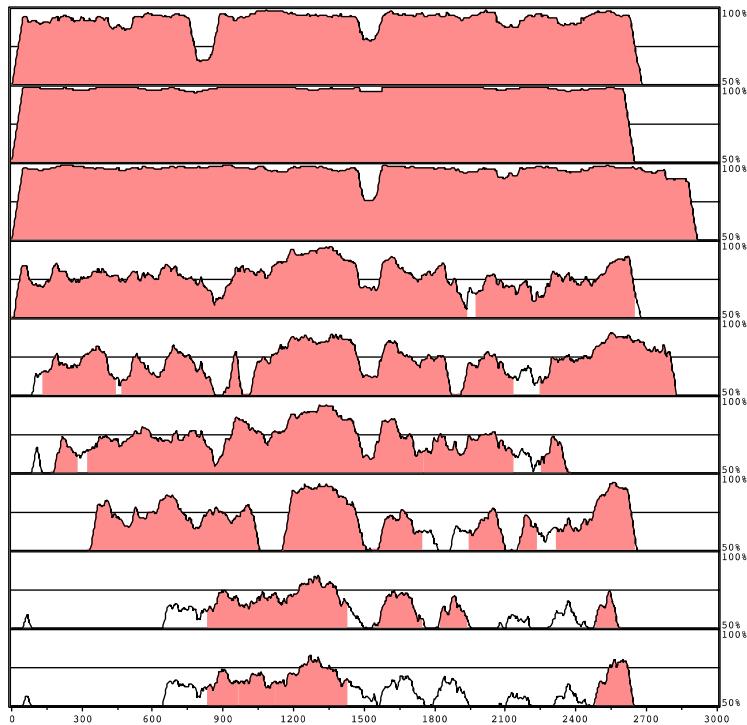
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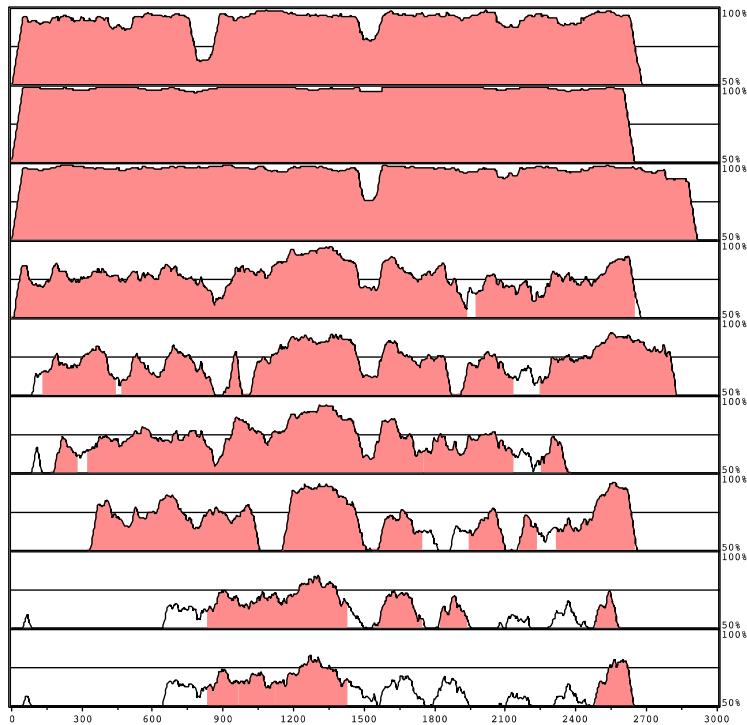
Pig
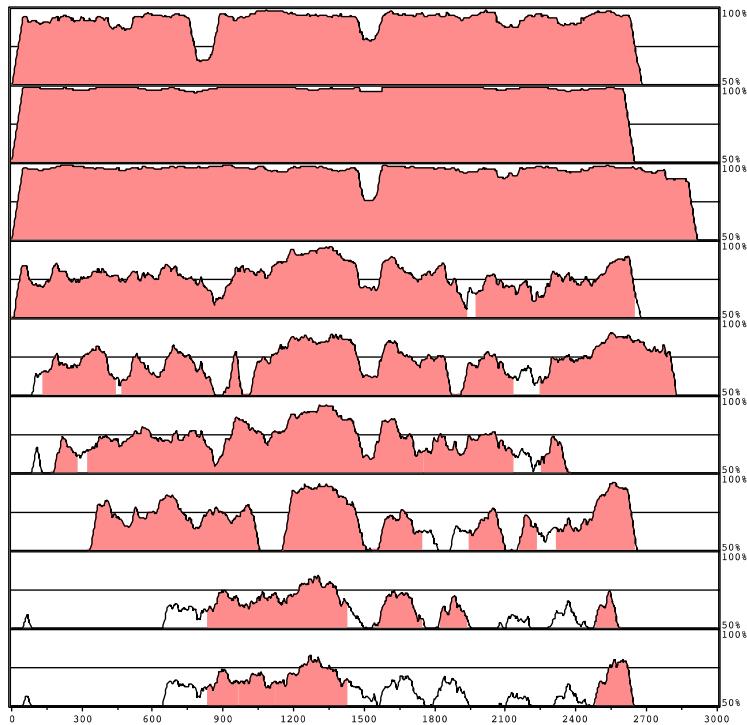
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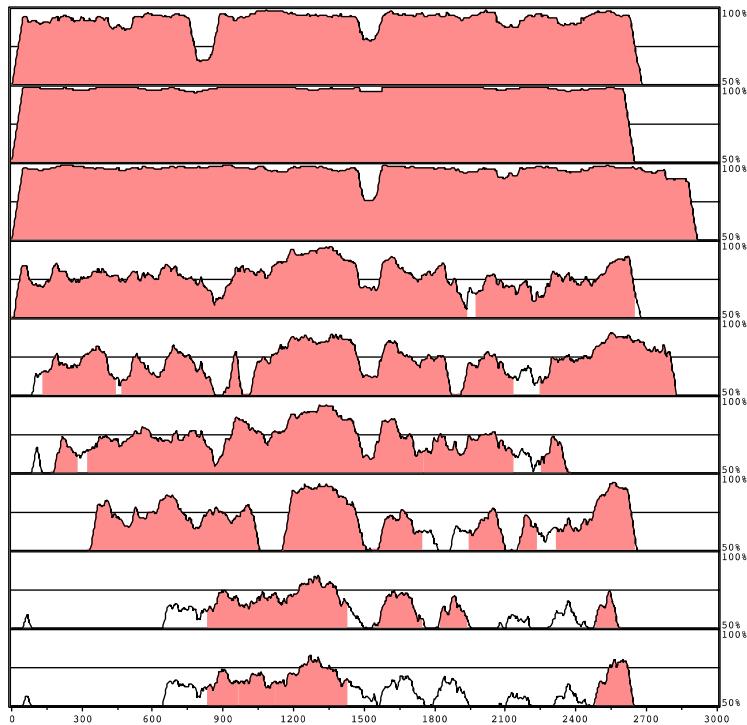
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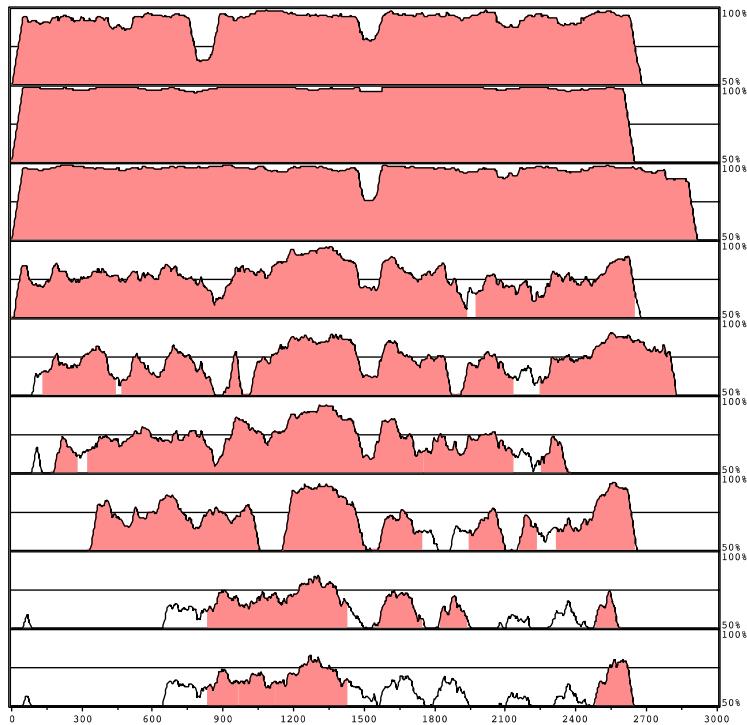
Rat
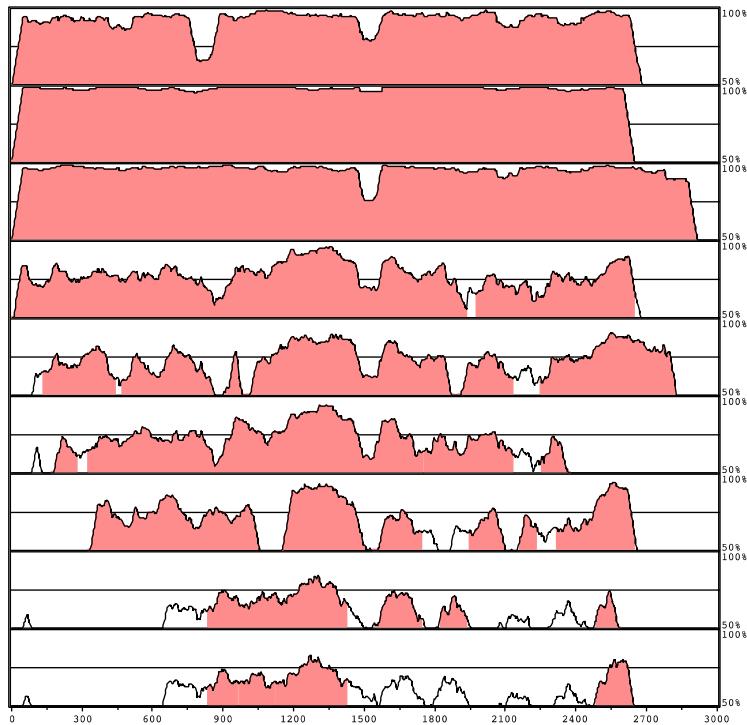
B.

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                                     -1239Twist
Human       000002058 AACTC-TAACACTT------GAGACCTCAGGTAGTT----CGGTTGGTT 000002095
Macaque     000002144 AACTCTTAACACTT------GAGACCTCAGGTAGTT----CGGTTGGTT 000002182
Chimpanzee  000002112 AACTCTTAACACTT------GAGACCTCAGGTAGTT----CGGTTGGTT 000002150
Orangutan   000001739 AACTC-TAACGCTT------GAGACCTCAGGTAGTT----CGGTTGGTT 000001776
Horse       000002139 AACAC-TGACACTT-------CAGGCCACAGACAGTTCA--CAGTTTGTT 000002178
Dog         000002151 GGATTCTGACACCG------GAGGCCACAGACAGTTCAC--AGTTGGTC 000002191
Pig         000001808 AACTT-TAACACTT------GAGGCTATACAGAGATAATTCAGTTTGTT 000001849
Squirrel    000001470 AACT---CACACTT------GAAGCCACTGATAGTTC----AGTTTCTT 000001505
Mouse       000002160 --------GCATCTCATCAGGAAGGCTACTGATAGTTTATTTAATTTGTA 000002201
Rat         000002104 --------GCATCTCTTAAGAAAAGCTACTGATAACTTATTAAGTTTGTA 000002145
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C.

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                                        -2661SREBP1 -2648SREBP1
Human       000000616 CTGTTTTATATTTTTACTGTGGTGGTGGTCACATGAATATACACATGCGA 000000665
Macaque     000000708 CTGTTCTATATTTTTACTGTGGTGGTGGTCACATGAATGTACACATGTGA 000000757
Chimpanzee  000000677 CTGTTCTATATTTTTACTGTGGTGGTGGTCACATGAATGTACACATGTGA 000000726
Orangutan   000000280 CTGTTTTATATTTTTACTGTGGTGGTGGTCACATGAATATACACATGTGA 000000329
Horse       000000722 CTGTTCAGTATCTTGATTGTGGCGCTGGTCACATGAACCCACACATGTGA 000000771
Dog         000000535 TTGTTCAGTATTCTGACTGTGGTGGTGGCCACATGAAGCAATACATGTGA 000000584
Pig         000000377 TTG--------TTGACTGTGGTGGTGATTGCATCAACCTACATATGTGA 000000417
Squirrel    000000096 CTGTTTTGTATCCTGACTGTGGTGGTGATCACCTGAACCTACCTATGTGG 000000145
Mouse       000000000 ------------------------------------------------- 000000000
Rat         000000000 ------------------------------------------------- 000000000
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                                         -2611Twist
Human       000000666 TAAAATTGCATAGAATTATATACACAGGCAAATGAGTGCATATAAAACTG 000000715
Macaque     000000758 TAAAATTGCATAGAATTATATACATAGGCAAATGACTGGATATAAAACTG 000000807
Chimpanzee  000000727 TAAAATTGCATAGAATTATATACATAGGCAAATGACTGGATATAAAACTG 000000776
Orangutan   000000330 TAAAATTGCATAGAATTATATACACAGGCAAATGAGTACATATAAAACTG 000000379
Horse       000000772 TAAAATTTCATAGAACTATACACATAGGCAAATGGGTGCACGTAAAAGTG 000000821
Dog         000000585 TAAAATTTCATATAACTATATACACAGGCAAATGGATGCATGCAAAACTG 000000634
Pig         000000418 TAAAATTTCCTAGAGCTATACGCATAGGCAGATGGGTGCGTATAAATG-A 000000466
Squirrel    000000146 TAAAATTGCGTAGAATTATACATGCAGGCAAATGAGTGCATGTAAAACTG 000000195
Mouse       000001075 -----------------------AGGCACA------------------ 000001081
Rat         000001023 -----------------------AGGCACA------------------ 000001029
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Supplementary Figure S2. Bioinformatic analysis of the 5' upstream region of the human *CHRDL1* gene to identify putative TF binding sites and sequence conservation A. mVISTA bioinformatic analysis of the upstream region (-3000 to +1) of the *CHRDL1* gene among selected mammals. Schematic representation of the global multiple sequence alignment of the different animals when compared to the human *CHRDL1* gene upstream sequence. Red areas indicate conservation based on mVISTA parameters. B) Multiple sequence alignment of the E-boxes found in probe #3 (from -1297 to -1148). C) Multiple sequence alignment of the E-boxes found in probe #5 (from -2697 to -2548). The region contained in probe #5 is the most conserved in most of the species considered in the mVISTA analysis. The -2661 site is conserved in human, macaque, chimpanzee, orangutan, horse, dog, and squirrel. The -2648 site is conserved in human, macaque, chimpanzee, orangutan, horse, and pig. Interestingly the -2611 TWIST site is conserved in 8 out of 10 species considered in this analysis.

A

T2 Q119X T1 Luc E12

C

Twist1	+	-	-	-	+	+	-	-	+	-	-
Twist2	-	+	-	-	-	-	+	+	+	-	-
E12	-	-	+	-	+	-	+	-	-	-	-
TCF3	-	-	-	+	-	+	-	+	-	-	-
Luciferase	-	-	-	-	-	-	-	-	-	+	-

E12 → homodimers

B

Twist1 Twist2 ADD1 Q65X Twist2ΔA Twist2ΔB Twist2ΔAB ADD1
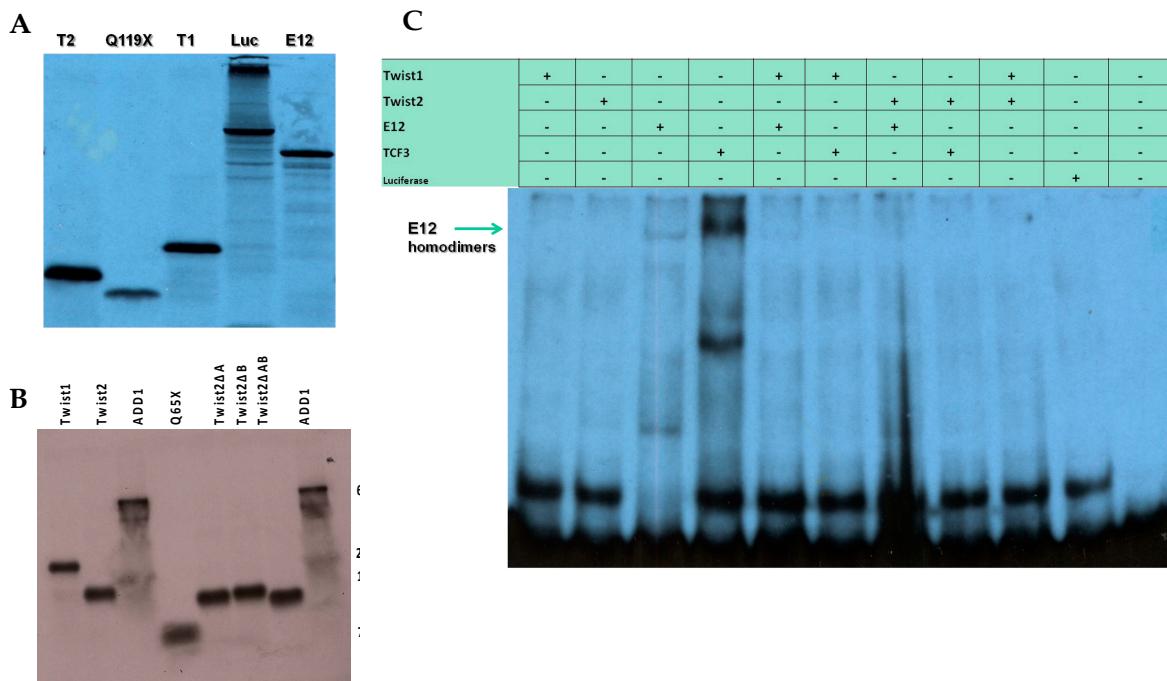
Supplementary Figure S3. SDS-PAGE of *in vitro* **transcribed/translated proteins and Electrophoretic Mobility Shift Assay for probe #1 of the** *CHRDL1* **gene. A.** This SDS-PAGE autoradiography represents an example of the typical results obtained for the proteins synthesized *in vitro* using the TnT Quick-Coupled Transcription/Translation kit (TnT) and labeled by incorporation of ^{35}S-Methionine to assess protein production. **B.** Western Blot of SDS -PAGE of TnT reactions of TWIST proteins and ADD1, detected with an anti-Myc antibody. Luciferase was used as a positive control for protein synthesis and as a negative control (mock) for EMSA binding reactions. In both A and B, similar amounts of protein were obtained for most proteins. Abbreviations used: T2=TWIST2; Q119X and Q65X= mutant forms of TWIST2 found in Setleis Syndrome patients, T1= TWIST1; Luc=Luciferase. **B.** EMSA carried out with *in vitro* synthesized proteins using probe #1. For this probe we detected binding of E12 and in the reaction with the TCF3 construct, which codes for E12 as well. The region in probe #1 contains a putative binding site for E12 at position -167 and a TWIST site at -194.
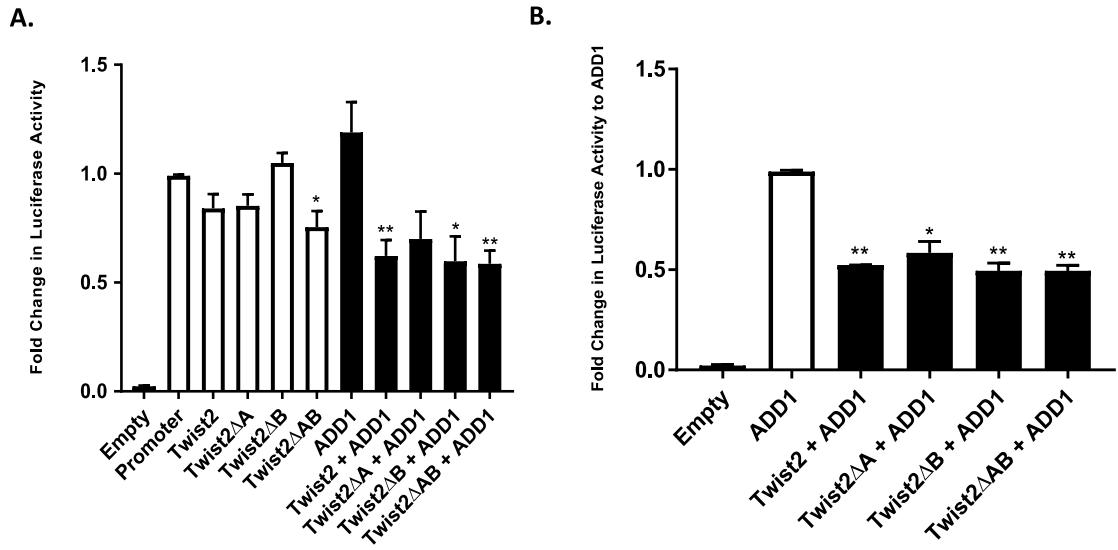
Supplementary Figure S4. Suppression of the transcriptional activity of ADD1/ SREBP1c by TWIST2 and its deletion mutant N-terminal forms in HeLa cells. Luciferase reporter gene assay using HeLa cells were used to assess the effect of the different proteins (TWIST2, TWIST2ΔA, TWIST2ΔB, TWIST2ΔAB and ADD1) on the *CHRDL1* gene upstream region. **A.** Comparison of the basal levels of reporter expression (promoter) signal to the expression signal obtained when transfected with the constructs observed in the figure. All comparisons were made relative to the basal promoter activity. **B.** The expression levels of the co-transfected reactions were compared to ADD1-dependent activation of the *CHRDL1* gene promoter (reporter expression activity was normalized against the expression of the reporter gene when transfected with ADD1). Transfection assays were performed in duplicate and repeated independently at least three times (N=3). Statistical analysis was done using one-way ANOVA followed by unpaired Student's T-test. Error bars represent standard error of the mean (SEM). *= p<0.05, **= p<0.01.