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Supplemental Data

Mutations Affecting the BHLHA9 DNA-Binding Domain Cause Mesoaxial Synostotic Syndactyly with Phalangeal Reduction, Malik-Percin Type, MSSD

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Supplemental Data

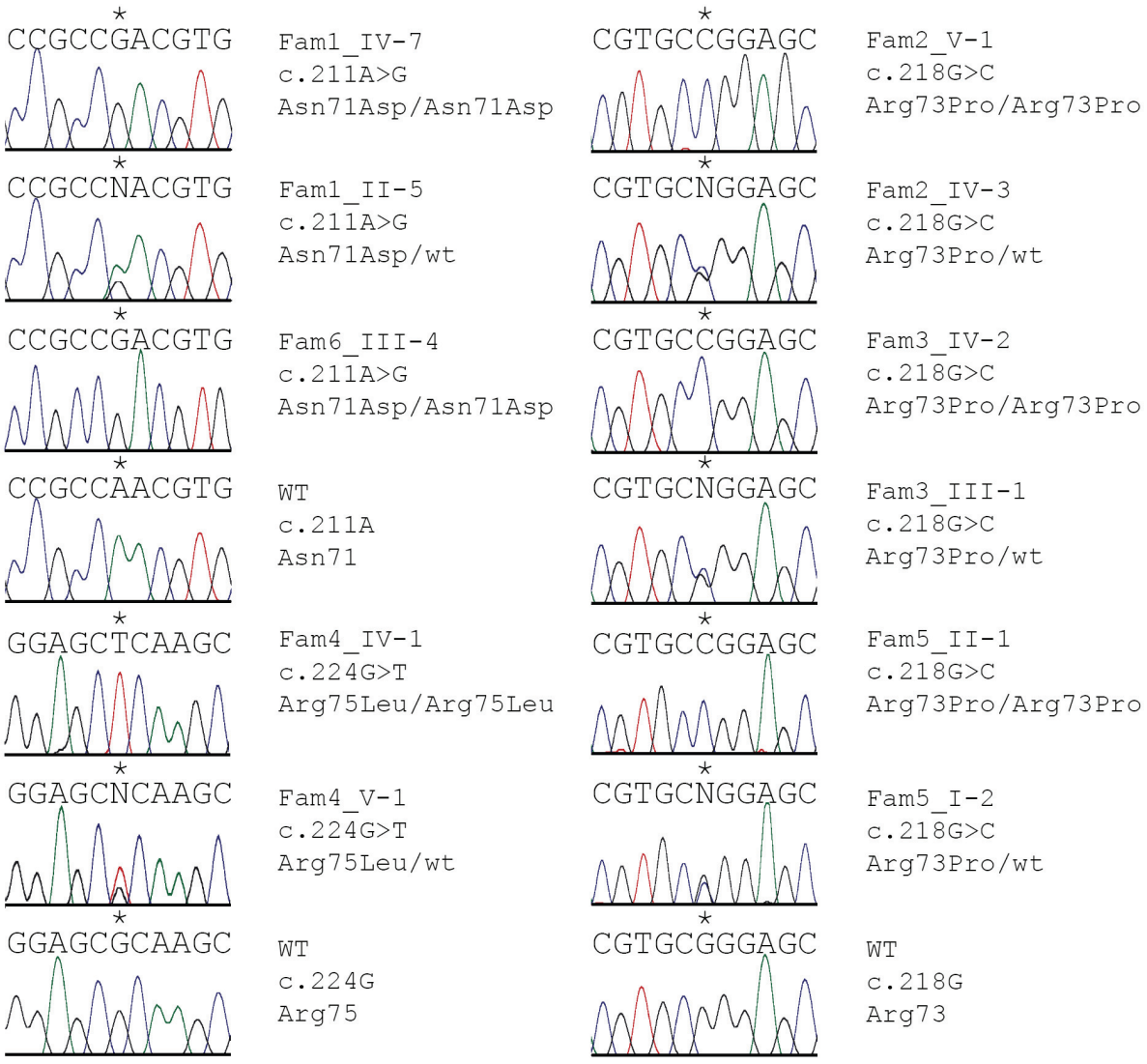

Figure S1

***BHLHA9* Mutations in MSSD Detected by DNA Sequencing**

DNA sequence electropherograms of wild type (WT) *BHLHA9* and mutations observed in syndactyly type IX individuals or heterozygous parents from 6 unrelated families. The positions of the mutant bases are indicated by asterisks. Phenotypes of the individuals analyzed are listed in Table1.

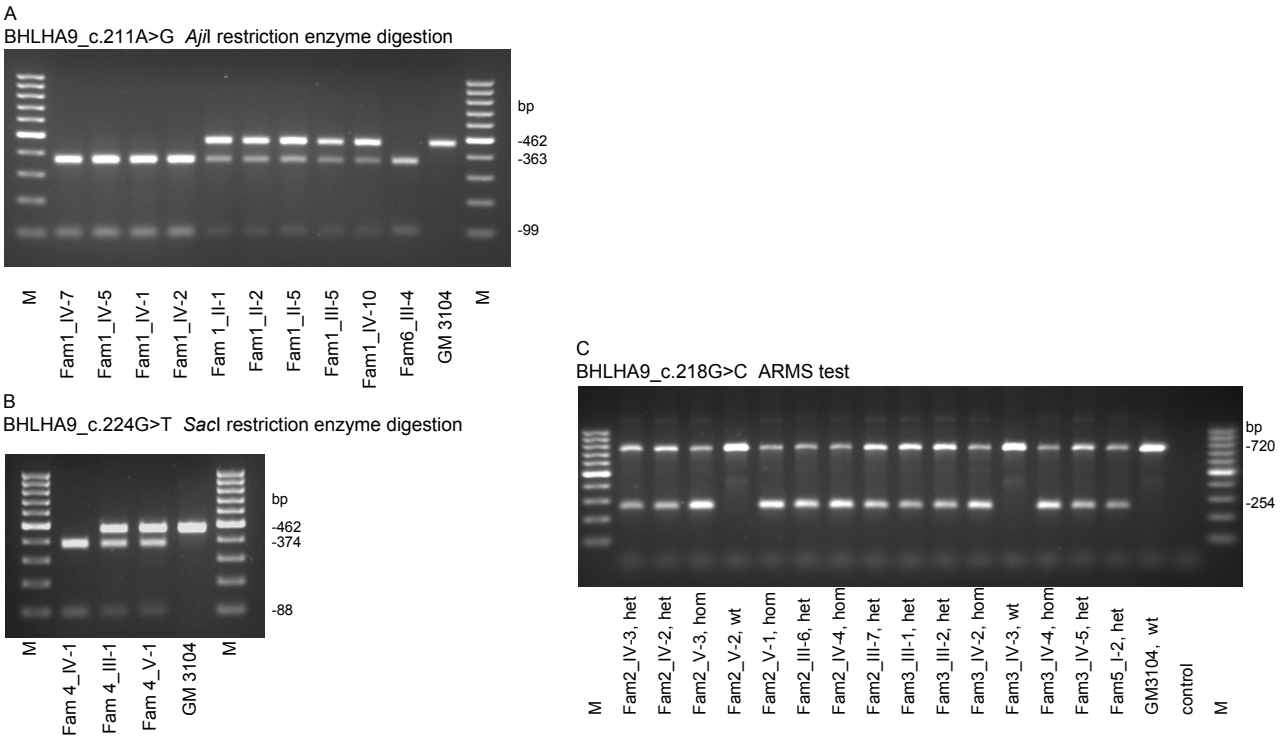

Figure S2

***BHLHA9* Mutations in MSSD Detected by Allele Specific Restriction Enzyme Digestion or an ARMS Test**

(A) Electropherogram of *Aji*I restriction enzyme digestion of a PCR fragment (primers BH 2-1for and rev, Table S3) of families 1 and 6 segregating *BHLHA9*_c.211A>G. In DNA from individuals with the mutation the wild type fragment of 462 bp is cut into fragments of 363 bp and 99 bp.

(B) Electropherogram of *Sac*I restriction enzyme digestion of a PCR fragment (primers BH 2-1for and rev, Table S3) of family 4 segregating *BHLHA9*_c.224G>T. In DNA from individuals with the mutation the wild type fragment of 462 bp is cut into fragments of 374 bp and 88 bp.

(C) Electropherogram of PCR products (254 bp) generated by an amplification-refractory mutation system (ARMS) test (primers BH2for and BHarms 2 rev Mut, Table S3) with DNA of families 2, 3, and 5 segregating *BHLHA9*_c.218G>C. The products generated from the mutated allele differ in quantity in homozygotes (hom) versus heterozygotes (het). A 720bp PCR product generated in the same reaction with primers BH2for and rev is shown for control. A parallel test (primers BH2for and BHarms1 rev WT) generating a product only with wild type alleles confirmed the result (data not shown).

M = 100 bp size marker. Phenotypes of the individuals analyzed are listed in Table 1. GM3104 = human unaffected control.

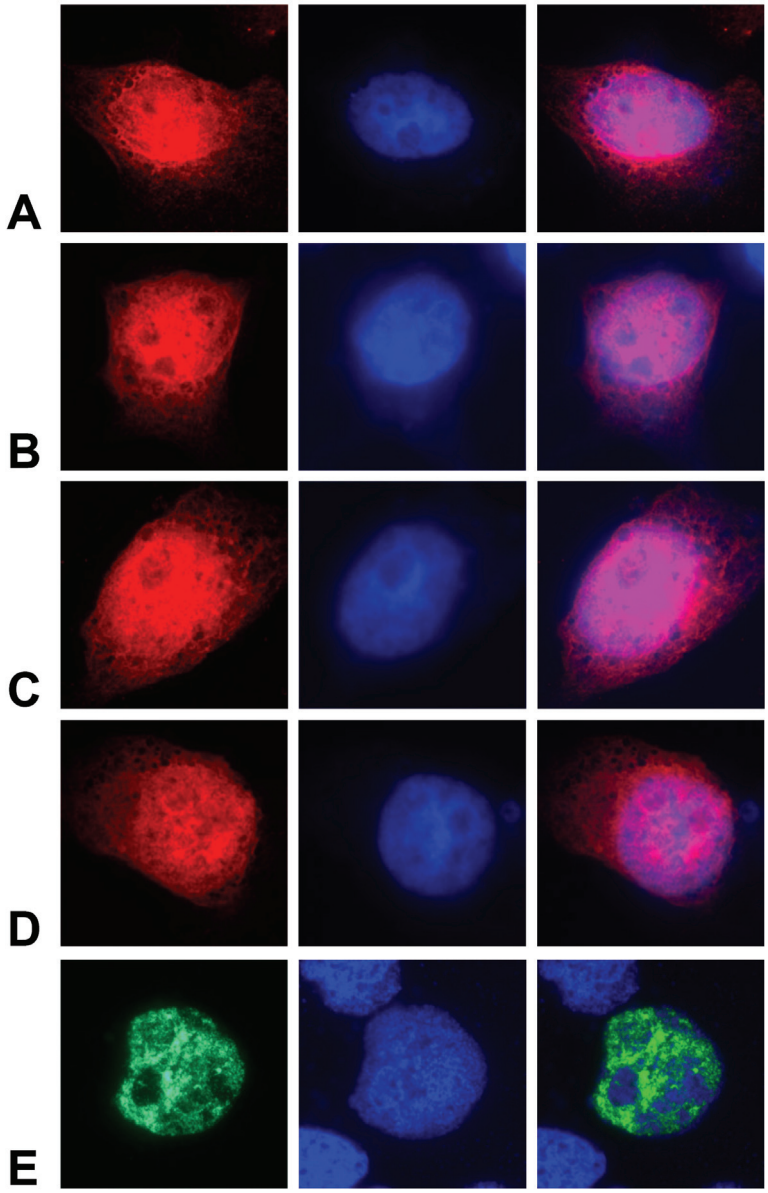

Figure S3

Histochemical Analysis of Cellular Expression of Wild Type and Mutant BHLHA9 and TCF4

Cos7 cells expressing (A) wild type *HsBHLHA9_MYC/DDK* or mutant *BHLHA9*, (B) *BH_N71D*, (C) *BH_R73P*, (D) *BH_R75L* in expression vector pCMV6-Entry/C-Myc_DDK, or (E) human *TCF4* in expression vector pcDNA6.2/N-YFP-DEST. First column: The MYC-tag of BHLHA9 was detected by immuno-fluorescence with anti-c-myc mouse IgG monoclonal antibody and Alexa Fluor 594 goat anti-mouse IgG (H+L). Expressed TCF4 was detected by fluorescence of the YFP tag. Second column: Nuclei are stained with DAPI. Third column: Overlay images. In contrast to TCF4 which is detected only in the nucleus, the BHLHA9 protein is localized both in the cytoplasm and the nucleus. With a mass of 24,142 Da, BHLHA9 may enter the nucleus by passive transport.

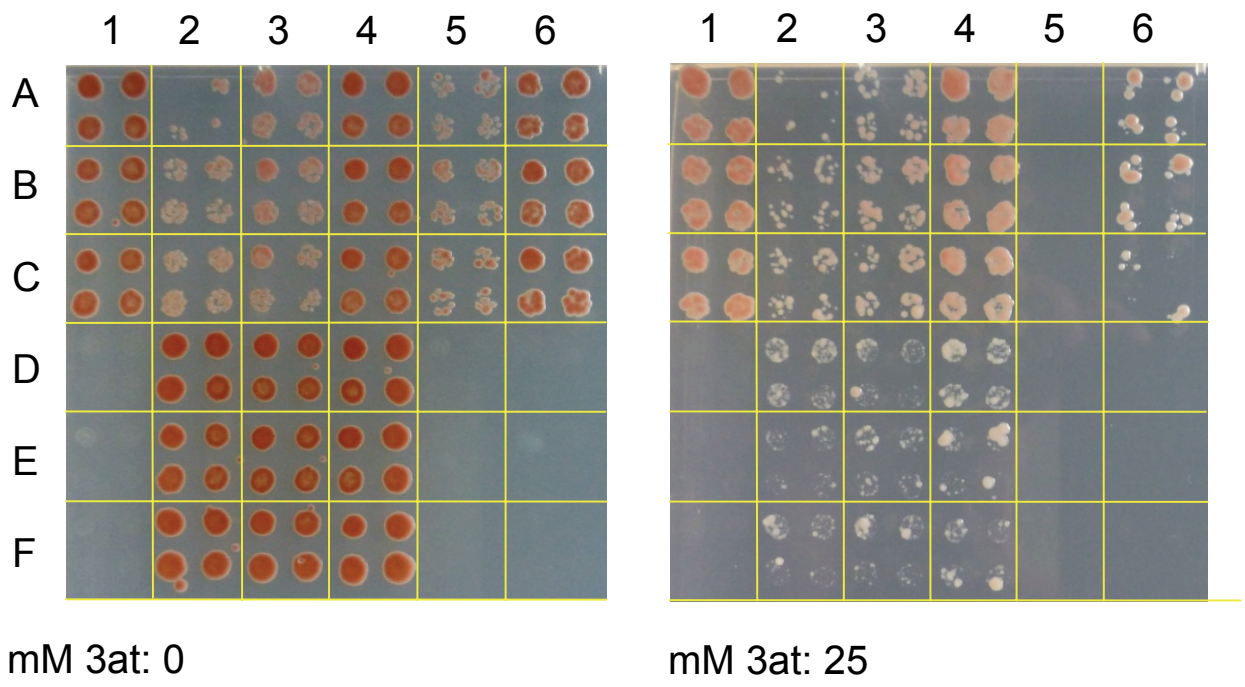

Figure S4

Pairwise Y2H Analysis of BHLHA9 with BHLH Domain Containing Bait Proteins

(A), (B), (C) Triplicate analyses of Y2H analyses at 0 mM 3-Amino-1,2,4-triazole (3at) and 25 mM 3at of *BHLHA9wt* as prey in vector pGADT7gw with baits: 1: *BHLHA9wt*, 2: *TCF4*, 3: *TCF3*, 4: *TCF12*, 5: *TWIST1*, 6: *HAND2* in vector pGBT9gw.

(D), (E), (F) Triplicate autoactivation controls with baits 1 – 6 in pGBT9gw and empty pGADT7gw. BHLHA9wt–homodimers show reproducible interaction and no autoactivation, whereas TCF3, TCF4, and TCF12 show strong autoactivation which can mostly be eliminated by addition of 25 mM 3at. TWIST1 interacts only weakly with BHLHA9wt (at 0 mM 3at), whereas HAND2 shows strong reproducible interaction (10-25 mM 3at) and no autoactivation.

Molecular Constructs for Y2H Analysis

The translated segment of Mus musculus (Mm) *Bhlha9* cDNA [NM_177182.4] was isolated from the potentially complete cDNA clone [BC048728/IRAVp968C09144D, Source Bioscience] and cloned into vector pGBT9_DNA_BD [Clontech; Genbank: U07646] to generate pGBT9_MmBhlha9. The translated segment of wild type human *BHLHA9* cDNA was inserted by TOPO cloning into pCR8/GW/TOPO and shuttled using gateway (gw) technology [Invitrogen] into pGBT9gw or pGADT7gw (kindly provided by F. Schwarz, DKFZ, Heidelberg) to generate pGBT9_HsBHLHA9/wt and pGADT7_HsBHLHA9/wt. The translated segments of human *TCF3*,

TCF4, and *TCF12* were isolated by PCR (Table S3) from the potentially complete cDNA clones [BC110580.1/IRCMp5012E0634D, BC125084.1/IRCMp5012C034D, and BC050556/IRATp970B1176D, Source Bioscience, Nottingham, UK] and inserted by TOPO cloning into pCR8/GW/TOPO [Invitrogen]. The inserts of the resulting constructs were shuttled using Gateway (gw) technology [Invitrogen] into pGBT9gw to generate pGBT9_HsTCF3, pGBT9_HsTCF4, and pGBT9_HsTCF12.

Plasmids pGBT9_HsTWIST1 and pGBT9_HsHAND2, harbouring the translated segments of human *TWIST1* and *HAND2* cDNAs were obtained from Source Bioscience.

Yeast-2-Hybrid (Y2H) Analysis Methods

Two mouse total embryonic cDNA libraries (d11 and d17, $3.8x10^6$ and $5.6x10^6$ clones, respectively) in vectors pACT2 and pGADT7 were screened at the German Cancer Research Center, Heidelberg, by Y2H analyses for clones encoding proteins interacting with mouse BHLHA9, expressed by vector pGBT9_MmBhlha9. Interaction was detected using appropriate *S. cerevisiae* yeast strains. cDNA library inserts of positive clones were retrieved by PCR, sequenced from the 5'-end, and identified by BLAST analysis. Reliable interactions were defined considering stringency (1mM 3 Amino-1,2,4 triazol, 3at), number of clones analyzed, signal intensity, and the frequency of occurrence together with the BHLHA9 bait in comparison with the frequency of occurrence with other baits in previous screens (German Cancer Research Center, Heidelberg). Interactions detected by library screening were confirmed by one-to-one Y2H analysis at 0 mM 3at and 25 mM 3at of *HsBHLHA9* in vector pGADT7gw as prey with human *TCF3*, *TCF4*, and *TCF12* in pGBT9gw as baits (German Cancer Research Center, Heidelberg). Likewise, interactions of BHLHA9 with itself or with human TWIST1 and HAND2 basic helix loop helix proteins were analyzed by one-to-one Y2H analysis. Each of these analyses was performed in triplicate.

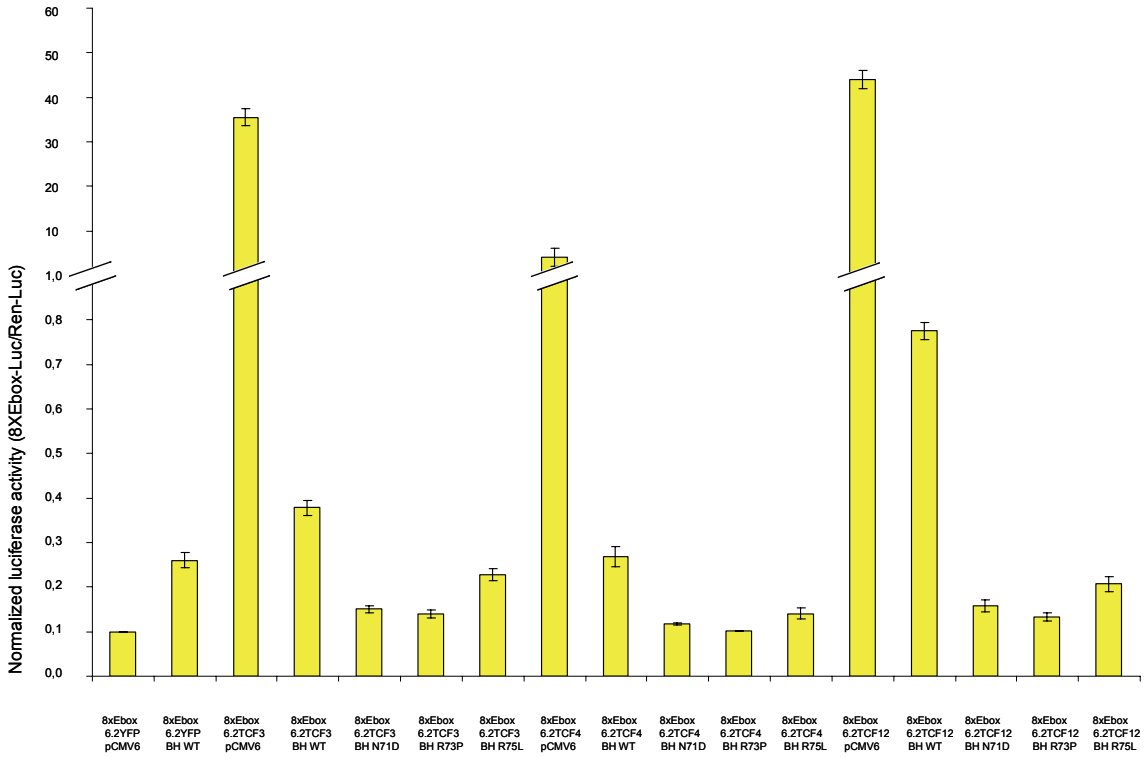

Figure S5

Luciferase Assays in U2OS Cells Demonstrate Regulatory Activity of Co-expressed BHLHA9 and TCFs

Transient cotransfection into U2OS cells of a construct coding for human wild-type (BH_WT) or mutant BHLHA9 (BH_N71D, BH_R73P, BH_R75L) in expression vector pCMV6-Entry/C-Myc_DDK (pCMV6), with human *TCF3* (TCF3), human *TCF4* (TCF4), human *TCF12* (TCF12) in expression vectors pcDNA6.2/N-YFP-DEST (6.2YFP), and a pGL3-derived luciferase reporter gene under transcriptional control of the mouse *fos*-promoter with an enhancer element encompassing eight E-box sequences (8xEbox). Transient expression of BH_WT alone has a small effect on luciferase expression which, however, is more pronounced than in Cos7 cells. The TCFs individually act as strong activators. For *TCF3* and *TCF4* cotransfection with *BHLHA9* reduces reporter activity highly. The luciferase expression in the combination BH_WT with TCF12 is about double as high as with the other two TCFs. Replacing expression of BH_WT with any of the three mutant proteins detected in affected individuals abolishes almost completely reporter gene activation in combination with TCF3, TCF4, and TCF12.

Transfection efficiency is normalized to cotransfected *Renilla* luciferase. The values obtained in transfection experiments with 8xEbox and the empty expression vectors (6.2YFP and pCMV6) are set at 0.1, the other values are adapted accordingly. The results shown for TCF3 and TCF12 are derived from three independent experimental series, the results for TCF4 from one series, each made up of three parallel transfection experiments. Normalized luciferase activity (8xEbox-Luc/Ren-Luc) data are represented as mean ± SD.

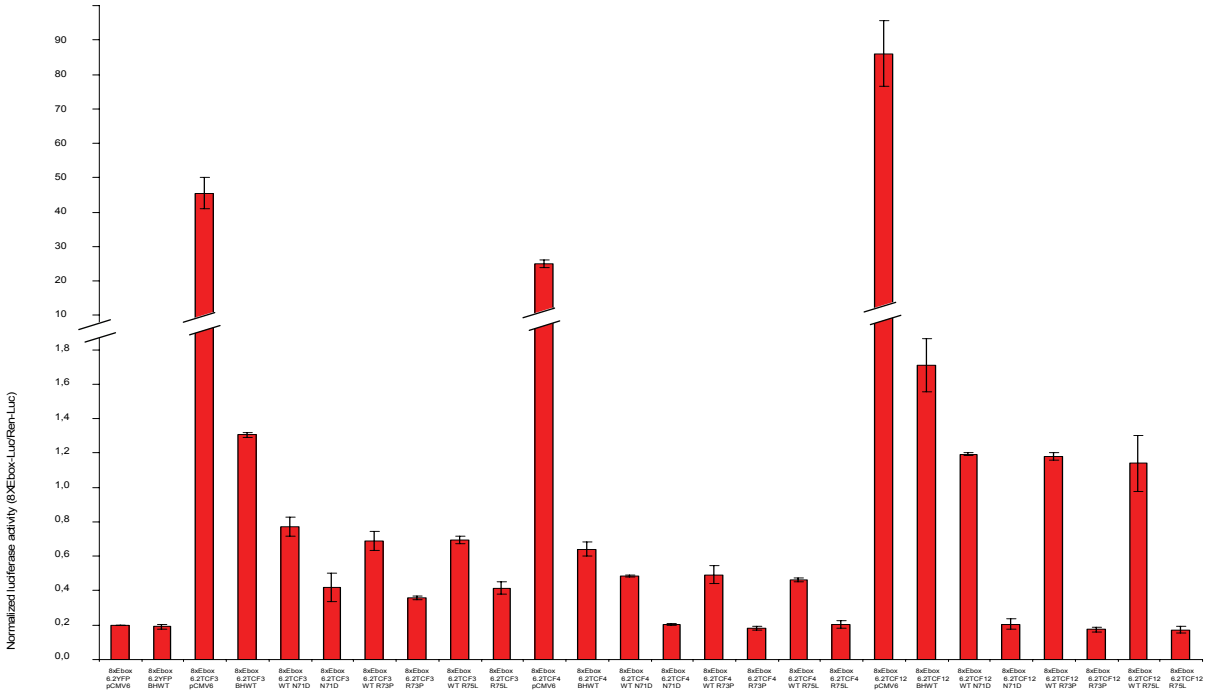

Figure S6

Luciferase Assays Demonstrate a Quantitative Effect of Mutant BHLHA9

Transient cotransfection into Cos7 cells of constructs coding for human TCF3 (6.2TCF3), TCF4 (6.2 TCF4), and TCF12 (6.2TCF12) in expression vector pcDNA6.2/N-YFP-DEST (6.2YFP) with wild-type *BHLHA9* alone (BHWT), mutant *BHLHA9* alone (N71D, R73P, R75L) or combined in equal amounts (WT_N71D, WT_R73P, WT_R75L), in expression vector pCMV6-Entry/C-Myc_DDK (pCMV6), and a pGL3-derived luciferase reporter gene under transcriptional control of the mouse *fos*-promoter with an enhancer element encompassing eight E-box sequences (8xEbox). Transient expression of BHLHA9_WT alone has no effect on luciferase expression. The TCFs individually act as strong activators. Cotransfection with wild-type *BHLHA9* reduces luciferase activity considerably. A 1: 1 mixture of wt and mutant *BHLHA9* in combination with *TCF3, TCF4, and TCF12* reduces the expression rate of the reporter by approximately one third, leaving a residual activity higher than the combination with mutant *BHLHA9* alone. This effect was particularly pronounced for *TCF12*.

Transfection efficiency is normalized to cotransfected Renilla luciferase. The values obtained in transfection experiments with 8xEbox and the empty expression vectors (6.2YFP and pCMV6) are set at 0.2, the other values are adapted accordingly. The results shown are representative of four independent experimental series, each with triplicate parallel transfection experiments. Normalized luciferase activity (8xEbox-Luc/Ren-Luc) data are represented as mean ± SD.

Table S1: Pair-wise LOD Scores Between MSSD Syndactyly Phenotype in Family 3 and Microsatellite Markers on Chromosome 17p13.3

Marker	Map position			Family 3 (θ)						Families 1—3 (θ)				
	Genetic [a]	Genetic [b]	Physical [c]	0.00	0.01	0.05	0.10	0.20	0.30	0.00	0.01	0.05	0.10	0.20
D17S849	0.63	4.52	432,537	0.693	0.671	0.586	0.484	0.301	0.157	4.104	4.000	3.559	3.003	1.969
D17S1308	0.63	4.52	622,950	0.546	0.528	0.459	0.377	0.231	0.120	3.119	3.020	2.661	2.209	1.378
D17S926	0.63	4.52	630,233	0.000	0.000	0.000	0.000	0.000	0.000	4.915	4.791	4.390	3.834	2.752
D17S695	3.67	-	746,134	0.000	0.000	0.000	0.000	0.000	0.000	4.968	4.858	4.429	3.882	2.782
D17S596	3.96	6.58	1,009,505	0.000	0.000	0.000	0.000	0.000	0.000	1.646	1.623	1.514	1.354	0.994
D17S1529	3.92	6.58	1,049,360	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D17S1533	3.99	-	1,540,534	0.786	0.762	0.669	0.555	0.349	0.184	5.153	5.026	4.535	3.911	2.692
D17S831	6.60	-	1,910,400	0.000	0.000	0.000	0.000	0.000	0.000	1.016	1.305	1.538	1.446	1.023
D17S654	6.60	-	1,910,574	0.000	0.000	0.000	0.000	0.000	0.000	-0.960	-0.570	-0.130	0.020	0.090
D17S1528	6.60	9.44	2,024,777	0.000	0.000	0.000	0.000	0.000	0.000	2.659	3.331	3.518	3.258	2.415
D17S1583	7.19	-	2,704,868	0.000	0.000	0.000	0.000	0.000	0.000	1.922	1.898	1.773	1.578	1.141

The markers depicted in bold-face are included in the disease interval. The LOD scores yielded by combining Family 1 and 2, reported earlier (Malik et al. 2005), are also provided (Families 1-3).
[a] Sex-averaged Kosambi map distance (cM) from the Marshfield map (Broman et al. 1998)
[b] Sex-averaged Kosambi map distance (cM) from the Rutgers map (Kong et al. 2004)
[c] Sequence-based physical map distance according to the Human Genome Browser (UCSC Santa Cruz; Feb. 2009; GRCh37/hg19 Assembly)

References Table S1

Broman, K.W., Murray, J.C., Sheffield, V.C., White, R.L., Weber, J.L. (1998). Comprehensive human genetic maps: individual and sex-specific variation in recombination. Am. J. Hum. Genet. *63*, 861-869.

Kong, X., Murphy, K., Raj, T., He, C., White, P.S., Matise, T.C. (2004). A combined linkage-physical map of the human genome. Am. J. Hum. Genet. *75*, 1143-1148.

Malik, S., Percin, F.E., Ahmad, W., Percin, S., Akarsu, N.A., Koch, M.C., Grzeschik, K-.H. (2005). Autosomal recessive mesoaxial synostotic syndactyly with phalangeal reduction maps to chromosome 17p13.3. Am. J. Med. Genet. *134*, 404-408.

Table S2: SNP Genotypes in a 10.7 kb Segment of Chromosome 17p13.3 Encasing BHLHA9

SNP	Alleles	Fam1_IV-7 Pakistan	Fam6_III-4 Pakistan	Fam2_V-1 Turkey	Fam3_IV-2 Turkey	Fam5_II-1 Pakistan
rs6502542	C,T	C/C	C/C	T/T	T/T	T/T
rs7216365	A,C	A/A	A/A	A/A	A/A	A/A
rs57016694	A,G	A/A	A/A	A/A	A/A	A/A
rs7220578	A,G	G/G	G/G	A/A	A/A	A/A
rs7218715	C,T	T/T	T/T	T/T	T/T	T/T
rs6502668	A,C	A/A	A/A	C/C	C/C	C/C
rs7224856	A,C	A/A	A/A	C/C	C/C	C/C
rs111986848	C,G	C/C	C/C	C/C	C/C	n.d.
rs8079493	T,C	T/T	T/T	C/C	C/C	n.d.
rs190953401	T,C	C/C	C/C	C/C	C/C	n.d.
rs28754558	A,G	A/A	A/A	A/A	A/A	n.d.
rs73283201	C,G	G/G	G/G	G/G	G/G	n.d.
rs112091126	C,T	T/T	T/T	T/T	T/T	n.d.
rs60062553	A,G	G/G	G/G	G/G	G/G	n.d.
rs5818793	-,G	G/G	G/G	G/G	G/G	n.d.
rs73975685	A,T	T/T	T/T	T/T	T/T	n.d.
rs2063188	A,G	G/G	G/G	G/G	G/G	G/G
BHLHA9 c.211A>G	A,G	G/G	G/G	A/A	A/A	A/A
BHLHA9 c.218G>C	C,G	G/G	G/G	C/C	C/C	C/C
rs3951819	A,G	G/G	G/G	G/G	G/G	G/G
rs8071123	G,T	G/G	G/G	G/G	G/G	n.d.
rs73975687	A,G	G/G	G/G	G/G	G/G	n.d.
rs77839307	A,C	C/C	C/C	C/C	C/C	n.d.
rs73975688	C,G	C/C	C/C	C/C	C/C	n.d.
rs112560750	C,T	C/C	C/C	C/C	C/C	n.d.
rs59168600	C,G	G/G	G/G	G/G	G/G	n.d.
rs111751562	A,C	A/A	A/A	A/A	A/A	A/A
rs6502732	A,G	A/A	A/A	A/A	A/A	A/A
rs114947760	G,T	T/T	T/T	T/T	T/T	T/T
rs73285306	G,T	T/T	T/T	T/T	T/T	n.d.
rs58541517	A,G	G/G	G/G	G/G	G/G	n.d.
rs111556356	C,T	C/C	C/C	C/C	C/C	n.d.
rs112921237	A,G	A/A	A/A	A/A	A/A	n.d.
rs113058253	A,G	G/G	G/G	G/G	G/G	n.d.
rs201047604	-,AAAG	-/-	-/-	-/-	-/-	n.d.
rs112648227	C,T	C/C	C/C	C/C	C/C	C/C
rs1472180	A,G	A/A	A/A	A/A	A/A	A/A
rs73975691	A,G	A/A	A/A	A/A	A/A	A/A
rs78022295	G,T	G/G	G/G	G/G	G/G	n.d.
rs115288747	A,G	G/G	G/G	G/G	G/G	n.d.
rs199551922	-,GA	-/-	-/-	-/-	-/-	n.d.
rs10577031	-,AA	-/-	-/-	-/-	-/-	n.d.
rs201932744	-,GTGA	GTGA/GTGA	GTGA/GTGA	GTGA/GTGA	GTGA/GTGA	n.d.
rs202010921	-,T	-/-	-/-	-/-	-/-	n.d.
rs10577032	-,GT	-/-	-/-	-/-	-/-	n.d.
rs62090185	C,T	C/C	C/C	C/C	C/C	T/T
rs62090186	C,T	C/C	C/C	C/C	C/C	T/T

SNP denominations are from dbSNP; (-) indicates a deletion; n.d. = alleles not analyzed because of restricted availability of DNA. Phenotypes of the individuals analyzed are listed in Table 1.

Table S3: Primers and PCR Details

Primer	Sequence (5'-3')	Length [bp]	% GC	Fragment length [bp]	PCR conditions	Cycles	Application
BH 2-1for	CAT AAA GCC CAG CTG GAA GG	20	55	462	Beads 57°C	34	Mutation detection
BH 2-1rev	GAT CTT GGA GAG CCT CTT GC	20	55				
BH 1for	GAG GGA GAT GGA TGG ACG GGA	21	62	970	Ampli Taq 60°C	33	Mutation detection
BH 1rev	GCA GTC CCG GGT TTT ATA GTC	21	52				
BH 2for	GGG AAG GCC ATG CTG CGG G	19	74	720	Ampli Taq 63°C	30	Cloning
BH 2rev	CGG TCA GGA GCG CGG ATG G	19	74				
BHarms1 rev (WT)	TTGTAGTCTAGGATGCGCTTGCGCTCTC	28	57	254 with BH2for	Ampli Taq 65°C	33	ARMS Test
BHarms2 rev (Mut)	TTGTAGTCTAGGATGCGCTTGCGCTCTG	28	57				
TCF3 for	AGA ATG AAC CAG CCG CAG AG	20	55	1995	Beads 56°C	19	Cloning
TCF3 rev	ACG GAG GCA TAC CTT TCA CA	20	50				
TCF3-1for	GGT CTT CCA TCC TCG GTG TA	20	55				Sequencing
TCF3-2for	GCT GGC CTC AGG TTT CAC	18	61				Sequencing
TCF4 for	GCT AAA ATG CAT CAC CAA CAG	21	43	2037	Beads 56°C	19	Cloning
TCF4 rev	GCA ACT TGG ACC CTT TTA CAT C	22	45				
TCF4-1for	TCC TTG GAG GTG ACA TGG AT	20	50				Sequencing
TCF4-2for	CAT CTC TCT CAG CAG GCA CA	20	55				Sequencing
TCF12 for	AAG ATG AAT CCC CAG CAA CA	20	45	2142	Beads 56°C	19	Cloning
TCF12 rev	TCT GGA ACT GGC TGA TGT TTA	21	43				
TCF12-1for	GTC GAT TAG GAG CCC ATG AA	20	50				Sequencing
TCF12-2for	TCA CAC ACT CCT CCC ATC AA	20	50				Sequencing
TCF12-3for	AGG TGG CTT GCA AAG TCA GT	20	50				Sequencing
VP1.5 for	GGA CTT TCC AAA ATG TCG	18	44	Vector primer for pCMV6			Sequencing
XL39 rev	ATT AGG ACA AGG CTG GTG GG	20	55	Vector primer for pCMV6			Sequencing
BH SgfI for	gaggcgatcgccATGCTGCGGGGCGCGCCAGGACT	35	74	Cloning of *BHLHA9* Mut in pCMV6			Cloning
BH MluI rev	gcgacgcgtGGAGCGCGGATGGCCCATCCCGGG	33	79				
V5 rev	ACC GAG GAG AGG GTT AGG GAT	21	57	Vector primer for pcDNA 3.2/V5-DEST			Sequencing
TK polyA rev	CTT CCG TGT TTC AGT TAG C	19	47	Vector primer for pcDNA 6.2/N-YFP-DEST			Sequencing
RV3 for	CTA GCA AAA TAG GCT GTC CC	20	50	Vector primer for pGL3			Sequencing
GL2 rev	CTT TAT GTT TTT GGC GTC TTC CA	23	39				
pGBT9gw 1for	TCA TCG GAA GAG AGT AG	17	47	Vector primer for pGBT9gw			Sequencing
pGADT7g for	TTC GAT GAT GAA GAT ACC CCA	21	43	Vector primer for pGADT7g			Sequencing
8xEbox for	TGC CAG AAC ATT TCT CTA TCG	21	43	291	Beads 56°C	18	Re-cloning into pGL4.23
8xEbox rev	ATA GAA GCG CTG TGA ATG GA	20	45				

Table S4: Consequences of Mutations in *BHLHA9* (Computational Analyses)

Name/ Exon	Mutation	Codon change	Change in amino acid	Type of change	SIFT Human Protein result:	PolyPhen-2 result:	Mutation Taster result:	MutPred result:	MutPred features of mutation:
Mut1, exon 1	c.211A>G	AAC > GAC	N71D	Missense mutation	DAMAGING - Low confidence predictions with Median conservation above 3.25	This mutation is predicted to be PROBABLY DAMAGING with a score of 0.998 (sensitivity: 0.27; specificity: 0.99)	disease causing	Probability of deleterious mutation 0.766	Gain of ubiquitination at K76 (P = 0.0314) Loss of methylation at R67 (P = 0.0831) Gain of helix (P = 0.132) Loss of loop (P = 0.2897) Loss of stability (P = 0.3857)
Mut2, exon 1	c.218G>C	CGG > CCG	R73P	Missense mutation	DAMAGING - Low confidence predictions with Median conservation above 3.25	This mutation is predicted to be PROBABLY DAMAGING with a score of 0.999 (sensitivity: 0.14; specificity: 0.99)	disease causing	Probability of deleterious mutation 0.831	Gain of ubiquitination at K76 (P = 0.0303) Loss of helix (P = 0.0558) Gain of glycosylation at R73 (P = 0.0591) Loss of MoRF binding (P = 0.0921) Gain of methylation at K76 (P = 0.1203)
Mut3, exon 1	c.224G>T	CGC > CTC	R75L	Missense mutation	TOLERATD - Low confidence predictions with Median conservation above 3.25	This mutation is predicted to be PROBABLY DAMAGING with a score of 0.999 (sensitivity: 0.14; specificity: 0.99)	disease causing	Probability of deleterious mutation 0.890	Loss of disorder (P = 0.1204) Loss of helix (P = 0.1299) Loss of methylation at K76 (P = 0.1387) Loss of ubiquitination at K76 (P = 0.1828) Gain of loop (P = 0.2045)

In silico analyses were carried out in order to predict the pathogenicity of identified mutations. The following tools were utilized: SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/), MutPred (http://mutpred.mutdb.org/). Conclusions: All tools utilized predict severe pathogenic consequences of all three mutations.

Table S5: TCFs Interacting with a BHLHA9 Bait in Y2H Screens of d11 and d17 Mouse Embryo Libraries

Bait	Prey gene symbol	Number of times this prey has been isolated	Number of times the fragment starts in the 5' UTR	Number of times the fragment starts in the coding sequence	Number of times the fragment starts in the 3' UTR	Prey promiscuity	Description	Link to genes	Number of diffrerent screens in which this pair has been found	Number of diffrerent cDNA libraries in which this pair has been found
BC048728	Tcf4	43	1	41	1	1	transcription factor 4	21413	2	2
BC048728	Tcf3	12	0	12	0	1	transcription factor 3	21423	2	2
BC048728	Tcf12	1	0	1	0	1	transcription factor 12	21406	1	1