

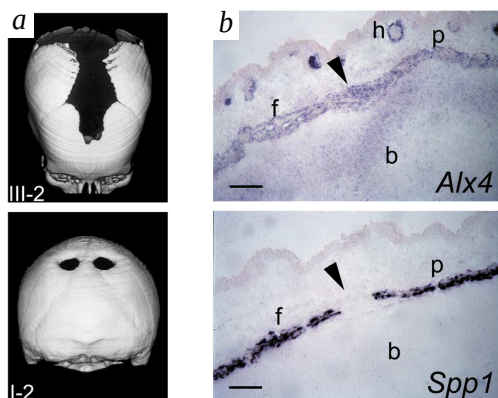


Fig. 2 Human skull phenotypes associated with *ALX4* mutations and expression of *Alx4* in developing mouse calvaria. **a**, Three-dimensional computed tomographic skull reconstructions from III-2 (age 1 y, antero-superior view) and I-2 (age 50 y, posterior view) in family 4. **b**, *In situ* hybridization of *Alx4* compared with *Spp1* (encoding osteopontin) in adjacent sections of the E16 mouse coronal suture. p, parietal bone; f, frontal bone; h, hair follicle; b, brain. Arrowheads indicate the mid-sutural region. Scale bar, 150 µm.

osteoblasts and osteoblasts¹⁵), in embryonic day (E) 16 mice revealed transcripts in the less-differentiated cells of the outer layers of the developing frontal and parietal bones and in the midsutural mesenchyme (Fig. 2b). This indicates a role for *Alx4* in controlling the proliferation-differentiation balance in the coronal suture.

We conclude that, as for *MSX2*, these *ALX4* mutations result in functional haploinsufficiency. This implies that correct *ALX4* dosage is critical for human skull ossification, supporting a recent proposal of Wu *et al.*¹⁴. It will be important to clarify whether *MSX2* and *ALX4* act hierarchically or in parallel developmental pathways. PFM and craniosynostosis (premature fusion of the cranial sutures) may result from opposite perturbations in osteogenic differentiation in the skull vault¹. Both clinical observations² and the expression studies in

the mouse validate *ALX4* as a candidate gene for craniosynostosis.

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X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy

To determine whether human X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome (IPEX; MIM 304930) is the genetic equivalent of the scurfy (*sf*) mouse, we sequenced the human ortholog (*FOXP3*) of the gene mutated in scurfy mice (*Foxp3*), in IPEX patients. We found four non-polymorphic mutations. Each mutation affects the forkhead/winged-helix domain of the scurfy protein, indicating that the mutations may disrupt critical DNA interactions.

IPEX is a recessive disorder characterized by the neonatal onset of insulin-dependent diabetes mellitus (IDDM), infections, enteropathy, thrombocytopenia and anemia, other endocrinopathy, eczema and cachexia¹. Mononuclear cell and plasma-macrophage infiltration is associated with the disappearance of pancreatic islet cells, loss

of small bowel mucosa and disruption of other tissues. IPEX is also known by several other names^{1,2} (O. Baud, manuscript submitted; see MIM 304930, 304790) and is usually lethal in infancy. It maps to chromosome Xp11.23–Xq13.3 and is not allelic to Wiskott–Aldrich syndrome/X-linked thrombocytopenia^{2,3}.

Mouse scurfy is an X-linked recessive disorder of immune regulation⁴. Affected males have scaly skin, apparent infection, diarrhea, progressive Coombs-positive anemia, thrombocytopenia, gastrointestinal bleeding, hypogonadism, leukocytosis, lymphadenopathy and cachexia^{5,6}. They die within four weeks after birth. The recently cloned gene *Foxp3* encodes a novel, highly conserved protein, scurfy, that is truncated in the scurfy mouse, eliminating a forkhead/winged-helix domain at the carboxy terminus⁷.

Because the scurfy locus is syntenic to the proximal short arm of the human X chromosome⁸, and because the mouse and human phenotypes overlap, we hypothesized that IPEX is the human equivalent of mouse scurfy. We identified five unrelated, ethnically diverse families in which at least two boys were affected with IPEX (Table 1) and in which X-linked recessive inheritance was probable. In one affected male from each family, we sequenced both strands of

Table 1 • IPEX cases and results of mutation analysis of *FOXP3*

Family	Reference	Clinical characteristics	Outcome	DNA variation ^a	Amino acid change ^b
1	1	IDDM, enteropathy, hypothyroidism, thrombocytopenia, peritonitis	death at 5 weeks	1189C→T	R397W
2	11	IDDM, enteropathy, anemia, lymphadenopathy, eczema, sepsis	death at 10 months	del1290–1309 /insTGG	GPter>VGKGGWTNR-GQTGGRQRWWGQG
3	unpublished	IDDM, enteropathy, anemia, exfoliative dermatitis	living after bone marrow transplant	1113T→G	F371C
4	unpublished	IDDM, enteropathy, hypothyroidism, thrombocytopenia, exfoliative dermatitis, sepsis	death at 4 months	1150G→A	A384T
5	2, 10 and unpublished	enteropathy and eczema partially responsive to CSA ^c , later onset IDDM, asthma, food allergies	living, on chronic CSA	none	–

^aNumbers represent the base location in the cDNA sequence, where base 1 is the first base in the initiation codon. ^bter, termination codon. ^cCyclosporin A.

human *FOXP3* PCR products from genomic DNA representing exons –1 to 11, the 5' and 3' untranslated regions, and 50 intron bases at each exon end.

Unique mutations in *FOXP3* are present in the proband from families 1 through 4 (Table 1). One mutation is in exon 10 and the remainder are in exon 11. We found no coding mutation in the boy from family 5. The available obligate carriers (families 1, 2 and 4) are heterozygous for their respective mutations. An unaffected brother from family 2 lacks the mutation.

To address the possibility that the mutations represent polymorphisms rather than disease-causing mutations, we sequenced exons 10 and 11 in 240 unaffected, ethnically diverse individuals. No sequence variations were found.

The observed mutations all fall within the winged-helix domain of scurfin⁷, indicating they may disrupt a property such as DNA binding. We compared their positions with the aligned sequences of two winged-helix proteins, Genesis and HNF3γ, whose DNA-bound structures have been experimentally determined (Fig. 1). Scurfin A384 abuts a base-contacting asparagine residue in the major-groove-occupying helix 3. The normal alanine diverges from the serine found at this position in most forkhead proteins, whereas the mutant residue (threonine) is more similar to the consensus amino acid.

This may indicate that scurfin may bind to a different type of DNA target, compared with other winged-helix proteins. R397 is a conserved basic residue that contributes to a β-sheet. The cognate residue in Genesis contacts the DNA phosphate backbone. Although it is predicted to face away from the DNA in both Genesis and HNF3γ, the phenylalanine at scurfin position 371 is very highly conserved among winged-helix proteins⁹. The scurfin carboxy terminus corresponding to another DNA-contacting domain of Genesis and HNF3γ (Wing-2; Fig. 1) is erroneously extended in the *FOXP3* deletion/insertion mutant. We conclude that each of the mutations has a high likelihood of altering the putative DNA-binding property of scurfin.

Although the disease in family 5 maps to the pericentromeric region of the human X chromosome², we failed to find mutations in the coding region of *FOXP3*. A later age of onset, waxing and waning clinical course, and occasional compatibility with prolonged survival have been reported in this family¹¹. This contrasts with the early, unrelenting course seen in most cases¹. We suggest that family 5 may harbor a non-coding *FOXP3* mutation that affects transcription regulation or RNA splicing, resulting in a milder, environmentally dependent phenotype.

We conclude that IPEX is generally

caused by mutations in *FOXP3*, and is therefore the human equivalent of the scurfy mouse. The association of this highly conserved gene with a disease of dysregulated immune function underscores its importance in the maintenance of normal immune homeostasis in humans. Because IPEX patients suffer from IDDM, hypothyroidism, eczema and immune thrombocytopenia, it is possible that variations in the scurfin protein or in its regulation may have a role in the development of the familiar, common forms of these and other autoimmune disorders. It is tempting to propose that its manipulation might prevent or treat them. Our understanding of immune modulation should be increased by further study of the new molecular system defined by scurfin and its associates.

GenBank accession numbers. Human *FOXP3* genomic sequence, AF235097; human *FOXP3* cDNA, AF277993.

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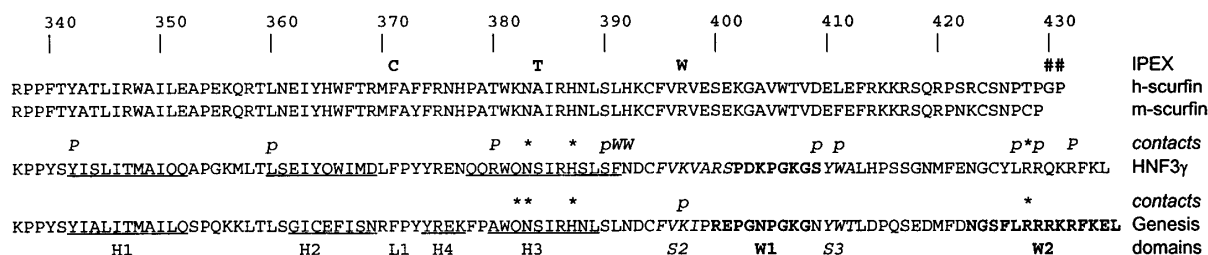


Fig. 1 Human (h) and mouse (m) scurfin, HNF3γ and Genesis winged-helix domain amino acid sequences with IPEX mutations. Numbering is for h-scurfin⁷. ##, See Table 1. HNF3γ (ref. 12) and Genesis¹³ structural features: underline, helix (H); bold, wing (W); italic, β-sheet (S); L, loop. DNA contacts: *, base; P, sense strand backbone; p, antisense strand backbone; W, water-mediated, to sense strand.



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The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of *FOXP3*

IPEX is a fatal disorder characterized by immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (MIM 304930). We present genetic evidence that different mutations of the human gene *FOXP3*, the ortholog of the gene mutated in scurfy mice (*Foxp3*), causes IPEX syndrome. Recent linkage analysis studies mapped the gene mutated in IPEX to an interval of 17–20-cM at Xp11.23–Xq13.3 (refs. 1,2).

The gene *WAS*, contained in this interval, was excluded as a candidate for IPEX (refs. 1,2). The scurfy syndrome in mice³ shares phenotypic features with IPEX and maps to a region of conserved synteny on the mouse X chromosome. Human and mouse *FOXP3* were recently identified by positional cloning and the 'scurfy' mutation was found to be a 2-bp insertion leading to a truncated *Foxp3* protein product⁴.

As scurfy mice share many phenotypic features with IPEX, we investigated the possibility that mutations in *FOXP3* might lead to IPEX. We examined three unrelated IPEX pedigrees for *FOXP3* mutations by direct sequencing of genomic DNA. A G→A transition (nt 1,338), resulting in a putative Ala→Thr substitution at residue 384 (A384T), segregated with the disease in all members tested from family 1 (Fig. 1a; ref.

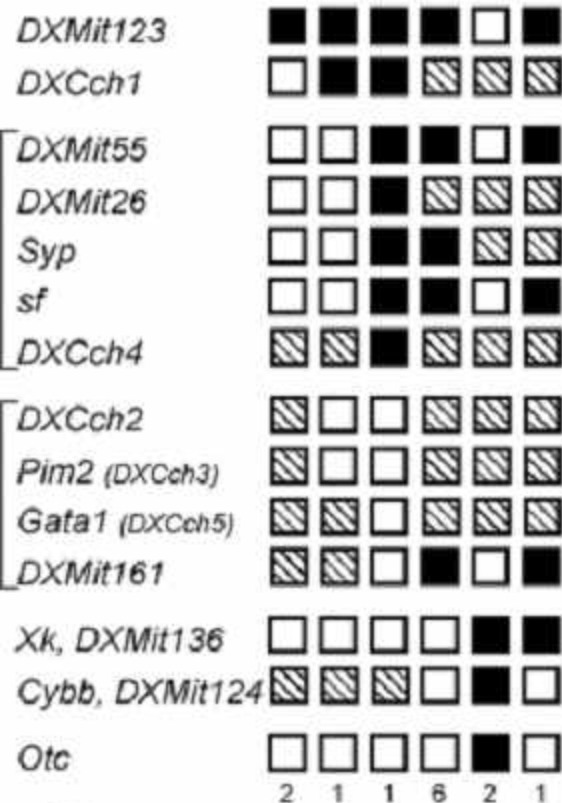
1). The two affected males tested (V-2 and V-7; Fig. 1a) were hemizygous for the mutation. Although the mutant residue threonine at 384 is chemically similar to serine (the most common amino acid found at this residue for human FOX proteins⁴), we hypothesize that the loss of the uniquely hydrophobic alanine residue of the scurfy protein causes IPEX in this family. We used a combination of standard and single-base sequence analyses of an additional 500 control X chromosomes and did not detect the A384T mutation, excluding the possibility that this mutation represents a polymorphism. In family 2 (Fig. 1b), we identified a CT dinucleotide deletion at 1481–1482 (delCT) within the *FOXP3* termination codon, predicting a frameshift and the addition of 25 amino acids (Z432T; Table 1). We did not detect mutation of *FOXP3* in family 3 (ref. 5). At this time, without additional studies, we cannot exclude the possibility of genetic heterogeneity within IPEX. These data, however, indicate that mutations of *FOXP3* can result in a severe clinical pheno-

Table 1 • Syndromes caused by mutations of human Fox-protein genes

Gene	Substitution/ deletion	Nucleotide	Phenotype	Disease transmission	Reference
<i>FOXP3</i>		MRPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRNHPATWKNAIRHNLSLHKCFVRVESE - - - - KGAVTVD - - - - Z			
<i>FOX1</i>		GKPPYSYIALIAMAIAHAPERRLTLGGIYKFITERFPFYRDNPQKQNSIRHNLTLDNCFKIPREAGRPQKGNWALD - - - - Z			
<i>FOX1</i>	♦ ↑↑	VKPPYSYIALITMAIQNAPDKKITLNGIYQFIMDRFPFYRDNPQKQNSIRHNLSLNECFVKVPRDDKKPKGSGYWTLD - - - - Z			
<i>FOXP3</i>	Ala→Thr(384)	GCC-ACC	IPEX (family 1)	X-linked recessive	—
<i>FOXP3</i>	Stop→Thr(432)	GCCcctGA	IPEX (family 2)	X-linked recessive	—
<i>FOX1</i>	Ala→Val(65)	GCC-GTC	thyroid agenesis	AR	7
<i>FOX1</i>	del(31–35)	CCGCGggcgccgggGGCG	Axenfeld-Rieger anomaly	AD	8
<i>FOX1</i>	Ser→Thr(82)	AGC-ACC	Axenfeld-Rieger anomaly	AD	8
<i>FOX1</i>	Ile→Met(87)	ATC-ATG	Axenfeld-Rieger anomaly	AD	8
<i>FOX1</i>	Phe→Ser(112)	TTC-TCC	anterior segment defect with glaucoma	AD	9
<i>FOX1</i>	Ile→Met(126)	ATC-ATG	Axenfeld anomaly, with glaucoma	AD	9
<i>FOX1</i>	Ser→Leu(131)	TCG-TTG	Rieger anomaly, with glaucoma	AD	9
<i>FOX1</i>	del(81–85)	CTGCgcacgcccagcAGTA	anterior segment defect with glaucoma	AD	9
<i>FOX1</i>	Gln→Term(23)	CAG-TAG	Axenfeld-Rieger syndrome	AD	10

Mutations identified in two human IPEX pedigrees are contrasted with previously reported mutations in *FOX1* and *FOX1*. The localization of mutations within the FOX-domain alignment (top) are indicated as follows: nonsense mutation, ♦, amino terminal of forkhead domain; deletion, ↑↑, N terminal of forkhead domain; and substitution, shaded amino acid residue. The bracketed amino acid residue numbering refers to its position in the specific proteins^{7–10}, and not the alignment shown.





musculus
 castaneus
 not typed

Supplementary InfoA. High resolution genetic mapping of the *sf* region. DNA samples from the *sf* x *M. spretus* backcross reported by Blair *et al.* (Mamm. Genome 5:652-654, 1994) were typed with additional microsatellite markers, whose order was ascertained from EUCIB map (Rhodes *et al.*, Genome Res. 8:531-542, 1998), to localize *sf* to the *DXMit123* - *Otc* interval. For the current study, 13 *DXMit123* - *Otc* recombinants were identified by typing 720 N2 animals obtained by crossing (*sf* *Otc*^{SPf} / + + x *M.m. castaneus*)F1 carrier females to normal *M. musculus* males. Sparse fur (*Otc*^{SPf}) results from a mutation in the *Otc* gene, and causes a visible phenotype in affected males (Veres *et al.*, Science 237:415-417, 1987). The scurfy phenotype was ascertained in recombinant N2 males directly, and in the male progeny of recombinant N2 females. These animals were typed further with microsatellite markers (*DXMit55*, -26, -161, -136, -124), as well as novel polymorphic markers developed for this study at the gene loci *Pim2* (*DXCch3*) and *Gata1* (*DXCch5*), and the BAC end-derived STS *DXCch1*, *DXCch2* and *DXCch4*. Markers are ordered from most proximal (top) to most distal (bottom); for markers inseparable in *sf* backcross (indicated by the half-brackets), order was confirmed by physical mapping and sequencing across BAC contig and/or by data from the EUCIB backcross map.

m	scurfy	Foxp3	RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFRR-----NHPATWKNA
h	Scurfy	FOXP3	RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFRR-----NHPATWKNA
m	Qrf	Foxp1	RPPFTYASLIRQAILESPEKQRTLNEIYNWFTRMFARFR-----RNAATWKNA
m	HNF3-β	Foxa2	KPPYSYISLITMAIQQSNNMLTLSEIYQWIMDLFPYR-----QNQQRWQNS
h	HNF3-γ	FOXA3	KPPYSYISLITMAIQQAGVLTSLSEIYQWIMDLFPYR-----DNQQRWQNS
z	fkf2	FoxA3	KPPYSYISLITMAIQQSQSMLTLNEIYQWIMDLFPYR-----ENQQRWQNS
x	XFD1	FoxA4a	KPPYSYISLITMAIQQAFNMMLTLNEIYQWIIDLFPYR-----QNQQRWQNS
d	fkf		KPPYSYISLITMAIQNNPTMLTLSEIYQFIMDLFPYR-----QNQQRWQNS
h	ILF-1	FOXK1a	KPPYSYAQLIVQAITMAFDKQLTLNGIYTHITKNYPYR-----TADKQWQNS
m	Whn	Foxn1	KEIYSYSILIFMALKNSKTGSLPVSEIYNFMTEHEPYFK-----TAPDGWQNS
h	FKHR	FOXO1a	WGNLSYADLITKAIESSAEKRLTLSEIYENMVKSVPYFKDKGDSNSSAGWKNS
c	daf-16		WGNMSYAEIITTAIMASPERLTLAQVYENMVQNVPMYFRDKGDSNSSAGWKNS

m	scurfy	Foxp3	IRHNLSLHKCFVRV-----ESEKG--AVWTV-----DEFEFRKKRSQR
h	Scurfy	FOXP3	IRHNLSLHKCFVRV-----ESEKG--AVWTV-----DEFEFRKKRSQR
m	Qrf	Foxp1	VRHNLSLHKCFVRV-----ENVKG--AVWTV-----DDVEFCRRRFPQK
m	HNF3-β	Foxa2	IRHSLSFNDCFLKVP-RSPDKPGKG--SFWTLHPDS--GNMFENG CYLRROK
h	HNF3-γ	FOXA3	IRHSLSFNDCFLKVA-RSPDKPGKG--SYWALHPSS--GNMFENG CYLRROK
z	fkf2	FoxA3	IRHSLSFNDCFLKVA-RSPDKPGKG--SYWALHPNS--GNMFENG CYLRROK
x	XFD1	FoxA4a	IRHSLSFNDCFLKVP-RSPEKPGKG--SYWTLHPES--GNMFENG CYLRROK
d	fkf		IRHSLSFNDCFLKIP-RTPDKPGKG--SFWTLHPDS--GNMFENG CYLRROK
h	ILF-1	FOXK1a	IRHNLSLNRYFIKVP-RSQEEPGKG--SFWRIDPASESK--LIEQAFRRKRFR
m	Whn	Foxn1	VRHNLSLNKCFEKVENKSGSSSRKG--CLNALNPSKIDK--MQEELQKWKRK-
h	FKHR	FOXO1a	IRHNLSLHSEFIRVQN-----EGTGKSSWMLNPEGGKSG-----KSPRR-R
c	daf-16		IRHNLSLHSEFMRIQN-----EGAGKSSWMLNPD-AKPG-----RNPRRTR

Supplementary InfoB. Multiple sequence alignment (using PileUp from GCG Wisconsin Package) of several members of the forkhead/winged-helix family demonstrate that scurf is most similar to "glutamine-rich factor", or Qrf (ref. 16). Residues identical in mouse and human scurf, as well as any other forkhead domains are highlighted with black background, while residues identical to either the corresponding mouse or human scurf residue are highlighted in gray. The letter preceding the gene name indicates species: m, mouse; h, human; x, xenopus; z, zebra fish; d, drosophila; c, c. elegans.