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MUTATION IN BRIEF

Identification of 34 Novel and 56 Known *FOXL2* Mutations in Patients With Blepharophimosis Syndrome

Diane Beysen[1], Sarah De Jaegere[1], David Amor[2], Philippe Bouchard[3], Sophie Christin-Maitre[3], Marc Fellous[4], Philippe Touraine[5], Arthur W. Grix[6], Raoul Hennekam[7], Françoise Meire[8,9], Nina Oyen[10], Louise C. Wilson[11], Dalit Barel[12], Jill Clayton-Smith[13], Thomy de Ravel[14], Christian Decock[8], Patricia Delbeke[8], Regina Ensenauer[15], Friedrich Ebinger[16], Gabriele Gillessen-Kaesbach[17], Yvonne Hendriks[18], Virginia Kimonis[19], Rachel Laframboise[20], Paul Laissue[4], Kathleen Leppig[21], Bart P. Leroy[1,8], David T. Miller[22], David Mowat[23], Luitgard Neumann[24], Astrid Plomp[25], Nicole Van Regemorter[26], Dagmar Wieczorek[17], Reiner A. Veitia[4], Anne De Paepe[1], and Elfride De Baere[1]

[1] Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium; Genetic Health Services Victoria, Royal Children's Hospital, Parkville, Australia; [3] Service d'Endocrinologie, Hôpital Saint-Antoine, Paris, France; [4] INSERM U709 Génomique et Epigénétique des Pathologies Placentaires and Universités Paris V & VII, Paris, France; [5] Department of Endocrinology and Reproductive Medicine, Pitie-Salpetrière Hospital, Paris, France; [6] The Permanente Medical Group, Department of Medical Genetics, Sacramento, California, USA; [7] Department of Pediatrics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [8] Department of Ophthalmology, Ghent University Hospital, Ghent, Belgium; [9] Ophthalmology Department, HUDERF-ULB, Brussels, Belgium; [10] Center of Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway; [11] Clinical and Molecular Genetics Unit, Institute of Child Health and Great Ormond Street Hospital, London, UK; [12] The Genetic Institute, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; [13] Academic Department of Medical Genetics, St. Mary's Hospital, Manchester, United Kingdom; [14] Center for Human Genetics, Catholic University of Leuven, Belgium; [15] Departments of Laboratory Medicine & Pathology and Medical Genetics, Mayo Clinic College of Medicine, Rochester, USA; [16] Department of Pediatric Neurology, University Children's Hospital, Heidelberg, Germany; [17] Universitätsklinikum Essen, Institut für Humangenetik, Essen, Germany; [18] Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands; [19] Department of Pediatrics, University of California, Irvine, Orange, California, USA; [20] Clinical Genetics Division, Department of Paediatrics and Medicine, CHUL, CHUQ, Faculty of Medicine, Laval University, Quebec, Canada; [21] Genetic Services, Group Health Cooperative (K.A.L.), Seattle, USA; [22] Division of Genetics, Children's Hospital, Boston, USA; [23] Sydney Children's Hospital, Dept of Clinical Genetics, Randwick, Australia; [24] Humangenetik, Charite Campus Virchow, Berlin, Germany; [25] Netherlands Institute for Neuroscience, an institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, The Netherlands; [26] Medical Genetics Department, Hôpital Erasme-ULB, Brussels, Belgium.

*Correspondence to Elfride De Baere, MD, PhD, Center for Medical Genetics, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium; Phone: +32-9-2405186; Fax: +32-9-2404970; E-mail: Elfride.DeBaere@UGent.be

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Blepharophimosis syndrome (BPES) is caused by loss-of-function mutations in the single-exon forkhead transcription factor gene *FOXL2* and by genomic rearrangements of the *FOXL2* locus. Here, we focus on 92 new intragenic *FOXL2* mutations, 34 of which are novel. Specifically, we found 10 nonsense mutations (11%), 13 missense mutations (14%), 40 deletions or insertions leading to a frameshift (43%), and 29 in-frame changes (32%), of which 28 (30%) lead to a polyalanine expansion. This study confirms the existence of two previously described mutational hotspots. Moreover, we gained novel insights in genotype-phenotype correlations, emphasizing the need to interpret genotype-phenotype correlations individually and always in the context of further clinical observations. © 2008 Wiley-Liss, Inc.

KEY WORDS: BPES, *FOXL2*, phenotype, genotype-phenotype correlations

INTRODUCTION

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; MIM# 110100) is an autosomal dominant developmental disorder that is characterized by a malformation of the eyelids, associated (BPES type I) or not (BPES type II) with premature ovarian failure (POF) (Zlotogora, et al., 1983). Loss-of-function mutations in the *FOXL2* gene (MIM# 605597), existing of a single exon and encoding a forkhead transcription factor located at 3q23, underlie both types of BPES (Crisponi, et al., 2001; De Baere, et al., 2001).

In previous studies, we reported on 43 *FOXL2* mutations and showed that intragenic mutations account for 70% of all molecular defects found in BPES patients (De Baere, et al., 2001; 2003). Our studies showed the existence of two mutational hotspots and demonstrated genotype-phenotype correlations for a subset of intragenic mutations. Mutations predicted to result in proteins with truncation before the poly-Ala tract may lead to BPES type I, whereas poly-Ala expansions were rather found in BPES type II. For missense mutations and mutations leading to a truncated or extended protein containing an intact forkhead domain and poly-Ala tract, no correlations were possible as they were found in both types of BPES and display intra- and interfamilial variability (De Baere, et al., 2003).

To date, more than 200 mutations have been reported in BPES patients, of which 100 unique (i.e. different) mutations. Most mutations reported so far have been collected in the Human *FOXL2* Mutation Database, available at http://medgen.ugent.be/foxl2/ (Beysen, et al., 2004). Here, we report on 92 newly identified *FOXL2* mutations, of which 34 are novel.

PATIENTS AND METHODS

Patients

Samples from 164 consenting probands with a tentative diagnosis of BPES were analysed. As most patients were external referrals, the following criteria were used to accept a diagnosis of BPES (for calculations of mutation detection rates): (1) availability of a facial picture of the proband confirming presence of four diagnostic criteria of BPES, including blepharophimosis, ptosis, epicanthus inversus and telecanthus; (2) patients for whom at least three of the four diagnostic criteria of BPES were mentioned on the clinical questionnaire. POF was defined as amenorrhea for a duration of $\geq$ 6 months at the age < 40 years and a concentration of follicle-stimulating hormone (FSH) of > 40 IU/L. Mutation screening of *FOXL2* was performed in 6 affected individuals from type I families, 10 from families with type II, 13 from families with unknown type, 86 individuals with sporadic disease, and 49 patients with atypical blepharophimosis. None of these patients have been included in previous mutation studies of *FOXL2*.

Mutational analysis

Genomic DNA was isolated from peripheral blood leukocytes by standard methods. Amplification and subsequent sequencing of the entire coding region of *FOXL2* was performed as described (De Baere, et al., 2003). Sequencing was carried out on an ABI Prism 3100, 3130 and 3730XL Genetic Analyser, according to the manufacturer's instructions (Applied Biosystems). All mutations found were confirmed by an independent analysis (i.e. sequencing of a second PCR amplicon). For all sequence variants found segregation analysis in parents or

other family members was performed when possible. If needed, 300 control chromosomes of Caucasian origin were screened for a particular sequence variation.

Mutation nomenclature

Mutation nomenclature is based on GenBank entry AF301906.1, with +1 corresponding to the A of the translation initiation codon ATG in the cDNA nomenclature, according to HGVS nomenclature guidelines (http://www.hgvs.org/mutnomen) (den Dunnen and Antonarakis, 2000). The gDNA numbering is also provided with the first nucleotide of mRNA sequence AF301906.1 as +1.

RESULTS AND DISCUSSION

In our study, a total of 164 unrelated patients with a tentative diagnosis of BPES were screened for mutations in the *FOXL2* gene by direct sequencing. This revealed 92 newly identified mutations, of which 34 have not been previously described (i.e. novel). The pedigrees of familial cases in whom a *FOXL2* mutation was found are depicted in Figure 1. Each patient or family has a unique identifier indicated with a number and the prefix "FOXL2_" that corresponds with the FOXL2 database identifier (FOXL2db-Id; http://medgen.ugent.be/foxl2/). An overview of their clinical features is given in Table 1.

Specifically, we found 10 nonsense mutations (11%), 13 missense mutations (14%), 40 frameshift mutations (43%), and 29 in-frame changes (32%), of which 28 (30%) lead to a poly-Ala expansion. These percentages are in agreement with our previous mutation studies (De Baere, et al., 2001; 2003). These mutations are summarized in Table 1, in which they are classified into 7 previously proposed groups according to their effect on the predicted protein that is likely to be produced as *FOXL2* is a single-exon gene (De Baere, et al., 2003).

A novel mutation c.40delG (p.Ala14fs) was classified in group A, representing mutations predicted to lead to a truncated protein without a forkhead domain. This mutation was found in three siblings whereas it was absent in their unaffected parents, suggesting germline mosaicism (FOXL2_00235). Haplotype analysis was performed to determine the parental origin of the mutation, but no informative results could be obtained (data not shown). This is the third reported case of germline mosaicism in BPES (Beysen, et al., 2005; Piemontese, et al., 2002).

Group B contains mutations predicted to lead to a truncated protein with a partial forkhead domain. In previous mutation studies, these mutations were mainly found in BPES type I (De Baere, et al., 2003). Interestingly, in a 2-generation BPES family (FOXL2_00142) *FOXL2* mutation c.290_294del5 (p.Gly97fs) was found in an affected mother and her son, which is suggestive of type II BPES. Another interesting family is FOXL2_00172, where both females with and without BPES suffer from POF. In individuals with BPES and POF a nonsense mutation c.244C>T (p.Gln82X) was found, while in the females with isolated POF this mutation was absent. It is thus unclear whether the POF phenotype in the females with BPES is due to the *FOXL2* mutation or representing a phenocopy.

Group C harbours nonsense mutations leading to predicted truncated proteins with complete forkhead domain and without the poly-Ala tract. It is interesting that the recurrent mutation c.655C>T (p.Gln219X) was found in a 1-year old patient with severe developmental delay and a complex heart defect (FOXL2_00174), whereas the same mutation was also found in patients with typical BPES without associated features (FOXL2_00144 and FOXL2_00145). Group D comprises new frameshift mutations leading to a truncated protein with a complete forkhead and poly-Ala domain. For this group, no clear-cut genotype-phenotype correlations could be made previously, as these mutations were found in both types of BPES (De Baere, et al., 2003). This is also corroborated by the current study: the recurrent mutation c.843_859dup (p.Pro287fs), located in a mutational hotspot in *FOXL2*, was found in a family with BPES type II (FOXL2_00188), as well as in three patients with BPES type I (FOXL2_00107; FOXL2_00241; FOXL2_00246). Moreover, this is the second *FOXL2* mutation with interfamilial variable expressivity of POF (De Baere, et al., 2003).

For group E mutations, comprising frameshift mutations leading to an elongated FOXL2 protein with a complete forkhead and poly-Ala domain, no genotype-phenotype correlations could be demonstrated (De Baere, et al., 2003). In the current study, two mutations belonging to this group c.855_871del17 (p.Pro287fs) and c.872dupA (p.His291fs) were found in two female patients suffering from fertility problems (FOXL2_00228 and FOXL2_00157 respectively). The other new mutations of this group occurred in sporadic male patients of which the BPES type could not be assessed.

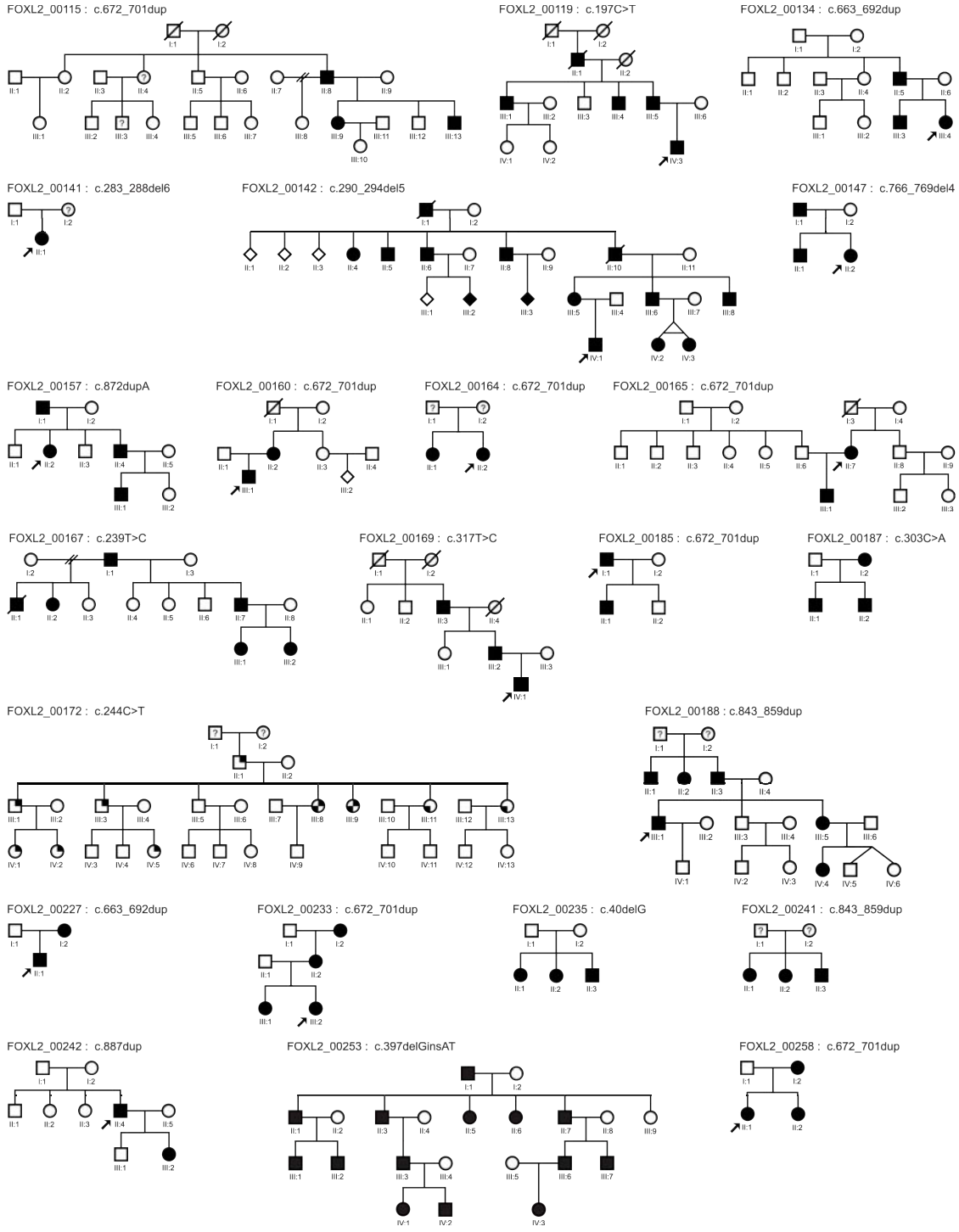

Figure 1: Pedigrees and respective *FOXL2* mutations of patients with BPES.
Affected individuals are indicated with filled symbols. Index patients are indicated with an arrow. In family FOXL2_00172, the patients with BPES are depicted by a black square in the right upper part of their symbol (◪ or ◔), the female patients with isolated POF have a black square in the left lower part of their symbol (◶) and female BPES patients who suffer from POF are represented by a symbol with black squares in the right upper and left lower corner (◕).

Table 1: New *FOXL2* mutations identified in BPES patients

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
TRUNCATION							
A No forkhead domain							
Fs, truncated to 148 aa	c.40delG	p.A14fs	g.277delG	FOXL2_00235	AU	F	BPES in 9-y and 6-y old sisters and their 3-year old brother. Their parents are unaffected, which is suggestive of germline mosaicism
B Partial forkhead domain							
Fs, truncated to 148 aa	c.167delC	p.P56fs	g.404delC	FOXL2_00105	DE-TR	S	BPES in 1-y old female. Father has small eyes and aunt bilateral ptosis
Fs, truncated to 148 aa	c.179delT	p.V60fs	g.416delT	FOXL2_00104	BE	S	BPES in 1-y old sporadic female
Fs, truncated to 81 aa	c.244C>T	p.Q82X	g.481C>T	FOXL2_00139	US	S	BPES in 14-y old sporadic female
Fs, truncated to 81 aa	c.244C>T	p.Q82X	g.481C>T	FOXL2_00172	AU	F	Three-generation BPES family. Affected and non-affected females have POF
Fs, truncated to 90 aa	c.273C>G	p.Y91X	g.510C>G	FOXL2_00140	IE-UK-PL	S	BPES in 1-y old sporadic male patient
Fs, truncated to 98 aa	c.290_294del5	p.G97fs	g.527_531del5	FOXL2_00142	CA	F	Two-generation BPES type II family, affected mother transmits BPES to son
Fs, truncated to 97 aa	c.294G>A	p.W98X	g.531G>A	FOXL2_00143	MA-BE	S	BPES in 2-y old sporadic male, low-set rotated ears
Fs, truncated to 117 aa	c.352G>T	p.E118X	g.589G>T	FOXL2_00223	BE	S	BPES in 41-year old sporadic female patient, oligomenorrhea
Fs, truncated to 237 aa	c.397delGinsAT	p.A133fs237ter	g.634delGinsAT	FOXL2_00253	FR	F1	Three-generation BPES family. Two affected females have POF
C Complete forkhead domain, no polyalanine tract							
Fs, truncated to 237 aa	c.556dupT	p.Y186fs	g.793dupT	FOXL2_00224	Sl	S	BPES in 53-year old male patient
Fs, truncated to 237 aa	c.576dupC	p.K193fs	g.813dupC	FOXL2_00256	US	S	BPES in 7 mo-old female patient. She has a cupped left ear, high arched eyebrows and very faint lower lid eyelashes
Fs, truncated to 193 aa	c.582C>G	p.Y194X	g.819C>G	FOXL2_00234	BE	S	BPES in 17-y old female patient with hypergonadotropic hypogonadism, primary amenorrhea and streaked ovaries.
Fs, truncated to 203 aa	c.611G>A	p.W204X	g.848G>A	FOXL2_00173	AU	S	BPES in 1-y old sporadic female patient
Fs, truncated to 218 aa	c.655C>T	p.Q219X	g.892C>T	FOXL2_00174	DE	S	BPES in 1-y old sporadic female, complex heart defect, severe mental retardation

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
Fs, truncated to 218 aa	c.655C>T	p.Q219X	g.892C>T	FOXL2_00144	FR	-	-
Fs, truncated to 218 aa	c.655C>T	p.Q219X	g.892C>T	FOXL2_00145	BE	S	BPES in 2-y old sporadic male patient. No associated anomalies.
D Complete forkhead domain and polyalanine tract							
Fs, truncated to 269 aa	c.710delG	p.G237fs	g.947delG	FOXL2_00129	BE	S	BPES in 1-y old sporadic female
Fs, truncated to 268 aa	c.766_769del4	p.G256fs	g.1003_1006del4	FOXL2_00147	-	F	Unilateral ptosis?
Fs, truncated to 269 aa	c.804delC	p.P268fs	g.1041delC	FOXL2_00148	NO	-	BPES in 1-y old female patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00106	TR	S	BPES in 4-y old sporadic female
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00107	US	S	BPES in 20-y old sporadic female with oligomenorrhea. Small uterus
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00108	DE	S	BPES in 2-y old sporadic male
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00150	FR	-	-
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00151	LK	S	BPES in 4-y old sporadic male
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00152	FI	S	BPES in 1,5-y old sporadic male
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00153	NL	-	BPES in 34-y old male
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00175	BE	S	BPES in 3-y old sporadic female patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00186	BE	S	BPES in 1-year old sporadic female patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00188	US	F2	Four-generation BPES type II family
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00226	CA	S	BPES in 3,5-y old sporadic female patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00225	BE	S	BPES in 5-y old sporadic male patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00241	FR	F1	BPES in a 30-y old female patient with ovarian insufficiency. Her father, four brothers and sisters also have the BPES-phenotype
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00246	DE	S1	BPES in 17-y old sporadic female patient with primary amenorrhea
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00252	FR	S	BPES in a 18-mo old female patient
Fs, truncated to 360 aa	c.843_859dup	p.P287fs	g.1080_1096dup	FOXL2_00257	US	S	BPES in 12-y old female sporadic patient

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
Fs, truncated to 362 aa	c.843_865dup	p.H289fs	g.1080_1102dup	FOXL2_00109	FR	S1	BPES in 31-y old sporadic female. Secondary amenorrhea at 17 y
Fs, truncated to 360 aa	c.855_871dup	p.H291fs	g.1092_1108dup	FOXL2_00176	CA	S	BPES in 4-y old sporadic female
Fs, truncated to 360 aa	c.855_871dup	p.H291fs	g.1092_1108dup	FOXL2_00237	BE	S	BPES in 11-y old female patient
Fs, truncated to 162 aa	c.887dup	p.H297fs	g.1124dup	FOXL2_00242	FR	F	Two generation BPES family. A de novo mutation was found in a 38-y old male patient, which was transmitted to his daughter.
Fs, truncated to 357 aa	c.912_919dup	p.P307fs	g.1149_1156dup	FOXL2_00110	UK	S	BPES in 1-y old sporadic female
Fs, truncated to 354 aa	c.959delG	p.G320fs	g.1196delG	FOXL2_00154	FR	-	-
Fs, truncated to 338 aa	c.997_1045del49	p.P333fs	g.1234_1282del49	FOXL2_00155	SE-CA	S	BPES in 2-y old sporadic female
Fs, truncated to 354 aa	c.1056delG	p.E352fs	g.1293delG	FOXL2_00156	NL	S	BPES in 11-y old sporadic female with psychomotor retardation
ELONGATION							
E Complete forkhead domain and polyalanine tract							
Fs, extended to 526 aa	c.855_871del17	p.P287fs	g.1092_1108del17	FOXL2_00228	IL	S1	BPES in 30-year old sporadic female BPES patient, oligomenorrhea
Fs, extended to 532 aa	c.872dupA	p.H291fs	g.1109dupA	FOXL2_00157	NL	F1	BPES in 56-y female with secondary amenorrhea before 40 y
Fs, extended to 528 aa	c.947_957del11	p.A316fs	g.1184_1194del11	FOXL2_00158	-	-	-
Fs, extended to 529 aa	c.949_956del	p.P317fs	g.1186_1193del	FOXL2_00245	FR	S	BPES in 21-y old sporadic male patient
Fs, extended to 527 aa	c.971_984del	p.P324fs	g.1208_1221del	FOXL2_00244	CA	S	BPES in 17-y old sporadic male patient
Fs, extended to 528 aa	c.1127_1139del insCG	p.L376fs	g.1364_1376del insCG	FOXL2_00240	SE	S	BPES in 6-mo old sporadic male patient
F IN-FRAME MUTATION							
In-frame deletion in FKH domain	c.283_288del6	p.K95_K96del	g.520_525del6	FOXL2_00141	FR	F	Affected female, unaffected mother carries the mutation in less than 50% of the DNA from peripheral blood leukocytes, which is suggestive of somatic and germline mosaicism in this patient
Polyalanine expansion	c.663_692dup	p.A221_A231dup	g.900_929dup	FOXL2_00134	AU	F	Two-generation BPES pedigree

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
Polyalanine expansion	c.663_692dup	p.A221_A231dup	g.900_929dup	FOXL2_00166	UK	S	BPES in 4-y old male patient with developmental delay, unilateral conductive hearing loss (probably due to glue ear), dysmorphic appearance
Polyalanine expansion	c.663_692dup	p.A221_A231dup	g.900_929dup	FOXL2_00227	-	F2	Seven-year old male BPES patient with cleft palate (Pierre Robin syndrome) and his 29-year old mother, who has very long menstrual cycles (3 months) and polycystic ovaries
Polyalanine expansion	c.663_692dup	p.A221_A231dup	g.900_929dup	FOXL2_00238	IT	S	BPES in 1-y old sporadic male patient with microtia, hypospadias and a small unilateral retinal coloboma
Polyalanine expansion	[c.664_701dup; 701_702insT	p.A221_A234dup	g.901_938dup; g.938_939insT]	FOXL2_00189	NO	S	BPES in 4-y old female patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00111	UK	S	BPES in 8-y old female patient. Associated feature: mild 2/3 skin syndactyly
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00112	NL	S	BPES in 3-y male sporadic patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00113	BE	S	BPES in 31-y sporadic female. Pregnancy after IVF treatment at 31 y. No hypergonadotrophic hypogonadism
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00114	NL	S	BPES in 12-y old male patient, pediatric Burkitt lymphoma
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00115	NO	F2	Two-generation BPES pedigree; affected female has unaffected child at 30 y, oligomenorrhea, irregular cycles, FSH borderline high
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00132	BE	S	BPES in 3-y old female sporadic patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00160	IT-BE	F2	Two-generation BPES type II pedigree. Affected female transmits disease
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00161	mix	S	BPES in 18-m old sporadic female patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00162	BE	S2	BPES in 31-y sporadic female.
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00163	UK	S1	BPES in 37-y old sporadic female, menarche at 13 years, very infrequent and irregular menses, one spontaneous conception at 34 y
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00164	AT	F	One-y old and 4-y old sisters with BPES
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00165	FI	F2	Two-generation BPES type II family, affected mother transmits BPES to son

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00177	SE	-	-
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00178	FR	S1	BPES and POF in 35-y old female
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00184	BE	S	BPES in a 1-year old sporadic female. She also has a small apical muscular ventricular septal heart defect
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00185	NL	F	BPES in 4-year old male patient and his 29-year old father
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00231	BE	S	BPES in 3-y old sporadic female patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00233	BE	F2	Three-generation BPES family. No fertility problems reported
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00236	NO	F2	BPES in 10-y old female, and at least seven affected family members, including her brother and father. No fertility problems have been reported
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00247	BE	S	BPES in 11-y old sporadic female patient
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00250	SA	F	BPES in 10-y old male patient and his father
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00255	PT	S	A child with sporadic BPES
Polyalanine expansion	c.672_701dup	p.A224_A234dup	g.909_938dup	FOXL2_00258	US	F	Two-generation BPES family. Affected 36-y old male transmits BPES to his 2 daughters
G MISSENSE MUTATION							
Missense in FKH domain	c.173C>T	p.S58L	g.410C>T	FOXL2_00118	NO	S	BPES in 1-y old female, *de novo*
Missense in FKH domain	c.193A>G	p.M65V	g.430A>G	FOXL2_00249	UK	S?	A child with sporadic BPES
Missense in FKH domain	c.197C>T	p.A66V	g.434C>T	FOXL2_00119	DE	F	Three-generation BPES pedigree (male-to-male transmission)
Missense in FKH domain	c.197C>T	p.A66V	g.434C>T	FOXL2_00120	UK	S	BPES in 3-y old female, *de novo*
Missense in FKH domain	c.205G>A	p.E69K	g.442G>A	FOXL2_00121	US (GR)	S	BPES in 5-y old male, *de novo*. Associated features: bilateral vocal cord nodules and VSD
Missense in FKH domain	c.239T>C	p.I80T	g.476T>C	FOXL2_00167	MA	F1?	Three-generation BPES family
Missense in FKH domain	c.251T>A	p.I84N	g.488T>A	FOXL2_00179	NO	-	-

Predicted protein change [a]	cDNA mutation name [b]	Protein mutation name	gDNA mutation name [c]	FOXL db identifier	Geographic origin [d]	BPES type [e]	Clinical information
Missense in FKH domain	c.269T>C	p.F90S	g.506T>C	FOXL2_00180	BE	S	BPES in 2-y old sporadic female
Missense in FKH domain	c.292T>G	p.W98G	g.529T>G	FOXL2_00168	US	S	BPES in 1-y old male, *de novo*
Missense in FKH domain	c.303C>A	p.S101R	g.540C>A	FOXL2_00187	BE	F	Mother and two sons with mild phenotype
Missense in FKH domain	c.305T>C	p.I102T	g.542T>C	FOXL2_00222	US	S	BPES in 17-y old male with laterally protruding ears, cleft lip and mild syndactyly
Missense in FKH domain	c.307C>T	p.R103C	g.544C>T	FOXL2_00232	BE	S	BPES in 1-y old female
Missense in FKH domain	c.317T>C	p.L106P	g.554T>C	FOXL2_00169	UK	F	Three-generation BPES pedigree (male-to-male transmission)

a Mutations are classified into 7 groups according to their effect on the predicted protein as described by De Baere et al., 2003.

b The systematic mutation nomenclature is in accordance with the most recent HGVS guidelines (www.hgvs.org/mutnomen), in which +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (GenBank AF301906.1).

c gDNA numbering is in relation to the same genomic reference sequence with the A of the initiator methionine codon as nucleotide 238 (Crisponi et al., 2001).

d Geographic origin: AU Australia; DE Germany; TR Turkey; BE Belgium; US United States; IE Ireland; UK United Kingdom; PL Poland; CA Canada; MA Morocco; FR France; Sl Slovenia; NO Norway; LK Sri Lanka; FI Finland; NL The Netherlands; SE Sweden; IL Israel; IT Italy; AT Austria; SA Saoudi Arabia; PT Portugal; GR Greece.

e BPES type: F familial; S sporadic; F1 familial type 1; F2 familial type 2; S1 sporadic type 1; S2 sporadic type 2

\- Data not available

Table 2: Evaluation of pathogenic potential of missense mutations in the forkhead domain

Missense mutation in FKH	Segregation (see *FOXL2* Mutation Database)	Polyphen	SIFT	Grantham score	Localization studies (Beysen, et al. 2008b)	Transactivation assays (Beysen, et al. 2008b)	Overall scoring of clinical significance
p.S58L	*De novo* (FOXL2_00118)	Possibly damaging	Affect protein function	145 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation function	Disease-causing
p.M65V	Not performed	Probably damaging	Affect protein function	21	Not performed	Not performed	Probably disease-causing
p.A66V	*De novo* (FOXL2_00120); segregation in 3-generation family (FOXL2_00119)	Predicted to be benign	Affect protein function	64 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation (suggestive of weak dominant negative effect)	Disease-causing
p.E69K	*De novo* (FOXL2_00121)	Predicted to be benign	Tolerated	56	Nuclear aggregation	Normal transcriptional activation	Disease-causing
p.I80T	Segregation in 3-generation family (FOXL2_00167)	Possibly damaging	Affect protein function	33	Nuclear and cytoplasmic aggregation	Impaired transactivation (suggestive of weak dominant negative effect)	Disease-causing
p.I84N	Not performed	Probably damaging	Affect protein function	149 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation function	Disease-causing
p.F90S	Not performed	Probably damaging	Affect protein function	155 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation function	Disease-causing
p.W98G	*De novo* (FOXL2_00168)	Probably damaging	Affect protein function	184 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation function	Disease-causing
p.S101R	Segregation in 2-generation family (FOXL2_00187)	Possibly damaging	Affect protein function	110 (> 60)	Nuclear aggregation	Impaired transactivation function	Disease-causing
p.I102T	Not performed	Possibly damaging	Affect protein function	33	Nuclear and cytoplasmic aggregation	Impaired transactivation (suggestive of weak dominant negative effect)	Disease-causing
p.R103C	Not performed	Probably damaging	Affect protein function	180 (> 60)	Nuclear and cytoplasmic aggregation	Normal transcriptional activation	Disease-causing
p.L106P	Segregation in 3-generation family (FOXL2_00169)	Probably damaging	Affect protein function	98 (> 60)	Nuclear and cytoplasmic aggregation	Impaired transactivation (suggestive of weak dominant negative effect)	Disease-causing

In group F, with mutations leading to in-frame changes, a 6-bp deletion within the forkhead domain c.283_288del6 (p.Lys95_Lys96del) was found in a female patient (FOXL2_00141). Surprisingly, her apparently unaffected mother carries the same mutation in less than 50% of DNA from peripheral blood leukocytes (data not shown), demonstrating somatic mosaicism and assuming gonadal mosaicism. Although it was postulated that poly-Ala expansions may lead to BPES type II, fertility problems have been observed in four unrelated females (FOXL2_00113; FOXL2_00115; FOXL2_00163; FOXL2_00178) with the recurrent mutation c.672_701dup (p.Ala224_Ala234dup). In addition, the poly-Ala expansion c.663_692dup (p.Ala221_Ala231dup) was found in a 7-year old male and his 29-year old mother who suffers from oligomenorrhea and polycystic ovaries. In those patients in whom hormonal investigations could be performed, no hypergonadotrophic hypogonadism was found. These and two additional cases previously described (FOXL2_00051; FOXL2_00071) (Crisponi, et al., 2002; De Baere, et al., 2001), suggest that poly-Ala expansions might lead to BPES with a mild ovarian phenotype characterized by late-onset ovarian failure. This is further discussed in the “Mutation Update” of this issue (Beysen, et al. 2008a).

Group G, the last one, contains missense mutations. In this study, 13 missense mutations have been identified, all located in the forkhead domain of *FOXL2* (Figure 2). They are all presumed to be disease-causing based on some or all of several arguments, which are outlined in Table 2: (1) they all affect amino acid residues that are highly conserved in a wide range of FOXL2 orthologues; (2) some of them occur *de novo* or (3) co-segregate with the BPES phenotype; (4) none of them have been found in more than 300 matched control chromosomes; (5) Polyphen and SIFT predictions (Ramensky, et al. 2002; Ng and Henikoff, 2001), Grantham score calculations (score of > 60) (Grantham, 1974) suggest an effect on protein function; (6) localization and/or transactivation studies suggest a deleterious effect (Beysen, et al. 2008b). No genotype-phenotype correlations can be made for these mutations, as they were found in prepubertal sporadic female patients, sporadic male patients or families in which the BPES type cannot be specified.

Intragenic *FOXL2* mutations usually lead to BPES type I or II without developmental delay or other associated features. In this study however, several unusual features have been described in association with BPES. A ventricular septal defect (VSD) has been described in a 1-year old sporadic female patient (FOXL2_00184) with a poly-Ala expansion (c.672_701dup; p.Ala224_Ala234dup) and a 5-year old sporadic male patient (FOXL2_00121) with missense mutation c.205G>A (p.Glu69Lys). In a 1-year old sporadic female patient (FOXL2_00174) with nonsense mutation c.655C>T (p.Gln219X), BPES is associated with a complex heart malformation and severe developmental delay. Developmental problems were also observed in an 11-year old sporadic female patient (FOXL2_00156) with mutation c.1056delG (Glu352fs) and a 4-year old sporadic male with unilateral conductive hearing loss (FOXL2_00166), carrying a poly-Ala expansion (c.663_692dup; p.Ala221_Ala231dup). Other associated features are mild syndactyly in an 8-year old female (FOXL2_00111) with c.672_701dup (p.Ala224_Ala234dup) and a 17-year old male (FOXL2_00222) carrying c.305T>C (p.Ile102Thr). In FOXL2_00222 a cleft lip was present in addition to BPES. A 7-year old male (FOXL2_00227) with c.663_692dup (p.Ala221_Ala231dup) displayed a Pierre Robin syndrome. Finally, a paediatric Burkitt lymphoma was reported in a 12-year old sporadic male (FOXL2_00114) who carries the recurrent poly-Ala expansion c.672_701dup (p.Ala224_Ala234dup). In general, these associated symptoms are presumed not to be the result of a wider pleiotropic effect of FOXL2 in development but rather of other genetic or environmental factors.

In conclusion, sequencing of the coding region of *FOXL2* in a cohort of 116 unrelated patients with the clinical diagnosis of BPES revealed 92 new *FOXL2* mutations, including 34 novel ones. This approach allowed us to detect a causal mutation in approximately 80% of typical BPES, which is in agreement with previous mutation studies (De Baere, et al., 2001; 2003). The current study confirms the presence of two mutational hotspots described by us (De Baere, et al., 2003). The mutation c.843_859dup (p.Pro287fs) was found in 18% of the mutations identified here (17/92), while 30% (28/92) of the mutations in the coding region lead to poly-Ala expansions. In addition, we described several additional atypical features in BPES patients, possibly occurring independently from *FOXL2* mutations. Moreover, several exceptions to the current genotype-phenotype correlations were found, as well as intra- and interfamilial variable expressivity of POF. These data emphasize that molecular testing alone is not sufficient as a predictive marker for female infertility, and that genotype-phenotype correlations need to be interpreted individually and always in the context of further clinical observations.

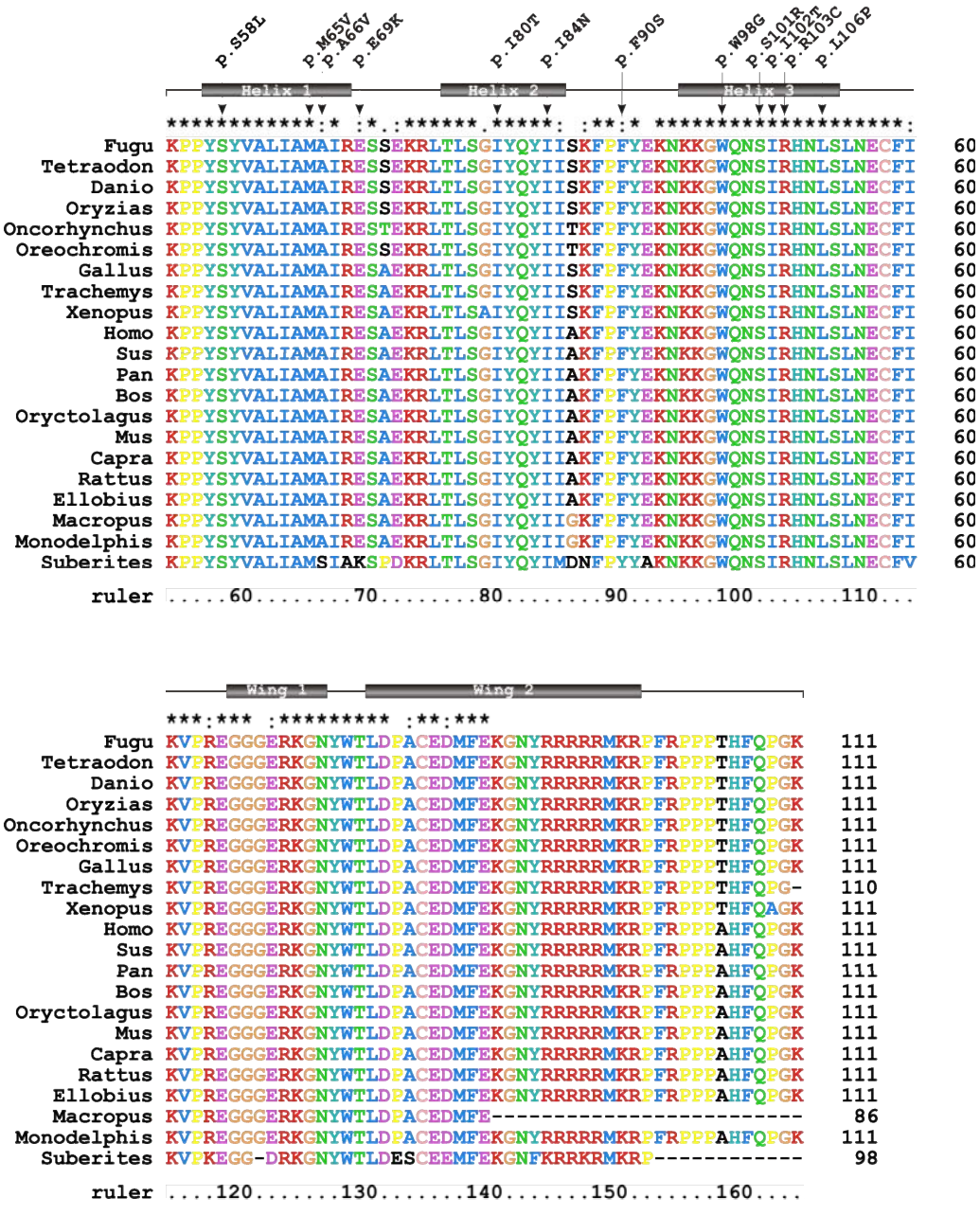

Figure 2: Multiple-sequence alignment of the forkhead domain of *FOXL2* orthologues and missense mutations in *FOXL2*

At the top, a consensus line indicates conservation of the amino acid residues: an asterisk (*) indicates fully conserved sites, a colon (:) points to a conserved substitution and a point (.) to a semi-conserved substitution. Amino acids are coloured according the consensus Clustal X colour scheme. The position of predicted 'helix' and 'wing' segments are indicated at the top of the alignment. Missense mutations detected in this study, are indicated with an arrow above the sequence at their respective amino acid positions.

Fugu: *Takifugu rubripes*, Torafugu (Scaffold_8165/ProtJGI_24134); Tetraodon: *Tetraodon nigroviridis*, tetraodon (Cocquet, et al., 2003); Oreochromis: *Oreochromis niloticus*, nile tilapia (AY554172); Oryzias: *Oryzias latipes*, medaka (AB252055) (Nakamoto, et al., 2006); Oncorhynchus: *Oncorhynchus mykiss*,

rainbow trout (AY507927); Danio: *Danio Rerio*, zebra fish (XP_685310); Xenopus: *Xenopus tropicalis*, frog (ENSXETG00000008253); Gallus: *Gallus gallus*, chicken (NM_001012612); Bos: *Bos taurus*, domestic cow (AY340970); Capra: *Capra hircus*, domestic goat (AY112725); Sus: *Sus scrofa*, wild boar (AY340971); Homo: *Homo sapiens*, human (AF301906.1); Pan: *Pan troglodytes*, chimpanzee (ENSPTRG00000015453); Mus: *Mus musculus*, house mouse (AF522275); Rattus: *Rattus norvegicus*, rat (AC105826); Ellobius: *Ellobius lutescens*, Transcaucasian mole vole (AY623815); Oryctolagus: *Oryctolagus cuniculus*, European rabbit (AY340972); Macropus: *Macropus eugenii*, tammar wallaby (AY340969); Monodelphis: *Monodelphis domestica*, short-tailed opossum (ENSMODG00000018597); Trachemys: *Trachemys scripta*, red-eared slider turtle (AY155535); Suberites: *Suberites domuncula*, sponge (AJ585266)

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REFERENCES

Beysen D, De Paepe A, De Baere E. 2008. FOXL2 mutations and genomic rearrangements in BPES. Hum Mutat 29; online publication ahead of print.

Beysen D, Moumné L, Veitia RA, Peters H, Leroy BP, De Paepe A, De Baere E. 2008. Missense mutations in the forkhead domain of *FOXL2* lead to subcellular mislocalisation, protein aggregation and impaired transactivation. Hum Molec Genet, doi: 10.1093/hmg/ddn100.

Beysen D, Raes J, Leroy BP, Lucassen A, Yates JR, Clayton-Smith J, Ilyina H, Brooks SS, Christin-Maitre S, Fellous M and others. 2005. Deletions involving long-range conserved nongenic sequences upstream and downstream of FOXL2 as a novel disease-causing mechanism in blepharophimosis syndrome. Am J Hum Genet 77(2):205-18.

Beysen D, Vandesompele J, Messiaen L, De Paepe A, De Baere E. 2004. The human FOXL2 mutation database. Hum Mutat 24(3):189-93.

Cocquet J, De Baere E, Gareil M, Pannetier M, Xia X, Fellous M, Veitia RA. 2003. Structure, evolution and expression of the FOXL2 transcription unit. Cytogenet Genome Res 101(3-4):206-11.

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S and others. 2001. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27(2):159-66.

Crisponi L, Uda M, Chiappe F, Delana M, Usala GL, Maronqiu M, Amati P, Bonneau D, Faravelli F, Tolmie J and others. 2002. Genetic basis for non-syndromic and syndromic, blepharophimosis/ptosis and epicanthus inversus syndrome (BPES)-associated premature ovarian failure (POF). American Journal of Human Genetics 71(4):324-324.

De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon M, Fellous M, Fryns JP and others. 2003. FOXL2 and BPES: mutational hotspots, phenotypic variability, and revision of the genotype-phenotype correlation. Am J Hum Genet 72(2):478-87.

De Baere E, Dixon MJ, Small KW, Jabs EW, Leroy BP, Devriendt K, Gillerot Y, Mortier G, Meire F, Van Maldergem L and others. 2001. Spectrum of FOXL2 gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES) families demonstrates a genotype--phenotype correlation. Hum Mol Genet 10(15):1591-600.

den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 15(1):7-12.

Grantham R. 1974. Amino acid difference formula to help explain protein evolution. Science 185(4154):862-864

Nakamoto M, Matsuda M, Wang DS, Nagahama Y, Shibata N. 2006. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, Oryzias latipes. Biochem Biophys Res Commun 344(1):353-61.

Ng PC, Henikoff S. 2001. Predicting deleterious amino acid substitutions. Genome Res 11(5):863-874

Piemontese M, Gasparini P, Zelante L. 2002. Paternal constitutional mosaicism in familial BPES. European Journal of Human Genetics 10:131-131.

Ramensky V, Bork P, Sunyaev S. 2002. Human non-synonymous SNPs: server and survey. Nucleic Acids Res 30(17):3894-3900

Zlotogora J, Sagi M, Cohen T. 1983. The blepharophimosis, ptosis, and epicanthus inversus syndrome: delineation of two types. Am J Hum Genet 35(5):1020-7.