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Reduced TFAP2A function causes variable optic fissure closure and retinal defects and sensitizes eye development to mutations in other morphogenetic regulators

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Abstract

Mutations in the transcription factor encoding *TFAP2A* gene underlie branchio-oculo-facial syndrome (BOFS), a rare dominant disorder characterized by distinctive craniofacial, ocular, ectodermal and renal anomalies. To elucidate the range of ocular phenotypes caused by mutations in *TFAP2A*, we took three approaches. First, we screened a cohort of 37 highly selected individuals with severe ocular anomalies plus variable defects associated with BOFS for mutations or deletions in *TFAP2A*. We identified one individual with a de novo *TFAP2A* four amino acid deletion, a second individual with two non-synonymous variations in an alternative splice isoform *TFAP2A2*, and a sibling-pair with a paternally inherited whole gene deletion with variable phenotypic expression. Second, we determined that *TFAP2A* is expressed in the lens, neural retina, nasal process, and epithelial lining of the oral cavity and palatal shelves of human and mouse embryos—sites consistent with the phenotype observed in patients with BOFS. Third, we used zebrafish to examine how partial abrogation of the fish ortholog of *TFAP2A* affects the penetrance and expressivity of ocular phenotypes due to mutations in genes encoding *bmp4* or *tcf7l1a*. In both cases, we observed synthetic, enhanced ocular phenotypes including coloboma and anophthalmia when *tfap2a* is knocked down in embryos with *bmp4* or *tcf7l1a* mutations. These results reveal that mutations in *TFAP2A* are associated with a wide range of eye phenotypes and that hypomorphic *tfap2a* mutations can increase the risk of developmental defects arising from mutations at other loci.

Introduction

The rare dominantly inherited branchio-oculo-facial syndrome (BOFS [MIM 113620]) manifests predominantly as anomalies affecting first and second pharyngeal arches, including ocular (anophthalmia, microphthalmia, coloboma, or cataract), cervical, or periauricular cutaneous defects, and distinctive craniofacial features (including cleft lip, often incomplete, cleft/high arched palate, and malformed ears). Other manifestations include ectodermal anomalies (hypodontia, dysplastic nails, and prematurely grey hair), conductive hearing loss, inner ear malformation, nasolacrimal duct obstruction, ectopic dermal thymus, scalp cysts, renal anomalies, short stature, upper lip pits, and mild developmental delay (Lin et al. 1995; Tekin et al. 2009).

Mutations or deletions in *TFAP2A* have recently been identified in seven individuals with BOFS syndrome (Milunsky et al. 2008; Tekin et al. 2009). An affected mother and son pair had a 3.2 Mb deletion at chromosome 6p24.3, encompassing the *TFAP2A* locus. Four additional cases had de novo missense mutations located within the highly conserved basic region of the *TFAP2A* DNA binding domain (Milunsky et al. 2008) and one further case a complex mutation within the DNA binding and dimerization domains (Tekin et al. 2009) (Fig. 1). Deletions at chromosome 6p24 have also been described in two patients with ocular findings: one with anterior chamber anomalies and a 6p24–p25 interstitial deletion, and the other with sclerocornea associated with a 6p22.3–p24 interstitial deletion (Davies et al. 1999; Moriarty and Kerr-Muir 1992). These cases raise the possibility that *TFAP2A* might

be more broadly involved in eye development, and that disruption of *TFAP2A* is associated with phenotypes other than BOFS.

TFAP2A is a member of the AP2 family of transcription factors that direct craniofacial and ocular development (Ahituv et al. 2004; Schorle et al. 1996; Zhang et al. 1996). In mice and zebrafish, *Tfap2a* is expressed in tissues that contribute to the developing eye, including lens primordium and neural crest (Bosserhoff et al. 1997; Knight et al. 2003; Mitchell et al. 1991; O'Brien et al. 2004). *Tfap2a* mutants in mice and zebrafish share similar craniofacial defects, but while *Tfap2a* null mice exhibit ocular phenotypes ranging from anophthalmia to defects in the developing lens and optic cup (Makhani et al. 2007; Schorle et al. 1996; West-Mays et al. 1999), no obvious eye abnormalities have been described in zebrafish *tfap2a* mutants (Knight et al. 2003). However, a link between *tfap2a* and eye development in zebrafish is suggested because simultaneous inhibition of *tfap2a* and *tfap2c* gives rise to embryos with abnormal eye and lens morphology (Li and Cornell 2007).

TFAP2A is a retinoic acid (RA)-responsive gene that acts in concert with other signals to regulate eye morphogenesis. For instance, development of the ventral eye is regulated by interactions between Hh (Hedgehog), RA, and fibroblast growth factor (Fgf) signals (Lupo et al. 2005; Takeuchi et al. 2003). The Wnt and Bmp (bone morphogenetic protein) pathways are also involved in eye formation and regionalization. In fish, enhanced Wnt signalling in the neural plate leads to anophthalmia (Cavodeassi et al. 2005; Heisenberg et al. 2001; Houart et al. 2002) and in mice, loss of function of the Wnt co-receptor *Lrp6* leads to microphthalmia and coloboma (Zhou et al. 2008). Overexpression of the Bmp antagonist Noggin induces microphthalmia, coloboma, and other ventral abnormalities in chick, suggesting that endogenous Bmps have significant effects on the development of the optic cup (Adler and Belecky-Adams 2002). Indeed, in *Bmp4*^{+/-} mice, the optic cup forms, but mature eyes show anterior segment abnormalities and an increased incidence of microphthalmia (Chang et al. 2001). Furthermore, recently we reported two mutations in the *BMP4* gene that compromise eye development: a frameshift mutation in a family with anophthalmia-microphthalmia, retinal dystrophy and myopia, and a missense mutation in an individual with anophthalmia-microphthalmia and brain anomalies (Bakrania et al. 2008). In addition, mutations in the BMP family protein, *GDF6*, in humans and fish cause variable ocular anomalies including coloboma, microphthalmia, and retinal mis-patterning (Asai-Coakwell et al. 2009; Gosse and Baier 2009).

In order to elucidate the role of *TFAP2A* in eye development, we determined whether *TFAP2A* mutations are associated with congenital eye defects in humans outside the confines of BOFS and also investigated the embryonic expression pattern of the human gene. In human and animal models, many congenital eye defects are variably expressed suggesting that genetic and environmental factors can significantly affect penetrance. To explore this idea, we established a zebrafish model for studying *tfap2a* function in eye development to determine the phenotypic consequences of disrupting *tfap2a* function in zebrafish heterozygous or homozygous for mutations affecting Wnt and Bmp signalling. Our findings indicate that *TFAP2A* is expressed in developing neural and craniofacial tissues in humans as in other vertebrates, plays a critical role in eye morphogenesis in both humans and fish, that *TFAP2A* mutations are associated with a wide spectrum of ocular phenotypes, and compromised *tfap2a* function sensitizes the developing eye to the effects of deleterious mutations in *bmp4* and *tcf7l1a*.

Materials and methods

Thirty-seven cases were selected from a larger cohort of patients with developmental eye anomalies recruited as part of a national anophthalmia study based at Moorfields Eye

Hospital, London and Birmingham Children's Hospital, Birmingham, UK. Informed consent for genetic analyses was obtained from all families, in accordance with Ethics approval 04/Q0104/129 by the Cambridgeshire 1 Ethics Committee. Three individuals, including one affected sibling-pair, demonstrated at least three typical BOFS features. The other cases had anophthalmia or microphthalmia associated with a partial phenotype of BOFS including facial anomalies (including cleft lip/palate or other facial clefting); deafness; skin defects (cutis aplasia in neck region, or upper lip pits); or a first degree relative with cleft lip/palate (one individual). A further 92 randomly selected cases with developmental eye anomalies from the cohort were screened for variations in the alternatively spliced exon 5.

We screened for *TFAP2A* mutations using PCR to amplify all coding exons from genomic DNA using primers located in the flanking introns. *TFAP2A* has three alternative transcription start sites, designated exons 1a (NM_003220.2), 1b (NM_001032280.2), and 1c (NM_001042425.1). In addition, there is an alternative carboxyl terminus due to alternative splicing of exon 5, designated 5a (M61156) (Buettner et al. 1993). PCR amplicons were generated for exons 1a, 1b, 1c, and 2–7 and alternative exon 5a (primer sequences and PCR conditions available on request). PCR products were treated with ExoSapIT (USB) and bi-directionally sequenced using standard techniques. Mutations were confirmed on a PCR product generated from a second genomic DNA sample. DNA from 192 healthy adults (European collection of cell cultures [ECACC]) was screened as controls.

We used the dosage-sensitive Multiplex Ligation-Dependent Probe Amplification (MLPA) technique (Schouten et al. 2002) to look for deletions or duplications of the whole *TFAP2A* gene or exons within the gene. Probes were designed to interrogate *TFAP2A* exons 2, 4, 5, 6 and 7 (probe sequences available upon request). The size of exons 1 and 3 and the presence of known polymorphisms restricted probe design for those two exons. Probes were diluted to 1.33 fmol/μl and 1.5 μl of this was added to a commercially available MLPA probe mix (Holoprosencephaly-P187; MRC-Holland, Amsterdam) to provide control peaks. The standard MLPA protocol was used, except the initial DNA denaturation volume was reduced by 1.5 μl to make room for the extra probe mix. MLPA PCR products (1 μl) were separated on an ABI3100 Sequencer and analysed using Genotyper (v3.7) software (Applied Biosystems, Foster City, CA, USA). *TFAP2A* copy number was determined by exporting peak heights into an Excel spreadsheet, specifically designed to assess the ratio of each test peak relative to all other peaks for the given individual. Further, ratios of test:control peaks and control:control peaks for each sample were compared to two normal individuals included in each run (Bunyan et al. 2004). The dosage quotient for a normal individual with two copies of *TFAP2A* is expected to be 1.0. Abnormal dosage quotients are 0.5 for a deletion, 1.5 for a duplication.

In order to characterize the *TFAP2A* deletion detected by MLPA, array Comparative Genomic Hybridization (CGH) was performed on cases 3 and 4 and both parents (father is case 5) using a custom-designed high resolution microarray. The array was designed using the online tool eArray 5.3.5 (Agilent Technologies Inc.). A 4 9 44k format was selected to cover chromosome 6p24.3 (chr6: 10,413,482–10,599,386 NCBI Build 36). All high-density oligonucleotides available in the eArray CGH library in this region were selected (1,725 probes in total). A second probe group of oligonucleotides at 7 bp spacing was generated for the same target region using the genomic tiling function (23,231 probes in total). A group of control probes, for normalization purposes, was generated from the high-density oligonucleotide library covering all autosomes at 300 kb spacing (10,257 probes in total). The arrays were hybridized according to the manufacturer's instructions with slight modification: 300 ng male reference DNA (NA10851) and patient DNA samples were differentially labelled in Cy3 and Cy5, respectively, as previously reported (Redon et al. 2006), but reaction volumes were halved and only half of the labelled DNA was hybridized

to the microarray. Array data were extracted using Agilent Feature Extraction 10.9.5.1. Array data were normalized using a robust quadratic spline algorithm. A correction method based on the GC content around each probe was applied to remove the presence of genomic waves (Marioni et al. 2007). The log₂ intensity ratio for each oligonucleotide on the array was then plotted against its genomic position to generate a CGH profile using the freely available software R (<http://www.gnu.org/software/r/R.html>). The colour of each point corresponds to the Agilent score of each oligonucleotide. Scores range from 0 to 1 and increasing score indicates increasing reliability; scores are grouped and coloured as listed, 0–0.2 red, 0.2–0.4 yellow, 0.4–0.6 green, 0.6–0.8 blue, 0.8–1.0 black. Only oligonucleotide probes with a score greater than 0.4 were used to identify breakpoint regions.

Zebrafish embryos were raised at 28.5°C and staged according to Kimmel et al. (1995). Phenylthiourea (PTU) was applied to embryos to prevent melanization when necessary. Cartilages were stained with Alcian Blue (Kimmel et al. 1998). Mutant lines were *bmp4^{st72}* (Stickney et al. 2007) and *tcf7l1a^{m88l}* (Kim et al. 2000). Genotyping of *tcf7l1a^{m88l/m88l}* and *bmp4^{st72/st72}* was performed as previously described (Kim et al. 2000; Stickney et al. 2007). The following morpholino sequence (GeneTools, Inc.) was used: *tfap2a*-splice 5'-GAAATTGCTTACCTTTTTTGATTAC-3' (O'Brien et al. 2004). Morpholinos were reconstituted in Danieaux buffer (Nasevicius and Ekker 2000) then diluted to 0.125 or 0.5 mg/ml. Embryos were injected with 1 nl of diluted morpholino at the 1–4 cell stage into the yolk immediately below the blastomeres. The phenotypes reported here come from three or more independent experiments. We observed comparable pharyngeal phenotypes to those reported in previous experiments, thus validating the specificity of the morpholino (O'Brien et al. 2004; Fig. 4a; iii/iv). Standard control random 25-mer morpholinos (Gene Tools) fail to give any phenotypes in our experimental conditions. All experiments were performed in accordance with regulatory standards and where appropriate regulated by the Animals (Scientific Procedures) Act 1986.

For in situ hybridization studies, digoxigenin (DIG)-labelled *TFAP2A* (NM_003220.2 nts 916–1253) probes were prepared by amplification from pCMV-SPORT6 vector containing full-length *TFAP2A* using primers 5'-TAATACGACTCACTATAGGGCTTGCTCACTTGCTCATTG-3' and 5'-ATTTAGGTGACACTATAGAGCTCCACCTCGAAGTAC-3'. The primers included T7 and SP6 primer sites. PCR was carried out using: 100 mM dNTPs, 0.1 mM each primer, 5 µl of 10× PCR amplification buffer and 1 U HotStar *Taq* DNA polymerase (Qiagen). Thermocycling was performed as follows: step 1, 94°C for 15 min; step 2.1, 94°C for 30 s; step 2.2, 72°C for 1 min (35 cycles); step 3, 72°C for 10 min. The 376 bp gel-purified PCR amplicon was used as a template to generate RNA probe. Digoxigenin-UTP was incorporated into riboprobes during in vitro transcription using the DIG RNA labelling mix (Roche) according to the manufacturer's instructions. Antisense and sense probes were generated using T7 and SP6 polymerase, respectively. In situ hybridization was carried out on 8 µm microtome sections on 12.5 dpc mouse embryos, 15–22 Carnegie stage (CS) human embryos and foetal stage 2 (F2) human foetuses as described by Wilkinson (1992). Expression patterns were visualized using the NBT/BCIP system (Roche) and sections were mounted in VectaMount (Vector Labs) and analysed using the Axioplan 2 imaging system (Zeiss).

Results

Mutation analysis

We identified three mutations in the cohort of 37 selected patients. Case 1 had a heterozygous de novo *TFAP2A* four amino acid deletion c.697_708del12 (p.Glu233_Arg236del) in the basic domain and case 2 had a heterozygous paternally

inherited c.956T>C (p.Phe319Ser) and also a maternally inherited c.961C>T (p.Arg321Cys) variation in an alternative splice isoform, *TFAP2A2* (Buettner et al. 1993). The c.961C>T (p.Arg321Cys) was present in 2/189 control samples suggesting that this is likely to be a polymorphism, but the c.956T>C (p.Phe319Ser) was not present in any of the 189 control samples and falls within a conserved residue (Fig. 1). The alternative transcript has only been defined in humans so far; however, the genomic region containing the c.956T>C variant is highly conserved throughout mammalian species (Fig. 1). In order to further elucidate the role of the alternatively spliced exon 5 in eye development, we screened an additional 92 individuals with eye development anomalies, principally anophthalmia, microphthalmia or coloboma, but found no variants. Nucleotide and codon numbers for case 1 refer to the *TFAP2A* 1a isoform (NM_003220.2) and for case 2 the *TFAP2A2* isoform (M61156). We also identified further presumed non-pathogenic sequence variants in our 37 cases, including a heterozygous c.420C>T (p.Leu140-Leu) in an individual with unilateral anophthalmia and bilateral cleft lip and palate; further variants were c.-20C>G in the exon 1a 5'UTR, two known SNPs in intron 1 (rs654351 and rs654340), a heterozygous c.533_554delG in intron 3, previously described in normal controls (Klootwijk et al. 2003; Stegmann et al. 2001), a heterozygous c.1025 + 21G>A, c.1025 + 61G>A and a known SNP (rs303048) in intron 6, and a known synonymous SNP in exon 7 (rs3734391). The exonic and intronic variants are not predicted to disrupt 5' - or 3' -splice sites, or predicted exonic splice enhancer sequences (Cartegni et al. 2003; Gao et al. 2008).

Deletion analysis

Using MLPA, we identified two individuals (cases 3 and 4), a sibling-pair with classical features of BOFS, with a heterozygous deletion of all tested exons (see Fig. 1c). This deletion was also present in the father (case 5). The MLPA results were confirmed using a high-resolution oligonucleotide custom array (Fig. 1d). The array design is comprehensive in coverage and has a probe on average every 7 bp across a 185 kb region on chromosome 6p24.3 encompassing the *TFAP2A* gene. The array refined the telomeric breakpoint to within ~550 bp (chr6:10,474,246–10,474,793 NCBI36) and the centromeric breakpoint within ~265 bp (chr6:10,541,899–10,542,164 NCBI36). Only two genes are included in the deletion, *TFAP2A* and *C6orf218*. *C6orf218* is a predicted gene that has no identified protein domain (Letunic et al. 2009) or known function.

Case histories

The clinical features of all cases are summarized in Table 1.

Case 1, born to unrelated Caucasian parents, had classical features of BOFS including a high arched palate, prominent philtrum, narrow ear canals, abnormal pinnae, scalp, and eyebrow nodules (Fig. 2; Table 1). His ocular features included a right cystic remnant (disorganized globe/cyst), a mildly microphthalmic left eye with a reduced corneal diameter (6–7 mm), iris coloboma, primary aphakia, and a large posterior chorioretinal coloboma. There was no family history of eye or other developmental anomalies.

Case 2 was born with congenital heart and eye anomalies to unrelated parents (mother Caucasian, father Afro-Caribbean). The maternal great uncle (uncle of the grandfather, and now deceased) and his twin brother, the great grandfather of case 2 were both blind in one eye (aetiology unknown), the maternal grandmother has an abnormal kidney and there is a strong family history of postaxial polydactyly on the paternal side (paternal grandmother, aunt, and her two children). Case 2 had a right microphthalmic eye with sclerocornea, primary aphakia, and localized tractional retinal detachment and an extremely microphthalmic left eye with sclerocornea. Her systemic features, which are not classical for BOFS are listed in Table 1.

Case 3, previously described (Fielding and Fryer 1992), had bilateral orbital cysts associated with microphthalmic remnants, pronounced features of BOFS, severe learning difficulties with an autistic spectrum disorder and seizures, treated with carbamazepine (Table 1; Fig. 2).

Case 4 (younger sister of case 3) has also been previously described (Fielding and Fryer 1992), had relatively mild systemic features (Table 1; Fig. 2). She had a right orbital cyst, partially excised, associated with a microphthalmic eye with a subluxed cataractous lens, shallow anterior chamber and no fundal view, and left mild microphthalmia with a shallow anterior chamber, persistent pupillary membrane, iris hypoplasia, mild cataract, and an extensive chorioretinal coloboma involving optic disc and macula and bilateral nystagmus.

Case 5 (father of 3 and 4) demonstrates some mild, classical features of BOFS, including premature ageing changes (Table 1; Fig. 2). His ocular features are also subtle: he has normal anterior segments, but the right optic disc is dysplastic with an unusual blood vessel pattern; the left disc showed mild dysplasia.

Expression of *TFAP2A* in human eye

TFAP2A expression was analysed in mouse 12.5 dpc and human embryos CS 15–22 and foetal stage F2. Expression in the mouse was seen in the nasal process, palate and within the CNS (Fig. 3a). Expression was found to be broadly comparable in the human with expression in the nasal process, epithelial lining of the oral cavity, palatal shelves, tooth buds, and within the CNS (data not shown). During human eye development, *TFAP2A* was first seen in the anterior epithelium of the lens at CS 15 (Fig. 3b). At CS 18, *TFAP2A* was expressed more strongly in the anterior epithelium of the lens and also in the ganglion layer of the neural retina (Fig. 3c). At CS 22, *TFAP2A* was expressed in the equatorial region of the lens epithelium, and secondary lens fibres and throughout the ganglion cell layer of the neural retina (Fig. 3d). *TFAP2A* expression was still visible, but weaker in the retina of F2 human eyes (data not shown).

Knockdown of zebrafish *tfap2a* function leads to eye and craniofacial defects

The zebrafish ortholog of *TFAP2A* is expressed in embryonic tissues that contribute to the eye, including the surface ectoderm and neural crest, and is required for craniofacial development (Barrallo-Gimeno et al. 2004; Knight et al. 2004; Knight et al. 2003; O'Brien et al. 2004). In order to address the role of zebrafish *tfap2a* in eye development, we used a morpholino (MO) anti-sense approach to knockdown *tfap2a* function (O'Brien et al. 2004). We found a range of morphological anomalies in the eyes of *tfap2a* morphant embryos, while overall embryo morphology was comparable to sibling control embryos (Fig. 4a; i, ii). Eye phenotypes were frequently asymmetric (Fig. 4a; vi, vii) and included microphthalmia (53%, Fig. 4a; vi–viii), mild coloboma (68%, Fig. 4a; vii, viii) and severe coloboma whereby ventral retinal tissue, including retinal pigment epithelium (RPE) protruded from the back of the eye towards the midline of the brain (13%, Fig. 4a; vii, viii). These phenotypes are not mutually exclusive and individual eyes can have all these features (Fig. 4a; vii, viii). We presume that variability of phenotype is due to incomplete abrogation of *tfap2a* function in all expressing cells.

Supporting previous studies (Barrallo-Gimeno et al. 2004; Knight et al. 2003, 2004; O'Brien et al. 2004), pharyngeal cartilages are affected in *tfap2a* morphants with the ceratohyal reduced in size and oriented medially instead of rostrally (Fig. 4a; iii, iv). Less severe defects were observed in more posterior arches (data not shown). We noted that there was concordance of severity between asymmetric eye and jaw phenotypes (arrow in Fig. 4a iv points to ceratohyal and palatoquadrate defects with an ipsilateral small eye and coloboma

phenotype). These results demonstrate that, as in human and mice, zebrafish *tfap2a* is required for both craniofacial and eye development.

Combined abrogation of *tfap2a* and either *bmp4* or *tcf7l1a* leads to eye phenotypes of enhanced severity

Many human congenital conditions are likely to be a consequence of mutations in two or more genes affecting the same pathway or developmental process. We therefore assessed if reduced *tfap2a* function sensitizes zebrafish embryos to the reduction or loss of function of other genes implicated in eye development. We selected mutations in the Bmp and Wnt signalling cascades, since both pathways are implicated in eye morphogenesis and in specification of *tfap2*-expressing neural crest cells (Adler and Belecky-Adams 2002; Kim et al. 2000). To test for genetic interaction, we reduced *tfap2a* function in embryos heterozygous or homozygous for mutations in *tcf7l1a*, which encodes a transcriptional effector of Wnt signalling, and *bmp4*, which encodes a Bmp ligand. Both *bmp4*^{st72/st72} mutants and *tcf7l1a*^{m881/m881} mutants lacking zygotic activity of Tcf3 (*tcf7l1a*^{m881/m881}) have no overt eye phenotype (Dorsky et al. 2003; Stickney et al. 2007). We injected a concentration of *tfap2a* morpholino that does not give rise to any phenotype in a wild-type background (Fig. 4b-i; c-i) and assessed the phenotypic consequences in the mutant backgrounds. In both heterozygous and homozygous conditions, the m881 mutation in *tcf7l1a* enhanced *tfap2a* partial loss of function phenotypes. In heterozygotes, reduction of *tfap2a* gave a consistent coloboma phenotype (Fig. 4b; ii, iv) and in homozygotes, embryos showed fully penetrant anophthalmia (Fig. 4b; iii, iv). Homozygosity of the st72 mutation in *bmp4* enhanced the severity of eye phenotypes upon reduction of *tfap2a* (Fig. 4c; ii, iii). In 100% of these embryos, eyes showed coloboma phenotypes and/or mild microphthalmia. These eye phenotypes are also present in 40% of st72 heterozygotes while the other 60% of embryos showed no overt eye phenotypes (Fig. 4c; i). Increasing the concentration of the *tfap2a* morpholino in the st72 background gave rise to body patterning defects (data not shown). Taken together, these results indicate that both overactivation of Wnt signalling due to the *tcf7l1a*^{m881} mutation and reduced Bmp signalling due to the *bmp4*^{st72} mutation sensitize embryos to reduced *tfap2a* function.

Discussion

We describe four cases of classical BOFS: one associated with a four-amino acid deletion (case 1), and three associated with a small chromosome deletion including the *TFAP2A* gene (cases 3–5). The whole gene deletion cases exhibited considerable phenotypic variability, ranging from severe (case 3), moderately severe (case 4) to mild in the father (case 5). Indeed, the father's phenotype was so mild as to be unrecognized in the original report (Fielding and Fryer 1992), but with hindsight included several features, notably calcified styloid processes, related to second arch development and, more recently, features of premature ageing (Table 1). A previously described mother-son pair with a deletion encompassing *TFAP2A* and a less severe phenotype led to the suggestion that deletion cases might be milder due to complete haploinsufficiency. This is not supported here since our proband had very severe ocular, skin, and developmental anomalies, including autism, although the severity may be related to adverse first trimester pregnancy or genetic background effects.

Case 2, in whom we identified variations in the alternatively spliced exon 5a of *TFAP2A2* (M61156) (Buettner et al. 1993), had severe eye defects, but did not exhibit a classical BOFS phenotype. She had a paternally inherited heterozygous c.956T>C (p.Phe319Ser) mutation in a conserved base, not present in controls, which segregated with polydactyly on the paternal side and a likely polymorphism c.961C>T (p.Arg321Cys) on the maternal side, also present in the great grandfather (with blindness in one eye), but not the grandmother

who had an abnormal kidney. The TFAP2A2 isoform lacks the TFAP2A dimerization domain necessary for DNA binding, but reduces TFAP2A transcriptional activation by inhibiting interaction of TFAP2A with DNA (Buettner et al. 1993). However, it is unclear whether the mutation(s) constitutively increase or decrease function.

The eye phenotypes in individuals with *TFAP2A* mutations or deletions are extremely variable, from extreme microphthalmic remnants to dysplastic optic discs, probably influenced by mutations in other regulatory eye genes and/or gestational factors. Intermediate phenotypes, such as cataractous lenses and coloboma or cyst formation, indicate an important role for *TFAP2A* in lens formation and optic fissure closure. This is supported by our in situ hybridization studies which demonstrate *TFAP2A* expression in the developing human embryonic lens and ganglion cell layer of the neural retina, consistent with mouse expression studies (Bassett et al. 2007; Mitchell et al. 1991), and evidence from mouse knockout models. Germline knockout of mouse *Tfap2a* causes defects in lens development with persistent adhesion of the lens to the overlying surface ectoderm (Peters' anomaly) and retinal defects including RPE transdifferentiation to neural retina and lack of a ganglion cell layer (West-Mays et al. 1999). However, although mouse conditional knockouts with selective reduction of *Tfap2a* in lens placode derivatives display similar lens defects, there are no retinal anomalies, suggesting these occur only if retinal *Tfap2a* levels are additionally compromised (Pontoriero et al. 2008). This appears to be in slight contrast to humans with mutations in the *FOXE3* lens transcription factor gene, which is expressed predominantly in the developing human embryonic lens (Ugur Iseri et al. 2009). These individuals display not only severe lens defects, including primary aphakia (similar to Case 2) and Peters' anomaly, but also colobomas, suggesting an inductive role for the lens in optic fissure closure (Ugur Iseri et al. 2009).

Zebrafish *tfap2a* morphants also have retinal and optic nerve colobomas. Despite comparable ocular defects in humans and zebrafish *tfap2a* morphants, including microphthalmia and retina pigment epithelial abnormalities, the phenotypes are less severe in fish probably due to partial redundancy of function of different zebrafish *tfap2* genes. Abrogation of both *tfap2a* and *tfap2c* (Li and Cornell 2007) gives rise to very severe ocular malformation in zebrafish (GG and SW, unpublished data), suggesting a strong conservation of function of *tfap2* genes across vertebrates.

The importance of genetic background to *TFAP2A* mutant phenotypes is reinforced by our zebrafish data showing strong genetic interactions between *tfap2a* knockdown and *bmp4* or *tcf7l1a* mutations. Neither *bmp4* nor *tcf7l1a* heterozygotes or homozygotes alone display overt eye phenotypes, but show strong ocular abnormalities upon partial abrogation of *tfap2a*, implying that human hypomorphic *TFAP2A* mutations might contribute to eye developmental disorders when on a background of additional mutation(s) in other genes regulating eye formation. Parents carrying such mutations are consequently at higher risk of having offspring with severe eye abnormalities.

The combined abrogation of zebrafish *tfap2a* and *tcf7l1a* causes anophthalmia, which is almost certainly an early phenotype affecting specification on field or formation of the optic vesicles. A similar phenotype is seen upon combined maternal and zygotic loss of *tcf7l1a* function and other situations where canonical Wnt/ β -catenin signalling is enhanced in the neural plate eye-forming region (Cavodeassi et al. 2005; Dorsky et al. 2003; Heisenberg et al. 2001; Houart et al. 2002; Kim et al. 2000). Abrogation of *tfap2a* may enhance activation of the Wnt pathway when combined with reduced *tcf7l1a* function. Both *tfap2a* and *bmp4* are expressed during gastrulation in non-neural ectoderm and later, either in the forming eye or surrounding crest cells, providing a broad period during which the combined genetic lesions could have deleterious consequences.

The extraocular phenotypes observed in BOFS provide a further opportunity to consider the role of interacting pathways. We observed digit abnormalities, including partial syndactyly, in our BOFS cases suggesting a role for *TFAP2A* in digit development. Interestingly, reduced expression of the mouse *Tfap2a* in limb bud mesenchyme causes postaxial polydactyly (Feng et al. 2008). Furthermore, the c.956T>C (p.Phe319Ser) mutation in *TFAP2A2* was associated with polydactyly in two relatives of Case 2. We have previously described individuals with *BMP4* mutations with eye anomalies combined with poly- and syndactyly (Bakrania et al. 2008). Evidence for a direct interaction of *tfa2a* with *Smad2/3* (part of the *Bmp4* signalling pathway) has been shown by chromatin immunoprecipitation studies (Koinuma et al. 2009). Further possible sites for interaction may include the oral cavity, nasal prominence and palatal shelves where there is expression of *TFAP2A* in human embryos, consistent with studies in other animals (Knight et al. 2003; Mitchell et al. 1991; Moser et al. 1997; O'Brien et al. 2004), and corresponding anomalies in individuals with BOFS, presumably due to reduced *TFAP2A* activity in these areas. Recently, the involvement of *BMP4* in cleft lip, both overt and microform types, not unlike that seen in BOFS, has been reported (Suzuki et al. 2009). These findings, together with our zebrafish evidence of interacting pathways, suggest this phenotypic overlap may reflect an underlying genetic interaction.

This study reveals that *TFAP2A* is critical to eye, brain, and craniofacial development in humans in the context of classical BOFS, and may also play a role in isolated eye anomalies. We propose that the mutations and deletions described here would cause a reduction of *TFAP2A* in the developing lens and retina and subsequent degeneration of these structures leading to the various ocular defects seen in these patients. Furthermore, we demonstrate oligogenic effects between the *tfa2a* pathway and both the *bmp* and *wnt* signalling pathways in the zebrafish that are likely to be important mechanisms in the non-Mendelian origin of human developmental eye anomalies.

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Fig. 1.

Results of molecular analysis of *TFAP2A* showing gene and protein structure, MLPA and custom array CGH. **a** Schematic representation of *TFAP2A* mRNA showing exon breakdown and number of bases encoded by each exon; splice indicators above depict *TFAP2A* full-length mRNA (NM003220.2 isoform 1a), while those below describe exon 5 alternatively spliced in the *TFAP2A2* isoform (M61156); *TFAP2A* protein representation showing full-length protein (*upper*) and M61156 isoform (*lower*) showing domains. *PG* proline/glutamine rich domain (for transactivation), *B* basic domain of DNA binding region, *HSH* helix-span-helix domain (mediates dimerization and site specific DNA binding). *Small arrows* show previously reported mutations, *large arrows* show our cases. **b** Chromatograms from Cases 1 and 2 and normal controls showing position of variations (*arrows*). Comparative amino acid sequence alignment of vertebrate *TFAP2A* and genomic sequence alignment of *TFAP2A2* encompassing the regions altered in Cases 1 and 2. **c** MPLA traces for Cases 3, 4, and 5 showing a 50% reduction in peak height for exons 2, 4, 5, 6 and 7 of *TFAP2A* compared to maternal and control peaks (no probes were available for exons 1 and 3). **d** High resolution comparative genomic hybridization (CGH) microarray analysis of the *TFAP2A* region. The data show an approximate 70 kb deletion in (i) Case 3, (ii) Case 4, and (iii) Case 5 (father), but not in (iv) mother. The position of the *TFAP2A* gene is shown in *green* and *C6orf218* in *blue*. Oligonucleotides are colour coded in order of increasing reliability according to their Agilent probe score (where <0.4 is poor and >0.4 is good, >0.8 is very good): 0–0.2 *red*, 0.2–0.4 *yellow*, 0.4–0.6 *green*, 0.6–0.8 *blue*, 0.8–1.0 *black*. Chromosome co-ordinates are according to NCBI36

Fig. 2.

Photographs of external features of Cases 1, 3, 4, and 5. Case 1 showing **a** full face with frontal bossing, R anophthalmia (wearing prosthesis), prominent philtrum; **b** left iris coloboma **c** right periorbital subcutaneous lump (presumed dermoid); **d** right and left external ears showing malformed pinnae; and **e** close-up of oral cavity demonstrating abnormal dentition (three inferior incisors) and prominent philtrum (**f**) view of chest showing widely spaced nipples. Case 3: **g** Full face in early life showing bilateral orbital cysts; **h** full face showing patient wearing bilateral prosthetic eyes and prominent philtrum; **i** close-up of microphthalmic remnants; **j** close-up of oral cavity demonstrating abnormal dentition; **k** right and left cervical skin defects; **l** pits in posterior pinna. Case 4: **m** Full face showing bilateral microphthalmia with R esotropia and hypertropia and inferior orbital cyst; **n** close of right and left microphthalmic eyes; **o** feet showing bilateral 2/3 partial syndactyly; **p** showing ears with mild malformed pinnae; **q** mild webbing of fingers. Case 5 showing **r** full face with prominent philtrum; **s** bilateral 2/3 partial syndactyly feet; **t** bilateral malformed pinnae

Fig. 3.

In situ hybridization of *TFAP2A* in mouse and human developing embryos showing expression of *TFAP2A*. **a** Sagittal section of 12.5 dpc mouse showing expression of *Tfap2a* in the nasal process, palate, cerebellum, thalamus, and spinal cord. **b** Coronal section of CS 15 human eye showing expression of *TFAP2A* in the anterior lens epithelium. **c** Coronal section of CS 18 human eye showing expression of *TFAP2A* in the anterior lens epithelium, and ganglion cell layer of the neural retina. **d** Coronal section of CS 22 human eye showing expression of *TFAP2A* in the equatorial region of the lens epithelium, throughout the secondary lens fibres and ganglion cell layer of the neural retina. *np* nasal process, *ce* cerebellum, *pa* palate, *th* thalamus, *sc* spinal cord, *nr* neural retina, *le* lens

Fig. 4.

Zebrafish studies demonstrating eye and craniofacial defects in *tfap2a* morpholino-injected larvae and enhanced phenotypes in Wnt and Bmp morphants. **a** Abrogation of *tfap2a* function leads to eye and craniofacial defects. (*i, ii*) Wild-type and *tfap2a* morpholino-injected larvae at 3.5 dpf; *arrow* in (*ii*) points to the expansion of the retinal pigmented epithelium (RPE) towards the midline (*black line*) due to ventral eye coloboma. (*iii, iv*) Pharyngeal cartilages from 3.5 dpf wild-type (*iii*) and *tfap2a* morpholino-injected larvae (*iv*), stained with Alcian Blue and shown in ventral view. Palatoquadrate (roof of mouth) and ceratohyal (part of the hyoid bone) (*iv, arrows*) are severely disorganised in the morphant. Higher magnification of 3.5 dpf embryos showing wild-type (*v*) and morphant eyes with ectopic RPE (*vi*) and coloboma (*arrow* in *vii*). (*viii*) Table showing the percentage of the abnormal phenotypes in the *tfap2a* morpholino-injected larvae (ectopic RPE, coloboma, small eye, jaw defects). *m* Meckel, *pq* palatoquadrate, *ch* ceratohyal. **b** Synthetic and enhanced coloboma and anophthalmia phenotypes are revealed by combined abrogation of *tfap2a* and *tcf7l1a*. Lateral view of 3.5 dpf larva injected with a low concentration of *tfap2a* morpholino that does not give rise to any phenotypes in a wild-type background (*i*), gives rise to a coloboma phenotype in *tcf7l1a*^{m881/+} heterozygotes (*ii*) and anophthalmia in the *tcf7l1a*^{m881/m881} homozygote (*iii*). PCR-based confirmation of predicted genotypes based upon phenotypes (*iv*). PCR products were amplified from individual 3.5 dpf larvae with a wild-type phenotype as in (*i*) (*lanes 1–4* 16/16 embryos are homozygous for the wild-type allele), a mild phenotype as in (*ii*) (*lane 5–12*; 32/36 embryos are heterozygous for the mutant allele as shown in *lane 5, 7, 9–12* and 4/36 are homozygous for the mutant allele as in *lanes 6 and 8*) and an anophthalmia phenotype as in (*iii*) (*lane 13–16*; 16/16 embryos are homozygous for the mutant allele). **c** Synthetic and enhanced eye phenotypes are revealed by combined abrogation of *tfap2a* and *bmp4*. Lateral views of 3.5 dpf larvae injected with a low concentration of *tfap2a* morpholino that does not give rise to any phenotypes in a wild-type background. In 60% of the *bmp4*^{st72/+} heterozygotes (*i*) there is no phenotype (wild-type and heterozygote embryos are indiscernible), whereas in the *bmp4*^{st72/st72} homozygotes (*ii*) there is a prominent coloboma (*black brackets*). PCR-based confirmation of predicted genotypes based upon phenotypes (*iii*). PCR products were amplified from individual 3.5 dpf larvae with a wild-type phenotype as in (*i*) (*lanes 1–4*), both wild type (*lanes 1 and 4*) and heterozygote genotype are present (*lanes 2 and 3*), and a coloboma phenotype as in (*ii*) (*lane*

5–14). Most affected individuals are *bmp4*^{st72/st72} homozygotes although 2 (*lanes 5 and 7*) are *bmp4*^{st72} heterozygotes. The differences in intensity of the PCR bands can result from a difference in the efficiency of the genomic DNA extraction or PCR reaction, or, less likely, from partial DNA digestion

Summary of molecular and clinical findings

Table 1

Case	1	2	3	4	5
TFAP2A molecular findings	c.697_708del12 (p.Glu233_Arg236del)	Alt transcript 5a c.956T > C (p.Phe319Ser); c.961C > T (p.Arg321Cys)	Whole gene deletion	Whole gene deletion	Whole gene deletion
Sex	M	F	M	F	M
Age at last exam	5 years	10 months	22 years	19 years	57 years
Gestation; B wt (kg)	FT; 3.260	FT; 3.425	42/40; 3.685	41/40; 3.470	34/40; 2.620
Pregnancy	APH	ROC 16/40	App; HE	Normal	Normal
Height (percentile)	50th	2–9th	9th	2nd	25th
Development	Mild speech delay	Delayed motor development	Severe learning difficulties	Above average intelligence	Mild dyslexia
Vision	R NPL; L 6/95	R PL; L NPL	NPL R & L	R PL; L 2/60	6/5 R & L
Eye findings	R CyR AL 17 mm; L Mi, AL 18.9 mm, ICo, CRCo, Aph; NLDO	R Mi, Sc, Aph, tract RD AL 13 mm; L Mi, Sc	R CyR Cy; L CyR, Sc	R Mi, Cy, Sub Ca; L Mi, Cy	Dys OD
Abnormal philtrum/pseudocleft	Y	N	Y	Y	Y
High arched palate	Y	N	Y	Y	Y
Pinna anomalies and narrow ear canals	Y	N	Y	Y	Y
Skin	Periorbital and scalp cysts	N	Branchial cysts; cyst natal cleft	N	N
Premature greying	N/A	N/A	Y	Y	Y
Dental anomalies	Missing lower central incisor	N	Small widely spaced teeth	N	N
Hand/foot	5th finger clinodactyly	N	Toe 2/3 partial syndactyly	Mild hand syndactyly; toe 2/3 partial syndactyly	Short tips of fingers, brittle nails, toe 2/3 partial syndactyly
Other	Widely spaced nipples; secretory otitis media	Congenital heart disease ^a , facial capillary hemangioma	Autistic spectrum disorder; seizures	Calcified styloid processes, deafness, MI, degenerative arthritis, ischaemic lesions in cerebral cortex/cerebellum	

FT full term, *B w* birth weight, *APH* antepartum haemorrhage, *ROC* ruptured ovarian cyst, *App* appendicectomy and oophorectomy first trimester, *HE* hyperemesis and oculogyric crisis with prochlorperazine, *R* right, *L* left, *AL* anophthalmia, *M*/microphthalmia, *CyR* cystic ocular remnant, *Cy* orbital cyst, *Sub* subluxed lens, *Ca* cataractous lens, *ICo* iris coloboma, *CRCo* chorioretinal coloboma, *Aph* primary aphakia, *Sc* sclerocornea, *AL* axial length, *NPL* no perception of light, *PL* perception of light, *NLDO* nasolacrimal duct obstruction, *Dys OD* dysplastic optic discs, *MI* myocardial infarction

Atrial septal defect with enlarged anomalous blood vessel draining into the RA^a