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Novel cases with biallelic variants in *Wnt ligand secretion mediator* (*WLS*) further support its role in structural eye anomalies

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Background

Anophthalmia (absent eye), microphthalmia (small eye) and coloboma (gap in the eye structures) (AMC) are rare congenital eye anomalies affecting 11.9/100,000 live births[1] and are associated with systemic features in >50% of cases. More than 130 genes are diagnostically tested in UK patients, including members of the Wnt signalling pathways.

Wnt Ligand Secretion mediator Wntless (*WLS*) is essential to the trafficking and secretion of all Wnt ligands. Biallelic *WLS* variants cause a rare multi-systemic condition (Zaki syndrome), to date described in 7 families with a range of systemic phenotypes[2,3,4]. Among these, AMC was reported in 3 families which led us investigate the role of *WLS* in developmental eye anomalies.

Methods

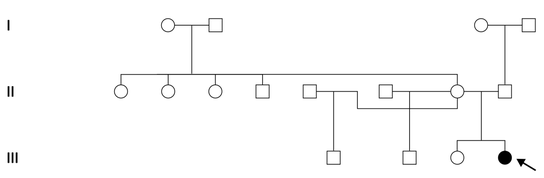
***WLS* screening:** We screened three cohorts using whole exome/genome sequencing (WES/WGS) data to identify rare biallelic variants affecting *WLS* coding regions and canonical splice sites. A) Ragge AMC cohort - 172 individuals with AMC from 167 families (REC 04/Q0104/129); B) CAP69 cohort – 486 individuals with developmental eye anomalies from 484 families selected from the Deciphering Developmental Disorders (DDD) study (REC 10/H0305/83); C) 100,000 Genomes Project (100K) – Rare Disorders cohort (Tiering data v14). The study was conducted in accordance with the Declaration of Helsinki.

Abrogation of zebrafish *wls*: We generated F0 *wls* crispants (wls F0) by injecting guide RNA (gRNAs) in 1-cell embryos to target three *wls* exons (1, 11, 12). We confirmed loss of wildtype *wls* alleles with MiSeq sequencing.

Zebrafish screen for genetic interactions: We also abrogated *wls* in zebrafish backgrounds with mutations in *lrp5*, *lrp6*, *taz*, *mab21l2* and *lama1*, in which fish are sensitised to ocular malformations[5,6,7,8]. We confirmed *wls* disruption in fish with anomalous phenotypes using T-ARMS-PCR.
All zebrafish experiments were conducted in accordance with standard protocols and UK Home Office regulations (PIL: PP4300676).

Two new families with syndromic AMC and biallelic *WLS* variants

FAMILY A

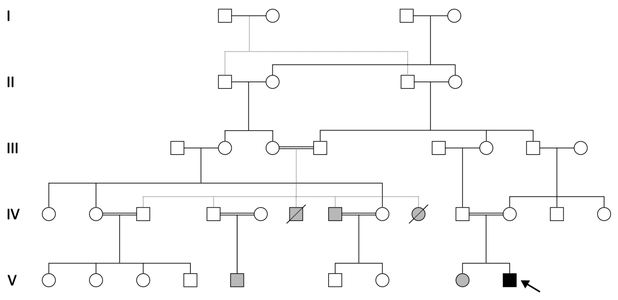


AMC phenotypes:
Bilateral microcornea, iris and chorioretinal coloboma

Systemic features:
Micrognathia, abnormal left ear, small left kidney, mild 2-3 toe syndactyly, spinal lordosis, osteopenia

***WLS* (NM_024911): compound heterozygous variants**
- Exon3:c.443G>A:p.Trp148* (maternal)
  MAF (gnomAD v4.1): 0.00001735; CADD=40, GERP_RS=5.94
- Exon3:c.473G>A:p.Arg158Gln (paternal)
  MAF (gnomAD v4.1): 0.00007745; CADD=29, GERP_RS=5.94

FAMILY B



AMC phenotypes:
Bilateral microphthalmia, iris and chorioretinal coloboma

Systemic features:
Turricephaly, right lip and alveolar cleft, preauricular skin tags, slightly upturned earlobes, irregular dentition, cryptorchidism, small scarred left kidney, square fingertips and flat feet, hypotonia, osteopenia

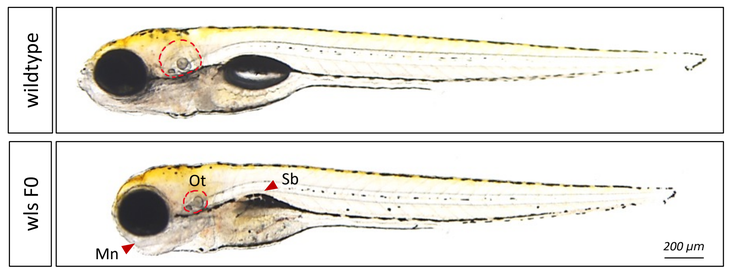
***WLS* (NM_024911): homozygous variant**
- Exon9:c.1261C>T:p.Arg421Trp (homozygous)
  MAF (gnomAD v4.1): 0.000003098; CADD=34, GERP_RS=5.36

Black symbols: individuals carrying biallelic *WLS* variants; grey symbols: individuals with Bardet-Biedl syndrome.

	Family A (n=1) This study	Family B (n=1) This study	Family 1 (n=5) Chai et al 2021	Family 2 (n=2) Chai et al 2021	Family 3 (n=1) Chai et al 2021	Family 4 (n=1) Chai et al 2021	Family 5 (n=1) Chai et al 2021	Family 6 (n=1) Yu et al 2023	Family 7 (n=1) Abdel-Salam et al 2023
WLS variant NM_024911.7	c.443G>A; p.W148X c.473G>A; p.R158Q	c.1261C>T; p.R421W	c.1175A>G; p.Y392C	c.1433A>G; p.Y478C	c.1433A>G; p.Y478C	c.1592T>C; p.I531T	c.1606C>T; p.R536C	c.421C>T; p.R141C c.1433A>G; p.Y478C	c.1579G>A; p.G527R
Consanguinity	-	+	+	+	(suspected)	(suspected)	+	-	+
Gender	F	M	4 F, 1 M	2 F	F	F	F	M	M
Short stature	-	-	+	+	-	n/a	+	-	+
Microcephaly	Relative microcephaly in early childhood	-	+ (*)	+	+	n/a	+	-	+
AMC	+ (BL iris and chorioretinal coloboma, BL microcornea)	+ (BL iris and chorioretinal coloboma, BL microphthalmia)	-	+ (iris coloboma and microcornea)	+ (BL iris coloboma)	n/a	-	n/a	+ (BL optic disc coloboma, BL microphthalmia, L cyst)
Other ocular features	L divergent squint, nystagmus, visual impairment	L convergent squint, upslanting palpebral fissure	Strabismus (2/5), no visual impairment	Strabismus, nystagmus, visual impairment	Strabismus, visual impairment	n/a	Strabismus, no visual impairment	Refraction error	BL vitreous floaters
Mouth	Micrognathia, wide mouth	R cleft lip and alveolar cleft, wide mouth, abnormal dentition	Micrognathia, wide mouth, short philtrum, high arched palate	Median pseudocleft lip, wide mouth, short philtrum, high arched palate	n/a	Micrognathia, high arched palate	Median pseudocleft lip, wide mouth, short philtrum	Median pseudocleft lip, high arched palate, delayed eruption of deciduous teeth	Wide mouth, median pseudocleft lip, BL transverse cleft, short philtrum, hypodontia, abnormal teeth shape and spacing
Ears	Abnormal shape, anomalous L pinna	Abnormal shape, preauricular tags	Cupped ears	Cupped ears	n/a	n/a	Cupped ears, preauricular tags	Abnormal shape	Cupped ears, preauricular tags
Hair	Sparse hair	Thin hair	Sparse hair	Sparse hair and eyebrows	n/a	n/a	Sparse hair and eyebrows	n/a	Sparse hair, absent eyebrows
Limb and skeletal anomalies	Mild 2-3 toe syndactyly, osteopenia, lordosis	BL short index finger, curving of R index, flat feet, osteopenia	Partial toe syndactyly (3/5), scoliosis (1/5)	Toe syndactyly, broad distal phalanx of finger, hypoplasia of toenails and phalanges of toes (1/2)	n/a	Broad distal phalanx of finger, scoliosis	Broad distal phalanx of finger, hypoplasia of toenails and phalanges of toes	-	3rd-4th finger syndactyly (L complete, R: partial), thin cortex of long bones, severe osteopenia (frequent fractures), mild scoliosis
Kidneys	L small	Small scarred L kidney	-	-	n/a	Renal agenesis, BL hydronephrosis	-	Renal agenesis (R), CKD stage 2	R small, but normal function
Genital	n/a	BL undescended testes, buried penis	n/a	n/a	n/a	n/a	n/a	BL undescended testes (hypoplastic R testicle)	BL undescended testes, epispadius
Brain	- (normal MRI)	MRI n/a	CV hypoplasia and enlarged 4th ventricle (MRI:1/5)	CC hypoplasia, CV hypoplasia (1/2)	n/a	CC and CV hypoplasia, enlarged 4th ventricle	Intracranial arachnoid cyst (3rd ventricle)	Slight dilation of lateral ventricles	CC and CV hypoplasia, enlarged 4th ventricle, Dandy-Walker malformation
Neurological findings	Seizures	Hypotonia	Tone, gait, tendon reflexes (1/5)	Tone and gait (1/2), tendon reflexes	-	Seizures	Tone and gait	-	-
Developmental delay	Speech delay, mild learning difficulties	Motor delay, mild ID	Motor delay (3/5), ID (4/5)	Motor delay (1/2), ID	Motor delay, ID	Motor delay, ID	Motor delay, no ID	Delayed global milestones	Motor and speech delay
Other features	Nasolacrimal duct obstruction	BL punctal agenesis, widely spaced nipples, Vitamin D deficiency	Widely spaced nipples (1/5)	Widely spaced nipples, patent foramen ovale (1/2)	Congenital diaphragmatic hernia		Widely spaced nipples, patent foramen ovale, patent ductus arteriosus	Nasolacrimal duct obstruction	Craniosynostosis

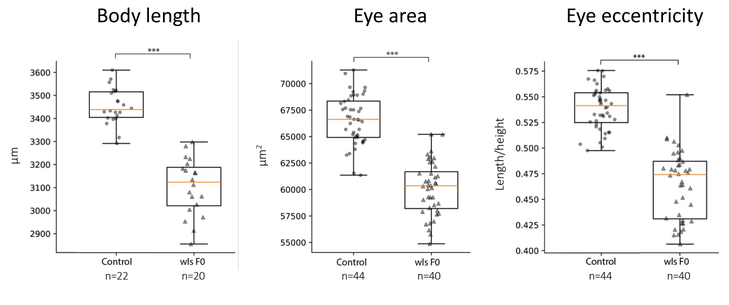
Clinical features of the individuals described in this and previous studies.[2,3,4] In families 1-2, phenotypes shared across all affected individuals unless specified.
Abbreviations: BL: bilateral, CC: corpus callosum, CKD: chronic kidney disease, CV: cerebellar vermis, F: female, ID: intellectual disability, L: left, M: male, n: number of affected individuals, n/a: not available, R: right. *1 individual was not assessed.

Zebrafish *wls* crispants show reduced eye, brain and body size



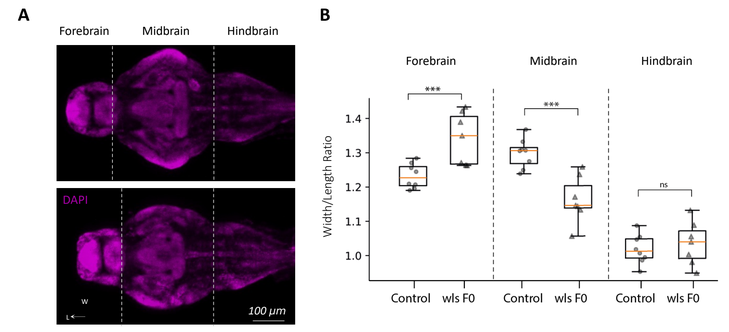
F0 *wls* crispants (wls F0) larvae at 4 days post-fertilization (dpf) exhibit visibly reduced:

- Body length
- Eye size (and altered shape)
- Otic vesicles (*Ot*) size (*indicated by dashed line*)
- Mandible (*Mn*) size
- Swim bladder (*Sb*) inflation



Quantification of body length, eye area and eccentricity in wls F0 at 4 dpf.

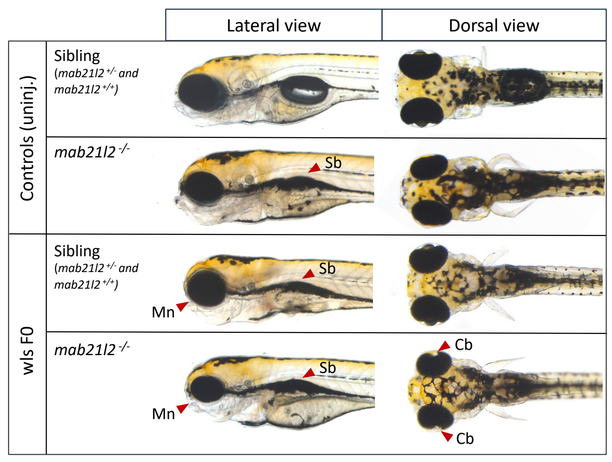
wls F0 larvae display significant (*** $p < 0.001$):
- Reduction in body length
- Reduction in eye size
- More spherical eye shape



A) Forebrain, midbrain and hindbrain of 5 dpf larvae, separated by white dashed lines (ventral view).
Wls F0 brains display visibly reduced:
- Forebrain length
- Midbrain width

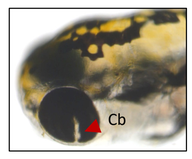
B) Quantification of width/length ratios in forebrain, midbrain and hindbrain of wildtypes (n=8) and wls F0 (n=7).
wls F0 brains display significant (*** $p < 0.001$) differences in:
- Forebrain (increased width/length ratio)
- Midbrain (decreased width/length ratio)

Zebrafish *mab21l2* mutants display more severe eye phenotypes upon abrogation of *wls* function



Phenotypes of *mab21l2* larvae at 4 dpf with and without abrogation of *wls*

- wls control (uninjected):
  - *mab21l2*$^{+/+}$ and *mab21l2*$^{+/-}$ were phenotypically normal
  - *mab21l2*$^{-/-}$ exhibited microphthalmia and variable swim bladder (*Sb*) defects
- *wls* F0 crispants:
  - All wls F0 larvae exhibited consistently reduced Mn size and Sb defects, independent of the *mab21l2* genotype
  - *mab21l2*$^{-/-}$ with abrogated *wls* exhibited an additional ocular coloboma (*Cb*) phenotype in 100 % of the embryos



58/58 *mab21l2*$^{-/-}$ fish with abrogated *wls* displayed bilateral coloboma

	Average	Controls (unjected)	Wls F0 (injected)	Effect (% change)
Siblings (*mab21l2* $^{+/+}$ *and* *mab21l2* $^{+/-}$)	Eye Length (µm)	319	296	Reduction (7.20%)***
	Body Length (µm)	3492	3134	Reduction (10.23%)***
	Eye/Body Ratio	0.091	0.095	Increase (3.46%)***
	Head/Body Ratio	0.2296	0.2304	Increase (0.35%, ns)
mab21l2 $^{-/-}$	Eye Length (µm)	299	276	Reduction (7.70%)***
	Body Length (µm)	3579	3244	Reduction (9.36%)***
	Eye/Body Ratio	0.084	0.086	Increase (1.83%, ns)
	Head/Body Ratio	0.2230	0.2216	Reduction (0.62%, ns)

Comparison of average eye length, body length, eye/body ratio and head/body ratio across groups.
Mab21l2 mutants with abrogated *wls* show a significant (*** $p<0.001$) reduction in:
- Eye size
- Body length

Discussion

- We describe two novel cases with rare biallelic *WLS* variants which are predicted damaging. Both displayed AMC, kidney defects and osteopenia, in addition to facial, ear and digit dysmorphisms. Case 2 also presented with genital anomalies.
- Our findings provide further evidence for the inclusion of eye anomalies among the recurrent features of the *WLS* spectrum, being now observed in 50% of examined individuals (n=6/12), while also highlighting additional phenotypes such as kidney defects (n=5/13), osteopenia (n=3/14) and genital anomalies (n=3/4 males).
- Our clinical findings are supported by zebrafish *wls* F0 crispants, which recapitulate phenotypic features observed in patients such as reduced eye, jaw and body size, and altered brain size and shape.
- Disrupting *wls* function in *mab21l2*$^{-/-}$ zebrafish additionally resulted in coloboma, a phenotype not observed in other AMC-sensitised fish (*lrp5*, *lrp6*, *taz*, and *lama1),* suggesting that the impact of *wls* dysfunction on eye development depends on specific genetic backgrounds, specifically demonstrating an interaction between *mab21l2* and *wls.* Variability with genetic background might help explain the variability in ocular (and other) anomalies observed in patients with *WLS* variants.
- By highlighting the critical role of genetic interactions, our study marks a shift in the understanding of these disorders from the prevailing single-gene paradigm.

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