

## **Novel *FOXA2* Mutation Causes Hyperinsulinism, Hypopituitarism with Craniofacial and Endoderm-Derived Organ Abnormalities**

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1      **Abstract**  
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4      Congenital hypopituitarism(CH) is characterised by the deficiency of one or more pituitary  
5      hormones and can present alone or in association with complex disorders. Congenital  
6      hyperinsulinism(CHI) is a disorder of unregulated insulin secretion despite hypoglycemia  
7      that can occur in isolation or as part of a wider syndrome. Molecular diagnosis is unknown in  
8      many cases of CH and CHI. The underlying genetic etiology causing the complex phenotype  
9      of CH and CHI is unknown. In this study, we identified a *de novo* heterozygous mutation in  
10     the developmental transcription factor, forkhead box A2, *FOXA2* (c.505T>C,p.S169P) in a  
11     child with CHI and CH with craniofacial dysmorphic features, choroidal coloboma and  
12     endoderm-derived organ malformations in liver, lung and gastrointestinal tract by whole  
13     exome sequencing. The mutation is at a highly conserved residue within the DNA binding  
14     domain. We demonstrated strong expression of *Foxa2* mRNA in the developing  
15     hypothalamus, pituitary, pancreas, lungs and oesophagus of mouse embryos using *in situ*  
16     hybridization. Expression profiling on human embryos by  
17     immunohistochemistry showed strong expression of hFOXA2 in the neural tube, third  
18     ventricle, diencephalon and pancreas. Transient transfection of HEK293T cells with Wt(Wild  
19     type) hFOXA2 or mutant hFOXA2 showed an impairment in transcriptional reporter activity  
20     by the mutant hFOXA2. Further analyses using western blot assays showed that the *FOXA2*  
21     p.(S169P) variant is pathogenic resulting in lower expression levels when compared with  
22     Wt hFOXA2. Our results show, for the first time, the causative role of *FOXA2* in a complex  
23     congenital syndrome with hypopituitarism, hyperinsulinism and endoderm-derived organ  
24     abnormalities.

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1    **Introduction**

2    The pituitary gland is a master regulator of vital physiological functions such as growth,  
3    puberty, lactation, metabolism, stress response and reproduction. The development of the  
4    pituitary gland is tightly controlled by signaling molecules and transcription factors that  
5    dictate pituitary cell lineage specification, cell proliferation and terminal differentiation into  
6    hormone-producing cells (1, 2). Abnormal pituitary development can lead to congenital  
7    hypopituitarism (CH) resulting in deficiency in one or more pituitary hormones. CH  
8    comprises of a spectrum of disorders with variable phenotypes that can range in severity,  
9    from isolated hormone deficiency [isolated growth hormone deficiency being the most  
10   common] to combined pituitary hormone deficiency (CPHD) when two or more pituitary  
11   hormones are deficient. CH may present as part of a syndrome with abnormalities in  
12   structures that share a common embryological origin with the pituitary gland, such as the  
13   forebrain and eyes, leading to septo-optic dysplasia (SOD) or holoprosencephaly (HPE) (1).  
14   SOD is a rare condition with a prevalence of 1:10,000(3) live births and comprises the  
15   following features: optic nerve hypoplasia, midline forebrain defects and hypopituitarism (4,  
16   5). Mutations in transcription factors such as *HESX1* (6), *PROPI* (7), *POU1F1* (8), *LHX3* (9),  
17   *LHX4* (10), *PITX1*, *PITX2* (11), *OTX2* (12), *SOX2* (13) and *SOX3* (14, 15) have been  
18   associated with CH in mouse and humans. However, these mutations account only for a small  
19   proportion of CH patients with the majority of patients having an unknown genetic cause for  
20   their symptoms.

21   Congenital hyperinsulinism (CHI) is a rare condition with an estimated prevalence of 1 in  
22   50,000 live births, characterized by an inappropriate secretion of insulin from the  $\beta$ -cells of  
23   the pancreas during hypoglycemia (16). CHI is the most common cause of severe and  
24   persistent hypoglycemia in the neonatal period. The identification and appropriate  
25   management of this condition is very important to avoid hypoglycemic episodes and prevent

1 the consequent neurological impairment. Mutations in genes *ABCC8* (17-21), *KCNJ11* (17-  
2 21), *GLUD1* (22), *GCK* (23), *HADH* (24), *UCP2* (25), *HNF4A* (26), *HNF1A* (26), *MCT1*  
3 (27), *HK1* (28) and *PGM1* (29) have been associated with genetic forms of CHI (30).

4 However, the genetic cause for many CHI patients remains elusive.

5 *FOXA2* (formerly hepatocyte nuclear factor-3 $\beta$ , *HNF-3 $\beta$* ) belongs to the family of the  
6 forkhead class of transcription factors that has an essential role in embryogenesis during the  
7 formation of the node, notochord and floorplate (31, 32) which are important for the  
8 development of the vertebrate body axis. Thus, *Foxa2* null embryos die during early  
9 gestation, at embryonic day 9.5, and fail to form axial mesoderm (31). Later during  
10 organogenesis, *Foxa2* co-operates with *Foxa1* and are required for the formation of  
11 endoderm-derived organs such as the liver (33), lung (34), pancreas (35) and gastrointestinal  
12 tract(36). *Foxa2* has been shown to be important in the development of anterior forebrain  
13 structures, which have the same embryonic origin as the pituitary gland(37). Data from  
14 murine genetic studies have shown a genetic interaction between *Foxa2* and Sonic Hedgehog  
15 (*Shh*) signaling pathway with overlapping expression pattern of *Foxa2* and *Shh* in the  
16 notochord and floor plate at E8.5. *Foxa2* can modulate *Shh* signalling, contributing to the  
17 specification of ventral motor neuron progenitor identity(38). The secretion of Shh by the  
18 notochord and floor plate is an important morphogenetic signal that is required for the  
19 development of central nervous system including the pituitary gland(39). In the islet cells of  
20 mature pancreas, *Foxa2* has been shown to activate components of insulin secretion, such as  
21 sulfonylurea receptor1 [SUR1], encoded by *ABCC8* (40) and the inward rectifier potassium  
22 channel member 6.2 [Kir 6.2], encoded by *KCNJ11*(40). In humans, mutations in *ABCC8* and  
23 *KCNJ11* (17-21) are the most common causes of genetic forms of CHI. Notably, tissue-  
24 specific deletion of *Foxa2* from the pancreatic  $\beta$ -cells (*Foxa2*<sup>loxP/loxP</sup>; Ins:Cre) has been shown  
25 to the development of CHI in mouse(41).

1 Thus, *FOXA2* is an important developmental transcription factor required for the formation of  
2 ventral midline structures such as the floorplate and forebrain, as well as endoderm-derived  
3 organs including the pancreas, and regulating glucose homeostasis in mature pancreatic  $\beta$ -  
4 cells. To date, mutations in *FOXA2* causing disorders of glucose homeostasis, endoderm  
5 abnormalities and CH have not been described in humans. In this study, we report for the first  
6 time, a “*de novo*” heterozygous mutation in *FOXA2* in a patient with unique clinical  
7 phenotype of CH, persistent CHI, craniofacial dysmorphism, abnormalities in the liver, heart,  
8 lung and the gastrointestinal tract. This finding brings a new insight into the underlying  
9 molecular cause of a complex clinical phenotype that will serve as a tool to elucidate the  
10 pathogenesis of these congenital malformations and to better understand the development and  
11 function of the pituitary gland and the pancreas.

## 12 **Results**

### 13 **Clinical findings**

14 Our patient, a 5-year-old girl, was born to non-consanguineous Caucasian British parents at  
15 42 weeks’ gestation with a birth weight of 4.185 Kg (+1.72 SDS). The pregnancy was normal  
16 and the 20-week antenatal scan showed polyhydramnios. The delivery was complicated by  
17 shoulder dystocia, needing resuscitation. She was found to be persistently hypoglycemic  
18 (blood glucose<2.5mmol/L) requiring a total glucose load of 25 mg/kg/min (normal: 4-6  
19 mg/kg/min) to maintain normoglycemia (plasma glucose>3.5mmol/L). She had low free  
20 thyroxine (FT4) (5.3 pmol/L) and suppressed thyroid-stimulating hormone (TSH) (<0.03  
21 mu/L) that persisted even after the phase of acute severe illness. She also had an undetectable  
22 adrenocorticotropic hormone (ACTH) (<1.1 pmol/L) with no cortisol response to synacthen  
23 stimulation (peak cortisol to synacthen <50 nmol/L). **Hydrocortisone replacement**  
24 (**10mg/m2/day**) was commenced followed by levothyroxine therapy. The MRI scan of the  
25 brain showed a hypoplastic anterior pituitary, absent posterior pituitary, interrupted pituitary

1 stalk and a thin corpus callosum (Figure 1B). The hypoglycemia persisted and further  
2 investigations showed an inappropriately high plasma insulin (200 pmol/L) and c-peptide  
3 (1500 pmol/L) with suppressed plasma free fatty acid (<100 μmol/L) and beta hydroxyl  
4 butyrate (<100 μmol/L) during hypoglycemia (blood glucose: 1.2mmol/L) confirming the  
5 diagnosis of CHI. The GH was undetectable at the time of hypoglycaemia [<0.05μg/L]. A  
6 trial of diazoxide (5 mg/kg/day) was commenced along with chlorothiazide (7mg/kg/day).  
7 However, the patient suffered from significant fluid retention leading to discontinuation of  
8 diazoxide. Commencement of octreotide (10 mcg/kg/day) caused a derangement of liver  
9 enzymes and therefore had to be discontinued after which the liver enzymes returned to  
10 normal levels. She developed significant feed intolerance due to severe gastroesophageal  
11 reflux which persisted despite maximum medical treatment. A gastro-jejunostomy tube was  
12 inserted to support feeding. Normoglycemia was maintained by continuous feed via the  
13 gastro-jejunostomy tube. Genetic analysis was negative for *ABCC8*, *KCNJ11*, *HNF4A* and  
14 *GCK* mutations. The 18F-DOPA PET-CT scan of the pancreas suggested a diffuse uptake.

15 The facial dysmorphic features comprise of a single median maxillary central (SMMC)  
16 incisor (Figure 1A), congenital nasal pyriform aperture stenosis (CNPAS), which was  
17 conservatively managed, and a left choroidal coloboma. She does not have any vision  
18 abnormalities. The cardiac echocardiogram revealed pulmonary stenosis which required  
19 balloon dilatation. She had a persistent oxygen requirement(0.5-1L via nasal cannulae) of  
20 unknown etiology (negative for respiratory infections, normal chest imaging(CT) and  
21 bronchoscopy) from birth. At 1.5 years of age she was diagnosed with growth hormone (GH)  
22 deficiency (height<-3 SDS, IGF1<3.3 nmol/L and a peak GH of 1.1 μg/L (normal>7 μg/L) to  
23 arginine stimulation) and was commenced on rGH (recombinant GH) therapy. She  
24 demonstrated a good response to treatment with rGH (25 mcg/kg/day) with an improvement  
25 in the height velocity (Figure 1C). She developed persistently elevated liver transaminases

1 when she was 3 years old, with a negative autoimmune hepatitis and infection screen. The  
2 liver biopsy showed dense chronic inflammation with portal-portal bridging fibrosis. The  
3 clinical features are summarised in Table 1.

4 She is currently 5 years old, with persistent CHI, motor, speech and developmental delay and  
5 continues to be on rGH, levothyroxine and hydrocortisone replacements. There are no  
6 symptoms suggestive of diabetes insipidus and the biochemistry has been completely normal.  
7 She has shown response to the reintroduction of diazoxide (5 mg/kg/day) and chlorothiazide  
8 without any features of fluid retention, which has enabled her to come off continuous feeds  
9 for 6 hours.

10 **Mutation analysis**

11 A novel heterozygous *FOXA2* mutation (c.505T>C, p.S169P) was identified in the affected  
12 child but not in the parents by whole exome sequencing. To further validate our results, the  
13 mutation was confirmed by Sanger sequencing (Figure 1D). The variant is not present in  
14 control databases (ExAc, dbSNP, 1000 genome). Multiple sequence alignment shows that the  
15 serine residue at position 169 is highly conserved across different species, from drosophila,  
16 human, mouse, chicken to frog (Figure 1E), suggesting that this residue is functionally  
17 important and has been maintained throughout evolution in different species. The *FOXA2*  
18 mutation (c.505T>C, p.S169P) lies at the DNA binding domain of the transcription factor. In  
19 silico analysis using SIFT, PolyPhen, Mutation Taster predict this aminoacid substitution to  
20 have deleterious impact on the protein function.

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1   **Functional analysis**

2   To further characterize the possible role of *FOXA2* in the observed clinical phenotype, we  
3   studied the expression of *Foxa2* at the mRNA level during mouse embryonic development.  
4   *Foxa2* mRNA transcripts were detected in the midbrain, ventral forebrain, ventral hindbrain,  
5   epithelial structures lining the main bronchus, lungs and the esophagus from embryonic day  
6   e11.5 (Figure 2A). Importantly, the expression of *Foxa2* mRNA was detected in the pituitary  
7   gland from e13.5 (Figure 2C-C') in the anterior lobe. From e15.5, *Foxa2* mRNA expression  
8   was stronger with robust expression in the ventral diencephalon, posterior lobe and anterior  
9   lobe of the pituitary gland (Figure 2D-D'). At embryonic day e18.5, *Foxa2* mRNA transcripts  
10   were localized in the hypothalamic-pituitary axis, with transcripts strongly expressed in the  
11   ventral hypothalamus and anterior pituitary (Figure 2E-E'). Analysis of hFOXA2 expression  
12   in human embryos by immunohistochemistry revealed expression of hFOXA2 in the ventral  
13   neural tube (Figure 3A') and in the diencephalon (Figure 3A'') at six weeks (Carnegie stage  
14   16), and around the third ventricle at 8 weeks (Carnegie stage 23) (Figure 3B'). hFOXA2 was  
15   specifically localized in the cytoplasm of cells scattered in the pancreatic parenchyma at 13  
16   weeks of gestation (Figure 3C-C'-C''). In summary, our expression analysis shows  
17   expression of *Foxa2* mRNA in hypothalamic-pituitary axis and lungs during mice embryonic  
18   development. hFOXA2 expression was detected in midline neural tube and pancreas.

19   *FOXA2* has been previously shown to bind and activate the human glucose transporter type 2  
20   (*GLUT2*) (42). To determine the pathogenic effect of *FOXA2* p.S169P variant, we performed  
21   transcriptional activation assays using the *GLUT2* promoter coupled to luciferase (phGT2-  
22   294-promoter-luc). We used transiently transfected HEK293T cells with equal quantities of  
23   Wt hFOXA2 or mutant hFOXA2 p.S169P and demonstrated that the hFOXA2 p.S169P  
24   significantly impairs the transcriptional activation of the *GLUT2* luciferase reporter (Figure  
25   4A). We also performed quantification of protein expression using western blot and showed

1 that the mutant hFOXA2 p.S169P results in significantly reduced protein expression levels  
2 compared to the Wt hFOXA2 (Figure 4B). Using double immunofluorescence on transiently  
3 transfected HEK293T cells, we demonstrated that both the Wt hFOXA2 and mutant hFOXA2  
4 are expressed in the nucleus and the mutation did not result in changes to cellular localization  
5 (Figure 5). Together the results indicate that the hFOXA2 p.S169P variant results in lower  
6 transcriptional activity due to an effect of the mutation on the FOXA2 protein levels.

7 **Discussion**

8 We have characterized a “*de novo*” heterozygous mutation in the developmental transcription  
9 factor *FOXA2* that causes a rare and unique clinical phenotype of hypopituitarism, CHI,  
10 dysmorphic features, liver, pancreas, heart and gastrointestinal abnormalities. The c.505T>C,  
11 p.S169P genetic variant occurs at the conserved forkhead DNA binding domain of the  
12 *FOXA2*. This region binds to the DNA and may provide tissue-specific gene regulation  
13 important for the development of multiple organs. Our data has confirmed that the mutation  
14 impairs the transcriptional activation of *FOXA2*. Importantly, *FOXA2* is localised at the  
15 cytogenetic location 20p11.2 and some studies have linked chromosomal deletions within  
16 this region with the clinical phenotype of hypopituitarism, central nervous system (CNS)  
17 defects, hypoglycemia, facial dysmorphic features and congenital abnormalities of the heart,  
18 liver and gastrointestinal tract (43-47). Chromosomal deletions of the 20p11.2 are rare(46)  
19 and recent studies have mapped the minimal critical region which contains 20 genes,(43)  
20 including *FOXA2*. All the patients reported to have the 20p11.2 chromosomal deletion have  
21 hypopituitarism, CNS abnormalities and facial dysmorphic features as shared features,  
22 strongly indicating that a gene or multiple genes within this chromosome region have a key  
23 role in CNS, pituitary and facial development. We have identified the gene responsible for  
24 the clinical phenotype of hypopituitarism at the 20p11.2 region as *FOXA2* and confirmed the  
25 causative role with functional analysis.

1 In our study we show that *FOXA2* mutation causes the clinical phenotype of hypopituitarism,  
2 CHI and facial dysmorphic features that overlaps with the clinical data published in patients  
3 with 20p11.2 deletions(43-47). The mutation in hFOXA2 p.S169P is pathogenic, as it results  
4 in impaired transcriptional activation of the phGT2-294-promoter-luc reporter and significant  
5 reduction in the protein expression compared to wild type hFOXA2. Interestingly, *FOXA2*  
6 has been shown to regulate key signaling pathways important in ventral midline, pituitary and  
7 CNS development such as *Shh* signaling<sup>38</sup>. Data from in vivo studies using Wnt1:Cre;  
8 *Foxa2*<sup>flox/flox</sup> embryos showed that *Foxa2* has an early role in the initiation of Shh expression.  
9 *Foxa2*, in combination with *Foxa1*, downregulates the expression of intracellular transducers  
10 and downstream targets of Shh signalling such as Ptch1,Gli1 and Gli2, which regulate the  
11 patterning of the ventral midbrain(38). Hence, we hypothesise that hFOXA2 could mediate  
12 its role in pituitary development by regulating Shh expression. Moreover, the midline  
13 anomalies in our patient including SMMC, CNPAS are often associated with pituitary  
14 abnormalities, as described in an extensive literature review by Lo et al (48), where  
15 hypopituitarism or growth hormone deficiency were identified in 43-48% of patients with  
16 CNPAS or SMMC. This is consistent with the clinical presentation of our patient, who has  
17 hypopituitarism along with hypoplastic pituitary, thin corpus callosum and thin pituitary stalk  
18 on the MRI.

19 The detection of *Foxa2* mRNA transcripts from the early stages of mouse pituitary and brain  
20 embryonic development suggests a potential role in the development of these structures.  
21 Furthermore, the detection of hFOXA2 by immunohistochemistry in human embryos at  
22 various developmental stages, along with the biochemical experiments demonstrating that the  
23 variant p.S169P mutation in *FOXA2* impairs transcriptional activation and protein expression  
24 levels, strongly indicate that *FOXA2* has a pivotal role in hypothalamic-pituitary axis  
25 formation in humans.

1 The co-existence of hypopituitarism along with a persistent form of hyperinsulinism, as  
2 encountered in our patient, is extremely uncommon. Hypoglycemia in CHI is caused by  
3 unregulated insulin secretion while in hypopituitarism it is due to the lack of counter-  
4 regulatory hormonal response due to the deficiency of ACTH and GH. Diagnosis can often  
5 be challenging, as the hallmark of CHI is detectable insulin in the presence of hypoketotic  
6 hypoglycemia while hypopituitarism causes ketotic hypoglycemia. Almost half of the  
7 patients with persistent CHI do not have mutations in the already recognized genes known to  
8 cause CHI. Genetic diagnosis is important as it will inform the prognosis, recurrence risk and  
9 guide the medical management besides providing valuable insight into  $\beta$ -cell physiology. The  
10 negative mutations in the known CHI genes in our patient together with strong biochemical  
11 evidence of CHI, makes it highly likely that the CHI in our patient is due to a novel genetic  
12 aetiology (*FOXA2*). We have further confirmed this by demonstrating the expression of  
13 hFOXA2 in the developing human pancreas.

14 Glucose-stimulated insulin secretion occurs by the closure of ATP dependent KATP channels  
15 situated on the  $\beta$ -cell membrane with the resultant depolarization of the membrane causing  
16 the exocytosis of the insulin granules(16, 30). KATP channels consist of 2 subunits, SUR1  
17 and Kir6.2, encoded by *ABCC8* and *KCNJ11* respectively, the mutations of which cause  
18 defects in the channels resulting in the most common form of genetic CHI(16, 30). Lantz et.al  
19 demonstrated that when SUR1 or Kir6.2 promoter/luciferase reporter was transfected with  
20 *Foxa2* expression plasmids, *Foxa2*-Sur1 and *Foxa2*-Kir6.2 promoter constructs showed 6-  
21 fold and 4-fold activation respectively demonstrating a vital role of *Foxa2* in the  
22 transcriptional activation of the KATP subunits(40). Hence, a *FOXA2* mutation could  
23 potentially alter the expression of SUR1 and/or Kir6.2 leading to hyperinsulinism although  
24 the precise mechanism is yet to be explored. The other possible mechanism could be linked  
25 to *HADH* that encodes L-3-Hydroxyacyl-CoA-dehydrogenase (HADH), an enzyme involved

1 in the penultimate step of the beta-oxidation pathway(8). Mutations in *HADH* cause CHI in  
2 humans. It has been demonstrated in mice that *Foxa2* directly targets *HADH* causing its  
3 transcriptional activation(49). Sund et.al demonstrated that *Foxa2* knocked out from the β-  
4 cells in mice show a 3-fold downregulation of *Hadhd* mRNA leading to severe  
5 hyperinsulinaemic hypoglycaemia(41, 42) The third possibility could be linked to *GLUT2*,  
6 which is expressed in the plasma membrane of the pancreatic β-cells, liver, kidney and  
7 intestine to facilitate insulin secretion by transporting the glucose across the cell membrane  
8 (29). Wang et. al showed that *GLUT2* plays an important role in the insulin secretion from  
9 the β-cells as its mRNA level is influenced by the plasma concentrations of glucose and  
10 insulin(50). Cha et. al demonstrated that *GLUT2* has binding sites for *FOXA2* and showed  
11 that the promoter activity of *GLUT2* is synergistically activated by *FOXA2* in NIH3T3  
12 cells(42). *FOXA2* also plays a critical role in the tissue specific expression of *GLUT2*(42).  
13 The reduction in the transcriptional activation of the *GLUT2* reporter (phGT2-294-promoter-  
14 luc) activity by the mutant hFOXA2 (p.S169P) shown in our transcriptional assay  
15 experiment, could imply that the *GLUT2* tissue expression is reduced in the pancreatic β-  
16 cells of patients with *FOXA2* mutation. However, the precise mechanism by which this leads  
17 to hyperinsulinaemic hypoglycaemia is not yet understood.  
18 It is also plausible that *Foxa2* plays a role in the development of the pancreas. *Foxa2* has  
19 been shown to regulate *Pdx1*, a homeobox gene essential for pancreatic development(49).  
20 *Foxa2* has also been linked to regulating the mRNA levels of pancreatic transcription factors  
21 such as *Hnf4a* and *Hnfla*, mutations of which can cause monogenic forms of diabetes  
22 mellitus. However, some studies contradict that *Foxa2* is an upstream regulator of  
23 *Pdx1*,*Hnf4a* and *Hnfla*(50) . While it has been shown that β-cell-specific deletion of *Foxa2*  
24 in mice causes a phenotype of hypoglycaemia(41), it also has been demonstrated that it can  
25 cause downregulation of *Pdx1* mRNA causing the reduction of PDX-1 protein levels in the

1 pancreatic islets(51) and a targeted  $\beta$ -cell-specific deletion of *Pdx1* results in diabetes in  
2 transgenic mice(52). Thus, *FOXA2* is a crucial transcription factor that controls the  
3 expression of multiple genes involved both in glucose sensing and glucose homeostasis and  
4 therefore has a potential role in diseases involving insulin secretion and glucose homeostasis.  
5 Diazoxide is used as an effective treatment in majority of patients with CHI except in those  
6 with mutations abolishing the KATP channel activity (*ABCC8* or *KCNJ11*) or activating  
7 mutations in *GCK*. Our patient has shown response to diazoxide treatment which could  
8 potentially imply that the variant p.S169P has not completely abolished the KATP channel  
9 activity or increased the *GCK* expression.

10 Whilst it is difficult to speculate the progression of abnormalities in glucose homeostasis in  
11 patients with *FOXA2* mutation, screening more patients with similar phenotype will give  
12 further insights into the role of this transcription factor in the insulin secretion and in related  
13 diseases like neonatal diabetes mellitus and maturity onset diabetes of the young (MODY).  
14 The main limitation of our study is the lack of more patients with similar phenotype. As the  
15 combination of the phenotype comprising CHI and hypopituitarism is extremely rare, we  
16 were unable to recruit more patients for this study. However we are hopeful that the  
17 dissemination of the findings from this study will hopefully alert the researchers from across  
18 the world to screen for *FOXA2* mutations in patients with similar phenotype, thereby  
19 enabling a better understanding of genotype-phenotype correlations.

20 In conclusion, we have identified the first disease-causing mutation in *FOXA2* in an  
21 individual with an extremely rare complex phenotype of CHI, cranio-facial dysmorphic  
22 features, CH, cardiac, liver and gastrointestinal abnormalities. Identification of the genetic  
23 cause contributing to such a unique clinical phenotype will help medical management and  
24 provide valuable insights into molecular mechanisms underlying pituitary development and  
25  $\beta$ -cell physiology.

1   **Material and Methods**

2   **Patient enrolment**

3   The patient was recruited to the ‘Whole exome sequencing for rare endocrine disorders’  
4   study following written consent from the parents. The study was given favorable ethical  
5   opinion by the North West - Liverpool Central Research Ethics Committee (REC Reference:  
6   15/NW/0758) and site study approval was granted by the Clinical Research Business Unit at  
7   Alder Hey Children’s NHS Foundation Trust, Liverpool, UK.

8   **DNA extraction**

9   DNA was obtained from blood samples of the child and both the biological parents (trio)  
10   using the QIAamp DNA blood Midi Kit (Qiagen, Hilden, Germany) as per the manufacturer’s  
11   instructions and subjected to whole exome sequencing. The quality and the quantity of the  
12   genomic DNA were assessed using the Qubit (ThermoFisher Scientific) and the NanoDrop  
13   (ThermoFisher Scientific).

14   **Library preparation, exon capture and sequencing**

15   The samples (3 µg/sample) were sheared with the Picoruptor to a size of approximately 150-  
16   200 bp. The samples were cleaned with 1.8x AMPure beads (Agencourt) and end repaired at  
17   20°C for 30 minutes. The products were A tailed by incubation at 37°C for 30 minutes,  
18   cleaned with AMPure beads again and ligated to index adapters at 30°C for 10 minutes to  
19   make a pre-capture library using the Agilent Sureselect XT target enrichment system for  
20   Illumina. Enrichment was achieved by 5 rounds of PCR using Herculase II fusion DNA  
21   polymerase. The libraries were checked on an Agilent HS Bioanalyser chip and quantified by  
22   Qubit Assay. 750 ng of pre-capture library was used for the hybridization. Samples were  
23   lyophilized to attain the required volume. Libraries were then mixed with hybridisation  
24   buffer, baits from the Human All Exon 5 kit and incubated overnight (24h) at 65°C. The  
25   samples were then mixed with washed streptavidin beads (Dynabeads MyOne Streptavidin

1 T10) and the captured products were washed and pooled. The quantity and quality of the pool  
2 was assessed by Bioanalyzer and subsequently by qPCR using the Illumina Library  
3 Quantification Kit from Kapa on a Roche Light Cycler LC480II, according to manufacturer's  
4 instructions. The template DNA was denatured according to the protocol described in the  
5 Illumina cBot user guide and loaded at 300 pM concentration. Sequencing was carried out on  
6 one lane of an Illumina HiSeq4000 at 2x150 bp paired-end sequencing with v1 chemistry.

7 **Bioinformatics**

8 The sequence data were aligned to the reference genome (GRCh37/hg19). Reads were  
9 mapped to the reference sequences using BWA mem version 0.7.5a(53) with default  
10 parameters. The mean depth of the coverage was 100x. In order to retain only confidently  
11 aligned reads, alignments were filtered to remove reads with a mapping quality lower than  
12 10. The mapped reads were locally re-aligned to improve the alignments around small  
13 insertions/deletions (indels) using the Genome Analysis Tool Kit (GATK) version 2.1.13  
14 (54). Base quality scores were recalibrated using GATK Base Quality Score Recalibrator  
15 (BQSR). BQSR is a module of GATK to create more accurate base qualities, which in turn  
16 improves the accuracy of our variant calls. The variants identified were annotated using  
17 SnpEff. The variants present in at least 1% minor allele frequency in 1000 Genomes Project,  
18 Exome aggregation consortium (ExAC), dbSNP142, and NHLBI ESP exomes were  
19 excluded. The predicted deleterious variants included non-synonymous coding, splice site,  
20 frameshift, and stop gain variants. The analysis of variants was performed using the ingenuity  
21 variant analysis (Qiagen bioinformatics) software. The identified potential variant  
22 segregating with the patient's phenotype was subsequently confirmed by Sanger sequencing.

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1   **Mice**

2   All mice were housed with a 12h light/12h dark cycle in a temperature- and humidity-  
3   controlled room (21°C, 55% humidity) with constant access to food and water. Timed  
4   pregnancies were achieved by mating females and males overnight and, the presence of  
5   vaginal plug the following morning, was considered as embryonic day (e) 0.5. All  
6   experiments were conducted under the regulations, licenses and local ethical review of the  
7   UK Animals (Scientific Procedures) Act 1986.

8   **Immunohistochemistry**

9   Paraffin-embedded human tissue samples at 6, 8 and 13 weeks of gestation were obtained  
10   from the Human Developmental Biology Resource (Institute of Genetic Medicine,  
11   Newcastle, and Institute of Child Health, London; [www.hdbr.org](http://www.hdbr.org)). Immunohistochemistry  
12   was performed by deparaffinisation of the sections followed by rehydration through  
13   decreasing ethanol dilutions. Heat-induced antigen retrieval was performed with a microwave  
14   in 10 mM sodium citrate buffer (pH 6). Samples were left to cool down at room temperature  
15   before incubating them for 1hr in blocking buffer [1XPBS, 0.1% Triton X-100, 5% Normal  
16   Goat Serum (Vector Laboratories)]. Endogenous hFOXA2 was detected with a primary  
17   rabbit monoclonal antibody against hFOXA2 (Thermo Fisher Scientific; 701698; 1:250)  
18   followed by a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories; BA-  
19   1000; 1:300). Staining was achieved using DAB Peroxidase Substrate Kit (Vector  
20   Laboratories; SK-4100). The colorimetric reaction was stopped with washes in water and the  
21   sections were counterstained using Haematoxylin (Sigma-Aldrich). Images were acquired  
22   using a Leica microscope and figures were done with Adobe Photoshop CS6.

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1    ***In situ* hybridization**

2    Wild type mouse embryos were collected at different embryonic stages of mouse  
3    development (e.11.5, e12.5, e13.5, e15.5 and e18.5), fixed with 4% paraformaldehyde (PFA)  
4    and washed in PBS before proceeding with paraffin embedding. Paraffin-embedded mouse  
5    embryos were sectioned at 7 µm thickness for histochemical evaluation. In situ hybridisation  
6    was performed by processing the slides with a pre-hybridisation treatment. Sections were  
7    deparaffinised, rehydrated through decreasing ethanol dilutions, fixed with 4% PFA,  
8    incubated with proteinase K, fixed again with 4% PFA and finally incubated with 0.1 M  
9    triethanolamine, 0.1% acetic anhydride. The mouse Foxa2 gene fragment (1567 bp) plasmid  
10   was kindly provided by www.hdb.org. The digoxigenin-labeled anti-sense probe for mFoxa2  
11   was generated by in vitro transcription using T3 RNA polymerase (Roche). Hybridization  
12   with 100 ng of the digoxigenin-labeled probe was carried out overnight at 65°C. Sections  
13   were washed in 0.1 M Tris-HCl Buffer (pH = 7.5, 0.15 M Sodium) followed by 1 hour  
14   blocking at room temperature and overnight incubation at 4°C with anti-Dig antibody  
15   (Sigma-Aldrich). Detection of murine Foxa2 was achieved by colorimetric reaction using 4-  
16   Nitro blue tetrazolium chloride solution (NBT, Sigma-Aldrich) and 5-Bromo-4-chloro-3-  
17   indolyl phosphate disodium salt (BCIP, Sigma-Aldrich). Images were acquired using a Leica  
18   microscope and figures were done using Adobe Photoshop CS6.

19    **Plasmids and site-direct Mutagenesis**

20    Full length cDNA of human FOXA2 (GENE Bank RefSeq NM\_021784.4) was cloned in  
21    ORF mammalian expression vector pCMV3 (pCMV3-hFOXA2, Sino Biological Inc). E.coli  
22    DH5α competent cells were transformed with hFOXA2 (cDNA size: 1392 bp). The detected  
23    mutation was introduced by site-directed mutagenesis using QuikChange II XL Site-Directed  
24    Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions  
25    (primers used, Forward strand: 5'-AAAGCCGCCCTACCCGTACATCTCGCTC-3'. Reverse

1 strand: 5'-GAGCGAGATGTACGGTAGGGCGGCTTG-3'). Sanger sequencing  
2 confirmed the point mutation.

3 **Cell culture and Luciferase assays**

4 HEK293T cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented  
5 with 10% FBS.  $2.5 \times 10^5$  cells/well were seeded in 24-well plates. 200 ng of phGT2-294-  
6 promoter-luc reporter (kindly provided by Professor Yong-Ho Ahn) and 100 ng of Renilla  
7 SV-40 were transiently co-transfected with either i) equal amounts (50 ng and 75 ng) of Wt  
8 or mutant p.S169P hFOXA2 expression plasmids or ii) both Wt and mutant p.S169P  
9 hFOXA2 expression plasmid (25 ng or 35.5 ng of each plasmid) using Lipofectamine 2000  
10 (Life Technologies) according to the manufacturer's instructions. The total amount of DNA  
11 transfected was kept constant at 500 ng by adding pBluescript plasmid. The cells were  
12 harvested 24h after transfection and the luciferase activity was measured using the Dual-  
13 Luciferase Reporter Assay System (Promega) in a BMG LABTECH Microplate reader  
14 (Omega, Germany) according to manufacturer's instructions. Firefly luciferase activity was  
15 normalised to the Renilla luciferase expression from pRL-SV40 (Promega). The experiments  
16 were independently repeated four times in triplicates and statistical analysis was performed  
17 using one-way ANOVA.

18 **Western blotting**

19  $1.75 \times 10^5$  cells/well were seeded in 24-well plates and transiently transfected with equal  
20 amounts (200 ng) of Wt or mutant p.S169P hFOXA2 expression plasmids using  
21 Lipofectamine 2000 according to the manufacturer's instructions. 300 ng of pBluescript  
22 plasmid were added to each transfection mix to maintain the total amount of DNA constant at  
23 500 ng. The cells were harvested 24h after the transfection in a lysis buffer containing 50 mM  
24 Tris-Base (pH 7.6), 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Complete  
25 Mini, EDTA-free tablets, Roche) at a 1:6 ratio and 1% phosphatase inhibitor Cocktail3

1 (Sigma-Aldrich). Samples containing 20 µg of total proteins were loaded on 12%  
2 polyacrylamide gel. The proteins were transferred on a nitrocellulose membrane and  
3 nonspecific binding sites were blocked for 1h with 5% dried skimmed milk in PBS-T  
4 (1XPBS, 0.1% Tween 20). The membrane was incubated overnight at 4°C with the primary  
5 antibody (rabbit anti-FOXA2; Thermo Fisher Scientific; 701698, 1:5000 dilution in 5% dried  
6 skimmed milk in PBS-T), followed by one hour incubation with IRDye 800CW Donkey anti-  
7 rabbit antibody (LI-COR Biosciences; 1:5000). Anti-GAPDH (Santa Cruz; 1:5000, rabbit  
8 polyclonal) levels were used to normalise the total level of protein. Blots were analysed using  
9 Odyssey 2.1 Imaging System (LI-COR Biosciences). The experiments were independently  
10 repeated six times and the statistical analysis was performed using one-way ANOVA.

11 **Immunofluorescence**

12  $1 \times 10^5$  cells/well were seeded in 4-well cell culture slide (Millipore, Fisher Scientific) and  
13 transiently transfected with 200 ng of Wt or mutant p.S169P hFOXA2 expression plasmids  
14 and 300 ng of pBluescript plasmid using Lipofectamine 2000 according to the  
15 manufacturer's instructions. 24h after transfection, the cells were fixed in 2% PFA in 1X PBS  
16 for 10 min and washed with 1X PBS three times. Samples were permeabilised with 0.1%  
17 Triton X-100 in 1X PBS for 30 min and blocked with blocking buffer (5% Normal Goat  
18 Serum in 1X PBS) for 30 min. The staining was performed by incubating the samples with  $\alpha$ -  
19 FOXA2 antibody (Thermo Fisher Scientific; 701698, 2ug/ml) in blocking buffer for 1h,  
20 followed by a 30 min incubation with goat  $\alpha$ -rabbit Alexa fluor 594 (ThermoFisher  
21 Scientific; 1:250) and  $\alpha$ -PHALLOIDIN Alexa fluor 488 (Molecular Probes; 1:1000)  
22 antibodies. The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images  
23 were acquired using a fluorescence microscope (Leica microsystem, Germany) and processed  
24 using Adobe Photoshop CS6.

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12   **Conflict of Interest statement:**None declared

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1   **Figure legends**

2   **Figure 1:** Picture of the patient's face showing single central incisor tooth (A, arrowhead).  
3   (B) Sagittal view of the MRI scan of the brain: The normal pituitary gland cannot be  
4   identified, the sella turcica is shallow and poorly defined with possibly a very hypoplastic  
5   anterior pituitary gland (arrowhead). Also, there is no evidence of the normal high signal of  
6   the posterior pituitary. There is a very short and thin pituitary stalk in its superior third  
7   (arrow) which is suggestive of an interrupted pituitary stalk. The corpus callosum is also  
8   noted to be thin (arrowhead). (C) The patient's linear growth curve compared with British  
9   contemporary references. Recombinant GH was started at 1.5 years of age when the linear  
10   height was -3SDS. A good response to GH treatment is seen subsequently with an  
11   improvement in the height SDS. (D) Electropherograms show the wild type (Wt FOXA2 and  
12   the presence of the missense mutation (thymine to cytosine) in the patient at the nucleotide  
13   position 505. (E) The evolutionary conservation of the amino acid residue serine at position  
14   169 is shown across different species such as drosophila, human, mouse, chicken and frog.  
15   Abbreviations: standard deviation score, SDS; wild-type, Wt; growth hormone, GH;  
16   drosophila, DROME; frog, XENTR.

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19   **Figure 2. mRNA expression of *Foxa2* during mouse embryonic development.**

20   A-D represent sagittal sections, with anterior to the left side, and E is a coronal section. A',  
21   C', D', E' show higher-magnification views of the boxed areas in A, C, D, E, respectively. At  
22   embryonic day e11.5 (A) Foxa2 mRNA transcripts were expressed within the midbrain (MB)  
23   and ventral hindbrain (HB) (arrowheads) and also in a few cells localised in the forebrain  
24   (asterisk) (FB). At this stage of development no transcripts were detected in the primordium  
25   of the anterior pituitary gland, the Rathke's pouch (RP dotted line in A and A'), or in the  
26   infundibulum (Inf). A' shows an enlarged image of the squared area in A, confirming  
27   undetectable expression of Foxa2 at this stage in the RP and Inf. At e12.5 (B and B') Foxa2  
28   mRNA transcripts were detected in the epithelial structures lining the main bronchus (MBr)  
29   (B, arrowhead) and in the epithelium lining the lung (Lu) and oesophagus (OE) (B',  
30   arrowheads). By e13.5, expression of Foxa2 appears localised in the ventral side of the  
31   anterior lobe of the developing pituitary gland (AL, arrow) with transcripts localised in the  
32   ventral marginal zone (arrowheads in C'). Foxa2 mRNA expression become stronger at  
33   e15.5 (D) with robust expression in the ventral diencephalon (VD, asterisk), posterior lobe  
34   (PL) and anterior lobe (AL, arrowheads in D') of the pituitary gland. At embryonic day e18.5

1 (E), expression was found widely spread in the central nervous system, with strong  
2 expression in the lumen surrounding the lateral ventricles (LV, arrowheads) and the third  
3 ventricle (TV, asterisks). Enlarged image of the boxed area in E shows mRNA expression  
4 localised in the hypothalamic area (Hyp) (E', arrowheads) with distinct pattern in the luminal  
5 area where the hypothalamic precursors tanycytes reside (arrowheads in E'). mRNA  
6 transcripts were also localised in both the posterior (PL) and anterior (AL) lobes of the  
7 pituitary gland (arrows in E'). Abbreviations: midbrain, MB; hindbrain, HB; forebrain, FB;  
8 Rathke's pouch, RP; infundibulum, Inf; main bronchus, MBr; lung, Lu; oesophagus, OE;  
9 ventral diencephalon, VD; pituitary gland posterior lobe, PL; pituitary gland anterior lobe,  
10 AL; lateral ventricles, LV; third ventricle, TV; hypothalamus, Hyp. Scale bars represent: 50  
11 µm (A', B, C', D'); 100 µm (B'), 250 µm (A, C, D, E'); 500 µm (E).

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14 **Figure 3. Immunohistochemical analysis of human FOXA2 expression during human**  
15 **embryonic development.**

16 A-C represent coronal sections of human embryos at 6 weeks (Carnegie stage 16), 8 weeks  
17 (Carnegie stage 23) and 13 weeks of gestation, respectively. A'-C'' show higher-  
18 magnification views of the boxed areas in A, B, C, respectively. At 6 weeks of gestation (A)  
19 Foxa2 expression was observed in the developing neural tube (NT) (A') and diencephalon  
20 (Dc) (arrowheads in A''). At 8 weeks of gestation (B) its expression was localised in the  
21 epithelium surrounding the third ventricle (TV) (B') and in the cells lining the diencephalon  
22 (Dc) (arrowheads in B''). No expression of Foxa2 was detected in the primordium of the  
23 pituitary gland (Rathke's pouch, RP) at CS16 (A''') nor in the developing pituitary gland at  
24 CS23 (B'''). In the pancreas at 13 weeks of gestation (C) Foxa2 was specifically localised in  
25 the cytoplasm of cells scattered in the pancreatic parenchyma (cells pointed by arrowheads in  
26 C' and C''). Abbreviations: neural tube, NT; diencephalon, Dc; Rathke's pouch, RP; pituitary  
27 gland, P; third ventricle, TV. Scale bars represent: 50 µm (A', A'', A''', B', C''); 100 µm  
28 (B'', B'''), 250 µm (C').

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1 **Figure 4:** The serine to proline change in position 169 of hFOXA2 results in decreased  
2 protein expression levels leading to impairment of transcriptional activation of the human  
3 GT2 promoter. Dual luciferase assay (A) in HEK293T cells transiently transfected with 50 ng  
4 or 75 ng of Wt hFOXA2 or mutant hFOXA2 p.S169P indicates that Wt hFOXA2 is able to  
5 transactivate the human GT2 reporter, whilst the hFOXA2 p.S169P transcriptional activation  
6 is impaired (graph represents 4 independent experiments performed in triplicate, \* p<0.05,  
7 one-way ANOVA). 3 independent western blots (B) show that the levels of the variant  
8 hFOXA2 p.S169P protein are reduced compared to the Wt hFOXA2, indicating that the  
9 mutation is functional and affects protein levels. Graph of the quantification of the western  
10 blots (B'') as percentage of Wt hFOXA2 and hFOXA2 p.S169P normalised to GAPDH  
11 indicates that hFOXA2 p.S169P variant results in half of the protein expression levels  
12 compared to Wt hFOXA2 (results from 6 independent experiments; \*\*\*\* p< 0.0001, one-  
13 way ANOVA). Abbreviation: NT, non-transfected; Wt, wild-type

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16 **Figure 5:** The S169P mutation in hFOXA2 does not result in changes in cellular localisation  
17 of the protein. Double-immunofluorescence using anti-FOXA2 antibody (red A,B) and anti-  
18 PHALLOIDIN (green A',B') performed in HEK293 cells transiently transfected with either  
19 200 ng of Wt hFOXA2 (A-A'') or mutant hFOXA2 p.S169P (B-B'') shows nuclear  
20 expression of both Wt and mutant FOXA2 (A,B) which overlaps with the nuclear DNA  
21 marker DAPI staining (A'',B'') but not with the cytoskeletal marker phalloidin.  
22 Abbreviation: DAPI, 4',6-diamidino-2-phenylindole. Scale bars in A and B represent 10  $\mu$ m.

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1      **Table 1: Summary of clinical features**

2	Face	Single median maxillary central incisor, congenital nasal pyriform aperture stenosis
3	Eye	Left choroidal coloboma
	Heart	Supra-valvular pulmonary stenosis
	Gastrointestinal	Feed intolerance, severe gastro-esophageal reflux disease requiring gastro-jejunostomy feeding
	Liver	Portal-portal bridging fibrosis, elevated transaminases
	Lung	Persistent oxygen requirement of unknown etiology
	Pancreas	Persistent form of hyperinsulinism
	Pituitary	ACTH,GH and TSH deficiencies Thin pituitary stalk,hypoplastic anterior pituitary Thin corpus callosum
	Neuro-developmental	Speech and motor developmental delay

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## 8      Abbreviations

9      CH	Congenital hypopituitarism
10     CHI	Congenital hyperinsulinism
11     SOD	Septo-optic dysplasia
12     CPHD	Combined pituitary hormonal deficits
13     HPE	Holoprosencephaly

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