Identification of a novel variant of the ciliopathic gene FUZZY associated with craniosynostosis

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Craniosynostosis is a birth defect occurring in approximately one in 2000 live births, where premature fusion of the cranial bones inhibits growth of the skull during critical periods of brain development. The resulting changes in skull shape can lead to compression of the brain, causing severe complications. While we have some understanding of the molecular pathology of craniosynostosis, a large proportion of cases are of unknown genetic aetiology. Based on studies in mouse, we previously proposed that the ciliopathy gene Fuz should be considered a candidate craniosynostosis gene. Here, we report a novel variant of FUZ (c.851 G > C, p.(Arg284Pro)) found in monozygotic twins presenting with craniosynostosis. To investigate whether Fuz has a direct role in regulating osteogenic fate and mineralisation, we cultured primary osteoblasts and mouse embryonic fibroblasts (MEFs) from Fuz mutant mice. Loss of Fuz resulted in increased osteoblastic mineralisation. This suggests that FUZ protein normally acts as a negative regulator of osteogenesis. We then used Fuz mutant MEFs, which lose functional primary cilia, to test whether the FUZ p.(Arg284Pro) variant could restore Fuz function during ciliogenesis. We found that expression of the FUZ p.(Arg284Pro) variant was sufficient to partially restore cilia numbers, but did not mediate a comparable response to Hedgehog pathway activation. Together, this suggests the osteogenic effects of FUZ p.(Arg284Pro) do not depend upon initiation of ciliogenesis.

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INTRODUCTION
Craniosynostosis is the premature fusion of one or more sutures of the skull vault and has an incidence of (approximately) 1:2000 live births [1–3]. While there are syndromes that present with craniosynostosis (sometimes with known genetic aetiology), the genetic associations of the more common non-syndromic craniosynostosis (approximately 85%) is less well understood [4]. Craniosynostosis is closely linked to the development of the cranial bones and sutures. During embryogenesis, there are condensations of neural crest (broadly anterior) and mesodermally (broadly posterior) derived skeletal progenitors. These cells then proliferate and differentiate into osteoblasts that go on to lay down matrix (osteoid). This matrix is then mineralised to form bone. Between the growth front of these bones the mesenchymal sutures work to prevent premature fusion. Pathological fusion can result from aberrant specification of the sutural mesenchyme towards the bone lineage, increased osteoblastic/decreased osteoclastic activity, or from aberrant mechanical forces causing bone fusion at close approximating surfaces [5]. Craniosynostosis can lead to raised intracranial pressure, which can impair eyesight and mental development [1, 6, 7]. There are good surgical treatment options for craniosynostosis; however, genetic sequencing is crucial for our understanding of the developmental aetiology of this disorder.

Here, we report a novel variant in the human FUZZY gene as a candidate for craniosynostosis, identified from whole genome sequencing (WGS) of a pair of monozygotic twins with craniosynostosis (case 1 and 2, Table 1, Fig. 1A) and their consanguineous clinically unaffected parents. Just over a handful of pathological variants of FUZ have previously been identified (cases 3–6, Table 1); three cases with severe neural tube defects (cases 3–5, Table 1) [8] and one case with the embryonically lethal, short-rib polydactyly syndrome II-like phenotype (case 6, Table 1) [9]. In addition, Zhang et al. 2018 reports the identification of FUZ p.(Arg284Leu), described as an “unsolved case” diagnosed as asphyxiating thoracic dystrophy (ATD) with polydactyly (case 7, Table 1). The fact that so few variants are present in the literature suggests that full loss-of-function changes are lethal, and case 6 further supports this as this early truncation is likely loss-of-function.

The human FUZ protein is comprised of 418 amino acids and shares 86.364% sequence homology with mouse FUZ. A number of key protein-protein interactions have been identified, although no specific binding sites have been established (Fig. 1D). FUZ has 3 putative longin domains (Fig. 1E), which function in membrane and vesicular trafficking [10–15]. The FUZ gene was first identified as a planar cell polarity (PCP) effector in Drosophila [16, 17]. More recent research from our lab and others has highlighted the role

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Table 1. Human variants of Fuz and associated phenotypes.

| Case | Variant | Type | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | Missense | 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increase in skeletogenic precursors in the head [29]. Second, using an Osx-1::GFP-cre reporter line to lineage label the osteoblast precursors, we showed that the frontal bone mesenchyme was expanded at the expense of the parietal bone [30], which could manifest as craniosynostosis, or an absence of the coronal suture in late gestation animals. These studies identified a clear role for FUZ in early establishment of cell fate and cranial bone tissue boundaries. While mouse Fuz is expressed throughout craniofacial structures [31], FUZ itself had not previously been directly implicated in osteoblast-specific development or bone formation.

In this study, we report the first craniosynostosis associated variant of FUZ and propose a novel function of FUZ during the later stages of cranial bone development, using a mouse model to demonstrate that loss of FUZ leads to excessive ossification. Furthermore, we find that the novel FUZ p.(Arg284Pro) variant can partially rescue ciliogenesis function in Fuz mutant mouse.
embryonic fibroblasts, suggesting that this variant may reveal cilia dependent and independent functions of FUZ.

RESULTS
Novel missense mutation in FUZ identified in twins presenting with craniosynostosis
We performed whole genome sequencing and identified a novel homozygous variant in FUZ (c.851 G > C, p.(Arg284Pro)) (Table 1, Fig. 1) in monozygotic twinned, female infants presenting with craniosynostosis. The consanguineous parents (paternal grand-father and maternal grandmother are siblings) are both heterozygous carriers, suggesting that this variant is recessive. Other homozygous variants found that are not present in the control population are presented in Table 2 (12 variants total). SIFT and PolyPhen scores suggested that these other variants were not pathogenic (Supplemental Table 1). The only variant predicted to be involved in craniofacial osteogenesis was FUZ. In addition, this novel mutation has not been reported in allele frequency reference databases such as (GnomAD) but is in the same position as an “unsolved case” (p.(Arg284Leu), case 7, Table 1). Moreover, the calculated SIFT and PolyPhen scores were indicative for a pathogenic variant (Supplemental Table 1).

Both patients presented at 6 months old with metopic suture craniosynostosis (Fig. 1B–C, yellow arrowhead). Additionally, case 1 (Table 1) presented with unilateral right coronal synostosis (Fig. 1B, yellow arrowhead). While the metopic suture usually fuses between 3–9 months, the coronal suture should remain patent until 20+ years [1]. Both patients also had dilatation of the lateral brain ventricles, with agenesis of the corpus callosum present in case 1 (data not shown). The twins were not reported to have any other phenotypes associated with FUZ knockout models, suggesting that this allele may not be a complete loss of function. Further relevant clinical observations are presented in Table 3.

Table 3. Clinical observations and phenotypes.

|                  | Case 1                                                                 | Case 2                                                                 |
|------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|
| Craniosynostosis | Metopic and coronal suture synostosis                               | Metopic suture synostosis                                            |
| Eye              | Fundoscopy: papilledema OS > OD, no Drusen, no ophthalmologic cause | Fundoscopy: papilledema both eyes                                    |
|                  | Skiascopy right eye: S + 3.25 = C-1.0 AS 90;                         | Skiascopy right eye: S + 2.50 = C − 0.50 AS 6;                       |
|                  | Left eye: S + 6.50 = C-1.0 AS 180                                    | Left eye: S + 2.50 = C-0.50 AS 154                                   |
|                  | Ishihara OD 0.5/8 ft OS 0.5/8 ft                                      | Wears glasses                                                        |
| Ear              | Prominent ears                                                       | Prominent ears                                                       |
|                  | Right ear larger than left                                           | Frequent ear infections; tubes placed 3 times                        |
| Endocrine        | N/A                                                                  | Premature puberty, started hormonal therapy                          |
| Skull morphometrics notes | Skull circumference: initially −1, moving to −1.5 SD since age 5 years | Skull circumference: −1SD, moving to −1.5 SD since age 7 years         |
|                  | Skull length: −3SD                                                  | Skull length: −2.5 SD                                                |
| Other observations| Developmental delay                                                  | Developmental delay                                                  |
|                  | Low Hairline                                                         | Low hairline                                                         |
|                  | Narrow nose                                                          | Hypopigmentation caudally of the left nipple                         |
|                  | Ultrasound abdomen aged 6 years: normal                              | Ultrasound of abdomen aged 6 years: normal                           |
|                  | CT and MRI aged 6 months and MRI aged 18 months: agenesis of the callosal body, stable wide lateral and third ventricles | CT scan aged 6 months: stable dilatation of lateral ventricles       |
|                  | MRI scan aged 8½ years: unchanged dilatation of ventricles           | MRI scan aged 8½ years: dilatation of ventricles, mild agenesis of the callosal body |
|                  | Increased intracranial pressure requiring occipital expansion aged 8 years |                                                                                  |
| Birth observations | At birth 1870 grams, skull circumference 31.5 cm, gestation 36 weeks and 4 days | At birth 2125 grams, skull circumference 31 cm, gestation 36 weeks and 4 days |

Hedgehog signalling (Gli1, Gli2, Gli3 and Sufu) and in the CPLANE complex (Intu, Rsg1 and Wdpcp) (Fig. 1D). Within FUZ, the arginine residue at amino acid position 284 is conserved across human, Xenopus, mouse, and Drosophila and lies adjacent to the C-terminal longin domain (Fig. 1E), which is thought to mediate vesicular trafficking [10–12]. This mutation is predicted to be deleterious (CADD score 25.4 (CADD Exome (1.6.1)). Arg284 lies outside of the predicted α helices or β sheets in FUZ (Fig. 1, E) suggesting that any structural change from the Arg284Pro variant is likely to be in the tertiary structure of the protein rather than affecting secondary structure. Other reported FUZ gene variants (Table 1) are either missense or truncating and will be discussed further in discussion. However, none are associated with craniosynostosis.

The role of FUZ during craniofacial ossification
To date most research has focused on the requirements of FUZ during neural crest induction, and toward implications for patterning of the craniofacial skeleton. Specific roles during osteogenesis or bone formation have not been explored. Given the craniosynostosis observed in these patients, we set out to test whether mouse Fuz is required during craniofacial osteoblast mineralisation. Homozygous Fuz mutant mice exhibit 100% prenatal lethality with a progressive loss from embryonic day 13.5 (E13.5) onwards. Therefore heterozygous Fuz+/− mice were intercrossed to generate control wild-type, heterozygous and homozygous null animals, which were collected at embryonic day 18 (E18.5), just prior to birth. Primary osteoblasts were isolated from the skull vaults and mandibles for culturing in a mineralisation assay (Fig. 2). Pre-osteoblasts were assessed based on alkaline phosphatase levels (red staining), while von Kossa staining was used to determine overall mineralisation levels (black staining). Interestingly, Fuz mutant osteoblasts from both skull vault and mandible showed increased mineralisation compared to the wildtype and heterozygous controls (Fig. 2, black staining in D, E, I and J compared to A-C and F-H).
This suggests that the mineralisation potential of Fuz mutant osteoblasts is increased. Because the severe prenatal lethality seen in the Fuz\(^{-/-}\) mice limited our access to calvarial osteoblasts, we then turned to mouse embryonic fibroblasts (MEFs) to further confirm the pro-osteogenic phenotypes. MEFs can be induced to undergo osteogenesis when challenged with media containing bone morphogenetic protein-2 (BMP-2). Within 7 days, Fuz mutant cells had a more cobblestoned appearance compared to the more fibroblastic wildtype cells (Fig. 2K, L). In addition, an increase in the area of alkaline phosphatase positive cells was seen (red staining Fig. 2M and N). By 36 days in culture, the appearance of the cultures was very different, with Fuz mutant cells showing many refractive, mineralised alkaline phosphatase positive nodules (Fig. 2O–T).

FUZ\(\text{p.}(\text{Arg284Pro})\) can partially rescue FUZ mutant ciliogenesis

If the function of FUZ is impaired by the p.(Arg284Pro) variant, then we might expect to see that protein localisation, ciliogenesis,
or signalling function of the primary cilium is also impaired. We expressed GFP-fused versions of both variants in wildtype MEFS in order to test their localisation, and found that both forms localised to the cytoplasm (Fig. 3A, B). We then examined their effects on ciliogenesis. Normally, MEFs cultured in reduced serum media will each generate a single primary cilium. Using this assay we checked whether overexpression of FUZ could increase the length of the cilium as previously reported in MDCK cells; however, we did not observe any increase in length, based on staining for the ciliary marker Arl13b (Fig. 3C–E).

**Fig. 3 FUZ p.(Arg284Pro) partially rescues in vitro ciliogenesis phenotype.** Both GFP-FUZ-FLAG (+FUZ) or GFP-FUZ p.Arg284Pro-FLAG (+R284P) transfected in immortalised wildtype MEFS show similar cytoplasmic localisation (A–B, anti-GFP (green)). Transfection of GFP-FUZ-FLAG (+FUZ) or GFP-FUZ p.Arg284Pro-FLAG (+R284P) resulted in no significant change in primary cilium length (measured from anti-anti-Arl13b staining (magenta), quantified in (F)). Rescue experiments were performed in primary MEFS. Ciliary axoneme staining (G–J, anti-Arl13b, green) revealed significantly decreased numbers of cilia present in mutant (H and K, 3% of cilia positive cells) compared to control GFP transfected cells (G and K, 70% cilia positive cells, p = <0.0001, K). Mutant cells transfected with FUZ (I) or FUZ p.(Arg284Pro) constructs (J) exhibited 4% (K, p = 0.0555) and 7% (K, p = 0.0017) increase in cilia number compared to mutant cells transfected with GFP Cilia were present as dots (G–J, pink arrowhead) or lines (G–J, yellow arrowhead) and were both included in quantification. RT-qPCR relative expression for Hedgehog transcriptional readouts *Ptc1* and *Gli1* (L and M) from immortalised MEFS. Data grouped by genotype as indicated with DMSO [D] or 2 µM Purmorphamine [P] treatments and no transfection (Ctrl), transfection of GFP-FUZ-FLAG (+FUZ) or GFP-FUZ p.Arg284Pro-FLAG (+R284P). Statistics and P values (F) are student’s T tests and (K) are Fisher’s exact tests comparing conditions as indicated. Scale bars (A, B) = 10 µm, (C–E) = 3 µm, (G–J) = 50 µm.
We then used MEFs from Fuz knockout mice to assess whether the p.Arg284Pro variant could rescue ciliogenesis, due to the observation that MEFs from Fuz−/− mutant animals very rarely generate cilia (3% (Fig. 3H and K, n = 315, compared to 70% wildtype MEFs Fig. 3G, K, n = 168). Fuz−/− MEFs were transfected with either a control GFP plasmid, a full-length wild-type FUZ construct, or the variant FUZ-p.(Arg284Pro) construct and assessed for cilia formation. Transfection efficiencies were low; however, the wild-type FUZ construct rescued ciliogenesis in a significant proportion of cells (7% seen in Fig. 3I and K, n = 277, p = 0.0555 compared to Fuz−/−). Similarly, transfection with the FUZ-p.(Arg284Pro) variant construct increased the proportion of cells with a cilium to 10% (Fig. 3J, K, n = 403, p = 0.0017 compared to Fuz−/−). Altogether, this suggested that the p. (Arg284Pro) variant could function similarly to the wild-type protein during ciliogenesis.

Inefficient transduction of the Hedgehog signal response by FUZ p.(Arg284Pro)

While FUZ p.(Arg284Pro) appeared to restore some ciliogenesis, this is relatively unsurprising since the Fuz−/− mice do initially have functional cilia. A defect in Hedgehog signal transduction arises subsequently, likely due to poorly functioning of retrograde IFT leading to cilia degeneration [19]. To address the ciliary function of FUZ p.(Arg284Pro) we compared the ability of wildtype human FUZ and the FUZ p.(Arg284Pro) variant to increase Hh responsiveness in both control and mutant Fuz MEFs. We performed RT-qPCR for two transcriptional targets of Hedgehog, Patched-1 (Ptc1) and Gli1. Ptc1 is thought to be an immediate early response to Hh activation, while Gli1 requires a sustained and higher Hh cue. First, we note that the wildtype MEFs respond robustly to treatment with 2 μM purmorphamine, which binds to Smoothened and mimics Hh ligand activation (Figure L and M, Fuz+/−, P, Ctrl). In contrast, Fuz−/− MEFs do respond, but less robustly (Fig. 3L, M). In control MEFs, transfection with wildtype FUZ increases expression of Ptc1 over baseline levels. Transfection of the FUZ p.(Arg284Pro) variant also increases target gene activation, but less than the wild-type (Fig. 3L−M, Fuz+/−, P, Arg284Pro). The variability in these experiments is likely due to transfection efficiencies; therefore, future experiments should be performed using stable transgenic lines, with further confirmation by immunolocalisation of Hh pathway effectors. Nevertheless, our observations support the idea that normal MEFs carrying the FUZ p.(Arg284Pro) variant are less efficient when transducing Hedgehog signals. In Fuz−/− MEFs, we found that transfection of either human FUZ variant was sufficient to increase both Ptc1 and Gli1 transcription; however, response to FUZ p.(Arg284Pro) was increased with regards to Ptc1 while we saw almost no response in Gli1.

DISCUSSION

The genetic aetiology of craniosynostosis is poorly understood. Here, we report a novel craniosynostosis associated mutation in FUZ (c.851G>C, p.(Arg284Pro)). We found that loss of Fuz resulted in increased mineralisation in both in vitro embryonic primary osteoblast cultures and in fibroblasts undergoing an osteogenic challenge. A direct effect of FUZ mutation in the late stages of bone development and mineralisation has not been reported before. In addition, the novel variant also partially rescued the loss of primary cilia with a cilium to 10% (Fig.3J, K, n = 403, p = 0.0017 compared to Fuz−/−). Altogether, this suggested that the p. (Arg284Pro) variant could function similarly to the wild-type protein during ciliogenesis.

In the long term, it will be of interest to investigate ossification ability of the p.(Pro39Ser) variant in comparison to our p.(Arg284Pro) data.

Based on animal models and human case studies, it is clear that genetic mutations affecting the structure and function of the primary cilium can result in developmental abnormalities and skeletal dysplasias [33]. These often affect the limbs with polydactyly and the face with cleft lip/palate, micrognathia, facial width abnormalities and craniosynostosis. Several ciliopathies that predominantly affect the craniofacial skeleton present with craniosynostosis [22]. Together, this suggests an overlap between ciliopathic syndromes and craniosynostosis [29, 30] and attributes a subset of these to Hedgehog signalling changes. 

Hh signalling can affect both osteoblast differentiation and mineralisation. Indian Hedgehog (IHH) signalling is necessary for the early differentiation of osteoblasts, with upregulation of Hh signalling via the small molecule purmorphamine resulting in increased commitment to osteoblastic maturation [34]. A loss at this stage could leave osteoprogenitors in a proliferative and undifferentiated state, effectively increasing the number of precursors able to differentiate and mineralise. In contrast, loss of Hh signalling, via a conditional knockout of the Smoothed receptor in mature osteoblasts, results in increased bone mass and a disruption in bone homeostasis [35]. Thus, it is evident that Hh
signalling is necessary at several key time points during skeletal development and that loss of FUZ may reduce the Hh signalling capacity via the primary cilium at these critical stages.

However, simple non-syndromic craniosynostoses are often surgically treated and whether there is a genetic aetiology is not assessed. These cases could be caused by partial loss of function variants in multifunction genes, like FUZ. Where complete loss of function would cause systemic anomalies; here, a missense or partial loss of function mutation may affect a specific functional region of the protein giving a ‘microform’ presentation of a syndrome. Linking genetic variants to the functional regions of proteins will help us understand the phenotypic manifestation of the syndrome, as well as the relative severity. This will help with genetic counselling, diagnosis, and may determine what future follow-up is required.

MATERIALS AND METHODS

Sequencing

Whole genome sequencing (WGS) was performed on DNA from blood by Complete Genomics, (Mountain View, CA, USA) [36]. Variants were annotated using NCBI build GRCh37/hg19 and dbSNP build 137. Data were analyzed using cga tools version 1.8.0. An autosomal recessive disease model was tested. The analysis was restricted to novel non-synonymous variants, variants disrupting a splice site (±two basepairs), and insertions or deletions in the coding sequence (±50 bp). The remaining variants were analyzed with Annovar [37] and OpenCravat [38] to get an indication of the pathogenicity and allele frequency, and compared to those present in the welllender cohort [39]. The variant identified was described according to HGVS nomenclature [40], using reference sequence NM_025129.5, on GRCh37/hg19 and was submitted to the Leiden Open Variation Database. The variant identified by WGS was validated by dideoxy-sequence analysis.

Mouse lines and animal husbandry

Fuz mutants (MGI:3531090) in this study were previously reported [12]. All animal work was carried out in accordance with UK Home Office regulations under the project licence P8D5E2773 held by KJL. Immortalised MEFs were derived from another previously reported mutant line [8].

Cell culture

Primary mouse embryonic fibroblasts (MEFs) were isolated from E12.5 embryos using standard procedures (Fig. 2, Fig. 3 G-K), while immortalised MEFs have been previously described (used in Fig. 3 A-E and L, M) [8]. MEFs were passed every 3-4 days and primary MEFs were used up to passage 5. DNA transfections were performed with Lipofectamine LTX or 2000 (Invitrogen) and incubated at 37 °C. For MEF to osteoblast differentiation primary MEFs (primary osteoblast growth media, 50 ug/ml ascorbic acid, 5 mM β-glycerophosphate). For MEF to osteoblast differentiation primary MEFs were cultured in osteoblastic mineralisation medium (an ab initio and homology modelling tool) [43]. Variants were annotated using NCBI build GRCh37/hg19 and dbSNP build 137. Data were analyzed

Protein predictions and secondary structure prediction

STRING queries (https://string-db.org/) were rooted on human FUZ protein identifier ENSP00000313309 showing functional and physical protein associations. Human FUZ amino acid sequence was analysed using PHYRE2 (an ab initio and homology modelling tool) [43]

DATA AVAILABILITY

Data available within the article or upon reasonable request. The p.Arg284Pro variant is reported in the Leiden Open Variation Database (https://databases.lovd.nl/shared/individuals/00375537/).

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AUTHOR CONTRIBUTIONS

JACG, SMAS, MVdS, PVdS, IMJM contributed to clinical evaluation and sequencing analysis. WBB, HAA and KJL designed and performed experiments. ET provided cell lines. All authors contributed to the writing of the manuscript.

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ETHICAL APPROVAL

The clinical study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam (MEC-2012-140). Written informed consent to obtain samples for genetics research was given by the parents.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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