**Foxf2: A Novel Locus for Anterior Segment Dysgenesis Adjacent to the Foxc1 Gene**

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**Abstract**  
Anterior segment dysgenesis (ASD) is characterised by an abnormal migration of neural crest cells or an aberrant differentiation of the mesenchymal cells during the formation of the eye’s anterior segment. These abnormalities result in multiple tissue defects affecting the iris, cornea and drainage structures of the iridocorneal angle including the ciliary body, trabecular meshwork and Schlemm’s canal. In some cases, abnormal ASD development leads to glaucoma, which is usually associated with increased intraocular pressure. Haploinsufficiency through mutation or chromosomal deletion of the human FOXC1 transcription factor gene or duplications of the 6p25 region is associated with a spectrum of ocular abnormalities including ASD. However, mapping data and phenotype analysis of human deletions suggests that an additional locus for this condition may be present in the same chromosomal region as FOXC1. DHPLC screening of ENU mutagenised mouse archival tissue revealed five novel mouse Foxf2 mutations. Re-derivation of one of these (the Foxf2W174R mouse lineage) resulted in heterozygote mice that exhibited thinning of the iris stroma, hyperplasia of the trabecular meshwork, small or absent Schlemm’s canal and a reduction in the iridocorneal angle. Homozygous E18.5 mice showed absence of ciliary body projections, demonstrating a critical role for Foxf2 in the developing eye. These data provide evidence that the Foxf2 gene, separated from Foxc1 by less than 70 kb of genomic sequence (250 kb in human DNA), may explain human abnormalities in some cases of ASD where FOXC1 has been excluded genetically.

**Introduction**  
Anterior segment dysgenesis covers a spectrum of disorders affecting the iris, cornea, trabecular meshwork and Schlemm’s canal of the eye, which can result in abnormal aqueous humor drainage from the eye leading to raised intraocular pressure and glaucoma [1]. These abnormalities result from a primary defect in the migration and differentiation of neural crest cells that contribute to the development of the anterior segment structures [2]. Malformation of tissue specifically at the iridocorneal angle (iridogoniodygenesis anomaly) or in the anterior stroma of the iris - contribute to the glaucoma phenotype [3,4].  

Anterior segment dysgenesis (ASD) phenotypes are inherited as autosomal dominant traits with variable expressivity and incomplete penetrance, pointing to a complex etiology [5,6]. Nine different human genes have been associated with ASD or congenital glaucoma including FOXC1, PITX2, PITX3, FOXE3, Pax6, Maf, Cyp1b1 and Limx1b. Mutations in the FOXC1 gene [7], or dosage effects due to deletions [8] or duplications [9,10] in the 6p25 region that surrounds FOXC1 can all cause iridogoniodygenesis; as can mutations in the PITX2/RIEG1 gene [11]. Patients with FOXC1 mutations have a milder average prognosis for glaucoma development than do patients with any one of the known PITX2 mutations [12]. One common link between these genes, other than their expression in the neural crest cells of the pericellular mesenchyme [13,14]; is that their upregulation can be triggered by Tgfb2 activity. Inactivation of this growth factor in mouse neural crest cells results in malformed trabecular meshwork, ciliary body and corneal endothelial cells [15].  

Genetic evidence suggests that other genes near FOXC1 may also be involved in the underlying etiology of iridogoniodygenesis and other eye abnormalities associated with glaucoma. For example, deletion of 6p24-p25 proximal to the FOXC1 locus causes anterior segment abnormalities [16,17,18]. Recombination mapping in families linked to 6p25 excluded FOXC1 as the causative gene [19]. Furthermore, a patient with an unbalanced translocation between 6p25 and 4p14 was disomic for FOXF2 but may have been monosomic for FOXC1 [20].  

To investigate whether the nearby Foxf2 gene could be involved in anterior segment development and dysgenesis we took advantage of an ENU mutagenised DNA archive [21,22], that allowed recovery of identified Foxf2 mutant lineages. We describe the genetic analysis of an identified Foxf2 mutation and the phenotypic features of the affected animals. These analyses suggest Foxf2 is essential for normal anterior segment development, and that the FOXF2 gene should be considered as an additional candidate for anterior segment dysgenesis in humans.
Results

Identification of *Foxf2* sequence variants from archival DNA

Archival DNA from tail biopsies of the F1 progeny of mice that had undergone ENU mutagenesis, was screened by DHPLC analysis followed by sequencing of samples that produced heteroduplexes. This protocol identified 5 sequence variants in the *Foxf2* genomic DNA (Table 1). Two base changes did not alter amino acid sequence and are therefore silent variants. Individual mouse GSK14H3 carried a T→A transversion at position 821 of the *Foxf2* transcript (Figure 1A). This change results in a W174R amino acid substitution in the forkhead DNA binding domain of the protein. In mouse MRC18C1, a G→T transversion at position 1535 of the *Foxf2* transcript resulted in a conservative V412F amino acid substitution in the third sub-region of the AD2 transactivation domain [23]. An A→G transition was identified in mouse MRC31H8 at the third base of the intron. The six base region following the end of exons is generally highly conserved between eukaryotic 5' splice donors, but this third base is the least conserved of these positions. In an analysis of intron – exon boundaries within 1446 genes, 35% of splice sites contain an adenosine at this position and 60% a guanosine, whereas all of the other positions showed much greater levels of conservation [24] - so interference with normal splicing could be considered unlikely. However, 106 disease associated A→G splice site mutations at the equivalent position (IVS+3) in the donor regions of 79 genes, are present in the human gene mutation database (HGMD) [25]. Thus, the possibility remains that this mutation could result in aberrant splicing.

The *Foxf2* mutation rate within the ENU archives that was previously determined during the discovery of the *Foxf2*W174R mutation and one of the silent mutations [22] can now be updated to 5 mutations in 1340 bp of 7990 individuals.

Recovery of the *Foxf2*W174R mouse lineage

Analysis of inter-species conservation, physico-chemical implications of the amino acid substitutions and the position of the mutations in the protein structure suggested that *Foxf2*W174R was the mutation that was most likely to disrupt the function of the gene product. The tryptophan residue is conserved in all genes with a forkhead domain (Figure 1B) and occurs within a β-sheet structure. This mouse line was therefore re-derived for further examination.

 homozygous mutants die within 14 days of birth and in 16 individuals, none showed evidence of malformation in either the primary or secondary palate. This is in contrast to earlier findings in the homozygous *Foxf2* knockout mice which die within 18 hours with cleft palates and gas distended guts [26]. *Foxf2*W174R homozygotes appear normal at birth but fail to thrive and by 3 days are noticeably smaller than their wildtype littermates (Figure 2). As in the knockout, microscopic analysis did reveal any lung defects despite the gene’s intense expression in the lung [23] which, in common with the eye but not any of the other tissues that express *Foxf2*, continues to express the gene into adulthood [27]. Heterozygous mice appear to thrive normally and are fertile, as was the case for knockout mice.

*Foxf2*W174R eye phenotype analysis

The eyes from ten *Foxf2*W174R heterozygous mice that were 45 days of age were examined by light microscopy. The iris stroma showed irregular thinning of the tissue (compared to wildtype (Figure 3)) and a loss of structural organization. A number of unusual features were observed in the irido-corneal angle of all mice analysed (Figure 4). The canal of Schlemm was smaller in most of the mice (7/10) and was not seen at all in others (2/10); the trabecular meshwork showed signs of hypoplasticity (7/10); one individual had a hypoplastic ciliary muscle. In some mice the angle between the cornea and iris was significantly reduced (6/10) and in one individual the two tissues were adherent. The phenotype variability that was seen between different animals was also apparent between the eyes of individuals, although to a lesser degree. This type of variation is also seen in *Foxc1* heterozygous mice [28] as well as in human disease [29] and may be dependent on genetic background. Although this variability could be attributed to genetic modifiers it is also likely to be influenced by the presence of normal and abnormal tissue, probably reflecting stochastic events in which the spatiotemporal regulation of *Foxf2* downstream targets is critical to anterior segment development. Nevertheless, all mice exhibited two or more defects. Histological analysis showed no signs of damage to the cornea, optic nerve or retinal nerve fibres at 45 days of age (data not shown).

To investigate the effect of the W174R mutation in older mice (6 months), the retina, cornea and optic nerve of heterozygous mice were examined to determine if there was any apparent glaucomatous damage. Histological analysis of 18 mice showed a range of anterior segment defects as previously seen in younger mice. In addition two mice appeared to have bulging eyes that can be associated with raised intraocular pressure. However, on histological investigation there appeared to be extraneous amorphous tissue between the retina and the lens. Histological analysis in the majority of *Foxf2*W174R heterozygous mice (16/18) revealed no substantial damage to the optic nerve, retina or cornea (Figure 5). In two mice there was swelling of the optic nerve, which disrupted the outer nuclear layer of the retina (Figure 5B&C). In mice that were 12 months of age, the optic nerve appeared to be normal because there was no optic nerve cupping as would be expected at this age if glaucomatous damage had occurred [30].

To investigate whether homozygote *Foxf2*W174R embryos displayed iridocorneal defects, E18.5 embryos were examined by histology. Evagination of tissue from the anterior optic cup begins at E14 to form the future iris and ciliary body. At E18.5 finger-like
projections of tissue forming the ciliary body processes are clearly visible in wildtype littermates (Figure 6A). However, there was no evidence of tissue evagination in the homozygote embryos (Figure 6B). Heterozygous mice at this stage appear indistinguishable from wildtype mice.

Mouse subjects were from several divergent lineages that had been outcrossed to between G5 and G8 from the single mutagenised founder. This meant that the likelihood that the observed phenotype resulted from mutations in ASD associated genes on other chromosomes was negligible. However, due to the close genetic linkage of Foxc1 to Foxf2 and the similarity between the Foxf2W174R and Foxc1 mutant and knockout eye phenotypes, it was important to ensure that no mutations in Foxc1 were responsible for the observed phenotype. The Foxc1 coding sequence of the Foxf2W174R mouse was therefore sequenced. No differences between this sequence in the Foxf2W174R mouse and the Foxc1 mouse reference sequence were present.

Discussion

Chromosome 6p25 is a major locus for anterior segment dysgenesis (ASD). Previous reports of cytogenetic abnormalities are consistent with the notion that the eye is exquisitely sensitive to both reduced and increased dosage in this chromosomal region. Although FOXC1 dosage is a major contributor to eye defects localised to this region, we now provide evidence that Foxf2 is a novel locus for anterior segment dysgenesis. Heterozygous mutation of the forkhead binding domain of the Foxf2 gene is associated with anterior segment defects in the iridocorneal angle of mice, whereas homozygous defects are lethal. At E18.5 the development of the ciliary body is defective, suggesting that Foxf2 is essential for normal ciliary body formation. These data support the role of Foxf2 in normal anterior segment development.

Data from the characterisation of a 200 kb deletion located 1.2 Mb upstream of FOXC1 [31] suggested that mutations could induce a phenotype via long-range effects. It is therefore feasible that the observed phenotype in Foxf2W174R individuals could be the result of a mutation within a Foxc1 regulatory region. However,
other evidence to support the involvement of Foxf2 in anterior segment dysgenesis, including the patterning of its ocular expression [27,32], the high level of conservation and physicochemical changes of the mutagenised amino acid and the absence of Foxc1 coding mutations; in combination with the observed physical phenotype - all contribute towards a greatly strengthened candidacy of Foxf2.

Previous studies have shown that targeted deletion of Foxf2 caused palate malformations and an abnormal tongue [26]. Analysis of Foxf2 knockout mice subsequently revealed megacolon, colorectal muscle hypoplasia and agangliosis [33]. However, the colon was not analysed in the present study and therefore the effects of this mutation on the gut would seem like a promising focus of future investigations into the effects of the Foxf2W174R mutation.

Foxf2 is expressed in the absence of its closest paralogue (Foxf1) in the CNS, ear, and limb buds as well as the eye [27] so these systems are also worth prioritising in the search for other potential Foxf2-associated phenotypes.

The effect on eye development was not examined in previous analyses of Foxf2 knockouts [33]. Interestingly however, one study did demonstrate normal Foxf2 expression in the pericellular mesenchyme of the developing eye at about E12.5 [32]. Furthermore, in situ hybridisation established that there was continued Foxf2 expression from E13 through to adult stages [27]. High levels of Foxf2 expression at E17 were observed in the developing ciliary body and choroid. These data support the abnormal morphological finding in the developing ciliary body in homozygous Foxf2W174R embryos.

The difference in phenotype that was identified between targeted knockout and homozygous missense mutation could suggest that Foxf2W174R is a hypomorphic allele, However it is also possible that the differences are due to genetic background and that the mutation causes a complete loss of function. Molecular modelling of FOXC1 in a previous study revealed that a tryptophan residue (Trp152) - the direct homologue of Trp174 in Foxf2, is one of nine critical intramolecular interaction residues that maintain structural integrity of the forkhead winged helix structure [34]. It therefore seems likely that disruption of Trp174 in Foxf2 would lead to protein instability. Another example of an unstable forkhead transcription factor with a mutation in the DNA binding region is the I87M variant of FOXC1 [34]. Cos7 cells transfected with this mutant plasmid demonstrated markedly reduced levels of the protein at only 5% of levels observed for the wildtype, but the molecule retained its nuclear localisation function. A drastic reduction but not complete destruction of protein functionality, could explain the reduced severity of phenotype that is observed in association with the Foxf2W174R mutation and would be consistent with the hypothesis that haploinsufficiency plays a key role in the pathogenesis of Fox associated anterior segment anomalies.

The ocular abnormalities found in Foxf2W174R mice are variable in eyes from different individuals, recapitulating the variable...
expressivity observed in human patients with ASD. Schlemm’s canal was often smaller than typically seen in wild-type eyes and trabecular meshwork was either missing or was underdeveloped, suggesting abnormal migration of mesenchymal cells into the iridocorneal angle. The ciliary body malformations may affect aqueous humor production and secretion of antioxidant proteins into the aqueous humor [35]. Aqueous humor is drained through the trabecular meshwork, therefore alterations in aqueous humor homeostasis are likely to occur when these tissues are malformed and could contribute to changes in intraocular pressure [36]. The iridocorneal abnormalities observed in the FoxF2W174R mice are very reminiscent of those seen in mice that are heterozygous for Foxc1 or Foxc2 mutations [28]. Since Foxc1, Foxc2 and Foxf2 are all expressed in the developing periocular mesenchyme, this suggests that this tissue is particularly sensitive to gene dosage [9,37].

Despite the high level of conservation in their DNA binding domain, forkhead transcription factors are an extraordinarily diverse group of genes with roles as varied as development, homeostasis, stress response and cell cycle control [38]. Intriguingly, mutations in a number of forkhead genes can result in a variety of disorders affecting the eye. Mutations of the FOXE3 gene affect lens development and can be inherited as either an autosomal dominant or recessive trait [39]. The more severe recessive trait is associated with bilateral microphthalmia, aphakia, corneal defects and glaucoma, whereas the milder autosomal dominant trait is associated with iris hypoplasia, Peters’ anomaly, and isolated cataract. Mutations of the FOXC2 gene cause lymphedema-distichiasis syndrome [40] - characterised by double rows of eyelashes, ptosis, photophobia and anterior segment anomalies reminiscent of those caused by FOXC1 [7]. FOXL2 mutations cause blepharophimosis-ptosis-epicanthus inversus syndrome (a complex eyelid malformation) [41] and in some patients lacrimal duct anomalies, amblyopia, strabismus, and refractive errors. In addition, expression of three other forkhead genes; Foxg1 [42], Foxd1 [43] and Foxn4 [44], has also been shown in the developing retina. It is clear that forkhead transcription factors play a critical role in the developing eye, and now the Foxf2 gene can be added to this growing list.

The 6p25 region contains a forkhead cluster (FOXC1/FOXF2/FOXQ1) in which FOXC1 is separated from FOXF2 by less than

Figure 5. Representative images of eye phenotype of aged heterozygous Foxf2W174R mutant mice. A, 6 month old Foxf2W174R eye (m+/) showing normal retinal termination at the edge of the optic nerve fibre layer (black arrows). B&C, sections through two other 6 month old Foxf2W174R eyes showing abnormal bulge in the nerve fibre layer. The outer nuclear layer of the retina can be seen to continue at the surface of this bulging tissue (white arrows). D&E, retinal sections in 6 month eyes. F&G, corneal sections in 6 month eyes. H–J, 12 month Foxf2W174R optic nerves that do not show signs of glaucomatous change. v, vein; p, photoreceptors; onl, outer nuclear layer; inl, inner nuclear layer; ce, corneal epithelium; cs, corneal stroma. Scale bar = 50 μm.
doi:10.1371/journal.pone.0025489.g005

Figure 6. Comparison of iridocorneal development in homozygous Foxf2W174R embryos. A, The ciliary body is beginning to form in E18.5 wildtype embryos, showing two finger-like projections of evaginated tissue (white arrows). B, Formation of the ciliary body is absent in the E18.5 homozygous Foxf2W174R mutant mouse eye.
doi:10.1371/journal.pone.0025489.g006
250 kb of genomic DNA, and FOXQ1 is 470 kb proximal of FOXC1. Because duplication and deletions of this region in human disease often contain more than one of these genes, confirmation of pathogenicity has relied on specific mutations in animal models. Although gene knockouts [28,45] and naturally occurring mutations [14] recapitulate FOXC1 deletions or mutations, no model carrying an additional functional copy of FOXC1 has been developed to explore gain-of function effects seen in interstitial gene duplication events. Since our data provides evidence that Foxf2 in mice is also critically involved in anterior segment development, then duplications or deletions containing both FOXF2 and FOXC1 in patients may contribute to the phenotype. This is supported by clinical observations where interstitial duplication of FOXC1 alone causes an iris hypoplasia phenotype, whereas duplications containing both genes (plus several others depending on the extent of the duplication) cause microcornea and ptosis, without iris hypoplasia [46]. This suggests that different combinations of transcription factor gene dosage within cytogenetic abnormalities influence how eye development is affected.

**Materials and Methods**

**DHPLC mutation scanning**

We used ENU archival DNA that was generated as previously described [21,22] as a template for Foxf2 mutation scanning using DHPLC [47]. DNA concentrations were determined with a Spectramax 190 spectrophotometer (Molecular Devices). Five of six overlapping sets of primers were used for amplification of Foxf2 (Table 2). For each PCR reaction, 10 ul of pooled archive DNA (4 samples) was added at a concentration of 5 ng/ul. Following amplification of the DHPLC targets, thermal cycling using the WAVE™ DNA Fragment Analysis System (Transgenomic, Cheshire, UK), was used to denature and then re-anneal PCR products with the following parameters: 95°C for 4 min, 45 cycles of 93.5°C for 1 min with a reduction of 1.5°C per cycle down to 25°C.

**Sequencing**

PCR amplification products from pooled DNA that exhibited evidence of heteroduplexes in their DHPLC profiles, were individually PCR-amplified and screened by DHPLC. The single DNA heteroduplex that was identified was sequenced on both strands to determine the mutation. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequencing was carried out using BigDye 3.1 terminator chemistry on an ABI prism 377 DNA sequencer. Sequences were aligned and compared with consensus data obtained from the mouse genome database (http://genome.ucsc.edu).

**Mutant mouse recovery and genotyping**

Recovery of the mutant mouse lineage was achieved by in vitro fertilisation with archival sperm and C3H/HeH females using standard methodology. Genotyping of the Foxf2 W174R mice was performed by SfiI (which cuts the mutant locus) and BsrI (wildtype locus) restriction digestion of the exon1c PCR product to

**Table 2.** Primers used for the gene driven screen and mouse Foxf2 sequencing.  

| Primer Set | Forward | Reverse | Product Size |
|------------|---------|---------|--------------|
| Exon1a     | CTGCCGCGATTTGTGGAC | AGCCGAGTGACGAGTAAAG | 414           |
| Exon1b     | AGTTGAAGCAGACGACGTC | GGGGAAGGACCTTCAAAAC | 316           |
| Exon1c     | GTTCCCCCTTTTCCGTTTGG | TGGCCATATAGGTTGAGGC | 460           |
| Exon1d*    | TCAAGCGTGGATGGTGG | AGAGGAGTCAGAGGCCTGG | 513           |
| Exon1e*    | ACACCCACTCAGCCACAC | AGAGGATGCAGAGGCTCG | 433           |
| Exon2a     | AGTCGCTTACACCTCAG | ACAGTGATGTCGAGTTCG | 384           |

*Primer sets Exon1a-1e were used to screen the first exon and flanking regions, primer set Exon2a was used to screen exon 2. *Primer set Exon1e replaced Exon1d for the screen of the MRC archive. doi:10.1371/journal.pone.0025489.t002

**Table 3.** Primers used for sequencing mouse Foxc1.  

| Primer Set | Forward | Reverse | Product Size |
|------------|---------|---------|--------------|
| Foxc1_A    | AGTCCTCGGCTGGTGTGAC | CTGCCAGCCAGACTCAGTC | 401           |
| Foxc1_B1   | AGTTGAACGAGGCTTCTTC | GCCGCATAGCTGGCTCTGG | 387           |
| Foxc1_B2   | GCACACTCGGGTTCAG | CTCTGCTTACGGGGGAGTC | 286           |
| Foxc1_C    | GAAAGCTGGCCCATACAGTAC | CTGAGGATGGAGGACTAG | 552           |
| Foxc1_D    | AGACCCACAGACGGATGCTG | CTGAGGATGAGGACTAG | 528           |
| Foxc1_E    | AAGTCCGAGAAGCACTG | CTTGCGGAGAAGCAGAAC | 532           |
| Foxc1_F    | GATTCGGCGATGGACACTCC | TCCCGTCTTCCGACATTAGG | 540           |
| Foxc1_G    | CCTTCCTCGCTCATGTCCTTG | TTTGACAAAACGGGTAGG | 558           |
| Foxc1_H    | TGGCTATGCTCTGCTCAT | TTTGCGGCTTCTACGTCC | 600           |
| Foxc1_I1   | TTTGAGGACTTACACGCAATACGCAG | GTAATCAAAAACGGCTTCC | 240           |
| Foxc1_I2   | TTAGGTTGTCATCGCCTGT | TCCGGTCTATTAGTCTAGG | 280           |

doi:10.1371/journal.pone.0025489.t003
distinguish Foxf2<sup>W174R</sup> heterozygotes from homozygotes and wild-types. Because the C3H mice carry a <i>Pde6brd1</i> retinal mutation affecting the eye, the identified Foxf2<sup>W174R</sup> mice were outcrossed to C57BL/6 mice for 2 generations. To exclude <i>sid</i> carriers, genotyping was performed with the following two primers: F: 5′-ACCTGAGCTCGAAGAGGC-3′ and R: 5′-GCTTTCTAGGTTGCCAAGTGT-3′ as described previously [48]. The mutation was detected by DdeI restriction digest (which cuts the <i>Pde6brd1</i> mutant locus) and <i>Snai1</i> (wildtype locus) thus allowing differentiation between <i>Pde6brd1</i> heterozygotes, homozygotes and wildtypes. All subsequent analyses were carried out on mice with only the Foxf<sup>2</sup> mutation. Primers for sequencing the <i>Fox1</i> gene are in Table 3. All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. The Harwell ethical committee approved the study and the work was performed under UK Home Office project licence numbers 30/1517, 30/2049 and 30/2228.

**Histological analysis**

Eyes were enucleated and placed in 50% Karnovsky’s fixative for 45 minutes. Eyes were then washed 3×30 min in PBS, dehydrated through a graded ethanol series (50%, 70%, 90% and 100%) and then embedded in paraffin wax. Whole eye sections cut sagittally to a thickness of 3 µm were counterstained with hematoxylin and cosin. Retinal histology was imaged using a digital camera mounted on an Olympus 1×71 microscope.

**Acknowledgments**

Technical expertise provided by Martin Fray and the Frozen Embryo & Sperm Archive (FESA) team at MRC Harwell enabled the rederivation of the mutant mouse lines. Michael Cheeseman and Hilda Tatoessian assisted with palate analysis and Charlotte Dean assisted with lung analysis. Lucie Vizar, Sara Wells and the animal technicians at the MRC Mammalian Genetics and Unit and Mary Lyon Centre were responsible for animal care and husbandry. The histopathology group at MRC Harwell sectioned the lungs and palates. Lorraine Lawrence of Imperial College, London sectioned the eyes.

**Author Contributions**

Conceived and designed the experiments: PD RM HV CYG-E. Performed the experiments: RM. Analyzed the data: RM CYG-E. KD-G. Contributed reagents/materials/analysis tools: PD CYG-E. Wrote the paper: RM PD CYG-E.

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