Glaucma-associated WDR36 variants encode functional defects in a yeast model system

Tim K. Footz1, Jill L. Johnson2, Stéphane Dubois3, Nicolas Boivin3, Vincent Raymond3 and Michael A. Walter1,*

1Department of Medical Genetics, University of Alberta, 8-39 Medical Sciences Building, Edmonton, AB, Canada T6G 2H7, 2Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844-3052, USA and 3Laboratory of Ocular Genetics and Genomics, Laval University Hospital (CHUL) Research Center, 2705 Laurier Boulevard, Québec City, QC, Canada G1V 4G2

Received December 3, 2008; Revised January 7, 2009; Accepted January 13, 2009

Primary open-angle glaucoma (POAG) is a leading cause of blindness worldwide. POAG is associated with a characteristic progression of changes to ocular morphology and degeneration at the optic nerve head with the loss of visual fields. Physical mapping efforts identified genomic loci in which to search for causative POAG gene mutations. WDR36, at locus GLC1G, was initially identified as a gene with a low frequency of non-synonymous sequence variations that were exclusive to adult-onset POAG patients. It has since been shown that rare WDR36 sequence variants are also present in the normal population at similarly low frequencies. The lack of a consistent genotype:phenotype correlation prompted us to investigate the functional consequences of WDR36 sequence variations. WDR36 is involved in rRNA processing, a critical step in ribosome biogenesis, and is very similar to yeast Utp21p which is a member of the small subunit (SSU) processome complex responsible for maturation of 18S rRNA. We, therefore, developed a yeast model system to test the functional and phenotypic consequences of POAG-associated sequence variants introduced into UTP21. Alone, the POAG variants did not produce any significant defects in cell viability or rRNA processing. However, when combined with disruption of STI1 (which synthetically interacts with UTP21), 5 of the 11 tested variants had increased or decreased cell viability which corresponded to reduced or elevated levels of pre-rRNA, respectively. These results demonstrate that, in the correct genetic background, WDR36 sequence variants can lead to an altered cellular phenotype, supporting the theory that WDR36 participates in polygenic forms of glaucoma.

INTRODUCTION

Glaucoma is a clinically complex grouping of progressive optic neuropathies (1,2) and is the second leading cause of blindness worldwide (3). Primary open-angle glaucoma (POAG) is described as the most common subset of glaucoma whose progression is usually associated with elevated intraocular pressure (IOP) (4). The mechanical theory of POAG pathology suggests that high IOP, as a result of defective aqueous humor homeostasis, puts stress on the optic nerve head that activates apoptosis of retinal ganglion cells (RGCs) and leads to optic nerve atrophy (5).

POAG is genetically heterogeneous with at least 20 susceptibility loci mapped, but only three causative genes have been identified (6). MYOCILIN (MYOC), discovered at locus GLC1A, encodes a secreted protein of unknown function that is highly expressed in trabecular meshwork cells (7,8). Transgenic mouse models have been produced to demonstrate the association of the MYOC mutation Tyr437His with POAG symptoms such as elevated IOP, RGC death and axonal degeneration in the optic nerve (9,10). OPTINEURIN (OPTN), also of unknown function, is the causative gene at locus GLC1E (11). Expression of the OPTN mutation E50K was shown to selectively induce RGC death mediated by oxidative stress (12).

GLC1G is the POAG locus mapped to 5q22.1 (13–15). Analysis of the genes in the critical region initially revealed several sequence alterations in WDR36 that were exclusive to adult-onset POAG patients. It has since been shown that rare WDR36 sequence variants are also present in the normal population at similarly low frequencies. The lack of a consistent genotype:phenotype correlation prompted us to investigate the functional consequences of WDR36 sequence variations. WDR36 is involved in rRNA processing, a critical step in ribosome biogenesis, and is very similar to yeast Utp21p which is a member of the small subunit (SSU) processome complex responsible for maturation of 18S rRNA. We, therefore, developed a yeast model system to test the functional and phenotypic consequences of POAG-associated sequence variants introduced into UTP21. Alone, the POAG variants did not produce any significant defects in cell viability or rRNA processing. However, when combined with disruption of STI1 (which synthetically interacts with UTP21), 5 of the 11 tested variants had increased or decreased cell viability which corresponded to reduced or elevated levels of pre-rRNA, respectively. These results demonstrate that, in the correct genetic background, WDR36 sequence variants can lead to an altered cellular phenotype, supporting the theory that WDR36 participates in polygenic forms of glaucoma.

*To whom correspondence should be addressed. Tel: +1 780 4924172; Fax: +1 780 4921998; Email: mwalter@ualberta.ca

© The Author 2009. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
to POAG patients who encoded predicted amino acid substitutions in evolutionarily conserved residues (13). Variant D658G also segregated accordingly within a GLC1G-linked family (13). However, subsequent reports have collectively discovered that while there are several non-synonymous WDR36 sequence variants exclusive to POAG patients in various populations, many, including D658G are also found on normal control chromosomes (16–21). These data suggest that WDR36 participates in a polygenic form of glaucoma or acts as a glaucoma-modifier gene, causing glaucoma to occur in individuals with WDR36 mutation only if additional factors are also present.

WDR36 is a widely expressed protein in humans that contains several iterations of the WD40 repeat motif (13). WD40-containing proteins normally contain seven copies of the repeat arranged in a seven-bladed propeller, each blade composed of four meandering β-strands (22–24). Although WD40 proteins exhibit a broad range of diversity in cellular functions, as β-propeller fold proteins they are likely to act as scaffolds for multimolecular complexes (24,25). Zebrafish WDR36 has recently been shown to be functionally homologous to the essential yeast gene UTP21 (26). Utp21p is a small nucleolar ribonucleoprotein that is part of the SSU processome responsible for early processing of the 35S pre-ribosomal RNA molecule into mature 18S rRNA, which is destined for the 40S small ribosomal subunit (27,28). The immunoprecipitation of Utp21p with the U3 snoRNA and the 23S rRNA intermediate (28) and its inclusion in the stable Pwp2p-subcomplex of the SSU processome (27) established Utp21p’s function in pre-rRNA processing. However, no specific functional defect was associated with the gene until a study in zebrafish reported that reduced expression of the homologous Wdr36 resulted in reduced levels of 18S rRNA (26). In this study, loss of wdr36 function also resulted in ocular dysmorphology and activation of the p53 stress-response pathway, which are relevant to considering the causative or modifying roles that WDR36 sequence variants may play in the pathology of POAG (26).

To ascertain whether WDR36 sequence variants affect protein function, we developed a yeast model system to assay the function of altered Utp21p. Baker’s yeast offered the distinct advantage of the ease in which we could perform complex genetic manipulations to study an essential gene. As well, yeast are extremely useful for investigating basic eukaryotic cellular processes such as rRNA processing. Using a haploid up21 deletion strain but expressing recombinant Utp21p modified with POAG-associated sequence variations and under the control of the natural UTP21 promoter, we did not find any changes in cell viability or in the levels of pre-rRNA that would indicate defective processing. However, since a UTP21 point mutation was found to synthetically interact with disruption of the co-chaperone STT1 (by causing reduced viability in the double mutant) (29), we also tested the POAG-associated variants in an stt1 null background. In this system, we discovered that five Utp21p variants (homologous to L25P, R529Q, I604V, D658G and M671V in human WDR36) resulted in growth defects with associated changes in the levels of pre-rRNA. These investigations confirm the prediction that non-synonymous amino acid variations in WDR36 alter protein function and lead to deleterious cellular conditions that may contribute to the progression of glaucoma. This work also implicates chaperone/stress-response pathways involving STT1 as a new target of investigation for understanding the biochemical nature of glaucoma pathogenesis.

RESULTS

WDR36 is structurally homologous to UTP21

A recent report by Skarie and Link (26) established that mammalian Wdr36 is functionally homologous to Utp21p. To demonstrate that Utp21p and human WDR36 could also be structurally homologous, molecular modeling was performed with the double β-propeller actin-interacting protein from Caenorhabditis elegans (AIP1) as the template (Fig. 1). Based on the homology models and the computed secondary structure of WDR36, we discovered that Utp21p and WDR36 each contain 14 WD40 repeats (Fig. 2) which likely fold into two connected seven-bladed β-propellers as deduced for the structure of AIP1 (30). Each WD40 repeat forms the inner portion of its associated blade.

In an attempt to verify that human WDR36 is functionally equivalent to Utp21p, we created a plasmid-shuffle assay with a haploid yeast strain containing a chromosomal deletion of UTP21 and a counter-selectable (URA3+) high-copy plasmid expressing the essential UTP21 open reading frame (ORF). When this strain is transformed with a second low-copy (CEN, LEU2+) plasmid expressing functional Utp21p under the control of the natural UTP21 promoter (construct ‘pRS315-UTP21’ in Supplementary Material, Fig. S1) and grown on media containing 5-fluoroorotic acid (5-FOA), the initial plasmid is cured from the viable cells and subsequent growth of the culture relies on the ability of the gene on the second plasmid to complement the up21 deletion. After transforming the intermediate strain with the LEU2+ plasmid encoding WDR36 (Supplementary Material, Fig. S1) instead of Utp21p, no complementation was observed on 5-FOA media (data not shown). We also tested the expression of WDR36 under the control of different promoters, but these vectors also did not complement up21Δ. The WDR36 ORF facilitates the expression of correctly sized recombinant protein in mammalian cells as visualized by western analysis (construct ‘pcDNA4HisMaxA-WDR36’ in Supplementary Material, Fig. S1, data not shown). However, no protein product was detected in yeast transformed with any of the WDR36 expression vectors, so it is possible that yeast are unable to correctly synthesize the functional human protein product.

Characterization of POAG variants in UTP21

A panel of UTP21 mutant constructs was created to mimic POAG-associated sequence variants detected previously in WDR36. We chose several variants for functional analysis that were discovered by Monemi et al. (13), Hauser et al. (16), and Raymond et al. (31) that fall into three classes. The possible disease-causing variants (A163V, H212P, N355S, A449T, R529Q and I604V) are exclusive to POAG patients as they have not been found on normal chromosomes,
potential disease-susceptibility variants (L25P, D33E, D658G and M671V) exist at a low frequency in control individuals and common polymorphisms (I264V) are present in equally high frequencies in both POAG and control individuals (Table 1). Recently, the A163V, H212P and A449T variants were discovered on control chromosomes (18) so they were reclassified into the second group. Most of these residues have been conserved throughout evolution and are identical in human and yeast, except for I264 (L223 in yeast), A449 (I419) and M671 (F634) (Fig. 2). In addition to the POAG-associated variants, as an experimental control, we also created an S602F yeast construct which replicates the defect discovered in the UV-irradiated strain SL42 that synthetically interacts with disruption of the co-chaperone STI1 (29). The UTP21 variants were engineered into the LEU2 plasmid and introduced into the utp21D test strain via the plasmid-shuffle assay (see above).

After the URA3+ Utp21p plasmid was cured from the panel of yeast variants with 5-FOA counter-selection, only the strain carrying the STI1-interacting UTP21(S602F) mutation exhibited growth which was different from the wild-type construct-bearing strain (Fig. 3a and b). All of the POAG-associated variants were able to complement the chromosomal deletion of UTP21. Similar to the description by Flom et al. (29), the S602F strain grew slowly in a wild-type STI1 background. The panel of UTP21 variants was then subjected to northern blot analysis of total RNA to investigate potentially subtle defects in 35S-to-18S rRNA processing. Based on the observations of Pwp2p depletion (32) and Wdr36 zebrafish mutants (26), we expected functional defects of Utp21p to manifest as an accumulation of rRNA precursors. We measured the levels of the high-molecular weight rRNA species (unresolved 35S, 33S and 32S) in the panel of UTP21 variants and found that none of the strains exhibited significant elevation of these transcripts compared with the wild-type construct (Fig. 3c). Many of the mutants had reduced levels of pre-rRNA, but the fact that the growth-defective S602F strain did not have a significant alteration in pre-rRNA argues that cell viability assays are more sensitive for detecting functional defects in Utp21p.

Based on the relationship identified between the S602F mutation of UTP21 and disruption of STI1, we created a double-mutant yeast strain by mating utp21D (carrying the URA3+, UTP21 plasmid) with strain CN11 (sti1::HIS3, an insertion-based null mutant) (33) and selecting for the HIS3+/URA3+ recombinant haploid sporulation product (confirmed by PCR analysis and mating type tests), followed by transformation with the panel of UTP21 variants on LEU2 plasmids. After counter-selection with 5-FOA, the S602F mutant was not viable in this sti1 background [strain SL42 was similarly inviable at 37°C and exhibited slow growth at 23 and 30°C (29)]. Plate growth assays (Fig. 4a) and liquid growth curves (Fig. 4b) both revealed that some of the POAG-associated variants display growth defects in an sti1 background. Compared with the sti1/utp21D strain carrying wild-type recombinant Utp21p, mutation L5P resulted in an increased growth rate at all temperatures, whereas the R495Q, I567V, D621G and F634V strains exhibited slow growth in complete media at 23 and 30°C. The slow-growth mutants appeared to be inviable at 37°C in the plate assay (Fig. 4a).

The POAG-variant UTP21 panel in the sti1 background was then examined for defects in rRNA processing. As shown in Figure 4c, the slow-growth mutant D621G has significant accumulation of low-mobility rRNA in the 35/33/32S range (216 ± 29%), whereas the fast-growth mutant L5P has a...
Figure 2. Alignment of WDR36 and Utp21p. Identical amino acid residues are shaded black and structurally similar residues are shaded grey. The location of the 14 predicted WD40 domains are indicated by arrows. POAG-associated amino acid substitutions are boxed, with WDR36 variants shown above the alignment and Utp21p variants shown below.
significant reduction (50 ± 10%). The other slow-growth mutants I567V and F634V also appear to have slightly elevated levels of unprocessed rRNA (165 ± 25 and 166 ± 30%, respectively) albeit with only borderline significance (Table 1). These results, summarized in Table 1, suggest that there is an inverse relationship between the level of pre-rRNA (relative to actin mRNA) and the growth rate of the mutant strains in our sti1/utp21Δ panel.

WDR36 and STI1 are co-expressed in ocular cells

In order to verify that the synthetic interaction between WDR36 and STI1 is conserved in humans, we began to investigate their relationship in tissues of the eye. RT-PCR (Fig. 5a) and western (Fig. 5b) analyses show that both genes are expressed in ocular cells that are relevant to glaucoma (Fig. 5a) and western (Fig. 5b) analyses show that both genes are expressed in ocular cells that are relevant to glaucoma.

DISCUSSION

For the purposes of patient counseling and disease prediction, it is necessary to determine the contribution of WDR36 variants to the development of glaucoma. Collectively, studies have shown that some sequence variants of WDR36 are found exclusively in glaucoma patients, whereas other rare variants are also in wild-type control individuals, and that variants do not consistently segregate with the disease within pedigrees. We have now shown that certain WDR36-homologous UTP21 variants exhibit functional defects in an sti1 mutant background, strengthening the argument that WDR36 participates in polygenic forms of glaucoma. This study also implicates the involvement of STI1-related stress-response and molecular chaperone pathways, along with ribosome assembly, in glaucoma progression.

Although WDR36 was unable to functionally complement yeast lacking Utp21p, it has now been established that both proteins are involved in rRNA processing (26) and are thus likely to have functionally equivalent roles in the SSU processome. Our molecular modeling (Fig. 1) suggests that the yeast and human orthologs can adopt the same three-dimensional structure so it is reasonable to suggest that their physical interactions have been conserved throughout eukaryote evolution. For a fundamental cellular process such as ribosome biogenesis, there is considerable evolutionary conservatism of gene networks (34). As we have now revealed that POAG-associated homologous variants in Utp21p affect the function of an rRNA processing gene, it will be intriguing to approach the study of glaucoma pathogenesis in patients carrying those variants from the perspective that high metabolic demand in their ocular tissues may be putting stress on the ribosome assembly pathway in which defective WDR36 would not be able to overcome. Conceivably, defects in other SSU processome proteins could work separately or in concert with WDR36 mutations to contribute to glaucoma progression in complex cases.

None of the human-derived UTP21 variants exhibited a growth defect in yeast with the sti1Δ(utp21Δ) background (Table 1). However, six of these strains showed significantly decreased levels of pre-rRNA, suggesting they had altered SSU processome function. This observation was unexpected since we predicted that loss-of-function mutations in UTP21 would result in accumulation of pre-rRNA, based on previous observations from the depletion of the SSU processome protein Pwp2p (32) (which is normally complexed with Utp21p). The phenotypes of the S602F and L223V strains demonstrate that the relationship of rRNA processing to cell viability is not straightforward. L223V is homologous to the WDR36 variant I640V. The I640V variant is unlikely to contribute to glaucoma pathogenesis because the V264

<table>
<thead>
<tr>
<th>WDR36 variant</th>
<th>UTP21 variant</th>
<th>utp21Δ Background (30°C)</th>
<th>sti1/utp21Δ Background (30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth defect</td>
<td>Pre-rRNA level</td>
</tr>
<tr>
<td>Exclusive to glaucoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N355S</td>
<td>N317S</td>
<td>None</td>
<td>101 ± 10% (P = 0.95)</td>
</tr>
<tr>
<td>R529Q</td>
<td>R495Q</td>
<td>None</td>
<td>42 ± 5% (P &lt; 0.01)</td>
</tr>
<tr>
<td>I604V</td>
<td>I567V</td>
<td>None</td>
<td>38 ± 15% (P = 0.01)</td>
</tr>
<tr>
<td>Low normal frequency (&lt;5% of control chromosomes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L25P</td>
<td>L5P</td>
<td>None</td>
<td>99 ± 27% (P = 0.97)</td>
</tr>
<tr>
<td>D33E</td>
<td>D13E</td>
<td>None</td>
<td>64 ± 16% (P = 0.09)</td>
</tr>
<tr>
<td>A163V</td>
<td>A116V</td>
<td>None</td>
<td>87 ± 7% (P = 0.14)</td>
</tr>
<tr>
<td>H212P</td>
<td>H172P</td>
<td>None</td>
<td>52 ± 12% (P = 0.02)</td>
</tr>
<tr>
<td>A449T</td>
<td>A419T</td>
<td>None</td>
<td>75 ± 14% (P = 0.15)</td>
</tr>
<tr>
<td>D658G</td>
<td>D621G</td>
<td>None</td>
<td>34 ± 12% (P = 0.01)</td>
</tr>
<tr>
<td>n/a</td>
<td>S602F</td>
<td>Decreased rate</td>
<td>81 ± 14% (P = 0.25)</td>
</tr>
<tr>
<td>M671V (SNP: rs11956837)</td>
<td>F634V</td>
<td>None</td>
<td>48 ± 4% (P &lt; 0.01)</td>
</tr>
<tr>
<td>I264V (SNP: rs11241095)</td>
<td>L223V</td>
<td>None</td>
<td>35 ± 10% (P &lt; 0.01)</td>
</tr>
</tbody>
</table>

Variants are grouped according to cumulative allele frequencies from published results (13,16–21). Pre-rRNA levels were calculated as the mean of the ratios of unresolved 32S/33S/35S rRNA:ACT1 mRNA band intensities, relative to cells transformed with UTP21(WT). P-values were calculated using Student’s t tests.
Figure 3. Functional characterization of UTP21 mutants in the \textit{STI1}^+/utp21Δ background. (a) Plate growth assay of yeast diluted to a common starting concentration and then subjected to 10-fold serial dilutions in water. A sample of each dilution was spotted onto complete media plates and grown at the indicated temperatures. The asterisk (*) indicates S602F as the only strain with visibly altered viability. (b) Liquid growth curves of yeast diluted to a starting OD$_{600}$ of $\sim$0.2 and cultured at 30°C in complete media. Error bars for WT (thick black line) and S602F (grey line, circles) represent the standard error of the mean for two experiments. (c) Relative abundance of pre-rRNA calculated from northern blot analysis carried out on total RNA with the net band intensity (unresolved for 35S, 33S and 32S) normalized to actin (\textit{ACT1}) mRNA and expressed relative to the \textit{UTP21}(WT) strain. Error bars represent the standard error of the mean for three experiments. Asterisks (*) denote statistically significant differences from WT as calculated using Student’s $t$ tests ($P < 0.05$).
Figure 4. Functional characterization of UTP21 mutants in the sti1/utp21Δ background. (a) Plate growth assay of yeast diluted to a common starting concentration and then subjected to 10-fold serial dilutions in water. A sample of each dilution was spotted onto complete media plates and grown at the indicated temperatures. The parental strain ‘utp21Δ + WT’ was viable at all temperatures, whereas the parental strain ‘sti1’ was inviable at 37°C. Asterisks (*) indicate strains with visibly altered viability. (b) Liquid growth curves of yeast diluted to a starting OD<sub>600</sub> of ~0.2 and cultured at 30°C in complete media. The grey-shaded zone represents two standard deviations from the results for UTP21(WT), calculated from three experiments. The mean values for L5P (black line, closed squares), R495Q (black line, open diamonds), I567V (grey line, squares), D621G (grey line, triangles) and F634V (grey line, diamonds) fall outside this zone. (c) Relative abundance of pre-rRNA from northern blot analysis carried out on total RNA with the net band intensity (unresolved for 35S, 33S and 32S) normalized to actin (ACT1) mRNA and expressed relative to the UTP21(WT) strain. Error bars represent the standard error of the mean for three experiments. Asterisks (*) denote statistically significant differences from WT as calculated using Student’s t tests (P < 0.05).
In our yeast assays, the D658G-homologous mutation confers growth impairment upon Sti1p-deficient yeast, with the accumulation of unprocessed pre-rRNA (Fig. 4). Considering WDR36 as a modifier locus for POAG, this establishes D658G as a disease-susceptibility mutation, requiring an additional functional deficiency or unique environmental interaction to manifest the glaucoma phenotype. Observed non-penetrance in WDR36-associated POAG pedigrees (16) could be attributed to a lack of co-segregation with a second responsible locus. Alternatively, the D658G variation and other WDR36 variants tested here may act as modifiers by altering the severity, response to treatment or age-of-onset of the glaucoma phenotype when caused by a mutation in a primary disease gene such as MYOC or OPTN. Slow growth was also observed for the mutation homologous to the single nucleotide polymorphism M671V (SNP: rs11956837). The rare V671 variant was found in POAG patients at a low frequency (13,16) but was not reported to be in controls in the published studies of WDR36. The SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) records the rare V671 allele as having a frequency of up to 11% depending on the population. Our results suggest that, like D658G, M671V may increase the risk of developing glaucoma. The two other yeast mutants that grow slowly in the sti1 background, R495Q and I567V, also strongly correspond to disease susceptibility as the homologous human alterations, R529Q and I604V, are among the few glaucoma-exclusive WDR36 variants (13,16,20,21). The last of the UTP21 variants that exhibited an sti1-associated growth phenotype is L5P, which showed the unusual association of enhanced cell growth with significantly decreased levels of pre-rRNA. L5P was designed to mimic the WDR36(L5P) variant which was found in control individuals (13,16,18). However, the poor conservation of amino acid sequence and structure in the N-terminus of the Utp21p/WDR36 alignment (Fig. 2) casts doubt on whether L5P is truly homologous to L25P. Although the position L5 must be important to the genetic interaction of UTP21 with STI1, it would be necessary to test the WDR36(L5S) mutation in a model system more closely related to humans to more accurately assess its functional significance. The WDR36 variant N355S has been reported to be exclusive to glaucoma patients (13,19,20) but was not found to have a functional effect in our sti1 assays. For all the variants which did not affect culture growth rate, it remains to be seen if they will produce measurable functional defects in mutant backgrounds other than sti1. Since ribosome assembly and stress-response pathways are very complex and involve a diverse array of genetic and environmental components, certain WDR36/UTP21 variants might interact specifically with other potential, and yet undiscovered, glaucoma co-modifying genes. As the complex nature of glaucoma genetics becomes appreciated, it is becoming necessary to identify all the gene networks or pathways that are involved in disease progression to guide further research into the functional roles of the currently known glaucoma genes such as WDR36.

The association of the co-chaperone STI1 (also known as heat shock protein-organizing protein or HOP in humans) with HSP70/HSP90 complexes has been conserved in eukaryotes whereby STI1 is responsible for transferring client proteins between the two HSP chaperones (35). Of particular interest to its potential involvement in glaucoma, STI1 has...
been shown to modulate proliferation and neuroprotection of retinal explants (36,37) and also to promote neuritogenesis (38,39). These activities are situationally dependent on interaction with prion protein (PrP\(^\text{c}\)) and exemplify the evolved roles this protein has undertaken in higher eukaryotes.

The nature of the \textit{UTP21}–\textit{STI1} interaction and how it affects yeast growth has not yet been determined. There is no additional evidence of a link between Sti1p and ribosome biogenesis. Sti1p has been discovered to physically interact with a diverse set of proteins (http://db.yeastgenome.org/cgi-bin/interactions.pl?dbid=S000005553), but we did not find information to suggest that they are involved with SSU processome pathways. As WD40 repeat-containing proteins typically act as scaffolding for the assembly of protein complexes (24,25), it is possible that mutations in \textit{Utp21p}–\textit{WDR36} cause structural defects that inhibit protein–protein interactions. It is therefore interesting that the five \textit{Utp21p} variants that display a synthetic growth defect in the \textit{stil} background (including the non-POAG-related S602F mutation) are physically located in one region of the protein, specifically in or near the last four blades of the second \(\beta\)-propeller (WD repeats 11–14, see Figs 1 and 2). This clustering points to the possibility of these mutations altering physical interactions involving this particular domain of Utp21p/WDR36.

Prior to testing if human \textit{STI1} is also a co-modifier gene for glaucoma, it is important to establish that its genetic relationship with \textit{WDR36} has been evolutionarily conserved. \textit{STI1} and \textit{WDR36} are ubiquitously expressed in humans (http://www.ncbi.nlm.nih.gov/UniGene) and we confirmed their expression in several ocular cell line cultures. Endogenous protein was detected in NPCE, HTM and RGC5 cells (Fig. 5b), all of which are useful models for studying glaucoma because of their relevance to aqueous humor homeostasis or neurodegeneration. We have shown that these two proteins are expressed in the same cells, but there is currently no evidence to suggest that protein–protein binding is an explanation for their synthetic relationship in yeast. Since we were unable to demonstrate co-immunoprecipitation of WDR36 with STI1, implying that these two proteins do not physically interact, future studies of how WDR36 contributes to glaucoma pathogenesis should also focus on discovering the physical interactions of WDR36 that have a role outside of rRNA processing.

In summary, we have established that yeast is a useful model system for exploring the functional consequences of \textit{WDR36} sequence variation. Mutation of yeast \textit{UTP21} with homologous glaucoma-associated variants of \textit{WDR36} underlies cell growth defects resulting from altered protein function. These phenotypic consequences are the result of genetic interaction with the co-chaperone \textit{STI1}. The variants R529Q, I604V, D658G and M671V are likely to encode subtle defects in WDR36 that, in certain environmental or genetic contexts such as a mutation of \textit{STI1}, predispose to progression of glaucoma. Human \textit{STI1} has not previously been implicated in the genetics of glaucoma, but now warrants investigation as a potential co-modifier gene in patients suspected to have mutation of \textit{WDR36}. Due to its properties of retinal neuroprotection, and involvement in stress response, \textit{STI1} is an ideal candidate gene for glaucoma. Studying the various roles and interactions of \textit{STI1} may lead to identification of other co-modifying glaucoma genes. The \textit{WDR36} variants for which a functional defect was not discovered in this report need to be further examined for interaction with other co-modifying genes to help understand the mechanisms by which \textit{WDR36} contributes to glaucoma pathogenesis.

**MATERIALS AND METHODS**

**Homology modeling**

ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to generate the alignment between human \textit{WDR36} (NP_644810.1) and yeast \textit{Utp21p} (NP_013513.1) in Figure 2. WD40 repeat motifs were deduced from a consensus sequence (http://bmerc-www.bu.edu/projects/wdrepeat) and secondary structure prediction (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). For homology modeling, \textit{Utp21p} and \textit{WDR36} were first aligned with ClustalW (http://toolkit.tuebingen.mpg.de/clustalw), then exported to HHpred (http://toolkit.tuebingen.mpg.de/hhpred) where \textit{C. elegans} AIP (PDB# 1nro) was identified as the highest-scoring structural homolog (\(E\)-value = 8.2E–38). The \textit{WDR36-1nro} and \textit{Utp21p-1nro} alignments were exported to Modeller within the same web platform (http://toolkit.tuebingen.mpg.de/modeller) to automatically generate homology models that were saved and rendered using Swiss-PdbViewer (40) and POV-Ray (Fig. 1).

**Yeast strains**

The \textit{utp21}\(\Delta^{\pm}\) heterozygous diploid strain (BY4743 background, Clone ID 25318: \textit{MATa/MATa his3A1/ his3A1 leu2A0/leu2A0 ura3A0/ura3A0 met15A0/MET15 lys2A0/lys2A0}) and the haploid tester strains (W303 \textit{MATa} or \textit{a}: \textit{URA3-52} \textit{TRP1} \textit{LEU2-3,112} \textit{HIS3-11} \textit{ADE2-1} \textit{CAN1-100}) were purchased from Open Biosystems (Huntsville, AL, USA). CN11 (\textit{MATa} \textit{DTRP1 lys1} \textit{lys2} \textit{ura3-52 leu2-3,112 his3-11,15 \textit{STI1::HIS3}}) (33) was a gift from Dr Elizabeth Craig (Department of Biochemistry, University of Wisconsin, Madison, WI, USA).

**Plasmid construction**

See Supplementary Material, Table S1 for primer sequences and Supplementary Material, Figure S1 for partial vector maps. An epitope-tagged \textit{WDR36} yeast expression construct was assembled in pRS315 (41) comprised of subcloned \textit{ADH1} promoter (\textit{SacI}/\textit{BamHI} fragment), the \textit{WDR36} ORF cloned by RT–PCR from human lymphocyte RNA in two overlapping sections (primers \textit{WDR36-attB1}/\textit{WDR36-2R}; primers \textit{WDR36-2F}/\texti{WDR36-attB2}; \textit{BamHI}/\textit{SacI} fragment), the 3' end of the ORF modified by PCR (primers \textit{WDR36-Sca}/\textit{WDR36-KpnNot}) to contain \textit{KpnI} and \textit{NotI} sites and a \textit{C-terminal} V5 epitope tag (\textit{KpnI}/\textit{NotI} fragment from a previously described plasmid (42)). The promoter was later replaced with PCR-amplified versions of \textit{GAL1} (primers \textit{GAL1Prom-F/R}, from vector BG1805) or \textit{UTP21} (amplified with primers \textit{T7}/\textit{UTP21Prom-R} from plasmid pRS315–\textit{UTP21-5'}) promoters using \textit{SacI}/\textit{PacI} fragments. The \textit{BamHI}/\textit{NotI} fragment from plasmid pRS315–\textit{ADH-WDR36} was subcloned into pcDNA4HisMaxA© (Invitrogen,
Burlington, ON, USA) for mammalian expression of WDR36. This construct includes N-terminal 6× HIS and Xpress™ epitope tags.

The UTP21 ORF (YLR409C) in plasmid BG1805 (2μ, URA3+) was purchased from Open Biosystems. The pRS315-UTP21 (CEN, LEU2) yeast expression plasmid was constructed with the UTP21 promoter and 5′-end of the ORF amplified from yeast genomic DNA (primers UTP21-5′-F/R; SacII/EagI fragment), the remainder of the UTP21 ORF from BG1805-YLR409C (longest NdeI/XbaI fragment) and overlapping oligomers encoding the C-terminal Xpress™ epitope (primers Xpress-F/R; BamHI/HindIII compatible ends).

Substitutions in pRS315-UTP21 were introduced into subcloned gene fragments (in vector pGEM-T) using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), sequenced for verification, then re-inserted into the pRS315-UTP21 plasmid and re-sequenced. The forward complementary primers for each mutation are listed in Supplementary Material, Table S1.

**Yeast genetics**

Yeast were cultured in YP (10 g/l yeast extract, 20 g/l peptone and 2% d-glucose or galactose) or synthetic drop-out (6 g/l nitrogen base and 2% d-glucose or galactose) media at 30°C. Strain and plasmid identification was confirmed by standard PCR analysis of colony pellets by dispersing cells into 100 μl of 50 U/ml lyticase (#L4025; Sigma-Aldrich, Oakville, ON, USA) in TE buffer, incubating at 37°C for 30 min, then using 1 μl as template. Yeast transformation was performed by following a standard lithium acetate procedure (http://www.clontech.com/images/pt/PT3024-1.pdf).

For random spore analysis, diploid yeast were first grown in a patch on a GNA (10 g/l yeast extract, 30 g/l nutrient broth and 2% d-glucose) plate, then cells were transferred to supplemented sporulation media (2% potassium acetate) and incubated with shaking at 30°C for several days until tetrads were visible. Non-sporulated vegetative cells were killed by vortexing the suspension vigorously in 50% ether for 5 min, then asci were digested for 1 h in 500 U/ml lyticase plus 0.3 mM β-mercaptoethanol at 37°C. Washed cells were plated on selective media. Sporulation products were distinguished from surviving heterozygotes via PCR analysis and mating type tests.

**Growth assay**

To measure growth curves, yeast were cultured in 5 ml YPD media at 30°C overnight, with shaking at 225 rpm. Cultures were diluted in media to reach an OD 600 of ~0.20 in ~20 ml, then incubated again. One milliliter aliquots were extracted every 2 h to monitor the OD 600 as a function of cell concentration. For visual assessments of strain viability, the OD 600 of overnight-cultured yeast was measured and an aliquot equivalent to 0.05 OD 600 units was diluted in water to 100 μl total volume. Ten-fold serial dilutions in water were prepared and 5 μl of each was spotted onto complete media plates.

**Yeast RNA preparation and northern blot analysis**

Total RNA was prepared from yeast cultured in complete media using a hot-phenol protocol (43), separated on a 1.2% agarose-MOPS-formaldehyde gel (43) and transferred to Hybond-N membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). DNA oligomer probes specific for the 20S and 27SA2 pre-rRNA transcripts, which also detect 35S, 33S and 32S pre-rRNA (32), and the probe for ACT1, were end-labeled with 32P-γdATP and hybridized to the northern blots with ExpressHyb (Clontech, Mountain View, CA, USA) at 68°C. Alternatively, an ACT1 probe was generated by PCR amplification (primers ACT1-F/R) from yeast genomic DNA, gel purification and use of the Random Primers DNA Labeling System (Invitrogen) with 32P-odCTP. After overnight incubation, blots were washed at room temperature in 2 × SSC/0.05% SDS, and sometimes additionally at 50°C with 0.1 × SSC/0.1% SDS, and then exposed to SuperRX X-ray film (FUJIFILM). Autoradiograms were scanned on a UMAX Astra 2400S scanner (UTC transmissive setting), the image was saved as a TIFF file, and bands were quantified with Kodak Molecular Imaging Software v4.0.5. Net pixel intensities for the rRNA bands were normalized to actin (ACT1) and displayed relative to the recombinant wild-type strain. Statistical significance (P < 0.05) of deviation from wild-type values was calculated by two-tailed equal-variance Student’s t tests in Microsoft Excel.

**Mammalian molecular analyses**

COS-7 and NPCE (originally from Dr Miguel Coca-Prados, Department of Ophthalmology and Visual Science, Yale University, New Haven, CT, USA) cells were cultured in high-glucose Dulbecco’s modified Eagle’s Medium (DMEM) + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (for COS-7) or 1% antimycotic (for NPCE). HTM (from Dr Miguel Coca-Prados) and rat retinal ganglion (RGC5, originally from Dr Neeraj Agarwal, Department of Cell Biology and Genetics, UNT Health Science Center, Fort Worth, TX, USA) cell lines were cultured in low-glucose DMEM + 10% FBS + 1% penicillin/streptomycin. All cells were grown at 37°C with 5% ambient CO2. Whole-cell protein extracts were obtained by lysing in cationic buffer (20 mM HEPES pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl2, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF and 5 μg/ml Sigma Protease Inhibitor Cocktail) with three freeze–thaw cycles of 10 min in a dry-ice/isopropanol bath followed by 1 min in a 37°C water bath. Two hundred micrograms of extracts were separated in an 8% SDS–PAGE gel, blotted to nitrocellulose, blocked with 5% milk in TBST and probed with α-WDR36 (1:1000) (H00134430-M01; Abnova, Walnut, CA, USA), α-ST1 (1:5000) (H00010963-M11; Abnova) or α-TFIIH (1:2000) (sc-204; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in blocking solution at 4°C overnight. Following washes in TBST, blots were incubated with horseradish peroxidase-conjugated secondary antibodies α-Mouse(IgG) or α-Rabbit(IgG) (1:5000) (Jackson Immuno-Research, West Grove, PA, USA) in blocking solution at room temperature for 1 h, and detection was carried out with
a SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA) and SuperRX film. Total RNA was extracted from cells with TRIZol® Reagent (Invitrogen), and cDNA was synthesized with oligo-dT(n) and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer’s protocols. RT–PCR analysis was carried out with 1 μl of cDNA as template and 10 pmol of each of the appropriate primers (hWDR36-F/R, rWDR36-F/R and STI1-F/F).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

The authors thank Ms May Yu and Dr Robert Scott for technical assistance and Dr Moira Glerum and the Ocular Genetics Laboratory for critical analysis of the manuscript.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Canadian Institutes of Health Research (MOP77782 to M.A.W and V.R.); The Glaucoma Foundation (G700000350 to M.A.W) and Vision Research (MOP77782 to M.A.W and V.R.); The Glaucoma Network/Re´seau Vision of Fonds de la Recherche en Santé du Québec (travel grant to N.B.).

REFERENCES


