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Dominant *PRPF31* Mutations Are Hypostatic to a Recessive *CNOT3* Polymorphism in Retinitis Pigmentosa: A Novel Phenomenon of "Linked *Trans*-Acting Epistasis"

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Summary

Mutations in *PRPF31* are responsible for autosomal dominant retinitis pigmentosa (adRP, RP11 form) and affected families show nonpenetrance. Differential expression of the wildtype *PRPF31* allele is responsible for this phenomenon: coinheritance of a mutation and a higher expressing wildtype allele provide protection against development of disease.

It has been suggested that a major modulating factor lies in close proximity to the wildtype *PRPF31* gene on Chromosome 19, implying that a *cis*-acting factor *directly* alters *PRPF31* expression. Variable expression of *CNOT3* is one determinant of *PRPF31* expression. This study explored the relationship between *CNOT3* (a *trans*-acting factor) and its paradoxical *cis*-acting nature in relation to RP11.

Linkage analysis on Chromosome 19 was performed in mutation-carrying families, and the inheritance of the wildtype *PRPF31* allele in symptomatic–asymptomatic sibships was assessed—confirming that differential inheritance of wildtype chromosome 19q13 determines the clinical phenotype ($P < 2.6 \times 10^{-7}$).

A theoretical model was constructed that explains the apparent conflict between the linkage data and the recent demonstration that a *trans*-acting factor (*CNOT3*) is a major nonpenetrance factor: we propose that this *apparently cis*-acting effect arises due to the intimate linkage of *CNOT3* and *PRPF31* on Chromosome 19q13—a novel mechanism that we have termed "*linked trans-acting epistasis*."

Keywords: *PRPF31*, *CNOT3*, retinitis pigmentosa, epistasis

Introduction

It has long been recognized that a disease-causing mutation can have different effects in different individuals and this may be due to environmental factors, stochastic molecular variation, epigenetic variation between individuals, or epistatic interactions (Nabholz & von Overbeck, 2004; Fraga et al., 2005; Raj & van Oudenaarden, 2008). Epistasis is the phenomenon whereby the phenotype caused by a mutation (or polymorphism) is masked by an allele at another locus, hence the alternative use of the terms gene–gene or genetic interaction. Although epistasis was first described over 100 years ago, it has received relatively little attention, in part due to our limited knowledge of epistatic mechanisms at a molecular level, and also due to our almost nonexistent ability to predict epistatic interactions *de novo* (Bateson, 1909; Lehner, 2011). It is clear, however, that epistasis is a pervasive phenomenon, having been reported to determine the phenotypic outcome of genetic variations in all sorts of organisms, including plants, invertebrates, (e.g., flies) and vertebrates (e.g., birds, mammals) (Montooth et al., 2003; Kroymann & Mitchell-Olds, 2005; Carlborg et al., 2006; Shao et al., 2008). Several molecular mechanisms can underlie epistasis, including direct

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interaction between the protein products of the genes, functional redundancy, involvement of the two genes in a common pathway or interaction between separate molecular pathways (Lehner, 2011). A further possibility is that epistatic interactions affect the regulatory network of genes. The multifaceted nature of gene expression regulation means that epistatic interactions are frequent and complex, although this idea has not been explored in great detail to date (Carter et al., 2007; Gertz et al., 2010).

Retinitis pigmentosa (RP) is a genetically heterogenous group of retinal degenerations, characterized by progressive cell death of the rod photoreceptors. Autosomal dominant retinitis pigmentosa (adRP) accounts for approximately 30–40% of cases and causative mutations have been identified in over 20 genes. Amongst these is an unusual class of causative genes—the splicing factors—six of which have been defined as causative of adRP (McKie et al., 2001; Vithana et al., 2001; Chakarova et al., 2002; Keen et al., 2002; Zhao et al., 2009; Tanackovic et al., 2011a).

Mutations in the ubiquitous splicing factor *PRPF31* were found to underlie a major RP locus, termed RP11 (Al-Magtheth et al., 1996; Vithana et al., 2001). PRPF31 protein plays a key role in the process of mRNA splicing, through its interaction with the U4/U6.U5 tri-snRNP—the key ribonucleoprotein complex of both major and minor spliceosomes. The PRPF31 protein contains a Nop domain that allows binding of the U4 snRNP within the U4/U6 di-snRNP (Liu et al., 2007). PRPF31 then links the U4/U6 di-snRNP with the U5 snRNP to form the U4/U6.U5 tri-snRNP, this process being mediated by the binding of PRPF6 by PRPF31 (Weidenhammer et al., 1997; Makarova et al., 2002; Liu et al., 2006). RNA interference mediated knockdown of *PRPF31* expression inhibits formation of tri-snRNPs, with accumulation of U4/U6 di-snRNPs in the Cajal bodies (Schaffert et al., 2004).

Mutations in *PRPF31* are a relatively common cause of adRP, accounting for approximately 5–10% of cases (Waseem et al., 2007; Audo et al., 2010; Xu et al., 2012). Over 40 mutations have been identified to date, including nonsense, missense, and frameshift mutations, as well as large deletions (Audo et al., 2010; Utz et al., 2013). Most *PRPF31* mutations result in null alleles, either through complete or partial absence of gene (large deletions) or through mRNA that quickly undergoes degradation (Rio Frio et al., 2008a; Audo et al., 2010). Carriers of such mutations can, therefore, be considered to be functional hemizygotes (Rio Frio et al., 2008a; Audo et al., 2010). There has been one report of functional haploinsufficiency, where a single base pair deletion within the *PRPF31* promoter region reduced transcription of the gene (Rose et al., 2012).

One interesting aspect of RP11 is that, although *PRPF31* is essential for splicing in all cells, the disease phenotype is retina-specific but this is not due to the existence of retina-specific isoforms of *PRPF31* (Tanackovic & Rivolta, 2009). In patients carrying *PRPF31* mutations, there is a generalized defect in spliceosome assembly and pre-mRNA processing and, as the retina has a particularly high requirement for pre-mRNA splicing, it was suggested that retina—as compared to other tissues—is relatively more affected by mutations of splicing factors (Tanackovic et al., 2011b).

Another consistent feature of *PRPF31*-associated adRP (RP11) is phenotypic nonpenetrance, whereby an asymptomatic individual carrying a mutant allele can have affected siblings or children. It is known that nonpenetrance is determined by the expression level of the wildtype *PRPF31* allele, and that there is variable expression of the *PRPF31* gene in the general population (Rio Frio et al., 2008a). It has been shown that asymptomatic mutation carriers have more than twofold higher expression levels of wildtype *PRPF31* compared to symptomatic individuals (Vithana et al., 2003; Rivolta et al., 2006; Liu et al., 2008). Patients coinheriting a *PRPF31* mutant and a higher expressing wildtype allele are asymptomatic because the residual level of protein is still sufficient for normal retinal function—the wildtype allele is able to compensate for the mutant allele.

McGee et al. (1997) looked at phenotypic discordance between mutation-carrying siblings and observed that the affected siblings and unaffected siblings consistently inherited different wildtype Chromosome 19q13 alleles from the nonmutation-carrying parent. It is generally thought, therefore, that a *cis*-acting factor that directly alters wildtype *PRPF31* expression is responsible for the observed phenotypic nonpenetrance. Attempts to identify single nucleotide polymorphisms (SNPs) that might be responsible for altered gene expression have failed to identify any causative changes. One theory draws attention to the high-repeat content of Chromosome 19 and proposes that variability in repeat elements, such as *Alu* repeats, might play a role (Rose et al., 2011).

Another interesting aspect of this disease is that, prior to nonsense-mediated decay of the mutant PRPF31, there is increased expression of both *PRPF31* alleles in mutation-carrying asymptomatic individuals (Rio Frio et al., 2008a). As there is some increased expression of both the wildtype and mutant alleles of *PRPF31*, there must be at least one factor that is *trans*-acting. One possible *trans*-acting factor was identified through the association of higher *PRPF31* expression and an expression quantitative trait locus (eQTL) at 14q21–23 (Rio Frio et al., 2008b).

It has also been shown that *PRPF31* expression level is influenced by *CNOT3*, with increased levels of CNOT3 protein causing transcriptional repression of *PRPF31* (Venturini et al., 2012). CNOT3 is a component of the Ccr4-Not transcription complex, which is an evolutionarily conserved global regulator of RNA polymerase II-mediated transcription (Miller

& Reese, 2012). An inverse relationship has been shown between *PRPF31* expression and *CNOT3* mRNA levels, and siRNA-mediated silencing of *CNOT3* provoked an increase in *PRPF31* expression, these observations further confirm the repressive nature of the interaction (Venturini et al., 2012).

This paper explores an apparent paradox in the relationship between *PRPF31* and *CNOT3*: This paradox arises when trying to reconcile (i) the current knowledge that a *cis*-acting factor in close proximity of *PRPF31* is the major factor underlying nonpenetrance (McGee et al., 1997), whereas (ii) the *trans*-acting *CNOT3* appears to be one of the major factors controlling *PRPF31* expression (Venturini et al., 2012).

Methods

Two approaches were taken to address this paradox.

First, the conjecture of McGee et al. was reviewed—in which they suggest that inheritance of the wildtype allele of Chromosome 19q13.4 is responsible for determining phenotype in carriers of the *PRPF31* mutation (McGee et al., 1997). This was tested with the null hypothesis that, for mutation-carrying siblings, the wildtype gene is randomly inherited from the nonmutation-carrying parent and, therefore, each haplotype from the unaffected parent is equally represented in the "symptomatic" and "asymptomatic" offspring within a given sibship.

To test the null hypothesis, previously collected microsatellite linkage data from six families (AD5, AD11, AD24, AD29, ADC1, RP1907) were reanalyzed to examine the inheritance of the wildtype Chromosome 19q13 (Al-Maghtheh et al., 1996; Vithana et al., 1998; Abu-Safieh, 2003; Rose et al., 2012). Microsatellite data were also collected from one further family (ADB1), in which a *PRPF31* mutation had previously been identified, but without performance of linkage analysis (Chakarova et al., 2006). The genotype data for mutation-carrying siblings were phased before consideration of the wildtype gene: The left-hand-most (oldest) mutation-carrying sibling of each branch was treated as the reference wildtype haplotype; if they were symptomatic they were assigned "green," if they were asymptomatic they were assigned "orange." The wildtype allele in the remaining siblings was analyzed, and classed as being concordant with the reference haplotype or nonconcordant. Where meiotic recombination had occurred, the sibling was regarded as concordant if half or more of the markers were identical.

Second, a theoretical model was proposed to explain the relationship between *CNOT3* and *PRPF31* mutations, this model reconciling how a known *trans*-acting factor (CNOT3) could be a major nonpenetrance factor and yet be consistent with a "wildtype inheritance" theory that appears to require a direct *cis*-acting mechanism.

Microsatellite Analysis

The inheritance of the wildtype chromosome 19q13 was analyzed in individuals from unrelated families carrying known mutations in *PRPF31*, each individual being required to have at least one mutation-carrying sibling. All families had evidence of nonpenetrance, with both symptomatic and asymptomatic mutation carriers within sibships. The inheritance of the wildtype Chromosome 19q13 was analyzed for six such families (AD5, AD11, AD24, AD29, ADC1, RP1907) by re-examination of previously collected microsatellite linkage data (Al-Maghtheh et al., 1996; Vithana et al., 1998; Abu-Safieh, 2003; Rose et al., 2012) and by linkage analysis of microsatellite data for one further mutation-carrying family (ADB1) (Chakarova et al., 2006). The microsatellite markers covered the region surrounding *PRPF31* and *CNOT3* on Chromosome 19q13, this region including markers between D19s921 and D19S418 (Chromosome 19:53771240–55546128). Additional markers to north or south of this region were analyzed where data were available (Fig. 1).

To perform linkage analysis in family ADB1, ABI PRISM Linkage Mapping Set (Applied Biosystems, Paisley, UK) was used, using only the primers for the region of interest on Chromosome 19q13 (Fig. 1). The following procedure was used: 5-μl Absolut qPCR mix (Thermoscientific, Lougborough, UK) was combined with 1-μl genomic DNA, 300-μM primer (forward/reverse), and distilled water to a total volume of 10 μl. The mixture was denatured at 94°C for 15 min, followed by 30 cycles of 94°C, 15 s; 60°C, 30 s; 72°C, 30 s. Finally, the mixture was held at 72°C for 5 min. The product was then diluted by combining 1-μl PCR product with 11-μl HiDi formamide (Applied Biosystems) and 0.1-μl GeneScan 500 Liz Size Standard (Invitrogen, Paisley, UK). The mixture was denatured for 5 min at 95°C, then incubated on ice for 2 min. The sample was genotyped on ABI 3730 and the results analyzed using GeneMarker v1.8 (Softgenetics, Pennsylvania, USA). The marker primers used were from ABI Prism Linkage mapping set v2.5 (Applied Biosystems). Where possible, previously collected data were validated, using the same methods as described in the original manuscripts.

Statistical and Bioinformatic Analyses

The reference sibling was excluded from the dataset and the null hypotheses assessed using Fisher's exact test.

Polymorphism data was obtained from Ensembl (http://www.ensembl.org, hg19 genome sequence) and dbSNP databases (http://www.ncbi.nlm.nih.gov/SNP). Pathogenicity of nonsynonymous SNPs was analyzed using standard parameters in Polyphen (http://genetics.bwh.harvard.edu/pph2/) and Sift (http://sift.bii.a-star.edu.sg/).

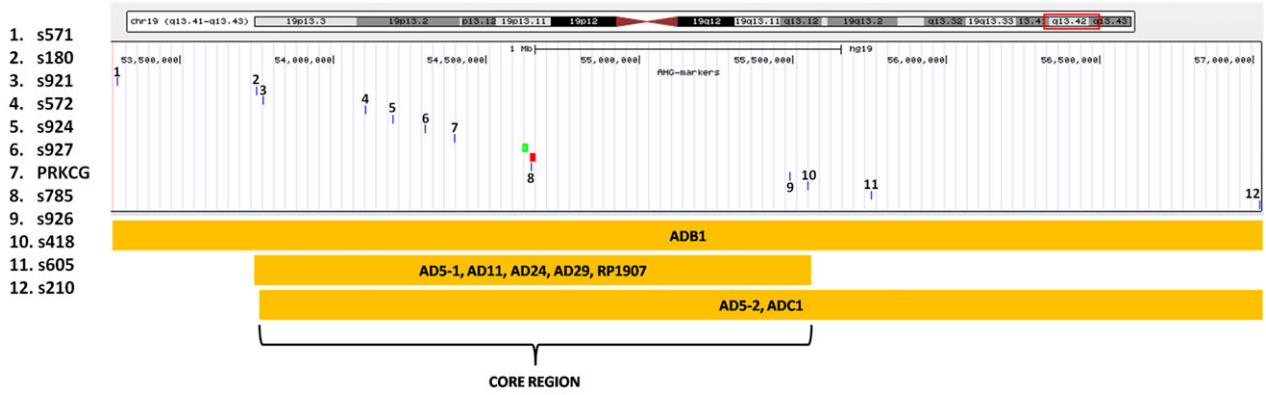

Figure 1 Marker map showing the relative position of markers used in linkage analysis (blue), *PRPF31* (green), and *CN0T3* (red). The regions covered in each family are shown by the orange boxes below, with the core region containing both genes of interest (D19s921-D19s418; chr19:53771240–55546128) being examined in all families.

Results

Microsatellite data were derived for six members of family ADB1 (Fig. S1) and this, together with previously derived data from other unrelated families, provided data for 53 individuals—both symptomatic and asymptomatic (Fig. 2). The inheritance pattern shows a very highly significant deviation from the null hypothesis ($\chi^2 = 21.5$; $P = 2.6 \times 10^{-7}$) (Table 1).

Discussion

The highly concordant—rather than random—inheritance of the wildtype allele in mutation-carrying sibships (Table 1) indicates that the factor controlling the clinical manifestations of the *PRPF31* mutation must be *cis*-acting. This is a necessary conclusion because, if it was *trans*-acting, the controlling allele could reside on either Chromosome 19 (or, indeed, any other chromosome) and thereby determine phenotype (Figs 3A and B): under these circumstances the inheritance of wildtype Chromosome 19 would be random and independent of phenotype—which is manifestly not the case (Table 1).

The simplest *cis*-acting factor would be one that directly affects the expression of the *PRPF31* wildtype allele (Fig. 3C). Alternatively, there might be a *cis*-acting factor that affects another gene lying immediately upstream to *PRPF31* and which cannot, therefore, be separated by recombination—an "indirect *cis*-acting" element.

CNOT3 and *PRPF31*: An Apparent Paradox?

The only known nonpenetrance factor affecting the RP11 phenotype is transcriptional repression of *PRPF31* by CNOT3, the latter being a *trans*-acting factor (Venturini et al., 2012). Furthermore, there is increased expression of both the mutant and wildtype *PRPF31* alleles prior to nonsense-mediated decay of the mutant transcripts (Rio Frio et al., 2008a), and this necessitates at least one nonpenetrance factor which is *trans*-acting—again, this observation being compatible with a *CNOT3* control mechanism. An apparent paradox appears to exist between this known, *trans*-acting control of *PRPF31* expression by CNOT3, and the results of our study that implicate a mechanism which is *cis*-acting. This paradox can, however, be resolved if a *trans*-acting factor lies in close proximity to its target gene—in this case, *PRPF31*—and is therefore not separated by meiotic recombination (Fig. 3D). Such a factor, although working through a *trans*-acting mechanism, would appear to be *cis*-acting because of its linkage and proximity to the target gene—a mechanism that might usefully be termed "linked *trans*-acting epistasis."

In the case of RP11, it is known that the *trans*-acting *CNOT3* gene is located only a short distance from *PRPF31* and the two genes are effectively linked during meiotic recombination. This linkage renders their epistatic interaction equivalent to a *cis*-acting mechanism (Fig. 3D) and we consider it likely that differential *CNOT3* expression is the *trans*-acting factor that modulates expression of both *PRPF31* alleles, but with degradation of the mutant PRPF31 mRNA (Rio Frio et al., 2008a).

A New Conjectural Model for "Linked *Trans*-Acting Epistatic" Inheritance

In the context of *CNOT3* control and *PRPF31* mutations, this novel "linked *trans*-acting epistatic" model of inheritance leads to some further theoretical consequences: First, it is known that lower expression of *CNOT3* leads to higher expression of *PRPF31*, and vice-versa (Venturini et al., 2012).

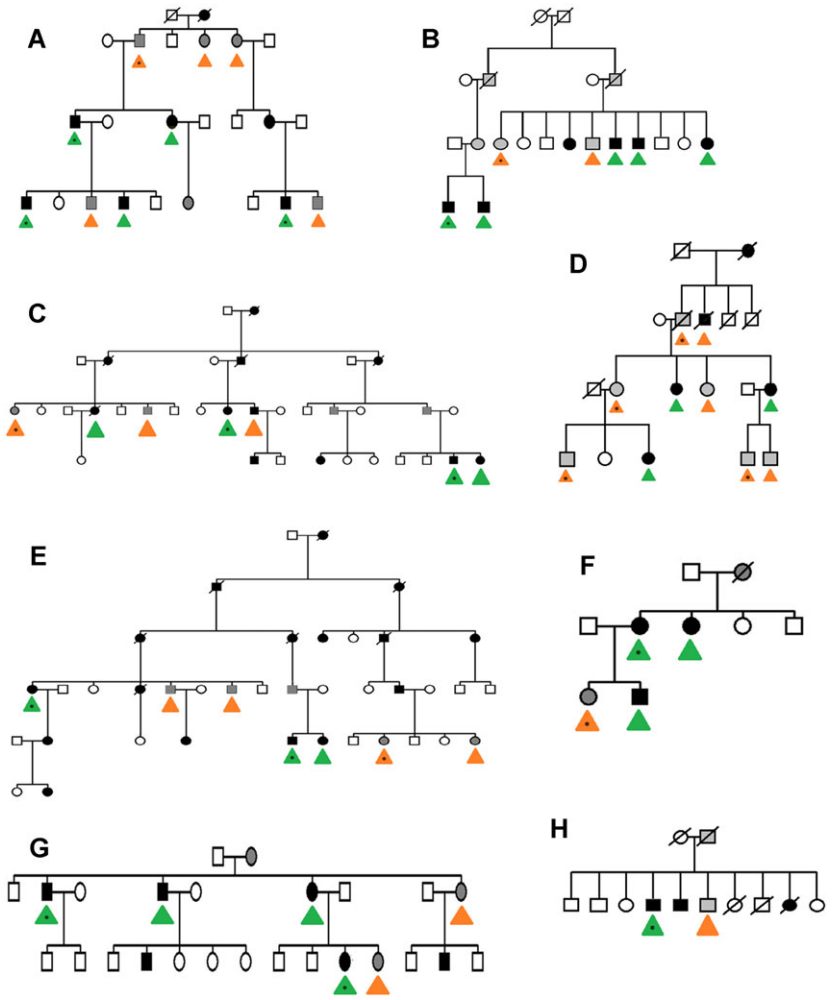

Figure 2 Inheritance of the wildtype allele of Chromosome 19 in seven families carrying a mutation in *PRPF31*. In each group of siblings, the oldest mutation-carrying sibling (asterisked) acts as reference wildtype and was denoted orange if symptomatic or green if asymptomatic. The other mutation-carrying siblings were then assigned a concordant color triangle if they inherited the same wildtype allele as the reference sibling, or a discordant color triangle if they inherited the different wildtype allele to the reference sibling. "A" represents family AD5–1; B = AD5–2; C = AD11; D = AD24; E = AD29; F = RP1907; G = ADB1; H = ADC1.

Table 1 The Nonreference Siblings were Counted and Classified as Asymptomatic or Symptomatic and Concordant or Nonconcordant—The Latter with Respect to the Reference Sibling (the Oldest Mutation Carrier). The Expected Values were Calculated Assuming Random Inheritance of Chromosome 19q—in which Case, there would be Equal Numbers of Concordant and Nonconcordant for both Asymptomatic and Symptomatic Individuals.

	Concordance of genotype with that of reference sibling			
	Observed concordance		Expected concordance	
	Concordant	Nonconcordant	Concordant	Nonconcordant
Symptomatic	2	16	9	9
Asymptomatic	14	0	7	7

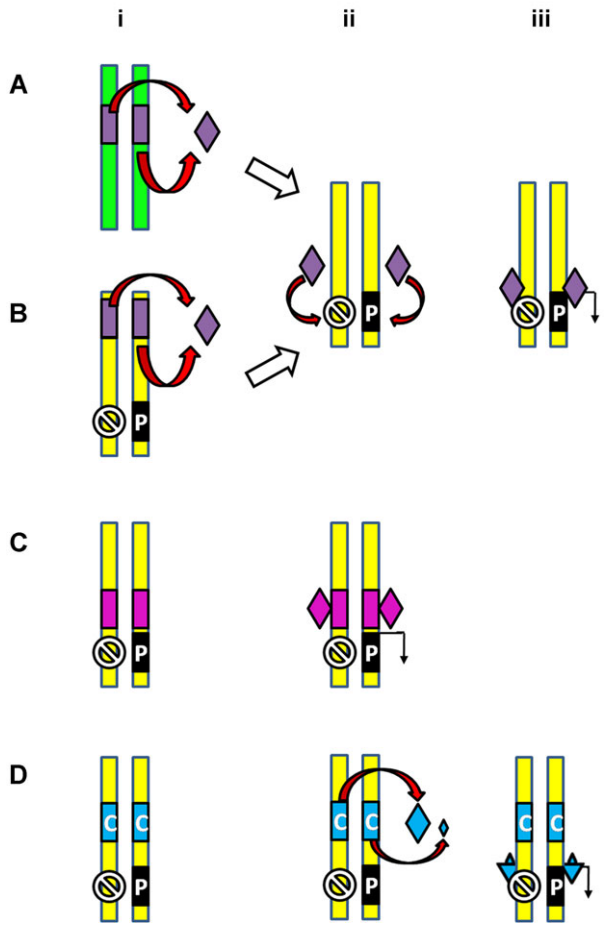

Figure 3 Four mechanisms for modulation of the expression of *PRPF31*. (A) Nonsyntenic modulation: there is a *trans*-acting factor (purple diamond) being transcribed from another chromosome (i), this binding to an element on Chromosome 19 (ii), and thereby modulating the expression of *PRPF31* (iii). This mechanism is not compatible with the observed nonrandom inheritance of wildtype alleles. (B) Distant modulation on Chromosome 19: a mechanism similar to (A) applies to a hypothetical *trans*-acting factor transcribed from a remote part of Chromosome 19. As the remote, controlling gene is liable to be separated from the *PRPF31* mutation during meiotic recombination, inheritance of the wildtype Chromosome 19 and the *trans*-acting factor are, effectively, independent. This mechanism is not compatible with the observed nonrandom inheritance of wildtype alleles. (C) "Direct *cis*-effect": there is a *cis*-element (pink) that lies in close proximity to *PRPF31* (i), this then being bound by a transcription factor (pink diamond), and the interaction directly affects *PRFP31* expression level (ii). This mechanism is compatible with the observed nonrandom inheritance of wildtype alleles. (D) "Linked *trans*-effect": there is a *cis*-element (turquoise) linked in close proximity to the *PRPF31* gene, such that the two are not separated by recombination (i). The *cis*-acting element causes differential expression of *CNOT3* (blue diamonds; larger size indicating transcription from a higher expressed allele) (ii), this thereby modulating the expression of both the mutant and wild-type *PRPF31* alleles (iii). This situation is compatible with the observed inheritance pattern of wildtype alleles, as long as the *cis*-element (turquoise) and *PRPF31* are not separated during meiotic recombination. The hypothetical *trans*-acting factor would bind and activate both *PRPF31* copies, but only the wildtype allele is functionally expressed—as *PRPF31* mutations generally result in null alleles (that is, large deletions or nonsense alleles that are immediately degraded; Rio Frio et al., 2008a).

In any mutation-carrying individual, there will be a higher or lower expressing *CNOT3* allele syntenic to the *PRPF31* mutation, and a higher or lower expressing one syntenic to the wildtype *PRPF31* (Fig. 4). In reality, as polygenic factors act to determine expression of the two genes, there will be a continuum of expression level at a population level. There will, however, be a threshold—above which the level is considered "high" and below which the level is considered "low." With this in mind, it is possible to construct a simplified model.

Linkage analysis has shown that the *CNOT3* and *PRPF31* loci are not separated by recombination, and so the mutation-carrying chromosome will always carry the same syntenic *CNOT3* allele; the *PRPF31* mutation will be linked either to a "lower expressivity" or to a "higher expressivity" *CNOT3* allele (Fig. 4). A necessary consequence is that, for any given sibship with two parents, individuals can only fall into one of two groupings—either "A" and "B," or else "C" and "D" (Fig. 4); for example, it is not possible for siblings to have genotypes "A" and "D." Further to this inference, the phenotypic difference between symptomatic and asymptomatic siblings can only be attributed to a difference in the *CNOT3* allele linked to the wildtype *PRPF31* allele.

Considering the four possible genotypes (Fig. 4), expression of *CNOT3* is the same for individuals "B" and "C" and, as *CNOT3* is *trans*-acting, this would result in similar *PRPF31* expression and, therefore, the same phenotype. It needs to be considered what the phenotype of "B + C" individuals is: asymptomatic or symptomatic? It is clear that "B + C" individuals cannot be symptomatic, as this would lead symptomatic "C + D" siblings pairs who inherit different wildtype alleles—which we have demonstrated does not occur. It must be the case, therefore, that "B + C" individuals are asymptomatic.

Further to the deduction of all affected families being in the "C+D" group, it is therefore necessary for symptomatic individuals to have two copies of a higher expressing *CNOT3* allele (that is, a homozygous recessive inheritance).

With this assumption in mind, it is possible to predict an approximate frequency of *CNOT3* alleles within the

Table 2 Ensembl and dbSNP Report Three Nonsynonymous SNPs as Rare Normal Variants within the Coding Region of the *PRPF31* Gene, these being Predicted to be Pathogenic by both Sift and Polyphen. This Raises the Issue that these Rare Changes might be Pathogenic on a Different Genetic Background.

SNP	Exon	Change	Sift	Polyphen	Heterozygote frequency
rs150280707	6	p.Val169Ile	0.1	0.946	<0.0001
rs119475042	7	p.Ala216Thr	0	1	<0.0001
rs151337240	13	p.Ala453Asp	0	0.999	0.001

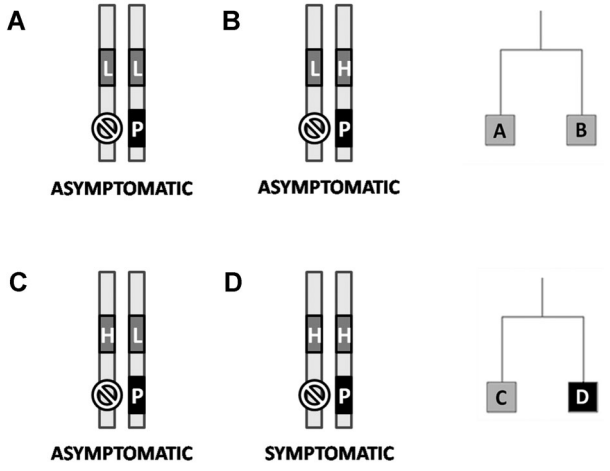

Figure 4 Combinations of *CNOT3* genotype in patients carrying the *PRPF31* mutation. The L indicates a low-expressing *CNOT3* allele—which, due to the repressive nature of the interaction, leads to high level of *PRPF31* expression. The H indicates a high-expressing *CNOT3,* which leads to lower level of *PRPF31*. Sibling pair "A + B" carries a *PRPF31* mutation with a syntenic low-expressing *CNOT3* (L), whereas sibling pair "C + D" carries a syntenic high-expressing *CNOT3* (H). Because of the intimate linkage of *CNOT3* and *PRPF31*, these alleles are effectively locked together during meiotic recombination—this dictating that all individuals from a given sibship will be either "A + B," or else "C + D." The level of CNOT3 protein in individuals "B" and "C" is identical, and they would therefore be expected to have the same asymptomatic phenotype. They cannot both be symptomatic, as the "C + D" sibling pair would be incompatible with the observed nonrandom inheritance of the wildtype allele. Individual "A" should also be unaffected, having the lowest levels of CNOT3 (with correspondingly highest level of PRPF31). The most important conclusion of this inheritance model is that the only symptomatic individual ("D") inherits two high-expressing *CNOT3* alleles—there is homozygous, recessive inheritance of the *CNOT3* trait.

population, given that approximately 30% of mutation carriers are asymptomatic. Using standard Hardy–Weinberg equilibrium, we can predict that 83.7% of *CNOT3* haplotypes lead to higher expressing alleles (which when inherited recessively lead to disease) and the remaining 16.3% of haplotypes lead to lower expressing alleles, with a level of *CNOT3* above our theoretical threshold. This is, of course, a gross oversimplification, as it assumes that *CNOT3* is the only factor affecting *PRPF31*.

A further necessary deduction from this hypothesis is that mutation-carrying families can occur with absolutely no clinically affected individuals, despite harboring *PRPF31* mutations (entirely "A and B" sibling pairs). As such families are clinically normal, they will never present to medical attention unless a meiotic recombination affecting the *CNOT3–PRPF31* interzone occurs on the mutation-carrying chromosome—this being a remotely improbable event given the tight linkage between *CNOT3* and *PRPF31*. To prove the existence of such "A and B" families would be arduous: RP affects 0.03% people, of which adRP accounts for 60%, and *PRPF31* mutations account for less than 10% of adRP cases—the population prevalence therefore being about 0.002%. To find a completely asymptomatic mutation-carrying ("A + B") family would require screening of a vast number of control individuals. It is possible, however, that some of the rare changes reported in the reference databases are mutations—but on a genetic background that masks the clinical phenotype: For example, there are three nonsynonymous coding region SNPs within the *PRPF31* coding sequence that both Sift and Polyphen predict to be deleterious (Table 2).

Conclusions

Coinheritance of a heterozygous *PRPF31* mutation and a homozygous higher expressivity variant of *CNOT3* appears to determine the clinical presentation of RP11, this being a classic example of epistasis, where a disease mutation is masked by genetic interaction with a second locus. In this case, a mutation in *PRPF31* is hypostatic to a trait acting on *CNOT3*, with the RP11 phenotype only being observed when there is homozygous (recessive) inheritance of the higher expressivity *CNOT3* ("symptomatic" or risk) allele.

Traditionally, genetic disease has been split into two groups—"Mendelian" and "complex"—but this is a gross oversimplification. *PRPF31*-associated adRP can usefully

be thought of as a "hybrid" genetic disease, with features of Mendelian and complex disorders: primarily, there is a *PRPF31* gene mutation (the monogenic "Mendelian" component)—and, superimposed on this, there is polygenic control of expression of the remaining, genetically active wildtype *PRPF31* gene—the "complex" component of the disorder.

Nonpenetrant traits have previously been largely regarded as monogenic, this greatly hindering research as most investigations have sought "all-or-nothing" phenomena. As shown in this study, it appears more appropriate to investigate phenotypic nonpenetrance as a polygenic trait, with interaction of major risk alleles (such as repression by *CNOT3*) and a number of minor alleles to determine the overall level of *PRPF31* expression and, consequentially, the clinical manifestation of the monogenic mutation. It should also be noted that given the continuous nature of *PRPF31* expression in the population, some mutation carriers will be "nearer" the threshold for normal retinal function and hence will have milder disease presentation. One might foresee the ability to quantify the odds ratios for such risk factors and—based upon the genome—produce a predictive model for development of the disease.

Although control of *CNOT3* expression has been discussed as if a single entity, it should be emphasized that this, too, is multifactorial with *cis*-acting factors on Chromosome 19q13 affecting *CNOT3* expression; such factors being within core promoter, intronic regulatory, or long-range regulatory elements. These factors will produce a haplotype that has a cumulative effect on *CNOT3*, seen as a continuous distribution of *CNOT3* expression level in the population.

Based on the evidence that approximately 30% of mutation carriers are asymptomatic, we predicted that approximately 16% of haplotypes result in *CNOT3* level that is lower than the theoretical threshold. It should be noted, however, that in some populations the frequency of asymptomatic mutation carriers is significantly lower than the average—for example, based on recent data in Chinese families, approximately 5% of mutation-carrying family members were asymptomatic (Xu et al., 2012; Yang et al., 2013). In these populations, the lower expressing *CNOT3* haplotypes would be very rare.

Several SNPs—such as rs4806718—lie close to, or within, the *CNOT3–PRPF31* region and correlate with the phenotype of some, but not all, sibling groups (unpublished data; Venturini et al., 2012); the importance of SNPs should, however, be viewed with caution until their functional effect has been demonstrated. Functional polymorphisms within the regulatory elements of both *PRPF31* and *CNOT3* might have a combined effect to determine the overall expression level of *PRPF31*. Likewise, *trans*-acting factors from other chromosomes—such as the eQTL described on Chromosome 14 (Rio Frio et al., 2008b)—might modulate *PRPF31* and *CNOT3* expression.

Identification of all polymorphisms with a role in the *CNOT3–PRPF31* regulatory network is daunting: It would be necessary to sequence the 19q13 region in a large cohort of control individuals with predetermined expression levels of *CNOT3* and *PRPF31*. Sequencing this region is, however, very complex as it is highly enriched with repetitive elements—especially Alu repeats, that may act as long-range regulators of gene expression (Tomilin, 1999). At present, high-repeat content presents an insurmountable barrier to sequencing, and currently available commercial sequencing technologies are not able to read through repeat elements of greater than 500 bp. After identification of SNPs, it would be necessary to statistically evaluate the individual risk contributed by each allele, followed by experimental validation of their functional effect on transcription—a mammoth task!

In summary, linkage data from seven unrelated families from different ethnic populations have unequivocally shown that inheritance of the wildtype Chromosome 19 is the major determinant for the *PRPF31* adRP mutation of RP11. When considered alongside recent work into *CNOT3* expression and function, it can be concluded that the RP11 phenotype is hypostatic to normal variant alleles of *CNOT3*, and that recessive coinheritance of the *CNOT3* "high-expressing" allele is one of the major determinants of the disease phenotype in *PRPF31* mutations—a classic example of epistasis affecting a Mendelian disorder.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Genotype data for family ADB1, covering a large region on Chromosome 19q13 (D19s571 – D19s210).

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