CNOT3 Is a Modifier of PRPF31 Mutations in Retinitis Pigmentosa with Incomplete Penetrance

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Abstract

Heterozygous mutations in the PRPF31 gene cause autosomal dominant retinitis pigmentosa (adRP), a hereditary disorder leading to progressive blindness. In some cases, such mutations display incomplete penetrance, implying that certain carriers develop retinal degeneration while others have no symptoms at all. Asymptomatic carriers are protected from the disease by a higher than average expression of the PRPF31 allele that is not mutated, mainly through the action of an unknown modifier gene mapping to chromosome 19q13.4. We investigated a large family with adRP segregating an 11-bp deletion in PRPF31. The analysis of cell lines derived from asymptomatic and affected individuals revealed that the expression of only one gene among a number of candidates within the 19q13.4 interval significantly correlated with that of PRPF31, both at the mRNA and protein levels, and according to an inverse relationship. This gene was CNOT3, encoding a subunit of the Ccr4-not transcription complex. In cultured cells, siRNA–mediated silencing of CNOT3 provoked an increase in PRPF31 expression, confirming a repressive nature of CNOT3 on PRPF31. Furthermore, chromatin immunoprecipitation revealed that CNOT3 directly binds to a specific PRPF31 promoter sequence, while next-generation sequencing of the CNOT3 genomic region indicated that its variable expression is associated with a common intronic SNP. In conclusion, we identify CNOT3 as the main modifier gene determining penetrance of PRPF31 mutations, via a mechanism of transcriptional repression. In asymptomatic carriers CNOT3 is expressed at low levels, allowing higher amounts of wild-type PRPF31 transcripts to be produced and preventing manifestation of retinal degeneration.

Introduction

The penetrance of a disease-causing mutation corresponds to the proportion of individuals who carry such variant and develop clinical symptoms. In the majority of Mendelian disorders penetrance is 100%, but incomplete penetrance is far from being uncommon [1]. Although in medical genetics penetrance is still largely uncharacterized at the molecular level, it is usually determined by genetic or epigenetic factors, and sometimes even by environmental modifiers [2].

Retinitis pigmentosa (RP) is a group of inherited degenerative diseases of the retina that cause the progressive death of photoreceptors, the neurons of the eye that are sensitive to light. Typically, patients affected by RP first suffer from night blindness, most often during adolescence. Rod and cone photoreceptor cells start to degenerate from the mid periphery to the far periphery and the center of the retina, resulting in the so-called tunnel vision. Later in life, central vision is also lost, leading to legal or complete blindness [3]. Clinically, RP is a highly-heterogeneous disease, reflecting not only genetic heterogeneity (mutations in different genes), but also inter-individual diversity (penetrance and expressivity) [4].

The PRPF31 gene encodes in humans a pre-mRNA processing factor. In autosomal dominant RP (adRP) due to mutations in PRPF31 penetrance of the disease can be incomplete. Specifically, in families with PRPF31 mutations it is not uncommon to observe the presence of asymptomatic individuals who have affected parents, affected children, or both [5–8]. Although they carry the same PRPF31 mutation as their affected relatives, asymptomatic subjects show no visual impairment, even at older ages, and normal to slightly reduced electroretinographic recordings [7]. PRPF31 mutations causing adRP are largely null alleles, such as deletions, nonsenses, or DNA changes leading to premature termination codons and to mRNA degradation [9–14]. Patients are therefore hemizygotes for PRPF31, suggesting that the molecular pathophysiology of the disease is due to the functional loss of one allele and to haploinsufficiency [10,12,15]. The ubiquitous expression of PRPF31 has allowed a number of functional studies to be performed in immortalized lymphoblastoid cell lines (LCLs) from patients and asymptomatic carriers of mutations [16–18]. In particular, it has been shown that penetrance of mutations is due to the differential expression of the PRPF31 allele that is not inactivated by mutations, in both symptomatic and asymptomatic individuals. Unlike affected persons, asymptomatic carriers naturally express high amounts of functional PRPF31 mRNA, a phenomenon that compensates for the mutation-induced loss of one allele and prevents manifestation of symptoms [16–18].
Author Summary

Retinitis pigmentosa (RP) is an inherited disorder of the retina that is caused by mutations in more than 50 genes. Dominant mutations in one of these, PRPF31, can be non-penetrant. That is, some carriers of mutations suffer from the disease while others do not display any symptoms. In these particular individuals, functional PRPF31 transcripts are expressed at higher levels compared to affected persons, thus compensating for the deleterious effects of the mutated allele. Up to now, the nature of such a stochastic and protective effect was unknown. In this work, we identify CNOT3 as the modifier gene responsible for penetrance of PRPF31 mutations. We show that CNOT3 is a negative regulator of PRPF31 expression and modulates PRPF31 transcription by directly binding to its promoter. In asymptomatic carriers of mutations, CNOT3 expression is lower, allowing higher amounts of PRPF31 to be produced and therefore inhibiting the development of symptoms. Finally, we find that a polymorphism within a CNOT3 intronic region is associated with the clinical manifestation of the disease.

This variable expression of PRPF31 seems to be present within the general population [16] and therefore asymptomatic carriers of mutations would be individuals that by chance are “high expressers”. Furthermore, protection from PRPF31 mutations (and therefore variable PRPF31 expression) is itself an inheritable character [16,19]. In an elegant meta-analytic study, McGee et al. [19] have shown that protective alleles, named isoalleles, are inherited by carriers of PRPF31 mutations from the parent who does not transmit the mutation (i.e. they are in trans with respect to the mutation). Furthermore, such isoalleles would be responsible for the majority of incomplete penetrance cases, and map to chromosome 19q13.4, in proximity to PRPF31 itself [19]. The same study also indicated that these isoalleles were not the only modulators of PRPF31 penetrance, since some individuals with discordant phenotypes carried an identical wild-type haplotype for the isoalleles on chromosome 19. Another genetic element potentially capable of influencing the penetrance of PRPF31 mutation was later mapped to chromosome 14q21–23 [16].

In this study, we search for and identify the major modifier gene responsible for penetrance of PRPF31 mutations, through the analysis of LCLs from a very large family with adRP due to a PRPF31 microdeletion [6,20].

Results

CNOT3 expression is inversely proportional to that of PRPF31 in asymptomatic and affected carriers of mutations

The region on chromosome 19q13.4 harboring the main modifier gene for PRPF31 penetrance was determined by McGee et al. to lie between microsatellite markers D19S572 and D19S926 [19]. This interval contains 118 genes, including 50 protein-coding genes, 50 miRNAs and 18 pseudogenes.

Based on data from lymphoblast studies describing the nature and the possible mechanism of action of the penetrance modifier gene [16–18], we selected protein-coding genes that were consistently expressed in LCLs, as detected by q-PCR (18 genes). We also excluded some of the genes that in this region belong to the leukocyte receptor cluster (LRC) and are implicated exclusively in leukocyte functions. We were left with 10 sequences, namely: NDUF3, TPPT, CNOT3, LENG1, MBOAT7, TSEN34, RPS9, LILRB3, IL7T, and XALP2. We then measured by q-PCR the mRNA expression levels of these genes in LCLs from 4 asymptomatic and 6 affected individuals from the RP856/AD5 family (Table S1 and Figure S1). All genes showed consistent expression across the family members. Of these, only CNOT3 showed a statistically significant difference in mRNA expression between the two groups of individuals (p<0.001) (Figure 1 and Figure S1). Unexpectedly, CNOT3 trend of expression was the opposite to that of PRPF31, as it showed lower expression in asymptomatic than in the affected carriers of PRPF31 mutations (Figure 1B). This phenomenon was particularly clear when expression of CNOT3 and PRPF31 were paired by cell lines and the relevant regression lines calculated (Figure 1C).

Assessment of CNOT3 protein by quantitative western blotting confirmed the differential expression detected by q-PCR (Figure 1D).

CNOT3 is a negative regulator of PRPF31 expression

CNOT3 belongs to the Ccr4-Not complex, a conserved multi-protein structure involved in the regulation of gene expression [21].

To investigate if CNOT3 could influence PRPF31 expression, we silenced its expression in ARPE-19 cell lines, by using two different siRNA sequences. Suppression of CNOT3 resulted in significant increase of PRPF31 mRNA and protein (p<0.001, Figure 2). This effect was very specific, as no influence was observed in negative controls and in TPPT expression, a neighboring gene sharing part of the promoter with PRPF31 (Figure S2).

CNOT3-dependent modulation of PRPF31 expression is achieved at the transcriptional level

CNOT3 can negatively regulate transcription by either directly binding to the promoter of target genes or by affecting their mRNA rate of degradation [22,23].

To understand which could be the mechanism through which CNOT3 modulates PRPF31 expression, we incubated LCLs from two asymptomatic-affected pairs with Actinomycin D, a drug that inhibits de novo transcription, and then measured the rate of decay of PRPF31 mRNA. No statistically significant difference was observed between the asymptomatic and affected individuals (Figure S3), suggesting that the modulation of PRPF31 expression happens most probably at the transcriptional level.

CNOT3 binds directly to the PRPF31 promoter

To test this hypothesis, we performed a Chromatin Immuno-Precipitation (ChIP) assay in LCLs from 3 healthy individuals, using an anti-CNOT3 antibody and serum IgG as a negative control. To confirm that CNOT3 enrichment of a target DNA region was due to a specific immunoprecipitation rather than to a random precipitation of DNA, we designed primers targeting genomic regions that were not supposed to be bound by CNOT3. Primers targeting CNOT3 promoter were used as a positive control, since it has been previously shown that CNOT3 self-regulates its expression by binding to its own promoter [23]. Both qualitative and quantitative PCR showed a statistically significant enrichment in PRPF31 promoter sequences in DNA that was immunoprecipitated by the CNOT3 antibody, compared to that exposed to serum IgG (Figure 3A, 3B).

CNOT3 rs4806718 alleles are associated with the clinical manifestation of the disease

In order to identify genetic markers that could be associated with variable expression of CNOT3 and therefore with penetrance
of PRPF31 mutations, we sequenced the entire CNOT3 genomic region by next-generation sequencing (NGS) in one asymptomatic-affected sibling pair. We identified five polymorphic variants (rs36643, rs56079424, rs36661, rs4806718, rs1055234) that differed between the two subjects. These five variants were subsequently analyzed in a second asymptomatic-affected sibling pair from the same pedigree, showing that only alleles of rs4806718, lying in intron 17 of CNOT3, segregated with the trait.

Figure 1. CNOT3 shows an opposite trend of expression with respect to that of PRPF31 between the asymptomatic (AS) and affected (AF) individuals of the AD5 family. (A) PRPF31 mRNA expression normalized to the housekeeping gene GAPDH. Error bars refer to the standard deviation of the mean for 5 independent experiments for each group. (B) CNOT3 mRNA expression from the same 5 experiments used to generate PRPF31 data. **, p < 0.01. (C) Linear regression analysis of PRPF31 and CNOT3 mRNA expression, which shows an inverse trend of the two genes in each cell line. Circles, asymptomatic subjects; triangles, affected individuals; open symbols, CNOT3 expression; filled symbols, PRPF31 expression. Data having the same value for the x axis have been obtained from the same individual. (D) Quantification of CNOT3 protein abundance relative to β-actin from 3 independent SDS-PAGE gels, after simultaneous detection of the two proteins by quantitative LI-COR western blot.

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Figure 2. CNOT3 silencing stimulates PRPF31 expression in ARPE-19 cells. (A) CNOT3 mRNA depletion by 2 different siRNA sequences and its effect on PRPF31 mRNA expression (B). ***, p < 0.001. (C) Representative western blot of CNOT3 silencing and effect on PRPF31 protein expression. siRNA_1 and siRNA_2, different CNOT3-specific siRNA sequences; Control, treatment with transfection reagent with no siRNA; Mock, treatment with transfection reagent and scrambled siRNA.

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This SNP was then sequenced in a total of 38 asymptomatic and affected individuals from the RP856/AD5 family, as well as from an unrelated family for which the modifier gene for PRPF31 penetrance was also found to be linked to chromosome 19q13.4 [24] (Figure 4). Association between the C allele of rs4806718 with the affected status and the T allele with the asymptomatic status was moderately significant \(p = 0.04\), by Fisher exact test.

Discussion

Despite penetrance being an old concept in genetics, little is known about its molecular causes, especially in inherited human diseases. Notable positive examples include dominant erythropoietic protoporphyria, caused by mutations in the FECH gene, and dominant elliptocytosis, due to mutations in SPTA1. In these disorders, an imbalance of expression between the wild-type and the mutated alleles causes the manifestation of the symptoms [25–27]. Similar mechanisms determine penetrance of PRPF31 mutations, since asymptomatic carriers are individuals who display increased levels of wild-type mRNA alleles, which in turn compensate for the deficiency caused by the mutation [16–18]. However, unlike erythropoietic protoporphyria and elliptocytosis, in PRPF31-linked adRP the molecular causes of such beneficial hyper-expression have remained, up to now, unexplained. Previous mapping studies have shown that the penetrance and expression of PRPF31 is influenced by at least two loci: one, likely having a major effect, lies within the same chromosomal region as

![Figure 3. CNOT3 binds to the PRPF31 promoter in cells. (A) CNOT3 ChIP-PCRs on different target sequences. Enrichment is visible only for PRPF31 promoter and CNOT3 promoter (positive control); DHFR 3' UTR, PTEN exon 8, and GAPDH promoter sequences are all negative controls. (B) CNOT3 ChIP-q-PCR on PRPF31 promoter sequence. Error bars indicate the standard deviation of the mean for three independent ChIP-qPCR experiments. Serum IgG is used as IP negative control. ***, p<0.001. doi:10.1371/journal.pgen.1003040.g003](#)

![Figure 4. Analysis of rs4806718 alleles in two unrelated pedigrees. (A) Family RP856/AD5. The individuals initially tested with NGS are marked with a star. The individuals marked with a triangle belong to a sibship pair, which was previously shown by McGee et al. to have the same isoallele haplotype but different phenotypes. (B) Family ADB1, a Bulgarian gypsy family carrying a heterozygous splice site mutation in PRPF31 (NM_015629.3:c.527+1G>T, or IVS6+1G>T). In both pedigrees carriers of mutations are either in black (affected individuals) or in grey (asymptomatic individuals). doi:10.1371/journal.pgen.1003040.g004](#)
**CNOT3 Modulates the Penetrance of PRPF31 Mutations**

**Materials and Methods**

**Patients and cell lines**

This study involved 10 individuals from the British family RP856/AD5, segregating an 11-bp deletion in exon 11 of PRPF31 (c.1115_1125del) [6,20]. Our research has been conducted in accordance with the tenets of the Declaration of Helsinki and has been approved by the IRBs of our Institutions. Lymphoblastoid cell lines derived from peripheral blood leukocytes of each individual were either obtained from the Coriell Cell Repositories or through the immortalization of peripheral blood leukocytes. Cells were grown and maintained as previously described [18].

The human retinal pigment epithelial cell line ARPE-19 (kindly provided by Dr. Yvan Arsenijevic) was grown and maintained at 37°C with 5% CO₂ in N1 medium (DMEM/F12 complemented with 2.5 mM L-glutamine, 56 mM NaHCO₃, and 10% fetal bovine serum).

**RNA extraction and cDNA synthesis**

Lymphoblasts were harvested during their exponential growth phase (500,000–1,000,000 cells/ml) and RNA was isolated from 10⁷ cells using the Qiagen RNeasy Mini Kit, following the manufacturer's instructions. The only modification to the protocol concerned the DNase treatment, since we used double the amount.
of enzyme compared to the suggested quantity. RNA concentration was measured with the Dropsonde 96 spectrophotometer (ThermoScientific). cDNA synthesis was carried out as previously described [10].

q–PCR primer design and optimization
Most of the primer sequences used in this study were annotated in the qPrimerDepot database (http://primerdepot.nci.nih.gov/). These sequences are specifically designed to span exon-exon junctions, thus avoiding genomic DNA to be amplified during q-PCR. To design other primer sequences, which were not present in the qPrimerDepot database, we used the Primer Blast tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To validate each primer pair for q-PCR we first optimized the primer amounts (50–200 nM), and then loaded 10 μl of the q-PCR product obtained on a 1% agarose gel, in order to check the specificity of the amplification product. Finally, a standard curve using a control cDNA template was used to test each primer pair’s efficiency. We considered as acceptable ranges of efficiency between 90 and 110%, corresponding to standard curve slopes between 3.6 and 3.1. All primer pairs used for this study are listed in Table S2. For GapDH and PRPF31 amplification we used primers and probes previously described [16].

Real-time quantitative PCR
All genes but PRPF31 and GapDH were amplified with the Sybr Green PCR Master Mix (Applied Biosystems). q-PCR reactions were performed as published [16]. After having assessed that PCR efficiencies for all genes were comparable, mRNA expression of each of them was normalized with respect to GapDH, using the ∆∆Ct method.

Protein extraction
Total protein was extracted from lymphoblastoid cell lines in RIPA buffer as reported before [10]. ARPE-19 whole cell lysate was obtained by scraping the cells into 150 μl of lysis buffer (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% glycerol, 2 mM EDTA, 1% TritonX-100) complemented with protease and phosphatase inhibitors, and incubated on ice for 15 minutes followed by a centrifugation at 14,000 rpm for 30 minutes at 4°C. Proteins concentration was measured with the BCA protein assay kit (Pierce), using BSA to generate a standard curve.

Western blot
Anti-PRPF31 antibody was raised in rabbit as previously described [10]. Rabbit anti-CNOT3 antibody was purchased by Bethyl Laboratories. This targets residues 525 to 575 of the human CNOT3 protein (NP_055331.1), allowing detection of a 117-kDa protein. Mouse anti-β-actin antibody (Sigma) was used as a loading control.

Equal amounts of proteins were loaded and run on an 8% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and blocked in 5% milk overnight at 4°C or alternatively for 1 hour at room temperature. The incubation of all primary antibodies was performed for 1 hour at room temperature using the following dilutions: anti-PRPF31 (1:500), anti-CNOT3 (1:2,000), and anti-β-ACTIN (1:2,500). The membrane was washed 3 times with 0.5% Tween-20 in TBS. Rabbit and mouse HRP-conjugated secondary antibodies were diluted 1:1,000 in 2% milk and incubated for 1 hour at room temperature, Bands were detected using enhanced chemiluminescence (Pierce).

Signal detection via the Odyssey infrared imaging system (LI-COR) was performed by using fluorescently-labeled secondary antibodies provided by LI-COR, diluted 1:5,000 in 0.5% milk and incubated in the dark, for 1 hour at room temperature. The membrane was then washed twice with 0.05% Tween-20 in TBS and once in PBS to remove residual Tween-20 prior to the laser scanning.

In vitro silencing experiments
We used two different siRNA sequences targeting CNOT3 (QIAGEN, FlexiTube siRNA, Hs_CNOT3_5 and Hs_CNOT3_8, 1 nmol) and a negative control siRNA for human genes (Santa Cruz Biotechnology). One day before transfection ARPE-19 cells were seeded at a concentration of 2×10^5 cells/well in a 6 well-plate, and transfection was achieved by using 5 μl Lipofectamine (Invitrogen) and 50 pmol siRNA. RNA was extracted 48 hrs after transfection.

Actinomycin D treatment of cells
Lymphoblasts grown at a concentration of ~8 million cells in a T75 flask were treated with Actinomycin D (5 μg/ml in DMISO (Sigma) by adding it directly to the medium. Cell pellets were collected at seven different time points (0–24 hrs) and total RNA was extracted and analyzed by q-PCR.

Chromatin immunoprecipitation (ChiP)
Three control lymphoblastoid cells from the Centre d’Etude du Polymorphisme Humain (CEPH) were grown to have 10^7 cells per ChiP experiment. DNA and proteins were cross-linked by adding 1% formaldehyde directly to the medium and by incubating the cells on a rotating hybridization oven at 37°C for 10 minutes. To quench cross-linking, we then added 125 mM glycine and incubated the cells at 37°C for 5 minutes. Cells were pelleted by centrifugation (800 g for 5 minutes at 4°C) and washed twice with cold PBS, supplemented with protease inhibitors. Optimization of the chromatin shearing was performed by using a Covaris sonicator, to obtain on average cross-linked DNA fragments of 150–400 bp. ChiP was performed using buffers provided with the Ep-iT Chromatin Immunoprecipitation kit (Bio-AAA). Immunoprecipitation was performed using three different antibodies: anti-CNOT3, anti-pol2 (Bio-AAA) as a positive control for IP, and serum IgG (Santa Cruz Biotechnology) as a negative control for IP. Antibody-protein-DNA complexes were collected on protein A agarose beads (2 hrs, 4°C), then washed with the low salt buffer, high salt buffer, LiCl buffer, and TE buffer (pH 8.0) provided in the kit to remove non-specific binding. Complexes were eluted from the beads by using the elution buffer (0.1 mM NaHCO3 and 1% SDS) in an orbital shaker. Cross-links were removed by an overnight incubation at 65°C. Ribonuclease and proteinase K digestion were added to remove specific contaminants, before the eluted DNA was extracted once in 25:24:1 phenol-chloroform-isooamyl alcohol and once in 24:1 chloroform-isooamyl alcohol. DNA was ethanol precipitated, washed in 70% ethanol, and finally eluted in TE.

ChiP-PCR was performed using the GoTaq DNA Polymerase (Promega) and 0.5 μl of the ChiP DNA, by using standard cycling conditions and primers described in Table S3. GapDH primer sequences are the ones provided by Millipore for the EZ-ChiP kit, while primers for DHFR have been previously described [38].

Two microliters of ChiP DNA were also amplified by q-PCR using Sybr Green PCR Master Mix (Applied Biosystems) and the PRPF31 promoter primer pair (Table S3).

Ultra-high-throughput sequencing
CNOT3 genomic region was amplified by 3 overlapping long-range PCRs (Table S4), for a total length of 34 Kb. PCR was
performed in 20 μl using TaKaRa LA Taq and GC buffer I (Takara Bio Inc.). Final primers concentration was 1 μM, and 200 ng of genomic DNA were used as template. PCR amplification conditions were: an initial step at 94°C for 1 minute, 30 cycles of denaturation at 96°C for 5 seconds and annealing/extension at 68°C for 15 minutes, and a final extension step at 72°C for 10 minutes. Long-range PCR products were sequenced with an Illumina HiSeq 2000 machine, to obtain coverage values in the range of thousands of reads. Mapping of the reads and variant detection was performed by using the CLCbio Genomics Workbench software.

Statistical analysis

Differences of gene expression between asymptomatic and affected individuals were tested by t-test, and likelihood computed by 100 Monte Carlo label-swapping simulations per each gene. One-way ANOVA followed by Bonferroni’s multiple comparison tests was used to analyze the effect of CNOT3 silencing on the expression of the target genes. The enrichment of PRPF31 promoter sequence after CNOT3 immunoprecipitation compared to the serum IgG was evaluated by using the Mann Whitney non-parametric statistical hypothesis test.

In figures, p<0.05 is indicated by one star, p<0.01 by 2 stars, and p<0.001 by 3 stars.

Supporting Information

Figure S1 Gene expression analysis of candidate genes in LCLs derived from asymptomatic (AS) and affected (AF) carriers of mutations. mRNA expression of each gene is normalized to the housekeeping gene GAPDH. Error bars refer to the standard deviation of the mean for each group.

Figure S2 Effect of CNOT3 silencing on the mRNA expression of two housekeeping genes and TFTP, in ARPE-19 cells. The data presented here are from the same experiments shown in Figure 2. Depletion of CNOT3 has no effects on the mRNA expression of these control genes. Mock, scrambled siRNA sequence; siRNA_1 and siRNA_2, sequences specific for CNOT3; Control, cells treated with no siRNA. Error bars refer to the standard deviation of the mean for three independent experiments.

Figure S3 PRPF31 mRNA decay in LCLs from asymptomatic and affected carriers of mutations, following treatment with actinomycin D. mRNA half-life is similar in both groups. Error bars refer to the standard deviation of the mean at different time points for at least three independent experiments.

Table S1 Lymphoblastoid cell lines from the RP856/AD5 family used in this work.

Table S2 Primers for q-PCR amplification. Annealing temperature for all primers is 60°C.

Table S3 Primers for ChIP-PCR.

Table S4 Primers for CNOT3 long-range PCR amplification.

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Author Contributions

Conceived and designed the experiments: GV AMR AZS CR. Performed the experiments: GV AMR AZS CR. Analyzed the data: GV CR. Contributed reagents/materials/analysis tools: AMR AZS SSB. Wrote the paper: GV CR.

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(PDF)

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