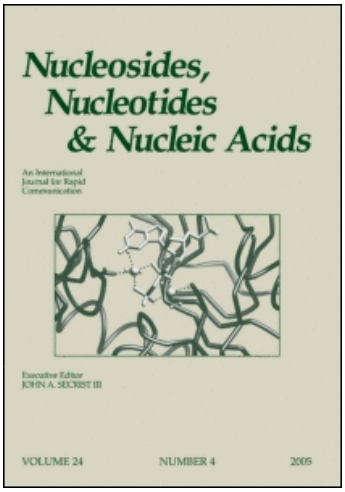

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IMP Dehydrogenase-Linked Retinitis Pigmentosa

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IMP DEHYDROGENASE-LINKED RETINITIS PIGMENTOSA

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□ *Many retinal diseases are caused by mutations in photoreceptor-specific proteins. However, retinal disease can also result from mutations in widely expressed proteins. One such protein is inosine monophosphate dehydrogenase type 1 (IMPDH1), which catalyzes a key step in guanine nucleotide biosynthesis and also binds single-stranded nucleic acids. The pathogenic IMPDH1 mutations are in or near the CBS domains and do not affect enzymatic activity. However, these mutations do decrease the affinity and specificity of single-stranded nucleic acid binding. These observations suggest that IMPDH1 has a previously unappreciated role in RNA metabolism that is crucial for photoreceptor function.*

Keywords CBS domains; Leber congenital amaurosis; IMPDH1; moonlighting enzymes; Bateman domains

Retinitis pigmentosa (RP) is the most prevalent hereditary retinopathy, affecting approximately 1 in 4,000 people. Autosomal dominant, recessive, X-linked, mitochondrial and digenic forms exist.[1] While symptoms can vary widely even among individuals carrying the same mutation, the apoptotic loss of photoreceptor cells usually causes blindness by middle age. No treatments are currently available for RP, and the prospects for treatment are poor due to the limited understanding of the molecular mechanisms of this disease. Many RP-associated genes encode proteins that are directly involved in visual transduction (http://www.sph.uth.tmc.edu/retnet/). It is reasonable to expect that alterations in these proteins would compromise photoreceptors. In contrast, several other RP-associated genes are widely expressed.[1] The photoreceptor-specific effects of these mutations are perplexing and the pathophysiological mechanisms are not understood.

One of the most curious genes in this group is RP10, which encodes the enzyme inosine monophosphate dehydrogenase type 1 (IMPDH1). Mutations in RP10 account for 2–3% of autosomal dominant RP (adRP).[2–4] IMPDH catalyzes a key step in guanine nucleotide biosynthesis and is an important target for immunosuppressive, antiviral and cancer chemotherapy

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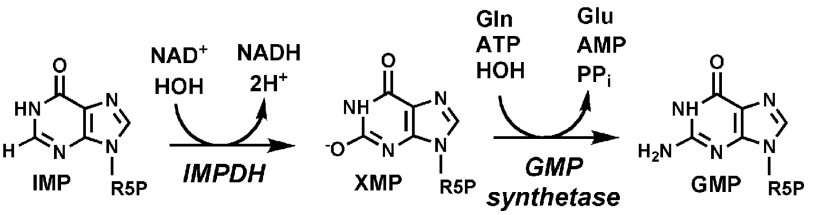
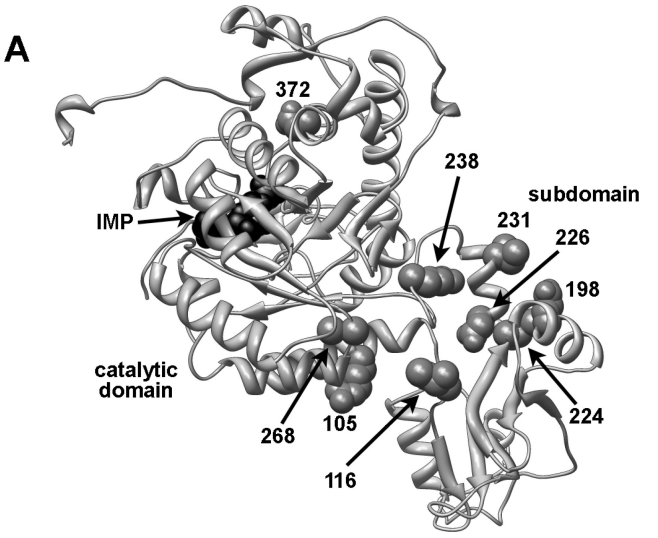


(Scheme 1).[5] Mammals contain two IMPDH isozymes, IMPDH1 and IMPDH2. While most tissues express both isozymes, only IMPDH1 expression has been observed in photoreceptors.[6–8] At least three IMPDH1 variants (Arg224Pro, Asp226Asn, and Arg231Pro) cause adRP and four others are likely to be pathogenic (Thr116Met, Arg238Glu, Val268Ile, and His372Pro).[2–4,9] Recent work from the Daiger laboratory has identified two additional mutations (Arg105Trp and Asn198Lys) that are associated with a more severe hereditary retinopathy, Leber congenital amaurosis (LCA).[10] It is likely that more pathogenic IMPDH1 mutations will be discovered as the search for hereditary retinopathy genes continues. Interestingly, no null alleles have been discovered,[4] and mice heterozygous with an IMPDH1 knockout display no phenotype, while homozygous knockout mice have only mild retinopathy.[6,11] In addition, IMPDH inhibitors are widely used in immunosuppressive chemotherapy, yet side effects involving impaired vision have not been reported.[12] Therefore, while guanine nucleotides are critical components of photoreceptor signaling, it is unlikely that IMPDH1-mediated adRP results from the loss of enzymatic activity and the consequential depletion of the guanine nucleotide pool.

The structure of IMPDH provides few clues into the pathophysiological mechanism of IMPDH1-associated adRP/LCA. IMPDH is a homotetrameric protein. Each monomer contains a catalytic domain with an α/β barrel fold and a subdomain consisting of two cystathionine β-synthase (CBS) domains (Figure 1A). The junction between the catalytic domain and the CBS domains is flexible and different domain orientations are observed in various crystal structures.[13] The adRP/LCA-associated mutations are found in or near the CBS domains (Figure 1A). The CBS domains are not required for enzymatic activity, so it would be surprising if any of these mutations altered the catalytic properties of IMPDH1.[14,15] Our work, and the work of others, confirms that these mutations do not affect the enzymatic activity of IMPDH1.[10,11,16] However, the adRP-causing mutations are well positioned to alter the function of the CBS domains.

What do the CBS domains do? CBS domains are also found in chloride channels, ABC transporters and AMP-activated protein kinases in addition to cystathionine β-synthase and IMPDH; mutations within the CBS domains of these proteins lead to a variety of other hereditary diseases.[17,18] Hardie and colleagues have proposed that CBS domains are AMP/ATP/AdoMet



B

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AAKG-1   IPTSSKLVVFDTSLQVKKAFFALVTN....GVRAAPLWDSK.....KQSFVGMLTITDFINILH
AAKG-3   IGTYANIAMVRTTTPVYVALGIFVQH....RVSALPVVDEK......GRVVDIYSKFDVINLAA
AAKG-4   SHYFEGVLKCYLHETLETIINRLVEA....EVHRLVVVDEN......DVVKGIVSLSDILQALV
IMD1-1   QGFITDPVVLSPSHTVGDVLEAKMRH....GFSGIPITETGTM...GSKLVGIVTSRDIDFLAE
CLC6-2   PYMNPSPFTVSPNTHVSQVFNLFRTM....GLRHLPVVNAV......GEIVGIITRHNLTYEFL
AAKG-2   QDSFKPLVCISPNASLFDAVSSLIRN....KIHRLPVIDPE.....SGNTLYILTHKRILKFLK
IMD1-2   MTPRIELVVAPAGVTLKEANEILQRS....KKGKLPIVNDC......DELVAIIARTDLKKNRD
CBS      ELGLSAPLTVLPTITCGHTIEILREK....GFDQAPVVDEA......GVILGMVTLGNMLSSLL
CLC6-1   DIMEPNLTYVYPHTRIQSLVSILRTT....VHHAFPVVTENRGN.EKEFMKGNQLISNNIKFKK
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FIGURE 1 Structure of IMPDH and CBS domains. A) A monomer of IMPDH from *Streptococcus pyogenes* is shown because it is the only IMPDH structure where the CBS domains are completely structured (PDB accession number 1ZFJ.[52]). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).[53] B) Alignment of CBS domains from various proteins. AAKG-1, human AMP-activated protein kinase gamma subunit, residues 42–96; AAKG-2, residues 123–177; AAKG-3, residues 198–251; AAKG-4, residues 270–323. IMD1-1, human IMPDH1, residues 112–168; IMD1-2, residues 179–232. CLC6-1, human chloride channel 6, residues 603–661; CLC6-2, residues 805–858. CBS, human cystathionine beta synthase residues 415–468. From Alex Bateman's CBS domain webpage: http://www.sanger.ac.uk/Users/agb/CBS/CBS.html.

binding modules,[19] but the persuasiveness of the evidence varies considerably for each protein. In the case of IMPDH, the CBS domains are reported to bind ATP and stimulate catalytic activity,[19] but we and others have failed to observe this phenomenon.[16,20,21] Moreover, an apparent "stimulation" of IMPDH activity by various nucleotides has previously been traced to the presence of contaminating nucleotidases.[21] Therefore, ATP regulation seems likely to result from the incomplete purification of the recombinant protein. Further, we note that the sequence identity of CBS domains from different proteins is essentially nonexistent (Figure 1B); therefore, while the CBS fold is conserved, the function has probably diverged.

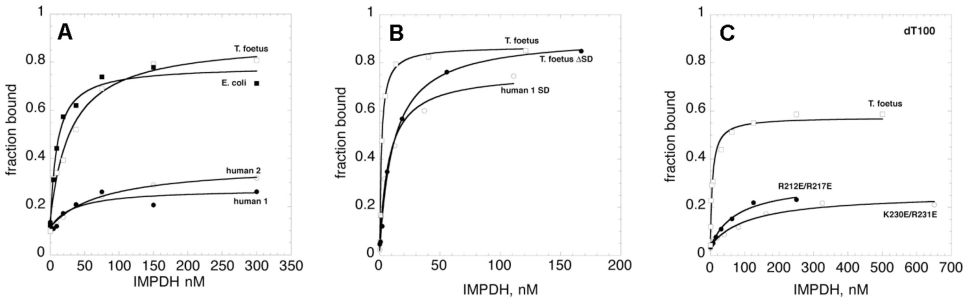

FIGURE 2 IMPDH binds ssDNA in a filter binding assay. 5′-[^{32}P]-labeled ssDNA was incubated with varying concentrations of IMPDH from different organisms (IMPDH concentration is given in tetramers). The fraction of bound oligonulcotide was determined from the ratio of the protein-associated radioactivity bound to the nitrocellulose filter and free radioactivity which binds to the Hybond filter. The lines represent the best fits of the data to a simple binding model. A) A random pool of ssDNA (0.2 nM, sequence: GGGAATGGATCCACATCTACGA-ATTC-N_{30}-TTCACTGCAGACTTGACGAAGCTT); human type I IMPDH (closed circles); human type II IMPDH (open circles); *T. foetus* IMPDH (open squares); and *E. coli* IMPDH (closed squares). B) A random pool of ssDNA as above (2 nM). Wildtype *T. foetus* IMPDH (open squares); subdomain deleted variant, *Tf*Δ*SD* (closed circles); and isolated human type I IMPDH subdomain (open circles). C) This experiment used dT_{100} (4 nM). Wildtype *T. foetus* IMPDH (open squares); R212E/R217E (closed circles); and K230E/R231E (open circles). Reproduced with permission from J. E. McLean et al. (2004) *Biochemical Journal*, vol. 379, pp. 243–251. © The Biochemical Society.

We have discovered that IMPDH binds nucleic acids with nanomolar affinity while selecting DNA aptamers for another project.[22] Both human isozymes, *Escherichia coli* and *Tritrichomonas foetus* IMPDHs all bind to a significant fraction of a random pool of single stranded DNA oligonucleotides in filter binding assays, which suggests that nucleic acid binding is a general property of IMPDH (Figure 2A). We have characterized the *T. foetus* enzyme in detail.[22] *Tf* IMPDH binds poly-dT, poly-U, poly-A and oligo-dT_{60} with high affinity, but little binding is observed to oligo-dA_{60}, oligo-dG_{60}, oligo-dC_{60}, and poly-C (where the subscript denotes the number of nucleotides in the oligonucleotide). The apparent preference for dT_{60} probably reflects the fact that poly-dT is less structured than the other homopolymers. Heparin and double stranded DNA do not bind to *Tf* IMPDH. The nucleic acid binding site appears to span ~100 nucleotides; oligo-dT_{100} interacts with all four subunits of the tetramer. While no binding is observed to oligonucleotides containing less than 20 nucleotides, we cannot rule out the existence of shorter oligonucleotides that can bind to IMPDH via a high affinity interaction with a single subunit.

The CBS domains mediate this interaction with nucleic acids.[22] IMP does not compete with oligo-dT_{100}, and nucleic acids do not inhibit IMPDH activity, which indicates that nucleic acids do not bind to the active site. Deletion of the CBS domains decreases the affinity of nucleic acids (Figure 2B). In addition, the isolated CBS domains bind nucleic acids. Lastly, substitution of Arg/Lys residues with Glu in the CBS domains has no effect on enzymatic

TABLE 1 Characterization of IMPDH1 mutations

Mutant	Disease/pathogenecity	K_d nM	Random oligo pool #1 Maximum % bound
Wildtype	n.a.	$6 \pm 2^{a,b}$	4.0 ± 0.2^{a}; 8.0 ± 0.4^{b}
R105W	LCA/likely[10]	$>50^{b}$	$>20^{b}$
T116M	adRP/likely[10]	110 ± 15^{b}	16 ± 2^{b}
N198K	LCA/pathogenic[10]	$> 73^{b}$	$>25^{b}$
R224P	adRP/pathogenic[2]	43 ± 5^{a}	7.0 ± 0.3^{a}
D226N	adRP/pathogenic[3]	200 ± 70^{a}	70 ± 20^{a}
R231P	adRP/pathogenic[9]	n.d.	n.d.
K238E	adRP/likely[4]	n.d.	n.d.
V268I	adRP/likely[3]	300 ± 100^{a}	50 ± 10^{a}
A285T	None[3]	3 ± 1^{a}	4.0 ± 0.3^{a}
H296R	None[4]	n.d.	n.d.
G324D	None[10]	6 ± 1^{b}	8.0 ± 0.3^{b}
H372P	adRP/likely[10]	12 ± 1^{b}	30 ± 1^{b}

[a]Random oligonucleotide pool #1.[16]
[b]Random oligonucleotide pool #2.[10]
n.a., not applicable; n.d., no data.

activity but decreases nucleic acid binding (Figure 2C). These experiments demonstrate that the CBS domains are involved in nucleic acid binding.

We have characterized the seven RP/LCA-associated variants of IMPDH1 and two of the nonpathogenic variants in vitro and in cell culture with the aim of identifying the functional property that correlates with disease.[8,16] These proteins were expressed in *E. coli* and purified to homogeneity. As expected, and in agreement with others, these mutations do not affect enzymatic activity.[10,11,16] Another laboratory has reported that the adRP-causing mutations induce the aggregation of IMPDH1.[11] While IMPDH1 does have an annoying tendency to aggregate, in our hands the adRP-causing variants are no more aggregation-prone than the wildtype enzyme. We believe that the reported aggregation derives from protein over-expression aggravated by the presence of His-tags. The Arg224Pro and Val68Ile mutations increase the proportion of IMPDH1 in the nucleus, but this effect is not observed with Asp226Asn, and therefore is unlikely to be involved in pathogenesis. In contrast, all of the pathogenic mutations perturb nucleic acid binding (Table 1). Our standard assay measures the fraction of a random pool of oligonucleotides associated with protein in a filter binding assay. Importantly, the concentration of protein is varied, and the unbound oligonucleotides are also analyzed by trapping on a Hybond filter, which avoids artifacts due to degradation. The wildtype IMPDH1 binds 4–8% of the random pool with $K_d = 6$ nM. The values of K_d increase by factors of 2–50 in six pathogenic or likely pathogenic variants.[10,16] The value of K_d also increased in potentially disease causing variant, Val268Ile, suggesting that this mutation is indeed pathogenic, while the nonpathogenic mutations have no effect on the nucleic acid affinity. We propose that nucleic acid

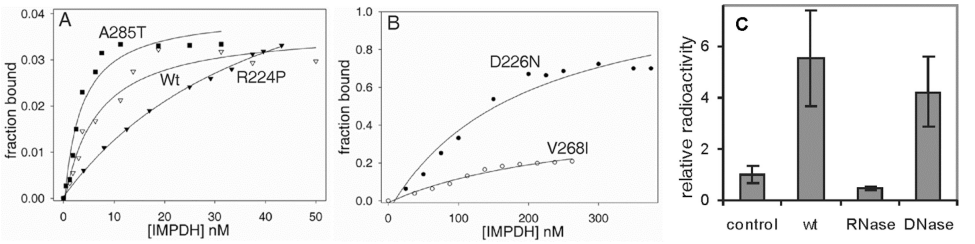

FIGURE 3 AdRP-linked mutations decrease affinity and specificity of nucleic acid binding. A) and B) Filter binding assays as described Figure 2. C) HeLa cells were transfected to enable expression of IMPDH1 tagged with GFP at the C-terminus. Cells were crosslinked with formaldehyde and immunoprecipitated with anti-GFP antibody. The immunoprecipitates were treated with phosphatase followed by polynucleotide kinase/[γ^{32}P]-ATP to label nucleic acids. The filter-bound radioactivity is shown relative to untransfected cells (control). The ^{32}P-labeled immunoprecipitates were treated with either RNase or DNase. Asterisks mark samples significantly different from wildtype ($P = 0.02$ for both control and RNase). Reproduced with permission from S. E. Mortimer and L. Hedstrom (2005) *Biochemical Journal*, vol. 390, pp. 41–47. © The Biochemical Society.

affinity presents a functional assay for the retinal pathogenicity of IMPDH1 mutations as well as the first clue into the mechanism of disease.

The RP-causing mutations also appear to decrease the specificity of the IMPDH1 interaction with nucleic acids (Table 1). IMPDH1 binds a maximum of ~4–8% of the random oligonucleotide pool[10,16,22] (Figures 2 and 3; note that this represents ~10^{11} sequences. We have cloned some of these binding sequences, but have not identified a physiologically relevant sequence). The maximum fraction bound increases by factors of 2–9 in five of the six pathogenic variants, and also in the potentially pathogenic variant Val268Ile. The exception, Arg224Pro, binds approximately the same number of sequences as the wildtype enzymes, which suggests that this mutation has little effect on specificity. However, since we do not know the identity of these sequences, this conclusion could be premature: it is possible that the sequences associated with Arg224Pro are very different from those associated with the wildtype IMPDH1. Therefore the specificity of nucleic acid binding may also provide a functional test for pathogenicity of IMPDH1 mutations. Since the Val268Ile mutation has similar effects as the pathogenic and likely pathogenic mutations, we have classified Val268Ile as a likely pathogenic mutation in Table 1.

The RP-linked mutations also decrease the association of IMPDH1 with RNA in vivo. We previously demonstrated that RNA co-immunoprecipitates with IMPDH.[22] To determine if the RP-linked mutations perturb RNA binding in vivo, we expressed C-terminally GFP-tagged IMPDH1 (IMPDH1-GFP) in HeLa cells. The addition of the GFP tag has no effect on either the enzymatic activity or nucleic acid binding properties of IMPDH1.[16] Western analysis demonstrated that the IMPDH1-GFP is expressed in amounts comparable to the wildtype IMPDH.[16] IMPDH1-GFP was isolated by immunoprecipitation with an anti-GFP antibody and the associated nucleic

acid was measured by labeling with polynucleotide kinase and [γ^{32}P]ATP (Figure 3). Significantly more nucleic acid is observed in immunoprecipitations from cells expressing IMPDH1-GFP than in the untransfected cells or cells expressing GFP alone. This immunoprecipitated nucleic acid is degraded by RNase but not by DNase (Figure 3). Therefore, like endogenous IMPDH, the IMPDH1-GFP binds RNA in vivo. The bound RNA appears to be heterogeneous and primarily much smaller than 80 nucleotides in length, although larger RNAs are also observed.[16] These co-precipitated RNAs are too small to be consistent with rRNAs or tRNAs (rRNAs, 28S (~5 kb) or 18S (~2 kb); tRNAs, 4S, 75–80 nucleotides). We suspect these small RNAs are remnants of larger RNA targets that were degraded during isolation, although it is possible that they are small regulatory RNAs.[23] Much less RNA is present in immunoprecipitations of the adRP-linked proteins. These results demonstrate that the pathogenic mutations decrease the association of IMPDH1 and RNA in vivo.

Our experiments demonstrate that the RP/LCA-linked mutations perturb nucleic acid binding, which provides a functional assay for the pathogenicity of IMPDH1 alleles.[10,16] However, these observations do not explain the photoreceptor-specific nature of the disease. The Daiger laboratory has recently discovered new IMPDH1 variants in photoreceptor cells that derive from alternative splicing,[8] and our preliminary experiments suggest the adRP-linked mutations also perturb the nucleic acid binding properties of these proteins. The presence of these alternative IMPDH1 spliceforms in the retina provide an attractive explanation for photoreceptor-specific apoptosis.

Since IMPDH binds single stranded nucleic acids and RNA coprecipitates with IMPDH, we propose that IMPDH1 regulates mRNA processing, nuclear transport, subcellular localization, translation, and/or degradation. Keene has proposed that specific mRNA binding proteins coordinate the expression of functionally related proteins, effectively creating a "posttranscriptional operon"[24] and evidence for such coordination of mRNA localization, translation, and degradation is accumulating.[25–29] Therefore, we hypothesize that IMPDH1 coordinates the expression of proteins critical for photoreceptor function by regulating mRNA translation, localization or degradation. The adRP mutations may perturb this regulatory function by relaxing the regulation of normal mRNA targets or by recognizing new targets. This hypothesis provides several appealing explanations for photoreceptor-specific apoptosis:

(1) *IMPDH1 regulates translation.* IMPDH1 may coordinate the translation of a set of photoreceptor proteins by regulating translational initiation, elongation or termination.[30] The RP-causing mutations might upset this coordinated translation, either by failing to recognize normal

targets or by recognizing new targets. Translational regulation plays an important role in stress response and apoptosis.[31] In addition, translation is closely coupled to protein translocation and proteins can be mislocalized when translational regulation is perturbed. Protein mislocalization is especially critical in highly polarized cells, and is known to cause apoptosis in photoreceptors and other neuronal cells,[32,33] also providing a potential pathophysiological mechanism for IMPDH1-mediated adRP.

(2) *IMPDH1 directs the localization of target mRNAs.* There are now many examples of mRNAs that localize to specific sites within eukaryotic cells.[34–37] mRNA localization concentrates protein synthesis to these sites. In addition, the same factors that direct mRNA localization often inhibit translation until the mRNA reaches its destination. The molecules involved in these processes have only begun to be identified. mRNA localization is a critical feature of polarized cells such as oocytes, budding yeast and neuronal cells; defects in mRNA localization can cause apoptosis in neuronal cells.[33] mRNA localization is also likely to be especially critical in photoreceptor function, so perturbation of mRNA localization provides another potential pathophysiological mechanism for IMPDH1-mediated adRP. Interestingly, IMPDH1 has recently been reported to associate with the N-terminus of RP1, although this interaction has not yet been confirmed in photoreceptors and must therefore be considered putative.[38] While the function of RP1 is unknown, RP1 is localized to the base of the connecting cilium where it could be involved in protein transport and deletion of RP1 causes rhodopsin mislocalization.[39,40]

(3) *IMPDH1 regulates mRNA degradation.* mRNA degradation is another critical, although comparatively uncharacterized, determinant of protein expression. While the major degradation pathways have been delineated, the molecular mechanisms regulating mRNA degradation remain largely obscure.[41,42] mRNA abundance is determined by the interplay of transcription and decay; inappropriate decay will change the accumulation of mRNA, causing an imbalance in protein expression that could lead to apoptosis. The over-expression of opsin causes apoptosis of photoreceptor cells,[43,44] as does the underexpression of RDS/peripherin.[45,46] Interestingly, RNA degradation appears to be localized to specific intracellular sites[47–50] and site-specific degradation is another mechanism for localizing mRNA and proteins.[51] Therefore, as above, perturbation of RNA degradation could also cause protein mislocalization, triggering apoptosis.

Summary. The RP/LCA-linked mutations of IMPDH1 decrease both the affinity of specificity of nucleic acid binding. Thus, perturbation of nucleic acid binding provides the first functional assay for pathogenicity,

and the first insight into the pathophysiological mechanism of IMPDH1-mediate RP/LCA. Understanding this mechanism may suggest new, urgently needed, strategies for therapy for RP and other hereditary diseases involving CBS domain proteins.

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