

# ABCA4 mutations causing mislocalization are found frequently in patients with severe retinal dystrophies

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**ABCA4, also called ABCR, is a retinal-specific member of the ATP-binding cassette (ABC) family that functions in photoreceptor outer segments as a flippase of *all-trans* retinal. Homozygous and compound heterozygous *ABCA4* mutations are associated with various autosomal recessive retinal dystrophies, whereas heterozygous *ABCA4* mutations have been associated with dominant susceptibility to age-related macular degeneration in both humans and mice. We analyzed a cohort of 29 arRP families for the mutations in *ABCA4* with a commercial microarray, ABCR-400 in addition to direct sequencing and segregation analysis, and identified both mutant alleles in two families (7%): compound heterozygosity for missense (R602W) and nonsense (R408X) alleles and homozygosity for a complex [L541P; A1038V] allele. The missense mutations were analyzed functionally in the photoreceptors of *Xenopus laevis* tadpoles, which revealed mislocalization of ABCA4 protein. These mutations cause retention of ABCA4 in the photoreceptor inner segment, likely by impairing correct folding, resulting in the total absence of physiologic protein function. Patients with different retinal dystrophies harboring two misfolding alleles exhibit early age-of-onset (AO) (5–12 years) of retinal disease. Our data suggest that a class of ABCA4 mutants may be an important determinant of the AO of disease.**

## INTRODUCTION

ABCA4 is a member of the ABC (ATP-binding cassette) transporters, which localizes to the rims of the outer segment (OS) discs in photoreceptors (1,2). ABCA4 acts as an outwardly directed flippase that transports *all-trans* retinal (*atRAL*) and/or its derivatives across the rod and cone OS discs in an energy-dependent manner. This action accelerates the recycling of *atRAL* released from photobleached rhodopsin via the retinoid cycle (3,4). It has been suggested that decreased transporter activity leads to the accumulation of toxic *atRAL* derivatives in rods and cones, which results in apoptosis of the supporting RPE cells and, finally, degeneration of the photoreceptors (4,5). ABCA4 insufficiency leads

to several autosomal recessive retinal dystrophies including retinitis pigmentosa (RP, OMIM no. 601718), cone-rod dystrophy (CRD, OMIM no. 604116), Stargardt disease (STGD, OMIM no. 248200) and a dominant susceptibility to age-related macular degeneration (AMD) in both human and mice (6–17).

Previous studies have hypothesized that the severity of the ocular clinical phenotype correlates inversely with ABCA4 activity, i.e. the lower the residual activity, the more severe the resultant phenotype, especially as monitored by age-of-onset (AO)/detection (7,18–20). Currently, there is no direct assay for the transporter activity of ABCA4. This limitation hinders the validation of functional effects for many *ABCA4* mutations including a huge array of missense and splicing

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**Table 1.** Clinical characteristics and genetic analysis of *ABCA4* mutations in seven autosomal recessive retinitis pigmentosa families

Pedigree	Patients	Age-of-onset (years)	Visual acuity	Diagnosis	Allele 1	Allele 2
AR168	168-03	30	N/A	RP	36 IVS+1G > A	WT
	168-05	24	20/25 OD; 20/30 OS; VF < 30° OU	RP	N/A	N/A
	168-06	26	N/A	RP	N/A	N/A
AR192	192-03	9	20/20 OD; 20/25 OS; VF < 5° OU	RP	D2177N	WT
	192-04	19	20/30 OD; 20/40 OS; VF < 5° OU	RP	WT	WT
	192-05	19	20/30 OD; 20/40 OS; VF < 5° OU	RP	WT	WT
AR194	194-03	30	N/A	RP	D2177N	WT
	194-05	Childhood	20/25 OD; 20/40 OS	RP	N/A	N/A
	194-06	5	N/A	RP	D2177N	WT
	194-07	4 or 5	20/80 OU	RP	N/A	N/A
AR197	197-05	7	CF 3 feet OD; CF 2 feet OS	RP	[L541P; A1038V]	[L541P; A1038V]
	197-06	9	CF 5 feet OD; HM OS	RP	[L541P; A1038V]	[L541P; A1038V]
AR554	554-03	2 10/12	20/60 OU	RP	V2050L	WT
	554-04	1 9/12	N/A	RP	N/A	N/A
AR591	591-03	20	20/25 OU	RP	N/A	N/A
	591-04	8	20/20 OD; 20/25 OS; VF < 10° OU	RP	G1961E	WT
AR689	689-03	7	N/A	RP	R408X	R602W
	689-08	7	N/A	RP	N/A	N/A

OD, right eye; OS, left eye; OU, both eyes; VF, visual field; RP, retinitis pigmentosa, WT, wild type; N/A, not available; CF, counting fingers; HM, hand motions.

mutations. However, the hypothesis, 'ABCA4 activity versus retinal dystrophy severity', is supported by a multitude of indirect observations including: (a) *Abca4*-knockout mice which partially recapitulate human retinal disease by manifesting the accumulation of lipofuscin, delayed dark adaptation and slow progression of photoreceptor degeneration (4,5,17,21), (b) human genetics studies correlating AO and/or phenotypes conveyed by the selective combination of mutant *ABCA4* alleles (9,18,22) and (c) increased susceptibility to AMD of *ABCA4* mutant-carrying grandparents of children with STGD (16,19).

Autosomal recessive retinitis pigmentosa (arRP) is the most severe retinal dystrophy associated with *ABCA4* mutations and is believed to be caused by complete loss of *ABCA4* activity. Since the mapping of a novel locus for recessive RP (RP19) to the *ABCA4* region, 12 RP19 families have been described with mutations in *ABCA4*, most of which were truncating or aberrant splicing mutations (6-8,23-27). To determine the prevalence of *ABCA4* alterations in arRP families, we used a commercially available microarray with APEX technology to search for >400 *ABCA4* mutations (28), and direct sequencing and segregation analyses to investigate 29 arRP families. Frameshift, missense and complex mutations were identified. Functional studies of missense alleles [L541P; A1038V], R602W and C1490Y in transgenic frogs demonstrate that they do not translocate to rod OSs (ROSs). This apparent mislocalization event prevents *ABCA4* from reaching its proper physiologic compartment (i.e. ROS) and produces functionally null alleles.

## RESULTS

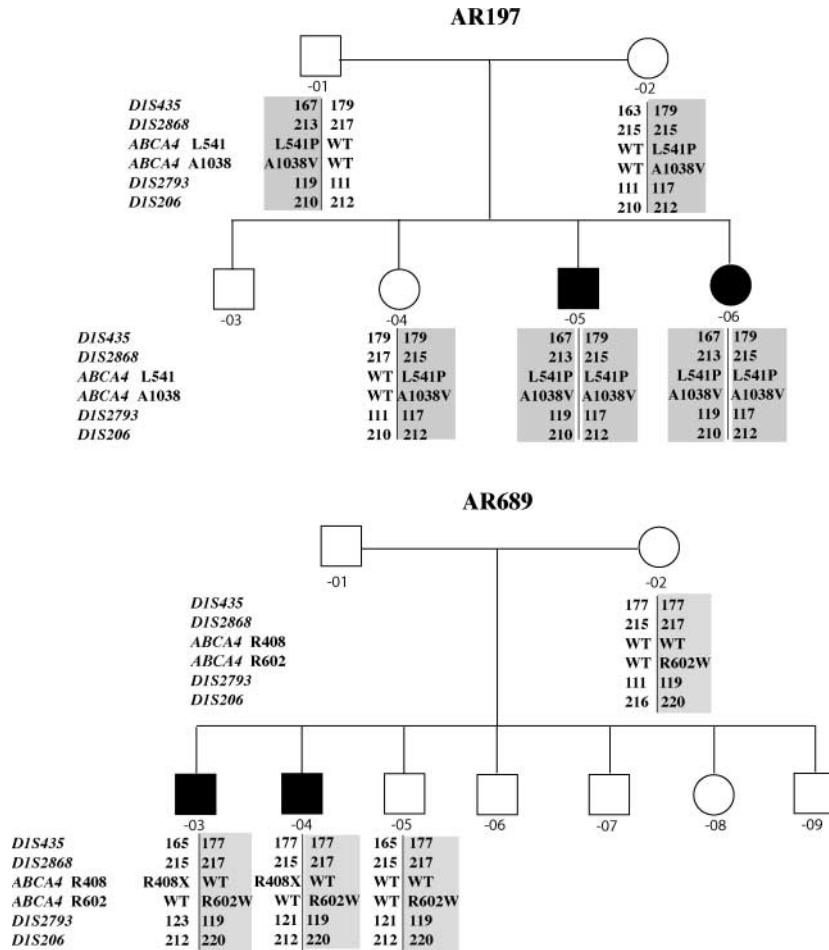
### Mutation screening and segregation analysis

To investigate the involvement of *ABCA4* mutations in autosomal recessive forms of RP, we performed mutational screening in 29 RP families with at least two affected siblings

and unaffected parents. Mutations were detected with a commercially available microarray chip, ABCR-400 (ASPER, Tartu, Estonia). We detected 9/58 (~16%) *ABCA4* mutant alleles in seven arRP (~24%) families (Table 1). In two families (~7%), both apparent disease-associated *ABCA4* mutant alleles were identified consistent with previous reports that *ABCA4* is responsible for ~2-6% of arRP (27).

In these two unrelated arRP families, AR197 and AR689, two mutant alleles were detected in all affected individuals and segregation was consistent with autosomal recessive inheritance. The sequence analysis of *ABCA4* in patients AR197-05 and AR197-06 revealed homozygosity for the complex *ABCA4* allele [L541P; A1038V]. There is no historical evidence for consanguinity in this family. Interestingly, each patient experienced early-onset, classic RP with macular involvement in both retinas at ages 7 and 9, respectively. Sequence analysis of patient AR689-03 revealed compound heterozygosity for the missense *ABCA4* alteration R602W and a nonsense allele, R408X. This patient also experienced early-onset RP at age 7. Ophthalmoscopic examination revealed diffuse retinal degeneration with bone spicules and extensive macular atrophy with some pigment clumps and profound vascular attenuation outside the major vascular arcades, consistent with the classic RP phenotype.

arRP patients from five families (AR168, AR192, AR194, AR554 and AR591) were heterozygous for various missense *ABCA4* mutations that by conceptual translation would lead to either an amino acid change (G1961E, V2050L and D2177N) or splicing alteration (36IVS+1G > A). Segregation analyses of single allele alterations in five arRP families (Table 1) revealed *ABCA4* mutations were inherited by affected siblings and by unaffected ones suggesting a carrier status. We implemented direct DNA sequencing of all 50 exons in the seven probands in whom *ABCA4* mutations



**Figure 1.** Haplotype and segregation analysis of *ABCA4* mutations in two arRP families. Pedigrees are drawn using standard conventions; filled symbols denote RP-affected individuals. Haplotypes for the *ABCA4* genomic region are given below for each family member. *ABCA4* mutations are abbreviated by '*ABCA4*' with their predicted amino acid changes shown. Segregation is denoted with background gray shading.

were identified and confirmed the results obtained with the ABCR-400 microarray.

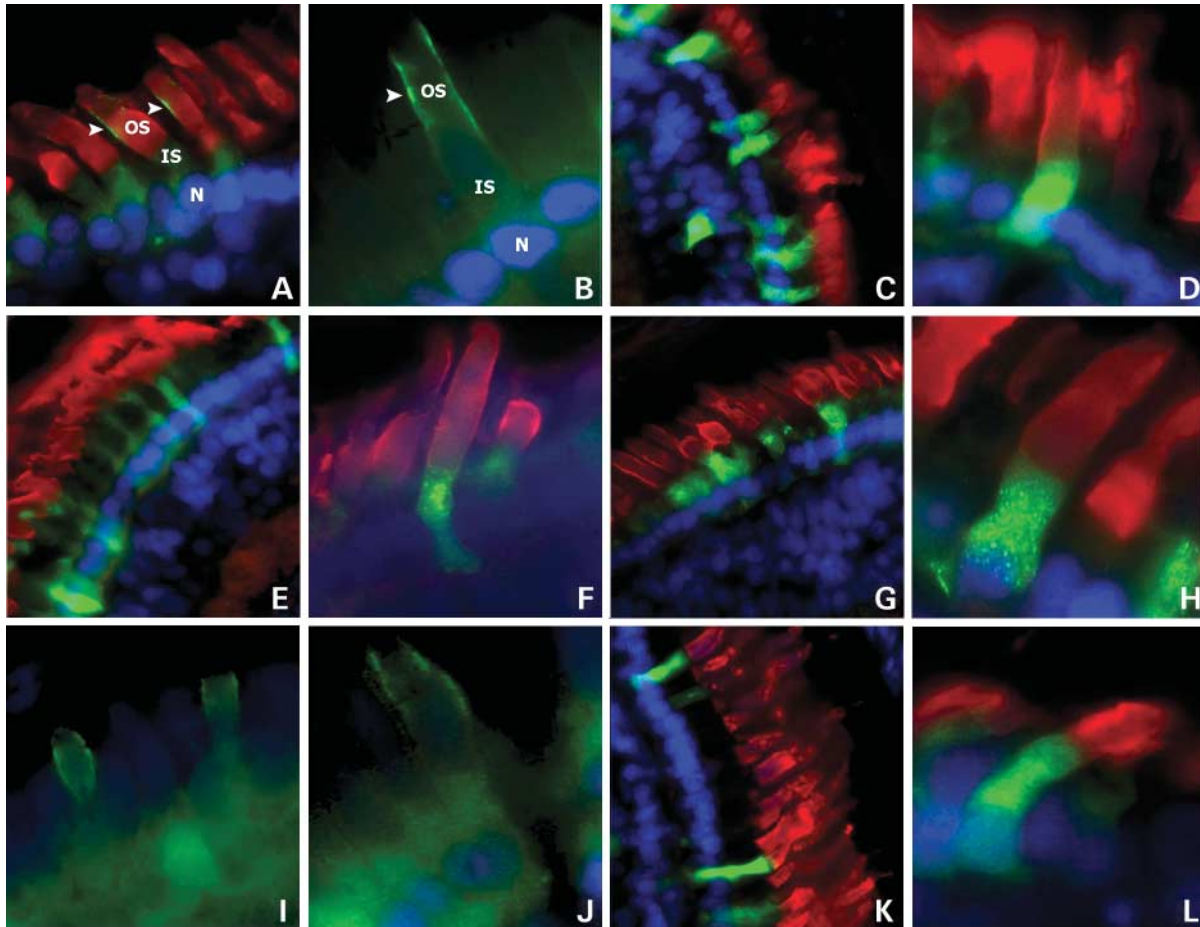
### *ABCA4* haplotype analysis

To evaluate the relationship between *ABCA4* mutations and the disease phenotype, we used haplotype analyses with four microsatellite markers and intragenic single nucleotide polymorphism (SNP) markers that span the *ABCA4* genomic locus in the 5'–3' direction: *DIS435*– ~0.8 cM –*DIS2868*– ~1.5 cM –*ABCA4* SNP– ~1.5 cM –*DIS2793*– ~4.8 cM –*DIS206* (Fig. 1). Haplotype analyses in families AR197 and AR689 were consistent with *ABCA4* mutation segregation analyses and revealed that arRP patients share common haplotypes inherited from each parent. However, we did not find a common haplotype for the *ABCA4* region that was shared among multiple affected siblings in any of the five arRP families with heterozygous *ABCA4* mutations. We noted several differences in the microsatellite markers and the *ABCA4* SNPs between haplotypes of arRP patients in the same family. In addition, several unaffected

siblings in families AR192 and AR194 have identical haplotypes to arRP subjects within the same family (data not shown). Thus, haplotype studies were consistent with mutation analysis suggesting a carrier state and inconsistent with a non-coding *ABCA4* mutation co-segregating with the mutant allele. Thus, the potential pathogenicity and contribution of those *ABCA4* mutations in these five arRP families could not be established under the assumption of a monogenic inheritance model.

### Expression of human *ABCA4* in *Xenopus laevis* rods

Limited functional studies have been performed on disease-associated *ABCA4* missense mutations. Previous studies with COS7 cells have shown that even wild-type *ABCA4* appears to be retained in the ER (29). We expressed wild-type *ABCA4* in COS7 cells and examined localization of the protein by immunofluorescence (IF) of transfected cells and western blot analysis of subcellular fractions. Indeed, both assays confirmed the retention of *ABCA4* to the endoplasmic



**Figure 2.** Transgenic expression of WT and mutated human ABCA4 in *X. laevis* tadpoles. (A and B) Cryosections of the retina from 2-week-old tadpoles expressing human ABCA4 were immunostained with anti-ABCA4 Rim 3F4 antibody and subsequently visualized with goat anti-mouse antibody conjugated to Alexa Fluor 488 (green). The sections were stained additionally with Texas Red—WGA (red) to label the photoreceptor outer segments (A). The nuclei in both sections were stained with DAPI (blue). Human ABCA4 is recognized by 3F4 monoclonal antibody and found exclusively in the ROS rims as shown by the yellow signal (marked with arrow). (C–L) Microphotographs of 2-week-old *X. laevis* tadpoles expressing R602W (C and D), [L541P; A1038V] (E and F), L541P (G and H), A1038V (I and J) and C1490Y (K and L). Rod photoreceptors expressing each of the mutant proteins, except A1038V, demonstrated localization of the transgenic protein to the rod inner segment and cell body. Misfolded proteins are homogeneously distributed in the inner segments and cell bodies, although the presence of fine aggregates was noted for L541P. Mutation A1038V does not influence the localization of ABCA4 and the mutant protein was found in the ROS. OS, outer segments; IS, inner segment; N, nucleus.

reticulum (data not shown) documenting that COS7 cells cannot be used to assay for effects of mutations on subcellular localization. This observation was a major impetus to develop an *in vitro* animal model to investigate ABCA4 expression and localization.

Transgenic *X. laevis* tadpoles were developed with a construct containing human ABCA4 cDNA under the control of the *X. laevis* 1.3 kb opsin promoter of *X. laevis*. Initially, ABCA4 constructs were tagged with EGFP at the C-terminus but the transgenic protein did not localize to the ROS and was retained exclusively in the inner segments (IS). The inability of ABCA4-EGFP to localize to the ROS is a serious experimental challenge because this limits the discrimination of transgenic from non-transgenic tadpoles under UV light during early developmental stages. The lack of this transgenic marker resulted in the requirement for IF analysis of all transgenic animals. Nevertheless, it was apparent from these

studies that transport of ABCA4 to the ROS could be assayed readily.

Constructs containing wild-type ABCA4 without an EGFP tag were then used as the transgene to make transgenic frogs. IF analysis of whole eye sections from 10- to 14-day-old animals revealed the expression of transgenic ABCA4 in rod photoreceptors only. The expression of ABCA4 was detected with Rim 3F4 mouse monoclonal antibody specific for the bovine and human nine amino acid epitope located at the C-terminus. The homologous fragment in *X. laevis* ABCA4 is remarkably different and therefore, not recognized by Rim 3F4 antibody (30) (data not shown). ABCA4 was identified primarily in the rims of ROSs (Fig. 2A and B), however, animals expressing high levels of the transgene demonstrated trace amounts of ABCA4 in the IS, especially in the ciliary stalk (data not shown). The level of ABCA4 expression varied among transgenic animals and among rods of the

same retina. Rods expressing human ABCA4 were usually clustered, two to five, and distributed throughout the retina. Typical rod morphology was preserved and the photoreceptors did not display signs of degeneration such as shortening of ROS.

### RP-associated mutants are misfolded and retained in the rod IS

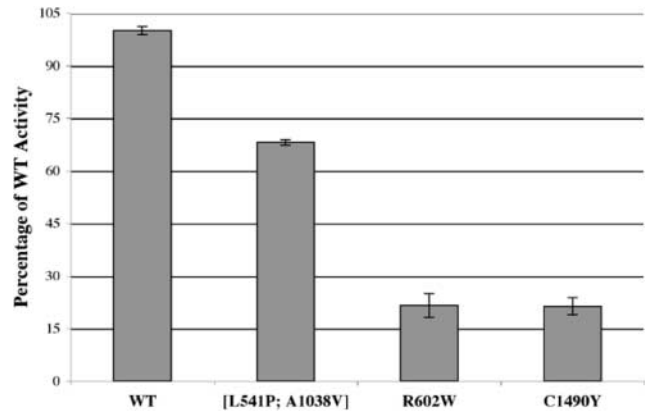
To investigate effects of the presumably severe arRP-associated R602W missense allele on ABCA4 localization, transgenic tadpoles were generated with the R602W construct. IF analysis of the retina demonstrated the retention of transgenic R602W in the IS (Fig. 2C and D). Staining of the IS was homogenous, suggesting misfolding of the mutated protein. The morphology of transgene-expressing rods was unchanged, and no signs of retinal degeneration were observed. Functional studies of the arRP-associated complex allele [L541P; A1038V] also showed abnormal localization to rod IS although the IF studies revealed a different staining pattern than R602W, because the mutant protein forms fine aggregates in the IS (Fig. 2E and F). The aggregate formation suggests a mechanism distinct from that observed for R602W may be responsible for the retention of [L541P; A1038V] in the IS. To determine which mutation prevents translocation of [L541P; A1038V] to ROS, we examined functionally the L541P and A1038V mutants independently. We observed retention of L541P in the IS (Fig. 2G and H) and correct localization of A1038V to ROS (Fig. 2I and J). These results suggest that L541P is a disease-causing mutation that affects ABCA4 processing.

### Mutation C1490Y causes retention of ABCA4 to rod IS

The ABCA4 C1490Y allele has been found in patients presenting with both STGD and CRD. The C1490 residue is located in the second extracellular domain loop (ECD II) and has been postulated to be responsible for the formation of a disulfide bond between the two loops. Therefore, we sought to explore the effect of C1490Y mutation on ABCA4 processing. We found that C1490Y causes retention of the mutated protein in the IS (Fig. 2K and L). Similar to the staining pattern found in R602W, the IS compartment was stained homogeneously suggesting impaired folding. No traces of ABCA4 were found in the ROS.

### ATPase activity

The measurement of ATPase activity has been the only assay available to study the effects of mutations on ABCA4 function. We employed this assay to examine the effects of [L541P; A1038V], R602W and C1490Y mutations on *in vitro* ATP hydrolysis. Constructs containing wild-type and mutated ABCA4 cDNAs, tagged with the eight amino acid bovine opsin C-terminal epitope (1D4), were expressed in COS7 cells and proteins were purified on a 1D4 affinity column. CHAPS-solubilized ABCA4 was incubated sub-



**Figure 3.** ATP hydrolytic activity assay. WT and mutant constructs [L541P; A1038V], R602W and C1490Y ABCA4 were expressed in COS7 cells. The proteins were affinity-purified and assayed for basal ATP hydrolytic activity at identical protein concentrations. All investigated mutants resulted in decreased ATPase activity.

sequently with ATP, and the hydrolysis rate was estimated with the charcoal method (31).

The rate of ATP hydrolysis of the complex allele [L541P; A1038V] was decreased to 68.1% of wild-type ABCA4 (Fig. 3). In addition, we observed a marked decrease in ATP hydrolytic activity compared with wild-type in both R602W (21.6%) and C1490Y (21.4%) alleles. Control experiments in which COS7 cells were transfected with constructs expressing ABCA4 mutants showed normal or nearly normal protein expression (data not shown) (29). Such findings suggest that the observed diminished hydrolytic activity of ABCA4 mutants is not related to expression or stability of the investigated proteins. Collectively, these observations indicate that these mutations have at least a dual effect on ABCA4 as they prevent the correct localization to the ROS *in vivo* and also perturb ABCA4 ATPase activity *in vitro*. Decreased ATPase activity does not likely contribute to the overall pathogenicity of [L541P; A1038V] because localization to the ROS is the primary event that determines placement of the protein in its natural environment and is presumably required for it to perform its physiological function.

### Mutations: R602W, [L541P; A1038V] and C1490Y are frequently detected in patients with retinal diseases

Mislocalization mutations R602W, [L541P; A1038V] and C1490Y have been reported as disease-associated mutations in patients with RP, CRD and STGD (18,20,32,33). These mutations have been reported both as homozygous and as one of compound heterozygous mutant alleles. We identified three RP, three CRD and five STGD patients with two mislocalization alleles and found that they share a similar mean AO, 7–9, 7–12 and 7–12 years, respectively (Table 2). We conclude that patients bearing two mislocalization-ABCA4 alleles can develop different retinal dystrophy phenotypes, but there is uniformly an early AO.

**Table 2.** Genotype–phenotype correlations among patients bearing one and two mislocalization—mutations

Two mislocalization—alleles			One mislocalization—allele		
RP			STGD		
Allele 1	Allele 2	AO	Allele 1	Allele 2	AO
[L541P; A1038V]	[L541P; A1038V]	7	[L541P; A1038V]	L2027F	13
[L541P; A1038V]	[L541P; A1038V]	9	[L541P; A1038V]	R1108H	13
			[L541P; A1038V]	G1961E	16
			[L541P; A1038V]	G1961E	28
			[L541P; A1038V]	L2027F	13
			[L541P; A1038V]	L2027F	12
			[L541P; A1038V]	P1380L	5
			[L541P; A1038V]	T1019M	6
			[L541P; A1038V]	G1961E	7
			[L541P; A1038V]	T1019M	6
			R602W	L2027F	9
			C1490Y	G1961E	28
			C1490Y	G1961E	13
			C1490Y	L2027F	10
			C1490Y	L2027F	18
			C1490Y	L2027F	18
			C1490Y	L2027F	18

AO—age of onset (in years).

We compared these results with those obtained for 16 STGD patients in whom one mislocalization-mutation was detected accompanied by a missense mutation on the other allele. The mean AO in this latter cohort was 13.6 with a range from 5 to 28 years. In those patients, the second allele consisted of substitutions present along the entire protein, including functional domains (e.g. Walker motifs) and, therefore, presumably having different effects on ABCA4 activity.

## DISCUSSION

We investigated the consequences of ABCA4 mutations on protein processing in the photoreceptors of transgenic *X. laevis* tadpoles and ATP hydrolytic activity *in vitro*. As mutations affecting expression often lead to complete loss of function, we selected missense mutations detected in patients with the most severe ABCA4-associated retinal phenotype, RP. This condition represents a clinically and genetically heterogeneous group of inherited retinal disorders. More than 39 genes have been identified to date, and among the known causative arRP genes, mutations in ABCA4 are of interest due to their high (7–8%) prevalence in the general population (34,35). To evaluate the relative frequency of ABCA4 mutations and their role in the pathogenesis of RP, we performed a comprehensive clinical and genetic analysis in a cohort of 29 arRP families and further characterized seven arRP families with ABCA4 mutations. We found a surprisingly high frequency (16%) of these alterations in arRP-affected individuals. In subjects from five RP families (9% of disease alleles), we found heterozygous missense ABCA4 mutations (G1961E, V2050L, D2177 N and 36IVS+1) that are suggested to have functional consequences for ABCA4 activity (29,36). Haplotype analysis of the ABCA4 genomic region did not identify a shared disease-associated haplotype in any of them. Moreover, a heterozygous ABCA4 mutation was not found in

all RP-affected family members. Thus, the severity of the RP phenotype did not appear to be influenced in a specific way by the presence of the mutation. The presence of ABCA4 alterations that do not segregate with the disease in five arRP families may suggest that retinal dystrophy in those individuals may be associated with (an)other locus or *loci*.

Co-segregating compound heterozygous mutant alleles (R602W/R408X) and a homozygous complex allele [L541P; A1038V] were identified in two (AR689 and AR197) arRP families (Table 1). We hypothesized that the disease-associated missense mutations [L541P; A1038V], R602W and C1490Y could exert a possible effect on protein processing as this mechanism, which prevents altered proteins from locating to its physiologic compartment, was documented for other ABC transporters in related diseases including cystic fibrosis (*CFTR*) and Tangier disease (*ABCA1*). To examine this hypothesis, we generated transgenic *X. laevis* tadpoles expressing WT, [L541P; A1038V], R602W and C1490Y mutants and documented that the three mutant alleles cause mislocalization of ABCA4. The biochemical findings for these mutant alleles revealed that ATPase activity and retinal stimulation are impaired to a varying degree *in vitro*, and on the basis of this test, they can be classified as either mild or severe. Nevertheless, *in vivo*, these mutants are retained in the IS and never fully reach the ROS where it may appropriately provide its transporter activity. Whereas ATPase activity provides one assay to document functional effects of disease-associated missense mutations, experiments with transgenic animals reveal that assessment of ABCA4 mutant alleles based solely on ATP activity can be misleading. ABCA4 expression in *X. laevis* tadpoles is the first *in vivo* assay that enables the examination of wild-type and mutant protein processing. *X. laevis* has been the model organism for studying other retinal expressed genes including rhodopsin and peripherin-2 (37,38).

In our experiments, we demonstrated the localization of human ABCA4 to the rims of frog OSs. Traces of ABCA4

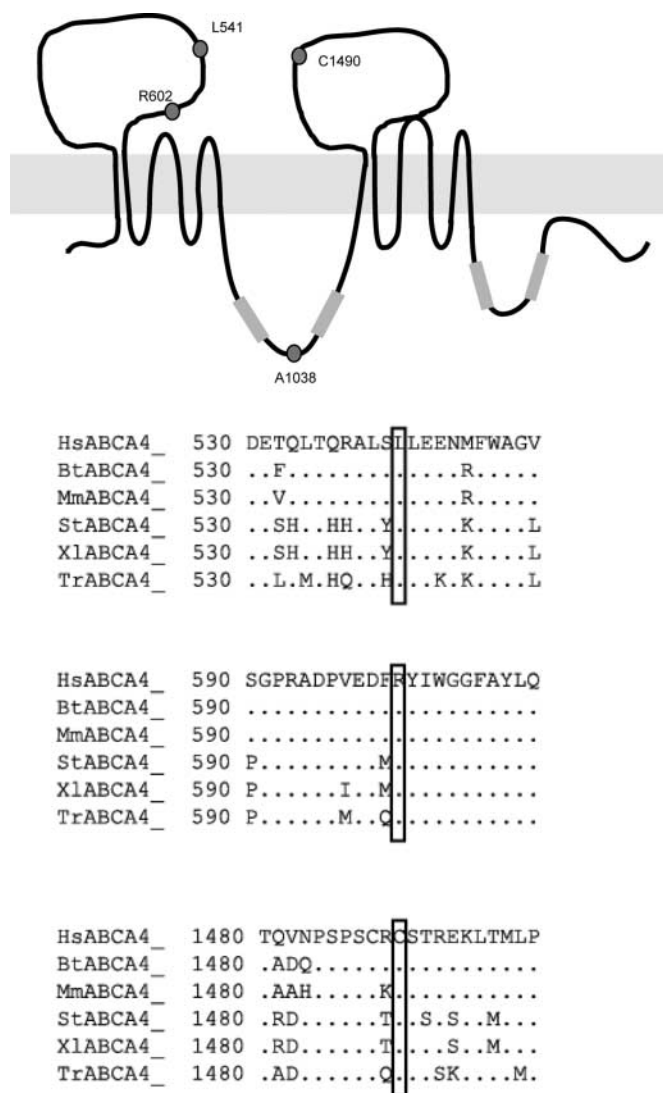
were detected in the IS especially in animals highly expressing this transgene. This observation was also recorded in experiments with other transgenes and may be explained by the saturation of the internal mechanism responsible for ABCA4 transfer from the IS to the ROS. However, these mechanisms for ABCA4 translocation remain largely unknown.

The results of IF studies of rods expressing [L541P; A1038V], R602W and C1490Y mutants were quite distinct from those observed for WT and ABCA4 EGFP. The mutant protein was found in the IS, staining the cell body homogeneously; typical for misfolded proteins that are unable to be processed through the ER and Golgi body (37,38). We also identified that L541P is responsible for the retention of the complex allele [L541P; A1038V], whereas A1038V seems to have no effect on ABCA4 processing. Interestingly, the mutants are localized to ECD loops whose function remains to be determined (Fig. 4). We speculate that the substitution of highly conserved L541 and R602 residues disturbs spatial organization and/or interaction within ABCA4 that results in misfolding of the protein. These amino acids potentially interact with other factors that mediate its processing and translocation to the ROS.

The R602 residue is highly conserved among *ABCA4* genes of different species and with other members of the *ABCA* family. Similar misfolding effects were observed for the analogous R587W mutation found in *ABCA1* in patients with Tangier disease. Experiments in which COS7 cells were transfected with a mutant *ABCA1* construct, R587W, demonstrated the retention of the misfolded protein in the ER (39). The R602 residue is localized in the ECD I and its significance for both structure and function is unknown. We speculate that the substitution of a positively charged arginine residue with non-polar tryptophan may have adverse effects on the spatial organization of the protein.

The C1490 is one of five cysteine residues localized in the ECD II. It has been found that extracellular loops are linked by one or more intramolecular disulfide bonds that influence the spatial organization of the molecule (31). The lack of cysteine may abrogate the formation of covalent bonds between two loops affecting protein organization and cause misfolding. This sequence of events was observed previously for other mutated transmembrane proteins including *ABCA1* C1478R (40) and peripherin-2 C214S (38). In addition, mutations of other cysteine residues from both *ABCA4* ECDs have been identified in patients with retinal disease (C54Y, C1488Y) (12,18,41).

Presumed folding mutations, especially [L541P; A1038V] and C1490Y, have been frequently detected in patients with autosomal recessive macular degenerations (18,20,32,33). We found that a combination of two mislocalization-alleles always result in early AO (7–12) of disease. However, the retinal phenotype including RP, CRD or STGD differed. From a histological standpoint, these retinal disorders primarily affect photoreceptors: rods in RP and cones in CRD and STGD. The type of primarily affected photoreceptors determines clinical symptoms at presentation. The survey of clinical reports demonstrates, in many patients, that one observes evolution of retinal disorders. Initially, one type of photoreceptors is involved and causes either cone- or rod-dependent early presenting symptoms, which determines the diagnosis. Catamnesis of those patients shows the evolution of retinal disease and



**Figure 4.** Folding mutations affect conserved residues from extracellular loops—ECD I and II. Shown is a proposed topological model of ABCA4 with its membrane spanning domains and the mutations: L541P, R602W, A1038V and C1490 analyzed. The comparison of ABCA4 sequences among species revealed that mutated residues: L541, R602 and C1490 are evolutionary conserved. Hs, human; Bt, bovine; Mm, murine; St, frog (*Silurana tropicalis*); Xl, frog (*Xenopus laevis*); Tr, fish (*Takifugu rubripes*) ABCA4.

the gradual involvement of the other photoreceptor type. The analysis of the clinical history of patients in whom two mislocalization-alleles were detected demonstrates they had different presenting symptoms that evolved to panretinal disease (20,32). All patients presented at an early age (5–12 years) and experienced severe progression of the disease. This observation may suggest that the lack of ABCA4 activity is a key element in pathogenesis of retinal disease that triggers both rod and cone involvements, but the course of the disease including primary involvement of rods or cones may be modified by other genetic and environmental factors specific to one group of photoreceptors. The potential genetic factors include enzymes involved in the retinoid cycle as they can regulate the amount of *atRAL* in photoreceptors. Experiments with *Abca4*-knockout mice treated with isotretinoin demonstrated

slowing of retinal disease due to decrease of *atRAL* in the photoreceptors (42). Other modulating factors could include genes encoding transcription factors, splicing factors (43) and regulators of apoptotic pathways. Collectively, these mechanisms may have a different efficiency when coupled with either group of photoreceptors and, therefore, vary among patients with respect to the primary involvement of either the cone or the rod photoreceptor.

We propose that severe retinal phenotypes including RP, CRD and STGD may have early AO associated with zero or very low activity of ABCA4. The ocular phenotype in this group of severe macular degenerations, including fundus morphology and progression of disease may be influenced by other genetic and stochastic environmental factors.

## MATERIALS AND METHODS

### Patients

Patients from arRP families ( $n = 29$ ) were diagnosed by clinical criteria (44) consistent with international standards and confirmed by review of ophthalmic records and retinal photographs. The clinical criteria included visual impairment at early age, progressive loss of peripheral visual functions and typical retinal changes of vascular attenuation, disc pallor and bone spicule accumulation. These studies were approved by The Institutional Review Board for Human Subject Research at Baylor College of Medicine.

### Genetic analysis of ABCA4

DNA was extracted from peripheral leukocytes with Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). *ABCA4* mutations were screened with a commercially available microarray chip, ABCR-400 (28). PCR for the region of interest was performed with the HotStarTaq PCR kit (Qiagen, Valencia, CA, USA). DNA sequencing used the BigDye Primer v3.1 reagent (PE-Applied Biosystems, Foster City, CA, USA). Genotyping analyses for the *ABCA4* locus used the ABI PRISM microsatellite markers: *DIS435*, *DIS2868*, *DIS2793* and *DIS206* (PE-Applied Biosystems). Previously identified *ABCA4* nucleotide changes were used as intragenic SNP markers.

### Generation of WT and mutant ABCA4 constructs

Plasmid pXOP-GFP was kindly provided by Dr David S. Papermaster. This plasmid contains a 1.3 kb fragment of the *X. laevis* opsin promoter and drives the expression of GFP in frog rod photoreceptors (37). Human ABCA4 cDNA was cloned into plasmid pXOP-GFP and two different constructs were generated: pXOP-ABCA4 in which unaltered ABCA4 cDNA is under control of the opsin promoter and pXOP-ABCA4-GFP with ABCA4 fused to GFP in frame. To generate the retinal dystrophy associated *ABCA4* alleles: [L541P; A1038V], L541P, A1038V, R602W and C1490Y ABCA4, the pXOP-ABCA4 plasmid was mutagenized with Quickchange PCR-based mutagenesis system (BioLabs, La Jolla, CA, USA). The sequences of WT and all mutant constructs were verified in both forward and reverse directions

with Big Dye Primer v3.1 chemistry to ensure that no random alterations were introduced. For ATPase activity experiments, pRK-ABCA4 plasmid (a generous gift from Dr J. Nathans) tagged at the C-terminus with the eight amino acid bovine opsin, 1D4 epitope was used.

### Transgenesis

Transgenic frogs were generated from the modified protocol of Kroll and Amaya (45). Sperm nuclei were isolated by a standard protocol (46). Partially decondensed *X. laevis* sperm nuclei were incubated with 100–150 ng of DNA construct linearized with *Apa* I endonuclease and purified using the Gel Extraction kit (Qiagen). The reaction was diluted 1:40 in sperm dilution buffer and DNA-treated sperm nuclei were then injected directly into dejellied oocytes. The four cell-embryos were transferred to 0.1× Marc's modified Ringer (MMR), 6% Ficoll and 1 mg/ml gentamicin solution and allowed to develop overnight at 16°C. Twenty-four hours after fertilization, embryos were transferred to 0.1× MMR solution and incubated in 16°C for an additional 48 h. At 3 day post-fertilization (dpf), tadpoles were transferred to water and kept at 18°C on a 12/12 h light/dark cycle. Tadpoles injected with the pXOP-ABCA4-GFP construct were examined and selected for further studies with an Olympus SZX12 dissecting microscope with UV epi-illumination and GFP filters.

### Immunocytochemistry

For immunocytochemistry studies, transgenic tadpoles were sacrificed between 10 and 14 dpf. The animals were immobilized in 0.02% Tricaine and fixed in 4% paraformaldehyde, saline phosphate buffer pH 7.5 at 4°C overnight. Fixed animals were protected in 4% sucrose, saline phosphate buffer pH 7.5 at 4°C for 1 h and then embedded in OCT tissue embedding medium (Tissue-Tek) and frozen. Cryostat sections (14 μm) were washed with PBS and blocked in 5% goat serum, 0.1% Triton X-100 in PBS for 1 h. The sections were labeled with diluted 1:10 monoclonal anti-ABCA4 Rim 3F4 antibody, 5% goat serum, 0.1% Triton X-100, PBS solution at 4°C overnight (3F4 hybridoma supernatant was kindly provided by Dr Robert S. Molday). After three washes in 0.1% Triton X-100 and PBS solutions, sections were labeled with secondary antibody, 0.2% goat anti-mouse antibody conjugated to Alexa Fluor 488 (Molecular Probes) mixed with 0.1 mg/ml Texas Red—conjugated wheat germ agglutinin—WGA (Molecular Probes) diluted in 5% goat serum, 0.1% Triton X-100, PBS solution for 3 h at room temperature. The nuclei were stained by the SlowFade Light Antifade Kit with DAPI (Molecular Probes), according to the manufacturer's instructions. The sections were analyzed by fluorescence microscopy with a Zeiss Axioscop microscope. The tadpoles generated with pXOP-ABCA4-GFP were sectioned and stained with WGA and DAPI as described earlier.

The images were obtained from at least two independent tadpoles expressing human ABCA4. For each retina, we analyzed (a) the localization of human ABCA4 in retinal cells, (b) the morphology of transgene-expressing photoreceptors and (c) the level of ABCA4 expression (number of expressing photoreceptors, the level of transgene expression per cell). To

further confirm our observations, we ‘traced’ the expression pattern by examining sections of the retina from the transgene-expressing animals, for additional independent images.

### ATPase activity assay

Transfections for ATPase assays were done with 24 µg of plasmid and 40 µl of Lipofectamine 2000 in 3 ml OptiMEM medium for each 10 cm (60 cm<sup>2</sup> surface area) plate (Invitrogen, Carlsbad, CA, USA). Mutant and wild-type constructs were transiently transfected in COS7 cells for 36–42 h. Cell harvesting and protein purification were performed, according to previously published protocols (29). The concentration of proteins used for ATPase activity assays was analyzed by western immunoblotting with 1D4 antibody. Densitometric analyses of developed hybridization films were measured via blot analysis software UN-SCAN IT (Silk Scientific Inc., Orem, UT, USA).

### Clinical correlations

To examine the genotype–phenotype correlations in a cohort of patients bearing at least one folding mutation, we compared clinical data together with molecular testing of subjects studied in our laboratory and those described in the literature (20,32,33). In particular, we searched for subjects with RP, CRD and STGD in whom two mislocalization-mutant alleles [L541P; A1038V], R602W and C1490Y were detected. We also searched for patients with STGD in whom one mislocalization-mutation was detected. For all patients studied in our laboratory, the anamnestic and ophthalmologic information for each individual was scored independently by a single observer (R.A.L.), who had no prior knowledge of the experimental studies.

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