Truncation mutations in ABCA1 suppress normal upregulation of full-length ABCA1 by 9-*cis*-retinoic acid and 22-*R*-hydroxycholesterol

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Abstract **Mutations in ABCA1 uniformly decrease plasma HDL-cholesterol (HDL-C) and reduce cholesterol efflux, yet different mutations in ABCA1 result in different phenotypic effects in heterozygotes. For example, truncation mutations result in significantly lower HDL-C and apoliprotein A-I (apoA-I) levels in heterozygotes compared with nontruncation mutations, suggesting that truncation mutations may negatively affect the wild-type allele. To specifically test this hypothesis, we examined ABCA1 protein expression in response to 9-*cis*-retinoic acid (9-*cis*-RA) and 22-*R*-hydroxycholesterol (22-*R*-OH-Chol) in a collection of human fibroblasts representing eight different mutations and observed that truncation mutations blunted the response to oxysterol stimulation and dominantly suppressed induction of the remaining full-length allele to 5–10% of wild-type levels. mRNA levels between truncation and nontruncation mutations were comparable, suggesting that ABCA1 expression was suppressed at the protein level. Dominant negative activity of truncated ABCA1 was recapitulated in an in vitro model using transfected Cos-7 cells. Our results suggest that the severe reduction of HDL-C in patients with truncation mutations may be at least partly explained by dominant negative suppression of expression and activity of the remaining full-length ABCA1 allele. These data suggest that ABCA1 requires a physical association with itself or other molecules for normal function and has important pharmacogenetic implications for individuals with truncation mutations.**—Wellington, C. L., Y-Z. Yang, S. Zhou, S. M. Clee, B. Tan, K. Hirano, K. Zwarts, A. Kwok, A. Gelfer, M. Marcil, S. Newman, K. Roomp, R. Singaraja, J. Collins, L-H. Zhang, A. K. Groen, K. Hovingh, A. Brownlie, S. Tafuri, J. Genest, Jr., J. J. P. Kastelein, and M. R. Hayden. **Truncation mutations in ABCA1 suppress normal upregulation of full-length ABCA1 by 9-*cis*-retinoic acid and 22-*R*-hydroxycholesterol. *J. Lipid Res.* 2002.** 43: **1939–1949.**

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Mutations in the ABCA1 gene have been identified as the underlying cause of Tangier Disease (TD) and of a dominantly inherited form of hypoalphalipoproteinemia (FHA) associated with reduced cellular cholesterol efflux (1–4). TD is a rare form of HDL cholesterol (HDL-C) deficiency in which patients have mutations in both alleles of ABCA1, resulting in greatly impaired cholesterol efflux and virtually no circulating HDL-C (5). Intracellular cholesterol accumulation in TD patients often results in clinical manifestations of orange tonsils, hepatosplenomegaly, and peripheral neuropathy (5).

Persons heterozygous for ABCA1 mutations have FHA, a less-severe clinical outcome of deficient cholesterol efflux (6). Generally, cells of FHA patients do not have excessive intracellular cholesterol ester accumulation, a feature attributed to the activity of the remaining wild-type allele. Compared with unaffected family members, heterozygotes have HDL-C and apolipoprotein A-I (apoA-I)

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; BMI, body mass index; 9-*cis*-RA, 9-*cis*-retinoic acid; FCS, fetal calf serum; FHA, hypoalphalipoproteinemia; GAPDH, glyceraldehyde phosphate dehydrogenase; 22-*R*-OH-Chol, 22-*R*-hydroxycholesterol; TD, Tangier Disease.

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levels that are reduced by approximately 40% accompanied by a mild decrease in apoA-II and decreased HDL-C particle size (6, 7). In some instances, certain FHA families have cholesterol efflux and HDL-C measurements that are considerably less than 50% of wild-type levels, raising the possibility that the function of the wild-type gene product is negatively affected by particular mutations (6). Understanding the molecular consequences of ABCA1 mutations on protein function will provide crucial information about the relationship between ABCA1 expression and HDL-C, which is a demonstrated protective factor against the development of cardiovascular disease.

To this end, we investigated the effect of eight different ABCA1 mutations on expression of ABCA1 mRNA and protein in primary fibroblasts in individuals from FHA and TD families (6). The mutations investigated in this study are dispersed throughout the ABCA1 gene and were specifically chosen to represent several different types, including missense, small in-frame deletions, frameshift, and nonsense mutations. ABCA1 expression is induced by 9-*cis*-retinoic acid (9-*cis*-RA) and 22-*R*-hydroxycholesterol (22-*R*-OH-Chol), which signal the presence of excess cholesterol (8–12). Here, we report that the normal upregulation of full-length ABCA1protein in response to 9-*cis*-RA, 22-*R*-OH-Chol is dominantly suppressed when one ABCA1 allele is prematurely truncated, resulting in ABCA1 protein levels that are approximately 5–10% of those observed in wild-type cells, despite comparable mRNA levels.

MATERIALS AND METHODS

Subjects

Subjects heterozygous for ABCA1 mutations from TD and FHA kindreds have been described (6). One new heterozygous subject is included in this study who is from a new TD kindred (JP2) and contains the truncation mutation R1851X (13). All subjects have informed consent for participation, and all received ethical approval by the University of British Columbia (6).

Cell culture and transfection

RAW murine macrophages and human fibroblasts were maintained in DMEM (Canadian Life Technologies) with 10% fetal calf serum (FCS), 50 U/ml penicillin-streptomycin, 2 mmol L-glutamine, 1× nonessential amino acids (Canadian Life Technologies), and 10 ng/ml epidermal growth factor (Sigma). ABCA1 expression was stimulated by the replacement of the growth medium with media containing delipidated serum (Sigma) with either ethanol (control) or 10 μmol/l 9-*cis* RA (10 mg/ml stock in ethanol, Sigma) and 4 μg/ml 22-*R*-OH-Chol (4 mg/ml stock in ethanol; Steraloids) for up to 24 h. Cos-7 cells were cultured in DMEM with 10% FCS, 50 U/ml penicillin-streptomycin, and 2 mmol/l L-glutamine. One day prior to transfection, cells were seeded at 1.6×10^5/well into 12-well plates coated with type I collagen. The next day, cells at approximately 95% confluence were transfected using Lipofectamine 2000 (Canadian Life Technologies) following the manufacturer's recommendations. Cells were transfected with wild-type or truncated human ABCA1 cDNA expression constructs tagged with the FLAG epitope (DYKDDDDK) at the C terminus to permit verification of expression in transfected cells as described (13).

Protein isolation and Western blotting

Cell pellets were lysed by trituration in 20 mmol/l HEPES, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5% (v/v) Triton X-100, and Complete protease inhibitor (Roche Molecular Biochemicals). Cell debris was removed by centrifugation at 5,000 rpm for 4 min at 4°C. The supernatant was collected and stored at −70°C. Protein concentration was determined by a Lowry assay. Equal amounts of protein were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride membrane (Millipore) prior to immunodetection with antibodies specific for the C terminus of ABCA1 (14), anti-glyceraldehyde phosphate dehydrogenase (GAPDH, Chemicon) as a loading control, anti-FLAG (Sigma), or anti-Xpress (Invitrogen), according to the manufacturers' recommendations. Immunoreactivity was detected by enhanced chemiluminescence (Amersham). Protein abundance was calculated by densitometry using National Institutes of Health Image 6.0 software and normalized to GAPDH levels. Densitometry was performed on duplicate or triplicate gels run from at least three independent experiments, and a wild-type control was included in each gel.

TD4 SNP analysis and Southern blotting

Genomic sequencing of the ABCA1 locus, including all exons, splice junctions, and promoter region was performed as previously described (1). Southern blot analysis of TD4 genomic DNA was performed as described (1), using DNA restricted with *Bam*HI, *Hind*III, or *Eco*RI and detection with either a probe encoding the entire human ABCA1 cDNA or corresponding to the promoter region of ABCA1 from −716 to intron 1.

RNA isolation

Cytoplasmic RNA was extracted by lysing cell pellets in 10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP40 followed by centrifugation for 30 s. The supernatant was mixed with an equal volume of 200 mM Tris, pH 7.5, 350 mM NaCl, 2% SDS, 25 mM EDTA, extracted twice with phenol-chloroform, once with chloroform, and precipitated with 1 ml ethanol. Pellets were resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1U RNase-free DNaseI (Roche Molecular Biochemicals), incubated for 30 min, phenol extracted, and precipitated with ethanol. Pellets were resuspended in RNase-free water and quantitated by absorbance spectrophotometry.

Northern blotting

Equal amounts (20 μg) of cytoplasmic RNA were electrophoresed through 1% agarose, 2 M formaldehyde gels in MOPS buffer, and then transferred to Hybond N+ membrane (Amersham). Membranes were prehybridized and hybridized in a buffer containing 50% formamide, 10% dextran sulfate (w/v), 0.2% BSA, 0.2% Ficoll, 50 mM Tris HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.5% polyvinylpyrrolidone, 1M NaCl, and 0.1 mg/ml denatured salmon sperm DNA. Murine ABCA1 was detected using a 605 bp fragment of spanning nt positions 1–605 of the murine ABCA1 sequence (Genbank accession X75926). Probe was randomly labeled with [α-^{32}P]dCTP using Rediprime (Amersham) and incubated with the membrane overnight at 42°C. Membranes were washed sequentially in 2× SSC at 42°C, and 2× SSC, 1% SDS at 60°C prior to exposure of film.

Quantitative PCR

Human ABCA1 primers (forward 5′ CCTGACCGGGTTGTTCCC 3′ and reverse 5′ TTCTGCCGGATGGTGCTC 3′ and the TaqMan probe (5′ACATCCTGGGAAAAGACATTCGCTCTGA 3′) were designed using Primer Express software (Applied Biosystems, Foster City, CA). RT-PCR reactions were performed on

an ABI P_{RISM} 7700 machine in a final volume of 50 μl, containing 40 ng of total RNA, 200 μM human ABCA1 primers, and 600 μM probe in 1× TaqMan One-Step RT-PCR Master mix (PE Biosystems, CA), according to the manufacturer's instructions. Ribosomal 18s rRNA primers and probe mix (PE Biosystems) were included in the reaction as an internal control. RT reactions were run at 48°C for 30 min and heat inactivated at 95°C for 10 min. PCR reactions were carried out for 30 cycles (denaturation at 95°C for 15 s, annealing and extention at 60°C for 1 min). Data quantification and analysis were performed according to the manufacturer's protocol (PE Biosystems). Values were calculated relative to the level of the control. Each sample was assayed in duplicate in at least two independent experiments.

For semiquantitative analysis of RNA extracted from transfected Cos-7 cells, RT-PCR reactions using murine-specific or human-specific ABCA1 and 18 S ribosomal primers were performed as previously described (14).

Cholesterol efflux

Lipid levels in TD and FHA patients were measured as described and HDL-C was expressed as mmol/l (1, 4). Cellular cholesterol efflux assays on primary human fibroblasts were performed as described (1, 4, 6). Briefly, efflux in the absence or presence of either 20 or 30 μg/ml apoA-I was measured over 24 h and calculated as the percent of free cholesterol in the medium relative to total cholesterol. Each experiment was performed in triplicate, averaged, and expressed as the percentage of efflux relative to the average efflux values obtained from primary fibroblasts of two healthy controls included as standards within the same experiment. Transfected Cos-7 cells were labeled with 1.0 μCi/ml ^{3}H-cholesterol on the day after transfection. After labeling for 18 h, the cells were washed three times with PBS and incubated for 2 h with DMEM plus 0.2% defatted BSA (Sigma). The media was then replaced with fresh DMEM and 0.2% BSA with or without 15 μg/ml apoA-I for 4 h. Radioactivity in the media was counted directly, cells were dissolved with 0.1 N NaOH and counted, and percent efflux was calculated as above. Each experiment was performed at least in triplicate.

Statistical analysis

Demographics, HDL-C, and apoA-I levels in truncation versus nontruncation heterzygotes were compared using a two-tailed Student's *t*-test. Analysis of the proportion of individuals within given HDL-C percentile ranges was performed by χ^2 analysis. Comparisons of ABCA1 mRNA and protein expression were performed using two-tailed unpaired Student's *t*-tests or, where applicable, one-way ANOVA with a Neuman-Keuls posttest. All statistical analyses were performed using Prism (version 3.0; Graphpad Software for Science Inc., San Diego, CA) or Systat (version 8.0, SPSS Inc, Chicago, IL).

RESULTS

HDL-C and apoA-I levels are significantly reduced in heterozygous carriers of mutations resulting in truncated compared with nontruncated ABCA1 alleles

We previously examined the phenotypes of 77 heterozygous individuals from 11 FHA and TD families that represent 13 different ABCA1 mutations and identified an approximately 40–50% decrease in HDL-C and apoA-I in heterozygotes compared with unaffected family members (6). In the present analysis, we excluded two individuals presumed to be heterozygous based on phenotype and cosegregation analyses because no mutation within the ABCA1 open reading frame has yet been identified. We also obtained one additional confirmed heterozygous individual (JP2) (13). The cohort analyzed in this study therefore consisted of 76 confirmed heterozygous individuals.

To determine the significance of mutation type on HDL-C and apoA-I levels, heterozygous individuals from these families were divided into two groups. One group consisted of all individuals with any mutation predicted to result in premature truncation of ABCA1, including nonsense, frameshift, and splice-site mutations. The other group consisted of individuals with missense or small in-frame deletions that would result in maintenance of a full-length allele and specific preservation of the C-terminal end of ABCA1. This division differed from a previous analysis (6) by separating the groups on the basis of predicted protein length (truncation vs. nontruncation) rather than on that of presumed severity (missense vs. truncation as well as in-frame deletions).

Comparison of the overall demographics between the truncation and nontruncation groups showed no differences in age, body mass index (BMI), or gender distributions (**Fig. 1A**). In contrast, apoA-I and HDL-C levels were

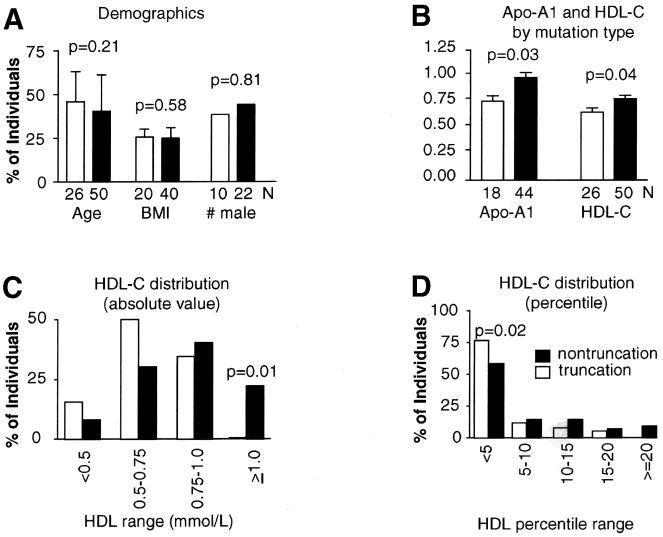

Fig. 1. Effect of ABCA1 mutation type on HDL cholesterol (HDL-C) and apolipoprotein A-I (apoA-I) levels in a cohort of 76 confirmed heterozygous individuals from 12 different hypoalphalipoproteinemia (FHA) and Tangier Disease (TD) families representing truncation (white bar) and nontruncation (black bar) heterozygotes. A: Age, body mass index (BMI), and sex are not significantly different between heterozygous carriers of truncation compared with nontruncation mutations. The truncation group contained 26 individuals, for whom BMI (kg/m^2) data were available for 20 persons. Ten of 26 persons were male in the truncation group. Similarly, the nontruncation group consisted of 50 individuals for whom BMI data were present for 40 persons, and 22/50 individuals were male. Comparison was done by Student's *t*-test. B: HDL-C (mmol/l) and apoA-I (g/l) levels are reduced in heterozygotes of truncation compared with nontruncation mutations. The HDL-C and apoA-I values from heterozygotes for truncation or nontruncation ABCA1 mutations were averaged and compared using a Student's *t*-test. C and D: HDL-C distributions by absolute level (C) and by age- and sex-normalized percentile (D) analyzed by χ^2 analysis.

significantly reduced in heterozygotes for truncation mutations compared with nontruncation mutations ($P = 0.03$ and 0.04, respectively) (Fig. 1B). Comparison of the distribution of HDL-C levels between the groups showed that HDL-C levels are shifted toward lower levels in truncation compared with nontruncation heterozygotes (Fig. 1C, D). Significantly, not a single heterozygote for a truncation mutation had an HDL-C level greater than 1.0 mmol/l, whereas these levels were observed in approximately 20% of nontruncation mutation heterozygotes ($P = 0.01$) (Fig. 1C). Finally, significantly more truncation heterozygotes had HDL-C levels below the 5th percentile for age and sex than nontruncation heterozygotes (Fig. 1D, $P = 0.02$). These results provide the first indication that the type of mutation in ABCA1 has a significant effect on HDL-C levels in vivo and that truncation mutations depress HDL-C levels significantly more than nontruncation mutations.

Dynamics of ABCA1 expression upon 9-*cis*-RA, 22-*R*-OH-Chol treatment

We then sought to determine the mechanism underlying the effect of mutation type on HDL-C levels using a collection of primary human fibroblasts representing eight different mutations in ABCA1. Because cAMP does not induce ABCA1 expression in fibroblasts (15), we began by characterizing the dynamics of ABCA1 expression in primary human fibroblasts obtained from healthy controls and RAW macrophages after treatment with 9-*cis*-RA, 22-*R*-OH-Chol (8). In primary human fibroblasts, ABCA1 mRNA expression peaks 8 h after stimulation with 9-*cis*-RA, 22-*R*-OH-Chol and diminishes slowly to baseline levels by 24 h (**Fig. 2A, C**). ABCA1 protein levels rise rapidly between 4 and 8 h, peak at 16 h, and are slightly lower at 24 h than at 16 h in fibroblasts (Fig. 2B, C). In RAW cells, ABCA1 mRNA peaked 4 h after stimulation, whereas protein levels were maximal by 8 h and both mRNA and protein levels declined to near baseline levels by 24 h (Fig. 2D).

Truncated ABCA1 dominantly suppresses protein expression of wild-type ABCA1 in human fibroblasts

Having determined appropriate conditions for detecting ABCA1 protein in primary fibroblasts, the effect of ABCA1 mutations was evaluated by Western blot analysis of protein extracted from primary, nontransformed human fibroblasts from eight FHA and TD families that were specifically selected to represent a variety of ABCA1 mutations distributed throughout the ABCA1 coding region. The fibroblasts examined contained several types of mutation, including missense, in-frame deletion of one or more amino acids, frameshift, and nonsense mutations (**Table 1**). Several of the mutations, including a missense splice site mutation, frameshift, and nonsense mutations, would be predicted to result in premature termination of translation of the mutated ABCA1 allele, resulting in the generation of a truncated ABCA1 fragment. Because both antibodies used in this study recognize the C terminus of ABCA1, Western blot analysis of the human fibroblasts

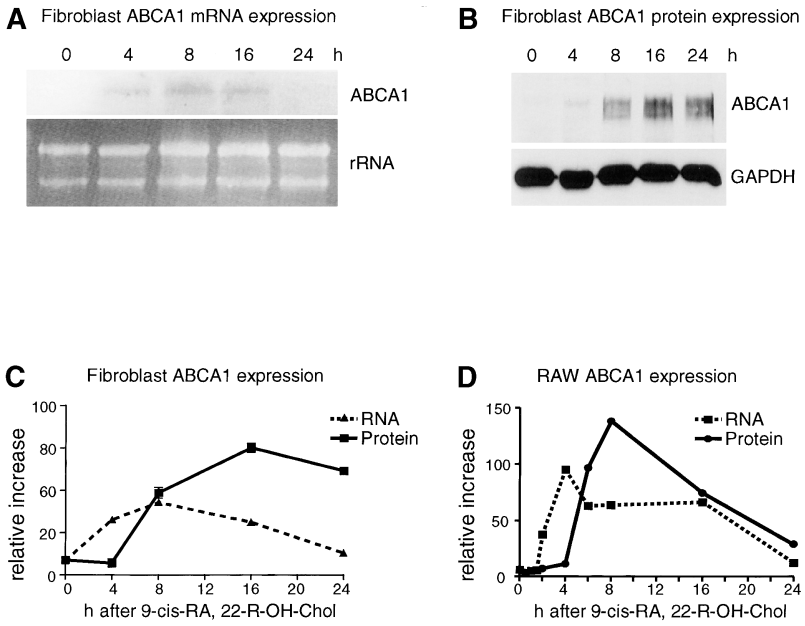

Fig. 2. Dynamics of ABCA1 expression in primary human fibroblasts and RAW murine macrophages in response to 9-*cis*-retinoic acid (9-*cis*-RA), 22-*R*-hydroxycholesterol (22-*R*-OH-Chol) stimulation. A: Northern blot showing induction of ABCA1 mRNA from 4 to16 h after stimulation. B: Western blot showing stimulated normal human fibroblasts contain abundant ABCA1 protein from 8 to 24 h after stimulation. C: Densitometric quantitation of expression dynamics in human fibroblasts. ABCA1 mRNA levels were expressed relative to rRNA levels, and ABCA1 protein levels were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) expression. Data are representative of two independent experiments. D: Densitometric quantitation of induction kinetics of murine ABCA1 mRNA and protein showing maximal mRNA expression at 4 h and maximal protein expression 8 h after stimulation.

TABLE 1. Expression data by mutation

Family	Mutation	Protein[c] Induced/Uninduced	HDL-C	Net Efflux[d]
			mmol/l	*% of control*
FHA1	Del L 693	5.95 (0.82)	0.4	79.47 (22.63)
FHA2	R2144X	2.45 (0.19)	0.18	64.01 (11.12)
FHA3	Del E,D 1893, 1894	7.82 (1.48)	0.39	60.03 (11.85)
FHA4	R909X	2.32 (0.52)	0.18	72.28 (18.01)
FHA5	M1091T	6.42 (0.29)	0.1	47.24 (3.79)
TD1	ivs2511G-C, C1477R	3.46 (0.50)	0.1	2.73 (1.05)
TD1-h[a]	C1477R	10.28 (1.07)	0.9	58.14 (5.49)
TD3	GG 5277C - 1636	2.89 (0.59)	0.09	23.3 (1.29)
TD3-h[b]	T9291	6.65 (0.10)	1.12	51.8 (1.30)
TD4	Del C 6825 - 2145X, unidentified	1.14 (0.13)	0.03	17.22 (0)
Control	None	11.31 (0.68)	1.63	100.00 (7.09)

[a] TD1-h is the heterozygous parent of the TD1 proband.
[b] TD3-h is the heterozygous parent of the TD3 proband.
[c] Protein values are pooled from all determinations (n = 3–7) and are represented as mean (standard error).
[d] Efflux values are pooled from at least three determinations expressed as % mean (standard deviation) relative to control fibroblasts from the same experiment.

containing truncation mutations would only detect the response of the remaining full-length allele to 9-*cis*-RA, 22-*R*-OH-Chol stimulation. In the case of heterozygous FHA fibroblasts, the remaining allele is wild-type and is predicted to be functional (Table 1). Cells from the TD probands are compound heterozygotes and contain one truncation and one nontruncation mutation (Table 1).

The pattern of ABCA1 protein expression in response to a 16 h treatment with 9-*cis*-RA, 22-*R*-OH-Chol fell into two groups. In one group, the abundance of ABCA1 protein in all fibroblasts with missense or small in-frame deletions was approximately 70% that observed in wild-type fibroblasts after 16 h of stimulation (**Fig. 3A**, **B**, Table 1). A second pattern was observed in FHA or TD fibroblasts that contained one truncated allele. In these cells, the abundance of the remaining full-length ABCA1 allele in response to 9-*cis*-RA, 22-*R*-OH-Chol treatment was markedly reduced (Fig. 3, Table 1). Densitometric quantitation of ABCA1 protein levels demonstrated that in carriers of one truncated allele, the remaining full-length allele was induced only approximately 1.2- to 2.5-fold by 9-*cis*-RA, 22-*R*-OH-Chol, compared with the 10-fold mean induction observed from pooled data from three different wild-type control fibroblasts (Fig. 3A, B, Table 1). Importantly, dominant suppression was observed in all cases with a truncation mutation regardless of whether the second allele was wild type (in FHA fibroblasts) or contained an additional mutation (in TD proband fibroblasts). Additionally, equivalent suppression of protein expression was observed regardless of whether protein was harvested at 16 or 24 h after stimulation (data not shown). These findings suggest that truncation mutations act to suppress or prevent expression of full-length ABCA1 protein by a dominant negative mechanism.

Because the TD fibroblasts contain two mutations, we next tested whether the decrease in protein abundance in these compound heterozygous cells was due to the missense rather than the truncation mutation. ABCA1 protein levels in heterozygous fibroblasts from the TD1 and TD3 kindreds that contained only the missense mutations were comparable to a wild-type control and were clearly more abundant than in the TD1 or TD3 compound heterozygous cells (Fig. 3C). Densitometic quantitation showed that the missense mutation alone resulted in a decrease of no more than 30% in fold induction of ABCA1 protein upon stimulation with 9-*cis*-RA, 22-*R*-OH-Chol, whereas the additional presence of a truncation mutation further decreased ABCA1 protein abundance by approximately 65% compared with wild-type control (Fig. 3D). Additionally, efflux assays on these primary fibroblasts demonstrated that the missense mutation alone slightly diminished apoA1-dependent cholesterol efflux compared with wild-type controls, whereas the combination of a missense plus a truncation mutation further decreased efflux activity nearly to baseline levels (Fig. 3E). These data confirm that truncation rather than missense mutations in ABCA1 dominantly suppress expression and functional activity of ABCA1.

As a negative control, we included TD4 fibroblasts, which are homozygous for a deletion of C6825 that would result in truncation of both ABCA1 gene products. Homozygosity in TD4 cells is supported by complete sequencing of the ABCA1 gene, a cSNP analysis that demonstrated homozygosity across the ABCA1 gene except at the extreme 5′ end, and Southern blot analysis demonstrating equivalent bands detected in both TD4 and wild-type control lanes (data not shown). Even in stimulated TD4 fibroblasts, ABCA1 protein is undetectable by either C-terminal antibody, which confirms the specificity of these antibodies to ABCA1 without detectable cross-reactivity to other ABC transporters.

Suppression of ABCA1 protein induction by 9-*cis*-RA, 22-*R*-OH-Chol occurs at the protein level in fibroblasts containing one truncated allele

The failure to observe upregulation of wild-type ABCA1 protein by 9-*cis*-RA, 22-*R*-OH-Chol in fibroblasts containing one truncated allele could occur at the mRNA or protein levels. To distinguish between these possibilities, cytoplasmic RNA was collected from fibroblasts before and after an 8 h treatment with 9-*cis*-RA, 22-*R*-OH-Chol and quantitatively analyzed for ABCA1 expression using real-

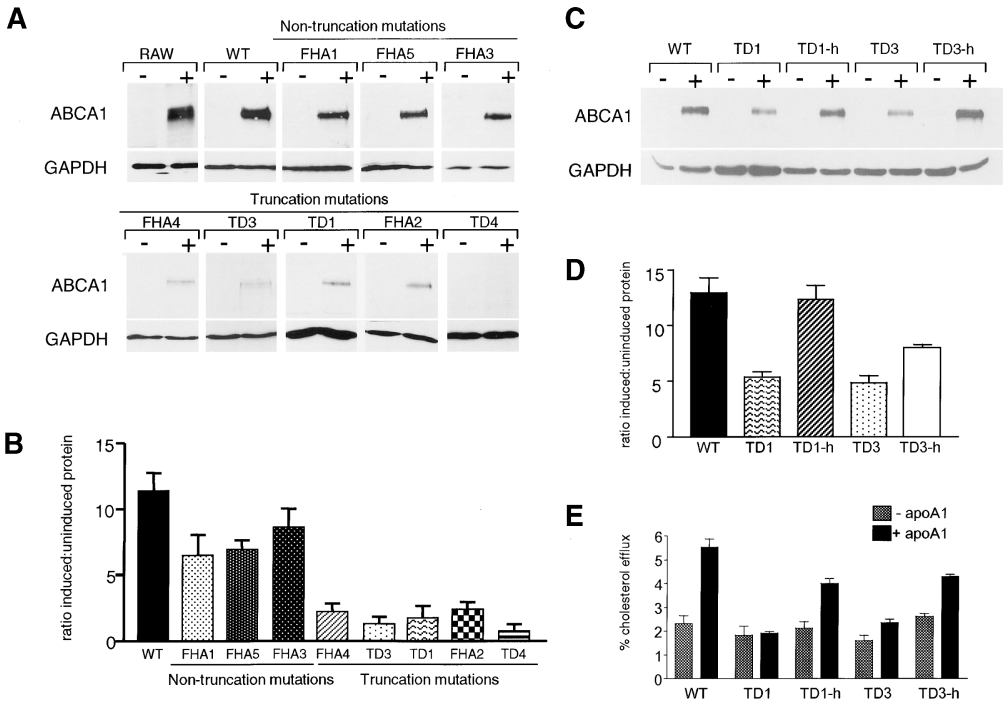

Fig. 3. ABCA1 protein expression in response to 9-*cis*-RA, 22-*R*-OH-Chol stimulation. A: RAW murine macrophages, wild-type human primary fibroblasts, and primary fibroblasts from eight TD or FHA families were harvested after a 16 h exposure to delipidated serum growth media without (−) and with (+) 10 μM each of 9-*cis*-RA, 22-*R*-OH-Chol. ABCA1 protein is markedly reduced in stimulated cells with truncation mutations compared with nontruncation mutations. Equivalent results were obtained using two different verified ABCA1 antibodies. B: Densitometric quantitation of ABCA1-GAPDH protein upregulation in stimulated fibroblasts, demonstrating reduced ABCA1 levels in stimulated fibroblasts with truncation mutations compared with nontruncation mutations. Values represent the mean of 3 to 5 independent experiments for each fibroblast cell type. Three different wild-type fibroblasts, each assayed in triplicate, were pooled to give an overall induction in wild-type fibroblasts. Statistical comparison is by one-way ANOVA with a Neuman-Kuels posttest. C: Western blot of ABCA1 protein in wild-type, TD1, and TD3 compound heterozygote, and TD1-h and TD3-h heterozygous fibroblasts stimulated as above. D: Densitometric quantitation of triplicate experiments from C, in which one wild-type fibroblast was used as a control. Data from the quantitations in B and D were pooled to give the values represented in Table 1. E: Cholesterol efflux activity over 24 h in wild-type, TD1, and TD3 compound heterozygote, and TD1-h and TD3-h heterozygous fibroblasts. Data represent the mean of triplicate measurements performed side by side with two wild-type controls (pooled) in the absence (−) or presence (+) of 20 μg/ml apoA-I.

time RT-PCR analysis using the Light Cycler (Roche). The 8 h time point was selected because it coincided with the midpoint of mRNA expression as determined by Northern blot analysis of wild-type fibroblasts. Real-time PCR quantitation showed that ABCA1 mRNA was present in each fibroblast type 8 h after stimulation (**Fig. 4A**). Although there are differences in the level of ABCA1 mRNA detected among fibroblasts from different genetic backgrounds, there were no significant differences between the truncation compared with nontruncation samples ($N = 5–8$, $P > 0.05$), confirming that ABCA1 mRNA is upregulated in response to 9-*cis*-RA, 22-*R*-OH-Chol in all cell types. Additionally, we determined the ratio of fold induction of ABCA1 protein relative to the fold induction of ABCA1 mRNA (Fig. 4B). Furthermore, wild-type cells and the nontruncation mutations were found to generate similar levels of ABCA1 protein per mRNA (Fig. 4B, $P > 0.05$), whereas all of the fibroblasts containing truncation mutations were impaired in their ability to generate ABCA1 protein (Fig. 4B, $P < 0.0001$). Additionally, the induction of ABCA1 mRNA is not significantly different between truncation and nontruncation fibroblasts ($P > 0.05$). Therefore, the failure to observe ABCA1 protein upregulation in cells containing truncation mutations is most likely due either to decreased translational efficiency of the full-length allele or increased turnover of the full-length protein in the presence of the truncated protein product.

Dominant negative suppression of ABCA1 activity in vitro

Data presented above support the hypothesis that truncation mutations in one ABCA1 allele dominantly suppress protein induction of the remaining full-length allele. To test this hypothesis further, we evaluated the effect of truncation versus missense mutations on ABCA1 expression and cholesterol efflux activity using Cos-7 cells cotransfected with wild-type and truncated or nontruncated human ABCA1 cDNA constructs.

Cells transfected with Xpress-tagged wild-type ABCA1 alone expressed ABCA1 protein and exhibited apoA-I-dependent cholesterol efflux compared with vector only (mock) transfected cells ($P < 0.001$, $n = 3$, **Fig. 5A, B**). Cotransfection of FLAG-tagged wild-type and Xpress-tagged wild-type ABCA1 resulted in a doubling of efflux activity ($P < 0.001$, $n = 3$), and Western blotting showed that wild-type ABCA1 protein was expressed from both

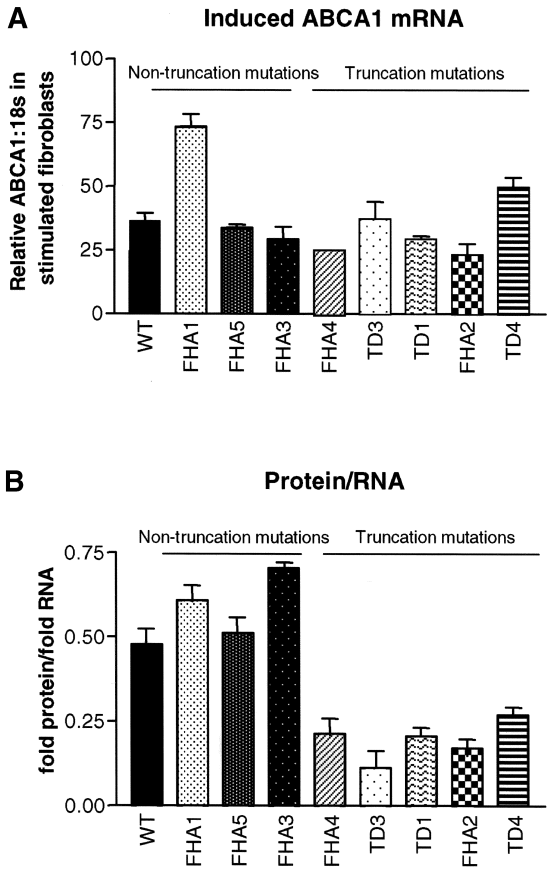

Fig. 4. ABCA1 mRNA expression in response to 9-*cis*-RA, 22-*R*-OH-Chol stimulation in human fibroblasts. Cytoplasmic RNA was collected from unstimulated cells as well as cells stimulated for 8 h and subjected to quantitative determination of ABCA1 mRNA using the Light-Cycler. A: Data from two independent experiments, each performed in triplicate, are pooled. Levels of ABCA1 mRNA in stimulated cells vary among individual fibroblasts, but are not consistently lower in truncation versus nontruncation samples. Statistical comparison is by one-way ANOVA with a Neuman-Keuls posttest. B: Relative protein per RNA was calculated by determining the ratio of fold induction of ABCA1 protein (normalized to GAPDH) relative to fold induction of ABCA1 mRNA (normalized to 18s rRNA) in stimulated compared with unstimulated fibroblasts. Values represent means from two independent experiments, with each measurement performed in triplicate. Statistical comparison is by one-way ANOVA with a Neuman-Keuls posttest.

constructs (Fig. 5A, B). It should be noted that because the Xpress and FLAG antibodies have different affinities, Western blotting cannot be used to estimate the relative levels of ABCA1 expressed from either tagged construct.

Next, cells were cotransfected with Xpress-tagged wild-type and FLAG-tagged mutant ABCA1. By Western blot, expression of the FLAG-tagged truncated construct is detectable but low, suggesting that truncation of ABCA1 may destabilize the protein (Fig. 5A). Additionally, abundance of the wild-type Xpress-tagged construct was reduced when coexpressed with truncated ABCA1. Coexpression of FLAG-tagged ABCA1 truncated at amino acid 1851 (R1851X) significantly inhibited efflux compared with cells transfected with a single copy of wild-type ABCA1 ($P < 0.001$, n = 3, Fig. 5B). Finally, cells were cotransfected with Xpress-tagged wild-type and FLAG-tagged missense ABCA1 (N1611D). Western blot analysis confirmed that both alleles expressed protein and that the presence of ABCA1 containing a missense mutation did not decrease the abundance of wild-type ABCA1 (Fig. 5A). As expected, efflux was comparable to cells singly transfected with wild-type ABCA1 (Fig. 5B, $P > 0.05$, n = 3). These data confirm that truncated ABCA1 interferes with the stable expression and functional activity of wild-type ABCA1.

To rule out the possibility that differences in mRNA expression between the FLAG-tagged wild-type, truncation, and missense constructs were responsible for the observed differences in protein level and efflux activity, semiquantitative RT-PCR analysis was performed on transfected cells. The results show that each of the FLAG-tagged constructs is similarly expressed in transfected cells (Fig. 5C), confirming that the dominant negative inhibition of ABCA1 protein levels and efflux activity occurs at a posttranscriptional level.

ABCA1 protein levels predict HDL-C levels

The clinical spectrum of TD and FHA patients results from defective ABCA1-mediated efflux of cholesterol and/or phospholipid, which in turn is a function of ABCA1 level and catalytic activity. Increasing ABCA1 protein levels in mice has recently been shown to increase HDL-C levels in vivo in two of three animal models (16–18). Because the sample size of fibroblasts is small, it is difficult to draw strong conclusions about the effect of ABCA1 protein level and HDL-C levels in vivo. Nevertheless, we did observe that protein induction and HDL-C correlated strongly (**Fig. 6A**, $r^2 = 0.67$, $P = 0.002$), suggesting that the degree of ABCA1 protein expression in primary fibroblasts predicted HDL-C levels in vivo. ABCA1 protein levels and cholesterol efflux activity in fibroblasts was also correlated and nearly reached statistical significance (Fig. 6B, $r^2 = 0.33$, $P = 0.06$). Strikingly, for both correlations, all of the fibroblasts expressing ABCA1 with truncation mutations clustered near the bottom of the curve, whereas all of the fibroblasts expressing nontruncation ABCA1 mutations clustered at an intermediate position between wild-type and truncation mutation cells.

DISCUSSION

ABCA1 has been identified as a key initiator of reverse cholesterol transport and plays an important role in mediating susceptibility to atherosclerosis (19). Using a large cohort of affected and unaffected family members that allows us to partly control for other genetic and environmental influences, we recently showed that ABCA1 heterozygotes have reduced HDL-C and apoA-I levels that are gene-dose dependent and demonstrate a critical role for ABCA1 in determining HDL-C levels (6). In this analysis, the effect of mutation type was assessed by comparing the HDL-C levels in carriers of missense versus frameshift, nonsense, and small in-frame deletions, and no significant effect was found (6). However, when separated by the ef-

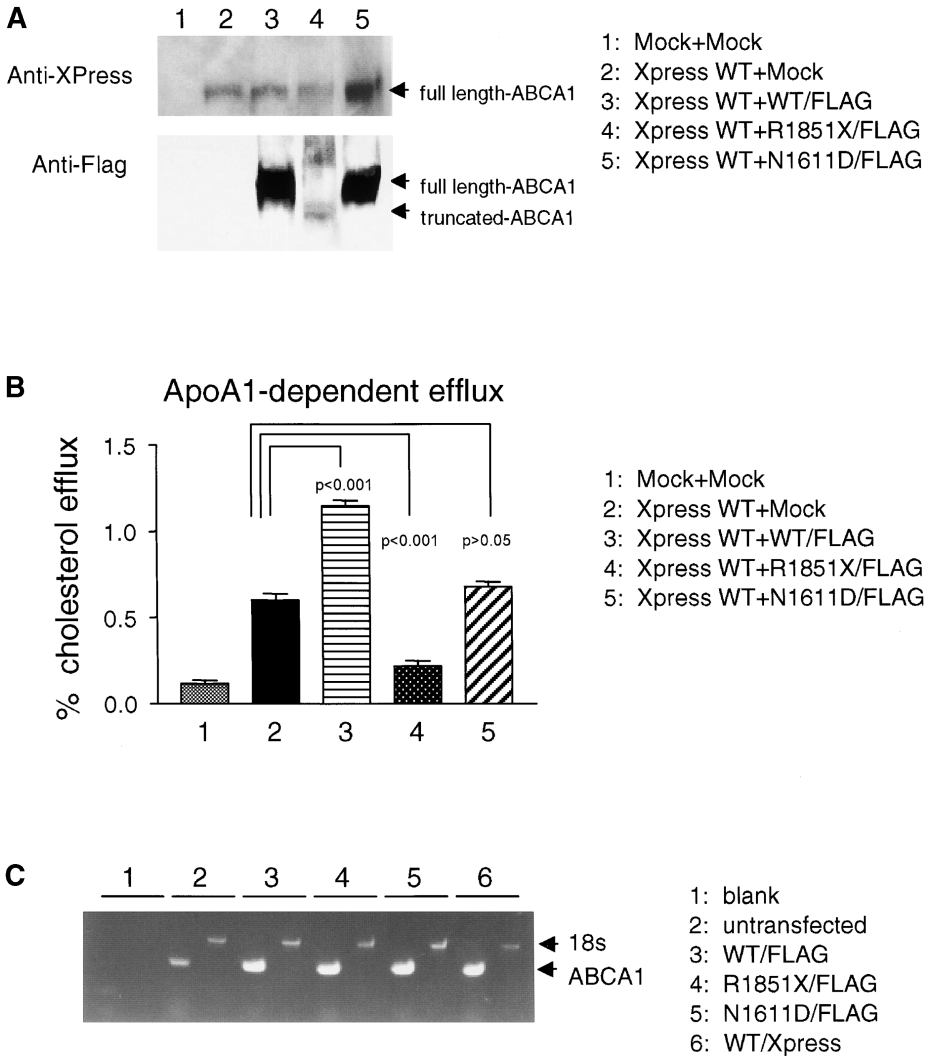

Fig. 5. Dominant negative suppression of ABCA1 activity in transfected cells. Cos-7 cells were transfected singly with vector (mock), Xpress-tagged wild-type human ABCA1 cDNA (WT/Xpress), or cotransfected with FLAG-tagged wild-type ABCA1 (WT/Xpress + WT/FLAG), FLAG-tagged ABCA1 containing the N1611D mutation (WT/Xpress + N1611D/FLAG), or with FLAG-tagged ABCA1 containing the R1851X mutation (WT/Xpress + R1851X/FLAG). A: Western blot analysis of vector or ABCA1-transfected cells using the Xpress or FLAG antibodies. The positions of full-length or truncated ABCA1 are marked. B: ApoA-I-dependent cholesterol efflux activity in vector or ABCA1-transfected cells. Efflux is expressed as the percent of counts in the medium relative to total counts in cells plus medium. Data are representative of three independent experiments. Statistical comparison is by one-way ANOVA with a Neuman-Kuels posttest. C: Semi-quantitative RT-PCR using human-specific ABCA1 primers as well as 18s rRNA primers as an internal control. RNA was prepared from untransfected Cos-7 cells or cells singly transfected with FLAG-tagged ABCA1 constructs.

fect of the mutation on protein length, heterozygotes for truncation mutations were found to have significantly lower HDL-C and apoA-I levels than heterozygotes for nontruncation mutations, suggesting that truncation mutations have a deleterious effect in vivo.

We now provide a molecular mechanism that may help to explain the clinical variation of individual phenotypes in FHA and TD kindreds through the analysis of ABCA1 expression in primary human fibroblasts representative of a variety of mutation types located throughout the ABCA1 protein coding region. We observed a profound effect of mutation type on the ability of ABCA1 protein to be up-regulated in response to oxysterol stimulation. Premature truncations of ABCA1 were found to dominantly inhibit expression of the remaining full-length allele, regardless of whether the full-length allele was wild type or whether it contained a second, nontruncation mutation. In contrast, mutations that maintained ABCA1 translation to its natural C terminus resulted in protein levels that were approximately 70% of those observed in wild-type cells. Furthermore, suppression of ABCA1 expression by truncation mutations occurred at the protein level because ABCA1 mRNA levels are comparable in these fibroblasts. Therefore, a reduction in the steady-state level of ABCA1 protein must be due either to decreased translation or to increased degradation of ABCA1 protein. These findings

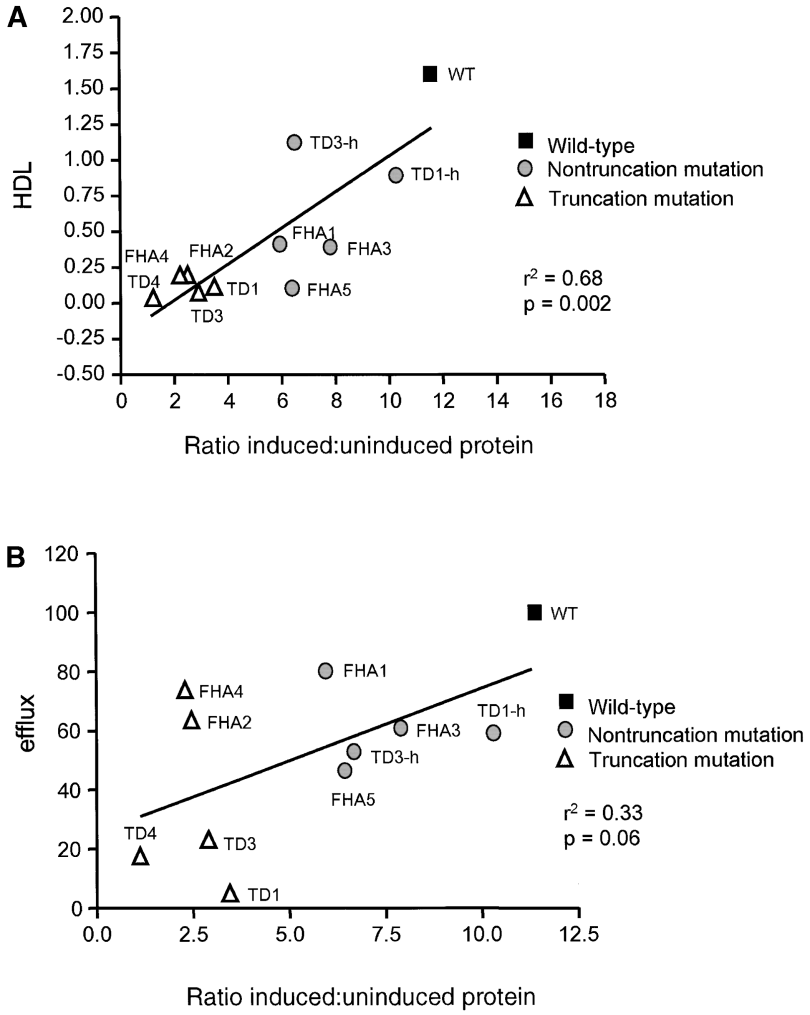

Fig. 6. ABCA1 protein levels correlate with plasma HDL-C levels and efflux activity. A: The fold increase in ABCA1-GAPDH levels observed upon stimulation of primary fibroblasts with 9-*cis*-RA, 22-*R*-OH-Chol (*x* axis) are plotted against the HDL-C levels from the patient from whom the fibroblasts were obtained (*y* axis), showing a strong, significant positive correlation ($r^2 = 0.68$, $P = 0.002$). Black square, wild-type; gray circle, nontruncation mutation; open triangle, truncation mutation. B: The fold increase in ABCA1:GAPDH levels (*x* axis) are plotted against the cholesterol efflux activity from the patient from whom the fibroblasts were obtained (*y* axis), showing a positive correlation that nearly reaches statistical significance ($r^2 = 0.33$, $P = 0.06$).

may explain why FHA or TD heterozygotes for truncation mutations are doubly disadvantaged; not only is one allele nonfunctional by virtue of a severe mutation, but in addition, expression of the remaining wild-type allele is also compromised.

Furthermore, our results suggest that ABCA1 protein levels strongly predict HDL-C and cholesterol efflux. Importantly, we observed that truncation and nontruncation mutations formed nonoverlapping clusters in correlation analyses between ABCA1 protein levels and either HDL-C or cholesterol efflux. For both correlations, all of the truncation mutations clustered near the bottom of these curves, consistent with dominant negative suppression of ABCA1 expression and activity by truncation mutations. These results confirm that truncation mutations are the most severe with respect to their effects on HDL-C levels, cholesterol efflux, and ABCA1 protein levels. In contrast, all of the nontruncation mutations clustered in an intermediate region of the curve, suggesting that nontruncation mutations do not generally have a dominant negative effect on expression of function of the remaining ABCA1 allele.

The dominant negative effect of ABCA1 truncation mutations was also confirmed in a synthetic in vitro system using Cos-7 cells transfected with tagged cDNA expression constructs encoding wild-type or truncated ABCA1. Coexpression of wild-type ABCA1 with ABCA1 containing a missense mutation did not inhibit expression or efflux activity of wild-type ABCA1. By contrast, coexpression of wild-type with truncated ABCA1 decreased cholesterol efflux from wild-type ABCA1 by approximately 65%, demonstrating that ABCA1 fragments dominantly interfere with ABCA1 functional activity.

Dominant negative effects are classically defined in terms of function and can manifest in several ways. Some dominant negative mutations inactivate catalytic activity while maintaining the ability to bind substrate (20–22).

Other common dominant negative mutations include truncated molecules that maintain ligand- or target-binding functions but lack other functional domains (23–26). Dominant negative mutations work by inhibiting the function of the wild-type allele, which often occurs by the mutated allele "poisoning" a molecular complex or titrating key components required for function. For example, many fibrillin-1 gene mutations generate fibrillin monomers that impair the global function of microfibrils and result in microfibrillopathies, including Marfan syndrome (27). Additionally, a truncation mutation in endoglin results in a protein fragment that dimerizes with wild-type endoglin protein and dominantly impairs its trafficking to the cell surface (28). Similarly, truncated V2 vasopressin receptors act as dominant negative regulators of wild-type V2 receptor function by inhibiting function and cell surface trafficking of full-length V2 receptors (29).

The finding that truncation mutations in ABCA1 can dominantly suppress protein expression of full-length ABCA1 suggests that association of ABCA1 molecules with other proteins may be required for maintenance of normal ABCA1 protein levels in cells. It is possible that ABCA1 may form higher order complexes that may be critical for its stable accumulation or expression at the cell surface. ABCA1 is a full-size ATP-binding cassette transporter and as such contains all domains required for transporter function in a single molecule (30). Quarter-sized and half-size transporters necessarily form functional complexes for activity (31–34). What is less clear is whether full-sized ATP-binding cassette transporters also have the ability to form functional dimers or other higher order complexes. Our results suggest that higher order complexes may be necessary for maintenance of protein abundance and functional activity at the cell surface because coexpression of one truncated allele is sufficient to significantly reduce overall protein levels and diminish efflux activity.

Much current research aims to identify compounds that increase ABCA1 levels in cells. However, if these compounds act to increase ABCA1 expression through transcriptional mechanisms, we would predict that they would be less effective in patients with truncation mutations, because expression of ABCA1 would remain impaired by the effect of the truncation mutation on ABCA1 translation or protein stability. Conversely, compounds that act at post-transcriptional levels to enhance ABCA1 expression or that enhance ABCA1 activity, perhaps through modulating the interactions of ABCA1 with its protein partners, may be more desirable as potential therapeutics for these patients.

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