The autosomal recessive hypercholesterolemia protein (ARH) is a 32-kDa endocytic adaptor protein involved in low-density-lipoprotein receptor (LDLR) endocytosis. The N terminus of ARH contains a highly conserved sequence of \( \approx 40 \) amino acids with unidentified function, followed by a phosphotyrosine-binding domain of \( \approx 130 \) residues. The C terminus is less conserved, but it contains a clathrin box sequence (LDLLE) and a binding motif for the \( \beta2 \) subunit of adaptor protein 2 (AP-2) and a PDZ binding motif with the potential to bind to many signaling proteins (reviewed by Soutar et al\(^1\)).

The uptake of plasma low-density lipoprotein (LDL) by eukaryotic cells occurs via clathrin-dependent LDLR-mediated endocytosis. Structure-function studies have shown that ARH regulates endocytosis of LDLR through binding of its phosphotyrosine-binding domain to the NPXY motif in the cytoplasmic tail of the LDLR and other members of its family and binding of its C-terminal motifs to clathrin heavy chain (CHC) and adaptor protein 2. This role is well established from studies in homozygous ARH patients, whose fibroblasts in culture fail to internalize LDLR and who exhibit severe hypercholesterolemia and premature atherosclerosis because LDL uptake is defective in the liver.\(^2,3\) ARH knock-out mice also show impaired endocytosis of LDLR and hypercholesterolemia.\(^4\) Surprisingly, cultured skin fibroblasts from ARH patients exhibit normal internalization of LDLR,\(^1\) possibly because Dab2 can compensate for the absence of ARH in these cells.\(^5,6\)

Although it is clear that ARH functions as an adaptor protein for LDLR internalization, there is substantial evidence to suggest that ARH may have additional functions. For example, hepatocytes express much higher levels of LDLR protein than other cells, yet the level of ARH protein in liver is similar to that in other tissues.\(^7\) Additionally, the subcellular distribution of ARH is the same in the presence or absence of the LDLR,\(^4\) suggesting that only a fraction of cellular ARH binds to the LDLR. ARH also binds to the LDLR-related receptor and megalin, but ARH deficiency does not affect the endocytosis of \( \alpha2 \)-macroglobulin, the major ligand of LDLR-related receptor,\(^4\) and in the case of megalin, ARH appears to be involved both in its intracellular trafficking and in presenting it at the cell surface.\(^8,9\) Finally, an ARH homologue has been found in the squid,\(^8\) but no LDLR family members have been identified in this species.

While studying the function of ARH in different cell types,\(^5\) we found that fibroblasts from ARH patients (ARH\(^{-/-}\) cells) grew more slowly than normal fibroblasts...
and more readily showed signs of premature senescence. This could be because ARH plays a role in cytokinesis through interaction with the centrosome, as demonstrated recently. However, in addition to premature senescence, we also observed misshapen nuclei and mitotic defects in ARH−/− cells. The aim of this study was to determine whether ARH functions in mitosis and to elucidate the molecular mechanisms underlying growth defects in cells from ARH-deficient patients.

Materials and Methods

Reagents

Nocodazole (Sigma-Aldrich) was dissolved in dimethyl sulfoxide at 1 mg/mL and stored at −20°C. Lambda protein phosphatase was obtained from New England BioLabs, Ro-3306 was from Alexis Biochemicals (Nottingham, United Kingdom). The 5-bromo-2′-deoxyuridine labeling and detection kit was purchased from Roche. Dynabeads, protein G, protein A, Lipofectamine 2000, and cell culture reagents were obtained from Invitrogen. 3xFLAG peptide was from Sigma-Aldrich. Cdc2/cyclinB kinase was obtained from Cell Signaling Technology. 4,6-Diamidino-2-phenylindole was from Vector Laboratories Inc.

Antibodies

The sources of antibodies were as follows: anti-FLAG (M2) and −/−/γ-tubulin, Sigma; anti-γ-H2AX, Upstate; anti-cdc2 and anti-phospho-cdc2, Cell Signaling Technology; horseradish peroxidase-labeled antibodies to mouse/rabbit/goat IgG, Dako; Alexa 488/568/633 labeled antibodies to mouse/rabbit/goat IgG, Dako; horseradish peroxidase-labeled antibodies to mouse/rabbit/goat IgG, Dako; Alexa 488/568/633 goat anti-mouse/rabbit IgG or donkey anti-goat/mouse/rabbit, Molecular Probes; anti-p16, p21, p53, lamin B1, Santa Cruz Biotechnology. The sources of antibodies were as follows: anti-FLAG (M2) and −/−/γ-tubulin, Sigma; anti-γ-H2AX, Upstate; anti-cdc2 and anti-phospho-cdc2, Cell Signaling Technology; horseradish peroxidase-labeled antibodies to mouse/rabbit/goat IgG, Dako; Alexa 488/568/633 goat anti-mouse/rabbit IgG or donkey anti-goat/mouse/rabbit, Molecular Probes; anti-p16, p21, p53, lamin B1, Santa Cruz Biotechnology. The sources of antibodies were as follows: anti-FLAG (M2) and −/−/γ-tubulin, Sigma; anti-γ-H2AX, Upstate; anti-cdc2 and anti-phospho-cdc2, Cell Signaling Technology; horseradish peroxidase-labeled antibodies to mouse/rabbit/goat IgG, Dako; Alexa 488/568/633 goat anti-mouse/rabbit IgG or donkey anti-goat/mouse/rabbit, Molecular Probes; anti-p16, p21, p53, lamin B1, Santa Cruz Biotechnology. The sources of antibodies were as follows: anti-FLAG (M2) and −/−/γ-tubulin, Sigma; anti-γ-H2AX, Upstate; anti-cdc2 and anti-phospho-cdc2, Cell Signaling Technology; horseradish peroxidase-labeled antibodies to mouse/rabbit/goat IgG, Dako; Alexa 488/568/633 goat anti-mouse/rabbit IgG or donkey anti-goat/mouse/rabbit, Molecular Probes; anti-p16, p21, p53, lamin B1, Santa Cruz Biotechnology.

Cell Culture and Synchronization of Cells

Fibroblasts from control subjects and ARH patients have been described previously. Cells were cultured at 37°C with 5% CO2. Skin fibroblasts and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with GlutaMAX, 10% fetal calf serum, and 100 units/mL penicillin and 100 μg/mL streptomycin. For synchronization in G2/M phase, cells were seeded into 6-cm diameter dishes and treated with nocodazole (50 ng/mL) for 16 to 20 hours. To obtain late mitotic HeLa cells, cells were shaken off the culture flasks and collected by centrifugation.

Expression Vectors, Site-Directed Mutagenesis, and Transfection
cDNA for wild-type or variants of ARH with a c-terminal myc or Flag tag was subcloned from pcDNA3 into pBabePuro for viral infection and expression of ARH or into pCherry for cellular localization of ARH. The W22X mutation was generated by site-directed mutagenesis (QuickChange kit, Stratagene) according to the manufacturer’s instructions and subsequently verified by nucleotide sequencing.

RNA Interference

For knocking down ARH in IMR90 cells, producer cells were transfected with retroviral vectors (pRetroSuper) encoding either a control or 2 different ARH short hairpin RNA sequences (listed in Supplemental Table I, available online at http://atvb.ahajournals.org). Viral particles were used to infect IMR90 cells, which were selected in medium containing puromycin to obtain a stable cell line depleted of ARH. Other cells were transfected as described previously with the following small interfering RNA (siRNA) oligonucleotides purchased from Dharmacon: D-001210-01-05 for control; D-013025-01-0020 for siARH1 and D-013025-02-0020 for siARH2; LQ-004001-01 for human CHC.

Growth Measurements

For measuring cell population doubling, cells were seeded at a constant density (3×104 per 5×6-cm flask) and at 5-day intervals were trypsinized, counted, and reseeded at the same density. Cell doubling was calculated according to the formula $F = (\log N - \log NO)/\log 2$, where NO is the number of cells on the day cells were seeded and N is the number of cells of each sample. For measuring the growth rate of IMR90 cells infected with siRNA expression virus, cells were seeded at density 5×103/well in 24-well plates. At time 0 and at 2- to 3-day intervals thereafter, cells were fixed with 0.5% glutaraldehyde for 10 minutes and stained with 0.2% crystal violet for 1 hour. After being washed 5 times with water, the crystal violet was dissolved in 1 mol/L acetic acid, and absorbance at 595 nm was measured. All measurements were in triplicate.

Immunoblotting and Coimmunoprecipitation

For whole cell extracts, cells were harvested and lysed in 500 μL of cold buffer containing 20 mmol/L Tris-HCl, pH 7.4, 137 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 2 mmol/L Na3VO4, 100 mmol/L NaF, 10 mmol/L Na4P2O7, and Complete protease inhibitor cocktail set III (CALBIOCHEM). For preparation of nuclei, cells were lysed in hypotonic buffer (50 mmol/L Tris, pH 7.4, 50 mmol/L mannitol, 2 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail); the nuclear pellet was collected by centrifugation at 500g for 10 minutes and solubilized in immunoprecipitation lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 100 mmol/L NaF, 200 μmol/L Na vanadate, and protease inhibitor cocktail). Immunoblotting was performed as described previously.

Immunoprecipitation experiments were performed with protein G magnetic beads (Dynabeads, Invitrogen). The beads were washed with immunoprecipitation lysis buffer and incubated with antibodies for 15 minutes at room temperature. The complexes were washed in immunoprecipitation lysis buffer, incubated for 45 minutes at room temperature with cytosolic or nuclear extracts, and then washed with 50 mmol/L Tris, pH 7.5, 20 mmol/L NaCl, 1 mmol/L MgCl2, 10% glycerol, 0.5 mmol/L EDTA, and protease inhibitor cocktail; bound proteins were eluted by incubation for 5 minutes at 98°C in SDS sample buffer containing 5% (vol/vol) mercaptoethanol.

Immunofluorescence

Cells were seeded onto coverslips at approximately 80% confluence. For immunofluorescence analysis, cells were fixed in 3% (wt/vol) paraformaldehyde for 20 minutes at room temperature and permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and blocked in phosphate-buffered saline containing 1% (wt/vol) bovine serum albumin. For better detection of nuclear proteins, cells were fixed and permeabilized in cold methanol (−20°C) for 10 minutes. For detection of proteins with antibodies, cells were incubated with primary antibodies for 1 hour (diluted 1/40 to 100) and then with Alexa 488/568/633–conjugated goat anti-mouse/rabbit or donkey anti-goat/mouse/rabbit IgG (highly cross-absorbed; diluted 1/100) for 1 hour, both at ambient temperature. Coverslips were mounted on slides with Vectashield antifade mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc). Cells were viewed on a Leica confocal microscope using the ×60 oil objective, and the images were analyzed using Leica confocal software (Leica Microsystems Ltd, Milton Keynes, United Kingdom).

5-Bromo-2′-Deoxyuridine Incorporation Assay

Cells (1×103/well) were plated onto coverslips to give ~50% confluence. After 24 hours, cells were incubated with 5-bromo-2′-deoxyuridine for 23 hours using the 5-bromo-2′-deoxyuridine labeling and detection kit (Roche Applied Science); all procedures were performed according to the protocol supplied.
Mass Spectrometry

Mass spectrometry (MS) for phosphorylation sites and ARH W22X protein sequence analysis were performed by NextGen Sciences (Cambridge, United Kingdom).

Results

ARH−/− Fibroblasts Grow Slowly and Display Premature Senescence

Serial passage of cultured skin fibroblasts from control subjects and ARH patients revealed that population doubling was reduced in ARH−/− cells (Figure 1A). ARH−/− cells also incorporated much less 5-bromo-2′-deoxyuridine than their normal counterparts (35.1 ± 5.01% 5-bromo-2′-deoxyuridine-positive cells versus 85.8 ± 7.98% during a 23-hour pulse, n > 200 cells in each of 3 independent experiments, P < 0.05; Figure 1B). ARH−/− cells appeared enlarged and flattened, and they overexpressed p16 (Figure 1C); they also exhibited larger DNA damage foci, as detected with anti-γ-H2AX (Figure 1D).

To confirm that the reduced growth rates in patients’ cells were due to the absence of ARH, we depleted ARH from IMR90 cells with viral vectors expressing 2 different siRNAs directed against ARH (pSuper-ARH-1 and -2). IMR90 cells were infected with supernatants from transfected packaging cells to produce stable lines of ARH-depleted cells; control cells were treated in the same way with a vector expressing control siRNA (pSUPER-con). ARH protein was depleted efficiently, as determined by immunoblotting (Figure 2A), and the growth rate was reduced in both these cell lines compared with a cell line infected with the control siRNA vector (Figure 2B).

ARH Is Phosphorylated By cdc2

The premature senescence and increase in p16 expression in ARH−/− cells reflect molecular changes that occur in ageing fibroblasts, and thus we investigated whether ARH expression decreases with age in control fibroblasts. Although this did not appear to be the case, we observed that ARH protein sometimes appeared as a doublet on immunoblots, suggesting that it might be phosphorylated. We also noted that its level fluctuated from one cell preparation to another, prompting us to investigate whether these changes were related to the cell...
Figure 2. Growth of autosomal recessive hypercholesterolemia (ARH)-depleted IMR90 cells. A, Knockdown of ARH with small interfering (si) RNA. Shown is the immunoblot of ARH protein in IMR90 cells stably expressing ARH-myc transfected with vector control or 2 different siRNA ARH constructs as indicated. Total cell extracts (30 µg of protein/lane) were fractionated on nondenatured SDS-polyacrylamide gels (13%), transferred to nylon membranes, and immunoblotted with antibodies to ARH and γ-tubulin. B, Growth rate of IMR90 cells. Cells were treated with control siRNA or 2 different siRNAs to ARH or were transfected with a construct expressing Ras (as a control for growth inhibition); growth rate was determined as described in Methods. 

When we synchronized fibroblasts in G2/M phase with nocodazole, a slowly migrating band of ARH was observed in lysates of the cells (Figure 3B), whereas triple bands were detected with anti-ARH in lysates of HeLa cells in G2/M (Figure 3C). These more slowly migrating bands were abolished after treatment of the lysates with λ protein phosphatase (Supplemental Figure I), suggesting that ARH is phosphorylated during the cell cycle. ARH contains 2 sites that match the consensus S/T-P consensus motif for cdc2 kinases17: amino acids R13-S14-P15-S16 and S94-P95-R96-G97. MS analysis of ARH from HeLa cells transfected with ARH-flag (Figure 3D) revealed 2 phosphorylated peptides in ARH. One of these, ALIRS14PSLAK, contained the identified SP motif for cdc2, but no peptide containing S94 was found. The second phosphorylated peptide was RDKAS186QEGGDVLGAR, containing a QS recognition motif for ATM/ATR.18

We and others have observed that cells from patients with mutations in ARH that introduce a premature stop codon, due either to a single base substitution in the codon for residue W22 or to a frameshift caused by deletion of GC (nt 86 to 7, G23+8X), produce a truncated immunoreactive ARH protein (Supplemental Figure II). We expressed the cDNA for W22X ARH in HeLa cells and determined the N-terminal sequence of the truncated ARH protein produced by MS. The results showed that translation initiated at residue Met46, the next methionine downstream from the premature stop codon, and thus the mutant truncated protein lacks the phosphorylation site at S14. Figure 3E shows a diagram of ARH protein showing the position of the phosphorylated peptides relative to other features of the protein.

Many substrates of cdc2 are involved in mitosis. To verify that cdc2 phosphorylated ARH in vivo, we examined the effect of roscovitine, a cell-permeable inhibitor of cdc2 kinase. Inhibition was monitored by analyzing phosphorylation of Dab2, a known substrate for cdc2.19 Treatment of control fibroblasts with nocodazole to synchronize cells in G2/M induced phosphorylation of both Dab2 and ARH, as shown by the shift toward slowly migrating bands on gels of cell lysates (indicated as Dab2-p in Figure 4A or ARH-p in Figure 4B). No slowly migrating bands were seen in cells treated with both nocodazole and rescovitine.

Figure 3. (A) Western blot analysis of ARH protein content during the cell cycle. IMR90 cells were preincubated in medium containing low serum (0.2% vol/vol) to arrest the cells in G1, and then released by incubation with 10% serum. ARH protein content was significantly reduced 20 hours after the switch to 20% control or 2 different siRNA ARH constructs as indicated. Total cell extracts (30 µg of protein/lane) were fractionated on nonreduced SDS-polyacrylamide gels (13%), transferred to nylon membranes, and immunoblotted with antibodies to ARH and γ-tubulin. B, Growth rate of IMR90 cells. Cells were treated with control siRNA or 2 different siRNAs to ARH or were transfected with a construct expressing Ras (as a control for growth inhibition); growth rate was determined as described in Methods.

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Next, we characterized the cellular localization of ARH in cells undergoing mitosis. We confirmed the recent findings that endogenous ARH localizes in centrosomes in fibroblasts,10 but in addition we found that ARH also colocalizes with CHC on mitotic spindles (Supplemental Figure III) and with lamin B1 on the newly formed nuclear envelope in late telophase and the mitotic matrix (Figure 5A). The interaction between lamin B and ARH in cells in G2/M phase was confirmed by coimmunoprecipitation of lamin B with ARH from nuclear lysates of cells treated with nocodazole (Figure 5C).

To further confirm the localization of ARH in mitotic spindles, we expressed plasmid constructs of ARH-cherry in HeLa cells. As shown in Figure 5B, ARH-cherry (red) was clearly visible on mitotic spindles, where it colocalized with α-tubulin (green).

**ARH Is Localized to the Nuclear Envelope and Mitotic Spindles**

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**ARH−/− Fibroblasts Manifest Misshapen Nuclei, Microtubule Defects, and an Increased Percentage of Prometaphase Cells**

We have observed various morphological abnormalities in the nuclei of fibroblasts from ARH patients. We counted nuclei with abnormalities, including anaphase bridges, microtubule, and multinucleated or dented nuclei (Supplemental Figure IVA), and found that the percentage of these in ARH−/− fibroblasts from 4 patients homozygous for different ARH mutations was significantly greater that that in fibroblasts from control individuals (Supplemental Figure IVB). Fibroblasts from patients homozygous for W22X appeared to have the highest percentage of normal nuclei. We have also observed mitotic cells with disorganized microtubules in ARH−/− fibroblasts (Figure 6A). Analysis of the percentage of cells in each stage of mitosis revealed that there were significantly more prometaphase cells in ARH−/− fibroblasts than in control cells (Figure 6B).
ARH-Depleted Cells Exhibit Aberrant Nuclei and Mitotic Defects

To determine whether the misshapen nuclei and the increased prometaphase cells and mitotic defects were the direct results of ARH deficiency, we knocked down ARH in control human fibroblasts with RNA interference. About 80% of ARH protein was depleted in these cells, as judged by immunoblotting of lysates harvested 72 to 96 hours after transfection. The morphology and distribution of mitotic cells were analyzed by microscopy after staining with 4,6-diamidino-2-phenylindole and antitubulin. Mitotic defects reminiscent of the depletion of p150glued20 or clathrin21 were seen (Supplemental Figure V). The percentage of cells with an abnormal mitotic spindle was significantly higher after treatment with ARH siRNA than with control siRNA (19.6+/−8.4% versus 3.6+/−1.9%, P<0.05 in 3 separate experiments; n=70 mitotic cells in each condition).

The known interaction between ARH and CHC in the endocytic pathway led us to examine the possibility that these proteins also interact during mitosis. To test this, we transfected control fibroblasts with siRNA directed to ARH, CHC, or both. Immunoblot analysis of cell lysates 72 hours after transfection (Supplemental Figure VIA and VIB) showed a reduction in the levels of ARH (≈80%) and CHC (≈50%). Depletion of clathrin produced the same percentage of cells with defects in mitotic spindle formation as depletion of ARH, but the effects of ARH and clathrin depletion were not additive (CHC siRNA, 12.2±5.6%; ARH siRNA plus CHC siRNA, 12.0±7.7%) (Supplemental Figure VIC).
Discussion

In this report, we have confirmed the observations of Lehtonen et al.\(^\text{10}\) that cells deficient in ARH have a growth defect. These authors showed that in murine embryonic fibroblasts, ARH localizes sequentially to the nuclear membrane, kinetochores, the spindle pole, and the mid-body and suggested that ARH was involved in cytokinesis. We have extended these observations by confirming that the phenotype is present in fibroblasts from ARH deficient patients and in other human cells depleted of ARH, but we have in addition presented strong evidence that ARH is involved in mitosis. First, ARH localizes at microtubules and the nuclear envelope in mitotic cells. ARH is a clathrin binding partner in the pathway for LDLR endocytosis, and clathrin has been implicated in mitotic function. Knockdown of CHC or ARH in normal fibroblasts both resulted in increased prometaphase cells and mitotic spindle defects, but double knockdown of ARH and clathrin appeared to have no additive effect, suggesting that ARH and clathrin may work in the same pathway.

Lamin B is a nuclear envelope protein which has been found to localize in the “mitotic matrix,” a new concept based on the finding that many nonmicrotubule binding proteins are

![Figure 5.](http://atvb.ahajournals.org/)

**Figure 5.** The localization of autosomal recessive hypercholesterolemia (ARH) in cells during mitosis. A, HeLa cells were fixed and permeabilized with methanol and then labeled with fluorescent antibodies to ARH (green) and lamin B (red) and visualized by confocal microscopy as described in Methods. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). ARH colocalized (yellow) with lamin B at mitotic spindles (top) and the nuclear membrane (bottom). B, HeLa cells expressing ARH-cherry were stained with an antibody to α-tubulin (green). ARH colocalized (yellow) with α-tubulin at mitotic spindles. Scale bars=10 μmol/L. C, Nuclear (Nuc) or cytosolic (Cyt) extracts of HeLa cells treated with or without nocodazole for 24 hours were immunoprecipitated (Imm ppt) with either rabbit anti-ARH or control rabbit IgG, as indicated below the gel, and immunoblotted with anti-lamin B.

![Figure 6.](http://atvb.ahajournals.org/)

**Figure 6.** Mitotic defects in autosomal recessive hypercholesterolemia (ARH)–/– fibroblasts. A, Microtubule organization in mitotic fibroblasts (normal, upper 2 panels; ARH–/–, lower 3 panels); representative prometaphase (P) and metaphase (M) mitotic cells are shown. Cells were fixed and permeabilized with methanol and then incubated with antibodies to γ-tubulin (green) and α-tubulin (red) to stain mitotic spindles. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (top). B, Quantification of the percentage of mitotic cells in each mitotic phase. Data are given as the mean±SD of 5 independent experiments; >50 mitotic cells were analyzed in each cell line for each experiment. *P<0.05, **P<0.01.
important for maintaining the structure of mitotic spindles.\textsuperscript{22} Based on this model, it has been proposed that dynein and nudel promote assembly of the lamin B matrix during mitosis.\textsuperscript{23} It is possible that ARH is also a component of the mitotic matrix, as we observed that ARH colocalized with lamin B on the nuclear envelope at the onset of mitosis and on the newly formed nuclear membrane in telophase.

The second line of evidence that ARH plays a role in mitosis is the phosphorylation of ARH in G\textsubscript{2}/M phase. It is well established that phosphorylation of proteins regulates cell cycle progression,\textsuperscript{24} and the kinase cdc2 and its substrates have been implicated in many crucial functions in mitosis.\textsuperscript{25} The fact that cells from a patient expressing a mutant ARH protein lacking the first 45 residues appear to have the most severe mitotic defects supports the physiological importance of the S14/S16 site in mitosis.

At least 2 roles for ARH phosphorylation during mitosis can be envisioned. First, it has been shown that receptor endocytosis is inhibited during mitosis and resumes in the late stage of mitosis because endocytotic recycling membrane is essential for completion of cytokinesis.\textsuperscript{26} Phosphorylation of the adaptors involved could be the underlying inhibitory mechanism. For instance, phosphorylation of Epsin in mitotic phase inhibits endocytosis by reducing its binding with other adaptors in the pathway,\textsuperscript{27} and its binding to AP-2 is enhanced by dephosphorylation, which in turn induces clathrin-mediated endocytosis.\textsuperscript{28} Second, phosphorylation by cdc2 is important for activation of proteins involved in mitosis\textsuperscript{29}; for example, phosphorylation of the kinesin-related motor HsEg5 by cdc2 enhances its binding to the dynactin subunit p150Glued.\textsuperscript{30}

The morphological changes in nuclear shape and the defects in cytokinesis such as interphase/anaphase bridges and micronuclei seen in cells from ARH patients or cells depleted ARH by RNA interference further support a role for ARH in mitosis. Some of the phenotypic changes imply dysfunction in chromosome congression at the metaphase plate\textsuperscript{31} or resemble defects seen in cells with lamin A/C mutations ("blebbing," "denting")\textsuperscript{32} and suggest a nuclear membrane abnormality. Cells unable to accomplish mitosis might be expected to arrest in mitosis because of the mitotic checkpoint mechanism, but we did not see an increase in mitotic index (mitotic cells/total cells) in ARH-depleted cells; those cells with abnormal nuclei appeared to be in interphase, suggesting that mutant cells can bypass the mitotic checkpoint.

An alternative explanation is that the nuclear morphology changes are caused by lack of interaction of ARH with nuclear membrane proteins such as lamin B1. The defects in nuclear morphology are reminiscent of those seen in cells from patients with progeria caused by mutations in lamin A/C,\textsuperscript{33} but less severe.

It is clear from our studies that ARH fibroblasts more readily undergo replicative or cellular senescence than control cells and that the mechanism is clearly specific for ARH deficiency, rather than due to reduced uptake of LDL, because LDLR function is normal in ARH skin fibroblasts. Two main underlying mechanisms of replicative senescence have been identified: genetically programmed and stress induced.\textsuperscript{34} DNA damage has been well documented as an underlying cause of stress-induced senescence, and Matsuoka and colleagues\textsuperscript{35} used quantitative SILAC MS to identify sites in proteins which are phosphorylated in response to DNA damage induced by ionizing radiation. It is interesting that one of the peptides they identified was RDKAS186QEGGDVLGAR in ARH. We also identified phosphorylation at S186 in ARH by MS. If ARH plays a role in the DNA damage response, this could explain the premature senescence exhibited by ARH deficient cells. However, other mechanisms are also possible. That mitotic factors affect senescence was experimentally proven long ago,\textsuperscript{36} but only recently have specific mitotic proteins been implicated in cellular senescence, such as Mad2\textsuperscript{27} or CENP-A\textsuperscript{37}, which supports a role for mitotic defects caused by ARH depletion as the underlying molecular mechanism for the premature senescent phenotype.

Most importantly, ARH deficiency being linked to cellular senescence could be physiologically significant in vivo, because there is already evidence that some gene defects that cause vascular senescence impair endothelial cell function and in turn affect vascular function.\textsuperscript{38} Vascular cell senescence contributes to atherosclerosis,\textsuperscript{40} and although mitotic defects or senescence have not been reported to occur in ARH knockout mice, it is unlikely that this phenotype has been explored. Apart from severe premature atherosclerosis and coronary heart disease, the signs of premature aging seen in patients with early onset progeria\textsuperscript{33} have not been reported to occur in young ARH patients,\textsuperscript{11,41} and thus we are not suggesting that ARH should be considered as another progeria gene. However, it is possible that the severity of atherosclerosis in ARH patients is influenced not only by the defect in clearance of LDL caused by defective LDLR internalization but also by defects in the cell cycle that drive cells into premature senescence. ARH patients do generally exhibit less elevated serum LDL cholesterol levels than patients homozygous for LDLR defects and are considered less severely affected, but in some cases they do experience equally severe premature atherosclerosis.\textsuperscript{41} Our results suggest that some mutations in ARH gene may have a greater effect on the LDLR pathway, whereas others have a more deleterious effect on cell growth/senescence in the vascular wall.

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**Disclosures**

None.

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Premature Senescence in Cells From Patients With Autosomal Recessive Hypercholesterolemia (ARH): Evidence for a Role for ARH in Mitosis
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Supplement Material

Premature senescence in fibroblasts from patients with autosomal recessive hypercholesterolaemia (ARH): evidence for involvement of ARH in mitosis

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Abbreviations: ARH, autosomal recessive hypercholesterolemia; LDL, low density lipoprotein; CHC, clathrin heavy chain; LMB1, Lamin B1;
Legends to the Supplementary Figures

**Supplementary Figure I.** Effect of λ-phosphatase on the mobility of ARH.

Whole cell lysates of Hela cells were incubated for 1 h at 30°C with 5 µl of λ-phosphatase in a buffer supplied by manufacturer (New England Biolabs) according to the supplier's instruction, and then analysed by immunoblotting with anti-ARH.

**Supplementary Figure II.** Immunoblot of ARH protein in cells from ARH patients.

Epstein Barr-transformed lymphocytes from different homozygous (Hmz) or compound heterozygous (Htz) ARH patients and their heterozygous relatives (Eden et al, 2002) were preincubated for 16h in medium containing lipoprotein depleted serum. Whole cell lysates were separated by PAGE and immunoblotted with anti-ARH.

**Supplementary Figure III.** Colocalisation of ARH and clathrin in cells at different stages of the cell cycle.

Fibroblasts were fixed and permeabilised with methanol, then incubated with antibodies to ARH and clathrin heavy chain (CHC). ARH (green) is colocalised with CHC (red) at mitotic spindles. The insert in the interphase panel shows ARH, but not CHC, at the nuclear envelope; inserts in the panels for prophase, metaphase and telophase cells show ARH colocalised with CHC at mitotic spindles (yellow). DNA was stained with DAPI.

**Supplementary Figure IV.** Irregular nuclei in ARH-/- fibroblasts.

(A) Examples of misshaped or deformed nuclei in ARH-/- fibroblasts. Cells were fixed and permeabilised with methanol, then incubated with antibodies to lamin A/C to stain
the nuclear envelope. The asterisks indicate micronuclei or dented nuclei. (B). Quantification of odd nuclei in ARH-/- cells. Passage number (p) of cells was as follows: control cells, p18-20; ARH1 (homozygous for c.432insA), p5-9; ARH2 (homozygous for W22X), p6-19; ARH3 (homozygous for Q136X), p14-17; ARH4 (compound heterozygous for c.599insC and del exons 1-7), p7-11. *p<0.05; **p<0.01. More than 200 cells were counted in 5 different experiments.

**Supplementary Figure V.** Mitotic defects in control fibroblasts depleted of ARH or CHC, or both. Cells were treated with siRNA by oligofectamine transfection as described in Material and Methods. Seventy-two hours after transfection, cells were analysed by immunofluorescence microscopy. Representative images showing examples of chromosome alignment defects after depletion of ARH (A) or reduction of CHC (B). Cells were stained with DAPI to detect DNA (blue), and antibodies to α-tubulin (red) and γ-tubulin (green). The arrows in (A) indicate examples of chromosomes adjacent to spindle pole (a and b), DNA at the pole (c) and DNA out of the mitotic plate (d). The arrows in (B) show chromosomes adjacent to the spindle pole (a-c) and a thin spindle (d). (C) Quantification of mitotic cell fraction in each mitotic phase. Mean±/− 3 independent experiments and >75 mitotic cells were analyzed in each condition in each experiment (*p<0.05; **p<0.01).

**Supplementary Figure VI.** Immunoblot of cells treated with siRNA against ARH or CHC.
Expression of ARH and CHC was determined by immunoblotting of whole cell extracts of fibroblasts prepared 72h after transfection with siRNA as described in the legend to supplementary Figure IV.
**Supplementary Table I.**
Oligonucleotides for cloning short hairpin RNAi into pSUPER.

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Supplementary Figure I

\( \lambda \) phosphatase - +

ARH-p

ARH
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Supplementary Figure IV

A

Normal | ARH1 | ARH2

DAPI

Lamin A/C

B

![Graph showing abnormal nuclei percentage across different samples](image)

- Normal
- ARH1
- ARH2
- ARH3
- ARH4

The graph illustrates the percentage of abnormal nuclei across different samples, with significant differences indicated by asterisks (*) for ARH1 and ARH2, and ** for ARH3 and ARH4.
**Supplementary Figure V**

**A.**

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**B.**

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Supplementary Figure VI

A. siRNA: C C ARH1 ARH2

-ARH
-γ-tubulin

B. siRNA: C CHC ARH

-CHC
-γ-tubulin

C. Cells in each Mitotic phase (%)

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