**Background:** The biological function of insertion/deletion sequences associated with AMD has not been fully characterized.

**Results:** The *HTRA1* regulatory region contains an insertion/deletion sequence that is significantly up-regulated in retinal neuronal cell lines.

**Conclusion:** *HTRA1* expression is enhanced by a mutation in the insertion/deletion in the *HTRA1* regulatory region.

**Significance:** This is the characterization of the *HTRA1* regulatory elements and the effect of insertion/deletion sequences associated with AMD.

Dry age-related macular degeneration (AMD) accounts for over 85% of AMD cases in the United States, whereas Japanese AMD patients predominantly progress to wet AMD or polypoidal choroidal vasculopathy. Recent genome-wide association studies have revealed a strong association between AMD and an insertion/deletion sequence associated with AMD. Transcription regulator activity was localized in mouse retinas using heterozygous knock-out mice in which *HtrA1* exon 1 was replaced with β-galactosidase cDNA, thereby resulting in dominant expression of the photoreceptors. The insertion/deletion sequence significantly induced *HTRA1* transcription regulator activity in photoreceptor cell lines but not in retinal pigmented epithelium or other cell types. A deletion construct of the *HTRA1* regulatory region indicated that potential transcriptional suppressors and activators surround the insertion/deletion sequence. Ten double-stranded DNA probes for this region were designed, three of which interacted with nuclear extracts from 661W cells in EMSA. Liquid chromatography-mass spectrometry (LC-MS/MS) of these EMSA bands subsequently identified a protein that bound the insertion/deletion sequence, LYRIC (lysine-rich CEACAM1 co-isolated) protein. In addition, induced pluripotent stem cells from wet AMD patients carrying the insertion/deletion sequence showed significant up-regulation of the *HTRA1* transcript compared with controls. These data suggest that the insertion/deletion sequence alters the suppressor and activator *cis*-elements of *HTRA1* and triggers sustained up-regulation of *HTRA1*. These results are consistent with a transgenic mouse model that ubiquitously overexpresses HtrA1 and exhibits characteristics similar to those of wet AMD patients.

Recent genome-wide association studies have identified more than 19 susceptibility genes associated with age-related macular degeneration (AMD)\(^2\) (1). Among these genes, two loci have been highly associated with AMD, and these include the *CFH* (complement factor H) gene on chromosome 1q32 and the *ARMS2/HTRA1* (age-related macular degeneration susceptibility 2/high temperature requirement A serine peptidase 1) genes. These results are consistent with a transgenic mouse model that ubiquitously overexpresses HtrA1 and exhibits characteristics similar to those of wet AMD patients. The biological function of insertion/deletion sequences associated with AMD has not been fully characterized.

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\(\dag\) The abbreviations used are: AMD, age-related macular degeneration; LD, linkage disequilibrium; RPE, retinal pigment epithelium; PCV, polypoidal choroidal vasculopathy; iPSC, induced pluripotent stem cell; TLR, Toll-like receptor.
Characterization of HTRA1 Regulatory Elements

EXPERIMENTAL PROCEDURES

Subjects—A total of 226 Japanese patients with typical wet AMD without PCV (average age, 74.68 ± 8.86 years) were classified as 5b according to Seddon et al. (20). In addition, 228 non-AMD Japanese individuals (average age, 75.22 ± 7.23 years) were recruited as controls for this study (Table 1). All patients were diagnosed by fundus observation or by fluorescein or indocyanine green angiographic findings. For the controls, no signs of early AMD, such as soft drusen or alterations of the retinal pigment epithelium in the macula area, were observed ophthalmoscopically. Informed consent was obtained from all of the patients and controls, and the procedures performed conformed to the tenets of the Declaration of Helsinki.

DNA Isolation and Sequencing—Human DNA was extracted from blood samples using a Magtration System (Hidex, Turku, Finland). Peripheral blood mononuclear cells were obtained from donors by the centrifugation system, and human RPE and ARPE19 cells were seeded in 96-well plates (104 cells/well) (Table 2). Insertion/deletion variants were also confirmed in both control and AMD patient samples using the primers listed in Table 1. PCR amplifications for all primer sets were performed in a 20-μl volume containing 100 ng of genomic DNA and 1 unit of Taq polymerase (PrimerStar, Takara Bio Co. Ltd., Japan) for 35–40 cycles of amplification. Amplified PCR products were then purified using an ExoSAP-IT kit (GE Healthcare) and were sequenced using a BigDye Terminator version 3.1 sequencing kit (Invitrogen) and an ABI 3130 Genetic Analyzer.

Cell Culture—The mouse photoreceptor cell line, 661W, and the rat retinal ganglion cell line, RGC5, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO2. The human RPE cell line, ARPE19, was cultured in DMEM/F-12 containing 15% FBS at 37 °C in 5% CO2.

Transcription Regulator Activity Assay—The ARMS2 regulatory region and the HTRA1 regulatory region were amplified and cloned into the pGL4.10 (Luc2) luciferase vector (Promega, WI) (Table 2). Insertion/deletion variants were also constructed using a KOD mutagenesis kit (Toyobo, Osaka, Japan). For the luciferase assays performed, 661W cells, RGC5 cells, and ARPE19 cells were seeded in 96-well plates (1 × 104 cells/well) 24 h before transfection. Transfections were performed using Lipofectamine LTX with Plus reagent (Invitrogen). A firefly luciferase gene was integrated into a reporter vector to normalize the activity of both transcription regulators. Luciferase activity was detected using the Dual-Glo luciferase assay system (Promega) and a microplate reader (Plate Chameleon, Hidex, Turku, Finland).

Preparation of Induced Pluripotent Stem Cells (iPSCs) from Controls and AMD Patients—Human iPSCs were established by infecting circulating T cells obtained from the peripheral blood of human AMD patients with the Sendai virus as described previously (21, 22). Briefly, peripheral blood mononuclear cells were obtained from donors by the centrifugation
of heparinized blood over a Ficoll-Paque PREMIUM (GE Healthcare) gradient, according to the manufacturer’s instructions. The peripheral blood mononuclear cells were then cultured at 37 °C in 5% CO₂ with plate-bound anti-CD3 monoclonal antibodies (BD PharMingen) in GT-T502 medium (Kohjin Bio, Sakado, Japan) supplemented with recombinant interleukin-2 (IL-2) at 175 Japan reference units/ml. After 5 days, activated peripheral blood mononuclear cells were collected and transferred to 6-well plates (1.5 × 10⁶ cells/well) coated with anti-CD3 monoclonal antibodies, and the cells were incubated for an additional 24 h. A solution containing Sendai virus vectors, anti-CD3 monoclonal antibodies, and the cells were incubated with blocking buffer and streptavidin-horseradish peroxidase conjugates. Bound conjugates were detected using a molecular imager (ChemiDoc XRS, Bio-Rad).

**EMSA**—Nuclear extracts were prepared with a Celllytic nuclear extraction kit (Sigma), according to the manufacturer’s protocol. The nuclear protein concentration of each sample was determined using a BCA protein assay kit (Thermo Scientific).

EMSA probes were 40 bp in length and are listed in Table 3. Probes 1–3 were designed to cover non-insertion/deletion and insertion/deletion regions (non-insertion/deletion, bp −4320 to −4220; insertion/deletion, bp −3936 to −3836). Probes 4–8 were designed to cover the non-insertion/deletion regulatory region (non-insertion/deletion, bp −3936 to −3777). Probes 9 and 10 were designed to cover the insertion/deletion region (insertion/deletion, −3836 to −3782 bp). Double-stranded DNA probes were biotin-labeled using a LightShift chemiluminescent EMSA kit (Thermo Scientific) and then were mixed with nuclear extracts. After 20 min, the samples were subjected to electrophoresis on 7% EMSA gels. After electrophoresis, the samples were transferred onto Biodyne B precut modified nylon membranes (Thermo Scientific). The nuclear protein concentration of each sample was determined using a BCA protein assay kit (Thermo Scientific). The membranes were cross-linked in a UV transilluminator for 15 min and then were incubated with blocking buffer and streptavidin-horseradish peroxidase conjugates. Bound conjugates were detected using a molecular imager (ChemiDoc XRS +, Bio-Rad).

**RT-PCR**—The iPSCs were removed from the feeder cells and were rinsed twice with PBS. Total cellular RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer-
er’s instructions. RNA concentrations were determined by measuring absorption values at 260 nm/280 nm. Reverse transcription was performed using a SuperScript first strand synthesis system for RT-PCR kit (Invitrogen). Primer sequences for human OCT3/4, NANO2, SOX2, and GAPDH as well as the associated PCR detection methods were created and performed according to Seki et al. (21). The primers used to detect human HTRA1 included AACTTTATCGCCGACTTGGTTGAG (forward) and TGATGGCGTGCGTGTGGAG (reverse) (10 μm). In situ hybridization was performed using a Quantigene line viewRNA system (Affymetrix, Santa Clara, CA) using HtrA1 and LacZ probes according to the manufacturer’s instructions.

Western Blotting—Total proteins were extracted from mouse brain tissues in ice-cold TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40) containing protease and phosphatase inhibitors (Roche Applied Science). Protein concentrations of the extracts were determined using a BCA assay kit (Thermo Scientific). Equal amounts of protein (10 μg/lane) were separated by 7.5% SDS-PAGE and were transferred to PVDF membranes (Trans-Blot Turbo, Bio-Rad). The membranes were then incubated with an anti-mouse Htra1 antibody (1:100; Abcam) and an anti-α-tubulin antibody (1:1000; Abcam). A FluorChem Western blot imaging station (ChemiDoc XR5+, Bio-Rad) and image analysis software (Image Lab, Bio-Rad) were used to calculate and normalize the pixel value of each protein band.

RESULTS

Sequencing of ARMS2-HTRA1 Loci in Controls and AMD Patients—To determine the genomic sequence of the LD block represented by the SNP, rs10490924, in association with AMD (6, 19, 24), ~10.5 kbp of the LD block (NC_000010.10) was sequenced for 228 controls and 226 AMD patients. Two unique sequences were identified in the regulatory region of ARMS2 and HTRA1 (Fig. 1). A C-to-A (C/A) variant was identified in the regulatory region of ARMS2, ~550 bp upstream of exon 1 (Fig. 1, A and B), and an insertion/deletion variant was identified immediately following the ARMS2 exon 2 in the regulatory region of HTRA1, ~3777 bp upstream of exon 1 (Fig. 1, A and C). Sequence variations in both transcription regulators were in complete LD with SNP rs10490924, thereby suggesting that AMD and PCV pathogenesis are directly associated with these variants.

The Effect of C/A and Insertion/Deletion Variants on ARMS2 and HTRA1 Regulatory Region Activity—To date, two hypotheses have been proposed to account for the increased risk of AMD that is observed. These involve an increase in the transcription of HTRA1 and/or the presence of unstable HTRA1 mRNA (7, 19, 24). To investigate the first hypothesis, regulator activity for ARMS2 was assayed using constructs covering the regulatory region from ~1,000 to +1 bp and from ~600 to +1 bp. Both of these regions contain the C/A repetitive variant (Fig. 1D). Regulatory region activity was not detected for either the non-C/A or C/A variants of differing length for retinal cell lines (ARPE19, 661W, and RGC5 tested) (Fig. 1, E–G). Next, regulator activity of HTRA1 was assayed for the ~4,320 to +1 bp region of the non-insertion/deletion regulatory region and for the ~3,936 to +1 bp region of the insertion/deletion regulatory region (Fig. 1H). Both regions had common sequences on both ends of the regulatory region. Transcription regulator activity was not found to be affected by the insertion/deletion variants in the ARPE19 cell line (Fig. 1I). However, activity of the insertion/deletion regulator was significantly up-regulated compared with the activity of the non-insertion/deletion regulator in both the 661W and RGC5 cell lines, by ~2- and 3-fold, respectively (Fig. 1, J and K). Moreover, HTRA1 non-insertion/
deletion regulator activity was not detected in the latter, similar to the empty vector. However, when the non-insertion/deletion regulator was replaced with the insertion/deletion regulator in the 661W and RGC5 cell lines, HTRA1 regulatory element activity increased 2- and 3-fold, respectively (Fig. 1 J).

To test whether C/A variants in the transcription regulatory region of ARMS2 influence HTRA1 gene expression, both normal and risk type ARMS2 C/A variant (Fig. 2 A) were cloned and fused to the HTRA1 transcription regulator of the non-insertion/deletion and insertion/deletion constructs. Luciferase levels were subsequently measured following transfection of these vectors (Fig. 2 A). Neither the normal nor the risk type, C/A variants affected HTRA1 transcription regulator activity (Fig. 2, B and C).

Characterization of HTRA1 Regulatory Elements

**FIGURE 2. The effect of the ARMS2 C/A variant on the HTRA1 transcription regulator.** A, schematic diagram of the C/A variant plus HTRA1 regulatory element in the constructs used for luciferase assays (blue region, 600-bp ARMS2 transcription regulatory region (contains C/A variant); orange region, region that contains the HTRA1 regulatory element; yellow region, the luciferase reporter gene). B, C/A variant plus non-insertion/deletion type HTRA1 regulatory element activity in 661W cells. C, C/A variant plus insertion/deletion type HTRA1 regulatory element activity in 661W cells. Error bars, S.D. EV, empty vector; Normal, normal type C/A variant; Risk, risk type C/A variant; NI, non-insertion/deletion type HTRA1 regulatory element; I, insertion/deletion type HTRA1 regulatory element.

deletion regulator activity was not detected in the latter, similar to the empty vector. However, when the non-insertion/deletion regulator was replaced with the insertion/deletion regulator in the 661W and RGC5 cell lines, HTRA1 regulatory element activity increased 2- and 3-fold, respectively (Fig. 1 J). To test whether C/A variants in the transcription regulatory region of ARMS2 influence HTRA1 gene expression, both normal and risk type ARMS2 C/A variant (Fig. 2 A) were cloned and fused to the HTRA1 transcription regulator of the non-insertion/deletion and insertion/deletion constructs. Luciferase levels were subsequently measured following transfection of these vectors (Fig. 2 A). Neither the normal nor the risk type, C/A variants affected HTRA1 transcription regulator activity (Fig. 2, B and C).

**FIGURE 1. Transcription regulatory region of ARMS2 and HTRA1.** A, a schematic diagram of the transcription regulatory region of ARMS2 and HTRA1. Sequencing of the indicated transcription regulatory region spanned 10.5 kbp and was performed for both AMD and non-AMD controls. Two unique sequences, a C/A repeat variant in the ARMS2 transcription regulator (indicated with a blue box) and an insertion/deletion variant downstream of exon 2 of ARMS2 (indicated with a red box), were identified in a patient with AMD that carried the SNP, rs10490924. B, characteristic C/A mutations present in the ARMS2 transcription regulatory region (blue, ARMS2 exon 2 region; red, point mutations). D–G, analysis of ARMS2 transcription regulator activity using a luciferase assay system. D, four luciferase vectors were generated to analyze ARMS2 transcription regulator activity: normal (1,000 bp), normal (600 bp), risk (1,000 bp), and risk (600 bp). The 1,000-bp sequence contained a C/T SNP in the upper 730-bp region from ARMS2 exon 1.

ARMS2 transcription regulator activity detected in ARPE19 cells (E), RGC-5 cells (F), and 661W cells (G). Error bars, S.D. H–K, HTRA1 transcription regulator activity detected in various retinal cell lines. H, schematic diagram of the ARMS2-HTRA1 constructs used in the luciferase assays performed (black line, common regulator sequence; blue line, non-insertion/deletion regulator unique sequence; red line, insertion/deletion regulator unique sequence). Full-length HTRA1 transcription regulator activity detected in ARPE19 cells (I), RGC5 cells (*, \( p = 7.5 \times 10^{-4} \)) (J), and 661W cells (**, \( p = 1.07 \times 10^{-5} \)) (K). Error bars, S.D. EV, empty vector; NI, non-insertion/deletion regulator; I, insertion/deletion regulator.
Characterization of HTR1 Regulatory Elements

HtrA1 expression was observed to localize to the photoreceptor cell layer (outer segment (OS) and inner segment (IS) in Fig. 3), the outer plexiform layer (OPL), and the ganglion cell layer (GCL). In contrast, HtrA1 expression in eye tissues from heterozygous HtrA1 mice was mainly localized to the photoreceptor cell layer and the outer plexiform layer, whereas minor expression of HtrA1 was observed in the ganglion cell layer.

To localize HtrA1 transcription regulator activity in the retina, a heterozygous HtrA1 knock-out mouse construct that included β-galactosidase (β-gal) cDNA in place of exon 1 of Htra1 was used. β-Gal protein was abundantly expressed in the outer nuclear layer (ONL), and lower levels of expression were observed in the RPE, the inner nuclear layer (INL), and the retinal ganglion cell layer (GCL) (Fig. 3B). In comparison, native HtrA1 protein was abundantly expressed in the OS and IS, with lower expression levels detected in the RPE, the outer plexiform layer (OPL), the inner plexiform layer (IPL), and the ganglion cell layer (Fig. 2B). Furthermore, abundant transcription of HtrA1 mRNA was detected by in situ hybridization in retinal sections from a HtrA1 heterozygous knock-out mouse (Fig. 3C). Taken together, these results suggest that HtrA1 is transcribed mainly in the outer nuclear layer, outer plexiform layer, and ganglion cell layer, and then the protein product is transported to the photoreceptor layer.

Identification of Suppressing and Activating cis-Elements in the HTRA1 Regulatory Element—To analyze the mechanism of insertion/deletion function in the HTRA1 regulatory region, both non-insertion/deletion (I, bp −4,320 to +1; 2, bp −4,238 to +1; 3, bp −4,022 to +1; 4, bp −3,936 to +1; 5, bp −3,777 to +1) and insertion/deletion (6, bp −3,936 to +1; 7, bp −3,854 to +1; 8, bp −3,788 to +1; 9, bp −3,836 to −3,788 defect mutant) regions were cloned into a pGL4.10[luc2] luciferase assay vector (Fig. 4A). These cloned vectors were then transfected into 661W cells and were analyzed for HTRA1 transcription regulator activity. The activity of the number 2 region (bp −4,320 to −4,239) non-insertion/deletion defect HTRA1 transcription regulator was 1.8–2-fold higher than that of the number 1 region, and transcription regulator activity did not differ between the number 2, 3, and 4 variants (Fig. 4B). In contrast, the activity of the number 5 transcription regulator was 20–30% higher than that of the number 4 variant (Fig. 4B).

In addition, activity of the number 6 insertion/deletion type HTRA1 transcription regulator was 2- and 3-fold higher than that of the number 1 non-insertion/deletion type transcription regulator in 661W cells (Figs. 1K and 4C). Furthermore, activity
of the number 7 (bp −3,936 to −3,855 defect) region was 10% higher than that of the number 6 region, and activity of the number 8 (bp −3,936 to −3,789 defect) region was lower than that of the number 7 region (Fig. 4C). In addition, the activity of the number 9 (insertion/deletion defect) region was 10–20% lower than that of the number 6 insertion/deletion type transcription regulator (Fig. 4C). Taken together, these results suggest that both of the non-insertion/deletion type regions (including bp −4,320 to −4,239 and bp −3,936 to −3,778 bp) may be regulated by suppressor factors, whereas the insertion/deletion type region from bp −3,836 to −3,789 may bind an enhancer.

**Endogenous HTRA1 Expression Is Enhanced by the Insertion/Deletion Type Regulatory Element in AMD Patient iPSCs**—To determine the level of HTRA1 expression in individuals with a non-insertion/deletion type regulatory element sequence,
Identification of the transcription factors binding to non-insertion/deletion and insertion/deletion regions of the HTRA1 transcription regulators. 

A, location of the double-stranded DNA probes, 1–10, in the region upstream of the HTRA1 coding region that were designed for EMSAs. Both non-insertion/deletion and insertion/deletion HTRA1 transcription regulatory regions were targeted. 

B, EMSAs of probes 1–10 using nuclear extracts from 661W cells cultured in 2 and 10% FBS. Red arrowheads, detected signals. 

C, increased expression of HtrA1 was detected in Western blot assays performed following treatment of 661W cells with LPS (1 μg/ml, 0–60 min) using anti-mouse HtrA1 antibodies. Detection of α-tubulin was used as an internal control. 

D, a Venn diagram shows the number of proteins found to bind the non-insertion/deletion versus insertion/deletion regions of the HTRA1 transcription regulator based on LC-MS/MS data. 

E, gene ontology term of non-insertion/deletion- and insertion/deletion-binding protein (categorized by molecular function).
iPSCs heterozygous and homozygous for the insertion/deletion type regulatory element sequence were subjected to genotyping. From both control and AMD patients, iPSCs were collected and categorized as having a non-insertion/deletion or insertion/deletion genotype (Fig. 5A). These cells were also assayed for expression of the iPSC markers, OCT3/4, NANOG, and SOX2 (Fig. 5B), and levels of HTRA1 mRNA were measured by RT-PCR and quantitative RT-PCR (Fig. 5, B and C). Individuals with heterozygous or homozygous HTRA1 insertion/deletion type sequences showed an approximately 6- and 7-fold increase in HTRA1 mRNA levels, respectively, compared with individuals having the non-insertion/deletion type sequence (Fig. 5C). In contrast, expression of HTRA1 mRNA was not influenced by the heterozygous or homozygous state of the insertion/deletion type sequence.

Analysis of Regulatory Element-binding Proteins—To detect proteins that bind the HTRA1 regulatory element, EMSAs were performed. Ten 3’-biotin-labeled double-stranded DNA probes were generated in order to assay the transcriptional activity of each region of the HTRA1 regulatory element (Figs. 4B and C) and 6A and Table 3). In addition to the use of 661W nuclear extracts in these assays, extracts of 661W cells grown in 10% FBS versus 2% FBS were also assayed; the latter conditions were included based on the observation that HTRA1 family proteins are known to mediate various stress response signaling pathways (25–28). Thus, we tested 661W cell protein-probe binding activity in a cell-stressful environment. Protein-probe binding signals were detected for probes 1–3 and 6–10 (Fig. 6A) in the presence of nuclear extracts from normally cultured 661W cells (Fig. 6B, left). These results suggest that the binding pattern of the insertion/deletion sequence (included in probes 4–8) may differ greatly from that of the non-insertion/deletion type sequence (number 9 and 10 probes). When the culturing conditions for the 661W cells were changed from 10% to 2% FBS, this drastically altered the protein-probe binding signal pattern obtained (Fig. 6B, right). For example, a reduction in FBS concentration resulted in the loss of two of three signals for the number 2 probe, and it altered the probe-protein binding pattern of probes 5–9. Detection of HtrA1 expression further demonstrated that HtrA1 expression was enhanced in 661W cells following starvation stress (Fig. 6C). Signaling pathways involving the Toll-like receptor (TLR) family of proteins have been shown to be associated with AMD pathogenesis (29), and 661W cells are known to express TLR-4 (30). Therefore, HtrA1 expression was assayed in 661W cells following stimulation with a TLR-4 ligand, lipopolysaccharide (LPS). However, HtrA1 expression was unaffected by this stimulation (Fig. 6C).

To identify the proteins that bind the regulatory element region of HTRA1, both non-insertion/deletion and insertion/ deletion sequence-binding factors were analyzed by LC-MS/ MS. Non-insertion/deletion-binding protein samples were incubated with probes 4–8, whereas the insertion/deletion-binding protein samples were incubated with probes 9 and 10 (Fig. 6A). A total of 172 common binding proteins, 43 non-insertion/deletion probe-specific binding proteins, and 60 insertion/deletion probe-specific binding proteins were identified (Fig. 6D). Using Scaffold 4 software, 19 transcriptional regulator proteins were detected (according to gene ontology (see the Gene Ontology Consortium Web site)) (Fig. 6E). These 19 factors were then classified according to their binding sequence (Tables 4–6). Six non-insertion/deletion-binding probes were found to bind PURB, NFIC, RUNX2, PEBB, APEX1, and RBM14 proteins (Table 4), whereas three insertion/deletion-binding probes were found to bind LYRIC (lysine-rich CEACAM1 co-isolated) protein, MED4, and PHF2 (Table 5). Ten additional proteins were found to bind both the non-insertion/deletion and insertion/deletion probes: ROAA, SHOX2, CUX1, DDX5, DDX1, MED24, RBM39, JMY, TCP4, and DDX17 (Table 6). In combination, these results suggest that expression of HtrA1 is influenced by factors that specifically bind this region and affect gene expression.

**DISCUSSION**

To the best of our knowledge, the present study provides the first detailed characterization of regulatory elements for HTRA1 and the effect of insertion/deletion sequences associated with wet AMD. The insertion/deletion variant that exhibited complete association with the SNP, rs10490924, is the only major sequence change in this LD block and is likely to play a major role in disease onset. Moreover, the insertion/deletion variant in close proximity to ARMS2 and HTRA1 has been at the center of the discussion of whether one or both genes are involved in AMD (19, 24). In the present study, the ARMS2 transcription regulator exhibited only marginal activity in each of the cell lines tested, whereas robust up-regulation of HTRA1 transcription regulator activity was observed in iPSCs derived from AMD patients containing heterozygous or homozygous forms of the insertion/deletion. Up-regulation of HTRA1 in the earliest stages of development is predicted to affect the entire body (31).

HTRA1 is known to be a TGF-β suppression factor, and some reports have suggested that HtrA1 is expressed in skeletal, brain, lung epithelium, heart, and skin tissues in fetal mice (31). Other reports have suggested that HTRA1 is involved in bone remodeling (e.g. RANK/RANKL signal-derived osteoclast bone absorption and osteoblast differentiation) (32) and oncogenesis in liver and lung cancers (33, 34). Thus, HTRA1 may play an important role in cell growth and differentiation, tissue and/or organ formation, and the onset of disorders. Correspondingly, a transgenic mouse with expression of mouse HtrA1 driven by a chicken actin (CAG) promoter was used to establish a model for choroidal neovascularization, and this model exhibited a significant reduction in tolerance to smoking (23).

Detailed characterization of the transcription regulator activity that surrounds the HTRA1 insertion/deletion sequence showed that suppressive regions are located between bp −4,320 and −4,239 and between bp −3,936 and −3,778 in the non-insertion/deletion sequence and between bp −3,936 and −3,854 for the insertion/deletion sequence. In addition, the insertion/deletion located between bp −3,836 and −3,783 exhibited transcription regulator activity unique to the insertion/deletion sequence (Fig. 4). An analysis of these results showed that the insertion/deletion variant interrupts a suppressor cis-element and replaces it with an activator, and this significantly alters HTRA1 transcription in the photoreceptor.
### TABLE 4
Non-insertion/deletion probe binding proteins

| MS/MS view: identified proteins | Symbol | Molecular mass | Taxonomy | Biological regulation | Binding | Molecular function | Transcription regulator activity | Peptide hit score (non-indel) | Peptide Hit Score (indel) |
|---------------------------------|--------|----------------|----------|------------------------|---------|-------------------|-------------------------------|-------------------------------|--------------------------|
| Transcriptional activator protein Pur-β | PURB    | 34             | *M. musculus* | Regulation of transcription, DNA-dependent | Protein binding | Transcription factor activity | Transcription factor activity | 3                             | 0                        |
| Nuclear factor 1 C-type          | NFIC   | 49             | *M. musculus* | Regulation of transcription, DNA-dependent | Protein binding | Transcription factor activity | Transcription factor activity | 3                             | 0                        |
| Runt-related transcription factor 2 | RUNX2  | 66             | *M. musculus* | Regulation of transcription from RNA polymerase II promoter | DNA binding | Transcription coactivator activity | Transcription coactivator activity | 1                             | 0                        |
| Core-binding factor subunit β   | PEBB   | 22             | *M. musculus* | Regulation of transcription from RNA polymerase II promoter | Nucleus | Transcription coactivator activity | Transcription coactivator activity | 1                             | 0                        |
| DNA-(apurinic or apyrimidinic site) lyase RNA-binding protein 14 | APEX1  | 35             | *M. musculus* | Cell redox homeostasis | Chromatin DNA binding | Transcription coactivator activity | Transcription coactivator activity | 1                             | 0                        |
|                                   | RBM14  | 69             | *M. musculus* | Regulation of transcription, DNA-dependent | Nucleic acid binding | Ligand-dependent nuclear receptor transcription coactivator activity | Transcription coactivator activity | 1                             | 0                        |

*indel, insertion/deletion.

### TABLE 5
Insertion/deletion probe binding proteins

| MS/MS view: identified proteins | Symbol | Molecular mass | Taxonomy | Biological regulation | Binding | Molecular function | Transcription Regulator Activity | Peptide Hit Score (non-indel) | Peptide Hit Score (indel) |
|---------------------------------|--------|----------------|----------|------------------------|---------|-------------------|-------------------------------|-------------------------------|--------------------------|
| Protein LYRIC                   | LYRIC  | 64             | *M. musculus* | Positive regulation of NF-κB transcription factor activity | Nucleus | Protein binding | Transcription coactivator activity | 0                             | 20                        |
| Mediator of RNA polymerase II transcription subunit 4 | MED4   | 30             | *M. musculus* | Androgen receptor signaling pathway | Thyroid hormone receptor binding | Transcription coactivator activity | Transcription coactivator activity | 0                             | 2                        |
| Lysine-specific demethylase PHF2 | PHF2   | 121            | *M. musculus* | Regulation of transcription, DNA-dependent | Methylated histone residue binding | Transcription coactivator activity | Transcription coactivator activity | 1                             | 1                        |

*indel, insertion/deletion.
Table 6: Non-insertion/deletion-probe and insertion/deletion probe-binding proteins

| Non-insertion/deletion-probe binding proteins | Insertion/deletion-probe binding proteins | MS/MS view | Peptide hit score (non-insertion/deletion) | Peptide hit score (insertion/deletion) |
|---------------------------------------------|------------------------------------------|------------|------------------------------------------|----------------------------------------|
| Heterogeneous nuclear ribonucleoprotein A/B | Heterogeneous nuclear ribonucleoprotein B | 31         | 24                                      | 1                                      |
| H3K9 methylation reader | H3K9 methylation reader | 35         | 1                                       | 6                                      |
| Probable ATP-dependent RNA helicase II | Probable ATP-dependent RNA helicase II | 69         | 25                                      | 2                                      |
| ATXN1 | ATXN1 | 83         | 6                                        | 1                                      |
| DNA-dependent Dof zinc finger protein 1 | DNA-dependent Dof zinc finger protein 1 | 110        | 2                                        | 1                                      |
| Probable ATP-dependent RNA helicase | Probable ATP-dependent RNA helicase | 389        | 4                                        | 1                                      |
| Probable ATP-dependent RNA helicase | Probable ATP-dependent RNA helicase | 59         | 1                                        | 2                                      |
| Probable ATP-dependent RNA helicase | Probable ATP-dependent RNA helicase | 111        | 12                                       | 1                                      |
| Probable ATP-dependent RNA helicase | Probable ATP-dependent RNA helicase | 72         | 1                                       | 2                                      |

Characterization of HTRA1 Regulatory Elements

The EMSA and LC-MS/MS data also suggest that HTRA1 regulatory element activity is regulated by a number of transcription factors (Fig. 6 and Tables 4–6).

When the proteins that bound the regulatory elements of the non-insertion/deletion and insertion/deletion sequences of HTRA1 were analyzed using LC-MS/MS, LYRIC (also known as MTDH/AEG1) was a high hit score insertion/deletion-binding protein that was identified (Table 5). LYRIC is known to promote hepatocellular carcinoma and to activate the transcription factor, nuclear factor κ-B (NF-κB), and also has an effect on bone and brain metastasis (35–37). Regarding the latter, LYRIC may enhance the seeding of tumor cells at the target organ endothelium. LYRIC also contributes to HIF-1α-mediated angiogenesis (38), with HIF-1α playing an important role in the activation of VEGF signaling in response to oxidative stress (39). Recently, Oka et al. (25) reported that HTRA1 gene expression is enhanced by oxidative stress. This result is consistent with the observation that AMD onset is associated with a variety of stresses, and stress response factors may play an important role.

Both common sequences (non-insertion/deletion (bp −4,320 to −4,220) and insertion/deletion (bp −3,936 to −3,836 bp)) and the insertion/deletion sequence (bp −3,836 to −3,782 bp) are partially associated with ARMS2 exon 2, thereby indicating that this exon represents a protein-coding region and a HTRA1 regulatory element region. Recent reports have suggested that both an increase in HTRA1 transcription and a decrease in ARMS2 transcription confer an increased risk of AMD (8). These insights suggest that ARMS2 gene expression may be regulated by HTRA1 transcription regulator activity via the ARMS2 exon 2 region.

AMD pathogenesis and previous HTRA1 experiments related to AMD have been performed and discussed in relation to the RPE (8, 16, 17). However, immunostaining of HTRA1 in mouse retinas in the present study showed that the majority of transcription occurs in the photoreceptor cell layer (Fig. 3). This result was confirmed when higher levels of transcription from the HTRA1 insertion/deletion regulator were observed in the photoreceptor cell line: 661W cells versus the RPE cells (Fig. 1, I–K). Photoreceptors are densely concentrated in the macula (40), which predicts that HTRA1 will also be concentrated in the macula. A recent report using transgenic mouse overexpressing human HTRA1 in the RPE showed that PCV-like capillary structures could be observed in the choroid. Vierkotten et al. (17) also observed fragmentation of the elastic layer in Bruch’s membrane, and Jones et al. (16) reported branching of chorioidal vessels, polypoidal lesions, and severe degeneration of the elastic lamina or tunica media of chorioidal vessels in the same model. When Htra1 expression was driven by the CAG promoter in a transgenic mouse model, an even more severe phenotype was induced compared with previous reports of AMD patient-like chorioidal neovascularization (23). These results indicate that overexpression of Htra1 alone can evoke chorioidal vasculopathy or neovascularization, and they also provide supporting evidence for the association of the insertion/deletion variant of HTRA1 regulatory element with wet AMD (7, 8).
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