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Chtop (Chromatin target of Prmt1) auto-regulates its expression level via intron retention and nonsense-mediated decay of its own mRNA

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ABSTRACT

Chtop (chromatin target of Prmt1) regulates various aspects of gene expression including transcription and mRNA export. Despite these important functions, the regulatory mechanism underlying Chtop expression remains undetermined. Using Chtop-expressing human cell lines, we demonstrate that Chtop expression is controlled *via* an autoregulatory negative feedback loop whereby Chtop binds its own mRNA to retain intron 2 during splicing; a premature termination codon present at the 5′ end of intron 2 leads to nonsense-mediated decay of the mRNA. We also show that Chtop interacts with exon 2 of *Chtop* mRNA *via* its arginine-glycine-rich (RG) domain, and with intron 2 *via* its N-terminal (N1) domain; both are required for retention of intron 2. In addition, we show that hnRNP H accelerates intron 2 splicing of *Chtop* mRNA in a manner dependent on Chtop expression level, suggesting that Chtop and hnRNP H regulate intron 2 retention of *Chtop* mRNA antagonistically. Thus, the present study provides a novel molecular mechanism by which mRNA and protein levels are constitutively regulated by intron retention.

INTRODUCTION

Chtop, the chromatin target of protein arginine methyltransferase (PRMT) 1, localizes mainly in nuclear speckles and tightly associates with facultative heterochromatin in vertebrate interphase cells (1,2). As indicated by its alternative names SRAG (small protein rich in arginine and glycine (RG)) (3) and FOP (Friend of PRMT1), Chtop has an RG-rich domain, which is methylated by PRMT1 (1). Chtop is expressed in a variety of tissues and cell types and is highly conserved in all vertebrates, although no ortholog has been identified in yeast, worm or fly.

Chtop is involved in activation of estrogen–receptor target genes (1) and in the downregulation of fetal γ-globin during the developmental transition from fetal to adult hemoglobin (4). Chtop is therefore a potential therapeutic target for β-thalassemia and sickle cell disease via its ability to regulate γ-globin gene expression (4,5).

Chtop is also involved in mRNA export as a component of the TREX complex (2,6,7); it drives remodeling of this complex during mRNP (mRNA-ribonucleoprotein complex) formation for mRNA export through its ability to bind the pre-mRNA splicing factor Uap56, which activates both its ATPase and RNA helicase activities. Methylation of an arginine residue in Chtop is required for this process (6). In addition, Chtop is recruited to 5-hydroxymethylcytosine-containing DNA sequences, which are common in glioblastoma cells and required for tumorigenicity (8). Chtop also promotes PRMT1-mediated methylation of arginine 3 of histone H4, resulting in acti-

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vation of many cancer-related genes (8). For these reasons, Chtop is a potential therapeutic target for certain cancers including glioblastoma.

Finally, Chtop is a component of the nuclear SMN (survival of motor neuron protein) complex that contains U5 and U6 but lacks U1 and U2 small nuclear RNAs (snRNAs) (9). Chtop is required for association of the SMN complex with heterogeneous nuclear RNPs (hnRNPs), histones and various RNA-binding proteins (9). Despite accumulating evidence that Chtop is involved in various aspects of gene expression, the underlying mechanism by which the expression level of Chtop itself is regulated remains undetermined. Here, we show that Chtop, by binding to exon 2 and intron 2 of its own mRNA, controls its protein level by negatively affecting the splicing of its own pre-mRNA. This, in turn, facilitates the formation of an intron 2 (Int2)-retained *Chtop* mRNA that is subsequently degraded via nonsense-mediated mRNA decay (NMD).

MATERIALS AND METHODS

RNA Isolation and RT-PCR

Total RNA from the cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 5 μg RNA was treated with three units of Baseline-ZERO DNase (Epicentre Biotechnologies) for 1 h at 37°C, and purified using TRIzol reagent. NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, USA) was used to estimate concentration and the RNA purity (A260/A280 ratio > 1.8). Electrophoresis on denaturing 8M urea PAGE was run to check for RNA degradation. For reverse transcription coupled quantitative PCR (RT-qPCR), first-strand cDNA was reverse-transcribed with random hexamers and oligo d(T) using the PrimeScript RT Reagent kit (Takara, Japan). RT-qPCR utilized SYBR premix, Ex Taq ll DNA polymerase (Takara) and the Thermal Cycler Dice Real Time System (Takara) according to the manufacturer's instructions. The CT values were obtained using second derivative maximum methods (Thermal Cycler Dice Real Time System, Takara). Relative gene expression was determined by the ΔΔCT method, and values were normalized to the value obtained for *GAPDH* mRNA. For RT-PCR, cDNA was generated with oligo d(T) using PrimeScript II RT reagents (Takara), and PCR was then performed using KOD-Plus-Neo (TOYOBO, Japan) or Ex Taq II DNA polymerase. Amplified DNA was subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and signals were detected with an ImageQuant LAS4000 (Fujifilm) and quantified by MultiGauge software (Fujifilm). Supplementary Table S1 lists sequences of primers used in this study.

RNAi

RNAi (siRNA/scRNA; Invitrogen) or MISSION RNAi (siRNA/control RNA; Sigma) transfection was performed using Lipofectamine RNAiMAX (Life Technologies). In 35-mm Petri dishes, 5×10^5 of 293T cells were transfected with 5 μl Lipofectamine RNAiMAX and 100 pmol RNA. Supplementary Table S1 lists each RNA sequence.

Transfection for the *in vivo* splicing assay

Transfection of FLAG-Chtop pcDNA5FRT/TO (minigene for splicing assay), GFP minigene and HA-DsRed2 into 293T cells (cultured on a 35-mm collagen-coated Petri dish) was performed using Lipofectamine 2000 (Life Technologies). After 24 h of transfection, cells were harvested and extracts subjected to Western blotting or RT-PCR analysis.

In vitro splicing assay

To prepare a pre-mRNA substrate (biotin-labeled Ex2-(Int2-1)-Ex3) for *in vitro* splicing assay, we amplified a DNA fragment with Ex1-(Int2)-Ex4-GFP minigene as a template using a primer set, *Mlu*I-4244-for and 3138-MluI-rev (Supplementary Table S1), digested the PCR product with MluI and self-ligated with T4 DNA ligase. Using the resultant vector (ΔInt2-2/ΔInt2-3 minigene) as a template, the region corresponding to Ex2-(Int2-1)-Ex3 (2591-3138/4244-4388) was amplified with a primer set, T7-Ex2-for and 4388-rev (Supplementary Table S1), separated by 1% agarose gel electrophoresis and extracted from the stained gel band using the QIAquick Gel Extraction kit. Biotin-labeled Ex2-(Int2-1)-Ex3 was transcribed from the extracted DNA template (0.25 pmol) in the presence of 10 mM of Ribo m^7G Cap Analog (Promega), 5 mM of ATP, 5 mM of CTP, 5 mM of UTP, 0.5 mM GTP, 0.5 mM of biotin-UTP (Roche) using CUGA® 7 *in vitro* transcription kit (NIPPON GENE, Japan) and purified by denaturing (8M urea) PAGE and subsequent gel extraction.

DAP-Chtop and DAP-Lyar (a negative control) were pulled down with 100 μl of Ni-NTA (Qiagen) in 8 ml xTractor buffer (Clontech) containing 1% (v/v) Empigen-BB (SIGMA) from T-Rex 293 cells (1.0×10^8 cells) expressing DAP-Chtop (9), and DAP-Lyar (10) after induction with doxycycline for 24 h, respectively. The pulled down DAP-tagged proteins were purified further with 100 μl of FLAG-M2 agarose (SIGMA) using a solution containing 50 mM Tris-HCl pH7.4, 150 mM NaCl, 0.5% IGEPAL CA-630 and 1% Empigen-BB, dialyzed in buffer D (20 mM Hepes (pH7.9), 100 mM KCl, 0.2 mM EDTA, 20% Glycerol, 1 mM DTT) for over 12 h, and used for *in vitro* splicing assay.

In vitro splicing assay was performed as described (11), with some modification. *In vitro* splicing reaction was carried out at 30°C for 0–180 min in a solution containing 4 μl of HeLa nuclear extract (Promega) in 15 μl buffer D consisting of 25 fmol of biotin-labeled *Chtop* mRNA described above, 20 mM Hepes (pH7.9), 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, 0.4 U/μl RNase inhibitor (Promega), 2.7% (w/v) polyvinyl alcohol (SIGMA) and either DAP-Chtop or DAP-Lyar. The reaction volume was set to 25 μl. The splicing reaction was stopped by adding 300 μl of TRIzol reagent, and RNA was extracted according to the manufacturer's instructions. The biotin-labeled (BT) RNA was separated by denaturing (8M urea) 6% PAGE and detected by ImageQuant LAS4000 after binding to stabilized Streptavidin-HRP Conjugate (Thermo Scientific, 89880D).

Northern blotting

Denaturing 8M urea-PAGE of RNA and subsequent Northern blotting were performed as described by Izumikawa *et al*. (9). A Biotinylated (BT)-oligonucleotide (5′-GACTATATTGCAAGTCGTCACGGC-3′) probe was used to detect U4 snRNA (12).

Synthesis of BT-RNA

The template DNAs for *in vitro* transcription of Int2-1, Int2-2, Int2-3, Int2-1-2-1, Int2-1-2-2, Int2-1-2-2a, Int2-1-2-2b and Int2-1-2-2pSL RNA were amplified with KOD-Plus-Neo DNA polymerase using the Ex1-(Int2)-Ex4-GFP minigene as a template for Int2-1, Int2-2, Int2-3, Int2-1-2-1, Int2-1-2-2, Int2-1-2-2a and Int2-1-2-2b and Int2pSL for Int2-1-2-2pSL, respectively. Supplementary Table S1 lists the primer sets. Amplified DNA was separated by 1% agarose gel electrophoresis and purified from gel pieces using the QIAquick Gel Extraction kit. *In vitro* transcription was performed with 0.2 pmol of the template DNA using CUGA® 7 *in vitro* transcription kit (NIPPON GENE). Transcripts were collected with the RNeasy Mini kit (Qiagen) (Int2-1, Int2-2, Int2-3) or isopropanol precipitation (Int2-1-2-1, Int2-1-2-2, In2-1-2-2a, Int2-1-2-2b, Int2-1-2-2pSL) and purified using reverse-phase liquid chromatography. Biotin (BT) was added to the 5′ end of RNA using the 5′ EndTag Nucleic Acid End Labeling System and Biotin (Long Arm) Maleimide (Vector Labs). BT-RNA was collected by ethanol precipitation.

RNA–protein binding assay

Nuclear extract from 1.0×10^7 cells expressing FLAG-Chtop was used for this assay. BT-RNA (1 pmol) was added to the extract and subjected to immunoprecipitation using anti-FLAG magnetic beads as described in Supplementary Data. RNA isolated from FLAG-tagged protein complexes was subjected to denaturing urea-PAGE and Northern blotting (9,12). BT-RNA was detected with stabilized Streptavidin-HRP Conjugate (Thermo Scientific, 89880D). Chemiluminescence was detected with an ImageQuant LAS4000.

Expression of TF-N1

To construct HA-N1-pcDNA3.1, a DNA fragment of HA-N1 was amplified using KOD-Plus-Neo DNA polymerase with FLAG-Chtop pcDNA5FRT/TO as a template. The amplified DNA was inserted at the *Kpn*I/*Xho*I site of pcDNA3.1 to produce HA-N1-pcDNA3.1. To construct N1-pCold TF DNA, a DNA fragment of HA-N1 was generated by digestion of HA-N1-pcDNA3.1 with *Kpn*I/*Xba*I and the fragment inserted at the *Kpn*I/*Xba*I site of pCold TF DNA (Takara). TF-N1 was expressed in *Escherichia coli* Rosetta2 (DE3) via a 24-h induction at 15°C in the presence of 0.1 mM isopropyl β-D-1-thiogalactosylparanoside. Cells were extracted with xTractor Buffer (Clontech), and TF-N1 was pulled down using TALON Magnetic Beads (Life Technologies). The eluted solution containing TF-N1 was dialyzed with Tris-buffered saline for 6 h at 4°C, and purified TF-N1 was quantified with the BCA protein assay (Thermo Scientific).

Electrophoretic Mobility Shift Assay

Each binding reaction was performed at room temperature for 20 min. Each reaction was performed in 10 μl binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 μg of yeast tRNA (Ambion), 1 pmol of BT-RNA, 100 ng of recombinant protein) and electrophoresed on a 6% non-denaturing polyacrylamide gel at 100 V for 120 min in 0.5 × Tris borate/EDTA buffer (44.5 mM Tris-borate and 1 mM EDTA). The separated RNA–protein complexes were transferred to a Hybond N+ membrane that was dried and UV-crosslinked using the FS-1500 crosslinker (Funakoshi) at 120 mJ/cm^2. The BT-RNA was detected using stabilized Streptavidin-HRP conjugate and visualized with an ImageQuant LAS4000.

RESULTS

Exogenous expression of Chtop reduces its endogenous protein level

Using a HEK293 cell line that could inducibly express a single copy of the FLAG-tagged Chtop (FLAG-Chtop) transgene (T-REx 293 cell) (9,12–14), we confirmed that doxycycline (Dox) induced FLAG-Chtop expression (Supplementary Figure S1A), and FLAG-Chtop localized to nuclear speckles coinciding with the speckle marker SC35 (Supplementary Figure S1B) (2). Western blotting revealed that FLAG-Chtop expression decreased the level of endogenous Chtop over time, i.e. ≥50% decrease after 48 h compared with uninduced cells, whereas the level of FLAG-Chtop increased steadily, leveling off after 24 h (Figure 1A). This decrease in endogenous Chtop correlated with a decrease in its endogenous mRNA level as measured by RT-qPCR (Figure 1B). The endogenous *Chtop* mRNA level was reduced upon induction of FLAG-Chtop for 48 h compared with uninduced cells, whereas induction did not affect the level of endogenous *Chtop* mRNA in T-REx 293 cells that did not carry the FLAG-Chtop transgene (Figure 1B). In contrast, total *Chtop* mRNA including exogenous *Chtop* mRNA increased >15-fold after 48 h induction compared with uninduced cells (Figure 1B). These results suggested that the observed reduction in the level of endogenous Chtop upon the exogenous expression of FLAG-Chtop is caused by an autoregulatory mechanism at the mRNA level.

Exogenous expression of Chtop causes intron 2 retention in *Chtop* mRNA

The levels of several proteins such as the splicing factors ASF/SF2 (15), polypyrimidine tract–binding protein (PTB) (16), hnRNP L (17) and the fragile X mental retardation 1 protein (18) are modulated via regulation of alternative splicing of their pre-mRNAs, and therefore we examined whether the level of alternatively spliced *Chtop* mRNA changed upon the expression of FLAG-Chtop in T-REx 293 cells. We performed RT-PCR of *Chtop* mRNA with a primer set amplifying regions covering exon 1 (Ex1) to Ex6 (Ex1-Ex6) (Figure 2A) and detected DNA bands of ∼2000, ∼3500 and ∼7000 bp (Figure 2B, asterisks). Although the exon/intron makeup of the 7000-bp DNA fragment could not be determined, the 2000-bp DNA corresponded to the

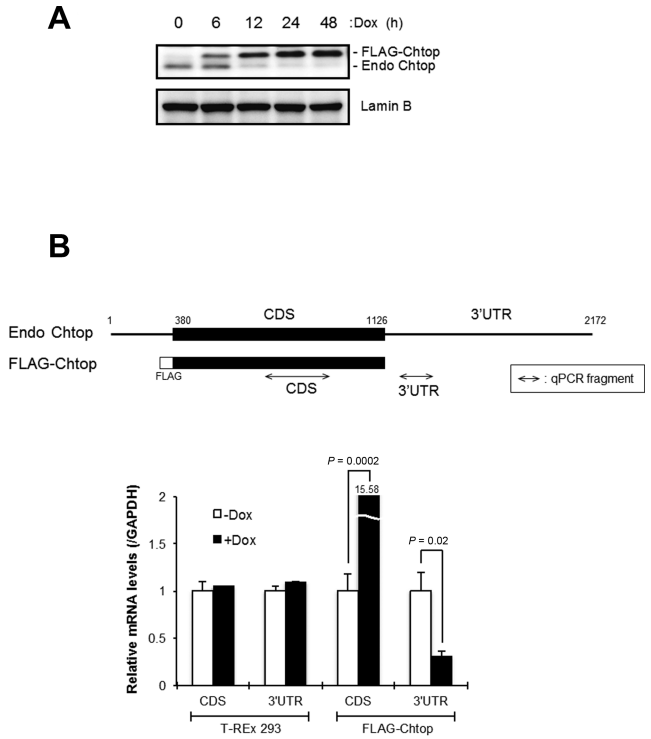

Figure 1. Exogenous Expression of FLAG-Chtop Represses the Expression of Endogenous Chtop. (A) Dox-induced expression of FLAG-Chtop. Whole-cell extracts were subjected to Western blotting with anti-Chtop (KT64) to reveal FLAG-Chtop and endogenous Chtop. Lamin B was used as a loading control. (**B**) Schematic representation of endogenous Chtop (NM_015607) and FLAG-*Chtop* mRNA. Double-headed arrows indicate the mRNA coding sequence (CDS: 705–940) (black) and the 3′ untranslated region (3′ UTR: 1138–1322) amplified by RT-qPCR. Endogenous *Chtop* mRNA and total Chtop (Endo *Chtop* + FLAG-*Chtop*) mRNA were measured by RT-qPCR with primer sets specific for the 3′ UTR or CDS of *Chtop* mRNA in T-REx 293 or FLAG-Chtop cells with (+Dox) or without (–Dox) Dox. Each value of *Chtop* mRNA was normalized by that of *GAPDH* mRNA, and relative *Chtop* mRNA levels were scaled to those without Dox treatment. Data represent the mean ± SEM of three independent experiments. *P* -values were calculated using the unpaired t-test.

size of Ex1-Ex6 without introns. The 3500-bp DNA fragment suggested the inclusion of intron 2 (Int2, i.e. 2163 + 1641 = 3804) or Int5 (2163 + 1699 = 3862) in addition to Ex1-Ex6. We subjected the DNAs to direct sequencing and confirmed that the 2000-bp DNA fragment had the intronless sequence Ex1-Ex6 (Supplementary Figure S2A) and that the 3500-bp DNA fragment contained Int2 (Supplementary Figure S2B); we therefore denoted this DNA as Ex1-(Int2)-Ex6. We noted that Int2 contained an in-frame stop codon at its 5′ end (Supplementary Figure S2B).

Dox induction of FLAG-Chtop after 48 h increased the amount of Ex1-(Int2)-Ex6 and reduced that of Ex1-Ex6, and the percent spliced in (PSI) values of Ex1-(Int2)-Ex6 increased ∼4-fold relative to that without Dox treatment (Figure 2B). Using two other primer sets to amplify regions covering Ex1-Ex4 and Ex4-Ex6, we confirmed that FLAG-Chtop induction increased the PSI values of the 2000-bp Ex1-(Int2)-Ex4 band, and reduced the PSI values of the 600-bp (Ex1-Ex4) and 1000-bp (Ex4-Ex6) bands (Supplementary Figure S2C). The increase of Ex1-(Int2)-Ex4 was detected in both the cytoplasmic and the nuclear fractions prepared by cell fractionation of the FLAG-Chtop induced cells (Supplementary Figure S2D), indicating that Int2-retained *Chtop* mRNA is delivered to the cytoplasm. A methyltransferase inhibitor (5′-methylthioadenosine, MTA) did not show any effect on Int2-retention of *Chtop* mRNA (Figure 2C). Since methylation of an arginine residue in Chtop is required for its role in the TREX complex, this suggests that the cytoplasmic localization of Int2-retained *Chtop* mRNA is not linked to the TREX complex (6).

To investigate Int2 retention further, we constructed a minigene containing Ex1-(Int2)-Ex4 positioned upstream of the GFP-coding DNA (wild-type Ex1-(Int2)-Ex4-GFP mini gene) (Supplementary Figure S2E). By using this minigene in which Int2 was the only intron, we monitored Int2 retention by RT-PCR with a primer set spanning Ex1 to GFP (Supplementary Figure S2E). In this analysis, the proportion of Ex1-(Int2)-Ex4-GFP increased with increasing FLAG-Chtop level compared with cells lacking FLAG-Chtop expression (Supplementary Figure S2F). In addition, the Chtop knockdown reduced the PSI value of Int2-retained transcript compared with that measured for cells transfected with scrambled/nonspecific RNA (scRNA) (Supplementary Figures S2G and S2H). We also used the E1A minigene (19) to distinguish between splicing inhibition and regulated alternative splicing, and showed that the increase of Chtop expression did not affect splicing of this minigene (Supplementary Figure S2I), suggesting that Chtop causes Int2 retention of *Chtop* mRNA as regulated alternative splicing. Collectively, these data strongly suggested that Chtop promotes Int2 retention during splicing of *Chtop* mRNA.

Chtop auto-regulates its expression by nonsense-mediated mRNA decay

Given the fact that a stop codon is present within *Chtop* intron 2, we predicted that the Int2-retained *Chtop* mRNA would be degraded by NMD (20). We therefore assessed the effect of the translation inhibitor cycloheximide, which also inhibits NMD (21), on Int2 retention in T-REx 293 cells expressing FLAG-Chtop. In agreement with our prediction, cycloheximide increased the level of Int2-retained *Chtop* mRNA in both FLAG-Chtop and control cells (Figure 3A). We also assessed the effect of siRNA-mediated knockdown of UPF1 (an essential component of the NMD machinery) (22) on Int2 retention, revealing that UPF1 knockdown increased Int2 retention (Figure 3B). In addition, using the Ex1-(Int2)-Ex4-GFP minigene, we showed that the amount of GFP protein decreased with increased FLAG-Chtop expression (Supplementary Figure S3). Collectively, these data support the notion that Int2-retained *Chtop* mRNA is degraded via NMD.

Both the N-terminal domain (N1) and RG-rich domain of Chtop are required for Int2 retention

To examine the mechanism by which Chtop causes Int2 retention during *Chtop* mRNA splicing, we first determined the region in Chtop responsible for Int2 retention. We pre-

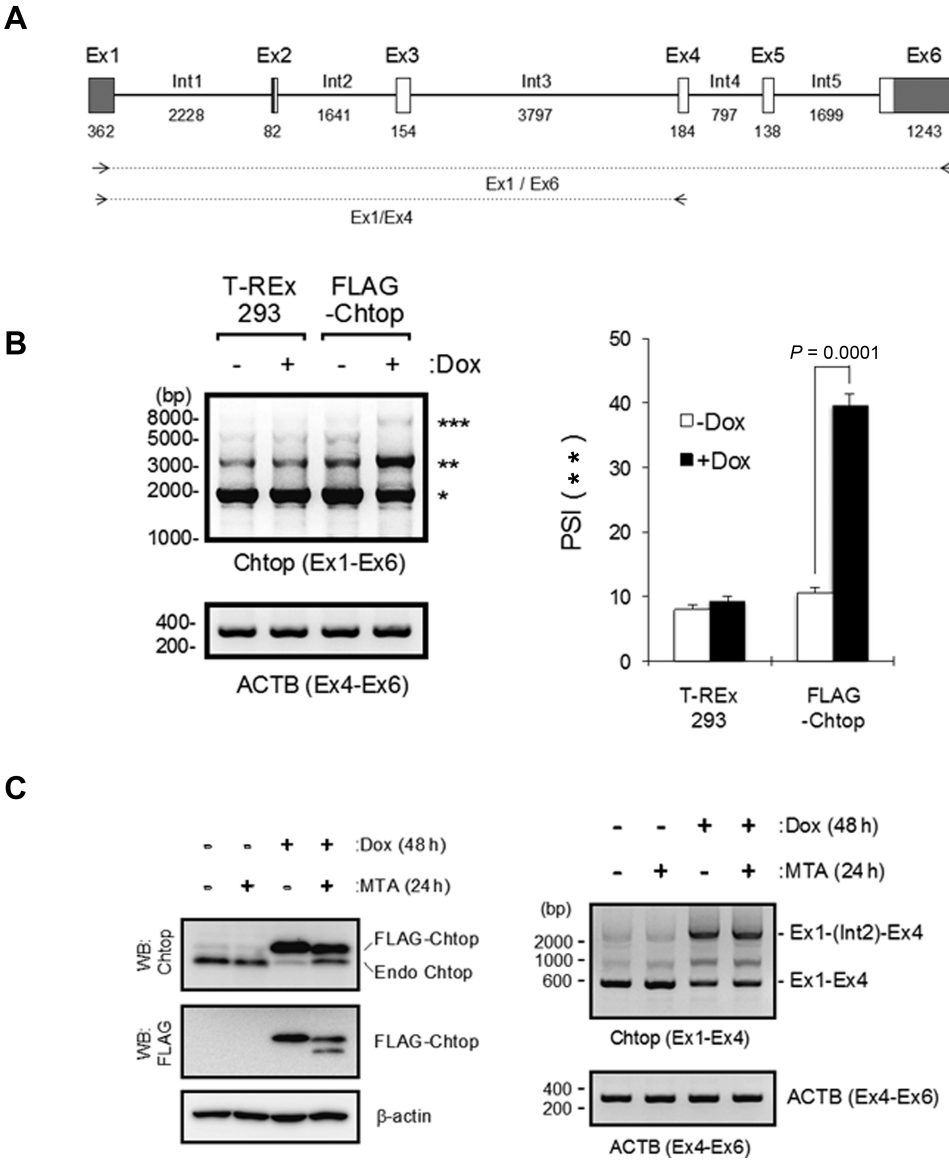

Figure 2. Chtop Causes Int2 Retention. (**A**) Schematic representation of *Chtop* gene. Exons (Ex, boxes) and introns (Int, lines) are indicated. Gray boxes denote non-coding regions in Ex1, Ex2 or Ex6. The sequence length is indicated under each exon and intron. Primers (arrows) and the PCR-amplified region (dotted line) are shown. (**B**) RT-PCR of *Chtop* mRNA upon FLAG-Chtop expression. DNA fragments (*, **, ***) were amplified from endogenous *Chtop* mRNA by using a primer set specific for Ex1 to Ex6 (Ex1-Ex6) of *Chtop* mRNA before (–) and 24 h after (+) Dox induction of T-REx 293 cells expressing FLAG-Chtop (left). T-REx 293 cells lacking FLAG-Chtop expression were used as control cells. *ACTB* mRNA was used as a loading control. The bar graph shows the percent spliced in (PSI) values of the ∼3500-bp DNA fragment (**) in total cDNA (right). Data represent the mean ± SEM of three independent experiments. The *P*-value was calculated with the unpaired t-test. (**C**) Effect of methyltransferase inhibitor on Int2 retention induced by Chtop overexpression. T-REx 293 cells expressing FLAG-Chtop were treated with (+) or without (–) 5′-deoxy-5′-methylthio-adenosine (MTA) for 24 h after induction (+) or not (–) with Dox for 24 h. Chtop protein (left) was detected by Western blotting with the indicated antibodies, and the DNA fragments (right) were detected with RT-PCR with the indicated primer sets.

pared additional Dox-inducible T-REx 293 cell lines expressing various deletion mutants of Chtop (ΔC1, ΔRG, ΔN1 or NLSΔN1), each containing an N-terminal FLAG tag (Figure 4A). When the four mutants were individually expressed in T-REx 293 cells (Figure 4B), only ΔC1 reduced the proportion of endogenous *Chtop* mRNA (Supplementary Figure S4A). Conversely, ΔC1 increased the proportion of the Int2-retained transcript (indicated as Ex1-(Int2)-Ex4 in Figure 4C). By contrast, ΔRG did not cause Int2 retention although the mutant protein localized mainly to the nucleus (Figure 4C, Supplementary Figure S4B). In addition to this result, expression of ΔN1 and NLSΔN1, which contain the RG domain, did not result in Int2 retention despite of the nuclear localization of NLSΔN1 (though ΔN1 seemed to be dispersed throughout the cell) (Supplementary Figure S4B). Consistent with these results, ΔC1 decreased the endogenous Chtop level, but the other mutants did not (Figure 4B). Collectively, these experiments suggest that the RG and N1 domains are both required for Int2 retention.

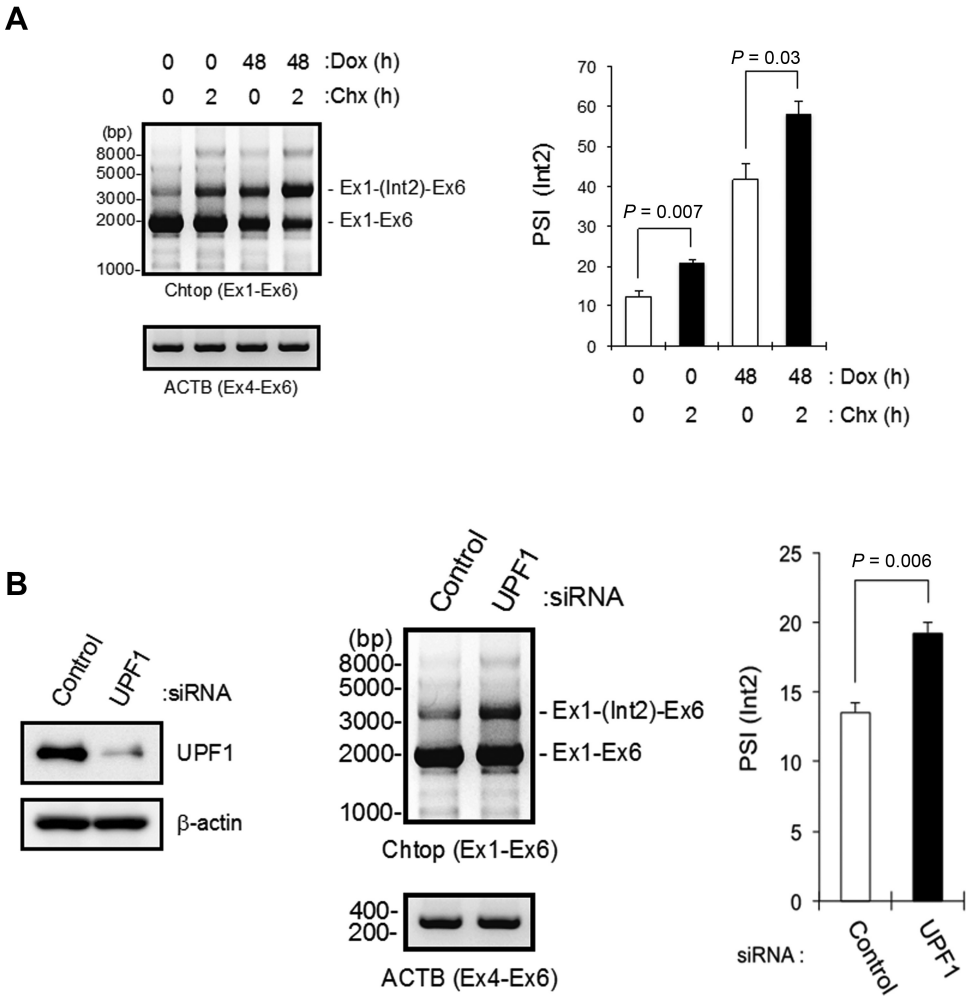

Figure 3. Int2-retained *Chtop* mRNA is degraded via NMD. (**A**) RT-PCR of *Chtop* mRNA upon cycloheximide (Chx) treatment. After Dox-mediated induction of FLAG-Chtop cells for 48 h, cells were treated with Chx for 2 h. *Chtop* mRNA was analyzed by RT-PCR with a primer set specific for Ex1 to Ex6 (Ex1-Ex6) of *Chtop* mRNA. Ex1-Ex6 and Ex1-(Int2)-Ex6 are indicated to the right of the figure. *ACTB* mRNA was used as a loading control. Bar graph shows the PSI values of Ex1-(Int2)-Ex6 mRNA. Data represent the mean ± SEM of three independent experiments. The *P*-values were calculated with the unpaired t-test. (**B**) RT-PCR of *Chtop* mRNA upon UPF1 knockdown. siRNA-mediated UPF1 knockdown in 293T cells is shown by Western blotting (left). β-actin was used as a loading control. *Chtop* mRNA in UPF1-knockdown cells was analyzed by RT-PCR with a primer set specific for Ex1-Ex6 of *Chtop* mRNA (middle). *ACTB* mRNA was used as a loading control. The bar graph reports the PSI values of Ex1-(Int2)-Ex6 mRNA. Data represent the mean ± SEM of three independent experiments. The *P*-values were calculated with the unpaired t-test.

Ex2 and 5′-region of intron 2 in *Chtop* mRNA are involved in intron 2 retention

To determine the region in *Chtop* mRNA that is involved in Int2 retention, we constructed a series of Ex1-(Int2)-Ex4-GFP minigenes that lacked Ex1 (56-362)(ΔEx1), Int2-1 (2697-3138) (ΔInt2-1), Int2-2 (3139-3970) (ΔInt2-2) or Int2-3 (3971-4243) (ΔInt2-3) (Supplementary Figure S5A). All of the minigenes tested displayed increased Int2 retention upon increased expression of Chtop (Supplementary Figure S5B). Next, we constructed minigenes in which two regions of Ex1-(Int2)-Ex4-GFP were altered by combination of deletion and replacement; i.e. ΔEx1-2/ΔInt2-1 had a replacement of Ex1 and Ex2 by Ex2 of the *INS2* gene and a deletion of Int2-1, ΔEx1-2 had replacement of Ex1 and Ex2 by Ex2 of the *INS2* gene, ΔEx1/ΔInt2-1 had deletions of Ex1 and Int2-1, and ΔEx2/ΔInt2-1 had a replacement of Ex2 by Ex2 of the *INS2* gene and a deletion of Int2-1 (Figure 5A). Of these minigenes, ΔEx1-2/ΔInt2-1 and ΔEx2/ΔInt2-1 did not display Int2 retention upon increased Chtop expression, whereas ΔEx1-2 and ΔEx1/ΔInt2-1 did retain Int2 upon increased Chtop expression (Figure 5B). Collectively, these data suggest that *Chtop* Ex2 and Int2-1 are directly involved in Int2 retention of *Chtop* mRNA, and thus are responsible for autoregulation of Chtop expression.

Based on these results, we synthesized an RNA containing the Int2-1 sequence between Ex2 and Ex3 (Ex2-(Int2-1)-Ex3), and used it as a substrate for an *in vitro* splicing assay to examine the effect of Chtop on Int2 splicing. The addition of purified Chtop increased the proportion of Int2-retained substrate when compared with reactions carried out in the absence of purified Chtop (Figure 5C), suggesting that Chtop regulates Int2 splicing directly.

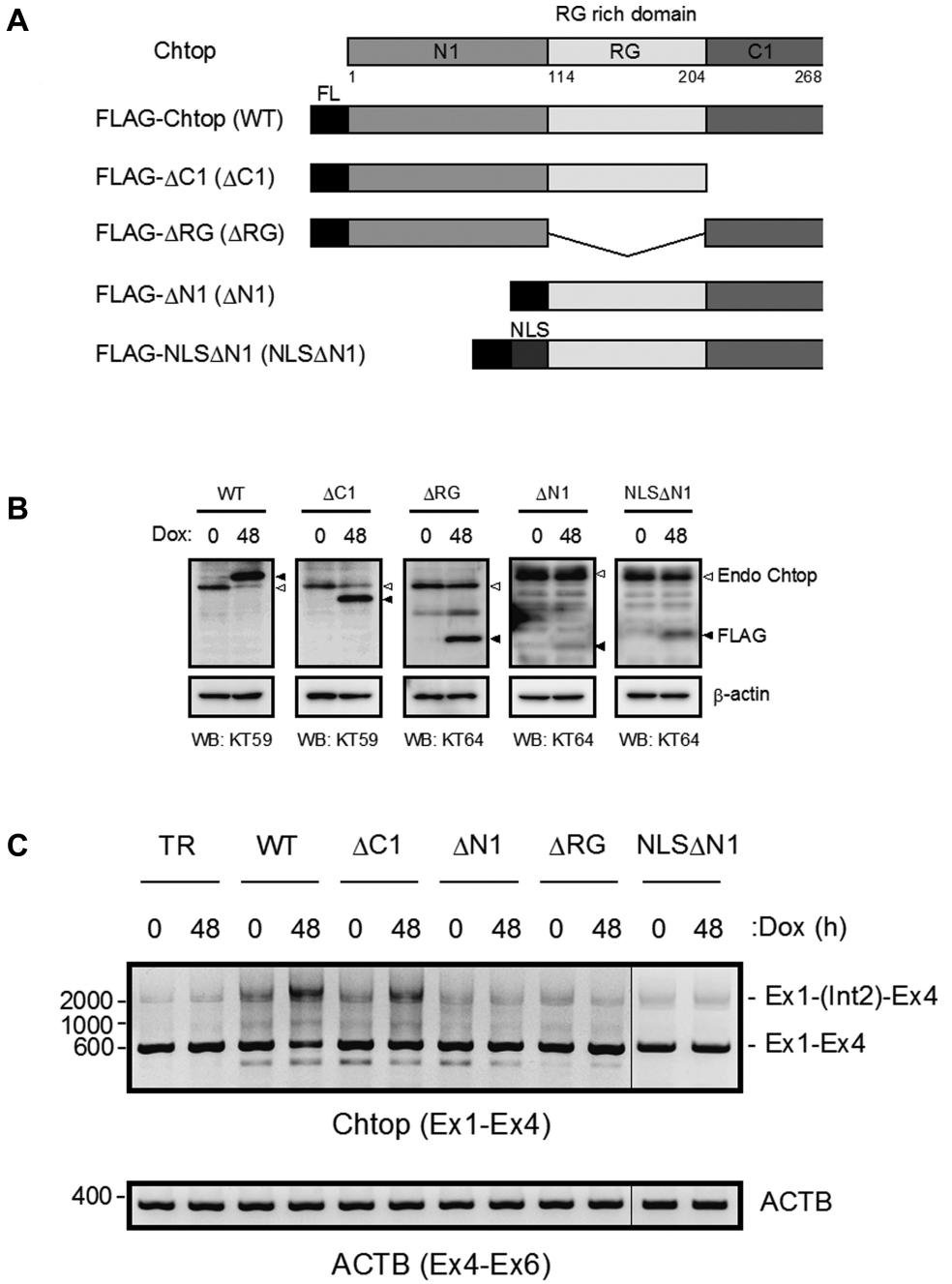

Figure 4. The N1 and RG regions of Chtop are responsible for Int2 Retention. (**A**) Schematic representation of the Chtop deletion mutants ΔC1, ΔRG, ΔN1 and NLSΔN1. FL: FLAG; NLS: Nuclear localization signal. (**B**) Mutant-expressing cells were induced with Dox for 48 h, and endogenous Chtop was analyzed by Western blotting with the antibodies indicated at the bottom of each blot (KT59, KT64). Each Chtop deletion mutant is indicated by 'FLAG' (closed arrowhead), and endogenous Chtop by open arrowhead. β-actin was used as a loading control. (**C**) RT-PCR of *Chtop* mRNA in Dox-inducible Chtop deletion-mutant cells. Each of the mutant cells was treated with Dox for 48 h, and *Chtop* mRNA was analyzed by RT-PCR with a primer set specific for Ex1 to Ex4. Ex1-(Int2)-Ex4 and Ex1-Ex4 of *Chtop* mRNA are indicated to the right of the figure. *ACTB* mRNA was used as a loading control.

N1 and RG domains bind Ex2 and putative stem loop region of Int2 in *Chtop* mRNA

Given that Chtop binds Int2-retained *Chtop* mRNA *in vivo* as determined by a combination of pulldown and RT-PCR analyses (Supplementary Figure S6A), we examined whether Chtop interacts with Ex2 and Int2-1 regions of *Chtop* mRNA. Since Ex2 has a relatively small length of 81 nucleotides, we synthesized a biotin labeled-RNA containing the entire region of Ex2 (BT-Ex2), and examined its binding to each of the Chtop domain mutants (ΔC1, ΔRG or NLS-ΔN1) by RNA–protein binding assay. This assay indicated that the RG domain is responsible for the binding to Ex2 (Figure 6A). Int2-1 has a length of 441 nucleotides, so we divided Int2-1 into two regions to syn-

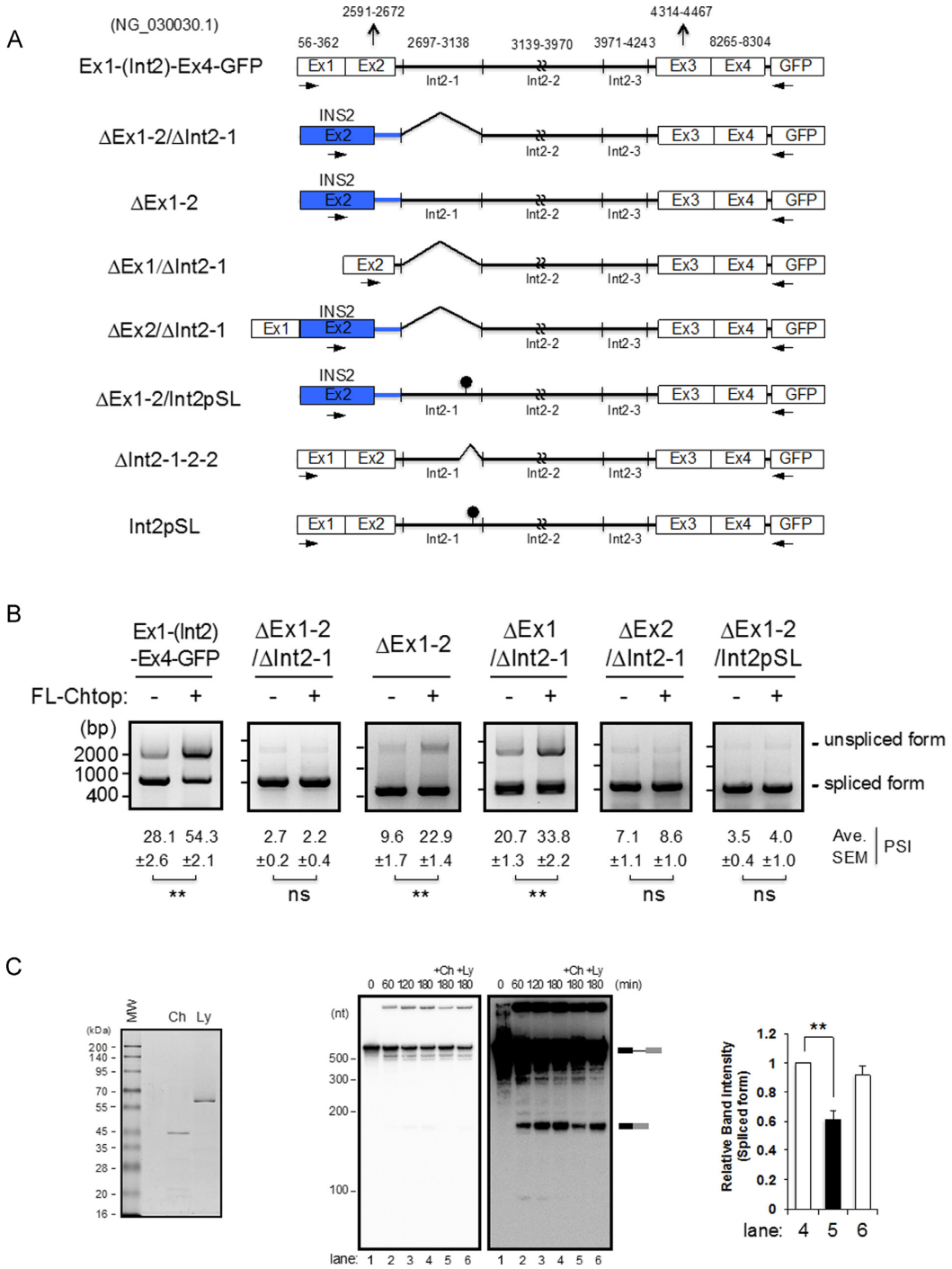

Figure 5. The Ex2 and Int2-1 region of *Chtop* mRNA is responsible for Int2 retention. (**A**) Schematic representations of Ex1-(Int2)-Ex4-GFP and its mutant minigenes constructed for *in vivo* splicing assays. Each Exon (Ex) and Intron (Int) of *Chtop* or Ex2 of *INS2* mRNA is shown. Nucleotide residue numbers corresponding to those of CHTOP gene (NG_030030.1) are shown on top of the figure. Primers (arrows) used for *in vivo* splicing assay are shown. (**B**) *In vivo* splicing assay using Ex1-(Int2)-Ex4-GFP and its deletion mutant minigenes with (+) and without (−) FLAG-Chtop expression. The PSI values of Int2 retained mRNA are shown under each of the figures. Data represent the average (Ave.) ± SEM of at least three independent experiments. The *P*-values were calculated with the unpaired t-test. (**C**) *In vitro* splicing assay using Ex2-(Int2-1)-Ex3 RNA in the presence of DAP-Chtop (+Ch) or DAP-Lyar (+Ly). The purified Ch and Ly were separated by SDS-PAGE and detected by CBB staining (Left). Ex2-(Int2-1)-Ex3 RNA was incubated with the nuclear extract of HeLa cells in the presence (200 ng) or absence of Ch or Ly for the indicated time periods. Overexposed picture is shown at the right side. Unspliced form and spliced form are indicated. Relative band intensities of spliced form in lane 4–6 were indicated in the graph. Data represent the average (Ave.) ± SEM of four independent experiments. The *P*-values were calculated with the paired t-test (**$P < 0.01$).

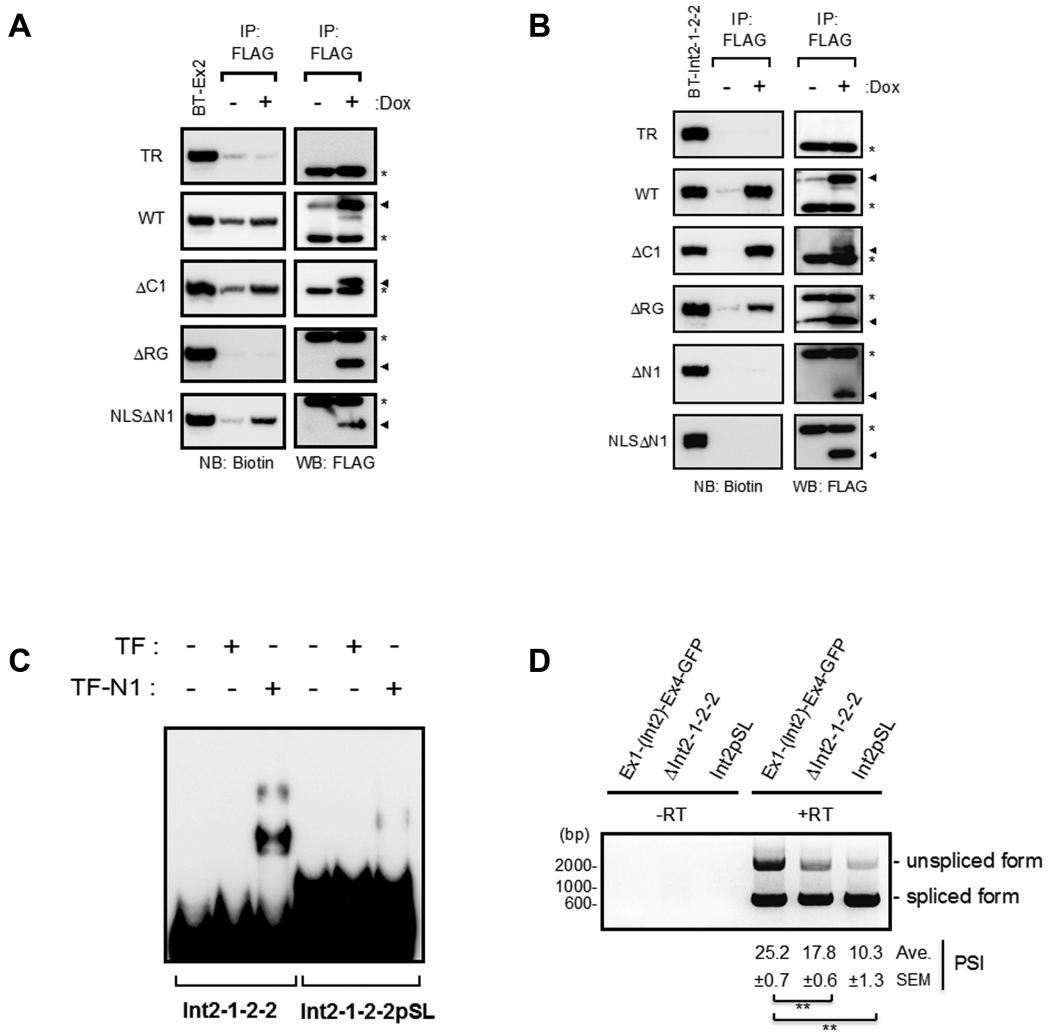

Figure 6. Identification of Chtop domains responsible for binding to *Chtop* mRNA. (**A** and **B**) RNA–protein binding assay. (**A**) BT-Ex2 or (**B**) BT-Int2-1-2-2 was incubated with the nuclear extracts of T-REx 293 cells (control; TR) or T-REx 293 cells expressing wild type (WT), ΔC1, ΔRG, ΔN1 or NLSΔN1 mutant with (+) or without (−) Dox induction, immunoprecipitated with anti-FLAG magnetic beads, and detected by Northern blotting. FLAG-tagged proteins were detected by Western blotting (arrowhead). IgG light chain of anti-FLAG antibody was also detected as shown by asterisk (loading control). (**C**) Electrophoretic mobility shift assay (EMSA) was performed using recombinant TF-N1 (or control TF) with BT-Int2-1-2-1 or BT-Int2-1-2-2pSL RNA. (**D**) *In vivo* splicing assay using Ex1-(Int2)-Ex4-GFP, ΔInt2-1-2-2 or Int2pSL minigene. PCR using reverse-transcribed RNA. (-RT) was used as a control of RT-PCR (+RT). The PSI values of unspliced form are shown under the figure. Data represent the average (Ave.) ± SEM of at least three independent experiments. The *P*-values were calculated with the unpaired t-test (**$P < 0.01$).

thesize their corresponding RNAs, taking the highly conserved regions of the Int2 sequence in *Chtop* mRNA among 100 vertebrates into account (UCSC Genome browser; http://genome.ucsc.edu/) (Supplementary Figure S6B and S6C). The synthesized BT-RNAs (BT-Int2-1-1 and BT-Int2-1-2) corresponded to the following regions of *Chtop* mRNA*:* 2668–2873 (206 nucleotides) and 2893–3169 (277 nucleotides), respectively (Supplementary Figure S6B and S6C). A third BT-RNA, BT-Int2-3, was also synthesized as a negative control representing a region not responsible for Int2 retention (3971–4314, 344 nucleotides) (Supplementary Figure S6B). The RNA–protein binding assay indicated that Chtop was bound tightly to BT-Int2-1-2 RNA and very weakly to BT-Int2-1-1 RNA, whereas BT-Int2-3 did not bind Chtop at all (Supplementary Figure S6D). To further narrow the region responsible for Chtop binding, we synthesized two additional BT-RNAs, namely BT-Int2-1-2-1 (2893–2994) and BT-Int2-1-2-2 (2997–3138) (Supplementary Figure S6C). The RNA–protein binding assay showed that BT-Int2-1-2-2 was bound by FLAG-Chtop (Supplementary Figure S6E).

A RNA–protein binding assay using BT-Int2-1-2-2 RNA and extracts of T-REx 293 cells expressing Chtop mutants (ΔC1, ΔRG, ΔN1 or NLSΔN1) showed that BT-Int2-1-2-2 bound to wild-type, ΔC1, and ΔRG but not to ΔN1 and NLSΔN1 Chtop (Figure 6B), suggesting that domain N1 is responsible for binding Int2-1-2-2. We confirmed the direct binding between N1 and BT-Int2-1-2-2 by electrophoretic mobility shift assay (EMSA) using recombinant N1 domain fused to trigger factor (TF) (Supplementary Figures S6F and S6G). TF-N1 caused a shift of BT-Int2-1-2-2 but not Bt-Int2-1-2-1, whereas TF alone (control) did not cause any

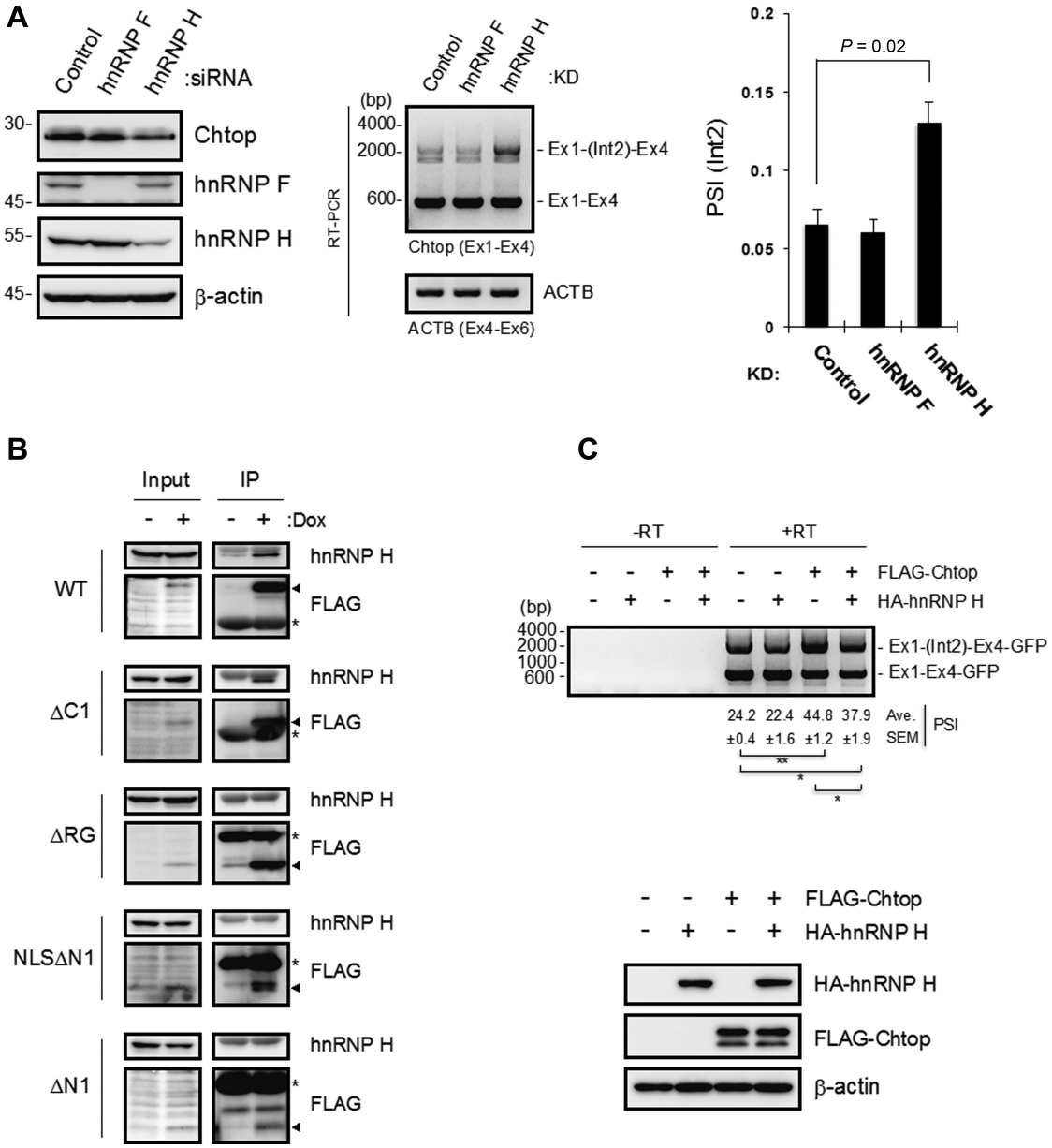

Figure 7. hnRNP H promotes excision of *Chtop* Int2. (**A**) hnRNP F or hnRNP H was knocked down with its corresponding siRNA in 293T cells and detected by Western blotting (left). β-actin was used as a loading control. *Chtop* mRNA in hnRNP-knockdown cells was detected by RT-PCR with a primer set specific for Ex1-Ex4 of *Chtop* mRNA (middle). *ACTB* mRNA was used as a loading control. The bar graph reports the PSI values of Ex1-(Int2)-Ex4 RNA (right). Data represent the mean ± SEM of three independent experiments. The *P*-values were calculated with the unpaired t-test. (**B**) FLAG-Chtop or each of its deletion mutants was immunoprecipitated from the nuclear extract of T-REx 293 cells expressing wild type; WT, ΔC1, ΔRG, NLSΔN1 or ΔN1 with (+) or without (−) doxycycline (Dox) induction and analyzed by Western blotting with anti-FLAG (arrowhead) and anti-hnRNP H antibodies. IgG light chain of anti-FLAG antibody is indicated with an asterisk. (**C**) Effects of FLAG-Chtop and/or HA-hnRNP H expression on Int2 retention in *in vivo* splicing assay using Ex1-(Int2)-Ex4-GFP minigene (upper panel). Expression vectors for FLAG-Chtop and/or for HA-hnRNP H (+) or empty vector (−) were transfected into 293T cells. PCR was performed using reverse-transcribed RNA (+RT); reactions lacking reverse transcriptase (-RT) were used as controls. The PSI values of Ex1-(Int2)-Ex4-GFP are shown below. Data represent the average (Ave.) ± SEM of at least three independent experiments. The *P*-values were calculated with the unpaired t-test. HA-hnRNP H or FLAG-Chtop was detected by Western blotting (bottom panel). β-actin was used as a loading control.

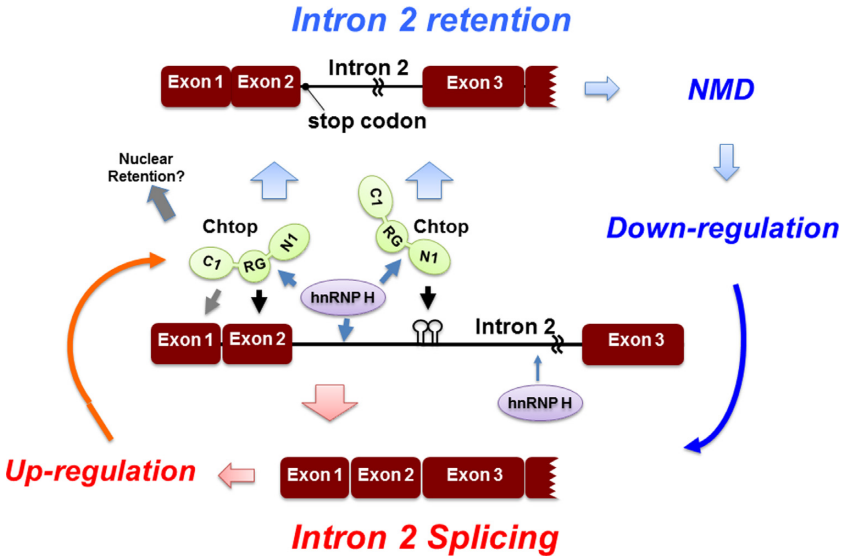

Figure. 8. Proposed mechanism by which Chtop regulates Int2 Exclusion. Chtop binds to the stem-loop region in Int2 of *Chtop* mRNA via its N1 domain and Ex2 via its RG region, and inhibits the excision of Int2. Int2-retained *Chtop* mRNA contains a stop codon at the 5′ side of Int2 and is degraded by NMD. hnRNP H binds Int2 of *Chtop* mRNA and N1-RG of Chtop protein, and promotes excision of Int2. Chtop and hnRNP H regulate Int2 splicing antagonistically.

shift (Supplementary Figure S6H). These data indicated that Chtop, via domain N1, binds the Int2-1-2-2 region of its mRNA.

To predict the secondary structure of the Int2-1-2-2 region, we used the RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (23). This analysis predicted that Int2-1-2-2 could form two stable stem-loops (Supplementary Figure S6I). Because the sequence of region Int2-1-2-2 is highly conserved among vertebrates, we assumed that these two predicted stem-loops are important for binding to Chtop and for Chtop-mediated Int2 retention. To further narrow the region responsible for Int2 retention, we constructed two additional Int2-containing minigenes, ΔInt2-1-2-2a and ΔInt2-1-2-2b, each lacking one of the predicted stem-loops of region Int2-1-2-2 (Supplementary Figure S6J). Mutant ΔInt2-1-2-2a decreased the ratio of Int2-retained mRNA to unretained mRNA by ∼40% compared with that of Ex1-(Int2)-Ex4-GFP, and ΔInt2-1-2-2b reduced the ratio by ∼77% (Supplementary Figure S6K). This result indicated that each of the predicted stem-loops contributes to Int2 retention with a more important role for Int2-1-2-2a. In agreement with this notion, our RNA–protein binding assays also showed that both BT-Int2-1-2-2a and BT-Int2-1-2-2b bound to FLAG-Chtop, but BT-Int2-1-2-2b bound very weakly (Supplementary Figure S6L). To disrupt the two predicted stem-loop structures of region Int2-1-2-2, we synthesized a mutant BT–RNA having a sequence corresponding to region Int2-1-2-2 but containing a G-to-C switch at nine positions in the two repeat sequences in Int2-1-2-2 (BT-Int2-1-2-2pSL) (Supplementary Figure S6I). BT-Int2-1-2-2pSL did not bind significantly to recombinant TF-N1 as revealed by EMSA (Figure 6C). We then constructed an Ex1-(Int2)-Ex4-GFP minigene containing a G-to-C switch at nine positions in region Int2-1-2-2 (Int2pSL) (Figure 5A and Supplementary Figure S6I). Int2pSL caused only marginal Int2 retention (Figure 6D). To assess the importance of these mutations further, we constructed a ΔEx1-2/Int2pSL minigene in which the Ex1 and Ex2 of *Chtop* mRNA were replaced by Ex2 of *INS2* mRNA and Int2-1-2-2 regions of *Chtop* mRNA by Int2pSL (Figure 5A), and showed that this construct did not cause Int2 retention (Figure 5B). Collectively, these results indicate that the two predicted stem-loops containing conserved repeat sequences in region Int2-1-2-2 of *Chtop* mRNA are responsible for Chtop-mediated Int2 retention and suggest strongly that the two regions Ex2 and In2-1-2-2 in *Chtop* mRNA are responsible for Int2 retention via binding to the RG and N1 domain of Chtop, respectively.

hnRNP H negatively regulates Int2 retention

Previous proteomic analysis identified a number of nuclear proteins that associate with Chtop, including proteins involved in mRNA splicing and mRNA export such as SRSF6, hnRNP F and hnRNP H (9). Because hnRNP F and hnRNP H are reportedly involved in splicing regulation (24–26), we examined the possible involvement of these two hnRNPs in Int2 retention by using RNAi-mediated knockdown. hnRNP H knockdown increased the proportion of Int2-retained forms ∼2-fold compared with cells treated with scRNA (control), whereas hnRNP F knockdown did not change the proportion (Figure 7A). Consistently, hnRNP H knockdown reduced the level of endogenous Chtop relative to β-actin when compared with control cells treated with scRNA (Figure 7A). In hnRNP H knockdown cells, cycloheximide increased the Int2-retained *Chtop* mRNA compared with control (Supplementary Figure S7A), suggesting that hnRNP H is involved in Int2 removal. In addition, the hnRNP H knockdown also increased Int2-retention when using the Ex1-(Int2)-Ex4-GFP minigene (Supplementary Figure S7B). These data suggest

that hnRNP H promotes Int2 exclusion and thus negatively affects Int2 retention.

To study how hnRNP H negatively impacts Int2 retention, we utilized antibody-mediated pulldown of endogenous hnRNP H. Association of endogenous hnRNP H with the Int2-retained *Chtop* mRNA increased in concert with increased FLAG-Chtop expression compared with cells lacking FLAG-Chtop expression (Supplementary Figure S7C). We did not detect association between hnRNP H and Int2-spliced *Chtop* mRNA (Ex1-Ex4 in Supplementary Figure S7C) even with FLAG-Chtop expression. These data suggest that hnRNP H increased its interaction with the Int2 region of Int2-retained *Chtop* mRNA in conjunction with increased Chtop expression, or that increased Chtop interaction with its mRNA results in the detection of hnRNP H interaction with the mRNA. To further narrow the region responsible for the binding of hnRNP H, we examined RNA–protein binding assay using the synthetic BT-RNAs and showed that hnRNP H bound Int2 in the regions corresponding mainly to Int2-1 and to a lesser extent to Int2-2. In contrast, hnRNP H did not have interactions with BT-RNAs corresponding to Int2-3 and exon 2 of *Chtop* mRNA (Supplementary Figure S7D).

We next examined interaction between hnRNP H and Chtop by pulldown analysis in combination with RNase treatment, revealing that FLAG-Chtop associated with hnRNP H independently of RNA (Supplementary Figure S7E). As a control, RNase activity was confirmed by the disappearance of U4 snRNA. The pulldown analysis was also carried out using the domain mutants ΔC1, ΔRG, ΔN1 and NLSΔN1 revealing that only ΔC1 associated with hnRNP H (Figure 7B). Because ΔRG and NLSΔN1 localized to the nucleus (as did wild-type FLAG-Chtop and ΔC1) but did not interact with hnRNP H (Figure 7B), these results suggested that the N1 and RG domains are responsible for Chtop binding to hnRNP H.

Finally, we examined the effect of overexpression of hnRNP H on Int2 retention, and found that the Chtop-induced Int2 retention was reduced by the overexpression of hnRNP H. Since overexpression of hnRNP H as such did not affect splicing and retention of Int2 of *Chtop* mRNA *in vivo* (Figure 7C), we conclude that Chtop and hnRNP H have an antagonistic effect on Int2 retention of *Chtop* mRNA.

DISCUSSION

Here, we demonstrate that the cellular level of Chtop is autoregulated via Int2 retention of its own mRNA, which subsequently undergoes NMD. We also show that domains RG and N1 of Chtop interact with Ex2 and the putative stem loop structure in Int2, respectively, and that both interactions are required for Int2 retention of *Chtop* mRNA (Figure 8). In addition, we show that hnRNP H is involved in Int2 removal. Given that deficiency of hnRNP H increases Int2-retained *Chtop* mRNA (Figure 7A), and that Chtop-induced Int2 retention is reduced by overexpression of hnRNP H (Figure 7C), we suggest that Int2 retention of *Chtop* mRNA is balanced by antagonistic action of Chtop and hnRNP H. Since Chtop and hnRNP H act on Int2 retention and splicing independently of each other (Figure 8), the proportion of Int2 retention is probably determined by the relative amount of Chtop to that of hnRNP H in the cell. In this case, we do not exclude the possibility that Chtop associates with hnRNP H without binding to regions in Ex2 and Int2-1-2-2; this association may also suppress the ability of hnRNP H to promote Int2 removal. Because Chtop bridges hnRNPs, including hnRNP H and the nuclear SMN complex, it is also possible that the hnRNP H–Chtop interaction is mediated by the SMN complex (9). It would therefore be interesting to determine whether Chtop anchors both hnRNP H and the SMN complex on Int2.

A number of studies have reported negative feedback mechanisms in which protein levels are autoregulated by modulating alternative mRNA splicing followed by NMD (22,27–31), but the molecular mechanisms underlying such autoregulation have been elucidated for only a few proteins (15–17,31). One such case is PTB, for which a high concentration increases its binding to intron 10 of its mRNA; this increases exon 11 skipping, producing an mRNA that then undergoes NMD (16). Another example is hnRNP L, which, at high concentration, increases its binding to intron 6 of its mRNA, thereby increasing exon 6a inclusion that also leads to NMD (17). However, these two examples of autoregulatory mechanisms do not involve intron retention.

Intron retention is generally thought to occur because of mis-splicing resulting in failed intron excision (32,33). Because intron-retained mRNAs often contain premature a stop codon, they are expected to undergo NMD (20,34). Recently, Wong *et al*. (33) reported that intron retention coupled with NMD is a physiological mechanism of gene expression control in normal granulopoiesis, but the molecular mechanism by which intron retention occurs is largely unknown. The present study provides a novel autoregulation mechanism involving intron retention followed by NMD.

Finally, because upregulation of Chtop is required for tumorigenicity of glioblastoma cells (8), it would be interesting to investigate whether Int2 retention-coupled NMD of *Chtop* mRNA is downregulated in glioblastoma cells. In addition, an increase in γ-globin level mitigates the severity of β-thalassemias (because γ-globin can mediate the replacement of adult hemoglobin with fetal hemoglobin) and sickle cell disease (because γ-globin can counteract polymerization of sickle hemoglobin) (35). Because Chtop is a critical modulator of γ-globin gene expression (4), Int2 retention during splicing of *Chtop* mRNA may be a therapeutic target for the treatment of these two diseases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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