An RNA *cis*-element upstream the HMGA1a binding site affects exon exclusion caused by HMGA1a

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In addition to its characteristics as a DNA binding transcription factor, HMGA1a is known to bind RNA, sequence specifically and regulate aberrant splicing of the Presenilin-2 gene in sporadic Alzheimer's disease[1,2]. We show here that an RNA *cis*-element, immediately upstream the HMGA1a binding site, induces exon inclusion upon mutating a GU sequence to CA. Psoralen mediated UV crosslinking showed U1 snRNP did not bind this sequence but oligonucleotide-mediated RNase H digestion showed it important for U1 snRNP to recognize the authentic 5' splice site. This sequence (5'-AAGUAC-3') was tested for hnRNPA1 binding and function. Coimmunoprecipitation of endogenous HMGA1a and hnRNPA1 in HeLa nuclear extract, implicated hnRNPA1 to be involved in HMGA1a mediated dysfunction of U1 snRNP. Though hnRNPA1 binding was weak, there was decreased binding when GU

was mutated to CA. hnRNPA1 alone had no effect of splicing of a 5' splice site adjacently downstream the HMGA1a binding site, but significantly attenuated HMGA1a mediated splicing suppression of this 5' splice site. Thus, hnRNPA1, known as the human homolog of hrp48 in the PSI model, attenuates HMGA1a mediated U1 snRNP dysfunction.

Alternative splicing is a critical step for higher eukaryotes to create a vast proteome from a limited amount of genes. Recent advances in this field have been focused an auxiliary sequences and proteins nearby the splice sites[3,4]. Nearby 5' splice sites which do not function, namely pseudo 5' splice sites, seem to affect the function of the authentic 5' splice site by simultaneous binding of U1 snRNP and hnRNPA1 to the same silencer motif[5]. Recently, the polypyrimidine tract-binding protein PTB has been shown to exert its function in alternative splicing depending on a positional effect of a splice site and PTB binding site[6].

During a search for the *trans*-acting factor responsible for aberrant exon exclusion of *Presenilin-2 (PS2)* exon 5 found in sporadic Alzheimer's disease (PS2V literature), HMGA1a was identified to bind a motif (5'-GCUGCUACAAG-3') found adjacently upstream the 5' splice site of *PS2* exon 5[1]. HMGA1a induced exon exclusion *in vitro* and *in vivo* through this HMGA1a binding RNA motif. A 2'-*O*-methyl RNA oligonucleotide of the HMGA1a binding site would act as a decoy and inhibit exon exclusion[2]. HMGA1a interacted with U1-70K, and expression of U1-70K but not U1A, could titrate HMGA1a induced exon exclusion. U1-70K and U1A are components of U1 snRNP, the complex which is commited to the 5' splice site at the earliest steps of spliceosome assembly. The mechanism was postulated that HMGA1a recruited U1 snRNP (through binding with U1-70K) to an upstream pseudo 5' splice site and leading to dysfunction of the authentic 5' splice site. In this way, the intron downstream the exon with the HMGA1a binding site would show suppressed splicing by HMGA1a, which was indeed the case[2].

We show here that an upstream pseudo 5' splice site showed no recruitment of U1 snRNP by HMGA1a but the authentic 5' splice site was influenced by HMGA1a binding. An RNA *cis*-element upstream the HMGA1a binding site is necessary for HMGA1a mediated exon exclusion and that hnRNPA1 is a strong candidate for

binding to this *cis*-element. The mechanism by which HMGA1a disrupts authentic 5' splice site function will be discussed.

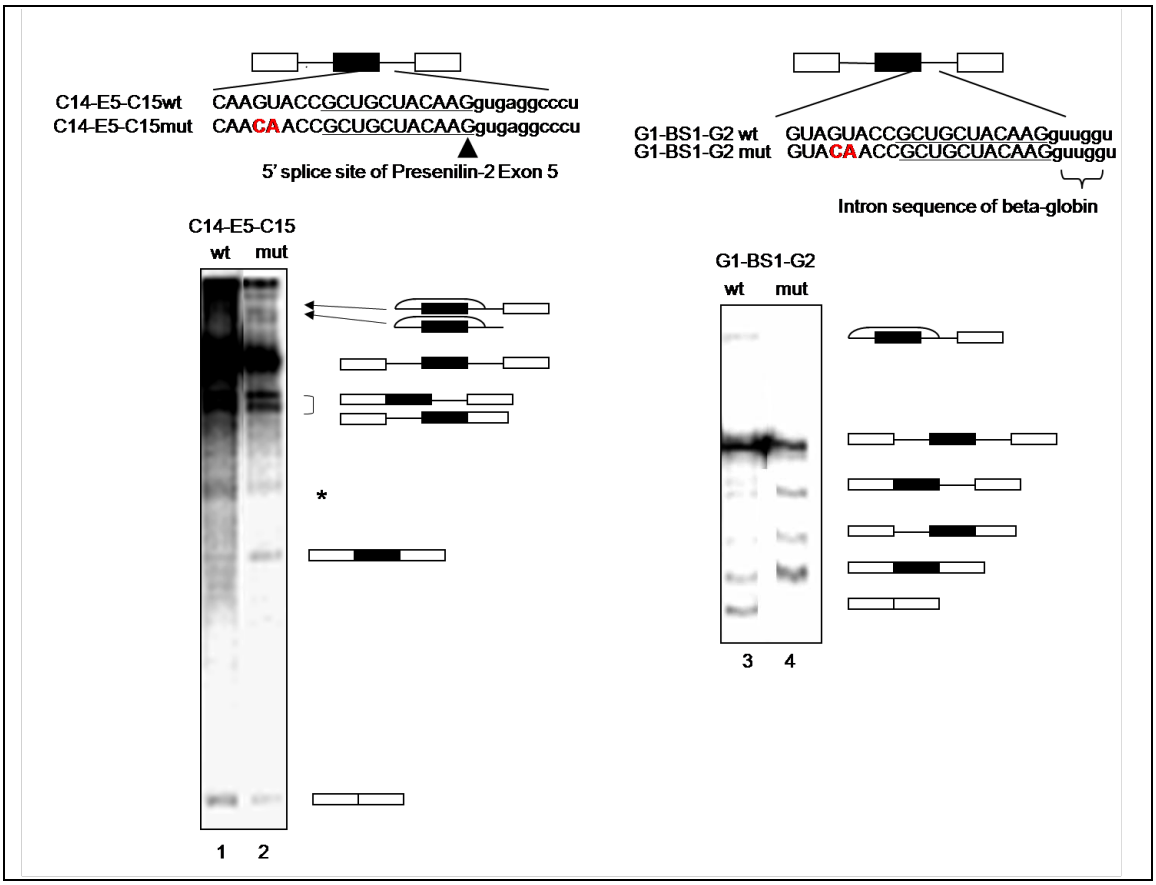

Fig.1 A *cis*-element upstream the HMGA1a binding site alters exon exclusion. In vitro splicing of C14-E5-C15 pre-mRNA (left panel) and G1-BS1-G2 pre-mRNA (right panel). The mutation is indicated in red. Mut showed more exon inclusion and less exon exclusion compared to wild type. HMGA1a binding site is underlined. Notice the decrease of splicing intermediate only found in exon exclusion. * indicates a non-specific band.

In order to test whether an upstream pseudo 5' splice site would influence HMGA1a mediated exon exclusion, two pre-mRNA substrates (C14-E5-C15 and G1-BS1-G2 [2]) with a mutation of GU to CA was introduced to an upstream GU site. The MAXENT scores (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)[7] are 1.75 (upstream pseudo 5' splice site) and 9.16 (authentic 5' splice site) for the middle exon of C14-E5-C15 pre-mRNA and -7.12 (upstream GU site) and 8.46 (authentic 5' splice site) for the middle exon of G1-BS1-G2 pre-mRNA. The GU sequence in G1-BS1-G2 pre-mRNA was created to resemble the putative hnRNPA1 binding UAG in the UAG motif[8]. As shown in figure 1, mutation of the pseudo 5' splice site in C14-E5-C15 pre-mRNA induced exon inclusion (Fig. 1 compare lane 2 to lane 1) in an *in vitro* splicing assay. This was in line with the presumption that an upstream pseudo 5' splice site would function in HMGA1a mediated exon exclusion. We tested G1-BS1-G2 pre-mRNA, where the upstream GU is too weak to be a pseudo 5' splice site, hoping that

the mutation in this pre-mRNA would not influence exon exclusion. But, on the contrary, this mutation also induced exon inclusion (Fig. 1 compare lane 4 to lane 3). Thus, both a pseudo 5' splice site and upstream GU site seemed to be involved in HMGA1a mediated exon exclusion.

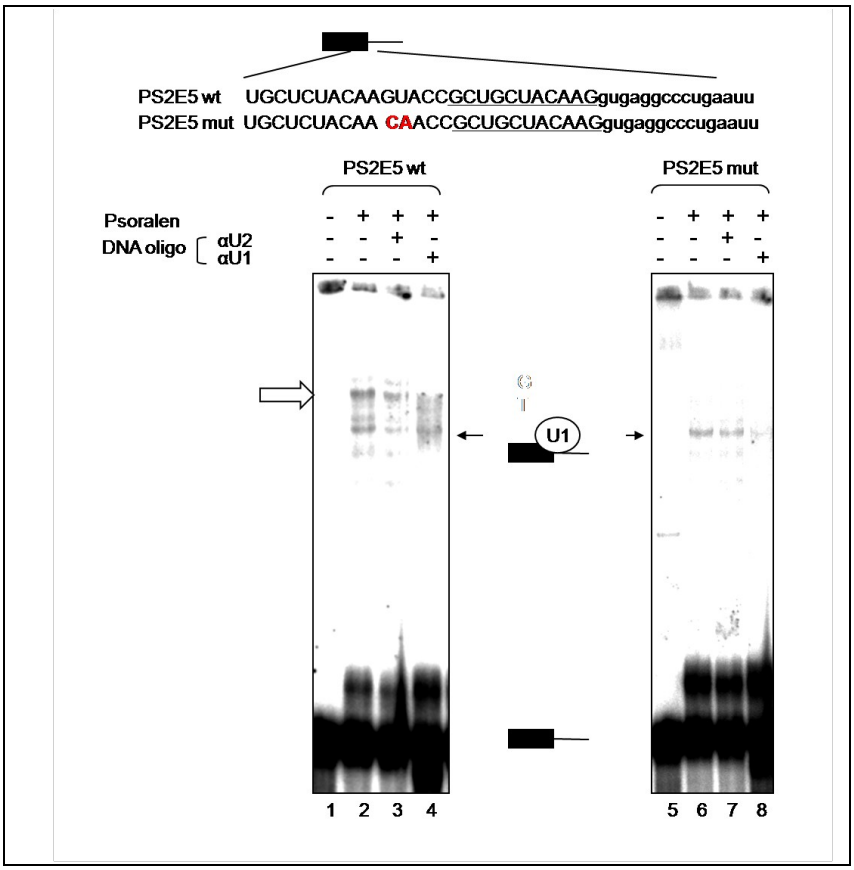

Fig.2 Psoralen mediated UV crosslinking of U1 snRNP to PS2E5 RNA. The native upstream sequence (shown in upper part (HMGA1a binding site is underlined.) (Mutation is in red.)) of Presenilin-2 was used to psoralen UV crosslink U1 snRNP of HeLa nuclear extract.

We could not determine whether a pseudo 5' splice site or hnRNPA1 binding site was involved in HMGA1a mediated exon exclusion by *in vitro* splicing. So, we checked U1 snRNP binding to the 5' splice sites by psoralen mediated UV crosslinking. We used the native sequence upstream the 5' splice site of PS2 exon 5. As shown in figure 2, two bands were found crosslinked when we used the wild type PS2E5 RNA (PS2E5 wt) (Fig. 2 lane 2), the band migrating faster was also found in the mutant PS2E5 RNA (PS2E5 mut) (Fig. 2 lane 6) which was digested by antisense-U1 DNA

oligonucelotide / RNase H digestion (Fig. 2 lane 8) but not by anti-U2 (Fig. 2 lane 7) implicating that this was U1 snRNA annealed and crosslinked to the authentic 5' splice site. However, the bands of wild type RNA were resistant to anti-U1 (Fig. 2 lane 7). Even if U1 snRNP was recruited to the upstream pseudo 5' splice site, it should be digested by anti-U1 since digestion was performed prior to psoralen mediated UV crosslinking. It may be U1 snRNP annealed and crosslinked to the 5' splice site through other parts of U1 snRNA. A candidate would be (5'-agataccat-3'), which starts from the 19th nucleotide of U1 snRNA. The antisense has a weak 5' splice site score of 3.16 (MAXENT)[7], which is in the stem of U1 snRNA and unlikely to unfold. Analysis of this is beyond the aim of this study, and lack of thorough RNase H digestion may be the answer.

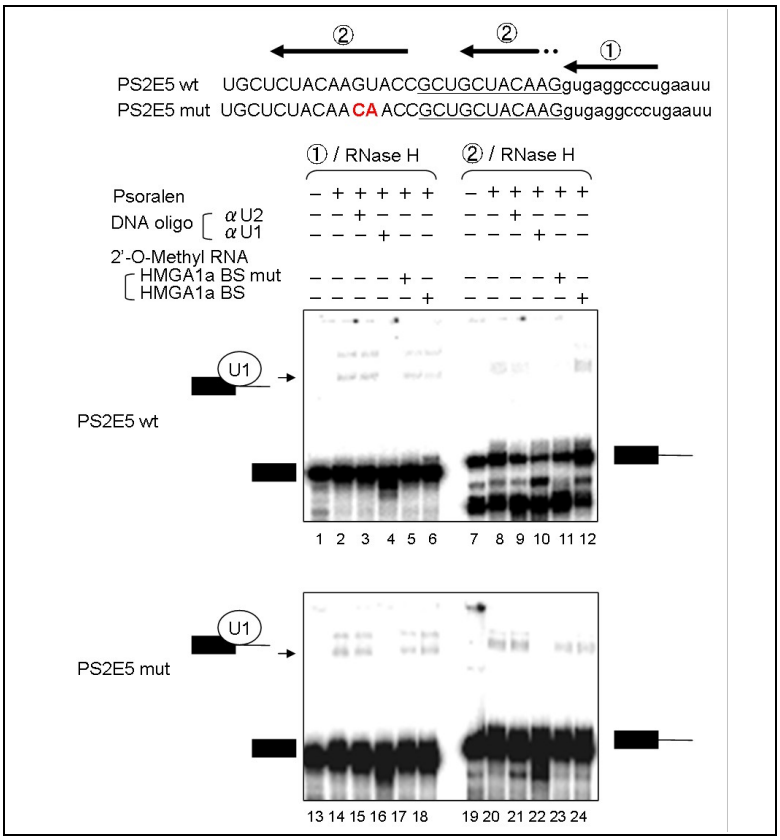

Fig.3 Psoralen mediated UV crosslinking of U1 snRNP in combination with RNase H sensitivity assay. After psoralen UV crosslinking, antisense DNA oligonucleotide and indicated in the upper part were used for RNase H digestion. 2'-O-methyl RNA of the HMGA1a binding site (HMGA1a BS) was used to titrate endogenous HMGA1a in HeLa nuclear extract.

When we combined a RNase H sensitivity assay with psoralen mediated UV crosslinking (Fig. 3), there was no difference of U1 snRNA/authentic 5' splice site crosslinking in PS2E5 wt and PS2E5 mut (Fig. 3 lanes 2 and 14). If U1 snRNP was recruited to the upstream pseudo 5' splice site, a difference should be observed between these two lanes. The slow migrating band found in PS2E5 wt in figure 2 was

also observed in both the PS2E5 wt and PS2E5 mut, thus these two bands seem to be U1 snRNP annealed and crosslinked to the authentic 5' splicing site. One of these bands may be an internal crosslink of U1 snRNA itself, which is already known[9,10]. But this is unlikely because these reactions were done in splicing conditions where U1 internal crosslinks tend not to appear[10]. Resistance to anti U1 digestion in figure 2 lane 4 could not be observed in figure 3 lane 4. In figure 3, antisense and DNA oligonucleotide / RNase H digestion was performed which means RNase H was added twice in anti U1 and U2 digestion samples, which may have ensured the RNase H digestion leading to anti U1 digestion in figure 3 but not figure 2. The most logical interpretation for the slow migrating band of U1 snRNA/authentic 5' splice site crosslinking in PS2E5 wt may be that an unusual form of U1 snRNP was induced by the upstream GU binding protein in these limited conditions. In figure 2, U1 snRNP may have crosslinked to internal crosslinks of PS2E5 which can be recognized as a slightly slower migrating band in figure 2 lanes 2,3,4 and 6,7,8. But since, internal crosslink of PS2E5 can be observed in both wt and mut, this can not explain the differential U1 snRNP crosslinking to PS2E5 wt and mut. And in figure 3, which has additional RNase H cleavage do not show internal crosslinks of PS2E5 and still two U1 snRNP related bands can be observed in PS2E5 wt. At least, what can be said from this data is that the upstream GU is important for this slower migrating band, and seems to be unrelated to HMGA1a since it can not be observed in HMGA1a BS lanes (Fig. 3 lanes 12 and 24).

When 2'-*O*-methyl RNA of the wild type HMGA1a binding site (HMGA1a BS) was added, wild type PS2E5 showed a band resistant to cleavage by antisense oligonucleotide / RNase H (Fig. 3 lane 6, band with slightly slower mobility than cleaved exon, corresponding to exon with intron). HMGA1a BS is known to prevent exon exclusion and induce exon inclusion, as shown in previous studies[2]. Thus, this resistant band may mean proper U1 snRNP binding to the authentic 5' splice site was induced in HMGBS, while it was not in PS2E5 mut (Fig. 3 lane 18). The upstream GU to CA mutation did not induce U1 snRNP occupancy, which is a resistant band to anti digestion, found in figure 3 lane 6 (faint top band) but not found in figure 3 lanes 14,15,17 and not even 18. So, exon inclusion induced by the GU to CA mutation seems to be related to HMGA1a induced exon exclusion. This is also evidenced by anti digestion. When HMGA1a BS was added to PS2E5 wt, an increase of U1 snRNP/authentic 5' splice site crosslink was detected, with resistance to anti digestion (Fig. 3 lane 12). This was not found in the PS2E5 mut (Fig. 3 lane 24). The upstream cleavage site may not be cleaved by anti digestion because the site is mutated, but the downstream cleavage site should be cleaved as in the wild type. Concerning U1 snRNP/ authentic 5' splice site crosslink, there was no difference with

anti digestion (Fig. 3 compare lanes 14,15 to 2,3) but more crosslink in anti digestion (Fig. 3 compare lanes 20,21 to 8,9). U1 snRNP seems to anneal to more nucleotides upstream the authentic 5' splice site, and becomes unrelated to HMGA1a mediated U1 snRNP dysfunction when the upstream GU is mutated to CA.

In summary, the upstream GU is involved in U1 snRNP/authentic 5' splice site dysfunction through HMGA1a mediated mechanism, but how HMGA1a disturbs authentic 5' splice site function can not be explained.

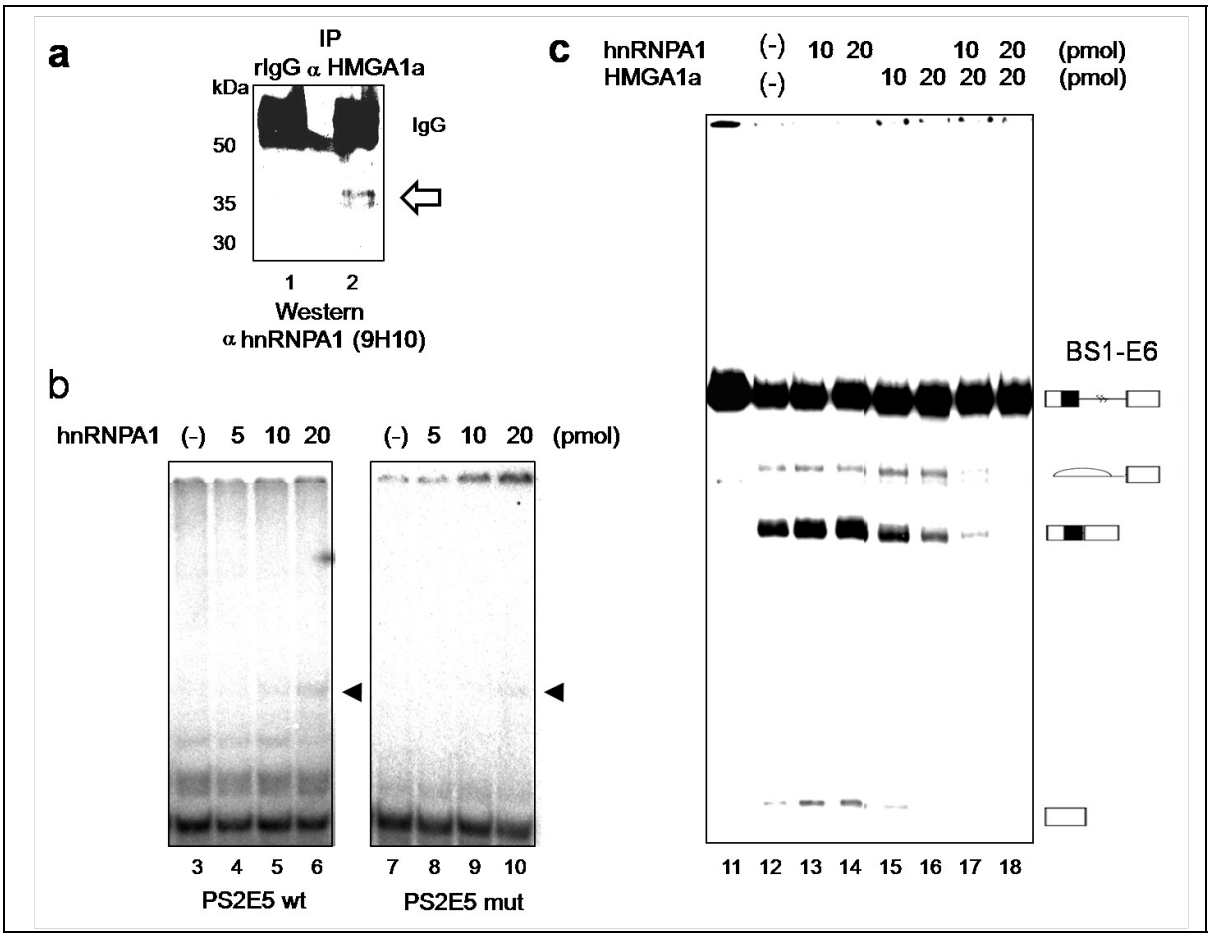

Fig.4 hnRNPA1 interacts with HMGA1a and attenuates HMGA1a mediated splicing suppression. **a,** Endogenous HMGA1a was immunoprecipitated by anti-HMGA1a antibody and revealed by anti-hnRNPA1 antibody (left panel, white arrow is hnRNPA1). **b**, RNA EMSA of hnRNPA1 using PS2E5 wt and mut as probe. Black arrowhead is hnRNPA1 binding to RNA. **c**, hnRNPA1 did not affect splicing of BS1-E6 pre-mRNA in an *in vitro* splicing assay, but when combined with HMGA1a, splicing suppression was attenuated (right panel). (HMGA1a binding site is depicted as black box in right side)

Since the upstream GU did not bind U1 snRNP (Fig. 3 lanes 2 and 14), we checked

whether hnRNPA1 is involved. First, interaction between hnRNPA1 and HMGA1a was checked. We were able to immunoprecipitate endogenous hnRNPA1 protein with anti-HMGA1a antibody (Fig. 4a). hnRNPA1 had a slightly better affinity to PS2E5 wt than mut RNA in an RNA electrophoretic mobility shift assay (Fig. 4b, compare lanes 5,6 to 9,10). This sequence (5'-AAGUAC-3') has low homology to exon splicing silencer 3 of HIV-*Tat* exon 3 (5'-UAGUGA-3') where hnRNPA1 binds and functions[11]. And also different from the putative hnRNPA1 binding UAG motif[8],(5'-UAGGG-3'). The core of the sequence (5'-AGUA-3') is part of UAG in tandem (5'-U**AGUA**G-3') (common sequence in bold).

hnRNPA1 alone did not affect splicing of BS1-E6 pre-mRNA (Fig. 4 compare lanes 13 and 14 to lane 12), but attenuated the splicing suppression by HMGA1a (Fig. 4 compare lanes 17 and 18 to lane 16). Thus, hnRNPA1 has low affinity to the upstream GU sequence, but functions in concert with HMGA1a.

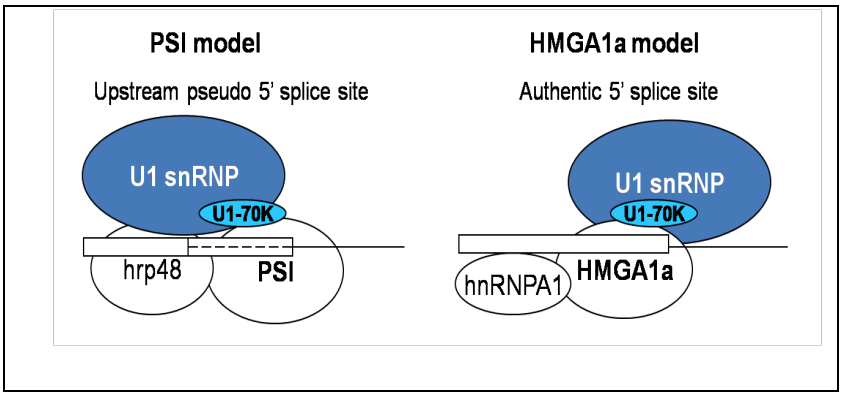

Fig.5 Comparison of the PSI model and the HMGA1a model. HMGA1a mediated exon exclusion is reminiscent of the PSI model except that hnRNPA1 functions as a synergistic *trans*-cofactor of HMGA1a.

From the results above, hnRNPA1 and HMGA1a possibly interact, and hnRNPA1 attenuates HMGA1a mediated splicing suppression. This is quite reminiscent but different from the PSI model in *Drosophila*[12-22] (Fig. 5) in two aspects. First, the human homolog of hrp48 is known to be hnRNPA1[21], but while hrp48 plays an essential role in splicing of P-element third intron (IVS3), hnRNPA1 alone does not affect splicing of pre-mRNA containing an HMGA1a binding site adjacently upstream the 5' splice site. Second, while U1 snRNP is recruited to an upstream pseudo 5' splice site in IVS3 splicing, U1 snRNP is binding the authentic 5' splice site in HMGA1a mediated splicing suppression. The mechanism for HMGA1a mediated dysfunction of the authentic 5' splice site has to be elucidated, but at least what we can say here is that hnRNPA1 is an important factor to stabilize HMGA1a mediated splicing suppression. HMGA1a is not an essential splicing factor, as it is barely expressed in normal cells. It is involved in aberrant splicing of *PS2* gene and resulting product[23,24] in pathological

stimulus such as hypoxia [1,25,26], chronic aluminum exposure[27] in sporadic Alzheimer's patients brain and neural cells. From its potential as an oncogenic product[28], HMGA1a induced aberrant splicing of Estrogen receptor alpha was checked in MCF-7 breast cancer cells, and indeed an aberrant 46 kDa isoform was found to be induced by HMGA1a RNA binding (data not shown). It would be interested to check global aberrant splicing induced by HMGA1a in cancer cells. Recently, the essential splicing factor Fox2 has been analyzed in cancer related splicing[29]. It would also be interesting to check the relationship of Fox2 induced and HMGA1a induced alternative splicing in cancerous cells[30].

How HMGA1a abrogates the adjacently downstream authentic 5' splice site can not be explained from our data. From the RNase H sensitivity assay, it seems HMGA1a changes the pattern of U1 snRNA annealing to the authentic 5' splice site. When we did a time course of U1 snRNA/5' splice site annealing, HMGA1a decreased normal dissociation of U1 snRNP from the 5' splice site, which is necessary for U5 and U6 snRNP to anneal near the 5' splice site in later steps of spliceosome assembly (data not shown). How U1 snRNA unwinding is disrupted by HMGA1a awaits structural and further biochemical studies.

Methods

Antibodies, plasmids and Oligonucleotides. Anti HMGA1a antibody (FL95) was purchased from Santa Cruz. Anti hnRNPA1 antibody (9H10) was a kindly provided by G. Dreyfuss. To construct δ-crystallin based heterologous plasmid (for C14-E5-C15 pre-mRNA), exon 5 and flanking intron sequences (221 bp for 5' and 51 bp for 3' intron sequences) of the *Presenilin-2* (PS2) exons 4–6 mini-gene plasmid[1] was amplified by PCR with flanking *Apa*I and *Nco*I sites, and the amplified fragment was subcloned into a PCR product which amplified pSP14-15[30] digested with *Sac*I by oligonucleotides with flanking *Apa*I and *Nco*I restriction sites (51 bp downstream from the 3' end of exon 14). The resulting C14-E5-C15 pre-mRNA includes, 77 bp of pSP14-15 first exon - 68 bp of pSP14-15 intron sequence – *Apa*I site – 221 bp of PS2 intron sequence – PS2 exon 5 – 51 bp of PS2 intron sequence – *Nco*I site – 185 bp of pSP14-15 intron sequence – 63 bp of pSP14-15 second exon. The plasmid for G1-BS1-G2 is previously described [2]. 2'-*O*-methyl RNA of HMG binding site (wt and mut) were purchased from FASMAC (Kanagawa, Japan). For RNase H sensitivity assay, antisense DNA oligonucleotide (5'-GGTACTTGTAG-3') and

(5'-GGTACTTGTAG-3') were used, purchased from Texas Genomics Japan (Tokyo, Japan).

***In vitro* splicing assay.** Plasmids were linearized by restriction enzymes (*Sma*I for C14-E5-C15, *Bam*HI for G1-BS1-G2, and *Xba*I for BS1-E6 plasmids) and used as template DNAs for *in vitro* transcription. Preparation of GpppG-capped ^{32}P-labeled pre-mRNA substrates by *in vitro* transcription with SP6 RNA polymerase was performed as described[32]. For C14-E5-C15, the full-length transcript was purified by preparative electrophoresis on a 5.5% polyacrylamide/7 M urea gel for the *in vitro* splicing assays as described [32].

Psoralen mediated UV crosslinking. A mixture of *in vitro* splicing was used to conduct psoralen mediated UV crosslinking according to the reported method with slight modifications[10,22]. Each reaction mixture (in a final volume of 12.5 l) containing ~20 fmol of ^{32}P-labeled pre-mRNAs and 200 ng of a psoralen derivative (4'-aminomethyl-trioxsalen hydroxy-chloride; Calbiochem) was incubated at 37°C for 15 minutes in the presence of 3 mM ATP, 20 mM creatine phosphate, 20 mM HEPES (pH 7.3), 3.5 mM MgCl2, 2% (w/v) of low-molecular-weight polyvinyl alcohol (Sigma), and 3.5 l of HeLa cell nuclear extract (CilBiotech). The mixture was irradiated with UV light (365 nm) for 15 min on ice at a distance of 3 cm from the light source (CSL-6A, Cosmo Bio). RNA was extracted with phenol, precipitated with ethanol, and fractionated on a denaturing 7% polyacrylamide gel. Dried gels were visualized using a Bio-imaging Analyzer (Fujix BAS1000; Fujifilm). To identify crosslinked products of RNA or pre-mRNA that interacted with U1 snRNA or RNase H sensitivity assay, we performed antisense oligonucleotide-directed RNase H digestion. Oligonucleotides (15 nt) complementary to the 5' ends of the U1 or U2 snRNAs were used as described previously[33]. To digest endogenous U1snRNA prior to psoralen UV crosslinking, 3.5 l of HeLa cell nuclear extract was incubated at 37°C for 20 min in a 12.5 l reaction mixture containing 50 pmol of each antisense oligonucleotide, 2 U RNase H (Takara Bio), and 3 mM $MgCl_2$.

RNA electrophoretic mobility shift assay. Each reaction mixture (in total 15 l) was incubated at 37°C for 20 min in a binding buffer containing 3 mM MgCl2, 50 mM KCl, 20 mM Tris-HCl (pH 7.5), 0.05 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 3 g of bovine serum albumin (BSA) and 1 g of tRNA. RNA-protein(s)

complexes were analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel [acrylamide:bisacrylamide = 30:1 (w/w)] at 4°C with 0.5 xTris-borate-EDTA (TBE) buffer. Dried gels were visualized by a Bio-imaging Analyzer (Fujix BAS1000; Fujifilm).

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K.O planned and performed all the experiments and wrote the manuscript. T.M., T.K., M.T. and A.M. coordinated the project. A.M. gave conceptual assistance.

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