Position-specific intron retention is mediated by the histone methyltransferase SDG725

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Abstract

Background: Intron retention (IR), the most prevalent alternative splicing form in plants, plays a critical role in gene expression during plant development and stress response. However, the molecular mechanisms underlying IR regulation remain largely unknown.

Results: Knockdown of SDG725, a histone H3 lysine 36 (H3K36)-specific methyltransferase in rice, leads to alterations of IR in more than 4700 genes. Surprisingly, IR events are globally increased at the 5′ region but decreased at the 3′ region of the gene body in the SDG725-knockdown mutant. Chromatin immunoprecipitation sequencing analyses reveal that SDG725 depletion results in a genome-wide increase of the H3K36 mono-methylation (H3K36me1) but, unexpectedly, promoter-proximal shifts of H3K36 di- and tri-methylation (H3K36me2 and H3K36me3). Consistent with the results in animals, the levels of H3K36me1/me2/me3 in rice positively correlate with gene expression levels, whereas shifts of H3K36me2/me3 coincide with position-specific alterations of IR. We find that either H3K36me2 or H3K36me3 alone contributes to the positional change of IR caused by SDG725 knockdown, although IR shift is more significant when both H3K36me2 and H3K36me3 modifications are simultaneously shifted.

Conclusions: Our results revealed that SDG725 modulates IR in a position-specific manner, indicating that H3K36 methylation plays a role in RNA splicing, probably by marking the retained introns in plants.

Keywords: Intron retention, Histone H3K36 methylation, Shift, SDG725, Rice

Background

Intron retention (IR), a specific form of pre-messenger RNA (pre-mRNA) alternative splicing (AS), has attracted increasing attention given its role in global gene expression regulation in both animals and plants [1–6]. Notably, IR is the most prevalent form of AS in higher plants, possibly due to the shorter intron length in plants than in animals [7, 8]. Genome-wide analyses revealed that greater than half of the AS events in rice belong to IR [9, 10].

Several studies have highlighted the functional importance of IR in plants. For example, IR has been shown to associate with abiotic stress response in barley [5, 11]. In strawberry, the level of IR is significantly reduced in post-fertilization compared to pre-fertilization, suggesting the involvement of IR in fruit maturation [12]. Retention of an intron in the 5′ UTR of the Zinc-Induced Facilitator 2 gene (ZIF2) enhances zinc tolerance in Arabidopsis [6]. Two intron-retained transcripts in Arabidopsis have been shown to remain in the nucleus to avoid nonsense-mediated degradation (NMD) [13]. During Arabidopsis gametophyte development, IR regulates translation in a transcription-independent and spliceosome-dependent manner [14]. Taken together, all these findings underscore the importance of IR in plant growth and development.

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Epigenetic regulators are known to be involved in AS regulation [4, 15, 16]. Histone modifications are particularly interesting because of their potential links between chromatin structure and co-transcriptional pre-mRNA splicing. Chromatin immunoprecipitation sequencing (ChIP-seq) analyses in human and mouse cells showed that H3K36me3 (tri-methylation of histone H3 at lysine 36) signals in introns and alternatively spliced exons are considerably lower than those in constitutive exons, suggesting that H3K36me3 modification likely acts as a mark for exons in animal cells [17]. Furthermore, H3K36me3 participates in AS by recruiting the splicing factor polypyrimidine tract-binding protein (PTB) via MRG15, an H3K36me3 reader protein in human [16]. BS69, a specific reader protein for H3.3K36me3, is involved in pre-mRNA splicing, especially IR, by interaction with the U5 small cytoplasmic fractionation extraction ribonucleoprotein (snRNP) in human cells [4]. Pajoro et al. discovered that H3K36me3 played a role in AS and flowering control in Arabidopsis, wherein mutants of SDG8 and SGD26, two methyltransferases of H3K36, affect temperature-dependent flowering [18]. Among all these findings support the notion that H3K36me3 plays a direct role in regulating AS.

However, it remains unclear whether all three forms of H3K36 methylation (mono-, di-, and tri-) are involved in AS regulation, especially IR, in plants. We previously reported that two H3K36-specific methyltransferases, SDG725 and SDG708, modulate gene transcription and affect rice growth and development [19–21]. In this study, we investigated splicing alterations in the SDG725- and SDG708-knockdown rice mutants by RNA sequencing (RNA-seq). We found that knockdown of SDG725 led to altered IR in thousands of genes. In addition, IR events tend to increase at the 3’ portion but decrease at the 5’ portion of the gene body when comparing the SDG725-knockdown mutant to the wild-type (WT) plants. The results coincided with a higher H3K36me2 occupancy at the 5’ part but a lower one at the 3’ part of the gene body. In contrast, SDG708 knockdown did not cause either these promoter-proximal shifts of IR or histone modification. Our work discovered a previously unknown shift of IR and its possible epigenetic regulator in rice.

Results

**SDG725 regulates a global shift of intron retention in rice** Since H3K36 methylation has been proposed for splicing regulation in animals, we sought to investigate whether a similar mechanism is also employed in plants. We took advantage of two transgenic rice lines previously generated, in which SDG725 or SDG708 was efficiently knocked down by RNA interference [19, 21]. Quantitative mass spectrometry showed that knockdown of SDG725 led to an increased level of H3K36me1 modification, but decreased levels of both H3K36me2 and H3K36me3 (Additional file 1: Figure S1) [12]. Two biological replicates of RNA-seq libraries were constructed, sequenced, and analyzed for 725Ri-1 (a stable RNAi line of SDG725), 708Ri-1 (a stable RNAi line of SDG708), and WT rice plants (Additional file 2: Table S1) [19, 21]. As the result of SDG725 knockdown, RNA-seq analyses revealed that 462 and 496 genes were up- and down-regulated, respectively (Additional file 1: Figure S2a). Gene ontology analysis showed that the differentially expressed genes (DEGs) in 725Ri-1 are enriched in metabolic and biosynthetic processes (Additional file 1: Figure S2b). The DEGs in 708Ri-1 (245 up- and 222 down-regulated) also are enriched in metabolic processes, but only a small fraction of them overlapped with those found in 725Ri-1 mutant plants [21], indicating the distinct biological roles of these two H3K36-specific methyltransferases in rice.

The splicing changes in rice were further analyzed by the SplAdder approach [22]. Consistent with previous findings [23], IR is the predominant form of AS events identified by comparing to rice genome annotation (Additional file 2: Table S2). To perform a quantitative investigation on IR, we used the intron retention index (IRI) to estimate the extent of retention for each annotated intron [3]. We found that 4714 genes contain one or more altered IR, including 2089 IRI-up introns (≥ twofold increase in IRI) and 4214 IRI-down introns (≥ twofold decrease in IRI) by comparing the 725Ri-1 plants to the WT rice plants. Ten IRI-altered introns were selected for validation by reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR). Primer pairs were designed to detect either spliced or intron-retained transcripts for each IR event (Additional file 2: Table S3). For all 10 cases, qRT-PCR results confirmed the RNA-seq data (Fig. 1a). To our surprise, we found that the IRI-up introns were favored at the 5’ portion of the gene body while the IRI-down introns were preferred at the 3’ portion of the gene body when comparing 725Ri-1 to WT rice plants using stringent coverage requirements (Fig. 1b, Additional file 1: Figure S3). The conclusion remained the same when the requirements of intron length coverage were relaxed (Additional file 1: Figure S4). In contrast, no location bias was observed between the 3326 IRI-up and 2497 IRI-down introns in 708Ri-1 (Fig. 1c, Additional file 2: Table S4). For exon skipping, no obvious position-specific splicing alteration was found by comparing 725Ri-1 with WT rice. Our results showed that knockdown of SDG725 but not SDG708 alters IR in a position-specific manner.

**Genes with increased IR show reduced expression levels** Among all the genes with one or more retained introns, 1399 genes only contain IRI-up introns, 2908 genes only contain IRI-down introns, and 407 genes contain both
IRI-up and IRI-down introns (Fig. 2a). IR may regulate gene expression through several different mechanisms, including but not limited to the following: (1) intron-retained transcripts are unstable, being degraded by nuclear RNA surveillance machinery or cytoplasmic NMD [3, 13, 24–26]; (2) intron-retained transcripts are stable and can be translated into new protein variants, where those introns are known as “exitrons” [27–29]; (3) a retained intron in a 5′ UTR regulates translation initiation [30] while IR in a 3′ UTR can either repress mRNA stability and translation by introducing more regulatory cis elements [31] or stabilize mRNA by

Fig. 1 SDG725, but not SGD708, affects global intron retention shift. a RNA-seq tracks (log2 transformed) and qRT-PCR validation for 10 differentially retained introns between 725Ri-1 and WT plants. Two biological replicates (rep1 and rep2) of RNA-seq data of 725Ri-1 and WT are shown. IRI and read numbers (in parentheses) are shown in the corresponding introns. Rice genes are shown below RNA-seq tracks. *, **, and *** indicate p values < 0.05, < 0.01, and < 0.001, respectively (t test). b, c Distribution of up-regulated (red) and down-regulated (blue) intron retention (IR) events between 725Ri-1 and wild-type (WT) rice (b), and between 708Ri-1 and WT (c). A twofold change in IRI was applied as a cutoff to define differential IR events. The retained introns also must be supported by at least 3 reads, length coverage ≥80%, and host gene expression ≥1 FPKM. Average IRI value of two biological replicates of both WT and mutant was used for location distribution Kolmogorov-Smirnov (KS) test. TSS transcription start site, TTS transcription termination site
avoiding NMD [32]. Therefore, global IR coupled with RNA stability could serve as a novel mechanism to fine-tune gene expression in rice. RNA-seq data were then used to determine the effect of altered IR on gene expression. We found that the expression level of the genes with only IRI-down introns tends to be up-regulated compared to those with only IRI-up introns, and the expression changes of the 407 genes with both IRI-up and IRI-down introns fall in between (Fig. 2b). These observations agreed with the notion that IR may serve as a post-transcriptional mechanism to reduce gene expression [1–4]. Consistent with Fig. 1b, in the 407 genes, IRI-up introns show a similar preference at the 5′ part of the gene body and IRI-down introns are enriched at the 3′ end of the gene body (Fig. 2c). The same occurs with the genes with IRI-up only and IRI-down only introns (Fig. 2d).

**Intron retention shift correlates with distribution shifts of H3K36 methylations**

We next asked why IR is shifted as the result of SDG725 knockdown. Considering the role of H3K36 methylation in IR in animals, we acquired H3K36 mono-, di-, and tri-methylation (H3K36me1/me2/me3) profiles of the 725Ri-1, 708Ri-1, and WT rice plants using ChIP-seq (Additional file 2: Table S5). By looking at gene-dense regions we found that H3K36me2 and H3K36me3 profiles exhibit apparent promoter-proximal shifts in 725Ri-1 but not in 708Ri-1 compared to those in WT rice plants (Fig. 3a). These observations were further confirmed by genome-wide analyses (Fig. 3b–d). It is worth noting that H3K36me1 levels are increased in nearly the entire gene body (Fig. 3b, left panel) upon SDG725 knockdown. In contrast, SDG708 knockdown led to a global decrease of all three H3K36 methylation marks (Fig. 3a, b).

To obtain a detailed view of H3K36 methylation changes at the individual gene level, we computed the difference of ChIP-seq tag density gene by gene between knockdown and WT plants (see details in Methods, Additional file 1: Figure S5). For almost all transcribed loci in the 725Ri-1 plants, H3K36me1 levels are increased across the gene body (Fig. 3c, left panel), while H3K36me2 (Fig. 3c, middle panel) and H3K36me3 (Fig. 3c, right panel) levels are increased at the 5′ end but decreased at the 3′ end of the gene body. However, this histone methylation positional bias was not detected in the 708Ri-1 plants (Fig. 3d), suggesting that the promoter-proximal shifts of H3K36me2/me3 are specific for the 725Ri-1 plants.

**H3K36me2/me3 shifts positively correlate with IR shifts caused by SDG725 knockdown**

SDG725 knockdown caused global shifts of H3K36me2 and H3K36me3, providing us with a unique opportunity to examine the potential contributor for IR shift. We
Fig. 3 (See legend on next page.)
therefore divided the transcribed genes into four groups. For type I genes, only H3K36me2 show a promoter-proximal shift. Type II genes are designated for H3K36me3 shift towards the 5′ end, while type III genes exhibit pattern shifts for both H3K36 methyl marks. Type IV genes show no shift for either H3K36me2 or H3K36me3. We noticed that H3K36me2 shift alone led to a considerable effect on the location bias of IR events (Fig. 4a). Similarly, H3K36me3 shift alone has an impact on IR shift (Fig. 4b). Intriguingly, when both H3K36me2 and H3K36me3 profiles are simultaneously shifted, IR showed a more significant shift compared to either H3K36me2 or H3K36me3 alone (Fig. 4c). As a control, unshifted H3K36me2/me3 exhibit no significant effect on IR switch (Fig. 4d). These results suggested that both H3K36me2 and H3K36me3 are involved in modulating IR shift, although a collaborative mechanism may exist in regulating IR.

However, the signals of H3K36 mono-, di-, and trimethylation were also compared in differentially retained introns. Interestingly, both the levels of H3K36me2 and H3K36me3 at IRI-up introns were higher than those at IRI-down introns (Additional file 1: Figure S6), supporting the notion that H3K36me2/me3 probably demarcate IR in rice.

Validation of the association between intron retention and H3K36me2 modification

The candidate gene approach was used to validate the potential link between H3K36me2 and IR. Two genes (LOC_Os01g24680, encodes putative 3-hydroxyacyl-CoA dehydrogenase; LOC_Os08g01760, encodes putative nutrition dehydrogenase) were selected from the 407 genes containing both IRI-up and IRI-down introns. For each gene, we selected six regions along the gene body to test H3K36me2 occupancy by ChIP-PCR. In addition, two IR

![Image](https://example.com/image.png)
events (one IRI-up and one IRI-down) for both genes were also examined by qRT-PCR in the same samples by multiple primer pairs spanning both intronic location and donor/acceptor sites (Fig. 5a, b, Additional file 2: Table S6). The results confirmed that the chromatin regions with increased levels of H3K36me2 modification produce increased IR events; accordingly, the chromatin regions with reduced H3K36me2 occupancy are associated with decreased IR events (Fig. 5).

**Transcripts with changed IR mainly accumulate in the nucleus**

Cellular fractionation was performed to examine whether the IR transcripts are accumulated in the nucleus or cytoplasm. The success of cellular fractionation was confirmed by qRT-PCR of genes specifically expressed in the nucleus or cytoplasm (Additional file 1: Figure S7) [33, 34]. RNA-seq analysis on each fraction showed that transcripts with changed IR in rice dominantly accumulated in the nucleus (Fig. 6a, b, Additional file 2: Table S7). To further examine whether cytoplasmic NMD contributes to the transcript abundance, both 725Ri-1 and WT plants are treated with cycloheximide (CHX) to stabilize transcripts that are otherwise degraded by NMD, followed by RNA-seq analysis.

Transcripts with a premature termination codon (PTC) showed an increased steady-state expression level upon CHX treatment (Additional file 1: Figure S8), indicating the successful inhibition of NMD. In contrast, genes with IR did not show an obvious expression increase upon CHX treatment (Additional file 1: Figure S9), suggesting cytoplasmic NMD has limited contribution to overall expression of IR loci in rice. These results indicate that intron-retained transcripts in rice are mainly accumulated in the nucleus rather than the cytoplasm and contribute to steady-state gene expression.

**Discussion**

IR is thought to play a critical role in gene expression regulation in animals and plants. However, how IR is regulated in plants remains largely unknown. In this study we revealed that SDG725 may regulate global IR through H3K36me2/me3 modifications. We previously reported that SDG725 acts as an H3K36 methyltransferase and functions in promoting gene transcription [19, 20]. In both WT and 725Ri-1 plants, a positive correlation was observed between transcript abundance and H3K36me1/me2/me3 levels (Additional file 1: Figure S10a), extending previous findings obtained from other species [17, 35]. We discovered that changes in average
H3K36me2/me3 occupancy level positively correlates with expression changes between 725Ri-1 and WT plants (Additional file 1: Figure S10b, c, left panels). The shift of H3K36me2/me3 affects IR but not expression level if the overall H3K36me2/me3 level does not change (Additional file 1: Figure S10b, c, right panels; Additional file 1: Figure S11). Taken together, we propose that SDG725 may control gene expression through two different mechanisms: (1) by modulating gene transcription via changing the overall levels of H3K36me2/me3, and (2) by regulating IR shift through H3K36me2/me3 shifts. How H3K36me2 or H3K36me2/me3 modulates IR in rice remains an open question. It might be achieved by reducing Pol II elongation rate and/or through a chromatin adaptor mechanism. In the first model, increased H3K36me2/me3 levels may slow down the elongation of Pol II. A longer dwell time of Pol II on introns may recruit splicing repressive factors or inhibit positive splicing factors to promote IR [2]. Alternatively, H3K36me2/me3 may be recognized by a specific “reader” protein, which interacts with splicing repressive factors to promote IR [4, 16]. Notably, the two models are not mutually exclusive and may act in concert to recruit splicing regulators. While significant expression change was not detected for known splicing factors between 725Ri-1 and WT rice (Additional file 2: Table S8), further investigations are warranted to identify H3K36me2/H3K36me3 reader(s) as well as downstream factors functionally involved in splicing regulation in rice.

The question of why the SDG708 knockout shows a global decrease of H3K36me2/me3 while the SDG725 mutant displays a pattern shift is intriguing. One possible explanation is the different enzyme specificity between SDG725 and SDG708. Although both are H3K36 methyltransferases, SDG725 plays a major role in mono- to di- and di- to tri-methylation, while SDG708 functions more on me0 to mono-methylation according to our previous studies [19, 38]. As expected, knockdown of SDG708 reduced the level of H3K36me1 around the transcription start site (TSS) and transcription termination site (TTS) (Fig. 3b). The occupancy of H3K36me2 and H3K36me3 was also reduced (Fig. 3b), possibly due to the lack of H3K36me1. In addition, SDG725 but not SDG708 contains a CW domain, which can bind to H3K4 methylation that is enriched at promoter regions [39]. When SDG725 became limiting under the...
that deserves further molecular characterization.

for the first time that a histone methyltransferase can regu-
late transcription and post-transcriptional gene
ordinate transcription and post-transcriptional gene

Several lines of evidence support the notion that
H3K36me2 in rice probably serves as the functional
counterpart of H3K36me3 in animal cells [4, 16, 17].
H3K36me2 in plants and H3K36me3 in animals share
similar occupancy profiles at transcribed regions (Fig. 3)
[21]. The H3K36me3 patterns in both rice and Arabidop-
sis resemble the H3K4me3 or H3K79me3 profile in ani-
imals (Additional file 1: Figure S15) [40–43]. These
observations suggest that plants and animals may employ
distinct epigenetic factors (e.g., writers and reads) to co-
ordinate transcription and post-transcriptional gene
regulation, although the underlying regulatory principles
are conserved during evolution. Our study demonstrated
for the first time that a histone methyltransferase can regu-
late the position preference of IR, a unique phenomenon
that deserves further molecular characterization.

Conclusions
We found that depletion of the histone methyltransferase
SDG725 in rice leads to position-specific alteration of in-
tron retention (IR), a phenomenon that has not been dem-
onstrated previously in any species. Further analyses
support the model that H3K36me2/me3 but not
H3K36me1 contribute to the IR alteration. As IR plays an
important role in regulated gene expression of both plants
and animals, the position-specific IR regulation revealed
by this study further extends our knowledge regarding the
complexity of gene regulatory networks.

Methods
Plant materials
Plants of Oryza sativa L. cv. Nipponbare were used in the
present study. Seedlings used for RNA extraction and
ChIP experiments were grown in artificial growth cham-
ers under a long day (LD) photoperiod (14 h 30 °C: 10 h
28 °C, light: dark). For a rational association between
ChIP-seq data and the RNA-seq data, the same set of
plant samples was divided into two parts, one part for
ChIP-seq library construction, and the other part for total
RNA extraction followed by RNA-seq library preparation.

Determination of H3K36 methylation level by mass
spectrometry
The 14-day-old rice shoots were subjected to histone
preparation based on an extraction method described
previously [44]. Then, the prepared histones were
separated according to molecular weight with sodium
dodecyl sulfate-polyacrylamide gel electrophoresis
(SDS-PAGE) using a standard procedure. The gels were
excised at a certain molecular weight and derivatized by
In-gel NHS and tested by mass spectrometry (MS) based
on a method published recently [45].

ChIP-seq and RNA-seq library construction and sequencing
We prepared ChIP-seq libraries using 14-day-old rice
shoots with the published ChIP protocol [46]. Anti-
monomethyl-H3K36 (ab9048), anti-dimethyl-H3K36
(ab9049), and anti-trimethyl-H3K36 (ab9050) were pur-
chased from Abcam, Cambridge, UK. The resulting
DNA and input control were subjected to ChIP-seq
library construction as previously described [40]. For
RNA-seq library construction, total RNA was extracted
from 14-day-old rice shoots using TRIzol reagent
(Invitrogen). PolyA+ RNA was enriched by selection
with two rounds of Oligo(dT)25 Dynabeads (Invitrogen).
A strand-specific RNA-seq library was constructed ac-
ording to a dUTP-based protocol [47] adapted from
Parkhomchuk et al. [48]. Both libraries were sequenced
on an Illumina HiSeq2000 instrument to generate 50-bp
single-end reads (for ChIP-seq) and 101-bp paired-end
reads (for RNA-seq). All related ChIP-seq and RNA-seq
data sets are summarized in Additional file 2: Table S9.

Bioinformatics analysis of ChIP-seq data
The sequence tags for H3K36me1/2/3 were aligned
against the complete reference genome of Nipponbare
(japonica) rice (the MSU Rice Genome Annotation Pro-
ject, Release 7 [49]) using Burrows-Wheeler Aligner
(BWA) v0.6.1 [50], with the uniquely mapped reads then
extracted with SAMtools [51]. To globally visualize the
level of each histone modification along and around rice
genes, genes longer than 500 bp were split into 300
blocks, with the upstream and downstream 2-kilobase
(kb) regions each split into 100 blocks, respectively. The
tag coverage of the aligned nonredundant ChIP-seq
reads was then calculated for each block, normalized to
10 million reads, and calibrated to the input. A normal-
ized tag intensity matrix was then obtained with custom
scripts, and the average tag intensity was used to con-
struct an aggregation plot. The normalized tag intensity
of each gene feature, such as exons and introns, was also
calculated as needed. For the heatmaps in Figs. 3 and
4, the x-axis denotes the relative position of a gene from 2
kb upstream of the TSS to 2 kb downstream of the TTS,
and the y-axis denotes the normalized coverage of ChIP-
seq reads calibrated by the input. The gene body (from
TSS to TTS) was split into 300 bins, and the 2-kb up-
stream TSS and downstream TTS regions were split into
100 bins, respectively; thus, a total of 500 bins for each
gene was used to calculate the coverage of ChIP-seq
reads. To define the pattern shift of H3K36me2 or H3K36me3 in Fig. 4, we require that the front half (150 bins) of the gene body should have at least 30% of bins (45 bins) with a difference value (725Ri-1 minus WT) greater than 0; at the same time, the latter half should have at least 30% of bins with a difference value smaller than 0. The rest are then considered as not having a pattern shift.

Bioinformatics analysis of RNA-seq data
To calculate the expression abundance of transcripts derived from each gene, we used a series of three programs: Bowtie v1.0.0 [52], TopHat2 v2.09 [53], and Cufflinks v2.1.1 [54, 55]. Briefly, the adaptor-removed raw RNA-seq reads were first aligned to Nipponbare (japonica) rice annotated transcripts from Release 7 of the MSU Rice Genome Annotation Project [49] with Bowtie to estimate insert fragment sizes and standard deviations, which were in turn used as parameter values in TopHat2. TopHat2 was then used to align the paired-end reads to the complete reference genome as mentioned above. Quantification of transcripts and genes, normalized for gene length, was performed with Cufflinks, as represented by fragments per kilobase of exon per million fragments uniquely mapped (FPKM). A differential expression analysis was performed using Cuffdiff, a subpackage of Cufflinks. A cutoff of at least twofold change, FPKM greater than 1 in at least one sample in a sample pair, and a p value smaller than 0.01 was used as the threshold to define DEGs.

Determination of changes in intron retention
To determine the IR and minimize the interference from exons, a new set of introns was acquired so that only introns or intron fragments that do not overlap with any exons were used as corresponding actual introns. In a similar way, we get a new set of exons that do not overlap with any other introns. To determine the IR events, the two exons neighboring an intron must be expressed (i.e., each with an FPKM value greater than 1), and there should be at least three reads supporting the IR events. To evaluate the IR level for a given intron, we introduced the intron retention index (IRI) for an individual intron to quantify its IR level. We obtained the IRI in the following way. The IR level (determined by FPKM) for a given intron was divided by the mean value of the expression level of its neighboring exons (determined by FPKM). To evaluate the IR changes between 725Ri-1 mutants and WT plants, the IRI ratio between the two samples was used, and those exceeding a twofold change were considered as differentially changed (up-regulated or down-regulated) IR events. To compare the location distribution difference between mutant and WT rice, we applied the two-sample Kolmogorov-Smirnov test, which is one of the most useful and general nonparametric statistical methods for comparing two samples without replication, as it is sensitive to differences in both location and shape of the empirical cumulative distribution functions of the two samples [56].

qRT-PCR for validation of intron retention
Total RNA was extracted from 14-day-old rice shoots using TRIzol reagent (Invitrogen). Reverse transcription was performed using Improm-II Reverse Transcriptase (Promega, Madison, WI, USA) according to the standard protocol provided. Quantitative PCR was performed using the gene-specific primers listed in Additional file 2: Tables S3 and S6.

ChIP-PCR for validation
The 14-day-old rice shoots were used in ChIP-PCR assays as previously described [46]. Anti-dimethyl-H3K36 (ab9049) was purchased from Abcam. ChIP assays were performed according to a previous method [19]. Quantitative PCR was performed to determine the enrichment of immunoprecipitated DNA using a kit from Takara, Otsu, Japan. The gene-specific primers are listed in Additional file 2: Table S6.

Nuclear/cytoplasmic fractionation extraction and quality assay
To determine whether intron-retained transcripts accumulate in the nucleus or cytoplasm, nuclear/cytoplasmic fractionation was performed according to a published method [33]. As quality controls for the fractionation, we evaluated the relative expression abundance of a cytoplasmic marker (Actin1) and nuclear markers (Pri-miR156d, Pri-miR156h, and Pri-miR156b) in the isolated fractions [33, 34]. Strand-specific RNA-seq libraries were constructed using both nuclear and cytoplasmic RNA according to a dUTP-based protocol [47] adapted from Parkhomchuk et al. [48].

Cycloheximide treatment and prediction of potential targets of nonsense-mediated mRNA decay
To evaluate the extent that NMD may be involved in the metabolism of intron-retained transcripts, we treated both 725Ri-1 and WT 2-week-old rice plants with cycloheximide (CHX) (which can block the translation process and rescue the NMD-targeted transcripts that would otherwise be degraded [57]) according to a published method with minor modifications [58, 59]. The same batch of dimethylsulfoxide (DMSO) treatments was used as the control. Then the treated rice samples were used for RNA extraction, followed by RNA-seq library preparation and sequencing as described above. After quality control, the raw sequenced reads were aligned to the rice reference genome as described above, then StringTie was used to assemble transcripts for each
sample and combine them together with Cuffcompare, a subpackage of Cufflinks [55]. Then the annotated unique start codon located on the assembled transcript was used for the open reading frame (ORF) prediction. A stop codon was defined as a premature termination codon (PTC) if it was located more than 50 nucleotides upstream of the last exon-exon junction, which is a well-known feature of NMD targets [60]. Lastly, a PTC-containing transcript was defined as a potential NMD target if its accumulation abundance increased more than twofold in 725Ri-1 rice compared to WT plants, with a p value smaller than 0.05, determined by Cuffdiff [55].

Analysis of splice site strength
MaxEntScan [37] was used to calculate maximum entropy scores for 9 bp spanning the 5′ (donor) splice sites (3 bp in the exon and 6 bp in the intron) and 23 bp spanning the 3′ (acceptor) splice sites (20 bp in the intron and 3 bp in the exon), respectively.

Additional files

**Additional file 1:** Figure S1. The quantification of H3K36 methylation in 725Ri-1 and WT plants. Figure S2. Differential gene expression between 725Ri-1 and WT rice. Figure S3. Accumulative plot of up-regulated (725Ri-1_UP) and down-regulated (725Ri-1_DOWN) IR events with different intron length coverage. Figure S4. Distribution of up-regulated (red) and down-regulated (blue) intron retention (IR) events between 725Ri-1 and wild-type (WT) rice (a) and between 780Ri-1 and WT (b). Figure S5. Schematic diagram illustrating the way to transform the H3K36 methylation changes between the 725Ri-1 and wild-type plants at individual gene level. Figure S6. Box plot for levels of H3K36 methylation at IR-up or IR-down introns. Figure S7. qPCR validation of quality of cellular fractionation in wild-type rice. Figure S8. Box plot for expression level of transcripts with premature termination codon. Figure S9. Box plot of gene expression before and after cycloheximide (CHX) treatment in either wild-type or 725Ri-1 rice. Figure S10. H3K36363636 methylation associates with gene expression levels. Figure S11. Distribution of up-regulated (red) and down-regulated (blue) intron retention (IR) events in genes with no obvious changes (≤ 5-fold) in total levels of H3K36me2 (a) or H3K36me3 (b). Figure S12. Box plot for intron length in two groups of introns by distinct methods in calculating the degree of intron retention. Figure S13. Box plot for GC percentage in two groups of introns. Figure S14. Box plot for maximum entropy score of 5′ splice site (a) and 3′ splice site (b) in two groups of introns. Figure S15. Comparison of H3K36me2/me3 distribution across gene body between rice (a) and Arabidopsis (b). (DOCX 43 kb)

**Additional file 2:** Table S1. Summary of the RNA-seq libraries. Table S2. Alternative splicing events discovered between rice RNA-seq data sets in this study compared with annotation by using SplAdder. Table S3. Primers used for validating intron-retention events. Table S4. The numbers of IR changed introns and genes. Table S5. Summary of the ChiP-seq libraries. Table S6. Primers used to validate intron retention in the same gene by qRT-PCR and those used to validate H3K36me2 by ChiP-PCR. Table S7. Number of retained introns for previously identified IR changed introns in nucleus or cytoplasm. Table S8. Expression changes of annotated rice splicing factors in our data. Table S9. Detailed information for ChiP-seq and RNA-seq data sets. (DOCX 43 kb)

Acknowledgements
We thank Dr. Wenhui Shen for his critical reading of the manuscript. Thanks also go to Dr. Li Jin and Hillary Sussman for suggestions on the data analysis. We thank Genery Biotechnology (Shanghai) Co, Ltd. for the deep sequencing service.

Funding
This work was supported by the National Key Basic Research Program of China (973 program 2015CB943000 to T.N) and the National Natural Science Foundation of China (31370752, 31570315, and 91519308 to A.D.; 31471192, 31521003, and 31771336 to T.N).

Availability of data and materials
The RNA-seq and ChiP-seq raw data are summarized in Additional file 2: Table S8 and can be found at the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRP136689.

Authors’ contributions
GW, MH, HF, and YS performed the bioinformatics analysis. TS constructed the RNA-seq and ChiP-seq libraries. KL, JS, BL, and MP performed experimental validation. JZ, AD, and TN designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 12 February 2018 Accepted: 5 April 2018
Published online: 30 April 2018

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