The exosome controls alternative splicing by mediating the gene expression and assembly of the spliceosome complex

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The exosome is a complex with exoribonuclease activity that regulates RNA surveillance and turnover. The exosome also plays a role in regulating the degradation of precursor mRNAs to maintain the expression of splicing variants. In *Neurospora*, the silencing of *rrp44*, which encodes the catalytic subunit of the exosome, changed the expression of a set of spliceosomal snRNA, snRNP genes and SR protein related genes. The knockdown of *rrp44* also affected the assembly of the spliceosome. RNA-seq analysis revealed a global change in bulk splicing events. Exosome-mediated splicing may regulate alternative splicing of NCU05290, NCU07421 and the circadian clock gene frequency (*frq*). The knockdown of *rrp44* led to an increased ratio of splicing variants without intron 6 (I-6) and shorter protein isoform small FRQ (s-FRQ) as a consequence. These findings suggest that the exosome controls splicing events by regulating the degradation of precursor mRNAs and the gene expression, assembly and function of the spliceosome.

Most eukaryotic genes contain coding exons and non-coding introns. During mRNA biogenesis, the introns are removed from the precursor mRNA (pre-mRNA), and the exons are joined in a process called splicing. Splicing is mediated by the spliceosome complex, a large RNA-protein complex. The spliceosome is comprised of U1, U2, U5, and U4/U6 small nuclear ribonucleoprotein particles (snRNPs) and an additional group of non-snRNP proteins. The budding yeast spliceosome contains approximately 100 different proteins, and the spliceosome complex in mammals contains over 300 different snRNP proteins and spliceosome-associated proteins. The structure and function of the spliceosome are under dynamic control.

The splicing of an exon can be either constitutive or alternative. In constitutive splicing, the exon is always included in the mature mRNA; while in alternative splicing (AS), an exon is not always included in the mRNA. AS occurs in multicellular organisms and has contributed to the evolution of highly complex proteomes by diversifying the gene expression products. Alternative splicing is essential for many important physiological processes in eukaryotes and is often regulated in tissue- or developmental stage-specific manners.

The regulation of mRNA degradation is crucial for the temporal control and fidelity of gene expression. The exosome complex, which consists of ten subunits, is a 3′→5′ exoribonuclease machinery that controls the turnover of all classes of RNAs. The structures and functions of the exosome are under dynamic control.

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highly conserved in eukaryotes. In budding yeast, the exosome core itself lacks RNase activity; instead, RNA degradation is achieved by Rrp44/Dis3 or Rrp6; these two proteins exert 3′ → 5′ exoribonuclease activity. Rrp44 is a processive hydrolytic exoribonuclease related to E. coli RNase R, a member of the RNase II (RNase B) family of hydrolytic exonucleases7,8. Rrp6 is a distributive hydrolytic exoribonuclease that mainly regulates the decay of nuclear RNAs9. In fungi and invertebrates, the genome only contains one copy each of the rrp6 and rrp44 genes. However, plants and vertebrates contain multiple copies of analogues of these genes10. The exosome has little RNase activity in the absence of cofactors, which may prevent the cell from inappropriate RNA degradation11. As a cofactor, the TRAMP (Trf4/5–Air1/2–Mtr4 polyadenylation) complex recruits the exosome to the RNA substrates and stimulates the exonucleolytic activity of the exosome11.

Most organisms possess circadian clocks that synchronize daily rhythmicities in metabolism, physiology, and behavior12. In recent decades, a growing body of evidence has suggested that alternative splicing plays a critical role in the regulation of circadian clocks in multiple species13–18. In the circadian clock of Neurospora crassa, WHITE COLLAR 1 (WC-1) and WC-2 are two positive components that form the WHITE COLLAR COMPLEX (WCC) to activate the transcription of the negative component FREQUENCY (FRQ) encoded by the frq gene19,20. The exclusion or inclusion of I-6 of the frq pre-mRNA leads to the production of small FRQ (s-FRQ) or large FRQ (l-FRQ) isoforms, respectively19,22. The ratio of s-FRQ to l-FRQ negatively correlates with the ambient temperature and is crucial for the fine-tuning of circadian periodicity and temperature compensation of the circadian clock22–24. The exosome is also implicated in the regulation of the circadian clock. Neurospora RRP44 directly regulates the turnover of frq mRNA and might account for the prolonged circadian period in the rrp44 knockdown strain25. In Neurospora, FRQ-interacting RNA helicase (FRH) is the homolog of yeast Mtr4, which plays multiple roles in regulating the circadian clock at both transcriptional and post-translational levels25–29.

During splicing, the exosome also regulates the degradation of pre-mRNAs. In yeast, the poorly spliced pre-mRNAs as well as the RNAs that contain longer introns are more susceptible to degradation30,31. The yeast exosome has been previously shown to regulate the processing and maturation of snRNAs5,32, suggesting that the exosome might be involved in the regulation of splicing. However, whether and how the exosome regulates alternative splicing remains elusive.

In this work, we showed that the exosome regulates the gene expression of splicesomal components and assembly of the splicesosome. Furthermore, in addition to the known role during pre-mRNA elimination, the exosome mediates splicing via the splicesome complex. This work also sheds light on the role of the exosome in the regulation of pre-mRNA splicing and the circadian clock.

Results
Exosome mediates expression of splicing factors. Rrp44 encodes the catalytic subunit of the exosome complex which regulates gene expression at the post-transcriptional level. To identify genes controlled by the exosome genome-wide, we conducted transcriptomic RNA sequencing (RNA-seq) in the dsrrp44 strain, in which the expression of rrp44 is silenced by the expression of a double-stranded RNA complementary to the rrp44 upon the addition of 0.01M QA25. The same strain without QA treatment was also subjected to RNA-seq as a control. Upon rrp44 knockdown, 2521 genes were up-regulated and 1180 genes were down-regulated. To identify the biological processes regulated by the exosome, we conducted bioinformatic analysis of the genes differentially expressed with or without QA treatment by searching the DAVID database33. KEGG pathway analysis indicated that the knockdown of exosome components of the spliceosome complex and spliceosome (Table 1), suggesting that the expression of spliceosomal genes might be controlled by the exosome.

The splicesome contains numerous small nuclear ribonucleoproteins (snRNPs) and small nuclear RNAs (snRNAs)34. The RNA-seq analysis showed that the levels of 16 mRNAs (snpl, prp39, snu71, prp40, prp5, snu14, brt2, prp8, prp46, snu13, prp3, snu66, luc7, snu23, isyl and syf1) that encode the components of the splicesome complex and protein arginine methyl transferase (prmt5) mRNA that encodes a splicesome-associated protein were significantly increased in the dsrrp44 strain (Fig. 1B; Table 2). PRMT5 is a type II protein arginine methyltransferase that regulates splicing by transferring methyl groups to arginine residues in splicesomal Sm proteins16. The expression of yeh1 was slightly but not significantly increased, and we also included this gene in the verification experiments. An increase in the expression of each of these genes was observed by qRT-PCR (Fig. 1B), which confirmed the RNA-seq results. However, the values of fold change largely differ from the qRT-PCR results, and this deviation can be explained by the fact that the RNA-seq results were obtained from only one pooled sample. The frq RNA levels were also elevated in the rrp44 knockdown strain using qRT-PCR analysis, consistent with a previous report25. The level of NCU01231 mRNA decreased in the presence of QA, which served as another control. We also evaluated the expression of snRNA genes (U1-1, U1-2, U1-3, U2-1, U4-1, U4-2, U4-5, U5 and U6-2), and the qRT-PCR results demonstrated that the expression levels of all tested snRNA genes were significantly elevated in dsrrp44 strain (Fig. 1A). These data imply that the expression of these snRNA genes is under the control of RRP44, which is consistent with the data obtained from yeast32.

To identify the splicesomal genes that are directly degraded by the exosome, we investigated the mRNA turnover of these 18 genes in the dsrrp44 strain with or without the addition of QA. To analyze
mRNA decay, the transcription inhibitor thiolutin was added to block the transcription\textsuperscript{25}. The silencing of \textit{rrp44} led to a marked decrease in the degradation rates of \textit{snp1}, \textit{snu71}, \textit{brr2}, \textit{prp40}, \textit{yhc1}, and \textit{prmt5} mRNAs, which suggested that the transcripts of these genes are direct targets of exosome-directed degradation. As expected, \textit{frq} mRNA displayed a reduced turnover when \textit{rrp44} expression was repressed, which agrees with previously reported results\textsuperscript{25}. In contrast, the stabilities of \textit{srp53}, \textit{isy1}, \textit{snf23}, \textit{luc7}, or \textit{prp8} mRNA did not significantly change (Fig. 1C). Unexpectedly, the degradation rate of \textit{prp39} increased upon \textit{rrp44} knockdown (Fig. 1C), which indicated that the mRNA decay of these genes is not under the direct control of RRP44. These data reflect that mRNA decay is redundantly and competitively regulated by different complexes, e.g., the 3′→5′ and 5′→3′ machineries. Thus, the exosome both directly and indirectly acts on the expression of a set of spliceosomal genes.

### Spliceosomal components regulate alternative splicing.

\textit{Neurospora} contains 1.7 introns per gene, and the average intron size is 134 nucleotides; both of these values are much lower than those in higher eukaryotes\textsuperscript{35}. To verify the roles of \textit{Neurospora} spliceosomal components in the regulation of splicing, we generated knockdown strains that expressed lower levels of \textit{prp5} (\textit{dsprp5}), \textit{prmt5} (\textit{dsprmt5}), and the snRNA gene \textit{U4-2} (\textit{dsU4-2}). PRP5 is an RNA-dependent ATPase that regulates pre-spliceosome formation and the release of spliced mRNA from the spliceosome\textsuperscript{36}. \textit{U4-2} is one member of the \textit{Neurospora} U4 snRNA gene family; U4 snRNA pairs with U6 snRNA in the U4/U6:U5 tri-snRNP complex\textsuperscript{4}.

The addition of QA induced the silencing of the targeted gene in these knockdown strains\textsuperscript{36}. The down-regulation of \textit{prp5}, \textit{U4-2}, and \textit{prmt5} expression in the knockdown strains upon QA addition was validated by northern blot analyses (Fig. 2A). To analyze the effect of the knockdown of these genes on splicing, the levels of the spliced and unspliced species of the three selected genes, \textit{NCU07995}, \textit{NCU08166} and \textit{frq}, were analyzed by RT-PCR. The \textit{frq} gene comprises eight splicing variants in total, and those variants with or without I-6 can be translated into I-FRQ or S-FRQ proteins, respectively. We selected these genes because the RNA-seq results revealed that the alternative splicing of these genes was differentially regulated in the \textit{dsrrp44} knockdown strain. As a control, the splicing of \textit{NCU04486}, which was not altered in the \textit{dsrrp44} strain, was also evaluated. The level of mature \textit{NCU07995} mRNA, but not \textit{NCU04486} mRNA, was decreased in the \textit{dsprp5}, \textit{dsU4-2}, and \textit{dsprmt5} strains in the presence of QA, while the mature \textit{NCU08166} mRNA level was also reduced in \textit{dsprp5} and \textit{dsU4-2}. The spliced variants of \textit{frq} without I-6 was decreased in \textit{dsU4-2} and \textit{dsprp5} strains and increased in \textit{dsprmt5} (Fig. 2B–D). The levels of \textit{frq} transcripts oscillate in constant dark (DD)\textsuperscript{30}. We further measured the splicing of \textit{frq} I-6 from DD12 to DD42 in increments of 6 h in WT and \textit{dsprp5} strains, and the results showed that the ratio of \textit{frq} variants without I-6 oscillated and the level of variants without I-6 in \textit{dsprp5} was significantly lower than that in WT (Fig. 2E).

Arginine/serine-rich non-snRNP splicing factors (SR proteins) contribute to the regulation of splicing specificity\textsuperscript{37}. The RNA-seq data also indicate an increase in the levels of several SR protein genes or SR protein related genes (data not shown). We measured the expression of four SR protein genes (\textit{srsf4}, \textit{sr140}, \textit{rhm39} and \textit{srsf15}), and in agreement, the results showed induction of levels of all the four genes. The results from mRNA degradation showed a significant decrease in the mRNA degradation of \textit{srsf4} and \textit{sr140}, suggesting they are direct targets of exosome. By contrast, the stabilities of \textit{rhm39} and \textit{srsf15} showed no significant change (Supplemental Fig. 1). Taken together, these data suggest that the spliceosome plays an important role in the regulation of alternative splicing in \textit{Neurospora}.

### Exosomes mediates the assembly of spliceosome complex.

The induction of spliceosomal genes observed in the \textit{dsrrp44} strain treated with QA suggests that the exosome might regulate the assembly and function of the spliceosome. To address this possibility, we performed sucrose density gradient sedimentation to fractionate the total protein extracts from \textit{dsrrp44} grown in the presence or absence of QA.

### Table 1. KEGG pathway analysis of differentially expressed genes (DEGs) in \textit{dsrrp44} strain (QA+ vs. QA −). Note: Enrichment of pathways was analyzed by DAVID; \textit{P}-values were adjusted using multiple hypothesis testing and the Benjamini and Hochberg correction\textsuperscript{60}.

| Pathway                               | DEGs with pathway annotation (2384 in total) | \textit{P}-value | Benjamini and Hochberg correction |
|---------------------------------------|---------------------------------------------|-----------------|----------------------------------|
| Pyrimidine metabolism                 | 23 (0.96%)                                  | 5.8E-5          | 4.2E-3                           |
| RNA polymerase                        | 13 (0.5%)                                   | 3.3E-4          | 1.2E-2                           |
| Spliceosome                           | 28 (1.2%)                                   | 7.5E-4          | 1.8E-2                           |
| Purine metabolism                     | 23 (1.0%)                                   | 2.1E-3          | 3.7E-2                           |
| Base excision repair                  | 10 (0.4%)                                   | 3.1E-3          | 4.5E-2                           |
| DNA replication                       | 13 (0.5%)                                   | 5.2E-3          | 6.1E-2                           |
| RNA degradation                       | 15 (0.6%)                                   | 1.5E-2          | 1.4E-1                           |
| Basal transcription factors           | 8 (0.3%)                                    | 3.2E-2          | 2.5E-1                           |
The distribution of PRP5 protein in the sucrose density gradient fractions was evaluated by western blot analysis, and the results showed that the PRP5 levels increased in the fractionated samples from organisms grown in QA, suggesting that the knockdown of \textit{rrp44} not only affects the levels of spliceosomal mRNA but also the protein level of this spliceosomal component. In addition, the distribution of PRP5 in fractions number 3–6 was significantly higher in response to QA treatment compared to untreated samples (Fig. 3A).

We then investigated the distribution patterns of snRNA \textit{U5} and \textit{U6-2} in samples fractionated by sucrose sedimentation. We extracted the total RNAs from the fractions and conducted RT-PCR to examine the \textit{U5} and \textit{U6-2} snRNA levels in the sucrose sedimentation samples. The RT-PCR results demonstrate the differential distribution patterns of \textit{U5} and \textit{U6-2} snRNAs in \textit{dsrrp44} grown in the presence or absence of QA; the levels of both snRNAs increased in higher-density fractions upon QA treatment (Fig. 3B,C). Because snRNAs exert their functions in complex with snRNP proteins\textsuperscript{38}, this change in the distribution of an snRNA upon the silencing of \textit{rrp44} supports our hypothesis that the spliceosome assembly is altered in RRP44-deficient organisms.

**Knockdown of \textit{rrp44} leads to extensive changes in AS events.** To further address our hypothesis that the exosome regulates splicing, we compared the alternative splicing (AS) events in the \textit{dsrrp44}
Because the exosome is known to be responsible for eliminating pre-mRNAs\(^{30,31}\), these data were almost undetectable in the absence of QA, but their expression was elevated in the presence of QA at a series of time points after thiolutin addition. The unspliced mRNAs of NCU02181 and NCU01776 included NCU07421, NCU02181, NCU01776 and NCU05290. Of these genes, NCU07421 encodes an orotate phosphoribosyltransferase. After rrp44 knockdown, while the unspliced transcripts in the ds\(^{-}\)strain treated with QA compared to the strain without QA treatment (Fig. 3C; supplemental Table 1). Because the exosome is implicated in pre-mRNA degradation\(^{30,31}\), some of the 179 IR events might result from changes in alternative splicing. These observations demonstrate that the exosome contributes to bulk splicing events.

We conducted RT-PCR to validate the RNA-seq results. Four representative genes were evaluated, including NCU07421, NCU02181, NCU01776 and NCU05290. Of these genes, NCU07421 encodes a nuclear transport protein, NCU02181 and NCU01776 encode ribosomal protein components, and NCU05290 encodes an orotate phosphoribosyltransferase. After rrp44 knockdown, NCU07421 showed a change in the selection of an alternative 3′ splice site (Fig. 3E). NCU02181 and NCU01776 showed higher levels of intron retention (Fig. 3F, G), while NCU05290 showed an increase in splice site (Fig. 3D). NCU02181 and NCU01776 showed a change in the selection of an alternative 3′ splice site (Fig. 3H), in agreement with the RNA-seq data.

Changes in IR events predominated among the total 918 changed splicing events, although differences in ES and A5′/3′SS were detected (Fig. 3C; supplemental Table 1). As in other eukaryotes, such as the fungus *Fusarium graminearum* and the plant *Arabidopsis thaliana*\(^{39,40}\), the most common AS events in *Neurospora* appear to be IR.

The analysis revealed that 179 IR events decreased while 681 IR events increased in the ratio of unspliced transcripts in the dsrp44 strain treated with QA compared to the strain without QA treatment (Fig. 3C, supplemental Table 1). Because the exosome is implicated in pre-mRNA degradation\(^{30,31}\), some of the 681 IR events might result from the stabilization of pre-mRNAs due to rrp44 knockdown, while some of the 179 IR events might result from changes in alternative splicing. These observations demonstrate that the exosome contributes to bulk splicing events.

To further investigate the degradation of the pre-mRNAs and spliced variants of the genes of interest, we treated the samples with thiolutin and examined the levels of pre-mRNA and spliced variants at a series of time points after thiolutin addition. The unspliced mRNAs of NU02181 and NCU01776 were almost undetectable in the absence of QA, but their expression was elevated in the presence of QA (Fig. 4A). Because the exosome is known to be responsible for eliminating pre-mRNAs\(^{30,31}\), these data suggest that these unspliced species might represent the pre-mRNAs of the tested genes, and their rapid degradation is controlled by RRP44, which requires less than 15 min to complete. Instead, the degradation of both the unspliced and spliced species of NCU05290 is likely controlled by RRP44 (Fig. 4A). As such, the decrease in its unspliced species might be attributed to the altered splicing upon rrp44 knockdown. Regarding NCU05290, the knockdown of prp5 decreased the ratio of the spliced species, and the increased level of prp5 in the dserp44 strain might consequently at least partially account for the increase in the spliced species (Fig. 4B).

Regarding NCU07421, which bears A3′SS, both the levels of the unspliced and spliced species showed a significant decrease upon rrp44 inhibition (Fig. 4A), suggesting that the change in its splicing ratio is not due to the degradation of pre-mRNA. Though QA treatment also induced a decrease in the unspliced transcripts of NCU07421 in the WT strain for unknown reason, the ratio of the unspliced transcripts of NCU07421 was significantly lower in dsU4-2 (Fig. 4C). These data suggest that the ratio of

### Table 2. snRNP and spliceosome-associated genes up-regulated in dserp44 strain (QA+ vs. QA−).

Note: The fold changes of expression between QA+ and QA− were calculated from RNA-seq data.

| Gene ID   | Gene name | Fold change | P-value | Functional classification |
|-----------|-----------|-------------|---------|--------------------------|
| NCU07548  | snp1      | 2.65586     | 3.09E-75 | U1 related               |
| NCU10810  | prp39     | 2.71543     | 0       | U1 related               |
| NCU06959  | smu71     | 2.140219    | 4.1E-87 | U1 related               |
| NCU05645  | luc7      | 3.219791    | 3.36E-55 | U1 related               |
| NCU03062  | prp40     | 2.861535    | 1.9E-262 | U1 related              |
| NCU02696  | prp5      | 2.223039    | 1.8E-169 | U1 related               |
| NCU02572  | smu14     | 2.068642    | 6.61E-86 | U5 related               |
| NCU02685  | brr2      | 2.156961    | 4E-307  | U5 related               |
| NCU07832  | prp8      | 2.185044    | 0       | U5 related               |
| NCU07265  | smu23     | 3.634172    | 1.71E-26 | U5 related               |
| NCU11222  | smu66     | 2.632671    | 1.3E-154 | U4/6 related             |
| NCU01637  | prp3      | 2.538265    | 4.39E-78 | U4/6 related             |
| NCU01331  | smu13     | 3.047531    | 2.7E-178 | U4/6 related             |
| NCU008036 | snf1      | 2.183291    | 7.7E-135 | PRF19 complex            |
| NCU00664  | isy1      | 2.748446    | 2.7E-55  | PRF19 complex            |
| NCU02966  | prp46     | 2.143234    | 9.91E-51 | PRF19 complex            |
| NCU01613  | prmt5     | 1.6094078   | 0       | Spliceosome-associated   |
the NCU07421 splicing variants might be controlled by exosome-mediated splicing via the spliceosome. Despite the induction of U4-2 (Fig. 1A), the level of the unspliced variant of NCU07421 was markedly decreased in dsrrp44 (Fig. 4C), which might be attributed to a disturbance of snRNA maturation due to exosome inhibition3,32. Taken together, these data, as exemplified by NCU05290 and NCU07421, suggest that in addition to its role in regulating the pre-mRNA degradation, the exosome may control alternative splicing by mediating the gene expression, assembly and function of the spliceosome.

**Regulation of alternative splicing of circadian clock gene frq.** frq transcripts with and without I-6 encode I-FRQ and s-FRQ, respectively, and both I-FRQ and s-FRQ are critical for the circadian clock in Neurospora21-24,41. The degradation of total frq RNA is controlled by the exosome (Fig. 4A)25. In addition to the increase in the level of total frq transcripts, the ratio of splicing variants with I-6 was significantly decreased (Fig. 4A), which agrees with the RNA-seq data (Bayes factor = 1E.twelve, supplemental Table 1) and suggests that the exosome may regulate frq splicing in addition to its degradation.

The frq gene comprises eight splicing variants in total, and variants I, II, III, V and VI, but not others, contain I-623. As such, the decrease in the level of frq variants that contain I-6 upon rrp44 knockdown reflects the influence of the exosome on the mixture of different variants. We conducted RT-PCR with primers that spanned all the introns (1–6) to analyze the influence of the exosome on individual splicing variants in dsrrp44, and the results showed that the profiles of splicing variants consistently and dramatically differed between QA-treated and -untreated tissues at three temperatures (Fig. 5A). To eliminate the effect of the differential total levels of frq mRNA between the QA-treated and -untreated tissues, the amount of each spliced species was normalized to the total amount, and the statistics indicate that the ratios of variants V and VII significantly increased, while the ratio of variant III decreased (Fig. 5B). These results suggest that the exosome plays differential roles in the regulation of these variants by differentially regulating the decay, splicing, or a combination thereof.

In the samples treated with thiolutin for 0 and 15 min, the decay rates of variants III, VI and VII, but not that of variant IV, were significantly slower in the dsrrp44 strain treated with QA (Fig. 5C). These results suggest that the degradation of frq variants are differentially regulated, and the degradation of...
variants III, VI and VII might be under the control of the exosome, while variant IV might be controlled by alternative mRNA decay pathways. Moreover, because variant III primarily determines the level of I-6-containing \(frq\) transcripts, an increase in the stability of variant III upon \(rrp44\) knockdown suggests an increase in the ratio of I-6-containing \(frq\) transcripts. However, the level of variant III in the presence of QA was less than that in the absence of QA (Fig. 5A,B), suggesting that the exosome participates in the regulation of splicing in addition to the degradation of variant III.

Because the ratio of l-FRQ to s-FRQ is critical for clock function, we next investigated whether the exosome regulates the levels of l-FRQ/s-FRQ by affecting \(frq\) splicing. We measured the levels of both the transcript variants of \(frq\) mRNA and the protein isoforms, and the strategy is schematically shown in Fig. 5D. RT-PCR with primers flanking \(frq\) I-6 was performed to detect the levels of mRNAs either excluding or including I-6 in the \(dsrpp44\) and WT strains at three different temperatures with or without QA addition. The RT-PCR results showed that the level of mRNA excluding I-6 was increased after \(rrp44\) knockdown (Fig. 5E,F). The alternative splicing of \(frq\) determines the ratio of the FRQ protein isoforms l-FRQ and s-FRQ. Because FRQ is so extensively phosphorylated that the phosphorylated l-FRQ and s-FRQ proteins cannot be easily distinguished, we conducted FRQ-IP followed by \(\lambda\) PPase treatment to...
compare the dephosphorylated l-FRQ and s-FRQ. The results showed that the ratio of s-FRQ/l-FRQ was increased in the dsrrp44 strain in the presence of QA (Fig. 5G,H), which agrees with the RNA analysis. These data demonstrate that the exosome is involved in the regulation of the alternative splicing of the circadian clock gene frq and consequently the ratio of FRQ isoforms.

Discussion

The exosome plays important roles in the regulation of a variety of molecular and physiological processes in different organisms, including mitotic regulation, growth, oxidative stress responses and carcinoma progression42–44. The aberrant activity of the exosome may cause various diseases. For example, the over-expression of Dis3/Rrp44 is associated with adenoma to carcinoma progression in colorectal cancer42, and the over-expression of Rrp46 contributes to chronic myelogenous leukemia (CML)46. At the molecular level, the nuclear exosome regulates the processing of a number of RNA species including rRNAs and sn/snoRNAs, and surveillance and degradation of RNA precursors, including pre-mRNAs, pre-sn/snoRNAs, pre-rRNAs and pre-tRNAs in the nucleus. Whereas, the cytoplasmic exosome regulates the degradation of mRNAs in the cytoplasm5,31.

The exosome is closely associated with complexes involved in mRNA processing to regulate gene expression at the post-transcriptional level in a coordinated manner. The components of the Ccr4-Not complex directly interact with the exosome, which may serve as a platform for the association between the exosome and TRAMP47. In the non-sense mediated mRNA decay (NMD) pathway, the exosome is responsible for the 3′ → 5′degradation of the premature termination codon (PTC)-containing transcripts48. The core NMD factor Upf1 physically interacts with the N-terminal domain of Ski7, which mediates the exosome-directed decay of NMD targets49. These facts suggest that the post-transcriptional regulation is under the control of a complex network in which the functions of different regulators may be mutually controlled. The exosome also likely cross-talks with the spliceosome. The exosome components Rrp6 and Mtr4 interact with tri-snRNP-associated proteins in HeLa and HEK293 cells50. In yeast, more than half of the intron-containing genes are degraded by the exosome, and the poorly spliced pre-mRNAs and the RNAs contain the longer introns are more susceptible to degradation51. In addition, the TRAMP complex promotes optimal pre-mRNA splicing by facilitating the recruitment of splicing factors and the rapid degradation of the spliced-out introns by the nuclear exosome51. These facts suggest that the exosome may be involved in the regulation of splicing; however, whether and how the exosome regulates alternative splicing remains unclear.

In this work, we first demonstrated that RRP44, the catalytic subunit of the exosome, modulates a variety of cellular processes, including the spliceosome pathway (Table 1). We further showed that the exosome regulates the gene expression of a number of spliceosome components at the RNA level. Among these components, the RNA decay of several genes is controlled by the exosome, suggesting that they are direct targets for the exosome (Fig. 1). More importantly, we showed that the knockdown of rrp44 resulted in changes in the assembly of the spliceosome U5 and U6 snRNPs (Fig. 3B). The U5 snRNA
Figure 5. RRP44 differentially controls the degradation and splicing of frq variants. (A) RT-PCR showing the splicing profiles of the dsrrp44 strain at three temperatures in the presence or absence of QA. Except for variants I and II, which were almost undetectable, variants III-VIII are labeled. (B) Quantification of the ratios of spliced variants versus total transcripts of the results from panel A (25°C). (C) RT-PCR results showing the degradation of frq variants (left) and densitometric quantification of variants III, IV, VI and VII at 0 and 15 min (right). The RNA levels at 0 min were set to be 1. (D) Schematic representation of frq 1-6 splicing and the corresponding protein products I-FRQ and s-FRQ. Horizontal arrows denote the relative location of PCR primers flanking frq 1-6. Vertical arrows denote the locations of the three initiation codons. (E,F) RT-PCR results and densitometric quantification of frq 1-6 splicing in dsrrp44 (E) and WT (F) strains grown in the presence or absence of QA. (G,H) Comparison of s-FRQ/I-FRQ ratios in dsrrp44 (G) and WT (H) strains grown in the presence and absence of QA. FRQ was immunoprecipitated and treated with λ PPase prior to analysis. The samples in the absence of QA without λ PPase treatment served as loading controls. Data represent means ± SD of three independent experiments. *p < 0.05, **p < 0.01.
contains a 11-nt loop, which functions to align the 5′ and 3′ exons for ligation during the second step of pre-mRNA splicing52. The U6 snRNA pairs with U4 snRNA to promote the assembly of pre-spliceosome, and it pairs with U2 to bind with the 5′ss of an intron to facilitate the first step of splicing1. These functions suggest that the knockdown of rrp44 may affect the assembly and functions of spliceosomal components. The analysis of RNA-seq data indicates that RRP44 is required for the regulation of bulk AS events (Fig. 3C).

Previous studies concentrated on the analysis of changes in the IR events caused by the impairment of the exosome, which provided evidence that the exosome plays a role in the degradation of pre-mRNAs30,31. In this work, the increase in the ratio of spliced forms of the 179 IR events (Fig. 3C, Supplemental Table 1) suggests that the exosome may play additional roles in the regulation of splicing events. An analysis of the AS events of NCU05290, NCU07421 and frq(Fig. 3–5) provided evidence that the exosome may directly influence splicing via the spliceosome. The detailed mechanisms by which the exosome selectively regulates splicing and degradation remain to be further investigated. In all the tested genes, we only identified a few whose splicing is controlled by exosome, and this specificity might be attributed to the SR proteins which are also affected by exosome.

The exosome has been known to regulate the circadian clock in a post-transcriptional fashion by facilitating the decay of frq transcripts25. Accumulating evidence has demonstrated that alternative splicing is involved in the regulation of the circadian clock13,14,18. Here, we demonstrate that the exosome is involved in the regulation of the alternative splicing of the circadian clock gene frq and consequently the ratio of FRQ isoforms. Of the FRQ isoforms, l-FRQ supports a shorter period, whereas s-FRQ supports a longer period22,24. In addition to the stabilization of frq transcripts, the increased splicing of frq I-6 may explain the longer circadian period of the ds rrp44 strain. These data also support that the exosome may exert its function by mediating alternative splicing.

In summary, we showed that in addition to the regulation of pre-mRNA degradation, the exosome regulates gene expression and consequently the assembly of the spliceosome complex. Thus, the exosome may regulate the levels of spliced species via spliceosome-dependent and -independent pathways. In the spliceosome-dependent pathways, the exosome regulates the gene expression and assembly of spliceosome and the processing and turnover of snRNAs, which further modulates the function of spliceosome. In the spliceosome-independent pathways, the exosome regulates the degradation of pre-mRNAs in the nucleus and the turnover of both the unspliced and spliced transcripts in the cytoplasm (Fig. 6). These pathways also most likely synergistically regulate the levels of spliced transcripts. Nuclear and cytoplasmic forms of exosome complex might function differently in these processes, the former regulates the processing and degradation of snRNAs and the surveillance of pre-mRNAs, while the latter mediates the mRNA degradation of a set of snRNP and SR protein related genes (Fig. 6).
Methods

Strains and growth conditions. The 301-5 (bd, a) strain was used as the wild-type (WT) strain. The 301-6-6 strain (bd, his-3, A) was used as the host strain for his-3-targeting constructs. Liquid cultures were grown in minimal a media (1 × Vogel's, 2% glucose). When quinic acid (QA) was used, the liquid cultures were grown in media with 0.01 M QA (pH 5.8), 1 × Vogel's, 0.1% glucose, and 0.17% arginine. The race tube media contained 1 × Vogel's, 0.1% glucose (0% when QA was used), 0.17% arginine, 50 ng/mL biotin, and 1.5% agar.

Generation of knockdown strains. The knockdown strains dsrrp5, dsU4-2 and dsprmt5 were generated via the introduction of plasmids that expressed RNA hairpins with complementarity to the gene to be inhibited into the 301-6-6 strain. The hairpin sequence was complementary to ~500 bp of the gene of interest downstream of the qa-2 promoter. The resulting plasmids were targeted to the his-3 locus via transformation into 301-6-6 (bd, his-3, A). The dsrrp44 strain was generated as described previously and allows the expression of rrp44 to be suppressed by the addition of QA.

RNA and protein analyses. RNA extraction and northern blot analyses were conducted as described previously. Equal amounts of total RNA (10 μg) were loaded onto agarose gels for electrophoresis; the gels were subsequently blotted and probed with RNA probes specific to the genes of interest. RNA degradation was detected by the addition of thiolulin (final concentration 10 μg/ml), which is an inhibitor of RNA polymerase II. The samples were harvested 15 min, 30 min, and 45 min after the addition of thiolulin, and the total RNA was extracted for subsequent RT-PCR detection. For qRT-PCR, the samples were treated with RNase-Free DNase I (NEB) and then subjected to reverse transcription using M-MLV (Invitrogen) and random primers. The synthesized cDNAs were amplified with SYBR Green Master Mix (Takara) using a LightCycler 480 (Roche). The protein extraction, western blot analysis, and immunoprecipitation assays were performed as previously described. Equal amounts of total protein (40 μg) were loaded onto gels (7.5% gels containing a ratio of 37.5:1 acrylamide/bisacrylamide) for SDS-PAGE. The FRQ protein was de-phosphorylated via treatment with Lambda phosphatase (λ PPase, NEB).

Generation and validation of PRP5 antibody. The entire ORF of the Neurospora prp5 gene was cloned into pET-28a (+) and expressed in the BL21 (DE3) Escherichia coli strain. Purified His-PRP5 protein was used to immunize rabbits. The obtained antibodies were validated by western blot analysis with the dsprp5 strain.

Sucrose fractionation analysis. Sucrose density gradients (10–30%) were prepared, and 4 mg total protein samples were loaded for each analysis. The gradients were centrifuged at 175,000 × g for 18 h in a SW-40 Rotor (Beckman) at 4 °C. Twelve equal fractions were collected, and 450 μl of each fraction was subjected to RNA analysis. The samples were treated with DNase I prior to RT-PCR to determine the levels of U5 and U6-2. Western blot analyses were utilized to determine the distribution of PRP5.

RNA-seq and analysis. The RNA samples from dsrrp44 strains grown with or without QA were pooled and processed to prepare the mRNA-seq library using the standard Illumina protocol. The sequencing was performed on an Illumina HiSeq2500 at BGI, China. Tophat was used as an aligner to map the reads to the reference genome (N. crassa OR74A (NC12)). Cufflinks was used to reconstruct the transcripts and estimate the gene expression levels. The SplicingViewer tool was used to annotate alternative splicing events. Mixture of Isoforms (MISO) was used to estimate and compare the AS event frequencies between samples. The Bayes factors were calculated by MISO as the presentation of evidence against no difference between the samples. The sashimi plots were graphed for visualization of the analyses of isoform expression with the build-in package sashimi plot of MISO (Supplemental information).

Statistical Analyses. Statistical significance was calculated using Student’s t-test. The significance values are *p < 0.05, **p < 0.01 and ***p < 0.001. n ≥ 3.

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Author Contributions

L.Z., G.H. and J.G. designed the experiments and wrote the manuscript. L.Z., Y.W., G.H., D.W. and X.Y. performed the experiments and analyzed the data. All authors reviewed the manuscript.

Additional Information

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