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Antisense oligonucleotide technology can be used to investigate a circular but not linear RNA-mediated function for its encoded gene locus

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As a class of powerful molecular tool, antisense oligonucleotides (ASOs) are not only broadly used in protein and RNA biology, but also a highly selective therapeutic strategy for many diseases. Although the concept that ASO reagents only reduce expression of the targeted gene in a post-transcriptional manner has long been established, the effect and mechanism of ASO reagents on RNA polymerase II (Pol II) transcription are largely unknown. This raised question is particularly important for the appropriate use of ASOs and the valid interpretation of ASO-mediated experiments. In this study, our results show that linear RNA ASO attenuates transcription of nascent transcripts by inducing premature transcription termination which is combinatorially controlled by Integrator, exosome, and Rat1 in *Drosophila*. However, circular RNA (circRNA) ASO transfection does not affect transcription activity of the encoded gene. These data suggest that the ASO technique can be applied to study a circRNA-mediated but not linear RNA-mediated function for its encoded gene locus.

antisense oligonucleotide, premature transcription termination, circular RNA, linear RNA

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INTRODUCTION

Antisense oligonucleotides (ASOs) are a class of short strands of deoxyribonucleotide analogue (15–20 nt) that are designed to bind to the complementary RNA through Watson-Crick hybridization, thereby reducing expression of the targeted transcripts. As a powerful molecular tool, ASOs are not only broadly used in protein and RNA biology (Do et al., 2018; Horwich and Zamore, 2008; Hu et al., 2016; Li et al., 2015), but also a highly selective therapeutic strategy for many diseases that are linked to dysregulated gene expression (Havens and Hastings, 2016; Kordasiewicz et al., 2012; Malerba et al., 2011; Passini et al., 2011; Rinaldi and Wood, 2018). For example, Nusinersen (Spinraza) is an approved ASO drug that can be used to promote inclusion of exon 7 in the survival motor neuron (SMN1) gene for the treatment of spinal muscular atrophy (SMA) (Corey, 2017).

There are several functional mechanisms that can be exploited to inhibit the function of the targeted RNA. The major pathway is that an ASO can form an RNA-DNA hybrid which becomes a substrate for RNase H-mediated cleavage, resulting in hydrolysis of the RNA strand of the hybrid (Chan et al., 2006; Liang et al., 2017b; Lima et al., 2007; Wu et al., 2004). Formation of an ASO-RNA heteroduplex also leads to splicing inhibition or exon skipping events by sterically blocking canonical splicing sites (Havens and Hastings, 2016; Heemskerk et al., 2010; Malerba et

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al., 2011; Sierakowska et al., 1996). For instance, treatment of human cells stably expressing the aberrant spliced β-globin mRNA with ASOs targeting the aberrant splicing sites can restore correct splicing, thereby generating correct mRNA and protein (Sierakowska et al., 1996). Moreover, ASO transfection can cause translational arrest by steric hindrance of ribosomal subunit binding and assembly (Baker et al., 1997). Although there has been remarkable progress in the development of antisense technology over the past decades, it is still unclear whether ASO could influence the early stages of transcription elongation of the targeted gene. For example, if the ASO-targeted gene (RNA or protein product) has a potential *cis* role in transcription of the encoded gene itself, the ASO cannot be used as a loss-of-function tool for a research of this *cis* effect. Clarification of this question will provide key insights into the appropriate use of ASOs and the valid interpretation of ASO-mediated experiments.

Using the *Drosophila* system, we demonstrate that ASO transfection attenuates transcription of newly-synthesized transcripts by inducing premature transcription termination, and ASO-directed premature transcription termination is combinatorially controlled by Integrator, exosome, and Rat1. Thus we conclude that ASO technique cannot be applied to study a linear RNA-mediated function for its encoded gene locus, such as the *cis* transcriptional regulation of a long non-coding RNA (lncRNA). In contrast to linear RNA ASO, the ASO targeting the circular junction of a circular RNA (circRNA) does not trigger premature transcription termination, suggesting that ASO technology can be used to establish a circRNA-mediated function for an encoded gene locus. In total, these findings reveal new insights into the appropriate application of ASO technique in various studies for experimental and therapeutic purposes.

RESULTS

ASO attenuates transcription of newly-synthesized transcripts by inducing premature transcription termination

The *Drosophila* Metallothionein is a type of metal responsive gene which can be rapidly and robustly activated in response to a heavy metal environment so that expression of the nascent Metallothionein transcripts could be easily labeled and examined in the cells treated with copper (Günther et al., 2012). Therefore, the *Drosophila* Metallothionein A (MtnA) and Metallothionein B (MtnB) were selected to investigate the effect of ASO treatment on transcription activity of their gene loci. The ASO targeting the coding region of MtnA transcript was transfected into copper-stressed *Drosophila* S2 cells for 2 days and qRT-PCR experiments were then performed to examine expression of the steady-state MtnA RNA in unlabeled cells or expression of the newly-synthesized MtnA RNA in the cells that had been labeled with 4-thiouridine (4sU) for the final 5 min (Figure 1A). As expected, the steady-state MtnA RNA level was found to be significantly decreased upon MtnA ASO transfection (Figure 1B, higher panel). Surprisingly, MtnA ASO treatment also resulted in a robust reduction of the nascent MtnA RNA level (Figure 1B, lower panel). This phenotype was not due to the possibility that the ASO binds to the DNA to inhibit transcription, since transfection of MtnA sense stain oligo (MtnA SO) had little effect on the steady-state or nascent MtnA RNA level (Figure 1B). A similar phenotype was also observed in the MtnB ASO treated cells (Figure S1A in Supporting Information). Thus we concluded that ASO could impair production of newly-synthesized transcripts.

To understand the underlying basis for ASO-induced transcription attenuation, we therefore asked whether ASO transfection affects recruitment of RNA polymerase II (Pol II) to the targeted gene locus. Chromatin immunoprecipitation (ChIP) was performed to investigate Pol II occupancy in the ASO transfected cells that were treated with copper sulfate and 4sU. Sites within the gene body of MtnA or MtnB were examined by qPCR subsequently (Figure 1A). Notably, we found that MtnA ASO transfection resulted in dissociation of Pol II from 3′ end of the MtnA gene body (Figure 1C, PCR amplicon E, F), whereas Pol II occupancy in the region upstream of the ASO targeting site was little affected (Figure 1C, PCR amplicons A–D). In line with Figure 1B, transfection of MtnA SO did have little effect on Pol II occupancy at the MtnA gene locus. A similar Pol II binding pattern was also observed in the MtnB ASO treated cells (Figure S1B in Supporting Information). Altogether, our data demonstrated that ASO triggers premature transcription termination by releasing Pol II from the region downstream of the ASO targeting site, thereby leading to degradation of the premature transcript.

Integrator is involved in ASO-mediated cleavage

As a nuclear complex, Integrator has been implicated in many biological processes, such as transcriptional regulation and RNA cleavage. It consists of an endonuclease and interacts with RNA Pol II (Baillat et al., 2005; Baillat and Wagner, 2015; Rienzo and Casamassimi, 2016). In addition, it is particularly important to note that the Integrator complex can attenuate expression of a subset of protein-coding genes by catalyzing premature transcription termination in the nucleus (Elrod et al., 2019; Tatomer et al., 2019). We thus hypothesized that Integrator might be involved in ASO-mediated premature transcription termination. We first applied RNA interference (RNAi) strategy to reduce expression of several Integrator components in the ASO treated cells and qRT-PCR was performed to examine the nascent

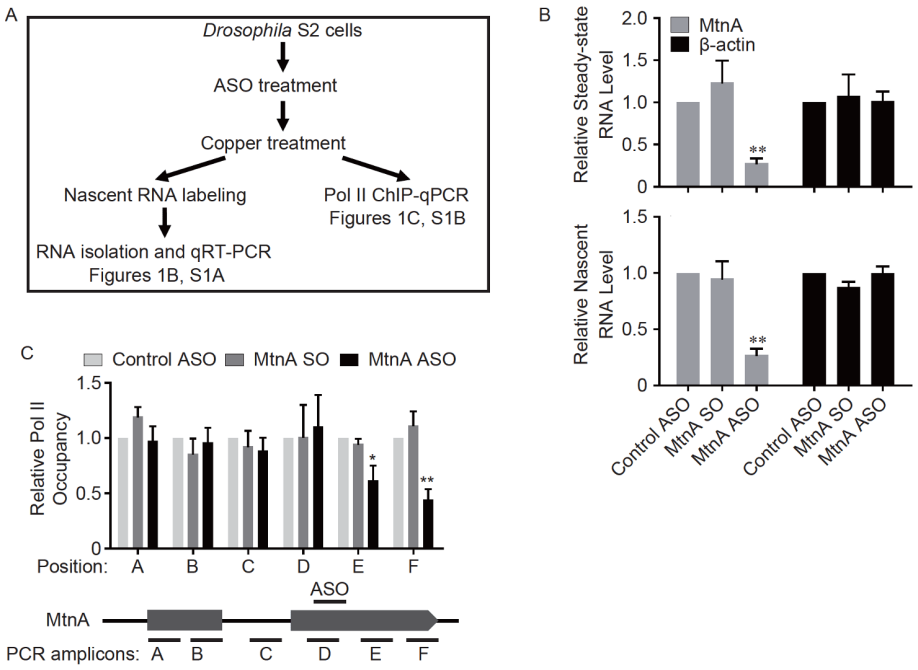

Figure 1 ASO impairs transcription of nascent transcripts by inducing premature transcription termination. A, Flow chart of the experimental setup for the data presented in Figure 1B, C and Figure S1 in Supporting Information. *Drosophila* S2 cells were transfected with the indicated ASO or SO for 2 days and treated with $CuSO_4$ for the final 10 min. B, 250 μmol L^{-1} 4sU was added to label nascent transcripts for 5 min. qRT-PCR was then performed to measure the steady-state or nascent MtnA RNA levels. β-actin served as a negative control. Data were normalized to the control ASO sample. C, RNA Pol II recruitment to the MtnA gene locus was measured using ChIP-qPCR. Pol II binding sites were defined as regions enriched over the input DNA. Data were normalized to the control ASO sample. The MtnA gene locus with the locations of ChIP amplicons is shown below. All data are shown as mean±SEM. n=3. **, P<0.01; *, P<0.05.

MtnA RNA level (Figure 2A). Indeed, the result showed that depletion of IntS1, IntS4, IntS9, or IntS11 significantly restored MtnA expression in the MtnA ASO transfected cells to levels similar to the control ASO treated sample (Figure 2B; Figure S2 in Supporting Information). Consistently, MtnB expression in the MtnB ASO transfected cells was also rescued in absence of IntS1, IntS4, IntS9, or IntS11 (Figure S3A in Supporting Information).

To further define how the Integrator complex functions in ASO-mediated premature RNA degradation, we asked whether the endonuclease activity of Integrator plays a role in this event. IntS11 is the key component of Integrator and harbors RNA endonuclease activity; therefore, IntS11 was chosen as our target for the following rescue experiments (Figure 2A). The wild-type (WT) and mutant (MUT) IntS11 expression plasmid that only harbor the coding sequence of IntS11 are insensitive to double-strand RNA (dsRNA)-directed knockdown (Figure 2C and D). Expression of the IntS11 WT plasmid in the MtnA ASO transfected cells that were depleted from endogenous IntS11 significantly attenuated expression of the nascent MtnA transcript, whereas expression of the IntS11 MUT plasmid that can generate the catalytically dead IntS11 protein had little effect (Figure 2E, left panel). The β-actin transcription served as a negative control and a similar phenotype was also observed in the MtnB ASO treated cells (Figure 2E, right panel; Figure S3B in Supporting Information). Taken together, these results clearly demonstrated that the endonuclease activity of Integrator is required for ASO-mediated transcription termination and premature RNA degradation.

Exosome and Rat1 are required for ASO-mediated premature transcription termination

Upon showing the endonuclease IntS11 cleaves nascent transcript in ASO-mediated premature transcription termination, we thus reasoned that the event may generate a free 3′ end of upstream segment and a free 5′ end of downstream segment, and these segments may provide an entry point for 3′ degradation by the exosome complex or 5′ exonuclease degradation by Rat1, respectively (Figure 3A). The RNA quality control is in a process that is tightly linked to transcription. Defect in RNP assembly results in exosome-mediated accumulation of the RNA in association with the site of transcription followed by RNA degradation. Moreover, Rat1-mediated degradation can be triggered by RNA cleavage that generates a nascent transcript segment with a phosphorylated 5′ end (Houseley and Tollervey, 2009; Schoenberg and Maquat, 2012). To test the model, Flag-Mtr4 (exosome-associated RNA helicase) and Flag-Rat1 stable cell lines were generated and used for RNA immunoprecipitation (RIP) to assess recruitment of Mtr4 or

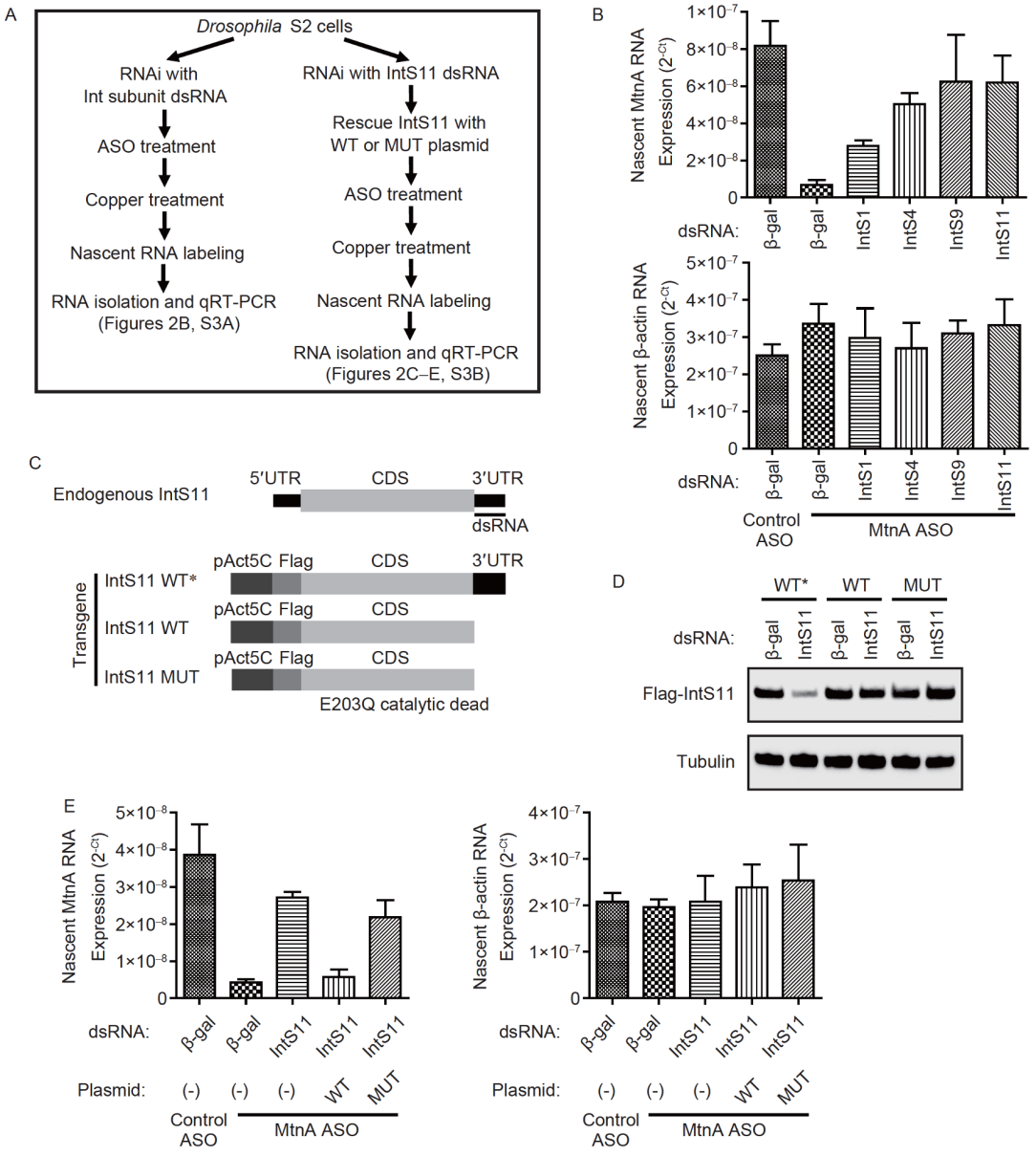

Figure 2 The Integrator complex is involved in ASO-induced premature transcription termination. A, Flow chart of the experimental setup for the data presented in Figure 2B–E and Figure S3 in Supporting Information. B, *Drosophila* S2 cells were treated with the indicated dsRNAs on day 1, transfected with the indicated ASOs on day 2, and collected for RNA purification on day 4. Copper was added for the final 10 min to activate MtnA transcription and 4sU was added for the final 5 min to label nascent transcripts. qRT-PCR was then used to measure the 4sU-labeled MtnA RNA levels. β-actin served as a negative control. C and D, Schematic of IntS11 depletion/plasmid rescue strategy. IntS11 WT* harbors the wild-type protein coding sequence (CDS) as well as the 3′ UTR and is used to confirm knockdown efficiency of the IntS11 dsRNA. IntS11 WT only harbors the wild-type CDS. IntS11 MUT harbors the catalytically dead mutant CDS with a point mutation (E203Q). The IntS11 dsRNA targets the 3′ UTR region, thereby only depleting the endogenous IntS11 and IntS11 WT* but not IntS11 WT or MUT. E, *Drosophila* S2 cells were treated with the IntS11 dsRNA on day 1, transfected with the IntS11 rescue plasmids (WT or MUT) on day 2, treated with the indicated ASOs on day 3, and collected for RNA purification on day 5. Copper was added for the final 10 min to activate MtnA transcription and 4sU was added for the final 5 min to label nascent transcripts. qRT-PCR was then performed to measure the nascent MtnA RNA levels. β-actin served as a negative control. The absolute levels of the indicated nascent RNAs were represented as 2^{-Ct} (*Ct*: qPCR cycle threshold). All data are shown as mean±SEM. *n*=3.

Rat1 to the ASO-induced MtnA or MtnB segments (Figure 3B–G; Figure S4A and S4B in Supporting Information). We isolated nuclear samples from the ASO transfected stable cells that had been activated with copper sulfate. RIP assays were then performed with an antibody against Flag-Mtr4 or Flag-Rat1 to pull down each binding RNA followed by Western blotting and qRT-PCR analysis (Figure 3B–G). Two primer sets were used to assess 5′ or 3′ segments of the premature RNAs (Figure 3C, 5′ MtnA segment: PCR amplicon A, 3′ MtnA segment: PCR amplicon F; Figure S4A in Supporting Information, 5′ MtnB segment: PCR amplicon A, 3′ MtnB segment: PCR amplicon D). Indeed, Mtr4 recruitment to 5′ RNA segment was drastically elevated in the MtnA or MtnB ASO treated cells, whereas Mtr4 recruitment

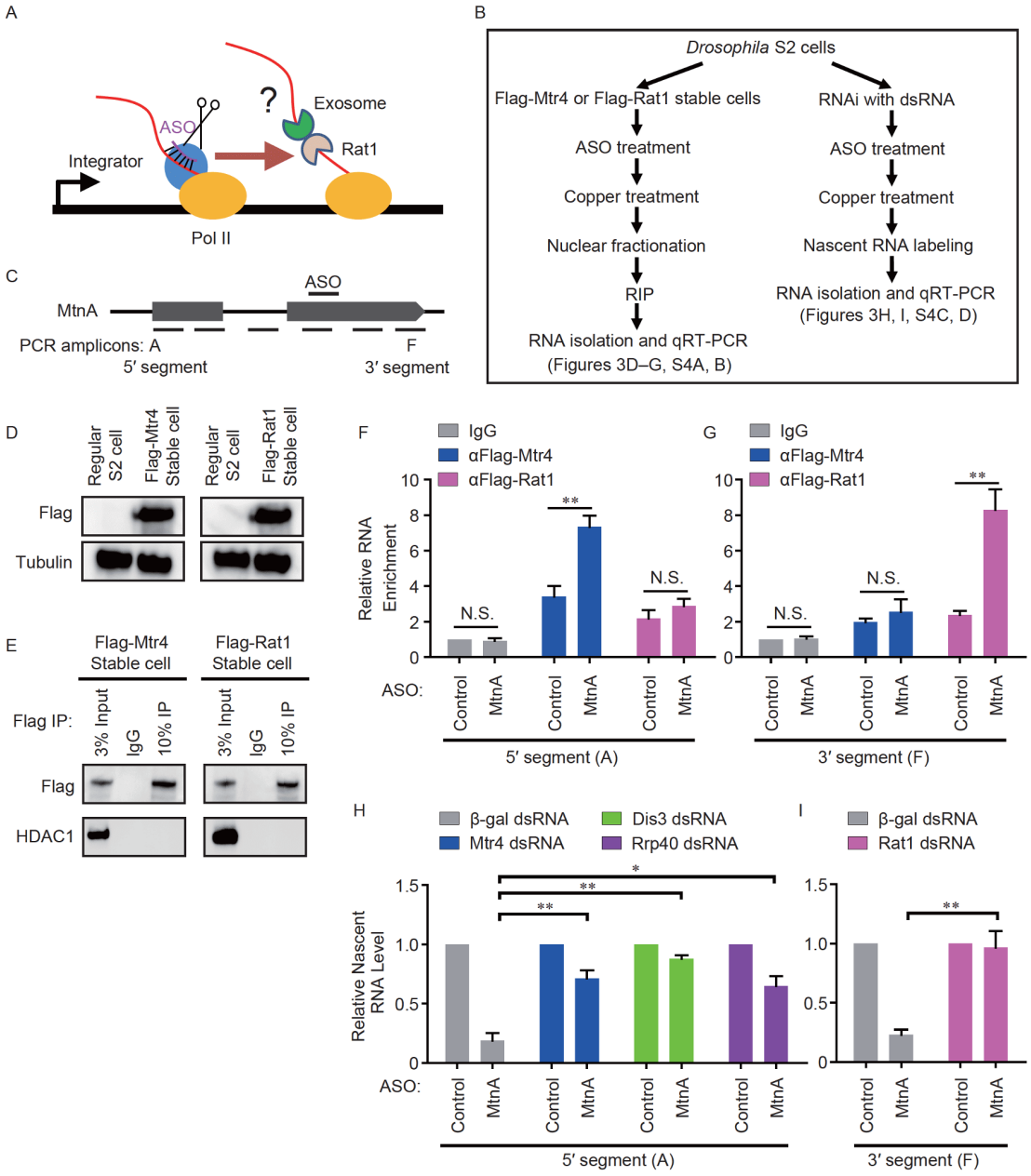

Figure 3 The exosome complex and Rat1 are required for ASO-induced nascent RNA degradation. A, The possible working model of ASO-induced premature transcription termination. Integrator cleaves nascent transcript and generates a free 3′ end of the upstream segment and a free 5′ end of the downstream segment which may provide an entry point for 3′ degradation by the exosome complex or 5′ exonuclease degradation by Rat1. B, Flow chart of the experimental setup for the data presented in Figure 3D–I and Figure S4 in Supporting Information. C, The MtnA gene locus with the locations of PCR amplicons is shown. Two primer sets were used to assess the 5′ or 3′ segments of the premature MtnA RNA (5′ segment: PCR amplicon A, 3′ segment: PCR amplicon F). D–G, In brief, Flag-Mtr4 or Flag-Rat1 stable cells were transfected with the indicated ASOs for 2 days, and copper was added for the final 10 min to activate MtnA transcription. Nuclear extracts from the treated cells were then used for RNA immunoprecipitation (RIP) with an antibody against flag tag. The negative IgG served as a control. D and E, Western blotting was performed to examine expression of Flag-Mtr4 or Flag-Rat1 in each stable cell line and the immunoprecipitation efficiency. F and G, qRT-PCR was then performed to measure recruitment of Mtr4 or Rat1 to the MtnA segments that were induced by ASO-mediated cleavage. Data were normalized to the control ASO sample immunoprecipitated with a negative IgG. H and I, *Drosophila* S2 cells were treated with the indicated dsRNAs on day 1, transfected with the indicated ASOs on day 2, and collected for RNA purification on day 4. Copper was added for the final 10 min to activate MtnA transcription and 4sU was added for the final 5 min to label nascent transcripts. qRT-PCR was then performed to measure the MtnA segment levels in the treated cells. Data were normalized to the control ASO sample. All data are shown as mean±SEM. n=3. **, P<0.01; *, P<0.05.

to 3′ RNA segment was largely unaffected (Figure 3F; Figure S4B in Supporting Information). On the other hand, Rat1 recruitment to 3′ RNA segment was significantly elevated in the MtnA or MtnB ASO treated cells, whereas Rat1 recruitment to 5′ RNA segment was little affected (Figure 3G; Figure S4C in Supporting Information).

To further evaluate whether depletion of the exosome components or Rat1 affects the degradation of premature

MtnA or MtnB segments, S2 cells were transfected with the indicated dsRNAs targeting β-gal, Mtr4, Dis3, Rrp40 or Rat1, and the MtnA or MtnB ASO was introduced 24 hours later. Cells were treated with 4sU prior to nascent RNA capture. qRT-PCR was performed to measure the levels of premature segments (Figure 3B, H, I; Figures S2, S4D and S4E in Supporting Information). As observed, the level of 5′ segment was rescued upon depletion of the exosome components (Figure 3H). Likewise, the level of 3′ segment was restored to a level similar to the control ASO treated sample in Rat1-depleted cells (Figure 3I). Collectively, these data suggested that ASO-induced premature transcription termination depends on the activity of exosome and Rat1. Considering that the *cis* effect of a linear lncRNA on its encoded gene transcription is now becoming increasingly an important field of gene regulation, our work demonstrated that ASO reagents cannot be used in such studies due to ASO-induced premature transcription termination.

ASO can be used to establish an RNA-mediated function for a circRNA locus

It is worth noting that a myriad of eukaryotic protein-coding genes can be spliced to generate not only mature linear RNAs, but also circRNAs via backsplicing, whereby a splicing donor is joined to an upstream splicing acceptor (Figure 4A). For example, *Drosophila* circular dati or laccase2 RNA is generated when splicing machinery "backsplices" to join the end of exon 2 to the beginning of exon 2 (Huang et al., 2018; Jia et al., 2019). The first circRNA was identified over 40 years ago, but it was only recently appreciated that these covalently closed molecules are common outputs of eukaryotic genome and what cellular functions they may have remains elusive. (Chen et al., 2015; Cheng et al., 2018; Huang et al., 2020; Huang and Shan, 2015; Li et al., 2019; Liu et al., 2020; Tan et al., 2019; Wilusz, 2017; Wilusz, 2018; Xiao and Wilusz, 2019). Therefore, emerging studies are raised to study the potential functions of circRNAs, and ASO technology is commonly applied to interrogate the function of circRNAs. Despite circRNA ASOs being typically designed to target the circular junction site (Figure 4B), it is unclear whether circRNA ASO triggers premature transcription termination of its gene locus since circRNA ASO is partially base-paired to the encoded pre-RNA (Figure 4B).

To this end, we designed a circRNA ASO targeting the junction sequence of circular dati or laccase2 RNA and a linear RNA ASO targeting the uncircularized exon of linear

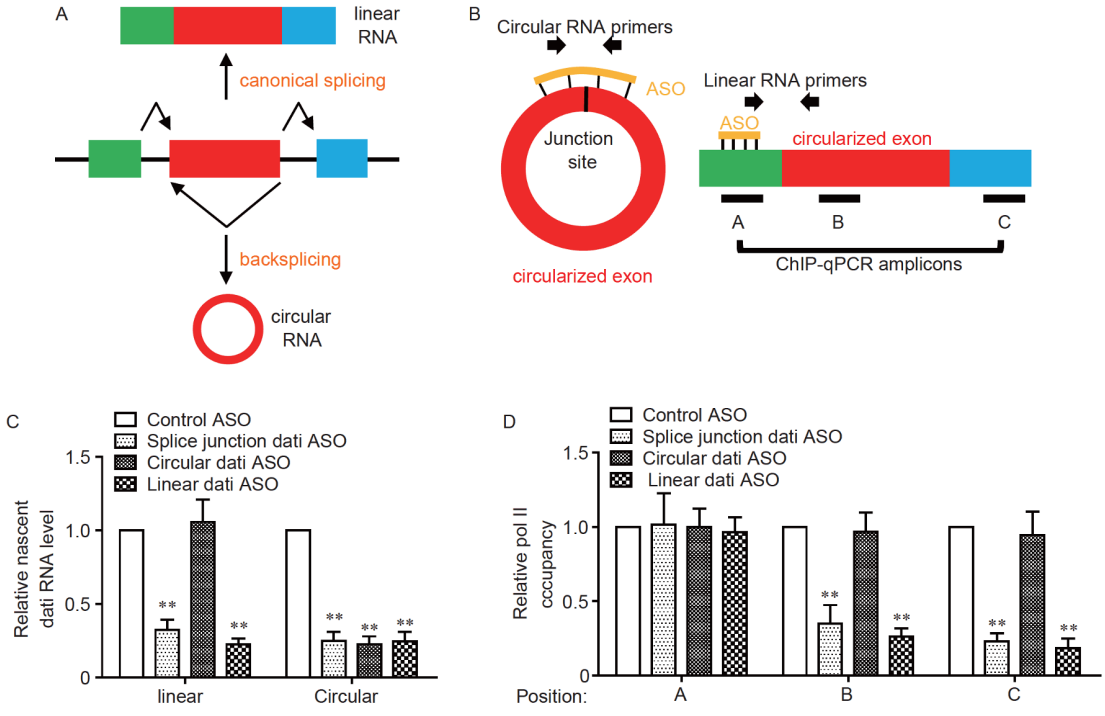

Figure 4 ASO can be used to establish an RNA-mediated function for a circRNA locus. A, The model of pre-RNA splicing. Pre-RNAs can be spliced to generate a linear RNA or a circRNA. B, The ASO strategy for a loss-of-function study (e.g. *Drosophila* dati). A circRNA ASO is designed to target the circular junction site that is solely generated from a backsplicing event, whereas a linear RNA ASO is designed to target the uncircularized exon. A splicing junction ASO is designed to target the dati splicing junction of intron 1 and exon 2. Primer sets for qRT-PCR or ChIP-qPCR are also shown. C, *Drosophila* S2 cells were transfected with the indicated ASOs for 2 days. 250 μmol L^{-1} 4sU was then added to label nascent transcripts for 5 min. qRT-PCR was then employed to measure the levels of nascent linear or circular dati RNA. D, *Drosophila* S2 cells were transfected with the indicated ASOs for 2 days, and recruitment of Pol II to the dati gene locus was measured using ChIP-qPCR. Pol II binding sites were defined as regions enriched over the input DNA. Data throughout C and D were normalized to the control ASO sample and are shown as mean±SEM. n=3. **, P<0.01; *, P<0.05.

dati or laccase2 RNA (Figure 4B). The effect of ASO targeting the dati or laccase2 splicing junction of intron 1 and exon 2 was also tested as a control. qRT-PCR was then performed to examine the 4sU-labeled nascent transcripts. The levels of linear and circular RNAs were significantly decreased in the splicing junction ASO or linear RNA ASO treated cells (Figure 4C), indicating both splicing junction ASO and linear RNA ASO impair transcription of the gene locus. When the cells were transfected with the circRNA ASO, the nascent circRNA level was drastically decreased, whereas the nascent linear RNA level was largely unaffected (Figure 4C; Figure S5A in Supporting Information), suggesting that circRNA ASO does not impair production of the encoded linear RNA. Furthermore, ChIP experiment was performed to compare the Pol II occupancy in each ASO treated sample. In line with Figure 4C, splicing junction ASO and linear RNA ASO transfection resulted in dissociation of Pol II from the gene region downstream of the ASO targeting site, whereas circRNA ASO transfection had no effect on the Pol II occupancy at the dati or laccase2 gene body (Figrue 4D; Figure S5B in Supporting Information). Taken together, these data provided evidence that circRNA ASO does not impair transcription activity of the encoded gene; therefore, ASO can be used to establish an RNA-mediated function for a circRNA locus.

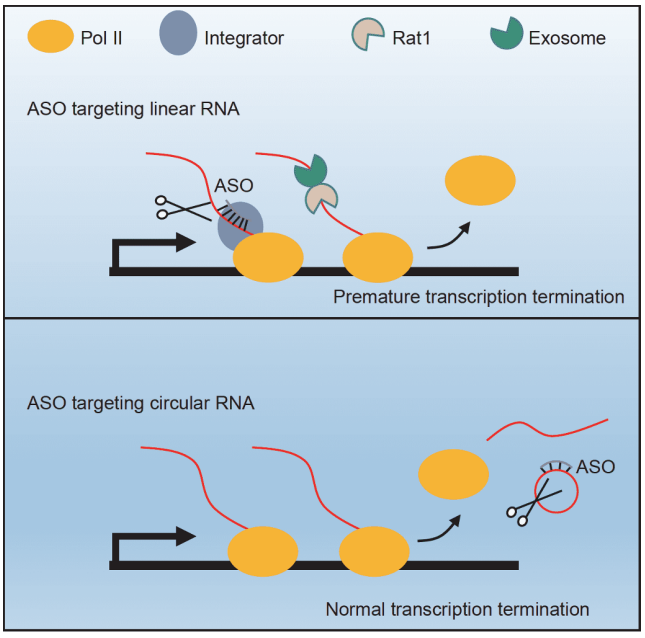

Figure 5 The working model of ASO-induced premature transcription termination in *Drosophila* system. Integrator, exosome, and Rat1 combinatorially control linear RNA ASO-induced premature transcription termination in *Drosophila*, but circRNA ASO does not affect transcription activity of the encoded gene. Therefore, ASO technique can be applied to study a circRNA-mediated but not linear RNA-mediated function for its encoded gene locus.

DISCUSSION

Although the concept that ASO reagents only affect gene expression in a post-transcriptional manner has long been established, the effect of ASOs on transcription of newly-synthesized RNAs is underappreciated. In addition, the question whether ASO reagents have a side-effect on transcription of their targeted gene is largely unknown. In this study, we point towards a novel mechanism whereby Integrator, exosome, and Rat1 combinatorially control ASO-induced premature transcription termination in *Drosophila* system (Figure 5). Furthermore, we compare the effect of a linear RNA ASO and a circRNA ASO on transcription activity of the gene locus. Informed by the results, we conclude that ASO technique can be applied to study a circRNA-mediated but not linear RNA-mediated function for the encoded gene locus itself, revealing novel insights into the appropriate application and the design strategy of ASO reagents. In line with our data, two very recent studies proved that ASO-mediated transcript knockdown induces cleavage of nascent transcripts and efficient pre-mRNA degradation on chromatin during the preparation of our manuscript (Lai et al., 2020; Lee and Mendell, 2020). In this event, human XRN2 is required for ASO-directed RNA Pol II transcription termination (Lai et al., 2020; Lee and Mendell, 2020). Given that the exonuclease XRN2 is the human homologue of *Drosophila* Rat1, our data indicate that the mechanism of ASO-induced premature transcription termination is evolutionarily conserved from *Drosophila* to human.

Transcriptional regulation by premature termination

It is widely established that premature transcription termination, an important aspect of transcriptional regulation, can negatively regulate expression of the full-length transcript in many cases across species, as well reviewed in (Kamieniarz-Gdula and Proudfoot, 2019). In general, premature transcription termination of a protein-coding gene can be divided into two types of termination events based on where it occurs: (i) *transcription start site linked premature transcription termination*, most premature terminations occur at the stage that Pol II terminates prematurely in the vicinity of the transcription start site (within the first exon of a nascent transcript) (Almada et al., 2013; Brannan et al., 2012; Krebs et al., 2017; Nechaev et al., 2010; Wagschal et al., 2012). In fact, a recent study demonstrated that most transcription initiation events result in premature transcription termination at the promoter region, whereas only 1% give rise to further elongation (Steurer et al., 2018). (ii) *intragenic premature transcription termination*, if Pol II overcomes the elongation checkpoint and goes downstream into the gene body, a cryptic polyadenylation site can trigger a co-transcriptional

cleavage reaction (Hoque et al., 2013; Lianoglou et al., 2013; Yao et al., 2012). In this case, the prematurely terminated transcripts are either fed into a rapid degradation pathway or polyadenylated for generation of truncated proteins.

Our research presented here is unique in that we prove that an exogenous molecular can cause premature transcription termination that mirrors the natural termination mechanism, and three regulators are identified in this event. Integrator has been reported to not only cleave multiple noncoding RNA classes, including small nuclear RNAs (snRNAs), enhancer RNAs, telomerase RNAs, and microRNA precursors (pre-microRNAs) (Baillat et al., 2005; Cazalla et al., 2011; Lai et al., 2015; Rubtsova et al., 2019; Xie et al., 2015), but also affect transcription elongation of many protein-coding genes (Elrod et al., 2019; Tatomer et al., 2019). Informed by these studies, we focused on the role of Integrator in ASO-induced nascent RNA degradation and found that Integrator can aid in catalyzing premature transcription termination of the ASO-targeted gene (Figure 5). Once the targeted nascent transcripts have been cleaved, they are rapidly degraded by the exosome complex and Rat1. Rat1 may further trigger dissociation of elongating Pol II from the transcription site (Figure 5). Altogether, our study points towards a novel mechanism of transcriptional control.

The right chooses of ASOs

There are many theoretical approaches for rational design of a valid and efficient ASO, such as chemical modification selection, binding energy calculation, and prediction of the secondary structure of the targeted RNA, as reviewed in (Chan et al., 2006). Above this rational design strategy, our work focuses on the appropriate use of ASOs and the valid interpretation of ASO-mediated experiments for both experimental and therapeutic purposes. Take the lncRNA research as an example. LncRNA is a class of untranslated RNA, defined as being transcripts with lengths exceeding 200 nt (Rashid et al., 2016; Yu and Shan, 2016). According to the molecular structure, lncRNA can be divided into two groups: linear lncRNA that usually consists of a 5′ cap as well as a 3′ polyA tail and circular lncRNA that are covalently closed. LncRNAs were reported to have diverse roles in development and disease, and one of the prominent roles is to affect their host gene transcription in *cis*, which is an interesting and important field of transcriptional control (Yu and Shan, 2016). Notably, we prove that ASO cannot be used in such studies as a supplement for loss-of-function of a linear lncRNA due to ASO-induced premature termination. However, circRNA ASO does not affect transcription activity of the encoded gene due to the specificity of circRNA ASO (only designed to target the circular junction). In total, prior to ASO design, we should keep two points in mind for the appropriate application of this technique: (i) What is the knockdown target of the ASO (linear RNA vs. circular RNA)? (ii) Is the ASO used to study the *cis* effect of targeted gene on transcription activity?

MATERIALS AND METHODS

Culture conditions for *Drosophila* S2 cells and copper treatment

Drosophila S2 cells were cultured at 25°C with Schneider's *Drosophila* medium (Sigma, USA) plus 10% fetal bovine serum (HyClone, USA) and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific, USA). For transcription activation of the MtnA mRNA, a final concentration of 500 μmol L^{-1} $CuSO_4$ was added for the last 10 min.

dsRNA and RNAi in *Drosophila* S2 cells

DsRNAs from the *Drosophila* RNAi Screening Center (DRSC: https://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl) or previously published works were generated by *in vitro* transcription of PCR templates containing the T7 promoter sequence on both ends using MEGAscript Kit (Thermo Fisher Scientific) (Liang et al., 2017a; Tatomer et al., 2019). Details for all dsRNAs are provided in Table S1 in Supporting Information. For dsRNA treatment, 1.5 million S2 cells in 12-well dishes were bathed with 8 μg of dsRNA for 3 days. RNA was isolated using Trizol (Thermo Fisher Scientific) according to the manufacturer's instructions.

ASOs and ASO transfection

ASOs were modified by changing the nucleotides on both ends into locked-nucleic-acid nucleotides. All bases of ASOs were converted into phosphorothioate oligonucleotides. For ASO transfection, 0.5 million *Drosophila* S2 cells in 6-well plate were transfected with the indicated ASO (final concentration: 100 nmol L^{-1}) for 2 days using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. All ASO sequences are provided in Table S2 in Supporting Information.

Plasmids and plasmid transfection

To generate plasmids for transfections into *Drosophila* S2 cells, the indicated sequences were inserted into a pMK33/pMtHy-based vector (https://www.addgene.org/69911/) as previously described (Huang et al., 2018; Jia et al., 2019). Cloning details for all plasmids are provided in the Supplementary Plasmid Information in Supporting Information. For expression plasmid transfection, 0.5 million *Drosophila* S2 cells in 6-well plate were transfected with 1 μg of plasmid using Effectene transfection reagent

(QIAGEN, Germany).

RNA immunoprecipitation

RIP was performed as previously described (Huang et al., 2015; Yu et al., 2019; Zhu et al., 2019). In this study, nuclear fraction was used for RIP experiments. In brief, Flag-Mtr4 or Flag-Rat1 stable cells were transfected with the indicated ASOs for 2 days, and copper was added for the final 10 min. The cells were washed twice with PBS and resuspended with slow pipetting in 1 mL of lysis buffer B (10 mmol L^{-1} Tris-HCl pH 8, 140 mmol L^{-1} NaCl, 1.5 mmol L^{-1} MgCl2, 0.5% IGEPAL CA-630, 1 mmol L^{-1} dithiothreitol, and 80 U mL^{-1} RNase inhibitor (Thermo Fisher Scientific)). The pellet was collected by centrifugation at 1,000×g for 3 min at 4°C, and resuspended in 1 mL of lysis buffer B with 100 µL of detergent (3.3% (w/v) sodium deoxycholate, 6.6% (v/v) Tween 40). Samples were slowly vortexed for 10 s and incubated on ice for 5 min. Nuclei were then collected by centrifugation at 1,000×*g* for 3 min and washed with 1 mL of lysis Buffer B. The final pellet (purified nuclear fraction) was resuspended in 1 mL of RIPA buffer (50 mmol L^{-1} Tris-HCl pH 7.4, 150 mmol L^{-1} NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100). The nuclear extract was then precleared, and immunoprecipitated with Protein A+G Agarose beads (Beyotime, Shanghai) bound to mouse anti-FLAG antibody (1:300 dilution, Beyotime) for 6 h at 4°C. The beads were washed six times with RIPA buffer, and then subjected to qRT-PCR analysis to examine the associated RNAs following RNA isolation.

Chromatin immunoprecipitation

ChIP was performed using ChIP Assay Kit (Beyotime) according to the manufacturer's instructions. Briefly, cells were fixed with formaldehyde, and sonicated in SDS lysis buffer to obtain chromatin solution containing 400–800 bp DNA fragments. The chromatin solution was precleared, and immunoprecipitated with an antibody against Pol II (1:200 dilution; Abcam, UK) for 8 h. Eluted DNA was then subjected to qPCR analysis to examine the enriched genomic DNA regions.

Metabolic labeling of nascent transcripts

Metabolic labeling of nascent RNAs with 4sU and nascent RNA purification was performed as previously described (Huang et al., 2018; Jia et al., 2019). *Drosophila* S2 cells, treated with the indicated dsRNA for 3 days, were incubated with 250 µmol L^{-1} 4sU (Sigma) for 5 min to label newly transcribed RNAs. 20 µg of 4sU labeled RNA was incubated in 500 µL of biotinylation buffer (10 mmol L^{-1} Tris pH 7.4, 1 mmol L^{-1} EDTA) with 10 µg mL^{-1} MTSEA biotin-XX (dissolved in dimethylformamide; Biotium, USA) at room temperature for 1.5 h. To verify that there was not significant variation across the biotinylation reactions, 2 ng of synthetic RNA was included in each reaction (Table S3 in Supporting Information). Unbound MTSEA biotin was removed by equal volume Chloroform: Isoamyl alcohol (Sigma) extraction, and RNA was precipitated at 12,000×*g* for 30 min at 4°C with 1:10 volume of 5 mol L^{-1} NaCl and an equal volume of isopropanol. The RNA pellet was washed with 80% ethanol and resuspended in 100 µL of DEPC treated water. 4sU-labeled and unlabeled RNA was separated using Dynabeads™ MyOne™ Streptavidin T1 beads (Thermo Fisher Scientific). Biotinylated RNA was incubated with 100 µL of Streptavidin beads in hybridization buffer (5 mmol L^{-1} Tris pH 7.5, 0.5 mmol L^{-1} EDTA, 1 mol L^{-1} NaCl) at room temperature for 1.5 h. Beads were washed 5 times with high salt washing buffer (100 mmol L^{-1} Tris pH 7.4, 10 mmol L^{-1} EDTA, 1 mol L^{-1} NaCl, 0.1% Tween 20). Nascent RNA was eluted with 100 µL of 0.1 mol L^{-1} dithiotheitol (DTT) twice, and purified using RNeasy MinElute Cleanup Kit (QIAGEN).

qRT-PCR

RNA extracts were reverse-transcribed to the complementary DNA (cDNA) using PrimeScript RT Master Mix (TaKaRa, Japan) according to the manufacturer's instructions. qPCR experiments were then performed in triplicate using FastSYBR Mixture (CWBIO, Beijing). All qPCR primer sequences are provided in Table S4 in Supporting Information.

Western blotting

Protein samples from cell extracts or RIP samples were prepared using RIPA buffer (50 mmol L^{-1} Tris-HCl pH 7.4, 150 mmol L^{-1} NaCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100). The same amount of protein from each sample was examined as described previously (Huang et al., 2018; Jia et al., 2019). These antibodies were used in Western blots: mouse monoclonal anti-α-Tubulin antibody (1:10,000 dilution, Sigma), rabbit polyclonal anti-HDAC1 (1:1,000 dilution, Abcam), and rabbit polyclonal anti-FLAG antibody (1:1,000 dilution, Beyotime). Blots were viewed with a Bio-Rad ChemiDoc Imaging System.

Statistical analyses

Statistical significance for comparisons of means was assessed by Student's *t*-test. Statistical details and error bars are defined in each figure legend: **, $P<0.01$ and *, $P<0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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