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Title	Slow decrease in temperature produces readthrough transcripts in mammalian hibernation
Author(s)	Sone, Masamitsu; Yamashita, Junpei; Shigenobu, Shuji et al.
Citation	Biochemical and biophysical research communications, 709, 149837 https://doi.org/10.1016/j.bbrc.2024.149837
Issue Date	2024-03-30
Doc URL	https://hdl.handle.net/2115/94361
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	2024_ReadThrough_manuscript_fin2.pdf



Title

Slow decrease in temperature produces readthrough transcripts in mammalian hibernation

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Abstract

Accumulating evidence suggests that various cellular stresses interfere with the end processing of mRNA synthesis and lead to the production of abnormally long transcripts, known as readthrough transcripts (RTTs), which extend beyond the termination sites. Small mammalian hibernators repeatedly enter a state referred to as deep torpor (DT), where the metabolic rate, respiration rate, and core body temperature become extremely low, which produces various types of cellular stresses and therefore induces RTTs. However, the types of stresses and processes around the DT that cause RTTs are unclear. In the present study, we showed that RTTs are produced from different gene loci in the livers of Syrian hamsters under DT and summer-like conditions. Moreover, *in vitro* analysis using hamster primary hepatocytes revealed that DT-specific RTTs are induced by a slow decline in temperature, as seen in body temperature in the entrance phase of DT, but not by rapid cold treatment or hypoxia. In addition, it was observed that RTTs are not elongated under a significantly cold temperature (4 °C). These results indicate that DT-specific RTTs are produced during the entrance phase of torpor by a slow decrease in body temperature.

Keywords

Hibernation, Readthrough transcription, Cold stress, Syrian hamster

Abbreviations

RTT, readthrough transcript; DT, deep torpor; PA, periodic arousal; Tb, body temperature; DoG, downstream-of-gene containing transcript; GRO-seq, global run-on sequencing; TTS, transcription termination site; LDW, long-day warm condition; FPKM, fragments per kilobase of exon per million mapped reads

1. Introduction

Hibernation is an adaptive strategy used by a wide variety of mammalian species to survive the cold and food-scarce seasons. The mammalian hibernators (hereafter referred to as hibernators) with small body sizes repeat several cycles of deep torpor (DT), in which their body temperature (Tb) declines to approximately 1 °C above ambient temperature for several days to weeks, and periodic arousal (PA), in which their Tb rapidly recovers to a euthermic state and is retained for <1 d, to save energy expenditure. In DT, both heart and respiration rates are repressed by >90% compared to euthermia [1], which causes hypoxic stress in non-hibernators such as humans, mice, and rats. However, hibernators possess extraordinary resistance to various stresses such as cold, hypoxia, and reperfusion [1]. Therefore, mammalian hibernation offers an excellent model for studying how organisms respond to cellular or systemic stresses that occur during their natural life cycles.

Recent studies have revealed that various cellular stresses, such as osmotic stress, heat shock, and viral infection, lead to the synthesis of inefficiently terminated RNAs downstream of gene-containing transcripts (DoGs) [2]. Comparative transcriptomic analysis of a mouse cell line subjected to several stresses revealed that different stresses induced largely overlapping but distinct sets of DoGs, suggesting that transcriptional readthrough may be regulated in a stress-specific manner [3]. As a mechanism, it is proposed that these cellular stresses interfere with the binding of the integrator complex, which cleaves the 3' end of nascent RNAs through endonuclease activity, at the C-terminal domain of RNA polymerase II (Pol II), leading to uncomplete termination of transcription at poly-adenylation signals [4,5]. However, the questions remain as to why the genes that produce DoGs differ among stresses as well as what the biological significance of readthrough transcription under cellular stresses is. To address these questions, it is important to choose appropriate stress models that reflect naturally occurring biological events; otherwise, the significance of the experimental outcomes may be compromised by the particularity of the model systems.

In a recent report on global run on sequence (GRO-seq) analysis of livers from thirteen lined ground squirrels in different time points during hibernation, the transcripts derived from 3' of annotated transcriptional termination sites (TTSs) further elongate in 3' direction in a subset of genes in late DT stage compared to PA or even to early DT stages [6]. These results indicate that the stresses that mammalian hibernators experience during DT may allow RNA polymerase readthrough TTS and continue to transcribe RNA under low Tb conditions. However, it is unclear what kinds of stress during hibernation cause readthrough transcription. In the present study, we addressed this question using a Syrian hamster (*Mesocricetus auratus*, hereafter referred to as hamster) and primary cultured hepatocytes derived from it as model systems.

2. Materials and methods

2.1 Animals and housing

Four-week-old Syrian hamsters were acquired from a breeder (SLC, Inc., Japan) and provided with an MR standard diet (Nihon Nosan, Japan). They were housed in a long day (photoperiod) and warm (LDW) condition with a light-to-dark ratio of 14:10 h and an ambient temperature range of 22–25 °C. For animals subjected to hibernation induction, approximately 8-week-old hamsters were implanted Tb loggers (iButton, Maxim Integrated, USA) into their abdominal cavity under isoflurane anesthesia, and after 1–2 weeks, were moved to a short day (photoperiod) and cold condition with a light-to-dark ratio of 8:16 h and an ambient temperature of 4–5 °C. In our laboratory, most hamsters enter hibernation within 3–4 months in this setting. The onset of DT was determined when the animals became immobile and exhibited a low Tb, whereas they were considered in periodic arousal (PA) when they spontaneously became active following DT. Animals were euthanized either by reperfusion with 4% isoflurane for hepatocyte isolation or by decapitation under anesthesia for liver sampling. Six male hamsters and nine female hamsters were used for RNA-seq analysis (N = 3 for each condition). 12 female hamsters were used for qRT-PCR analysis of livers (N = 4 for each condition). Six male hamsters were used for hepatocyte culture. All animal care and experimental procedures were approved by the Ethics Committee of Hokkaido University (ethical approval no. 18-0140) and conducted in accordance with the ethics guidelines of Hokkaido University.

2.2 RNA extraction

Total RNAs from hamster livers or hepatocytes were extracted as previously described [7].

2.3 RNA sequencing (RNA-seq)

RNA-seq analysis of female samples was performed as previously described [7]. Sequencing libraries were prepared using the TruSeq Sample Preparation Kit v2 (Illumina; RS-122-2001) and sequenced using the HiSeq1500 (Illumina) system with 101-bp paired-end readings. For RNA-seq analysis of male samples, sequencing libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs) and sequenced using a Novaseq 6000 (Illumina) with 150-bp paired-end readings.

2.4 RNA-seq data analysis

Assembled genome sequence data for Syrian hamsters were provided by Siomi [8]. The gene annotation file was created by transferring the BCM_Maur_2.0 annotation file to this genome using LiftOff (<https://github.com/agshumate/LiftOff>). The readthrough transcripts (RTT) annotation file was created by setting 3000-bp downstream regions of each annotated gene as RTTs on R software and removing RTTs that overlapped with the annotated genes on the same strand (for male RNA-

seq) or on either strand (for female RNA-seq) using bedtools (<https://bedtools.readthedocs.io/en/latest/index.html>). Raw sequencing reads were adaptor removed and filtered by sequence quality using TrimGalore software (<https://github.com/FelixKrueger/TrimGalore>). Trimmed reads were mapped to the hamster genome using TopHat2 (<https://ccb.jhu.edu/software/tophat/index.shtml>) for female hamsters and Hisat2 (<https://daehwankimlab.github.io/hisat2/main/>) for male hamsters. The resulting SAM files were converted to sorted BAM files using samtools (<https://github.com/samtools/>). FeatureCounts (<https://subread.sourceforge.net/featureCounts.html>) was used to calculate the read count of genes using the gene annotation file and RTTs using RTT annotation files from sorted BAM files with strand-specific settings (male hamsters) or unstranded settings (female hamsters). The FPKM of each gene or RTT and statistical significance of differences between animal conditions were calculated using the edgeR tool (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) in R software.

2.5 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

cDNA was synthesized from 300–1000 ng of total RNA using the PrimeScript RT Reagent Kit (Takara Bio) and diluted 20-fold with 100 ng/mL sonicated salmon sperm DNA (BioDynamics Laboratory). Real time PCR of 15 μ L reaction (7.5 μ L of TB Green Premix ExTaq II [Takara Bio], 0.4 μ M of primers, and 5 μ L of diluted cDNA) was performed on using a LightCycler 480 (Roche) system. Relative expression levels were calculated using the delta Ct method for the data in Figs. 1–3. For the data shown in Fig. 4, expression levels were calibrated using a standard prepared by serial dilution of hamster genomic DNA and normalized to Hprt1 expression as an internal control. The primer sequences are listed in Supplementary Table S1.

2.6 Primary culture of hepatocytes

Hamster hepatocytes were isolated and cultured as previously described [9]. Hepatocytes were seeded in 6-well plates at 2.5×10^5 cells/well.

2.7 Stress treatment of hepatocytes

On the day after seeding, the hepatocytes were treated with various stressors. For hypoxic stress, cells were incubated under 1% O₂, 5% CO₂ at 37 °C for 8 h. When incubated under this condition for 16 h, the hepatocytes detached from the bottom and could not be used for subsequent analysis. For osmotic stress, the medium was replaced with the medium containing 84 mM KCl and incubated under 5% CO₂ at 37 °C for 4 h. For cold stress, the medium was replaced with the medium containing 100mM HEPES pH 7.4 prewarmed to 37 °C. For rapid cold treatment, the cells in a culture plate were directly placed at 4 °C in refrigerator (PHCbi). For slow cold treatment, the cells

in a culture plate were placed in Styrofoam box (22 × 26 × 17 cm size, 2.3 cm wall thickness) containing a Tupperware filled with 300mL water prewarmed to 37 °C and incubated in a temperature-programable chamber (Shinfactory, Japan) that is programmed as 37 °C, 30 min; 4 °C, 122 h; 37 °C, 18 h. To monitor the temperature change during the experiment, the iButton was placed in the empty well of the culture plate.

3. Results and Discussion

3.1 Candidates of readthrough transcripts in hamster liver

We analyzed two series of RNA-seq datasets that were obtained from hamster livers of different sex and conditions in our laboratory using different sequencing methods (Fig. 1A): [Exp.1] strand-specific RNA-seq from DT male hamsters in short-day cold (5 °C) condition and age-matched 7-month old active male hamsters in long-day warm (approximately 25 °C) condition (LDW-7mo); [Exp.2] strand-unspecific RNA-seq from young active female hamsters in LD warm condition (LDW-4mo) and 6-month old female hamsters that were at DT phase or at euthermic PA phase. When investigating RNA-seq data with genome browser, we found that significant amount of sequencing reads were mapped at the 3' region beyond the TTS of *Apolipoprotein C1 (Apoc1)* gene in DT but not in LDW-7mo hamsters (Supplementary Fig. S1A). Strand-specific sequencing indicated that these sequencing reads were derived from transcripts continuing from *Apoc1*, but not from transcripts of an adjacent gene in the opposite direction (Supplementary Fig. S1A). The RTTs of *Apoc1* share characteristics such as splicing, as indicated by junctional reads, with previously reported DoG transcripts induced by other stresses [3]. However, the RTTs of *Apoc1* extend only approximately 2.7 kb from the TTS, which is significantly shorter than previously reported DoG transcripts that extend >5 kb, or sometimes even >100kb, and do not meet the criteria of DoG (>5kb) [2]. These transcripts are referred to as readthrough transcripts (RTTs). The identification of RTTs at *Apoc1* locus in DT hamsters prompted us to survey genes that produce RTTs in a condition-specific manner using conventional RNA-seq datasets. To list up candidate genes that produce RTTs specifically in DT or LDW, we defined transcripts derived from 3kb regions from 3' end of annotated genes as RTTs and set three criteria the condition-specific RTTs should meet (Fig. 1B, Supplementary Fig. S1B): (1) FPKM of RTT > 0.5; (2) the ratio of FPKM between RTT and the upstream gene was significantly higher than in the other condition (fold change >2, FDR <0.05); and (3) RTT should meet (1) and (2) consistently in both Exp.1 (male) and Exp.2 (female) datasets. As a result, 37 and 16 candidate genes that specifically produce RTTs in DT and LDW, respectively, were identified.

3.2 Identification of RTTs specifically expressed in DT or LDW

To confirm the results of the primary screening for condition-specific RTTs, we inspected each candidate gene using a genome browser. Most of these genes possessed RTTs that were significantly expressed under either DT or LDW conditions, as exemplified by *Tyrosine aminotransferase (Tat)* and *RNA binding motif protein 3 (Rbm3)*, respectively (Fig. 1C and D). Among the 37 DT-specific and 16 LDW-specific RTT-producing candidate genes, we selected ten and nine genes that showed relatively high RTT expression in the genome browser inspection, respectively, for further confirmation of their expression in the liver using qRT-PCR in individuals other than those used in RNA-seq. As seen in genome browser views, the expression levels of RTTs in most of the genes were significantly different among animal conditions, whereas transcripts from the gene body were not, except for *Hal*, *LOC101837099*, and *Rbm3* (Supplementary Fig. S1C). All DT-specific RTTs exhibited significantly higher expression levels in DT than in LDW tissues. Some LDW-specific RTTs (*Htatip2*, *Kyat3*, *LOC101837099*, and *Lrig2*) did not exhibit significantly higher expression compared to DT, possibly due to individual-to-individual fluctuations in expression levels (Fig. 1E, Supplementary Fig. S1C). We also investigated the expression of RTTs from these gene loci in the livers of PA animals sampled within 4 h after recovering euthermia using qRT-PCR (Supplementary Fig S1C and D). The expression of these RTTs in the PA animals was intermediate between those in the DT and LDW animals.

3.3 Rapid cold, hypoxic or osmotic stress did not induce DT-specific RTTs

A global transcriptional survey identified both DT-specific- and LDW-specific RTTs, but we mainly focused on the former for further analysis because DT status induces cellular stresses and therefore offers a good model for studying the relationship between cellular stresses and RTT production. Next, we set up *in vitro* experiments using primary cultured hepatocytes from male hamsters under LDW conditions to determine the types of cellular stresses that induce RTTs from DT-specific RTT-producing gene loci, such as *Apoc1* and *Tat*. We treated cultured hepatocytes with different types of stresses that occur during DT or are expected to induce RTTs and examined the expression levels of several DT-specific RTT-producing genes (Fig. 2A). After 1 d of culture under normoxic condition at 37 °C, hepatocytes were subjected to three types of stresses: (1) rapid cold exposure by transferring culture plates of hepatocytes directly into 4 °C refrigerator and subsequent prolonged cold storage for one or 5 d. Prolonged cold exposure experiments are possible because of the remarkable cold resistance of hamsters [9,10]. (2) hypoxic stress under 1% O₂ at 37 °C for 8 h, (3) osmotic stress with 84mM KCl at 37 °C for 4 h. Contrary to our expectations, cold exposure did not induce RTTs in most of the analyzed gene loci, except for *Tat* locus (Fig. 2B). Similarly, hypoxia and osmotic stress did not consistently increase RTTs (Fig. 2B).

3.4 Slow decrease of temperature induced DT-specific RTTs

We hypothesized that the temperature drop in the previous experiment was too fast to cause up-regulation of DT-specific RTTs, considering that Tb decline during the entrance into DT from PA is significantly slower than the temperature shift caused by transferring culture plates to 4 °C refrigerator. To mimic the slow Tb decline during entrance into DT, we incubated the hepatocytes at 4 °C in an Styrofoam-insulated container with a water heat storage prewarmed to 37°C, which successfully recapitulated the slow decline of Tb in hibernation (Fig. 3A). This “slow shift” method to decrease the ambient temperature significantly increase the expression of RTTs from several gene loci including *Apoc1*, *Insulin-like growth factor binding protein 4 (Igfbp4)*, *Insulin induced gene 1 (Insig1)* and *Proline-rich nuclear receptor coactivator 1 (Pnrc1)*, all of which are DT-specific RTTs in livers (Fig. 3B). In addition, RTTs from *Igfbp4*, *Insig1*, *Pnrc1* and *Tat* were reduced by rewarming hepatocytes (Fig. 3B). These results clearly indicate that a slow but not a rapid decrease in temperature is key to the induction of DT-specific RTTs. We further analyzed one of LDW-specific RTT producing loci, *Rbm3*, and found that expression of its RTT was reduced by approximately 50% at 4 °C compared to 37 °C similarly by both rapid and slow shift methods (Fig. 3B). This suggests that the mechanism by which RTTs are regulated may differ between DT-specific- and LDW-specific RTT-producing genes in terms of dependency on temperature kinetics.

Although the experimental setup mimicking the slow decline in Tb significantly upregulated RTTs from several DT-specific RTT-producing genes, the magnitude of the upregulation was still smaller than that observed in the livers of DT animals. For example, the increase of RTTs from *Apoc1* and *Insig1* in 4 °C by slow shift method were only two to three-fold compared to 37 °C, whereas those in DT were approximately 10- and 30-fold compared to LDW, respectively (Supplementary Fig. S1C, Fig. 3B). This difference in the extent of RTT induction between *in vivo* and *in vitro* systems may be due to other stresses we have not examined *in vitro*. For instance, in hibernating animals, the systemic metabolic rate, measured by the oxygen consumption rate, decreases during PA before entering DT [11], which may cause a transient energy shortage and lead to cellular metabolic stress. One of the proposed mechanisms that decrease the metabolic rate before entering torpor is the repression of mitochondrial electron transport chains (ETC) [12]. The possibility that metabolic stress contributes to RTT induction could be addressed in the future by inhibiting ETC or depriving metabolic substrates of mitochondrial activity in primary hepatocyte cultures in combination with cold stress using the slow shift method.

3.5 Elongation of RTTs pauses in cold temperature

GRO-seq analysis of 13LGS revealed that the peak binding positions of Pol II downstream of TSSs shifted by several kb in the 3' direction from the early to late phases of DT [6]. This observation raises an intriguing possibility that Pol II keeps transcription beyond TSS under a notably low temperature (approximately 5 °C) in hibernators. Taking advantage of our *in vitro* model to precisely

control the time and temperature, we addressed this possibility indirectly using the following method: *Insig1* locus was chosen for investigation of RTT elongation under low temperature because it has longest “tail” of RTT in DT liver among DT-specific RTT producing loci (Fig. 4A). qPCR primer sets were designed at 3' UTR of the annotated *Insig1* gene and at five points downstream of TTS with an interval of approximately 2.8 kb (Fig. 4A). The expression levels of transcripts from this locus were quantified by qRT-PCR and calibrated by standards using genomic DNA to make it possible to compare the transcriptional levels among the different points assessed by the different primer sets (Fig. 4B). As a result, transcriptional levels of RTTs has decreasing trend in 3' direction from the point at 2.8 kb downstream of TSS in cold temperature (compare expression levels of #1–4), but more importantly, they did not differ between 1- and 5-d incubation in cold temperature (Fig. 4B). The absence of a downstream shift in transcription from the early to late phases of cold incubation suggests that the transcriptional process of RTT is completed and ceases at least 1 d after the cold shift in this locus. Given that a steep decline in temperature did not induce RTTs from most of the examined DT-specific RTT-producing loci, including *Insig1* (Fig. 2), *in vivo* transcription of RTTs may only occur during the slow transition from euthermia to hypothermia, but not after Tb reaches significantly cold temperatures approximately 4°C.

Based on our observations, we propose a working hypothesis to explain how RTTs are synthesized from DT-specific RTT-producing gene loci such as *Apoc1* and *Insig1* in Fig. 4C: (1) in euthermic conditions, Pol II completes transcription at TTSs, and Cleavage and polyadenylation (CPA) or Integrator complexes that are loaded at the C-terminal domain of Pol II catalyze the end processing of transcripts. (2) When the temperature decreases, the CPA or Integrator first detaches from Pol II [2]. (3) When the temperature drops rapidly, Pol II stops transcription before reading through the TTS. (4) When the temperature decreases slowly as the entrance phase of torpor, Pol II continues to transcribe and sometimes goes through the TTS at mildly cold temperatures. (5) When temperature decreases to approximately 4 °C, Pol II ceases transcription but may keep moving in 3' direction [6]. (6) When the temperature recovers to euthermia, Pol II downstream of TTS detaches from chromatin, and RTTs are degraded.

4. Conclusion

Our transcriptome and qRT-PCR analyses of hamster livers identified RTTs that were expressed specifically in the DT or LDW. Moreover, our *in vitro* analysis using hamster primary hepatocytes showed a slow decrease in temperature induced RTTs from DT-specific RTT-producing loci while a rapid decline in temperature or a hypoxic stress did not. These data indicate that the slow decline in Tb observed in the entrance phase of DT is an important element in the production of RTTs. In addition, we did not obtain any evidences of RTT elongation in cold temperature, suggesting that Pol II pauses transcription of RTTs under notably cold temperature (approximately 4 °C). In summary,

our new simple experimental setup to mimic the slow decrease in Tb revealed a mechanism by which RTTs are produced during hibernation. This method would be useful for studying how cellular processes such as transcription, translation, and protein degradation are regulated during hibernation and could contribute to solving the question of how hibernators protect themselves from damage by cold stress.

CRedit authorship contribution statement

Masamitsu Sone: Conceptualization, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft. **Junpei Yamashita:** Data curation, Formal analysis, Software, Visualization. **Shuji Shigenobu:** Methodology, Data curation, Investigation. **Yoshifumi Yamaguchi:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Acknowledgements

We thank Chayama Y, Ando L, Miura M (Univ. of Tokyo), members of the Functional Genomics Facility and NIBB Core Research Facilities (NIBB), Enju S, Sone K, and all members of the Yamaguchi Laboratory for their supports in this study. This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT)/Japan Society for the Promotion of Science KAKENHI (16H05127, 20H05766, 23H04940, 23H03322 to YY; 22K19320 to MS), Japan Agency for Medical Research and Development (AMED) (23gm6310019 to YY), NIBB Collaborative Research Program (16-408, 19-101), Toray Science Foundation, Takeda Science Foundation, Inamori Research Institute for Science to YY, and Akiyama Life Science Foundation to MS.

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Figure legends

Fig. 1. RTTs are produced from more gene loci in DT than in LDW. (A) Schematic diagram of sampling points and representative plots of body temperature (Tb) of hamsters in SD-Cold condition. (B) Male hamster liver RNA-seq data of genes with RTTs that are > 0.5 FPKM are shown as ratios of FPKM between RTT and gene regions. Genes significantly different in both experiments (see main text) are shown in red. (C, D) (left) genome browser view of female liver RNA-seq data of a representative individual for each condition and (right) qRT-PCR analysis of *Tat* (C) and *Rbm3* (D) loci in male livers. Gene and RTT expression are normalized to *Hprt1* as an internal control (one-way ANOVA with the Tukey's multiple comparison test, $p < 0.05$). (E) qRT-PCR analysis of male livers. Data is represented as ratios between RTT and gene regions, mean $\pm$ SD, $n=4$.

Fig. 2. Rapid cold, hypoxic, or osmotic stress did not induce DT-specific RTTs in primary hepatocytes. (A) Schematic diagram of experimental settings. (B) qRT-PCR analysis of RTT expressions normalized to *Hprt1* in the indicated genes in hepatocytes (one-way ANOVA with Dunnett's multiple comparison test compared to 37 °C, *** $p < 0.001$).

Fig. 3. Slow decrease in temperature induced RTTs in primary hepatocytes. (A) (left) Schematic diagram of experimental settings. (right) Temperature shift in these experimental settings and a representative Tb shift in a hamster in an entrance phase of DT. (B) qRT-PCR analysis of RTT expressions normalized to *Hprt1* in the indicated genes in hepatocytes (one-way ANOVA with Dunnett's multiple comparison test compared to 37 °C, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 4. Elongation of RTTs pauses in hepatocytes under cold temperature. (A) Genome browser view of *Insig1* locus of female liver RNA-seq data of a representative individual for each condition with the positions of primer sets used for qRT-PCR. (B) qRT-PCR analysis of expressions quantified with the indicated primer sets, calibrated to a standard using genomic DNA, and normalized to *Hprt1* (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). (C) Working hypothesis of RTT production in rapid and slow temperature decline.

Figure 1

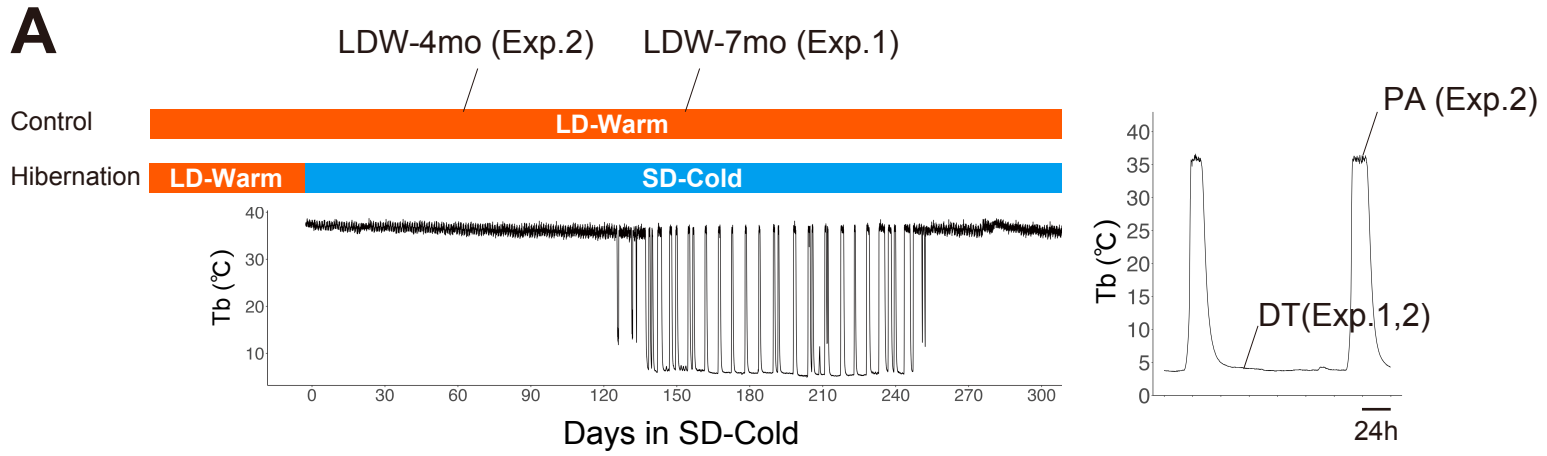
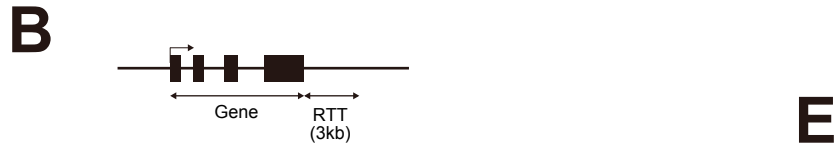
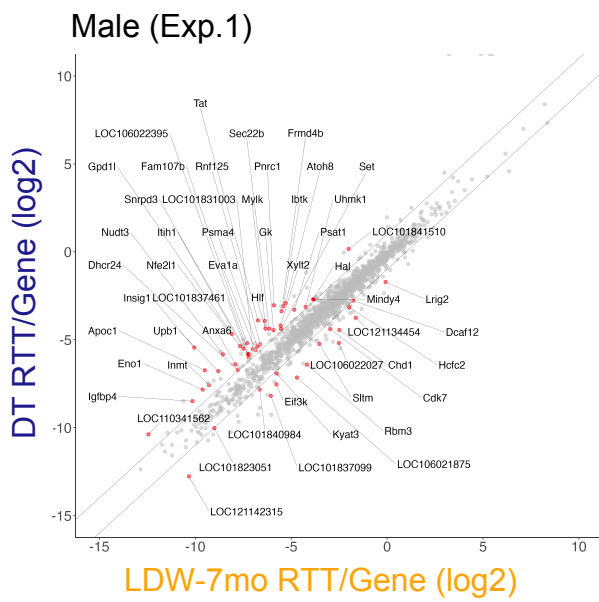
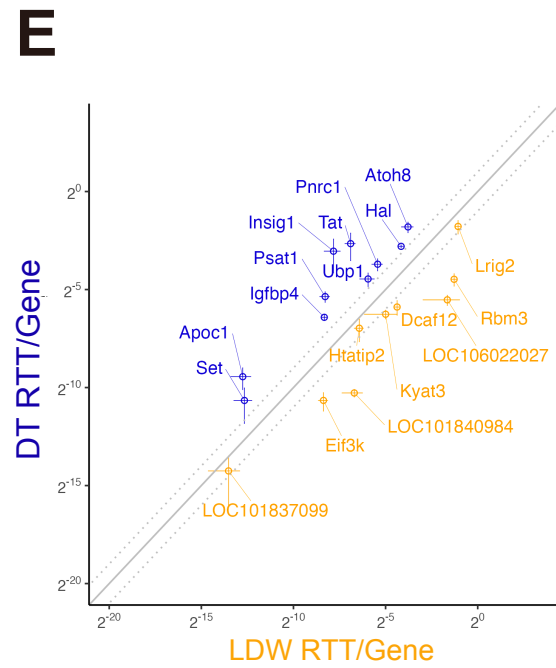
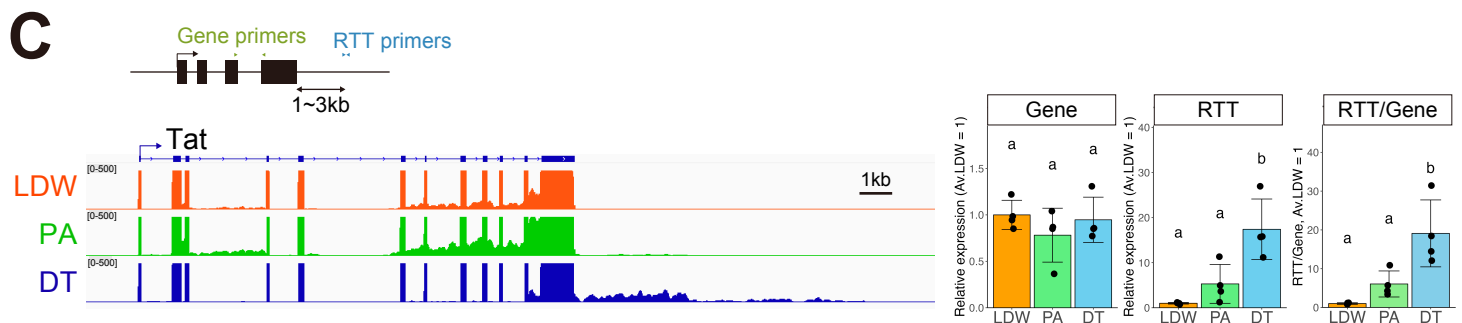
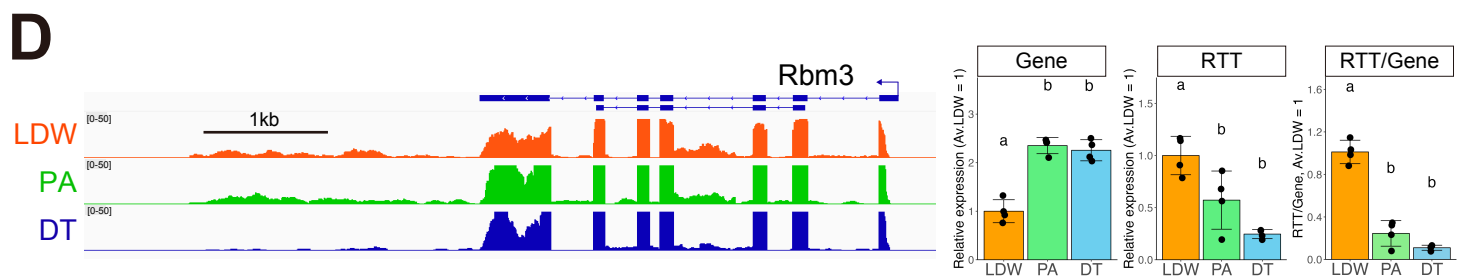
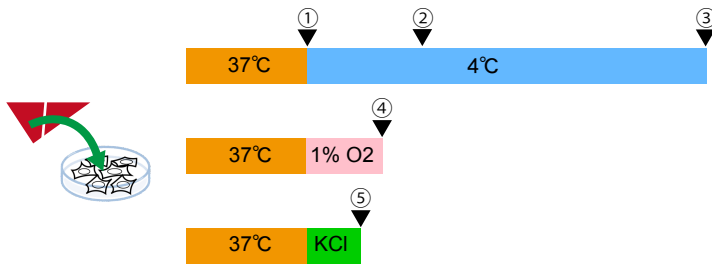


Figure2

A



B

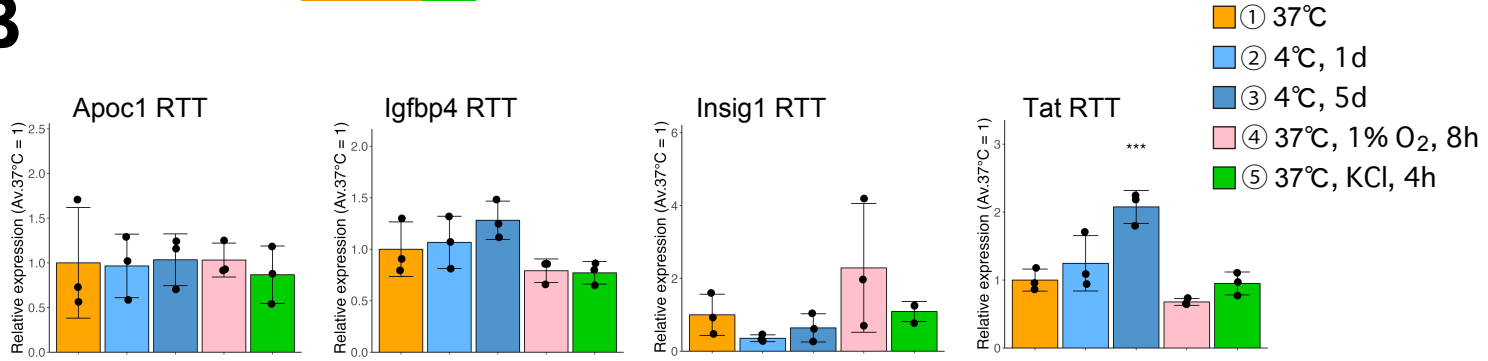
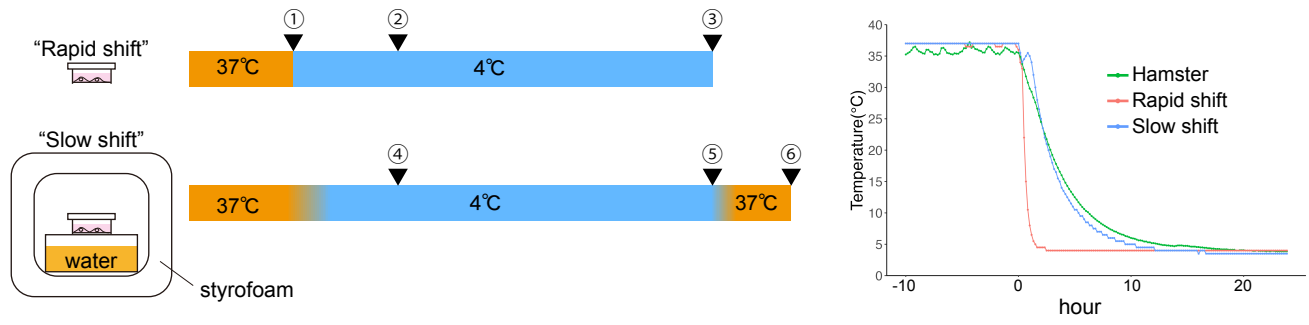


Figure3

A



B

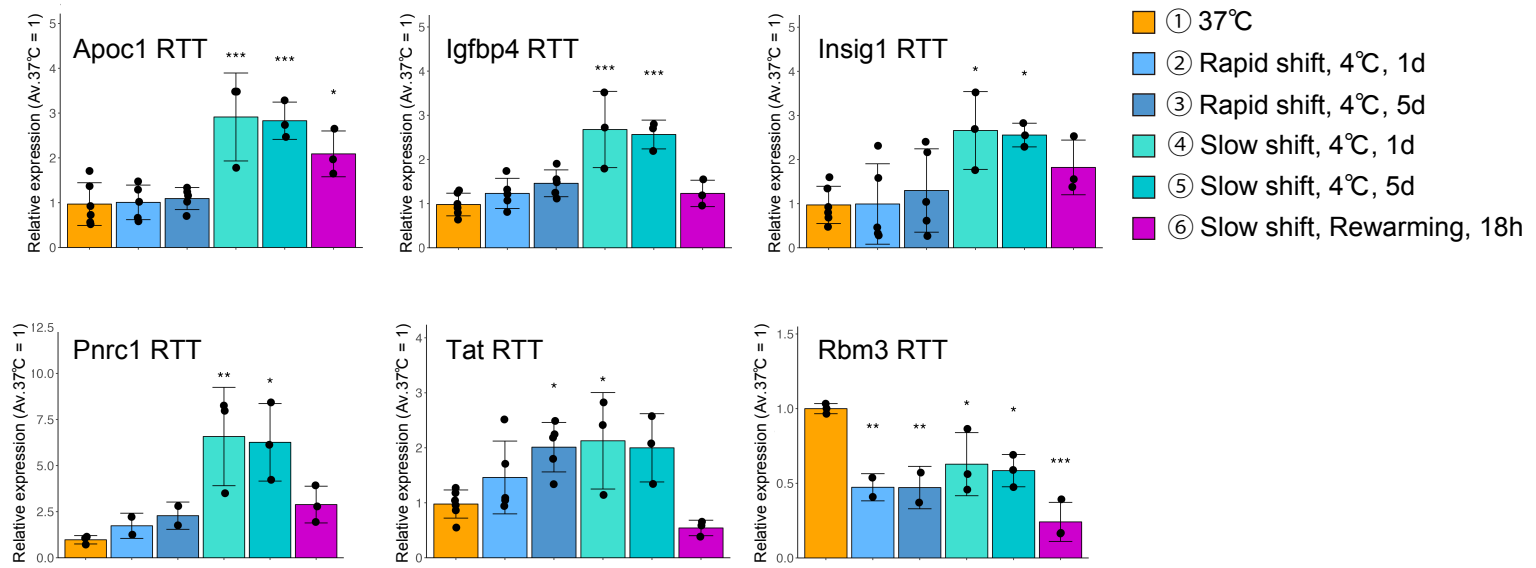


Figure 4

