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RESEARCH ARTICLE

Figures and figure supplements

Efficient termination of nuclear lncRNA transcription promotes mitochondrial genome maintenance

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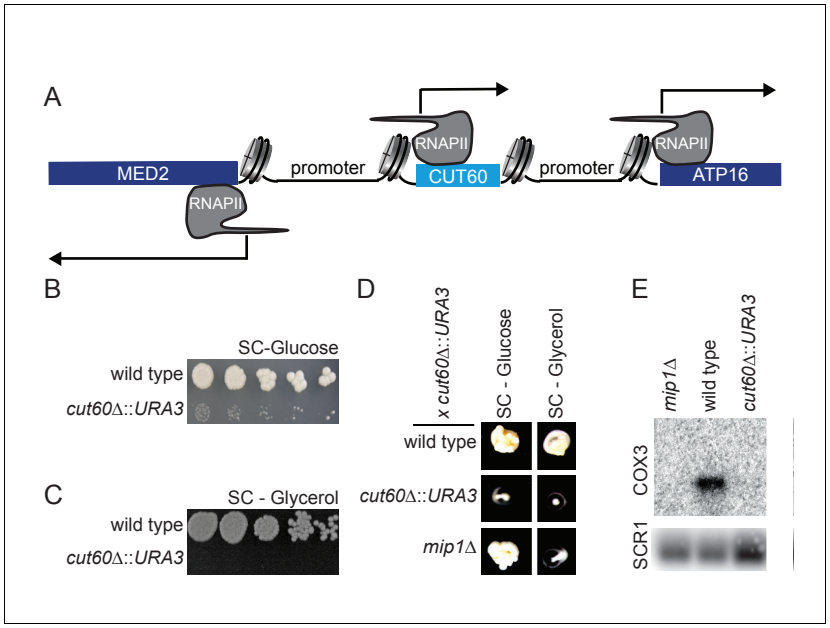

Figure 1. The divergent non-coding transcript *CUT60* promotes growth and mitochondrial genome maintenance. (**A**) Schematic representation of the bidirectional promoter of *MED2*. *CUT60* is a divergent lncRNA transcript originating from the *MED2* promoter, and it is located upstream in tandem of *ATP16*. (**B**) Serial dilution growth assay of wild type and *cut60Δ::URA3* strains on SC-Glucose medium (n = 3). (**C**) Serial dilution growth assay of wild type and *cut60Δ::URA3* strains on SC-Glycerol medium (n = 3). (**D**) Growth of wild type, *cut60Δ::URA3* and *mip1Δ* on SC-Glucose and SC-Glycerol after mating with *cut60Δ::URA3* strain (n = 3). (**E**) Northern blot analysis of *COX3* and *SCR1* (as loading control) transcripts in *mip1Δ*, wild type and *cut60Δ::URA3* (n = 3).

DOI: https://doi.org/10.7554/eLife.31989.003

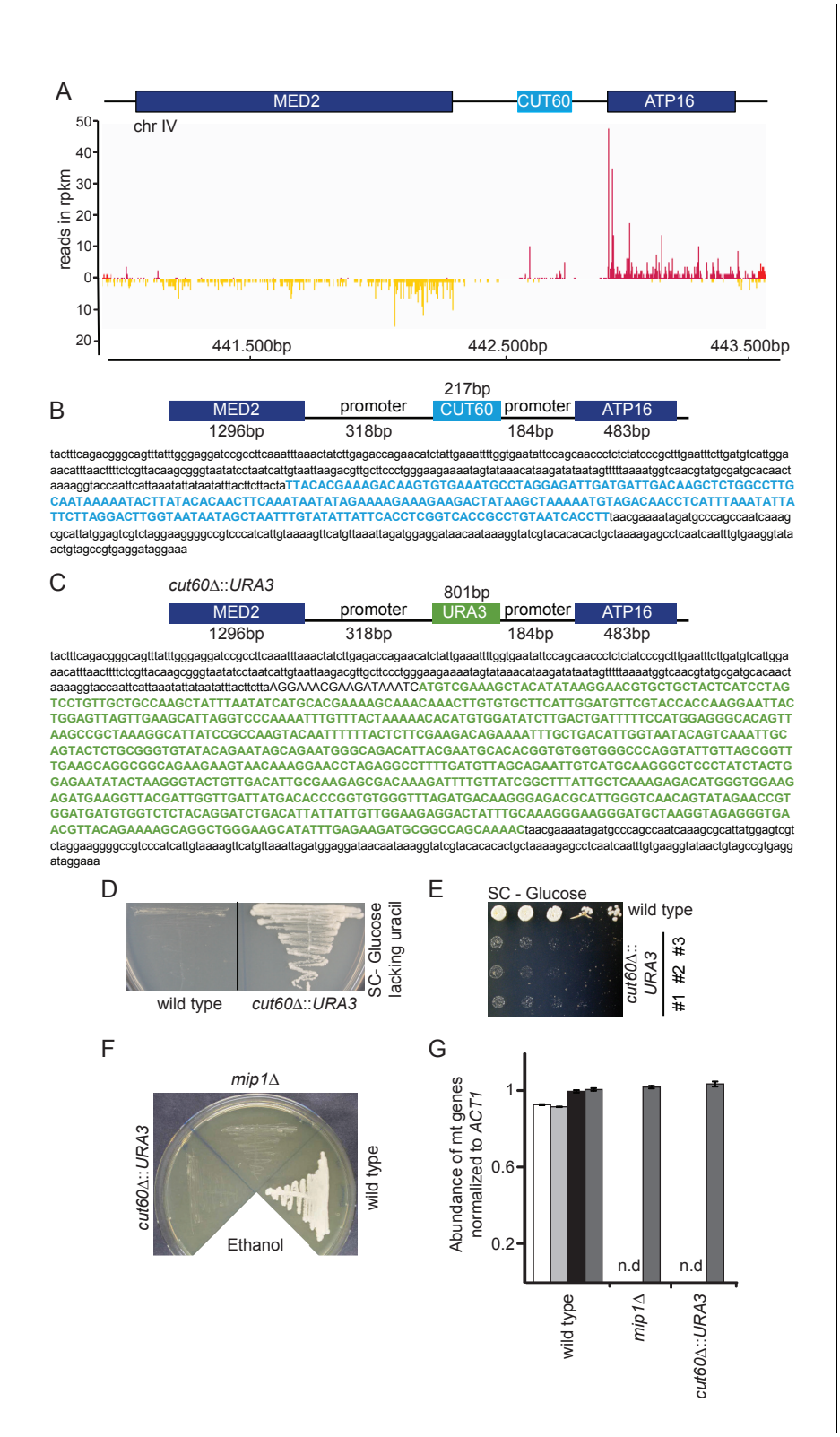

Figure 1—figure supplement 1. NET-seq data showing low transcription from *CUT60* locus compared to *MED2* and *ATP16* coding regions. (**A**) Reads in rpkm shown for a 2.5 kb window of chromosome IV. Schematic representation of *MED2*, *CUT60* and *ATP16* transcripts is shown (top). NET-seq reads for the +strand containing *CUT60* and *ATP16* indicated in red, reads from the – strand containing *MED2* indicated in yellow (n = 1). The data

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were described in (**Marquardt et al., 2014**). (**B**) Schematic representation of the locus between *MED2* and *ATP16*. DNA sequence of the individual parts of this locus is shown in the lower panel. ((**C**)) Schematic representation of the locus between *MED2* and *ATP16* when the coding sequence of *URA3* (green) replaces *CUT60 (cut60Δ::URA3)*. DNA sequence of the individual parts is shown in the lower panel. (**D**) Left panel shows lack of wild type cell growth, right panel shows growth of *cut60Δ::URA3* cells on SC-Glucose media lacking uracil (n = 3). (**E**) Serial dilution growth assay on SC-Glucose media of wild type, *cut60Δ::URA3*#1, *cut60Δ::URA3#2* and *cut60Δ::URA3#3* strains (n = 3). These strains represent independently generated isolates of the *cut60Δ::URA3* mutation. (**F**) Growth of wild type, *cut60Δ::URA3*, *mip1Δ* strains on plates with the non-fermentable carbon source ethanol (n = 3). (**G**) Mitochondrial DNA content in wild type, *mip1Δ*, *cut60Δ::URA3* strains assayed by qPCR. qPCR analysis of three genes encoded by mitochondrial genome *COX2* (white), *COX3* (grey), *ATP8* (black) and nuclear encoded household gene *ACT1* (dark grey) in wild type, *mip1Δ*, *cut60Δ::URA3* strains. Gene expression normalized to expression of the nuclear *ACT1* transcript. Background level transcript-abundance is indicated by 'n.d.' (not detectable). Error bars are s.e.m. of three biological repeats.

DOI: https://doi.org/10.7554/eLife.31989.004

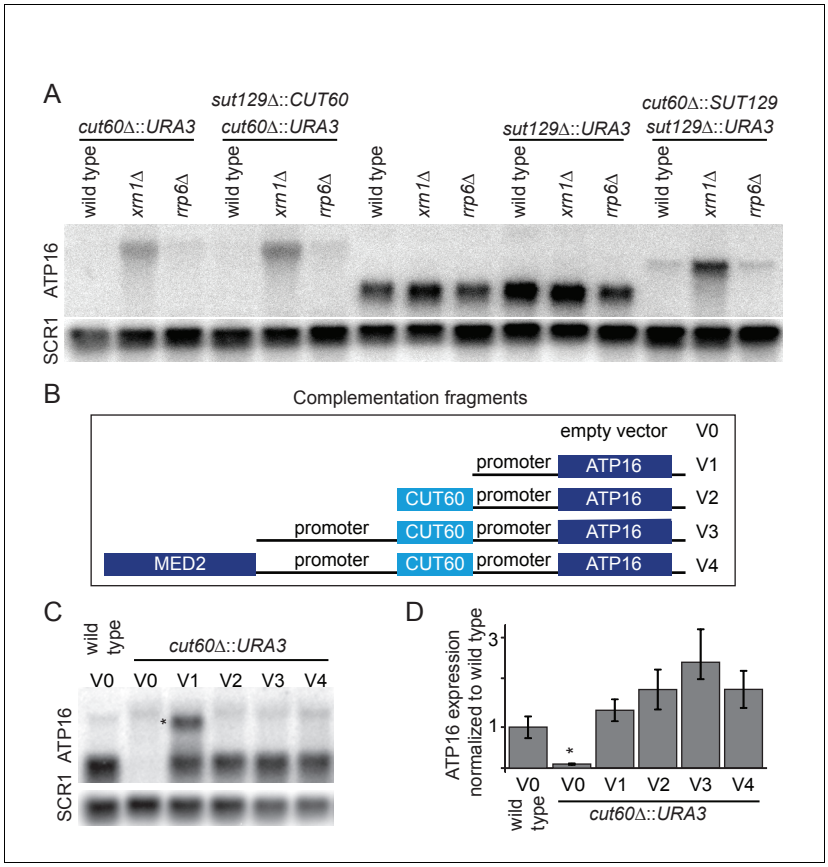

Figure 2. *CUT60* promotes *ATP16* expression as cis-acting transcript. (**A**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in *cut60Δ::URA3*, *sut129Δ::CUT60 + cut60Δ::URA3*, wild type, *sut129Δ::URA3* and *cut60Δ::SUT129 + sut129Δ::URA3* strains. For all strains the transcripts were analyzed in three different backgrounds: Wild type or respective decay pathway mutant background indicated above blot (n = 1). (**B**) Schematic representation of the five different fragments used for complementation in ***Figure 2C***. V0 is the empty vector control, V1 contains the *ATP16* promoter sequence excluding *CUT60*, V2 expands V1 with *CUT60*, V3 expands V2 with the *MED2* promoter, V4 expands V3 with the sequence of *MED2*. (**C**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcript in wild type +empty vector (V0, as shown in ***Figure 2B***), *cut60Δ::URA3* with complementation vectors (V0-4) (n = 3). * indicates extended transcript in *cut60Δ::URA3* + V1 strain. (**D**) Quantification of *ATP16* expression of ***Figure 2C***. Error bars are s.e.m. of three biological replicates. Statistical significance assessed by t-test, wild type compared to *cut60Δ::URA3* is significant ($p<0.02$). All complementation vectors show no statistical significant increase of *ATP16* expression compared to wild type *ATP16* expression.

DOI: https://doi.org/10.7554/eLife.31989.005

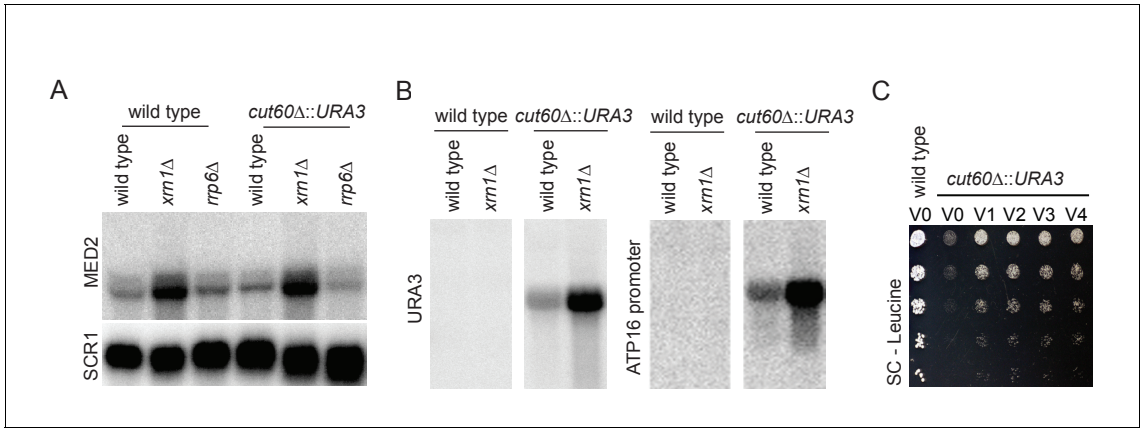

Figure 2—figure supplement 1. *CUT60* influences downstream *ATP16* locus in cis. (**A**) *cut60Δ::URA3* mutants show no detectable effect on *MED2* expression. Northern blot analysis of *MED2* and *SCR1* (as loading control) transcripts in wild type and *cut60Δ::URA3* strains. Genetic backgrounds indicated on top (n = 1). (**B**) Northern blot analysis of *URA3* and transcripts derived from expression of the *ATP16* promoter region in wild type and *cut60Δ::URA3* strains. Genetic backgrounds indicated on top (n = 1). Samples are run on same gel, but the gel was spliced to show the indicated genotypes. (**C**) Serial dilution growth assay of wild type +V0 and *cut60Δ::URA3* with V0-4 on SC-Glucose–Leucine media (constructs shown in ***Figure 2B***) (n = 3).

DOI: https://doi.org/10.7554/eLife.31989.006

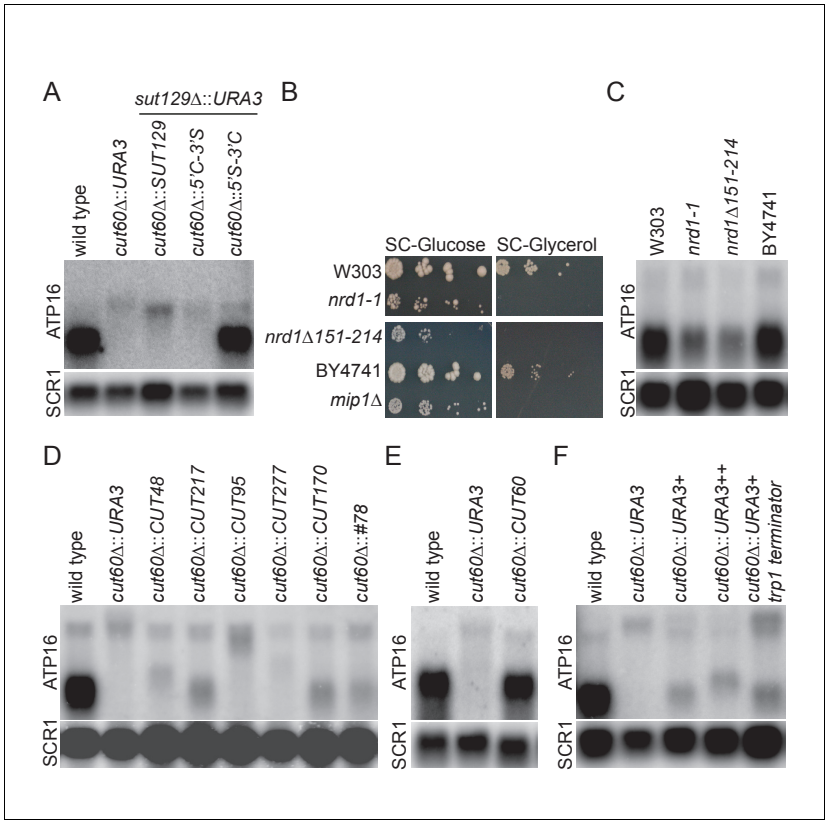

Figure 3. Upstream transcriptional termination promotes *ATP16* expression. (**A**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in wild type, *cut60Δ::URA3*, *cut60Δ::SUT129 + sut129Δ::URA3*, *cut60Δ::5'C-3'S + sut129Δ::URA3*, *cut60Δ::5'S-3'C + sut129Δ::URA3* strains (n = 4). (**B**) Serial dilution growth of W303 (wild type for *nrd1-1*), *nrd1-1*, *nrd1Δ151–214* and BY4741 (wild type for *nrd1Δ151–214*) strains on SC-Glucose and SC-Glycerol plates (n = 2). (**C**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in W303 (wild type for *nrd1-1*), *nrd1-1*, *nrd1Δ151–214* and BY4741 (wild type for *nrd1Δ151–214*) (n = 3). (**D**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in wild type, *cut60Δ::URA3*, *cut60Δ::CUT48*, *cut60Δ::CUT217*, *cut60Δ::CUT95*, *cut60Δ::CUT277*, *cut60Δ::CUT170* and *cut60Δ::#78* strains (n = 3). (**E**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in wild type, *cut60Δ::URA3* and *cut60Δ::CUT60* strains (n = 3). (**F**) Northern blot analysis of *ATP16* and *SCR1* (as loading control) transcripts in wild type, *cut60Δ::URA3+*, *cut60Δ::URA3++* and *cut60Δ::URA3+trp1 terminator* strains (n = 3).

DOI: https://doi.org/10.7554/eLife.31989.007

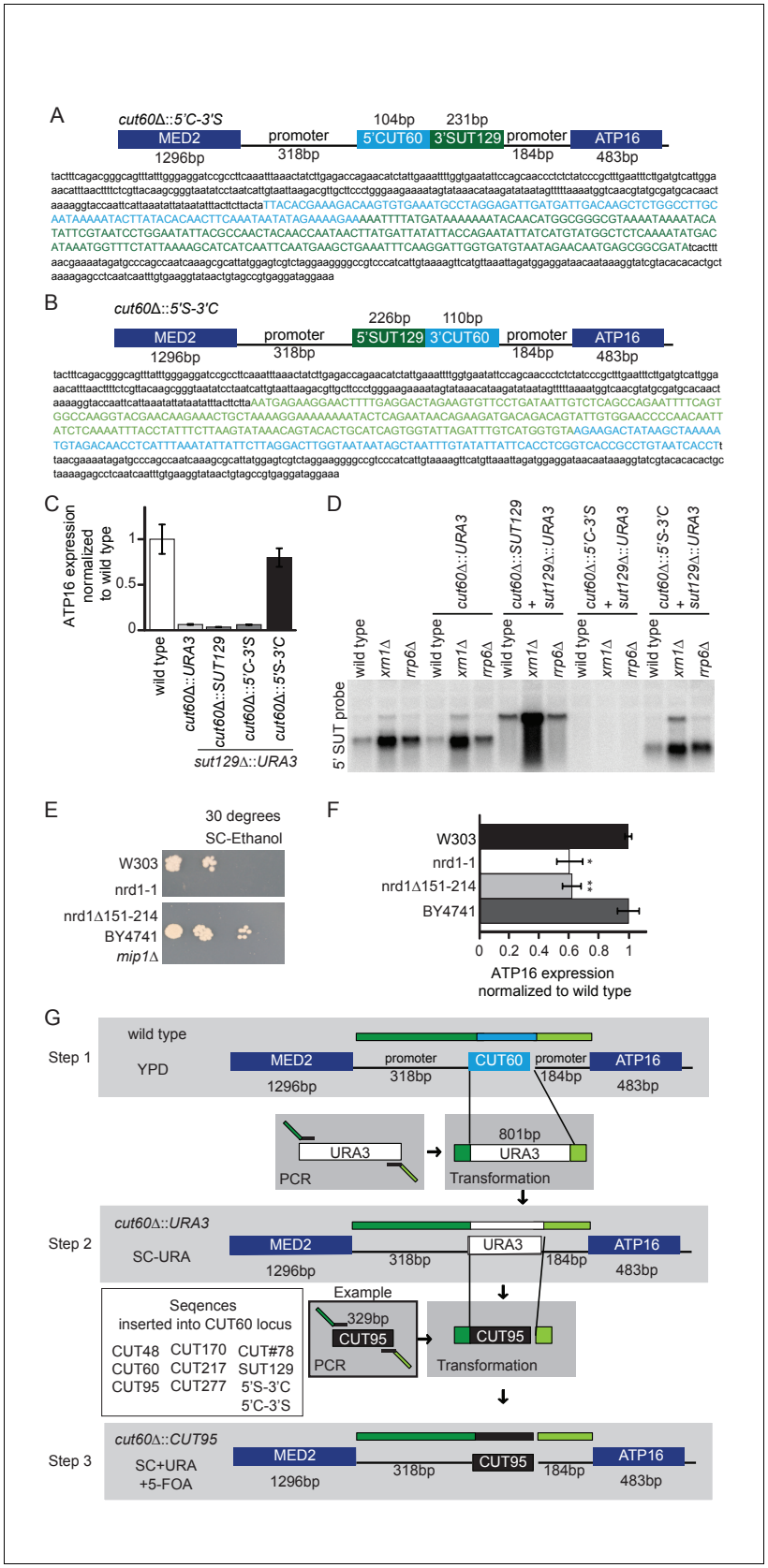

Figure 3—figure supplement 1. Genomic replacement of *CUT60*. (**A**) Schematic representation of the locus between *MED2* and *ATP16* when the 5' sequence of *SUT129* is fused to the 3' sequence of *CUT60*, and this fusion

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sequence is inserted into the location of *CUT60*. The DNA sequences of the individual parts are shown in the lower panel. (**B**) Schematic representation of the locus between *MED2* and *ATP16* when the 5' sequence of *CUT60* is fused to the 3' sequence of *SUT129*, and this fusion sequence is inserted into the location of *CUT60*. The DNA sequences of the individual parts of this locus are shown in the lower panel. (**C**) Quantification of *ATP16* expression in northern blot shown in ***Figure 3A*** normalized to wild type. Data of expression in wild type, *cut60Δ::URA3*, *cut60Δ::SUT129 + sut129Δ::URA3*, *cut60Δ::5'C-3'S + sut129Δ::URA3* and *cut60Δ::5'S-3'C + sut129Δ::URA3* strains. Error bars are s.e.m. of 4 biological replicates (**D**) Northern blot analysis with a probe against the 5'-region of *SUT129* transcripts in wild type, *cut60Δ::URA3*, *cut60Δ::SUT129 + sut129Δ::URA3*, *cut60Δ::5'C-3'S + sut129Δ::URA3* and *cut60Δ::5'S-3'C + sut129Δ::URA3* strains. For all strains the transcripts were analyzed in three different backgrounds: wild type, *rrp6Δ* and *xrn1Δ* indicated on top (n = 1). (**E**) Serial dilution growth of W303 (wild type for *nrd1-1*), *nrd1-1*, *nrd1Δ151–214* and BY4741 (wild type for *nrd1Δ151–214*) strains on SC-Ethanol plates (n = 2). (**F**) Quantification of *ATP16* expression in northern blot shown in ***Figure 3C***. *ATP16* expression in W303 (wild type for *nrd1-1*) and *nrd1-1* are normalized to *W303*. *ATP16* expression in BY4741 and *nrd1Δ151–214* are normalized to BY4741 (wild type for *nrd1Δ151–214*). Error bars are s.e.m. of 3 biological replicates. (**G**) Schematic representation of the three steps needed to generate precise site-specific genomic replacements of *CUT60*. In step1 wild type yeast needs to be transformed with a PCR product containing the coding sequence of *URA3* with overhangs homologous to the sequences upstream and downstream of *CUT60*. These transformants need to be selected on plates lacking uracil (SC-URA). In step two these transformants need to be transformed with a PCR product containing the sequence of the non-coding sequence for desired *CUT60* replacements (*CUT95* is shown as example) with overhangs homologous to the sequences upstream and downstream of *CUT60*. In Step 3, transformants are selected on plates containing 5-FOA and additional uracil (SC + URA + 5 FOA) and confirmed by genotyping.

DOI: https://doi.org/10.7554/eLife.31989.008

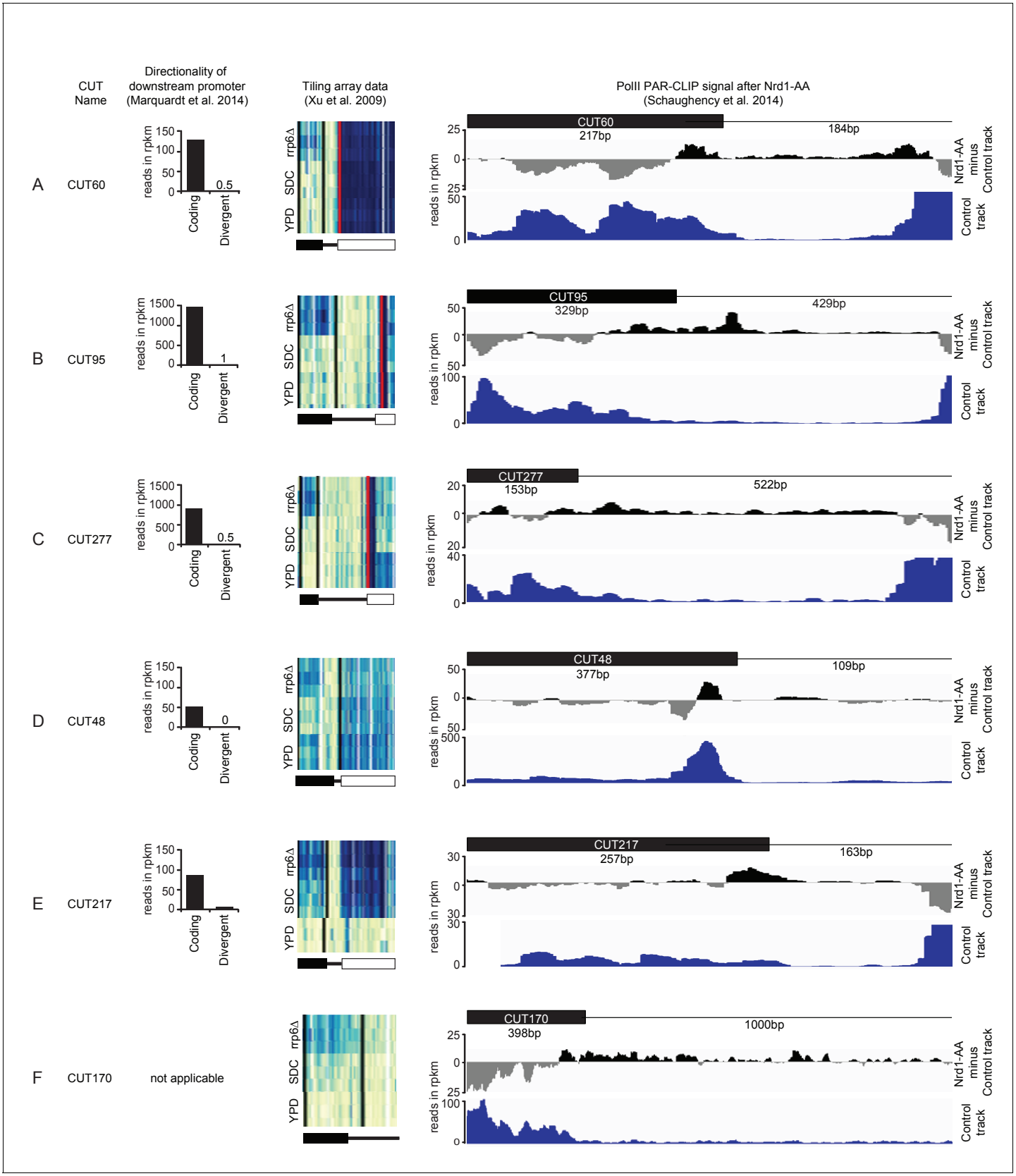

Figure 3—figure supplement 2. Genomic configuration of CUTs selected for *CUT60* replacement. (**A–F**) Visualization of three features of *CUT60* and CUTs selected for genomic replacement of *CUT60*. From left to right the name of the CUT, a plot showing the directionality of the promoter of the

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Figure 3—figure supplement 2 continued

downstream protein coding gene, a genome browser snapshot of the *rrp6Δ*, SDC and YPD track described in (***Xu et al., 2009***) and a genome browser snapshot visualizing differential Pol II PAR-CLIP signal after Nrd1 Anchor-away. The directionality of the downstream protein coding gene is calculated using NET-seq data from (***Marquardt et al., 2014***) and shown for the coding and the divergent direction of the promoter. Screenshots of the browser described in (***Xu et al., 2009***) show the level of transcription intensity across the CUTs and the respective downstream sequence by tiling array analysis. The transcription intensity is shown for three replicates in *rrp6Δ* background, for wild type yeasts grown in SDC medium and wild type yeast grown in YPD medium. Dark blue lines indicate high transcription, white lines indicated low transcription. The schematic underneath the three tracks indicate the starts of the CUTs (black box), the start and end of the downstream promoter (black line) and the start of the downstream gene (white box). The last screenshots visualizing the change of Pol II PAR-CLIP signal upon Nrd1 depletion consist of two tracks: (i) Upper: the difference track where the Pol II PAR-CLIP signal in the negative control (baseline) sample was subtracted from the Nrd1-anchor away (AA) sample. Genomic regions covered by the positive (black) difference values are interpreted as intervals where the transcription read-through is suppressed by Nrd1-dependent termination in the wild type, but becomes visible in Nrd1-AA samples; (ii) Lower: the original baseline (negative control) track to show the overall magnitude of nascent transcription at given region.

DOI: https://doi.org/10.7554/eLife.31989.009

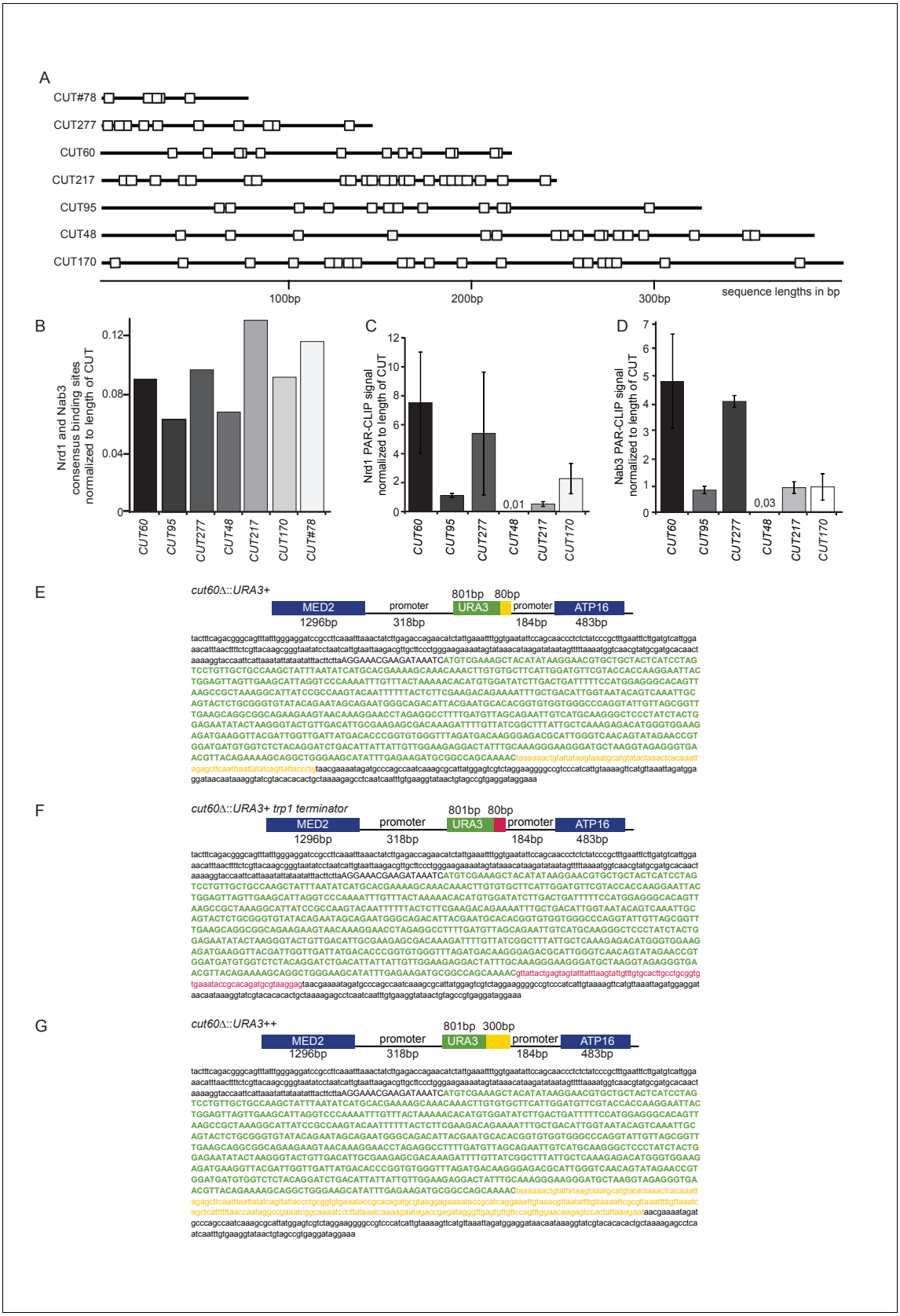

Figure 3—figure supplement 3. Features preventing Transcriptional Interference highlighted in *CUT60* and CUTs selected for genomic replacements. (**A**) Schematic representation of all CUTs inserted at the *CUT60* locus, their length and their Nrd1p/Nab3p binding sites. Binding sites from

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(***Jamonnak et al., 2011***; ***Carroll et al., 2004***; ***Wlotzka et al., 2011***; ***van Nues et al., 2017***; ***Delan-Forino et al., 2017***; ***Schulz et al., 2013***) are shown and indicated as white boxes. (**B**) Number of consensus Nrd1 and Nab3 binding sites normalized to the length of the individual CUTs are shown for *CUT60, CUT95, CUT277, CUT48, CUT217, CUT170* and CUT#78. (**C**) Nrd1 PAR-CLIP signal (***Schulz et al., 2013***) normalized to length of the CUTs is shown for *CUT60, CUT95, CUT277, CUT48, CUT217 and CUT170* (n = 2). (**D**) Nab3 PAR-CLIP signal (***Schulz et al., 2013***) normalized to length of the CUTs is shown for *CUT60, CUT95, CUT277, CUT48, CUT217 and CUT170* (n = 2). (**E**) Schematic representation of the locus between *MED2* and *ATP16* when the *URA3* including an 80 bp endogenous *URA3* terminator as present in pRS316 (indicated in yellow) is inserted into the location of *CUT60*. The DNA sequences of the individual parts of this locus are shown in the lower panel. (**F**) Schematic representation of the locus between *MED2* and *ATP16* when the *URA3* including an 80 bp terminator derived from the *TRP1* locus (indicated in red) is inserted into the location of *CUT60*. The terminator in this sequence is homologous to the *TRP1* terminator found in pRS314. The DNA sequences of the individual parts of this locus are shown in the lower panel. (**G**) Schematic representation of the locus between *MED2* and *ATP16* when the *URA3* including a 300 bp (indicated in yellow) terminator as present in pRS316 is inserted into the location of *CUT60*. The DNA sequences of the individual parts of this locus are shown in the lower panel.
DOI: https://doi.org/10.7554/eLife.31989.010

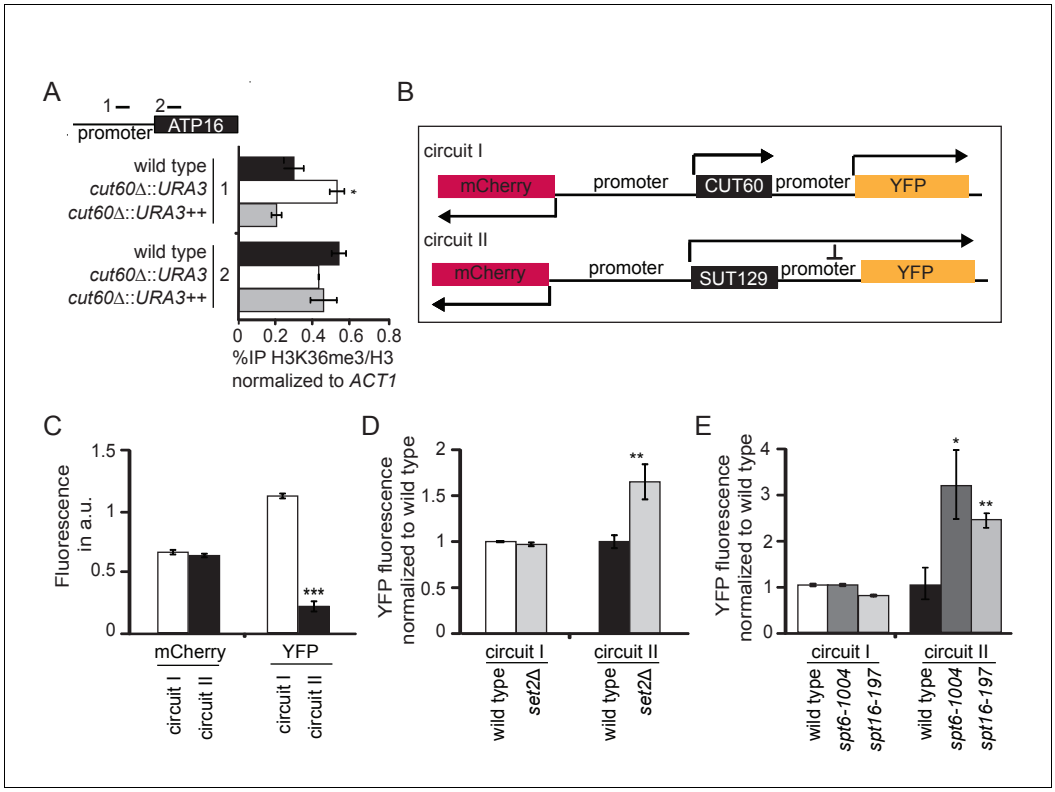

Figure 4. *CUT60* prevents *ATP16* repression by Transcriptional Interference. (**A**) ChIP-qPCR experiments for histone H3K36 trimethylation (H3K36me3) relative to the histone H3 levels at the promoter and gene body of *ATP16* in wild type (black), *cut60Δ::URA3* (white) and *cut60Δ::URA3++* (grey) strains. Error bars are s.e.m. of three biological replicates. * indicates p values of p<0.05 comparing %IP of H3K36/H3 in *cut60Δ::URA3* to the %IP of H3K36me3/H3 in wild type. Position of ChIP-qPCR probes indicated in top panel. (**B**) Schematic representation of synthetic circuits. *mCherry* replaces *MED2*, *YFP* replaces *ATP16*. Circuit I represents wild type circuit containing *CUT60*, circuit II represents the *cut60Δ::SUT129* mutant circuit. (**C**) Relative fluorescence of mCherry and YFP in circuit I (white) and circuit II (black). Error bars are s.e.m. of nine biological replicates, the mean was derived from multiple thousand individual measurements. *** indicates p values of p<0.0001. (**D**) Relative YFP fluorescence of circuit I in wild type (white) and *set2Δ* (light grey) background, and of circuit II in wild type (black) and *set2Δ* (light grey) background. YFP fluorescence levels are normalized to the corresponding wild-type values. Error bars are s.e.m. of six biological replicates. ** indicates p values p < 0.01 comparing YFP fluorescence in *set2Δ* to the respective wild type. (**E**) Relative YFP fluorescence in circuit I in wild type (white), *spt6*-1004 (dark grey) and *spt16*-197 (light grey) background, and in circuit II in wild type (black), *spt6*-1004 (dark grey) and *spt16*-197 (light grey) background. YFP fluorescence is normalized to the corresponding wild-type values. Error bars are s.e.m. of six biological replicates. * and ** indicate p values of p<0.05 and p<0.01 respectively comparing YFP fluorescence in *spt6*-1004 and *spt16*-194 to the respective wild type.

DOI: https://doi.org/10.7554/eLife.31989.012

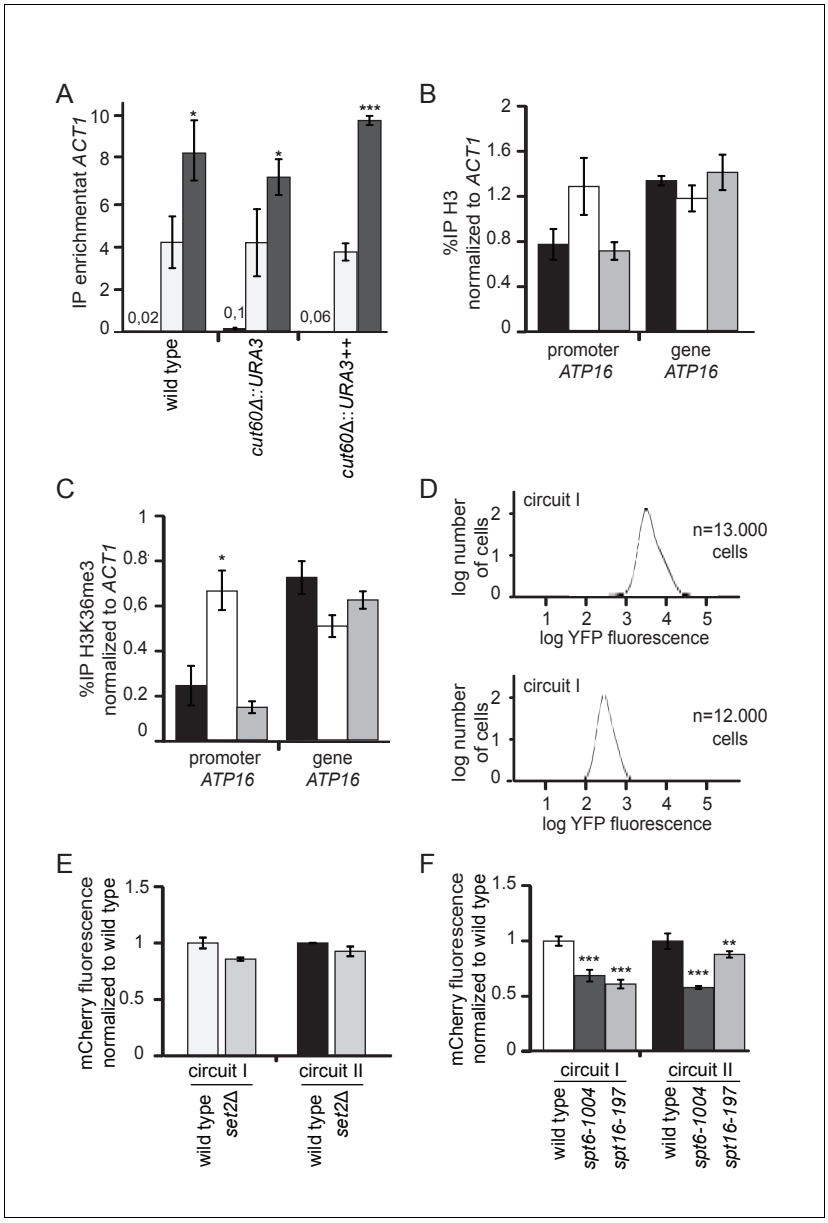

Figure 4—figure supplement 1. Set2p, Spt6p and Spt16p participate in Transcriptional Interference of *ATP16*. (**A**) Enrichment of DNA corresponding to the Actin control gene for H3 (white) and H3K36me3 (grey) antibodies relative to no antibody control (black, values indicated) in wild type, *cut60Δ::URA3* and *cut60Δ::URA3 ++* strains. Error bars are s.e.m. of 3 biological replicates. *and *** indicate p values of $p<0.05$ and $p<0.001$ comparing H3K36me3 to no antibody control. (**B**) ChIP-qPCR experiments for histone H3 at the promoter and gene body of *ATP16* in wild type (black), *cut60Δ::URA3* (white), *cut60Δ::URA3++* (grey) strains. Enrichment is normalized to *ACT1*. Error bars are s.e.m. of three biological replicates. (**C**) ChIP-qPCR for H3K36me3 levels at the promoter and gene body of *ATP16* in wild type (black), *cut60Δ::URA3* (white), *cut60Δ::URA3++* (grey) strains. Enrichment is normalized to *ACT1* locus. Error bars are s.e.m. of three biological replicates. * indicates p values of $p<0.05$ comparing H3K36me3 at the promoter of *ATP16* in *cut60Δ::URA3* to wild type. (**D**) Histograms showing the frequency of YFP fluorescence values of individual cells of cell populations containing circuit I (top panel, n = 13.000 cells) or circuit II (bottom panel, n = 12.000 cells). The distributions suggest variation around one distinct fluorescence state rather than a bi-modal distribution. (**E**) Relative mCherry fluorescence of circuit I in wild type (white) and *set2Δ* (light grey) background, and of circuit II in wild type (black) and *set2Δ* (light grey) background. mCherry fluorescence levels are normalized to the circuit I or circuit II wild type data. Error bars are s.e.m. of six biological replicates. (**F**) Relative mCherry fluorescence of circuit I in wild type (white), *spt6*-1004 (dark grey) and *spt16*-197 (light grey), and of circuit II in wild type (black), *spt6*-1004 (dark grey) and *spt16*-197 (light

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grey). mCherry fluorescence levels are normalized to the circuit I or circuit II wild type data. Error bars are s.e.m. of six biological replicates. ** and *** indicate p values of $p<0.01$ and respectively $p<0.001$ comparing mCherry fluorescence in *spt6*-1004 and *spt16*-194 to the respective wild types.

DOI: https://doi.org/10.7554/eLife.31989.013

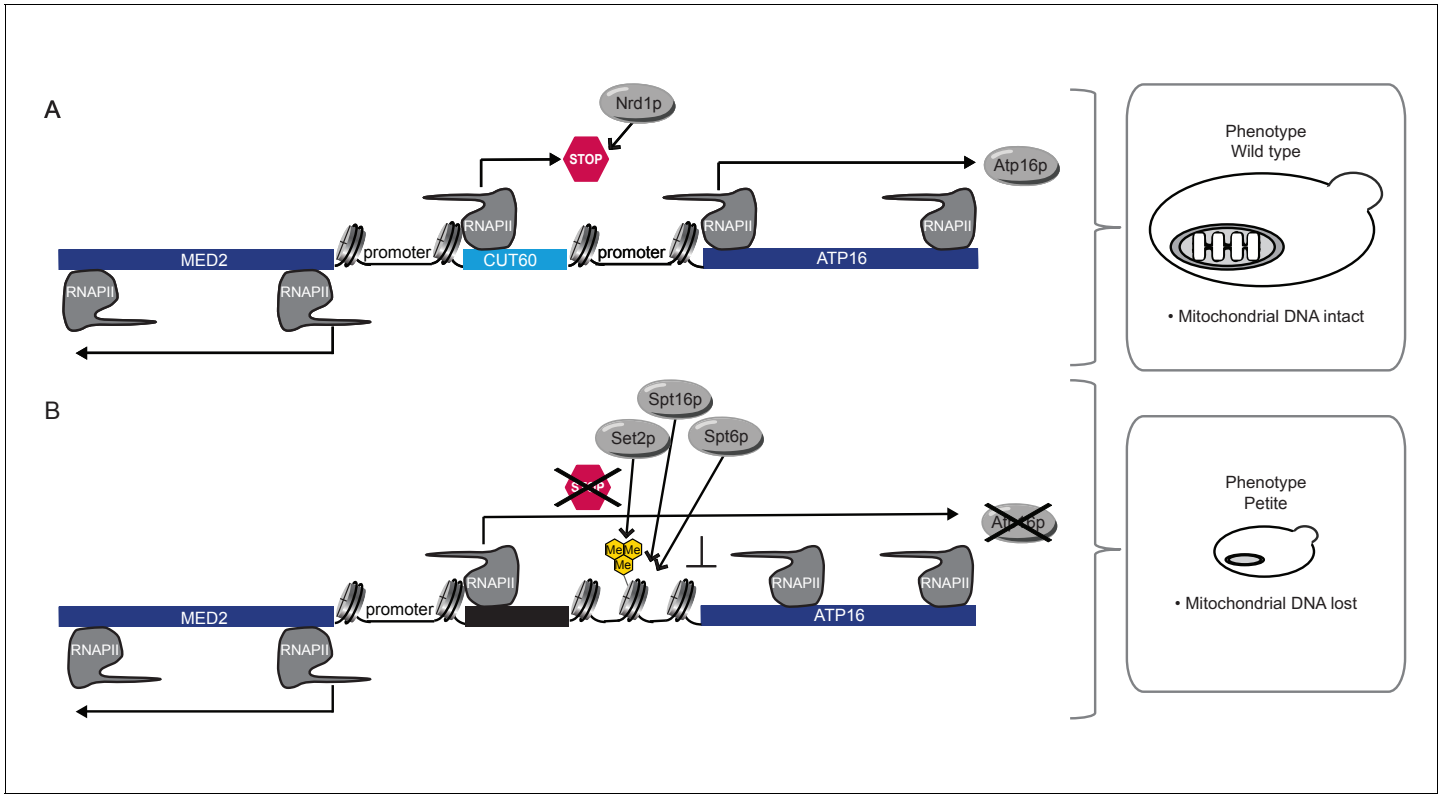

Figure 5. Model for *CUT60* function as insulator from Transcriptional Interference. (**A**) In the presence of *CUT60* transcription initiating from the bidirectional *MED2* promoter terminates efficiently with the help of Nrd1p to prevent Transcriptional Interference of *ATP16*. Atp16p promotes the maintenance of mitochondrial DNA. (**B**) *ATP16* is repressed by Transcriptional Interference if *CUT60* is replaced with sequences terminating inefficiently. Interfering transcripts initiate from the bidirectional *MED2* promoter. The interference mechanism is enforced by Set2p, Spt6p and Spt16p. This leads to the loss of mitochondrial DNA triggered by the lack of Atp16p.

DOI: https://doi.org/10.7554/eLife.31989.014