Supplementary Information

Single-cell, single-mRNA analysis of Ccnb1 promoter regulation

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Supplementary Results

Expression of the Ccnb1::Luc-MS2 transgene mimics the native Ccnb1 gene in synchronized cells

To reproduce and validate our findings on Ccnb1 promoter regulation during the cell cycle, we performed cell cycle synchronization experiments to examine cells that were blocked at G1 and progressed into G2 (6 hours after release) and into the next G1 phase (10 hours after release) (Supplementary Fig. S7). We then examined Ccnb1::Luc-MS2 transgene and native Ccnb1 expression by single molecule RNA FISH (Supplementary Fig. S7). As expected, G2 cells had higher mRNA counts for both the Ccnb1::Luc-MS2 transgene and the native Ccnb1 gene than G1 cells (Supplementary Fig. S7). We note that mRNA counts in synchronized G2/M or G1 cells were lower than mRNA counts in G2/M-arrested or G1-arrested cells (Fig. 4b, e), likely due to the prolonged cell cycle arrest induced by nocodazole/mimosine that may increase cell volumes resulting in higher mRNA counts.1,2 Importantly, we observed reduced transcription site (TS) brightness at both the Ccnb1::Luc-MS2 transgene and the native Ccnb1 gene in G1 than in G2/M (Supplementary Fig. S7). Under the same assumptions used in this study, we estimated that the average Pol II density at the native Ccnb1 gene was 1.42 kb⁻¹ and 1.74 kb⁻¹ in G1 and G2/M-synchronized cells, respectively and that the average Pol II firing rate at the native Ccnb1 gene was 1.57 ± 0.22 min⁻¹ and 1.93 ± 0.28 min⁻¹ in G1 and G2/M-synchronized cells, respectively. We observed that a higher fraction of cells expressed both the Ccnb1::Luc-MS2 transgene and the native Ccnb1 gene in G2/M than in G1 (Supplementary Fig. S7). Therefore, we confirmed that expression of the Ccnb1 promoter transgene mimics the native Ccnb1 gene during the cell cycle. Additionally, about 40% of G2/M-synchronized cells had two
active *Ccnb1* alleles while only ~20% of G1-synchronized cells contained two active *Ccnb1* alleles (Supplementary Fig. S7).

**Distinct subcellular localizations of *Luc-MS2* mRNA correlate with *Ccnb1* promoter activities among G1 or G2/M cells.**

One of the explanations for the observed cell subpopulations with distinct *Luc-MS2* mRNA localizations among asynchronous cells (Fig. 5a) is that the subpopulation of cells with nuclear-enriched *Luc-MS2* mRNA are in G2/M phase when the *Ccnb1* promoter has a higher activity. To test this, we first examined FISH data of cells arrested at G1 and G2/M by mimosine and nocodazole treatment. We identified that 36% cells and 47% cells exhibited nuclear-enriched *Luc-MS2* mRNA after mimosine treatment (G1) and nocodazole treatment (G2/M), respectively (Supplementary Fig. S10). As found in asynchronous cells (Fig. 5), cells with nuclear-enriched *Luc-MS2* mRNA display higher mRNA counts and higher TS brightness than cells with uniformly-localized *Luc-MS2* mRNA in mimosine and nocodazole-treated cells (Supplementary Fig. S10), confirming that higher *Ccnb1* promoter activity correlates with mRNA nuclear retention among cells blocked at G1 or G2/M and suggesting that variable *Ccnb1* promoter activation states observed at individual cell level are cell cycle-independent.

Furthermore, we examined FISH images of cells synchronized in G2 and the next G1 phase. We confirmed the existence of two cell populations that differed in *Ccnb1* promoter activity and *Luc-MS2* mRNA localization among synchronized G1 or G2/M cells (Supplementary Fig. S11). We identified that 39% cells and 69% cells exhibited nuclear-enriched transgene RNA in synchronized G1 or G2/M cells, respectively (Supplementary Fig. S11). Cells with nuclear-enriched *Luc-MS2* mRNA generally had higher levels of mRNA counts
and TS brightness than cells with uniformly-localized Luc-MS2 mRNA (Supplementary Fig. S11). Taken together, our study identified cell-to-cell variations in \textit{Ccnb1} promoter activities and \textit{Luc-MS2} mRNA localizations among asynchronous cells, G1- or G2/M-arrested cells and among cells synchronized at G1 or G2/M. Therefore, variable activation states of the \textit{Ccnb1} promoter likely exist among individual cells during each cell cycle stage.

\textbf{Differential changes in \textit{Luciferase} and \textit{Ccnb1} expression detected by qPCR and by FISH during the cell cycle}

We noticed some differences in \textit{Ccnb1::Luc-MS2} transgene expression and native \textit{Ccnb1} gene expression detected by FISH or by RT-qPCR. Compared to asynchronous cells, \textit{Luciferase} mRNA levels decreased more than 2-fold in G1 and increased by \textasciitilde50\% in G2 as detected by RT-qPCR, resulting in an approximately \textasciitilde4-fold increase of \textit{Luciferase} mRNA in G2 than in G1 (Supplementary Fig. S5). In contrast, we observed by FISH that the median \textit{Luc-MS2} mRNA counts were 302, 335 and 426 in asynchronous, G1 and G2 cells, respectively (Figs. 2c, 4b). Thus, \textit{Luc-MS2} mRNA counts only increased by 30-50\% in G2 cells compared to asynchronous cells or G1 cells. Likewise, native \textit{Ccnb1} mRNA levels decreased by \textasciitilde25\% in G1 and increased by \textasciitilde2-fold in G2 (compared to asynchronous cells), resulting in an \textasciitilde2.5-fold increase of \textit{Ccnb1} mRNA in G2 than in G1 (Supplementary Fig. S5). In contrast, FISH detected about \textasciitilde50\% higher native \textit{Ccnb1} mRNA counts in G2 cells than asynchronous cells or G1 cells (Figs. 3c, 4e, median mRNA counts = 391, 406 and 593 in asynchronous, G1 and G2, respectively). Therefore, both RT-qPCR and FISH observed increased \textit{Luciferase} and \textit{Ccnb1} mRNA levels in G2 cells than in asynchronous cells or G1 cells. However, the two methods differed in that decreased \textit{Luciferase} and \textit{Ccnb1} mRNA levels in G1-arrested cells than in asynchronous cells were observed by RT-
qPCR (Supplementary Fig. S5) but were not detected by FISH (Figs. 2c, 3c, 4b, 4e). Because approximately two-thirds of asynchronous cells were in G1 (Supplementary Figs. S5, S12), a 2-3 fold decrease of *Luciferase* mRNA in G1 cells than asynchronous cells would require a 4-8 fold higher *Luciferase* mRNA in S/G2 cells than in G1 cells among the asynchronous population. Although MS2 RNA FISH demonstrated excellent dynamic range in detecting *Luc-MS2* transgene expression (Figs. 2a, d), we did not observe such a 4-8 fold higher *Luc-MS2* mRNA count in G2 than in G1 either in arrested cells (Fig. 4b) or synchronized cells (Supplementary Fig. S7), nor could we distinguish two cell populations differing in mean *Luc-MS2* mRNA counts among asynchronous cells (Fig. 2c). Because RT-qPCR can be subject to artifacts during cell lysis and RNA isolation while FISH quantitation is sensitive to enlarged cell volumes \(^1\), \(^2\), we note here that one should be cautious to interpret conflicting FISH and RT-qPCR data on gene expression changes.
Supplementary Methods

Splinkerette PCR

Splinkerette PCR was carried out as described. Briefly, genomic DNA was extracted and digested with BstYI at 60 °C. The purified digested genomic DNA was ligated to annealed Splinkerette oligonucleotide (Supplementary Table S5) and two rounds of PCR were carried out with Phusion Taq polymerase (NEB) and PCR primers (Supplementary Table S5). The final PCR products were resolved in agarose gel. The PCR products were then treated with Antarctic phosphatase (NEB) and Exonuclease I (NEB) and subjected to sequencing.

Luciferase reporter activity assays

C2C12 cells were plated in a 24-well plate at a density of $4 \times 10^4$ cells/well and transfected 24 hours later with 180 ng of pGL3 empty vector (Promega) or pGL3 vectors containing four Ccnb1 promoter deletion mutants (Supplementary Fig. S2). Renilla vector pRL (Promega) (20 ng) was co-transfected to normalize for the transfection efficiency. Luciferase activity was measured 48 hours after transfection using the Dual Luciferase Reporter Assay System according to the manufacturer’s instructions (Promega).

RNA extraction and real-time PCR analysis

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Four hundred nanograms of total RNA was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) in conjunction with oligo(dT) primers (Invitrogen). The resulting cDNA samples were subjected to real-time PCR using gene-specific primers and iTaq Universal SYBR® Green Supermix (Bio-
Rad). Real-time PCR was performed in a CFX-96 Touch Real-Time PCR Detection System (Bio-Rad) and the data were analyzed using the CFX Manager software (Bio-Rad).

Measurement of mRNA decay

mRNA half-lives were calculated by measuring mRNA levels at distinct time points after adding the transcription inhibitor 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) (Sigma) at a final concentration of 30 µg/ml into the culture media. Total RNA was extracted at the time points 0, 0.5 hour, 1 hour, 2 hours and 4 hours after adding DRB. RT-qPCR was carried out using 18S rRNA to normalize the expression. mRNA decay rates were determined as previously described 4. Briefly, \( \ln(2^{-\left(\text{Ct}_{\text{mRNA}} - \text{Ct}_{18S}\right)} \) at distinct time points after DRB treatment were plotted on the y-axis and the corresponding time points were plotted on the x-axis. \( k_{\text{decay}} \) was determined by linear regression, and mRNA half-life \( T_{1/2} \) was calculated as

\[
T_{1/2} = \frac{\text{Ln}2}{k_{\text{decay}}}
\]

Three independent experiments were carried out and the average \( T_{1/2} \) was calculated.

Propidium iodide (PI) staining and cell cycle analysis by flow cytometry

Cells were trypsinized and centrifuged at 1,200 rpm for 5 min and were washed with cold 1X PBS three times. The cell pellet was re-suspended in 400 µl 1X PBS and 1 ml cold 100% ethanol was added slowly while vortexing. The cell suspension was incubated overnight at 4°C. For PI staining, cells were pelleted, washed three times with 1X PBS and re-suspended in 1X PBS. Cells were then incubated with RNase A (final concentration 100 µg/ml) and PI (final concentration 50 µg/ml) for 30 min at room temperature (RT). The stained cells were immediately analyzed on a Stratedigm flow cytometer (FACS facility, Yale School of Medicine).
for relative DNA content. Collected data was analyzed using the FlowJo software (FlowJo, LLC).

**Cell cycle synchronization**

Cells were synchronized at G1 and G2/M phases according to Heintz et al. Briefly, cells were plated at around 40-60% confluency in growth media containing 2 mM thymidine and incubated for 12 hours. Cells were then released from the block by three washes in fresh media minus serum, trypsinized and suspended in growth media containing 24 µM thymidine and deoxycytidine. Cells were then plated in Nunc Lab-Tek CC2 two-well chamber slides (ThermoFisher Scientific) for single molecule RNA FISH and on 10 cm plates for flow cytometry analysis. 9 hours after plating cells, the media was replaced with media containing 5 µg/ml aphidicolin for an additional 12 hours. Cells were then released from the aphidicolin block by washing with fresh media minus serum and then grown in complete media. Cells synchronized at G2/M or G1 were analyzed by FISH or flow cytometry at 6 hours or 10 hours post-release, respectively.

**Combined RNA FISH and DNA FISH**

RNA FISH probes against MS2 were generated by nick translation of MS2 repeats purified from AluI digestion of the pGL3-MS2 vector. Nick translation was carried out using BioProbe Nick Translation DNA labeling system (Enzo Life Sciences) in presence of Cy3-dUTP labelling Mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.25 mM dTTP, 0.25 mM Cy3-dUTP (GE Healthcare), pH 7.5). The probes were then purified using MinElute PCR Purification Kit (Qiagen). DNA FISH probe against the transgene insertion site was generated by Atto488 NT
labeling Kit (Jena Bioscience) using bacteria artificial chromosome (BAC) clone RP23-370K1. DNA FISH was carried out in conjunction with RNA FISH according to the published protocol with slight modifications. Briefly, cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed once with 1X PBS, permeabilized with 0.5% Triton X-100 in 1X PBS for 10 min, followed by washing with PBST (1X PBS (DEPC treated) + 0.05% Tween-20) for three times and 2X SSC for 5 min. Cells were then denatured in 50% formamide and 2X SSC for 30 min at 80°C. Next, cells were incubated with 25 µl hybridization buffer containing 50% formamide, 2X SSC, 50 mM phosphate buffer, 10% dextran sulfate, 0.7 mg/ml sonicated salmon sperm DNA, 0.1 mg/ml mouse Cot1 DNA, 0.4 U/µl RNaseOUT and 200 ng labeled DNA FISH and RNA FISH probes (100 ng each). The hybridization mix was denatured at 80°C for 3 min. Slides were sealed with rubber cement, denatured at 80°C heat block for 3 min and incubated overnight at 37°C. Cells were then washed with 2X SSC + 50% formamide for 1 hour at 37°C, washed with 2X SSC at room temperature (RT) and then with PBST. Samples were mounted with Vectashield (Vector Laboratories) and imaged in an Olympus IX-81 inverted wide-field microscope. A 488nm laser was used to image DNA FISH signals. Green fluorescence was collected by a 525/50 Brightline bandpass filter and imaged in z-stacks (z-distance = 0.2 µm). RNA FISH signals were imaged in the red channel as described in the main text.

**Combined single molecule RNA FISH and immunofluorescence staining**

Single molecule RNA FISH was performed as described in the main text. To continue with immunofluorescence staining, cells were fixed again with 4% paraformaldehyde for 15 min. Cells were washed once with 1X PBS and then permeabilized with 1X PBS + 0.25% Triton X-
100 for 10 min. Cells were washed three times with PBST (1X PBS + 0.05% Tween-20) for 5 min each and blocked with 2% Ultrapure BSA (Ambion) in 1X PBS in presence of 0.4 U/µl RNaseOUT (Invitrogen) for 30 min at RT. Cells were then incubated with 1:500 goat-anti-Lamin B antibody (Santa Cruz Biotechnology, sc-6217) in 2% BSA in 1X PBS and 0.4 U/µl RNaseOUT for 1 hour at RT. Cells were washed three times in DEPC-treated 1X PBS before incubating with anti-goat Alexa Flour 488 conjugate (A-11055, ThermoFisher Scientific) at 1:1000 dilution for 1 hour at RT. Cells were then mounted in VectaShield (Vector Labs) and imaged in an Olympus IX-81 wide-field microscope (immunofluorescence in the green channel and RNA FISH in the red channel) as described above.

**Western blotting**

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) supplemented with 1X protease inhibitor mixture (Roche). Lysates were clarified by centrifugation for 20 min at 14,000 rpm and supernatants were collected. Protein concentration in the soluble fraction was determined by BCA protein assay (ThermoFisher Scientific). Proteins were separated by electrophoresis through 10% Tris-glycine gels (Bio-Rad) and transferred to nitrocellulose membrane. Primary antibodies used for western blot analysis were mouse monoclonal anti-NF-YA (Santa Cruz Biotechnology, sc-10779) at 1:500 dilution and rabbit polyclonal anti-α Tubulin (Abcam, ab18251) at 1:1000 dilution. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution). The blot was then developed using SuperSignal West Femto kit (ThermoFisher Scientific) and images were acquired with a ChemiDoc XRS+ Imaging (BioRad) according to the manufacturer's instructions.
Chromatin Immunoprecipitation

Cells were cultured to ~70-80% confluency in five 150 mm plates. Cells were fixed with 1% formaldehyde in serum-free media for 10 min at RT on a shaker. Formaldehyde crosslinking was quenched with a final concentration of 125 mM glycine. Cells on each plate were then washed in cold 1X PBS and scraped in 5 ml of 1X PBS. Cells were then pelleted by spinning at ~5,000 rpm for 5 min and lysed with 4 ml cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40). After incubating on ice for 5 min, the nuclei were washed with cell lysis buffer and spun down again. The nuclei were then lysed by nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) and incubated on ice for 10 min. The nuclear extract was transferred into the TPX plastic microtubes (Diagenode) for sonication. The nuclear lysate was then sonicated in a Bioruptor sonicator (Diagenode) at high settings for 20-minute sonication cycle, 30sec ON/30sec OFF at 4 ºC to yield DNA fragments with an average length of ~500 bp. The sonicated lysate was centrifuged at 14,000 rpm for 10 min at 4 ºC and the supernatant was collected as chromatin extract. The chromatin extract (15-25µg) was diluted 10-fold and precleared with Protein A/G Dynabeads (ThermoFisher Scientific) for 15 min at 4 ºC. Antibodies including anti-H3K4me2 (Abcam, ab32356, 6 µl), anti-H3K4me3 (Abcam, ab8580, 2 µl), anti-H3K79me2 (Abcam, ab3594, 2 µl) and anti-H3 (Abcam, ab1791, 2 µl) were added separately to the precleared chromatin extract and incubated overnight. Rabbit IgG antibody (2 µl) was added to a separate sample as the negative control. Fresh Protein A/G Dynabeads were then added for 3 hours at 4 ºC to immunoprecipitate chromatin and beads were sequentially washed in low salt, high salt, LiCl and TE buffer. DNA/protein complexes were eluted and treated with RNase A and crosslinking was reversed by heating overnight at 65ºC. Samples were subsequently treated with Proteinase K for 2 hours. DNA was extracted using Qiaquick PCR purification kit (Qiagen)
and eluted using 100 µl water. Each qPCR reaction was performed in duplicate in 20 µl reaction volumes containing 2 µl of ChIP-enriched or input DNA, iTaq™ Universal SYBR Green Supermix (Bio-Rad), and region-specific primer sets. Target amplification and detection were performed in a CFX-96 Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in Supplementary Table S4.
Supplementary References

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Suppl. Figure S1

a

![Image of gel electrophoresis with DNA sequence markers and bands labeled as 1D1 1D2 2B5 2B6 - 4C4 4C5 5A2 5A3 5A4 5A5. The gel shows the concatenated plasmid DNA sequence and the plasmid DNA sequence + insertion site.]

b

![Image of chromosome 19 (chr19) bands with scale bars indicating 5 kb. The bands are marked with letters from A to E, and the scale ranges from 53,324,000 to 53,332,000. UCSC Genes (RefSeq, GenBank, tRNAs & Comparative Genomics) are highlighted, with chr19: 53,326,337-53,326,438 indicated.]

c

![Image showing DNA FISH, MS2 RNA FISH, and Merge. The DNA FISH and MS2 RNA FISH images show fluorescent signals, and the Merge image combines both signals. The Chr19 BAC Probe is also shown.]
Supplementary Figure S1. Generating a stable Flp-In C2C12 cell line for site-specific integration of single-copy promoter transgenes. (a) An agarose gel image of splinkerette PCR products. The upper arrow indicates PCR products of identical sizes from several cell lines that contain concatenated pFRT/LacZeo plasmid DNA sequence. The lower arrow in the Flp-In cell line 5A5 indicates PCR product containing pFRT/LacZeo plasmid DNA sequence linked to the insertion site DNA sequence at the Scal restriction site. PCR products were sequenced to identify the insertion sites. (b) UCSC Genome Browser view of the insertion site of the pFRT/LacZeo vector in C2C12 Flp-in cell line 5A5. The insertion site (marked in vertical red lines) was found to be located within the first intron of a non-coding RNA gene (1700001K23Rik) on mouse chromosome 19. (c) Representative RNA-DNA FISH images of cells containing the Ccnb1::Luc-MS2 transgene (clone 96). RNA FISH probes target MS2 RNA and DNA FISH probes target the genomic DNA sequence at the integration site at chromosome 19. Merged images are shown on the right. Yellow arrowheads indicate the transgene loci and the cyan arrowhead indicates the other allele without the integrated transgene. Scale bars: 5 µm.
Suppl. Figure S2

**a**

Luciferase/Renilla activity

|   | pGL3 basic | Ccnb1::Luc-MS2-500 bp | Ccnb1::Luc-MS2-1000 bp | Ccnb1::Luc-MS2-1500 bp | Ccnb1::Luc-MS2-2000 bp |
|---|-------------|-----------------------|------------------------|------------------------|------------------------|
|   | 0.5         | 0.8                   | 0.7                    | 0.6                    | 0.5                    |

**b**

PCR1, PCR2, PCR3

Ccnb1::Luc-MS2 Clone 96

No Promoter::Luc-MS2 Clone 5

C2C12

**c**

Ccnb1::Luc-MS2-96

No Promoter::Luc-MS2-5

C2C12

Luciferase expression normalized to Gapdh

**d**

Cell clones with transgenes at the same locus

**e**

pFRT/LacZeo (5A5) C2C12 Ccnb1::Luc-MS2-96 No Promoter::Luc-MS2-5
Supplementary Figure S2. Characterizing C2C12 cell clones with integrated single-copy transgenes driven by the Ccnb1 promoter or without a promoter. (a) Dual-Glo luciferase reporter assay to measure activities of various mouse Ccnb1 promoter fragments. Mouse Ccnb1 promoter fragments (+1 to -454, -1014, -2210 and -2784) were cloned in the pGL3-Basic vector. pRenilla vector was co-transfected to normalize for transfection efficiency. Error bars represent standard deviations (n = 2). (b) Agarose gel images of genomic DNA PCR to verify transgene integration in these two cell clones: Ccnb1::Luc-MS2 (clone 96) and No Promoter::Luc-MS2 (clone 5). Primer locations are shown in Figure 1. Genomic DNA from wildtype C2C12 cells served as negative controls. (c) Agarose gel images of genomic DNA PCR to detect the presence of MS2 repeats and the Ccnb1 promoter in Ccnb1::Luc-MS2 (clone 96) cells and No Promoter::Luc-MS2 (clone 5) cells. (d) Luciferase mRNA expression in cells containing Ccnb1::Luc-MS2 (clone 96) and No Promoter::Luc-MS2 (clone 5 and clone 68) detected by RT-qPCR. Wildtype C2C12 cells served as a negative control. (e) β-Gal staining images of the following cell clones: pFRT/lacZeo Flp-In cell line (clone 5A5), wild-type C2C12, Ccnb1::Luc-MS2 (clone 96) and No Promoter::Luc-MS2 (clone 5).
Suppl. Figure S3

(a) Ccnb1::Luc-MS2-134

(b) mRNA counts

$M_{MS2} = 292$

$N = 64$

(c) Transcription site brightness

$T_{MS2} = 4.8$

$N = 50$
Supplementary Figure S3. Quantifying mRNA counts and transcription site brightness from an additional cell clone containing the *Ccnb1::Luc-MS2* transgene (clone 134). (a) Representative single molecule RNA FISH images of *Ccnb1::Luc-MS2* (clone 134) using MS2 FISH probes (120X magnification). The white arrow indicates the transcription site. Scale bar: 5 µm. (b) Histograms of mRNA counts of the *Ccnb1::Luc-MS2* transgene. M_{MS2} indicates the median mRNA count of the *Ccnb1::Luc-MS2* transgene measured by MS2 FISH. N indicates the measured cell number. (c) Histograms of transcription site (TS) brightness of the *Ccnb1::Luc-MS2* transgene. T_{MS2} indicates the median TS brightness of the *Ccnb1::Luc-MS2* transgene measured by MS2 FISH. N indicates the measured TS number. FISH images from one experiment were analysed.
Suppl. Figure S4

- **Luciferase mRNA t\textsubscript{1/2} = 2.78 h**
  - $y = -0.2489x - 6.1069$
  - $R^2 = 0.9486$

- **Ccnb1 mRNA t\textsubscript{1/2} = 3.85 h**
  - $y = -0.1797x - 4.5976$
  - $R^2 = 0.9335$

- **c-Myc mRNA t\textsubscript{1/2} = 1.37 h**
  - $y = -0.507x - 4.911$
  - $R^2 = 0.7427$

- **Table**

| mRNA t\textsubscript{1/2} | Mean     |
|---------------------------|----------|
| *Luciferase*              | 2.49 ± 0.29 h |
| *Ccnb1*                   | 3.83 ± 0.04 h |
| *c-Myc*                   | 1.01 ± 0.32 h |
Supplementary Figure S4. Measuring decay kinetics of Luciferase, Ccnb1 and c-Myc mRNA in the Ccnb1::Luc-MS2 cell clone. (a–c) Representative mRNA decay curves of Luciferase, native Ccnb1 and c-Myc in the Ccnb1::Luc-MS2 (clone96) cells after inhibiting transcription with 30 μg/ml DRB. 18S rRNA was used to normalize the expression. In the mRNA decay curves, $\ln\left(2^{-\left(Ct_{\text{mRNA}}-Ct_{18S}\right)}\right)$ values were plotted in the y-axis and the corresponding time points were plotted in the x-axis. mRNA half-lives were calculated as described in Supplementary Methods. (d) Table showing calculated average half-lives ($t_{1/2}$) of Luciferase, Ccnb1 and c-Myc mRNA ($n= 3, 2$ and $3$, respectively). Errors represent standard deviations.
Suppl. Figure S5

(a) Asynchronous and synchronized cells under different conditions:
- 40 ng/ml Nocodazole
- 2 mM Mimosine
- Propidium Iodide Intensity

(b) Expression of Ccnb1 and Luciferase mRNA normalized to Gapdh:
- Ccnb1 mRNA
- Luciferase mRNA

(c) Comparison of expression levels under different conditions:
- Asynchronous
- 2 mM Mimosine
- 40 ng/ml Nocodazole

(d) Percentage of cells with FISH signals:
- No promoter::Luc-MS2-5
- Mimosine: 57% (N=419)
- Nocodazole: 51% (N=320)

(e) Normalized frequency of mRNA counts:
- No promoter::Luc-MS2-5
- M1: mimosine (n=38)
- M2: Nocodazole (n=48)
- M1: 52
- M2: 38

P = 0.1015
P = 0.73
Supplementary Figure S5. Effects of cell cycle arrest on Ccnb1::Luc-MS2 transgene expression and No promoter::Luc-MS2 transgene expression. (a) Cell cycle profiles of asynchronous cells, mimosine-treated cells (G1 arrest) and nocodazole-treated cells (G2/M arrest) by flow cytometry. (b) Measuring native Ccnb1 mRNA and Luc-MS2 mRNA levels in asynchronous cells and cells arrested at G1 or G2/M by RT-qPCR. mRNA expression was normalized to Gapdh mRNA levels. Results are averages from three independent repeats. Error bars are standard deviations. (c) Measuring native Ccnb1 gene and Ccnb1::Luc-MS2 transgene expression upon induction of myogenic differentiation by RT-qPCR. mRNA expression was normalized to Gapdh mRNA levels. Error bars are standard deviations (n = 2). (d) Fractions of the No Promoter::Luc-MS2 transgene cells displaying MS2 FISH signals after being arrested by mimosine (G1) or nocodazole (G2/M). P-values were determined by Fisher’s exact test. (e) Histograms and medians of mRNA counts of cells containing the No Promoter::Luc-MS2 transgene after being arrested by mimosine (G1) or nocodazole (G2/M). mRNA counts were not significantly different between G1-arrested cells and G2/M-arrested cells as determined by students’ t-test. 40X (multiple cell) and 120X (single cell) FISH images from two experiments were analysed.
Suppl. Figure S6

**a**
- Mimosine (early G1)
- Nocodazole (G2/M)

**Ccnb1::Luc-MS2**

- One Transcription Site
- Two Transcription Sites

**Native Ccnb1**

- 40X Magnification

**b**
- Mimosine (early G1)

- One Transcription Site
- Two Transcription Sites

- Native Ccnb1

- Nocodazole (G2/M)

- 120X Magnification
Supplementary Figure S6. Expression of the *Ccnb1::Luc-MS2* transgene and number of active native *Ccnb1* alleles in G1 and G2/M. (a) Representative single molecule RNA FISH images of the *Ccnb1::Luc-MS2* transgene and native *Ccnb1* mRNA in mimosine-treated and nocodazole-treated *Ccnb1::Luc-MS2* cells (clone 96), respectively. Images were taken at 40X magnification. White arrows indicate cells with FISH signals. Grey arrows indicate cells without FISH signals. Scale bars: 10 µm. (b) Representative single molecule RNA FISH images of the native *Ccnb1* gene in *Ccnb1::Luc-MS2* cells (clone 96) treated with mimosine and nocodazole, respectively. Images were taken at 120X magnification. White arrowheads indicate the TSs. Scale bars: 5 µm.
Suppl. Figure S7

a) 6 hour

b) 6 hour

Ccnb1::Luc-MS2

Ccnb1

6 hour

10 hour

G1 G2

Counts

0 200 400 600 800 1000

Propidium Iodide Intensity

0

100

200

300

400

Counts

0

100

200

300

400

0 1000 2000 3000

G1 G2

G1 G2

M_{6hr} = 407

M_{10hr} = 257

TS_{6hr} = 4.3

TS_{10hr} = 4.0

M_{6hr} = 361

M_{10hr} = 301

TS_{6hr} = 4.9

TS_{10hr} = 4.1

N=51

N=270

P= 0.0008*

P= 0.0003*

% of cells with FISH signal

% of cells

6h 10h

Ccnb1::Luc-MS2

Native Ccnb1

Fisher's exact test

P= 0.058
Supplementary Figure S7. Expression of the Ccnb1::Luc-MS2 transgene and the native Ccnb1 gene in cells synchronized at G1 or G2/M measured by single molecule RNA FISH. (a) Flow cytometry profiles of cells synchronized with thymidine/aphidicolin and released for 6 hours and 10 hours. (b) Representative single molecule RNA FISH images using MS2 probes in Ccnb1::Luc-MS2 (clone-96) cells synchronized at G2/M (6 hours) or at G1 (10 hours). White arrowheads indicate the TSs. Scale bars: 5 µm. (c, d) Histograms and medians of mRNA counts and TS brightness of the Ccnb1::Luc-MS2 transgene. Asterisks (*) indicate statistically significant differences between G2/M cells and G1 cells. p < 1e-4 in panel C (n= 101 and 51 for G1 and G2/M cells, respectively). p = 0.03 in panel D (n = 96 and 51 for G1 and G2/M cells, respectively). (e) Representative single molecule RNA FISH images of native Ccnb1 mRNA in Ccnb1::Luc-MS2 (clone 96) cells synchronized at G2/M (6h) or at G1 (10h). White arrowheads indicate the TSs. Scale bars: 5 µm. (f, g) Histograms and medians of mRNA counts and TS brightness of the native Ccnb1 gene. Asterisks (*) indicate statistically significant differences between G2/M cells and G1 cells. p = 0.005 in panel F (n = 101 and 53 for G1 and G2/M cells, respectively). p = 0.005 in panel G (n = 117 and 62 for G1 and G2/M cells, respectively). In panels C, D, F and G, student’s t test was used to determine statistically significant differences. (h) Fractions of cells with Luc-MS2 FISH signals and native Ccnb1 FISH signals in Ccnb1::Luc-MS2 cells synchronized at G2/M (6h) or at G1 (10h). Fisher’s exact test was used to determine the statistical significance. Asterisks (*) indicate statistically significant differences (p < 0.05). (i) Fractions of cells with one or two TSs of the native Ccnb1 gene among cells synchronized at G2/M (6h) or at G1 (10h). Fisher's exact test was used to determine the P value. FISH images from one successful cell cycle synchronization experiment were analysed.
Suppl. Figure S8

10 hr post release (G1)

|            | One TS | Two TS |
|------------|--------|--------|
| Pol II density | 1.21 kb⁻¹ | 1.91 kb⁻¹ |
| Pol II firing rate | 1.34 ± 0.19 min⁻¹ | 2.11 ± 0.30 min⁻¹ |

6 hr post release (G2/M)

|            | One TS | Two TS |
|------------|--------|--------|
| Pol II density | 1.46 kb⁻¹ | 1.91 kb⁻¹ |
| Pol II firing rate | 1.61 ± 0.23 min⁻¹ | 2.11 ± 0.30 min⁻¹ |

P = 0.48

M₁TS = 278 (n=40)
M₂TS = 339 (n=11)

TS₁TS = 3.6 (n=40)
TS₂TS = 5.3 (n=22)

P = 1.3E⁻⁴*

M₁TS = 367 (n=53)
M₂TS = 359 (n=31)

TS₁TS = 4.2 (n=53)
TS₂TS = 5.3 (n=62)

P = 0.001*

P = 0.18
Supplementary Figure S8. mRNA counts, TS brightness and transcription rates from cells synchronized at G1 or G2/M with one or two active Ccnb1 alleles. Box plots show mRNA counts or TS brightness of the native Ccnb1 gene among cells synchronized at G1 or G2/M with 1 TS or 2 TS. Measured cell numbers and TS numbers are noted below each box plot. Asterisks (*) indicate statistically significant differences between the two experimental groups determined by student’s t-test (P values are noted in the plots). Tables show calculated Pol II densities and Pol II firing rates of the native Ccnb1 gene among cells synchronized at G1 or G2/M with 1 TS or 2 TS, respectively.
Suppl. Figure S9

**a**

*Cenob1::LucMS2-96*

Cytoplasm (4%)  Uniform (66%)  Nuclear (30%)

* Luciferase RNA FISH

**b**

![Graph showing mRNA counts distribution](image1)

- Cytoplasm (n=4)
- Uniform (n=52)
- Nuclear (n=23)

- $M_{\text{Cytoplasm}} = 277$
- $M_{\text{Uniform}} = 370$
- $M_{\text{Nuclear}} = 512$

- *$P=0.003$

**c**

![Graph showing transcription site brightness distribution](image2)

- Cytoplasm (n=4)
- Uniform (n=52)
- Nuclear (n=23)

- $TS_{\text{Cytoplasm}} = 1.5$
- $TS_{\text{Uniform}} = 5.0$
- $TS_{\text{Nuclear}} = 6.5$

- *$P<0.001$
- *$P=0.001$
Supplementary Figure S9. Distinct subcellular localizations of Luc-MS2 mRNA detected by Luciferase RNA FISH. (a) Representative images of single molecule RNA FISH using probes against Luciferase mRNA in asynchronous Ccnb1::Luc-MS2 (clone 96) cells. Cells display cytoplasmic, uniform or nuclear localization of Luc-MS2 mRNA. The percentages of cells with respective subcellular localizations of Luc-MS2 mRNA are shown in parentheses (N = 98). Scale bars: 5 µm. (b, c) Histogram and medians of mRNA counts (b) and TS brightness (c) of the Ccnb1::Luc-MS2 transgene among cells exhibiting distinct subcellular mRNA localizations. Measured cell numbers and TS numbers are noted in the panels. Asterisks (*) indicate statistically significant differences between the two experimental groups determined by student’s t-test (P values are noted in the panels). FISH images from one experiment were analysed.
Suppl. Figure S10

(a) Mimosine (early G1) Nocodazole (G2/M)

(b) Fisher's exact test

(c) Mimosine (early G1)

- Uniform (n=63)
- Nuclear (n=35)

M_{Uniform} = 276
M_{Nuclear} = 444

- TS_{Uniform} = 3.7
- TS_{Nuclear} = 5.8

N=188
N=63
N=51

(c) Nocodazole (G2/M)

- Uniform (n=47)
- Nuclear (n=46)

M_{Uniform} = 393
M_{Nuclear} = 502

- TS_{Uniform} = 4.6
- TS_{Nuclear} = 6.4

N=122
N=35
N=46

Uniform
Nuclear

Fisher's exact test
Supplementary Figure S10. Two cell populations differing in Ccnb1 promoter activities and Luc-MS2 mRNA localizations were found in cells arrested at G1 or G2/M. (a) Representative single molecule RNA FISH images of the Ccnb1::Luc-MS2 transgene among cells treated with mimosine or nocodazole displaying nuclear- or uniformly-localized Luc-MS2 mRNA. Images were taken at 120X magnification. Scale bars: 5 µm. (b) Fractions of cells with nuclear- or uniformly-localized Luc-MS2 mRNA in asynchronous, mimosine-treated and nocodazole-treated cells, respectively. P values were determined by Fisher's exact test. Cells with cytoplasmic localized Luc-MS2 mRNA were counted together with cells with uniformly localized Luc-MS2 mRNA. (c) Histograms and medians of mRNA counts and TS brightness of the Ccnb1::Luc-MS2 transgene among cells displaying uniformly- or nuclear-localized Luc-MS2 mRNA in mimosine-treated or nocodazole-treated cells. Measured cell numbers N are shown in each plot. Asterisks (*) indicate statistically significant differences in mean values of mRNA counts or TS brightness among cells with nuclear- or uniformly-localized Luc-MS2 mRNA determined by student’s t-test (p < 0.001 in all cases). FISH images from one successful cell cycle arrest experiment were analyzed.
Suppl. Figure S11

(a) 6h (G2/M) and 10h (G1) images showing Uniform and Nuclear staining.

(b)Normalized Frequency

(c) 6h mRNA counts showing Uniform (n=31) and Nuclear (n=70) with M_{Uniform} = 325 and M_{Nuclear} = 435.

(d) 10h mRNA counts showing Uniform (n=20) and Nuclear (n=31) with M_{Uniform} = 238 and M_{Nuclear} = 290.

(e) Transcription site brightness showing Uniform (n=31) and Nuclear (n=65) with TS_{Uniform} = 3.9 and TS_{Nuclear} = 4.4.

(f) Transcription site brightness showing Uniform (n=19) and Nuclear (n=31) with TS_{Uniform} = 3.7 and TS_{Nuclear} = 4.1.
Supplementary Figure S11. Cells synchronized at G1 or G2/M also displayed distinct *Luc-MS2* mRNA localizations and *Ccnb1* promoter activities. (a) Representative single molecule RNA FISH images (MS2 FISH probes) in *Ccnb1::Luc-MS2* cells (clone 96) synchronized at G2/M (6h) or at G1 (10h) displaying nuclear- or uniformly-localized *Luc-MS2* mRNA. Images were taken at 120X magnification. Scale bars: 5 µm. (b) Fractions of cells displaying uniformly- or nuclear-localized *Luc-MS2* mRNA in synchronized cells. Asterisks (*) indicate statistically significant differences determined by Fisher’s exact test (p < 0.05). (c–f) Histograms and medians of mRNA counts and TS brightness of the *Ccnb1::Luc-MS2* transgene among cells synchronized at G2/M (6h) or at G1 (10h) displaying uniformly- or nuclear-localized *Luc-MS2* mRNA. Measured cell and TS numbers are shown in the figure. Mann-Whitney U-test was used to compare mRNA counts or TS brightness among cells with uniformly- or nuclear-localized *Luc-MS2* mRNA. Asterisks (*) indicate statistically significant differences (p < 0.05 in all cases). FISH images from one successful cell cycle synchronization experiment were analysed.
Suppl. Figure S12

(a) Flow cytometry analysis of pSG5-1, NF-YAm29-1, NF-YAm29-5, and NF-YAm29-3.

|            | pSG5-1 | NF-YAm29-1 | NF-YAm29-5 | NF-YAm29-3 |
|------------|--------|------------|------------|------------|
| G1         | 66.7%  | 70%        | 78.6%      | 72.2%      |
| S          | 13.3%  | 11.6%      | 9.0%       | 13.6%      |
| G2         | 20%    | 18.3%      | 12.4%      | 12.7%      |

(b) Western blot analysis of Ccnb1::Luc-MS2:96, pSG5-1, and NF-YAm29-1. Anti-NF-YA and anti-α Tubulin.

(c) Fluorescence images of pSG5-1 and NF-YAm29-1.

(d) Bar graph showing expression normalized to Gapdh of Ctc12, Ccnb1::Luc-MS2:96, pSG5-1, NFYA-m29-1, NFYA-m29-5, and NFYA-m29-3.
Supplementary Figure S12. Cell cycle profiles and mRNA expression in stable cell lines overexpressing a dominant negative mutant NF-YAm29. (a) Flow cytometry cell cycle profiles of Ccnb1::Luc-MS2 (clone 96) cells stably transfected with the pSG5 vector (clone 1) or the pSG5NF-YAm29 vector (clone 1, clone 3 and clone 5). The percentages of cells in each cell cycle phase are presented in the table to the right. (b) Overexpression of NF-YA in the cell clone containing pSG5-NF-YAm29 vector (clone 1) was verified by western blot. Anti-α Tubulin was used as the loading control. (c) Detecting NF-YA expression in pSG5-1 control cells and NF-YAm29 (clone 1) cells by immunofluorescence staining. Greyscale images are shown in pseudocolor to provide contrast for fluorescence intensity. Scale bars: 20 µm. (d) Comparing levels of Luciferase mRNA, native Ccnb1 mRNA and NF-YA mRNA in C2C12 cells, Ccnb1::Luc-MS2 (clone 96) cells, pSG5 control cells (pSG5-1) and cell clones stably transfected with the pSG5-NF-YAm29 vector (clone 1, clone 3 and clone 5). mRNA expression was normalized to Gapdh mRNA levels. Error bars represent standard deviations (n=3).
Suppl. Figure S13

Luciferase RNA FISH

(a) pSG5-1

(b) NF-YAm29-1

(c) NF-YAm29-5

Ccnb1::Luc-MS2

0 100 200 300 400 500

0.0 0.1 0.2 0.3 0.4

Normalized Frequency

mRNA counts

0 100 200 300 400 500

0.0 0.1 0.2 0.3 0.4

Normalized Frequency

Transcription site brightness

pSG5-1

m29-1

m29-5

M_{pSG5-1} = 194

M_{m29-1} = 67

M_{m29-5} = 79

TS_{pSG5-1} = 3.9

TS_{m29-1} = 4.0

TS_{m29-5} = 3.7

*
Supplementary Figure S13. Effects of expressing the NF-YA m29 mutant on *Ccnb1::Luc-MS2* transgene expression measured by RNA FISH using the *Luciferase* probe. (a)

Representative single molecule RNA FISH images using *Luciferase* probes in cell clones with stably integrated pSG5-1 or pSG5-NF-YA m29 vectors (Clone 1 and Clone 5). Scale bars: 5 µm. Images were taken at 120X magnification. White arrowheads indicate the TSs. (b) Histograms and medians of mRNA counts. Measured cell numbers n = 89, 30 and 24 for pSG5-1, m29-1 and m29-5 cell clones, respectively. Asterisks (*) indicate statistically significant differences in mean values of mRNA counts between pSG5-1 cells and NF-YA m29-1 or NF-YA m29-5 cells determined by Student’s t-test (p < 1e-6 in both cases). (c) Histograms and medians of TS brightness. Measured TS numbers n = 71, 22 and 22 for pSG5-1, m29-1 and m29-5 cell clones, respectively. TS brightness are not significantly different between pSG5-1 and NF-YA m29-1 or NF-YA m29-5 cells (p = 0.34 and 0.20). FISH images from one experiment were analysed.
Suppl. Figure S14

MS2 RNA FISH

(a) Nf-YA29-3

Conb1::Luc-MS2

(b) NF-YA29-3

Conb1::Luc-MS2

Normalized Frequency

Normalized Frequency

M_{pSG5-1} = 124
M_{m29-3} = 38
M_{m29-5} = 58

TS_{pSG5-1} = 3.3
TS_{m29-3} = 2.7
TS_{m29-5} = 2.8

(c) Nf-YA29-5

Conb1::Luc-MS2

(d) NF-YA29-5

Conb1::Luc-MS2

Normalized Frequency

Normalized Frequency

M_{pSG5-1} = 342
M_{m29-3} = 262
M_{m29-5} = 250

TS_{pSG5-1} = 5.1
TS_{m29-3} = 4.8
TS_{m29-5} = 5.2

(g) % of cells with FISH signal

Conb1::Luc-MS2

Native Conb1

P < 0.0001*

P = 0.0598

P < 0.0001*

P = 0.0143*

(h) Normalized Frequency

Uniform

Nuclear

N=36
N=42
N=41

N=51
N=52
N=25

N=173
N=319
N=229

N=254
N=315
N=302

pSG5-1  Nf-YA29-5  Nf-YA29-3
Supplementary Figure S14. Effects of expressing the NF-YAm29 mutant on Ccnb1::Luc-MS2 transgene expression and native Ccnb1 gene expression in additional cell clones. (a) Representative single molecule RNA FISH images using MS2 probes in cell clones stably transfected with the NF-YAm29 vector (NF-YAm29-3 and NF-YAm29-5). (b) Histograms and medians of mRNA counts of the Ccnb1::Luc-MS2 transgene. Measured cell numbers N = 95, 88 and 67 for pSG5-1, m29-3 and m29-5 cell clone, respectively. (c) Histograms and medians of TS brightness of the Ccnb1::Luc-MS2 transgene. Measured TS numbers N = 90, 62 and 54 for pSG5-1, m29-3 and m29-5 clone, respectively. (d) Representative single molecule RNA FISH images of native mouse Ccnb1 mRNA in NF-YAm29-3 and NF-YAm29-5 cell clones. In panels A and D, images were taken at 120X magnification. White arrowheads indicate the TSs. Scale bars: 5 µm. (e) Histograms and medians of mRNA counts of the native Ccnb1 gene. Measured cell numbers N = 96, 100 and 93 for pSG5-1, m29-3 and m29-5 clone, respectively. (f) Histograms and medians of TS brightness of the native Ccnb1 gene. Measured TS numbers N = 130, 106 and 97 for pSG5-1, m29-3 and m29-5 clone, respectively. In panels b, c, e and f, student’s t-test was used to determine statistically significant differences in mean values of mRNA counts or TS brightness between pSG5-1 cells and m29-3 (or m29-5) cells. Asterisk (*) indicates that the difference is statistically significant (in b, p < 1e-6; in c, p = 0.02 and 0.03; in e, p < 1e-6; in f, p = 0.03 between pSG5-1 and m29-3, p = 0.64 between pSG5-1 and m29-5). (g) Fractions of cells detected with Luc-MS2 mRNA FISH signals and native Ccnb1 mRNA FISH signals in the control cell clone pSG5-1 and cell clones stably expressing the NF-YAm29 mutant (m29-5 or m29-3). (h) Fractions of cells displaying uniformly- or nuclear-localized Luc-MS2 mRNA in the control cell clone pSG5-1 and cell clones stably expressing the NF-YAm29 mutant (m29-1 or m29-5). In panels g and h, Fisher's exact test was used to determine the P
value. Asterisks (*) indicate statistically significant differences (p < 0.05). FISH images from one experiment were analysed.
Suppl. Figure S15

MS2 RNA FISH

a

pSG5

Uniform

Nuclear

NF-YAm29-1

Uniform

Nuclear

b

pSG5

NF-YAm29-1

NF-YAm29-5

Uniform (n=42) Nuclear (n=52)

M_{Uniform} = 104  M_{Nuclear} = 166  P=0.002*

M_{Uniform} = 44  M_{Nuclear} = 67  P=0.03*

M_{Uniform} = 51  M_{Nuclear} = 91  P=0.003*

Uniform (n=42) Nuclear (n=25)

Uniform (n=41) Nuclear (n=25)

Uniform (n=36) Nuclear (n=51)

Uniform (n=36) Nuclear (n=51)

M_{Uniform} = 44  M_{Nuclear} = 67  P=0.03*

M_{Uniform} = 2.6  M_{Nuclear} = 3.1  P=0.003*
Supplementary Figure S1. Distinct Luc-MS2 mRNA localizations and Ccnb1 promoter activities in cells expressing the NF-YAm29 mutant. (a) Representative single molecule RNA FISH images of Ccnb1::Luc-MS2 cells stably transfected with the control pSG5 vector (left) and the pSG5-NF-YAm29 vector (right) showing uniformly- or nuclear-localized Luc-MS2 transcripts. Scale bars: 5 µm. (b) Histograms of mRNA counts and TS brightness in each stable cell line. Measured cell numbers n of each experimental group are shown in each plot. Asterisks (*) indicate statistically significant differences in mRNA counts or TS brightness between cells with uniformly- or nuclear-localized Luc-MS2 mRNA determined by students’ t-test (p < 0.05). FISH images from one experiment were analysed.
Suppl. Figure S16

|          | mRNA t_{1/2} | mRNA decay rate k_{decay} |
|----------|--------------|----------------------------|
|          | pSG5-1       | NF-YAm29-1                 | pSG5-1       | NF-YAm29-1                 |
| Luc      | 1.51 ± 0.70 h| 5.8 ± 0.56 h               | 0.46 ± 0.15 h^{-1} | 0.12 ± 0.012 h^{-1}       |
| Ccnb1    | 2.40 ± 0.24 h| 2.0 ± 0.42 h               | 0.29 ± 0.03 h^{-1} | 0.35 ± 0.09 h^{-1}       |
| c-Myc    | 0.65 ± 0.09 h| 0.84 ± 0.27 h              | 1.07 ± 0.15 h^{-1} | 0.82 ± 0.27 h^{-1}       |
Supplementary Figure S16. Comparing stability of *Luciferase*, *Ccnb1* and *c-Myc* mRNA in control cells and cell clones overexpressing NF-YAm29 mutant. (a–f) mRNA decay plots of *Luciferase*, native *Ccnb1* and *c-Myc* in cells stably transfected with the pSG5 vector (a–c) or the pSG5-NF-YAm29 vector (d–f), respectively. 18S rRNA was used to normalize the expression. Decay curves were plotted and mRNA half-lives were calculated as described in Supplementary Methods. mRNA half-lives calculated from each mRNA decay curve are shown above each plot. (g) Table comparing average half-lives ($t_{1/2}$) and decay rates ($k_{decay}$) of *Luciferase* mRNA, native *Ccnb1* mRNA and *c-myc* mRNA from pSG5-1 and NF-YAm29-1 clones calculated from two biological replicates.
Suppl. Figure S17

(a) Ccnb1::LucMS2 and Native Ccnb1

- pSG5-1
- NF-YA29-1

40X Magnification

(b) Native Ccnb1 intron FISH probe

- 1 Transcription Sites
- 2 Transcription Sites

- pSG5-1
- NF-YA29-1

120X Magnification
Supplementary Figure S17. Detecting the fraction of cells expressing the *Ccnb1::Luc-MS2* transgene and active TSs of the native *Ccnb1* gene in the presence of NF-YAm29 mutant. *(a)* Representative single molecule RNA FISH images of *Luc-MS2* mRNA (using MS2 probes) and native *Ccnb1* mRNA (using exon probes) in pSG5-1 and NF-YAm29-1 cell clones. Images were taken at 40X magnification. White and grey arrows indicate cells with and without MS2 FISH signals, respectively. Scale bars: 10 µm. *(b)* Representative single molecule RNA FISH images using probes targeting mouse *Ccnb1* introns 1 to 4 (see Supplementary Table S3) in pSG5-1 and NF-YAm29-1 cell clones. Images were taken at 120X magnification. White arrowheads indicate the TSs. Scale bars: 5 µm.
Suppl. Figure S18

a

Ccnb1::LucMS2
Clone 96

No Promoter::LucMS2
Clone 5

C2C12

b

C2C12
Ccnb1::LucMS2/96
No Promoter::LucMS2-5

C2C12
Ccnb1::LucMS2/96
No Promoter::LucMS2-5

Ccnb1 promoter LP + Luciferase RP

500 bp
1000 bp
1500 bp
3000 bp

n

Ccnb1::LucMS2/96
PSGS1-1
NF-YA

Anti-NF-YA

Ccnb1::LucMS2/96
PSGS1-1
NF-YA

Anti-α Tubulin
Supplementary Figure S18. Full-size gel images of Supplementary Figure S2b (a), S2c (b) and full-size western blot image of Supplementary Figure S12b (c).
Supplementary Tables

**Supplementary Table S1.** Sequences of single molecule FISH probes of mouse *Ccnb1* mRNA.

| Probe | Position (bp) | Percent GC |
|-------|---------------|------------|
| gctaaacactaaccagcgggtt | 3 | 45.0% |
| cgattcgagaagacacccta | 40 | 50.0% |
| tcctccaaatcagaggttc | 62 | 45.0% |
| attttctgttctcatgac | 97 | 40.0% |
| tggagaacgacgtaactgctc | 171 | 50.0% |
| aatgtcctcaagagcagtttc | 209 | 45.0% |
| aacagtccctttccagttac | 293 | 40.0% |
| cttctctcacaggtttgta | 320 | 40.0% |
| tgagaaggagatatacaacca | 437 | 45.0% |
| gacacagatctctctgca | 486 | 45.0% |
| gtctctactgtaagagatta | 521 | 45.0% |
| attcactacagaggtttggg | 564 | 45.0% |
| gaggatacgctctcatggttt | 671 | 45.0% |
| cttgagagccttaaatcttc | 717 | 45.0% |
| ggcacacagctctgtataggg | 740 | 50.0% |
| gttctgcattggaaccgcatca | 764 | 45.0% |
| ataaacatggccgattacacc | 817 | 45.0% |
| tacatctcctcatatttgct | 841 | 35.0% |
| cacaaggcaagttcaacca | 872 | 50.0% |
| gcctagttgctgtagtgtta | 894 | 40.0% |
| aatctctcttcttctgctc | 923 | 40.0% |
| ggcctgaatgctcaacacagggcagcgg | 945 | 50.0% |
| cgacttttagactgctctcaggg | 993 | 50.0% |
| aggtatttgcgcagaagttgtg | 1033 | 45.0% |
| gcraaatgcaccattgctctgta | 1075 | 45.0% |
| ttccgtgctaagcagaaagc | 1117 | 50.0% |
| ccattcaccctgctcaagaa | 1139 | 45.0% |
| ggaagtctctctgttattagta | 1181 | 45.0% |
| gttcactcaagactacattc | 1229 | 40.0% |
| acaagtctatgtttgttgtag | 1255 | 45.0% |
| cttagcatcgttgtagtggtctg | 1289 | 45.0% |
| tagttgctcaagctcattgtc | 1322 | 45.0% |
| ccacccttcattgacaaatctt | 1346 | 45.0% |
| ttacttgaggttagctgttcttt | 1368 | 35.0% |
| caactgtcatctgcagattgta | 1392 | 45.0% |
| gtaggtctctctatgtcagg | 1425 | 45.0% |
| cctcggctcataactcagaaa | 1518 | 45.0% |
| cattagttgtgctcactcgttt | 1582 | 40.0% |
| ggaacaagatcctgcttgttt | 1697 | 50.0% |
| gttaaaagttggaaccagctg | 1723 | 50.0% |
| acccttacatctgttgagatgtg | 1879 | 40.0% |
| ttcttggagttatagcagt | 1929 | 45.0% |
| acacacagtgtggactccactc | 2024 | 40.0% |
| agaatgctcattgagaccccac | 2062 | 45.0% |
| taagacacagtggagagggca | 2087 | 50.0% |
| ctctcggctgtcagaaatcttc | 2165 | 45.0% |
| attaaagacaggagttggc | 2209 | 50.0% |
| tgagacaggattttttctca | 2235 | 40.0% |
Supplementary Table S2. Sequences of single molecule FISH probes of the 5’-portion of *Luciferase* mRNA.

| Probe                  | Position (bp) | Percent GC |
|------------------------|---------------|------------|
| catcttccagcgatagaat    | 39            | 45.0%      |
| gtatctctctcatagccttat | 80            | 35%        |
| aagcaatgttcaggaacc    | 105           | 45.0%      |
| taagtgatgtccacctcgat  | 139           | 45.0%      |
| ccgaacggacatcttggaagt | 167           | 50.0%      |
| tctgtcttatggctgatc    | 190           | 40.0%      |
| ctaaatccacctgcagccg   | 212           | 40.0%      |
| gtttttacatgatcagccg   | 234           | 45.0%      |
| cccacacccgatcataagaa | 262           | 50.0%      |
| cgcaactgcaactccgataa  | 290           | 50.0%      |
| gaaatgcacactgttttgta  | 344           | 45.0%      |
| aacgcaacaccacgtagccg  | 366           | 55.0%      |
| aatccccctggccatccttt  | 389           | 30.0%      |
| attgggagcttttttttgca  | 415           | 40.0%      |
| cctctatagctggctctttaa | 459          | 40.0%      |
| gacgaactgtgtacatcagct | 485          | 50.0%      |
| aacggggagtgatgagat    | 507           | 50.0%      |
| ctaatggcataaatagtctt  | 533           | 40.0%      |
| ttgtctttgtcctactggaag | 555          | 45.0%      |
| gagaggattctcatcagttgc  | 577          | 50.0%      |
| tttagcagacagtagtc     | 599           | 45.0%      |
| ttctatggcagacgacgaca  | 621           | 50.0%      |
| cttccagatctccacgcag   | 645           | 55.0%      |
| aatgcagcctgtcgggaatga | 692           | 45.0%      |
| cttccaaaccgatgatgga   | 728           | 45.0%      |
Supplementary Table S3. Sequences of single molecule FISH probes targeting intronic regions of the mouse *Ccnb1* gene.

|   | Probe                      | Percent GC |
|---|----------------------------|------------|
| 1 | cttcaccattaagagcagcag      | 45.0%      |
| 2 | aaggcgaagaattcacccttg      | 50.0%      |
| 3 | ataaaagctccgctgcagtaa     | 45.0%      |
| 4 | ccacccttaacatggtgaat      | 45.0%      |
| 5 | aagcaagtccccaccccaaat    | 40.0%      |
| 6 | aggaaagggttgtagcatctca    | 50.0%      |
| 7 | tcacgggttaattaagggcag     | 50.0%      |
| 8 | taatgggcttagaggagatcg    | 50.0%      |
| 9 | tagaagatgggcccccttcag     | 45.0%      |
|10 | acagaatgtttttcccaagg     | 45.0%      |
|11 | actctggtagaagggaga        | 50.0%      |
|12 | tgtcatgtcatacaagctga     | 45.0%      |
|13 | aatgtttttctagccactc      | 40.0%      |
|14 | ccagagtcgtaatgcagta      | 45.0%      |
|15 | catatatttcgccccacatt     | 35.0%      |
|16 | aggcttaaatgcttaacccat   | 40.0%      |
|17 | caggctacaacaaactcagcgaa | 50.0%      |
|18 | gttaaatctgtgctctcga      | 45.0%      |
|19 | tctttatcagggtgtatcca     | 35.0%      |
|20 | cccacgtgaacctgtatcaaa    | 45.0%      |
|21 | agtcatttatccccataggctc  | 40.0%      |
|22 | ttcagtaagcatgctcaact    | 40.0%      |
|23 | gaaaaggcagttactgtatcc    | 45.0%      |
|24 | tcctgaacacaggtcacatt    | 45.0%      |
|25 | gaagcagactgcgaatgaca    | 50.0%      |
|26 | attgtccaggaagctgcaag    | 50.0%      |
|27 | tgtatatattggcaccacclttta | 35.0%    |
|28 | atagttgagttggtgtcaca     | 40.0%      |
|29 | tttcactatgtgatcagtc       | 40.0%      |
|30 | catcttagaccacccccacagat  | 45.0%      |
|31 | acaaagaatgcctgggtggtg  | 50.0%      |
|32 | ccactggtcgtgacaagtaca    | 45.0%      |
|33 | aggccgaggtgatgaaacca    | 50.0%      |
|34 | aggctatagaatgccttgttt   | 35.0%      |
|35 | accccacacaaggttcgaaaaa  | 45.0%      |
|36 | tcaagctgtcctcttgaacc    | 45.0%      |
|37 | attcttcaagggacaccaact  | 40.0%      |
|38 | gaagggcaagggagaaacct    | 50.0%      |
|39 | tatgagcaacagcgctctcat   | 45.0%      |
|40 | cagctagtttcgaacccagat  | 45.0%      |
|41 | ccaagttctgagcgttagaa  | 45.0%      |
|42 | aacaccccacacagtgtaaca  | 45.0%      |
|43 | gctgctccatgttataaact  | 40.0%      |
|44 | tctgttagccccgaaactatt  | 40.0%      |
|45 | cagactgtgacagacgac     | 55.0%      |
|46 | tcaaaagctcatagtaggctc   | 45.0%      |
|47 | gggcagaaggttccaccaagaa | 50.0%      |
|48 | cacaacatctaccatcgggtg | 50.0%      |
Supplementary Table S4. PCR primers used in this study.

| Amplicon                      | Left Primer                        | Right Primer                        | Experiment          |
|-------------------------------|------------------------------------|-------------------------------------|---------------------|
| 1 PCR1                        | CCAGTTCCGCCCATTCTCC                | CTGTTATGCGGCGCATTGTCC               | Genomic DNA PCR     |
| 2 PCR2                        | TCGAGCAACACATGATAAGAC              | GTAACCGTGACATCTGCCAGT               | Genomic DNA PCR     |
| 3 PCR3                        | TAGTTGCCAGCCCATCTGTTT              | GTAACCGTGACATCTGCCAGT               | Genomic DNA PCR     |
| 4 MS2                         | GACCGCCTGAAGTCTCTGAT               | TAGAAGGCAACAGTCAGG                  | Genomic DNA PCR     |
| 5 Ccnb1 Promoter+Luc          | CAACAAAGCTTTTCGGGAACT              | CCGGGCCTTTCTTTATGTTT                | Genomic DNA PCR     |
| 6 Ccnb1 EE                    | GCTCAGCAAGTCCACCTCT                | AGCGAAGAGCTACAGGCAAG                | RT-qPCR             |
| 7 Luciferase                  | GAGGCAGAATGTTGTTGTTGAGA            | GTGGTCGTCTTCTGTCAGTTGAG            | ChIP and RT-qPCR    |
| 8 NF-YA                       | GTGATATGTGCAAGTCACTGGGA            | TCTGCTGTCACAACCTTGTGTGTC            | RT-qPCR             |
| 9 Gapdh                       | GCCAAAGGGGCTCATCATCT                | CTAAGCAGTTGGTGTGTCAGG               | RT-qPCR             |
| 10 c-Myc                      | TGACCTAACTCAGGGAGGGACTGCCACTC      | AAGGTTGAGGGCAGTTAAAATTGCTGAGCAGC    | RT-qPCR             |
| 11 18S rRNA                   | GTAACCCGTTGAACCCCCATT            | CCACTCAATCGGTAGTCGAGGG              | RT-qPCR             |
| 12 SV40 promoter              | CAGTTCCGCCCCATTCTGCC              | TCCTCACTACTTCTGGAATAGCTC            | ChIP                |
| 13 Hygromycin                 | GAGCCAGATGCGTGTGTCAGAA            | CGAAGCCCCACCTTTCATAG                | ChIP                |
| 14 No Promoter::Luc-MS2       | ATGTACGGGGCCAGATATTCG              | CCGGGCCTTTTTATGTTT                  | ChIP                |
| 15 Ccnb1::Luc-MS2 transgene   | GAGCGAGTGCGCCAGAAGT               | CCGGGCCTTTTTTATGTTT                 | ChIP                |
| 16 Chr. 19 intergenic region  | AACCATCATTCAAGCGGGT              | GGATGAGATGGGCAAAAACGC               | ChIP                |
| 17 Native Ccnb1 promoter      | TAAACCTAAGCCCAGGCAGAC            | CCCGATTGCAGAAGACACC                 | ChIP                |
| 18 Ccnb1 Exon 2               | TGGTACAGTTACTGGGCTGCTCC           | GCCTGTAGCTCTCCTGTCGAC               | ChIP                |
| 19 Ccnb1 intergenic region    | GACTCCACACTGGGCTCTG              | CTTCCCTCTCCAAGTGTA                  | ChIP                |
| (Chr.13)                      |                                    |                                     |                     |
**Supplementary Table S5. Oligonucleotides used in splinkerette PCR.**

| Name       | Sequence                                                                 | Experiment                          |
|------------|--------------------------------------------------------------------------|-------------------------------------|
| SPLNK-GATC-TOP | GATCCCACTAGTGTCGACACCAGTCTCTCTAACATTGTTTTTTTTTTTTTTTCAAA AAA             | Generate annealed splinkerette adaptors |
| SPLNK-BOT  | CGAAGAGTAACCCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTG GTGTCGACACTAGTGG          | Generate annealed splinkerette adaptors |
| spPCR-Us1R | CCAGTGCTGCAATGATACCGCGAGAC                                                | Splinkerette PCR                   |
| spPCR-Us2R | CACCGGCTCCAGATTTATCAAC                                                  | Splinkerette PCR                   |
| SPLNK #1   | CGAAGAGTAACCCGTTGCTAGGAGAGAC                                             | Splinkerette PCR                   |
| UsSeq      | AAGTGGTCTGCAACTTTTATCCG                                                  | Sequence PCR products               |