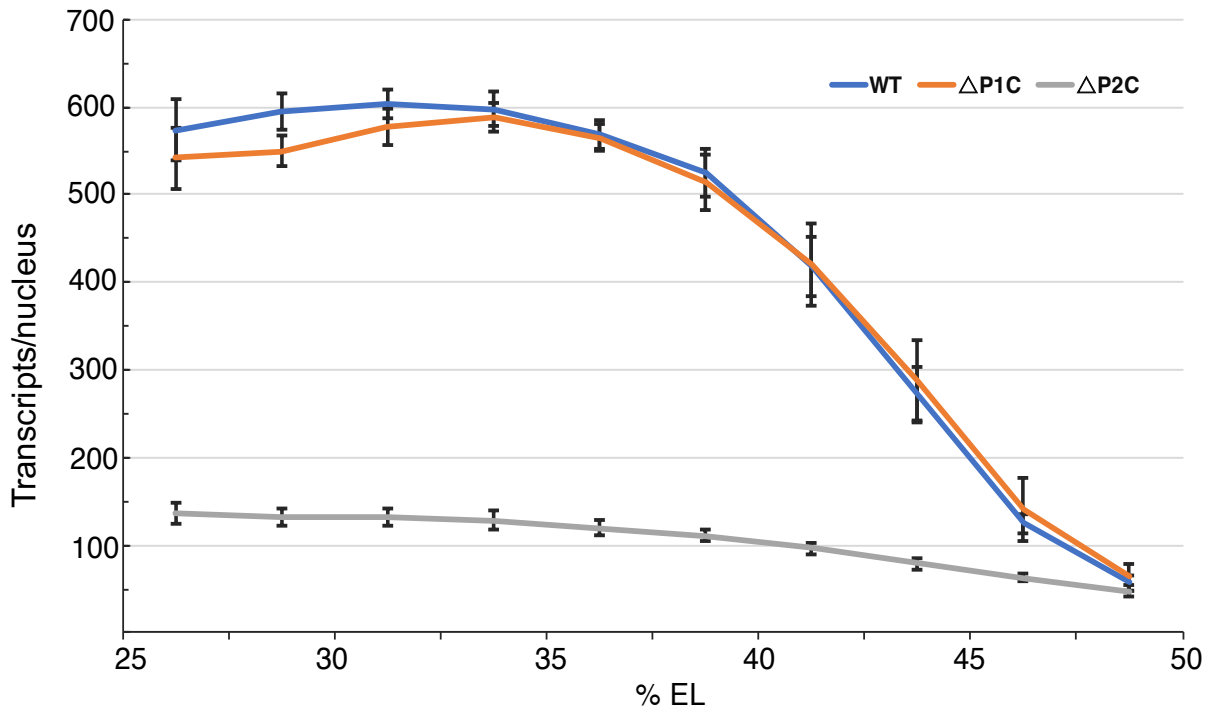

Figure S1. Related to Figure 2. Quantifying *hb* mRNA expression in wt, ΔP2C, and ΔP1C embryos

Numbers of transcripts per nucleus as a function of AP position (% Egg Length, 0% = anterior tip) are presented. Numbers of embryos measured for each AP bin are at least 5 for wt, 3 for ΔP2C and 4 for ΔP1C. Error bars represent standard errors of the mean (SEM).

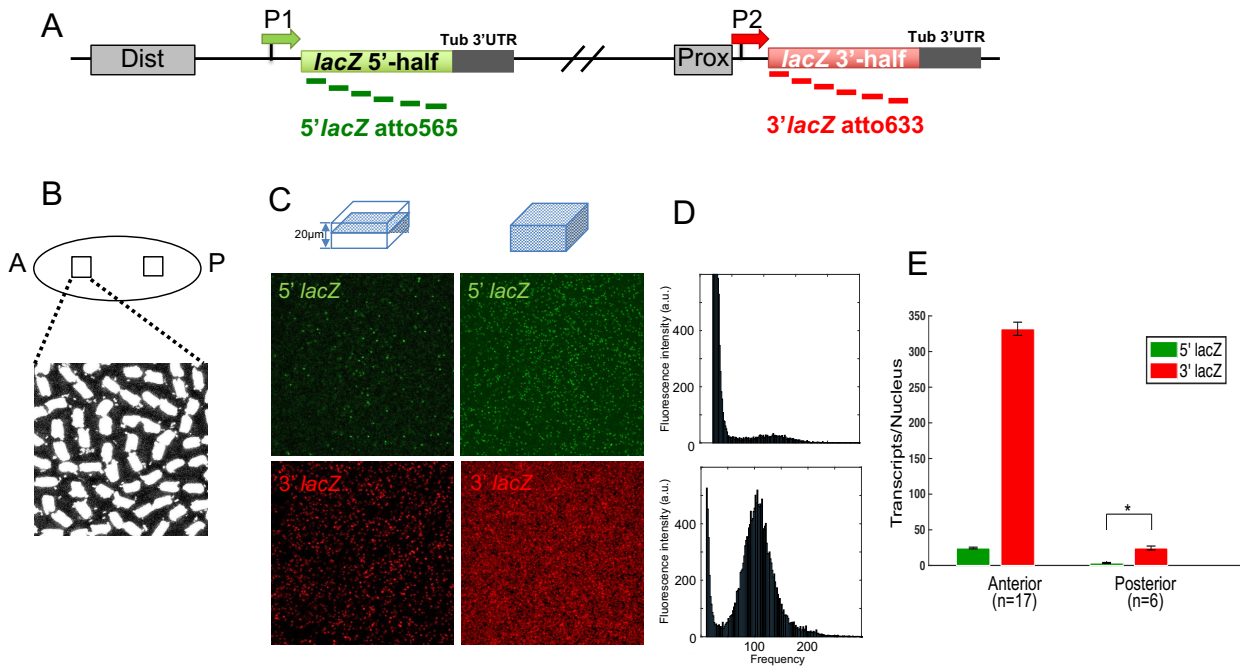

Figure S2. Related to Figure 2 and STAR Methods. A dual reporter system to quantify transcripts from P1 and P2

A. Schematic of the wt 5.6 kb *hb* dual reporter gene. The green and red bars represent smFISH probes complementary to the 5' *lacZ* and 3' *lacZ* reporters, respectively. **B.** Schematic of imaged region in an embryo and the DAPI channel of telophase nuclei from a NC13 embryo. **C.** Images showing cytoplasmic spots for a single frame (left) and for z-projection (right). **D.** Histograms of cytoplasmic spot distribution. X axis: number of spots. Y axis: spot intensity. **E.** Numbers of transcripts per nucleus in anterior and posterior regions of the embryo for the wt 5.6 reporter.

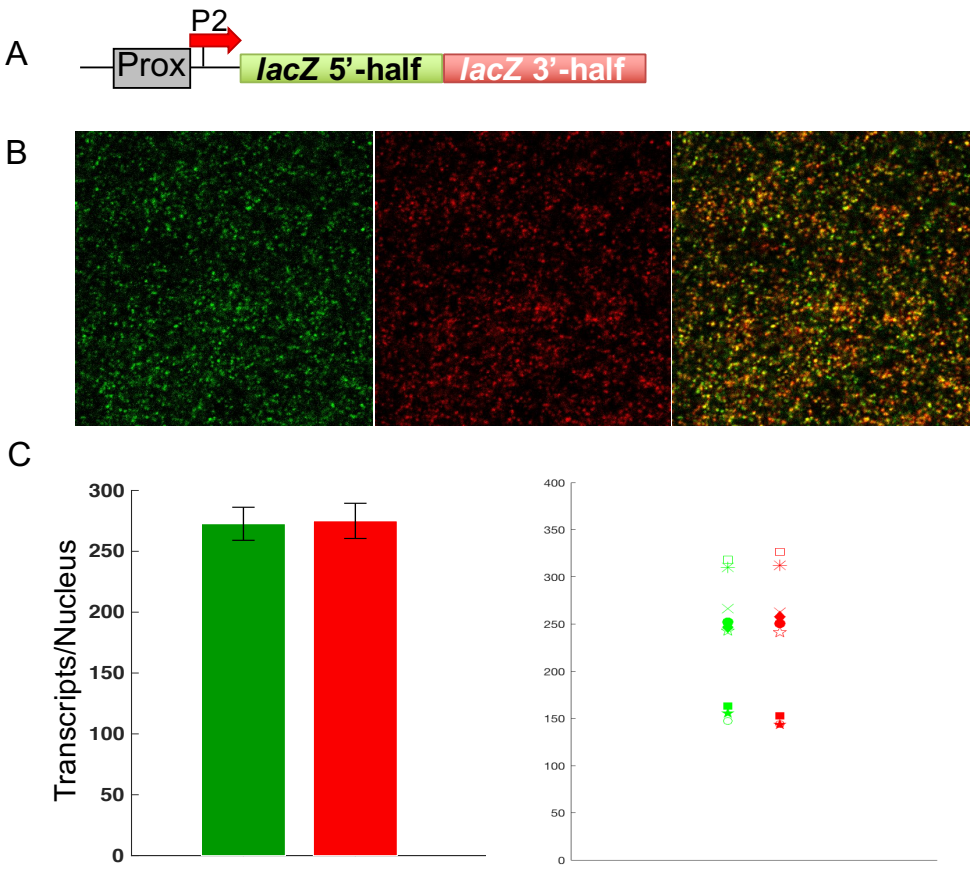

Figure S3. Related to STAR Methods. Testing detection efficiencies for the 5' and 3' *lacZ* probes

A. Schematic of the control reporter with full length *lacZ*. **B.** Images of spots for 5' *lacZ* (green), 3' *lacZ* (red), and overlapping channels. **C.** Left: number of transcripts per nucleus detected by Atto 565 for 5' *lacZ* (green) and by Atto 633 for 3' *lacZ* (red). Right: a plot showing that for every measured embryo (at varying nuclear cycles) the number of transcripts for 5' lacZ (green) is very similar to that detected for 3' *lacZ* (red). Datapoints with the same symbol represent data from the same embryo.

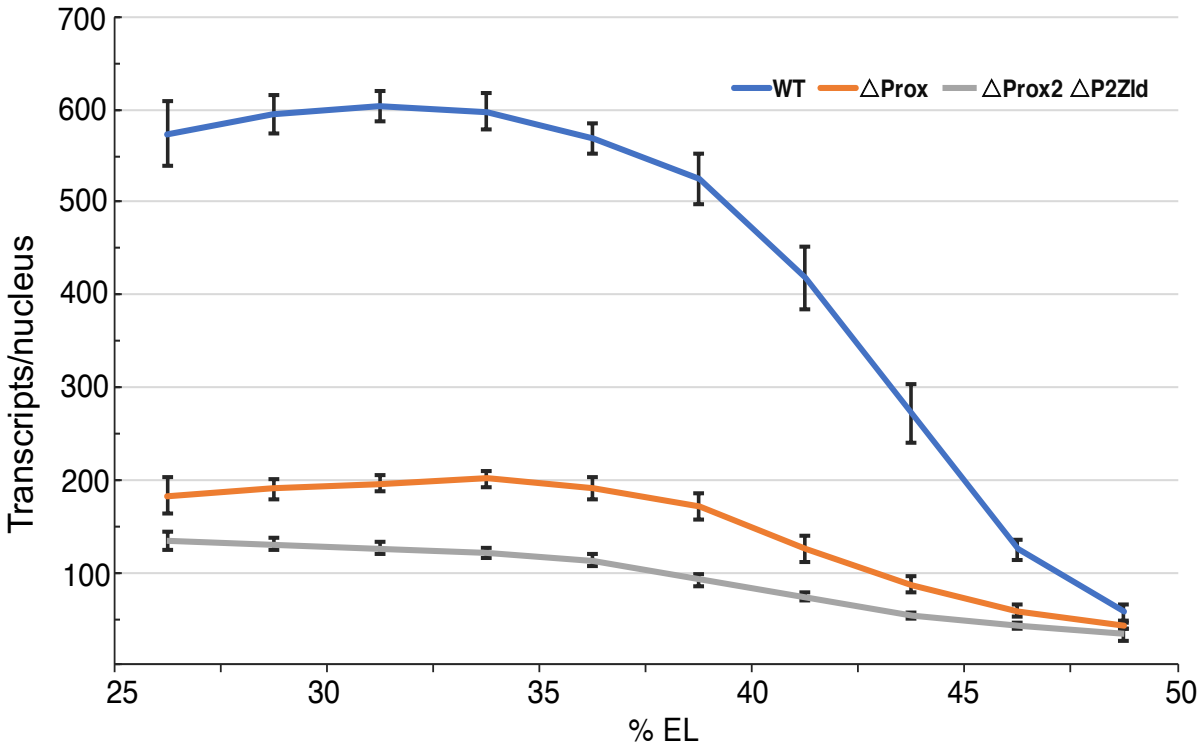

Figure S4. Related to Figure 5. Quantifying *hb* mRNA expression in wt, ΔProx, and ΔProx ΔP2Zld embryos

Numbers of transcripts per nucleus as a function of AP position (% Egg Length, 0% = anterior tip) are presented. Numbers of embryos measured for each AP bin are at least 5 for wt, 4 for ΔProx and 5 for ΔProx ΔP2Zld. Error bars represent standard errors of the mean (SEM).

Table S1. Related to Figure 2, 5, and STAR Methods. gRNA sequences used for CRISPR/Cas9 experiments and sequences at the junctions of deletion break point

ΔP2C gRNA 1	GTTATATATCGCTCAGGTAGA
ΔP2C gRNA 2	GATTTGTCGGGATTTTCGTA
ΔP1C gRNA 1	GTTGGCGAGTGCACTGTTGTG
ΔP1C gRNA 2	GCGTTTCGTCCTTTGGATGTT
ΔProx/ΔProx ΔP2Zld gRNA 1	GAGGAGTAGGCAGCTAGCGT
ΔProx gRNA 2	GGTAGACGGATGCACGCGTCA
ΔProx ΔP2Zld gRNA 2	GTTATATATCGCTCAGGTAGA
ΔDist gRNA 1	GTCTCAACAGAAGTTCCTTCG
ΔDist gRNA 2	GCCCAAATATTCCACATAAAC

>ΔP2C

----------attP-loxP-dsRed-loxP-attP----------

CGCGTGCATCCGTCT|**CATATGCACACCTGC--------CAGTTGGGGCACTAC**|**GAAAATCCCGACAAA**

>ΔP1C

----------attP-loxP-dsRed-loxP-attP----------

GGCGAGTGCACTGTT|**GTAGTGCCCCAACTG--------GCAGGTGTGCATATG**|**ATCCAAAGGACGAAA**

>ΔProx

----------attP-loxP-dsRed-loxP-attP----------

ACGAAACTGCCCACG|**GTAGTGCCCCAACTG--------CAGTTGGGGCACTAC**|**CGCGTGCATCCGTCT**

>ΔProx ΔP2Zld

----------attP-loxP-dsRed-loxP-attP----------

ACGAAACTGCCCACG|**GTAGTGCCCCAACTG--------CAGTTGGGGCACTAC**|**ACCTGAGCGATATAT**

>ΔDist

----------attP-loxP-dsRed-loxP-attP----------

CAATCTTCCCCTCGA|**GTAGTGCCCCAACTG--------GCAGGTGTGCATATG**|**AACAGGAGATTGAAT**

Table S2. Related to Figure 2, 3, 5, and STAR Methods. Sequences at the junctions of promoter deletions and replacements tested in dual reporter experiments

>P1 replaced by P2

----------516 bp----------

ACTGTTGTGAGGAGC|TGCATCCGTCTACCT--------GAACTGGGAGACGAC|CCATGGTGATCACCT

>P1C replaced by P2C

----------120 bp----------

ACTGTTGTGAGGAGC|TGCATCCGTCTACCT--------ATATACTTGCAAATC|CCAAACATCCAAAGG

>P1C/2 replaced by P2C/2

----------59 bp----------

ACTGTTGTGAGGAGC|TGCATCCGTCTACCT--------ATTGTTCAGTCAGTC|TCCGAGTCCCGAAAA

>P2 replaced by P1

----------546 bp----------

TAATCCCTTGACGCG|AGTGAGGAGATTTTC--------ACTGTCCGCAAGGTA|CTCGAGATCTGTCGA

>P2C replaced by P1C

----------120 bp----------

TAATCCCTTGACGCG|AGTGAGGAGATTTTC--------GAAATACAAAAATAA|CTTACGAAAATCCCG

Green represents sequence from P1 and red represents sequence from P2.

Table S3. Related to Figure 4 and STAR Methods. Core promoter sequences for constructs shown in Figure 4.

P1 core

+1

AGTGAGGAGATTTTCAGCTATTAGAAGAGCCCGCTGAGCGTGAGTTTGGTCAGTTGTGCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

P2 core

TGCATCCGTCTACCTGAGCGATATATAAACTAATGCCTGTTGCAATTGTTCAGTCAGTCACGAGTTTGTTACCACTGCGACAACACAACAGAAGCAGCACCAATAATATACTTGCAAATC

P2C △TATA at P1

TGCATCCGTCTACCTGAGCGATAGAAGAGCTAATGCCTGTTGCAATTGTTCAGTCAGTCACGAGTTTGTTACCACTGCGACAACACAACAGAAGCAGCACCAATAATATACTTGCAAATC

P2C △Zld(S) at P1

AGTGAGGAGATTTTCAAGCGATATATAAACTAATGCCTGTTGCAATTGTTCAGTCAGTCACGAGTTTGTTACCACTGCGACAACACAACAGAAGCAGCACCAATAATATACTTGCAAATC

P1 + Zld(S)

AGTGAGGAGCTACCTGGCTATTAGAAGAGCCCGCTGAGCGTGAGTTTGGTCAGTTGTGCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

P1 change Zld(W->S)

AGTGAGGAGATTTTCAGCTATTAGAAGAGCCCGCTGAGCGTGAGTTTGGTCAGTTGTGCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGTAGCCACTGAAATACAAAAATAA

P1 + Zld(S) + TATA

AGTGAGGAGCTACCTGGCTATTATAAGAGCCCGCTGAGCGTGAGTTTGGTCAGTTGTGCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

P1 change Zld(W->S) + TATA

AGTGAGGAGATTTTCAGCTATTATAAGAGCCCGCTGAGCGTGAGTTTGGTCAGTTGTGCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGTAGCCACTGAAATACAAAAATAA

P1 + Zld(S) + TATA + InrInr

AGTGAGGAGCTACCTGGCTATTATATAAACCCGCTGAGCGTGAGTTTGGTCAGTCAGTCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

P1 + Zld(W) + TATA + InrInr

AGTGAGGAGCTGCCTGGCTATTATATAAACCCGCTGAGCGTGAGTTTGGTCAGTCAGTCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

P1 + Zld(S) + 10bp + TATA + InrInr

AGTGCTACCTGTTTCAGCTATTATATAAACCCGCTGAGCGTGAGTTTGGTCAGTCAGTCTCCGAGTCCCGAAAACGAAAGTCGCCAGCATTGACAGGCAGCCACTGAAATACAAAAATAA

Green represents sequence from P1 and red represents sequence from P2. Motifs (TATA box, Inr, DPE, strong and weak Zld sites) are underlined. +1 indicates the position of TSS for each promoter.