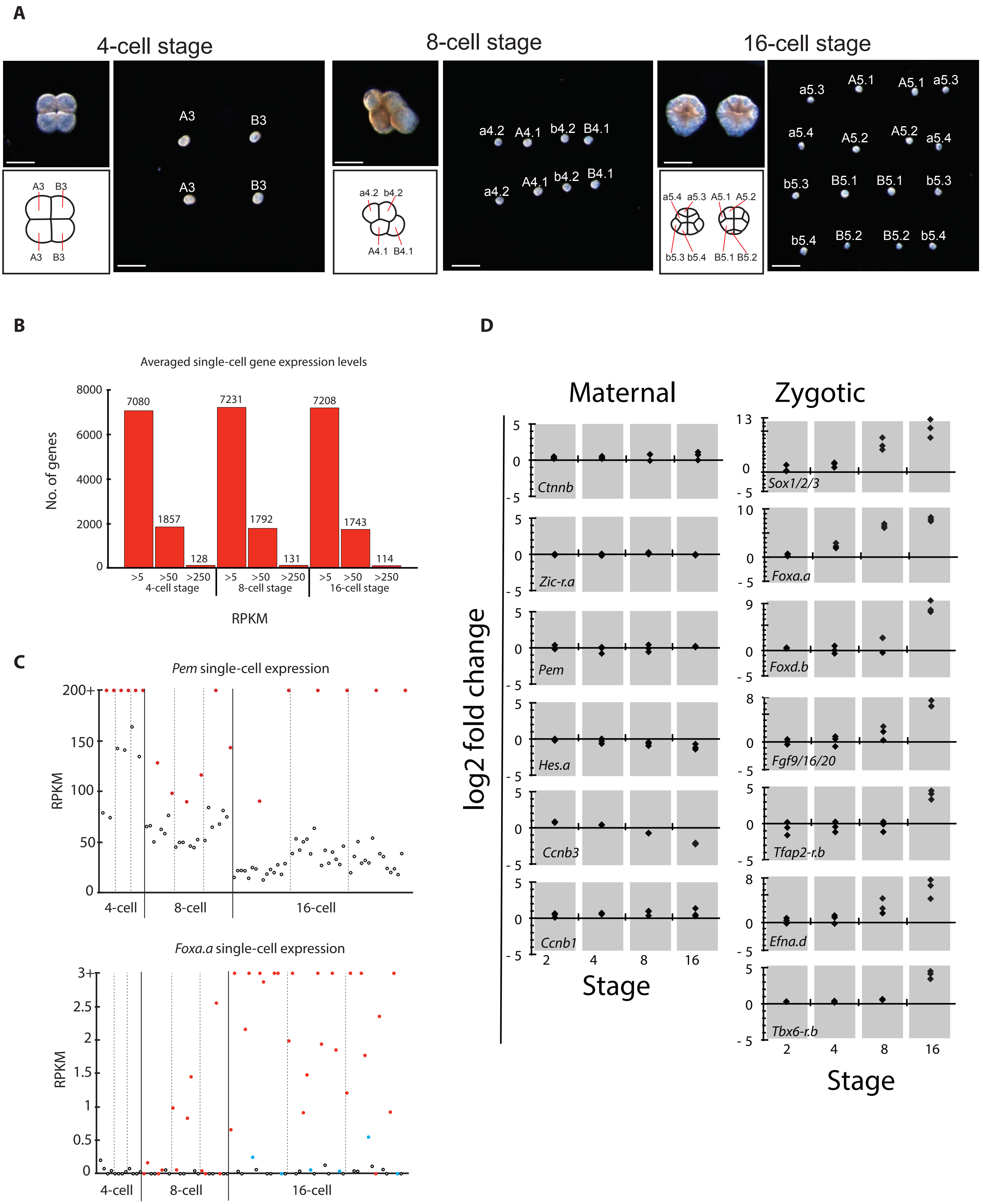
A
4-cell stage
A3
B3
A3
B3
A3
B3
A3
B3
8-cell stage
a4.2
b4.2
A4.1
B4.1
a4.2
A4.1
b4.2
B4.1
a4.2
A4.1
b4.2
B4.1
16-cell stage
a5.4
a5.3
A5.1
A5.2
b5.3
b5.4
B5.1
B5.2
a5.3
A5.1
A5.1
a5.3
a5.4
A5.2
A5.2
a5.4
b5.3
B5.1
B5.1
b5.3
b5.4
B5.2
B5.2
b5.4
B
Averaged single-cell gene expression levels
No. of genes
8000
6000
4000
2000
0
7080
1857
128
7231
1792
131
7208
1743
114
>5
>50
>250
4-cell stage
8-cell stage
16-cell stage
RPKM
C
Pem single-cell expression
RPKM
200+
150
100
50
0
4-cell
8-cell
16-cell
Foxa.a single-cell expression
RPKM
3+
2.5
2
1.5
1
0.5
0
4-cell
8-cell
16-cell
D
Maternal
Zygotic
log2 fold change
Ctnnb
Zic-r.a
Pem
Hes.a
Ccnb3
Ccnb1
Sox1/2/3
Foxa.a
Foxd.b
Fgf9/16/20
Tfap2-r.b
Efna.d
Tbx6-r.b
5
0
- 5
13
10
9
8
2
4
8
16
Stage
Stage

Figure S1. *Ciona* embryo single dissociation and gene expression. Related to Figure 1.
(A) Images of intact embryos and embryos that have been dissociated to single-cells at the 4, 8 and 16 cell stages. Each cell is labelled to indicate its identity. 4- and 8-cell intact embryos are oriented anterior left. Two individual 16-cell embryos are oriented anterior up with the embryo on the left showing the animal hemisphere and the embryo on the right showing the vegetal hemisphere. Scale bar = 100 µm.
(B) The number of genes that are expressed at or beyond specific RPKM cutoffs averaged over single-cell gene expression levels at the 4-, 8-, and 16-cell stages.
(**C)** Single-cell expression levels for Pem and Foxa.a. Cells from individual embryos are separated by dotted lines. Cells that are known to show a positive in situ hybridization signal for the gene is indicated by a solid red dot and cells that are known to not show in situ hybridization signals are indicated by hollow dots. For *Foxa.a*, the B5.1 cells, known to show a weaker in situ hybridization signal, are indicated by solid blue dots. The identities of the cells for each embryo on the graph are (from left to right) 4-cell: A3, B3, A3, B3 8-cell: a4.2, A4.1, b4.2, B4.1, a4.2, A4.1, b4.2, B4.1 and 16-cell: a5.3, a5.4, b5.3, b5.4, A5.1, A5.2, B5.1, B5.2, a5.3, a5.4, b5.3, b5.4, A5.1, A5.2, B5.1, B5.2.
(D) Expression levels of genes from the 2- to 16-cell stages relative to the expression level at the early 1-cell stage determined by qPCR. Individual diamonds denote a single measurement from pooled total RNA purified from eggs collected from a single adult.

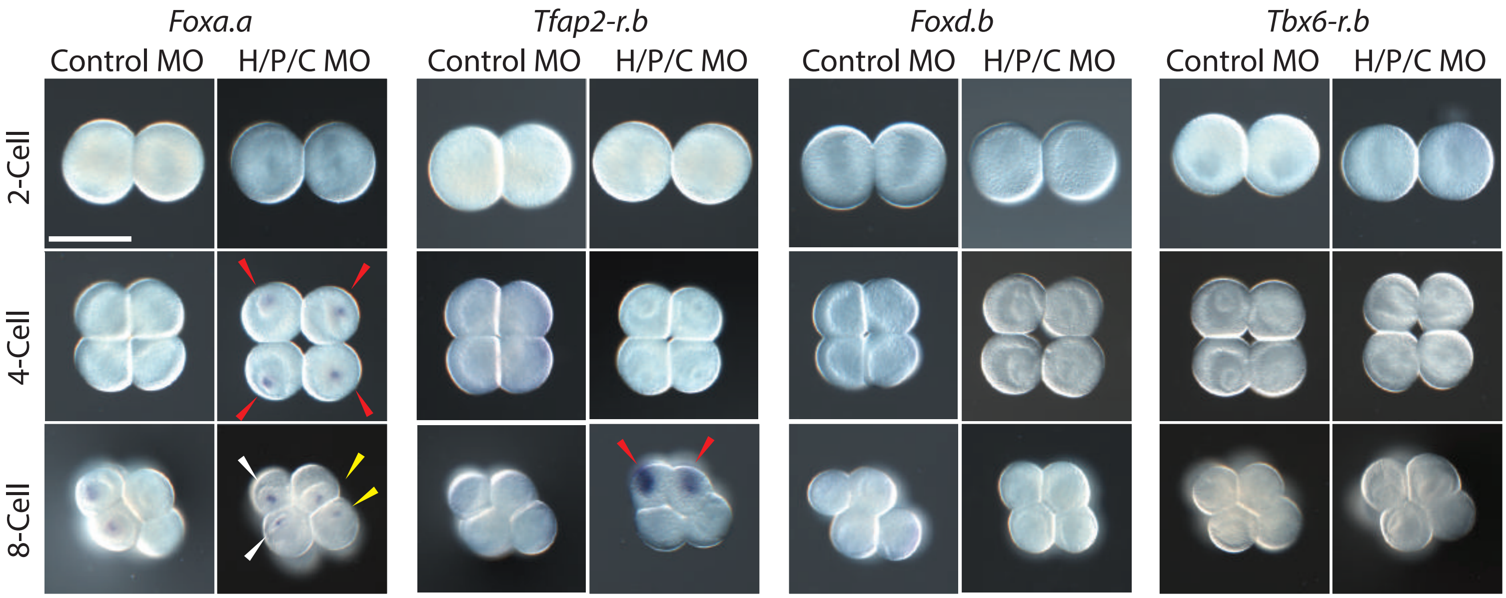

Figure S2. *Hes.a*, *Pem* and *Ccnb3* triple knockdowns. Related to Figure 2.
In situ hybridizations of *Foxa.a*, T*fap2-r.b*, *Foxd.b* and T*bx6-r.b* in early *Ciona* embryos simultaneously injected with MOs targeting *Hes.a*, *Pem* and *Ccnb3* (H/P/C). Expression in cells matching controls are indicated with a white arrowhead. The expansion of expression into additional cells is indicated with a yellow arrowhead. Precocious expression is indicated with a red arrowhead. 4- and 8- cell stage embryos are orientated anterior left. Scale bar = 50 µm.

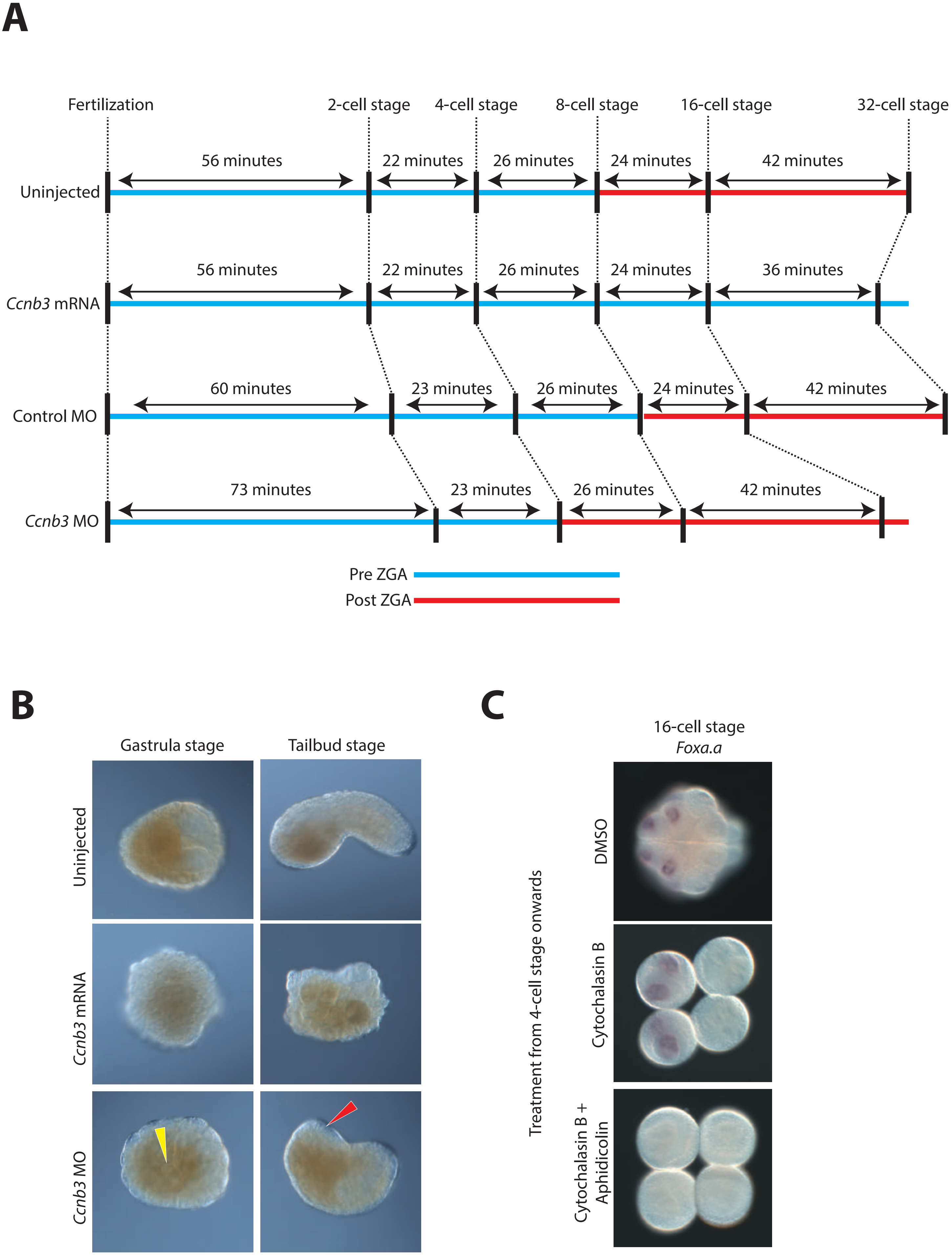
A
Fertilization
2-cell stage
4-cell stage
8-cell stage
16-cell stage
32-cell stage
Uninjected
56 minutes
22 minutes
26 minutes
24 minutes
42 minutes
Ccnb3 mRNA
56 minutes
22 minutes
26 minutes
24 minutes
36 minutes
Control MO
60 minutes
23 minutes
26 minutes
24 minutes
42 minutes
Ccnb3 MO
73 minutes
23 minutes
26 minutes
42 minutes
Pre ZGA
Post ZGA
B
Gastrula stage
Tailbud stage
Uninjected
Ccnb3 mRNA
Ccnb3 MO
C
16-cell stage
Foxa.a
Treatment from 4-cell stage onwards
DMSO
Cytochalasin B
Cytochalasin B + Aphidicolin

Figure S3. *Ccnb3* is essential for ZGA during early *Ciona* development. Related to Figures 2-3.
(A) A depiction of the cell cycle lengths for the first 170 minutes of development for normal *Ciona* embryos and in embryos where *Ccnb3* was perturbed. The start of each stage developmental stage is considered to be the point when cleavage is completed.
(B) Effects of *Ccnb3* perturbation at later gastrula and tailbud stages. All morphogenetic processes have failed in *Ccnb3* mRNA overexpressions. In *Ccnb3* MO knockdowns gastrulation succeeded but neurulation failed. The yellow arrowhead indicates the position of the blastopore. The red arrowhead indicates the unfused neural folds. Embryos are oriented anterior left except for *Ccnb3* mRNA injections where the embryos could not be oriented to any axis. Scale bar = 100 µm.
(C) *Foxa.a* expression in *Ciona* embryos treated from the 4-cell stage onwards with cyctochalasin B by itself or in combination with aphidicolin. The embryos are oriented anterior left.

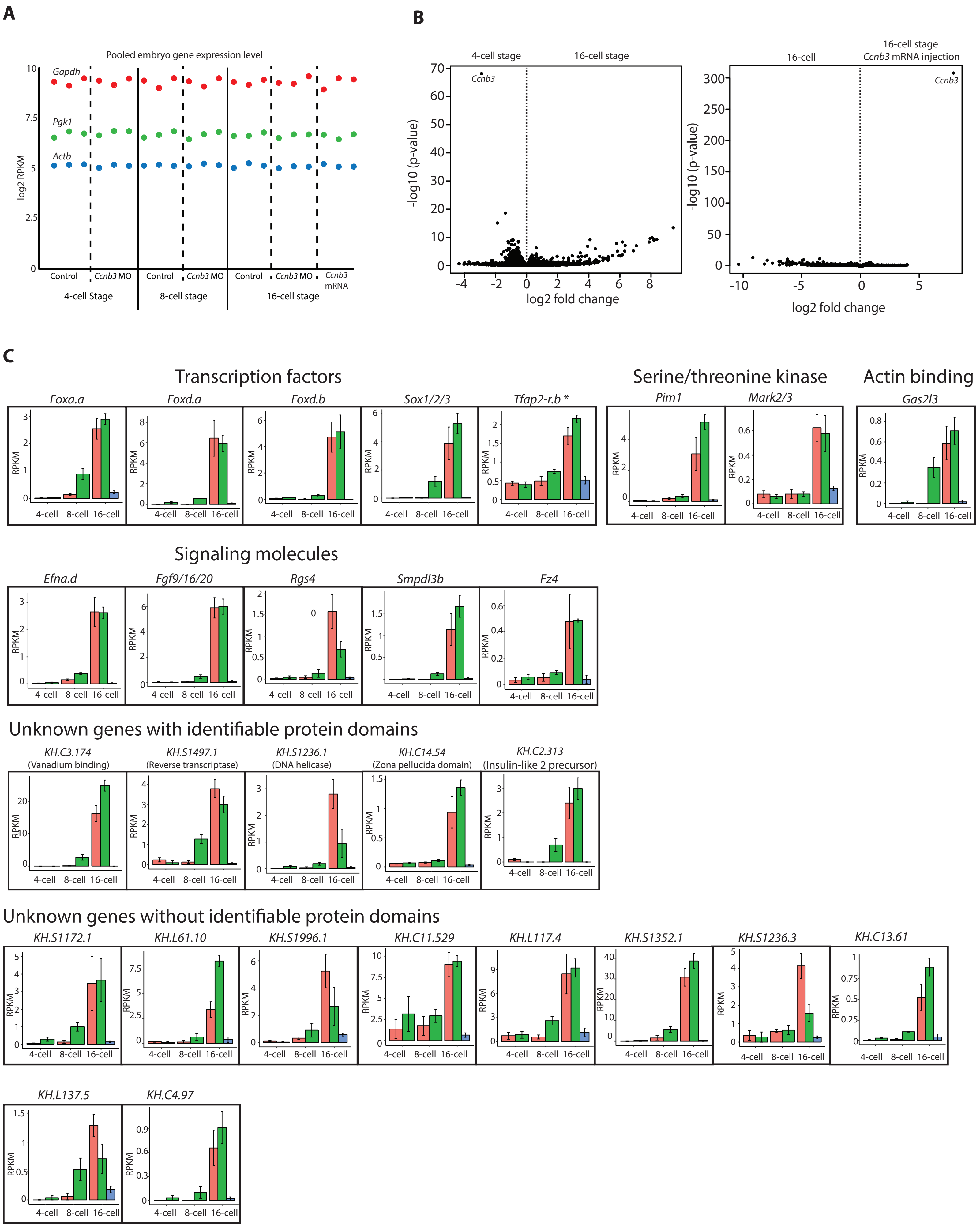
A
Pooled embryo gene expression level
log2 RPKM
10
7.5
5
2.5
0
Gapdh
Pgk1
Actb
Control
Ccnb3 MO
Control
Ccnb3 MO
Control
Ccnb3 MO
Ccnb3 mRNA
4-cell Stage
8-cell stage
16-cell stage
B
4-cell stage
16-cell stage
Ccnb3
-log10 (p-value)
70
60
50
40
30
20
10
0
-4
-2
0
2
4
6
8
log2 fold change
16-cell
16-cell stage
Ccnb3 mRNA injection
Ccnb3
300
250
200
150
100
50
0
-10
-5
0
5
log2 fold change
C
Transcription factors
Foxa.a
Foxd.a
Foxd.b
Sox1/2/3
Tfap2-r.b *
Serine/threonine kinase
Pim1
Mark2/3
Actin binding
Gas2l3
Signaling molecules
Efna.d
Fgf9/16/20
Rgs4
Smpdl3b
Fz4
Unknown genes with identifiable protein domains
KH.C3.174
(Vanadium binding)
KH.S1497.1
(Reverse transcriptase)
KH.S1236.1
(DNA helicase)
KH.C14.54
(Zona pellucida domain)
KH.C2.313
(Insulin-like 2 precursor)
Unknown genes without identifiable protein domains
KH.S1172.1
KH.L61.10
KH.S1996.1
KH.C11.529
KH.L117.4
KH.S1352.1
KH.S1236.3
KH.C13.61
KH.L137.5
KH.C4.97
RPKM
4-cell
8-cell
16-cell

Figure S4. Pooled embryo RNA-seq analysis of *Ccnb3* perturbations of zygotic genes in *Ciona*. Related to Figure 4.
(A) Pooled embryo expression levels of *Gapdh*, *Pgk1* and *Actb* from the 4 to 16-cell stages.
(B) Volcano plot depicting the same data as in Figure 4 of the main text but with *Ccnb3* included.
(C) Expression levels of genes identified as significantly affected by *Ccbn3* perturbation at the 16-cell stage or upregulated from the 4- to 16-cell stage in normal *Ciona* development based on differential expression anaylsis. Each stage and experimental condition was performed in biological triplicate (n=3) with embryos from different parent animals. *Tfap2-r.b* was not identified by the analysis but is indicated for reference.
Genes are grouped based on their known or predicted functions. Error bars indicate ± the standard error of the mean.

Species	Gene	Method	Reference
Human	Ccnb3	Single cell RNA-seq	[S1]
Mouse	Ccnb3	Single cell RNA-seq	[S2]
Xenopus	Ccnb1, Ccnb2	Developmentally staged RNA-seq	[S3]
Sea urchin	CycB, CycB3	Developmentally staged RNA-seq	[S4]
Drosophila	CycB	Fluorescent i*n situ* hybridization	[S5]
C. elegans	Cyb-2.1	Single cell RNA-seq	[S6]

Table S1. List of organisms that display diminishing levels of Cyclin B genes correlating with the onset of zygotic transcription. Related to Figure 4.

Gene names	Synonyms	KH gene model (2012)	MO	MO reference	qPCR Primer 1	qPCR Primer 2	qPCR reference	ISH clone
Foxa.a	*FoxA*	KH.C11.313			CACCATCAGTGAATGACACTA	GAGACGGAGTGGACACTGA	[S8]	GC13a23
Sox1/2/3	*SoxB1*	KH.C1.99			AGCAGAGAGTGAAACGACCA	CCGTTTCGCTTCGTCGATAA	this study	
Foxd.b	*FoxD-b*	KH.C8.396			GCCTTGTTTTGGCAGAACTTA	GGTTTCACAGCTGCCACTT	[S8]	GC29n08
Fgf9/16/20	*Fgf9*	KH.C2.125			CTTTCCGACAAGGAAGCTAT	GCGTTCGTCTGCTGTGAAA	[S8]	
Tfap2-r.b	*AP2*	KH.C7.43			CCAACGACCTCTTACACATTTCAG	GATAACGCAGCATCTCCGTTAAGT	this study	GC25n01
Efna.d	*EphA*	KH.C3.716			CGTTGCTGGTCATGCTTACA	AGGTCACTCTGACTCATGCC	this study	
Tbx6-r.b	*Tbx6b*	KH.S654.3			AACGACCACATCTCCGTTTTA	AGTGCCGCATGCAGTAGTAA	[S8]	GC09i19
Ctnnb	*Beta catenin*	KH.C9.53			AGCTCCGTCAGTTCAGTTCA	AAAGTCCATGGGTTGTTGGC	this study	
Zic-r.a	*Macho1*	KH.C1.727			CTGCATTCGTCGCATCAGCA	AGCGTCTGCGTTCTCGTACA	this study	
Pem	*PEM*	KH.C1.755	ATACTGTGCATGTTTACATTCATAT	[S7]	TTCTCCGGCAACTGGAATCT	TGAGGTGTTGAGGTAACGCT	this study	
Ccnb3	*Cyclin B3*	KH.L139.17	ACGAGGCATATTTATAGGCAGCTAT	this study	TTTATCGCCGTAGCCGCAGT	ACGGGAACTTTACCAGTGCGA	this study	
Hes.a	*HesA*	KH.C1.159	TTCTTCGTTCAACAGGCATGATTGT	[S8]	ACCCTAACGTTCCCACCAACC	CCGATGACGTAGGTGAGGGT	this study	
Ccnb1	*Cyclin B1*	KH.C4.213			AAACCCACAACTGTGTTCGG	GTGGTGAACTTGGACCAACC	this study	
Gapdh	*GAPDH*	KH.C11.121						
Pgk1	*PGK1*	KH.C14.296						
Actb	*Beta actin*	KH.C13.70						
		KH.C3.174						
Foxd.a	*FoxD-a*	KH.C8.890						
Smpdl3b	*Asm3a*	KH.C8.685						
Rgs4		KH.C11.577						
Ttll1		KH.C8.745						
Pim1		KH.C14.414						
		KH.C2.313						
		KH.S1497.1						
		KH.L61.10						
		KH.C8.299						
		KH.S1172.1						
		KH.S1236.1						
		KH.L117.4						
		KH.S1352.1						
		KH.S1996.1						
		KH.C11.529						
		KH.C14.54						
Mark2/3		KH.C11.697						
		KH.C4.97						
		KH.L137.5						
		KH.C13.61						
Fz4		KH.C6.162						
Gas2l3		KH.L152.12						

Table S2. List of gene names and reagents use. Related to STAR Methods.

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