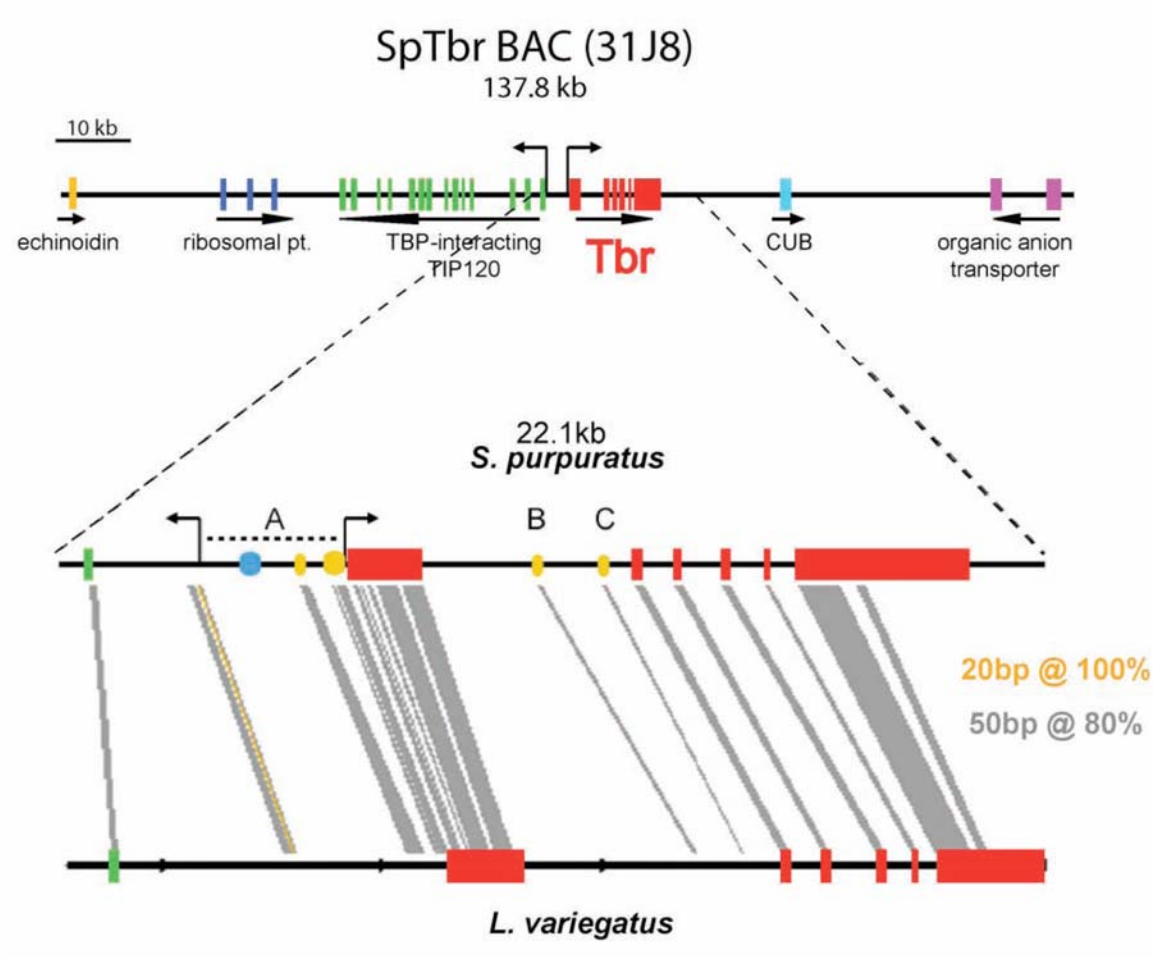
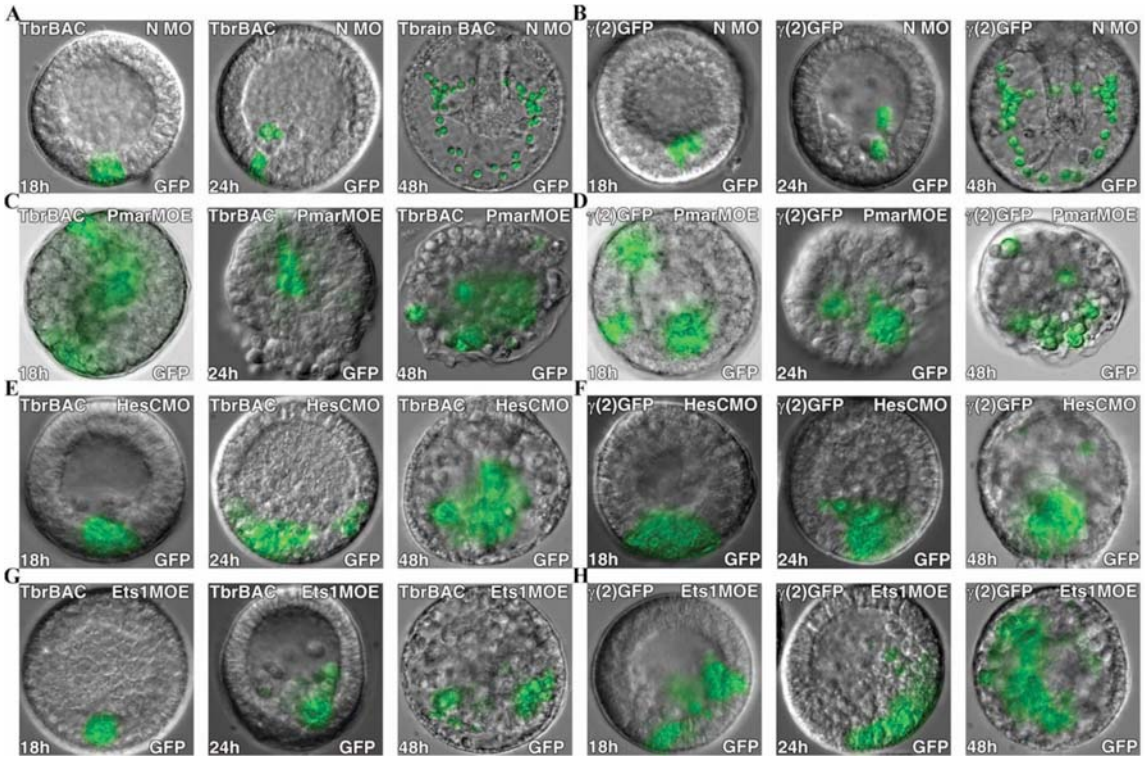
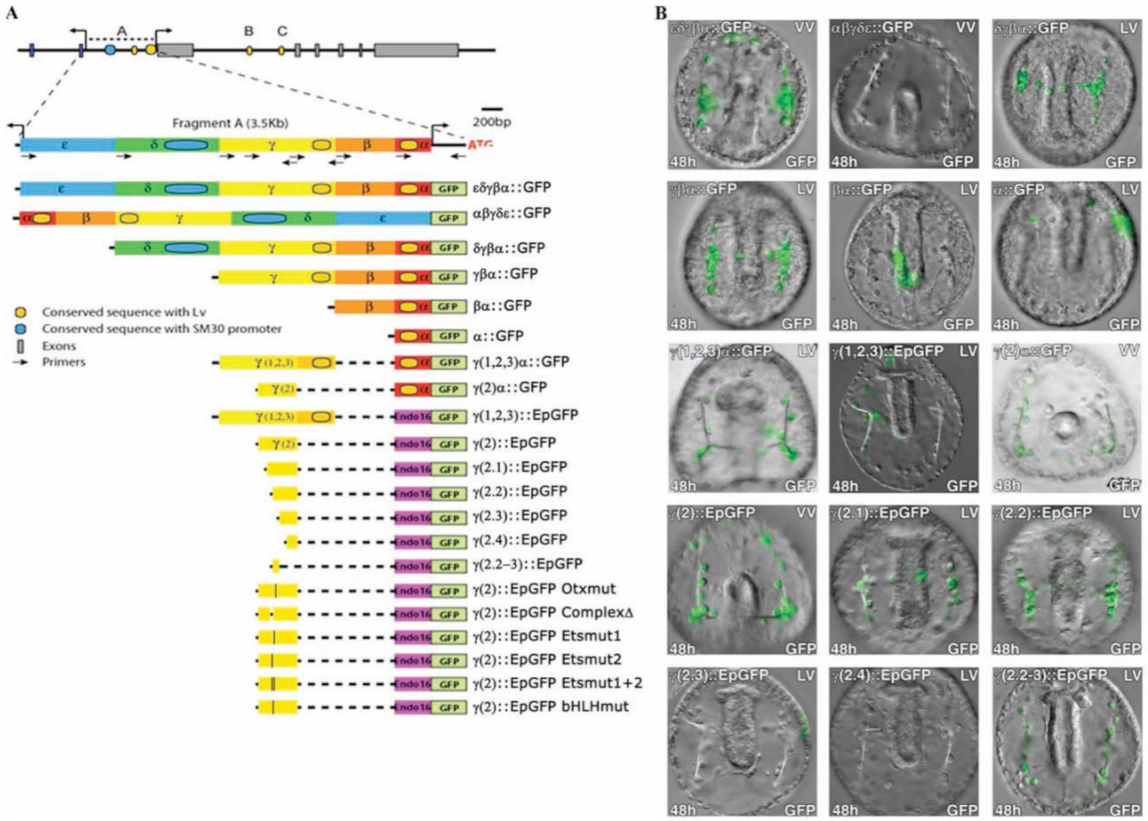


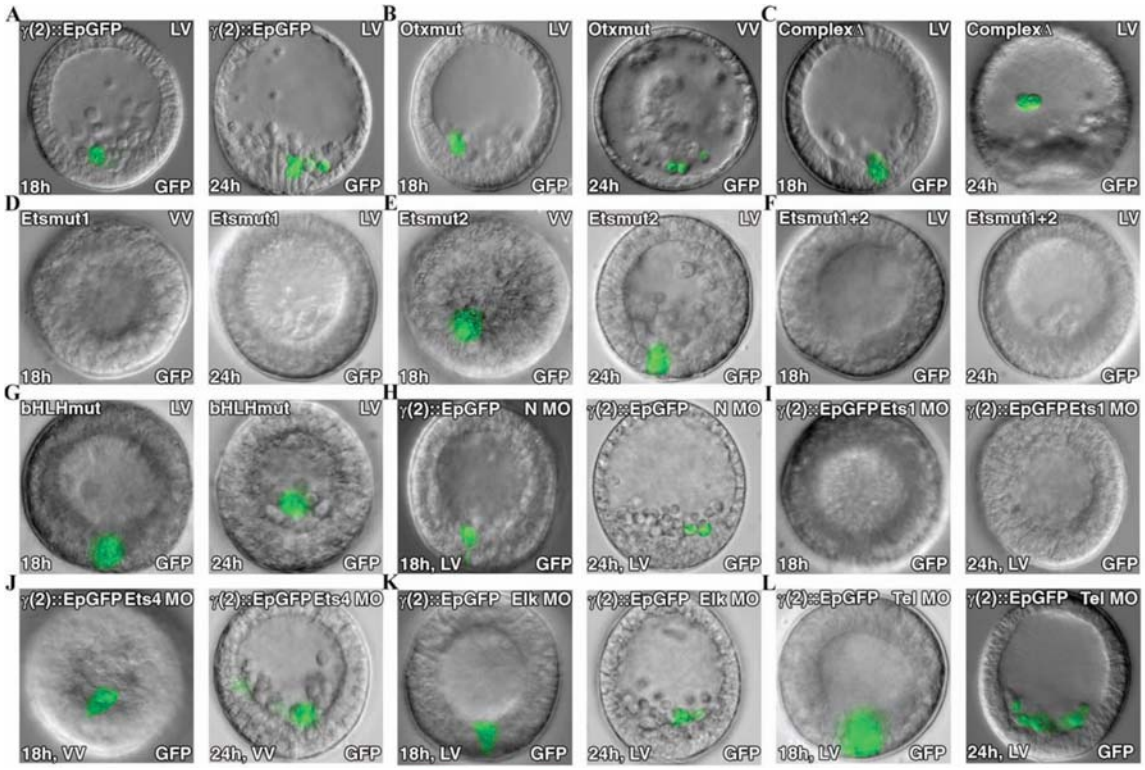
Supplementary Figure 1. Genomic sequence alignment between *S. purpuratus* and *L. variegatus*. The interspecific sequence comparison was done using FamilyRelationsII (see Materials and methods). The *tbrain* basal promoter and α region, and the predicted B and C regulatory modules, but not the γ(2) module (blue oval), are conserved at the criteria used. The B and C modules were identified from sequence alignment (Ochiai et al., 2008), while γ(2) was identified through sequential deletion from a reporter construct driven by the region denoted "A". A conserved region in "A" containing trimeric repeats (yellow oval right of γ(2)) was not found to have regulatory function (data not shown).



Supplementary Figure 2. Responses of *tbr::GFP* BAC and *γ(2)::EpGFP* to upstream perturbations. (A,C,E,G), *tbr::GFP* BAC; (B,D,F,H), *γ(2)::EpGFP;* times indicated in each panel. (A,B), Control (randomized) MASO (N MO); (C,D) global overexpression of *pmar1* mRNA (MOE); (E,F), *hesC* MASO (MO); (G,H), *ets* MOE. All views are lateral except where ectopic mesenchymal transition has obscured embryonic axes. Scoring data are tabulated in Supplementary Table 1.



Supplementary Figure 3. Isolation and functional characterization of γ(2) module. (A) Deletions, constructs and mutations; (B) Typical results of injection of indicated constructs; quantitative scoring data are tabulated in Supplementary Tables 2 and 3. The γ(2) module was identified through successive deletions of a reporter containing the entire 5' *tbrain* intergenic region (Fragment "A", top). Fragment A produced PMC-specific reporter expression in the sense orientation but had no transcriptional activation activity in the antisense orientation, indicating that it lacked regulatory function for the nearby upstream gene (see Fig. S1). Constructs including the γ region drove SM-specific GFP expression, so fragments of this region were examined for regulatory activity. Reporters were constructed using the basal promoter (Ep) of *endo16* (Yuh et al., 1996). We found that **γ**(2), and at lower levels γ(2.2-3), drove SM-specific GFP expression.



Supplementary Figure 4. *Cis*-regulatory analysis of *γ(2)::EpGFP*. (A, H-L), intact *γ(2)::EpGFP*; (B-G), mutated derivatives of *γ(2)::EpGFP*. Observations were at 18 and 24hpf as indicated; VV, vegetal view, LV, lateral view. See Fig. S3A and text Fig. 7 for mutations. Representative embryos are reproduced; scoring data are tabulated in Supplementary Table 3. (A), control; (B), Otx site mutation; (C), Mutation of 31bp region forming gel shift complex; (D), Ets1/2 site 1 mutation; (E), Ets1/2 site 2 mutation; (F), mutation of both Ets1/2 sites; (H), Control randomized MASO; (I), *ets1/2* MASO; (J), *ets4* MASO; (K), *elk* MASO; (L), *tel* MASO.

Supplemental Table 1. Response of Tbrain reporter constructs to perturbation of the Pmar-HesC double negative PMC specification gate

Stage	Construct	Perturbation	Total embryos scored	GFP+ embryos (% of total)			Embryos misexpressing GFP (% of GFP+)		
				#	%	SEM (%)	#	%	SEM (%)
Blastula (18h)	Tbrain BAC GFP	—	110	26	23.6	9.0	0	0.0	6.0
		Random MO	342	191	55.8	18.4	1	0.5	0.6
		Pmar1 MOE	110	88	80.0	11.4	30	34.1	12.4
		HesC MO	406	334	82.3	4.6	31	9.3	0.4
		Ets1/2 MOE	366	255	69.7	8.2	5	2.0	0.9
	γ(2)::EpGFP	—	186	45	24.2	18.7	6	13.3	7.2
		Random MO	334	144	43.1	23.7	2	1.4	1.5
		Pmar1 MOE	93	74	79.6	14.8	33	44.6	2.5
		HesC MO	339	258	76.1	11.2	18	7.0	6.4
		Ets1/2 MOE	286	216	75.5	9.9	65	30.1	36.2
Mesenchyme blastula (24h)	Tbrain BAC GFP	—	186	79	42.5	9.8	7	8.9	13.3
		Random MO	617	483	78.3	3.3	15	3.1	0.4
		Pmar1 MOE	93	79	84.9	9.6	39	49.4	9.4
		HesC MO	553	407	73.6	7.1	98	24.1	3.4
		Ets1/2 MOE	104	98	94.2	0.1	70	71.4	15.5
	γ(2)::EpGFP	—	185	59	31.9	11.2	2	3.4	14.3
		Random MO	244	129	52.9	9.2	4	3.1	2.1
		Pmar1 MOE	96	80	83.3	7.2	36	45.0	16.8
		HesC MO	173	148	85.5	10.1	63	42.6	4.4
		Ets1/2 MOE	303	280	92.4	0.2	265	94.6	2.6
Late gastrula (48h)	Tbrain BAC GFP	Random MO	124	96	77.4	11.4	7	7.3	1.0
		HesC MO	90	77	85.6	5.3	77	100.0	0.0
		Ets1/2 MOE	53	41	77.4	2.4	41	100.0	0.0
	γ(2)::EpGFP	Random MO	133	108	81.2	6.8	14	13.0	2.9
		HesC MO	90	83	92.2	9.1	83	100.0	0.0
		Ets1/2 MOE	86	80	93.0	6.8	79	98.8	1.3

Supplemental Table 2. Identification of γ (2) *cis*-regulatory module in the Tbrain 5′ intergenic region through serial deletion

Stage	Construct	Total embryos scored	GFP+ embryos (% of total)			Embryos misexpressing GFP (% of GFP+)		
			#	%	SEM (%)	#	%	SEM (%)
Mesenchyme blastula (24h)	εδγβα::GFP	411	137	33.3	17.5	42	30.7	5.9
	αβγδε::GFP	82	11	13.4	0.8	6	54.5	1.7
	δγβα::GFP	177	50	28.2	20.7	21	42.0	18.8
	γβα::GFP	149	113	75.8	8.0	39	34.5	7.4
	βα::GFP	134	108	80.6	19.4	35	32.4	2.7
	α::GFP	98	54	55.1	18.3	21	38.9	3.0
	γ(1,2,3)α::GFP	210	93	44.3	18.9	12	12.9	3.8
	γ(2)α::GFP	299	157	52.5	19.3	12	7.6	6.4
	γ(1,2,3)::EpGFP	390	79	20.3	24.0	9	11.4	3.0
	γ(2)::EpGFP	247	114	46.2	18.9	10	8.8	1.1
Late gastrula (48h)	εδγβα::GFP	316	161	50.9	17.5	48	29.8	8.0
	αβγδε::GFP	76	3	3.9	5.4	2	66.7	0.7
	δγβα::GFP	101	54	53.5	13.4	35	64.8	17.5
	γβα::GFP	213	191	89.7	9.0	46	24.1	13.5
	βα::GFP	213	170	79.8	17.3	92	54.1	9.8
	α::GFP	109	62	56.9	16.7	20	32.3	2.3
	γ(1,2,3)α::GFP	254	103	40.6	10.9	64	62.1	10.8
	γ(2)α::GFP	218	99	45.5	15.5	52	52.5	6.9
	γ(1,2,3)::EpGFP	199	67	33.7	17.9	32	47.8	6.7
	γ(2)::EpGFP	219	82	37.4	14.4	20	24.4	9.4

Supplemental Table 3. A 71bp fragment of γ (2) sufficient to drive PMC-specific expression contains two functional Ets factor binding sites

Construct	Total embryos scored	GFP+embros (%of total)			Embryos misexpressinf GFP (% of GFP+)		
		#	%	SEM (%)	#	%	SEM (%)
γ(2)::EpGFP	444	173	39.0	8.1	4	2.3	1.6
γ(2.1)::EpGFP	140	66	47.1	13.6	3	4.5	2.3
γ(2.2)::EpGFP	258	64	24.8	10.1	0	0.0	0.0
γ(2.3)::EpGFP	169	12	7.1	0.9	7	58.3	21.3
γ(2.4)::EpGFP	241	1	0.4	0.0	1	100.0	0.0
γ(2.2-3)::EpGFP	282	63	22.3	7.3	5	7.9	5.9
γ(2)::EpGFP Otxmut	268	78	29.1	3.5	0	0.0	0.0
γ(2)::EpGFP ComplexD	191	20	10.5	9.2	0	0.0	0.0
γ(2)::EpGFP Etsmut1	406	35	8.6	5.0	3	8.6	0.5
γ(2)::EpGFP Etsmut2	96	28	29.2	11.9	3	10.7	3.9
γ(2)::EpGFP Etsmut1+2	99	3	3.0	4.0	3	100.0	0.0
γ2)::EpGFP bHLHmut	320	161	50.3	8.0	13	8.1	3.3
γ(2)::EpGFP + N MO	448	206	46.0	12.6	3	1.4	2.0
γ(2)::EpGFP + Ets1/2 MO	282	13	4.6	7.3	2	0.7	10.0
γ(2)::EpGFP + Erg MO	199	157	78.9	0.2	48	24.1	0.9
γ(2)::EpGFP + Ets4 MO	95	59	62.1	11.7	0	0.0	0.0
γ(2)::EpGFP + Elk MO	200	47	23.5	9.5	7	14.9	12.1
γ(2)::EpGFP + Tel MO	212	57	26.9	10.5	1	1.8	1.4