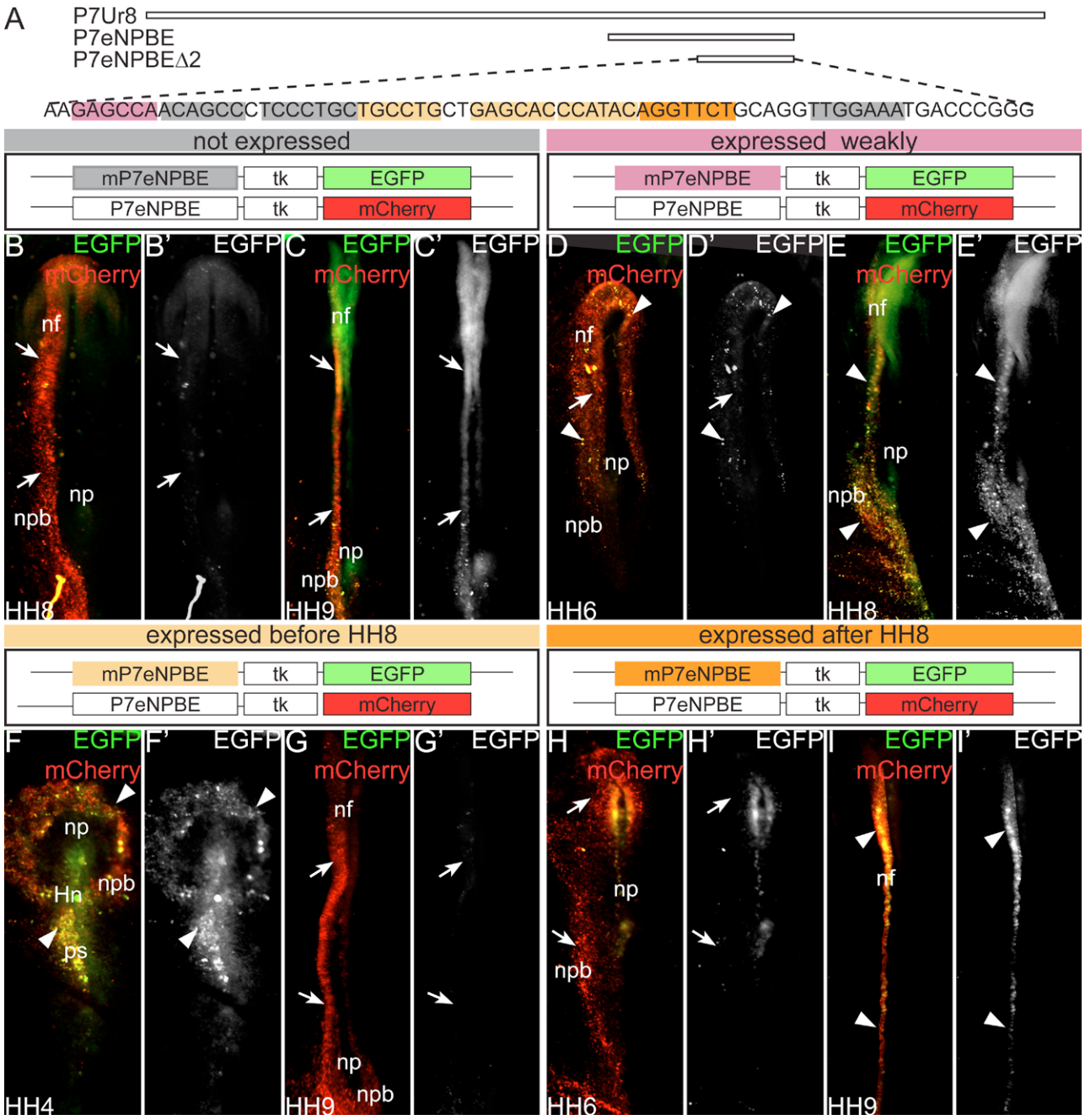

Fig. S1. Mutational analysis identified crucial bases for P7eNPBE enhancer activity. (**A**) A mutational analysis was performed to determine which binding sites are crucial for the enhancer activity of P7eNPBE. (B-I′) Embryos were co-electroporated with wild-type P7eNPBE driving mCherry and mutated P7eNPBE (termed mP7eNPBE) driving EGFP to directly compare wild type with mutated enhancer activity in the same embryo. (**B-C′**) The first type of mutation completely eliminated the activity of P7eNPBE at all stages screened. Although the wild-type enhancer (red) is expressed in the neural plate and neural plate border at HH8 (B) and HH9 (C), the mutated enhancers (green) are not expressed (B′,C′, arrows). (**D-E′**) The second type of mutation retains the spatiotemporal enhancer activity, but is generally expressed less brightly or in fewer cells. The wild-type enhancer (red) is robustly expressed in the neural plate and neural plate border at HH6 (D) and HH8 (E). The mutated enhancer (green) is also expressed in the neural plate and neural plate border (D′,E′, arrowheads), but often there are many cells not expressing the enhancer as robustly or even at all (D′, arrow). (F-I′) The remaining two types of mutations had stage-specific effects, with the mutated enhancers only expressed either before HH8 (F-G′) or after HH8 (H-I′). (**F-G′**) The wild-type (red) and mutated (green) enhancers are expressed at HH4 (F,F′) surrounding Hensen's node and in the primitive streak (arrowheads). But while the wild-type enhancer expression continues in the neural folds, neural plate and neural plate border at HH9 (G), the mutated enhancers are not expressed (G′, arrows). (**H-I′**) The final type of mutation is expressed only after HH8. The wild-type enhancer (red) is expressed in the neural plate border at HH6 (H) but the mutated enhancer (green) is not (H′, arrows). However, at HH9, both forms are expressed in the neural folds and neural plate border (I,I′, arrowheads). Hn, Hensen′s node; np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; ps, primitive streak.

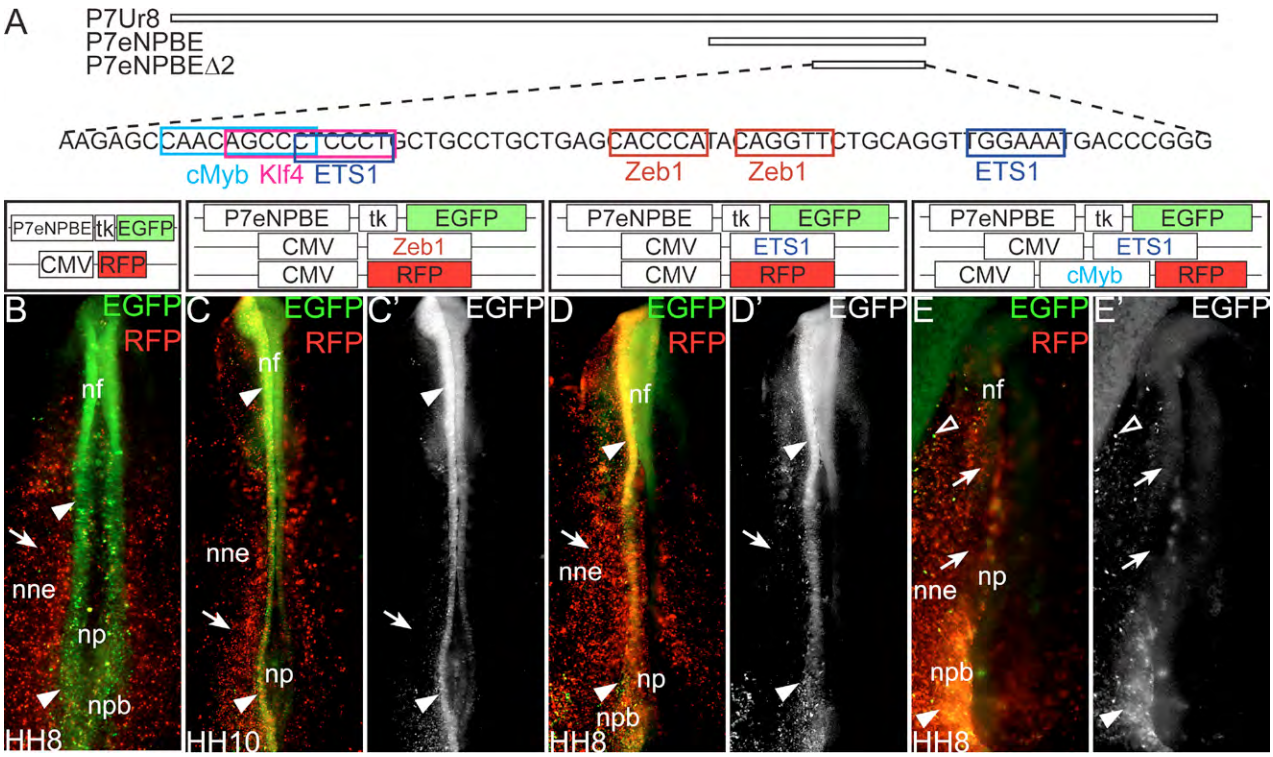

Fig. S2. Zeb1 and ETS1 are not sufficient to alter the enhancer activity of P7NCE. (**A**) Position size and sequence of the enhancer P7eNPBEΔ2 in comparison with the P7Ur8 and the P7eNPBE. Boxes in sequence from A indicate putative transcription factor-binding sites modulating enhancer activity, and include cMyb (light blue box), Klf4 (pink box), ETS1 (dark blue boxes) and Zeb1 (red boxes). (B-E′) These transcription factors were overexpressed by electroporation to assess possible effect on P7eNPBE enhancer activity. (**B**) At HH8, P7eNPBE (green) is normally robustly expressed in the neural plate and neural plate border (arrowheads) and is restricted from the non-neural ectoderm (arrows). (**C-D′**) Ectopic expression of either Zeb1 (C,C′, red) or ETS1 (D,D′, red) does not alter the normal expression of P7eNPBE (green), which is still present in the neural plate and neural plate border (arrowheads), and is restricted from the non-neural ectoderm (arrows). (**E**,**E′**) At HH8, when ETS1 and cMyb (red) are ectopically expressed together, P7eNPBE (green) expression is no longer restricted from the non-neural ectoderm (open arrowhead) and is in a ‘salt and pepper’ pattern. Although some cells in the neural plate and neural folds still express P7eNPBE (closed arrowhead), there are many cells that do not (arrows). np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds.

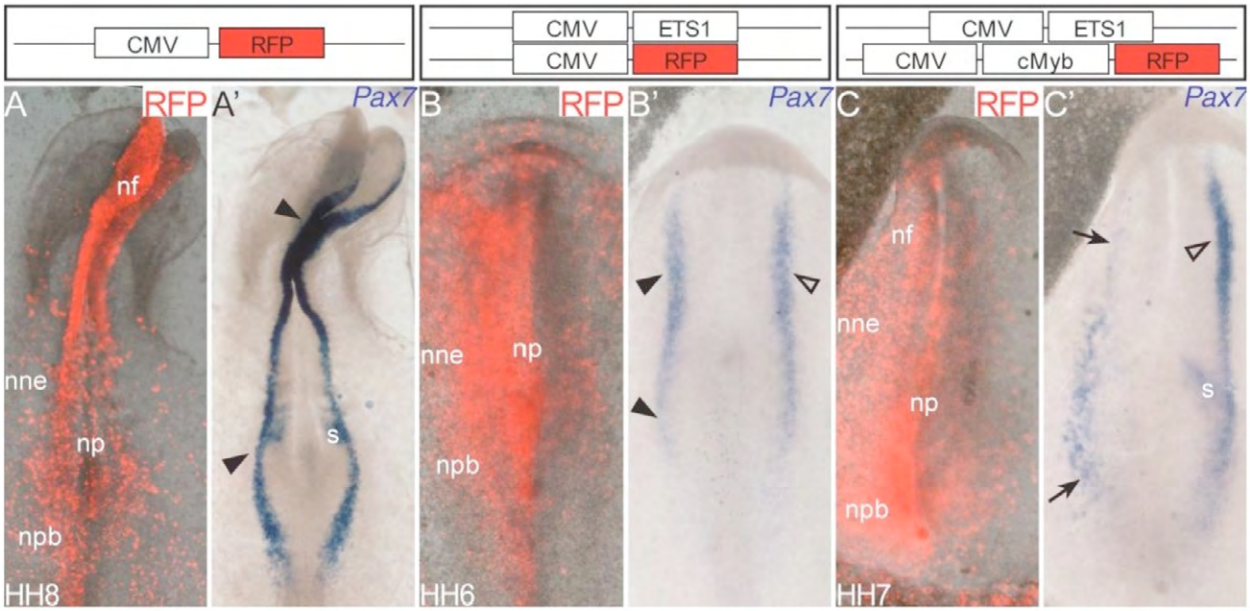

Fig. S3. Ectopic expression of ETS1 does not alter Pax7 mRNA expression. (A-C′) Empty vector control (A,A′), ETS1 (B,B′) or ETS1 and cMyb (C,C′) were ectopically expressed in embryos, which were subsequently stained for *Pax7* mRNA by in situ hybridization. (**A**,**A′**) At HH8, embryos misexpressing the control express *Pax7* (blue) in the neural folds and somites (closed arrowheads) as normal. (**B**,**B′**) At HH6, embryos misexpressing ETS1 display normal *Pax7* mRNA expression (blue) in the electroporated neural fold (closed arrowheads) when compared with the untreated neural fold (open arrowhead). (**C**,**C′**) At HH7, embryos treated with both ETS1 and cMyb show reduced levels of *Pax7* mRNA (blue) in the electroporated neural fold (arrows) and in the first forming somite (double arrowhead) compared with the untreated side (open arrowheads). The expression is not expanded into the neural plate or non-neural ectoderm. np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; s, somites.

Table S1. Summary of binding sites affected by mutagenesis

Mutation	1	2	3	4	5	6	7	8
Wild type sequence	GAGCCA	ACAGCC	TCCCTGC	TGCCTG	GAGCAC	CCATAC	AGGTTCT	TTGGAAA
Mutated sequence	TCTAAC	CACTAA	GAAAGTA	GTAAGT	TCTACA	AACGCA	CTTGGAG	GGTTCCC
Activity	+ (weak)	-	-	+/– (+ before HH8)	+/– (+ before HH8)	+/– (+ before HH8)	+/– (+ after HH8)	-
Sites deleted or moved	FOXA1 NFIC TFAP2A BRCA1 Mafb Myb	FOXA1 TFAP2A BRCA1 Mafb Myb BRCA1 Klf4	Klf4 ETS1 BRCA1 Mafb	SP1 BRCA1 Mfab Arnt::Ahr	INSM1 Arnt::Ahr Zeb1	INSM1 MZF1_5_13 Arnt::Ahr Zeb1 YY1 GATA2 FOXC1	Zeb1 SPIB	BRCA1 CEBPA ELF5 ETS1 NFIC HoxA5 NFATC2 REL REL
Sites added	GATA2 BRCA1	RUNX1 ZNF354C	ELK1 ELK1	FOXc1 AP1	ZNF354C FOXC1	RUNX1 En1	EBF1 Hltf	ESR1 En1

	GATA3	SOX10	E2F1	Arnt::Ahr	FOXL1	BRCA1	Zeb1	ESR2
	RUNX1	HoxA5	TFAP2	SPIB	Zeb1	SOX10	TFAP2A	NFKB1
	SOX10	GATA3	FEV	NKX3-1		Zfp423	NFIC	ETS1
		Pdx1	FOXC1			Zfp423	ZNF354C	
		ELF5	SP1			Arnt::Ahr	Znf143	
		FEV	NKX3-1			Hltf		
		SPI1	Prx2			ETS1		
			FOXL1			Nr2e3		
			GATA2			Nr2e3		

Sequence blocks of 6-7 bp were mutated in the enhancer P7eNPBE to determine the critical bases for enhancer activity. Putative binding sites were identified in wild-type and mutated forms of the enhancer using JASPAR (Bryne et al., 2008). All mutated forms of P7eNPBE modulated the activity, and all of them eliminated several overlapping binding sites while adding several new sites.

Table S2. Primers for *Pax7* upstream regions (putative enhancer regions)

Region	Primers (5′-3′)
P7Ur1	GAGCGGTACCCTCCTCTGAGCATTTCCACC GAGCCTCGAGTCACAACTTATTTCTCGGCG
P7Ur2	GAGCGGTACCCCTCTGAGCATTTCCACCC GAGCCTCGAGGAGCAGCATGGAAAATAGCC
P7Ur3	GAGCGGTACCATTTCCAGTTCCCATTCTGC GAGCCTCGAGACGTCCAAAGCAACTCTTCG
P7Ur4	GAGCGGTACCAGAGAAGGAAACCTCTCCCC GAGCCTCGAGGGACAAATCCTATCTGTAAGACGC
P7Ur5	GAGCGGTACCTGATGACCAAAACTGGGAGC GAGCCTCGAGCAATAATCACTGCTGCTTGGG
P7Ur6	GAGCGGTACCTAACCATGTCCCTCAGTGCC GAGCCTCGAGCACTTGGTTGTAGGAATGGG
P7Ur7	GAGCGGTACCCACCGAGTTTCACGTTAGGG GAGCCTCGAGGGAGGTTGCTACAATGAGGG
P7Ur8	GAGCGGTACCGCACAGAAAGGCAATAACCC GAGCCTCGAGAAAGCAACTATAAAACCCCGC
P7Ur9	GAGCGGTACCTTTTAGCAGTGTGTTTGCGG GAGCCTCGAGGACAGGGAAACACACCCAAC
P7Ur10	GAGCGGTACCGTTGTGTTCCATCACCTCCC GAGCCTCGAGGTTGGGTGAAAACACTTGCC
P7Ur11	GAGCGGTACCAAGGAAGAGGAAATGCAGGG GAGCCTCGAGTATTTCAGAGGGACTTCCGC
P7Ur12	GAGCGGTACCGAAGGAGCTCTCAAACACCG GAGCCTCGAGAATCACCCATACTTTCCCCC

Table S3. Primers used to generate deletions and smaller constructs from P7Ur8

Region	Primers (5'-3')
P7Ur8Δ1	AACATTTTTCCCTCTTCTTCCCCTGTGCT AGCACAGGGGAAGAAGAGGGAAAAATGTTT
P7Ur8Δ2	CCTCTCCCATTGGGGCCATCTGGGTTTTGG CCAAAACCCAGATGGCCCCAATGGGAGAGG
P7Ur8Δ3	TTTAACCTTTTCTTCGGCAGTTAGAAGGCA TGCCTTCTAACTGCCGAAGAAAAGGTTAAA
P7Ur8Δ4	CCAAAATTTCCATCTCAGGAGAGACGGAAC GTTCCGTCTCTCCTGAGATGGAAATTTTGG
P7Ur8Δ5	ATTGCAACGTGGCAGAGAGGATGAAAAGAG CTCTTTTCATCCTCTCTGCCACGTTGCAAT
P7Ur8Δ6	TGAATCTCACACAGGACAGGTTCTGCAGGT ACCTGCAGAACCTGTCCTGTGTGAGATTCA
P7Ur8Δ7	CACAAAAAGCAGAGGGAAACTCAACTCAAA TTTGAGTTGAGTTTCCCTCTGCTTTTTGTG
P7Ur8Δ8	GGAGAGCTCTGCTCGGACGAGTCCTAAGC GCTTAGGACTCGTCCGAGCAGAGCTCTTCC
P7Ur8Δ9	GAGCGGTACCGCACAGAAAGGCAATAACCC CTCAGAGCTCTGTGTGAGATTCACTGAT
P7Ur8Δ9G	CTCAGAGCTCTGTGTGAGATTCACTGAT CTGAGAGCTCTCGACTTCAAGGAGGACG CTGAGAGCTCTCCTCGATGTTGTGGCGGATCTTGAA
P7eNPBE	GACTGGTACCTTCATCCTCTGCTTTTTGTG GTAGCTCGAGTCACACAGGTCCAGGAGAGA
P7eNPBED1	GTAGCTCGAGTCACACAGGTCCAGGAGAGA ATTACCCGGGAGGTCCAGGAGAGACGGA
P7eNPBED2	ATTACDCCGGGAGCCAACAGCCCTCC GTAGCTCGAGTCACACAGGTCCAGGAGAGA

Primer pairs designed to generate and clone specific deletions (Δ) of Pax7 upstream region 8 (P7Ur8), as well as the smaller elements derived from it.

Table S4. Mutagenesis analysis of P7eNPBE

Region	Primers (5′-3′)
M1	GCTCAGCAGGCAGCAGGGAGGGCTGTGTTAGATTTTCATCCTCTGCTTTTTGTGATGC GCATCACAAAAAGCAGAGGATGAAAATCTAACACAGCCCTCCCTGCTGCCTGCTGAGC
M2	GGGTGCTCAGCAGGCAGCAGGGAGTTAGTGTGGCTCTTTTCATCCTCTGCTTTT AAAAGCAGAGGATGAAAAGAGCCACACTAACTCCCTGCTGCCTGCTGAGCACCC
M3	GCAGAACCTGTATGGGTGCTCAGCAGGCATACTTTCGGGCTGTTGGCTCTTTTCATCC GGATGAAAAGAGCCAACAGCCCGAAAGTATGCCTGCTGAGCACCCATACAGGTTCTGC
M4	GCAGAACCTGTATGGGTGCTCAGACTTACGCAGGGAGGGCTGTTGGCTCTTT AAAGAGCCAACAGCCCTCCCTGCGTAAGTCTGAGCACCCATACAGGTTCTGC
M5	CCAAACCTGCAGAACCTGTATGGTGTAGAAGCAGGCAGCAGGGAGGGCTGTT AACAGCCCTCCCTGCTGCCTGCTTCTACACCATACAGGTTCTGCAGGTTTGG
M6	TCGTTTCCAAACCTGCAGAACCTTGCGTTGTGCTCAGCAGGCAGCAGGGAGG CCTCCCTGCTGCCTGCTGAGCACAACGCAAGGTTCTGCAGGTTTGGAAACGA
M7	AGCCCGGGTCGTTTCCAAACCTGCCTCCAAGGTATGGGTGCTCAGCAGGCAGCAG CTGCTGCCTGCTGAGCACCCATACCTTGGAGGCAGGTTTGGAAACGACCCGGGCT
M8	TCTTACGCGTGCTAGCCCGGGTCGGGGAACCACCTGCAGAACCTGTATGGGTGCT AGCACCCATACAGGTTCTGCAGGTGGTTCCCCGACCCGGGCTAGCACGCGTAAGA

Mutations (red sequence) consisting of changes in groups of 6 to 7 bp at a time introduced in the P7eNPBE.

Table S5. Primers used to generate overexpression constructs

Clone	Primers (5′-3′)
Klf4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCGCCATGGCTGTCAGCGACGCG GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAATGCCTCTTCATGTG
Zeb1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCGCCATGGCGGATGGCCCCAGGTGTAA GGGGACCACTTTGTACAAGAAAGCTGGGTAGGCTTCATTTGTCTTTTC
DN-cMyb	GCGCTCTAGAAATGGGCCGGAGACCC CTTACCCGGGTTAGGAATTCCAGTGGTTCTT