nature cell biology

DOI: 10.1038/ncb1998

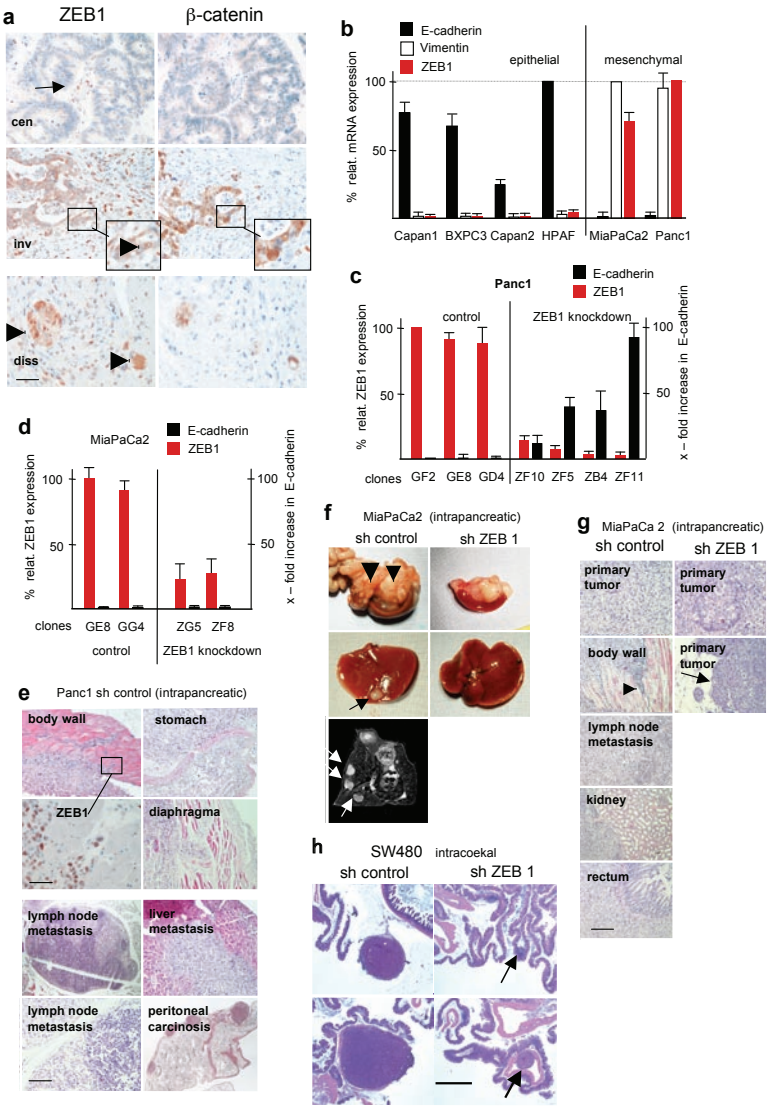


Figure S1 Loss of ZEB1 reduces aggressiveness and metastasis of cancer cells. (**a**) Immunohistochemical detection of ZEB1 and β-catenin in human colorectal cancer. (Upper row) Central (cen) differentiated areas of colorectal adenocarcinomas lack expression of ZEB1 and nuclear β-catenin. Stroma cells express ZEB1 (arrow). In contrast undifferentiated invasive (inv) tumor cell clusters (middle row) and tumor cells disseminated (diss) into surrounding tissue (Lower row) coexpress both ZEB1 (arrowheads) and nuclear β-catenin, as shown in serial section stainings of the same tumor. Boxes indicate regions shown in magnification. Size bar 50µm. (**b**) Expression of ZEB1 correlates with a mesenchymal phenotype of pancreatic cancers cells, as shown by high vimentin and low E-cadherin mRNA levels. (**c**) Stable knockdown of ZEB1 in the undifferentiated line Panc1 correlates with increased E-cadherin expression in the analysed cell clones (clone GF2 set to 100%). (**d**) Reduction of ZEB1 mRNA expression after stable knockdown of ZEB1 in the undifferentiated pancreatic cancer cell line MiaPaCa2. Note that in contrast to Panc1, E-cadherin expression is not increased. (**e**) After intrapancreatic injection in nude mice, tumors from Panc1 control knockdown clones are highly infiltrative into the indicated peritumoral organs as shown by immunohistochemistry for ZEB1 or HE stainings. Moreover, they metastasize to regional lymphnodes, the liver and make peritoneal carcinosis. ZEB1 knockdown tumors are not shown, because they are neither infiltrative nor metastasize. Size bars 50µm and 150µm. (**f**) Orthotopic (intrapancreatic) injection of 10^6 MiaPaCa2 control clones (shown for clone GE8) into nude mice results in a large, highly invasive primary tumor (arrowheads). The tumors made distant metastases to the liver, which are also shown by NMR (arrows). In contrast, tumors from ZEB1 knockdown clones (shown for clone ZF8) had reduced primary tumor size, almost no local infiltration and no distant metastasis. (**g**) HE sections of the MiaPaCa2 tumors shown in (**e**): Control tumors were undifferentiated, highly invasive (arrowhead), metastases to lymph nodes, kidney and rectum are shown. In contrast shZEB1 derived tumors are more differentiated and show an expansive, less invasive growth pattern (arrows) without metastasis. Size bar 80µm. (**h**) Orthotopic xenograft tumors of the colorectal cancer cell line SW480 are much smaller after ZEB1 knockdown (arrows) compared to controls, shown are each two different tumors. Size bar 200µm. All quantitative values show mean ± s.d., n = 3.

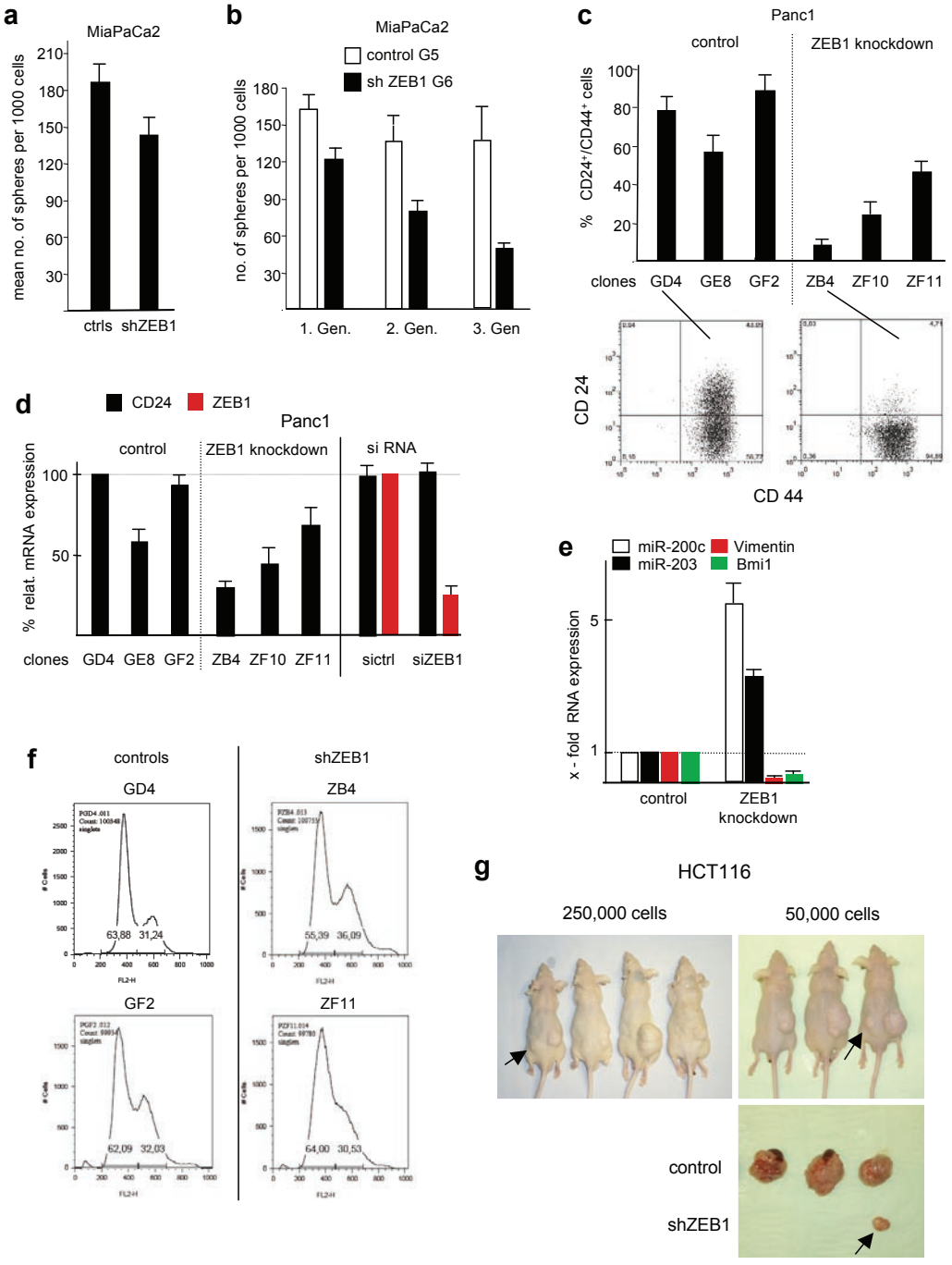
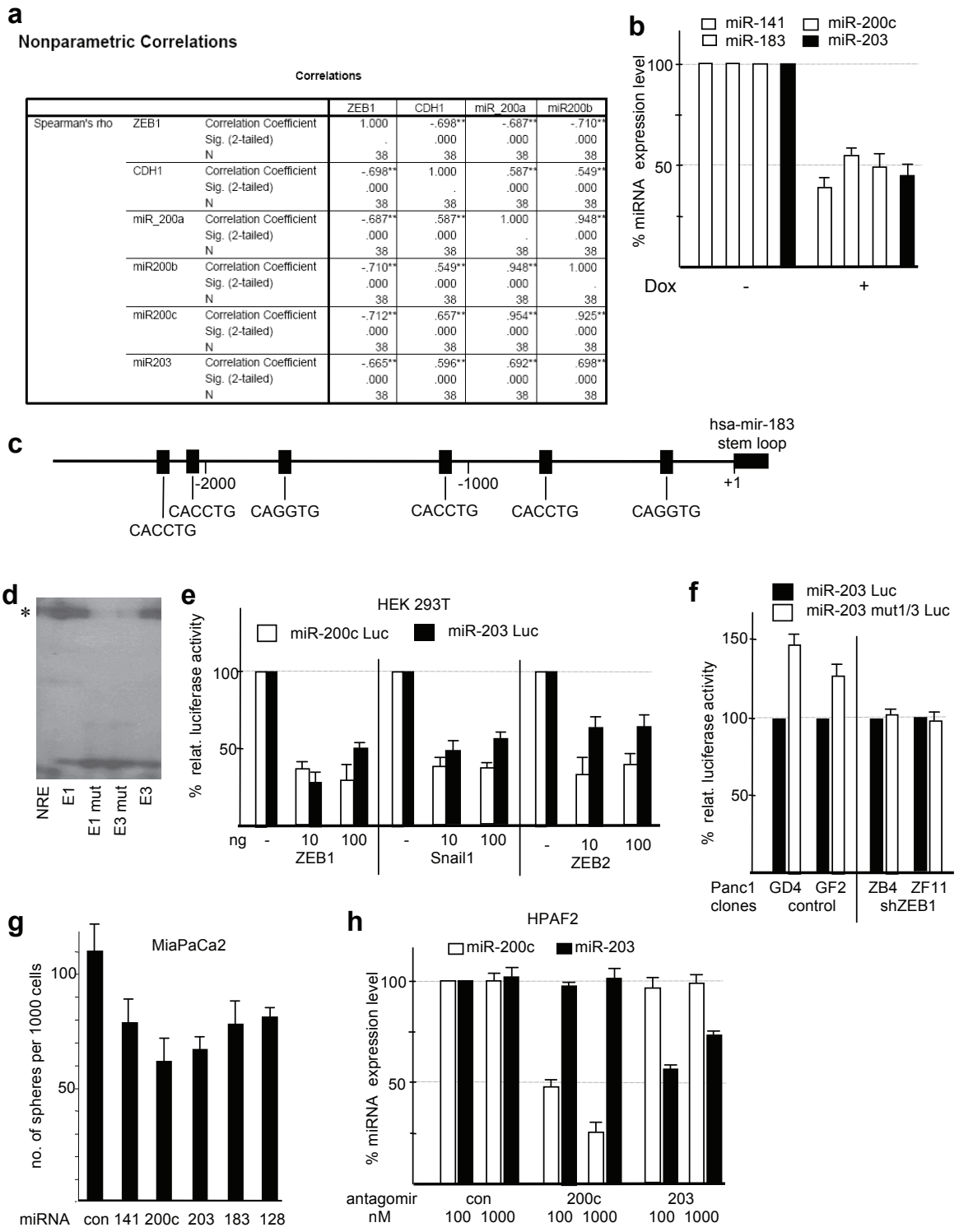


Figure S2 ZEB1 is crucial for properties attributed to cancer stem cells. (**a**) ZEB1 knockdown clones of MiaPaCa2 have a slightly reduced sphere forming capacity. Shown are mean values of three independent clones. (**b**) Sphere numbers after *in vitro* propagation of spheres in subsequent generations is almost constant in MiaPaCa2 control transfectands, but further reduced in ZEB1 knockdown clones. (**c**) ZEB1 knockdown clones show reduced numbers of the CD24/CD44 double positive population, as measured by FACS analysis (example shown below). (**d**) Average CD24 mRNA expression levels are lower in stable ZEB1 knockdown clones compared to controls. In contrast transient siRNA-mediated ZEB1 knockdown for five days had no effect on CD24 mRNA levels. (**e**) Expression levels of miR-200c and miR-203 are increased and of Vimentin and Bmi1 are decreased in xenograft tumors of ZEB1 knockdown clones (mean levels of each six Panc1 derived tumors). (**f**) No significant differences in cell cycle distribution of Panc1 shZEB1 knockdown clones versus control clones. (**g**) Example of tumor growth after subcutaneous injection of different numbers of tumor cells: shZEB1 clones are injected on the left flank, control clones on the right flank. Note the reduction in tumor number and size (arrows) after ZEB1 knockdown. All quantitative values show mean ± s.d., n = 3.



			ZEB1	CDH1	miR_200a	miR200b
Spearman's rho	ZEB1	Correlation Coefficient	1.000	-.698**	-.687**	-.710**
		Sig. (2-tailed)	.	.000	.000	.000
		N	38	38	38	38
	CDH1	Correlation Coefficient	-.698**	1.000	.587**	.549**
		Sig. (2-tailed)	.000	.	.000	.000
		N	38	38	38	38
	miR_200a	Correlation Coefficient	-.687**	.587**	1.000	.948**
		Sig. (2-tailed)	.000	.000	.	.000
		N	38	38	38	38
	miR200b	Correlation Coefficient	-.710**	.549**	.948**	1.000
		Sig. (2-tailed)	.000	.000	.000	.
		N	38	38	38	38
	miR200c	Correlation Coefficient	-.712**	.657**	.954**	.925**
		Sig. (2-tailed)	.000	.000	.000	.000
		N	38	38	38	38
	miR203	Correlation Coefficient	-.665**	.596**	.692**	.698**
		Sig. (2-tailed)	.000	.000	.000	.000
		N	38	38	38	38

Figure S3 ZEB1 controls miR-203 expression. (**a**) Table showing inverse correlation between ZEB1 and miR-200-family members, miR-203 and E-cadherin, as well as positive correlations between miR-200-family members, miR-203 and E-cadherin. (**b**) Doxycyclin-induced upregulation of ZEB1 (42-fold) in DLD1 colorectal cancer cells leads to reduction of miR-141, miR-200c and miR-183 as well as the stemness-repressing miR-203. (c) Putative ZEB1 binding sequences in the human miR-183 promoter region. (**d**) Electromobility shift assay using recombinant DNA-binding domain of ZEB1 and the indicated labelled probes. Mutation (mut) of the two conserved E-boxes (E1, E3) strongly reduced the specific binding complex (*).The known ZEB1 binding site of the interleukin-2 promoter (NRE) was used as positive control. (**e**) Overexpression of the EMT inducers ZEB1, Snail1 and ZEB2 decrease the activity of the miR-203 promoter construct. (**f**) The relative activity of the miR-203 promoter construct with mutated E-box 1 and 3 is increased in control but not shZEB1 clones of Panc1. (**g**) Overexpression of the indicated miRNAs reduced the sphere forming capacity of MiaPaCa2 cells. (**h**) Antagomirs against the indicated miRNAs specifically inhibit their expression in differentiated cancer cells. All quantitative values show mean ± s.d., n = 3.

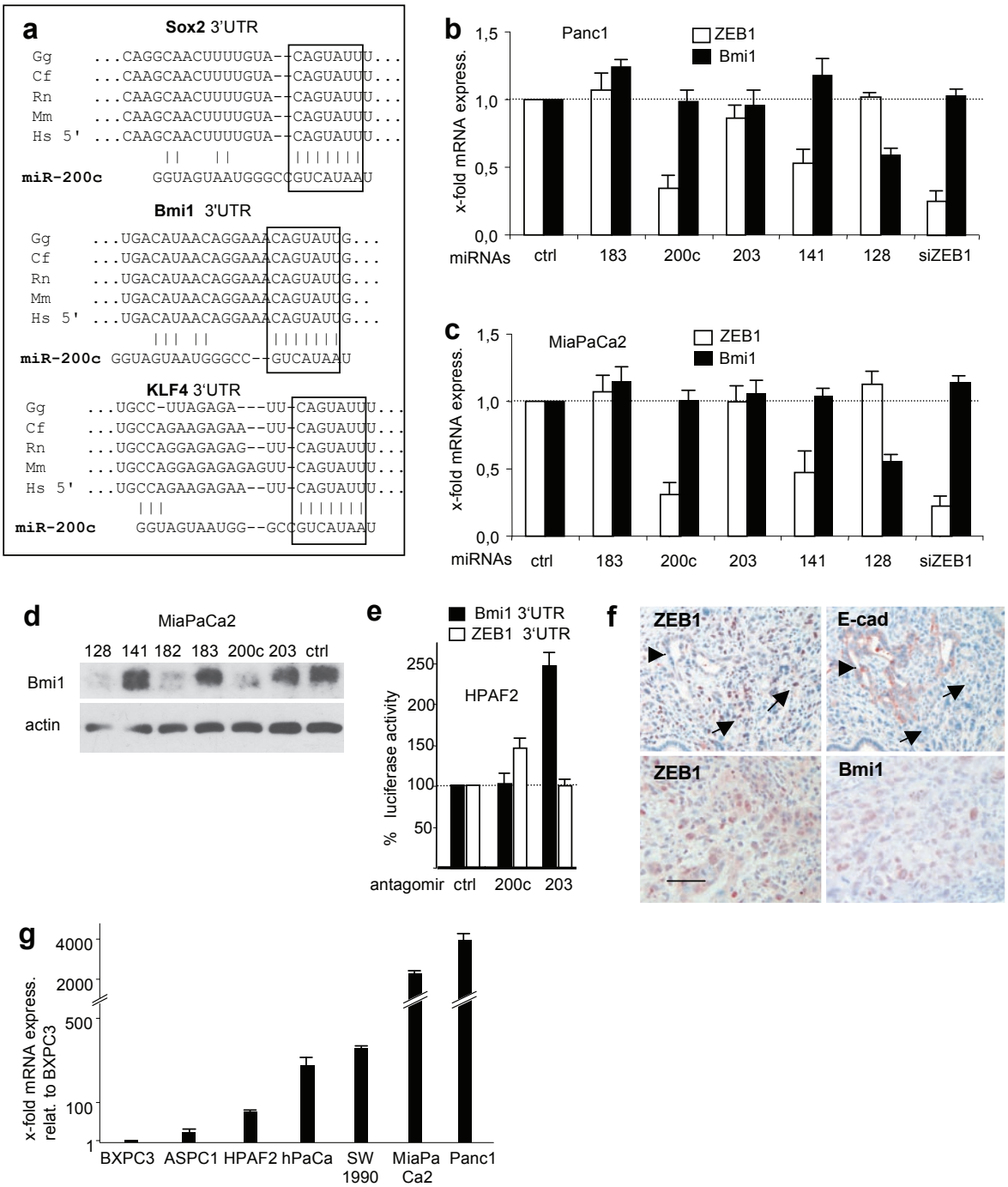


Figure S4 miRNAs targeted by ZEB1 control stem cell factors. (**a**) Highly conserved, predicted binding sites for the seed sequences of miR-200c in the 3'UTRs of the ES stem cell factors Sox2, Bmi1 and KLF4. (b,c) mRNA expression levels of Bmi1 and ZEB1 after overexpression of the indicated miRNAs in Panc1 and MiaPaCa2. Note that in contrast to miR-128 all other miRNAs and siRNA to ZEB1 did not reduce Bmi1 mRNA, whereas ZEB1 mRNA is reduced by miR-141 and miR-200c as expected. (**d**) Immunoblot showing the protein levels of Bmi1 after overexpression of the indicated miRNAs in MiaPaCa2. (**e**) Antagomirs against the indicated miRNAs increase the activity of reporter constructs. Note that the antagomir against miR-203 increases the luciferase/Bmi1 3'UTR reporter activity. (**f**) Serial section immunohistochemistry of a pancreatic cancer from *Pdx1-Cre, LSL-Kras$^{G12D/+}$, LSL-Trp$^{53R172H/+}$* mice. Like in human tumors, ZEB1 is well expressed in undifferentiated tumor cells lacking E-cadherin (arrows) and only weakly in differentiated tumor areas (arrowheads). Higher magnifications show co-expression of ZEB1 and Bmi1 in undifferentiated tumor cells. Size bar 50µm. (**g**) ZEB1 levels of isolated human pancreatic cancer cells (hPaCa, median of two isolated lines) compared to established cancer cell lines. All quantitative values show mean ± s.d., n = 3. Full scans of the blots in d are available in SI Fig. S5.

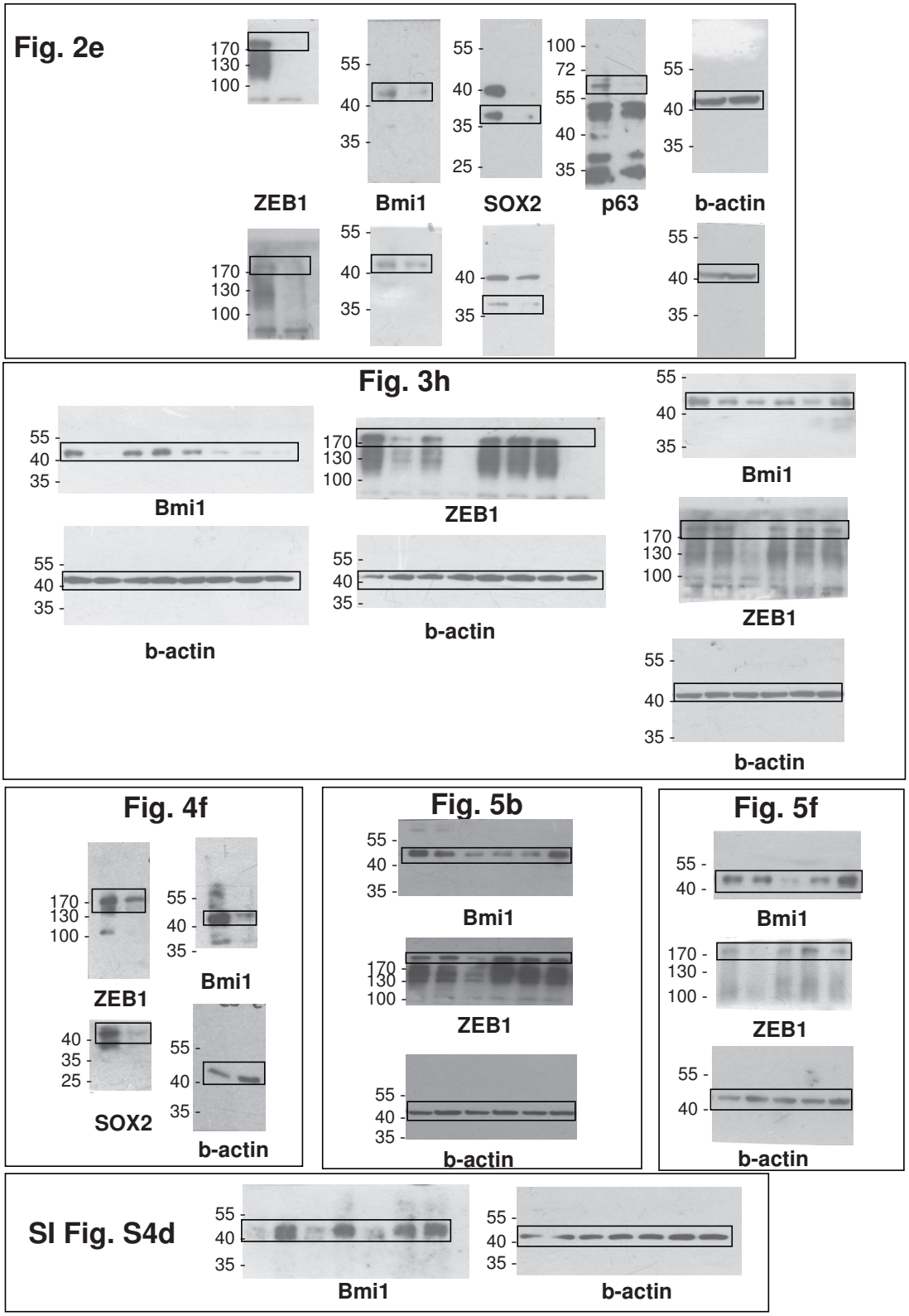


Figure S5 Full scans of Western blot data shown in the indicated Figures (frames indicate excised bands).

Supplemental Tables

Table S1: Orthotopic injection of 10^6 tumor cells in nude mice.

cell line		tumor numbers		mean prim. tumor vol. (µl)			metastases	
		ctrl	shZEB1	ctrl	shZEB1		ctrl	shZEB1
pancreatic	MiaPaCa2	7/7	3/8	1064	145	(14 %)	+	-
	Panc1	6/6	4/6	650	180	(24 %)	+	-
colorectal	SW480	5/7	2/7	46	0.05	(0.1%)	-	-
	HCT116	4/4	4/4	1360	380	(28%)	++	(+)

Table S2: Subcutaneous injection of tumor cells in nude mice (each 5 mice, numbers indicate mice with tumors).

		no. of cells injected		
cell line	clones	8000	40000	200000
Panc1	monolayer	0	0	3
	sphere	2	4	5
MiaPaCa2	monolayer	0	1	3
	sphere	1	5	5

Table S3: Correlation of ZEB1 expression with disease recurrence in pancreatic cancer patients

No.	disease recurrence	sex	localisation	TNM-status	Grading	R-Classif.	recurrence	ZEB1 staining
1	no > 2 years	m	pancreas head	pT4pN0Mx	2	0		-
2	no > 2 years	f	pancreas head	pT3pN1pMx	2	0		-
3	no > 2 years	m	pancreas head	pT3pN1Mx	2	0		-
4	no > 2 years	m	pancreas head	pT3pN1pMx	2	0		-
5	no > 2 years	f	pancreas head	pT3pN0pMx	2	0		-
6	no > 2 years	f	pancreas corpus	pT3pN1pMx	2	0		-
7	no > 2 years	f	pancreas head	pT3pN1pMx	2	0		-
8	no > 2 years	m	pancreas head	pT3pN0pMx	2	0		-
9	no > 2 years	f	pancreas head	pT3pN1pMx	2	0		-
10	no > 2 years	m	pancreas corpus	pT3pN0pMx	2	0		-
11	no > 2 years	m	pancreas head	pT3pN1pMx	2	0		+
12	yes <6 months	m	pancreas head	pT3pN1pM0	2	0	local	+
13	yes <6 months	m	pancreas head	pT3 pN1Mx	2	0	met	-
14	yes <6 months	f	pancreas head	pT3pN1Mx	2	0	met	+
15	yes <6 months	m	pancreas head	pT3pN1Mx	2	0	local	+
16	yes <6 months	m	pancreas head	pT3pN1pMx	3	0	local	+
17	yes <6 months	f	pancreas head	pT3pN1pMx	3	0	local	-
18	yes <6 months	m	pancreas head	pT3pN1pMx	2	0	local	-
19	yes <6 months	m	pancreas head	pT3pN1pMx	2	0	local	-
20	yes <6 months	m	pancreas corpus	pT3pN1pMx	2	0	local	-
21	yes <6 months	f	pancreas head	pT3pN1pMx	3	0	local, met	+
22	yes <6 months	f	pancreas head	pT3pN1pMx	3	0	local, met	+