Bmi1 is Expressed *in Vivo* in Intestinal Stem Cells

Eugenio Sangiorgi and Mario R. Capecchi

Supplementary methods

Generation of *BmiIRESCreER* mice. A BAC clone containing the *Bmi1* coding region was isolated from a RPCI-129 library (CHORI) using a probe amplified with the following oligos Eug83 and Eug84 (all primer sequences used in this study are in Supplementary Figure1). The BAC DNA was digested with SnaB1 and an 11kb band containing the Bmi1 coding region was subcloned into the Eco47III site of a pTK1TK2 vector. A BamHI-XhoI fragment from the pTK1TK2BMI plasmid, containing the 3 prime UTR of BMI1, was subcloned in pZero2 (Invitrogen, Carlsbad CA) and an IRESCreERTFrtMC1NeoFrt cassette, previously engineered, was inserted into the PshA1 site located 85bp after the stop codon and before the first putative polyadenylation signal (Supplementary Figure1 a). After verifying the whole construct by sequencing we replaced a NheI-PacI fragment with the engineered 3 prime untranslated region into the original pTK1TK2BMI vector. The final targeting vector was linearized with AhdI and electroporated in 129R1 ES cells. After positive-negative selection 144 clones were screened by Southern blot for homologous recombination using 5 prime and 3 prime external probes amplified with the following oligos: Eug89 and Eug85 and Eug90 and Eug97. To exclude the presence of randomly inserted vectors the positive clones were tested with an internal probe. One of 8 correctly targeted clones (Supplementary Figure1 b) was injected into blastocysts and generated germline chimeric mice. Heterozygous mice were crossed with a Flpe deleter mouse to remove frt-flanked Neo. Mice Neo and Flpe negative were subsequently used for the experiments. To genotype the mice, a tail snip is lysed in 75ul of Hot shot Lysis buffer

in boiling water for 30 minutes and then neutralized with 75ul of Neutralizing buffer. Two microliters of this solution are used as a DNA template to be amplified with the oligos Eug161, Eug162 and Eug96. Oligos Eug161 and Eug162 amplify the wild-type band of 421bp, oligos Eug161 and Eug96 amplify a band of 365bp corresponding to the first 200bp of the targeted IRESCreER cassette (Supplementary Figure1 c). Between postnatal days 30 (P30) and P50, double heterozygous mice *Bmi1*$^{CreER/+}$; *Rosa26*$^{LacZ/+}$ (or *Rosa26*$^{YFP/+}$) were given a single intraperitoneal injection of TM. We analyzed the following mice (*Bmi1*$^{CreER/+}$; *Rosa26*$^{YFP/+}$) at different time points after one single TM injection: 5 days (n=4), 15 days (n=1), 30 days (n=1), 45 days (n=2), 2 months (n=4), 4 months (n=2), 6 months (n=2), 7 months (n=2), 8 months (n=3), 12 months (n=2). We analyzed the following mice with this genotype *Bmi1*$^{CreER/+}$; *Rosa26*$^{LacZ/+}$ after one single TM injection: 20 hours, 24 hours, 30 hours (mice n=2 each time point), 2, 3, 4, 5, 6, 7, 10, 13, 17, 19, 22, 24, 30 days (mice n=2 each time point), 8 days with three consecutive daily injections (n=2), 20 days with three 5-day interval injections (n=2), 2 months (n=1), 4 months (n=1), 6 months (n=1), 7 months (n=1), 9 months (n=2). As a control for "leakiness" we analyzed 4 mice *Bmi1*$^{CreER/+}$; *Rosa26*$^{LacZ/+}$ at 2, 3, 4 and 6 months, without treating them with tamoxifen.

Mice expressing a stable version of *Beta-catenin* were obtained from Makoto Taketo and were genotyped according to the published oligos and conditions. We analyzed the following mice at different time points after TM injection: 5 days (n =1), 15 days (n=2), 21 days (n=1), 30 days (n=2), 45 days (n=2), 50 days (n=3).

Mice carrying a *Rosa26*Diphtheria-toxin allele were previously generated in our laboratory and genotyped following the published oligos and conditions. We analyzed the following mice at different time points after TM injection: 5 days (n =1), 7 days (n=2 three consecutive injections), 10 days (n=1), 30 days (n=3), 60 days (n=2), 210 days (n=2), 270 days (n=2), 450 days (n=1).

Immunohistochemistry analysis. The small intestine was fixed in 4% PFA for 4 hours, cryopreserved in 30% sucrose and then embedded in O.C.T. (TissueTek) and stored at -80°C. 8μm cryosections were rinsed in PBS and directly mounted in Vectashield with DAPI (Vector Laboratories, Burlingame CA) to visualize the YFP signal. We used the following antibodies with the corresponding dilutions: rabbit anti-Bmi1 (Abgent, 1:100), rabbit anti-Bmi1 (Abcam, 1:50), rabbit anti-Cre (Covance, 1:2000), rabbit anti-Lysozyme (Zymed, 1:200), rabbit anti-ChromograninA (Epitomics, 1:100), mouse anti-Ki-67 (Bd Pharmingen, 1:100). To visualize Goblet cells we used Rhodamine labelled Dolichus Biflorus Agglutinin (DBA) (Vector Laboratories, 1;100). We used secondary antibodies conjugated with AlexaFluor 488 and AlexaFluor 594 (Molecular Probes). For the Bmi1 and Cre antibodies we microwaved the sections in 1 mM EDTA pH8 or in 10mM Na-citrate pH6 for 20 minutes for antigen retrieval, then incubated the sections overnight at 4°C. We used an ABC kit for signal amplification and a DAB kit for the staining reaction (Vector Laboratories) following manufacturer's recommendations. All the fluorescent images were captured using a Leica TCS SP5 laser scanning confocal microscope.

LacZ staining. Small intestines were fixed for 2 hours in PBS with 2% PFA, 0.2% Gluteraldehyde, 2mM MgCl2, 25mM EGTA pH7.5 and 0.02% NP40. Rinsed three times in 2mM MgCl2 PBS, and then stained for 24 hours at 4°C with X-Gal or Red-Gal (Research Organics) 1mg/ml, 2mM MgCl2, 5mM K3Fe(CN)6, 5mM K4Fe(CN)6, 0.01% Na-deoxycholate and 0.02% NP40 in PBS. The organs were rinsed three times in PBS and then postfixed in 4% PFA at 4°C for four hours. Before sectioning, the stained intestine was cryopreserved in 30% sucrose, embedded in OCT, frozen on dry ice and then stored at -80°C.

In situ hybridization. The *Bmi1* probe was PCR amplified with the following oligos 164 and 165. The PCR product was then subcloned in pCR2.1-TOPO vector

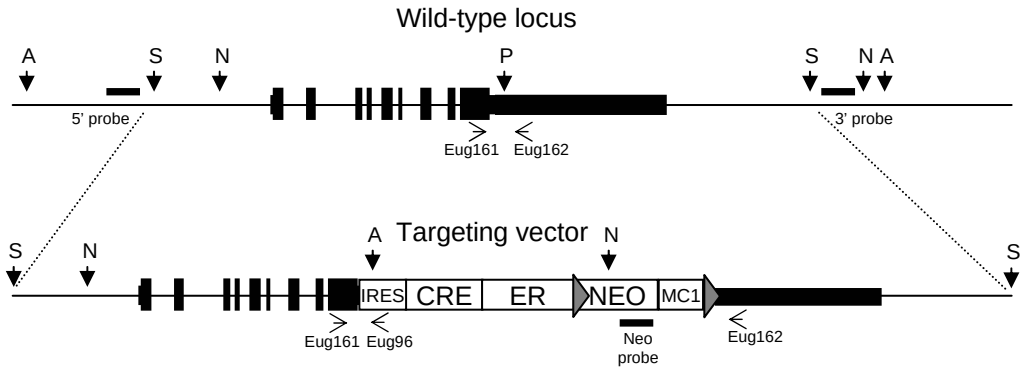
(Invitrogen) following manufacturer's instructions. The *Cryptydin4* control probe was amplified with the following oligos 168 and 169 and then subcloned in the pCR2.1-TOPO vector. The probes were transcribed with the appropriate T3 or T7 promoter with the DIG RNA labelling kit (Roche) and then hybridized following standard procedures.

Paraffin processing. Small intestines were fixed in 4% PFA for 24 hours then processed and embedded in paraffin using the MVP automatic tissue processor (Ventana). 4 μm sections were dewaxed, rehydrated and stained with hematoxylin and eosin, using standard procedures (www.ihcworld.com).

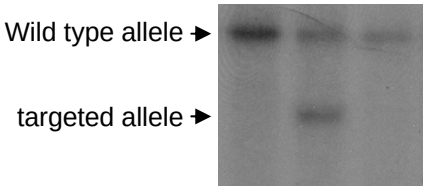
Supplementary table 1

Eug83	5’GTAGGAAGTGCTGTTGTCCC3’
Eug84	5’TTGGGTAGGCATGCTGGTGT3’
Eug89	5’CGAGAGCGGCACTTGTGTCT3’
Eug85	5’GTCTCTCTCCTTGTTTATTTCC3’
Eug90	5’TGCGAGGGCGCACGGGCGCA3’
Eug97	5’GCAACATGGCAGCCGGGACTG3’
Eug161	5’ACCAGCAACAGCCCCAGTGC 3’
Eug162	5’TAGGCATTAATTGAGATTAACAAACTA 3’
Eug96	5’AAAGACCCCTAGGAATGCTC3’
164	5’ TGGCCTTGTCACTCCCAGAG3’
165	5’AAATAAAGAGAAGCCTAAGG3’
168	5’AAGAGACTAAAACTGAGGAGCAGC3’
169	5’GGTGATCATCAGACCCCAGCATCAGT3’

a



b

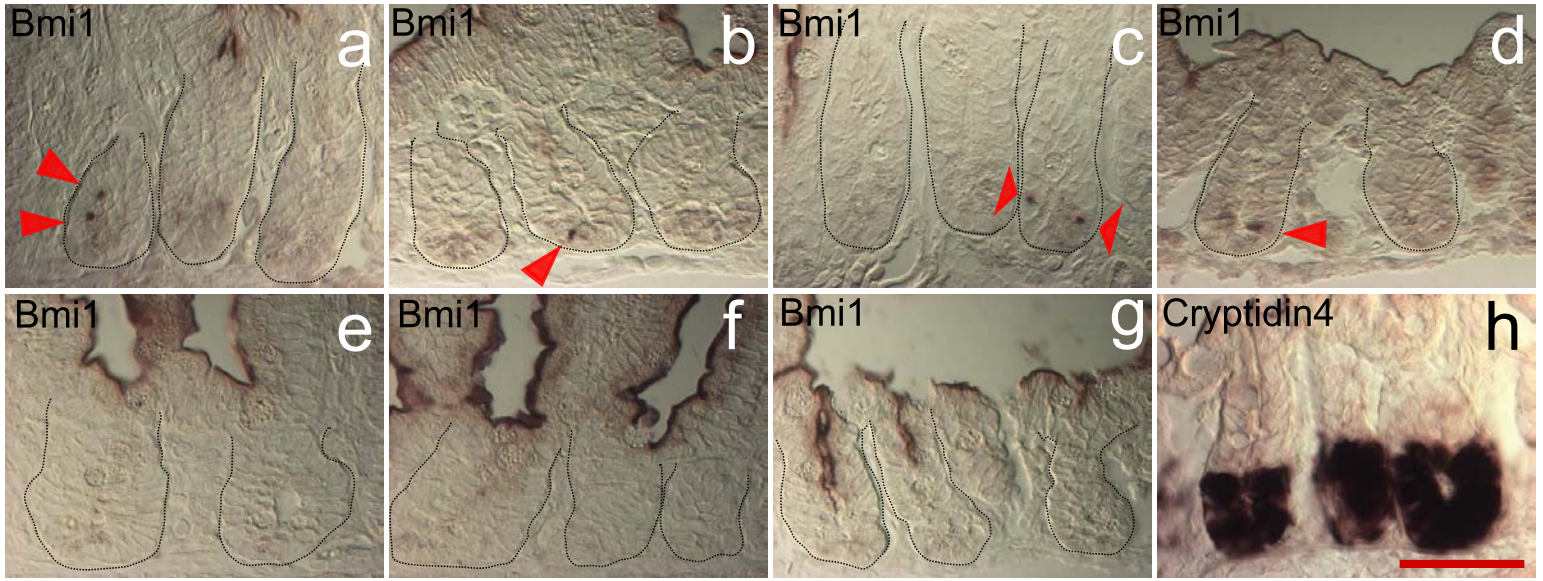


WT allele NcoI 10 kb
Targeted allele 7 kb
3' probe

c

Supplementary Figure 1

Supplementary Figure 1. Schematic representation of the Bmi1CreER targeting vector (a). The region between two SnaBI (S) sites, containing the *Bmi1* coding region, was subcloned from a BAC clone and subsequently modified with an IRESCreERfrtMC1Neofrt cassette. This cassette was inserted between the stop codon and the first putative polyadenylation site at a PshAI (P) site. The restriction sites AvrII (A), NcoI (N) and the 5 prime, 3 prime and Neo probes were used to identify the correctly targeted clones by Southern blotting. (b) Southern blot analysis of 3 G418 resistant ES cells, showing one homologous recombination event identified with the 3 prime probe. (c) PCR genotyping of adult tail DNA showing the 365bp band amplified with the oligos Eug161 and Eug96 located inside the IRES and the 421bp WT band amplified with the oligos Eug161 and Eug162.



Supplementary Figure 2

Supplementary Figure 2. Bmi1 mosaic expression. To evaluate Bmi1 expression in wild type mice, a Bmi1 RNA probe was made and tested on different segments of the small intestine. (a-d) Histological sections from the first 10cm of the small intestine. Representative pictures of crypts showing Bmi1 staining (red arrowheads) around position +4 and occasionally among Paneth cells (b). Adjacent crypts do not show evidences of Bmi1 staining. (e-g) Sections were cut from small intestine located between 10 and 20 cm from the pylorus, even though in this segment there are crypts YFP/LacZ^{+}(as shown in Figure 2 a-e), many of the crypts were negative for Bmi1 expression as shown in those three representative figures. (h) Control probe labelling Paneth cells. Scale bar 50μm.