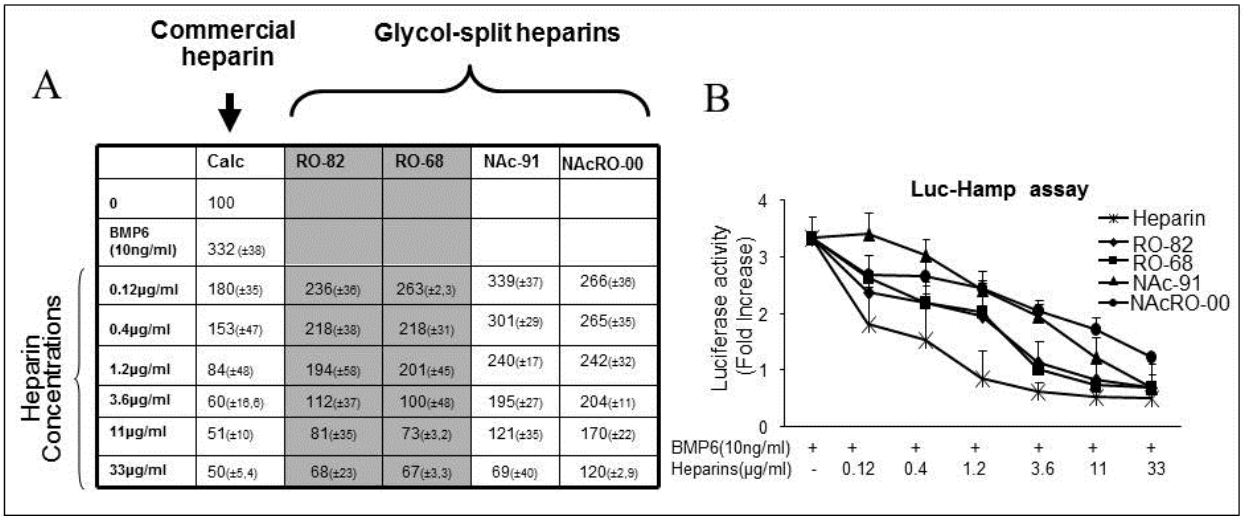
Supplementary

Figure S1.



	Calc	RO-82	RO-68	NAc-91	NAcRO-00
0	100				
BMP6 (10ng/ml)	332 (±38)				
0.12µg/ml	180(±35)	236(±36)	263(±2,3)	339(±37)	266(±36)
0.4µg/ml	153(±47)	218(±38)	218(±31)	301(±29)	265(±35)
1.2µg/ml	84(±48)	194(±58)	201(±45)	240(±17)	242(±32)
3.6µg/ml	60(±16,6)	112(±37)	100(±48)	195(±27)	204(±11)
11µg/ml	51(±10)	81(±35)	73(±3,2)	121(±35)	170(±22)
33µg/ml	50(±5.4)	68(±23)	67(±3,3)	69(±40)	120(±2,9)

Figure S1. RO-heparins affected the HAMP promoter activity. HepG2 cells were transfected with pGL2Hamp-Luciferase and pGL2TK-Renilla. 24h after transfection, the cells were treated for 16h with different heparin derivatives in presence of BMP6 (10ng/ml). Luciferase and Renilla activity were mesured. (A) Luciferase activity, normalized for Renilla activity, is expressed as means (of more than three independent experiments) ± SD. 100 is untreated cells (0); (B) Graphical representation of the data listed in (A). The values are expressed as fold increase of untreated cells (1).

Transfection and Luciferase Assay. HepG2 cells were plated onto 12-well plate ($15x10^4$ cells/ well). After 24h, the transfectant agent (GenMute, SignaGen Laboratories) and the plasmids pGL2-HAMP-Luciferase (kid gift of Dr. Silvestri) and pGL2 Thymidine Kinase Renilla luciferase plasmid (pGL2TK-RL) (Promega) were added. After 16h, the medium was replaced with fresh medium containing RO-heparins (0.12 to 33 εg/ml) and BMP6 (10 ng/ml). After 6h, the cells were lysed and bioluminescence was measured using plate luminometer (BERTHOLD). The firefly luciferase signal was measured by adding Luciferase Assay Reagent II, followed by the addition of Stop&Go substrate to measure Renilla luciferase bioluminescence. The firefly/Renilla luciferase ratio was used to normalize for transfection efficiency. Results were expressed as percentage of untreated cells (100%).

Figure S2.

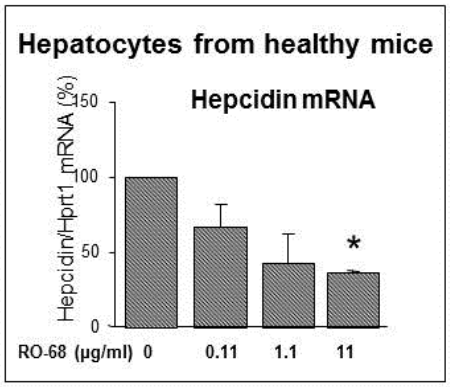


Figure S2. Heparin RO-68 inhibition of hepcidin expression in hepatocytes from healthy mice: Murine hepatocytes were isolated from three healthy mice and seeded in collagen I plate. After 16h the cells were treated with RO-68 (0.11-1.1-11 ε g/ml) for 6h and hepcidin mRNA levels were quantified with qRT-PCR in relationship to Hprt1 mRNA. Data are presented as percentage of untreated cells (100%) and as means ± SD. * $p < 0.05$

Primary cell cultures. Primary hepatocytes were isolated from three healthy mice (C57BL/J6) as previously described [47,48] with some modifications. The resulting cells suspension had high purity (>99%) as determined by light microscopy and the viability of purified hepatocytes was higher than 90% as determined by trypan blue exclusion. Cells were plated on 12 or 24-well tissue culture plate coated with collagen I (Becton Dickinson Labware; Gibco by Life Technologies) in Williams E Medium (Sigma-Aldrich) supplemented with 5% FBS. After an initial 16h attachment period, the hepatocytes were treated with different RO-heparins at different concentrations (0.11 to 11.0 ε g/ml) for 6h and then harvested for hepcidin mRNA evaluation by qRT-PCR.

Figure S3.

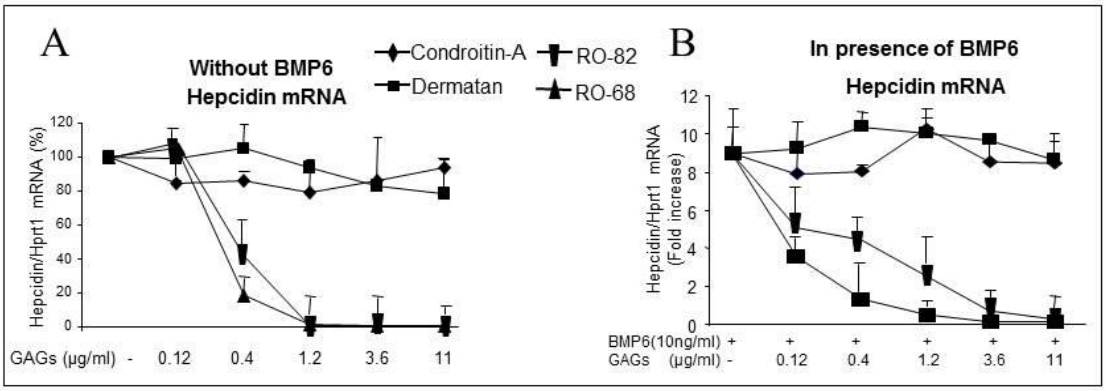


Figure S3. Condroitin-A and Dermatan (GAGs) do not affect basal and BMP6-stimulated hepcidin expression. HepG2 cells were treated with different concentrations (0.12-0.4-1.2-3.6-11 ε g/ml) of Condroitin-A, Dermatan sulfate, RO-82 and RO-68 without (A) or in presence of BMP6 (10ng/ml) (B) for 16h. Hepcidin mRNA level were quantified by qRT-PCR in relationship to Hprt1 mRNA. The values are means of three different experiments and are expressed as percentage (%) in (A) and fold increased in (B).

Figure S4

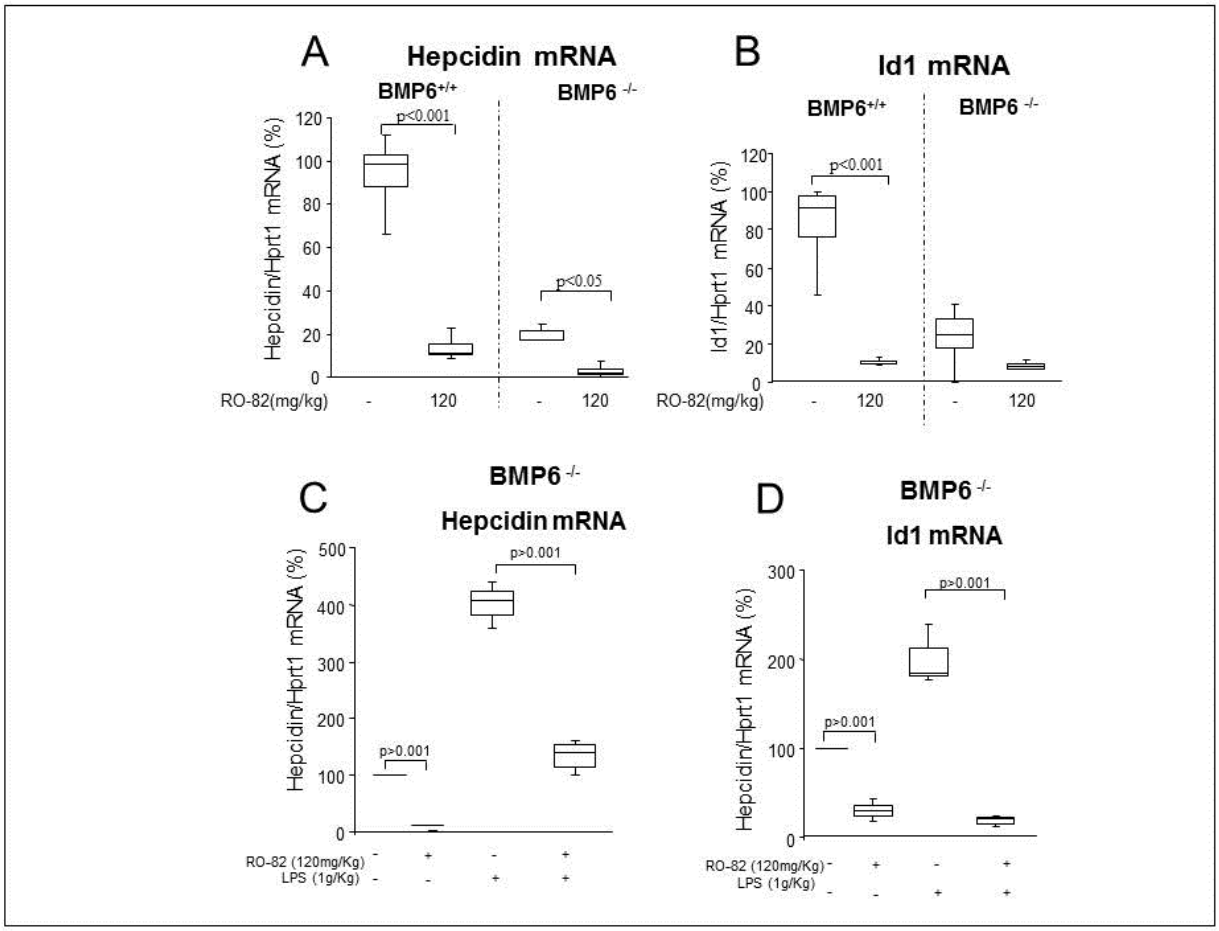


Figure S4. Treatments of Bmp6-/- mice. Panels A and B: 8-week old female mice were treated with single dose of saline or RO-82 (120 mg/Kg) and sacrificed 6h after the treatment (four mice per group). Hepcidin (A) and Id1(B) mRNA were quantified by qRT-PCR in relationship to Hprt1 mRNA. Hepcidin and Id1 level are expressed as percentage of untreated mice (100%). Panels C-D: 9-week old female Bmp6-/- mice were treated for 6 h with 120 mg/kg RO-82 heparin and for 4 h with 1 mg/kg LPS, then sacrificed and the liver analyzed for level of transcript with qRT-PCR in relationship to Hprt1 mRNA. The heparin blunted hepcidin induction by LPS and suppressed Id1 mRNA. Data are representative of four mice/point and are expressed with Box Plot histograms as percentage of untreated mice.