SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. Promoter PCR assay in colon cancer cell lines. **A.** FACS staining of cancer cell lines for the AC133 and 239C epitopes. All colon cancer cell lines were found positive for CD133 protein expression, except for T84. **B.** PCR analysis of CD133 mRNA and promoter region of CD133 in colon cancer cell lines. All lines were found positive for CD133 mRNA, except for T84. All promoters, excluding promoter 4, were active in these cell lines. Promoter 5 was also active in the CD133- cell line T84, questioning its functional role. For promoter 3 and 5, all bands were cut out of the gel and sequenced. **C.** Testis was used as a positive control for the PCR on the corresponding exons of promoter 4. To detect CD133 mRNA in the testis, the PCR required 40 cycles of amplification. **D.** Corresponding splice variants of the PCR products of promoter 3 and 5. The three different bands correspond to three different splice variants; the highest band containing exons C1, C2 and C3, the middle band containing exons C1 and C2 and the lowest band representing only exon C1. For promoter 5, four different bands were found that correspond to four different splice variants.

Supplementary figure 2. Clonogenic assay for CD133 sorted cells. **A.** CSCs acquired from primary colon carcinomas, were cultured in vitro as spheroids or differentiated for 14 days. Cells were adhered to a 24-well plate by drying overnight at 37°C, fixed in 2% paraformaldehyde for 20min at 37°C, permeabilized with PBS-0.1% Triton-X-100 for 10min and blocked with 10% FCS in PBS for 1h. First and second antibody were incubated in blocking buffer for 1h at RT. Stainings were kept at 4°C in PBS containing 5ng/ml DAPI (Invitrogen). Pictures were taken on an Axiovert 200-M microscope (Zeiss). Differentiation was confirmed by immunofluorescence staining for differentiation markers CK-20 and Muc-2 and IAP (red). Nuclei were counterstained with DAPI (blue). Scale bar represents 100 μm. Brightfield pictures are taken under 10x magnification. **B.** C001 cells were

sorted into 96-well plate based on either low or high AC133 detection. Either 1, 2, 4, 8, 16 or 32 cells were plated per well. After two weeks, the clonogenic outgrowth of these cells was monitored. The graph represents the fraction of plated wells per cell number that contain spheres.

Supplementary figure 3. Several antibodies clones, like AC133, 293C, AC141 have been developed to detect extracellular localized epitopes of CD133. It has been suggested, but never formally shown that AC133 (also referred to as CD133/1) recognizes a distinct epitope compared to 293C and AC141 (also referred to as CD133/2) which are thought to recognize the same epitope on the CD133 protein. To confirm this, we performed cross blocking studies with the AC133, 293C and AC141 antibodies. CSCs were pre-incubated with for 30min with 500ng/ml unlabeled antibodies to block their specific epitope, then stained with fluorescent labeled AC133, 293C or AC141 antibody and analyzed by FACS. Binding of AC133–PE wasblocked by pre-incubation with the AC133 antibody, but not by 293C and AC141 showingthat the AC133 epitope is located on a different part of the protein than the 293C and AC141epitope. On the other hand, 293C and AC141 could cross block each other, confirming that they recognize the same or closely linked epitope(s).

Supplementary figure 4. Glycosylation of CD133. **A.** The CD133 protein has eight predicted N-glycosylation sites on its two extracellular loops, five sites on the first extracellular loop, three on the second loop. The complete open reading frame of human CD133 (accession number AY449689) was amplified by PCR from a colon cancer cell line and cloned into pcDNA3.1-V5-His-TOPO vector (Invitrogen) introducing a stop-codon to exclude the vector-encoded C-terminal tag. To show that CD133 is normally glycosylated, 293T cells were transfected with pcDNA3.1-CD133-V5-HIS-TOPO and lysates of these cells were treated

with PNGaseF to remove N-linked glycans from the protein. Treatment with PNGaseF reduced the apparent molecular weight of the CD133 approximately 15-20 kD, confirming that CD133 is normally highly glycosylated, as has been published earlier (21, 23). **B** All 8 predicted N-glycosoylation sites of the mature protein (accession number O43490) were mutated into alanine. Mutations were performed using the Quick-Change II site-directed mutagenesis kit (Stratagene) according to manufacturers' instructions. All final constructs were verified by sequencing. Mutated CD133 was transfected into 293T cells and lysates were loaded on a SDS-PAGE. Detection was performed with AC133, W6B3C1 and a His antibody. Mutation of single site affected mobility in the gel, but not binding of the CD133 antibodies, indicating that antibody recognition does not depend on glycosylation.