FIGURE 1

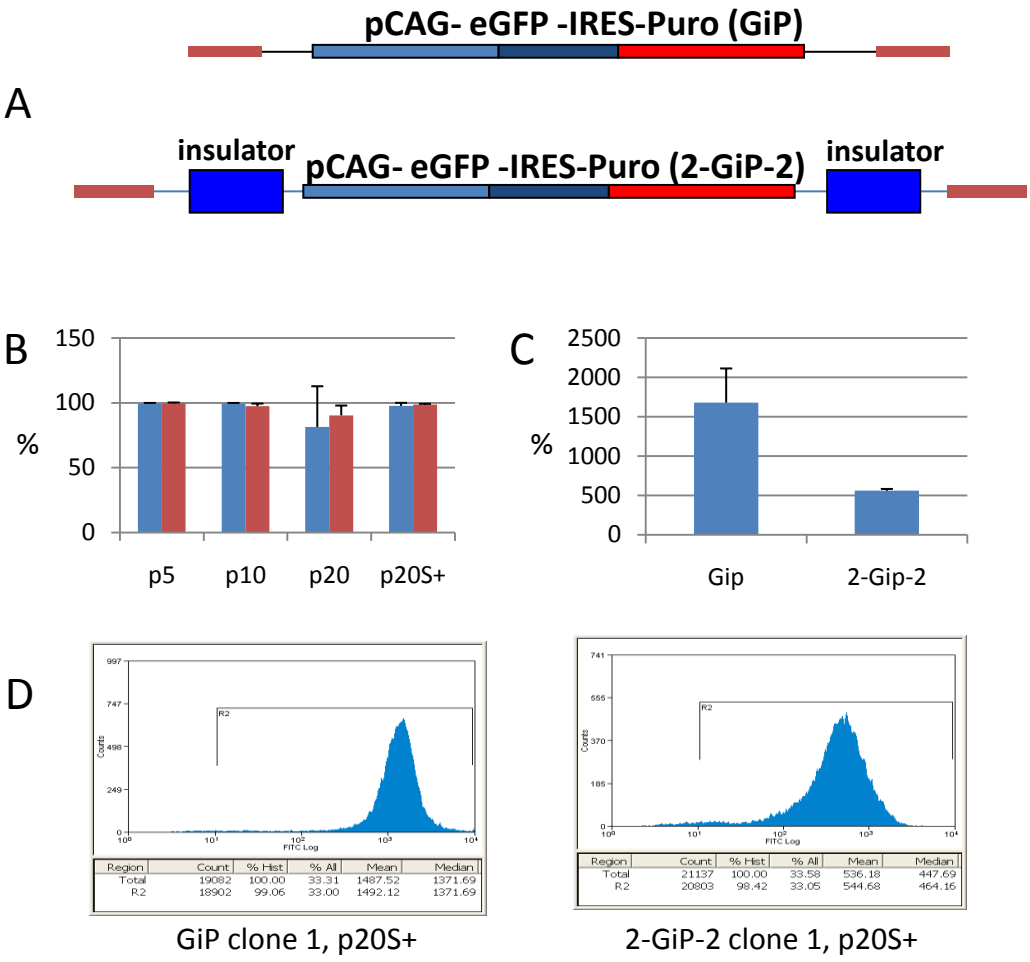

Figure 1. As a positive control for FACS analyses and for testing the insulator sequences with known stable promoter the pCAG test constructs were generated. A) The schematic representation of the pCAG-eGFP-IRES-Puro (GiP) constructs. The CAG promoter drives the expression of both eGFP and puromycin resistance. In the insulated version the insulator sequences flank the whole expression unit potentially isolating it from exogenous chromosomal interference. B) The proportion of the GFP positive cells in stably transfected non-insulated (blue bars) and insulated (brown bars) GiP clones. After initial clone selection the cells were cultured without antibiotic selection and analysed at passages 5, 10 and 20. In general, the CAG promoter maintained its activity over the culture period independent of the insulator sequences. The p20S+ cells were selected 2 passages (p18-p20) with puromycin prior FACS analyses The results are expressed as a mean of three independent clones. C) The average expression level of the GFP was remarkably lower in insulated clones as indicated by lower median intensity of the fluorescent signal (average of two independent clones at p20S+) suggesting that the insulation as such may interfere with the insulated promoter and lower its activity. D) In general, both constructs produce stable and uniform transgene expression pattern. GiP = non-insulated, 2-GiP-2 = insulated construct.

FIGURE 2

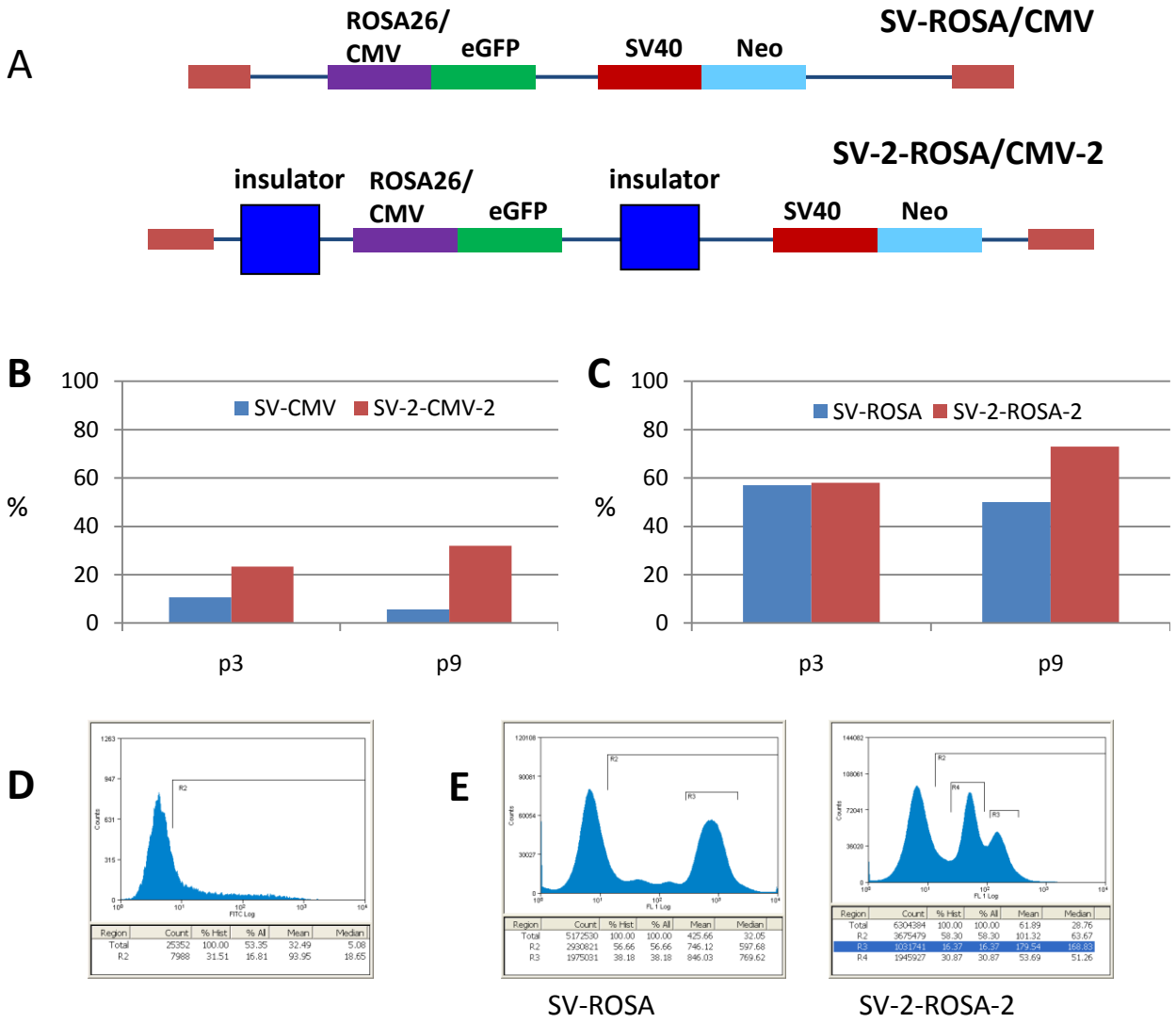

Figure 2. Insulators in HeLa cells. To evaluate the insulators with weak promoters known to be silenced in hESC two pairs of test constructs were generated (A). The expression of eGFP was driven either by insulated (blue boxes) or non-insulated CMV or ROSA26 promoter. The neomycin resistence for transgene selection was expressed from independent SV40 promoter. Prior hESC transfections the plasmids were tested in HeLa cells to confirm the functionality of the constructs. The activity of the CMV promoter in HeLa cells was weak evan in the beginning of the culture and totally silenced in few passages (B; blue bars). In contrats, the insulated construct maintained the original expression pattern over the 10 week (9 passage) study period (B; brown bars). The ROSA26 promoter showed strong and stable expression in HeLa cells independent of the insulator sequences (C). The CMV promoter produced with or without insulators a long tail-like projection of GFP-positive cells with variable intensity (D). The ROSA26 promoter produced one main positive peak (E), that was sorted and further cultured without loss of positivity (not shown). The insulated construct produced two separate peaks of cells (E), that upon sorting and further culturing mainatined their expression levels at the initial levels (not shown). Both peaks produced by insulated ROSA26 promoter have lower intensity than that produced by uninsulated ROSA26 further suggesting that insulation may downregulate the promoter activity (see Fig 1). Data shown is from pools of 30-50 clones.

FIGURE 3

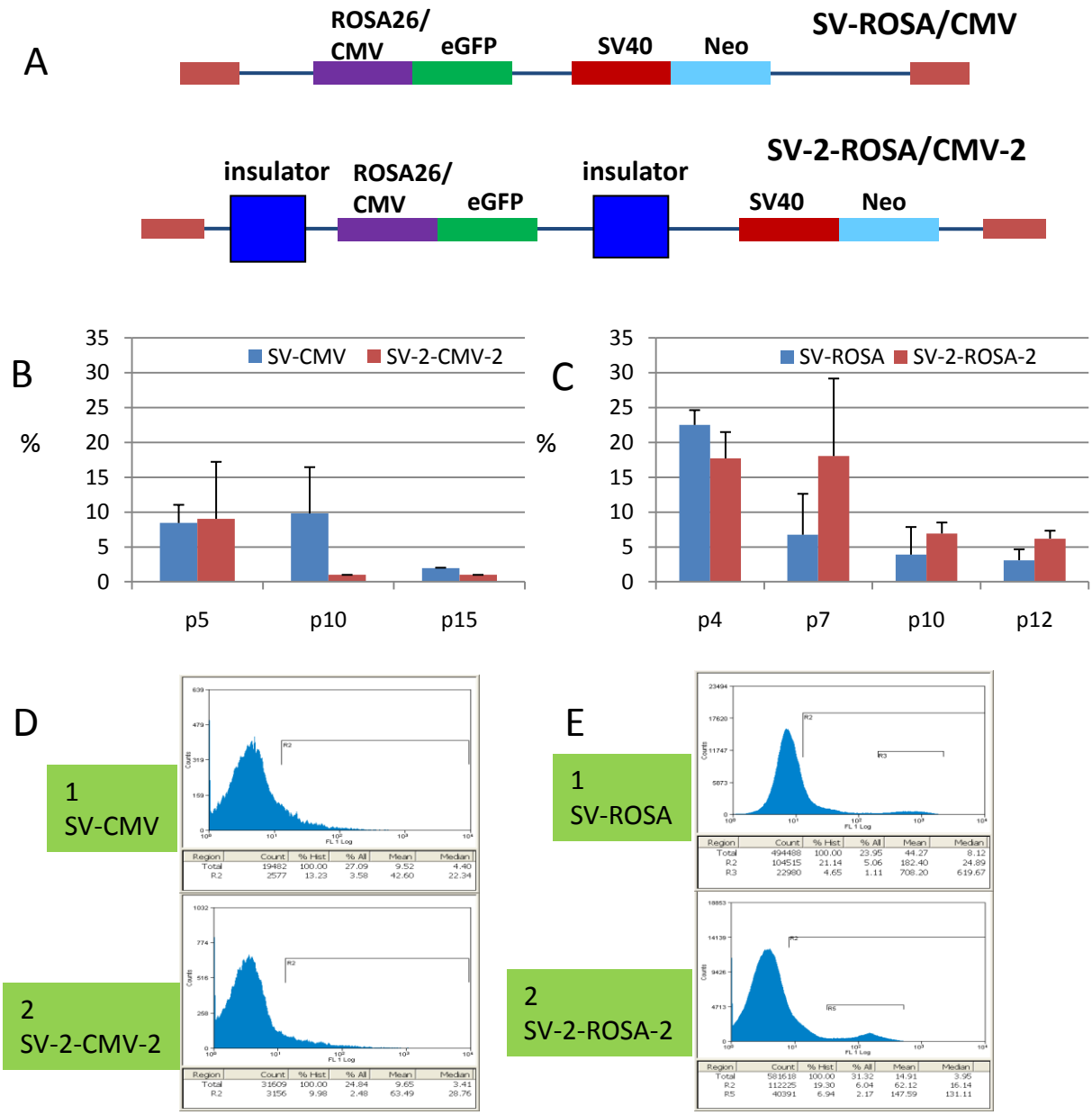

Figure 3. A) The test contsructs with either insulated or un-insulated ROSA26 or CMV promoters were next transfected in hESC. The stability of the CMV (B) or ROSA26 (C) promoters was not increased with insulator sequences. After initial antibiotic selection of the transfected clones the cells were cultured for up to 15 passages and analysed as indicated. At the end of the culture periods the amount of GFP positive cells were negligible in all cultures independent of the insulators. Data shown is an average of three independent clones. The FACS histograms of representative transgenics clones (D and E) indicate similar expression patterns for tested promoters as in HeLa cells. The CMV was in general weak and produced long tail like projections with variable expression levels independent of the insulators (D1 and 2). The ROSA26 formed a distinct relatively bright population of positive cells (E1 and 2).

FIGURE 4

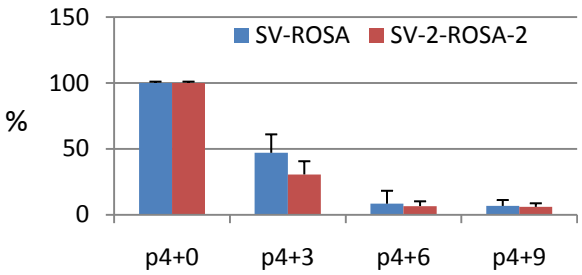

Figure 4. The insulated and non-insulated ROSA26 transfected clones were FACS-sorted at passage 4 and the positive cells cultured 9 further passages without antibiotic selection. Again, indpendent of the insulation the clones lost the GFP expression within the next six passages. The results shown are mean of two (SV-ROSA) and three (SV-2-ROSA-2) clones.

FIGURE 5

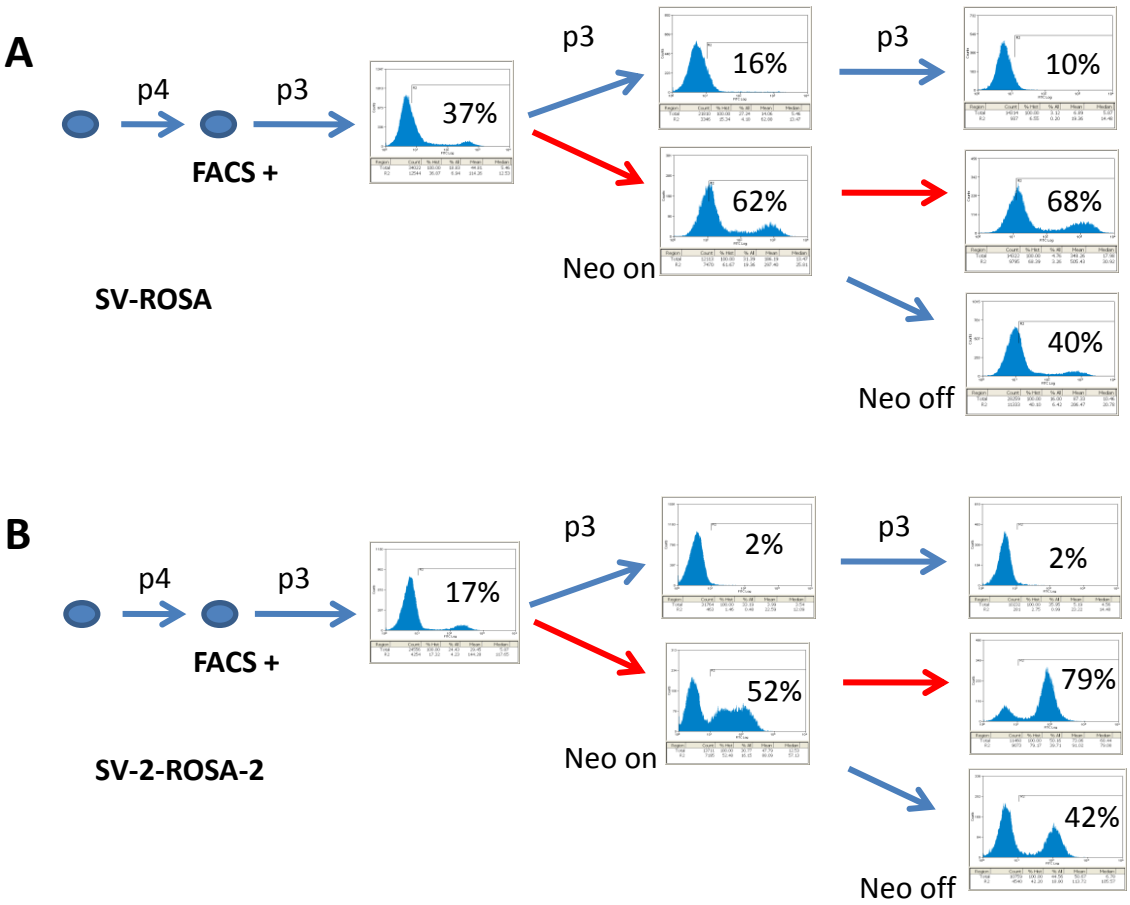

Figure 5. The effect of antibiotic selection on GFP expression levels. The GFP postive cells were divided into two subgroups (with or without Neomycin selection) three passages after FACS sorting. In both test groups (with or without insulation) the antibiotic selection increased the proportion of the GFP positive cells remarkably. The withdrawal of the selection reversed the effect and the number of GFP positive cells started to decrease. The results shown are data from single clones from both test groups.

FIGURE 6

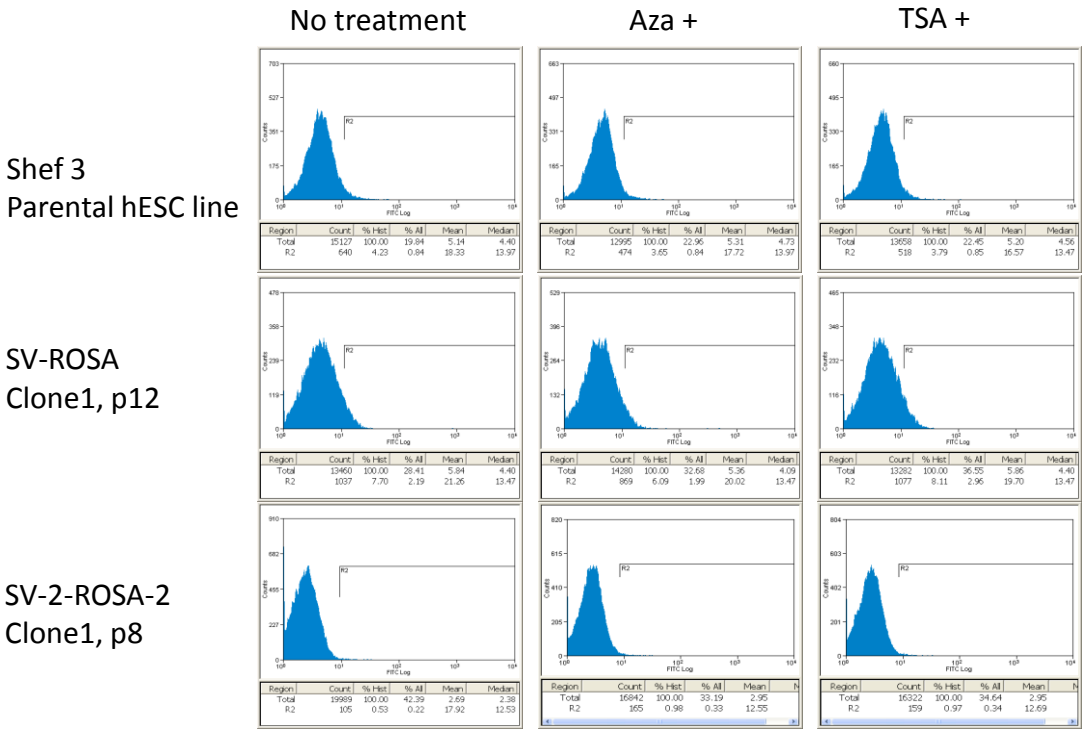

Figure 6. The effect of epigenetic manipulation on GFP expression. The FACS sorted GFP-positive cells were cultured 12 or 8 passages without antibiotic selection and then treated either with DNA methylation blocker 5-aza-2'-deoxycytidine (Aza) or histone deacetylace inhibitor trichostatin A (TSA) for 4 days. After treatments the samples were analysed by FACS. Neither of the treatments were able to reactivate the eGFP expression.