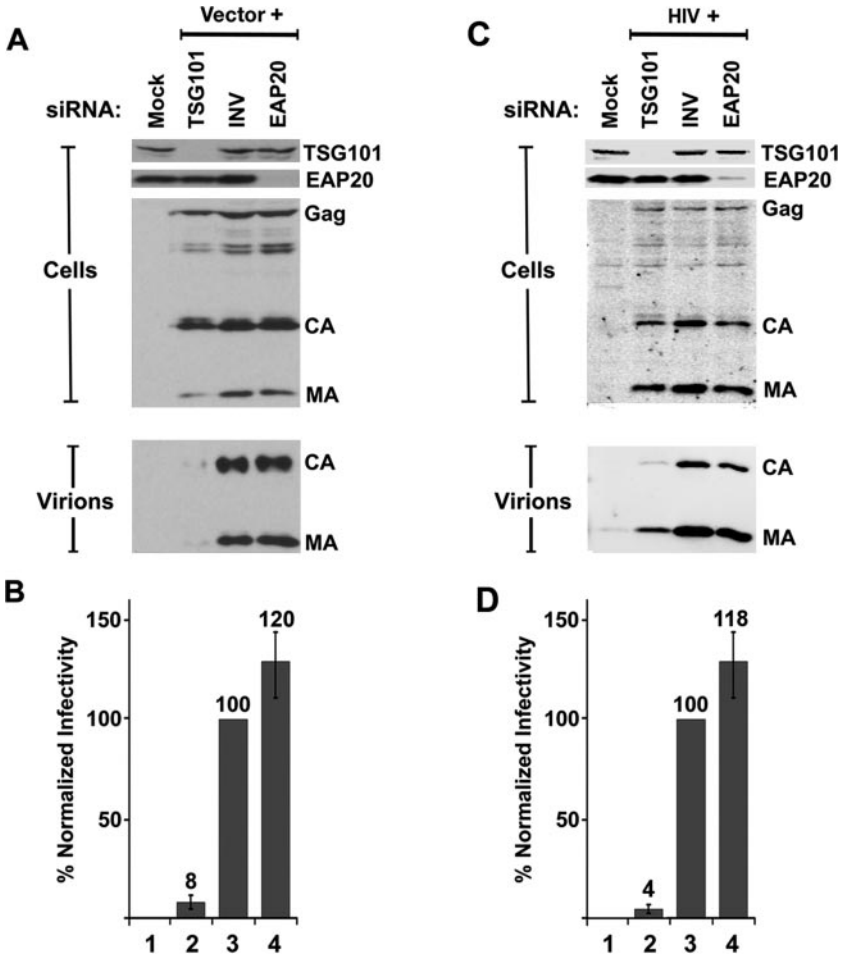

FIG. 7. siRNA depletion of EAP20 does not diminish HIV-1 vector release or transduction efficiency. (A) Western blot analysis of 293T cell extracts showing the efficiency of siRNA depletion of TSG101 (positive control, lane TSG101 in top gel) or EAP20 (lane EAP20 in second gel from top) and the effects on Gag protein expression (third gel from top) and virus-like particle release (virions, bottom gel) from a cotransfected HIV-1 vector system. Lanes Mock and INV are negative controls showing cells that were mock transfected without the HIV-1 vector or cotransfected with an inverted siRNA against TSG101 (INV), respectively. Cellular Gag, CA, and MA levels were monitored to ensure equal transfection and protein expression levels. Extracellular CA and MA levels reflect the relative efficiencies of virus-like particle release. The TSG101 signal intensity for TSG101-depleted cells was 1% of that of the control, and the EAP20 signal intensity for EAP20-depleted cells was 2% of that of the control. Quantification of at least three repetitions of this experiment showed the following levels of MA and CA release: TSG101 siRNA, 9% ± 2% of the control; inverted siRNA, 100% (defined as the control); EAP20, 108% ± 12%. The data were collected 48 h after the second siRNA transfection. (B) HIV-1 viral-vector titers produced by cells depleted of EAP20 or TSG101 (positive control). Vector transduction levels were normalized to the negative control (INV) and averaged from six independent experiments. Control vector titers were 2.5×10^5 to 3.2×10^5/ml. The data were collected 48 h after the second siRNA transfection. (C) Western blot analysis showing the efficiency and effects of siRNA-mediated silencing of EAP20 in analogous experiments with wild-type (wt) HIV-1_{NL4-3} virus. The experiment was similar to that shown in panels A and B, except that wt HIV-1_{NL4-3} virus was utilized. Quantification of at least three repetitions of this experiment showed the following levels of MA and CA release: TSG101 siRNA, 12% ± 9% of the control; inverted siRNA, 100% (defined as the control); EAP20, 75% ± 4%. The data were collected 26 h after the second siRNA transfection. (D) HIV-1 titers produced by cells depleted of EAP20 or TSG101 (positive control). Control viral titers were 0.9×10^6 to 4×10^6/ml of supernatant. The values represent the average and standard error of eight independent experiments. The data were collected 24 to 26 h after the second siRNA transfection.

signals. Following high levels of EGF stimulation, the receptor is internalized, trafficked through the MVB pathway, and degraded in the lysosome, thereby attenuating the growth signal (32, 43). The lack of a requirement for EAP20 and CHMP6 in HIV-1 budding was surprising, and we therefore tested whether depletion of these proteins altered EGFR downregulation, as would be expected for proteins that function in MVB vesicle formation and protein sorting. These experiments also served as controls for our ability to deplete TSG101, EAP20, and CHMP6 to functionally significant levels.

As shown in Fig. 9, depletion of TSG101/ESCRT-I, EAP20/ESCRT-II, and CHMP6/ESCRT-III significantly reduced the efficiency of EGFR degradation in response to EGF stimulation, although the block was not complete and was somewhat greater for TSG101/ESCRT-I than for EAP20/ESCRT-II and CHMP6/ESCRT-III. Total cellular EGFR protein levels and localization were assayed by immunofluorescence, using an anti-EGFR antibody. In the absence of EGF stimulation, EGFR was located almost exclusively on the surfaces of 293T cells (Fig. 9A, column 1 [from left] and inset). Following EGF