Supplementary Information

X-linked Inhibitor of Apoptosis Protein negatively regulates neuronal differentiation through interaction with cRAF and Trk

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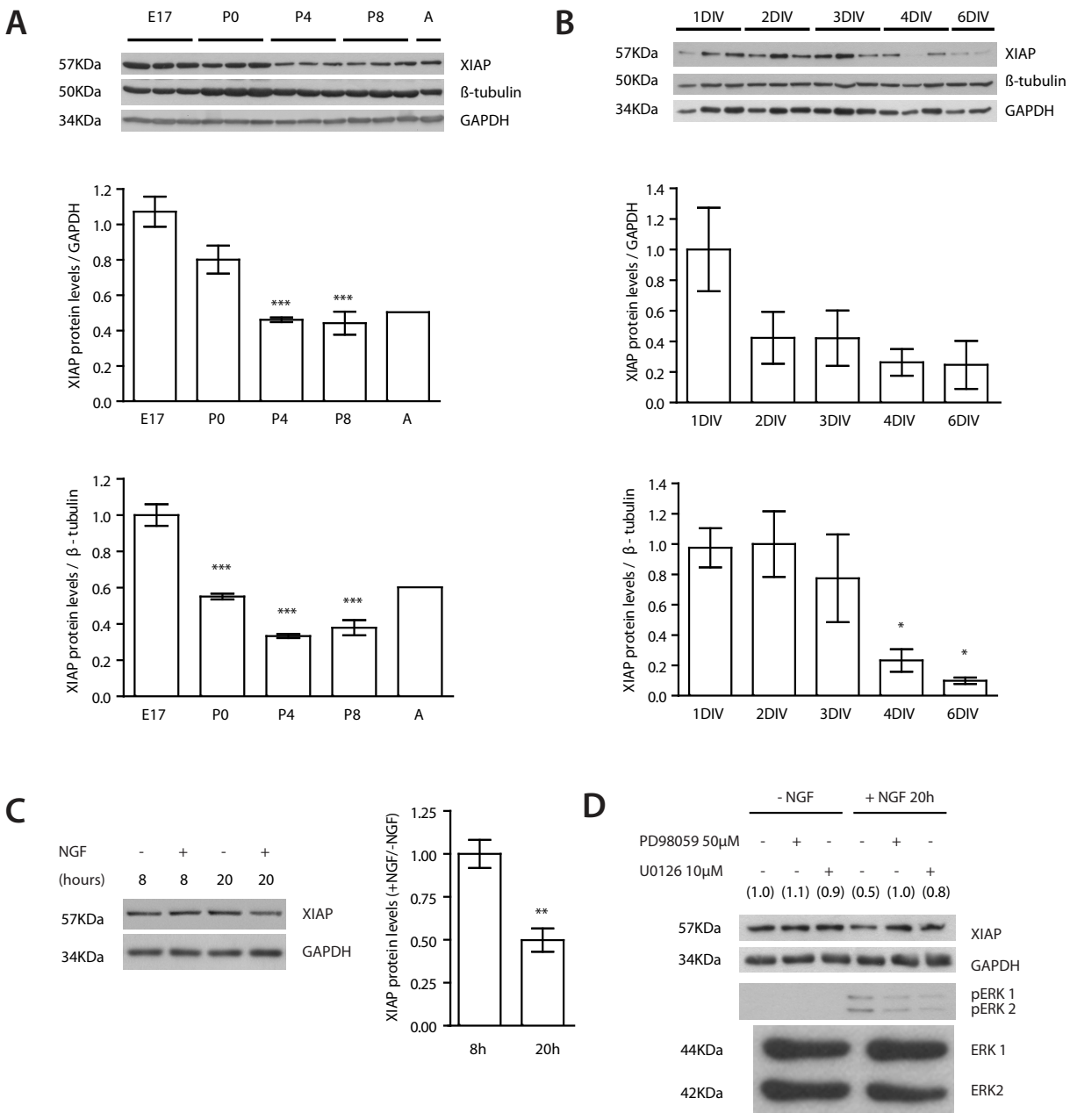
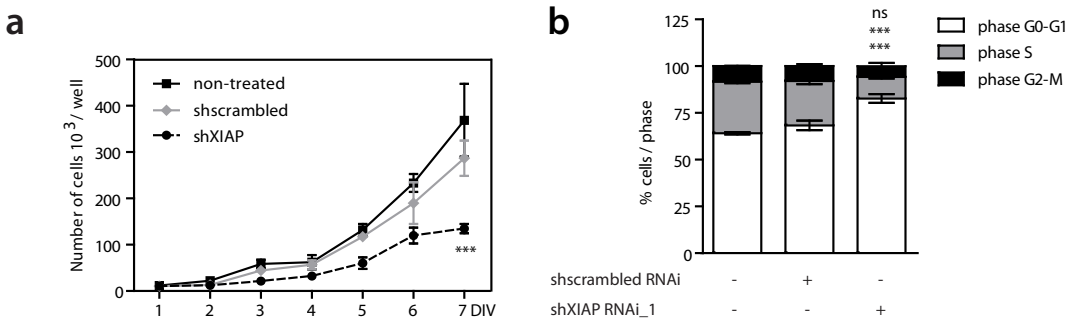
SUPPLEMENTARY FIGURES

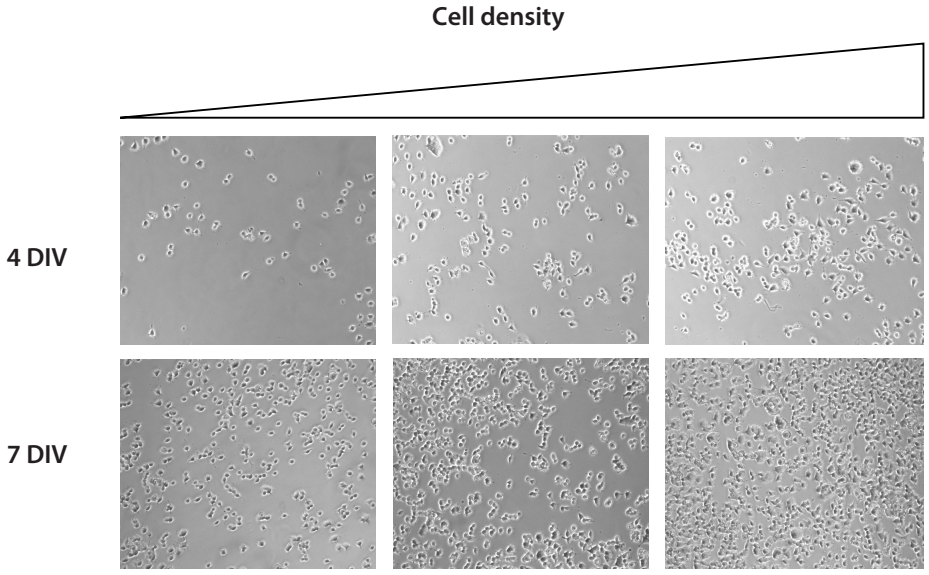


Supplementary Figure S1. XIAP regulation during neuronal development.
A, XIAP protein levels were analysed in cerebrocortical homogenates from embryonic (17 days of gestation; E17), postnatal (P) 0, 4 and 8 days and adult rats by western blot. Results shows protein levels normalized vs. loading control (GAPDH or tubulin) and are the mean ± SEM of 3 different animals. ***p<0.001 vs. E17. *B*, XIAP protein levels were determined in cultured cortical cells harvested at different days *in vitro* (DIV). Results represent normalised protein levels and are the mean ± SEM of 3 independent cultures. **p<0.01 vs. 1DIV. *C*, XIAP protein levels in PC12 cells were analysed by western blotting after NGF treatment. At 3DIV, cells were serum deprived for 2 hours and then treated with NGF for indicated times. Results are given as the ratio between

+NGF/-NGF protein levels of XIAP (normalized with GAPDH levels) and are the mean ± SEM from 3 independent experiments. **p<0.01 vs. 8 hours. *D*, MEK inhibition, with PD98059 or U012610, blocks NGF-mediated XIAP protein levels reduction. Western blotting was used to detect XIAP, phospho-ERK and total ERK protein levels. Values of the ratio XIAP/GAPDH are shown between brackets.



Supplementary Figure S2. XIAP down-regulation affect cell cycle progression.
A, Number of cells were analysed by trypan blue at different days post infection. Values are the mean ± SEM of 3 independent experiments performed in duplicate. *B,* Cell cycle analysis was carried out measuring cellular DNA content stained with propidium iodide by flow cytometry. ModFit FL Software showed the results as the percentage of cells in G_1-G_0, S or G_2-M phase. Results are the mean ± SEM of 3 independent experiments. ***p<0.001 vs. scrambled



Supplementary Figure S3. Neurite formation and length is not related with cell density in culture. PC12 cells were initially seeded at 1,800 cells/cm_2 (left column), 3,600 cells/cm_2 (middle column) or 7,200 cells/cm_2 (right column). Representative photographs from 4 and 7 DIV cultures are shown.

SUPPLEMENTARY METHODS

Measurement of cell proliferation.

PC12 proliferation was analysed by counting cell number. Cells were seed at a concentration of $1{,}25x10^4$ cells/well in 24-well plates and grown in DMEM containing 7% FBS and 7% HS. Cells were infected as explained in methods section. At indicated times, cells were collected and the number of viable cells was determined by the trypan blue exclusion method.

Cell cycle analyses.

To evaluate the percentage of cells in different phases of the cell cycle, cellular DNA content was measured by flow cytometry. Cells were seeded ($1{,}25x10^4$ cells/well in a 60mm plates), infected with lentivirus and incubated for 3 days. Then, cells were collected, washed twice with PBS and centrifuged at 200 g for 6 minutes. Cells were carefully resuspended with PBS, diluted with ethanol (final proportion 1:0.63) and incubated for 2 hours at 4ºC. Fixed cells were centrifuged at 200 g for 5 minutes. Ethanol was discarded and pellets were washed with PBS and suspended in 0.5 ml of propidium iodide (PI) solution (20 µg/ml of PI in PBS buffer containing 0.1 % of Triton X-100). After incubation for 30 minutes at RTª, PI-stained nuclei were analysed by flow cytometry (FACSCalibur; Becton Dickinson). The rate of the cell cycle within G_0/G_1, S and G_2/M phase was analysed with ModFit LT program.

Viability assay.

The cell viability was estimated by MTT assay, a reagent which is converted to formazan blue by metabolically active mitochondria. Cells were treated with MTT (Sigma; 0.2mg/ml) during 45 minutes at 37ºC and after medium removal formazan was dissolved in dimethylsulfoxide. Samples were read at a wavelength of 630nm and 570nm. The data were calculated as the absorbance at 570nm minus the absorbance at 630nm.