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Supplemental materials and methods

Luciferase assay

The luciferase reporter construct (m67-luc) was cotransfected with mammalian expression vectors and the pRL-TK (thymidine kinase promoter-dependent Renilla luciferase) as an internal control for transfection efficiency into PC12 cells. Cells were starved of serum for 24 h before stimulating with 40 ng/ml IL-6 for 8 h. Cells were subsequently lysed, and a Dual Luciferase Reporter assay (Promega) was performed according to the manufacturer's instructions using the Ascent luminoskan (ThermoLab Systems).

Immunoprecipitation and immunoblotting

Total cell lysates were prepared in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1 mM EDTA, 1% wt/vol sodium deoxycholate, 1% vol/vol Triton X-100, 0.2% wt/vol NaF, and 100 μM Na_3VO_4) supplemented with protease inhibitors. After 10 min on ice, cell debris was removed by centrifugation. Immunoprecipitation and Western blot analysis were performed as previously described (Lufei et al., 2003).

Retroviral infection

Full-length murine Stat3α cDNA was cloned into pLNCX2 retroviral vector (CLONTECH Laboratories, Inc.) by restriction digest with XhoI and HindIII. pLNCX2 vector and pLNCX2-mu Stat3α were transfected into a 293T-based Phoenix-Eco packaging cell line provided by G.P. Nolan (Stanford University School of Medicine, Stanford, CA). Ecotropic retroviruses were harvested after 48 h, filtered through a 0.45-μM cellulose acetate membrane, and diluted with fresh 10% FBS/DME without antibiotics. ΔSt3 MEFs were infected overnight with retrovirus supplemented with 4 μg/ml polybrene, washed, and replaced with fresh medium.

References

Lufei, C., J. Ma, G. Huang, T. Zhang, V. Novotny-Diermayr, C.T. Ong, and X. Cao. 2003. GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. EMBO J. 22:1325–1335.

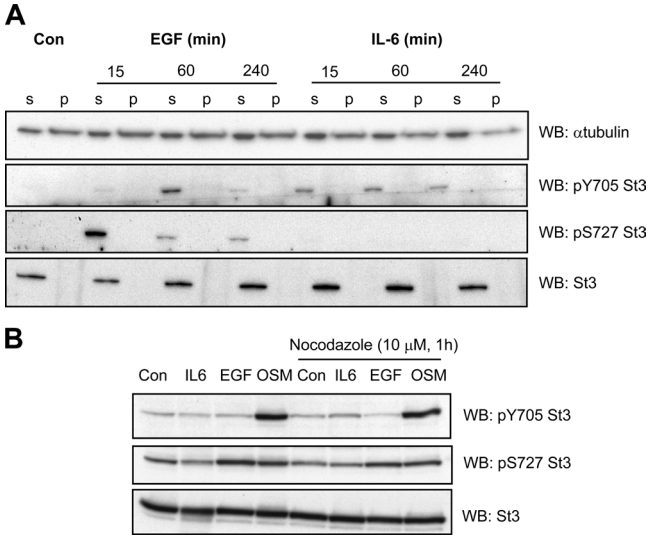


Figure S1. **MT polymerization state is independent of Stat3 activation.** (A) WT MEF cells were stimulated with 100 ng/ml EGF or 40 ng/ml IL-6 for the indicated times. Polymerized tubulin (p) was differentially sedimented from soluble tubulin (s), and fractions were blotted for α-tubulin, Stat3 Y705, and S727 phosphorylation and total Stat3. (B) WT MEF cells were pretreated with 10 μM nocodazole for 1 h before stimulating with 100 ng/ml EGF, 40 ng/ml IL-6, or 5 ng/ml oncostatin-M (OSM) for 15 min. Stat3 Y705 and S727 phosphorylation and total Stat3 protein levels were then assessed by immunoblotting. Con, control.

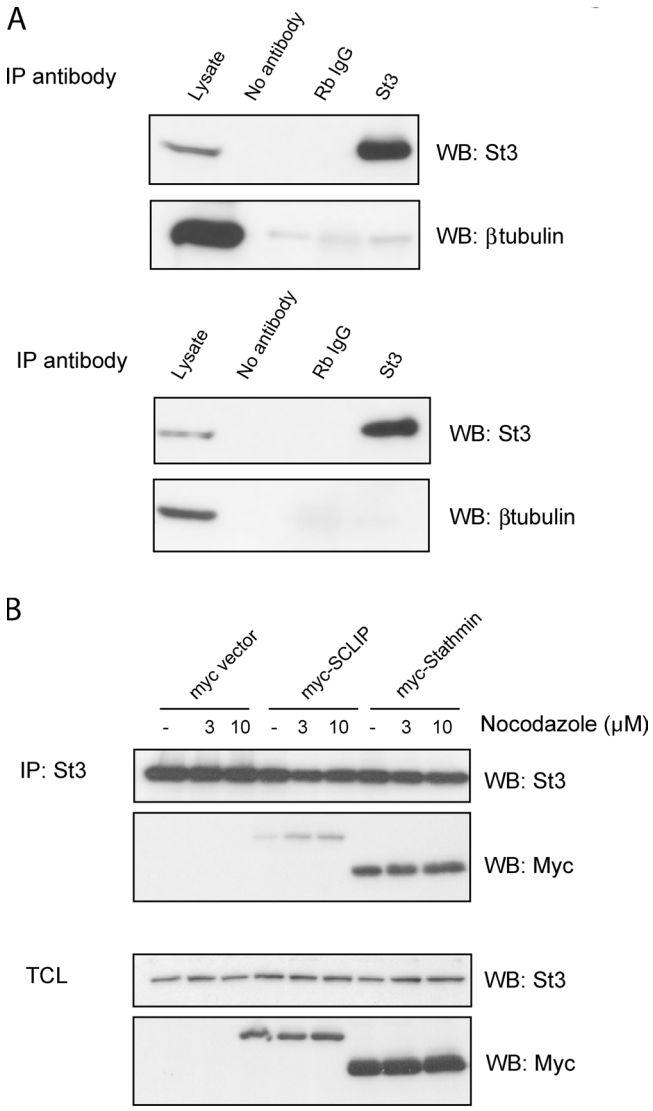
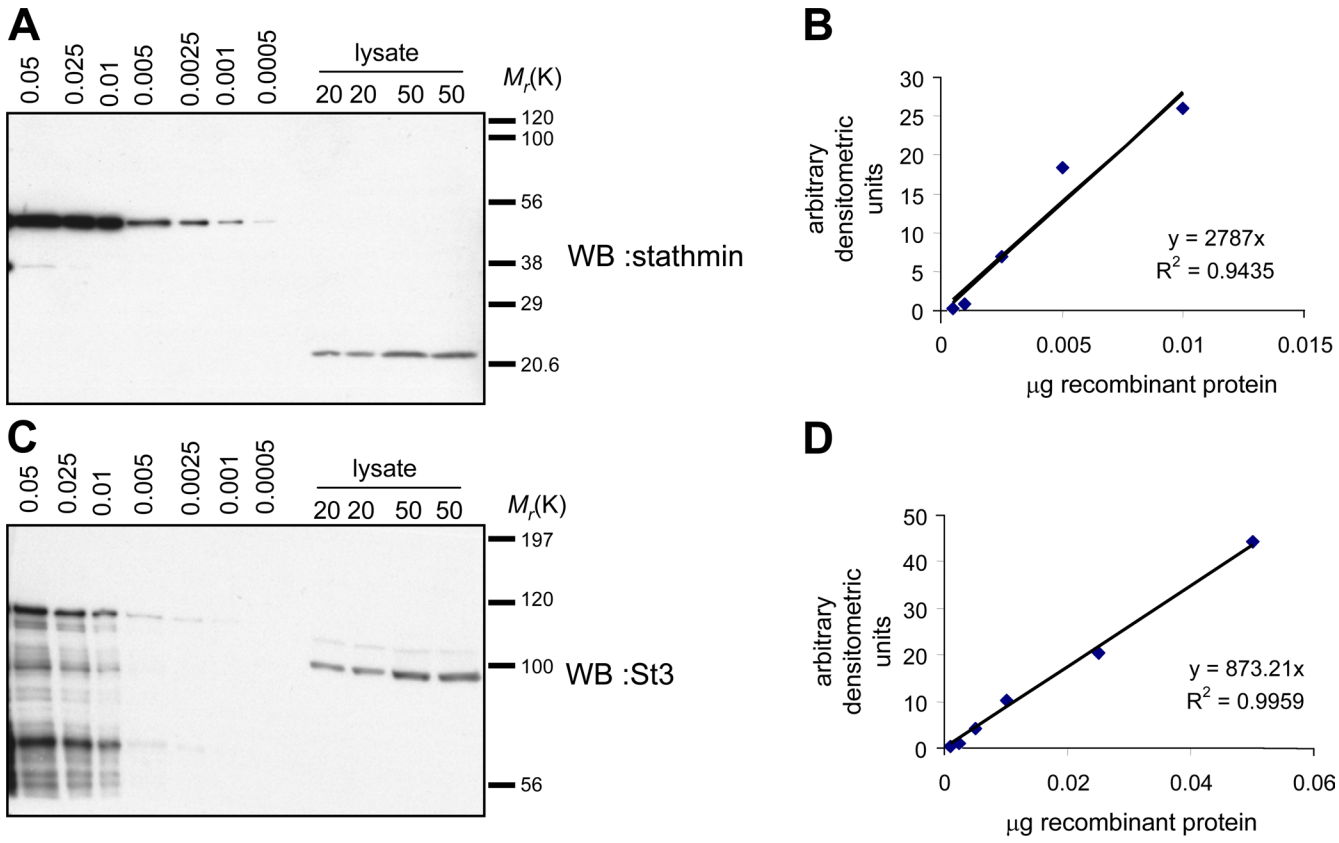


Figure S2. **Stat3 does not associate with tubulin, and binding to stathmin does not require the MT network.** (A) Endogenous Stat3 was immunoprecipitated from PC12 cell lysates, and the association of α-tubulin and β-tubulin was determined by immunoblotting. As controls, the Stat3 immunoprecipitation antibody was substituted with a rabbit IgG antibody control (Rb IgG) or no antibody. Whole cell lysates were also included in the immunoblot analysis to indicate endogenous protein expression. (B) PC12 cells expressing myc-tagged SCLIP, stathmin, or an empty vector were pretreated with nocodazole (3 or 10 μM). Protein lysates were then immunoprecipitated with anti-Stat3 and probed for the myc-tagged SCLIP or stathmin and Stat3. Whole cell lysates were similarly blotted.



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	GST-Stathmin	GST-STAT3
50 μg densitometric units (A)	3.26	7.36
50 μg densitometric units (B)	3.46	7.01
μg/50 μg lysate	0.0012	0.0082
Ratio	1	6.83
Mr(K)	44	118
Ratio Normalized for *Mr*(K)	**1**	**2.5**

Figure S3. **Quantification of Stat3 and stathmin protein in PC12 cells.** (A) Known amounts (μg) of GST-stathmin and PC12 protein lysates were blotted with anti-stathmin. (B) Values obtained by densitometric quantitation of GST-stathmin bands were then used to construct a linear standard curve. (C) Known amounts (μg) of GST-Stat3 and PC12 protein lysates were blotted with anti-Stat3. The GST-Stat3 recombinant protein contained a second major band (~75 kD) that also cross reacted with the Stat3 antibody. Therefore, the two major bands in the Stat3 blot represent full-length GST-Stat3 and cleavage products. This was taken into account when calculating the cellular levels of Stat3. (D) GST-Stat3 bands were quantitated and used to construct a standard curve. (E) Densitometric values from endogenous stathmin and Stat3 bands (50 μg) were used to extrapolate the absolute protein amounts present in the PC12 lysates. A molar ratio of Stat3 to stathmin was then determined by taking into account the molecular mass difference between GST-Stat3 (118 kD) and GST-stathmin (44 kD).

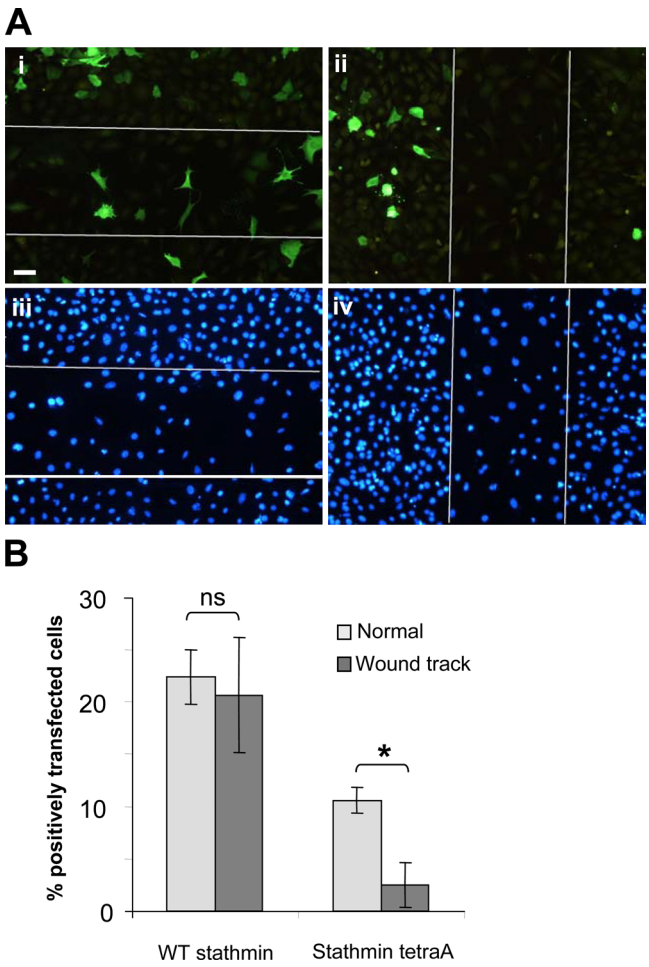

Figure S4. **Constitutive stathmin activity attenuates cell migration.** (A) WT MEFs were transfected with myc-tagged WT stathmin (i and iii) or myc-tagged stathmin tetraA (ii and iv) and subjected to in vitro wounding. Cells were cultured for a further 48 h before being fixed and stained with anti-myc for protein expression (I and ii) and with Hoechst for all nuclei (iii and iv). The field in between the white lines indicates the wound track. Bar, 20 μm. (B) The numbers of cells positively expressing stathmin or stathmin tetraA (a gift from M. Gullberg, University of Umeå, Umeå, Sweden) were counted between (wound track) and outside the white lines (normal) and expressed as a percentage of the total number of nuclei counted in the same area. Five random fields were counted, and the average ± SD (error bars) is shown. *, $P < 0.01$ as determined by a paired *t* test analysis.