Supplementary Materials and Methods

G-CSF binding studies

Studies of G-CSF binding were done with the Human G-CSF Phycoerythrin Fluorokine Kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Competitive binding of unlabeled G-CSF was performed using 100-fold excess filgrastim (Neupogen, Amgen Inc., Thousand Oaks, CA).

Antibodies

The following primary antibodies were used: PE-conjugated anti-CD114, APC-conjugated anti-CD114, APC-conjugated anti-CD56, PerCP-conjugated anti-CD45 (BD Biosciences, San Jose, CA), and APC-conjugated anti-CD133 (Miltenyi Biotech Inc., Auburn, CA).

Flow cytometry and fluorescence-activated cell sorting

Reactivity to mouse and human Fcγ receptors was blocked by 15-minute pre-incubation with mouse Fc-block (1:1,000; BD Biosciences) and polyclonal human IgG (2 mg/ml; Sigma-Aldrich, St. Louis, MO), respectively. Dead cells were excluded by staining with 4',6-diamidino-2-phenylindole (DAPI) 5 minutes before analysis (Invitrogen Corp., Carlsbad, CA). Fluorochrome- and isotype-matching mAbs suggested by BD Biosciences were used as negative controls. Analysis was performed on an LSR II 5-laser flow cytometer (BD Biosciences). FACS was performed on a DAKO Cytomation MoFlo 9-color cell sorter. BD FACSDiva v6.1.2 (BD Biosciences) was used to analyze flow data and Summit v4.3 (Cytomation) software was used to analyze FACS data.

The neuroblastoma patients participating in this study were recruited from the Texas Children's Cancer Center at Texas Children's Hospital in Houston, TX, USA. All of the procedures were approved by the Institutional Review Board of Baylor College of Medicine (IRB protocol H-26515). Fresh tumor tissues were collected according to IRB protocol from patients with pathologically and clinically confirmed neuroblastoma. In performing FACS analysis of patient tumor samples, neuroblastoma cells were distinguished from hematopoietic and other cells as CD45-negative, CD56-positive as previously described (Song et al, 2009).

Cell cycle analysis

The APC BrdU flow kit (BD Biosciences) was used according to the manufacturer's instructions for cell cycle analysis. Briefly, neuroblastoma cells (NGP, CHLA-255, IMR-32, LA-N-5) were analyzed forty-eight hours after plating. Cells were incubated with 10 μM BrdU for 1 hour prior to harvesting. Surface staining for CD114 was carried out followed by staining for BrdU. DAPI was used for total DNA staining. Analysis was performed on an LSR II flow cytometer (see ***Flow cytometry and fluorescence-activated cell sorting*** for further details regarding instruments).

G-CSF treatment assay

Neuroblastoma cells were harvested and 0.5 x 10^6 cells were either untreated or treated with 10 or 100 ng/ml of G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA) for 30 min, 2h and 24 h. Treated and untreated cells were fixed at specific time points with 1% paraformaldehyde for 10 min at 37^0C followed by two washings with FACS buffer (PBS, 1% BSA, 0.1% NaN3). Fixed cells were then surface stained with PE-conjugated anti-CD114 antibody (BD Biosciences) for 30 min, washed twice with FACS buffer, and analyzed on an LSR II 5-laser flow cytometer (BD Biosciences). All experiments were performed in independent duplicates and repeated three times.

Stat3 reporter assay

The Stat3 activation reporter used in the present study has an eGFP gene cloned downstream of a M67 responsive promoter, which contains four M67 binding motifs (TTCCCGTAA) and a TATA box. This reporter cassette was then cloned into a self-inactivating lentiviral vector plasmid and named Stat3.eGFP reporter (kindly provided by Dr. Michael T. Lewis, Baylor College of Medicine). The Stat3 reporter lentivirus was packaged in 293T cells by co-transfecting pVSVG, pCMV-dR8.2-dvpr and Stat3.eGFP construct. Viral supernatant was collected and used fresh to transduce several neuroblastoma cell lines. Transduction efficiencies were monitored by fluorescent microscopy 48 h post-infection. Stable GFP-expressing cells were selected by flow cytometry (DAKO Cytomation MoFlo 9-color cell sorter) and cultured routinely.

Stat3.eGFP reporter-transduced neuroblastoma cells were harvested and 0.5 x 10^6 cells were either untreated or treated with 10 or 100 ng/ml of G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA) in combination with 0, 3, 10 µM Stattic (Calbiochem, EMD Millipore, Darmstadt, Germany) for different time points. Treated and untreated cells were fixed with 1% paraformaldehyde for 10 min at 37^0C followed by two washings with FACS buffer (PBS, 1% BSA, 0.1% NaN3). Fixed cells were then surface stained with PE-conjugated anti-CD114 antibody (BD Biosciences) for 30 min, washed twice with FACS buffer, and analyzed for GFP expression in the CD114+ subpopulation using an LSR II 5-laser flow cytometer (BD Biosciences). FACS data were further analyzed by FlowJo ver. 7.6.4. All experiments were performed in independent duplicates and repeated three times.

Small RNA isolation and amplification

We used FACS to isolate CD114+ and CD114- cells from the IMR-32, NGP, and LA-N-5 neuroblastoma cell lines using a PE-conjugated anti-CD114 antibody (BD Biosciences, San Jose, CA). We used 40,000 cells from each group (pooled from three sorts) to generate small RNA sequencing libraries. Total RNA was isolated using the miRVANA kit (Ambion, Grand Island, NY). Small-RNA library preparation and sequencing was performed as previously described (Creighton, Reid et al. 2009) using the Illumina Solexa platform (Illumina Inc., San Diego, CA). We generated between 700,000 and 1.3 million unique sequencing tags for each sample. Sequence read mapping and quantification was performed using the optimized genomic mapping and data analysis pipeline described previously (Creighton, Reid et al. 2009; Shohet, Ghosh et al. 2011).

Gene sequencing

Total RNA was prepared from about 1 million cells from each cell line the using RNeasy Mini Kit (Qiagen Inc., Valencia,

CA). Five micrograms total RNA was used for each reverse transcription using random hexamer primers to generate cDNA with SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. Gene-specific primers (shown below) were used to PCR amplify 0.25 µl of cDNA sample using OneTaq Hot Start DNA Polymerase (New England Biolabs Inc., Ipswich, MA) with the following parameters: 94ºC 30 seconds, 59ºC 30 seconds, 68ºC 3 min, repeat for 30 cycles. Amplified products were run on a 1% agarose gel from which the 2500 base pair band was purified using QIAquick Gel Extraction Kit (Qiagen Inc.). The purified RNA was then sequenced with the following primers (shown below) and analyzed using Clone Manager v 9.0 (Science and Educational Software, Cary, NC).

CSF3R Amplification Primers: (2451 Bps)
Left: ATCAAGTTGGTGCTATGGCAAGGCTG
Right: CCATGACTAAAAACTACCCCAGCCCAGG
Sequencing primers:
(Bp 1880 Forward) CCCAGCTCTGACCAGACTCCATGC
(GCSFR Reverse End) CCTGGGCTGGGGTAGTTTTTAGTCATGG
GGGTCGGAGCTACACATCATCCTGG
CCAGGATGATGTGTAGCTCCGACCC
(Bps 1460 Reverse): CCCGTGGCTCTCCCATTCTG
(Bps 640 Reverse): CTGCCTGCACCCAGATGCCC
(Bps 1200 Forward): GAGGAAGACAGCGGACGGATC
(Bps 950 Forward): CTCTGCAACACCACAGAGCTCAG
(Bps 668 Reverse): CATGGGATCAAGACACAGTTGTGG
(Bps 1351 Reverse): CCATGGATTGGGGGGCTC
(Bps 607 Forward): GCCTGACTTGGGCTGCCCTG
(Bps 1091 Reverse): GGTCTGACAGTTGCCCCGGC
(Bps 1136 Forward): CCACTGCTGCATCCCACGCA
(Bps 1910 Reverse): AGGGTCTCGGGCCATGGCAT

RNA extraction and quantitative real time RT-PCR

RNA was extracted from FACS-sorted 0.5 x 10^5 CD114+ and CD114- neuroblastoma cells using Arcturus PicoPure RNA isolation kit (Applied Biosystems Inc., Carlsbad, CA) according to the manufacturer instructions. The amount of RNA obtained was determined spectrophotometrically at 260 nm (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). Total RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY) according to the protocol provided by the manufacturer and using oligo(dT)$_{20}$ primers. The cDNA prepared from 250 ng of total RNA isolated from CD114+ and CD114- cells was further diluted (1:10) and loaded on the RT^2 Profiler PCR Array **Human Cancer Pathway Finder** (PAHS-033, SABiosciences, Valencia, CA) and RT^2 Profiler PCR Array **Human Stem Cell** (PAHS-405, SABiosciences). The real time RT-PCR reaction was performed using RT^2 SYBR Green/ROX

PCR Master Mix (SABiosciences) on a StepOnePlus Real-Time PCR System (Applied Biosystems Inc.). Data obtained from the PCR array plates were further analyzed using RT² Profiler PCR array data analysis software ver 3.5 (SABiosciences). PCR array results were further validated by designing PCR primers (see below) for individual genes and performing real-time reactions in triplicates for each sample using QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA). The relative expression was normalized to β-actin and GAPDH. The p values were calculated by Student's t-test for expression fold difference of individual gene between CD114+ and CD114- cells.

Primers for qPCR validation of overexpressed genes in CD114+ and CD114- subpopulations

Gene	Primer Forward (5' to 3')	Primer Reverse (5' to 3')
ANGPT1	AGGGTGCAGCAATCAGCGCC	CCAGCATGGTAGCCGTGTGGT
ASCL1	GCTGCAAACGCCGGCTCAAC	GCGGATGTACTCGACCGCCG
CCND1	GCCCGAGGAGCTGCTGCAAAT	TGCCACCATGGAGGGCGGAT
CCND2	CCACCTGGATGCTGGAGGTCTGT	GGAGTCGGGACCCCAGCCAA
CDH2	TCCACGCCGAGCCCCAGTAT	TCAGCCGCTTTAAGGCCCTCAT
CDK1	ACTGGCTGATTTTGGCCTTGCC	TGAGTAACGAGCTGACCCCAGCA
E2F1	GCTGCTCGACTCCTCGCAGA	GTCGACGACACCGTCAGCCG
EP300	GCCAGCGATGGCACAGATTTTGG	ACCTGGGCCACCAACTCCCAT
EZH2	AACACCGGTTGTGGGCTGCAC	AGCCAGGTAGCACGGGCACT
FOS	AGCCAAATGCCGCAACCGGA	CTGGCAGGCCCCCAGTCAGA
HDAC2	GGCGTACAGTCAAGGAGGCGG	GCTCCAGCAACTGAACCGCCAG
HIF2a	GCTCCACGCCCAATAGCCCT	AGGGACTGTGCGGGGCTACA
ITGB1	GCGCGTGCAGGTGCAATGAA	ACACACTGTCCGCAGACGCA
JUN	CGAGATGCCCGGCGAGACAC	CTTGGAGGCAGCGATGCGGT
NME1	AGGCGAGGGGCCTCCTATCTCA	TGCAGGGTTGGTCTCCCCGA
NOTCH1	GTGCCAACCCCTGCGAGCAT	ACACCCTCGTAGCCGGGCAT
NOTCH2	CTGGCTTTGCTGGGGAGCGT	TGCCGGCCAGTAAAGGCACT
PAX6	GGGCCGAACAGACACAGCCC	GAAGTGGTGCCCGAGGTGCC
S100B	ATGGTGGCCCTCATCGACGTT	CGCCGTCTCCATCATTGTCCAGT

SOX10	GCTCCGTCCTAACGAGGCCGT	CAACGCCCACCTCCTCGGACC
SOX2	TAAATACCGGCCCCGGCGGA	GCTCGCCATGCTATTGCCGC
STAT1	GGCCAAAGGAAGCACCAGAGCC	CCTCAGGAGACATGGGGAGCAGG
STAT3	CCCAGGTAGCGCTGCCCCAT	GGGGAGGTAGCGCACTCCGA
TUBB3	TGAGGGAGATCGTGCACATCCAGG	CGAGTCGCCCACGTAGTTGCC
GAPDH	GGTCGTATTGGGCGCCTGGTC	GCCAGCATCGCCCCACTTGA
ACTB	TGATATCGCCGCGCTCGTCGTC	CCATGCCCACCATCACGCCCTG

Establishment of transduced cell lines

To generate cell lines expressing either GFP or tandem dimer (td) Tomato we used the lentiviral pCCL vector, a gift of Dr. J. Kandel and Dr. D. Yamashiro (Columbia University, New York, NY). Recombinant viruses were produced by transient transfection into 293T cells with packaging plasmids pMDL g/p RRE (purchased from Addgene Inc., Cambridge MA), and pRSV-REV and pVSVG, donated by Dr. G. Dotti (Baylor College of Medicine, Houston, TX). Infectious lentivirus particles were collected after 36-48 hours and filtered through a 0.45 µm HT Tuffryn® filter (Pall Corporation, Ann Arbor, MI). NGP, NB-1691, LA-N-5 and CHLA-255 cells were infected for 24 hours before removal of viral supernatant. Stably transduced cells were selected by fluorescence-activated cell sorting (FACS) 96 hours after transduction.

Phospho-flow assay

Neuroblastoma cells from the NGP cell line were harvested, and 0.5 x 10^{6} cells were either untreated or treated with 10 ng/ml of G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA). Treated and untreated cells were fixed at specific time point with 1% paraformaldehyde for 10 min at 37^{0}C followed by washing twofold with FACS buffer. Fixed cells were then surface stained with PE-conjugated anti-CD114 antibody (BD Biosciences) for 30 min on ice followed by twofold washing with FACS buffer. Cells were then treated with ice cold methanol for 30 min and washed twice with FACS buffer. Permealized cells were then incubated with Pacific Blue-conjugated anti-STAT3 (pY705) antibody (BD Biosciences) for intracellular staining, washed twice with FACS buffer and analyzed on an LSR II 5-laser flow cytometer (BD Biosciences). All experiments were performed in independent duplicates and repeated three times.

Side population analysis

For side population (SP) analysis, the NB cell lines CHLA255, IMR32 and SH-SY5Y were stained as previously described (Shafer, Cruz, et al., 2010). Briefly, cells were re-suspended at 1x10^{6} cells per ml in pre-warmed RPMI with 2% FBS and 10mM HEPES. 5 µg/ml of Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO) with or without 50 µM Verapamil were added and cells were incubated from 90-120 minutes at 37 C, with brief mixing every 30 minutes. Cells were then washed in cold RPMI with 2% FBS and 10 mM HEPES, and stained with CD114-PE or the IgG1k-PE isotype control (BD

Bioscience, San Jose, CA) as previously described. Immediately before samples were run, 1 µg/ml of propidium iodide (PI)(Sigma, St. Louis, MO) was added to each sample to stain for dead cells. Samples were analyzed using a BD LSRII analyzer (BD Bioscience). For the analysis of SP, samples were first gated on PI-negative cells and then analyzed based on Hoechst Red vs. Hoechst Blue staining to identify both the SP and the non-SP. Further analysis was done to determine the percentage of CD114+ cells in each subpopulation.

Additional antibodies and Western Blot analysis

Primary antibodies for Western blot analysis are the following: anti-N-Myc Mouse mAb (clone NCM II 100; EMD Chemicals, Gibbstown NJ), anti-CyPB (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used are anti-mouse IgG IRDye800 and anti-goat IgG IRDye700 (Rockland Immunochemicals, Gilbertsville, PA). One hundred microgram aliquots of protein were electrophoresed on a 4-15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA) and transferred as described (Slack, Chen et al. 2005). Human Cyclophilin B was used for loading control in the experiments. The Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) with the above secondary antibodies was used for detection and densitometry (Odyssey software v3.0, Li-Cor).

Supplementary references:

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