Supplemental Methods

Establishment of Instrument Compensation Settings

1. Add 50μl healthy donor whole blood to each tube labeled as Unstained, Alexa488, FITC, PE, Qdot605, PerCP, PacBlue, Alexa647, and Alexa700.
2. Add 20μl of appropriate compensation cocktail to corresponding tube.
3. Incubate 20 minutes at ambient temperature in the dark.
4. Lyse samples by adding 1mL of BD Pharmlyse (prepare fresh with 18mL DI H2O and 2mL 10X stock BD Pharmlyse) and incubating 15 minutes at room temperature.
5. Centrifuge 5 minutes at 400x*g*.

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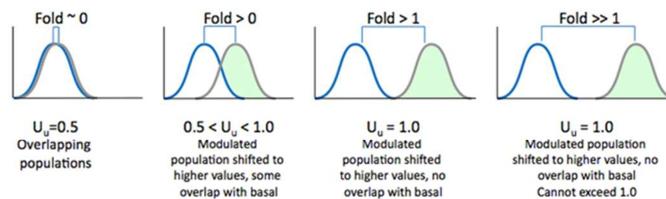
6. Decant tubes to ~100µl and gently rack rake to resuspend cell pellets.
7. Add 2mL 1X PBS + 2% FBS to tubes and centrifuge 5 minutes at 400x*g*.
8. Decant tubes to ~100µl and gently rack rake to resuspend cell pellets.
9. Resuspend tubes in 1mL 1% PFA.
10. Transfer 150µl of the unstained tube and compensation tubes to a 96-well plate.
11. Place the plate on the instrument HTS (High Throughput Sampler) unit.
12. Under the instrument setup folder click the 96-well plate icon titled "Compensation Plate".
13. Use appropriate settings for the HTS unit, "Parameter" and "Threshold" in the cytometer. Ensure that all compensation values are set to 0.0.
14. After the plate has finished acquisition all the instrument setup files must be exported.
15. In the FACSDiva "Browser" window, click "Instrument Setup" and chose export FBS files.
16. Export the data for Winlist analysis and compensation determination.

Aqua Compensation

1. Prepare Aqua positive and Aqua negative cells for compensation setup. Peripheral blood mononuclear cells were used and split into two conditions: 1) unstained cells (Aqua negative): cells were fixed/permeabilized in paraformaldehyde/methanol; 2) Aqua stained cells (Aqua positive): cells were fixed/permeabilized in paraformaldehyde/methanol, washed out of methanol in fluorescence-activated cell sorting buffer, and then Aqua stained in PBS. After Aqua staining, cells were quenched with RPMI 10% FBS, fixed with paraformaldehyde, and permeabilized with methonal. The two conditions were then mixed 1:1 and store at -80°C.
2. Add 250µl fixed 1:1 Aqua positive: Aqua negative cells (~250,000 cells) to a tube.
3. Wash cells by adding 2mL PBS + 2%FBS.
4. Centrifuge tube 5 minutes at 400x*g.*
5. Decant tube to ~100µl and gently rack rake to resuspend cell pellets.
6. Resuspend cells in 1ml PBS + 2%FBS.
7. In FACSDiva "Browser" window, select the "AquaComp" specimen in the "Instrument Setup" folder and ensure that the "AquaComp" tube is active.
8. Place the Aqua comp tube on the instrument, acquire and record the data.
9. Export the data for Winlist analysis and compensation determination.

Supplemental Figure S1: Metrics



Several classes of metrics are employed to measure basal activities or modulated activities on the myeloid, CD34$^+$ and nRBC populations. These metrics are calibrated for each instrument, ensuring rigorous comparisons and longitudinal stability of results. Three classes of metrics have been evaluated for this study.

Fold Change: Measure of the shift in the median value of the population of cells that display modulation of signaling relative to the basal state. Calculated as log_2Fold.

U$_u$: Measure of proportion of cells that display induction of signaling relative to basal activity. Rank based metric based on Mann-Whitney U statistic.

Total Phospho: Measure of the shift in the median value of the population of cells that display modulation of signaling relative to the autofluorescence state. Calculated as log_2Total Phospho.

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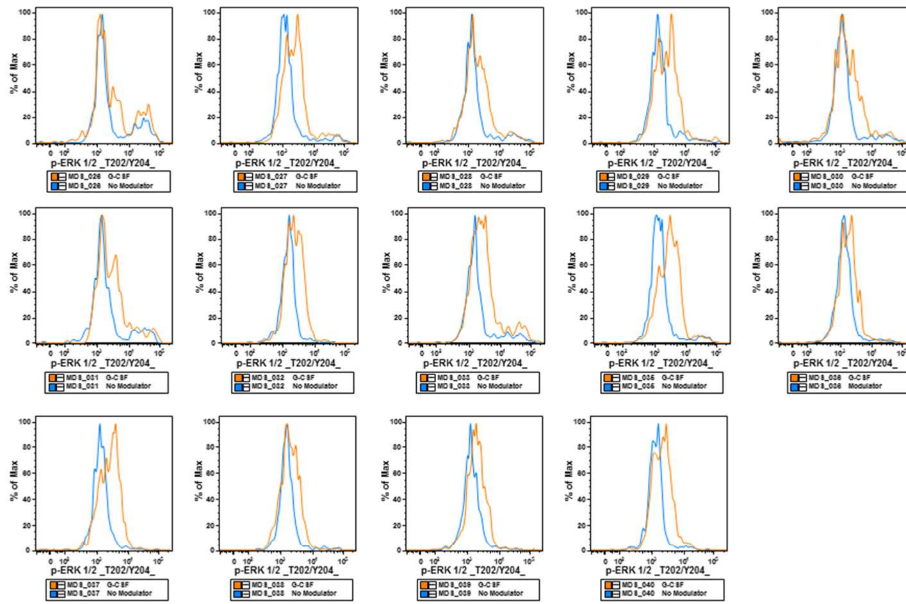
Supplemental Figure S2: GCSF Modulated p-Erk1/2 Activity in Healthy BM Samples



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Supplemental Table S1: Characteristics of samples that failed minimum viability/signaling criteria

Screening Failures

Donor ID	Age	Gender	IPSS	FAB	WHO	Cyto	WBC (K/uL)	Hgb (g/dL)	Plt (K/ul)	BM Blast (%)	Reason for Failure
MDS_006	72	M	INT-1	RAEB	RAEB-1	dip	2.8	10.3	56	8	Poor viability
MDS_007	62	F	INT-1	RAEB	RAEB-1	miscellaneous	11.4	11.8	3	5	Poor signaling
MDS_008	75	M	INT-1	RAEB	RAEB-1	dip	4.1	8.2	23	6	Poor viability
MDS_011	62	M	INT-1	RARS	RARS	+8	3.1	13	141	3	Poor viability
MDS_012	61	M	INT-1	RARS	RARS	dip	2.5	9.3	50	3	Poor signaling

Poor Viability: live and non-apoptotic cells (i.e. Aqua and c-PARP negative) < 50%

Poor Signaling:
MDS_007: no GCSF response (Uu=0.44-0.49);
no data for EPO nodes (numbers of events recorded for the nodes were below the cut-off, <100)
MDS_012: no EPO response (Uu=0.49-0.51);
no data for GCSF nodes (not enough cells for the assay, sample fell off)

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Supplemental Table S2: Modulators and Nodes Tested

A) List of Nodes Tested

Functional Signaling	Modulator	Node: Readout		
STAT Pathway	EPO	p-STAT1	p-STAT3	p-STAT5
STAT Pathway	GCSF	p-STAT1	p-STAT3	p-STAT5
PI3K Pathway	GCSF	p-Akt	p-Erk1/2	p-S6

B) Modulators and Technical Conditions

Modulator	Final Concentration	Modulation Time	Manufacturer (Location)
EPO	3 IU/mL	15 min	R&D Systems (Minneapolis, MN)
GCSF	50 ng/mL	15 min	R&D Systems (Minneapolis, MN)

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Supplemental Table S3: Antibodies and Non-Antibody Reagents

Antibody	Species & Isotype	Clone	Fluorophore	Manufacturer (Location)
CD34	Mouse IgG1	8G12	PerCP	BD (San Jose, CA)
CD45	Mouse IgG1	HI30	Alexa Fluor 700	Invitrogen (Carlsbad, CA)
CD71	Mouse IgG2a, k	M-A712	Biotin	BD (San Jose, CA)
CD235a	Mouse IgG2b, k	HIR2	PE	eBioscience (San Diego, CA)
p-STAT1 (Y701)	Mouse IgG2a	4a	Alexa Fluor 488	BD (San Jose, CA)
p-STAT3 (Y705)	Mouse IgG2a, k	4/P-STAT3	Pacific Blue	BD (San Jose, CA)
p-STAT5 (Y694)	Mouse IgG1	47	Alexa Fluor 647	BD (San Jose, CA)
p-Akt (S473)	Rabbit IgG	193H12	Alexa Fluor 647	CST (Danvers, MA)
p-S6 (S205/236)	Rabbit IgG	2F9	Alexa Flour 488	CST (Danvers, MA)
p-Erk1/2 (T202/204)	Mouse IgG1	20A	Pacific Blue	BD (San Jose, CA)
cleaved PARP (Asp214)	Mouse IgG1, k	F21-852	FITC	BD (San Jose, CA)

Non-Antibody	Manufacturer (Location)
Live/Dead Fixable Aqua Dead Cell Stain	Invitrogen (Carlsbad, CA)
Streptavidin-Qdot605	Invitrogen (Carlsbad, CA)

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Supplemental Table S4: Antibody Staining Panels and Compensation Settings

A) List of Antibody Staining Panel

Staining Panel	Alexa488	PE	Qdot605	PerCP	Pacific Blue	Aqua	Alexa647	Alexa700
STAT Pathway	p-STAT1	CD235a	CD71	CD34	p-STAT3	Aqua	p-STAT5	CD45
PI3K Pathway	p-S6	CD235a	CD71	CD34	p-Erk1/2	Aqua	p-Akt	CD45

Staining Panel	FITC	PerCP	Aqua	Alexa700
Viability	Cleaved PARP	CD34	Aqua	CD45

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B) List of Compensation Matrix

Compensation Matrix of STAT Staining Panel

	Alexa Fluor 488-A	PE-A	Qdot 605-A	PerCP-A	Pacific Blue-A	Aqua-A	Alexa Fluor 647-A	Alexa Fluor 700-A
Alexa Fluor 488-A		16.98	4.05	1.50	0.00	0.40	0.00	0.00
PE-A	0.99		40.97	22.23	0.00	7.08	0.00	0.00
Qdot 605-A	0.23	2.17		1.28	0.00	8.85	0.00	0.00
PerCP-A	0.05	0.00	0.00		0.00	0.00	10.42	3.50
Pacific Blue-A	0.10	0.00	0.00	0.00		11.60	0.00	0.00
Aqua-A	0.13	0.00	0.00	0.00	15.26		0.00	0.00
Alexa Fluor 647-A	0.10	0.00	0.00	2.44	0.05	0.05		40.61
Alexa Fluor 700-A	0.57	0.21	0.14	3.26	0.07	0.15	5.61	

Compensation Matrix of PI3K Staining Panel

	Alexa Fluor 488-A	PE-A	Qdot 605-A	PerCP-A	Pacific Blue-A	Aqua-A	Alexa Fluor 647-A	Alexa Fluor 700-A
Alexa Fluor 488-A		13.86	3.61	1.37	0.00	0.51	0.00	0.00
PE-A	1.34		35.61	20.12	0.00	5.39	0.00	0.00
Qdot 605-A	0.09	4.33		1.01	0.00	8.03	0.06	0.00
PerCP-A	0.00	0.00	0.00		0.00	0.00	16.88	5.04
Pacific Blue-A	0.00	0.00	0.00	0.00		11.10	0.00	0.00
Aqua-A	0.68	0.16	0.06	0.00	25.50		0.00	0.00
Alexa Fluor 647-A	0.00	0.00	0.00	2.83	0.06	0.00		34.15
Alexa Fluor 700-A	0.57	0.20	0.13	3.28	0.13	0.15	6.63	

Compensation Matrix of Viability Staining Panel

	FITC-A	PerCP-A	Aqua-A	Alexa Fluor 700-A
FITC-A		2.21	1.57	0.00
PerCP-A	0.05		0.00	3.50
Aqua-A	0.13	0.00		0.00
Alexa Fluor 700-A	0.57	3.26	0.15	

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