SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Phosphorylation of Akt^{Thr308} is diminished in L-GMPs. A. Lineage^{low}, Sca-1^{-}, cKit^{hi}, CD34^{+} cells purified from healthy and MLL-AF9-induced leukemic mice were subjected to flow cytometry with phospho-AKT^{Thr308} (CD34+ myeloid progenitors (MP) vs. CD34+ leukemic progenitors (LP)) and **B.** phospho-S6$^{Ser235/236}$ (MP vs. LP p=0.0674). For B. the right panel is a histogram from a single experiment with bar graphs representing aggregate data from 2 experiments. **C.** MLL-AF9-expressing leukemia BM cells were treated with 0, 1, 10 or 100nM Rapamycin (Cell Signaling Technology) *ex vivo* for 24 hours. Following treatment cells were lysed and subjected to western blot with indicated antibodies. **D.** MLL-AF9-expressing leukemia BM cells stably transduced with control or myr-Akt-expressing recombinant retroviruses were lysed and subjected to western blot with the indicated antibodies. **E.** Mononuclear bone marrow (MNBC) cells recovered from MLL-AF9 leukemic mice were infected with MSCV-IRES-GFP control (Ctrl) or myr-Akt-expressing retroviruses. Following infection, cells from each condition were treated with either vehicle or 1nM rapamycin and then cells from all conditions were evaluated for size and granularity by flow cytometry six days post-infection.

Figure S2. Deletion of FoxO1/3/4 promotes myeloid maturation and reduces leukemic cell growth on supportive stroma. A and B. Leukemic BM cells expressing MLL-AF9 and bearing floxed alleles for FoxO1, FoxO3 and FoxO4 (FoxO1/3/4^{floxed};MLL-AF9 cells) were infected with Ctrl or CreER expressing recombinant retroviruses. Stably transduced Ctrl and CreER cells were then

treated with vehicle, 50, 100, 200 or 400nM 4-hydroxytamoxifen (4-OHT) for 4-6 hours. Five days following treatment, cells from each condition were assessed for CD11b and Gr-1 expression by flow cytometry (* CreER + 4-OHT vs. CreER + vehicle, Ctrl + vehicle, or Ctrl + 4-OHT $p<0.0001$). **B.** FoxO1/3/4^{floxed};MLL-AF9;Ctrl and FoxO1/3/4^{floxed};MLL-AF9;CreER cells from each CreER +/- 4-OHT were stained with May-Grünwald Giemsa. **C and D.** MLL-AF9-expressing leukemic BM was stably transduced with lentiviruses that carry a loxP-STOP-loxP-human CD34 cassette (LSL-hCD34). These cells were then transduced with control or CreER-expressing recombinant retroviruses. MLL-AF9;LSL-hCD34;CreER or control cells were treated with vehicle of 4-OHT as described above and then assessed 5 days later for mature myeloid surface marker expression, CD11b and Gr-1 by flow cytometry. **C.** Flow cytometric plot of hCD34 expression and **D.** graphical represenation of CD11b and Gr-1 expression in each condition. **E.** Vehicle and 4-OHT-treated FoxO1/3/4^{floxed};MLL-AF9;CreER and control cells were layered on top of pre-plated OP-9 cells 24 hours following treatment. Colony formation was assessed after 14 days of co-culture. **F.** FoxO1/3/4^{floxed};MLL-AF9;CreER and control cells were co-cultured with OP-9 stroma. Seven days later, cobblestones area forming colonies (CAFC) were counted and subsequently administered vehicle or 400nM 4-hydroxytamoxifen (4-OHT) for 4-6 hours. Seven days following 4-OHT or vehicle treatment CAFC were again counted and tabulated.

Figure S3. Depletion of FOXO3 promotes myeloid maturation and apoptosis of human AML cells. A. NOMO1, THP-1, U-937, NB4 and K562 were stably

transduced with recombinant lentiviruses expressing either non-targeting (NT shRNA) or FOXO3 (FOXO3 shRNA-1 & -2) shRNAs. Following stable infection, cells were lysed and subjected to western blot with FOXO3 and Tubulin antibodies. **B.** NOMO1, THP-1, U-937, NB4 and K562 cells were counted every 1-2 days following stable expression of designated shRNAs (at Day 4 NOMO1 1 NT vs. F3-1 *p<0.0001; at Day 6 THP-1 NT vs. F3-1 *p<0.0001 1 NT vs. F3-2 **p<0.0001; at Day 4 U-937 NT vs. F3-1 *p=0.0003, NT vs. F3-2 **p<0.0001; at Day 4 NB4 NT vs. F3-1 *p=0.0003 NT vs. F3-2 **p=0.0058). **C. SKM-1, NB4, HL-60 and THP-1** cells expressing either NT, FOXO3-1 and FOXO3-2 shRNAs were analyzed for human CD11b expression by flow cytometry eight days post-infection (SKM-1 NT vs. F3-1 *p<0.0001, NT vs. F3-2 **p=0.0115, **F.** NB4 NT vs. F3-1 *p<0.0001, NT vs. F3-2 **p=0.0008 **G.** HL-60 NT vs. F3-1 *p=0.0005; THP-1 NT vs. F3-1 *p<0.0001, NT vs. F3-2 **p<0.0001). **D.** Mono-Mac-6 and **L.** K562 cells stably expressing either NT, FOXO3-1 and FOXO3-2 shRNA molecules were incubated with pHrodo fluorescent-labeled *E. coli* particles and subsequently analyzed by flow cytometry for pHrodo+ cells. **E.** Cytospins of NOMO1, THP-1, MonoMac-6 (MM6), U-937 and HL-60 cells were stained with May-Grünwald Giemsa 7-8 days following infection with lentiviruses expressing either NT or FOXO3-1 shRNAs. **F.** THP-1, MOLM-14 and NB4 cells expressing either NT, FOXO3-1 or FOXO3-2 shRNAs were stained with Annexin V and CD11b and assessed for $CD11b^{-}$Annexin V^{+} and $CD11b^{+}$Annexin V^{+} staining (THP-1 * NT vs. F3-1, CD11b+ p<0.0001, ** NT vs. F3-2, CD11b+ p<0.0001; MOLM-14 * NT shRNA vs. FOXO3 shRNA-1, CD11b+ p<0.0001, ** NT shRNA

vs. FOXO3 shRNA-2, CD11b+ p<0.0001; NB4 (* NT shRNA vs. FOXO3 shRNA-1, CD11b+ p<0.0001, ** NT shRNA vs. FOXO3 shRNA-2, CD11b+ p<0.0001). (NT=NT shRNA, F3-1=FOXO3 shRNA-1 and F3-2=FOXO3 shRNA-2).

Figure S4. FOXOs are active in primary AML samples derived from patients. **A.** BM cells derived from patients with AML (designated as patient #5) were stained with human lineage cocktail and human CD34 (both available from BD biosciences) and then lineage low, CD34+ cells were isolated by flow cytometry. Total bone marrow (TBM) cells, lineage high, CD34- and lineage low, CD34+ cells were biochemically fractionated to recover nuclear (N) and cytoplasmic (C) extracts, which were then subjected to western blot with FoxO3-specfic antibodies (75D8). Both nuclear and cytoplasmic fractions from each cell line were also subjected to western blot with Orc2- (nuclear) and Tubulin- (cytoplasmic) specific antibodies to determine the efficiency of biochemical separation. **B.** Whole BM aspirates (TBM) from 4 patients diagnosed with AML were fractionated and blotted as described in panel A. **C.** Distribution of the FOXO gene signature clusters among various chromosomal abnormities and geneotypes commonly seen in human AML (p<0.0001). **D.** Distribution of FLT3-ITD-positive and –negative AMLs within each FOXO gene signature defined cluster (p<0.0001).

Figure S5. Excision efficiency of pI-pC treatement in FoxO1/3/4^{floxed};MLL-AF9;Mx1-Cre+ mice. A. WBC analysis of peripheral blood recovered from mice examined in Figure 5B and 5C (Mx1-Cre+ (pI-pC) vs. Mx1-Cre+ (saline), p<0.0001; n=4). **B.** Left schematic depicts the experimental outline of the Kaplan-

Meier survival curve analysis to the right. As described in Figure 5, we generated MLL-AF9-induced AML in a FoxO1/3/4^{floxed};Mx1-Cre+ genetic background that was subsequently transplanted into secondary mice. Fourteen days post-transplant, secondary recipients were administered with saline or pI-pC and monitored for the development of AML (Figure 5). BM cells recovered from saline (Ctrl) and pI-pC treated mice that succumbed to AML were then transplanted into tertiary recipients and evaluated for the development of AML (p=0.0061; n=8). **C-E.** RNA recovered from BM was converted to cDNA and then analyzed for FoxO1 (**C.**), FoxO3 (**D.**), FoxO4 (**E.**) and GAPDH expression by quantitative Real Time PCR. Each FoxO gene expression is normalized with GAPDH expression. **F.** BM recovered from one saline-treated FoxO1/3/4^{floxed};MLL-AF9;Mx1-Cre+ mouse (lane 1) five FoxO1/3/4^{floxed};MLL-AF9;Mx1-Cre+ mice treated with pI-pC (lanes 2-6) that succumbed to AML-related death were subjected to western blot with FoxO3 (75D8) and Tubulin antibodies. **G.** Seven days following the administration of saline or pI-pC, secondary mice transplanted with MLL-AF9-positive FoxO1/3/4^{floxed};Mx1-Cre+ leukmic BM cells were euthanized. Leukemic BM cells were then isolated from each treatment condition (Saline (Ctrl) versus pI-pC (pI-pC)) were analyzed for the proportion of mature myeloid cells (**H.** lineage^{hi}, CD11b^{+}, p=0.0098; n=3) **H.** BM cells recovered from MLL-AF9 leukemic mice were infected with MSCV-IRES-GFP control (Ctrl) or myr-Akt-expressing retroviruses. Following infection, 100,000 GFP+ cells from each condition were purified and separately injected into syngeneic recipients. Mice were monitored for AML-related death.

Figure S6. JNK inhibitor, SP600125 cooperates with AKT activation or FOXO inhibition in driving myeloid maturation. A. MOLM-14 and SKM-1 cells stably expressing either non-targeting (NT) or FOXO3 (FOXO3-1) shRNA were lysed and subjected to western blot with the indicted antibodies. **B.** SKM-1 cells stably expressing either non-targeting (NT) or FOXO3 (FOXO3-1) shRNA were lysed and subjected to western blot with the indicted antibodies. **C.** SKM-1 cells stably expressing non-targeting (NT) or FOXO3 (F3-1) shRNA were treated with 10μM SP600125 (JNK inhibitor) or vehicle. Forty-eight hours after treatment cells from each condition were assessed for Annexin V staining (NT (SP600125) vs. F3-1 (vehicle) *p<0.0001, F3-1 (vehicle) vs. F3-1 (SP600125) **p=0.0001). **D.** MOLM-14 and SKM-1 cells stably expressing non-targeting (NT) or FOXO3 (F3-1) shRNA were treated with 10μM SP600125 (JNK inhibitor) or vehicle. Forty-eight hours after treatment cells from each condition were assessed for CD11b expression (MOLM-14 NT (SP600125) vs. F3-1 (vehicle) *p=0.0008, F3-1 (vehicle) vs. F3-1 (SP600125) **p<0.0001; SKM-1 NT (SP600125) vs. F3-1 (vehicle) *p<0.0001, F3-1 (vehicle) vs. F3-1 (SP600125) **p=0.0001). **E.** MLL-AF9-expressing leukemia BM cells stably transduced with control or myr-Akt-expressing recombinant retroviruses were treated with 10μM SP600125 (JNK inhibitor) or vehicle. Forty-eight hours after treatment cells from each condition were assessed CD11b and Gr-1 expression. **F.** GFP+, $CD11b^{int}$, $Gr\text{-}1^{int}$ cells were purified by flow cytometry from vehicle- and SP600125-treated control cells as well as SP600125-treated myr-Akt-positive cells. Additionally, flow cytometry was used to isolate GFP+, $CD11b^{+}$, $Gr\text{-}1^{int}$ cells from vehicle- and SP600125-

treated myr-Akt-positive cells and GFP+, CD11b^{+}, Gr-1^{+} cells from SP600125-treated myr-Akt-positive cells. Purified cells were subjected to cytospin followed by Wright-Giemsa staining.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibody Information (all antibodies are for mouse unless otherwise indicated): To analyze and isolate L-GMPs and GMPs, total BM cells were recovered from flushing the tibias and femurs of leukemic and healthy mice respectively with PBS supplemented with 2% FBS and 1% Penicillin/Streptomycin (F-PBS). Bone marrow mononuclear cells (BMMCs) were then stained with a lineage cocktail comprised of antibodies targeting CD3, CD4, CD8, CD19, B220, Gr-1, Ter119, and IL-7RαPE-Cy5 conjugates). Cells were also stained with antibodies targeting cKit-Ax750, Sca-1-APC or -Pacblue, FcγRII/III-PE-Cy7, and CD34-FITC. L-GMP and GMP populations were analyzed and sorted with a FACSAria instrument (Becton Dickinson). For Phosphoflow experiments cells were stained with cKit-Ax750 and CD34- antibodies in combination with phospho-AKT^{Ser473}-PE, phospho-AKT^{Thr308}-APC or phospho-S6$^{Ser235/236}$-APC antibodies and analyzed on a FACSAria instrument (See supplementary experimental procedures for details). Mature myeloid markers of murine MLL-AF9-expressing leukemia cells were assessed with mouse CD11b-APC and Gr-1-PE-Cy5 antibodies. Human AML, APL and CML cell lines were stained with human CD11b-APC antibodies. Primary AML patient BM cells were stained with human CD34-APC and Lineage cocktail-FITC. Antibody information tabulated below:

ANTIBODY	APPLICATION	COMPANY	CLONE# OR CATALOG#	DILUTION FACTOR
CD3	Flow	Biolegend	145-2c11	1:40
CD4	Flow	BD Biosciences	RM4-5	1:100
CD8	Flow	Biolegend	53-6.7	1:100
CD19	Flow	Biolegend	6D5	1:100
B220	Flow	eBioscience	RA3-6B2	1:100
Gr-1	Flow	eBioscience	RB6-8C5	1:100
Ter119	Flow	Biolegend	TER-119	1:400
IL-7Ra	Flow	eBioscience	A7R34	1:30
CD11b	Flow	Biolegend		1:100
cKit	Flow	eBioscience	2B8	1:200
Sca-1	Flow	Biolegend	D7	1:200
CD34	Flow	eBioscience	RAM34	1:50
FcgRII/III	Flow	eBioscience	93	1:200
phospho-AKT^{Ser473}-PE	Flow	Cell Signaling Technology (CST)	D9E	1:25
phospho-AKT^{Thr308}-APC	Flow	CST	C31E5E	1:25
phospho-S6$^{Ser235/236}$-APC	Flow	CST	D57.2.2E	1:100
Human CD11b	Flow	BD Biosciences	ICRF44	1:100
Human CD34-APC	Flow	BD Biosciences	8G12	5ul/ 1x10^6 cells
Human Lineage cocktail	Flow	BD Biosciences	340546	5ul/ 1x10^6 cells
FOXO3	Western	CST	9467	1:1000
FOXO3 (75D8)	Western IF	CST	2497	1:1000 (W) 1:100 (IF)

Tubulin	Western	CST	9F3	1:2500
ORC2	Western	Santa Cruz Bio-technology	H-300	1:1000
Phospho-FOXO3 (Ser256)	Western	CST	9466	1:1000
Phospho-ERK1/2 (Thr202/Tyr204)	Western	CST	197G2	1:1000
Phospho-AKT (Ser473)	Western	CST	193H12	1:1000
Phospho-AKT (Thr308)	Western	CST	244F9	1:1000
AKT	Western	CST	9272	1:1000
Phopho-4E-BP1 (Thr37/46)	Western	CST	236B4	1:1000
Phospho-S6 (Ser235/236)	Western	CST	2F9	1:1000
Phospho-cJUN (Ser63)	Western	CST	54B3	1:1000
cJUN	Western	CST	9162	1:1000
Phospho-JNK (Thr183/Tyr185)	Western	CST	98F2	1:1000
JNK	Western	CST	9252	1:1000

Phophoflow experiments

1. Euthanize mice and recover tibias and femurs.
2. Use a scapel to cut the ends of each bone off and use a syringe with a 271/2G needle (filled with PBS supplemented with 2%FBS and 1%P/S =F-PBS) flush into a 50ml conical tube covered with a 70uM nylon filter.
3. Lyse cells with RBC lysis buffer (BD Biosciences) for 5-10minutes on ice. Wash with 10.0ml F-PBS and centrifuge 5min at 1500rpm.
4. Aspirate supernatant and wash one more time with 5.0ml F-PBS. Centrifuge 5min at 1500rpm.

5. Aspirate supernatant and resuspend cells in 400ul of F-PBS containing lineage antibodies conjugated with PE-Cy5 at the dilutions tabulated above. Incubate on ice in the dark for 20min.
6. Wash cells with 4.0ml F-PBS. Centrifuge 5min at 1500rpm.
7. Wash cells with 4.0ml F-PBS. Centrifuge 5min at 1500rpm.
8. Aspirate supernatant and resuspend cells in 400ul of F-PBS.
9. Sort lineage low (10-15%) into IMDM supplemented with 2%FBS (F-IMDM).
10. Following sort rest cells in IMDM supplemented with 2% FBS for 1 hour at 37°C.
11. Treat cells with 10ng/ml mSCF (or 1ng/ml GM-CSF, 10ng/ml IL-3 or 10ng/ml IL-6) for 5 and 15 minutes and then immediately fixed with 1.6% paraformaldehyde (PFA) for 10 minutes at 37°C.
12. Centrifuge 5min at 1500rpm.
13. Aspirate supernatant and resuspend pellet in 1.0ml of ice-cold 95% methanol while vortexing. Incubate on ice for 10 minutes.
14. Transfer cells to a 1.5ml eppendorf tube and centrifuge 3min at 6000rpm.
15. Aspirate and wash by adding 1 mL of 0.50%BSA/0.02%NaN_3/PBS.
16. Pellet cells (as above), aspirate.
17. Add another 1 mL of 0.50%BSA/0.02%NaN_3/PBS.
18. Pellet cells, aspirate.
19. Stain cells for 20min in the dark on ice with cKit-Ax750 and CD34-FITC or Pacblue antibodies in combination with phospho-AKT^{Ser473}-PE, phospho-

AKT^{Thr308}-APC or phospho-S6$^{Ser235/236}$-APC antibodies. NOTE: Alcohol permeabilization destroys the FcγRII/III epitope and therefore these specific antibodies were excluded (Kalaitzidis and Neel, 2008)).

20. Wash by adding 1 mL of 0.50%BSA/0.02%NaN_3/PBS.
21. Pellet cells (as above), aspirate.
22. Resuspend cells in 300-400ul and analyze on a FACSAria instrument.

Western Blotting

1. Protein lysates were generated by counting cells with trypan blue staining and then lysing cells directly in 1x LDS buffer supplemented with 1x reducing agent (Invitrogen) at a ratio of $1x10^6$ cells per 100ul of 1xLDS.
2. Lysates were boiled 10min and then centrifuged for 5min at 14000rpm.
3. Lysates were then loaded onto either 7% Tris-Acetate gels (Invitrogen) or 4-20% Tris-Glycine gels (Invitrogen) and run at 100V for approximately 90-150min.
4. Gels were then transferred onto nitrocellulose membranes using the invitrogen wet gel transfer system (120min at 30V).
5. Nitrocellulose membranes were then blocked in 5% BSA TBS-T for 1 hour at room temperature.
6. Membranes were then dissected based on protein size markers and placed in the appropriate antibodies diluted in 1% BSA/TBS-T. Incubate overnight on a rocker at 4 degrees centigrade.
7. Wash membranes three times in 15-25ml 1X TBS-T for 10 minutes each.

8. Incubate with HRP-conjugated anti-rabbit (or anti-mouse depending on the primary antibody) secondary antibody (Amersham, GE biosciences) at dilution of 1:2500 for 1 hour at room temperature.
9. Wash membranes three times in 15-25ml 1X TBS-T for 10 minutes each.
10. Incubate membranes with pico ECL kit available from Pierce Technologies.
11. Expose to film and develop in an X-OMAT developer.

Cell Fractionation Protocol

1. Resuspend cells in 2-3 pellet volumes of Buffer A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 0.4% NP-40, 10% glycerol, 1mM DTT and Protease Inhibitors). Smaller volumes generate more concentrated cytoplasmic fractions.
2. Incubate cells on ice for 20 minutes.
3. Pellet nuclei by centrifugation (4 minutes @ 1,300x*g* (~3500rpm on a microcentrifuge), 4 degrees centigrade).
4. Recover supernatant **(Cytoplamic fraction)**
5. Wash nuclei 1X with Buffer A and centrifuge 4 minutes @ 1,300x*g*, 4 degrees centigrade.
6. Aspirate Supernatant and Resuspend the pellet in 1-2 pellet volumes of buffer C (20mM HEPES, pH7.9, 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.5mM EDTA, 0.5mM PMSF, 0.5mM DTT). Incubate on ICE for 30 minutes with vortexing for 10 sec every 5 minutes **OR** spin at 60rpm @ 4 degrees centigrade.

OR

7. Lyse pellets in 1X LDS + reducing agent 10min @ 95 degrees Celsius.
8. Spin at max speed (20,000x*g*) for 5 minutes.
9. Recover supernatant **(Nuclear fraction)**
10. Nuclear and cytoplamic fractions were then subjected to western blot with antibodies targeting FOXO3 (1:1000, clone 75D8, Cell Signaling Technology), Tubulin (1:2500, Cell Signaling Technology) and ORC2 (1:1000, Santa Cruz Biotechnology). Antibodies were resuspended in TBS-T supplanted with 1% BSA.

Immunofluorescence

1. Euthanize mice and recover tibias and femurs.
2. Use a scapel to cut the ends of each bone off and use a syringe with a 271/2G needle (filled with PBS supplemented with 2%FBS and 1%P/S =F-PBS) flush into a 50ml conical tube covered with a 70uM nylon filter.
3. Lyse cells with RBC lysis buffer (BD Biosciences) for 5-10minutes on ice. Wash with 10.0ml F-PBS and centrifuge 5min at 1500rpm.
4. Aspirate supernatant and wash one more time with 5.0ml F-PBS. Centrifuge 5min at 1500rpm.
5. Aspirate supernatant and resuspend cells in 400ul of F-PBS plus lineage antibodies conjugated with PE-Cy5, cKit-Ax750, Sca-1-APC, FcgRII/III-PE-Cy7 and CD34-FITC with the appropriate dilutions tabulated above. Incubate on ice for 20 min in the dark.

6. Wash cells with 4.0ml F-PBS. Centrifuge 5min at 1500rpm.
7. Wash cells with 4.0ml F-PBS. Centrifuge 5min at 1500rpm.
8. Sort cells using FACS Aria (Becton Dickinson).
9. Purified L-GMPs and GMPs were placed on microscope slides via cytospin (4 minutes at 450rpm).
10. Cells were then fixed with 1% PFA for 10minutes at room temperature.
11. Aspirate PFA and permeabilize with 100% methanol.
12. Wash cells two times with TBS-T.
13. Block cells with 5%BSA at room temperature.
14. Incubate cells with FOXO3 (75D8) antibodies (1:1000; Cell Signaling Technology) overnight at 4 degrees centigrade.
15. FOXO3 stained cells were then incubated with an anti-rabbit FITC-conjugated secondary antibody (1:2000; Sigma).
16. Cells were also stained with DAPI to visualize nuclei.
17. Cells were then visualized under 100X magnification using a Nikon fluorescence microscope.

Viral Production and Infections

Day 1:

1. Seed 3-4x10^6 293FT or 293TL cells/10cm dish. The number is low because you want the cells to be ~60-80% confluent the following day.

Day 2: ~4:00-5:00pm

2. Transfect cells with DNA:Fugene Mix as follows.

a. Add 18ul of fugene to 282ul of plain DMEM (No serum or P/S). Tap tube to mix and incubate 5min at RT.
b. Add 3ug of lentiviral backbone (i.e. pKLO shRNA), 3ug of each packaging plasmid (i.e. 3ug of VSV-G + 3ug of pCMVΔ8.2 **OR** 3ug of MD + 3ug of pSPAX) to fugene:DMEM mix. Incubate 15-30 minutes at RT (We usually wait 15min).
c. Add DNA:Fugene:DMEM mix to 5ml of DMEM/10%FBS (No P/S).
d. Aspirate media from 293FT cells and replenish with ~5.5ml DNA:Fugene:DMEM:Full Serum mix. Incubate O/N in a 37 degree incubator.

Day 3 (~9-10:00am)

3. Aspirate Transfection mixture from each 10cm plate and replenish with 3.5ml DMEM/30%FBS. Incubate O/N in a 37 degree incubator and ensure the plates are on a flat surface and all cells are covered with a thin layer of media.

Day 4 (~9-10:00am)

4. Collect ~3.5ml viral supernatant and place in a 50ml conical tube and store O/N in the dark at 4 degrees Celsius (Viral Sup#1).

5. Replenish with 3.5ml DMEM/30%FBS. Incubate O/N in a 37 degree incubator and ensure the plates are on a flat surface and all cells are covered with a thin layer of media.

Day 4 (~9-10:00pm) OPTIONAL

6. Collect ~3.5ml viral supernatant and place in a 50ml conical tube and store O/N in the dark at 4 degrees Celsius (Viral Sup#2).
7. Replenish with 3.5ml DMEM/30%FBS. Incubate O/N in a 37 degree incubator and ensure the plates are on a flat surface and all cells are covered with a thin layer of media.

Day 5 (~9-10am.)

8. Collect ~3.5ml viral supernatant and combine with Viral Sup#1 & 2 in a 50ml conical tube. Centrifuge combined viral supernatant 5min @ 1200rpm.
9. NO NEED to filter if freezing the virus. However, it should be filtered if you are going to use the virus immediately to remove 293T contamination.
10. Aliquot centrifuged virus in to cryovials and store at -80 degrees Celsius.

For infection:

11. We typically infect 1x106 suspension cells in a 6-well plate with a total volume of 2.0ml which is composed of 500ul of viral supernatant + 8ug/ml polybrene + 1.5ml of media.
12. For puromycin selection we would use the following hypothetical schedule:
    a. **Monday (or Thursday):** Infect cells sometime before 11:00am. BE SURE to include a no infection control.

b. **Tuesday (or Friday):** Sometime after 4:00pm, add 3.0ml of fresh media to each infection (for a total of 5.0ml per well) supplemented with puromycin (the majority of our suspension cells lines tolerate 2ug/ml puromycin, but you should check each individual cell line personally).

c. **Friday (or Monday):** All no infected cells including no infection control should be dead and you should have your stable cell line. Stable cell lines are maintained in media supplemented with 0.5ug/ml Puromycin.

d. You can use Trypan Blue to assess infection efficiency (live to total cell ratio).

Bo**ne Marrow Transplanation (BMT) Assay**

1. Both *FoxO1/3/4*^{floxed};Mx1-Cre+ and *FoxO1/3/4*^{floxed};Mx1-Cre- mice were administered 150mg/kg 5-flurouracil (5-FU, Sigma).
2. Six days following 5-FU treatment mice were euthanized and mononuclear bone marrow (BM) cells recovered were subjected to retroviral transduction with recombinant MLL-AF9 expressing retroviruses.
3. After transduction, infected cells were transplanted into lethally irradiated (2 doses of 450rad gIR separated by 12 hours) F1 FVB/C57/Bl6 mice.
4. Recipient mice developed leukemia within 70-80 days with a median survival of 77 days.
5. Mononuclear BM cells recovered from leukemic mice were then transplanted into secondary sub-lethally irradiated recipients.

6. Fourteen days following transplant, secondary recipients were administered intra-peritoneally three doses of saline or 12.5mg/kg pI-pC (Amersham) every 2 days.
7. Mice were then monitored for external (i.e. moribund) and internal (white blood cell counts) signs of leukemia.

May-Grunwald or Wright-Giemsa

1. To examine morphological changes associated with myeloid differentiation, cells from respective conditions were centrifuged (4 minutes at 450rpm) onto microscope slides.
2. Cells were then permeabilized in 100% methanol for 4 minutes and subsequently stained with May-Grünwald dye (Sigma) for 10minutes.
3. Cells were then stained 1 minute with Giemsa dye, washed with distilled water and allowed to air dry.

OR

4. For cells stained with Wirght-Giemsa, cells were adhered to microscope slides as described above.
5. Cells were permeabilized and stained in 100% Wright-Giemsa stain for 4 minutes.
6. Cells were then stained for 10 minutes with 20% Wright-Giemsa/80% Cell wash buffer and subsequently washed with distilled water and allowed to air dry.

Colony Assays

For murine myeloid colony assays, three replicates of 1000 cells were plated in 1.5ml M3434 cytokine enriched methylcellulose according to manufacturer's instructions (Stem Cell Technologies). For human myeloid colony assays, three replicates of 10000 cells were plated in 1.5ml H4034 cytokine enriched methylcellulose according to manufacturer's instructions (Stem Cell Technologies). For CAFC assays, murine leukemia cells were co-cultured on OP9 stroma cell for 7-14 days.

Drug Treatments

1. Rapamycin (Cell signaling Technology or Calbiochem) was resuspend in 100% MeOH or DMSO at a stock concentration of 100 micromolar.
2. SP600125 (Selleck chemicals or Calbiochem) was resuspended in 100% DMSO at a concentration of 100mM.
    a. Prior to the addition of SP600125 at a final concentration of 10 micromolar, culture media was supplemented with 0.1% DMSO (due to the extreme hydrophobicity of SP600125)
3. Either drug was added to culture media prior to infections or 48 hours following infections.

FOXO GENE SET LIST

Probe set	Gene symbol
1449526_a_at	Gdpd3
1453109_at	Arsk
1456753_at	
1443504_at	E330022O07
1455292_x_at	
1428719_at	2010309G21Rik
1428720_s_at	2010309G21Rik
1436491_at	OTTMUSG00000000601
1423890_x_at	Atp1b1

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