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# AMPK/FIS1-Mediated Mitophagy Is Required for Self-Renewal of Human AML Stem Cells

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## SUMMARY

**Leukemia stem cells (LSCs) are thought to drive the genesis of acute myeloid leukemia (AML) as well as relapse following chemotherapy. Because of their unique biology, developing effective methods to eradicate LSCs has been a significant challenge. In the present study, we demonstrate that intrinsic overexpression of the mitochondrial dynamics regulator FIS1 mediates mitophagy activity that is essential for primitive AML cells. Depletion of FIS1 attenuates mitophagy and leads to inactivation of GSK3, myeloid differentiation, cell cycle arrest, and a profound loss of LSC self-renewal potential. Further, we report that the central metabolic stress regulator AMPK is also intrinsically activated in LSC populations and is upstream of FIS1. Inhibition of AMPK signaling recapitulates the biological effect of FIS1 loss. These data suggest a model in which LSCs co-opt AMPK/FIS1-mediated mitophagy as a means to maintain stem cell properties that may be otherwise compromised by the stresses induced by oncogenic transformation.**

## INTRODUCTION

Despite decades of research, effective therapies for acute myeloid leukemia (AML) remain extremely limited. A majority of patients relapse following conventional chemotherapy or, in some cases, never achieve a meaningful response (Dombret and Gardin, 2016). Analysis of AML biology has shown that disease pathogenesis is mediated by a subset of the tumor population termed leukemia stem cells (LSCs). Multiple studies have demonstrated that LSCs are biologically distinct from bulk tumor, have differential sensitivity to chemotherapy, and are likely a major cause of therapy resistance and relapse (Jordan et al., 2006; Mikkola et al., 2010). This concept is further supported by recent studies focusing on the clinical significance of LSCs. These studies demonstrate that the frequency and phenotypic diversity of LSCs increase substantially in relapse versus diagnosis AML (Ho et al., 2016). In addition, AML patients who have high expression of LSC gene expression signatures demonstrate significantly poorer treatment outcomes (Eppert et al., 2011; Gentles et al., 2010), suggesting that enrichment of LSC activities correlates with decreased efficacy of conventional therapy. Despite strong laboratory and clinical evidence emphasizing the need to improve anti-LSC regimens, the ability to effectively target such populations in patients remains a major challenge (Guzman and Allan, 2014; Pollyea and Jordan, 2017).

Although LSCs were first described as within the CD34+/CD38− subpopulation of human AML cells (Bonnet and Dick, 1997; Lapidot et al., 1994), later studies have shown significant inter- and intra-patient immunophenotypic heterogeneity in them, making CD34/CD38-based LSC isolation and characterization a significant challenge (Eppert et al., 2011; Ho et al., 2016; Ng et al., 2016; Sarry et al., 2011). As an alternative approach to characterization of cancer stem cell (CSC) populations, we and others have turned to the biochemical property of oxidative state as a means to identify and isolate primitive populations (Diehn et al., 2009; Lagadinou et al., 2013). As previously demonstrated for many types of stem cells, including normal hematopoietic stem cells (HSCs) (Jang and Sharkis, 2007), the level of reactive oxygen species (ROS) can be used to enrich for primitive populations. In all known instances, somatic stem cells reside in a relatively reduced condition, termed ROS-low, whereas more differentiated cells demonstrate a net increase in oxidative state, termed ROS-high. The ROS-low physiological state is thought to be required by normal and malignant stem cell populations as a means to alleviate both intrinsic and extrinsic stresses and to sustain their self-renewal and proliferation potential (Zhou et al., 2014). Thus, we have employed the ROS-low phenotype as an alternative means to enrich LSC populations for molecular, biochemical, and cellular analyses.

Using the oxidative state to characterize primary human AML specimens, we previously compared the gene expression

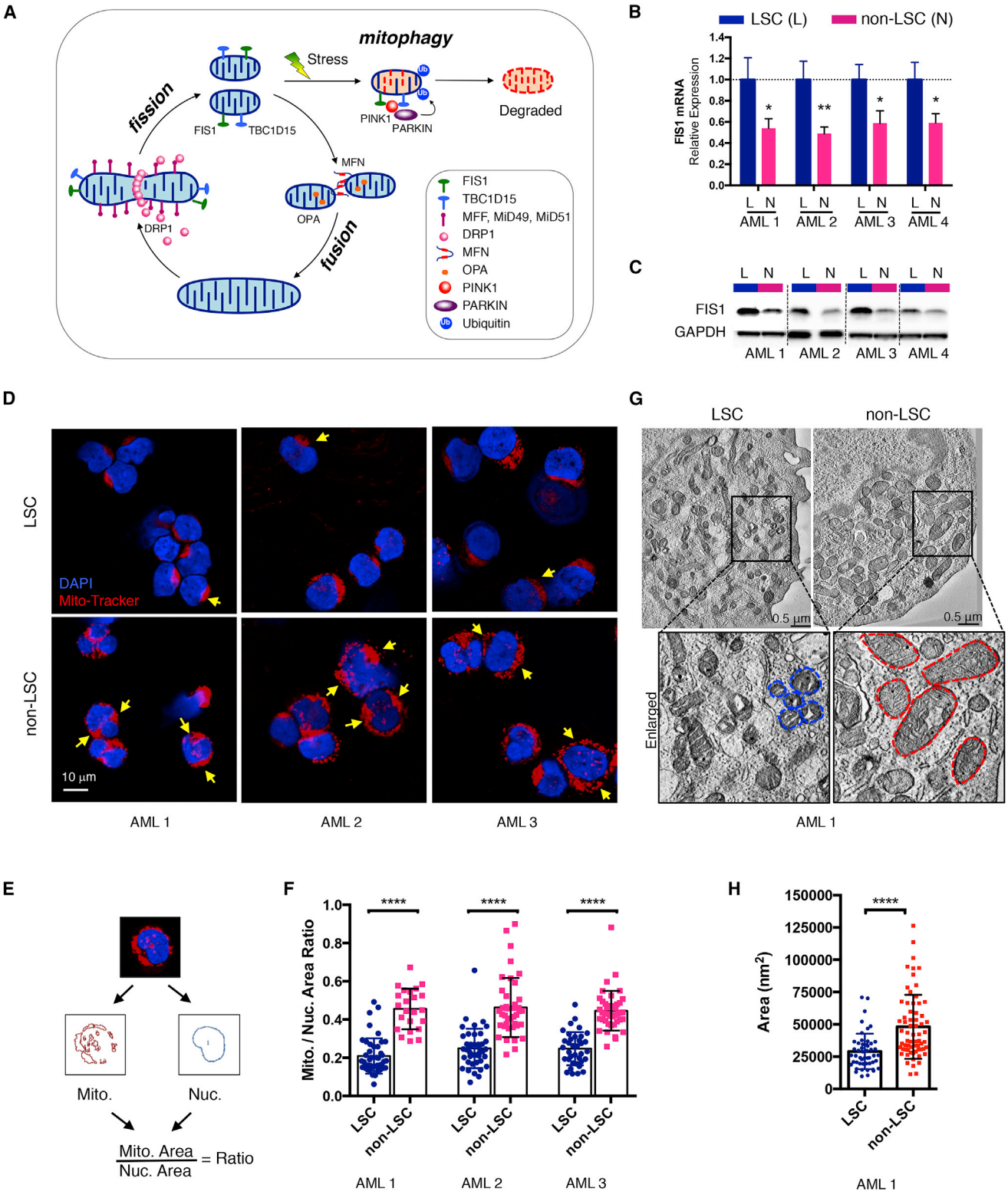


**Figure 1. AML LSCs Have Higher Expression of FIS1 and Distinct Mitochondrial Morphology**

(A) A diagram showing regulation of a healthy mitochondrial network through mito-fusion, mito-fission, and mitophagy.

(B) qPCR results showing relative expression of the *FIS1* gene in sorted ROS-low LSCs (L) versus ROS-high non-LSCs (N). Mean ± SD (n = 3), type 2, two-tailed t test.

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profile between ROS-low LSCs versus ROS-high non-LSCs. These studies show that genes related to mitochondrial biology are upregulated in the ROS-low LSC population, including FIS1 (Mitochondrial fission 1), a gene known to be critical for mitochondrial dynamics. The term mitochondrial dynamics describes the interconnected processes of mito-fusion, mito-fission, and mitophagy (Chen and Chan, 2017). Briefly, the mito-fusion process functions to fuse individual mitochondria together and is balanced by the opposite process of mito-fission to allow proper formation of the mitochondrial network. When defective mitochondria accumulate upon stress, mito-fission and mitophagy can function together to isolate and degrade damaged mitochondria. These processes thus function to organize mitochondria into healthy networks and maintain a homeostatic balance required for proper cell function (Wai and Langer, 2016).

Despite a number of recent studies of the mechanisms that regulate mitochondrial dynamics, the functional role of processes such as mito-fusion, mito-fission, and mitophagy in human cancers and/or CSCs is largely unknown. In the present study, we dissect mitochondrial dynamics in human AML with a focus on LSC biology. We show that LSC-specific activation of the metabolic stress regulator AMPK induces upregulation of FIS1, which, in turn, regulates mitophagy activity that functions to sustain AML LSCs. Inhibition of FIS1-mediated mitophagy induces myeloid differentiation, reduction in cell cycle activity, and loss of leukemic stem and progenitor cell potential.

## RESULTS

### LSCs Have Higher Expression of FIS1 and Distinct Mitochondrial Morphology

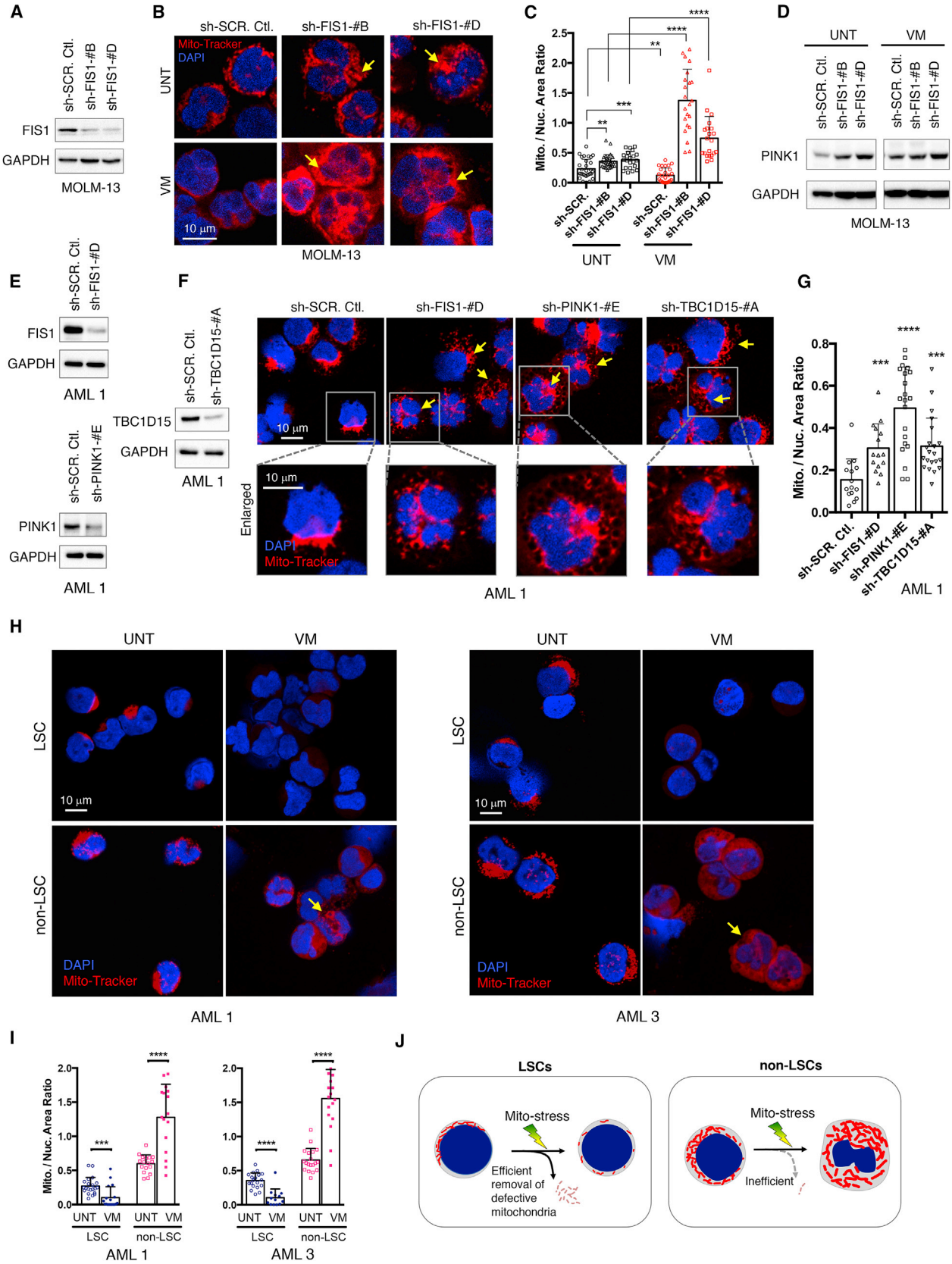
We previously utilized the ROS-low phenotype to enrich for LSCs and then employed RNA sequencing (RNA-seq) to identify molecular properties that are unique to the LSC population. Those studies show upregulation of the mitochondrial dynamics regulator FIS1 (Lagadinou et al., 2013). Further analysis of those data identified a trend toward increased expression of many mitochondrial dynamics regulators in the ROS-low LSC population, including *FIS1*, *TBC1D15*, *PINK1*, *MFF*, *MiD49*, and *MiD51* (Figures 1A, S1A, and S1B). Among the genes known to regulate mitochondrial dynamics, FIS1 shows the clearest and most consistent differential expression in comparison with the ROS-high non-LSC population (Figure S1B). These data suggest that FIS1 activity is elevated in primitive AML cells and may drive a distinct state of mitochondrial dynamics as a component of LSC growth and survival.

To test this hypothesis, in the current study we isolated both ROS-low and ROS-high cells from an independent cohort of primary AML specimens by flow cytometric sorting (Figure S1C). We confirmed in multiple primary AML specimens that ROS-low AML cells are significantly enriched for both colony-forming and engraftment abilities in comparison with ROS-high AML cells (Figures S1D–S1G), demonstrating ROS-low as a reliable functional state to enrich for LSCs in AML, as we have reported previously (Lagadinou et al., 2013). Importantly, in this new cohort of primary AML specimens, both mRNA and protein expression of FIS1 are significantly higher in LSCs relative to non-LSCs (Figures 1B and 1C). Given the role of FIS1 in mitochondrial dynamics, we next examined mitochondrial morphology in LSCs versus non-LSCs using confocal and transmission electron microscopy (TEM). For confocal studies, we labeled mitochondria and nuclei of freshly sorted LSCs and non-LSCs with the mitochondrial dye Mito-Tracker and the DNA dye DAPI. Compared with non-LSCs, LSCs demonstrate a significantly lower mitochondrial to nuclear area ratio, suggesting that they have less mitochondrial content (Figures 1D–1F; Figure S1H). In addition, the majority of mitochondria in LSCs are located in a single compact crescent-shaped area, whereas non-LSCs show a distinctly more diffuse labeling pattern (Figure 1D; Figure S1H). To further investigate mitochondrial morphology at individual mitochondrion resolution, TEM was performed on freshly isolated LSC and non-LSC populations. The TEM images revealed that the cross-sections of mitochondria in LSCs consistently demonstrate a significantly smaller area compared with non-LSCs (Figures 1G and 1H), suggesting that LSC mitochondria have a smaller volume. Together, these data indicate that AML LSCs have a unique mitochondrial morphology indicative of a distinct state of mitochondrial dynamics relative to non-LSCs.

### Inhibition of FIS1 Disrupts Mitochondrial Dynamics in AML

To test whether FIS1 is responsible for the distinct mitochondrial morphology, we next performed shRNA-mediated knockdown studies in both the AML cell line MOLM-13 and primary AML cells to examine the effect of FIS1 loss on mitochondrial dynamics. We observed that FIS1-depleted MOLM-13 cells have significantly increased mitochondrial content relative to control cells (Figures 2A and 2B, top row, and 2C, left). Notably, a recent study has shown FIS1 to be a regulator of mitophagy in mammalian cells (Shen et al., 2014). Thus, we hypothesized that increased mitochondrial content may be caused by a deficient state of mitophagy in FIS1-depleted AML cells. To test

(C) Western blot (WB) results showing expression of FIS1 protein in sorted ROS-low LSCs (L) versus ROS-high non-LSCs (N). Dashed lines separate paired samples from multiple experiments compiled together for representation in the same figure.
(D) Representative confocal images showing the morphology of mitochondria in ROS-low LSCs versus ROS-high non-LSCs. Yellow arrows highlight distinct mitochondrial morphology.
(E) A diagram showing the method used to quantify the mitochondrial to nuclear area ratio.
(F) Mitochondrial to nuclear area ratio in LSCs versus non-LSCs in 3 primary AML specimens. Each dot represents an individual cell. Mean ± SD, type 3, two-tailed t test.
(G) Representative TEM images showing the morphology of mitochondria in ROS-low LSCs and ROS-high non-LSCs. Blue and red dotted lines outline the mitochondrial shape.
(H) Quantification of the mitochondrial cross-section area from the TEM images of AML 1. Each dot represents a single mitochondrion. Mean ± SD, type 3, two-tailed t test.
*$p < 0.05$; **$p < 0.01$; *****$p<0.0001$; ns, not significant. See also Figure S1.

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this hypothesis, we induced mitophagy using the previously reported mitochondrial stress inducer valinomycin (Ashrafi and Schwarz, 2013). Valinomycin is a $K^+$ inonphore that can permeabilize the mitochondrial membrane to $K^+$, inducing mitochondrial membrane potential loss, activating mitophagy (Rakovic et al., 2010; Seibler et al., 2011). We observed that, although control cells responded to valinomycin treatment with loss of mitochondria, the shFIS1 cells further accumulated mitochondrial content, suggesting an inability to perform efficient mitophagy in response to mitochondrial stress (Figures 2B, bottom row, and 2C, right). To further test this hypothesis, we probed for expression of PINK1, a kinase stabilized upon mitophagy activation and involved in the clearance of damaged mitochondria (Lazarou et al., 2015). We observed that, although control cells had increased PINK1 expression upon valinomycin treatment, shFIS1 cells showed no change in PINK1 level despite the same insult (Figure 2D). These data further suggest that FIS1-depleted AML cells are unable to activate mitophagy upon mitochondrial stress increase.

To extend the above findings from MOLM-13 cells, shFIS1 knockdown studies were also performed in primary human AML cells (Figure 2E), where we observed that reduction of FIS1 also induced accumulation of mitochondria, which led to a significant increase in the mitochondrial to nuclear area ratio in AML 1 (Figures 2F, 2G, and S2A) and AML 7 (Figures S2B–S2E). In addition, depletion of the known mitophagy regulators PINK1 and TBC1D15 (Nguyen et al., 2016; Yamano et al., 2014) reproduced the same mitochondrial accumulation phenotype in shFIS1 AML cells (Figures 2E–2G), further supporting the role of FIS1 in regulating mitophagy. Notably, we also observed that the mitophagy defect phenotype is distinctly different from a fission defect phenotype observed in shDRP1 MOLM-13 (Figures S2F–S2H) and primary AML cells (Figures S2I and S2J), where longer and more thread-like mitochondria are prevalent (Figure S2K). Last, shFIS1 primary AML cells recapitulated the diffuse mitochondrial distribution phenotype seen in non-LSCs, where FIS1 expression is low (Figure 2F, enlarged insets).

To determine whether primary AML cells with a higher expression of FIS1 have increased mitophagy activity, we performed valinomycin treatment experiments on freshly sorted LSCs and non-LSCs from human AML specimens. We found that, consistent with the knockdown experiments, LSCs also responded to valinomycin treatment with efficient clearance of mitochondria, whereas non-LSCs showed further accumulation of mitochondrial mass (Figures 2H and 2I).

Together, these data demonstrate that FIS1 plays an important role in regulating the mitochondrial dynamics of leukemic cells, largely through mitophagy, and suggest the model illustrated in Figure 2J. LSCs or AML cells expressing higher levels of FIS1 possess fewer mitochondria and show localization to a defined region of the cell. In contrast, non-LSCs or AML cells engineered to reduce FIS1 expression have increased mitochondrial mass and a more diffuse mitochondrial morphology. Elevated FIS1 expression in LSC populations mediates increased mitophagy activity, whereas non-LSCs with less FIS1 expression are inefficient in mitophagy, resulting in further accumulation of mitochondria under stress conditions (Figure 2J).

## Loss of FIS1 Impairs the Stem and Progenitor Potential of AML

Given the profound effect of FIS1 loss on AML mitochondrial dynamics and the unique mitochondrial morphology present in AML LSCs, we next investigated the functional role of FIS1 and other mitophagy regulators in AML stem and progenitor cells. To this end, knockdown of *FIS1*, *TBC1D15*, and *PINK1* was performed in both the MOLM-13 cell line and primary AML specimens (Figure S3A). Depletion of FIS1 using shRNA strongly reduced the colony-forming ability of MOLM-13 and primary AML cells (Figures 3A and 3B). In addition, shRNA-mediated knockdown of TBC1D15 and PINK1 also caused a significant loss of colony-forming ability in these specimens (Figure S3B), suggesting that mitophagy in general might be required for the stem and progenitor potential of AML. To directly assess the role of FIS1 in LSC growth and survival, we performed xenograft studies using shFIS1-transduced MOLM-13 and primary AML specimens. Importantly, given the known heterogeneity of LSCs, knockdown studies were performed using unfractionated primary AML specimens to avoid any possible bias that may be introduced using a subpopulation of cells defined using a specific phenotype (e.g., CD34+, ROS-low, etc.). We observed that loss of FIS1 almost completely abolished the engraftment ability of MOLM-13 and AML specimens in immune-deficient mice (Figures 3C–3E and S3C). This depleting effect was evident

**Figure 2. Loss of FIS1 Induces Mitophagy Defects in AML**

(A) WB images showing the knockdown efficiency of sh-FIS1 in MOLM-13 cells.

(B) Representative confocal images showing the mitochondrial morphology of MOLM-13 cells on day 6 following knockdown with or without 5 μM valinomycin treatment for 3 hr. Arrows highlight distinct mitochondrial morphology.

(C) Mitochondrial to nuclear area ratio in MOLM-13 cells. Each dot represents an individual cell. Mean + SD, type 3, two-tailed t test.

(D) WB images showing the expression of PINK1 in MOLM-13 cells on day 6 following shRNA-mediated knockdown of FIS1 with or without 5 μM valinomycin treatment for 3 hr.

(E) WB images showing the knockdown efficiency of sh-FIS1, sh-PINK1, and sh-TBC1D15 in primary AML 1.

(F) Representative confocal images showing the mitochondrial morphology of primary AML cells on day 6 following shRNA-mediated knockdown of FIS1, PINK1, and TBC1D15. Arrows highlight distinct mitochondrial morphology.

(G) Mitochondrial to nuclear area ratio in primary AML 1 with knockdown of FIS1, PINK1, or TBC1D15. Each dot represents an individual cell. Mean + SD, type 3, two-tailed t test.

(H) Representative confocal images showing the mitochondrial morphology of ROS-low LSCs versus ROS-high non-LSCs freshly sorted from primary AML specimens and then treated with or without 5 μM valinomycin for 3 hr. Arrows highlight accumulated mitochondria induced by valinomycin treatment.

(I) Mitochondrial to nuclear area ratio in LSCs versus non-LSCs isolated from primary AML 1 and AML 3 under basal and valinomycin-treated conditions. Each dot represents an individual cell. Mean + SD, type 3, two-tailed t test.

(J) A diagram summarizing the mitochondrial morphology changes seen in LSCs versus non-LSCs with or without mitochondrial stress.

**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant. See also Figure S2.

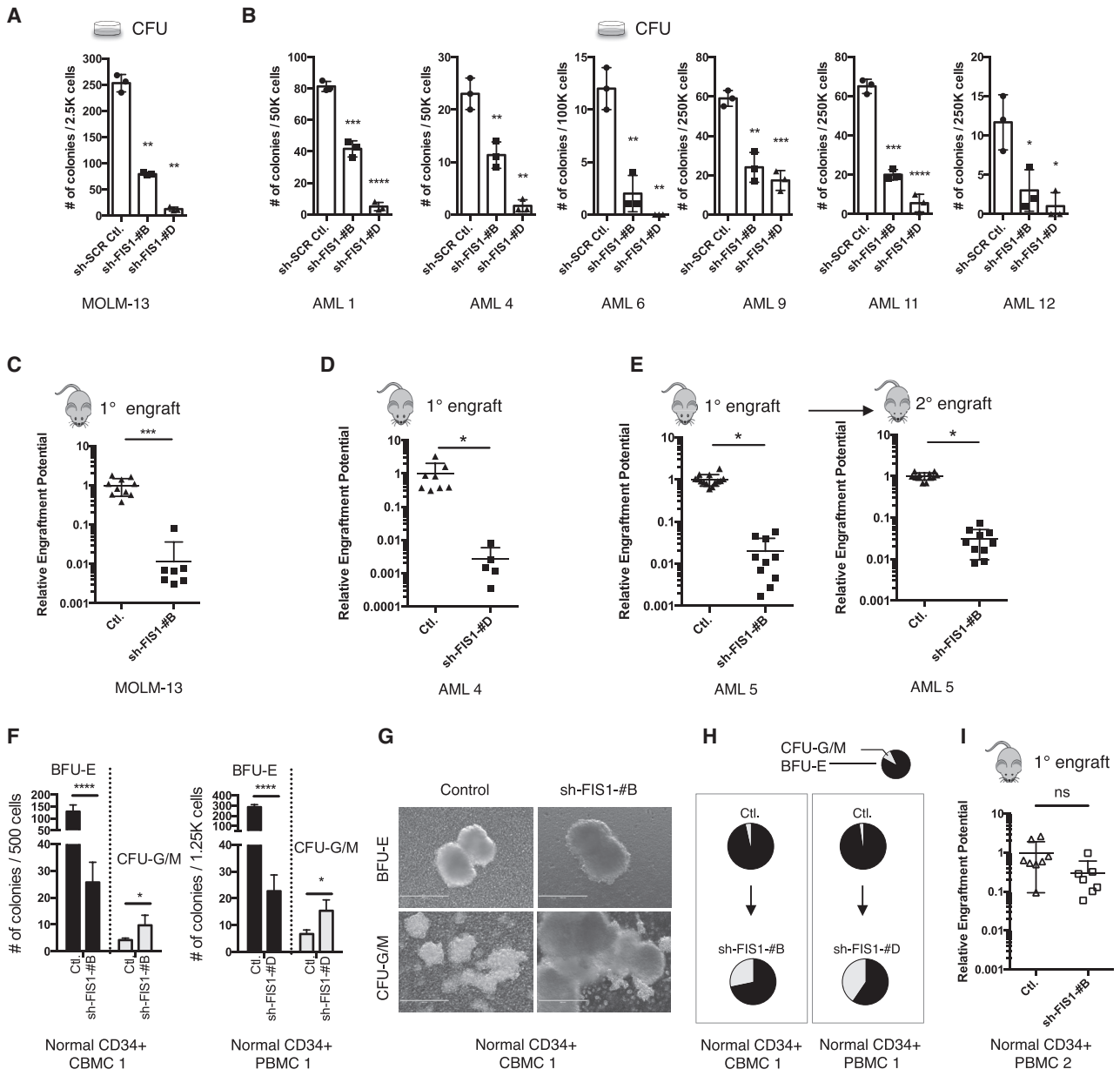


**Figure 3. Depletion of FIS1 Impairs the Stem and Progenitor Potential of Primary AML but Relatively Spares Normal HSPCs**

(A) Colony-forming ability of MOLM-13 cells following shRNA-mediated knockdown of *FIS1*. Mean ± SD (n = 3), type 2, two-tailed t test.

(B) Colony-forming ability of primary AML cells following shRNA-mediated knockdown of *FIS1*. Mean ± SD (n = 3), type 2, two-tailed t test.

(C) Normalized relative engraftment potential of MOLM-13 cells with or without FIS1 knockdown.

(D) Normalized relative engraftment potential of primary AML 4 with or without FIS1 knockdown.

(E) Normalized relative engraftment potential of primary AML 5 with or without FIS1 knockdown. 1° and 2° indicate primary and secondary xenograft experiments, respectively.

(F) Number of BFU-E and CFU-G/M colonies produced by normal CD34+ CBMCs or PBMCs in methylcellulose. Mean ± SD (n = 3), type 2, two-tailed t test.

(G) Representative images showing the morphology of BFU-E and CFU-G/M colonies.

(H) Pie charts showing changes in proportion of CFU-G/M and BFU-E colonies induced by FIS1 knockdown.

(I) Normalized relative engraftment potential of normal CD34+ PBMCs with or without FIS1 knockdown.

In (C)–(E) and (I), each dot represents an individual mouse, and lines represent mean ± SD; type 2, two-tailed t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. See also Figure S3.

in both primary and secondary transplantation assays (Figures 3E and S3C), indicating that FIS1 is required for LSC self-renewal potential. Finally, CRISPR/Cas9-mediated disruption of the FIS1 gene also significantly impaired both the colony-forming and engraftment ability of MOLM-13 cells (Figures S3D–S3F), corroborating the results obtained with shRNA approaches.

Together, these findings indicate a central role of FIS1 in the maintenance of LSC populations.

### FIS1 Loss Attenuates Erythropoiesis without Significantly Affecting the Engraftment Ability of Normal HSPCs

A recent study has shown that loss of MFN2 (mitofusin 2), another mitochondrial dynamics regulator, can impair the lymphoid potential of normal hematopoietic stem and progenitor cells (HSPCs) (Luchsinger et al., 2016). To investigate the role of FIS1 in normal hematopoiesis, CD34+ normal cord blood mononuclear cells (CBMCs) and peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and subjected to FIS1 knockdown using the same shRNA strategy employed for AML studies. We first investigated the effect of FIS1 loss on the mitochondrial morphology of normal HSPCs. We found that FIS1-depleted CD34+ cells had negligible change in their mitochondrial mass compared with control cells (Figures S3G and S3H), suggesting that FIS1 is largely dispensable in the regulation of mitochondrial morphology in normal HSPCs. In addition, in contrast to the mitochondrial loss phenotype seen in valinomycin-treated AML, the same treatment induced mitochondrial accumulation in both control and shFIS1 CD34+ cells, suggesting a slower rate of mitophagy in normal HSPCs (Figure S3G and S3H). We next performed functional assays to evaluate the effect of FIS1 loss on the stem and progenitor potential of normal HSPCs. Analysis of the colony-forming ability showed two notable phenotypes. First, although the number of burst-forming unit-erythroid colonies (BFU-E) was markedly reduced (Figure 3F, left), the size and morphology of BFU-Es were normal (Figure 3G, top). In contrast, granulocyte/macrophage myeloid colonies (CFU-G/M) were more abundant and much larger upon FIS1 depletion (Figures 3F, right, and 3G, bottom). As a result of this lineage skewing, FIS1 depletion substantially increased the proportion of CFU-G/M colonies compared with the control (Figure 3H). Together, these data suggest that FIS1 loss predisposes normal hematopoietic progenitors to favor myelopoiesis over erythropoiesis. Consistent with this hypothesis, analysis of FIS1 expression during normal human hematopoiesis in the publicly available dataset GSE24759 (Bagger et al., 2016; Novershtern et al., 2011) shows that, among all stages of normal hematopoiesis, the expression of FIS1 is highest in the erythroid lineage (Figure S3I). This observation provides a possible explanation for the shFIS1-mediated selective effect on erythropoiesis.

To measure the effect of FIS1 loss in the HSC compartment, xenograft assays were performed to measure the engraftment ability of normal CD34+ PBMCs transduced with control versus shFIS1 vectors. As shown in Figure S3J, we achieved a level of FIS1 knockdown comparable with AML experiments. The engraftment efficiency of total human cells in recipient mice was 14% ± 18% (n = 15) 12 weeks post-transplant. As shown in Figures 3I and S3K, FIS1 knockdown caused a modest loss of engraftment potential (~3-fold) (Table S1). This is in sharp contrast to the ~50- to 350-fold drop in relative engraftment potential seen in primary AML cells (Figures 3D, 3E, and S3C). Together, these data suggest that the functional role of FIS1 is critical to survival and self-renewal of AML LSCs but that its role in normal mitochondrial morphology and self-renewal is much more subtle.

### Loss of FIS1 Induces GSK3 Inhibition and Myeloid Differentiation in AML

To investigate the mechanism underlying shFIS1-induced loss of stem and progenitor potential in AML, we performed two independent RNA-seq experiments to characterize global gene expression changes induced by shFIS1 in MOLM-13 and a cohort of 3 primary AML specimens (Figure S4A), respectively. Several previous studies have identified a link between GSK3B and DRP1 (Chou et al., 2012; Wu et al., 2013), suggesting that the key stem and progenitor potential regulator GSK3 signaling and mitochondrial dynamics could be interconnected. Using gene set enrichment analysis (GSEA), we identified that FIS1 loss in both MOLM-13 and primary AML specimens strongly enriches a list of gene sets representing inactivation of GSK3 signaling in leukemic cells. These gene sets include the WANG_GSK3I_SB216763 gene set, which is generated by treating human leukemia cell line RS4;11 cells with the GSK3 inhibitor SB216763 (Wang et al., 2010; Figure 4A). The list also includes the BANERJI_GSK3A_KD and BANERJI_GSK3B_KD gene sets, which are produced by specifically knocking down GSK3A and GSK3B in leukemic cell line MOLM-14 cells (Banerji et al., 2012; Figure S4B). GSK3A and GSK3B are inactivated by phosphorylation at S21 and S9, respectively (McCubrey et al., 2014). To confirm GSK3 inactivation, we probed phospho-S21-GSK3A and phospho-S9-GSK3B in AML cells following FIS1 depletion using both shRNA and CRISPR/Cas9 strategies. Both MOLM-13 and primary AML cells with depleted FIS1 expression showed elevated phospho-S21-GSK3A and phospho-S9-GSK3B (Figures 4B and S4E), indicating that GSK3 inactivation occurs as a result of losing FIS1.

Inactivation of GSK3 signaling can lead to myeloid differentiation and impaired growth in various subtypes of primary AML (Banerji et al., 2012). Given that both enrichment of the GSK3 inhibition signature and inhibitory phosphorylation of GSK3 proteins are observed, we next investigated whether shFIS1-induced GSK3 inactivation would also result in a differentiation phenotype. First, we observed that shFIS1 enriches the KEGG_HEMATOPOIETIC_CELL_LINEAGE gene set and induces global upregulation of many hematopoietic lineage markers, including CD33, CD22, CD14, and CD11b (Figures S4C and S4D). Next, flow cytometry analysis verified a strong increase in the myeloid differentiation marker CD11b in FIS1-depleted MOLM-13 cells grown in culture (Figure 4C) and methylcellulose (Figure S4F) and engrafted in immune-deficient mice (Figure S4G). Interestingly, knockdown of other mitophagy regulators, such as TBC1D15 and PINK1, also strongly upregulated CD11b expression (Figure 4D), suggesting that inhibition of mitophagy activity in general can trigger myeloid differentiation in AML. Finally, using Giemsa staining, we observed that FIS1-depleted MOLM-13 and primary AML cells displayed crumpled cytoplasmic membranes, nuclear condensation, and a decreased nuclear to cytoplasmic area ratio, indicative of clear myeloid differentiation (Figures 4E and S4H). Last, despite a strong global upregulation of hematopoietic lineage genes, we did not detect significant changes in histone H3K27ac marks (Figure S4I), an event often associated with activation of gene expression during stem cell differentiation (Atlasi and Stunnenberg, 2017).

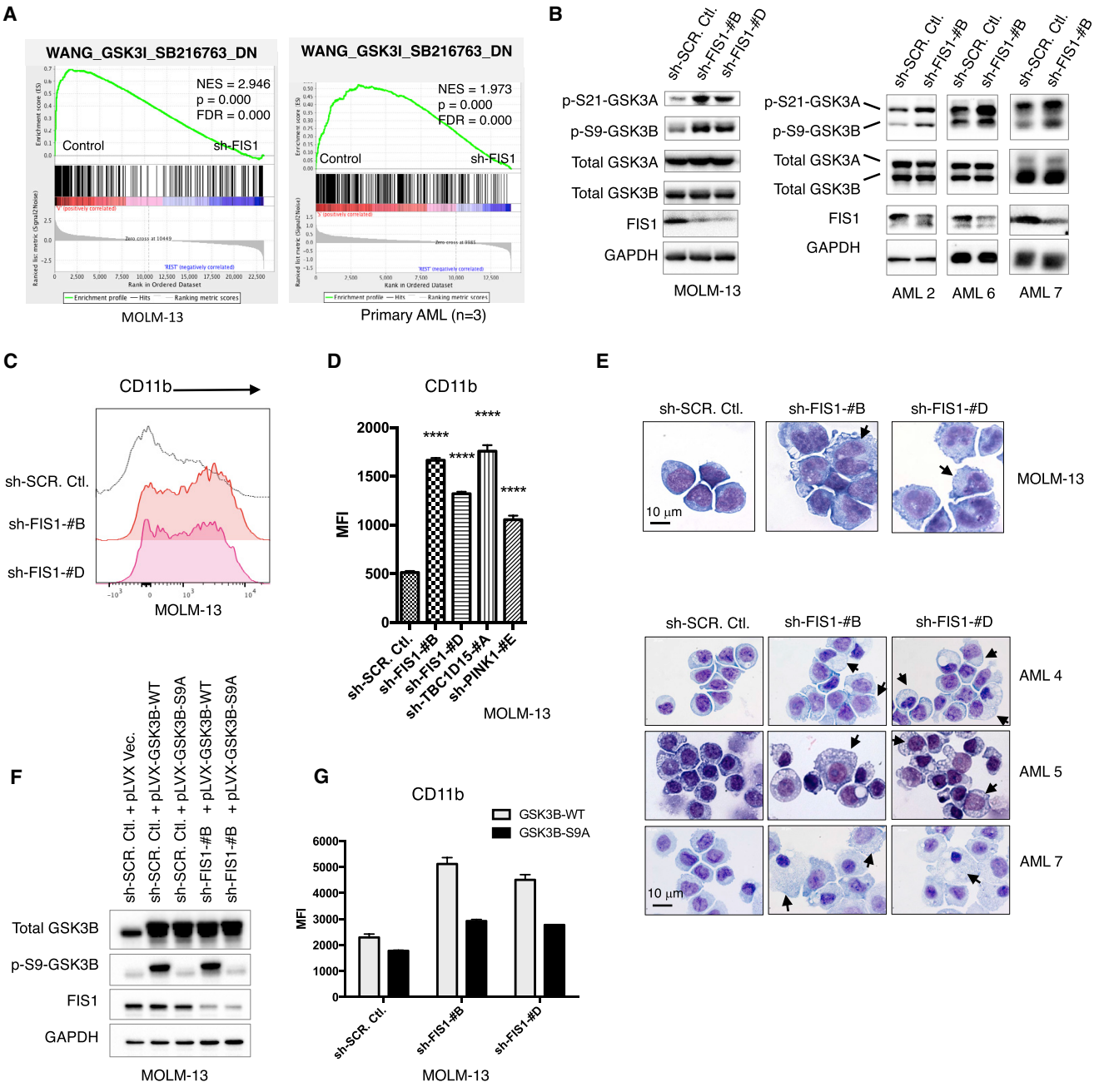


**Figure 4. Loss of FIS1 Induces GSK3 Inhibition and Myeloid Differentiation in Primary AML**

(A) GSEA enrichment plots showing that loss of FIS1 in MOLM-13 (3 technical replicates) and primary AML cells (3 biological replicates) results in downregulation of the WANG_GSK3I_SB216732_DN gene set. sh-FIS1 represents sh-FIS1-#B and sh-FIS1-#D together.

(B) WB images showing the activity of GSK3 signaling in MOLM-13 and primary AML cells on day 6 following FIS1 knockdown.

(C) Histogram showing flow cytometry analysis of CD11b expression in MOLM-13 cells on day 6 following FIS1 knockdown.

(D) Quantification of the CD11b flow stain signal in MOLM-13 cells on day 6 following knockdown of each target gene. Mean ± SD, (n = 3), type 2, two-tailed t test.

(E) Representative Giemsa staining images showing the morphology of MOLM-13 and primary AML cells on day 6 following FIS1 knockdown. Arrows highlight distinct morphology indicative of differentiation.

(F) WB images showing expression of total GSK3B, p-S9-GSK3B, and FIS1 in MOLM-13 cells engineered to express GSK3B-WT or GSK3B-S9A alleles with or without FIS1 knockdown.

(G) Quantification of the CD11b flow stain signal in MOLM-13 cells on day 6 following simultaneous expression of GSK3B-WT or GSK3B-S9A alleles and knockdown of FIS1. Mean ± SD, n = 3.

****p < 0.0001; ns, not significant. In (D) and (G), MFI stands for median fluorescence intensity. See also Figure S4.

To investigate whether GSK3 inhibition is required for the differentiation phenotype seen in FIS1-depleted leukemic cells, we performed genetic rescue experiments. GSK3B wild-type (GSK3B-WT) or GSK3B-S9A alleles were successfully expressed in combination with or without shFIS1 knockdown in MOLM13 cells (Figure 4F; Figure S4J). The GSK3B-S9A allele cannot be inhibited through phosphorylation of its Ser9 residue (Figure 4F); thus, is commonly used as a constitutively active allele of GSK3B (Lang et al., 2013). As shown in Figure 4G, co-expression of the GSK3B-S9A but not the GSK3B-WT allele suppressed the shFIS1-induced CD11b increase, suggesting that the shFIS1-induced differentiation phenotype is mediated, at least in part, by GSK3B inactivation. Expression of the GSK3B-S9A allele, however, did not rescue shFIS1-induced CFU loss (Figure S4K), suggesting that multiple mechanisms exist downstream of FIS1 that control the stem and progenitor potential of AML in addition to GSK3B. Last, expression of the constitutively active GSK3B-S9A allele failed to rescue the shFIS1-induced mitochondrial accumulation phenotype (Figures S4L and S4M), suggesting that GSK3B inactivation is likely downstream of the shFIS1-induced mitophagy defect event.

### Depletion of FIS1 Induces Cell Cycle Arrest in AML

In addition to its role in maintaining leukemia transformation and suppression of differentiation (Banerji et al., 2012; Wang et al., 2010), active GSK3 signaling is also known to promote cell cycle activity in AML (Wang et al., 2008). Consistent with this observation, we also found that FIS1 loss in both primary AML specimens and MOLM-13 cells acted to strongly modulate cell cycle activity. As shown in Figures 5A and S5A, cell cycle inhibition is clearly evident from GSEA analysis of multiple cell cycle-related gene sets. In addition, comparative analysis of control versus FIS1 knockdown showed a trend toward global inhibition of many representative cell cycle-related genes at all major stages (G1, G1/S, S, G2/M, and M) upon FIS1 loss (Figure 5B). The consequence was substantially reduced cell cycle activity, as shown by Ki-67/DAPI staining (Figures 5C and 5D), and a significantly lower *in vitro* proliferation rate, as shown by cell count in primary AML cells (Figure 5E; Figure S5B). Together, the data in Figures 4 and 5 indicate that the strong effect of FIS1 loss on the stem and progenitor potential of AML is related to its downstream GSK3 inactivation and cell cycle arrest effects.

### AMPK Is Upstream of FIS1

Although the abovementioned studies demonstrated that FIS1-mediated mitophagy activity is important for the stem and progenitor potential of LSCs, the upstream signaling that maintains FIS1 activity in LSCs has not been characterized. A recent study in a mouse model reported that AMPK signaling is upregulated in response to energetic stress in mouse LSCs (Saito et al., 2015). In addition, a separate study in osteosarcoma cells showed that AMPK is upstream of MFF (mitochondrial fission factor), which is another mitochondrial dynamics regulator (Toyama et al., 2016). Thus, we hypothesized that AMPK signaling might also regulate the activity of FIS1 in human LSCs as a means to manage mitochondrial stress (Figure 6A). To test this hypothesis, we first compared the activity of AMPK signaling in freshly sorted ROS-low LSCs versus ROS-high non-LSCs. Our results showed that AMPK is constitutively activated in LSC relative to non-LSC populations (Figure 6B), suggesting that AMPK may play an important role in human LSCs as well. To directly test whether AMPK is upstream of FIS1, we knocked down the *AMPKa1(PRKAA1)* subunit in both MOLM-13 and primary AML cells and measured its effect on FIS1 expression (Figures S6A–S6D). Our data clearly show that knockdown of *AMPKa1* resulted in significant FIS1 mRNA and protein loss (Figures 6C and S6C), providing strong evidence that AMPK is upstream of FIS1. As a master regulator of energy metabolism, modulation of AMPK activity is expected to have a plethora of downstream effects. Thus, to measure how much the downstream effects of AMPK loss overlap with FIS1 loss, we examined whether shAMPK also affects mitochondrial morphology in primary AML cells. Using confocal microscopy, we observed that shAMPK primary AML cells also display a mitochondrial accumulation phenotype (Figures 6D and 6E) similar to that observed for FIS1 knockdown. These data further support AMPK as an upstream regulator of FIS1 in AML cells and suggest that inhibition of AMPK may largely recapitulate the biological effects of FIS1 loss in AML. To investigate this hypothesis, we first performed RNA-seq experiments to characterize global gene expression changes induced by *AMPKa1* knockdown in primary AML cells. Using a minimal p value of 0.05, we identified a list of significantly downregulated genes induced by shAMPKa1. These genes were used to generate a gene set termed AML_AMPK_KD_DN (Table S2) to represent the biological effect of AMPK loss in AML. Interestingly, this gene set is significantly enriched by the shFIS1 gene expression profiles obtained from both the primary AML cohort and MOLM-13 cells (Figure 6F), suggesting that AMPK loss indeed largely recapitulates the biological effects of FIS1 loss in AML.

Given that AMPK is upstream of FIS1, we next wanted to assess the effect of AMPK loss on AML LSC function. To this end, the engraftment ability of AML cells following shRNA-mediated depletion of *AMPKa1* was measured in immune-deficient mice. Upon knockdown of *AMPKa1*, both MOLM-13 cells (Figure S6B) and primary AML specimens (Figures S6D) show greatly reduced engraftment potential (Figures 6G and 6H). Notably, AMPK depleted primary AML cells show further loss of engraftment potential in secondary transplantation assays (Figure 6H, right), suggesting that AMPK loss, like FIS1 depletion, can also impair long-term LSC potential. Together, these data demonstrate that AMPK regulates the activity of human AML LSCs and indicate that the mechanism involves downstream modulation of FIS1 activity.

## DISCUSSION

In AML, mitochondrial translation, mitochondrial DNA copy number, basal oxygen consumption rates, and many other properties of mitochondria have been found to be differentially regulated in comparison with normal hematopoietic cells (Cole et al., 2015; Liyanage et al., 2017; Schimmer and Skrtić, 2012; Skrtić et al., 2011; Yeung et al., 2015), suggesting that altered mitochondrial activity is important for leukemogenesis. Further, emerging studies have now described the highly dynamic nature of mitochondria and their increasingly evident role in multiple cellular processes (Wai and Langer, 2016). Mito-fission, mito-fusion, and mitophagy appear to co-exist in a homeostatic balance that is unique to varying cell types and physiological conditions.

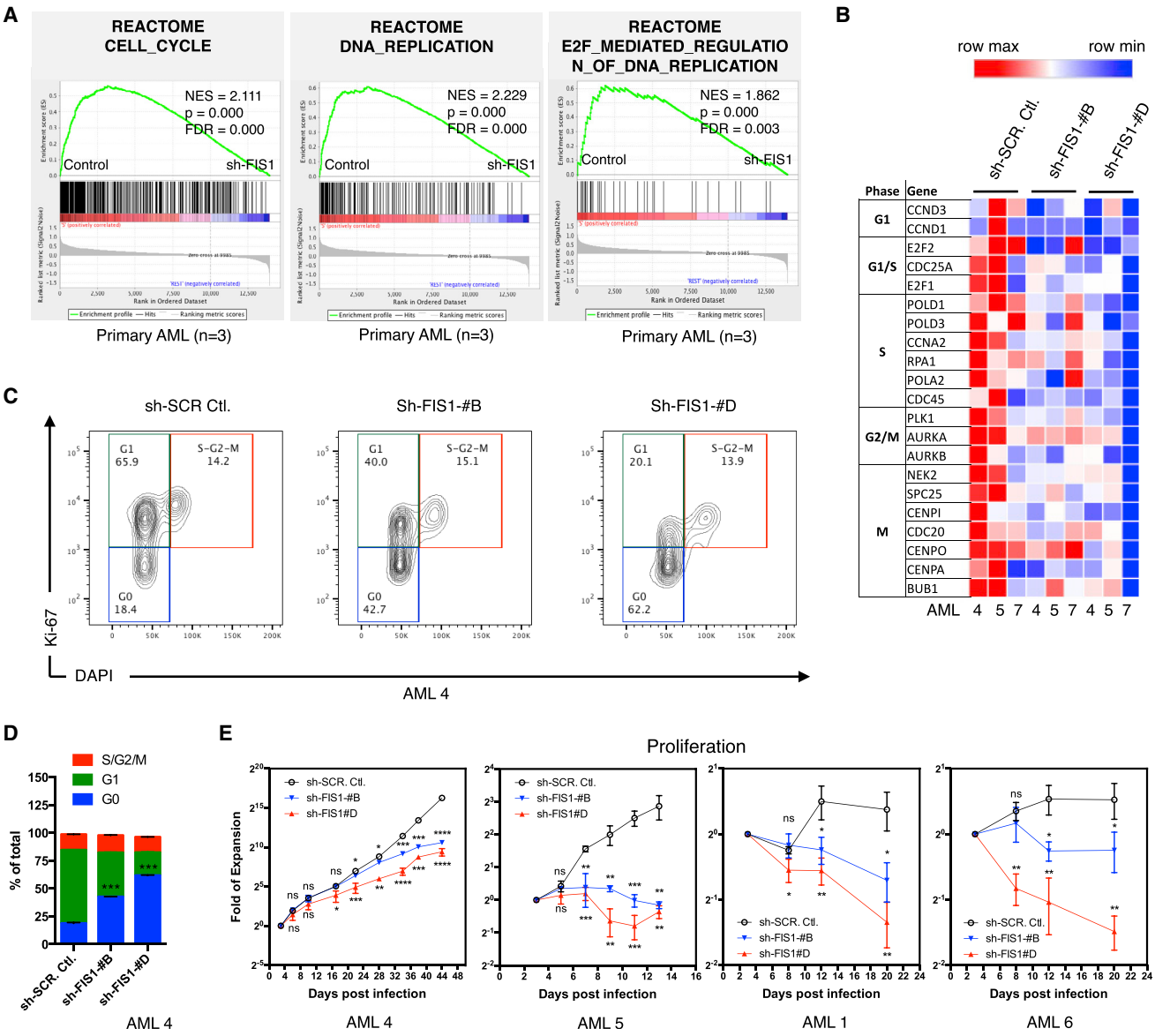


**Figure 5. Depletion of FIS1 Induces Cell Cycle Arrest in AML**

(A) GSEA enrichment plots showing that loss of FIS1 in primary AML cells (3 biological replicates) results in downregulation of 3 cell cycle-related gene sets from the GSEA Reactome collection. sh-FIS1 represents sh-FIS1-#B and sh-FIS1-#D together.

(B) A heatmap showing the expression of representative cell cycle-related genes in primary AML cells with or without FIS1 knockdown. Data are generated from RNA-seq analysis. Note that the genes included in this heatmap do not necessarily meet the adjusted p value of 0.05.

(C) Flow cytometry analysis of cell cycle status in primary AML cells on day 6 following FIS1 knockdown. The cell cycle profile is revealed by Ki-67 and DAPI staining.

(D) Quantification of the cell cycle profile shown in (C). Mean ± SD, n = 3; type 2, two-tailed t test was used to compare %G0 between control and knockdowns.

(E) Fold of expansion of primary AML cells cultured in complete serum-free medium, plotted in log2 scale. Mean ± SD, n = 3. For each time point, knockdown clones are compared with the control using type 2, two-tailed t test.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant. See also Figure S5.

However, the biological significance of mitochondrial dynamics in CSCs is still largely unknown (Chen and Chan, 2017). In the present study, we report that AML LSCs reside in a unique state of mitochondrial dynamics mediated by activation of AMPK and FIS1. This axis functions to inhibit differentiation via a GSK3-mediated downstream mechanism and supports self-renewal of primitive AML cells.

We show that the expression of FIS1 is significantly higher in AML LSCs versus non-LSCs. To date, only one previous report has linked FIS1 to AML biology. That study sought to identify poor prognosis markers of AML and found that expression of FIS1 is higher in AML patients who are refractory to chemotherapy in comparison with drug-responsive patients (Tian et al., 2014). Notably, several reports have indicated that LSC

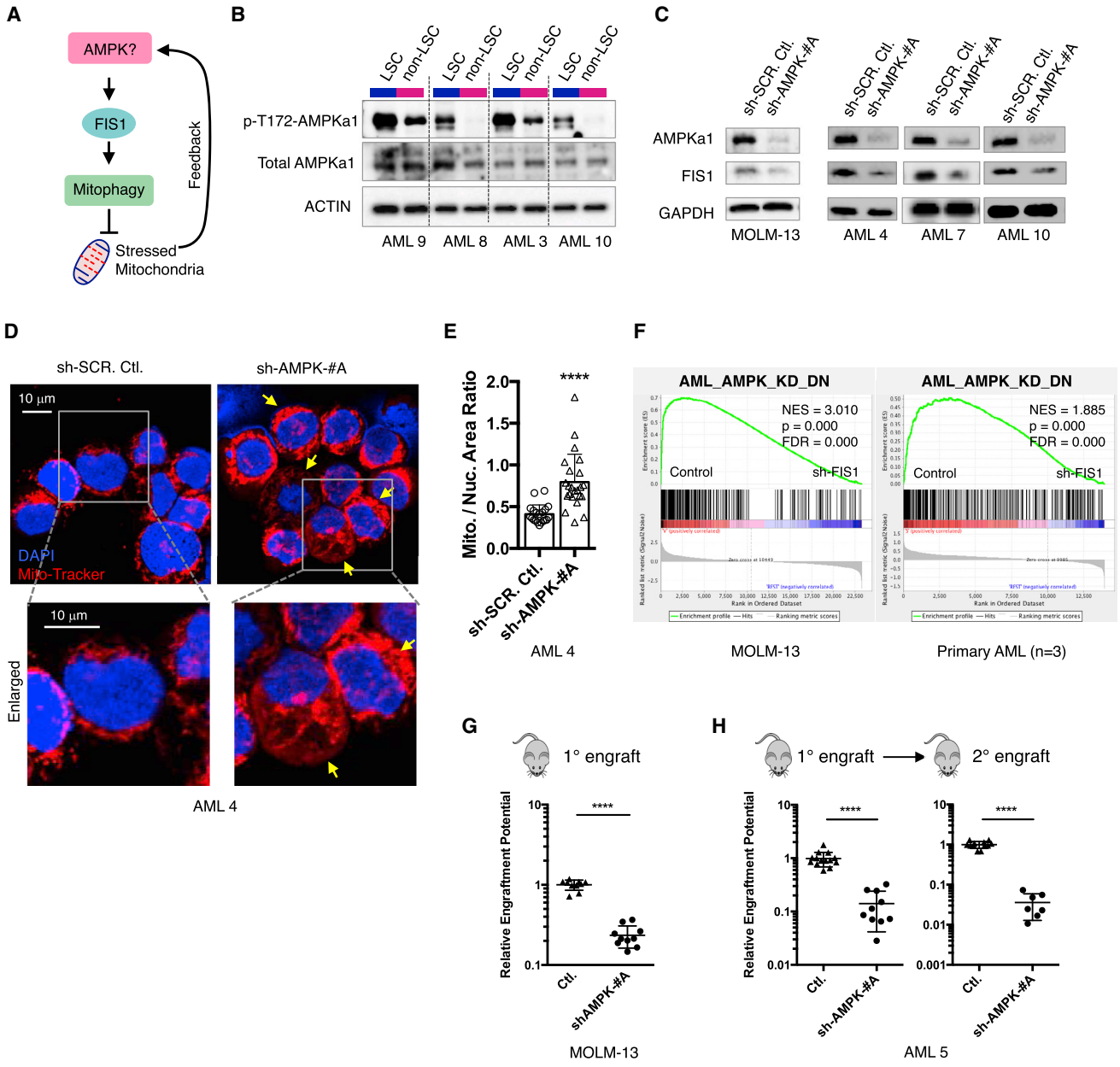


**Figure 6. Inhibition of AMPK Signaling Upstream of FIS1 Depletes the LSC Potential of Primary AML**

(A) A diagram showing the rationale underlying the AMPK hypothesis.

(B) WB images showing the expression of p-T172-AMPKa1 and total AMPKa1 in ROS-low LSCs (L) versus ROS-high non-LSCs (N).

(C) WB images showing the expression of FIS1 in MOLM-13 and primary AML cells on day 6 following AMPKa1(PRKAA1) knockdown.

(D) Representative confocal images showing the mitochondrial morphology of primary AML cells on day 6 following shRNA-mediated knockdown of *AMPKa1*. Arrows highlight mitochondrial accumulation.

(E) Mitochondrial to nuclear area ratio in control and shAMPK AML cells. Each dot represents a single cell. Mean + SD, type 3, two-tailed t test.

(F) GSEA enrichment plots showing that loss of FIS1 in MOLM-13 (3 technical replicates) and primary AML cells (3 biological replicates) results in downregulation of the AML_AMPK_KD_DN gene set. sh-FIS1 represents sh-FIS1-#B and sh-FIS1-#D together.

(G) Normalized relative engraftment potential of MOLM-13 cells with or without AMPK knockdown.

(H) Normalized relative engraftment potential of primary AML 5 with or without AMPK knockdown. 1° and 2° indicate primary and secondary xenograft experiments, respectively.

In (G) and (H), each dot represents an individual mouse, and lines represent mean ± SD. Type 2, two-tailed t test. ****p < 0.0001. See also Figure S6.

activity correlates with chemo-resistance in AML (Eppert et al., 2011; Gentles et al., 2010), suggesting a link between overall FIS1 expression, LSC biology, and therapy resistance. Further, our studies have recently demonstrated that relapsed AML patients increase the frequency of LSCs by 9- to 90-fold (Ho et al., 2016). Thus, increased expression of FIS1 in refractory or relapsed AML may be a reflection of increased LSC burden.

The present study also observed a distinct mitochondrial morphology in AML LSCs. The compact crescent-like localization pattern of mitochondria in LSCs may suggest asymmetrical division, an activity that has been observed in HSCs (Ito et al., 2012). Upon depletion of FIS1 in AML cells, dramatic accumulation of mitochondria and more diffuse mitochondria localization were clearly evident, suggesting that FIS1 is required to maintain the unique mitochondrial morphology seen in AML LSCs.

Importantly, our data clearly show that inhibition of FIS1 expression impairs the stem and progenitor potential of AML and that this phenotype is largely recapitulated by inhibition of other mitophagy players, PINK1 and TBC1D15, suggesting that mitophagy activity is critical for sustaining AML LSCs. Our results also show that FIS1 activity is less important for normal HSPCs, as evidenced by the limited effect of FIS1 knockdown on the mitochondrial morphology and engraftment ability of HSPCs. These results indicate a possible therapeutic index between HSCs and LSCs when targeting the FIS1 axis. It is important to note, however, that mitophagy has been found to be required for normal HSCs (Ito et al., 2016; Joshi and Kundu, 2013; Mortensen et al., 2011), suggesting that the dependency of stem cell function on mitophagy is evolutionarily conserved. A more recent study shows that loss of autophagy in HSCs can cause mitochondrial accumulation and an activated metabolic state, which impairs HSC self-renewal and regenerative potential (Ho et al., 2017). Thus, the possible therapeutic index may be specifically related to FIS1-mediated mitophagy. Given that multiple mitophagy pathways exist in addition to the FIS1/PINK1 axis described in the current study (Hamacher-Brady and Brady, 2016), we propose that the exact molecular mechanisms that maintain mitophagy activity might be different between HSCs and LSCs. In addition, our data suggest that normal HSPCs may have a slower mitophagy rate compared with LSCs, suggesting that they may better tolerate acute mitophagy inhibition. Last, our previous study showed that human AML LSCs preferentially rely on mitochondrial oxidative phosphorylation for energy production (Lagadinou et al., 2013). Given that mitophagy is a quality control mechanism for the health of mitochondria and that oncogenic transformation increases cellular stress and potentially damages mitochondria, then it is possible that clearance of dysfunctional mitochondria through mitophagy would be very important for LSCs that require mitochondrial oxidative phosphorylation for survival. Thus, despite a role of mitophagy in normal HSCs, our data suggest that targeting specific mechanisms such as the AMPK/FIS1 axis can provide selective eradication of LSCs.

Notably, studies of CSCs in other cancer types, including melanoma, glioblastoma, and pancreatic cancer, have also identified a dependence on oxidative phosphorylation (Roesch et al., 2013; Viale et al., 2014; Vlashi et al., 2011), suggesting that targeting mitochondrial stress response mechanisms such as mitophagy could be applicable for selective eradication of CSCs in multiple types of cancer.

The GSK3B-SA9 rescue experiments support the concept that GSK3 inhibition is necessary for the shFIS1-induced myeloid differentiation phenotype. The same allele, however, failed to rescue shFIS1-induced CFU loss and the mitochondrial accumulation phenotype. These data suggest the following: multiple mechanisms exist downstream of FIS1 that control the stem and progenitor potential of AML in addition to GSK3B; GSK3B inactivation likely happens downstream of the shFIS1-induced mitophagy defect event; and GSK3A inactivation may also contribute to multiple phenotypes induced by FIS1 loss.

It is unclear how FIS1-mediated mitophagy affects GSK3 phosphorylation. One possibility is through the PTEN/Akt/GSK3 axis. A recent study reported that an N-terminally extended form of PTEN, known as PTEN$\alpha$, specifically localizes to mitochondria, where it forms a complex with canonical PTEN to increase PINK1 expression and promote energy production (Liang et al., 2014). This study raises the possibility that, in shFIS1 AML cells, the increased expression of PINK1 could be induced by formation of the PTEN$\alpha$/PTEN complex in mitochondria. It would further suggest that shFIS1 can induce the translocation of canonical PTEN from the cytosol to mitochondria, resulting in PTEN activity loss in the cytosol, subsequent Akt activation, and GSK3 inhibition. This hypothesis is potentially interesting because it suggests that mitochondria have the ability to actively signal central metabolic pathways, including the Akt pathway, to "adapt" to differing physiological states.

In addition to GSK3 inhibition, we also observed strong cell cycle arrest in AML cells following FIS1 depletion. Cell cycle arrest often accompanies differentiation (Ruijtenberg and van den Heuvel, 2016); thus, this observation may simply be a consequence of induced differentiation. Obviously, cell cycle arrest will block the growth of bulk leukemia, but it may also have implications in targeting LSCs. Several recent studies have suggested that LSCs may also display a more actively cycling phenotype. For example, in MLL-rearranged leukemia, CD93 marks a subset of actively cycling, non-quiescent AML cells enriched for LSC activity (Iwasaki et al., 2015). Similarly, human AML LSCs may not be enriched following chemotherapy treatment, suggesting some degree of cell cycle activity in them (Farge et al., 2017). Thus, we believe that FIS1-induced cell cycle arrest may also contribute to the loss of LSC activity.

Last, although our data show that AMPK can regulate FIS1 at both the mRNA and protein levels, it remains a question whether the link between AMPK and FIS1 is direct or indirect. Importantly though, AMPK signaling is also preferentially active in ROS-low LSCs, and inhibition of AMPK results in loss of LSC potential, recapitulating the anti-LSC effect of FIS1 loss in AML. These data suggest that AMPK signaling could be a critical link between cellular and/or mitochondrial stress and activation of FIS1-mediated mitophagy (Figure 7). A recent study has convincingly demonstrated that AMPK activation is required to protect bone marrow LSCs from metabolic stress in a mouse model of MLL-AF9-induced leukemia (Saito et al., 2015). Others have shown that AMPK signaling can directly activate MFF, a critical regulator of mito-fission, in response to energy stress (Toyama et al., 2016). Our results suggest that AMPK can also regulate mitophagy activity under mitochondrial stress. Given

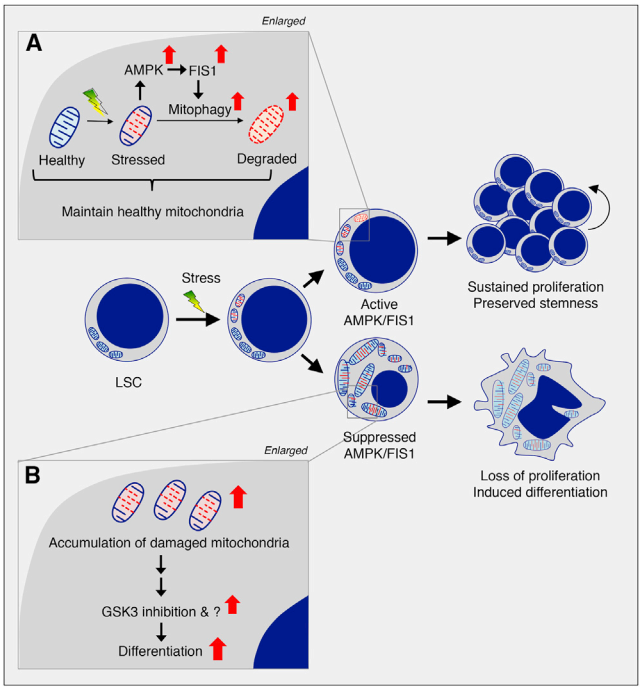


**Figure 7. Proposed Model for Dependency of AML LSCs on AMPK/FIS1-Mediated Mitophagy**

(A) In LSCs, mitochondrial stress generated from oncogenic transformation may activate AMPK signaling, which, in turn, drives FIS1-mediated mitophagy to degrade stressed mitochondria, maintaining a healthy mitochondrial network required for LSC potential.

(B) In LSCs with suppressed AMPK/FIS1 activity, accumulation of damaged mitochondria can lead to GSK3 inhibition and other unknown events, together inducing differentiation and impairing LSC function.

the fact that mito-fission and mitophagy are closely linked, it is reasonable to speculate that such dual abilities of AMPK in regulating both mito-fission and mitophagy make it an attractive target for therapies designed to selectively eradicate LSCs.

To date, the only other study that directly documents a role for mitochondrial dynamics in a CSC population was reported by Xie et al. (2015), who showed that aberrant activation of DRP1 promotes increased mito-fission to support the survival of brain tumor-initiating cells (BTICs) in glioblastoma (Xie et al., 2015). Intriguingly, in the BTIC model, AMPK signaling is downstream of DRP1 and can rescue the loss of DRP1 signaling, suggesting that, although mitochondrial dynamics may be broadly relevant to cancer-initiating cells, the way in which mitochondria dynamics mediate "stemness" may be specific to different tumor types.

In conclusion, as outlined in Figure 7, human AML LSCs show intrinsic activation of AMPK, which, we propose, is a response to increased cellular and/or mitochondrial stress arising as a consequence of oncogenic transformation. AMPK activation, in turn, upregulates FIS1-mediated mitophagy to facilitate the degradation of stressed mitochondria. This process likely functions as a quality control mechanism to maintain a healthy mitochondrial network, which is required to preserve stemness in LSCs (Figure 7A). Disruption of AMPK/FIS1-mediated mitophagy results in accumulation of defective mitochondria, inhibition of GSK3, and induction of differentiation (Figure 7B). This model suggests that targeting the AMPK/FIS1-mediated mitophagy axis may represent a promising strategy to eradicate AML LSCs.

## STAR★METHODS

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five tables and can be found with this article online at https://doi.org/10.1016/j.stem.2018.05.021.

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