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**Gaq signaling is required for the maintenance of MLL-AF9 induced AML**

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Title: Gαq signaling is required for the maintenance of MLL-AF9 induced AML

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Gαq signaling is required for the maintenance of MLL-AF9 induced AML

Self-renewal and differentiation block are two key features of leukemic stem cells (LSCs) in MLL-rearranged AML, which can originate from either hematopoietic stem cells (HSCs) or granulocyte macrophage progenitors (GMPs). We have previously demonstrated that β-catenin is essential for the establishment of MLL LSCs, where MLL-AF9 activates β-catenin, one role of which is to confer self-renewal capacity to GMPs necessary for malignant transformation (1). The effect of β-catenin appears to be largely context dependent and driven by its upstream components that fine-tune the signal to modulate different functional programs downstream of β-catenin (2). The targeting of critical upstream components could therefore serve as a potential strategy to effectively block β-catenin signaling. However, our understanding of upstream signaling events leading to β-catenin activation in leukemia still remains poor.

G proteins and G protein-coupled receptors have been implicated in playing crucial roles in multiple cancers, where specific members of this family influence self-renewal and tumorigenesis largely through activation of β-catenin signaling (2). The G protein subunit Gαq has recently been identified as a direct target of MLL-AF4 (3), and Dot1l-mediated H3K79 methylation marks are enriched at the Gαq locus in MLL-AF9 LSCs (4), indicating a role for Gαq in MLL leukemia.

To determine if Gαq is functionally important in MLL AML, we first examined the effects of Gαq inhibition on active β-catenin expression and colony-forming capacity of pre-LSCs derived from MLL-AF9 transduced KLS (HSC-enriched Lin⁻ CD127⁻ Sca-1⁺ c-Kit⁺) and GMPs (Lin⁻ CD127⁻ Sca-1⁻ c-Kit^{high} CD16/32^{high} CD34⁺) (1). These pre-LSCs are a recognized early stage of leukemia development that subsequently acquires additional events *in vivo* leading to a full-blown leukemia (1). Stable knockdown of Gαq in pre-LSCs was achieved by lentiviral-mediated delivery of short hairpin RNA (shGαq). We found that all three individual shGαq reduced Gαq expression in KLS-derived pre-LSCs (KLS^{pre-LSC}) and GMP-derived pre-LSCs (GMP^{pre-LSC}) and as a result, Gαq knockdown led to a significant decrease in the levels of active β-catenin and colony formation (**Figures 1a, 1b**).

We next explored the effect of pharmacological inhibition of Gαq on pre-LSCs and leukemic cells. In the presence of 40 μM GP antagonist 2A (GP), a selective inhibitory peptide of Gαq protein (5), KLS^{pre-LSC} exhibited a significant decrease in β-catenin expression associated with a 50% reduction in colony formation (**Figure 1c; Figure S1**). We then assessed the antagonistic effects of GP on GFP⁺ leukemic cells sorted from fully developed primary MLL AML originating from KLS or GMPs (KLS/GMP^{leukemic}). The inhibitory effects observed for KLS^{pre-LSC} and KLS^{leukemic} cells (**Figures 1c, 1d**) were similar while GMP^{leukemic} cells appeared to be more sensitive to GP treatment (**Figure 1e**).

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We next tested the leukemogenic inhibitory potential of GP inhibition *in vivo*, by pre-treating leukemic cells *ex vivo* with GP (40μM) for 2 - 4 days, and subsequently transplanting viable cells into sub-lethally irradiated recipient mice. *In vivo* BrdU labeling analysis at 8 days post-injection showed that 2-day GP pre-treatment led to impaired cell proliferation in both KLS^{leukemic} and GMP^{leukemic} cells (**Figures 1f, 1g**). Consistent with the *in vitro* clonogenic assays (**Figures 1d, 1e**) we observed a slightly more pronounced reduction in proliferation for GMP^{leukemic} cells. Further survival analysis showed that 4-day GP pre-treatment was able to promote a small but significant enhancement of survival in mice transplanted with GMP^{leukemic} cells (**Figure 1h**), and genetic depletion of Gαq also significantly delayed the onset of leukemia induced by GMP^{leukemic} cells (**Figure 1i**). Together, this provides the first evidence that Gαq signaling contributes to maintenance of fully-developed AML. Furthermore, we found that the inhibitory effect of GP on human MLL-AF9 AML cells might largely depend on the expression levels of endogenous β-catenin, as we observed that GP selectively suppressed the growth of human THP-1 AML cells (**Figure 1j**) that endogenously express β-catenin (6), but had no effects on MOLM-13 cells (**Figure 1j**) as well as other human AML cell lines (e.g. ML2 carrying the MLL-AF6 translocation, data not shown) in which β-catenin expression is undetectable (6). In contrast, ABT-199, a selective Bcl2 inhibitor, had a β-catenin-independent effect and inhibited the growth of these AML cell lines (**Figure 1j**).

In an attempt to dissect the mechanism by which Gαq inhibition causes impairment in MLL leukemogenesis, we performed gene-expression analysis using RNA from shGαq transduced KLS^{leukemic} cells and found that several mitochondrial complex 1 genes (i.e. *mt-Nd2*, *mt-Nd4l*, *mt-Nd5*) were down-regulated in Gαq knockdown cells, while the stress response and DNA damage repair gene *Gadd45a* was up-regulated (**Figure 2a**). The link between Gαq mRNA expression and the expression of mitochondrially-encoded *Nd2* and *Nd4l* genes was confirmed in a panel of primary human AML samples (n = 84; **Table S1**). Furthermore, activation of *Gadd45a* has been shown to occur as a result of activation of the FoxO family of transcription factors in response to oxidative stress (7). Taken together, these data suggest a possible link between Gαq and mitochondrial function; therefore, we next sought to test whether Gαq inhibition affects the response of leukemic cells to mitochondrial stress. We first treated leukemic cells with the mitochondrial complex 1 inhibitor rotenone, which has been reported to increase ROS production triggering apoptosis (8). Our results showed that ROS levels were slightly elevated in rotenone-treated KLS^{leukemic} cells and were dramatically increased in rotenone-treated GMP^{leukemic} cells (**Figure 2b**). Gαq knockdown also dramatically increased ROS levels in GMP^{leukemic} cells (**Figure 2c**), suggesting a crucial role for Gαq activation in protecting GMP^{leukemic} cells against oxidative stress. Gαq activity is important in GMP^{leukemic} cells for maintaining basal oxidative phosphorylation (OXPHOS) levels and mitochondrial ATP production as Gαq inhibition, by either GP treatment or Gαq knockdown, significantly reduced the oxygen consumption rate (OCR, indicative of OXPHOS) and ATP levels in these cells, with a slightly reduced effect in KLS^{leukemic} cells (**Figures 2d, 2e, 2f, 2g**). Such a functional role for Gαq is in keeping with the positive correlation between expression of Gαq and levels of mitochondrial complex 1 gene expression, supporting the hypothesis that Gαq expression and function may be closely

coupled to mitochondrial function in AML cells. It is worth noting that in MLL leukemic cells Gαq inhibition had similar effects to rotenone treatment on induction of ROS and suppression of OCR and colony formation (**Figures 2b, 2d, 2h**).

In this study, we report a previously unknown role for Gαq in maintenance of MLL leukemia. Previous studies implicate Gαq as a MLL fusion target whose expression correlates with H3K79 methylation in MLL LSCs (3, 4). The effect of Gαq inhibition on repression of MLL leukemic cell growth largely depends on endogenous β-catenin activity. This is consistent with our previous findings that aberrant activation of β-catenin is essential for self-renewal of MLL LSCs, whereas inhibition of β-catenin impairs LSC formation and MLL leukemia development (1, 9).

Here we describe a potential mechanism linking mitochondrial function to leukemogenesis via Gαq signaling activation (**Figure S2**). LSCs in AML rely primarily on OXPHOS to generate ATP for energy (10), and our results show that Gαq inhibition can impair the energy-generating capacity of MLL leukemic cells, increase ROS levels and lead to activation of a stress response associated with activation of Gadd45a. Our data revealing reduced β-catenin levels following Gαq inhibition are consistent with the effects of Gαq on mitochondrial activity being mediated via β-catenin activity, as recent studies have shown the crucial role for β-catenin in mitochondrial energy metabolism (11, 12), and β-catenin is also critical in HSC for suppression of ROS levels (12). MLL AML has a particular dependence on β-catenin activity (1) and the findings presented here raise the possibility that targeting of β-catenin and mitochondrial metabolism via Gαq may be a potential approach to reduction of leukemogenesis in MLL AML. Of note, while human AML represents a heterogeneous disease, the correlation of Gαq expression with expression of mitochondrially-encoded *Nd2* and *Nd4l* genes provides support that Gαq-regulation of mitochondrial function may also be important in other AML subtypes. Whether activity of Gαq and/or OXPHOS in human AML cells, or levels of mitochondrial complex 1 genes, are predictive of response to chemotherapy is still under investigation. Gαq-mediated signaling is likely to cooperate with other pathways to drive leukemogenesis. In particular, FLT3-ITD mutation accelerates the onset of MLL-AF9-induced AML and correlates with poor outcome in AML patients (13). We have previously demonstrated that down-regulation of Gadd45a via FLT3-ITD contributes to the myeloid differentiation block in AML (14), and strikingly the regulator of G protein signaling 2 (Rgs2), a potent negative regulator of Gαq function (15), has also been shown to be a critical target gene down-regulated in FLT3-ITD-induced AML (16).

It is essential to investigate the mechanisms involved in G protein-associated regulation of β-catenin signaling in AML LSCs, as a number of studies have now shown that G-protein coupled signaling can be targeted therapeutically (15) and such an approach may provide clinical benefit for MLL AML patients. Future studies will identify predominant transducers in Gαq signaling and further define the role of β-

catenin down-regulation, FoxO transcription factors and Gadd45a in the stress response associated with Gαq inhibition.

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Figure Legends

Figure 1: Gαq is required for maintenance of MLL leukemia.

(a) Western blot analysis of Gαq knockdown efficiency and β-catenin expression (total β-catenin versus phosphorylated/inactive β-catenin) in scrambled control (Scr) versus 3 independent Gαq shRNAs (shGαq#1, shGαq#2, shGαq#3) transduced KLS^{pre-LSC} and their respective colony formation. A reduced level of total β-catenin (β-cat) protein, concomitant with no change in phosphorylated/inactive β-catenin (p-β-cat), indicates the activation of β-catenin. The percentage of colonies at the third round of replating is shown.

(b) Western blot analysis of Gαq and β-catenin expression in Scr, shGαq#1, shGαq#2 or shGαq#3 transduced GMP^{pre-LSC} and their respective colony formation.

(c) Colony formation of KLS^{pre-LSC} treated with control (water, H₂O), 20 μM GP or 40 μM GP for 5 days, and western blot analysis of β-catenin expression in KLS^{pre-LSC} treated with 40 μM GP.

(d, e) Colony formation of KLS^{leukemic} cells (d) or GMP^{leukemic} cells (e) treated with 20 μM GP or 40 μM GP for 5 days. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.0005; ****, *P* < 0.0001.

(f, g, h) Bar graphs representing quantitative analysis of the data obtained from *in vivo* BrdU incorporation cell proliferation assays, indicative of 5 independent experiments for KLS^{leukemic} cells (f) and 6 independent experiments for GMP^{leukemic} cells (g). GFP⁺ leukemic cells, which were sorted from fully developed primary MLL AML originating from KLS or GMPs, were pre-treated *ex vivo* with 40 μM GP for 2 - 4 days and subsequently transplanted into 2nd recipient mice for *in vivo* BrdU cell proliferation assays at 8 days post-injection (f, g), and Kaplan–Meier analysis of mouse survival (h).

(i) Kaplan-Meier survival curves of mice transplanted with GMP^{leukemic} cells expressing Scr versus shGαq#2.

(j) Growth inhibition in human AML cell lines after treatment with 40 μM GP or 1 μM ABT-199 for 72 hr, determined by AlamarBlue cell proliferation assay.

, $P < 0.01$; *, $P < 0.0005$; ****, $P < 0.0001$; NS, not significant ($P > 0.05$).

Figure 2: Gαq inhibition target leukemic cells by impairing mitochondrial OXPHOS.

(a) Heat map showing differential expression of genes in Scr versus shGαq transduced KLS^{leukemic} cells (Blue = down-regulated, Red = up-regulated) with p -value ≤ 0.01 and fold change cutoff of ≥ 1.9 .

(b, c) Mitochondrial ROS, as evaluated by the mitochondrial-specific redox probe MitoSox in KLS^{leukemic} or GMP^{leukemic} cells treated with control (DMSO), 25 nM rotenone or 50 nM rotenone for 24 h (b), or transduced with Scr versus shGαq (c).

(d, e) KLS^{leukemic} or GMP^{leukemic} cells treated with 40 μM GP or 1 μM rotenone for 48 h (d), or transduced with Scr versus shGαq (e). OCR was measured over a 2 h period and plotted as normalized % values of baseline (control) values for each sample.

(f, g) ATP levels in KLS^{leukemic} or GMP^{leukemic} cells treated with 40 μM GP for 48 h (f), or transduced with Scr versus shGαq (g).

(h) Colony formation of KLS^{leukemic} or GMP^{leukemic} cells treated with 25 nM rotenone or 50 nM rotenone for 5 days.

Figure 1.

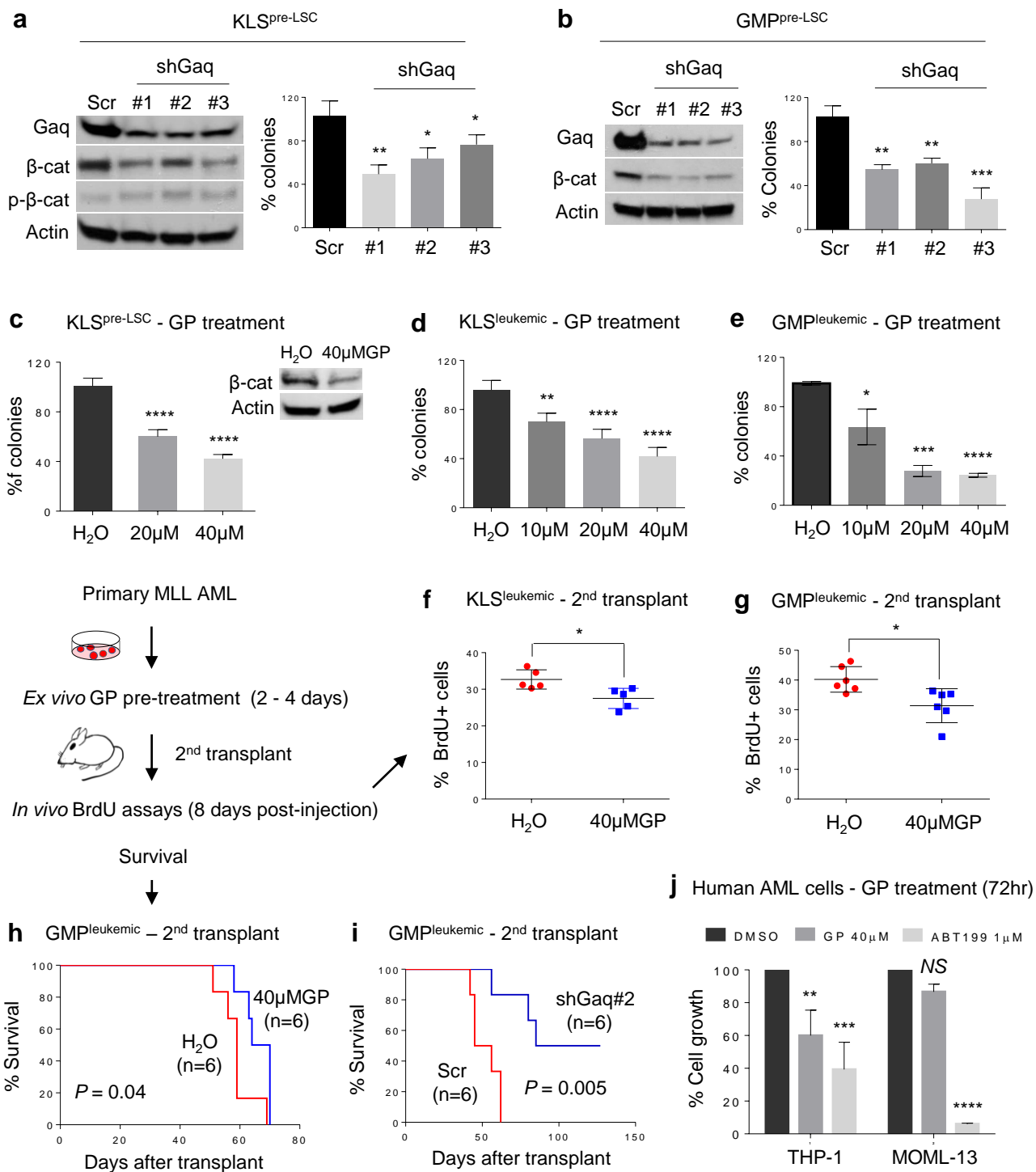


Figure 2.