Aurora kinase A promotes trained immunity via regulation of endogenous S-adenosylmethionine metabolism

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Abstract

Innate immune cells can acquire a memory phenotype, termed trained immunity, but the mechanism underlying the regulation of trained immunity remains largely elusive. Here, we demonstrate that inhibition of Aurora kinase A (AurA) dampens trained immunity induced by β-glucan. ATAC-seq and RNA-seq analysis reveals that AurA inhibition restricts chromatin accessibility of genes associated with inflammatory pathways such as JAK-STAT, TNF and NF-κB pathways. Specifically, AurA inhibition promotes nuclear localization of FOXO3 and the expression of glycine N-methyltransferase (GNMT), a key enzyme responsible for adenosylmethionine (SAM) consumption. Metabolomic analysis confirms a reduction in SAM level upon AurA inhibition. As a result of SAM deficiency, trained mouse macrophages exhibit decreased H3K4me3 and H3K36me3 enrichment on gene regions of *Il6 and Tnfα*. Additionally, the tumor inhibition effect of β-glucan is notably abolished by AurA inhibition. Together, our findings identify an essential role of AurA in regulating trained immunity via a methylation-dependent manner by maintaining endogenous SAM level through mTOR-FOXO3-GNMT axis.

Keywords

Aurora Kinase A, trained immunity, epigenetics, S-adenosylmethionine, GNMT, FOXO3

Introduction

Trained immunity is depicted as a memory state of innate immune cell, independent on adaptive immunity(Naik & Fuchs, 2022; Ochando, Mulder, Madsen, Netea, & Duivenvoorden, 2023). It can be induced by microbial polysaccharide component such as β-glucan, candida albicans and Bacillus Calmette-Guérin vaccine(Cirovic et al., 2020; Kalafati et al., 2020; Quintin et al., 2012). Trained immune cells exhibit a rapid and enhanced response to secondary related or unrelated stimulus, relying on chromatin remodeling and metabolic rewiring(Bekkering et al., 2018; Cirovic et al., 2020; Su et al., 2021b). In trained cells, histone modification associated with active transcription such as H3-histone-lysine-4 trimethylation (H3K4me3) is enriched in promoter regions of genes encoding inflammatory cytokines, supporting fast gene induction in response to secondary stimulation(Su et al., 2022). Trained cells display metabolic rewiring from oxidative phosphorylation to aerobic glycolysis, which depends on mTOR signaling pathway(Cheng et al., 2014). Intermediate metabolites in tricarboxylic acid (TCA) cycle such as fumarate and succinate function as DNA or histone demethylase inhibitors(Arts et al., 2016), whereas methionine and SAM in one carbon metabolism act as substrates for methyltransferases(Ampomah et al., 2022;

Yu et al., 2019). Therefore, metabolic processing and metabolites intertwined with epigenetic regulation by directly supplying methylation substrates or regulating methylation enzyme activities. SAM, a ubiquitous methyl donor in biological process, can be converted into S-adenosylhomocysteine (SAH) for the synthesis of cysteine in glutathione metabolism. The ratio of SAM/SAH is critical in support histone H3 trimethylation at lysine 36(H3K36me3) to enhance IL-1β transcription(Yu et al., 2019). However, it has not been clear how SAM level is regulated in the trained immunity process.

Aurora kinase A (AurA), a serine/threonine kinase, plays a critical role in mitosis and is frequently overexpressed in tumor tissues. Inhibition of AurA in tumor cells restricts cell proliferation, migration and induces cell death(Donnella et al., 2018; Jingtai et al., 2023; Tham et al., 2024; Wang-Bishop et al., 2019). Despite the well-established role of AurA on tumor cells growth and tumorigenesis, its function in innate immune cells like macrophages or in inflammation is not well understood(L. Ding, Gu, Gao, Xiong, & Zheng, 2015). Recent study reports that alisertib, a specific inhibitor of AurA, upregulates repressive histone methylation at lysine 27 via suppressing demethylase *KDM6B* gene expression during THP-1 cell (a human monocyte-like cell line) differentiation(Park, Cho, Oh, Kim, & Seo, 2018). Whether AurA plays a similar role in regulating trained immunity in macrophages remains unknown. Trained immunity can lead to aberrant inflammatory activity as well as enhanced anti-tumor effect(T. Wang et al., 2023). Understanding the role of AurA in

trained immunity would enable us to further exploit AurA for clinical application in cancer therapy.

In this study, we screened an epigenetic drug library and found that AurA inhibitors notably attenuated the trained immunity induced by β-glucan. Genetic disruption of AurA also showed a similar inhibitory effect on trained immunity. Mechanistically, AurA inhibition reduced the activation of mTOR signaling, thus leading to the nuclear localization of transcription factor FOXO3. Nuclear FOXO3 promoted the expression of GNMT to decrease the intracellular level of SAM, thereby inhibiting H3K4me3 and H3K36me3 enrichment on the promoters of inflammatory genes such as IL-6 and TNF-α. Moreover, pretreatment with alisertib abolished the anti-tumor effect conferred by β-glucan training in vivo. Thus, we conclude that AurA is an essential kinase required for the epigenetic regulation and antitumor activity of trained immunity in macrophages.

Results

Inhibition of Aurora kinase A suppresses trained immunity in macrophages

Trained immunity is orchestrated by epigenetic reprogramming, while the specific epigenetic modulators involved in regulating trained immunity remain incompletely understood. To uncover the key epigenetic modulators in trained immunity, we performed a small molecule inhibitor screening by using an epigenetic drug library (**Figure 1—figure supplement 1A, Appendix 1—table 1)**. Briefly, bone marrow-

derived macrophages (BMDMs) were trained by β-glucan in the presence of different inhibitors for 24 h, rested for 3 days, and then restimulated by LPS for secondary response. As trained immunity in macrophages strongly induces cytokines production including IL-6 and TNF-α upon restimulation by pathogen- or damage- associated molecular patterns (PAMPs or DAMPs)(Chakraborty et al., 2023; Netea et al., 2020), we used IL-6 level from the supernatant as a readout in our screening. Among a total of 305 inhibitors in the compound library, 8 AurA inhibitors showed inhibitory effect on trained immunity-induced IL6 production in both primary and secondary screening with different concentrations **(Figure 1—figure supplement 1B)**. To further confirm whether AurA identified in our screening could modulate trained immunity, we optimized β-glucan training protocol and used three AurA-specific inhibitors, and found that all these AurA inhibitors significantly inhibited IL-6 production in β-glucan-trained mouse macrophages **(Figure 1—figure supplement 1C)**. Among these inhibitors, alisertib is an orally active and highly selective inhibitor for AurA and has been applied in preclinical investigation(Bavetsias & Linardopoulos, 2015; Mosse et al., 2019; O'Connor et al., 2019). We further observed that alisertib obviously downregulated IL-6 and TNF-α in trained BMDMs in a concentration-dependent manner without affecting cell viability **(Figure 1A-B)**. Moreover, alisertib also decreased the transcriptional level of *Il6* and *Tnfα* in trained BMDMs **(Figure 1C)**. Furthermore, the phosphorylation of AurA was increased by β-glucan training but was blocked by alisertib **(Figure 1D)**. To further confirm the role of AurA in trained immunity, we knocked down the expression of AurA by small interfering RNAs

(siRNAs) in BMDMs **(Figure 1E)**. Consistently, knocking down of AurA also inhibited the production levels of IL-6 and TNF-α in trained BMDMs **(Figure 1F)**. Consistent with a recent report showing that tumor cells or tumor cell culture supernatant could function as the second stimulus for trained BMDMs(C. Ding et al., 2023), we also observed increased TNF-α and IL-6 production of trained macrophages upon secondary stimulation using MC38 tumor cells culture supernatant, and such effect was also inhibited by AurA knockdown or inhibition **(Figure 1G)**. To verify whether AurA regulates trained immunity in different cell models, we trained J774A.1 cells as well as THP-1 cells with β-glucan and also observed a reduction of IL-6 and TNF-α levels under AurA knockdown or inhibition **(Figure 1—figure supplement 1D)**. Moreover, intraperitoneal administration of alisertib together with β-glucan also attenuated trained immunity in vivo **(Figures 1H-I)**. Collectively, these results suggest that AurA inhibition suppresses β-glucan-induced trained immunity both in vitro and in vivo.

Aurora kinase A inhibition remodels chromatin landscape of inflammatory genes

Epigenetic reprogramming towards an open chromatin status is considered the basis for innate immune memory(Arts et al., 2016; Jeljeli et al., 2019; Moorlag et al., 2024; Novakovic et al., 2016). To clarify the status of chromatin landscape under AurA inhibition, we performed an assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) in trained BMDMs. In detail, mice were trained by intraperitoneal injection with β-glucan with or without alisertib, and the bone

marrow cells were isolated and differentiated into BMDMs for ATAC-seq analysis **(Figure 2—figure supplement 1A)**. Principal component analysis showed divergent distribution between AurA-treated and -untreated trained BMDMs **(Figure 2A).** And the erased regions by AurA inhibition were mapped into cellular processes like "regulation of growth", "myeloid leukocyte activation" and "MAPK cascade" **(Figure 2B)**. Analysis of enriched transcriptional binding motifs indicated that the erased peaks by alisertib were transcriptionally regulated by IRF1/2, FOS and STAT1/2, while the written peaks were transcriptionally regulated by PPARG, KLF4, ELF4 and FOXO1/3 **(Figure 2C)**. Recent report shows that PPARG and KLF4 were critical transcription factors in controlling genes of macrophage M2 polarization and FOXO3 regulates the expression of multiple genes involved in pathway such as anti-inflammation and oxidative stress resistance(Allemann, Lee, Beer, & Saeedi Saravi, 2023). Consistent with the reduced production of IL-6 and TNF-α proteins by alisertib, we observed a decreased chromatin accessibility of proinflammatory gene such as *Il6*, *Tnfα*, *Cxcl2* and *Il1a* by alisertib intervention **(Figure 2D)**. In contrast, the peaks of genes encoded M2 marker *mrc1* as well as *chil3* were enhanced **(Figure 2E)**. These results support that AurA regulate epigenetic changes in β-glucan-induced trained immunity and its inhibition restricts the chromatin accessibility for inflammatory genes in macrophages.

Meanwhile, we also performed RNA-seq with the trained BMDMs after LPS restimulation. Analysis of the transcriptome revealed that alisertib inhibited genes

associated with pro-inflammatory pathway including “JAK-STAT signaling pathway”, “TNF signaling pathway” as well as “NF-kappa B pathway” in trained BMDMs **(Figure 2F)**. In contrast, the up-regulated genes by alisertib were enriched in anti-inflammation pathway such as “FOXO signaling pathway” **(Figure 2G)**. Additionally, differentially expressed transcription factors were analyzed and mapped into Gene Ontology (GO) with enrichment in “negative regulation of Toll-like receptor signaling pathway”, “negative regulation of NLRP3 inflammasome complex assembly” and “negative regulation of interleukin-6 production” **(Figure 2—figure supplement 1B-C)**. Moreover, multiplex chemokine/cytokines array showed that alisertib decreased the chemokine/cytokines in trained BMDMs such as IL-6, TNF-α, CXCL2 and IL-1α **(Figure 2—figure supplement 1D)**. Collectively, these results demonstrate that alisertib restrict chromatin accessibility in genes associated with inflammation activation in trained BMDMs.

Alisertib inhibits glycolysis and remodel TCA cycle

Various studies show that metabolic rewiring such as glycolysis is critical for macrophage memory both in meeting energy demands and epigenetic modification(Bekkering et al., 2018; Bhargavi & Subbian, 2024; Liu et al., 2024). To determine the metabolic pathway that AurA may regulates in trained immunity, we analyzed the glucose metabolism in trained BMDMs. As expected, β-glucan increased glycolysis as indicated by higher extracellular acidification rate (ECAR) **(Figure 3A)**, with enhanced basal and maximum extracellular acidification rate **(Figure 3B)**, both

of which were inhibited by alisertib. In accordance, U-[^{13}C]-glucose tracer showed that β-glucan treatment upregulated the glucose incorporation into lactate, and this induction was significantly inhibited by alisertib **(Figure 3—figure supplement 1A)**. Meanwhile, metabolites in TCA cycle, including malate, citrate, α-KG, fumarate and succinate, were all decreased in alisertib-treated trained BMDMs **(Figure 3—figure supplement 1A)**. Previous study showed that fumarate was accumulated after β-glucan training, and it induced epigenetic reprogramming to facilitate trained immunity(Arts et al., 2016). However, alisertib treatment did not affect fumarate accumulation induced by β-glucan training **(Figure 3C)**, but increased tyrosine level **(Figure 3—figure supplement 1B)**. As a recent report shows tyrosine could be interconverted to fumarate(J. Li et al., 2023), we speculated the decreased fumarate from TCA cycle by alisertib may be compensated from tyrosine metabolism. Taken together, AurA inhibition inhibits glycolysis and remodels glucose metabolism in trained immunity.

Aurora kinase A regulates S-adenosylmethionine level in trained BMDMs

Next, we planned to identify the specific metabolites that support the function of AurA in trained immunity. KEGG analysis by using the differently expressed genes (DEGs) showed that glutathione (GSH) metabolism was significantly enriched **(Figure 3D)**. Consistently, the intracellular level of GSH induced by β-glucan training was notably reduced after AurA inhibition **(Figure 3E)**. It has been reported that β-glucan training induces a modified cellular redox status as elevation of ROS level and

enhanced synthesis of GSH(Ferreira et al., 2021; Ferreira et al., 2023). Genetic deletion of genes involved in GSH synthesis disrupts the cellular redox balance and dampens trained immunity(Su et al., 2021a). To investigate whether AurA functions on trained immunity directly though decreasing GSH, we measured the level of serine and SAM, both of which are precursors of GSH **(Figure 3—figure supplement 1C)**. By targeted liquid chromatography-tandem mass spectrometry (LC-MS) analysis, we found a significant reduction of SAM level under Aura inhibition, while the serine level remained unchanged **(Figure 3F)**. Next, we further detected the downstream products of SAM such as SAH and HCY. Consistently, we observed that alisertib also significantly decreased SAH and HCY in trained BMDMs **(Figure 3G)**. The decreased intracellular level of these precursors of GSH indicated that GSH was probably not a metabolite that AurA directly acted on, and its reduction was a result from SAM deficiency. SAM is directly linked to epigenetics as a methyl donor. For example, SAM induction upregulates the total intracellular level of histone lysine 36 trimethylation (H3K36me3) and thus increase H3K36me3 modification at the gene region of *Il1β* to promote IL-1β expression(Rodriguez et al., 2019; Yu et al., 2019). Since AurA inhibition resulted in intracellular SAM reduction, we wondered whether SAM upregulation would rescue trained immunity inhibited by alisertib or by AurA knocking down. GNMT is a key enzyme responsible for the conversion from SAM to SAH, and its deficiency blocked the conversion from SAM to SAH and thus leading to the increased ratio of SAM to SAH(Hwang et al., 2021; C. H. Li et al., 2015; Yen, Lin, Chen, Chen, & Chen, 2013). Furthermore, we found that AurA inhibition

upregulated GNMT protein level in trained BMDMs **(Figure 3H).** To verify whether intracellular SAM level was responsible for AurA-mediated trained immunity, we knocked down GNMT in BMDMs **(Figure 3I)**, and found that knockdown of GNMT increased the level of SAM while decreased the level of SAH, leading to increased ratio of SAM/SAH under AurA inhibition **(Figure 3J).** Furthermore, knockdown of GNMT rescued the IL-6 and TNF-α production in trained immunity under AurA inhibition in response to MC38 cell culture supernatant and LPS **(Figure 3K)**. These results suggest that AurA promotes trained immunity by supplying endogenous SAM level which is controlled by GNMT.

Inhibition of Aurora kinase A impairs histone trimethylation at H3K4 and H3K36

SAM is a well-known methyl donor for nearly all cellular methylation events including DNA, RNA and histone methylation(Keen & Taylor, 2004). Recent study reports that SAM deficiency limits histone methylation by phosphorylation of Rph1, which is a demethylase for H3K36me3(Ye et al., 2019). Meanwhile, SAM prefers to induce histone methylation changes in targeted sites like K4 without global changes to DNA and RNA(Pham et al., 2023). Therefore, we asked whether changes in histone methylation occurred when intracellular SAM level was inhibited by AurA inhibition. Among the major active histone methylation markers and repressive markers, we found that trimethylation at H3K4 and H3K36, induced by β-glucan training, were decreased by alisertib. Meanwhile, alisertib did not affect trimethylation at H3K9 and

monomethylation at H3K4, accompanied with a modest change but no significant difference in trimethylation at H3K27 **(Figure 4A, Figure 4—figure supplement 1 A).** Epigenetic modification including H3K4me3 and H3K36me3 promotes chromatin accessibility thus promoting rapid transcription of genes in response to second stimulation in trained immunity. This prompted us to investigate whether alisertib dampened β-glucan-induced epigenetic modification in genes like *Il6* and *Tnfα*. Chromatin immunoprecipitation (ChIP) assays demonstrated that both H3K4me3 and H3K36me3 enrichment on IL-6 and TNF-α promoters induced by β-glucan were notably decreased by alisertib **(Figure 4B)**. Consistently, knockdown of GNMT recovered the expression level of histone methylation and the enrichment level of H3K4me3 as well as H3K36me3 on gene promoter of *Il6* and *Tnfα*, which was inhibited by alisertib **(Figure 4C)**. Therefore, suppression of trained immunity caused by AurA inhibition is a consequence of decreased trimethylation events in histone by SAM deficiency.

Aurora kinase A regulates GNMT through transcription factor FOXO3

Given the role of GNMT in affecting SAM level, we questioned how AurA inhibition mediated GNMT expression in trained immunity. It was reported that nuclear FOXO3 facilitated GNMT expression to regulate the cell redox response(Hwang et al., 2021). Motif analysis of the ATAC-seq indicated that AurA inhibition promoted FOXO signal **(Figure 2C).** Thus, we hypothesized that AurA inhibition upregulated GNMT expression via FOXO3. Firstly, we found that knockdown of FOXO3 inhibited

GNMT protein level in BMDMs **(Figure 5A)**. Meanwhile, FOXO3 knockdown prevented the enhanced GNMT expression under AurA inhibition and knockdown **(Figures 5B-C)**, indicating the involvement of FOXO3 in AurA function. Moreover, knockdown of FOXO3 also restored the trained immunity as indicated by elevation of IL-6 level, which was inhibited by alisertib **(Figure 5D)**. To further investigate how AurA regulates FOXO3, we detected the phosphorylation level of FOXO3. The result showed that β-glucan training induced the phosphorylation of FOXO3 at Ser315 and AurA inhibition prevented the phosphorylation induction by β-glucan **(Figure 5E)**. Consistently, immunofluorescence detection showed a higher ratio of nuclear enrichment of FOXO3 by AurA inhibition compared with β-glucan training **(Figure 5F)**. Nuclear enrichment of FOXO3 is inhibited by AKT-mTOR activation(Liu et al., 2018; van der Vos et al., 2012). To demonstrate whether the increased FOXO3 nuclear localization by alisertib was associated with AKT-pathway activation, we checked the activation of AKT-mTOR in trained BMDMs. As expected, both pharmacological and genetic disruption of AurA significantly inhibited β-glucan-induced phosphorylation of AKT-mTOR-S6K-S6 **(Figure 5G)**. Additionally, mTOR activation by its agonist MHY1485 promoted trained immunity under aurora kinase A inhibition **(Figure 5H)**, suggesting that AKT-mTOR-FOXO3 signaling pathway act downstream of AurA-mediated trained immunity. In conclusion, these results show the role of transcription factor FOXO3 in AurA-mediated GNMT expression in trained immunity, which depending on the activation of AKT-mTOR pathway.

Alisertib abrogates the anti-tumor effect induced by trained immunity

Trained immunity has been implicated in anti-tumor immunity and is considered as a new branch for cancer immunotherapy development (Bird, 2023; C. Ding et al., 2023; T. Wang et al., 2023). We speculated about whether inhibition of AurA would disrupt the anti-tumor effect induced by β-glucan. Therefore, we conducted a tumor model in mice preinjected with β-glucan (**Figure 6A)**. Training with β-glucan inhibited MC38 tumor growth, but the administration of alisertib abolished the antitumor effect induced by β-glucan **(Figure 6B, Figure 6—figure supplement 1 A-B)**. To investigate the role of macrophages in the anti-tumor effect induced by β-glucan, we analyzed the population of myeloid cells ($CD45^+CD11b^+$) as well as the population of myeloid derived macrophages ($CD45^+CD11b^+F4/80^+$) in tumor microenvironment. The population of myeloid cells or macrophage induced by β-glucan or by alisertib intervention showed no changes **(Figures 6C, Figure 6—figure supplement 1C)**. Studies have shown that GNMT is a tumor suppressor gene and its expression is downregulated in tumor tissue(DebRoy et al., 2013; Simile et al., 2022). Considering that AurA inhibition enhanced GNMT expression in BMDMs in our experiment, we next detected GNMT expression in tumor associated macrophages (TAMs). We found that TAMs from alisertib inhibited mice exhibited a higher expression of GNMT, compared with β-glucan trained mice **(Figure 6D)**. Moreover, compared with alisertib-treated mice, the $CD45^+CD11b^+F4/80^+$ cells in tumor tissue from β-glucan trained mice showed a higher intracellular phospho-S6, suggesting that AurA inhibition inhibited mTOR activation in macrophages **(Figure 6E)**. Cytokines in

tumor microenvironment have profound effects on tumor progression. β-glucan adjuvant as well as trained immunity can act as modulator for TME(Sui & Berzofsky, 2024; M. Zhang, Kim, & Huang, 2018) Indeed, β-glucan training induced a higher level of IL-1β, IL-6 and IL-12p70 in tumor microenvironment, and the enhanced cytokines production was inhibited by alisertib. **(Figure 6F)**. Taken together, these results demonstrate that AurA inhibition dampens anti-tumor effect of trained immunity in mTOR-GNMT axis dependent manner.

DISCUSSION

Trained immunity defines the memory ability of innate immune cells in response to second challenge. Despite the fact that trained immunity has been described decades ago, the mechanism that supports trained immunity is still elusive. In this study, we identified that AurA was required for trained immunity. AurA deficiency or inhibition decreased Il-6 and TNF-α production in β-glucan-trained BMDMs upon rechallenge by LPS or supernatant from tumor cells. Moreover, alisertib impaired the anti-tumor effect induced by β-glucan. We also discovered that alisertib induced GNMT expression via mTOR-FOXO3 axis and thus decreased SAM production, resulting in decreased H3K36me3 and H3K4me3 modification on the promotors of inflammatory genes, highlighting the role of AurA kinase as hub for methionine metabolism and epigenetics.

S-adenosylmethionine, is one of the most ubiquitous metabolites in host. It contains

methyl group, adenosyl group and ACP group, all of which can be transferred by corresponding transferases(Lee, Ren, Jeon, & Liu, 2023). It is reported that SAM supplement in mammals is consumed for methylation events on DNA or histone proteins, which affects biological process like tissue differentiation and gene expression(Dai, Ramesh, & Locasale, 2020). In macrophages, DNMT transfers methyl group from SAM to DNA and leads to gene repression such as *Il6* and *Dusp4* (Ampomah et al., 2022; Ji et al., 2019; Jung, Park, & Ko, 2020). However, SAM is also reported to play an active role in primary macrophage to increase IL-1β expression in response to LPS, and its precursor methionine can promote M1 macrophage activation(Dos Santos et al., 2017; Yu et al., 2019). While, whether SAM is involved in trained immunity is not investigated. In our study, AurA inhibition reduced endogenous SAM level in trained BMDMs. It also inhibited the trimethylation at H3K4 and histone H3K36 via upregulating GNMT. GNMT is a glycine methyltransferase, which transfers methyl group from SAM to glycine to lower intracellular SAM level(Luka, Mudd, & Wagner, 2009). It is well-known that inhibition of AurA would cause abnormal chromatin segregation and chromatin instability. Previous research report that chromatin instability make sensitivity to SAM and polyamine related gene including GNMT, which might also explain the function of AurA inhibition on SAM(Islam et al., 2023). Additionally, SAM is the precursor for glutathione. It has been reported that methionine and SAM reduce oxidative damage by increasing glutathione(Bandyopadhyay et al., 2022). However, we found that AurA inhibition didn’t increase the level of glutathione, which suggests

that alisertib inhibited trained immunity in a ROS independent manner, but through SAM-mediated epigenetic regulation.

AurA belongs to serine and threonine kinase family. It plays critical role in centrosome and chromosome segregation and cell mitosis. Activation of AurA is associated with tumor progression and drug resistance(Zhao, Zhang, Yang, & Yang, 2023). During past two decades, there have been developed more than twenty agents for AurA including alisertib and VIC-1911(Attwood, Fabbro, Sokolov, Knapp, & Schioth, 2021; Bavetsias & Linardopoulos, 2015). These agents have been evaluated across metastatic breast cancer, lung cancer, gastro-oesophageal adenocarcinoma and peripheral T-cell lymphoma in clinical trails(Canova et al., 2023; Haddad et al., 2023; Melichar et al., 2015). Among these inhibitors, alisertib is a highly selective inhibitor for AurA. However, alisertib has limited efficacy against both solid and hematological tumors (Beltran et al., 2019; Mosse et al., 2019; O'Connor et al., 2019). In recent years, there are researches trying to explain the resistance to AurA-targeted therapy and reveal feedback loops existed in tumor cells, which contributes to drug resistance. For example, AurA inhibition by alisertib upregulates PD-L1 expression in tumor cells and allows immune escape(X. Wang et al., 2023). AurA inhibition also enhances fatty acid oxidation to overcome the glycolysis attenuation in tumor cells (Nguyen et al., 2021). However, these studies mainly focus on investigating the role of AurA in tumor cells while its role in immune cells is rarely understood. In this study, we demonstrated that AurA regulated the trained immunity in macrophages. And AurA

inhibition significantly decreased the trained immunity induced by β-glucan in vitro and in vivo. AurA is described to be expressed and activated in stem-cell like cells, bone marrow and epithelium (Qi et al., 2016; Rio-Vilarino et al., 2024; Zhou et al., 2018).Clinical data show that alisertib by oral administration of rapidly distributes in bone marrow, making bone marrow a site susceptible to side effect(Oh, Power, Zhang, Daniels, & Elmquist, 2022). Due to the inhibition effect of alisertib on trained immunity in bone marrow, this may be the potential explanation for the poor therapeutic efficacy or drug resistance of alisertib on patients with cancer. Trained immunity can reprogram the myeloid cells like neutrophils and enhance its anti-tumor activity(Moorlag et al., 2020). Thus, our findings provide a potential guidance for AurA inhibitor application in clinical cancer therapy, and the clues for design of new inhibitors such as avoiding targets on bone marrow.

In conclusion, our findings demonstrate that AurA supports trained immunity by maintain SAM level. As a result of SAM deficiency under AurA inhibition, H3K4m3 and H3K36me3 level are reduced and trained immunity is inhibited. Our finding reveals a novel AurA-SAM metabolic axis as a new mechanism for trained immunity. Furthermore, our findings also identify a potential guidance for the clinical application of AurA, and new clues for the design of next-generation AurA inhibitors in the future.

Materials and methods

Mice

C57BL/6J mice (male, 6~8 weeks old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were kept in specific pathogen-free (SPF) conditions. For all animal studies, mice were randomly assigned to experimental groups, and no statistical method was used to predetermine the sample size. All Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Mice were injected intraperitoneally with 1 mg β-glucan (Cat#G6513, Sigma-Aldrich, USA) suspended in 100 μL PBS. For drug administration, alisertib (Cat#A4110, ApexBio, shanghai, dissolved in 10% 2-hydroxypropyl-β-cyclodextrin and 1% sodium bicarbonate, oral gavage) was delivered once daily at 30 mg/kg for 7 days before MC38 inoculation. MC38 (1×10^6 cells) were subcutaneously inoculated into the mice. Mouse tumor volumes were measured every 3 days. The tumor volume was calculated as follows: $1/2 \times \text{length} \times \text{width}^2$.

Cell Culture

THP-1 cells were cultured in 1640 medium with 10% FBS, 1% Pen/Strep and 1% Glutamax. J774A.1 cells were cultured in DMEM medium with 10% FBS, 1% Pen/Strep. For primary macrophages, bone marrows were isolated from C57BL/6J mice (6~8 weeks) and cultured in 1640 medium with 10% FBS, 1% Pen/Strep, 10% supernatant from L929 cells. For transfection with siRNAs (synthesized by RuiBiotech, Guangzhou) using RNAiMAX (Invitrogen, Cat#13778150) according to

the manufacturer's instruction, bone marrows cultured for 4 days were digested with trypsin and seeded into cells plates followed by transfection at day 5. Cells was maintained in 95% humidified air and 5% CO2 at 37 ℃. All cells were authenticated tested for mycoplasma contamination.

The siRNAs sequences were as follows:

siRNA for mouse AurA#1: CGAGCAGAGAACAGCUACUUATT

siRNA for mouse AurA#2: GCACCCUUGGAUCAAAGCUAATT

siRNA for mouse GNMT#1: GGACAAAGAUGUGCUUUCATT

siRNA for mouse GNMT#2: CGUCAGUACUGACAGUCAATT

siRNA for mouse FOXO3#1: CGGCAACCAGACACUCCAATT

siRNA for mouse FOXO3#2: CUGUAUUCAGCUAGUGCAATT

Scramble siRNA: UUCUCCGAACGUGUCACGUTT

Drug screening

Drugs screening was performed using a drug library from TargetMol (Shanghai, China). A detailed drugs list was provided in **Appendix 1—table 1**. For the primary screening, 1×10^5 BMDMs were seeded into 96-wells plate. Following overnight incubation, the BMDMs were trained with β-glucan (100 μg/mL, Sigma-Aldrich, Cat#G6513) in the presence of drugs at a concentration of 5 μM for 24 h while the secondary screening was performed with drugs at two concentrations of 0.2 and 1 μM. Control cells received vehicle control DMSO. After 24 h training, cells were washed

with 1×PBS and further cultured in fresh complete medium (Containing 10% L929 supernatant) for 3 days, followed by LPS stimulation (100 ng/mL). The culture supernatant was subjected to ELISA to measure IL-6. The relative amount of IL-6 was calculated as the fold change of the drugs-treated trained cells compared with the glucan only-treated cells. The drugs that showed suppressing effects with fold changes of 0.8-fold or lower, were considered to be inhibitors for trained immunity.

Cell viability assay

The effect of alisertib on cell viability was examined by CCK8 (Cat#40203ES60, Yeasen). In brief, BMDMs were seeded in to 96-wells plate and trained with β-glucan (50 μg/mL) in the presence of alisertib at a different concentration. After 24 h treatment, CCK8 reagent was added to the wells for 4 h, the cell viability was measured at 450 nm.

Western blot analysis

To evaluate intracellular protein expression, total cellular protein was extracted with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% Na-deoxycholate supplemented with Protease Inhibitor Cocktail (Selleck, Cat#B14001) and Phosphatase inhibitors (Roche). Proteins were resolved by SDS-PAGE and subjected to Western blot as described elsewhere. The primary antibodies were incubated at 4 ℃ overnight and HRP-conjugated secondary antibodies was incubated for 1 h at room temperature. Enhanced chemiluminescence

was used to detect the specific blot bands. The specific bands were quantified using Image Lab (v6.0) and the relative expression was normalized to the internal control.

The following primary antibodies were used: anti-β-actin (1:10000, Cat#A2228, Sigma-Aldrich), anti-Aurora A (1:1000, Cat#ab108353, Abcam), anti-Phospho Aurora A (Thr288) (1:500, Cat#2913T, Cell Signaling Technology), anti-GNMT (1:500, Cat# PA5-100018, Invitrogen), anti-H3K4me1 (1:3000, Cat#ab8895, Abcam), anti-H3K9me3 (1:3000, Cat#ab8898, Abcam), anti-H3K36me3 (1:3000, Cat# ab282572, Abcam), anti-H3K4me3 (1:3000, Cat# ab213224, Abcam), anti-H3K27me3 (1:3000, Cat#9733, Cell Signaling Technology), anti-H3 (1:5000, Cat#14269, Cell Signaling Technology), anti-FOXO3 (1:500, Cat#ab23683, Abcam), anti-Phospho FOXO3 (Ser315) (1:500, Cat# 28755-1-AP, Proteintech), anti-AKT (1:1000, Cat# 2920, Cell Signaling Technology), anti-Phospho-AKT (Ser473) (1:1000, Cat# 4060, Cell Signaling Technology), anti-mTOR (1:1000, Cat# 2972, Cell Signaling Technology), anti-Phospho-mTOR (Ser2448) (1:1000, Cat# 5536, Cell Signaling Technology), anti-p70 S6 Kinase (1:1000, Cat# 9202, Cell Signaling Technology), anti-Phospho-p70 S6 Kinase (Thr389) (1:1000, Cat# 9205, Cell Signaling Technology), anti-S6 (1:1000, Cat# 2217, Cell Signaling Technology), and anti-Phospho-S6 (Ser235/236) (1:1000, Cat# 4858, Cell Signaling Technology). Goat anti-rabbit HRP-linked antibody (1:3000, 7074, Cell Signaling Technology) or goat anti-mouse HRP-linked antibody (1:3000, 7076, Cell Signaling Technology) served as the secondary antibody.

Bulk RNA sequencing

Total RNA was extracted by TRIzol™ reagent and purified. Library construction and sequencing were performed by Annoroad Gene Technology (Guangzhou). After removal of rRNA and filtering of clean reads, the paired-end reads were mapped to the GRCm38/mm10 genome using HISAT2 2.2.1(Florea, Song, & Salzberg, 2013). FPKM was obtained for each gene in the RNA-seq data using HTSeq 2.0.2. RNA differential expression analysis was performed by DESeq2 software(Love, Huber, & Anders, 2014). The genes with FDR (false discovery rate) below 0.05 and absolute fold change (FC) not less than 1.2 were considered as differentially expressed genes (DEGs). All DEGs were mapped to GO terms in the Gene Ontology database for GO enrichment analysis and were mapped to KEGG for pathway enrichment analysis using ClusterProfiler 4.6.6.

ATAC-seq analysis

1×10^5 cells were prepared for ATAC-seq library construction with Novoprotein Chromatin Profile Kit for Illumina as the manufacturer's instructions. The solubilized DNA fragments were amplified with Novoprotein NovoNGS Index kit for Illumina. The reaction was monitored with qPCR to prevent GC bias and oversaturation. Finally, the library was purified by Novoprotein DNA Clean Beads and sent to Annoroad Gene Technology (Guangzhou) to sequence with Illumina Novaseq 6000 (150 bp, paired-end). ATAC-seq data were analyzed following guidelines of Harvard FAS Informatics Group. Optimally, reads were aligned to the reference mouse genome

(GRCm38/mm10) using HISAT2 with X, no-spliced-alignment, and no-temp-splice site parameters according to the manual. Quality control was performed with FastQC (Version 0.12.1) in Linux and with ATACseqQC (Version 1.16.0) in R (Version 4.1.0)(Wilbanks & Facciotti, 2010). The peaks were called using MACS3 software with default settings tailored for the genome(Y. Zhang et al., 2008). For all remaining samples, peaks with low counts (consensus peaks with a median across treatments of counts-per-million less than 1) were filtered out from further analysis submitted to Diffbind~3.8.4 to perform further analysis(Ross-Innes et al., 2012). The read distribution of PCA plot was generated by dba.plotPCA with the normalized count matrix. And the differential analysis were performed by dba.analyze function with DBA_EDGER. The differentially accessible regions (DARs) were defined as log fold changes >1 or <−1, meanwhile the $p<0.05$. In order to determine whether differentially accessible chromatin peaks localized near genes with shared functional biological pathways, we performed peak annotation by Genomic Regions Enrichment of Annotations Tool (GREAT)(Heinz et al., 2010). We used 500kb as the maximum absolute distance to the nearest transcriptional start site and q-values less than 0.01 as statistically significant. TF Motif Enrichment Analysis was performed using HOMER's findmotifsGenome. Integrative Genomics Viewer (IGV) track plots of chromatin accessibility were generated with the bam files.

ECAR and Glutathione assay kit

Extracellular acid ratio was determined by Seahorse Flux Analyzer XF96 (Agilent)

according to the manufacturer's instructions. In brief, BMDMs were plated at a density of 5×10^4 cells per well into XFe96 cell culture microplates (Agilent) and cultured overnight followed by β-glucan (50 μg/mL) training or alisertib (1 μM) stimulated for 24 h. The stimulated cells were then washed with Seahorse XF RPMI Media (pH 7.4) supplemented with 2 mM glutamine and incubated for 1 h at 37 ℃ without CO2, followed by sequential injection of glucose (10 mM), oligomycin (1 μM) and 2-DG (50 mM). ECAR was measured with the Agilent Seahorse XF96 Analyzer and analyzed using XFe Wave software according to the manufacturer's instructions. For intracellular GSH detection, a total of 8×10^6 BMDMs were seeded into 10 cm dishes. Cells were trained with β-glucan (50 μg/mL) with or without alisertib (1 μM) for 24 h. Following that, cells were collected by trypsinization and washed once with 1×PBS. Cell pellet was resuspended in 1 mL of PB buffer before sonication. The lysate was centrifuged at 14,000 rpm at 4 ℃ for 10 min, and supernatants was collected for measurement of glutathione according to the manufacturer's instructions (Cat#DIGT-250, BioAssay Systems). The relative GSH level was calculated by normalization to mock group.

U13-glucose tracing

Labelled compounds U-^{13}C-glucose were added to customized RPMI medium lacking glucose. And BMDMs were trained with β-glucan (50 μg/mL) and inhibited by alisertib (1 μM) for 20 h in this customized medium with labelled U-^{13}C-glucose. Following that, cells were washed with 1× PBS twice by aspirating the medium and

immediately adding −80 ℃ methanol and pre-cold water containing norvaline. After 20 min of incubation on dry ice, the resulting mixture was scraped, collected into a centrifuge tube, mixed with pre-cold chloroform and centrifuged at 14,000×g for 5 min at 4 ℃. The supernatant was evaporated to dryness in a vacuum concentrator. The dry residue was completely dissolved in 20 μL of 2% (w/v) methyoxyamine hydrochloride in pyridine and incubated for 60 min at 37 ℃. N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane was used for the preparation of N-tert-butyldimethylsilyl ethanolamines (TBDMs) and sample was incubated with TBDMS for 30 min at 45 ℃ for derivatization. After centrifugation for 3min at 12000rpm, the derived samples were detected and analyzed using a Thermo1310 coupled to an IQ QD MS system with DB-35 (30 m×0.25 mm×0.25 μm). The ion source is an ionization energy of 70 eV and temperature of 300 ℃. The detection was in a mode of full scan, ranging between 100-650 m/z. The inlet temperature was 270 ℃, and helium was used as the carrier gas, in a flow rate of 1.2 ml/min. Polar metabolite ramp-up procedure: The initial temperature of the column heater was maintained at 100 ℃ for 2 min, and the temperature was raised to 255 ℃ at a rate of 3.5 ℃/min and to 320 ℃ at a rate of 15 ℃/min, with a total run time of approximately 50 min.

Targeted metabolomics analysis

To identify SAM, SAH, serine, fumarate and HCY, liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis was performed. Briefly, BMDMs were

trained with β-glucan (50 μg/mL) and inhibited by alisertib for 24 h. For transfection with siRNAs targeting GNMT, BMDMs was transfected with siRNA for 48 h and was followed by β-glucan and alisertib treatment for another 24 h. Metabolites were extracted by using cold 80% methanol (HPLC Grade, Sigma-Aldrich) from 3 million cells with vortex, followed by centrifugation for 15 min at 15000×g at 4°C to collect supernatant. The supernatant was then evaporated to dryness in a vacuum concentrator. Analysis of targeted metabolites was conducted on a A6495 triple quadrupole system interfaced with a 1290 UHPLC system (Agilent Technologies). The resulting MS/MS data were processed using Agilent Quantitative analysis software.

Chromatin immunoprecipitation (ChIP) assay

The SimpleChIP® Enzymatic Chromatin IP Kit (Cat#9003, Cell Signaling Technology, USA) was used to perform ChIP according to the manufacturer's instructions. Samples were subjected to immunoprecipitation using either Rabbit anti-H3K4me3 antibody, anti-H3K36me3 antibody or a control IgG antibody (Cell Signaling Technology). Fragmented DNAs were purified using spin columns (Axygen) and was used as the templates for qPCR using indicated primer sets spanning the *Tnf* and *Il6* promoters.

The following primers for Chip-PCR were used:

Chip IL-6 forward: TCGATGCTAAACGACGTCACA

Chip IL-6 reverse: CGTCTTTCAGTCACTATTAGGAGTC

Chip TNF-α forward: TGGCTAGACATCCACAGGGA

Chip TNF-α reverse: AAGTTTCTCCCCCAACGCAA

Immunocytochemistry and immunohistochemistry staining

For intracellular staining of FOXO3 (1:200, Cat#ab23683, Abcam), BMDMs was grown on glass bottom dishes (Nest, Wuxi) overnight. After indicated treatment, BMDMs were washed twice with 1×PBS, fixed in 4% paraformaldehyde in room temperature for 10 min, permeabilized with 0.25% Triton X-100 for 30 min at room temperature (RT). Cells were incubated with blocking buffer (1% BSA, 0.1% Tween 20 in TBS) for 30 min at RT. Primary antibody were incubated at 4°C overnight, and the appropriate fluorescent secondary antibody (1:500, Cat# A-11008, Thermo Fisher Scientific) for 1 h at RT. The dishes were then washed three times with 0.1% TBST with DAPI staining during the first wash. For tumor tissue staining, fresh tumor tissue was fixed in 4% paraformaldehyde in room temperature for 24~48 h and cut into ~8 μM section after embedded in OCT. The slice was blocked with normal goat serum with 1% Triton X-100 and incubated with primary GNMT (1:200, Cat# PA5-100018, Invitrogen), F4/80 (1:200, Cat#ab90247, Abcam) antibody overnight at 4C, followed by incubation with Alexa 488 anti-rabbit (1:200, Cat# A-11008, Thermo Fisher Scientific) and Alexa 555 anti-rat (1:200, Cat# 4417, Cell Signaling Technology) for 1 h at room temperature. Nuclei were counterstained with DAPI (1 mg/mL) and mounted with Prolong GLASS antifade mountant (P10144, Thermo Fisher Scientific). Images were obtained using a NIKON confocal microscope. The nuclear localization

of FOXO3 in trained BMDMs in vitro was compared by calculated the ratio of mean nuclear intensity to cytoplasmic intensity(Kelley & Paschal, 2019). For analysis the GNMT expression in tumor associated macrophages, we randomly counted 150~200 $DAPI^{+}F4/80^{+}$ cells and draw ROI to report intensity. The representative data in our result showed the mean intensity of counted macrophages.

FACS

Fresh tumor tissue was harvested at the end of time point and crushed with the barrel of a syringe to form homogenate on a 100 μM cell strainer with Phosflow lyse/fix buffer (Cat#558049, BD Biosciences) immediately. Subsequently, the suspended cells were washed twice with BD perm/wash buffer (Cat#554723, BD Biosciences). Cells were then stained with the desired antibodies for 30 min at room temperature in dark. For phospho-S6 staining, cells were incubated with secondary antibody. Cells were analyzed using a BD LSRFortessa flow cytometer.

The following primary antibodies were used: anti-CD45 BV421 (1:200, Cat#563890, BD Biosciences), anti-CD11b PerCP Cy5.5 (1:200, Cat# 45-0112-82, Thermo Fisher Scientific), anti- F4/80 FITC (1:200, Cat# 11-4801-82, Thermo Fisher Scientific), and anti-Phospho-S6 (Ser235/236) (1:200, Cat# 4858, Cell Signaling Technology). Anti-rabbit IgG (Alexa Fluor 555 Conjugate) (1:200, Cat# 4413, Cell Signaling Technology) served as the secondary antibody for anti-phospho-S6.

Statistical analysis

The GraphPad Prism version 8.0 was used for statistical analysis. Data are presented as mean ± SEM (Standard Error of Mean) of indicated biological replicates. For statistical significance analysis, unpaired one-tailed t-test was used for comparing two mean values; one-way ANOVA was applied for comparisons of multiple mean values and two-way ANOVA was applied for comparisons of multiple mean values under different conditions. Sample size was not determined using a specific statistical method, and no data were excluded from the analyses. The data distribution was assumed to be normal, although this assumption was not formally tested.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Figure source data files contain the numerical data used to generate the figures.

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Funding

National Key R&D Program of China (2021YFC2400601)

- Xiaojun Xia
- Zining Wang

National Natural Science Foundation of China (82073140)

- Zining Wang

National Natural Science Foundation of China (82102874)

- Hongxia Zhang

National Natural Science Foundation of China (32400716)

- Yongxiang Liu

Acknowledgements

This work was supported by grant (No. 2021YFC2400601) from the National Key R&D Program of China, grant (82073140, 82102874, 32400716) from the National Natural Science Foundation of China. We thank the Metabolic Center at Sun Yat-sen University for providing technical support.

Ethics

All animal experiments and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (2024002174).

Figure Legends

Figure 1. Inhibition of Aurora kinase A suppresses trained immunity in macrophages. (A) BMDMs were trained with β-glucan at a dosage of 50 μg/mL in the presence of different concentration of alisertib for 24 h. The viability of BMDMs was measured by CCK8. **(B)** Supernatant levels of IL-6 (left) and TNF-α (right) in trained BMDMs with alisertib (0.5 μM or 1 μM), followed by restimulation with LPS (20 ng/mL) for 24 h. **(C)** Relative mRNA expression of *Il6* and *Tnfα* in trained BMDMs with alisertib (0.5 μM or 1 μM), followed by restimulation with LPS (20 ng/mL) for 6 h. **(D)** Immunoblotting analysis of AurA phosphorylation after the treatment of β-glucan (50 μg/mL) or alisertib (1 μM) for 90 min. **(E)** Immunoblotting analysis of AurA in BMDMs transfected with siRNA targeting AurA for 48 h. **(F)** The BMDMs were firstly transfected with siRNAs for 48 hours and then followed by β-glucan training. Supernatant levels of IL-6 and TNF-α were detected by ELISA after 3 days rest and restimulation with LPS. **(G)** The BMDMs was trained with BMDMs (50 μg/mL) or in combination with alisertib or with AurA knocking down, followed by a rest for 3 days and restimulation with cell culture supernatant from MC38. **(H)** Graphical outline of in vivo training model (mice n=3 per group). **(I)** Supernatant levels of IL-6 (left) and TNF-α (right) in trained BMDMs as shown in **H;** each point in the graph represents an individual mouse. Data are representative of three independent experiments (except in **I**) and presented as the mean ± SEM. *P* values were derived from one-way ANOVA test with a Dunnett's multiple-comparison test (A, F, G, I) or two-way ANOVA with a Tukey's multiple-comparison test (B, C). Related to **Figure 1—figure supplement 1**, **Figure 1—source data 1-2.**

Figure 1—figure supplement 1. Targeting aurora A inhibits β-glucan-induced trained immunity. (A) Schematic of the assay protocol for the molecule screening in BMDMs. **(B)** Fold change of IL-6 production in drug screening inhibited by AurA inhibitors compared to the β-glucan only group. **(C)** BMDMs were trained with β-glucan (50 μg/mL) in the presence of Tozasertib (1 μM), alisertib (1 μM) or MLN8054 (1 μM) for 24 h, followed by LPS (20 ng/mL) stimulation for 6 h after a rest for 3 days. **(D)** Supernatant levels of IL-6 in trained J774A.1 cells and THP-1 cells. J774A.1 cells were transfected with small interfering RNAs to knock down AurA followed by β-glucan (50 μg/mL) training. After rest for 3 days, trained J774A.1 cells was counted and seed into cell culture plate with LPS (20 ng/mL) rechallenge for 6 h. THP-1 cells were trained with β-glucan (50 μg/mL) for 24 h, and were centrifuged and washed once to remove medium. The THP-1 cells were then cultured with fresh medium containing 10 ng/mL PMA for 48 h and rest 1 day followed with LPS rechallenge (20 ng/mL). Data in **C** and **D** are representative of three independent experiments and presented as the mean ± SEM. *P* values were derived from one-way ANOVA test with a Dunnett's multiple-comparison test. Related to **Figure 1**.

Figure 2. Aurora kinase A inhibition remodels chromatin landscape of inflammatory genes. (A) Principal component analysis (PCA) of gene peaks in ATAC-seq. **(B)** GO enrichment analysis of erased peaks by alisertib in trained

BMDMs. **(C)** Representative motifs in the erased (n=15,431) and written (n=19,733) peaks respectively. **(D and E)** Genome browser views of ATAC-seq signal of representative genes inhibited by alisertib including *Cxcl2*, *Il1a*, *Tnfα* and *Il6* **(D)** and representative genes enhanced by alisertib including *Mrc1* and *Chil3* **(E)**. **(F and G)** KEGG enrichment of differentially expressed genes in trained BMDMs rechallenged with LPS; alisertib downregulated genes **(F)** and upregulated genes **(G)** were mapped into KEGG respectively. Related to **Figure 2—figure supplement 1**.

Figure 2—figure supplement 1. Aurora kinase A inhibition suppresses the expression of transcription factor involved in inflammation activation. (A) In vivo training model in C57BL/6J mice with intraperitoneal injection of β-glucan (1 mg per mice) and daily administration of alisertib (30 mg/kg/d) for 7 days (mice n=2 per group). **(B)** Heatmap from RNA-seq analysis showing the differentially expressed transcription factors (DETFs) from mice treated as described in Figure S2A. **(C)** GO enrichment analysis of differentially expressed transcription factors (DETFs) between alisertib treated and untreated trained BMDMs. **(D)** Multiplex immunoassay measuring 18 cytokines/chemokines in supernatant from trained BMDMs as described in Figure S2A, which were rechallenged with LPS (20 ng/mL) for 6 h. Related to **Figure 2**.

Figure 3. Aurora kinase A inhibition decreases glycolysis and SAM level. (A and B) Extracellular acidification rate (ECAR) after a glycolysis stress test upon

sequential addition of glucose (Gluc, 10 mM), oligomycin (Oligo, 1μM), and 2-deoxyglucose (2-DG, 50 mM), as indicated in BMDMs with different treatment for 24 h **(A)**; basal glycolysis and maximal glycolysis **(B)**. **(C, F and G)** LC–MS/MS measurements of fumarate **(C)**, serine and SAM **(F)**, SAH and HCY **(G)** in trained BMDMs treated with vehicle or alisertib for 24 h. **(D)** BMDMs were trained with β-glucan (50 μg/mL) or combined with alisertib (1μM) for 24 h. The BMDMs were collected for RNA extraction and followed by RNA-seq. The TOP 10 enriched pathways identified by KEGG enrichment analysis of differentially expressed genes (Foldchange >1.2, FDR<0.05) by comparing alisertib inhibited with un-inhibited trained BMDMs. **(E)** Intracellular level of glutathione in trained BMDMs with vehicles or alisertib for 24 h. The level was normalized to untrained BMDMs. **(H)** Western blot analysis of GNMT in trained BMDMs treated with vehicles or alisertib for 24 h. β-actin was used as a loading control; * showed the position of GNMT blot. **(I)** Western blot to detect GNMT protein in wild type BMDMs that were transfected with small interferon RNA targeting GNMT. **(J)** LC–MS/MS measurements of SAM and SAH in alisertib-inhibited trained BMDMs with knockdown of GNMT. The SAM/SAH ratio is calculated by SAH normalization. *P* values were derived from unpaired one-tailed way t-test. **(K)** Supernatant level of IL-6 and TNF-α in trained BMDMs with AurA inhibition by alisertib or by siRNA targeting Aura, together with without GNMT deficiency. Data are representative of three independent experiments and presented as the mean ± SEM. *P* values were derived from one-way ANOVA test with a Turkey's multiple-comparison test (B) or with a Dunnett's multiple-comparison

test (C, E-G, K). Related to **Figure 3—figure supplement 1**, **Figure 3—source data 1-2.**

Figure 3—figure supplement 1. Alisertib inhibits glucose incorporation into glycolysis and TCA cycle. (A) Mass labelling of trained BMDMs in the absence or presence of alisertib for 24 h and administered U-^{13}C-glucose simultaneously. Training with β-glucan increased incorporation of ^{13}C-glucose into glycolysis and TCA cycle intermediates; this was reversed by alisertib. (**B)** peak area of tyrosine and comparing of the sum of peak areas for unlabeled and labeled tyrosine between different treatment groups. **(C)** Diagram illustrating the cross link between glycolysis, TCA cycle, glutathione and SAM. Data are representative of three independent experiments and presented as the mean ± SEM. *P* values were derived from one-way ANOVA (A) or two-way ANOVA (B) test with a Dunnett's multiple-comparison test. Related to **Figure 3**.

Figure 4. Inhibition of Aurora kinase A impairs histone trimethylation at H3K4 and H3K36. (A) Western blot analysis of histone methylation modifications in trained BMDM treated with vehicles or alisertib. Histone 3 (H3) was used as a loading control. BMDMs were trained with β-glucan (50 μg/mL) or combined with alisertib (1μM) for 24 h, then BMDMs were washed and cultured in fresh medium for 3 days, followed by protein extraction. **(B)** ChIP-qPCR analysis of H3K4me3 and H3K36me3 enrichment in IL-6 and TNF-α in trained BMDMs treated with vehicles

or alisertib for 24 h and rest for 3 days. **(C)** Western blot analysis of total H3K4me3 and H3k36me3 upon GNMT deficiency in BMDMs. The BMDMs were transfected with siRNA targeting GNMT for 48 h, followed by β-glucan (50 μg/mL) or combination with alisertib (1 μM) treatment for 24 h. Then the BMDMs were washed and cultured in fresh medium for 3 days and the protein was extracted for western blot analysis of H3K4me3 and H3K36me3. Data are representative of three independent experiments and presented as the mean ± SEM. *P* values were derived from one-way ANOVA test with a Turkey's multiple-comparison test. Related to **Figure 4—figure supplement 1**, **Figure 4—source data 1-2.**

Figure 4—figure supplement 1. Inhibition of Aurora kinase A impairs histone trimethylation at H3K4 and H3K36. (A) quantification of H3K4m1, H3K9me3, H3K36me3, H3K4me3 and H3K27me3 protein level was determined by Image Lab software; data are represented as mean values ± SEM, *P* values were derived from one-way ANOVA with Turkey's multiple-comparison test of n = 3 independent biological experiments. Related to **Figure 4**.

Figure 5. Aurora kinase A regulates GNMT through transcription factor FOXO3. (A) Protein level of GNMT under FOXO3 deficiency in BMDMs without treatment was detected by western blot; * showed the position of FOXO3 blot. **(B and C)** Western blot analysis of GNMT downregulation by si*Foxo3* in trained β-glucan with AurA inhibition. BMDMs were transfected with smalling interferon RNA targeting

FOXO3 for 48 h, followed by β-glucan training and alisertib for 24 h (B); BMDMs were transfected with smalling interferon RNA targeting FOXO3 and AurA for 48 h, followed by β-glucan training for another 24 h (C). **(D)** Supernatant levels of IL-6 and TNF-α in BMDMs. The cells were treated with β-glucan training and aurora A inhibition in combination with FOXO3 deficiency by siRNA. **(E)** Western blot analysis of phosphorylation level of FOXO3 at ser 315 in BMDMs treated with β-glucan (50 μg/mL) or combined with alisertib (1 μM) for 12 h**. (F)** Immunofluorescence staining of FOXO3 in trained BMDMs for 12 h with or without alisertib. Scale bars: 10μM (left). The nuclear localization of FOXO3 was compared by calculated the ratio of mean nuclear intensity to cytoplasmic intensity and the representative data (right) showed the mean intensity of counted macrophages. **(G)** Western blot analysis of activation of AKT-mTOR-S6 pathway in β-glucan trained BMDM. BMDMs were transfected with smalling interferon RNA targeting AurA for 48 h, followed by β-glucan training for 6 h (left); BMDM was trained with β-glucan in the absence or presence of alisertib for 6 h (right). **(H)** Supernatant levels of IL-6 and TNF-α in BMDMs. The cells were treated with β-glucan training and aurora A inhibition in combination with mTOR agonist, MHY1485 (2 μM), and restimulated with MC38 culture supernatant for 24 h. Data are representative of three independent experiments and presented as the mean ± SEM. *P* values were derived from one-way ANOVA test with a Turkey's multiple-comparison test (D, H) or with Dunnett's multiple-comparison test (F). Related to **Figure 5—source data 1-3.**

Figure 6. Alisertib abrogates the anti-tumor effect induced by trained immunity. **(A)** Experimental scheme of mouse experiment. 6~8 weeks old mice was injected with β-glucan and administrated with alisertib, followed by 1×10^6 MC38 cells inoculation (n=5 per group). **(B)** Tumor growth curve of MC38 in mice as shown in A. **(C)** Flow cytometric analysis of myeloid cells ($CD45^+CD11b^+$) and macrophages ($CD45^+CD11b^+F4/80^+$) cells in MC38 subcutaneous tumors in A. Gating strategy was shown in Fig S5C. **(D)** Co immunofluorescence staining of DAPI, F4/80 and GNMT tumor section; Scale bars: 20 μM. **(E)** FACs for intracellular phospho-S6 in gated macrophage. **(F)** Tumor tissue was lysed and the supernatant were collected for detection of cytokines production in tumor microenvironment. Data are represented as the mean ± SEM. *P* values were derived from one-way ANOVA test with Dunnett's multiple-comparison test. Related to **Figure 6—figure supplement 1, Figure 6—source data 1.**

Figure 6—figure supplement 1. Alisertib abrogates the anti-tumor effect induced by trained immunity. (A) Tumor growth curve for individual mice (n=5 per group). **(B)** Tumor images and tumor weight; data are represented as mean values ± SEM, P values were derived from one-way ANOVA with Dunnett's multiple-comparison test. **(C)** The gating strategy used for analyzing tumor-infiltrating macrophages. Related to **Figure 6**.

Source data files

Figure 1–source data 1 Uncropped and labeled blots for Figure 1D and Figure 1E.

Figure 1–source data 2 Raw unedited blots for Figure 1D and Figure 1E.

Figure 3—source data 1 Uncropped and labeled blots for Figure 3H and Figure 3I.

Figure 3—source data 2 Raw unedited blots for Figure 3H and Figure 3I.

Figure 4—source data 1 Uncropped and labeled blots for Figure 4A and Figure 4C.

Figure 4—source data 2 Raw unedited blots for Figure 4A and Figure 4C.

Figure 5—source data 1 Uncropped and labeled blots for Figure 5A-C, E and G.

Figure 5—source data 2 Raw unedited blots for Figure 5A-C, E and G.

Figure 5—source data 3 original images for Figure 5F.

Figure 6—source data 1 intensity analysis for Figure 6D.

Figure 1. Inhibition of Aurora kinase A suppresses trained immunity in macrophages

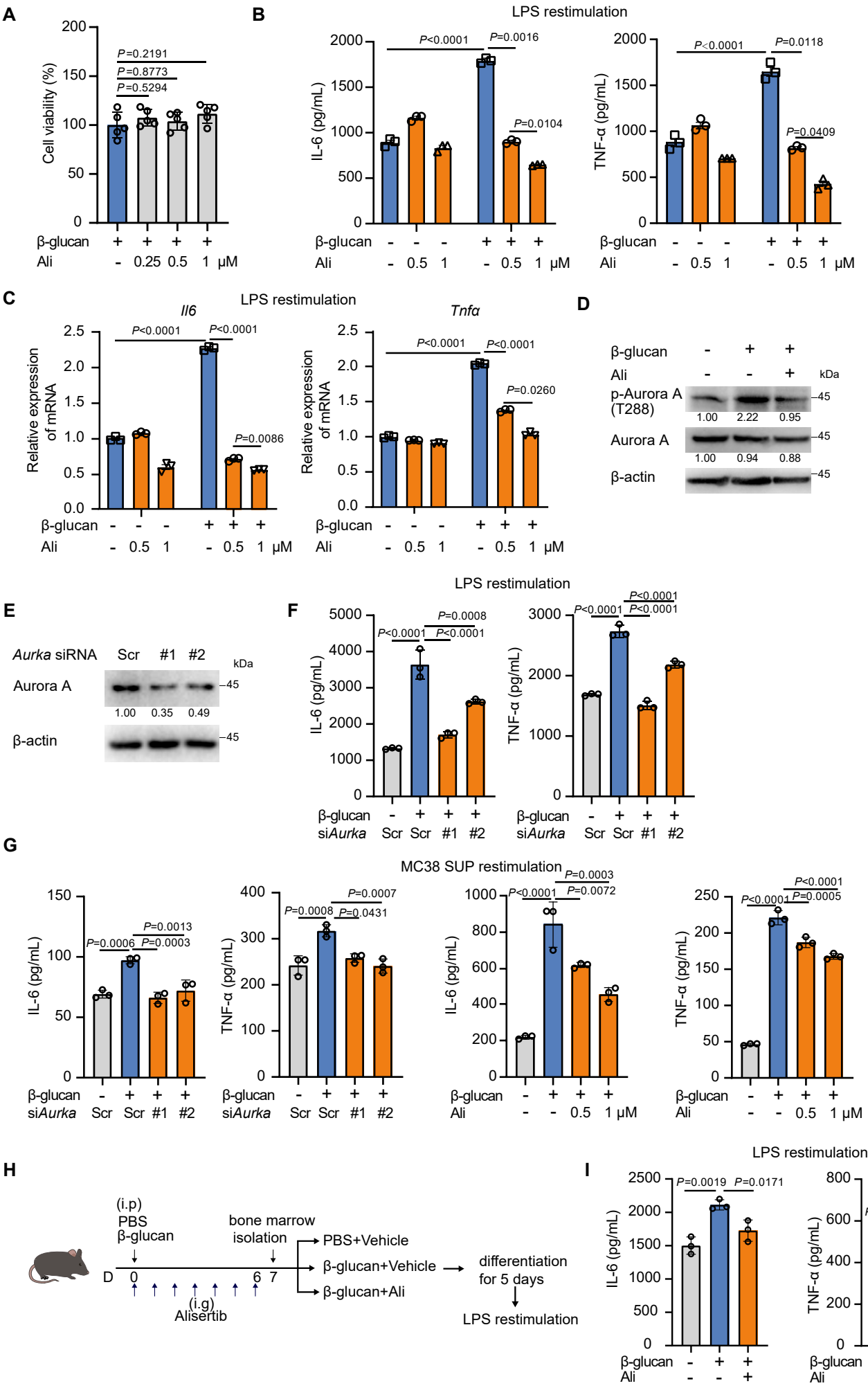

Figure 1—figure supplement 1. Targeting Aurora kinase A inhibits β-glucan-induced trained immunity

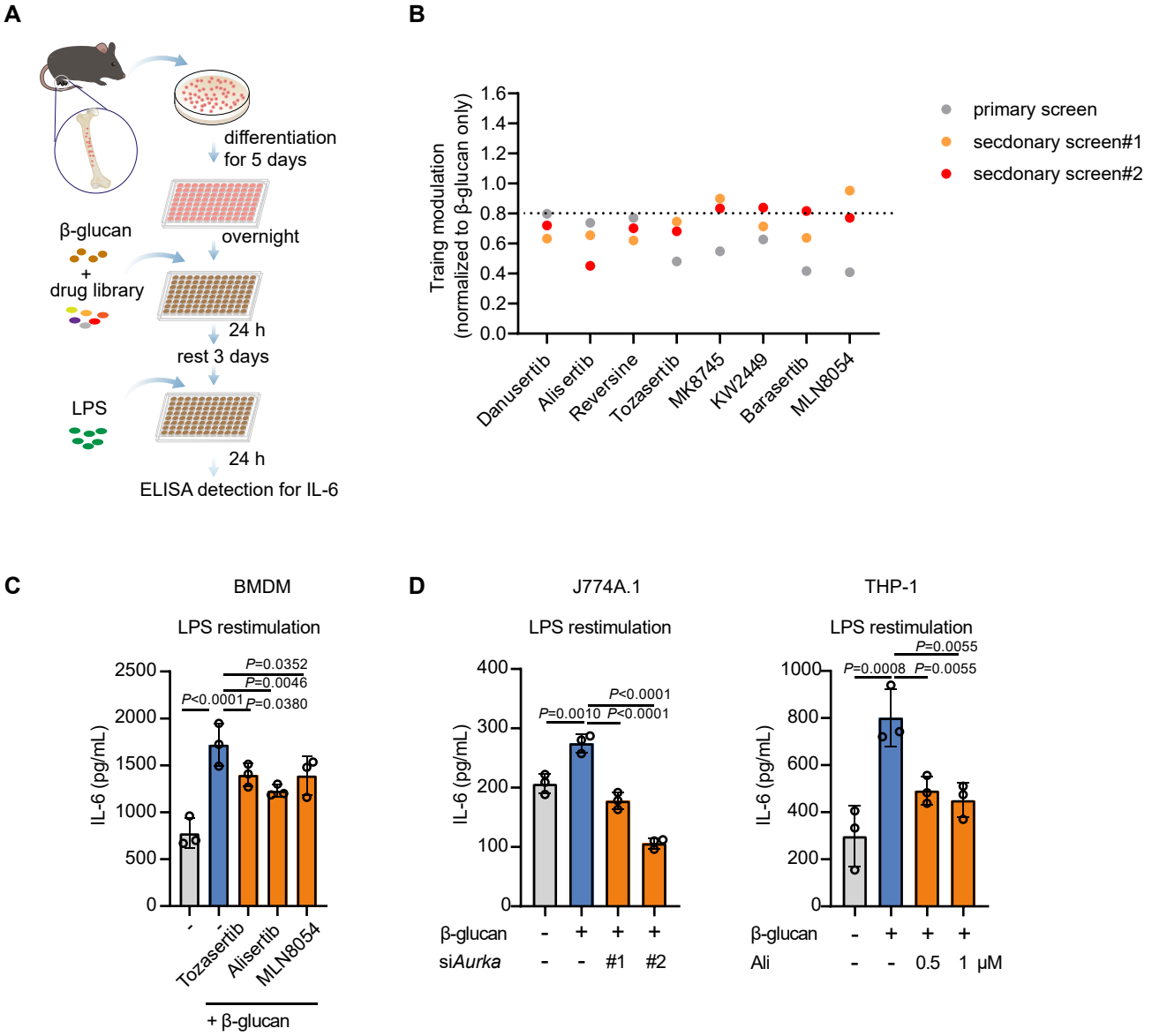

Figure 2. Aurora kinase A inhibition remodels chromatin landscape of inflammatory genes

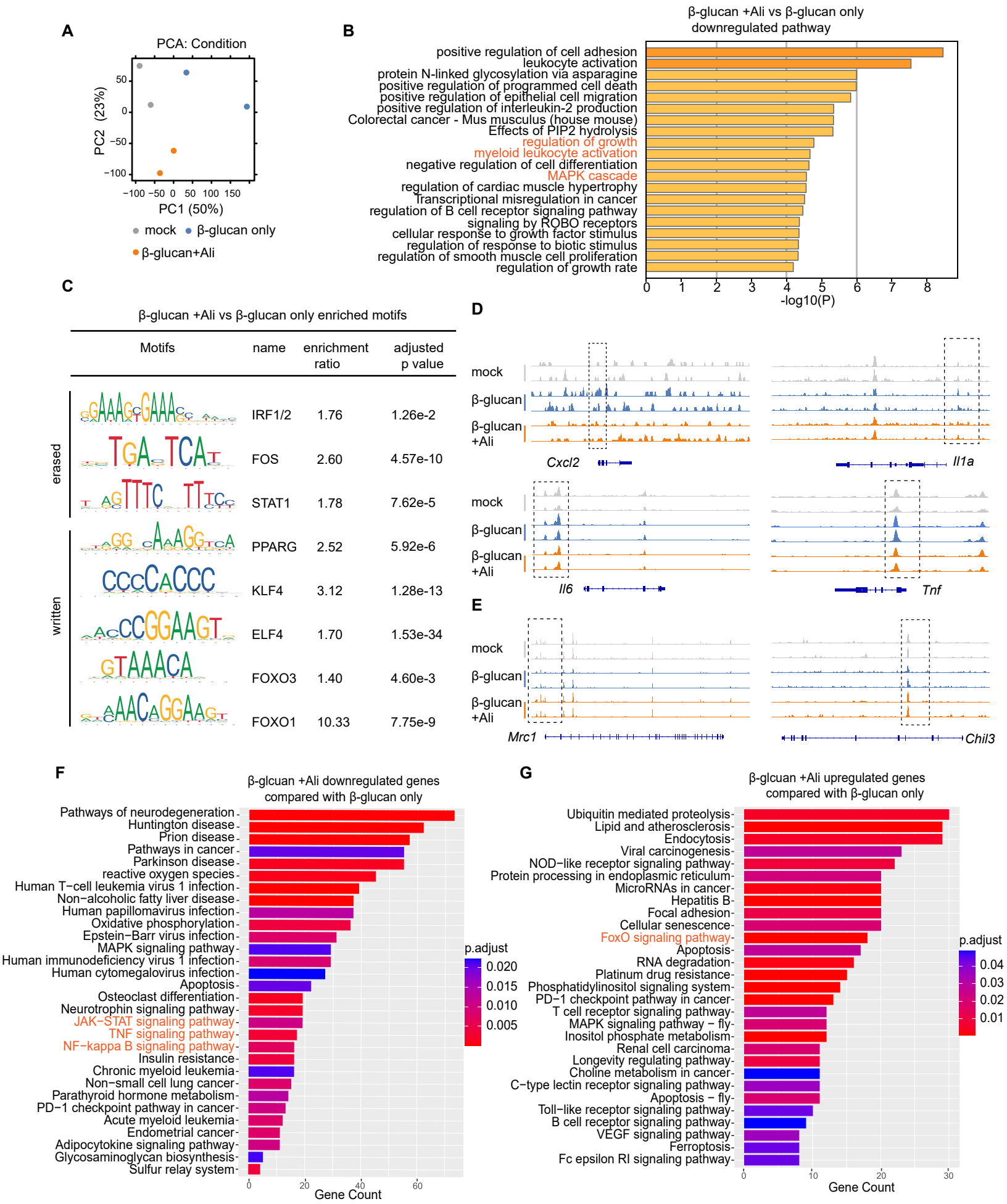

Figure 2—figure supplement 1. Aurora kinase A inhibition suppresses the expression of trancription factors involved in inflammation activation

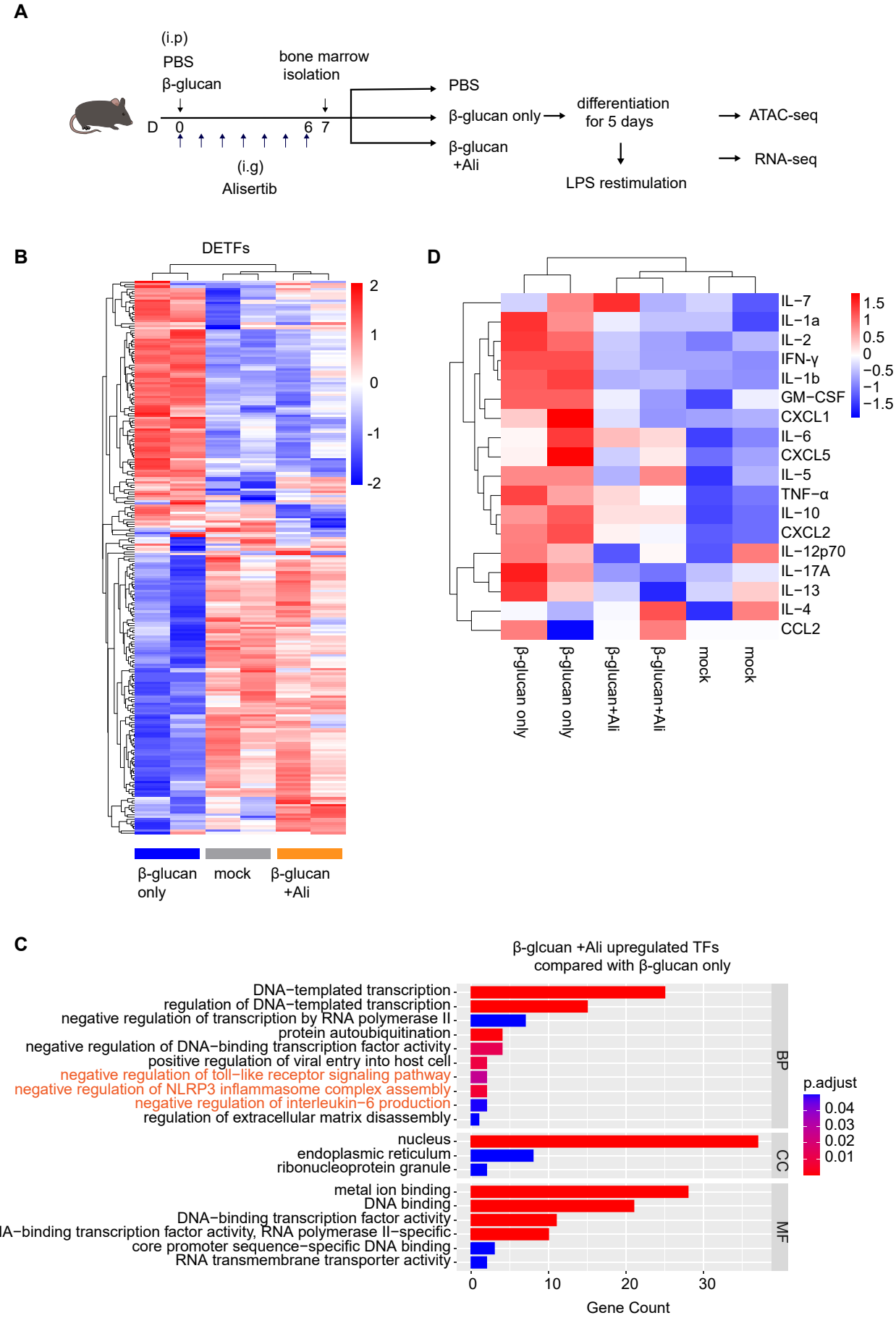

Figure 3. Aurora kinase A inhibition decreases glycolysis and SAM level

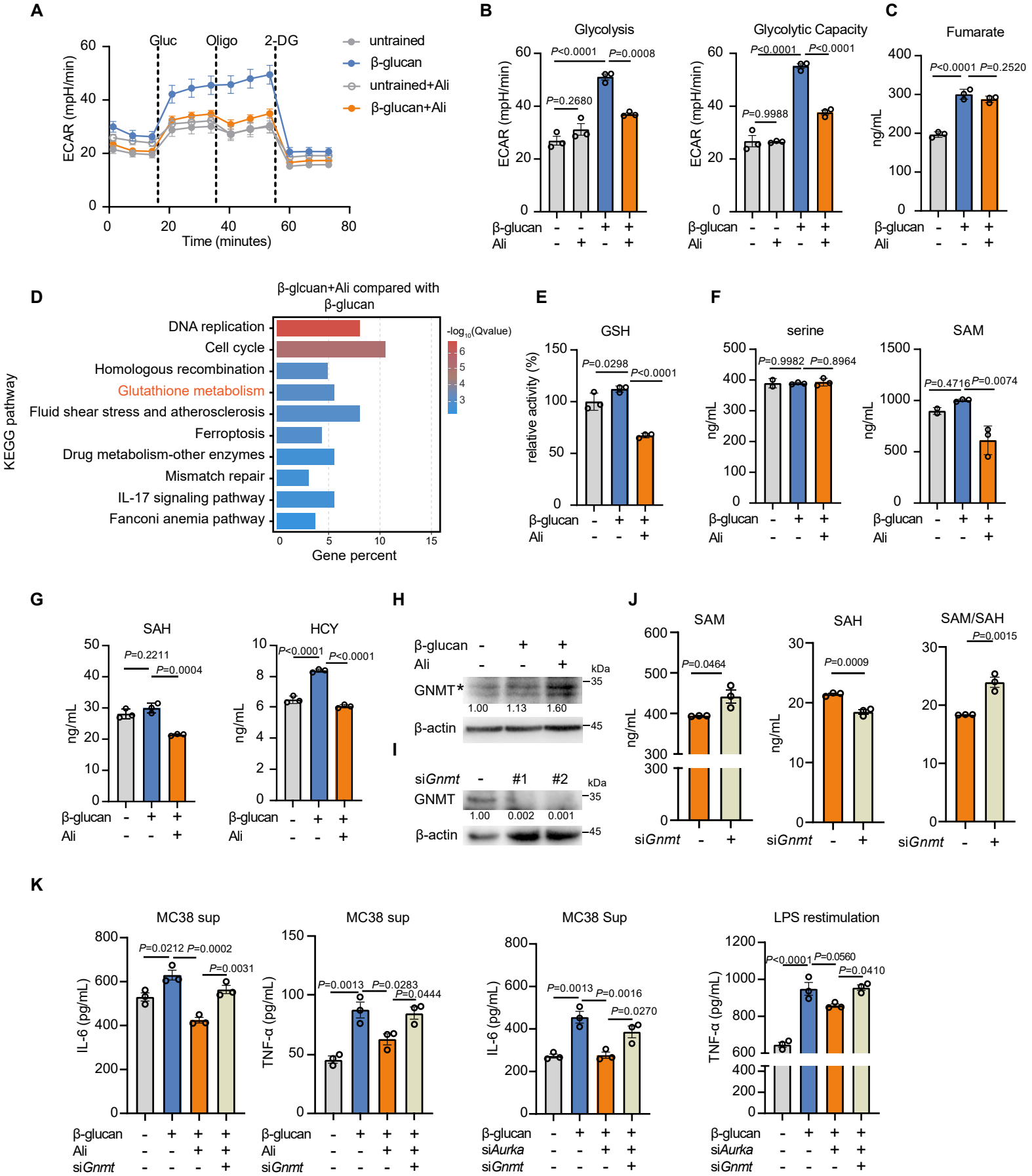

Figure 3—figure supplement 1. Alisertib inhibits glucose incorporation into glycolysis and TCA cycle

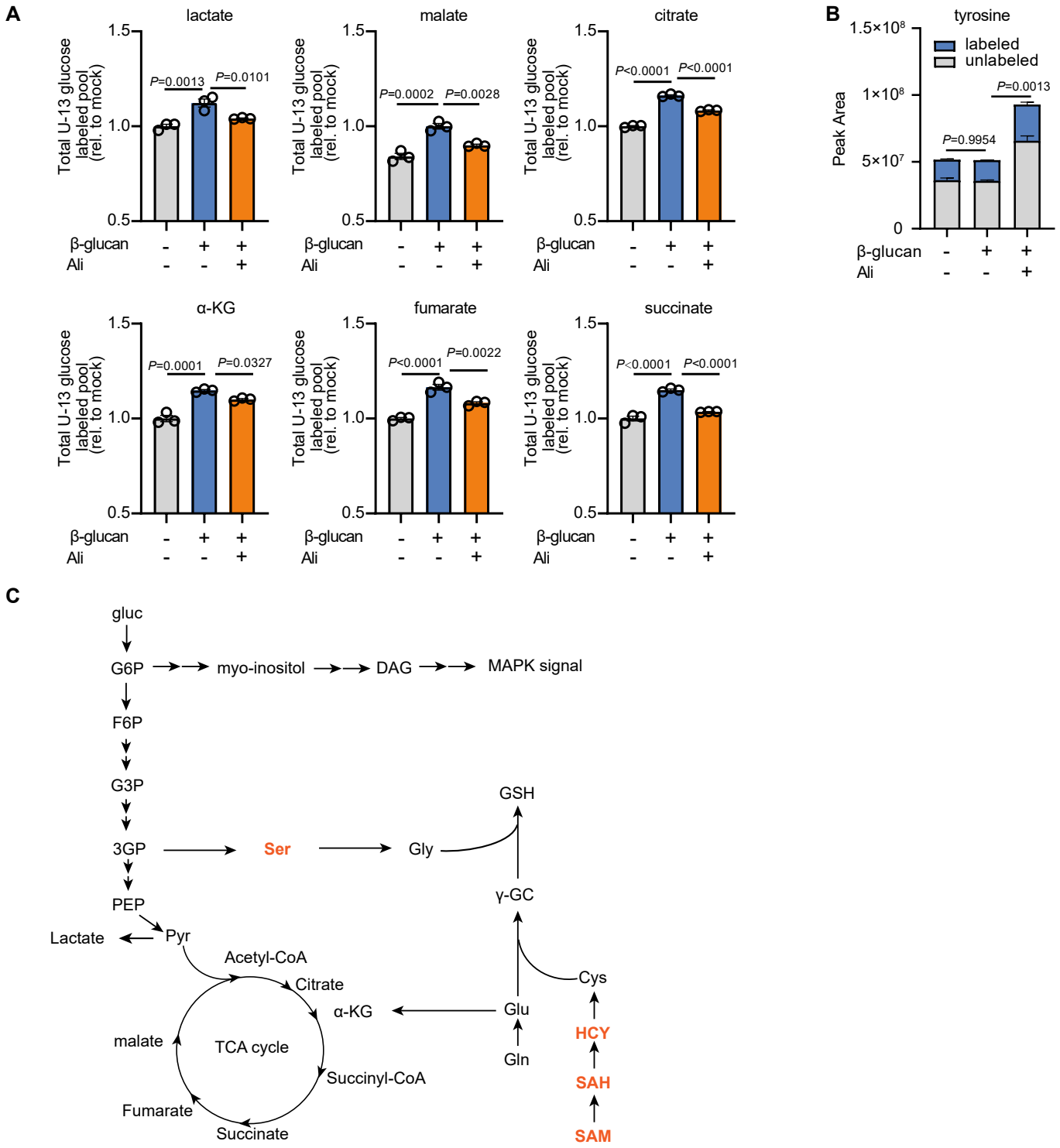

Figure 4. Inhibition of Aurora kinase A impairs histone trimethylation at H3K4 and H3K36

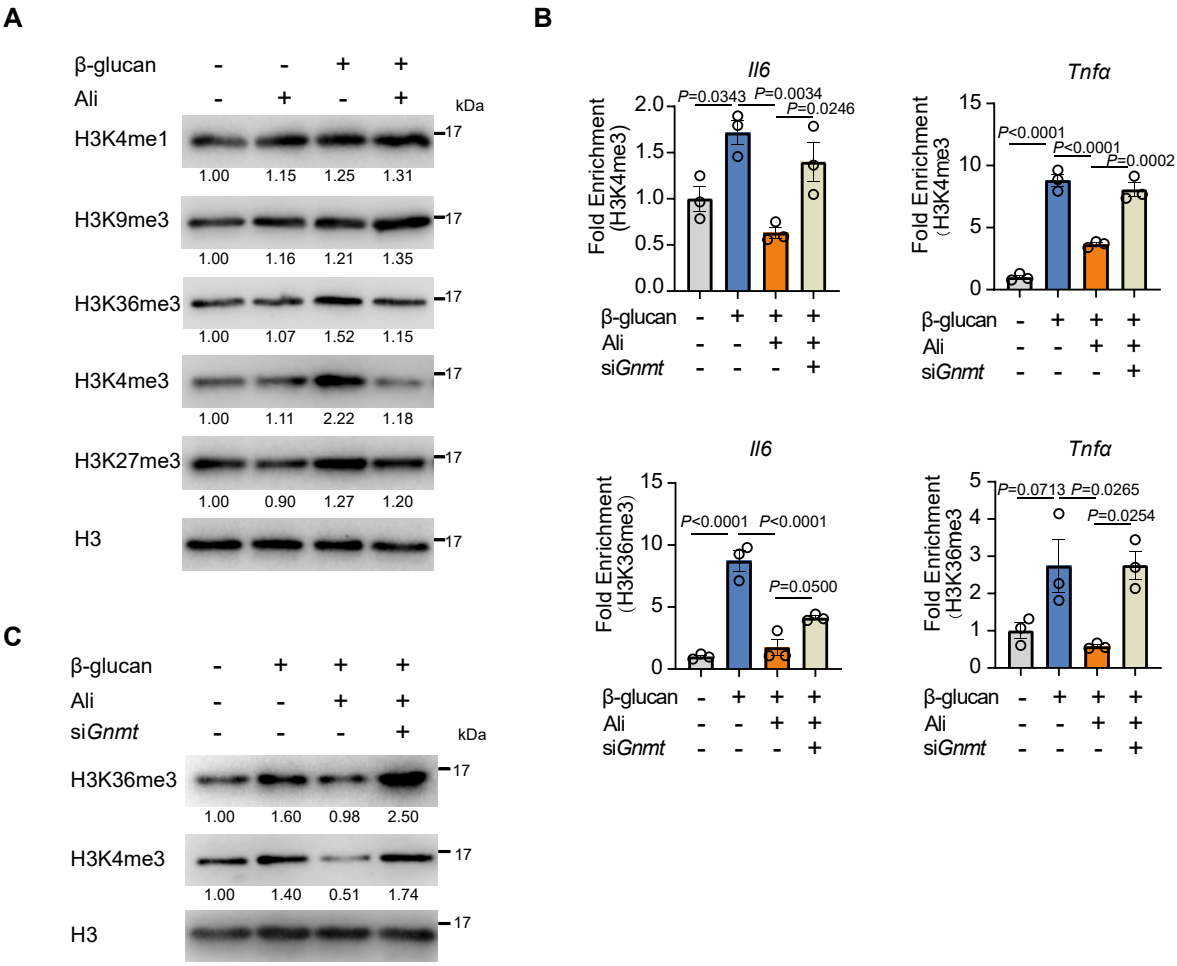

Figure 4—figure supplement 1. Inhibition of Aurora kinase A impairs histone trimethylation at H3K4 and H3K36

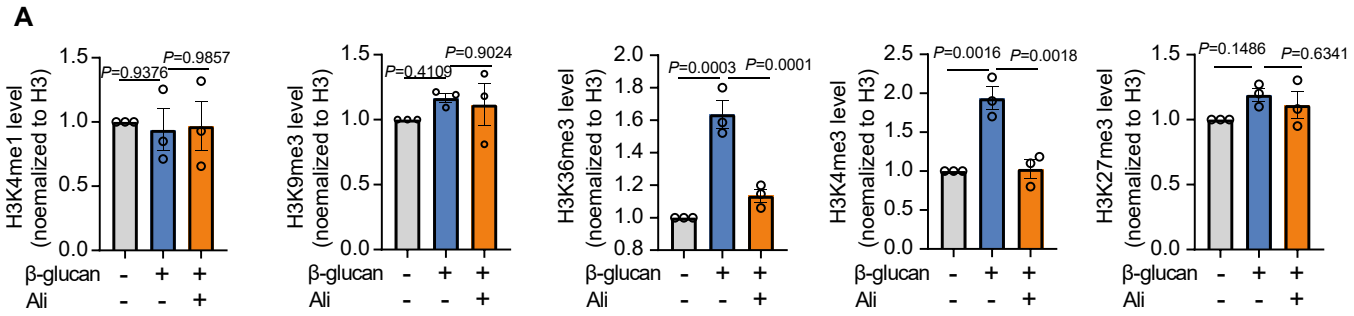

Figure 5. Aurora kinase A regulates GNMT though transcription factor FOXO3

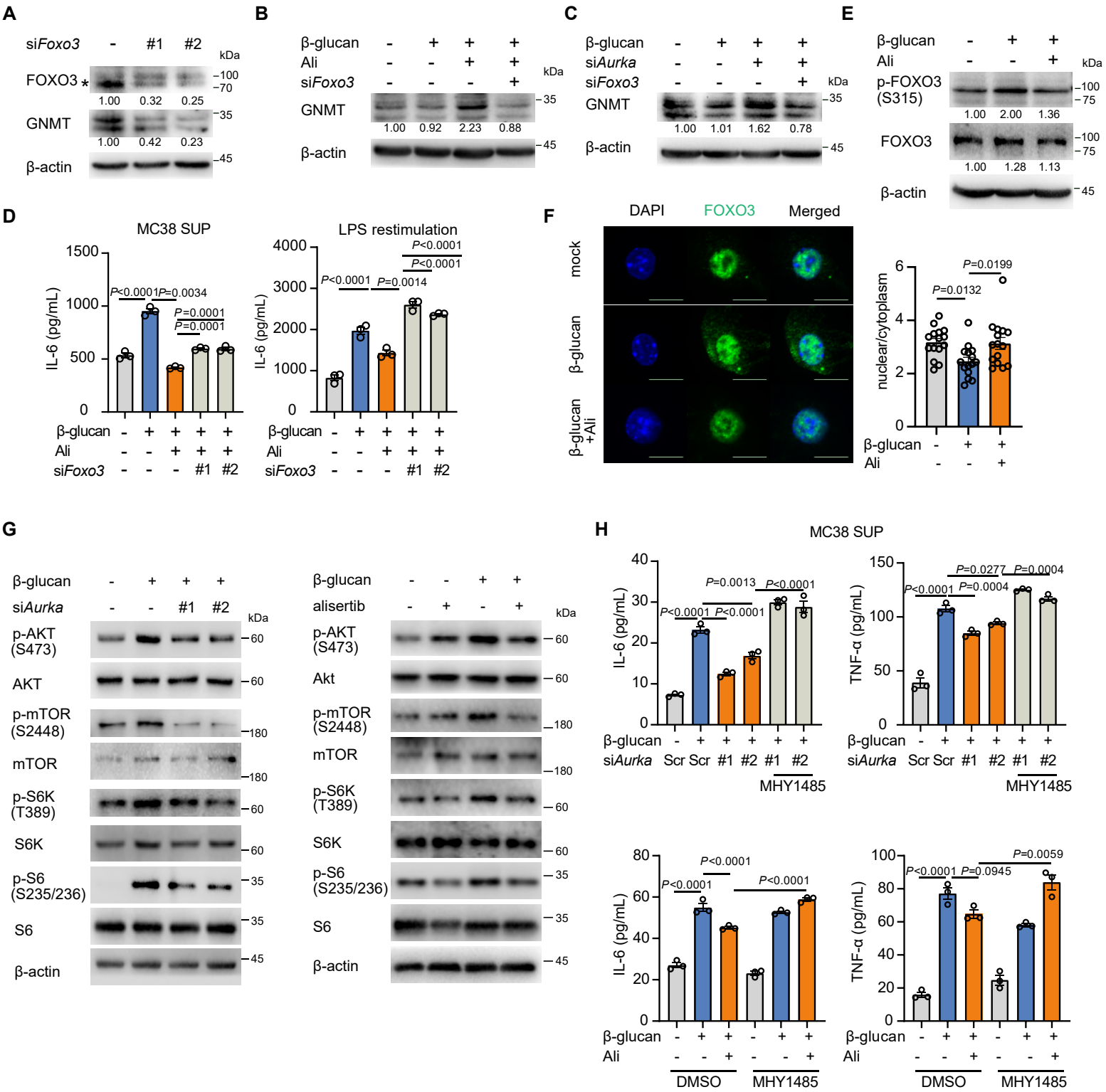

Figure 6. Alisertib abrogates the anti-tumor effect induced by trained immunity

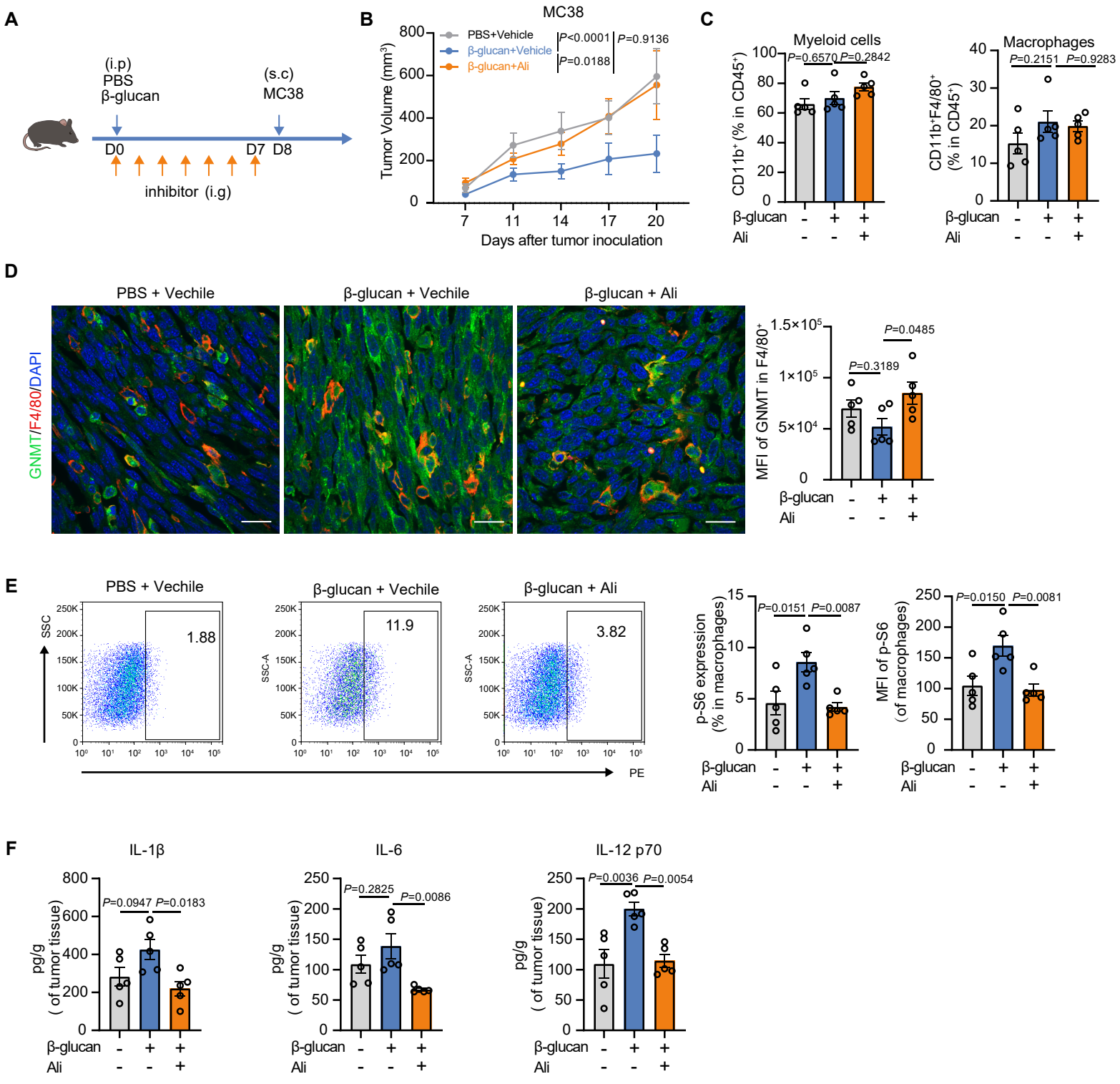
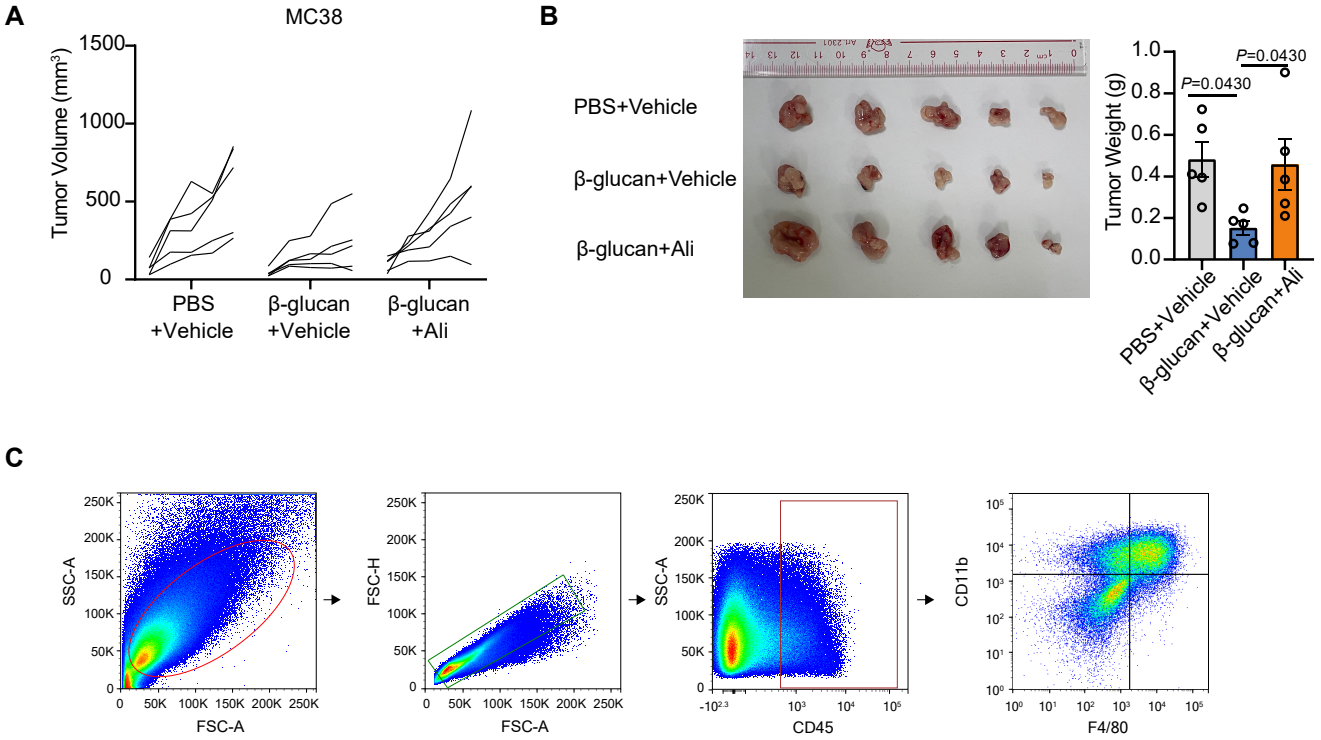
Figure 6—figure supplement 1. Alisertib abrogates the anti-tumor effect induced by trained immunity