A post-transcriptional program of chemoresistance by AU-rich elements/TTP in cancer quiescence

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Summary

Quiescent (G0) cells are transient cell-cycle-arrested subpopulations in cancers that include dormant cancer stem cells. While leukemic stem cells/minimal-residual-disease were studied transcriptionally, their translation profile and post-transcriptional mechanisms that control their proteome—and thereby, survival—are unknown. We find G0 leukemic cells are chemoresistant, with altered canonical translation and similar proteome and translatome—rather than transcriptome—to cells isolated post-chemotherapy, implicating post-transcriptional regulation in chemoresistance. Mechanistically, we find DNA-damage-responsive-ATM and stress-activated-p38-MAPK/MK2 alter post-transcriptional mechanisms, regulating mRNA-decay-factor, TTP, to increase AU-rich-element-bearing mRNAs. This permits translation of AU-rich-element-bearing pro-inflammatory-cytokine TNFα, and immune modulators that promote survival. Co-inhibiting p38-MAPK/MK2 and TNFα—prior to/alongwith chemotherapy—decreases chemoresistance in vivo. Disrupting TTP regulation reduces TNFα and chemoresistance, revealing TTP as a regulator of inflammation-mediated chemoresistance.

Significance

Leukemic stem cells/minimal-residual-disease that are chemoresistant have been studied transcriptionally; however, their translation profile and post-transcriptional mechanisms that control their proteome and survival are unknown. Our data suggest that G0 cancer cells are chemoresistant. We find alternate post-transcriptional mechanisms in G0 cancer cells, which may regulate a distinct translatome to mediate their resistance. Our data uncover a pro-inflammatory subpopulation in cancer that enables chemoresistance via DNA-damage- and stress-regulated post-transcriptional mechanisms. These studies reveal the significance of post-transcriptional regulation of inflammatory/immune response in chemoresistance, mediated by AU-rich-elements, mRNA-decay-regulator TTP, and non-canonical translation. The results reveal DNA-damage/stress-induced TTP as a key regulator of inflammation-mediated chemoresistance and developed a new combination therapy to reduce resistance in cell lines, patient samples and *in vivo*.

Introduction

Quiescent (G0) cells are an assortment of reversibly arrested cells, including dormant stem cells, which are found as a clinically relevant subpopulation in cancers (Coller et al., 2006; Ng et al., 2016; Meacham and Morrison, 2013; Crews and Jamieson, 2013). Such cells are anti-proliferative, anti-differentiation, and anti-apoptotic, and show distinct properties including resistance to harsh conditions (Bhola et al., 2016; Tavaluc et al., 2007; Coller et al., 2006; Gupta et al., 2009; Salony et al., 2016; Li and Bhatia, 2011; Giles et al., 2008; Ng et al., 2016). G0 cells show specific gene expression that may underlie their resistance and other properties (Coller et al., 2006; Salony et al., 2016; Li and Bhatia, 2011; Giles et al., 2008; Ng et al., 2016). Analyses from multiple groups revealed some genes up-regulated at the transcriptional level (Coller et al., 2006; Salony et al., 2016; Liu et al., 2007). Altered polyadenylation site selection on mRNAs produces longer 3′-untranslated regions (3′UTRs) in G0 compared to proliferating cells— which increases 3′UTR elements that can mediate post-transcriptional gene expression regulation (Sandberg et al., 2008). Our previous data demonstrated that translation mechanisms are distinct in G0 leukemic cells, with decreased canonical translation mechanisms and increase in mRNA translation by alternative mechanisms that involve non-canonical translation initiation factors (Lee et al., 2014) and 3′UTR mediated specific mRNA translation (Bukhari et al., 2016). These data suggest that alternate post-transcriptional mechanisms in G0 cancer cells may regulate a distinct translatome to mediate their resistance. Translated genes, post-transcriptional mechanisms involved, and outcomes on cancer persistence remain to be investigated.

We analyzed the translatome and proteome of chemotherapy-surviving G0 cancer cells, focusing on acute monocytic leukemia (AML), to provide comprehensive information that complement and expand

previous transcriptome analyses (Salony et al., 2016; Liu et al., 2007; Ng et al., 2016; Coller et al., 2006; Ebinger et al., 2016; Saito et al., 2010), (Fig. 1A-B), to reveal critical genes that are post-transcriptionally regulated for chemo-survival. G0 can be induced by growth factor-deprivation or serum-starvation and other conditions that isolate dormant cancer stem cells in distinct cell types (Tavaluc et al., 2007; Coller et al., 2006; Gupta et al., 2009). Our data demonstrate that serum-starvation induced G0 AML cells are chemoresistant—similar to surviving AML cells, isolated after chemotherapy. Chemoresistant cells isolated via serum-starvation, or as surviving cells post-chemotherapy, show inhibition of canonical translation mechanisms, indicating that non-canonical mechanisms express specific mRNAs when these cells are chemoresistant. Consistently, the translatomes and proteomes of serum-starved G0 and chemo-surviving cells show greater similarity than the transcriptome alone. These data indicate a specific post-transcriptional program common between serum-starvation induced G0 cells that exhibit resistance, and chemosurviving cells—which may underlie their shared property of chemoresistance.

We find that chemotherapy and serum-starvation induce DNA damage response (DDR) and p38 MAPK stress signaling that lead to up-regulation of specific genes via post-transcriptional mechanisms (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009) (Holohan et al., 2013; Cuenda and Rousseau, 2007; Lalaoui et al., 2016; Claudio et al., 2013; Senft and Ronai, 2015). Our data reveal AU-rich elements (AREs) enriched in mRNAs that are up-regulated in G0 and in chemo-surviving cells—due to p38 MAPK activation (Damgaard and Lykke-Andersen, 2013; Cannell et al., 2015; Lalaoui et al., 2016) of MAPKAP2 (MK2) kinase (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016). MK2 regulates ARE binding mRNA decay and translation repression factor, Zinc finger 36 homolog

(ZFP36) or Tristetraprolin (TTP) (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016; Clement et al., 2011). We find that p38 MAPK/MK2 mediated regulation of TTP leads to increased ARE bearing mRNA levels and translation in chemoresistant G0 cells. DNA damage-induced ATM kinase and stress signaling—inhibit canonical translation mechanisms in chemoresistant cells, which permits specific gene translation by non-canonical mechanisms that we observed previously (Lee et al., 2014; Bukhari et al., 2016). The post-transcriptionally regulated genes expressed in G0 resistant cells, include pro-inflammatory genes of the TNFα/NFκB pathway—which we find increase cell survival genes and are required for chemo-survival. Consistently, decreasing expression of TNFα, by expressing a non-regulatable, active mutant TTP (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016), reduces chemoresistance. Importantly, correlating with their early induction in serum-starved G0 or chemotherapy-treated cells, pharmacological inhibition of stress signaling and the key translated inflammatory response gene TNFα—prior to or along with chemotherapy—significantly reduces chemoresistance in AML cell lines, patient samples, and *in vivo*. Our data reveal that DNA damage and stress signaling cause post-transcriptional alterations to produce a specialized gene expression program of pro-inflammatory, immune effectors that elicit chemo-survival.

Results

Serum-starvation or AraC treatment induces a quiescent and chemoresistant state of leukemic cells

To study clinical resistance in cancer, THP1 human AML cells were used as they show significant resistance to cytarabine (Forbes et al., 2015) (cytosine arabinoside, AraC, Fig. S1A), a standard anti-

leukemic chemotherapeutic that targets DNA replication and thus proliferating cells (referred to as S+). Our data and others find that serum-starvation of THP1 (Lee et al., 2014) and other cell lines (Salony et al., 2016; Liu et al., 2007; Loayza-Puch et al., 2013; Coller et al., 2006) induces a transient G0 state with known G0 and cell cycle arrest markers expressed (Fig. 1C-D, S1B-C). Such serum-starvation induced G0 cells (referred to as SS) can be returned to the cell cycle upon serum addition (Fig. 1D), verifying that they are quiescent and transiently arrested, unlike senescence or differentiation that are not easily reversed. We find that serum-starvation induced G0 SS cells show resistance to AraC chemotherapy. Serum-grown S+ cells show a dose-dependent decrease in cell viability with AraC as expected, while SS cells persist, indicating their chemoresistance (Fig. 1E).

Chemoresistant cancer cells include cancer stem cells, and are a subpopulation that can be isolated from cancers after treatment with chemotherapy (Tavaluc et al., 2007; Gupta et al., 2009; Salony et al., 2016; Li and Bhatia, 2011; Giles et al., 2008; Ng et al., 2016) that targets and eliminates S+ cells. We find that AraC-surviving THP1 (referred to as AraCS) cells are transiently arrested, like serum-starved SS cells (Fig. 1C-D, S1B); both AraCS and SS cells survive chemotherapy. AraCS cells recover from their transient arrest upon AraC removal, proliferate (Fig. 1D) and give rise to resistance upon subsequent AraC treatment (Fig. 1E), affirming the reversible G0 arrest state of chemoresistant cells, similar to SS cells (Coller et al., 2006; Tavaluc et al., 2007; Gupta et al., 2009; Salony et al., 2016; Li and Bhatia, 2011; Giles et al., 2008; Ng et al., 2016).

Chemoresistant G0 cells induced by SS or AraC have similar translatomes and proteomes that recapitulate gene expression profiles of *in vivo* chemoresistant leukemic and G0 models

We asked whether the underlying gene expression is similarly regulated in both SS and AraC cells, leading to their common property of chemoresistance. We previously observed canonical translation shutdown that allowed non-canonical translation of specific genes in SS cells (Lee et al., 2014; Bukhari et al., 2016), indicating post-transcriptional changes in such cells. Therefore, to differentiate translationally-regulated from transcriptionally-regulated genes associated with chemoresistance, we profiled AraCS cells and SS cells—and compared them with S+ cells at the proteome, translatome and transcriptome levels using: multiplexed quantitative proteomics (Bukhari et al., 2016), microarray analysis of heavy polysome-associated mRNAs (Hsieh et al., 2012; Bukhari et al., 2016; Lee et al., 2014) for the translatome, and total RNA microarray for the transcriptome (Fig. 1A-B, S1D-F). Consistently, we find that AraCS and SS cells show significantly similar gene expression profiles in their proteomes and translatomes rather than transcriptomes (Fig. 1F). These data suggest that although these chemoresistant G0 cells are isolated via two different methods: therapy survival or serum-starvation, they exhibit a common set of specific genes expressed at the protein level, which could underlie their common characteristic of chemoresistance. These data indicate the relevance of examining the translatome and post-transcriptional regulation of gene expression, in addition to the transcriptome. Time-course translatome analysis revealed that early serum-starved G0 (4 h and 1 day SS) cells are distinct from late, 2-4 days G0 cells (2-4 days SS are more similar in their translatomes, R^2=0.81, Fig. S1E-F). This is consistent with G0 as a continuum of assorted, arrested states (Coller et al., 2006), with temporal differences in underlying gene expression in early G0 compared to more homogeneity at late G0.

SS and AraCS cells provide sufficient material to perform concurrent translatome, proteome and transcriptome profiling for post-transcriptional regulation analyses, compared to limited cells from in *vivo* resistance models. To test whether our G0 leukemic cells are relevant models to study

chemoresistance and G0, gene expression profiles of AraCS and SS cells were compared to published transcriptome profiles of *in vivo* chemoresistance models, including leukemic stem cells, dormant leukemic cells and chemosurviving cells, as well as to a published G0 model that were isolated by different methods: leukemia stem cells (LSC) from AML (Saito et al., 2010), dormant leukemic cells (LRC), and minimal residual disease (MRD) from chemotherapy surviving patient samples with acute lymphocytic leukemia (ALL) (Ebinger et al., 2016), as well as SS G0 fibroblasts (G0 HFF) (Coller et al., 2006). Importantly, we find that these published transcriptome signatures for *in vivo* chemoresistance and G0 models were significantly up-regulated in our SS and AraCS cells (referred to as resistant G0 leukemic cells), compared to S+ cells (Fig. 1G, S1G). These data indicate that our resistant G0 leukemic cells are relevant models to study post-transcriptional regulation in chemoresistance as they have similar gene expression profiles to known transcriptional profiles from *in vivo* chemoresistance models.

Altered translation with inhibition of canonical translation mechanisms in resistant G0 leukemic cells

As previously observed in SS cells (Lee et al., 2014; Sendoel et al., 2017; Holcik and Sonenberg, 2005; Zeenko et al., 2008; Lorsch and Dever, 2010; Ron and Walter, 2007; Zismanov et al., 2016), we find overall protein synthesis is reduced at least 2-fold in AraCS, compared to S+ cells (Fig. 2B, S1D). Mechanistically, both rate-limiting steps in canonical translation initiation: recruitment of initiator tRNA, and mRNA cap recognition to recruit mRNAs to ribosomes are inhibited in G0 leukemic cells (Fig. 2A-C). Recruitment of initiator tRNA by eIF2 can be blocked by eIF2α phosphorylation as a stress response (Lee et al., 2014; Sendoel et al., 2017; Holcik and Sonenberg, 2005; Zeenko et al., 2008; Lorsch and Dever, 2010; Ron and Walter, 2007; Zismanov et al., 2016). We find that two eIF2 kinases,

PKR and PERK, are activated and increase eIF2α phosphorylation (Fig. 2A, 2C) to inhibit canonical translation initiation in G0 leukemic cells. Our previous data revealed low mTOR activity (Bukhari et al., 2016), leading to dephosphorylated and active eIF4EBP (4EBP) (Fig. 2A, 2C) that inhibits cap-dependent translation initiation (Thoreen et al., 2012; Culjkovic et al., 2007). Low mTOR activity reduces translation of terminal oligopyrimidine tract (TOP) mRNAs such as ribosomal protein mRNAs (Han et al., 2014; Thoreen et al., 2012; Damgaard and Lykke-Andersen, 2011), which we find decreased in SS and AraCS cells (Fig. 2A, 2D). Decreased canonical translation can enable post-transcriptional regulation of specific genes, as observed previously (Lee et al., 2014; Bukhari et al., 2016) and lead to survival of G0 leukemic cells.

Global translatome analysis shows that inflammatory response genes are selectively translated in resistant G0 cancer cells

We measured the number of genes upregulated at the transcriptome, translatome and proteome levels in resistant G0 leukemic cells, compared to S+ cells. A significantly greater number of genes were upregulated in the translatome (580 genes, Table S1) and proteome (716 genes), compared to the transcriptome (318 genes) as shown in Fig. 2E. Importantly, 70% of upregulated genes (418 out of 580) were increased in the translatome (Fig. 2F) but not in the transcriptome, indicating a common set of specific genes that are post-transcriptionally/translationally expressed. To investigate the biological function of these differentially expressed genes, gene ontology (GO) analysis was performed with each gene set. Gene categories up-regulated in G0 translatomes include inflammatory response, immune modulators (receptors, antigen presentation and processing genes), cell adhesion, cell migration, lipid biosynthesis and cholesterol pathway genes (Fig. 2G, S2E). Down-regulated genes include RNA

processing and ribosome genes, consistent with the limited, specialized gene expression here, as well as decreased DNA repair genes that would permit DNA damage and stress signaling (Fig. 2G). To identify translationally up-regulated genes, we measured the change in ribosome occupancy, RO, which is the ratio of polysome-associated mRNA levels to total mRNA levels of each gene (Fig. 2F, Venn diagram, heat map). We find 180 genes are translationally up-regulated above RNA level changes in G0 ($\Delta RO = RO_{G0} - RO_{proliferating} > 1.5$-fold). These genes include antigen processing and presentation genes (Shukla et al., 2015) (HLA-G, HLA-E) and immune receptors (CD47, Fig. 2F-G, S2I) (Sosale et al., 2015; Soto-Pantoja et al., 2015; Zhang et al., 2015) that regulate anti-tumor immune response and are associated with leukemic stem cells and resistance (de Kruijf et al., 2010; Majeti et al., 2009).

We asked if this specific gene expression profile in resistant G0 leukemic cells is conserved in G0 cells of other tumors and in normal cells. Therefore, global translatome profiling was conducted in G0 cells from four different cells lines: breast cancer (MCF7, Fig. S2B-D), liver cancer (HEP-G2), and osteosarcoma (U2OS) as well as non-cancerous fibroblasts (HFF) (Fig. S2A, D). Their translatomes were compared with resistant G0 leukemic cells, using GSEA and DAVID tools (Fig. 2H-I, S2E-F). We find that 580 signature genes of resistant G0 leukemic cells (Table S1) were highly conserved at the translatome level in G0 cells of these other cell types (Fig. 2H). As expected for these arrested cells, genes related to cell cycle, ribosome biogenesis, and DNA replication were commonly down-regulated (Fig. 2I, S2E). Importantly, inflammatory response genes were commonly up-regulated in cancer G0 cells but not normal G0 fibroblasts and do not significantly overlap with senescence-associated secretory pathway (SASP) (Fig. 2I, S2G) (Serrano et al., 1997; Coppe et al., 2010), indicating a specific role in chemoresistant cancer cells. These results indicate that specialized post-transcriptional mechanisms are

enabled in these conditions of decreased canonical translation and lead to common expression of specific genes; in particular, of inflammatory response genes in chemoresistant G0 cancer cells.

Post-transcriptional up-regulation of ARE-bearing mRNAs is mediated by phosphorylation of TTP in resistant G0 leukemic cells

To identify RNA motifs that mediate post-transcriptional regulation, the untranslated regions (UTRs) of differentially expressed genes in G0 leukemic cells were examined. Three UTR elements were found: two 5′UTR elements and a key post-transcriptional regulatory 3′UTR AU-rich element. A GC-rich motif was enriched on 5′UTRs of translationally up-regulated genes (ΔRO > 1.5) and an AU rich motif, on 5′ UTRs of translationally suppressed genes (ΔRO < 1.5), indicating that mRNAs with structured 5′UTRs are highly translated in G0 cells (Fig. S3A-B). Importantly, 3′UTRs of genes in the up-regulated translatome have higher AU-rich elements (AREs) scores, compared to down-regulated translatome (Fig 3A). Over 30% of the translatome signature of G0 leukemic cells bear AREs, including key proinflammatory cytokines/chemokines, such as TNFα (Figs. 3B-D, Table S2) (Damgaard and Lykke-Andersen, 2013). AREs regulate mRNA stability and translation to control expression of critical growth factors and immune genes (Zhang et al., 2001; Griseri and Pages, 2014). AREs are involved in mediating specific mRNA decay; however, depending on their associated RNA binding proteins and cellular conditions that signal and modify these interactions, AREs can promote mRNA stability and translation, as in the case of TNFα and other mRNAs in G0 and other conditions (Bukhari et al., 2016; Nichols et al., 2011; Zhang et al., 2001; Mukherjee et al., 2011; Lal et al., 2004; Hitti et al., 2006). Consistently, we find that most ARE binding proteins that are RNA decay or translation repression factors (Fig. S3C, S3F) (Moore et al., 2014; White et al., 2013), are decreased in SS and AraCS cells.

Additionally, we find decrease of the exosome RNA decay complex (Fig. S3D) that is involved in ribosome processing (Zinder and Lima, 2017) and ARE mRNA decay (Hitti et al., 2006; Tiedje et al., 2016; Gherzi et al., 2004), and of proteasome factors (Laroia et al., 1999) (Fig. S3E) that are implicated in ARE mRNA decay. A key ARE mRNA decay factor, TTP, was surprisingly increased in G0 leukemic cells (Fig. S3F, 3B-C, 3E-F). TTP phosphorylation is established to increase its levels (Hitti et al., 2006), and results in inability of phospho-TTP to down-regulate ARE mRNA expression; such ARE mRNAs are stabilized and translated (Clement et al., 2011; Tiedje et al., 2016). Accordingly, we find that TTP is phosphorylated in SS and AraCS cells (Fig. 3F), accounting for its increased levels (Fig. S3F, 3B-C, 3E-F). To test whether phosphorylation of TTP was required for expressed ARE mRNAs in SS and AraCS cells, we over-expressed non-regulatable active mutant TTP that has its key phosphorylation sites (Ser 52, 178) mutated to alanine (TTP-AA). TTP-AA has been shown to dominantly maintain ARE mRNA decay activity and reduce pro-inflammatory cytokines like TNFα in immune cells (Clement et al., 2011; Tiedje et al., 2016; Hitti et al., 2006). Over-expression of myc-tagged TTP-AA compared to control vector significantly reduced TNFα mRNA in both THP1 and K562 AraCS cells (Fig. 3G), overturning the decay inactivity of endogenous phospho-TTP. These data indicate that inactivation of ARE mRNA decay by TTP phosphorylation (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016) is a key regulator of pro-inflammatory gene/TNFα expression in chemoresistant G0 cells. These results are consistent with our findings of increased levels and translation of ARE bearing mRNAs—including many pro-inflammatory cytokines (Fig. 2G, 3A-C)—due to decreased ARE decay activity in SS and AraCS cells (Fig. 3F-G, S3C-F).

Activation of p38 MAPK/MK2 phosphorylates TTP to promote expression of ARE-bearing mRNAs in resistant G0 leukemic cells

To investigate how TTP is phosphorylated in resistant G0 leukemic cells, we examined key signaling molecules involved in DNA-damage response (DDR) or stress (Holohan et al., 2013) since chemotherapies like AraC trigger DNA damage-mediated induction of ATM kinase and stress, which activate p38 MAPK (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009; Holohan et al., 2013). P38 MAPK signaling is also activated by G0 and serum-starvation (Sosa et al., 2011). As expected, AraC treatment induced rapid phosphorylation and activation of ATM (Fig. 3H-I) (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009; Holohan et al., 2013). Importantly, we find that ATM-mediated DDR leads to phosphorylation and activation of p38 MAPK and its downstream effector, MAPKAPK2 (MK2) (Cannell et al., 2015; Lalaoui et al., 2016) on AraC treatment (Fig. 3H-I). MK2 phosphorylates TTP to up-regulate inflammatory gene expression in immune cells (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016). To examine if MK2 kinase could phosphorylate TTP in resistant G0 leukemic cells, two different inhibitors of p38 MAPK/MK2 were tested. Inhibition of p38 MAPK, using the clinically tested p38 MAPKα/β inhibitor, LY2228820 (LY) (Lalaoui et al., 2016; Patnaik et al., 2016), or a pan p38 MAPK inhibitor that targets all isoforms, BIRB796 (BIRB) (Kuma et al., 2005), blocks phosphorylation of MK2, which prevents MK2-mediated TTP phosphorylation and reduces TNFα in AraCS cells (Fig. 3J). These results suggest that p38 MAPK/MK2 phosphorylates TTP, resulting in expression of ARE mRNAs such as TNFα in G0 leukemic cells (Fig. 3H). To test if p38 MAPK/MK/TTP regulates TNFα expression via its ARE, a Firefly luciferase reporter bearing the 3′ UTR ARE of TNFα was tested, with Renilla luciferase as co-transfected control. Luciferase activity increased by 2-fold in AraCS cells compared to S+ cells but not when p38 MAPK

was inhibited with LY (Fig. 3K). These data suggest that p38 MAPK/MK2/TTP axis up-regulates specific gene expression via AREs in G0 leukemic cells. These results are consistent with our findings of increased ARE bearing, pro-inflammatory factor mRNA levels and translation—due to decreased ARE mRNA decay by the ATM-p38 MAPK-MK2-TTP axis (Fig. 3H) that is induced by stress and DNA damage signaling due to serum-starvation and chemotherapy (Cannell et al., 2015).

Transiently activated p38 MAPK/MK2 in early G0—at early times of serum-starvation and chemotherapy treatment—promotes chemoresistance

We noted that p38 MAPK and MK2 were transiently phosphorylated, at early times upon induction of G0 cells with SS or AraC treatment (Fig. 3I and 4A-B). To investigate whether early activation of p38 MAPK/MK2 impacted AraC resistance, p38 MAPK was pharmacologically inhibited *before* (or along with) as well as *after* treatment with AraC—and then chemo-survival was measured using multiple assays, including cell death and two cell viability assays (Fig. 4C-E). Inhibition of p38 MAPK/MK2 with BIRB (Fig. 4D) or LY (Fig. 4E), one day *after* treatment with AraC, does not show any significant reduction in survival of AraC-resistant cells. Critically, inhibition of p38 MAPK/MK2 at early time points—4h-1 day *prior to* AraC treatment or *along with* AraC treatment—increased apoptosis and reduced survival of 70% of AraC-resistant cells (Fig. 4D-E). As a control, p38 MAPK inhibition alone does not affect viability of S+ cells that are not treated with AraC (Fig. 4D-E). These results suggest that p38 MAPK/MK2 is rapidly activated upon AraC treatment to turn on downstream survival genes at early times. Therefore, to overcome AraC resistance effectively, p38 MAPK/MK2 should be targeted at early time points before it induces downstream survival pathways that would dampen the effect of anti-inflammatory therapeutics. Consistently, sequential treatment with p38 MAPK inhibitor, LY, followed

by AraC showed a dramatic reduction (50-70%) in chemo-survival in multiple AML cell lines but not in non-cancerous cells (Fig. 4F, CD34+), as these pathways (MAPK, inflammatory genes) are not up-regulated in non-cancerous G0 (Fig. 2I). When treated with p38 MAPK inhibitor alone, viability of S+ cells in multiple AML cell lines remained unchanged, indicating the synergism of AraC and p38 MAPK inhibitors on resistant leukemic cells (Fig. 4F). Interestingly, p38 MAPK inhibition eliminated resistant cells more significantly at increasing concentrations of AraC (Fig. 4G). Treatment with high concentrations of AraC would increase the number of cells induced into the resistant G0 state with induction of p38 MAPK; therefore, inhibition of this pathway is more effective at high concentrations of AraC. Conversely, even low concentration of p38 MAPK inhibitor, BIRB, was sufficient to reduce chemoresistance (Fig. S4B). Activation of p38 MAPK/MK2 can induce resistance by arresting the cell cycle (Damgaard and Lykke-Andersen, 2013; Cannell et al., 2015; Lalaoui et al., 2016); however, p38 MAPK/MK2 inhibition did not affect the cell cycle in resistant G0 leukemic cells (Fig. S4A), indicating a distinct mechanism of resistance by p38 MAPK/MK2 in G0. These data uncover early and rapid activation of a p38 MAPK/MK2 pathway that enables chemo-survival of G0 leukemic cells—in part, through inhibition of TTP activity that enables expression of pro-survival, pro-inflammatory genes like TNFα (Fig. 5A). Pharmacological inhibition of this early-activated p38 MAPK/MK2 pathway *prior to* and along with chemotherapy reduces resistance; in contrast, post-chemotherapy inhibition of p38 MAPK/MK2 is not effective, as at later time points their downstream effectors of cell survival genes are already on.

Activation of p38 MAPK/MK2 promotes chemoresistance *via* inactivation of TTP and subsequent up-regulation of TNFα

We investigated the mechanism by which p38 MAPK/MK2 promotes chemo-survival in G0 leukemic cells. As shown in Fig. 3G-K, p38 MAPK/MK2 phosphorylates and inactivates TTP, leading to increased production of TNFα. TNFα activates the NFκB pathway that increases anti-apoptotic gene expression as a stress response to promote cell survival (Kagoya et al., 2014; Frelin et al., 2005; Chang and Vancurova, 2014). Importantly, our translatome analysis shows a significant increase in TNFα, TNFα receptors (Fig. S5A) and NFκB signaling (Fig. 5B), including anti-apoptotic BCL family members (Fig. S5B) (Chang and Vancurova, 2014; Haq et al., 2013; Kurosu et al., 2003), indicating that TNFα-mediated inflammatory response may cause chemoresistance. To examine the role of the TTP-TNFα axis in AraC resistance, we expressed non-phosphorylatable TTP mutant (TTP-AA) that is active in ARE mRNA decay and inhibits TNFα (Fig. 3G). Importantly, we find that TTP-AA expression reduces survival of AraC-resistant cells (Fig. 5C). These data suggest that TTP phosphorylation and inactivation—by p38 MAPK/MK2—enables chemo-survival through expression of ARE-bearing pro-inflammatory genes like TNFα.

Inhibition of TNFα/NFκB—prior to or at the same time as chemotherapy—decreases resistance, correlating with transient, early increase in expression of TNFα/NFκB in early G0

TTP may have other targets apart from TNFα. To verify that TTP regulates chemoresistance via targeting TNFα, we created a stable cell line expressing an inducible shRNA against TNFα. As shown in Fig. 5D, induction of TNFα depletion prior to AraC treatment effectively reduced AraC resistance, compared to after AraC treatment, while no effect was observed with TNFα depletion alone without AraC. In contrast, addition of recombinant TNFα enhanced survival of AraC-treated cells (Fig. 5D).

TNFα-mediated chemoresistance is not due to arrested cell cycle as TNFα treatment without subsequent AraC does not alter the cell cycle (Fig. S5C). These data suggest that phosphorylation of TTP and subsequent expression of TNFα, which are induced by p38 MAPK/MK2, are responsible for survival of G0 leukemic cells.

TNFα can also be inhibited pharmacologically with the inhibitor Pirfenidone (PFD, Fig. 5A) that can block TNFα translation, reduce inflammatory factors, and is clinically used for inflammatory disease like fibrosis (Grattendick et al., 2008; Nakazato et al., 2002; Lalaoui et al., 2016). PFD decreases TNFα at the translatome and proteome levels in G0 leukemic cells (Fig. 5E). Our observation of early activation of p38 MAPK/MK2 (Fig. 4A-B) suggested that TNFα could be rapidly up-regulated upon G0 induction. Time-course translatome analysis affirmed that TNFα is most significantly increased (16-fold) at the earliest time point of 4 h after serum-starvation or AraC treatment (Fig. 5B), causing early expression of NFκB target genes including cell survival genes (Fig. 5B and S5B). Consistently, PFD treatment at least 18 hours *prior to* or along with AraC or serum-starvation significantly reduced viability of G0 leukemic cells but failed to reduce resistance when added *after* AraC treatment (Fig. 5F, S5D). PFD treatment alone does not affect the viability of untreated S+ cells, indicating that the cytotoxic effect of PFD is specific to G0 leukemic cells (Fig. 5F). Similar results were observed in MCF7 cells, where PFD reduced doxorubicin resistance (Fig. S5H). These data indicate that activation of the inflammatory pathway is an early event in G0 cells, which leads to resistance, and needs to be inhibited early to preclude downstream survival regulators.

PFD treatment reduced chemotherapy survival in multiple AML cell lines but not all leukemia types (Fig. 5G, AML cell lines versus chronic myelogenous leukemia K562). Since PFD could affect targets other than TNFα to reduce resistance (Grattendick et al., 2008; Nakazato et al., 2002; Lalaoui et al.,

2016), we tested the effect of PFD along with shTNFα depletion together, prior to AraC chemotherapy, in comparison to individual treatments: an additive effect would not be observed if PFD affects chemosurvival through TNFα and not via other targets. Co-treatment with PFD and TNFα shRNA prior to AraC did not show an additive effect of reducing survival compared to PFD treatment alone, indicating that the effect of PFD was mediated through TNFα inhibition (Fig. S5E). Similarly, we find that inhibition of TNFα-induced NFκB, with NFκB inhibitor, BAY11-7082 (Rushworth et al., 2010) to block downstream anti-apoptotic response, prior to or along with AraC or serum-starvation, decreases survival; treatment after chemotherapy or serum-starvation had no effect as observed with upstream TNFα inhibitor PFD (Fig. 5H). These data suggest that the TNFα/NFκB inflammatory pathway is upregulated as an early survival pathway in G0 cells, which increases chemo-survival. In accord with the transient increase of inflammatory genes at early times in these G0 cells after chemotherapy or serum-starvation, we find that treatment with inflammation inhibitors (PFD or BAY11-7082) or TNFα shRNA *prior to* and continued with chemotherapy significantly reduces chemoresistance—while treatment post-chemotherapy, fails to reduce resistance.

Co-inhibition of p38 MAPK and TNFα sensitizes resistant leukemic cells to AraC treatment by reducing TNFα and activating a pro-apoptotic JNK pathway

Although chemoresistant cells are sensitive to individual inhibition of either TNFα or p38 MAPK by PFD or LY respectively, a substantial proportion of cells still survived (Fig. 4F, 5G). Therefore, we asked if co-inhibition of p38 MAPK and TNFα with LY *and* PFD respectively, could eliminate the remaining resistant cells. We find that individual treatment, with low concentrations of LY or PFD (half of the concentrations

used in Fig. 4-5), prior to or along with AraC, reduces approximately 50% of surviving leukemic cells (Fig. 6A-B). Importantly, the combination of PFD *and* LY2228820 prior to AraC treatment–called PLA therapy–eliminates about 90% of chemoresistant cells in multiple AML cell lines (Fig. 6A-C). Furthermore, PLA therapy decreased colony formation capacity of leukemic cells on methylcellulose by 10-fold, compared to AraC-treatment alone (Fig. 6D). These data indicate a severe loss of stem cell capacity of leukemic cells treated with PLA therapy. In contrast, in the absence of AraC treatment, the combination of PFD and LY2228820 did not affect cell viability, apoptosis and colony formation capacity, indicating the synergistic effect between AraC and anti-inflammatory drugs (Fig. 6A-D). Despite the fact that stromal niche cells have been shown to protect leukemic cells from chemotherapy (Li and Dalton, 2006), we find that AML cells co-cultured with stromal cells remained sensitive to PLA therapy (Fig S5F).

We investigated the molecular mechanism by which PLA therapy enhanced chemosensitivity. First, TNFα depletion reduces chemoresistance (Fig. 5D). LY destabilizes TNFα mRNAs by TTP dephosphorylation (Hitti et al., 2006) (Fig. 3J), while PFD was shown to suppress TNFα selectively at the translation level (Nakazato et al., 2002); consistently, PFD treatment in chemoresistant G0 leukemic cells reduced TNFα at the translatome level but not at the RNA level (Fig. S5G). Therefore, in PLA therapy, phosphorylation of p38 MAPK target MK2 is decreased by LY, diminishing TTP phosphorylation and reducing TNFα mRNA levels (Fig. 3G-J) (Hitti et al., 2006)—and additionally, due to inhibition of TNFα translation by PFD, TNFα remains more effectively blocked, compared to individual drug treatments (Fig. 6E). Second, we investigated c-Jun N terminal kinase (JNK) that can promote apoptosis depending on stimulus and cell type (Liu and Lin, 2005). Phosphorylation of JNK was enhanced in cells treated with PLA therapy, compared to single-drug treatments (Fig. 6E). JNK inhibitor, JNK-IN-8 partially reversed apoptosis of PLA therapy-treated cells, but did not affect the

viability of cells not treated with PLA (Fig. 6F). Together, these results suggest that PLA therapy reduces TNFα and promotes a pro-apoptotic JNK pathway, leading to apoptosis of chemoresistant cells.

PLA therapy reduces chemoresistance in primary AML cells *ex vivo* and *in vivo*

To test the anti-leukemic activity of PLA therapy in primary AML (Townsend et al.,), primary cells from AML patients as well as two murine AML models driven by Hoxa9/Meis1 or MLL/AF9 (SI Methods), were used. When either p38 MAPK or TNFα was inhibited prior to AraC treatment, moderate apoptosis of chemoresistant cells was observed in primary AML cells (Fig. 7A-B). Importantly, co-inhibition of p38 MAPK and TNFα by PLA therapy (pre-treatment before AraC) significantly reduced AraC resistance in fourteen out of fifteen AML patient samples as well as in primary cells from two AML mouse models *ex vivo* (Fig. 7A-B). In contrast, the viability of normal CD34+ cells from healthy donors was not affected by treatment with LY or PFD (Fig. 4F, 7A-B), consistent with clinical studies that have shown that PFD and LY have acceptable safety and tolerance (Patnaik et al., 2016; Grattendick et al., 2008). To further investigate the therapeutic potential of PLA therapy *in vivo*, human AML cells expressing luciferase (MOLM13-Luc, SI Methods) were intravenously or subcutaneously injected into NSG mice. After confirmation of engraftment by measuring tumor volume or bioluminescent imaging (BLI), the mice were treated with PLA therapy or AraC for two weeks. Consistent with *ex vivo* results (Fig. 6B), PLA therapy significantly decreased the leukemic burden and tumor volume by 6-fold, compared to AraC treatment alone (Fig. 7C-D). Next, we tested PLA therapy in C57BL/6 mice engrafted with primary Hoxa9/Meis1 or MLL-AF9 leukemia cells expressing luciferase. These mice were treated with PLA therapy or AraC. Consistently, BLI shows that PLA therapy eliminated 78% or 96% of chemoresistant cells in a dosage-dependent manner (Fig. 7E-F) and extended mice survival (Fig. 7H and S5I). In the absence of AraC treatment, the combination of PFD and LY2228820 did not affect

leukemic burden, suggesting that cytotoxic effects of this combination are limited to AraC-resistant cells, rather than proliferating cells (Fig. 7G). Together, these results suggest PLA therapy has potential for improving AraC-mediated apoptosis in AML.

Discussion

G0 cells are a transiently arrested, clinically relevant subpopulation in cancers (Bhola et al., 2016; Tavaluc et al., 2007; Coller et al., 2006; Gupta et al., 2009; Salony et al., 2016; Li and Bhatia, 2011; Giles et al., 2008; Ng et al., 2016). Our previous data and others, revealed altered gene expression mechanisms in G0 leukemic cells, at the post-transcriptional (Salony et al., 2016; Sandberg et al., 2008) and translational levels (Bukhari et al., 2016; Lee et al., 2014; Loayza-Puch et al., 2013). This would lead to a distinct gene expression profile to enable G0 cell survival in harsh conditions. G0 cells are resistant to stress conditions like serum-starvation, with transient inhibition of apoptosis, and proliferation (Coller et al., 2006; Loayza-Puch et al., 2013; Liu et al., 2007), which are features required for cells to survive chemotherapy. Importantly, we find that serum-starved leukemic SS cells exhibit chemoresistance (Fig. 1E); consistently, true chemo-surviving AraCS cells are transiently arrested and chemoresistant (Fig. 1D-E, S1B-C). In accord, we find that SS cells are similar in translatome and proteome to AraCS cells (Fig. 1F), indicating that consistent with their common features of G0 arrest and chemosurvival, they show similar post-transcription gene expression. Published transcriptional signatures of G0 fibroblasts, and of leukemic stem cells and other in vivo chemoresistance leukemic models (Salony et al., 2016; Coller et al., 2006; Liu et al., 2007; Ng et al., 2016; Ebinger et al., 2016; Saito et al., 2010), are also highly expressed in SS and AraCS cells (Fig. 1G, S1G). Thus, the common G0 resistance gene expression profile observed in AraCS and SS G0 cells—distinct from neighboring

proliferating tumor cells—likely comprises genes that control survival and resistance. These data revealed that in addition to known transcriptional profiles, altered post-transcriptional and translation mechanisms in G0 resistant cells contribute to their unique gene expression profile that underlies their chemoresistance.

Our findings reveal the importance of DNA damage and stress signaling that can initiate a pro-inflammatory response that causes survival instead of cytotoxicity (Fig. 4-6, S4-S6). Differential genomic instability in cancers would lead to distinct subpopulations within a tumor with disparate DDR and stress signaling (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009) that we find, enables their chemotherapy survival via pro-inflammatory cytokines. Cytokines upregulated in SS and AraCS cells do not significantly match SASP (Serrano et al., 1997; Coppe et al., 2010) (Fig. S2G). This is consistent with differences between G0 and senescence (Coller et al., 2006)—with low mTOR activity (Fig. 2A-C, I, S2E) (Bukhari et al., 2016) and mutated p53 in these G0 cells—unlike high mTOR activity and p53 in senescence (Loayza-Puch et al., 2013). These data indicate that a quiescence- and resistance-specific set of pro-inflammatory genes are expressed in these resistant cells. These include inflammatory cytokines (Fig. 2F-G) that promote downstream NFκB activated pro-survival target genes (Kagoya et al., 2014; Frelin et al., 2005; Chang and Vancurova, 2014) including BCL family members of anti-apoptotic genes (Chang and Vancurova, 2014; Kurosu et al., 2003; Haq et al., 2013) (Fig. 5A-B, S5A-B). Treatment with reagents against these resistance-enabling immune regulators *after* chemotherapy is not very effective as the downstream survival effectors have already been induced; thus, targeting their upstream cytokine regulators would not be effective at this later time (Fig. 4A-B, 4D-E, 5F-H, S5D, 6B). Thus, treatment with reagents that block these resistance pathways *prior*

to (and continued with) or along with chemotherapy, enables the most effective reduction of resistance, as they prevent further enrichment of such resistant cells by blocking induction of pro-survival signaling.

AraC is a nucleotide analog and inhibits replication, which triggers DDR (Forbes et al., 2015). Increasing the concentration of AraC would cause increased DDR and downstream p38 MAPK signaling (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009) and should lead to more cells expressing this inflammatory pathway that enables resistance—and thus pushes more cells into the inflammatory phase that can be targeted by inhibitors. Consistently, increased AraC treatment leads to more cells in the inflammatory phase that can be targeted by LY, leading to more significant loss of resistance (Fig. 4G). Non-cancerous cells do not show this pathway (Fig. 2I) and are not affected by inhibitors (Fig. 4F, 7A). These data suggest that certain chemotherapies and stresses like serum-starvation induce stress signaling (Fig. 4A-C) and enrich for resistant G0 cells—in addition to pre-existing subpopulations with genomic instability that trigger DDR and stress (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009). Importantly, this resistance mechanism can be blocked to significantly decrease resistance to AraC, not only in different AML cell lines (Fig. 4C-G, 6B) but also in vivo in AML xenograft and mouse models (Fig. 7C-G) as well as in multiple patient-derived AML samples—without affecting normal cells (Fig. 7A)—supporting their potential applicability as a therapeutic target against chemoresistance in AML.

We find key signaling pathways (Fig. S6), mediated by AraCS and SS treatments (Fig. 4A-B, 5A-B), which alter post-transcriptional and translational gene expression to enable resistance. These include: 1. DNA damage ATM (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009) and stress activated p38 MAPK signaling that in turn promotes (Fig. 4A-B) MK2 (Cannell et al., 2015; Lalaoui et al., 2016); MK2 post-transcriptionally upregulates ARE bearing mRNAs (Brooks and Blackshear, 2013; Hitti et al., 2006; Tiedje et al., 2016) (Fig. 3G-K, 4A-B), including proinflammatory cytokines like TNFα (Kagoya et al., 2014; Frelin et al., 2005) that activate downstream anti-apoptosis signals (Fig. 5A-B, S5A-B) (Chang and Vancurova, 2014; Haq et al., 2013; Kurosu et al., 2003) to promote resistance (Fig. 5-7). 2. ATM mediated suppression of mTOR activity (Shiloh and Ziv, 2013; Tee and Proud, 2000) to inhibit canonical translation via 4EBP dephosphorylation that enables non-canonical translation (Bukhari et al., 2016) (Fig. 2A-C); this results in specific translation of pro-inflammatory cytokines (Fig. 3A-D) and immune modulators (Shukla et al., 2015) (HLA-G, CD47, Fig. 2F-G, S2I) (Sosale et al., 2015; Soto-Pantoja et al., 2015; Zhang et al., 2015) that regulate anti-tumor immune response and are associated with resistance (de Kruijf et al., 2010; Majeti et al., 2009). 3. UPR stress signaling that can be induced by p38 MAPK (Sosa et al., 2011) and DNA damage (Claudio et al., 2013; Senft and Ronai, 2015); this inhibits canonical translation via PERK phosphorylation of eIF2α and enables non-canonical specific mRNA translation (Fig. 2A-D, S2H)—which also increases inflammation and blocks apoptosis to enable chemoresistance (Claudio et al., 2013; Senft and Ronai, 2015). Blocking the p38 MAPKα/β pathway with LY (Lalaoui et al., 2016; Patnaik et al., 2016), in combination with the anti-inflammatory PFD (Grattendick et al., 2008; Nakazato et al., 2002; Lalaoui et al., 2016) that blocks downstream TNFα expression (Grattendick et al., 2008; Nakazato et al., 2002) (Fig. 5A, E, S5G)—prior to (and continued with) AraC chemotherapy—lead to effective loss of chemoresistance in multiple AML cell lines (Fig. 6B), in tumors *in vivo* in AML mouse models (Fig. 7C-G), and in patient samples (Fig. 7A), validating

their ability to reduce resistance and tumors *in vitro* and *in vivo*. LY destabilizes TNFα mRNA by TTP dephosphorylation (Fig. 3J) (Hitti et al., 2006), while PFD suppresses TNFα selectively at the translation level (Nakazato et al., 2002) (Fig. S5G) and thus enables PLA combination therapy to more effectively sustain reduction in resistance than the individual drugs. Apart from its effect on TNFα translation, PFD blocks inflammation regulator (Cuenda and Rousseau, 2007; Korb et al., 2006) p38 MAPKγ that can be increased upon p38MAPKα/β inhibition, preventing feedback reactivation of inflammation, and enabling PLA combination therapy to remain more efficacious than the individual drugs. Consistently, in the combination treatment with LY and PFD, LY decreases phosphorylation of MK2 by p38 MAPK, reducing TTP phosphorylation and TNFα RNA levels (Fig. 3G-J) (Hitti et al., 2006); additionally, TNFα translation is inhibited by PFD, resulting in chemo-survival being more effectively blocked, compared to individual drug treatments (Fig. 6B, E). In addition, we find that upon inhibition of p38 MAPK and TNFα, the JNK pathway (Liu and Lin, 2005) is activated to promote apoptosis (Fig. 6E-F).Therefore, the combination of PFD and LY suppresses the inflammatory and stress response more effectively as it blocks upstream and downstream steps in the pathway to circumvent feedback reactivation or alternate pathways, which leads to the enhanced decrease in chemoresistant cell survival and cancer persistence *in vitro* and *in vivo* (Fig. 6, 7). These data indicate that blocking pro-inflammatory effectors—that are induced by chemotherapy mediated DNA damage and stress signaling—leads to increased chemosensitivity and decreased resistant cell survival.

EIF2α phosphorylation and mTOR inhibition reduces both rate limiting steps of canonical translation initiation (Fig. 2A-C). ATM and p38 MAPK can lead to activation of PKR and UPR stress-activated PERK, which phosphorylate eIF2α to decrease canonical tRNA recruitment and translation (Fig. 2B-C),

leading to specific mRNA translation (Han et al., 2014; Hsieh et al., 2012; Ron and Walter, 2007). These include cell cycle arrest factor, p27 KIP1(Cuesta et al., 2009) (Fig. S1C, S2A-B, p27), and GADD34 (Fig. S2H)—that are translated when canonical translation mechanisms are reduced (Han et al., 2014; Hsieh et al., 2012) as in G0 (Bukhari et al., 2016; Lee et al., 2014). ATM activates AMPK which inhibits mTOR (Shiloh and Ziv, 2013; Tee and Proud, 2000; Jackson and Bartek, 2009), leading to dephosphorylated, active 4EBP (Fig. 2B-C). 4EBP blocks canonical translation via eIF4E inhibition (Thoreen et al., 2012; Holcik and Sonenberg, 2005), and permits non-canonical translation mechanisms of specific genes , including immune modulators like TNFα and CD47 (Fig. 2F-G, S2I, 3A-C, 5A-C,-D, S5A) (Bukhari et al., 2016), which activate survival genes (Fig. S5B).

Our findings revealed that these inflammatory genes upregulated in G0, have AREs and other UTR sequences that regulate mRNA levels and translation (Fig. 3A-C, S3A). The ATM-p38 MAPK-MK2 axis stabilize these ARE bearing pro-inflammatory cytokine mRNAs by phosphorylating ARE binding mRNA decay factor, TTP to prevent its mRNA decay activity on pro-inflammatory cytokine mRNAs like TNFα (Fig. 3D-K, 4A-B, 4F, 5A-C). This is consistent with previous studies on the role of AREs in cancers (Damgaard and Lykke-Andersen, 2013; Nichols et al., 2011; Bukhari et al., 2016; Zhang et al., 2001; Mukherjee et al., 2011; Lal et al., 2004; Hitti et al., 2006). All AML cell lines tested respond to the combination treatment that inhibits the p38 MAPK/MK2/TTP/TNFα axis leading to reduced TTP phosphorylation and inhibited TNFα (Fig. 6B, 6E-F) (Clement et al., 2011; Tiedje et al., 2016; Hitti et al., 2006). These data suggest that the phospho-TTP and thus TTP activity, may be a key regulator of pro-inflammatory gene mediated chemoresistance. In support, overexpression of TTP-AA—that cannot be phosphorylated and is a dominant active form that restores ARE mRNA decay (Clement et al., 2011;

Tiedje et al., 2016; Hitti et al., 2006)—causes decrease in TNFα expression, and leads to reduced chemoresistance in both cell lines (Fig. 3G, 5C). These data suggest that phospho-TTP level is an important regulator of inflammatory response mediated chemoresistance, which can be harnessed as a marker and target against clinical resistance. Consistently, published in vivo leukemia resistance models show similar increased TTP and ARE bearing genes including inflammatory genes expressed (Ebinger et al., 2016; Kesarwani et al., 2017), as in our studies (Fig. 3A-F); additionally, inhibition of these pathways curtails chemoresistance *in vivo* in primary AML patients and tumor models (Fig. 7). Together, these pathways that are up-regulated in resistant cells (Fig. 4A, 5A) via chemotherapy and stress induced signaling—decrease canonical translation and permit non-canonical translation and post-transcriptional regulation of specific genes (Fig. S6)—to induce a pro-inflammatory response that promotes chemo-survival of G0 cancer cells.

Methods

Methods including cell culture, patient samples, tumor models, profiling, plasmids, cell viability assays, flow cytometry, protein analysis, drugs, and motif analysis are described in Supplemental Information.

Statistical analyses & data availability are described in Supplemental Information.

Statement on Human Data All human samples (de-identified) were handled in accordance with IRB protocols to SV (2015P000998/MGH), approved by the Partners Human Research Committee Institutional Review Board /MGH IRB, to DAS, and to TG (DF/HCC 13-583), approved by DF/HCC Office for Human Research Studies. Details in Supplemental Information.

Statement on animal data nod-scid-gamma (NSG), C57Black/6 mice, 10-12 weeks, are obtained from MGH Cox-7 Gnotobiotic animal facility of the AAALAC-accredited Center for Comparative Medicine and Services at MGH. These facilities are supervised by veterinarians in the Center for Comparative Medicine and the MGH Subcommittee for Animal Research (SRAC) and maintained according to the protocol approved by SRAC, and provide services for breeding, regular health checks, histopathology and macropathology.

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Author Contributions

SL conducted the research and bioinformatic analysis; SST, SIAB & DL contributed data; SL, YK, DM, BTN, ID-G, DTM, M-KC, DS, SM & DAH provided in vivo models & in vivo data; MAM, RR & RG did immune data; AL, NJH, & ML provided mRNA folding energies & patient gene signatures; MB & WH conducted proteomics; DAS & JL-S provided patient samples; SV supervised the project & wrote the manuscript.

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Statement on conflict of Interest Authors declare no conflict of interest.

List of Supplemental files:

1. **Supplemental Information**

   Methods (related to main text, main figures 1-7 & supplemental figures S1-S6)

   Supplemental References (related to supplemental information, methods section)

   Supplemental Figure legends S1-S6, related to main figures 1-7

2. **Supplemental Figures S1-S6 (related to main figures 1-7)**

3. **Supplemental Tables 1-2 (related to main figures 1-7 & supplemental Figures S1-S6)**

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Figure legends

Figure 1. G0 leukemic cells induced by AraC or serum-starvation are chemoresistant and recapitulate gene expression programs of *in vivo* chemoresistant and G0 models. A. Transcriptome, translatome and proteome analyses in proliferating and G0 leukemic cells. G0 cells were induced by treatment of proliferating cells (S+) with 5 µM AraC or serum starved (SS). Total RNAs, polysome-associated mRNAs and protein were analyzed by comparative microarray and quantitative proteomics. **B.** Polysome profiles of S+, SS and AraC-surviving leukemic cells (AraCS) are shown. Heavy polysome-associated mRNAs were isolated and analyzed by microarray. **C**. Expression of Ki67 at the translatome level and flow cytometric quantification of G0/G1, S and G2/M phases, using BrdU and PI staining. **D**. Cell counting with trypan blue staining shows that SS or 5 µM AraC treatment on day 6 arrested cell proliferation reversibly. Cells resumed proliferation when serum was added to SS cells, or when AraCS cells were washed with PBS and resuspended in fresh media on day 11. **E**. Both AraCS and SS cells acquire resistance to AraC. S+, SS and AraCS cells were treated with various concentration of AraC for 3 days. Viable leukemic cells were measured by cell counting using trypan blue staining and IC_{50} values of AraC are shown. **F**. Comparison of transcriptomic, translatomic and proteomic changes in response to SS and 5 µM AraC treatments. **G**. Comparison of AraCS and SS with leukemic stem cells (LSC) (Saito et al., 2010) in AML, dormant leukemic cells (LRC) (Ebinger et al., 2016), minimal residual disease (MRD) (Ebinger et al., 2016) in ALL, and G0 fibroblasts (Coller et al., 2006). GSEA analysis was performed to determine whether previously published transcriptome signatures of LSC, LRC, MRD and G0 HFF are up-regulated in AraCS and SS cells, compared to S+ cells. 'N' marks the limited resolution of the proteome in the GSEA. $*P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S1 & Table S1.

Figure 2. Inflammatory response mRNAs are selectively translated in G0 leukemic cells, where canonical translation is inhibited. A. Altered mechanisms of translation with repression of canonical translation. **B**. Polysome to monosome ratios in S+, SS and AraCS. **C**. Western analysis of translation initiation factor, eIF2α, and regulators eIF4EBP, PERK and PKR, in S+, SS and AraCS cells. **D**. Boxplot of the transcriptome and translatome changes in known TOP mRNAs in response to SS or AraC treatment. **E.** Number of differentially expressed genes. **F.** Venn diagram shows 162 genes up-regulated at both the transcriptome and translatome levels and 180 genes translationally up-regulated, where ribosome occupancy, RO increases by 1.5-fold. Heatmap shows gene expression changes in RNA, translatome levels and RO. **G.** Gene ontology (GO) analyses of differentially expressed genes. Statistical significance of enriched GO categories is shown as a heatmap. **H**. GSEA was performed to determine whether gene signatures of G0 leukemic cells is conserved in other G0 cells. **I**. GSEA shows gene categories regulated commonly in the translatome of G0 cells from five different cell types. Heatmap of normalized enrichment score (NES) is shown. $*P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S2 & Table S1.

Figure 3. Phosphorylation of TTP by p38 MAPK/MK2 up-regulates ARE-bearing mRNAs in G0 leukemic cells. A. Boxplot of ARE scores (SI methods) in the 3′UTRs of genes, up- or down-regulated at the translatome level in G0 compared to S+ cells. **B**. Venn diagram shows genes that are up-regulated at the translatome level and have AREs in their 3′UTRs (left). List of such genes (right). **C**. Expression of indicated ARE genes at the RNA and translatome levels. **D.** Western analysis of TNFα. **E.** Scatter plot showing the expression of RNA binding protein genes from RBPDB database (SI methods). TTP is indicated with a green dot. **F.** Western analysis of TTP in lysates from S+, SS and AraCS cells in the absence or presence of alkaline phosphatase (AP). Phospho-TTP is indicated with an arrow. **G.** Bar

graph shows TNFα mRNA expression normalized to GAPDH mRNA upon over-expression of vector or c-myc tagged non-phosphorylatable mutant TTP (TTP-AA) in AraC-treated THP1 or K562 cells. Western analysis of TTP-AA with c-myc antibody (right). **H**. Activation of p38 MAPK/MK2 permits stabilization and translation of ARE-bearing mRNAs via TTP phosphorylation in chemoresistant G0 cells. LY2228820 (LY) and BIRB396 (BIRB) are p38 MAPK inhibitors. Western analysis of indicated proteins in lysates from THP1 cells **I**. at indicated time points after AraC treatment, and **J**. in S+ and AraCS cells treated with vehicle, 5 μM LY or 5 μM BIRB. **K**. Bar graph shows Firefly luciferase activity of a reporter bearing TNFα ARE in its 3′UTR normalized to activity of co-transfected Renilla luciferase in S+ and AraCS cells treated with vehicle, 5 μM LY. $*P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S3 & Table S2.

Figure 4. Targeting p38 MAPK/MK2 signaling that is transiently activated in early G0, reduces AraC resistance. **A**. Western analysis of indicated proteins in THP1 cells at time points after serum starvation or 5 μM AraC treatment. **B**. Ratio of phospho-p38 MAPK, phospho-MK2 or TTP to p38 MAPK, MK2 or tubulin (loading control), respectively. **C**. Sequential treatment with p38 MAPK inhibitors and AraC in leukemic cells. **D & E**. Effects of p38 MAPK/MK2 inhibitions on survival of AraC-resistant cells. THP1 cells were treated with 5 μM BIRB796 (BB), 5 μM LY and vehicle in the absence (top panels, S+) or presence (bottom panels, AraC) of 5 μM AraC treatment for three days. Bar graphs show relative cell viability and death assessed by cell counting, MTS and caspase 3/7 assays. In the presence of AraC, THP1 cells were treated with p38 MAPK inhibitors prior to AraC treatment (BB → AraC, LY → AraC), at the same time with AraC (AraC + BB) and 1 day after AraC (AraC → BB, AraC → LY). 'H' and 'D' indicate hour(s) and day(s), respectively. **F**. Effect of p38 MAPK/MK2 inhibition on survival of resistant cells from five AML cell lines (M5 FAB subtype). Cells were treated

with 5 μM LY or vehicle 4 hours prior to AraC treatment (top panel, AraC) or in the absence of AraC (bottom panel, S+). Human CD34+ cells from healthy donors were tested as a control. **G**. Effect of p38 MAPK/MK2 inhibition on survival of chemoresistant cells with various concentrations of AraC. MV4:11 leukemic cells were treated with 5 μM LY or vehicle prior to 0 μM, 0.2 μM, 0.5 μM or 1 μM AraC for 3 days. *$P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S4.

Figure 5. Targeting TTP-TNFα and downstream NF-kB signaling sensitizes resistant leukemic cells to AraC treatment. **A**. Phosphorylation of TTP by p38 MAPK/MK2 stabilizes ARE-bearing TNFα mRNA, resulting in activation of NF-kB signaling in resistant G0 leukemic cells. TNFα expression is inhibited by TTP-AA mutant, pirfenidone (PFD) or shRNAs, and NF-kB signaling by NF-kB inhibitor, Bay11-7082. **B**. Expression of TNFα and NF-kB target genes at the translatome level at indicated time points after SS or AraC treatment. **C**. TTP-AA mutant expression prior to 5 μM AraC treatment, depleted TNFα in THP1 or K562 cells as shown in Fig. 3G. Viability of cells with TTP-AA or control vector was assessed by cell count. **D**. Cell viability (left) and Western analysis of TNFα (right) in stable THP1 cell lines expressing doxycycline-inducible shRNA against TNFα or control shRNA, which were treated with doxycycline, recombinant TNFα (ReTNFα) or 5 μM AraC. Recombinant TNFα protein was added 1 day prior to AraC (ReTNFα → AraC) and shRNA against TNFα was induced prior to AraC (shTNFα → AraC) or after AraC (AraC → shTNFα). **E**. Sequential treatment with PFD and AraC (left). TNFα expression at the translatome (middle) and protein levels (right) in response to PFD treatment. **F**. Effect of pharmacological inhibition of TNFα by PFD on AraC resistance. THP1 cells were treated with 300 μg/ml PFD or vehicle in the absence of AraC (S+, top panel), in the presence of AraC (AraC, middle panel), or on serum starvation (SS, bottom panel). Bar graphs show cell viability and death assessed by cell counting, MTS and caspase 3/7 assays. In middle

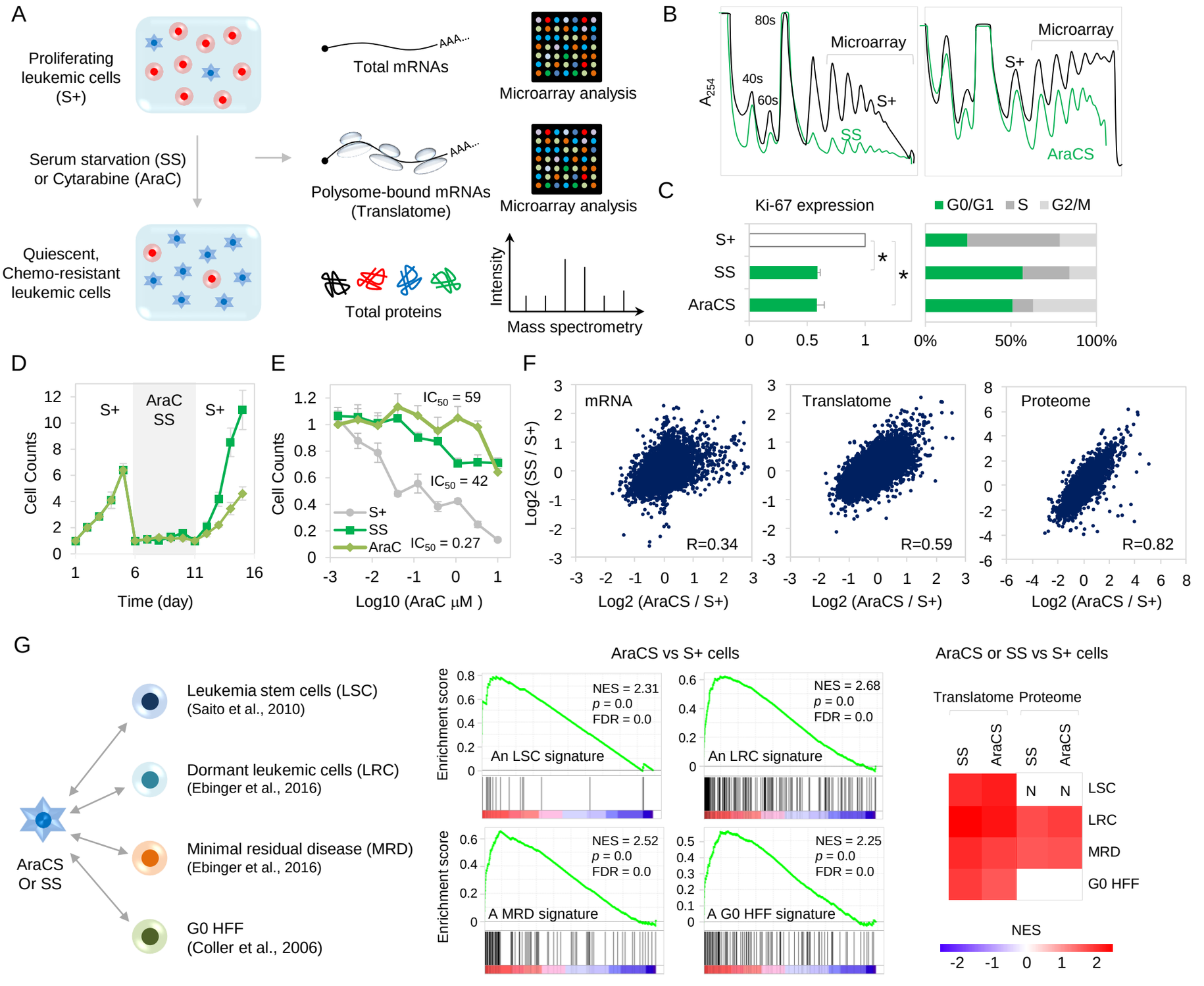
or bottom panels, THP1 cells were treated with PFD 1 day prior to AraC or SS (PFD → AraC, PFD → SS), at the same time with AraC or SS (AraC + PFD, SS + PFD), and 1 day after AraC or SS (AraC → PFD, SS → PFD). **G**. Effect of TNFα inhibition on AraC resistance from six different leukemic cell lines. Cells were treated with PFD or vehicle 1 day prior to AraC (top panel, AraC) or in the absence of AraC (bottom panel, S+). **H**. Effect of NF-kB inhibition on AraC resistance. THP1 cells were treated with 10 μM Bay11-7082 or vehicle in the absence of AraC (S+, top panel), in the presence of AraC (AraC, middle panel) or under serum starvation (SS, bottom panel). In middle or bottom panels, THP1 cells were treated with Bay11-7082 1 day prior to AraC or SS (Bay → AraC, Bay → SS), at the same time with AraC or SS (AraC + Bay, SS + Bay), and 1 day after AraC or SS (AraC → Bay, SS → Bay). *$P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S5.

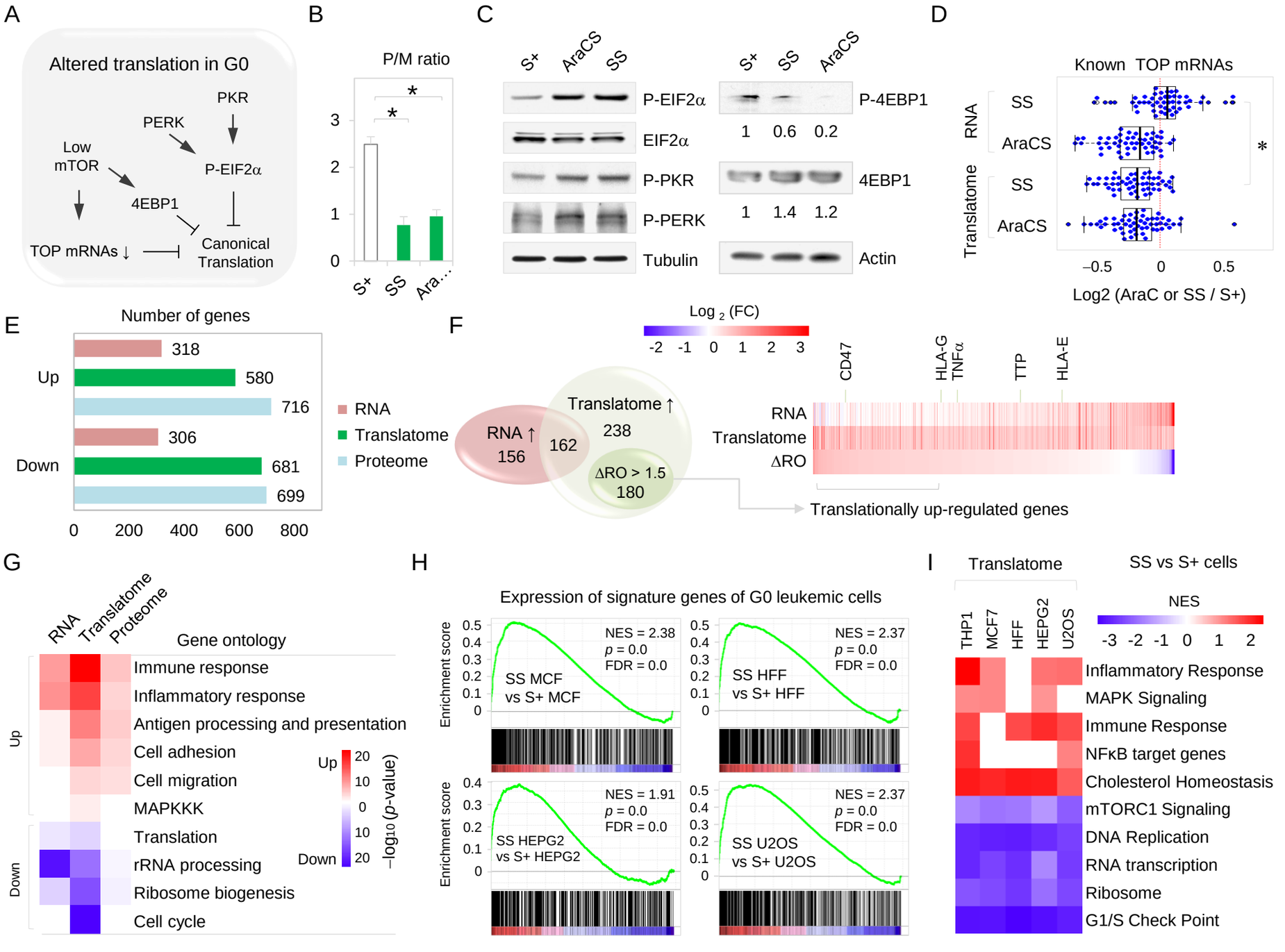
Figure 6. PLA combination therapy decreases AraC-resistant cells by reducing TNFα and activating pro-apoptotic JNK pathway. A. PLA therapy, involves pre-treatment of leukemic cells with PFD and LY for 1 day and 4 hours, respectively, followed by AraC treatment, using half of the concentrations used for individual drugs in Fig. 4 and 5. **B**. Three different AML cell lines were sequentially treated with indicated drugs, followed by assessment of cell viability and death. **C**. Ratio of apoptotic to living MOLM13 cells treated with indicated drug combinations (left). Flow cytometric profiles of cells stained with annexin V and propidium iodide (right). **D**. Quantification of colonies in MOLM13 cells treated with indicated drug combinations and then plated on methylcellulose media (left). Colony images are shown (right). **E.** Western analyses of indicated proteins in THP1 and MOLM13 cells treated with indicated drugs. Phospho-TTP is indicated with an arrow. **F**. Rescue of MOLM13 cells from PLA therapy-mediated apoptosis by inhibition of JNK pathway with JNK-IN-8.

Cell viability and death (left) and Western analyses of phospho-JNK, phospho-c-Jun and c-Jun (right) are shown *$P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S5.

Figure 7. PLA therapy significantly reduces AraC resistance in primary AML cells *ex vivo* and *in vivo*. A. Viability of primary cells from AML patients and normal CD34+ cells from healthy donors after indicated treatments. **B**. Viability and death of primary cells from AML mouse models driven by HoxA9-Meis1 and MLL-AF9 after indicated treatments. **C-G.** Bioluminescence images and quantification of tumor growth **C-D**. in NSG mice engrafted with MOLM13 cells and treated with PLA therapy or AraC, **E-F**. in C57BL/6 mice engrafted with primary HoxA9-Meis1/luciferase cells and treated with PLA therapy or AraC, and **G.** with PFD plus LY or vehicle as a control. **H**. C57BL/6 mice engrafted with primary MLL-AF9 cells and treated with PLA therapy or AraC. Kaplan-Meier survival curves of mice are shown. *$P \leq 0.05$. Data are represented as average ± SEM. See also Fig. S5-S6.

Figure 1


A
Proliferating leukemic cells (S+)
Serum starvation (SS) or Cytarabine (AraC)
Quiescent, Chemo-resistant leukemic cells
Total mRNAs
Microarray analysis
Polysome-bound mRNAs (Translatome)
Microarray analysis
Total proteins
Intensity
Mass spectrometry
B
80s
40s
60s
Microarray
S+
SS
AraCS
A254
C
Ki-67 expression
G0/G1
S
G2/M
S+
SS
AraCS
0
0.5
1
0%
50%
100%
D
S+
AraC SS
S+
Cell Counts
Time (day)
E
IC50 = 59
IC50 = 42
IC50 = 0.27
S+
SS
AraC
Cell Counts
Log10 (AraC μM)
F
mRNA
R=0.34
Translatome
R=0.59
Proteome
R=0.82
Log2 (SS / S+)
Log2 (AraCS / S+)
G
AraCS Or SS
Leukemia stem cells (LSC) (Saito et al., 2010)
Dormant leukemic cells (LRC) (Ebinger et al., 2016)
Minimal residual disease (MRD) (Ebinger et al., 2016)
G0 HFF (Coller et al., 2006)
AraCS vs S+ cells
NES = 2.31
p = 0.0
FDR = 0.0
An LSC signature
NES = 2.68
p = 0.0
FDR = 0.0
An LRC signature
NES = 2.52
p = 0.0
FDR = 0.0
A MRD signature
NES = 2.25
p = 0.0
FDR = 0.0
A G0 HFF signature
Enrichment score
AraCS or SS vs S+ cells
Translatome
Proteome
SS
AraCS
SS
AraCS
N
N
LSC
LRC
MRD
G0 HFF
NES
-2
-1
0
1
2


A
Altered translation in G0
PKR
PERK
Low mTOR
P-EIF2α
4EBP1
TOP mRNAs ↓
Canonical Translation
B
P/M ratio
S+
SS
Ara…
C
S+
AraCS
SS
P-EIF2α
EIF2α
P-PKR
P-PERK
Tubulin
P-4EBP1
4EBP1
Actin
1 0.6 0.2
1 1.4 1.2
D
Known TOP mRNAs
RNA
Translatome
SS
AraCS
Log2 (AraC or SS / S+)
E
Number of genes
Up
Down
318
580
716
306
681
699
RNA
Translatome
Proteome
F
Log 2 (FC)
CD47
HLA-G
TNFα
TTP
HLA-E
Translatome ↑
238
RNA ↑
156
162
ΔRO > 1.5
180
RNA
Translatome
ΔRO
Translationally up-regulated genes
G
RNA
Translatome
Proteome
Gene ontology
Immune response
Inflammatory response
Antigen processing and presentation
Cell adhesion
Cell migration
MAPKKK
Translation
rRNA processing
Ribosome biogenesis
Cell cycle
Up
Down
−log10 (p-value)
H
Expression of signature genes of G0 leukemic cells
Enrichment score
NES = 2.38
p = 0.0
FDR = 0.0
SS MCF vs S+ MCF
NES = 2.37
SS HFF vs S+ HFF
NES = 1.91
SS HEPG2 vs S+ HEPG2
SS U2OS vs S+ U2OS
I
Translatome
SS vs S+ cells
NES
THP1
MCF7
HFF
HEPG2
U2OS
Inflammatory Response
MAPK Signaling
Immune Response
NFκB target genes
Cholesterol Homeostasis
mTORC1 Signaling
DNA Replication
RNA transcription
Ribosome
G1/S Check Point

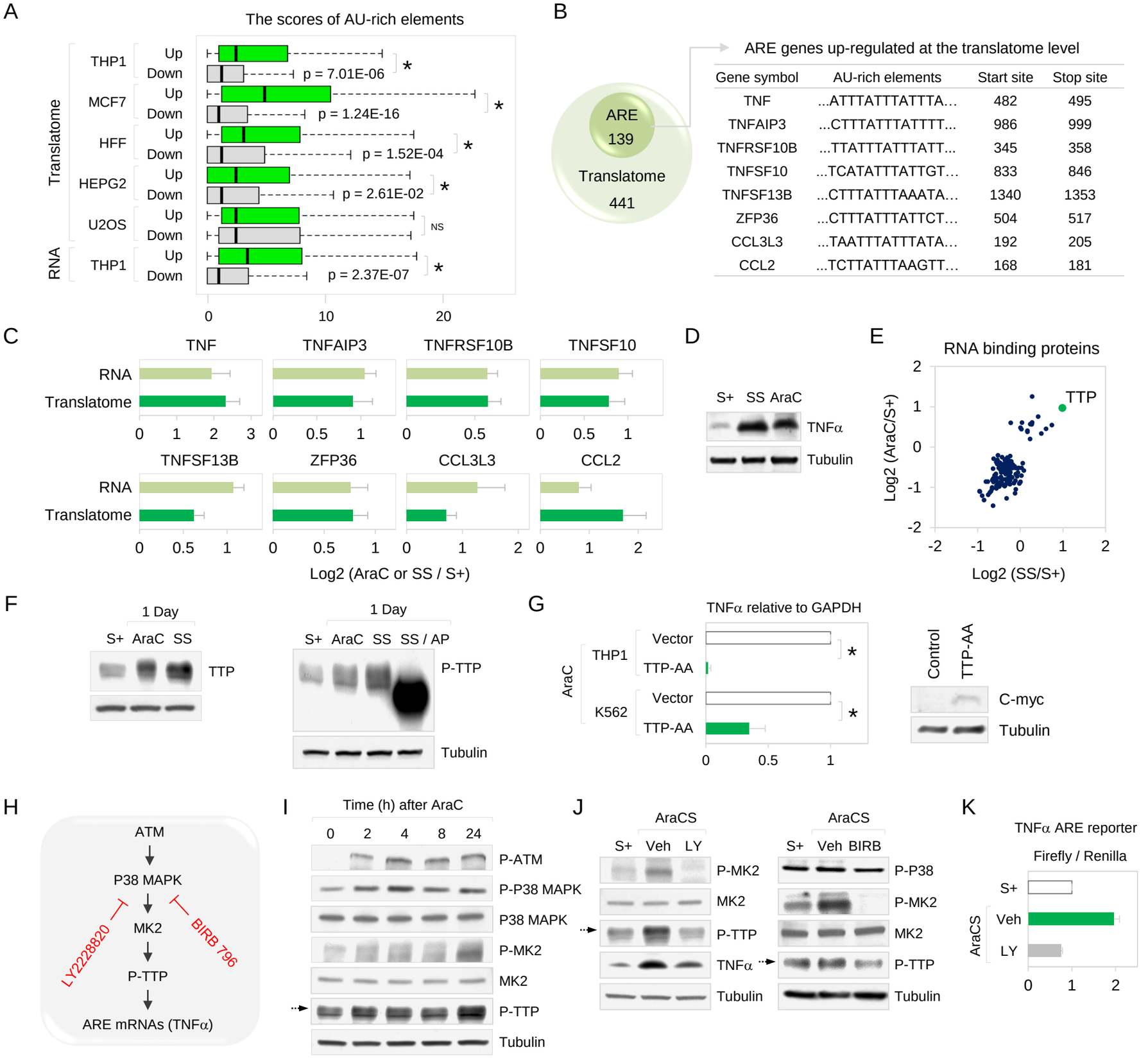
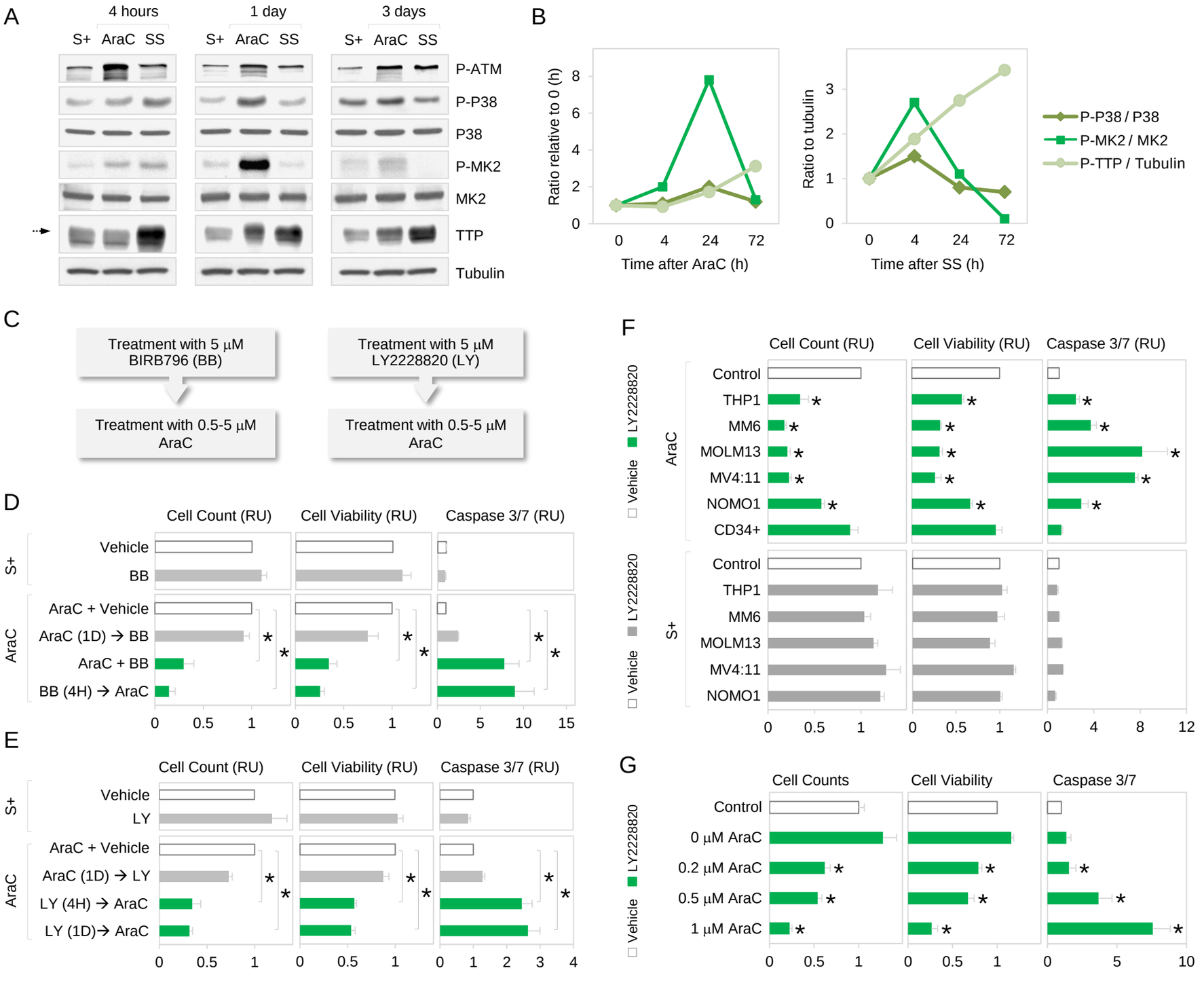

A
The scores of AU-rich elements
Translatome
RNA
THP1
MCF7
HFF
HEPG2
U2OS
THP1
Up
Down
p = 7.01E-06
p = 1.24E-16
p = 1.52E-04
p = 2.61E-02
NS
p = 2.37E-07
0
10
20
B
ARE
139
Translatome
441
ARE genes up-regulated at the translatome level
Gene symbol | AU-rich elements | Start site | Stop site
TNF | ...ATTTATTTATTTA... | 482 | 495
TNFAIP3 | ...CTTTATTTATTTT... | 986 | 999
TNFRSF10B | ...TTATTTATTTATT... | 345 | 358
TNFSF10 | ...TCATATTTATTGT... | 833 | 846
TNFSF13B | ...CTTTATTTAAATA... | 1340 | 1353
ZFP36 | ...CTTTATTTATTCT... | 504 | 517
CCL3L3 | ...TAATTTATTTATA... | 192 | 205
CCL2 | ...TCTTATTTAAGTT... | 168 | 181
C
TNF
TNFAIP3
TNFRSF10B
TNFSF10
TNFSF13B
ZFP36
CCL3L3
CCL2
RNA
Translatome
Log2 (AraC or SS / S+)
D
S+ SS AraC
TNFα
Tubulin
E
RNA binding proteins
TTP
Log2 (AraC/S+)
Log2 (SS/S+)
F
1 Day
S+ AraC SS
TTP
1 Day
S+ AraC SS SS / AP
P-TTP
Tubulin
G
TNFα relative to GAPDH
AraC
THP1
K562
Vector
TTP-AA
Control
TTP-AA
C-myc
Tubulin
H
ATM
P38 MAPK
MK2
P-TTP
ARE mRNAs (TNFα)
LY2228820
BIRB 796
I
Time (h) after AraC
0 2 4 8 24
P-ATM
P-P38 MAPK
P38 MAPK
P-MK2
MK2
P-TTP
Tubulin
J
AraCS
S+ Veh LY
AraCS
S+ Veh BIRB
P-MK2
MK2
P-TTP
TNFα
Tubulin
P-P38
P-MK2
MK2
P-TTP
Tubulin
K
TNFα ARE reporter
Firefly / Renilla
AraCS
S+
Veh
LY
0
1
2

Figure 4


A
4 hours
1 day
3 days
S+ AraC SS
P-ATM
P-P38
P38
P-MK2
MK2
TTP
Tubulin
B
Ratio relative to 0 (h)
Time after AraC (h)
Ratio to tubulin
Time after SS (h)
P-P38 / P38
P-MK2 / MK2
P-TTP / Tubulin
C
Treatment with 5 μM BIRB796 (BB)
Treatment with 0.5-5 μM AraC
Treatment with 5 μM LY2228820 (LY)
Treatment with 0.5-5 μM AraC
D
Cell Count (RU)
Cell Viability (RU)
Caspase 3/7 (RU)
S+
AraC
Vehicle
BB
AraC + Vehicle
AraC (1D) → BB
AraC + BB
BB (4H) → AraC
E
LY
AraC (1D) → LY
LY (4H) → AraC
LY (1D)→ AraC
F
Control
THP1
MM6
MOLM13
MV4:11
NOMO1
CD34+
LY2228820
G
Cell Counts
Cell Viability
Caspase 3/7
0 μM AraC
0.2 μM AraC
0.5 μM AraC
1 μM AraC

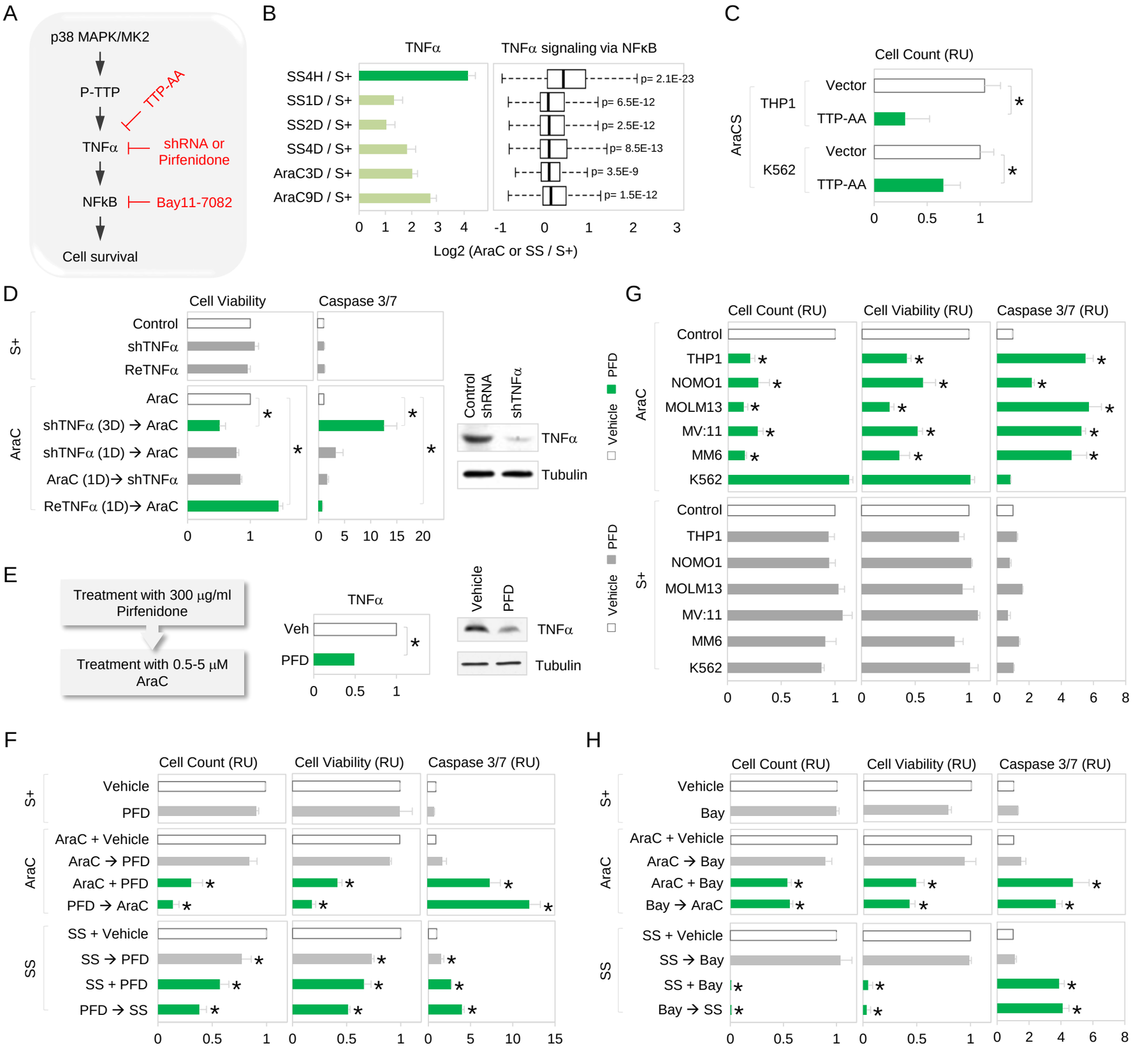

A
p38 MAPK/MK2
P-TTP
TTP-AA
TNFα
shRNA or Pirfenidone
NFkB
Bay11-7082
Cell survival
B
TNFα
TNFα signaling via NFκB
SS4H / S+
SS1D / S+
SS2D / S+
SS4D / S+
AraC3D / S+
AraC9D / S+
p= 2.1E-23
p= 6.5E-12
p= 2.5E-12
p= 8.5E-13
p= 3.5E-9
p= 1.5E-12
Log2 (AraC or SS / S+)
C
Cell Count (RU)
AraCS
THP1
K562
Vector
TTP-AA
D
Cell Viability
Caspase 3/7
S+
AraC
Control
shTNFα
ReTNFα
shTNFα (3D) → AraC
shTNFα (1D) → AraC
AraC (1D)→ shTNFα
ReTNFα (1D)→ AraC
Control
shRNA
shTNFα
TNFα
Tubulin
E
Treatment with 300 μg/ml Pirfenidone
Treatment with 0.5-5 μM AraC
TNFα
Veh
PFD
Vehicle
PFD
TNFα
Tubulin
G
Cell Count (RU)
Cell Viability (RU)
Caspase 3/7 (RU)
AraC
S+
PFD
Vehicle
Control
THP1
NOMO1
MOLM13
MV:11
MM6
K562
F
Cell Count (RU)
Cell Viability (RU)
Caspase 3/7 (RU)
S+
AraC
SS
Vehicle
PFD
AraC + Vehicle
AraC → PFD
AraC + PFD
PFD → AraC
SS + Vehicle
SS → PFD
SS + PFD
PFD → SS
H
Cell Count (RU)
Cell Viability (RU)
Caspase 3/7 (RU)
S+
AraC
SS
Vehicle
Bay
AraC + Vehicle
AraC → Bay
AraC + Bay
Bay → AraC
SS + Vehicle
SS → Bay
SS + Bay
Bay → SS

Figure 6

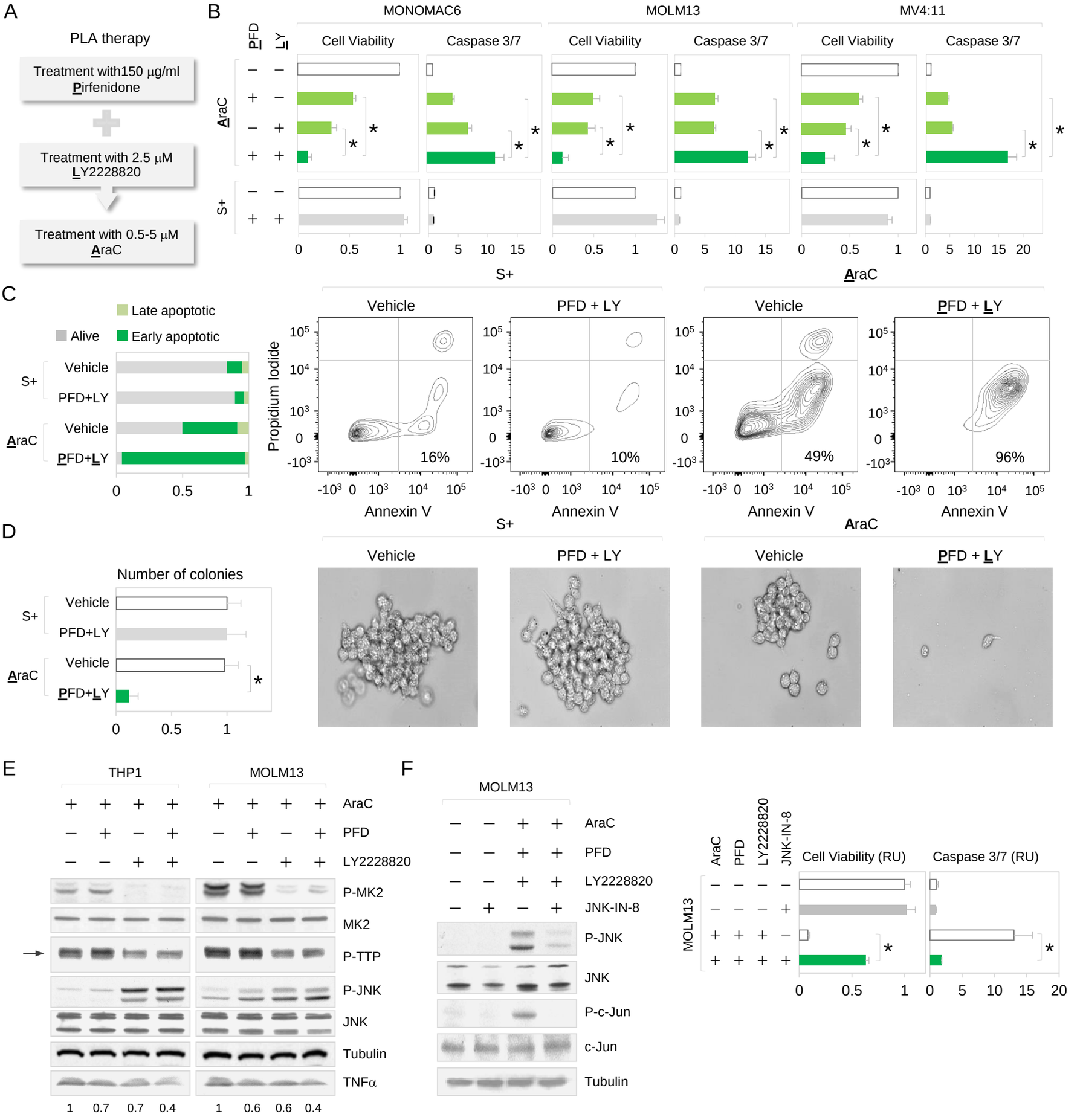

A
PLA therapy
Treatment with150 μg/ml Pirfenidone
Treatment with 2.5 μM LY2228820
Treatment with 0.5-5 μM AraC
B
MONOMAC6
MOLM13
MV4:11
PFD
LY
Cell Viability
Caspase 3/7
AraC
S+
S+
AraC
C
Late apoptotic
Alive
Early apoptotic
Vehicle
PFD+LY
Vehicle
PFD+LY
Propidium Iodide
Annexin V
16%
10%
49%
96%
D
Number of colonies
Vehicle
PFD+LY
Vehicle
PFD+LY
E
THP1
MOLM13
AraC
PFD
LY2228820
P-MK2
MK2
P-TTP
P-JNK
JNK
Tubulin
TNFα
1 0.7 0.7 0.4
1 0.6 0.6 0.4
F
MOLM13
AraC
PFD
LY2228820
JNK-IN-8
P-JNK
JNK
P-c-Jun
c-Jun
Tubulin
Cell Viability (RU)
Caspase 3/7 (RU)

Figure 7

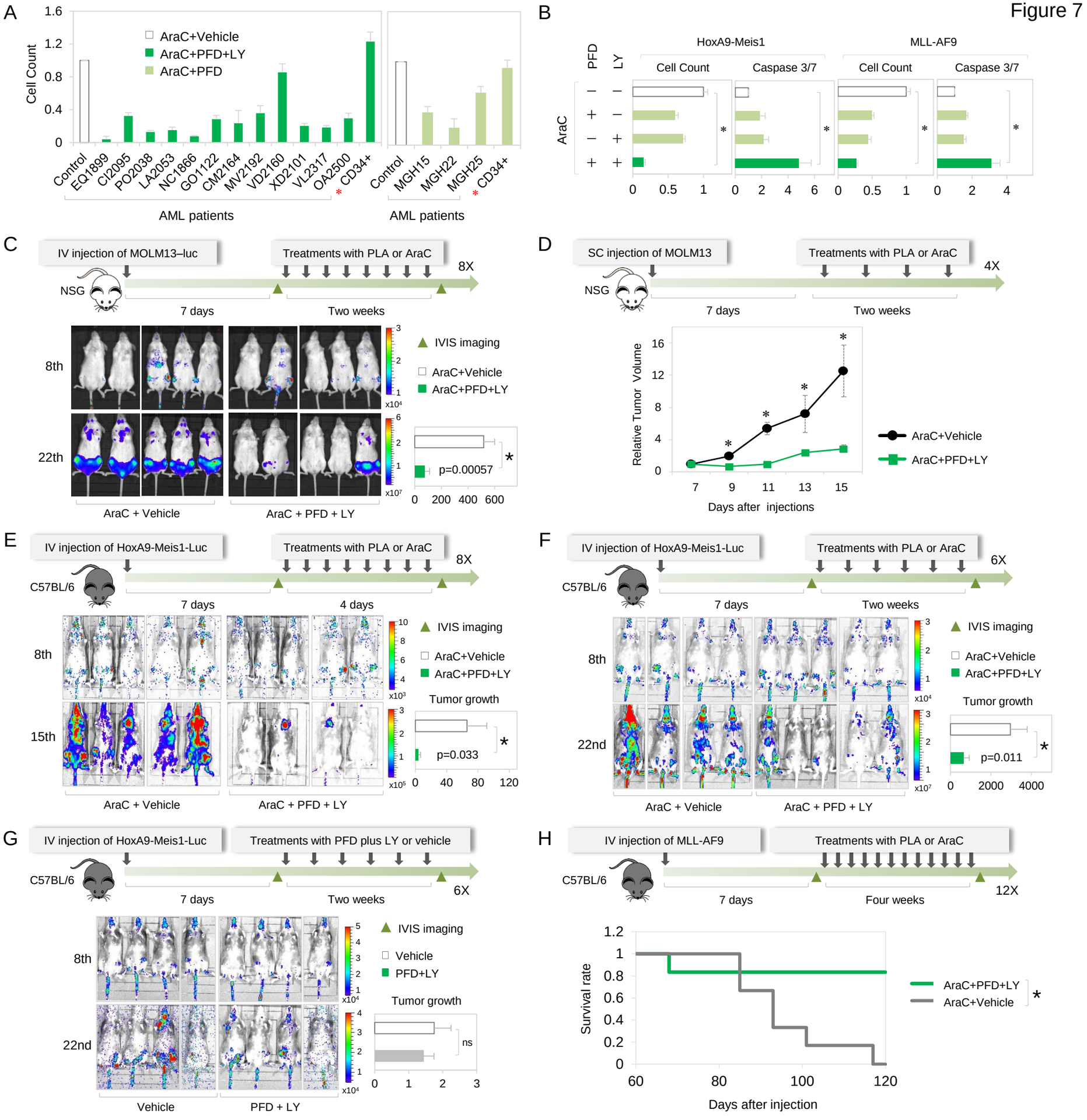

A
Cell Count
AraC+Vehicle
AraC+PFD+LY
AraC+PFD
Control
EQ1899
CI2095
PO2038
LA2053
NC1866
GO1122
CM2164
MV2192
VD2160
XD2101
VL2317
OA2500
CD34+
MGH15
MGH22
MGH25
AML patients
AML patients
B
HoxA9-Meis1
MLL-AF9
PFD
LY
AraC
Cell Count
Caspase 3/7
Cell Count
Caspase 3/7
C
IV injection of MOLM13–luc
Treatments with PLA or AraC
NSG
8X
7 days
Two weeks
8th
22th
IVIS imaging
AraC+Vehicle
AraC+PFD+LY
p=0.00057
AraC + Vehicle
AraC + PFD + LY
D
SC injection of MOLM13
Treatments with PLA or AraC
NSG
4X
7 days
Two weeks
Relative Tumor Volume
Days after injections
AraC+Vehicle
AraC+PFD+LY
E
IV injection of HoxA9-Meis1-Luc
Treatments with PLA or AraC
C57BL/6
8X
7 days
4 days
8th
15th
IVIS imaging
AraC+Vehicle
AraC+PFD+LY
Tumor growth
p=0.033
AraC + Vehicle
AraC + PFD + LY
F
IV injection of HoxA9-Meis1-Luc
Treatments with PLA or AraC
C57BL/6
6X
7 days
Two weeks
8th
22nd
IVIS imaging
AraC+Vehicle
AraC+PFD+LY
Tumor growth
p=0.011
AraC + Vehicle
AraC + PFD + LY
G
IV injection of HoxA9-Meis1-Luc
Treatments with PFD plus LY or vehicle
C57BL/6
6X
7 days
Two weeks
8th
22nd
IVIS imaging
Vehicle
PFD+LY
Tumor growth
ns
Vehicle
PFD + LY
H
IV injection of MLL-AF9
Treatments with PLA or AraC
C57BL/6
12X
7 days
Four weeks
Survival rate
Days after injection
AraC+PFD+LY
AraC+Vehicle

Figure S1

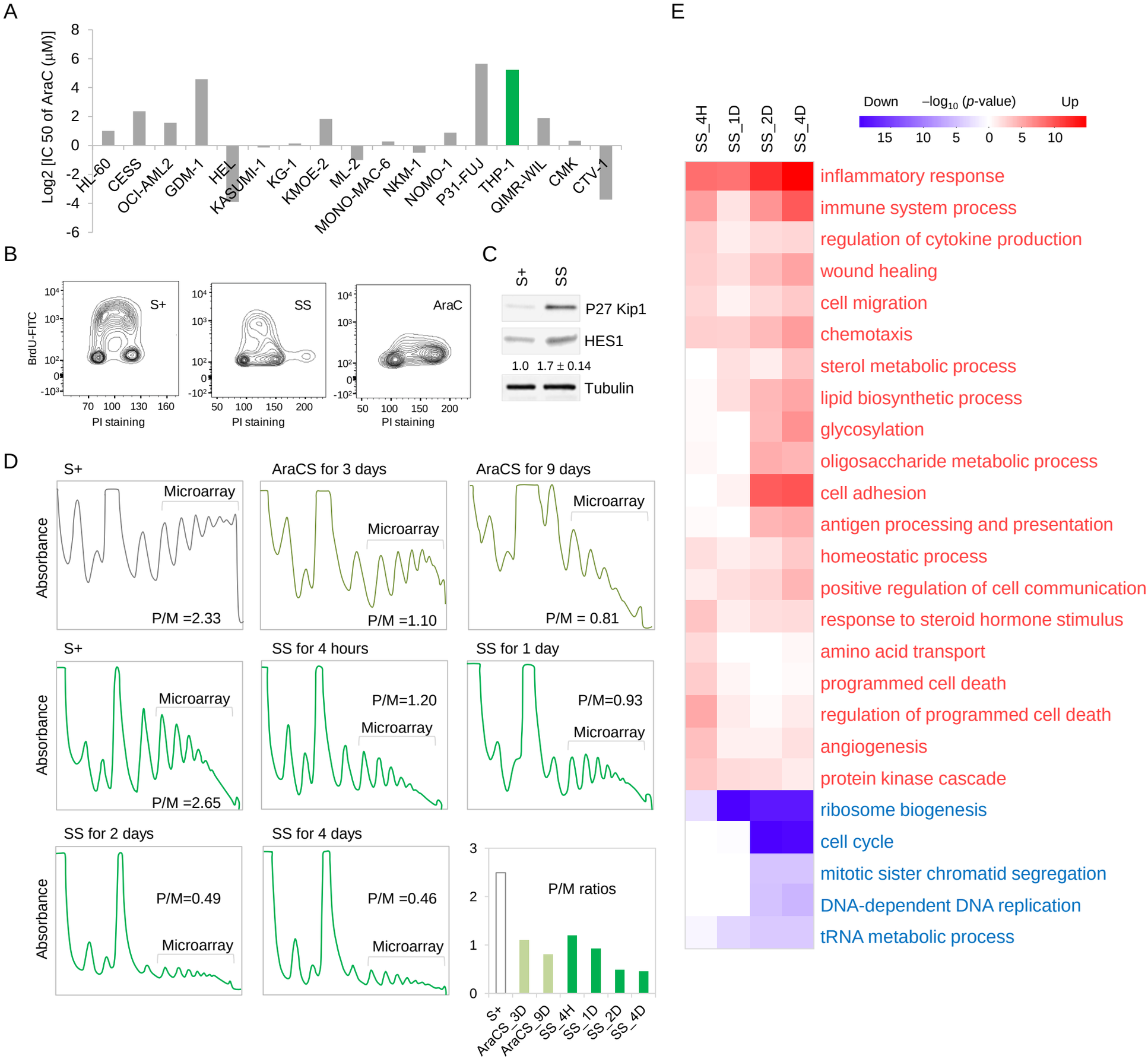
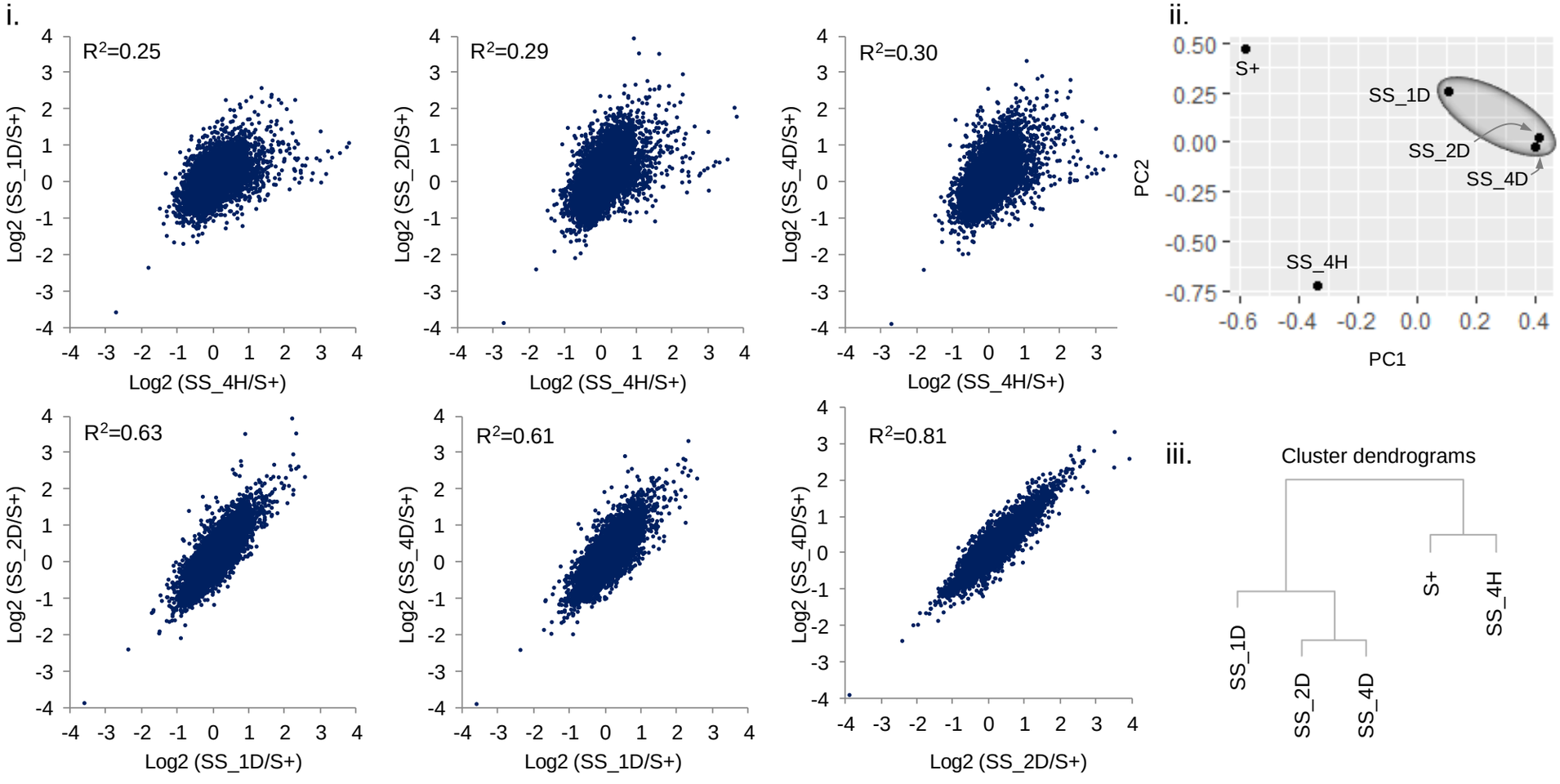

A
Log2 [IC 50 of AraC (μM)]
8
6
4
2
0
-2
-4
-6
HL-60
CESS
OCI-AML2
GDM-1
HEL
KASUMI-1
KG-1
KMOE-2
ML-2
MONO-MAC-6
NKM-1
NOMO-1
P31-FUJ
THP-1
QIMR-WIL
CMK
CTV-1
B
BrdU-FITC
S+
SS
AraC
PI staining
C
S+
SS
P27 Kip1
HES1
1.0
1.7 ± 0.14
Tubulin
D
S+
AraCS for 3 days
AraCS for 9 days
Microarray
P/M =2.33
P/M =1.10
P/M = 0.81
Absorbance
SS for 4 hours
SS for 1 day
P/M=1.20
P/M=0.93
P/M =2.65
SS for 2 days
SS for 4 days
P/M=0.49
P/M =0.46
P/M ratios
S+
AraCS_3D
AraCS_9D
SS_4H
SS_1D
SS_2D
SS_4D
E
Down
–log10 (p-value)
Up
15
10
5
0
5
10
inflammatory response
immune system process
regulation of cytokine production
wound healing
cell migration
chemotaxis
sterol metabolic process
lipid biosynthetic process
glycosylation
oligosaccharide metabolic process
cell adhesion
antigen processing and presentation
homeostatic process
positive regulation of cell communication
response to steroid hormone stimulus
amino acid transport
programmed cell death
regulation of programmed cell death
angiogenesis
protein kinase cascade
ribosome biogenesis
cell cycle
mitotic sister chromatid segregation
DNA-dependent DNA replication
tRNA metabolic process

Figure S1

E


i.
R²=0.25
R²=0.29
R²=0.30
R²=0.63
R²=0.61
R²=0.81
Log2 (SS_1D/S+)
Log2 (SS_2D/S+)
Log2 (SS_4D/S+)
Log2 (SS_4H/S+)
ii.
S+
SS_1D
SS_2D
SS_4D
SS_4H
PC1
PC2
iii.
Cluster dendrograms
S+
SS_4H
SS_1D
SS_2D
SS_4D

F

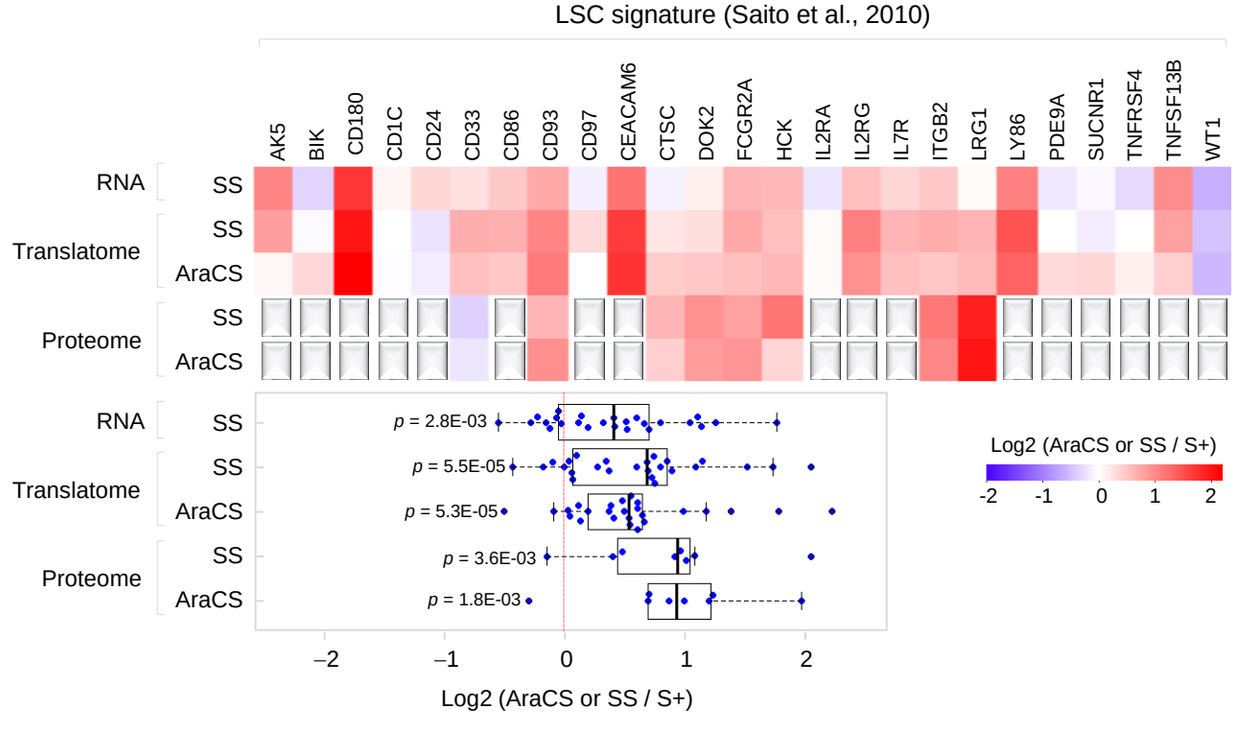

LSC signature (Saito et al., 2010)
AK5
BIK
CD180
CD1C
CD24
CD33
CD86
CD93
CD97
CEACAM6
CTSC
DOK2
FCGR2A
HCK
IL2RA
IL2RG
IL7R
ITGB2
LRG1
LY86
PDE9A
SUCNR1
TNFRSF4
TNFSF13B
WT1
RNA
Translatome
Proteome
SS
AraCS
p = 2.8E-03
p = 5.5E-05
p = 5.3E-05
p = 3.6E-03
p = 1.8E-03
Log2 (AraCS or SS / S+)

Figure S2

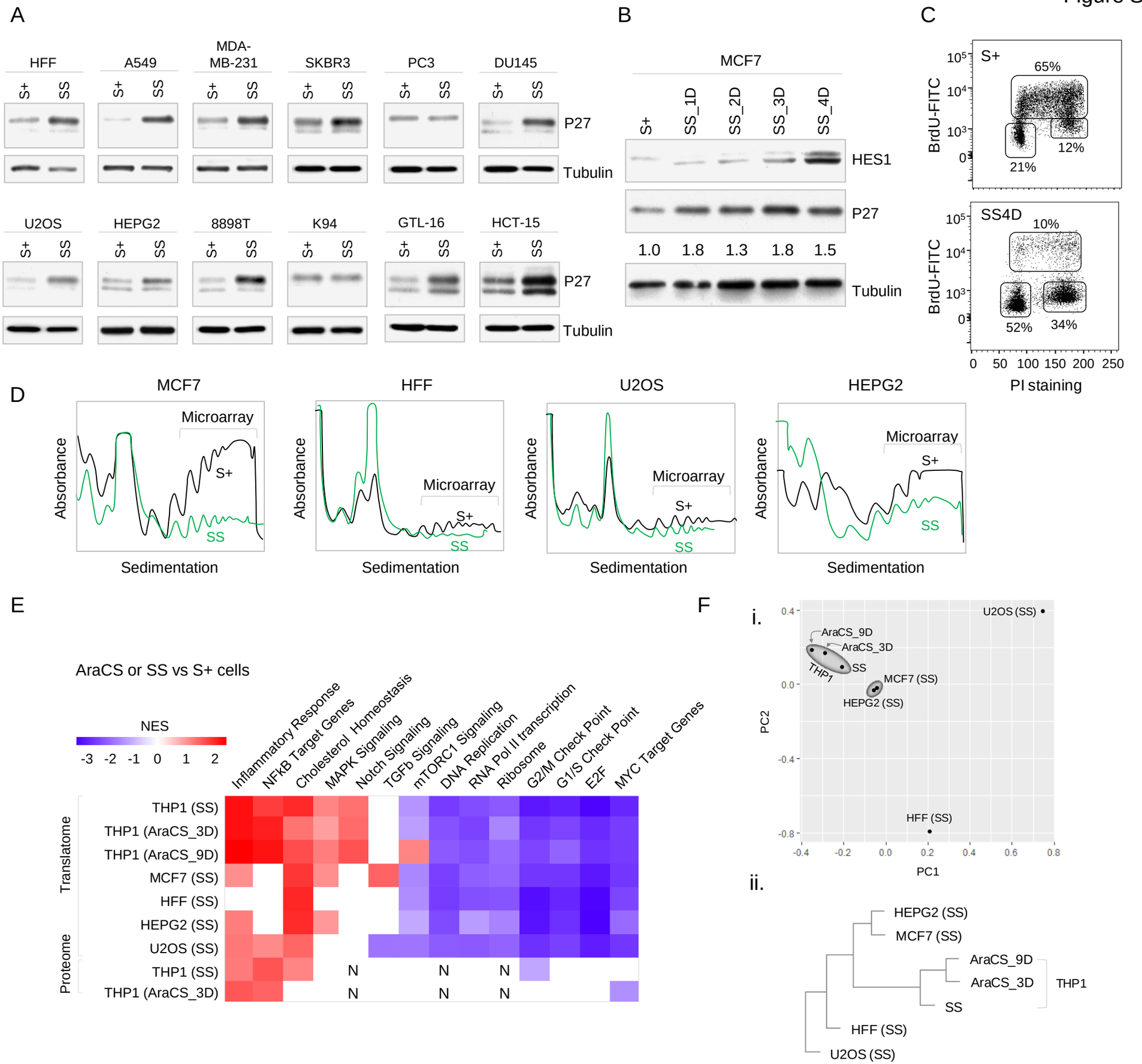

A
HFF
A549
MDA-MB-231
SKBR3
PC3
DU145
S+
SS
P27
Tubulin
U2OS
HEPG2
8898T
K94
GTL-16
HCT-15
B
MCF7
SS_1D
SS_2D
SS_3D
SS_4D
HES1
1.0 1.8 1.3 1.8 1.5
C
S+
65%
12%
21%
SS4D
10%
52%
34%
BrdU-FITC
PI staining
D
MCF7
HFF
U2OS
HEPG2
Microarray
Absorbance
Sedimentation
E
AraCS or SS vs S+ cells
NES
-3 -2 -1 0 1 2
Inflammatory Response
NFkB Target Genes
Cholesterol Homeostasis
MAPK Signaling
Notch Signaling
TGFb Signaling
mTORC1 Signaling
DNA Replication
RNA Pol II transcription
Ribosome
G2/M Check Point
G1/S Check Point
E2F
MYC Target Genes
Translatome
Proteome
THP1 (SS)
THP1 (AraCS_3D)
THP1 (AraCS_9D)
MCF7 (SS)
HFF (SS)
HEPG2 (SS)
U2OS (SS)
N
F
i.
U2OS (SS)
AraCS_9D
AraCS_3D
SS
THP1
MCF7 (SS)
HEPG2 (SS)
HFF (SS)
PC1
PC2
ii.
HEPG2 (SS)
MCF7 (SS)
AraCS_9D
AraCS_3D
SS
THP1
HFF (SS)
U2OS (SS)

Figure S2

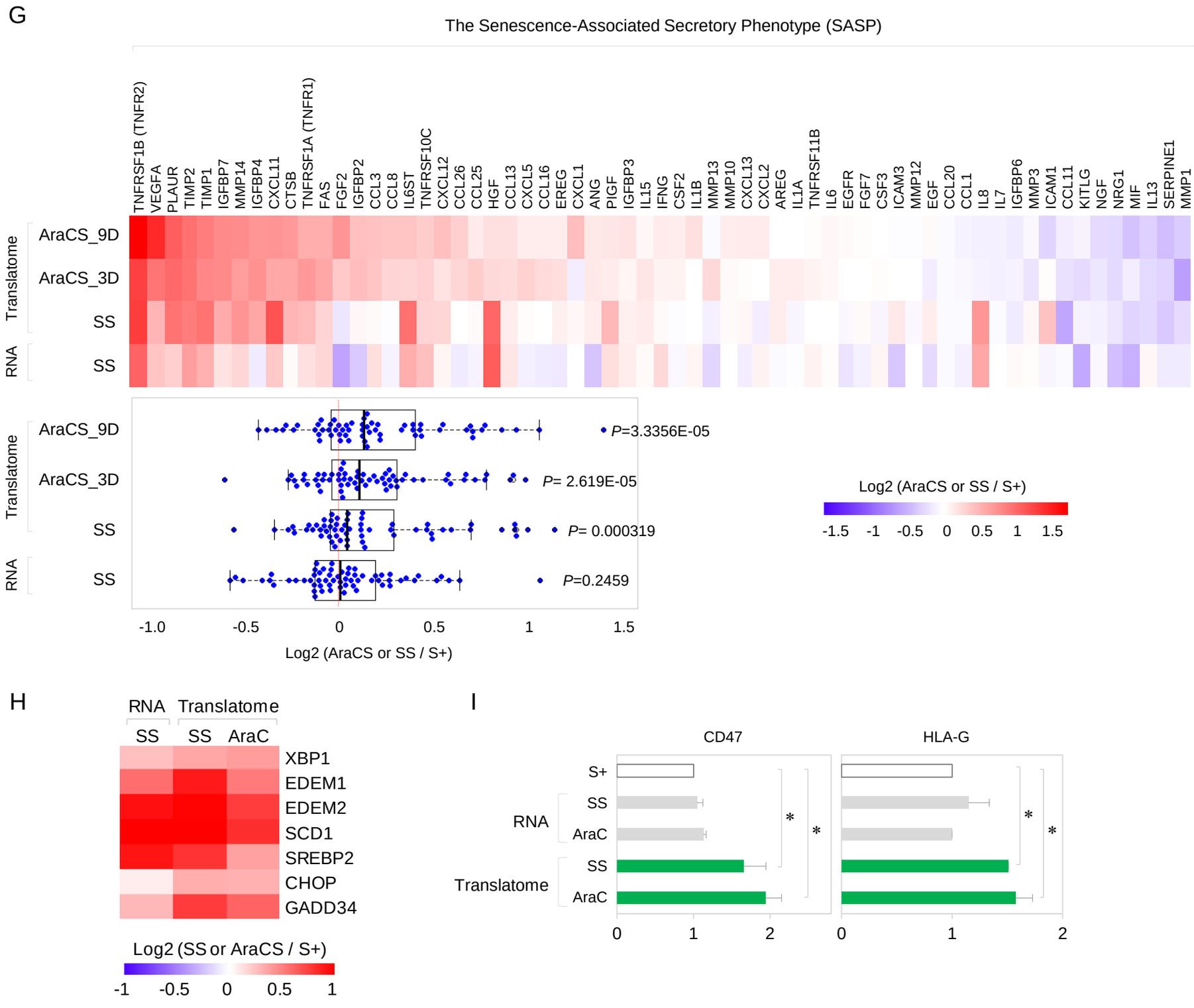

G
The Senescence-Associated Secretory Phenotype (SASP)
TNFRSF1B (TNFR2)
VEGFA
PLAUR
TIMP2
TIMP1
IGFBP7
MMP14
IGFBP4
CXCL11
CTSB
TNFRSF1A (TNFR1)
FAS
FGF2
IGFBP2
CCL3
CCL8
IL6ST
TNFRSF10C
CXCL12
CCL26
CCL25
HGF
CCL13
CXCL5
CCL16
EREG
CXCL1
ANG
PIGF
IGFBP3
IL15
IFNG
CSF2
IL1B
MMP13
MMP10
CXCL13
CXCL2
AREG
IL1A
TNFRSF11B
IL6
EGFR
FGF7
CSF3
ICAM3
MMP12
EGF
CCL20
CCL1
IL8
IL7
IGFBP6
MMP3
ICAM1
CCL11
KITLG
NGF
NRG1
MIF
IL13
SERPINE1
MMP1
Translatome
RNA
AraCS_9D
AraCS_3D
SS
SS
P=3.3356E-05
P= 2.619E-05
P= 0.000319
P=0.2459
-1.0
-0.5
0
0.5
1
1.5
Log2 (AraCS or SS / S+)
Log2 (AraCS or SS / S+)
-1.5
-1
-0.5
0
0.5
1
1.5
H
RNA
Translatome
SS
SS
AraC
XBP1
EDEM1
EDEM2
SCD1
SREBP2
CHOP
GADD34
Log2 (SS or AraCS / S+)
-1
-0.5
0
0.5
1
I
CD47
HLA-G
S+
SS
AraC
SS
AraC
RNA
Translatome
*
*
*
*
0
1
2
0
1
2

Figure S3

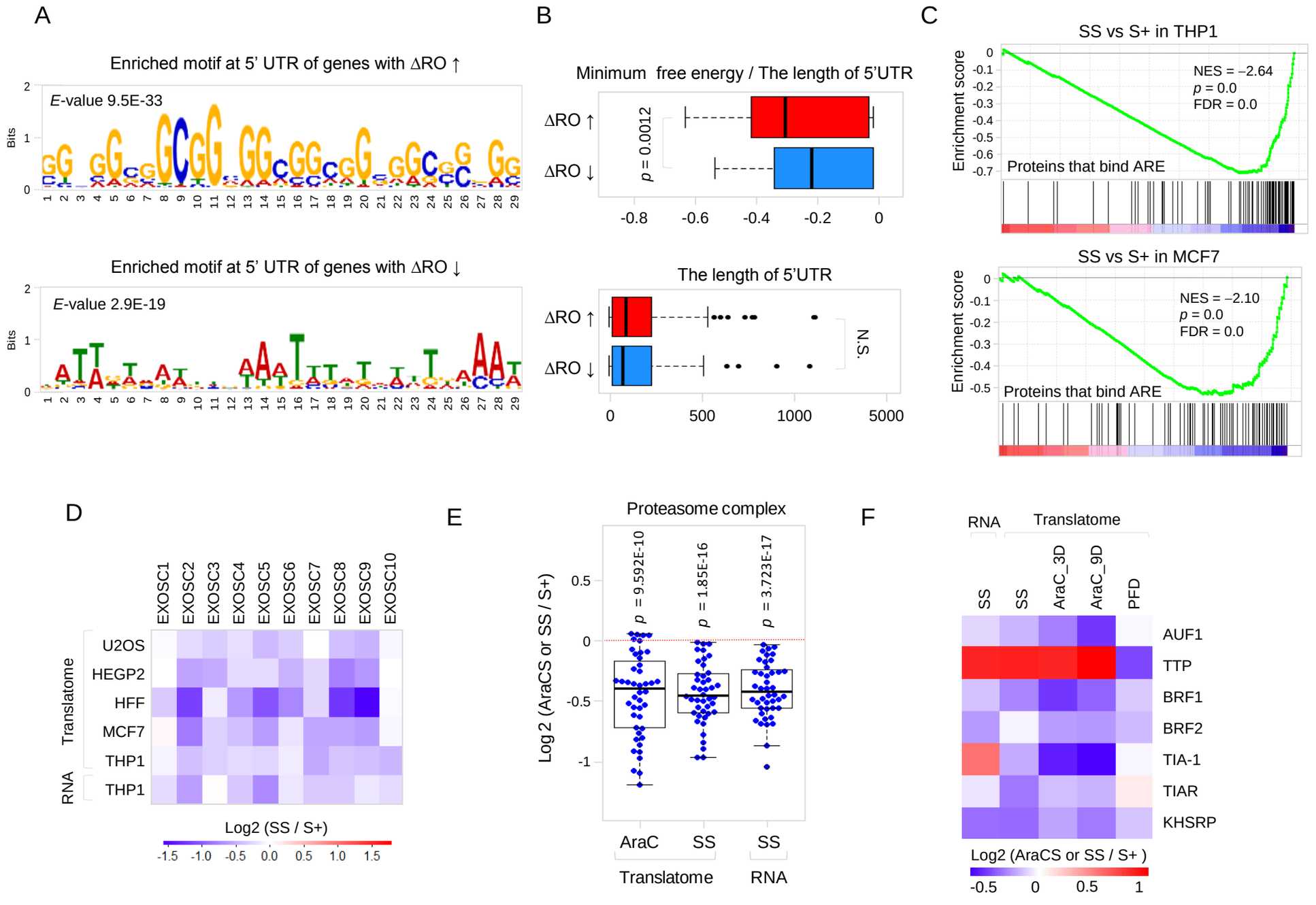

A
Enriched motif at 5' UTR of genes with ΔRO ↑
E-value 9.5E-33
Bits
Enriched motif at 5' UTR of genes with ΔRO ↓
E-value 2.9E-19
B
Minimum free energy / The length of 5'UTR
ΔRO ↑
ΔRO ↓
p = 0.0012
-0.8
-0.6
-0.4
-0.2
0
The length of 5'UTR
N.S.
0
500
1000
5000
C
SS vs S+ in THP1
Enrichment score
NES = –2.64
p = 0.0
FDR = 0.0
Proteins that bind ARE
SS vs S+ in MCF7
NES = –2.10
p = 0.0
FDR = 0.0
Proteins that bind ARE
D
EXOSC1
EXOSC2
EXOSC3
EXOSC4
EXOSC5
EXOSC6
EXOSC7
EXOSC8
EXOSC9
EXOSC10
Translatome
U2OS
HEGP2
HFF
MCF7
THP1
RNA
THP1
Log2 (SS / S+)
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
E
Proteasome complex
Log 2 (AraCS or SS / S+)
p = 9.592E-10
p = 1.85E-16
p = 3.723E-17
AraC
SS
SS
Translatome
RNA
F
RNA
Translatome
SS
SS
AraC_3D
AraC_9D
PFD
AUF1
TTP
BRF1
BRF2
TIA-1
TIAR
KHSRP
Log2 (AraCS or SS / S+)
-0.5
0
0.5
1

Figure S4

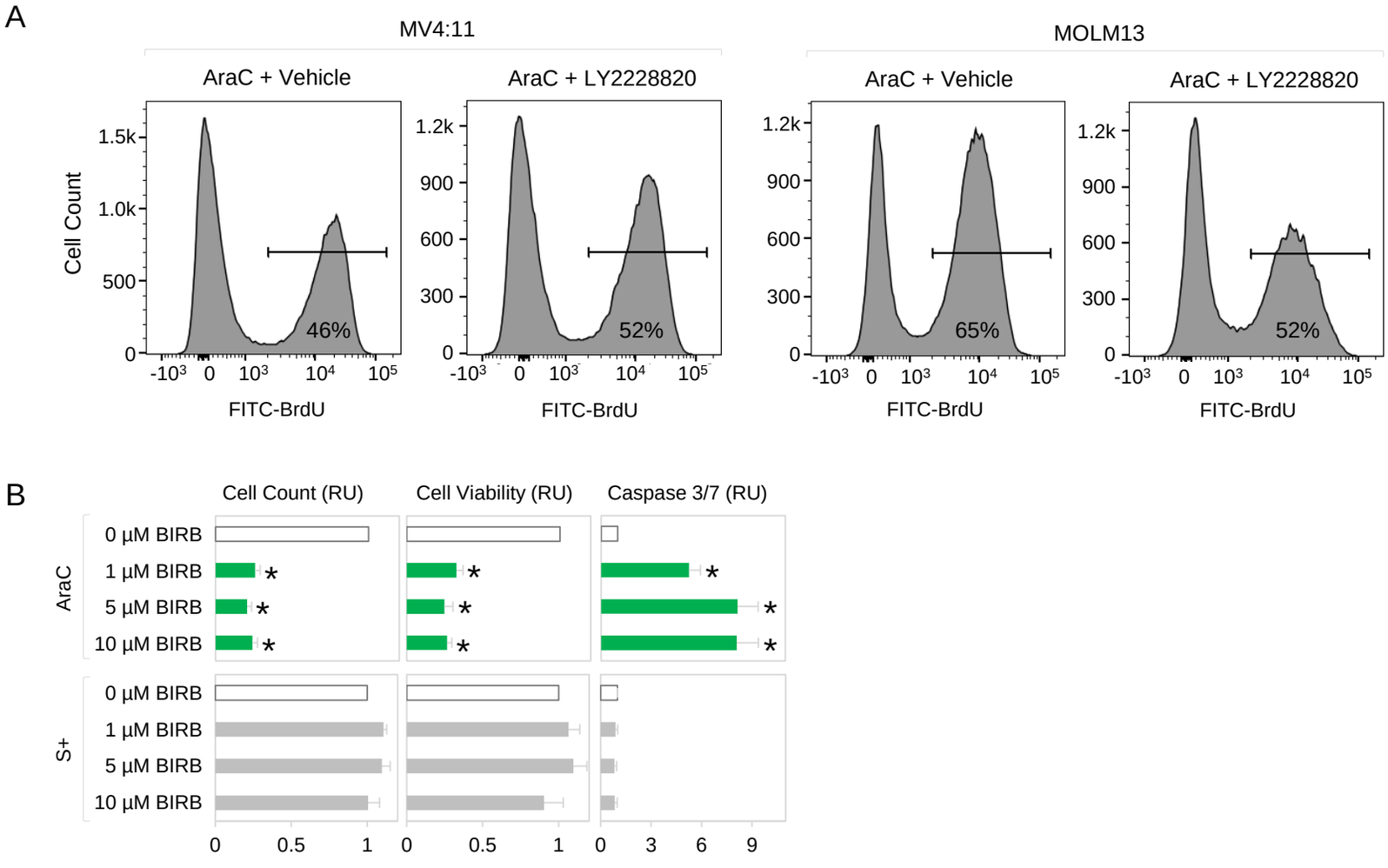

A
MV4:11
MOLM13
AraC + Vehicle
AraC + LY2228820
AraC + Vehicle
AraC + LY2228820
Cell Count
1.5k
1.0k
500
0
1.2k
900
600
300
46%
52%
65%
52%
-10³ 0 10³ 10⁴ 10⁵
FITC-BrdU
B
Cell Count (RU)
Cell Viability (RU)
Caspase 3/7 (RU)
AraC
S+
0 μM BIRB
1 μM BIRB
5 μM BIRB
10 μM BIRB
0 0.5 1
0 3 6 9

Figure S5

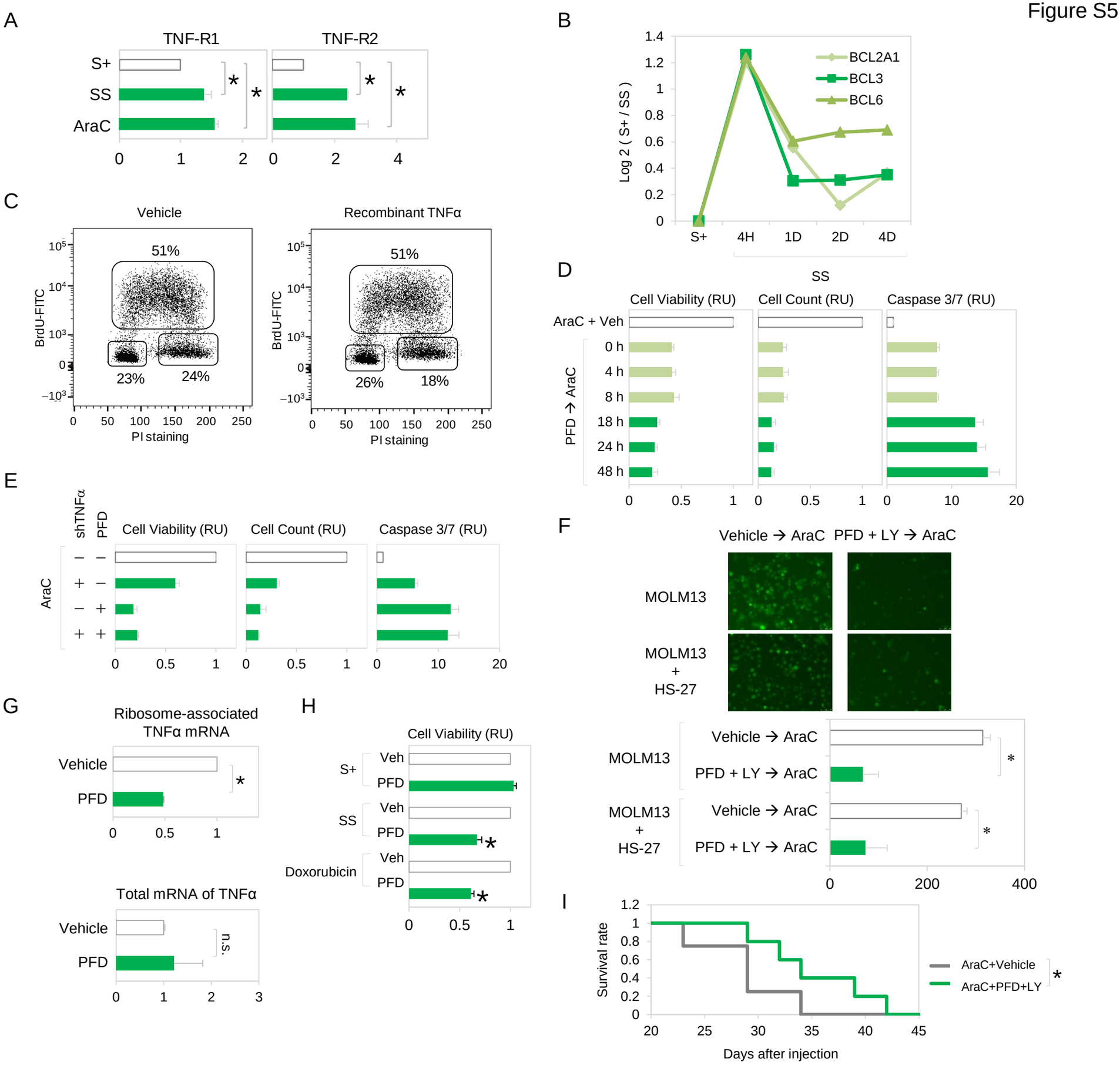
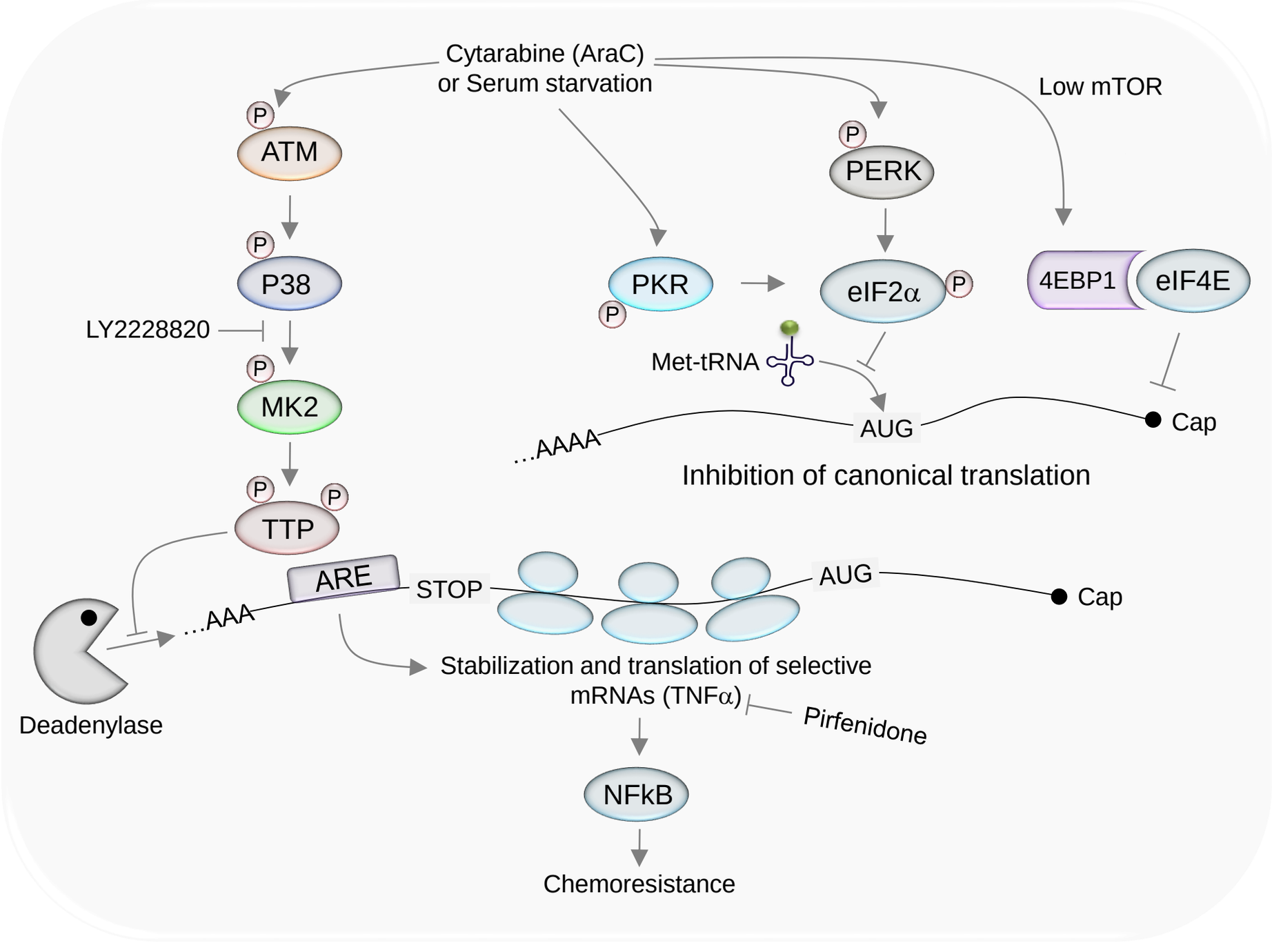

A
TNF-R1
TNF-R2
S+
SS
AraC
B
BCL2A1
BCL3
BCL6
Log 2 (S+ / SS)
S+
4H
1D
2D
4D
SS
C
Vehicle
Recombinant TNFα
51%
23%
24%
26%
18%
BrdU-FITC
PI staining
D
Cell Viability (RU)
Cell Count (RU)
Caspase 3/7 (RU)
AraC + Veh
PFD → AraC
0 h
4 h
8 h
18 h
24 h
48 h
E
shTNFα
PFD
AraC
Cell Viability (RU)
Cell Count (RU)
Caspase 3/7 (RU)
F
Vehicle → AraC
PFD + LY → AraC
MOLM13
MOLM13 + HS-27
G
Ribosome-associated TNFα mRNA
Vehicle
PFD
Total mRNA of TNFα
n.s.
H
Cell Viability (RU)
S+
SS
Doxorubicin
Veh
PFD
I
Survival rate
Days after injection
AraC+Vehicle
AraC+PFD+LY

Figure S6


Cytarabine (AraC)
or Serum starvation
Low mTOR
P
ATM
P
P38
LY2228820
P
MK2
P
P
TTP
P
PERK
PKR
P
P
eIF2α
4EBP1
eIF4E
Met-tRNA
AUG
Cap
...AAAA
Inhibition of canonical translation
ARE
STOP
AUG
Cap
...AAA
Deadenylase
Stabilization and translation of selective
mRNAs (TNFα)
Pirfenidone
NFkB
Chemoresistance