Targeting eIF4G1-dependent translation in melanoma

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Abstract

Elevated expression of components of eIF4F translation initiation complex has been documented in cancer, resulting in enhanced translation of mRNAs encoding pro-tumorigenic factors, including oncogenic proteins. We previously identified SBI-756, a small molecule that interferes with the eIF4F assembly and overcomes melanoma resistance to BRAF inhibitors. SBI-756 enhanced anti-tumor immunity in pancreatic cancer and was effective in the treatment of diffuse large B cell lymphoma. Here, we identified the eIF4G1 MA3 (4G1-MA3) domain as the target of SBI-756, attenuating eIF4F complex assembly. Melanoma cells expressing a mutant form of 4G1-MA3 exhibited polysome profiles resembling those of melanoma cells treated with SBI-756. A structure-based *in silico* screen against the eIF4G1 MA3 domain identified M19, a small molecule inhibitor that exhibited anti-melanoma effects. RNA sequencing (RNA-seq) revealed upregulation of UPR, mTOR, p53, and ROS signaling in M19-treated melanoma cells. Ribosome sequencing identified changes in ribosomal structure and electron transport chain components following M19-6 treatment of melanoma cells. Autophagy and histone deacetylase inhibitors were found to enhance anti-neoplastic activities of M19 or its analog, M19-6. M19-6 conferred a greater effect on melanoma than melanocytes and overcame melanoma resistance to BRAF or MEK inhibitors. Alone, M19-6 reduced melanoma growth and metastasis in a xenograft model. M19-6 offers a new therapeutic modality to overcome resistance and metastasis.

Introduction

Malignant melanoma is a rapidly metastasizing tumor that has served as a flagship model for cancer immunotherapy[1, 2], particularly following the limited success of chemo- or targeted therapies[3, 4]. Among novel factors proposed to enhance immunotherapy or overcome drug resistance are components of the eIF4F complex[5, 6, 7, 8]. However, a significant challenge in this effort is limiting excess eIF4F complex activity in tumors, given that this complex is essential for the viability of all cells.

Assembly of the eIF4F complex is the rate-limiting step in translation initiation[9]. Although eIF4F activity is required for cap-dependent translation of all cytoplasmic mRNAs, there is a a notable difference in requirements for eIF4F by housekeeping mRNAs (which are efficiently translated even when eIF4F complex activity or levels are low) versus cancer-promoting mRNAs (which require high eIF4F levels for optimal translation)[10, 11, 12]. Accordingly, eIF4F activity is frequently higher in malignant than in normal cells[10, 13, 14, 15, 16], providing a clinically exploitable therapeutic window[10, 13, 17, 18]. ElevatedeIF4F levels, commonly seen in tumors, including melanoma[19, 20], correlate with poor prognosis[7, 19], and increased eIF4F activity is linked to mTORC1 hyperactivation and 4E-BP inactivation[14]. As the major eIF4G paralogue, eIF4G1 is frequently overexpressed in malignancies, where it reportedly contributes in tumorigenesis and cancer progression, in part by regulating the DNA damage response and cell cycle activity[14, 21, 22, 23, 24,25]

Among small molecules that disrupt the eIF4F complex, we previously identified SBI-756, which binds to eIF4G1 and disrupts the eIF4F complex by targeting eIF4G1 independently of cellular 4E-BP status. This mechanism allows SBI-756 to act regardless of the eIF4E/4E-BP ratio in the cell[14]. Given that several oncogenes and tumor suppressors, including Myc and p53[7, 26], converge on the eIF4F complex, targeting this complex holds a promise in overcoming challenges associated with tumor heterogeneity, metastasis, and drug resistance[7, 8, 21, 27]. eIF4F inhibitors currently being tested in preclinical studies and clinical trials include 4EGI-1, which binds to eIF4E and prevents its association with eIF4G in a 4E-BP-dependent manner; the eIF4A inhibitors hippuristanol and rocaglates (e.g., silvestrol); and cap analogs and mimetics that inhibit eIF4E binding to the 5' mRNA cap[7, 28, 29]. Among the more recently developed eIF4F complex inhibitors is eFT226 (zotatifin), which inhibits eIF4A activity[30] and has demonstrated effectiveness against *Kras*-mutant lung cancer cells and triple-negative breast cancer[31, 32]. eFT226 has now reached clinical evaluation. Finally, a second-generation, more potent eIF4A inhibitor, MG-002, has recently been developed[33].

We recently demonstrated that SBI-756 inhibits melanomagenesis in several mouse models of human and mouse cell xenografts, including the genetic *Nras*^{Q61K}*::Ink4a*$^{-/-}$ melanoma model[34]. Independent studies have shown that SBI-756 is effective in a B-cell lymphoma model when combined with the BCL-2 inhibitor venetoclax[18]. Recent studies have revealed SBI-756's ability to promote anti-tumor immunity and attenuate the growth of pancreatic tumors in preclinical models[35]. SBI-756 treatment also promoted a gene expression signature resembling that caused by genetic ablation of eIF4G1[21]. Analysis of genetic changes in melanoma cells resistant to SBI-756 identified several genes associated with the cell cycle and DNA repair, hallmarks of eIF4G1

inhibition[21]. Nonetheless, suboptimal biophysical properties of SBI-756 have limited its further development, prompting the creation of a new generation of eIF4G1 inhibitors.

Here, we identify the MA3 domain of eIF4G1 as the target of SBI-756 and categorize a new class of small molecules that directly target the MA3 domain. Among the selected small molecules, M19 and its analog M19-6, disrupt the eIF4F complex and interfere with eIF4A/eIF4G1 interaction. Additionally, M19-6 demonstrated efficacy in eliminating melanoma as well as therapy-resistant cells, as a standalone treatment and, more effectively, when used in conjunction with an autophagy or histone deacetylase inhibitors. Preclinical evaluations reveal M19-6's ability to reduce the growth and metastatic spread of melanoma.

Results

Identifying the MA3 domain as an SBI-756 target.

To identify eIF4G1domain that binds SBI-756, we transduced human melanoma cell lines A375 and UACC903, both stably expressing Cas9 (see Methods for details), with an sgRNA library consisting of 947 guides to target the eIF4G1 gene. After selecting sgRNA-expressing cells, we treated them with increasing concentrations of SBI-756. We then harvested viable cells and extracted DNA for sequencing to identify the sgRNAs conferring SBI-756 resistance. That analysis identified two gRNAs that targeted sequences either within (gRNA-235) or proximal to (gRNA-622) the eIF4G1 MA3 domain [36] (Figure 1a, *Figure S1a*), suggesting that this domain is required for SBI-756 targeting of eIF4G1. To verify that these sgRNAs conferred resistance to SBI-756, we transduced A375 melanoma cells with either gRNA-235 or gRNA-622 and selected individual clones that were more resistant to SBI-756 treatment. The selected gRNA-235 transduced single clone had a 14-base pair deletion within the MA3 domain, resulting in early termination of the eIF4G1 transcript and protein. In contrast, the selected gRNA-622 transduced single clone led to 12-base pair deletion within a domain proximal to MA3, which did not result in early termination of the eIF4G1 transcript (*Figure S1b*). Previous studies suggest that the MA3 domain is important for the eIF4G1/eIF4A interaction[37, 38, 39, 40]. These findings are consistent with studies performed in *S. Cerevisiae* transduced with an eIF4F gene set library to identify components of the translation initiation machinery conferring SBI-756 resistance. That analysis revealed that the expression of truncated forms of eIF4G1 and eIF4A1 genes was sufficient to confer SBI-756 resistance (*Figure S1c*).

We next monitored the viability of single clones expressing either gRNA-235 or gRNA-622 following SBI-756 treatment. Compared to control (EV) sgRNA-transduced cells, melanoma cells expressing either gRNA-235 or gRNA-622 exhibited increased resistance to SBI-756 treatment (Figure 1b). Additionally, melanoma cells transduced with gRNA-235 showed reduced proliferation relative to EV or gRNA-622-transduced cells (*Figure S1d*).

Since SBI-756 disrupts eIF4F complex assembly[8], we investigated whether melanoma cells expressing either gRNA-235 or gRNA-622 show changes in eIF4F complex composition. Using pull-down analysis with the methyl-^{7}GTP cap analogue to capture complex components, we observed that the eIF4F complex levels were lower in melanoma cells expressing gRNA-235, as indicated by a notable decrease in eIF4G1 and increased abundance of 4E-BP1 in the pull-down material as compared to EV controls or gRNA-622 expressing cells (Figure 1c). Conversely, SBI-756 treatment disrupted the eIF4F complex formation in EV cells but did not appear to exert a major effect in gRNA-235-expressing cells. Interestingly, gRNA-622-expressing cells did not exhibit eIF4F complex dissociation and continued to respond to SBI-756 treatment, suggesting that a different mechanism may underlie melanoma resistance to SBI-756 in this context. Given that the MA3 domain is implicated in the eIF4A/eIF4G1 interaction, we investigated whether this interaction was disrupted in melanoma cells expressing mutant eIF4G1 forms. While melanoma cells expressing gRNA-235 showed clear inhibition of eIF4A/eIF4G1 interaction, the interaction was not disrupted in gRNA-622-expressing cells (Figure 1d), again suggesting that distinct mechanisms account for the effect of gRNA-235 and gRNA-622 on the resistance of melanoma cells to SBI-756.

To characterize possible differences in translation activity betweenMA3-mutant and control cells, we performed polysome profiling[41, 42] of single-cell clones harboring the MA3 mutation within eIF4G1. Notably, heavy polysome-to-monosome ratio in MA3-mutant melanoma cells largely overlapped with that induced by SBI-756-treated parental cells (Figure 1e). Moreover, relative to a vehicle control, SBI-756 induced dramatic reduction in heavy polysome-to-monosome ration in parental, but not gRNA-235- and gRNA-622-expressing melanoma cells (Figure 1e). This observation suggests that MA3 mutations generated in our CRISPR screen likely play a role in the effects of SBI-756 on the eIF4F complex.

In Silico screen identifies small molecules that bind the MA3 domain.

To identify small-molecule inhibitors of MA3 protein, we conducted a structure-based *in silico* screen targeting a binding site on the surface of a crystal structure of the C-terminal portion of human eIF4GI(PDB:1UG3)[43]. The selected binding site included residues Ala1292, Ile1293, Glu1343, Thr1346, Gln1350, Leu1384, and Lys1386 within the MA3 domain, predicted to be essential for drug binding. This selection was informed by blind docking experiments with SBI-756 and an assessment of the druggability of the MA3 domain surface. A molecular dynamics simulation (30ns, using the Desmond suite from Schrodinger[44]) also revealed that SBI-756 formed stable interactions with these residues (Figure 2a). The stability of these interactions, alongside the depth and hydrophobic characteristics of the identified binding cavity (Figure 2a), provided compelling evidence of the druggability of this selected pocket.

Next, we performed a virtual screening of the MA3 binding pocket to identify potential small-molecule inhibitors of eIF4G1. To accomplish this, we screened a library of 10 million drug-like compounds through molecular docking followed by filtering steps[45]. The initial docking phase

identified 54,500 compounds that exhibited more favorable docking scores than the benchmark compound SBI-756. From this pool, we selected a chemically diverse subset of 500 compounds using various filtration tools described in the Methods section. Final selection was carried out to exclude compounds with undesirable properties such as toxicity, promiscuity, and low solubility, resulting in 150 compounds. These were then clustered based on chemical diversity to yield 67 potential inhibitors of MA3 protein (*Figure S2a*).

Assessment of lead MA3 interacting compounds in melanoma.

Of those, 63 were available and purchased from ChemBridge (CB), Enamine (E), and Molport (M). We then assessed all 63 for (i) toxicity against melanoma and other tumor cell lines, (ii) effect on melanoma lines relative to non-transformed melanocytes, and (iii) impact on gRNA-235-transduced melanoma cell lines. Initially, we tested compounds for toxicity against six melanoma cell lines representative of diverse genetic mutations, as well as prostate cancer and a pancreatic cancer cell line. Of the 63 compounds evaluated, 4 (CB6, E8, E10, and M19) were selected for further analyses (*Figure S2b*), and, among them, E10 and M19 exhibited selectivity for melanoma cells relative to the immortalized melanocyte line H3A (Figure 2b). Compared to EV controls, the toxicity of the M19 compound was reduced in A375 cells expressing gRNA-235 (*Figure S2c*). Reducing serum levels further sensitized A375 melanoma cells to M19 treatment (*Figure S2d*). Finally, M19 was the most potent amongst the selected compounds in disrupting eIF4G1:eIF4A binding and eIF4F complex assembly (Figures 2c, 2d). Collectively, these data led us to select M19 for further experimental validation.

Melanoma cells treated with M19 exhibit altered gene expression.

To identify possible changes in gene expression following M19 treatment, we performed RNA-Seq using A375 melanoma cells treated for 24 hours with either control vehicle or M19 compound (20 μM). Differential expression analysis identified 633 genes upregulated and 543 downregulated following M19 treatment (Figure 3a). Gene set enrichment analysis (GSEA) of these genes showed enrichment of pathways involved in apoptosis, UPR, ROS, as well as mTORC1 and p53 signaling (*Figure S3a*). Ingenuity pathway analysis (IPA) of these gene sets further identified activation of oxidative stress response pathway genes associated with NRF2 as well as UPR and WNT/beta-catenin signaling pathway genes (*Figure S3b*, *Table S1*). Quantitative PCR of 14 upregulated and 2 downregulated genes confirmed these expression changes (*Figure S3c*). Among the downregulated pathways were E2F target genes and genes involved in the G2M cell cycle control, also confirmed by qPCR analysis (*Figure S3d*). Pathways identified as upregulated by M19 treatment may explain the toxicity that we observed in cultured tumor cells and are consistent with pathways altered by inhibition of translation initiation complex components[21].

This analysis also guided us in mapping possible pathways that may complement M19 effects to better sensitize tumor cells to eIF4F inhibition.

Characterization of select M19 analogs.

Following the identification of M19, we initiated a similarity search using the M19 scaffold as the basis to find analogs with potentially greater efficacy. This search involved screening the entire ZINC20 library, retrieving 3000 compounds with a Tanimoto similarity score above 0.65. We eliminated compounds with undesirable characteristics by employing the same filtration process used in the initial structure-based screen. The next step involved comparing the docking scores of these analogs against the parent M19 compound to identify those with superior scores. Through this rigorous selection process, we successfully identified and subsequently acquired 13 M19 analogs, all predicted to have enhanced binding affinity to the MA3 domain.

Subsequently, our experimental evaluation of 13 M19 analogs revealed that four of them (M19-5, M19-6, M19-12, and M19-13) demonstrated enhanced potency compared to the original M19 compound in assays carried out in A375 cells (*Figure S4a*). Of these, M19-6 was the most potent compound when tested in colony-forming assay (CFE, *Figure S4b*) or spheroid formation (Figure 3b). M19-6 showed superior efficacy in eIF4F complex dissociation compared to M19 (Figure 3c). Thus, we selected M19-6 for further characterization, given its greater potency against melanoma relative to melanocytes and based on changes in gene expression seen in treated cells, which resembled those seen after M19 treatment, the most notable being in UPR (ATF4, DDIT3, and ATF3) and ROS (ERN1, HERPUD1, HHMOX, and c-Jun) signaling (*Figure S4c*). Overall, when we monitored mRNA translation in the presence of M19-6 or other protein synthesis inhibitors, we observed dose-dependent inhibition by M19-6, which was lower compared with the commercial inhibitor 4EGI-1 that blocks eIF4E-eIF4G1 binding and is known to be toxic (*Figure S4d*). Treatment with M19-6 for three hours effectively induced A375 melanoma cell death (*Figure S4e*).

We further assessed M19-6 effects against melanoma lines grown in either 2D or 3D (spheroid) cultures. Analysis performed in both conditions revealed select effectiveness of M19-6 against A375, WM793, Mel-5, ZR75-1, and H1693 cells, as well as against BRAF inhibitor-resistant A375 cells, but not against the IGR37, IGR39, Mel28, WM3629 or MeWO cells (*Figure S4f*). To determine whether Nras or Braf mutations might confer greater sensitivity to M19-6, we treated melanoma cells harboring these genetic driver mutations with M19-6 and observed that Nras-mutant melanoma cells were more sensitive to M19-6 than Braf-mutant melanoma cells (*Figure S4g*). Comparable to changes observed in melanoma cells treated with SBI-756 and with M19 parental compounds, M19-6 induced S1 arrest and apoptosis in melanoma cells, effects that exhibited time dependency (Figure 3d). Spheroids formed from different BRAF or MEK inhibitor-resistant melanoma cell lines were also sensitive to M19-6 (Figure 3e, 3f).

Translatome Analysis of M19-6 Treated Cells

To investigate how M19-6 influences mRNA translation, we conducted ribosome profiling (Ribo-Seq) on A375 cells treated with M19-6 for eight hours, along with a control treatment (Figure 4A). Ribo-Seq, a sequencing-based method, enables genome-wide assessment of mRNA translation efficiency when paired with RNA sequencing (RNA-Seq)[41]. On average, we obtained approximately 37 million and 10 million mapped reads for Ribo-Seq and RNA-Seq, respectively, targeting coding regions of the transcriptome. Micrococcal Nuclease (MNase) digestion generated ribosome footprints with a central peak around 33 nucleotides (nt) (Figures S5A). Consistent with expectations, metagene plots of the Ribo-Seq data displayed a strong accumulation of ribosome footprints within the CDS regions of mRNAs (Figures S5B, C). Principal component analysis (PCA) of Ribo-Seq and RNA-Seq libraries indicated high reproducibility among biological replicates, with clear separation between M19-6-treated and control samples (Figure XB). We detected 11,468 and 11,255 highly expressed mRNAs (RPKM > 1) in Ribo-Seq and RNA-Seq datasets, respectively, using the longest CDS for each gene (Table S2).

To evaluate translation efficiency between control and M19-6-treated A375 cells, we applied the deltaTE (ΔTE) method[46], an enhanced version of DESeq2 optimized for assessing translation efficiency from the changes in Ribo-Seq and RNA-Seq data[46, 47] (Figure 4C; Table S3). Our analysis identified 46 mRNAs down-regulated in translation efficiency under M19-6 treatment (Figure 4C). In contrast, only three mRNAs—TUBA1A, HIST2H2AC, and HIST1H2AJ—showed increased translation efficiency in response to M19-6 treatment. Notably, most translation efficiency changes fell into the "Exclusive" category, where mRNA levels remained unchanged, but Ribo-Seq reads were significantly up- or down-regulated (Figure 4C). These findings suggest that M19-6 primarily affects gene expression through translational regulation.

Among the gene ontology (GO) groups translationally down-regulated by M19-6 in A375 cells (p-adj < 0.1 and log2 fold change in TE < -0.5) were mRNAs encoding proteins associated with the ribosomal structure and electron transport chain components (Figure 4D). The latter is consistent with the changes observed in viability of melanoma cells following treatment with M19-6, and resemble changes observed upon inhibition of other eIF4F inhibitors, which affected proteins involved in ribosomal structure[48].

M19-6 synergizes with autophagy and histone deacetylase inhibitors to eradicate melanoma cells.

To identify activities that might synergize with M19-6 in promoting melanoma cell death, we re-assessed cellular functions that were perturbed by M19 treatment in RNA sequencing analysis. Among the notable pathways upregulated upon M19 treatment was the signature of genes implicated in autophagy (*Figures S4c, S4d*). Indeed, increased LC3 processing was seen in melanoma cells subjected to M19 treatment. We thus assessed whether inhibition of autophagy, which is expected to augment survival pathways following M19 treatment, would enhance the death of melanoma cells in response to M19-6. To this end, we asked whether treatment with

the autophagy inhibitor chloroquine (CQ)[49] would increase the death of M19-6- -treated melanoma cells.

Notably, we observed a dose-dependent synergy between CQ and M19-6 in decreasing the survival of melanoma spheroids. An approximately 4-fold lower concentration of M19-6 (5 μM) relative to single drug treatment was required to achieve effective inhibition of melanoma spheroid growth in the presence of a relatively low dose (1.25 μM) of CQ (Figure 4a).

To further identify possible pathways that could augment the effect of M19-6 we performed screen of MedChemExpress, an FDA approved small molecules (see methods for details). Six of the 14 inhibitors that were confirmed in this screen were tested, of which HDAC inhibitors, Chidamide and CI-994, exhibited the most pronounced inhibition of melanoma cell viability, when combined with M19-6 (*Figures S4h, S4i*). Indeed, HDAC inhibitor FK-228 synergized with M19-6 (combination of 625 ng of M19-6 with 5nM of FK-228 achieved ~70% inhibition; Figure 4b). These finding point to the ability to augment M19-6 own effectiveness by co-targeting select cellular pathways including autophagy and histone deacetylases. In both cases, concentration of M19-6 required for this cooperation was markedly reduced.

Next, we assessed the stability of M19-6, using mouse hepatocytes assays. We observed that M19-6 exhibited greater T1/2 (19.27 min) and metabolic stability of 12.92% after 30 min, compared with 11.33 for Verapamil, a drug commonly used to treat hypertension (*Table S4*).

To evaluate M19-6 effectiveness against melanoma cells *in vivo,* we treated C3H mice inoculated with SW1 melanoma cells (harboring Nras mutation)[50, 51, 52] with M19-6 and monitored tumor growth weekly. While M19-6 decreased tumor growth by ~20% (Figure 4c), melanoma metastasis to the lungs was effectively inhibited (Figure 4d). These findings suggest that, as a single agent, M19-6 effectively inhibits melanoma metastasis.

Discussion

The eIF4F complex presents challenges as a therapeutic target in cancer since it is essential for the viability of all cells. However, this apparent disadvantage may be exploited, as complex components are generally overexpressed in tumors relative to normal cells[10,12,13]. Strong genetic evidence supporting the targeting of eIF4F has come from analysis of mice heterozygous for eIF4E, which are reportedly resistant to cancer development despite exposure to carcinogens[53]. Among the small molecule inhibitors designed to target this complex, a limited number have advanced to clinical trials, with the eIF4A inhibitor eFT226 being a notable example[54]. Thus, this study was designed to fill an unmet clinical need, leveraging our earlier analyses of SBI-756, a small molecule that interacts with eIF4G1 and dissociates the eIF4F complex[8].

Herein, we first discovered that a small molecule inhibitor, SBI-756, binds the MA3 domain of eIF4G1. This finding guided structure-based *in silico* screen that identified M19 and its analog

M19-6, novel small molecule inhibitors of eIF4G1. Notably, compared to SBI-756, these compounds demonstrate dose-dependent suppression of melanoma cell proliferation in 2D and 3D culture models. Furthermore, they effectively disrupted the eIF4F complex and inhibited the interaction between eIF4G1 and eIF4A, underscoring their potential therapeutic efficacy. RNAseq analysis of melanoma cells treated with M19 revealed ROS, UPR, and apoptosis signaling activation, indicating that M19 induces cell death programs. Further assessment using riboseq approach led to the identification of ribosomal structure and electron transport chain components that were inhibited following treatment with the 19-6 compound, consistent with the sensitization of melanoma cells to apoptosis, while resembling the requirement of other eIF4F inhibitors for structural ribosomal proteins[32]. In a search for pathways that may complement apoptotic signaling, we hypothesized that autophagy may serve as a salvage pathway to rescue tumors from cell death. Indeed, we found that pharmacological targeting of the autophagy pathway combined with treatment with M19 compounds enhanced the extent of melanoma cell death and reduced (4 to 5-fold) the concentration of compounds required to achieve a potent effect. An unbiased screen of >2000 FDA approved drugs was carried out to identify inhibitors that may augment M19-6 activity, leading to the identification of HDAC inhibitors among top candidates. Further assessment using three independent HDAC inhibitors confirmed their synergy with M19-6 in mediating melanoma cell death, while reducing amount of M19-6 inhibitor required to achieve effective inhibition. These observations are consistent with independent studies whereby single inhibitor may not be sufficient to inhibit tumor growth effectively despite their effectiveness. Given the risks to normal cells from overly inhibiting the eIF4F complex, achieving significant anti-tumor effects was shown to require combination therapies that target multiple pathways. Accordingly, combining eFT226 with MEK inhibitors reportedly augments eFT226 activity and is more effective than a single agent against Kras-mutant lung tumors [32]. Notably, Myc-driven overexpression of the components of translational machinery has been shown to sensitize NSCLC cell lines to eIF4A inhibitors[32].

Notably, M19-6 ability to attenuate eIF4F complex assembly enables it to overcome therapy resistance, consistent with findings made with the SBI-756[8], M19-6 is effective against BRAF- and MEK-resistant melanoma, confirming the need for complex dissociation to counteract therapy resistance.

Targeting of the eIF4F complex has been primarily achieved using small molecules that target eIF4E or eIF4A components to block respective cap-dependent translation or helicase activity[7, 13]. Moreover, reluctance to target eIF4G1 has been justified by a lack of structural data or based on its “adaptor-like” function[55]. Indeed, our earlier generation small molecule, SBI-756, is an effective inhibitor of the eIF4F complex, although its biophysical properties precluded its further development. Of the >140 SBI-756 analogs previously developed, none exhibited activity superior to SBI-756, while its pharmacokinetics was suboptimal. We have now overcome these limitations by identifying the eIF4G1 MA3 domain as the SBI-756 target and used structure-based analysis to identify and characterize a new generation of small molecule eIF4G1 inhibitors. Pharmacological eIF4G1 inhibition with these inhibitors was more effective than genetic inactivation of the MA3

domain, likely due to the presence of the eIF4G2 isoform, which also harbors the MA3 domain[36]. Indeed, siRNA-based eIF4G2 knockdown (KD) in melanoma cells harboring gRNA-235 potentiated cell death phenotypes seen in the presence of gRNA-235 alone. We anticipate that targeting the MA3 domain by M19-6 will impact both eIF4G isoforms and thus would also potentiate cell death seen in the absence of only one isoform. Moreover, decreasing the M19-6 dose when combined with an autophagy or HDAC inhibitor should confer greater efficacy and selectivity.

Previous studies demonstrated that eIF4G1 inhibition perturbed the cell cycle and DNA repair processes [21], changes also observed in the presence of SBI-756[8] and the M19 compound series. Disruption in cell cycling is a long-sought goal in cancer research that has guided the development of numerous inhibitors that have reached clinical evaluation; nonetheless, all must be used in combination with reagents that target other cellular pathways to achieve effective and sustained clinical outcomes, as exemplified by CDK4/6 inhibitors[56]

Could inhibition of eIF4E or eIF4A be combined with that of eIF4G1? Such a combination is unlikely to be advantageous since it would impact a single signaling pathway rather than complementary pathways, as seen in synthetic lethality interactions. Thus, the choice of a different small molecule to target eIF4F components may depend on (i) tumor type in terms of overexpression of a specific eIF4F component and (ii) the ability to reach synthetic lethality by a combination of eIF4G1 inhibitors with inhibitors to other key cellular regulatory pathways. These decisions will likely require the stratification of tumors based on the relative expression of eIF4F components and survival pathways that signify a given tumor. In agreement, recent studies demonstrated the effectiveness of SBI-756 in pancreatic cancer susceptibility to anti-tumor immunity, a tumor type in which eIF4G1 expression is upregulated[35].

Overall, our work describes the development of the new generation of eIF4G1 inhibitors that target the MA3 domain of the eIF4G1 gene. Exemplified by M19-6, this new class of inhibitors offers advantages over existing eIF4F inhibitors and merits further evaluation in preclinical and clinical models.

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Methods

Cell culture

All human and mouse melanoma cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Cytiva, Marlborough, MA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Cytiva). Cell cultures were maintained at 37^0C in 5% CO_2.

Reagents

Compounds used in this study were purchased from ChemBridge (San Diego, CA), Enamine (Monmouth Junction, NJ), and MolPort (Beacon, MA), as indicated. Chloroquine was obtained from Sigma-Aldrich. Compounds were dissolved in DMSO and stored as a 10 mM stock solution at -20°C. Each compound was freshly prepared from the stock solution before use. Compounds used in animal studies were synthesized by Pharmaron (Qindao, China) at >96% purity.

Cell viability assay

All cell lines were seeded in 96-well white plates (5,000 cells in 100 µl per well) or 384-well white plates (500 cells in 50 µl per well) and allowed to attach overnight. Test compounds were serially diluted from 10 mM stock solutions and added to cells. Assays were performed in triplicate, and each microplate included media and DMSO control wells. Cell viability was assessed 72 h after treatment using CellTiter-Glo (Promega) according to the manufacturer's protocol. Cell growth inhibition was calculated as a percentage of DMSO-treated controls.

For the spheroid growth inhibition assay, cells were seeded in 96-well (1000 cells in 100 µl per well) or 384-well (250 cells in 50 µl per well) white Corning spheroid microplates and allowed to grow 24 h to form spheroids. Test compounds were serially diluted and added to each well. Assays were performed in triplicate, and each microplate included media and DMSO control wells. Cell viability was assessed 5 days later using CellTiter-Glo 3D (Promega) according to the manufacturer's protocol. Cell growth inhibition was calculated as a percentage of DMSO-treated controls.

Colony formation assay

Cells were plated in triplicate (500 cells/well) in 6-well plates and allowed to grow overnight before compounds were added. After 1–2 weeks, depending on the cell line, colonies were stained with Crystal Violet solution (Sigma-Aldrich) for 30 min. Plates were rinsed with water, and images were acquired by scanning. Colony number and size were analyzed using Image J software. Colony formation efficiency was calculated relative to the number of colonies in control (DMSO)-treated wells.

Antibodies, Immunoprecipitation, and Western blot analyses

Cells were rinsed with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing protease inhibitors and a phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Protein concentration was determined using Coomassie Plus Protein Assay Reagent (Thermo Scientific). Immunoprecipitation (IP) of eIF4A was performed from cell lysates (300 μg) incubated overnight with 10 μg of anti-eIF4A antibodies (BioLegend, San Diego, CA, cat# 666202), followed by incubation with Protein A/G PLUS-Agarose beads (Santa Cruz, SC-2003) for 1 h. Beads were then washed 4 times with lysis buffer, and IP'd proteins were eluted with SDS loading buffer and analyzed by Western blot. Equal amounts of cell lysate proteins (20-50 μg) or proteins eluted from agarose beads were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences). Membranes were blocked 1 h with 3% BSA/TBST and incubated with primary antibodies overnight at 4°C, with shaking. Following three TBST washes, membranes were incubated 1 h at room temperature with secondary antibodies. Detection and quantification were made using an Odyssey Infrared Imaging System (LICOR Biosciences). Antibodies against eIF4G1, eIF4E, and 4EBP1 were purchased from Cell Signaling Technology (cat# 8701, cat# 2067, cat# 9644). Actin and HSP90 antibodies were obtained from Santa Cruz Biotechnology (cat# sc-58673, cat# sc-13119). Secondary antibodies were goat anti-rabbit Alexa Fluor 680 (cat# A21076), goat anti-mouse Alexa Fluor 680 (cat# A21057), and goat anti-rat Alexa Fluor 680) (cat# A21096) (Invitrogen). All antibodies were used according to suppliers' recommendations.

Construction of EIF4G1 tiling sgRNA library

The custom EIF4G tiling sgRNA library consisting of 947 unique sgRNAs was designed using the Broad Institute Genetic Perturbation Platform (GPP) sgRNA designer tool [57, 58, 59]. Library sequences were synthesized on an array platform (Genscript, NJ) and then cloned into the lentiGuide Puro vector (Addgene: #52963, a kind gift from Dr. Feng Zhang, MIT) using Golden Gate Assembly. The library plasmid was amplified using ElectroMAX™ Stbl4™ Competent Cells (Invitrogen, cat # 11635018). To ensure representation, the number of bacterial colonies was maintained at >500 times the number of sgRNA sequences in the library. sgRNA sequences in the library are deposited as indicated in the data availability section.

Selection of SBI-756-resistant clones and identification of corresponding gRNAs

The pooled tiling CRISPR library virus was produced using HEK293T cells transfected with pMD2.G and psPAX2 in the presence of Polyethylimine (PEI). A375 cells (2 x 10^6) were then transduced with the pooled library lentivirus in DMEM complete medium and 0.8 μg/ml polybrene. Twenty-four hours later, virus, polybrene, and medium were removed, and cells were plated in fresh culture medium. Forty-eight hours after that, puromycin was added at 1 μg/ml to select for sgRNA library-expressing cells. The multiplicity of infection (MOI) was adjusted to 0.3-0.4 to prevent >1

sgRNA infection per cell. Cells were cultured at >1,000X of the library size to ensure adequate sgRNA representation. Cells were treated with indicated SBI-756 concentrations for 2-4 weeks until resistant clones emerged. Genomic DNA extraction from drug-resistant cells was performed using the Zymo QuickDNA™ Midiprep Plus Kit (Zymo Research, Cat. D4068), according to the manufacturer's instructions.

gRNA sequencing data processing

A two-step PCR-amplification for sequencing library preparation was conducted with TaKaRa Ex Taq™ Polymerase (TaKara, Cat. RR001) and custom primers, to achieve adequate sequencing coverage in 1x75bp single-end reads, following published guidelines[60]. Barcoded libraries were pooled and sequenced using an Illumina Hiseq 500 platform.

CRISPR screen data analysis:

The sequence and Pfam domains of the longest transcript of EIF4G1 gene (ENST00000424196.5) was obtained from the Ensembl database. Raw reads were sequentially trimmed for 3' and 5' adapters using Cutadapt v2.8 with parameters "-j 4 -e 0.15 -a GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC" and "-j 4 -e 0.15 -m 18 -g TTTATATATCTTGTGGAAAGGACGAAACACCG" respectively. The trimmed reads were aligned to EIF4G1 longest transcript sequence using STAR v2.7.0d_0221[61]) with parameters "--runThreadN 8 --alignEndsType EndToEnd --outFilterMismatchNmax 0 --outFilterMultimapScoreRange 0 --outReadsUnmapped Fastx --outSAMtype BAM Unsorted--outFilterMultimapNmax 1 --outSAMunmapped None --outFilterScoreMinOverLread 0 --outFilterMatchNminOverLread 0 --outFilterMatchNmin 20 --alignSJDBoverhangMin 1000 --alignIntronMax 1". Signal bigwig files were generated using Deeptools bamCoverage v3.2.0[62] with parameters "—normalize using CPM --exactScaling".

Selection of single-cell gRNA clones

Individual gRNA235 or gRNA622 virus was produced using 293T cells following standard protocols and used to infect A375 cells following puromycin selection (1 µg/ml). Single-cell clones were generated by serial dilution (0.2 cell/100 µl) in 96-well plates. The resulting single-cell clones were treated with SBI-756, and resistant clones were selected. Genomic DNA was extracted from selected clones, and the region corresponding to the gRNA was PCR-amplified and sequenced to confirm specific deletion at the targeted region.

m^7GTP pull-down assay

Cells growing in 100 mm plates and treated with indicated compounds were washed with cold PBS, collected, and lysed in lysis buffer [50 mM MOPS/KOH (pH 7.4), 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% NP40, 1% Na-DOC, 7 mM β-mercaptoethanol containing protease

inhibitors and a phosphatase inhibitor cocktail). Lysates were incubated 20 min with m^7-GDP-agarose beads (Jena Bioscience, Jena, Germany) with rotation and washed three times with wash buffer (50 mM MOPS/KOH, pH 7.4, 100 mM NaCl, 50 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 7 mM β-mercaptoethanol, 0.5 mM PMSF, 1 mM Na3VO4 and 0.1 mM GTP). Bound proteins were eluted by incubating beads in a loading buffer. Materials pulled down by m^7-GDP-agarose were analyzed by Western blot.

Monitoring of newly synthesized proteins by puromycin labeling

For labeling experiments, A375 cells were seeded in 6-well plates (3 x10^5 cells per well). After 24 h, cells were pre-incubated 1 h with different compounds prior to treatment with 3 µg/ml puromycin for 30 min. Cells were washed 2 times with ice-cold PBS and lysed with lysis buffer. Cell lysates were subjected to Western blot analysis.

Flow cytometry analysis of the cell cycle.

A375 cells that express the gRNA constructs were subjected to FACS analysis following their trypsinization, fixation (60 min in 70% ice-cold ethanol), and washes in phosphate-buffered saline, followed by incubation for30 min at 25°C in propidium iodide (PI) buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 50 µg of PI per ml, and 10 µg of RNase A/ml). Stained cells were acquired using the FACSort flow cytometer (BD Biosciences), and DNA content was analyzed using FlowJo software.

Analysis of apoptosis

A375 cells were seeded in 96- or 384-well white plates, treated with different concentrations of indicated compounds, and apoptosis was measured at different time points using Caspase-Glo 3/7 (Promega, G8091), according to the manufacturer's protocol. Caspase-Glo 3/7 3D (Promega, G8981) was used to analyze A375 spheroid apoptosis induced by different treatments.

Reverse transcription and real-time qPCR

Total RNA from DMSO- and compound-treated cells was extracted using a total RNA miniprep kit (Zymo Research). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit from Life Technologies, according to the manufacturer's protocol. Real-time PCR was performed on a Bio-Rad CFX Connect Real-Time System using SsoAdvanced Universal SYBR®Green Supermix from Bio-Rad. H3A served as an internal control. Quantitative PCR reactions were performed in triplicate. PCR primers were designed using PrimerBank (http://pga.mgh.harvard.edu/primerbank) and are listed (*Table S3*).

Polysome profiling

A375-gRNA235 and A375-gRNA622 cells were seeded in 150 mm plates and treated with 0.2 µM SBI-756. After 24 h, cells were harvested and lysed in hypotonic lysis buffer (5 mM Tris HCl, pH

7.5, 2.5 mM MgCl2, 1.5 mM KCl, 100 µg/ml cycloheximide, 2 mM DTT, 0.5% Triton, 0.5% sodium deoxycholate). Polysome-profiling was carried out as described by Gandin et al.[42]. Fractions were collected, and RNA was extracted using TRIzol, based on the manufacturer's instructions. Experiments were done in independent triplicate samples.

RNA-seq

Total RNA was extracted using a total RNA miniprep kit (Sigma) with the supplied On-column DNase I for the digestion step. Libraries were prepared from isolated total RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Barcoded libraries were pooled and single end-sequenced (1X75) on the Illumina NextSeq 500 system, using the High output V2.5 kit (Illumina Inc., San Diego, CA). Read data was processed and multiplexed with the BlueBee Genomics Platform (BlueBee, San Mateo, CA).

RNA-seq data processing

The Illumina adapter, polyA/polyT and low-quality sequences were trimmed from raw RNA-seq reads using Cutadapt v2.3. The trimmed reads were then aligned to human genome version hg38 and Ensembl gene annotations version 84 using STAR v2.7.0d_0221[61] with parameters adopted from ENCODE long RNA-seq pipeline (https://github.com/ENCODE-DCC/long-rna-seq-pipeline). Gene-level counts and normalized expression (TPM) were obtained using RSEM v1.3.1[3]. Trimmed reads and alignment qualities were assessed using FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC v1.8[63]. Genes with RSEM-estimated counts equal to or greater than 5 times the total number of samples were retained for downstream analysis. Differential expression comparison was performed using DESeq2 v1.22.2[47]. Gene set enrichment analysis was performed using the GSEA app v4.3.2[64]

In silico screen for MA3-bound small molecules

To map SBI-756 binding to the MA3 structure, a blind docking technique (molecular docking without a defined target site) was first performed using Glide[65] and FRED docking software[66]. This analysis revealed a potential site where SBI-756 formed stable bonds during a 30ns molecular dynamics simulation performed with the Desmond suite from Schrodinger[44].

To identify small molecule inhibitors of eIF4GI, we carried out a virtual screen of the MA3 binding pocket. Based on molecular docking of a large library with subsequent filtering[45], a screen with a library of 10 million drug-like compounds (deposited in the ZINC20 database[67]) was carried out. Compounds were docked in the pocket based on three docking software systems, namely, GlideSP[65], FRED[66], and Autodock[68, 69, 70], which were shown to improve hit rates[71, 72]. Binding poses predicted by these systems were compared, and compounds with consensus predictions (binding poses within 3Å distances) were selected. These compounds were ranked based on Glide docking scores. A total of 54,500 compounds received a more favorable docking score than the

predicted SBI-756 with the docking score of -4.7. As SBI-756 presents an active scaffold targeting the MA3 pocket, we utilized its structure to build pharmacophore models to screen the filtered library to identify molecules with SBI-756-like binding features that lacked SBI-756 toxicity. Developed pharmacophore models contained three hydrophobic features corresponding to each of the three separate SBI-756 ring systems—one hydrogen donor and one hydrogen acceptor corresponding to the nitrogen and oxygen atom of the quinolone group. These features were used in different combinations to screen the filtered library and select 500 chemically diverse compounds. Finally, we selected compounds with drug-like physicochemical properties less likely to promote toxicity and exhibit off-target binding, based on empirical and machine learning-based methods. To this end, we first applied Andrew's scoring metric, which assesses how well a molecule binds to an average receptor, by assigning partial scores to each functional group in the molecule[71, 72]. Potentially promiscuous compounds with a high Andrew's score were eliminated. SBI-756 appeared in the top 10th percentile among the 500 scored compounds and thus is likely toxic due to its general receptor promiscuity, consistent with our earlier studies[73]. Second, we ran the remaining compounds through the Swiss-ADME platform to predict physicochemical properties and potential off-target effects[72]. Compounds predicted to inhibit 5 or 6 CYP family enzymes, which are critical for drug metabolism and clearance[74], were filtered out. Compounds receiving predicted log P values (lipophilicity) <5 were selected, as highly lipophilic compounds tend to be more promiscuous and are minimally soluble[75]. Compounds with low solubility in aqueous media (log S < -6) were excluded to avoid low bioavailability. SBI-756 was predicted to be on the borderline of lipophilicity and solubility scales, receiving 4.6 and -5.18 scores, respectively. The remaining 150 compounds were clustered, and cluster representatives were selected. Clustering procedures ensure that the purchased compound set is as chemically diverse as possible, maximizing the likelihood of finding an active scaffold. 67 potential inhibitors that bind the MA3 pocket were selected for biological evaluation.

Ribosome Profiling

Cultured cells were lysed in-dish using 250 µL of 2X lysis buffer, containing 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10 mM $MgCl_2$, 200 µg/mL cycloheximide (CHX), and 2 mM dithiothreitol (DTT), supplemented with cOmplete™ Protease Inhibitor Cocktail. Lysed cells were scraped into a eppendorf tube (~500µL), 5 µL of RiboLock RNase inhibitor (ThermoFisher Scientific, Cat#EO0382), and 5 µL of Turbo DNase (ThermoFisher Scientific, Cat#AM2238) were added to each lysate. The mixture was vortexed briefly (5 sec), incubated on ice for 10 minutes, and then centrifuged at 16,000× g for 10 minutes at 4°C. The optical density was recorded at A260 using a Nanodrop to assess concentration. Samples were stored at -80°C, with a portion reserved for RNA-Seq prior to Micrococcal Nuclease (MNase) treatment.

For ribosome profiling, lysates underwent MNase digestion (25U MNase (Sigma) per A260 unit) for 45 minutes at room temperature. The digestion was halted by adding EGTA. Monosomes were isolated by layering the digested polysomes over a 10-50% sucrose gradient, followed by centrifugation at 36,000 rpm for 3 hours at 4°C (Beckman Coulter). RNA was then extracted from the monosomes fraction using Trizol (Invitrogen) as per the manufacturer's guidelines. In parallel, total RNA was extracted with Trizol from a portion of the untreated lysate for RNA-seq analysis.

Ribo-seq library preparation followed the most recent ribosome profiling protocol, with slight modifications (PMID 28579404). RNA samples were size-selected on a 15% TBU-Urea gel, with fragments between 25–40 nt isolated. The gel slices containing these fragments were incubated in 700 µL of RNA gel extraction buffer (300 mM NaOAc pH 5.5, 1 mM EDTA, 0.25% SDS) on dry ice, then rotated overnight at room temperature. Footprint fragments were recovered via isopropanol precipitation. The ribosome footprints were dephosphorylated with T4 Polynucleotide Kinase (T4 PNK) for 1 hour at 37°C in 1X T4 PNK buffer, with the addition of 10 U SUPERase*In and 5U T4 PNK (10 U/µL). Ligation was achieved by adding the ligation mix (17.5% PEG-8000, 1X T4 RNA ligase buffer, 100 U T4 Rnl2(tr) K227Q (200 U/µL), and 1 µM pre-adenylated linkers) to the samples. Ligated RNA was separated on a 10% TBE-Urea gel, extracted, and purified using RNA extraction buffer and isopropanol. Reverse transcription was conducted with SuperScript III (Invitrogen), and samples were circularized with CircLigase I (Lucigen). A round of rDNA depletion was performed using biotin-labeled antisense probes and Dynabeads MyOne Streptavidin C1[76]. The final libraries were size verified on an 8% TBE gel, quantified, and sequenced on an Illumina Platform.

For RNA-seq library preparation, the same protocol was applied, with modifications. The NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) was used for rRNA removal, and the rRNA-depleted RNA was fragmented using the NEBNext® Magnesium RNA Fragmentation Module.

To process sequencing data, initial trimming of adapters from reads was conducted with Cutadapt 3.4 (See below for reference), allowing no mismatches in adapter sequences, and discarding reads shorter than six nucleotides. Trimmed reads were then demultiplexed by sample-specific barcodes anchored at the 5' end, with no mismatches permitted. Sequential alignment was performed against the human transcriptome, beginning with cytoplasmic and mitochondrial rRNAs and tRNAs, using Bowtie 1.3.0[77]. Reads with low quality or shorter than 25 nucleotides were removed. Remaining reads were aligned to the comprehensive transcriptome (Gencode v32[78]) with Bowtie 1.3.0, allowing two mismatches. The SAM files generated were converted to BAM files, sorted, indexed, and processed to remove PCR duplicates with UMI-Tools 1.0.1[79]. The final BAM files were converted into an SQLite format for analysis in Trips-Viz, a dedicated platform for Ribo-seq data[80].

For metagene profile generation, annotated start and stop codons of coding sequences were aligned across all principal transcript isoforms by read length. To estimate the A-site position of

each read, we calculated the nucleotide distance from the CDS start to the most prominent peak upstream, adding three nucleotides to this value.

Differential Expression Analysis

Count matrices for RNA-Seq and Ribo-Seq data were generated using the "Read Table" function in Trips-Vis[80], which quantifies reads that uniquely align to the CDS region of mRNAs based on inferred A-sites. A Ribo-Seq read contributes to the CDS count if its inferred A-site falls within the annotated start and stop positions of the CDS in a given transcript. The genomic coordinates for each read were assessed to ensure unambiguous mapping. Differential analysis was conducted with the deltaTE (ΔTE) package in R (version 1.34.0[46]), which extends DESeq2 by applying an interaction model. For each gene, adjusted p-values (p-adj) were computed to assess fold changes between two experimental conditions across Ribo-Seq, RNA-Seq, and TE levels, with a significance threshold of p-adj < 0.05 for each fold change. These fold changes allowed categorization of genes into eight groups, as described in the text. In the scatter plots for fold changes between Ribo-Seq and RNA-Seq (Figure 4C), genes with "NA" values in any of the comparisons (Ribo-Seq or RNA-Seq) were omitted. PCA plot values in Figure XB were also obtained using DESeq2.

Hepatocyte stability assay

A hepatocyte stability assay was performed by Pharmaron (Beijing, China). Briefly, mouse hepatocytes (0.5×10^6/ml) were dispensed into microtubes and 10 μL aliquots incubated (10 min) with the test compound (100 μM) or Verapamil (positive control; 100 μM), followed by incubation with 6 volumes of cold acetonitrile with IS (100 nM alprazolam, 200 nM labetalol, 200 nM caffeine and 200 nM diclofenac) to terminate the reaction. Samples were then centrifuged (45 minutes at 3,220 g), and 100 μL aliquots of supernatants were diluted with 100 μL ultra-pure H_2O, for LC/MS/MS analysis.

Screen for FDA approved drugs that augment M19-6 activity

The Cell Titer-Glo Luminescent Cell Viability Assay (Promega) was utilized to measure viability of A375 melanoma cells after their treatment with M19-6 in combination with compounds from the MedChemExpress, an FDA-Approved small molecule drug library. To optimize screening conditions, five different cell concentrations were tested alongside their tolerance to DMSO. Ultimately, a concentration of 1,000 cells per well with less than 2.5% DMSO was selected for the assay. A titration of M19-6 was then performed, to establish a baseline concentration for the screening process, which was set to achieve cell viability at 70% after 48 hours. The library was then screened at 6.25 μM (for each of the library compounds), using M19-6 at 40 μM. Cells treated solely with M19-6 were considered as the negative control (0% activity), while wells

containing only media (without cells) served as the positive control (100% activity). Compounds were ranked based on their percent activity, resulting in 81 hits selected for further testing. The selected compounds underwent a five-point dose-response assessment (25, 12.5, 6.25, 3.125, and 1.5625 µM) in triplicate, both with and without M19-6 at 40 µM. In this analysis, 14 compounds demonstrated dose-dependent activity and an increased IC50 in the absence of M19-6, leading to their selection for a more extensive dose-response evaluation. The latter consisted of a 15-point dose response with a 1:2 dilution factor starting at 100 µM (100, 50, 25, etc.), and was conducted in triplicate with and without M19-6 (40 µM).

Xenograft tumors

All animal studies were conducted at the SBP Animal Facility under the Institutional Animal Care and Use Committee guidelines (IACUC # 21-064). Six-week-old male C3H/HeNHsd mice were purchased from Inotiv and allowed to acclimatize for one week. SW1 cells (8×10^5, suspended in 200 µl sterile PBS) were injected subcutaneously into the flanks of mice. When tumor sizes reached ~150 mm^3, mice were sorted into different groups and treated with IP injection of Vehicle or M19-6 (100 mg/kg, twice per day). Mice were maintained in a pathogen-free environment with free access to food. Body weight and tumor volume were measured twice a week. Tumor size was measured with linear calipers and calculated using the formula: ([length in millimeters × (width in millimeters)^2]/2). Mice were sacrificed after 4 weeks, and tumors and lungs were fixed in Z-Fix and paraffin-embedded for immunohistochemistry analysis.

Statistics and Reproducibility

Statistical significance was assessed by ordinary one-way ANOVA and two-way ANOVA. GraphPad Prism 9 software (Graphpad, La Jolla, CA) performed all statistical calculations. All cell culture experiments were performed three times. Data is presented as mean ± SD (unless noted otherwise in figure legends). A P value <0.05 was considered statistically significant.

Primer information

ATF4: Forward, 5'-ATGACCGAAATGAGCTTCCTG-3'; Reverse, 5'-GCTGGAGAACCCATGAGGT-3'

XBP1: Forward, 5'-AGAAGTCCGTCCACTCTCTCA-3'; Reverse, 5'-GCTCGGGATTTTACCAGTTCAT-3'

DDIT3: Forward, 5'-GGAAACAGAGTGGTCATTCCC-3'; Reverse, 5'-CTGCTTGAGCCGTTCATTCTC-3'

HSPA5: Forward: 5'-CATCACGCCGTCCTATGTCG-3'; Reverse, 5'-CGTCAAAGACCGTGTTCTCG-3'

ERN1: Forward: 5'-AGAGAAGCAGCAGACTTTGTC-3'; Reverse, 5'-GTTTTGGTGTCGTACATGGTGA-3'

PPP1R15A: Forward, 5'-ATGATGGCATGTATGGTGAGC-3'; Reverse, 5'-AACCTTGCAGTGTCCTTATCAG-3'

EIF2AK3: Forward, 5'-GGAAACGAGAGCCGGATTTATT-3'; Reverse, 5'-ACTATGTCCATTATGGCAGCTTC-3'

CYP1A1: Forward, 5'-TCGGCCACGGAGTTTCTTC-3'; Reverse: 5'-GGTCAGCATGTGCCCAATCA-3'

HERPUD1: Forward, 5'-TGCTGGTTCTAATCGGGGACA-3'; Reverse, 5'-CCAGGGGAAGAAAGGTTCCG-3'

HMOX1; Forward, 5'-AAGACTGCGTTCCTGCTCAAC-3'; Reverse, 5'-AAAGCCCTACAGCAACTGTCG-3'

JUN: Forward, 5'-TCCAAGTGCCGAAAAAGGAAG-3'; Reverse, 5'-CGAGTTCTGAGCTTTCAAGGT-3'

TGFB2: Forward, 5'-CAGCACACTCGATATGGACCA-3'; Reverse, 5'-CCTCGGGCTCAGGATAGTCT-3'

PPP2R2B: Forward, 5'-CCACACGGGAGAATTACTAGCG-3'; Reverse, 5'-TGTATTCACCCCTACGATGAACC-3'

WNT7B: Forward, 5'-GAAGCAGGGCTACTACAACCA-3'; Reverse, 5'-CGGCCTCATTGTTATGCAGGT-3'

IL1B: Forward, 5'-ATGATGGCTTATTACAGTGGCAA-3'; Reverse, 5'-GTCGGAGATTCGTAGCTGGA-3'

ATF3: Forward, 5'-CCTCTGCGCTGGAATCAGTC-3'; Reverse, 5'-TTCTTTCTCGTCGCCTCTTTTT-3'

Author contributions:

YF, NS, IT, and ZAR designed the studies, and YF performed the experiments. RM and HK carried out bioinformatic analyses of RNAseq. MR and AC performed *in silico* screens. SO, ES, and MJ carried out small molecule screens and medicinal chemistry for select compounds. AD generated the eIF4G1 CRISPR library, and IL performed a screen for SBI-756 resistance in yeast. MA, CD, and NS carried out polysome sequencing. IT and PJ carried out polysome profiling. JV provided MEK-resistant melanoma cells. AM performed a pathological analysis of melanoma metastasis. NS, IT, AC, and ZAR provided critical data assessments. ZAR, IT, MA, NS, AC, and SO wrote the manuscript.

Competing Interests:

ZAR is a co-founder and scientific adviser of Pangea Biomed. No conflict is declared by any of the other authors.

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional information may be requested from the corresponding author. RNAseq raw data has been deposited in a public database (GSE-271201).

Figure Legends

Figure 1. Identifying MA3 domain as SBI-756 target on eIF4G1

CRISPR screen identifies eIF4G1 domains targeted by SBI-756. A CRISPR library of sgRNAs prepared against different eIF4G1 gene domains was used to transduce A375 and UACC903 human melanoma cells, which were then treated with puromycin to select for sgRNA-expressing cells and with increasing SBI-756 concentrations. (A) Genomic DNA from cells was PCR-amplified and Sanger-sequenced. Count #s of reads was mapped to each sgRNA. Two enriched sgRNAs (gRNA-235 and gRNA-622) were mapped to the eIF4G1 MA3 domain. gRNA-235 falls within the MA3 domain and results in early termination (aa 1322 of 1606) and thus excludes the W2 domain, which reportedly interacts with eIF4A, from this transcript. gRNA-622 is located proximal (upstream) of the MA3 domain. (B) MA3 mutant melanoma cells are more resistant to SBI-756 than parental MA3 domain WT melanoma cells. gRNA-235 and gRNA-622 melanoma cells obtained as described in Methods were treated with indicated concentrations of SBI-756, followed by the analysis of their viability (72h later) using CellTier-Glo. (C) M^7GTP pull-down assay. Proteins prepared from gRNA-235, gRNA-622 A375 melanoma cells that were treated with indicated SBI-756 concentrations were incubated with m^7GTP-agarose beads to capture the eIF4F complex. Indicated proteins were detected by Western blot analysis. Each protein band was quantified by ImageJ, normalized to eIF4E levels as indicated. Right panel shows the total cell lysate. Each protein band was quantified by ImageJ, normalized to actin levels as indicated. (D) Proteins prepared from EV, gRNA-235 and gRNA-622 A375 cells were immunoprecipitated with eIF4A antibody and detected with indicated antibodies. (E) Polysome profiling of human melanoma A375 cells harboring WT eIF4G1 or eIF4G1 with a mutant MA3 domain (sg235 and sg622), prior to and after SBI-756 treatment. Three replicates are shown, as is the polysome/monosome ratio (right panels).

Figure 2. In Silico screen to identify MA3 domain bound small molecules

(A) The proposed binding mode of SBI-756 to the surface of the eIF4G1 MA3 domain. Left, high-view of SBI-756 binding to the MA3 domain (PDB: 1UG3). Green areas, hydrophobic patches; red areas, solvent-exposed. Right, ligand-protein interactions of SBI-756 inside the proposed pocket. Blue shading, solvent-exposed; lines with arene symbols, hydrophobic interactions; arrowed lines, hydrogen bonds. Bonds shown were maintained throughout the 30ns molecular dynamics simulation. (B) A375 and H3A cells were seeded in 96-well plates and treated with different concentrations of indicated compounds. Cell viability was measured after 72 h. (C) A375 cells were treated as indicated. After 24 h, cells were harvested followed by proteins extraction, immunoprecipitation and Western blot analysis with indicated antibodies. Each protein band was quantified by ImageJ, normalized to eIF4A levels as indicated. (D) Proteins prepared from A375 cells treated as indicated were incubated with m^7GTP-agarose beads to capture the eIF4F

complex. Indicated proteins were detected by Western blot analysis. Each protein band was quantified by ImageJ, normalized to eIF4E levels as indicated.

Figure 3. RNAseq analysis of M19-treated melanoma and characterization of M19 analogs.

(A) Volcano plot comparing the transcriptomes of M19 treated and control A375 samples. The numbers of up- and down-regulated genes are indicated in the top corners. Representative genes that were up- or down-regulated upon treatment are denoted. (B) A375 and H3A cells were seeded in 96-well or 384-well Corning spheroid microplates. After 24h, indicated compounds were added to respective wells and cell viability was measured after 4 days using CellTiter-Glo 3D. (C) M^7GTP pull-down assay and immunoprecipitation assay. Left, proteins prepared from A375 cells treated with indicated compounds were incubated with m^7GTP-agarose beads to capture the eIF4F complex. Indicated proteins were detected by Western blot analysis. Each protein band was quantified by ImageJ, normalized to eIF4E levels as indicated blow blots. Right, proteins prepared from A375 cells treated with indicated compounds were incubated with anti-eIF4A antibody followed by Western blot analysis. Each protein band was quantified by ImageJ, normalized to eIF4A levels as indicated. (D) Left, A375 cells were treated with indicated concentrations of M19-6. After 24h, cells were collected and fixed followed by FACS analysis. Right, A375 cells were treated with indicated concentrations of M19-6. Apoptosis was measured using caspase-Glo 3/7 kit. (E) and (F) BRAF inhibitor-resistant and MEK inhibitor-resistant cell lines were grown as spheroids and treated with M19-6. Cell viability was measured after 4 days.

Figure 4: Translatome of M19-6 compound in A375 cells

(A) Schematic illustrating the experimental workflow for RNA-Seq and Ribo-Seq library preparation and sequencing in A375 cells. Biological replicates of A375 cells were treated with a control compound or M19-6 for eight hours prior to analysis. (B) Principal Component Analysis (PCA) of Ribo-Seq (left) and RNA-Seq (right) datasets, showing clear segregation by treatment. Each circle represents a biological replicate, with treatments color-coded for distinction. (C) Scatter plot depicting the relationship between fold changes (FC) in RNA-Seq and Ribo-Seq data for control versus M19-6-treated cells. Statistically significant mRNAs (p-adj < 0.05, Log2 FC > 0.2 or < -0.2) are categorized and color-coded as follows: *Intensified Up* (increased transcription and translation efficiency [TE]), *Intensified Down* (decreased transcription and TE), *Forwarded Up* (concurrent increases in mRNA and RPF with unchanged TE), *Forwarded Down* (concurrent decreases in mRNA and RPF with unchanged TE), *Exclusive Up* (increase in RPF and TE without mRNA change), *Exclusive Down* (decrease in RPF and TE without mRNA change), *Buffered Up* (decreased transcription/increased TE), and *Buffered Down* (increased transcription/decreased TE). (D) Gene Ontology (GO) enrichment analysis of translationally

down-regulated mRNAs in M19-6-treated cells compared to control, with p-values adjusted for multiple comparisons via the Benjamini–Hochberg method. Down-regulated mRNAs with p-adj < 0.1 and -0.5 < Log2 FC < 0.5 were included in this analysis, with n = 49.

Figure 5. M19-6 inhibits melanoma metastasis.

(A) A375 cells were seeded in 384-well Corning spheroid microplates and treated 24h later with indicated concentrations of M19 and chloroquine. Cell viability was measured after 4 days using CellTiter-Glo 3D. (B) Analyses of HDAC inhibitor (FK-228) with M19-6. A375 cells were seeded in 384-well plates and 24h later, M19-6 and FK-228 were added to respective wells at the indicated concentrations. Cell viability was measured after 72h days using CellTiter-Glo. (C) Volume of vehicle or M19-6 treated (Vehicle, n=8; M19-6, n=6) SW1 tumor grafted into C3H/HeNHsd mice and measurement were performed at the indicated time points. (D) analyses of lungs from SW1 inoculated mice by H&E staining for lung metastasis. The image depicts lungs with varying metastatic loads in control (a, b) and treated (c, d) mice. The black bar denotes 2mm (for all magnifications).

Supplemental Figures Legend

Supplemental Figure 1. Identifying MA3 domain as SBI-756 target on eIF4G1.

(A) A375 and UACC903 melanoma cells were subjected to indicated sgRNA infection followed by treatment with SBI-756. Genomic DNA was extracted from the cells followed by sequencing. eIF4G1_sgRNA-235 and sgRNA-622 counts were shown. (B) MA3 domain sequences of gRNA-235 and gRNA-622 A375 cells. sgRNA sequence shown in green and MA3 sequence in red. Deleted nucleotides are double strikethrough. (C) Left, KY775 strain was transformed with an empty vector or a vector coding for TIF1. Transformed cells were grown with DMSO or 1.25µM SBI-756 for 48h before assessing the cell number by optical density. Data were normalized to DMSO treated cells. Error bars represent sem, n=3. Significance was assessed by Student's T test: **: p-value < 0. Right, KY775 strain was transformed with a library of randomly mutated TIF1 expressing vectors. 9 clones resistant to SBI-756 were retrieved and DNA was extracted, transformed into TOP10 competent bacteria. Plasmids present in each clone were sequenced. All clones presented deletion or mutation of the helicase C-terminal domain. Stars indicate the presence of a stop codon. Q: Q motif, D: DEAD box. Bottom, TIF4631 deletions promote KY775 *S. cerevisiae* (pdr5D::hisG erg6D::hisG) strain resistance to SBI-756. KY775 cells were transformed with a library of randomly mutated TIF4631 expressing vectors. 12 clones resistant to SBI-756 were retrieved and DNA was extracted, transformed into TOP10 competent bacteria. Plasmids present in each clone were sequenced. All clones presented deletion of the C-terminal domains of TIF4631. Stars indicate the presence of a stop codon. MIF4G: middle domain of

eukaryotic initiation factor 4G. (D) gRNA-235, gRNA-622 and EV A375 cells were seeded in 96 well plates. Cell proliferation was measured daily for 4 days using CellTiter-Glo.

Supplemental Figure 2. Characterize MA3 domain bound small molecules selected from In Silico screen.

(A) Testing funnel of high-throughput screening campaign (details in Methods). (B) Indicated melanoma and prostate cancer cell lines were treated with the four selected compounds, followed by viability assay after 72h. (C) EV and gRNA-235 A375 cells were treated with M19, followed by viability assay. (D) A375 cells were cultured in a medium containing indicated concentrations of FBS, followed by treatment with M19. Cell viability was measured 72 h later.

Supplemental Figure 3. RNAseq analysis of A375 cells treated with M19.

(A) Gene set enrichment analysis of RNAseq data of A375 cells treated with M19 (20 µM). The top enrichment plots of indicated pathways and corresponding heatmaps of up-regulated genes are shown. (B) IPA analysis of RNAseq data of A375 cells treated with M19 (20 µM); top activated pathways are shown (C) qPCR validation of selected differentially expressed genes. (D) representative genes and related signaling that were downregulated upon M19 treatment (left panel) and their validation by qPCR (right panel).

Supplemental Figure 4. Characterization of M19 analogs.

(A) A375 melanoma and H3A melanocyte cells were treated with indicated M19 analogs. Cell viability was measured 72 h later. (B) A375 cells (300 cells/well) were seeded in 12-well plates and treated with indicated compounds. After one week, cells were stained with crystal violet. (C) A375 cells were treated with indicated concentrations of M19-6. qPCR analysis was performed on select differentially expressed genes. (D) A375 cells were pre-incubated with indicated concentrations of M19-6 for 2 h, followed by incubation with 3 µg/ml puromycin for 0.5 h. Cells were collected, lysed, and subjected to Western blot analysis using anti-puromycin antibody. The density of the whole lane was quantified by ImageJ and normalized to actin levels as indicated. (E) A375 cells were seeded in 384-well plate and treated with M19-6 after 24 h. The compound was removed from respective wells at indicated time points. Cell viability was measured 72h after the treatment. (F) Indicated BRAF mutant melanoma cells and spheroids were treated with M19-6, and cell viability was measured after 72 h for cell cultures and 96 h for spheroids. (G) Ten different NRas mutant melanoma cell lines were seeded in 384-well plates and treated with indicated concentrations of M19-6. Cell viability was measured after 72 h. (H-I) analyses of HDAC inhibitors with M19-6. A375 cells were seeded in 384-well plates and 24h

later, M19-6 and Chidamide (H) or CI-994 (I) were added to respective wells at the indicated concentrations. Cell viability was measured after 72h using CellTiter-Glo.

Supplemental Figure 5. Characterization of ribosome footprints

(A) Distribution of read lengths for ribosome footprints generated using MNase digestion. (B) Stacked bar charts showing the proportion of reads from RNA-Seq and Ribo-Seq libraries mapped to different mRNA regions: coding sequence (CDS), 5' untranslated region (5' UTR), and 3' untranslated region (3' UTR). (C) Metagene analysis of ribosome profiling libraries generated by MNase, with reads from all mRNAs aligned within ±300 nucleotides of the start and stop codons.

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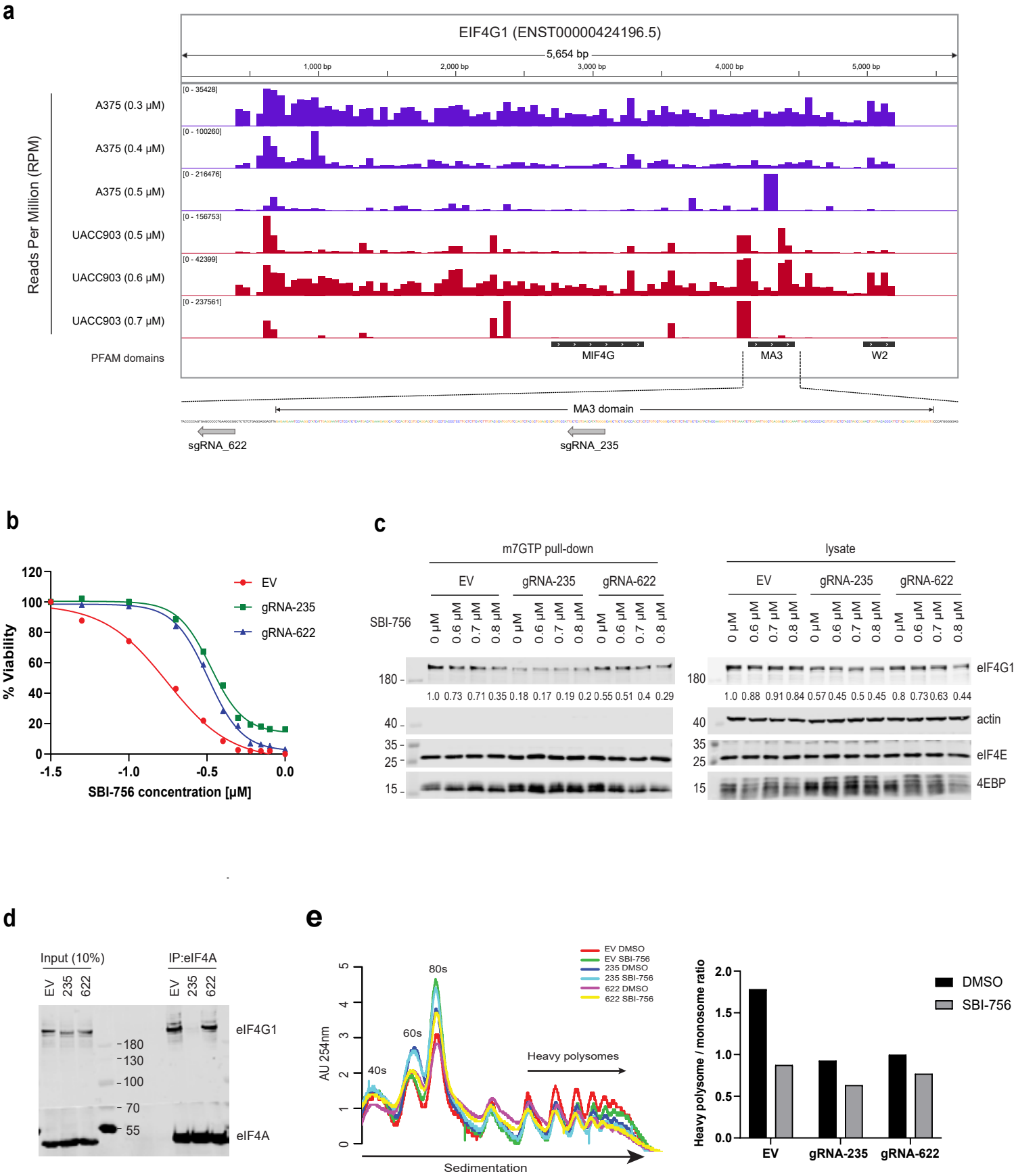

Feng et al. Fig. 1

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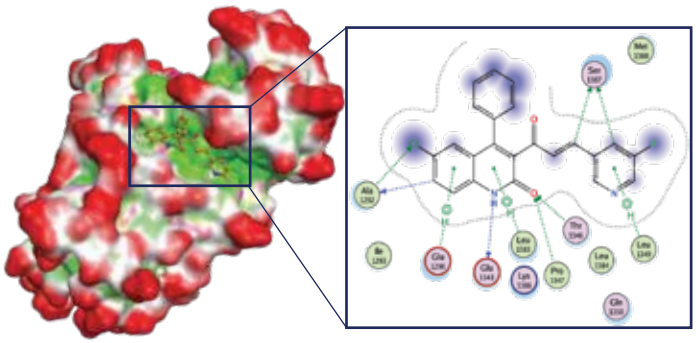

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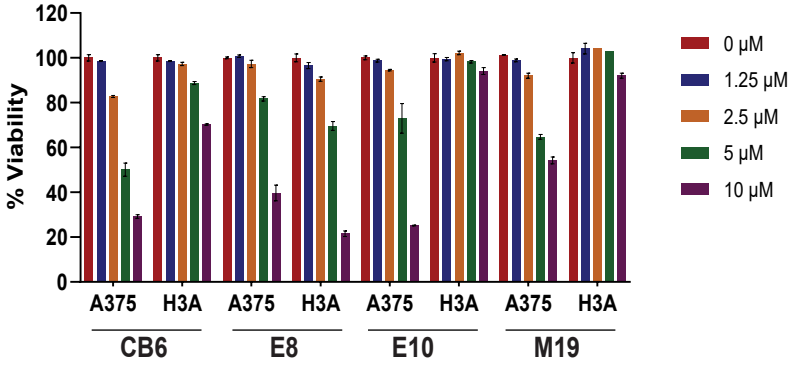
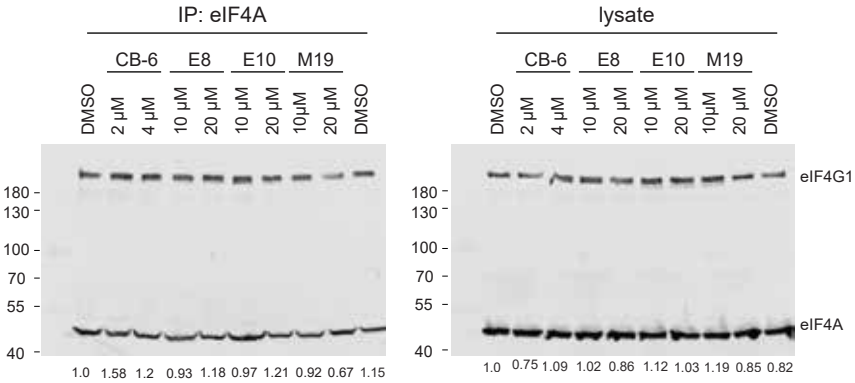

% Viability
120
100
80
60
40
20
0
A375
H3A
A375
H3A
A375
H3A
A375
H3A
CB6
E8
E10
M19
0 μM
1.25 μM
2.5 μM
5 μM
10 μM

c


IP: eIF4A
CB-6
E8
E10
M19
DMSO
2 μM
4 μM
10 μM
20 μM
10 μM
20 μM
10μM
20 μM
DMSO
180
130
100
70
55
40
1.0 1.58 1.2 0.93 1.18 0.97 1.21 0.92 0.67 1.15
lysate
CB-6
E8
E10
M19
DMSO
2 μM
4 μM
10 μM
20 μM
10 μM
20 μM
10μM
20 μM
DMSO
eIF4G1
eIF4A
180
130
100
70
55
40
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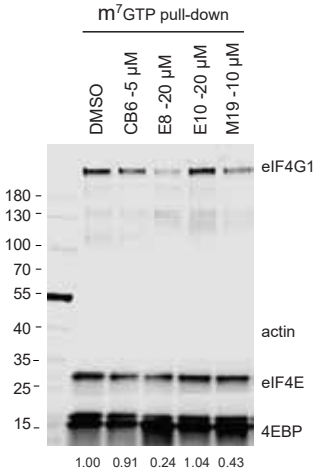
d


m7GTP pull-down
DMSO
CB6 -5 μM
E8 -20 μM
E10 -20 μM
M19 -10 μM
eIF4G1
actin
eIF4E
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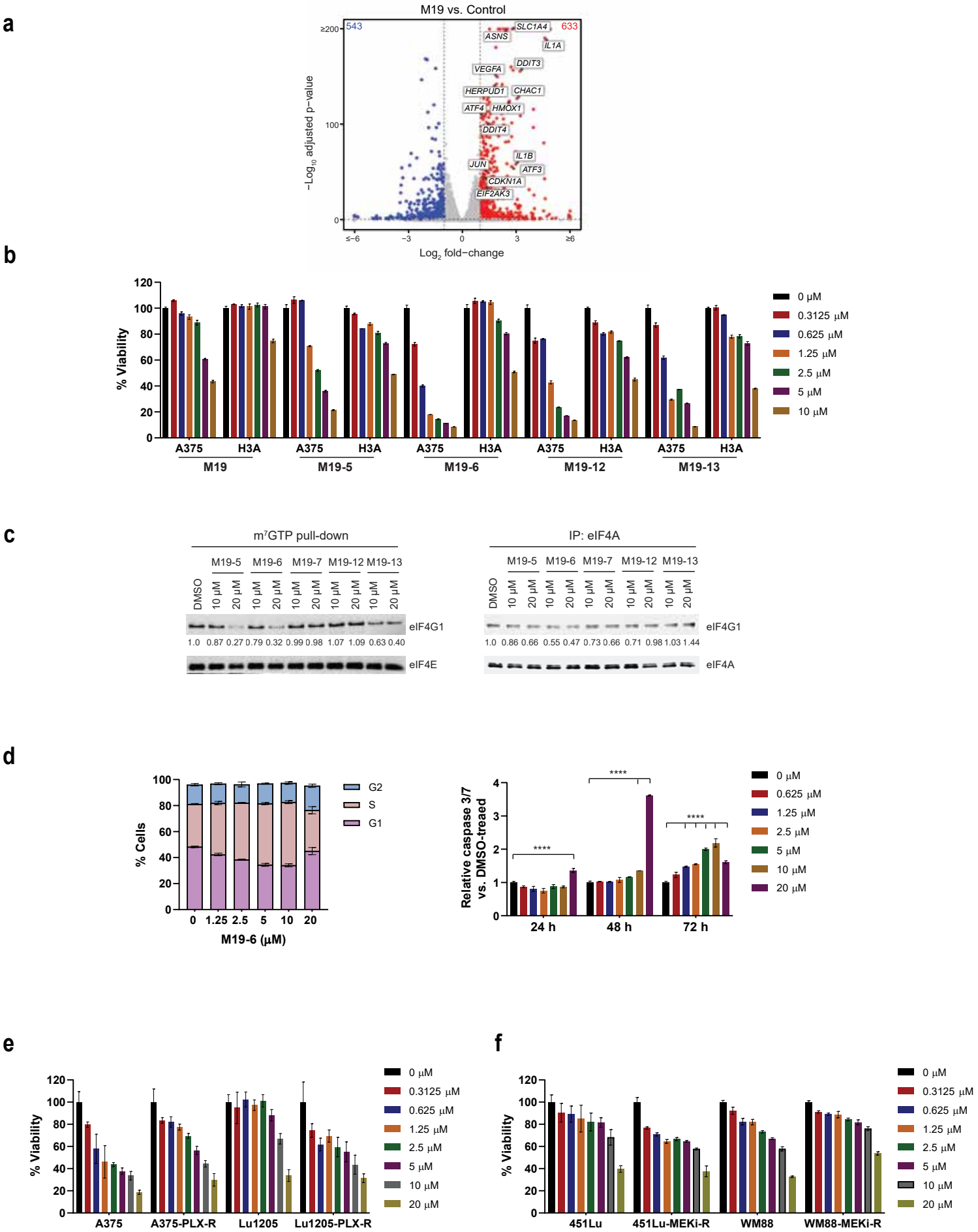
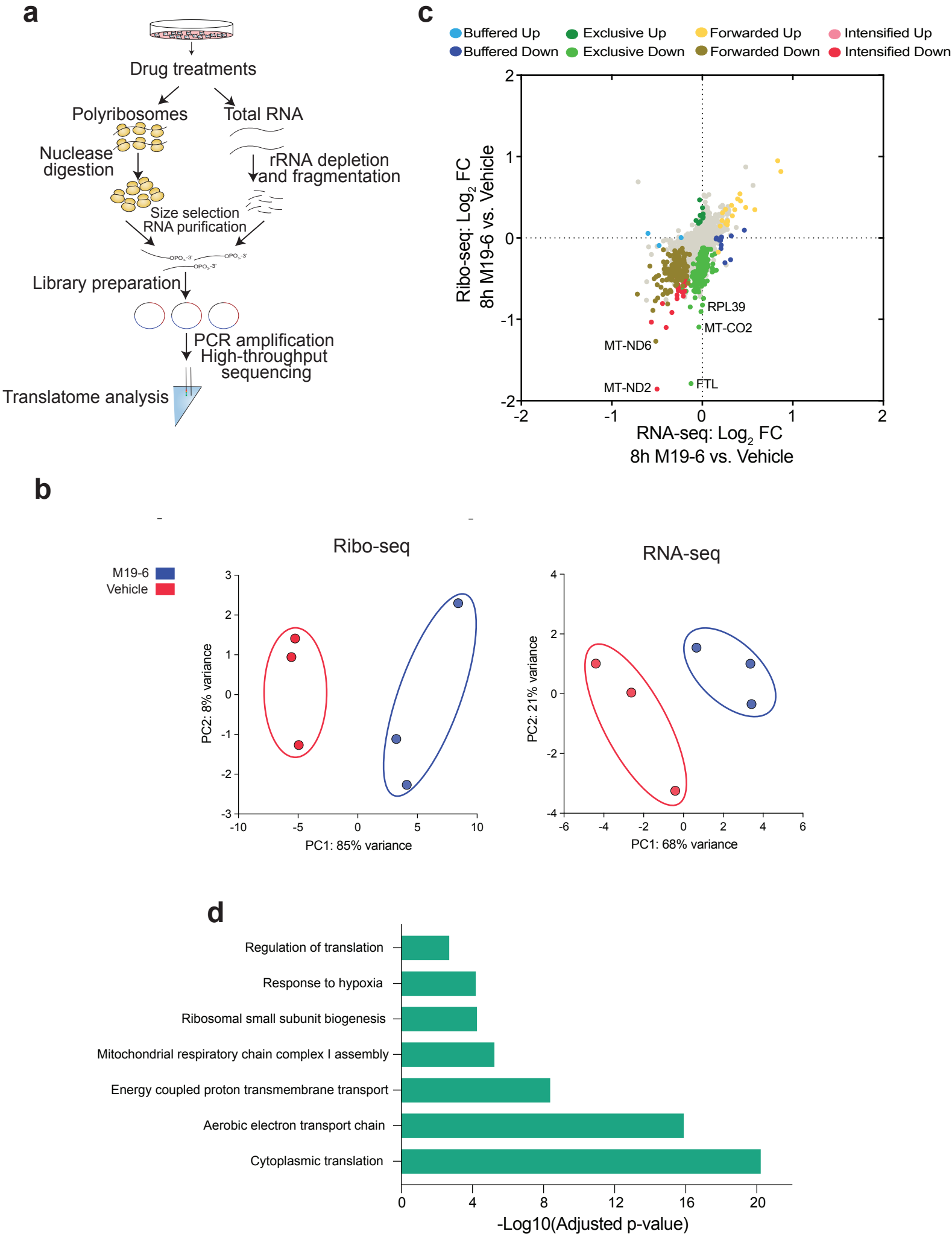
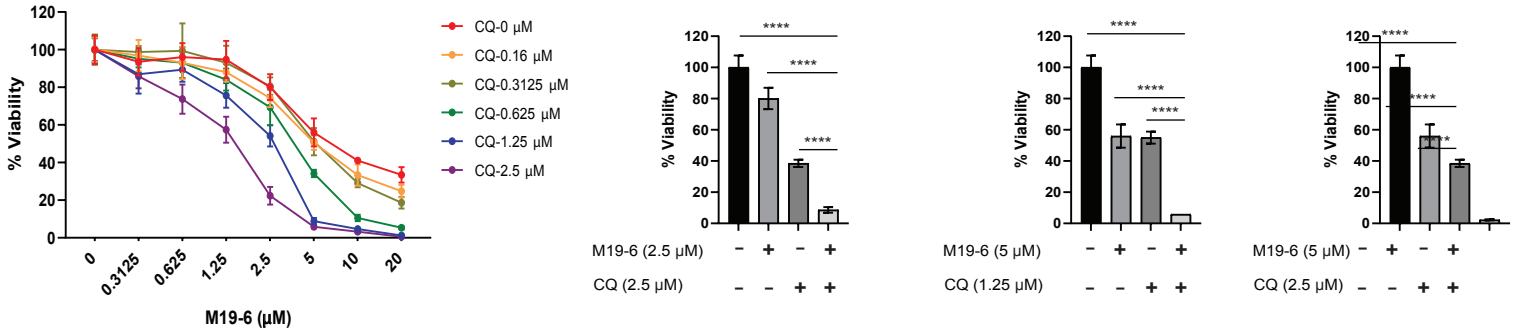


Feng et al. Fig. 3

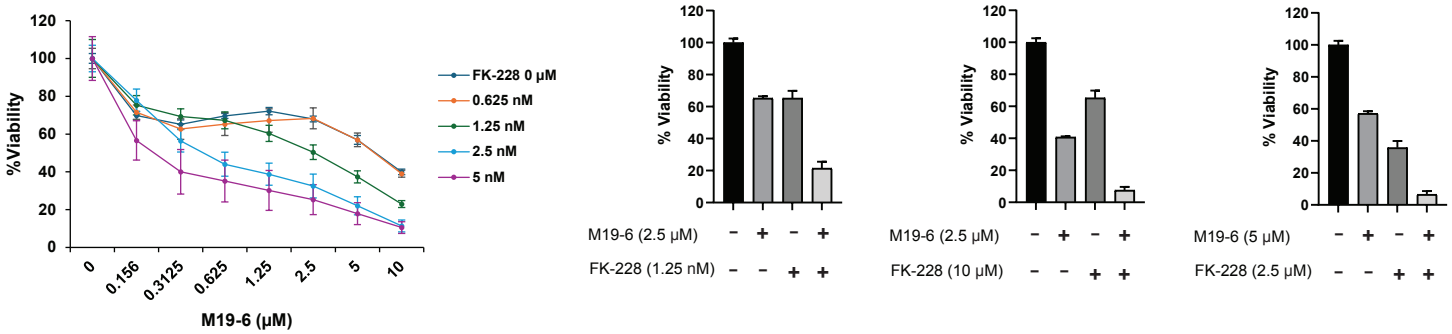


Feng et al., Figure 4

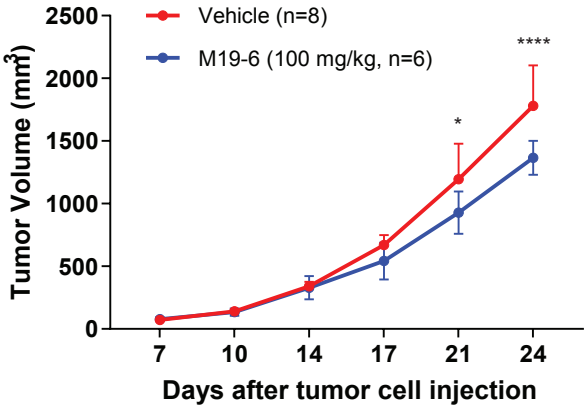
a


% Viability
M19-6 (μM)
CQ-0 μM
CQ-0.16 μM
CQ-0.3125 μM
CQ-0.625 μM
CQ-1.25 μM
CQ-2.5 μM
M19-6 (2.5 μM)
CQ (2.5 μM)
M19-6 (5 μM)
CQ (1.25 μM)
CQ (2.5 μM)
****

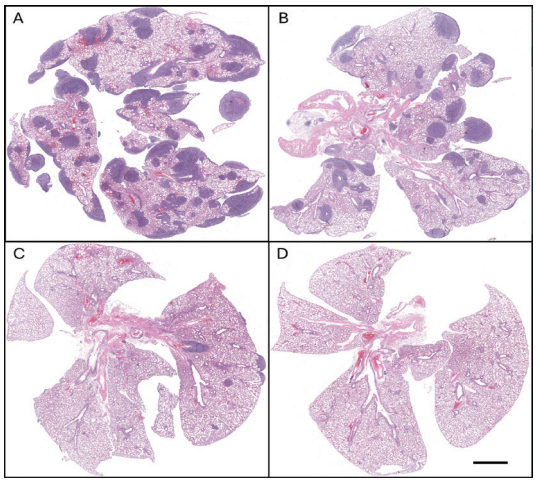
b


%Viability
M19-6 (μM)
FK-228 0 μM
0.625 nM
1.25 nM
2.5 nM
5 nM
% Viability
M19-6 (2.5 μM)
FK-228 (1.25 nM)
FK-228 (10 μM)
M19-6 (5 μM)
FK-228 (2.5 μM)

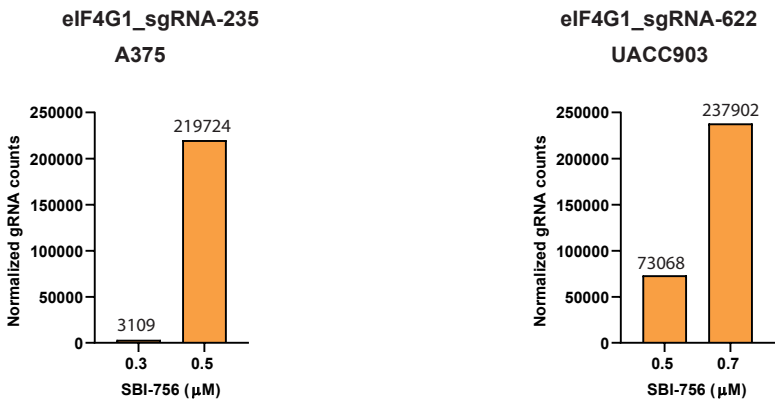
c


Vehicle (n=8)
M19-6 (100 mg/kg, n=6)
Tumor Volume (mm³)
Days after tumor cell injection
*
****

d


A
B
C
D

a



b

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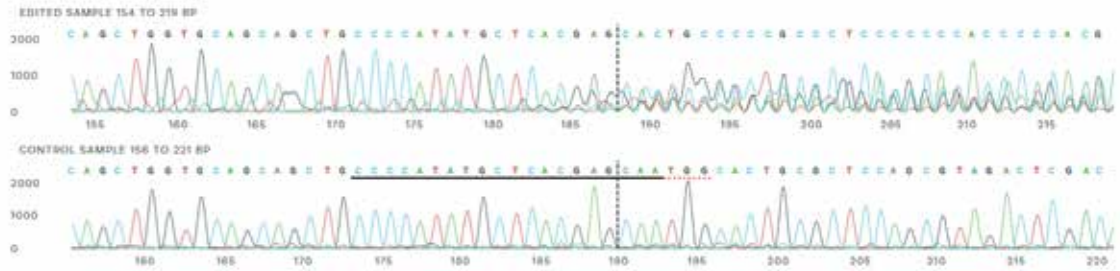
CCCTCCTTGCTCTTCATCTTTGTACGGCATGGTGTCGAGTCTACGCTGGAGCGCAGTGCC**ATTGCTCGTGAGCATATGGGG**CAGCTG
CTGCACCAGCTGCTCTGTGCTGGGCATCTGTCTACTGCTCAGTACTACCAAGGGTTGTATGAAATCTTGGAATTGGCTGAGGACATGG
AAATTGACATCCCCCACGTGTGGCTCTACCTAGCGGAACTGGTAACACCCATTCTGCAGGAA~~GGTGGGGTG~~

eIF4G1_sgRNA-235

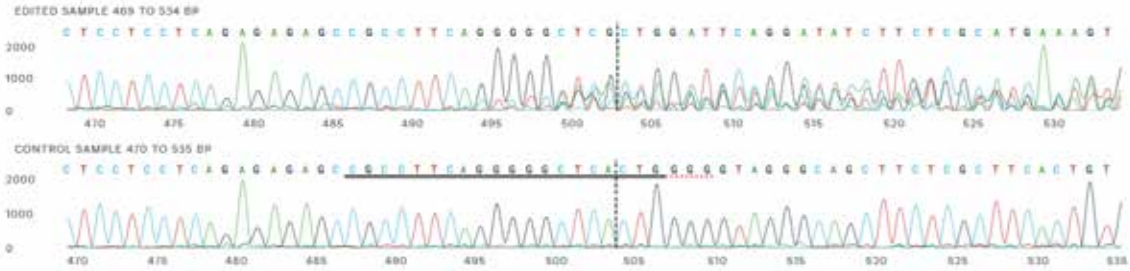
empty vector



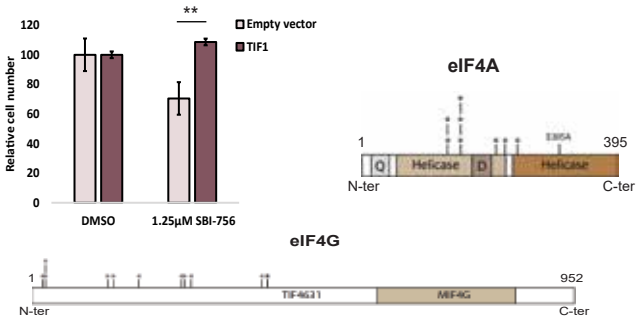
TGCGCAAGGCAGCTAGCCTCACGGAGGATCGGGACCGTGGGCGGGATGCCGTGAAGCGAGAAGCTGCCCTACCCC**CAGTGAGCCCCCTGAAGGCG**GCT
CTCTCTGAGGAGGAGTTAGAGAAGAAATCCAAGGCTATCATTGAGGAATATCTCCATCTCAATGACATGAAAGAGGCAGTCCAGTGCGTG
CAGGAGCTGGCCTCACCCTCCTTGCTCTTCATCTTTGTACGGCATGGTGTCGAGTCTACGCTGGAGC~~GCCAGTGCCAT~~TGCTCGTGAG
CATATGGGGCAGCTGCTGCACCAGCTGCTCTGTGCTGGGCATCTGTCTACTGCTCAGTACTACCAAGGGTTGTATGAAATCTTGG AAT
TGGCTGAGGACATGGAAATTGACATCCCCCACGTGTGGCTCTACCTAGCGGAACTGGTAACACCCATTCTGCAGGAAGGTGGGGTG

eIF4G1_sgRNA-622

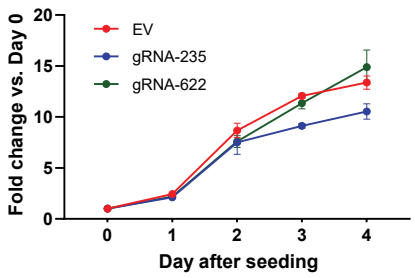
empty vector



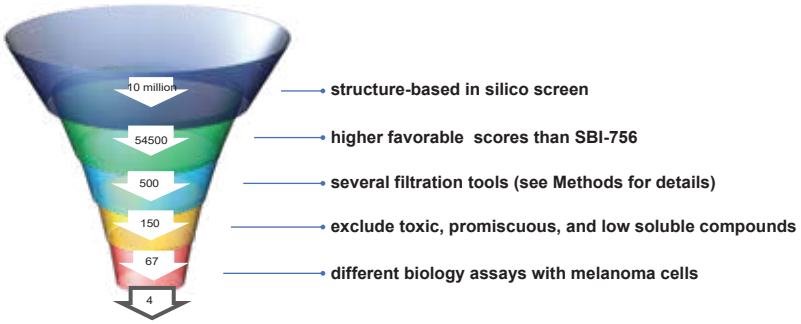
c



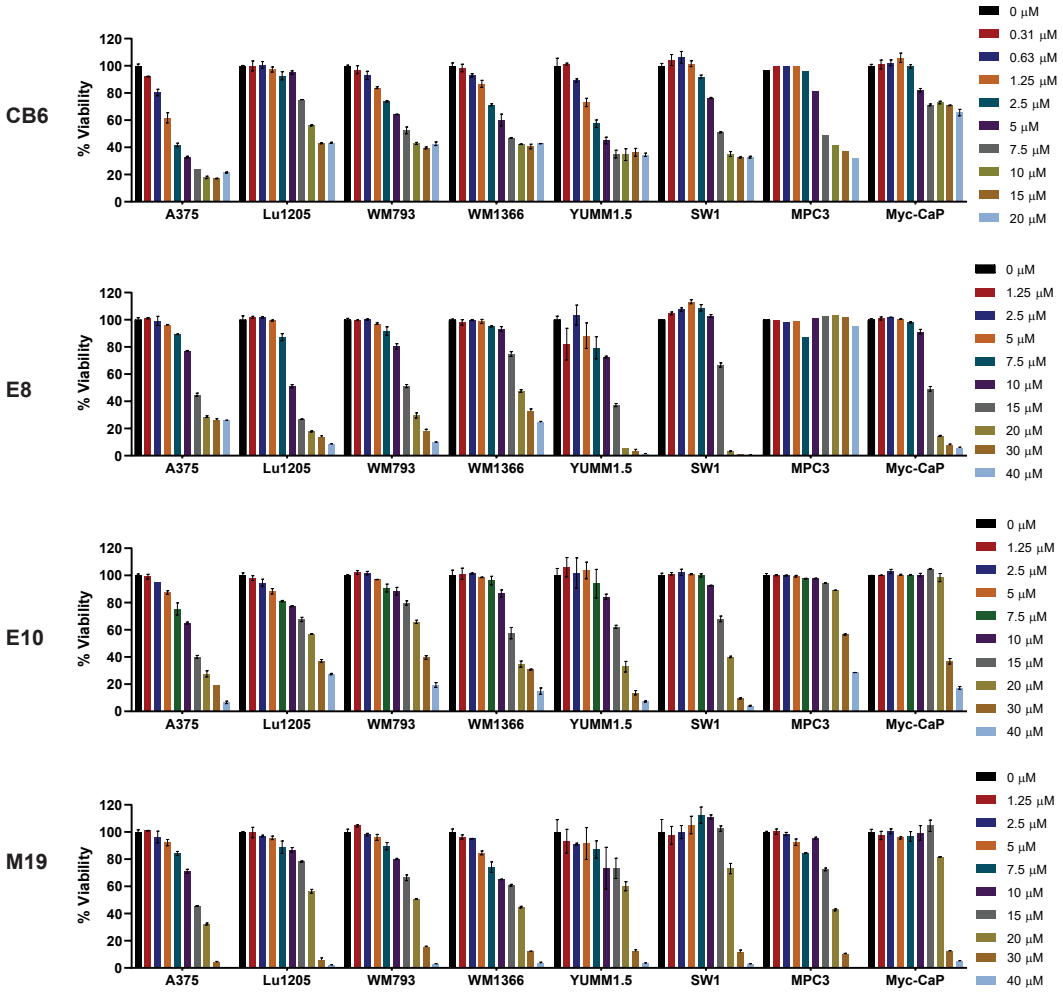
d



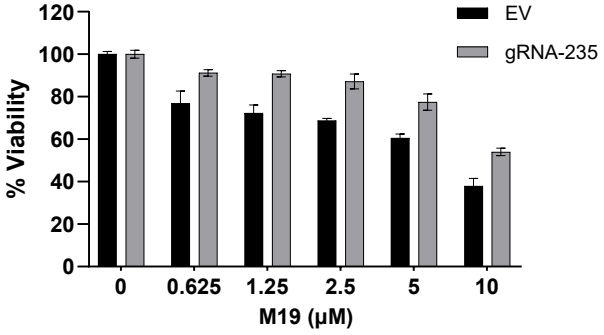
a


10 million
54500
500
150
67
4
structure-based in silico screen
higher favorable scores than SBI-756
several filtration tools (see Methods for details)
exclude toxic, promiscuous, and low soluble compounds
different biology assays with melanoma cells

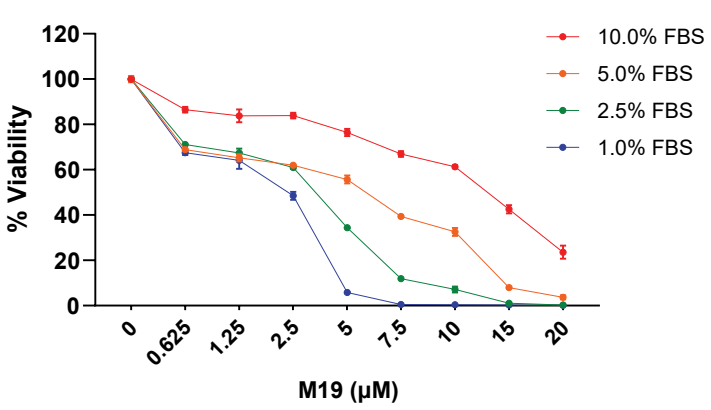
b


CB6
E8
E10
M19
% Viability
A375
Lu1205
WM793
WM1366
YUMM1.5
SW1
MPC3
Myc-CaP
0 μM
0.31 μM
0.63 μM
1.25 μM
2.5 μM
5 μM
7.5 μM
10 μM
15 μM
20 μM
30 μM
40 μM

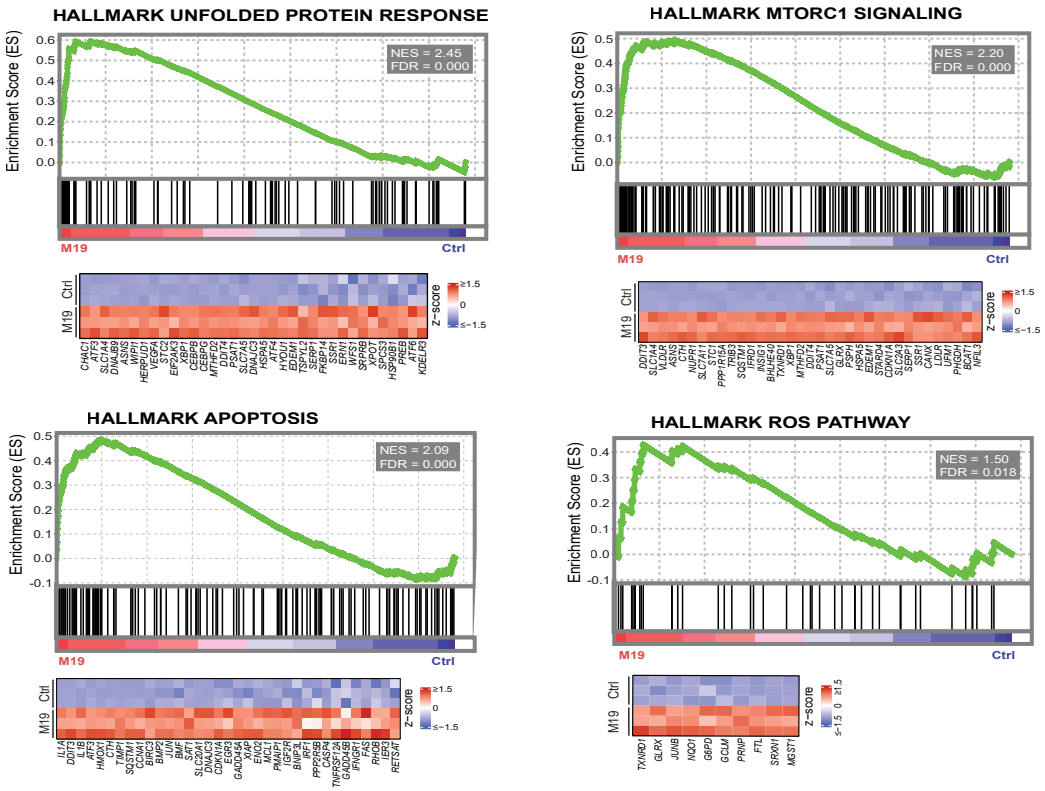
c


% Viability
EV
gRNA-235
0
0.625
1.25
2.5
5
10
M19 (μM)

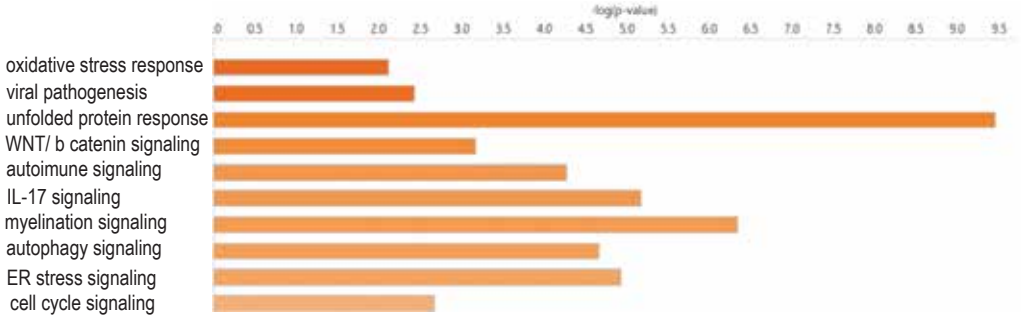
d


% Viability
10.0% FBS
5.0% FBS
2.5% FBS
1.0% FBS
0
0.625
1.25
2.5
5
7.5
10
15
20
M19 (μM)

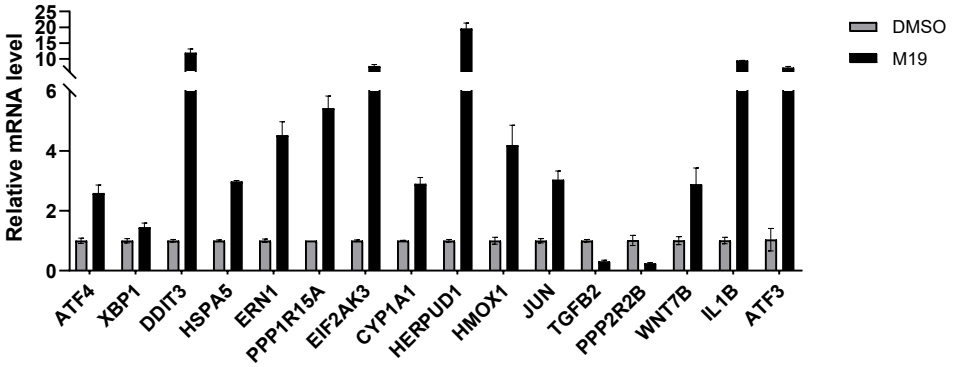
a



b



c



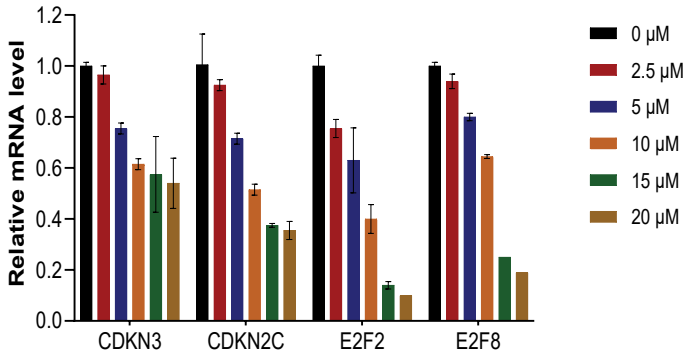
d

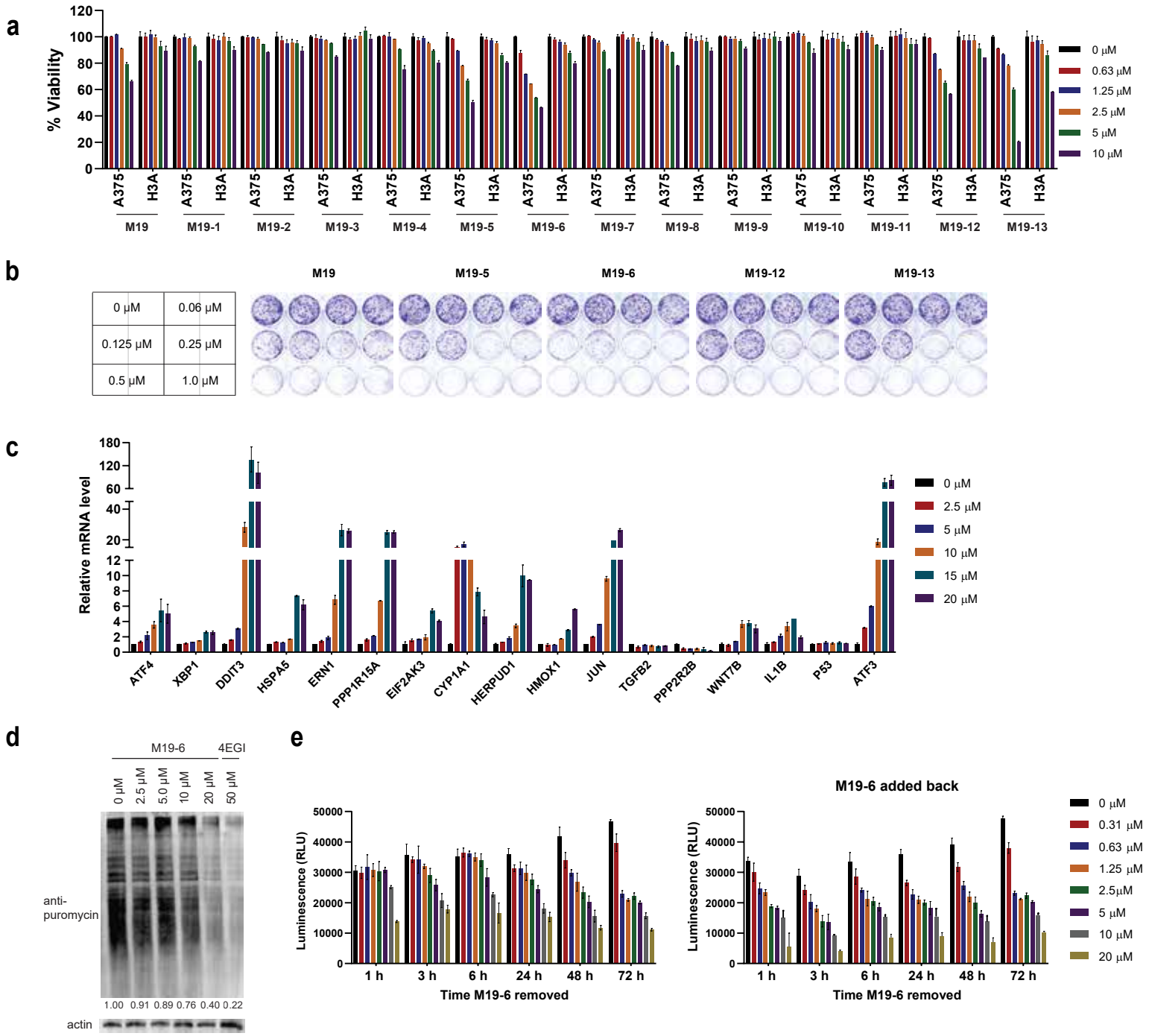
Top E2F targets genes

SYMBOL	RANK METRIC SCORE
CDKN2C	-2.328
E2F8	-2.262
CDKN3	-1.901
UNG	-1.809
BIRC5	-1.797
SPC25	-1.788
MCM6	-1.786
PCNA	-1.769
SPC24	-1.728
LMNB1	-1.694

Top G2M target genes

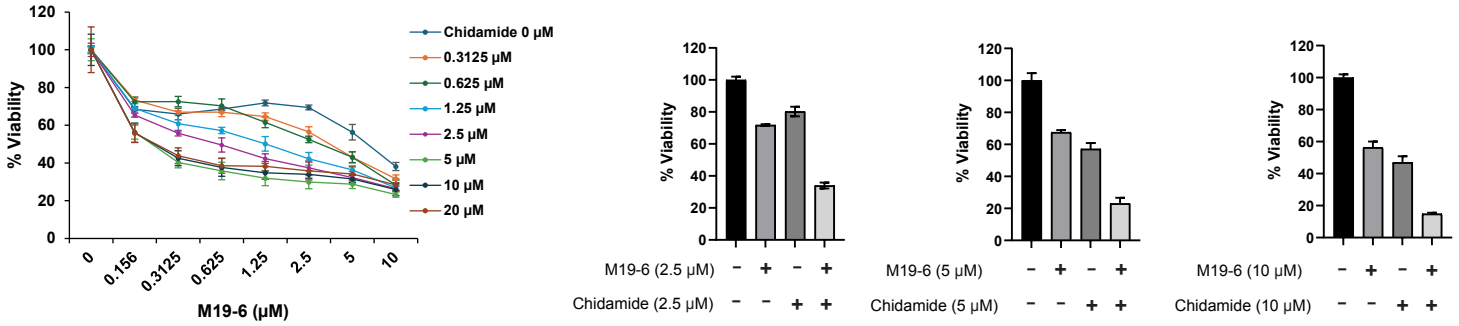
SYMBOL	RANK METRIC SCORE
CDKN2C	-2.328
E2F2	-2.177
CDKN3	-1.901
GINS2	-1.835
BIRC5	-1.797
MCM6	-1.786
LMNB1	-1.694
CCNF	-1.651
TOP2A	-1.646
HMMR	-1.607



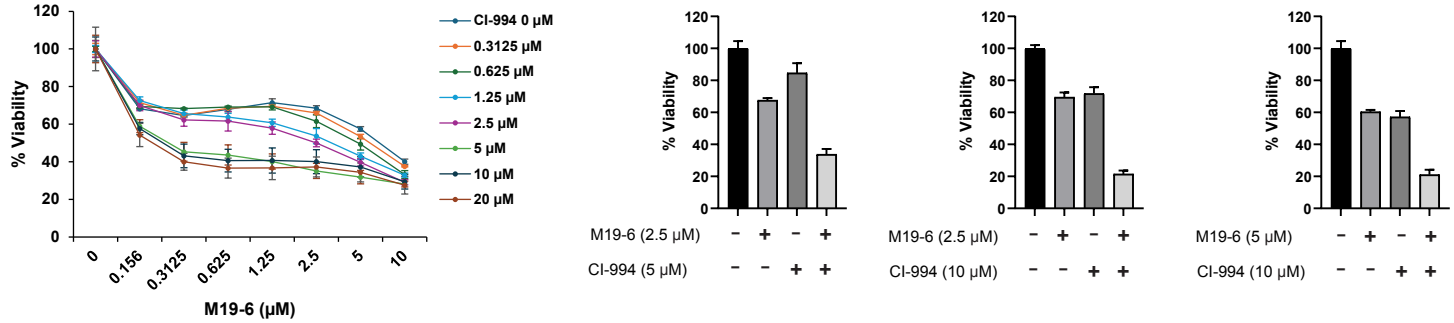


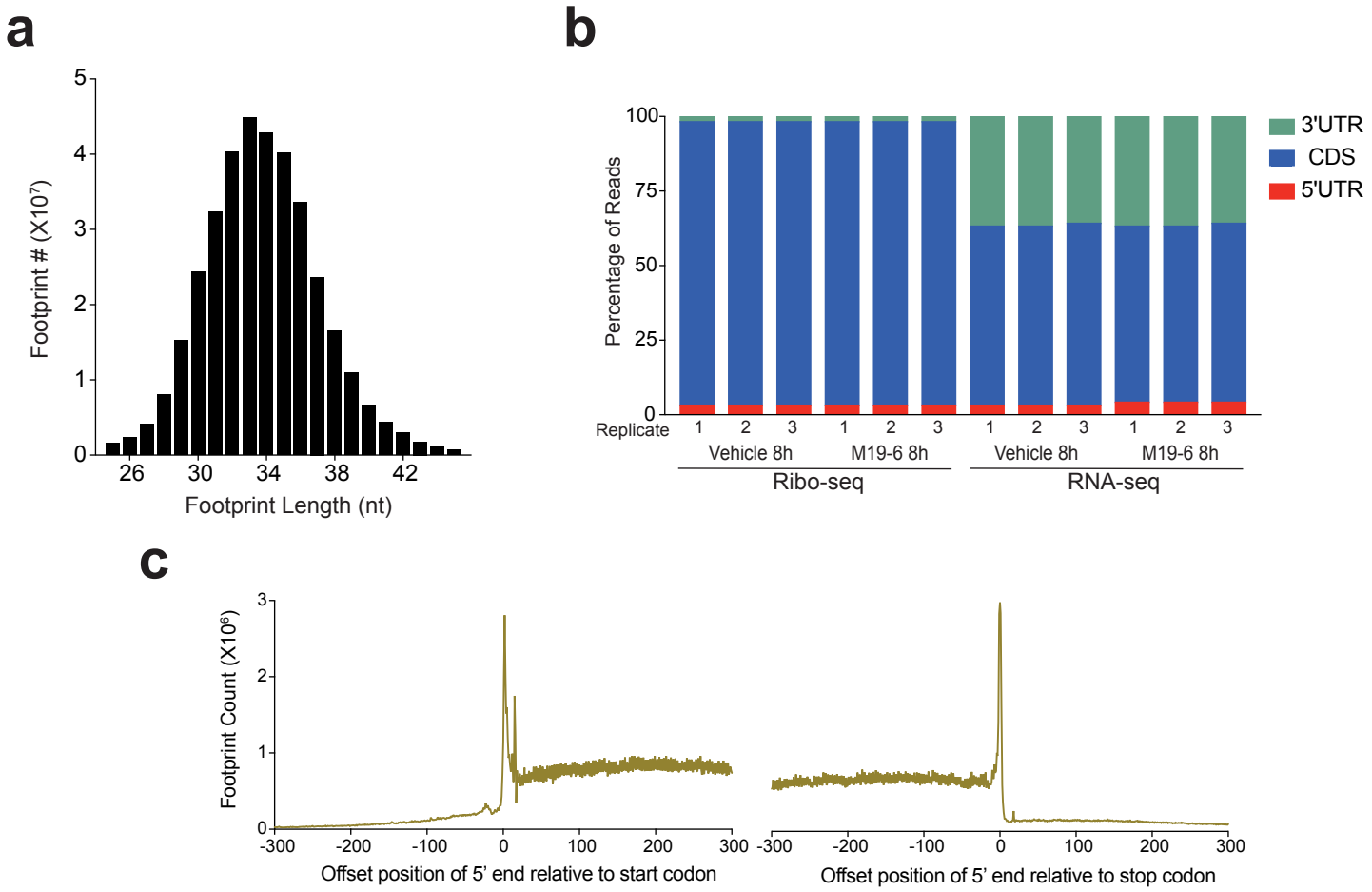
Feng et al. Supp Fig 4

h


% Viability
M19-6 (μM)
0
0.156
0.3125
0.625
1.25
2.5
5
10
Chidamide 0 μM
0.3125 μM
0.625 μM
1.25 μM
2.5 μM
5 μM
10 μM
20 μM
M19-6 (2.5 μM)
Chidamide (2.5 μM)
M19-6 (5 μM)
Chidamide (5 μM)
M19-6 (10 μM)
Chidamide (10 μM)

i


% Viability
M19-6 (μM)
0
0.156
0.3125
0.625
1.25
2.5
5
10
CI-994 0 μM
0.3125 μM
0.625 μM
1.25 μM
2.5 μM
5 μM
10 μM
20 μM
M19-6 (2.5 μM)
CI-994 (5 μM)
M19-6 (2.5 μM)
CI-994 (10 μM)
M19-6 (5 μM)
CI-994 (10 μM)



Feng et al., Supp Fig 5