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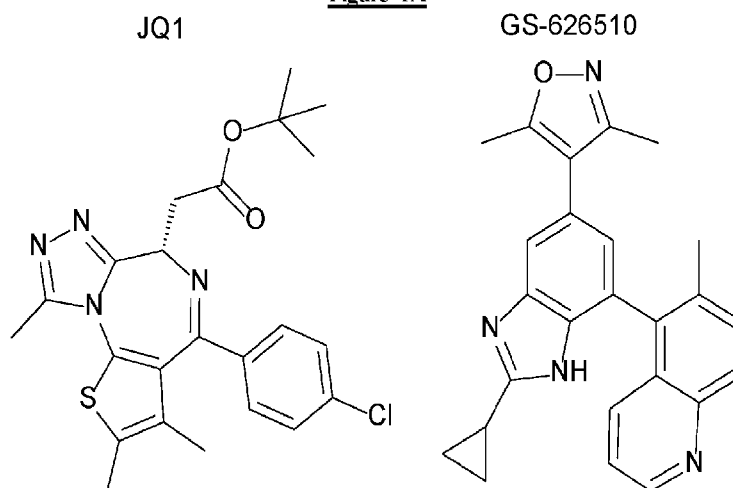
(54) Title: COMPOSITIONS AND METHODS OF RESENSITIZING CELLS TO BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN INHIBITORS (BETi)

Figure 1A



(57) Abstract: The present invention provides compositions and methods to increase anti-tumor sensitivity of a cell or tumor to bromodomain and extraterminal domain protein inhibitors (BETi). In one aspect, a composition comprises a BETi and a TGFβ pathway inhibitor, TRIM33 or a fragment thereof, or a nucleic acid encoding TRIM33. In another aspect, a method is described for increasing anti-tumor sensitivity to BETi. Methods for a tumor with BETi are also described.

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[Continued on next page]

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COMPOSITIONS AND METHODS OF RESENSITIZING CELLS TO BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN INHIBITORS (BETi)

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/353,424, filed June 22, 2016, which is incorporated herein by reference in its entirety.

10 **BACKGROUND OF THE INVENTION**

Epigenetic regulation of transcription is central to control of cell fate and proliferation. Enzymatic addition or removal of a variety of specific post-translational modifications of histones support the recruitment of epigenetic “readers,” proteins that selectively bind to modified sites and recruit transcriptional activators or repressors.
15 Alterations in this complex epigenetic code contribute to development of a range of diseases, including cancer. Consequently pharmacological modulation of enzymes that generate or remove epigenetic modifications and their readers offer new therapeutic opportunities for cancer treatment.

The bromodomain and extraterminal domain (BET) proteins are one class of
20 epigenetic readers involved in transcriptional control. The small family of BET proteins (BRD2, BRD3, BRD4 and BRDT) are characterized by tandem bromodomains, that bind acetylated lysine residues in histones and other proteins, and a C-terminal extraterminal domain responsible for interactions with chromatin regulators. BET proteins, in particular BRD4, have been implicated as general regulators of
25 transcription through recruitment of the elongation factor, P-TEFb, to gene promoters and through interaction with the mediator complex. In addition, high-level recruitment of BRD4 to enhancer regions has been implicated in gene-specific transcriptional activation. Evidence from a variety of approaches, including genetic screens in cultured cells, targeted gene deletion in mice, and analysis of human tumors, have
30 implicated BET proteins, in particular BRD2 and BRD4, in a range of cancers (Baratta et al., 2015, *Proc Natl Acad Sci U S A* 112(1):232-237; Zuber et al., 2011, *Nature* 478(7370):524-528).

Recent advances allowing small molecule inhibition of BET proteins generated considerable interest in the therapeutic potential of BET inhibitors (BETi). BETi are

small molecules that interact with the acetylated lysine binding pocket of the BET family bromodomains, interfering with BET protein binding to chromatin and consequently modulate transcription. BETi were initially shown to be effective in a mouse xenograft model of midline carcinoma, a rare cancer driven by a chromosomal translocation producing a BRD4-NUT fusion protein. BETi have subsequently proven to be effective in multiple models of hematologic malignancies and solid tumors that are not characterized by mutated oncogenic BET protein activation. One key mechanism by which BETi suppress growth and survival of at least some types of cancer cells is by preferentially repressing transcription of the proto-oncogene, MYC, which is often under the control of BRD4 (Dawson et al., 2011, *Nature* 478(7370):529-533; Delmore et al., 2011, *Cell* 146(6):904-917; Mertz et al., 2011, *Proc Natl Acad Sci U S A* 108(40):16669-16674). Thus, BETi may provide a new mechanism to target MYC and other oncogenic transcription factors, which lack obvious binding pockets for small molecules and are thus typically considered to be "undruggable".

The excitement surrounding the potential of targeting BET proteins in cancer has fueled the development of a variety of BETi, some of which are currently undergoing clinical trials (Filippakopoulos and Knapp, 2014, *Nat Rev Drug Discov* 13(5):337-356). However, lessons from other targeted cancer therapies suggest that acquired resistance will limit long-term responsiveness to BETi treatment. Acquired resistance to kinase inhibitors, for example, is often accompanied by outgrowth of clones harboring mutations to the kinase itself that disrupt inhibitor binding. However, in many cases resistance to targeted therapy occurs independently of mutations to the drug target, being driven by re-activation of signaling pathways suppressed by the drug or activation of bypass pathways that facilitate cell growth and survival despite inhibition of the target. A recent study showed that acquired resistance to JQ1 in cultured pancreatic cancer cells was associated with BRD4-independent MYC expression (Kumar et al., 2015, *Sci Rep* 5:9489). However, specific molecular lesions leading to BETi resistance have not been identified. Identifying such lesions may suggest specific therapeutic strategies for re-sensitizing cells to BETi. Therefore a need exists for methods that identify lesions for acquired resistance and for the development of therapeutics to resensitize tumor cells to BETi.

SUMMARY OF THE INVENTION

The present invention provides a method of increasing anti-tumor sensitivity to a bromodomain and extraterminal domain protein inhibitor (BETi) in a cell in a subject, the method comprising administering a composition comprising a TGFβ pathway inhibitor and a BETi to a subject in need thereof, wherein the TGFβ pathway inhibitor sensitizes the cell to the BETi.

The present invention further provides a method of treating a tumor comprising administering to a subject in need thereof a composition comprising a TGFβ pathway inhibitor and a BETi, wherein the TGFβ pathway inhibitor sensitizes the tumor to the BETi.

The present invention further provides a method of increasing anti-tumor sensitivity to a BETi of a cell in a subject, the method comprising administering a composition comprising a BETi and one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33 to a subject in need thereof, wherein the TRIM33 sensitizes the cell to the BETi.

The present invention further provides a method of treating a tumor comprising administering to a subject in need thereof a composition comprising a BETi and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein TRIM33 sensitizes the tumor to the BETi.

In certain embodiments, the TGFβ pathway inhibitor is selected from the group consisting of a small molecule inhibitor, an inhibitory nucleic acid, neutralizing antibody, and an antagonist. In other embodiments, the small molecule inhibitor is selected from the group consisting of SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299, R268712, D4476, SB525334, GW788388, TEW-7197, and any combination thereof. In other embodiments, the antagonist is selected from the group consisting of HtrA1, decorin, biglycan, fibromodulin, lumican, endoglin, somatostatin, follistatin, RAP-1332, pirfenidone (5-methyl-1-phenyl-2(1H)-pyridone), soluble ectodomains of TGFβ receptor type II (RII) or betaglycan (BG), and any combination thereof. In yet other embodiments, the neutralizing antibody specifically binds one selected from the group consisting of TGFβ and a TGFβ receptor.

In certain embodiments, the nucleic acid encoding TRIM33 is an expression vector comprising a TRIM33 gene. In other embodiments, the vector is a viral vector.

In certain embodiments, the BETi is selected from the group consisting of JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, TEN-010, and any combination thereof.

5 In certain embodiments, the subject has a cancer. In other embodiments, the cancer is selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer. In yet other embodiments, the method treats a tumor wherein the tumor is a cancer selected from 10 the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer.

In certain embodiments, the cell in the subject is BETi resistant. In other embodiments, the tumor is BETi resistant.

15 The invention further provides a composition comprising a TGFβ pathway inhibitor and a bromodomain and extraterminal domain protein inhibitor (BETi), wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

In certain embodiments, the the TGFβ pathway inhibitor is selected from the group consisting of a small molecule inhibitor, an inhibitory nucleic acid, a neutralizing 20 antibody, and an antagonist. In other embodiments, the small molecule inhibitor is selected from the group consisting of SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299, R268712, D4476, SB525334, GW788388, TEW-7197, and any combination thereof. In yet other embodiments, the neutralizing antibody specifically binds one selected from the group consisting of TGFβ and a TGFβ 25 receptor. In yet other embodiments, the antagonist is selected from the group consisting of HtrA1, decorin, biglycan, fibromodulin, lumican, endoglin, somatostatin, follistatin, RAP-1332, pirfenidone (5-methyl-1-phenyl-2(1H)-pyridone), soluble ectodomains of TGFβ receptor type II (RII) or betaglycan (BG), a neutralizing antibody against TGFβ or a TGFβ receptor, and any combination thereof.

30 The invention also provides a composition comprising a BETi and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

In certain embodiments, the nucleic acid encoding TRIM33 is a vector comprising a TRIM33 gene. In other embodiments, the vector is a viral vector.

In certain embodiments, the BETi is selected from the group consisting of JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075,
5 BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, TEN-010, and any combination thereof.

In certain embodiments, the compositions of the invention can be formulated in a pharmaceutical composition further comprising a pharmaceutically acceptable carrier. In certain embodiments, the pharmaceutical composition can be used in the
10 manufacture of a medicament for the treatment of a tumor or cancer in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For
15 the purpose of illustrating the invention, there are shown in the drawings embodiments, which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figures 1A-1H are images showing that the shRNA screening revealed TRIM33
20 as a regulator of BETi resistance in cancer cells. Figure 1A shows the structures of the two different BETi used in the study, JQ1 and GS-626510. Figure 1B shows the K_D values of GS-626510 for 40 bromodomains (Table 3) determined with a BROMOscan™ (DiscoveRx). The dendrogram image was generated using TREEspot™ Software Tool (DiscoveRx Corporation). Figure 1C shows the dose-
25 dependent inhibition of RKO cell proliferation by JQ1 and GS-626510 in a 5-day assay. Relative viable cell number was determined by CellTiter Glo assay. Figure 1D shows that both GS-626510 and JQ1 down-regulate MYC protein levels. RKO cells were treated with increasing concentrations of BETi for 3 h and MYC levels in whole cell lysates were assessed by immunoblotting. Actin was used as a loading control.
30 Figure 1E shows gene expression changes induced by JQ1 and GS-626510 in shCTRL cells were highly correlated. The log2 [fold change (BETi/DMSO)] of all gene expression following 3 h treatment with 1 μM JQ1 or 0.3 μM GS-626510 was fitted to a line. Two replicate experiments result in an R2 of 0.93 and 0.92 respectively. Figure 1F shows the scheme of the shRNA screening procedure. Cells infected by the pooled

shRNA library were propagated through 8 doublings in presence of either DMSO vehicle control or different concentrations of JQ1 or GS-626510. Genomic DNA was extracted from the T0 (reference) and T4 conditions for determination of proviral shRNA abundance. Figure 1G shows the top 10 enriched target genes revealed by RIGER analysis in each condition. TRIM33 was among the top 3 ranked genes in all four BETi conditions but not in the DMSO condition. Figure 1H shows multiple individual TRIM33 shRNAs were enriched in BETi-treated, but not in DMSO control treated conditions. Log_2 fold change (T4/T0) of each shRNA is plotted from the most depleted to the most enriched. Each red line represents a single shRNA targeting TRIM33.

Figures 2A-2J are images showing loss of TRIM33 conferred resistance to BETi. Figure 2A shows (top) a schematic of TRIM33 domain organization and positions of two pairs of RT-PCR primers, (middle) *TRIM33* mRNA levels determined by RT-PCR in shCTRL cell line and cell lines expressing four different TRIM33-targeting shRNAs, and (bottom) TRIM33 protein levels in these cell lines. Figure 2B shows shCTRL or shTRIM33 cells were seeded in a 6-well plate (3×10^5 cells per well) in the presence of DMSO, 100 nM JQ1 or 50 nM GS-626510 and cumulative cell numbers were assessed every 3 days for up to 15 days. Figure 2C shows the growth inhibition assay. shCTRL and shTRIM33 cells were cultured with different concentrations of JQ1 or GS-626510 for 120 h and relative cell numbers were determined using CellTiter Glo. Figure 2D shows the IC_{50} values (mean ± SEM) calculated from 5 independently performed growth inhibition assays using shCTRL and shTRIM33 cells. P values are based on paired t-test. Figure 2E is a set of graphs showing the effect of TRIM33 depletion on JQ1 or GS-626510 sensitivity in a panel of cancer cell lines; Figure 2E shows the IC50 values for each cell line expressing either shCTRL or shTRIM33 derived from 3 independent growth inhibition assays and the mean ± SEM of the fold change in IC50 (shTRIM33/shCTRL) calculated ($*P < 0.05$, paired t-test). Figure 2F shows 2×10^4 shCTRL or shTRIM33 cells plated in 6-well plates, treated with DMSO, 100 nM JQ1, or 50 nM GS-626510 for two weeks and then stained with crystal violet. The crystal violet staining was quantified at 590 nm absorbance. Figure 2G shows the cell proliferation assay of cell lines expressing two independent shRNAs (B5 and A12) targeting TRIM33. Cells were cultured in 100 nM JQ1, or 50 nM GS-626510 for two weeks and then stained with crystal violet. Figure 2H shows the shCTRL or shTRIM33 cells transduced with either an empty vector

control or TRIM33-expressing lentivirus and cell growth was assessed as in Figure 2F. Figure 2I shows TRIM33 expression levels in cells from Figure 2H assessed by immunoblotting. Figure 2J shows the crystal violet quantification measured at 590 nm absorbance corresponding to Figure 2H.

5 Figures 3A-3E are graphs showing RT-PCR quantification of mRNA levels of 15 selected genes whose expression was changed with shTRIM33 or BETi treatment (left graphs; error bars represent SD (n =3)), and normalized RNAseq reads of the 15 genes above from two replicate experiments (right graphs; error bars represent the SD between the two replicates).

10 Figures 3F-3J are images showing RNAseq analysis of vehicle or BETi-treated shCTRL or shTRIM33 cells. Waterfall plots show gene expression changes induced by 3 h treatment of shCTRL RKO cells with 1 μM JQ1 (Figure 3F) or 0.3 μM GS-626510 (Figure 3G). *MYC* is down-regulated by both JQ1 and GS-626510. Figure 3H shows the top 10 sequence motifs enriched in promoter regions of genes down-regulated >2-
15 fold by JQ1 and GS-626510 in shCTRL cells were determined by Gene Set Enrichment Analysis (Broad Institute). Figure 3I shows the gene expression changes induced by shTRIM33 in RKO cells. Figure 3J shows an immunoblot showing that BRD4 protein level is not changed by TRIM33 knockdown.

Figures 4A-4F are a panel of images showing TRIM33 modulated MYC
20 sensitivity to BETi. Figure 4A shows the normalized RNAseq reads of *MYC* mRNA from two replicate experiments before and after JQ1 or GS626510 treatment. Figure 4B shows the RT-PCR quantification of *MYC* mRNA in shCTRL, shTRIM33 and shTRIM33 rescued ($shTRIM33^{RES}$) cells, either untreated or treated with BETi for 3 h. Figure 4C shows cells treated similarly as in panel Figure 4B \ analyzed for MYC
25 protein. Figure 4D shows the MYC protein levels in control or MYC over-expressing cells before and after BETi treatment for 3 h. Figure 4E shows crystal violet staining of control or MYC over-expressing cells growing with DMSO, JQ1 or GS-626510 for two weeks. Figure 4F shows the cumulative cell growth of control or MYC-overexpressing cells over 15 days.

30 Figures 4G and 4H show the ChIP at MYC gene promoter region. Line threshold indicates IgG control level. Figure 4G shows the TRIM33 ChIP using 4 different primer pairs (#2, #14, #15 and #16) in the MYC promoter region. Figure 4H shows the BRD4 ChIP using the same set of primers as in Figure 4G.

Figure 5A shows the shCTRL or shTRIM33-transduced RKO and SK-CO-1 cells treated with 1 μM of JQ1 or 0.3 μM of GS626510 for 24 hours. MYC levels were determined by immunoblotting and ERK1/2 was used as a loading control.

Figures 5B-5E show the gene set enrichment analysis (GSEA) and down-regulation of TGFβ and MYC signatures by JQ1 was significantly decreased in shTRIM33 in comparison to shCTRL cells (NES: normalized enrichment score).

Figures 5F-5V are a set of images showing inhibition of TGFβ signaling potentiated the anti-proliferative effects of BETi. Figure 5F shows the TGFβ1 ligand stimulated phosphorylation of SMAD2 potentiated in shTRIM33 cells. shCTRL or shTRIM33 RKO cells were treated with increasing doses of TGFβ1 for 25 min (left panel) or with 2 ng/ml TGFβ1 for various times (right panel), cells were lysed and immunoblotted for phospho-SMAD2 (pSMAD2), total SMAD2 and TRIM33. Figure 5G shows the shCTRL or shTRIM33 cells untreated or treated with 100 pM of TGFβ1 for 25 min and SMAD4 was immunoprecipitated. Co-precipitating pSMAD2 was assessed by immunoblotting. Figure 5H shows the shCTRL or shTRIM33 cells infected with lentivirus encoding shCTRL or one of two hairpins targeting SMAD4 (shSMAD4-3 or shSMAD4-4). Cells were untreated or treated with 100 pM of TGFβ1 for 25 min SMAD4, pSMAD2 and total SMAD2 levels were assessed by immunoblotting. Figure 5I shows the TGFβ receptor II (*TβRII*) mRNA from RNAseq in shCTRL and shTRIM33 cells. Figures 5J and 5K show the ChIP at TGFBR2 (TβRII) gene promoter region. Line threshold indicates IgG control level. Figure 5J shows the TRIM33 ChIP using two different pair of primers (#4 and #3) amplifying TGFBR2 gene promoter region. Figure 5K shows the BRD4 ChIP using the same set of primers as in Figure 5J amplifying TGFBR2 gene promoter region. Figures 5L-5N show the inhibition of TGFβ pathway by silencing TβRII increases the magnitude of cell growth inhibition by BETi. Figure 5L shows the RT-PCR quantification of *TβRII* mRNA levels in shCTRL and shTRIM33 cells expressing control (shCTRL) or two different TβRII-targeting shRNAs (shTβRII-3 and shTβRII-4). Figure 5M shows cells from Figure 5L stimulated with 100 pM of TGFβ1 for 25 min and pSMAD2 levels assessed by immunoblotting. Figure 5N shows shCTRL cells (left) or shTRIM33 cells (right) expressing control and TβRII-targeting shRNAs cultured for 2 weeks with DMSO or different concentrations of BETi (as indicated) and then stained with crystal violet. Figure 5O shows the crystal violet quantification measured at 590 nm absorbance corresponding to Figure 5N. Figures 5P and 5Q show the TβRI inhibitor LY2157299 potentiated BETi-mediated

inhibition of cell proliferation. Figure 5P shows the shTRIM33 cells pre-treated with increasing doses of LY2157299 and then exposed to 100 pM TGFβ1 for 25 min. Immunoblotting shows dose-dependent inhibition of pSMAD2 by LY2157299. Figure 5Q shows the shCTRL and two shTRIM33 KD cell lines cultured in the presence of JQ1 or GS-626510, with or without LY2157299 for 2 weeks and stained with crystal violet. Figure 5R shows the crystal violet quantification measured at 590 nm absorbance corresponding to Figure 5Q. Figure 5S shows the shCTRL or shTRIM33 cells treated with 1 µM JQ1 or 0.3 µM GS-626510 with or without 5 µM LY2157299 overnight and MYC protein levels assessed by immunoblotting. Figures 5T, 5U and 5V show that the over-expression of TβRII was not sufficient to induce resistance to BETi. Figure 5T shows the pLentiCMV-EV or pLentiCMV-TβRII transduced stable cell lines treated with increasing doses of TGFβ1 for 25 min and pSMAD2 levels assessed by immunoblotting. Figure 5U shows the cells from Figure 10D cultured in the presence of DMSO, 100 nM JQ1 or 50 nM GS-626510 for 2 weeks and stained with crystal violet. Figure 5V shows the pLentiCMV-EV or pLentiCMV-TβRII stable cell lines treated with 1 µM JQ1 or 0.3 µM GS-626510 for overnight either in the presence or in the absence of 100 pM TGFβ1. MYC levels were determined by immunoblotting and actin was used as a loading control.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein may be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

As used herein, the articles “a” and “an” are used to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

As used herein when referring to a measurable value such as an amount, a temporal duration, and the like, the term “about” is meant to encompass variations of

±20% or within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the specified value, as such variations are appropriate to perform the disclosed methods. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

5 The term "antagonist" as used herein refers to a compound or molecule that inhibits or decreases a biological response. An antagonist has affinity for a target, such as a receptor, and binding to the target disrupts or prevents the interaction between the target and its cognate binding partner to inhibit or decrease activity of the target. An antagonist may also block the action of a stimulating ligand by binding to an allosteric 10 site that may "lock" the target in an inactive state or prevent the dimerization or oligomerizarion of the target protein

As used herein, "bromodomain and extraterminal domain" or "BET" proteins refer to epigenetic readers involved in transcriptional control. The small family of BET proteins are characterized by tandem bromodomains, that bind acetylated lysine 15 residues in histones and other proteins, and a C-terminal extraterminal domain responsible for interactions with chromatin regulators. Examples of BET proteins include, but are not limited to, BRD2, BRD3, BRD4 and BRDT.

As used herein, "bromodomain and extraterminal domain inhibitor" or "BETi" refers to a small molecule that interacts with the acetylated lysine binding pocket of the 20 BET family bromodomains and displaces the BET proteins from binding to chromatin. BETi demonstrate anti-tumor activity in a range of malignancies. Some BETi exert antiproliferative effects that disrupt oncogenic pathways. Examples of BETi include, but are not limited to, JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-25 3365, I-BET762, OTX015, and TEN-010. Additional examples of BETi include compounds disclosed in U.S. Patent Nos. 9,255,089 and 9,108,953, and U.S. Patent Publication No. 2014/0336190, all of which are hereby incorporated herein in their entireties.

The term "BETi resistant" as used herein refers to a loss or decrease in anti-30 tumor efficacy or response of a cellular target to a BETi.

The term "BETi sensitivity" as used herein refers to anti-tumor activity of a BETi in a cellular target. "Increasing anti-tumor sensitivity" as used herein refers to increasing an anti-tumor response in a cellular target to the BETi by increasing a basal level of the anti-tumor response or resensitizing the cellular target to the BETi.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian
5 cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, myeloma and the like.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists
10 essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "effective amount" is meant the amount required to reduce or improve at
15 least one symptom of a disease relative to an untreated patient. The effective amount of an active compound(s) used for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject.

The term "expression" as used herein is defined as the transcription and/or
20 translation of a particular nucleotide sequence driven by its promoter.

By "fragment" is meant a portion of a polynucleotide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acids. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000,
25 1500, 2000 or 2500 (and any integer value in between) nucleotides. The fragment, as applied to a nucleic acid molecule, refers to a subsequence of a larger nucleic acid. A "fragment" of a nucleic acid molecule may be at least about 15 nucleotides in length; for example, at least about 50 nucleotides to about 100 nucleotides; at least about 100 to about 500 nucleotides, at least about 500 to about 1000 nucleotides, at least about
30 1000 nucleotides to about 1500 nucleotides; or about 1500 nucleotides to about 2500 nucleotides; or about 2500 nucleotides (and any integer value in between).

As used herein, the term "functional fragment" refers to a truncated peptide or polypeptide of the parent that retains at least one biological, physiological, and/or pharmacological property of the parent.

As used herein, the term "inhibit" is meant to refer to a decrease in a biological state. For example, the term "inhibit" may be construed to refer to the ability to negatively regulate expression, stability or activity of an expression product, wherein such inhibition may affect expression of a gene, protein mRNA, stability of a protein mRNA, translation of a protein mRNA, stability of a protein, a protein post-translational modifications, and/or a protein activity.

As used herein, the term "inhibitory nucleic acid" refers to small RNAs that inhibit gene expression. Examples of inhibitory nucleic acids include, but are not limited to, microRNAs (miRNA) and siRNA.

"Instructional material," as that term is used herein, includes a publication, a recording, a diagram, or any other medium of expression that may be used to communicate the usefulness of the compounds of the invention. In some instances, the instructional material may be part of a kit useful for effecting alleviating or treating the various diseases or disorders recited herein. Optionally, or alternately, the instructional material may describe one or more methods of alleviating the diseases or disorders in a cell or a tissue of a mammal. The instructional material of the kit may, for example, be affixed to a container that contains the compounds of the invention or be shipped together with a container that contains the compounds. Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the instructional material and the compound cooperatively. For example, the instructional material is for use of a kit; instructions for use of the compound; or instructions for use of a formulation of the compound.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a

nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

5 By "microRNA" or "miRNA" or "miR" is meant a small non-coding RNA, which functions in transcriptional and/or post-transcriptional regulation of gene expression.

As used herein, "neutralizing antibody" refers to an antibody that binds an antigen and prevents the biological effects of the antigen. In one embodiment, the
10 neutralizing antibody binds TGFβ or another effector in the TGFβ signaling pathway.

"Pharmaceutically acceptable" refers to those properties and/or substances that are acceptable to the patient from a pharmacological/toxicological point of view and to the manufacturing pharmaceutical chemist from a physical/chemical point of view regarding composition, formulation, stability, patient acceptance and bioavailability.
15 "Pharmaceutically acceptable carrier" refers to a medium that does not interfere with the effectiveness of the biological activity of the active ingredient(s) and is not toxic to the host to which it is administered.

As used herein, the term "pharmaceutical composition" or "pharmaceuticaly acceptable composition" refers to a mixture of at least one compound or molecule
20 useful within the invention with a pharmaceutically acceptable carrier. The pharmaceutical composition facilitates administration of the compound or molecule to a patient. Multiple techniques of administering a compound or molecule exist in the art including, but not limited to, intravenous, oral, aerosol, parenteral, ophthalmic, pulmonary and topical administration.

25 As used herein, the term "pharmaceutically acceptable carrier" means a pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, stabilizer, dispersing agent, suspending agent, diluent, excipient, thickening agent, solvent or encapsulating material, involved in carrying or transporting a compound or molecule useful within the invention within or to the patient such that it
30 may perform its intended function. Typically, such constructs are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, including the compound useful within the invention, and not injurious to the patient. Some examples of materials that may serve as

pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and
5 suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; surface active agents; alginic acid; pyrogen-free water; isotonic saline;
10 Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. As used herein, "pharmaceutically acceptable carrier" also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound useful within the invention, and are physiologically
15 acceptable to the patient. Supplementary active compounds may also be incorporated into the compositions. The "pharmaceutically acceptable carrier" may further include a pharmaceutically acceptable salt of the compound or molecule useful within the invention. Other additional ingredients that may be included in the pharmaceutical compositions used in the practice of the invention are known in the art and described,
20 for example in Remington's Pharmaceutical Sciences (Genaro, Ed., Mack Publishing Co., 1985, Easton, PA), which is incorporated herein by reference.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the
25 general knowledge that nucleic acids are polynucleotides, which may be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides may be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences that are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a
30 recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means. The following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine. The term "RNA" as used herein is defined as ribonucleic acid. The term "recombinant DNA" as

used herein is defined as DNA produced by joining pieces of DNA from different sources.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

As used herein, the terms "prevent," "preventing," "prevention," and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

By "reduces" or "decreases" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By "reference" is meant a standard or control. A "reference " is a defined standard or control used as a basis for comparison.

As used herein, "sample" or "biological sample" refers to anything, which may contain the cells of interest (e.g., cancer or tumor cells thereof) for which the screening method or treatment is desired. The sample may be a biological sample, such as a biological fluid or a biological tissue. In one embodiment, a biological sample is a tissue sample including pulmonary arterial endothelial cells. Such a sample may include diverse cells, proteins, and genetic material. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s). Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like.

As used herein, the term "sensitivity" as used herein refers to the ability of a target to respond in a qualitative fashion to a pharmacologic action of a compound or agent.

The term "small molecule inhibitor" as used herein refers to a compound or agent that inhibits a target. In one embodiment, the small molecule inhibitor inhibits a target in the TGFβ signaling pathway. Examples of the small molecule inhibitor include, but are not limited to, SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299 R268712, D4476, SB525334, GW788388 and TEW-7197.

By "small interfering RNA" or "siRNA" is meant a short RNA molecule that may be double stranded, which interferes with the expression of a specific gene that includes a nucleotide sequence complementary to the RNA molecule.

By the term " specifically binds," as used herein with respect to an antibody, is meant an antibody or antibody fragment which recognizes and binds with a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

A "subject" or "patient," as used therein, may be a human or non-human mammal. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals. Preferably, the subject is human.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any

one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for
5 comparison.

As used herein, the term "TGFβ pathway inhibitor" refers to an agent that inhibits the TGFβ signaling pathway. Examples of a TGFβ pathway inhibitor include, but are not limted to, a small molecule, an inhibitory nucleic acid, neutralizing antibody, and an antagonist.

10 As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or improving a disorder and/or symptom associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely ameliorated or eliminated.

15 As used herein, "tripartitie motif-containing 33" or "TRIM33" refer to a transcriptional corepressor protein and tumor suppressor with three zinc binding domains, a RING, a B-box type 1, a B-box type 2 and a coiled-coil region. TRIM33 is also known as PTC7, RFG7, TF1G, TIF1G, FLJ32925, TIFGAMMA, ECTODERMIN or TIF1GAMMA. Three alternatively spliced transcript variants for this gene have
20 been described; however, the full-length nature of one variant has not been determined. TRIM33 regulates TGF-beta/BMP signaling cascade and promotes physiological differentiation of hematopoietic stem cells by associating with SMAD2 and SMAD3. TRIM33 also acts as an E3 ubiquitin-protein ligase to promote SMAD4 ubiquitination, nuclear exclusion and degradation via the ubiquitin proteasome pathway. An
25 exemplary embodiment includes TRIM33 nucleic acid sequence comprising GenBank Accession No. NM_015906 or NM_033020 for human TRIM33 or NM_001079830 or NM_053170 for mouse TRIM33. In another exemplary embodiment includes TRIM33 polypeptide sequence comprising GenBank Accession No. NP_056990.3 or NP_148980.2 human TRIM33 or NP_001073299.1 or NP_444400.2 for mouse
30 TRIM33.

A "vector" is a composition of matter that comprises an isolated nucleic acid and that may be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds,

plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression may be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Compositions

It has been discovered that acquired resistance to bromodomain and extraterminal domain protein inhibitors (BETi) limits long-term responsiveness to BETi treatment. It has also been discovered that loss of TRIM33 or over-activation of a TGFβ signaling pathway decreases sensitivity to BET inhibitors. As described herein, increased expression of TRIM33 or inhibition of a TGFβ signaling pathway increases anti-tumor sensitivity. The invention therefore includes compositions and methods to increase anti-tumor sensitivity in a cell or tumor.

BETi are small molecules that interact with BET family bromodomains and displace the BET proteins, such as BRD2, BRD3, BRD4 and BRDT, from binding to chromatin. BETi demonstrate anti-tumor activity, such as antiproliferative effects, to disrupt oncogenic pathways. Examples of BETi include, but are not limited to, JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, and TEN-010. In one embodiment, the BETi is selected from the group consisting of JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, TEN-010, and any combination thereof.

In one aspect, the present invention includes a composition comprising a TGFβ pathway inhibitor and a bromodomain and extraterminal domain protein inhibitor (BETi), wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

The TGFβ pathway inhibitor inhibits at least one TGFβ signaling pathway, such as TGFβ receptor signaling. In one embodiment, the TGFβ pathway inhibitor is selected from the group consisting of a small molecule inhibitor, an inhibitory nucleic acid, a neutralizing antibody, and an antagonist.

In some embodiments, the TGFβ pathway inhibitor is a small molecule inhibitor. The small molecule inhibitor is a compound or agent that inhibits TGFβ signaling pathway, such as inhibiting TGFβ receptor signaling. In one embodiment, the small molecule inhibitor is selected from the group consisting of SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299 R268712, D4476, SB525334, GW788388, TEW-7197, and any combination thereof.

In some embodiments, the TGFβ pathway inhibitor is an inhibitory nucleic acid. The inhibitory nucleic acid includes small RNAs that inhibit gene expression. In some embodiments, the inhibitory nucleic acid inhibits expression of at least one gene in a TGFβ signaling pathway, such as TGFβ or a TGFβ receptor. In one embodiment, the inhibitory nucleic acid is selected from the group consisting of a miRNA and a siRNA. In another embodiment, the siRNA inhibits expression of TGFβ or a TGFβ receptor.

In some embodiments, the TGFβ pathway inhibitor is a neutralizing antibody. The neutralizing antibody specifically binds TGFβ or another effector in the TGFβ signaling pathway. In some embodiments, the neutralizing antibody specifically binds TGFβ or a TGFβ receptor. In one embodiment, the neutralizing antibody specifically binds one selected from the group consisting of TGFβ and a TGFβ receptor. In another

embodiment, the neutralizing antibody is selected from the group consisting of ab66043, ab61213, ab31013, 341-BR, 241-R2, AF-241-NA, AF1003, 1600-R2, AF532, MAB1835, MAB240, MAB2411, MAB532, 3C11, V, D-12, 2E5, 500-M66, TB21, H-100, T-19, V-22, G-16, R-20, RM—10-3A11, C-4, D-2, E-6, I-20, L-21, S-20, T-20, H-567, C-16, A-4, C-20, H-280, F-20, ABF17, H-112, 3711, 56E4, LY3022859, and LY238770.

In some embodiments, the TGFβ pathway inhibitor is a TGFβ pathway antagonist. The TGFβ pathway antagonist has affinity for a TGFβ pathway target, such as a TGFβ receptor, and binding to the TGFβ pathway target disrupts or prevents the interaction between the TGFβ pathway target and its cognate binding partner to inhibit or decrease activity of the TGFβ pathway target. In one embodiment, the antagonist includes agents that bind TGFβ and prevent TGFβ from binding to a TGFβ receptor, such as blocking (neutralizing) antibodies specific for a TGFβ (NAbs) or TGFβ receptor (Types I, II or III) such as those described by Dasch et al. (J. Immunol. (1989) 142:1536) and Lucas et al. (J. Immunol. (1990) 145:1415), soluble TGFβ receptors, protease inhibitors that inactivate a protease responsible for activating a precursor TGFβ into mature TGFβ, and combinations thereof. Examples of such antagonists include monoclonal and polyclonal antibodies directed against one or more isoforms of TGFβ (U.S. Pat. No. 5,571,714 and PCT patent application WO 97/13844), TGFβ receptors, fragments thereof, derivatives thereof and antibodies directed against TGFβ receptors (U.S. Pat. Nos. 5,693,607, 6,008,011, 6,001,969 and 6,010,872 and PCT patent applications WO 92/00330, WO 93/09228, WO 95/10610 and WO 98/48024); latency associated peptide (WO 91/08291), large latent TGFβ (WO 94/09812), fetuin (U.S. Pat. No. 5,821,227), decorin and other proteoglycans such as biglycan, fibromodulin, lumican and endoglin (U.S. Pat. Nos. 5,583,103, 5,654,270, 5,705,609, 5,726,149, 5,824,655, 5,830,847, 6,015,693 and PCT patent applications WO 91/04748, WO 91/10727, WO 93/09800 and WO 94/10187). Further examples of such antagonists include follistatin, somatostatin (PCT patent application WO 98/08529), mannose-6-phosphate or mannose-1-phosphate (U.S. Pat. No. 5,520,926), prolactin (PCT patent application WO 97/40848), insulin-like growth factor II (PCT patent application WO 98/17304), IP-10 (PCT patent application WO97/00691), arg-gly-asp containing peptides (U.S. Pat. No. 5,958,411 and PCT patent application WO 93/10808 and), extracts of plants, fungi and bacteria (European patent application 813875, Japanese patent application 8119984 and U.S. Pat. No. 5,693,610), antisense

oligonucleotides (U.S. Pat. Nos. 5,683,988, 5,772,995, 5,821,234 and 5,869,462 and PCT patent application WO 94/25588), and a host of other proteins involved in TGFβ signaling, including SMADs and MADs (European patent application EP 874046, PCT patent applications WO 97/31020, WO 97/38729, WO 98/03663, WO 98/07735, WO 98/07849, WO 98/45467, WO 98/53068, WO 98/5,5512, WO 98/56913, WO 98/53830, and WO 99/50296, and U.S. Pat. Nos. 5,834,248, 5,807,708 and 5,948,639) and Ski and Sno (G. Vogel, Science, 286:665 (1999) and Stroschein et al., Science, 286:771-74 (1999)) and fragments and derivatives of any of the above molecules that retain the ability to inhibit the activity of TGFβ. In one embodiment, the antagonist is selected from the group consisting of HtrA1, decorin, biglycan, fibromodulin, lumican, endoglin, somatostatin, follistatin, RAP-1332, pirfenidone (5-methyl-1-phenyl-2(1H)-pyridone), soluble ectodomains of TGFβ receptor type II (RII) or betaglycan (BG), and any combination thereof.

In another aspect, the present invention includes a composition comprising a BETi and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

In some embodiments, the composition of the present invention comprises TRIM33. TRIM33 includes a polypeptide that is recombinantly or synthetically produced. In one embodiment, TRIM33 comprises an isolated TRIM33 polypeptide. In another embodiment, TRIM33 comprises a polypeptide having a GenBank Accession No selected from the group consisting of NP_056990.3, NP_148980.2, NP_001073299.1 and NP_444400.2. In some embodiments, TRIM33 includes at least one post-translational modification.

In some embodiments, the composition of the present invention comprises a functional fragment of TRIM33. The functional fragment of TRIM33 includes fragments that retain at least one biological, physiological, and/or pharmacological property of TRIM33. In one embodiment, the functional fragment of TRIM33 comprises about 10%, 25%, 30%, 50%, 68%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or any percent therebetween of TRIM33.

In some embodiment, the composition of the present invention comprises a nucleic acid encoding TRIM33. In one embodiment, the nucleic acid encoding TRIM33 comprises a nucleic acid sequence having a GenBank Accession No selected

from the group consisting NM_015906, NM_033020, NM_001079830, and NM_053170.

In another embodiment, the nucleic acid encoding TRIM33 is an expression vector comprising a TRIM33 gene. In another embodiment, the the vector is a viral
5 vector, such as an adenoviral vector, an adeno-associated virus vector, a retroviral vector, a lentiviral vector, and the like.

Methods

The present invention also includes a method for increasing expression of
10 TRIM33 or inhibiting a TGFβ signaling pathway to increase anti-tumor sensitivity. As described herein, loss of TRIM33 or over-activation of a TGFβ signaling pathway decreases sensitivity to BET inhibitors. Administering a composition that includes a TRIM33 or a TGFβ pathway inhibitor with a BETi to a subject in need thereof increases anti-tumor sensitivity in a cell or tumor in the subject.

15 In one aspect, the invention includes a method of increasing anti-tumor sensitivity to a bromodomain and extraterminal domain protein inhibitor (BETi) in a cell in a subject. The method comprising administering a composition comprising a TGFβ pathway inhibitor and a BETi to a subject in need thereof, wherein the TGFβ pathway inhibitor sensitizes the cell to the BETi.

20 In another aspect, the invention includes a method of treating a tumor comprising administering to a subject in need thereof a composition comprising a TGFβ pathway inhibitor and a bromodomain and extraterminal domain protein inhibitor (BETi), wherein the TGFβ pathway inhibitor sensitizes the tumor to the BETi.

In yet another aspect, the invention includes a method of increasing anti-tumor
25 sensitivity of a cell in a subject to a bromodomain and extraterminal domain protein inhibitor (BETi). The method comprising administering a composition comprising a BETi and one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33 to a subject in need thereof, wherein the TRIM33 sensitizes the cell to the BETi.

30 In still another aspect, the invention includes a method of treating a tumor comprising administering to a subject in need thereof a composition comprising a BETi and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein TRIM33 sensitizes the tumor to the BETi.

In one embodiment, the tumor is a cancer selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer. In another embodiment, the subject has a cancer, such as a cancer
5 selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer.

The methods and compositions of the present invention are useful to increase an anti-tumor response in a cellular target to a BETi by increasing a basal level of the anti-
10 tumor response or resensitizing the cellular target to the BETi. In another embodiment, the cell or tumor is BETi resistant. In yet another embodiment, cell or tumor has acquired decreased BETi sensitivity. In still another embodiment, administration of the composition to the subject increases an anti-tumor response to the BETi.

15 **Nucleic Acids**

The present invention includes, in some embodiments, a composition comprising a TGFβ pathway inhibitory nucleic acid, and in some embodiments, a composition comprising a nucleic acid encoding TRIM33. Such nucleic acids are introduced into a cell for the benefit of a subject.

20 Methods of introducing nucleic acids into a cell include physical, biological and chemical methods. Physical methods for introducing a polynucleotide, such as DNA like cDNA, into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. DNA, like cDNA, can be introduced into target cells using commercially available methods which include
25 electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendorf, Hamburg Germany). DNA can also be introduced into cells using cationic liposome mediated transfection using lipofection, using polymer encapsulation, using peptide mediated transfection, or using biolistic
30 particle delivery systems such as “gene guns” (see, for example, Nishikawa, et al. Hum Gene Ther., 12(8):861-70 (2001)).

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into

mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the nucleic acids in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and

PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Pharmaceutical Compositions

The invention also encompasses the use of a pharmaceutical composition of the invention to practice the methods of the invention. In one aspect, the invention includes a pharmaceutical composition comprising the composition as described herein and a pharmaceutically acceptable carrier. In another aspect, the composition described herein is used in the manufacture of a medicament for the treatment of a tumor or cancer in a subject in need thereof. In yet another aspect, the invention includes a pharmaceutical composition comprising the composition as described herein in combination with another therapeutic agent used in the treatment of a tumor or cancer. Such pharmaceutical compositions may be provided in a form suitable for administration to a subject, and may comprise one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. The composition described herein may comprise a physiologically acceptable salt, such as a compound contemplated within the invention in combination with a physiologically acceptable cation or anion, as is well known in the art.

Pharmaceutical compositions that are useful in the methods of the invention may be suitably developed for inhalational, oral, rectal, vaginal, parenteral, topical, transdermal, pulmonary, intranasal, buccal, ophthalmic, intrathecal, intravenous or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations. The route(s) of administration will be readily apparent to the skilled artisan and will depend upon any number of factors including the type and severity of the disease being treated, the type and age of the veterinary or human patient being treated, and the like.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if

necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

In one embodiment, the compositions of the invention are formulated using one or more pharmaceutically acceptable excipients or carriers. In one embodiment, the pharmaceutical compositions of the invention comprise a therapeutically effective amount of at least one compound of the invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers, which are useful, include, but are not limited to, glycerol, water, saline, ethanol and other pharmaceutically acceptable salt solutions such as phosphates and salts of organic acids. Examples of these and other pharmaceutically acceptable carriers are described in Remington's Pharmaceutical Sciences (1991, Mack Publication Co., New Jersey).

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", fourth edition (Sambrook, 2012); "Oligonucleotide Synthesis" (Gait, 1984); "Culture of Animal Cells" (Freshney, 2010); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1997); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Short Protocols in Molecular Biology" (Ausubel, 2002); "Polymerase Chain Reaction: Principles, Applications and Troubleshooting", (Babar, 2011); "Current Protocols in Immunology" (Coligan, 2002). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out embodiments of the present invention, and are not to be construed as limiting in any way.

Bromodomain and extraterminal domain protein inhibitors (BETi) hold great promise as a novel class of cancer therapeutics. As acquired resistance typically limits durable responses to targeted therapies, it is important to understand mechanisms by which tumor cells adapt to BETi. Described herein, through pooled shRNA screening of colorectal cancer cells, tripartite motif-containing protein 33 (TRIM33) was identified as a factor promoting sensitivity to BETi. Loss of TRIM33 was demonstrated to reprogram cancer cells to a more resistant state through at least two mechanisms. TRIM33 silencing attenuates downregulation of MYC in response to BETi. Moreover, loss of TRIM33 enhances TGFβ receptor expression and signaling, and blocking TGFβ receptor activity potentiates the anti-proliferative effect of BETi. Described herein is a mechanism for BETi resistance and combining inhibition of TGFβ signaling with BET bromodomain inhibition may offer new therapeutic benefits.

The Materials and Methods used in the performance of the experiments disclosed herein are now described.

Cell lines, antibodies and drugs. Cell lines 293T, RKO, HCT15, HCT116, LoVo, SW620, SW837, SK-CO-1, SW480, SW1463, MDA-MB-231, MDA-MB-415, MDA-MB-468, ZR-75-1, LNCap and PC-3 were obtained from ATCC and maintained as suggested. Antibodies were purchased from Cell Signaling Technology and Abcam: TRIM33 (#13387), SMAD2 (#5339), pSMAD2 (#3108), SMAD4 (#9515), BRD4 (#13440), actin (#3700) and MYC (ab32072). Recombinant human TGFβ1 was from Cell Signaling Technology (#8915LC). (+)-JQ1 (11187) was purchased from Cayman Chemical and LY2157299 (S2230) was purchased from Selleck Chemical. GS-626510 was synthesized at Gilead Sciences.

Stable knockdown and expression cell lines. Lentiviral expression vectors for shRNAs in the pLKO.1 puro vector (Sigma) were used to stably knockdown TRIM33, TβRII or SMAD4. For stable knockdown of two genes, the shTRIM33-B5 sequence was cloned into pLKO.1 blast (Addgene #26655) to silence TRIM33 expression. The shRNA target sequences used are listed in Table 1. For expression of TGFβRII and

TRIM33, cDNAs from Addgene #19147 and Addgene #15734 respectively, were cloned into pLentiCMV-hygro(DEST) (Addgene #17454)) through Gateway cloning (Invitrogen). Seven silent mutations were made to TRIM33 cDNA to render resistance to shTRIM33-B5. MYC lentiviral expression vector is from Addgene (#46970).

5

Table 1: shRNA target sequences.

shRNA	Target sequence	
shCTRL	CAACAAGATGAAGAGCACCAA	SEQ ID NO:1
shTRIM33-B5	GTACTAGTTGTGAAGACAATG	SEQ ID NO:2
shTRIM33-A12	GCTCCTGGTTATACTCCTAAT	SEQ ID NO:3
shTβRII-3	GCTCCCTAAACACTACCAAAT	SEQ ID NO:4
shTβRII-4	AATGACGAGAACATAACACTC	SEQ ID NO:5
shSMAD4-3	CAGATTGTCTTGCAACTTCAG	SEQ ID NO:6
shSMAD4-4	TACCATACAGAGAACATTGGA	SEQ ID NO:7

Cell lysis for immunoblotting and immunoprecipitation. For immunoblotting, cells in 6-well plates were quickly rinsed twice with PBS and directly lysed in 150 μL
10 SDS lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol). The lysate was then transferred to 1.5 mL Eppendorf tubes and heated for 10 min at 95-100 °C with intermittent vortexing. After spinning to remove any undissolved material and measuring the protein concentration using BCA assay, 20-40 μg total lysate was fractionated by SDS-PAGE and transferred to nitrocellulose membrane for
15 immunoblotting. For immunoprecipitation, cells were rinsed quickly with ice-cold PBS and lysed in buffer (50 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and Roche Complete Protease Inhibitor Cocktail) on ice for 15 min. Scraped cell lysate was centrifuged at 13,200 rpm for 10 min at 4 °C and 1 mg of supernatant was incubated
20 with 1-5 μg primary antibody overnight at 4 °C. 25 μL of protein A sepharose 4B (Invitrogen) was added to the tube for another 2 h, and the precipitate was washed 3 times and then eluted in 60 μL of Laemmli sample buffer. 20 μL of the elution was used for immunoblotting.

Quantitative RT-PCR analysis. Total RNA was extracted using an RNEASY®
25 mini kit (Source) with on-column DNA digestion. 1 μg of total RNA was used for cDNA synthesis with the ISCRIPT™ cDNA synthesis kit (Bio-Rad) as per manufacturer's suggestion. Real-time PCR was performed on a Bio-Rad CFX CONNECT™ Real-Time System and relative mRNA level was calculated in CFX

Manager software using the 2^ (-ΔΔCt) method. GAPDH mRNA was used as internal control. PCR primer sequences are listed in Table 2.

Table 2: PCR primer sequences (5'-3')

Gene	Forward primer	Reverse primer
GAPDH	GAAGGTGAAGGTCGGAGTCA SEQ ID NO:8	TTGAGGTCAATGAAGGGGTC SEQ ID NO:9
TRIM33	GGAGTGCTTGCATGTTGAG SEQ ID NO:10	CCAATTCACTTTCTAGATGCAGG SEQ ID NO:11
TRIM33	TTACAGCAATAGAGCTAATCCC SEQ ID NO:12	ACAACGTTTGCCTGTATGG SEQ ID NO:13
MYC	GGCTCCTGGCAAAAGGTCA SEQ ID NO:14	CTGCGTAGTTGTGCTGATGT SEQ ID NO:15
TGFBR2	GTAGCTCTGATGAGTGCAATGAC SEQ ID NO:16	CAGATATGGCAACTCCCAGTG SEQ ID NO:17
HBE1	ATGGTGCATTTTACTGCTGAGG SEQ ID NO:18	GGGAGACGACAGGTTTCCAAA SEQ ID NO:19
PDE4B	AACGCTGGAGGAATTAGACTGG SEQ ID NO:20	GCTCCCGGTTCAGCATTCT SEQ ID NO:21
ZNF474	ATATCGGAAAGCCAGCTTAGC SEQ ID NO:22	GACCCAAATTCTCGGCCAC SEQ ID NO:23
RGS4	ACATCGGCTAGGTTTCCTGC SEQ ID NO:24	GTTGTGGGAAGAATTGTGTTCAC SEQ ID NO:25
BCL2A1	TACAGGCTGGCTCAGGACTAT SEQ ID NO:26	CGCAACATTTTGTAGCACTCTG SEQ ID NO:27
MAP1LC3C	CCCAAGCGTCAGACCCTTC SEQ ID NO:28	GGGGAACTTTGCCCGGATT SEQ ID NO:29
FBXO8	AGCAAGGCTACCTCACCAGA SEQ ID NO:30	TCCTTCCTGTTCTTTCGATTTCC SEQ ID NO:31
CHCHD3	GAGGCGGACGAGAATGAGAAC SEQ ID NO:32	ACCAGAATACCGCTGAGACTTC SEQ ID NO:33
DUSP10	ATCGGCTACGTCATCAACGTC SEQ ID NO:34	TCATCCGAGTGTGCTTCATCA SEQ ID NO:35
ITGA6	ATGCACGCGGATCGAGTTT SEQ ID NO:36	TTCCTGCTTCGTATTAACATGCT SEQ ID NO:37
MYCT1	CAATCGGGCTGGTACTTGGAG SEQ ID NO:38	CGTGGGTGTAAGAAGACCTAGA SEQ ID NO:39
TRIML2	GCCACCGAGCTAGAGGAGAT SEQ ID NO:40	CTTGAGCAATGCCAAGGTGC SEQ ID NO:41
MARCH4	CTGTAAGGAGAAGACCGAGGA SEQ ID NO:42	ATCCACTTGATGAGGCAAGGC SEQ ID NO:43
ADRA2C	GCCTCAACGACGAGACCTG SEQ ID NO:44	CCCAGCCCGTTTTCGGTAG SEQ ID NO:45
ERRFI1	CTGGAGCAGTCGCAGTGAG SEQ ID NO:46	GCCATTCATCGGAGCAGATTTG SEQ ID NO:47

5

Cumulative cell growth assay. RKO cells (3 x 10^5) transduced with the indicated virus were plated in a single well of a 6-well plate at day 0 in the presence or absence of inhibitors. Three days later cells were detached, counted, and 3 x 10^5 cells were transferred to a new well. The process was repeated until day 15. The cumulative
5 cell number was then calculated from fold changes and the individual cell counts at each passage.

Crystal violet cell proliferation assay. Cells (5-20 x 10^3) were plated in each well of a 6-well plate with 3 mL of media with or without inhibitors and cultured for 14 days undisturbed. Medium was aspirated, and cells were stained with crystal violet
10 staining solution (0.05% w/v crystal violet, 1% formaldehyde, 1% methanol in PBS) for 30 minutes and washed with water several times. Stained plates were then air-dried and imaged with CHEMIDOC® using Image Lab software (Bio-Rad). To quantify the crystal violet staining, 1 mL of 10% acetic acid was added to each well to solubilize the stain for 20 min and the stain was diluted 1:4 in water and absorbance was measured at
15 590 nm.

Growth inhibition assay and IC_{50} value determination. Cells (1000 per well) were plated in 96-well plates in duplicate with 1:3 serial dilutions of BETi ranging from 0.169 nM to 10 μM or 0.1% DMSO vehicle and cultured for 120 h. The end point relative viable cell number was determined using CELLTITER GLO® by quickly
20 decanting the media, adding 100 μL of 1:2 CellTiter Glo reagent diluted in PBS to the well and incubating for 10 min. The luminescence of each well was read with a TECAN Infinite® M1000Pro plate reader. IC_{50} values were calculated with GRAPHPAD PRISM 6® by fitting the data to the "3-parameter log (inhibitor) vs response" equation. At least three independent growth inhibition assays were
25 performed for each pair of cell lines to derive mean IC_{50} values.

shRNA screening. The Mission human shRNA library (Luo et al., 2008, *Proc Natl Acad Sci U S A* 105(51):20380-20385) generated in pLKO lentiviral delivery vectors by The RNAi Consortium (TRC) was obtained as arrayed bacterial stocks (Sigma). All shRNAs targeting 517 genes annotated as protein kinases (5634 shRNAs
30 in total) and 85 non-targeting control shRNA vectors were picked from the library, cultured on LB-agar plates and plasmid DNA was prepared from a mixture of these cultures using GeneElute HP Endotoxin Free Plasmid Maxi-prep kit (Sigma). Lentiviral particles were generated by co-transfecting 293T cells with the pLKO plasmid mixture, pCMV dR8.91 packaging vector, and pCMV-VSV-G envelope vector in a 10:10:1

ratio. Viral supernatant was collected 48 and 72 h after transfection and stored at -80°C. RKO cells were transduced by incubating for 24 h with the pool of shRNA-expressing viruses diluted to give a MOI (multiplicity of infection) of ~0.3 to ensure that most of the cells received a single viral integration. Care was taken to ensure that the initial
5 number of infected cells exceeded 6 x 10^6 resulting in at least 1000-fold coverage of the ~6000 unique shRNAs in the pool, and 1000-fold coverage was strictly maintained at all steps of the screening protocol. Infected cells were selected with 1 µg/mL puromycin for 2 days, and 6 x 10^6 cells were removed for genomic DNA extraction to serve as the T0 reference sample. Remaining cells were split into 5 parallel 15 cm
10 plates each with 6 x 10^6 cells to be treated with 0.1 % DMSO vehicle, 0.1 µM JQ1, 0.3 µM JQ1, 0.05 µM GS-626510 or 0.1 µM GS-626510. When plates approached confluence, 6 x 10^6 cells were re-seeded into fresh plates until T4. Genomic DNA from T0 and the five T4 samples was extracted and shRNA integrants were PCR amplified with barcoded primers. All the samples were sequenced on an Illumina HiSeq
15 instrument and the relative abundance of each shRNA from T4 was compared to those of T0. To minimize error due to stochastic effects, hairpins with fewer than 50 raw reads in T0 were not considered. Each sample in the sequencing library preparation was normalized to a total read depth of 1 x 10^6 to correct for variation in read depth across samples. RIGER algorithm in the GENE-E java package from the Broad Institute was
20 used to rank each gene by their enrichment. The log fold change metric and second-best hairpin method was used to score the genes so that at least two hairpins against each gene were enriched in each condition.

RNAseq data analysis and gene set enrichment analysis. RKO cells expressing shCTRL or shTRIM33 were treated with 0.1% DMSO vehicle, 1 µM JQ1 or 0.3 µM
25 GS-626510 for 3 h, and mRNA was extracted using RNEASY® mini kit with on column DNase I digestion option (Qiagen) and submitted to the Yale Center for Genome Analysis for RNAseq analysis. Low quality reads and bases were trimmed, and filtered reads were then mapped to the human reference genome (hg19) using Tophat™ v2.0.13. Only reads that mapped to a single unique location within the
30 genome were reported. Tophat alignments from duplicate RNAseq experiments were then processed through DESeq to produce one differential expression data set. When a single RNAseq data set was analyzed, differential expression was calculated as fold change in the normalized raw counts of each transcript. For gene set enrichment analysis, the R software package edgeR was used to normalize the gene level read

counts across samples. Genes with less than one shortread count per million (CPM) in at least one sample were filtered out to remove genes with low levels of expression. Generalized linear regression in edgeR was then used to estimate $\log_2$ fold changes and *p* values. To identify the genes that respond differently to BETi in the shTRIM33 cells
5 relative to the shCTRL cells, the following contrast was specified in the edgeR analysis: (BETi in shTRIM33 - DMSO in shTRIM33) – (BETi in shCTRL - DMSO in shCTRL). Multiple testing was controlled by using false discovery rate (FDR). Next, the estimated *p* values of all the genes were converted using the zScores function in the R package gCMAP to Z scores to generate the ranked list of genes. The ranked list of
10 genes was then analyzed with GSEA Preranked included in the Broad GSEA java tool for Gene set enrichment analysis against MSigDB, C2 (curated gene sets), C6 (oncogenic signatures), and C7 (immunologic signatures) collections (total 5285 gene sets).

15 The Results of the experiments disclosed herein are now described.

Example 1: Pooled shRNA library screening identified TRIM33 as a negative regulator of BETi resistance

To identify genes whose loss confers resistance to the anti-proliferative effects of BET bromodomain inhibitors, a pooled shRNA screen was performed in a BETi-
20 sensitive colorectal cancer cell line (RKO). Screening was carried out in the presence of one of two structurally unrelated inhibitors: the widely used compound JQ1 and a novel BETi GS-626510 (Figure 1A). GS-626510 binds with high affinity and specificity to BET family bromodomains (Figure 1B, Table 3). Both JQ1 and GS-626510 potently inhibited growth of RKO cells with IC_{50} values of 81 nM and 33 nM respectively
25 (Figure 1C). As anticipated for BRD4 inhibition, both compounds strongly decreased MYC levels in RKO cells (Figure 1D). RNAseq analysis showed a strong correlation between genes up- and down-regulated following 3 h treatment of RKO cells with 1 μM of JQ1 or 0.3 μM of GS-626510 (Figure 1E), suggesting that growth suppression by these compounds is attributable to a common mechanism of action.

30

Table 3: Kd values of bromodomains to BETi GS-626510

Compound Name	DiscoveRx gene symbol	Entrex gene symbol	Modifier	Kd (nM)
GS-626510	ATAD2A	ATAD2	>	10,000
GS-626510	ATAD2B	ATAD2B	>	10,000
GS-626510	BAZ2A	BAZ2A	>	10,000
GS-626510	BAZ2B	BAZ2B	>	10,000
GS-626510	BRD1	BRD1	>	10,000
GS-626510	BRD2 (1)	BRD2	=	2.5
GS-626510	BRD2 (1, 2)	BRD2	=	1.1
GS-626510	BRD2 (2)	BRD2	=	0.59
GS-626510	BRD3 (1)	BRD3	=	1.7
GS-626510	BRD3 (1, 2)	BRD3	=	0.66
GS-626510	BRD3 (2)	BRD3	=	0.65
GS-626510	BRD4 (1)	BRD4	=	2.9
GS-626510	BRD4 (1, 2)	BRD4	=	1.3
GS-626510	BRD4 (2)	BRD4	=	3.2
GS-626510	BRD4 (full-length, short-iso)	BRD4	=	2.8
GS-626510	BRD7	BRD7	>	10,000
GS-626510	BRD8 (1)	BRD8	=	5,900
GS-626510	BRD8 (2)	BRD8	>	10,000
GS-626510	BRD9	BRD9	>	10,000
GS-626510	BRDT (1)	BRDT	=	19
GS-626510	BRDT (1,2)	BRDT	=	4.9
GS-626510	BRDT (2)	BRDT	=	5
GS-626510	BRPF1	BRPF1	=	4,100
GS-626510	BRPF3	BRPF3	>	10,000
GS-626510	CECR2	CECR2	=	8,200
GS-626510	CREBBP	CREBBP	=	170
GS-626510	EP300	EP300	=	220
GS-626510	FALZ	BPTF	>	10,000

GS-626510	GCN5L2	KAT2A	>	10,000
GS-626510	PBRM1 (2)	PBRM1	>	10,000
GS-626510	PBRM1 (5)	PBRM1	>	10,000
GS-626510	PCAF	KAT2B	>	10,000
GS-626510	SMARCA2	SMARCA2	>	10,000
GS-626510	SMARCA4	SMARCA4	>	10,000
GS-626510	TAF1 (2)	TAF1	=	8,000
GS-626510	TAF1L (2)	TAF1L	>	10,000
GS-626510	TRIM24 (Bromo.)	TRIM24	=	4,100
GS-626510	TRIM24 (PHD, Bromo.)	TRIM24	=	6,900
GS-626510	TRIM33 (PHD, Bromo.)	TRIM33	>	10,000
GS-626510	WDR9 (2)	BRWD1	>	10,000

A custom lentiviral shRNA library was generated containing 5634 shRNA constructs targeting 517 genes annotated as protein kinases and 85 non-targeting control shRNAs. RKO cells were infected with the pooled shRNA virus, and following puromycin selection for infected cells, 6 x 10^6 cells were removed for genomic DNA extraction to serve as a reference (T0) population. The remaining cells were placed into each of 5 different inhibitor conditions: DMSO vehicle control and low and high doses of either JQ1 or GS-626510 (Figure 1F). Cells were allowed to proliferate and were passaged when they approached confluence. This treatment was maintained until cells reached passage 4 (T4). Genomic DNA was extracted and the relative abundance of each shRNA in each treatment condition at T4, and in the reference T0 condition, was assessed by PCR amplifying the integrated shRNA followed by next generation sequencing (Figure 1F). This allowed calculation of the relative enrichment or depletion of each individual shRNA at T4 compared with T0.

As the library contained multiple shRNAs targeting each gene, RIGER analysis was used to identify and rank genes preferentially targeted by hairpins enriched upon drug treatment but not in the DMSO-treated control cells. These genes presumably encode proteins that promote susceptibility to BETi. Silencing expression of these genes thus causes drug resistance, resulting in cells harboring their respective hairpins being enriched at the end of the screen.

Strikingly, TRIM33 was the top ranked enriched target gene in all four BETi-treated conditions, but was not enriched in the absence of inhibitor (Figure 1G). Tracking individual shRNAs revealed clear enrichment of most shRNAs targeting TRIM33 at T4 in the presence of JQ1 or GS-626510 (Figure 1H). By contrast, TRIM33
5 hairpins appear to be preferentially depleted in the DMSO vehicle control sample.

An independent replicate of this screen, carried out to passage 5 (T5) produced very similar results with TRIM33 ranked in the top 3 of all four drug conditions (Figure 1G). Notably, TRIM24, the most closely related TRIM33 family member, was also highly enriched in all four inhibitor treated conditions but not in the DMSO control
10 (Figure 1G), supporting the potential functional relevance of TRIM33 to modulate BETi sensitivity.

Thus, data from two independent screens, each performed with two doses of two chemically unrelated BET bromodomain inhibitors, indicated that TRIM33 knockdown conferred a selective growth advantage in BETi-treated RKO cells.
15 TRIM33 and TRIM24 were included in the shRNA library on the basis of early reports identifying TRIM24 and TRIM28 as protein kinases, but the absence of a recognizable kinase catalytic domain and lack of subsequent verification suggests that these proteins are unlikely to have such activity.

20 **Example 2: BETi resistance in shTRIM33 cells was due to the specific loss of TRIM33 protein**

To verify the screening data suggesting that TRIM33 promotes sensitivity to BETi in cancer cells, a stable TRIM33 knockdown was established in RKO cells by lentiviral transduction and evaluated their sensitivity to JQ1 or GS-626510. Among
25 four individual shRNAs tested, shTRIM33-B5 (hereafter referred to as shTRIM33 unless otherwise noted) was chosen to silence expression of TRIM33 as it produced the most efficient TRIM33 knockdown at the protein level (Figure 2A).

Comparison of cell proliferation of shCTRL and shTRIM33 cells in 15-day cultures confirmed that knocking down TRIM33 conferred a growth advantage in the
30 presence of BETi (Figure 2B). Notably, consistent with the screening data, shTRIM33 cells cultured in the absence of inhibitor exhibited a growth disadvantage (Figure 2B), suggesting that the effect of TRIM33 on growth in the presence of BETi was not due to a basal increase in cell proliferation.

These studies were extended to compare the potency of JQ1 and GS-626510 in shCTRL and shTRIM33 cells. Cells were incubated with varying concentrations of JQ1 or GS-626510 for 5 days and the relative cell number was determined. TRIM33 knockdown produced a rightward shift in the growth inhibition curves for both JQ1 and 5 GS-626510 (Figure 2C). Multiple replicates revealed that the IC_{50} value of JQ1 and GS-626510 was increased by approximately 3-fold in shTRIM33 cells, suggesting the shTRIM33 cells were more resistant to BETi (Figure 2D). This effect was not limited to RKO cells as similar experiments performed in a panel of colorectal, breast and prostate cancer cell lines revealed that TRIM33 knockdown also decreased sensitivity 10 to JQ1 and GS-626510 in a subset of the cell lines tested (Figures 2E and Table 4).

Finally, in prolonged culture, TRIM33 knockdown facilitated outgrowth of BETi-treated RKO cells (Figure 2F). Similar effects were observed with a different shRNA targeting TRIM33 (A12) (Figures 2A and 2G), suggesting that the results were not due to off target effects.

15 To further confirm that BETi resistance caused by TRIM33-directed shRNA was due to the loss of TRIM33 protein and not due to off target silencing of other genes, rescue RKO cell lines were generated re-expressing a knockdown-resistant TRIM33 cDNA (Figures 2H and 2I). shTRIM33 cells re-expressing TRIM33 (pLenti-TRIM33), but not those infected with an empty vector (pLenti-EV), became more 20 sensitive to both JQ1 and GS-626510 in long-term culture assays (Figures 2H, bottom panel, and 2J). Furthermore, in these experiments, overexpression of TRIM33 in shCTRL cells increased sensitivity to both compounds (Figures 2H, top panel, and 2J). Together, these data support the idea that TRIM33 promotes sensitivity to BET bromodomain inhibition.

25

Table 4

Cell Line	**JQ-1 IC50 (µM)**		**GS510 IC50 (µM)**	
	ShCTRL	**shTRIM33**	**shCTRL**	**shTRIM33**
HCT15	0.098±0.019	0.076±0.012	0.147±0.039	0.092±0.021
HCT116	0.295±0.101	0.152±0.039	0.242±0.104	0.123±0.040
LoVo	0.056±0.029	0.047±0.022	0.044±0.024	0.032±0.017
SW620	0.293±0.005	0.407±0.032	0.136±0.025	0.264±0.055
SW837	0.056±0.008	0.093±0.013	0.023±0.003	0.037±0.004

SKCO1	0.495±0.050	1.404±0.231	0.259±0.035	0.649±0.120
SW480	0.172±0.060	0.376±0.147	0.161±0.066	0.285±0.142
SW1463	0.603±0.151	0.794±0.437	2.107±1.739	0.974±0.434
RKO	0.065±0.005	0.191±0.027	0.026±0.005	0.071±0.013
MDA-MB-231	0.114±0.019	0.267±0.025	0.085±0.022	0.216±0.024
MDA-MB-415	0.334±0.012	0.401±0.044	0.375±0.093	0.378±0.091
ZR75-1	0.215±0.100	0.210±0.099	0.145±0.069	0.150±0.072
MDA-MB-468	0.166±0.006	0.133±0.021	0.122±0.023	0.087±0.011
LNCap	0.033±0.006	0.049±0.019	0.011±0.001	0.031±0.012
PC3	0.12±0.008	0.111±0.011	0.05±0.011	0.055±0.013

Example 3: TRIM33 knockdown maintains MYC expression following BETi

Given the established role of both TRIM33 and BET proteins as transcriptional regulators, it was hypothesized that shTRIM33-mediated BETi resistance could be due
5 to deregulated gene transcription. RNAseq was used to investigate changes in gene expression resulting from treatment with BETi and with loss of TRIM33. RNAseq was performed in shCTRL and shTRIM33 cells after 3h treatment with JQ1 (1 μM), GS-626510 (0.3 μM) or vehicle control (DMSO). Results from two independent replicate experiments were analyzed by DESeq. Results consistent with RNAseq data were
10 obtained by measuring mRNA levels for 15 genes by qRT-PCR (Figures 3A-3E).

Similar to previous reports, 3-hour BETi treatment had a broad impact on gene expression: among the 11,277 genes reliably detected by RNAseq, approximately 1200 genes changed by greater than 2-fold (Figure 3F-3G). Consistent with prior studies in other cell types, BETi treatment of RKO cells strongly reduced levels of *MYC* (5 to 6-
15 fold). Furthermore, gene set enrichment analysis (GSEA) of transcripts down-regulated by both inhibitors revealed significant enrichment for genes having target motifs for MYC or the MYC co-activator MAZ in their promoter regions (20% of downregulated genes, Figure 3H).

In contrast to BET bromodomain inhibition, TRIM33 KD influenced the
20 expression of a relatively small fraction of genes (Figure 3I). Following TRIM33 knockdown, 272 transcripts were up-regulated by at least 2-fold, while only 84 were down-regulated by at least 2-fold, arguing that TRIM33 works preferentially as a transcriptional repressor rather than an activator. Notably, loss of TRIM33 had no

effect on expression of BET genes (BRD2, BRD3 and BRD4) themselves and did not affect BRD4 protein levels (Figure 3J).

Repression of MYC is believed to be a major mechanism by which BETi suppress growth of some cell types (10, 12). It was therefore examined whether there was a potential role for MYC in mediating the effect of TRIM33 knockdown. Consistent with the RNAseq data (Figure 4A), 3hr treatment with either JQ1 or GS-626510 strongly suppressed *MYC* mRNA levels as measured by qRT-PCR (Figure 4B). Furthermore, presumably due to the short (20-30 min) half-life of MYC protein, MYC protein levels were also strongly suppressed (Figure 4C).

While basal levels of *MYC* mRNA and protein were modestly increased in shTRIM33 cells, their downregulation by BETi was substantially attenuated (Figure 4B-4C). Furthermore, rescue of TRIM33 protein expression in shTRIM33 cells partially restored MYC sensitivity to JQ1 and GS-626510 (Figure 4B-4C). These results suggest that TRIM33 was required for the ability of BET inhibitors to maximally down-regulate MYC.

To determine whether stabilization of MYC may play a role in conferring resistance to BETi, MYC was stably over-expressed in RKO cells. Ectopically expressed MYC was resistant to BETi-mediated down regulation (Figure 4D). While RKO cells overexpressing MYC proliferated at the same rate as control cells, possibly reflecting the high basal levels of MYC expression in this cell line, MYC over-expressing cells had a growth advantage in long-term culture in the presence of JQ1 or GS-626510 (Figures 4E-4F). Thus, protection of MYC levels from downregulation is likely to contribute to BETi resistance in shTRIM33 RKO cells.

Consistent with a role for TRIM33 in regulation of MYC expression, chromatin immunoprecipitation (ChIP) revealed that TRIM33 associated with the MYC promoter in BETi-treated RKO cells (Figure 4G). Notably, BRD4 ChIP showed that BRD4 associated with similar sites in the MYC promoter and that BRD4 was displaced following BETi treatment (Figure 4H). These data suggest that BETi may suppress MYC expression by displacing BRD4 from the MYC promoter to allow recruitment of the transcriptional repressor TRIM33 at that site. In the absence of TRIM33, this negative regulation would be lost, rendering cells less sensitive to BETi

Example 4: TRIM33 knockdown potentiates TGFβ signaling and inhibition of TGFβ pathway increased BETi sensitivity

While the efficacy of BETi has been linked to down-regulation of MYC expression in hematopoietic cancers and a subset of solid tumors, in other tumor cells BETi-mediated growth suppression is independent of MYC. Notably, in contrast to what was observed in RKO cells, MYC levels in another colorectal cancer cell line, SK-CO-1, were much less sensitive to either BETi treatment or TRIM33 knockdown (Figure 5A). Nonetheless, in this cell line TRIM33 knockdown conferred resistance to BETi (Figure 2E and Table 4). This observation suggests that other pathways in addition to MYC signaling can contribute to shTRIM33 cell resistance to BETi.

Gene set enrichment analysis (GSEA) of the RNAseq data revealed that the two signatures most differentially regulated by BETi-treatment in shCTRL vs. shTRIM33 RKO cells corresponded to genes targeted by TGFβ signaling (Figures 5B-5E). Modulation of TGFβ target genes in the context of BET inhibition was of interest because TRIM33 has been implicated as a regulator of TGFβ signaling. Furthermore, as TGFβ signaling can promote resistance to other targeted therapies, it was investigated how the pathway was altered in shTRIM33 RKO cells.

Canonical TGFβ signaling involves TGFβ ligand-induced formation of heterotetramers containing dimers of the TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII) serine-threonine kinases. Receptor clustering promotes TβRII phosphorylation of TβRI, leading to recruitment and phosphorylation of regulatory SMADs (SMAD2/3) by TβRI. Phosphorylated SMAD2/3 then binds to SMAD4 to form a complex that enters the nucleus to drive transcription of target genes. Stimulation of control and shTRIM33 cells with recombinant TGFβ1 ligand revealed that phosphorylation of SMAD2 was dramatically potentiated in the absence of TRIM33 (Figure 5F). Thus, under conditions where control cells exhibited barely detectable responses to TGFβ1, SMAD2 was robustly phosphorylated in shTRIM33 cells. These changes were not due to differences in the expression level of SMAD2, which appeared uniform in control and shTRIM33 cells (Figure 5F).

TGFβ1-induced phosphorylated SMAD2 (pSMAD2) seen in shTRIM33 cells co-immunoprecipitated with SMAD4, suggesting that the pSMAD2 enters functional complexes with SMAD4 (Figure 5G). Previous reports have suggested that TRIM33 antagonizes TGFβ signaling by negatively regulating SMAD4 through either mono-ubiquitinating SMAD4 or competing with SMAD4 for phosphorylated SMAD2/3. However, knockdown of SMAD4 in shTRIM33 cells had no impact on the TGFβ1-mediated induction of pSMAD2 (Figure 5H). These results suggest that loss of

TRIM33 in RKO cells potentiates TGFβ signaling upstream of SMAD4, at the level of SMAD2 phosphorylation.

The RNAseq data showed that the TβRII mRNA was upregulated ~2 fold in shTRIM33 cells (Figure 5I). Furthermore, ChIP experiments revealed that TRIM33
5 association with the TβRII promoter was increased by BETi, while BRD4 association was decreased (Figures 5J-5K), similar to the manner that MYC is regulated by TRIM33 and BRD4.

To investigate whether TβRII up-regulation could underlie the potentiation of TGFβ signaling that accompanies loss of TRIM33, two different shRNAs were used to
10 knockdown TβRII and assess SMAD2 phosphorylation. Both shRNAs efficiently reduced TβRII mRNA levels (Figure 5L) and in shTRIM33 cells they dramatically reduced TGFβ1-induced pSMAD2 levels (Figure 5M). Notably, when the sensitivity of these cells to JQ1 or GS-626510 growth inhibition was assessed, the loss of TβRII re-sensitized the shTRIM33 cells to the BET bromodomain inhibitors (Figures 5N, right
15 panel, and 10B). TβRII knockdown also increased sensitivity of control cells to BETi (Figures 5G, left panel, and 5O).

These data suggest that a combination of TGFβ pathway inhibitors and BET bromodomain inhibitors may provide a more potent inhibition of cell growth and may provide a means to overcome resistance to BET bromodomain inhibitors. To test this
20 possibility directly, the small molecule TβRI inhibitor, LY2157299 (galunisertib), was used.

Treatment with LY2157299 at a dose that can substantially block TGFβ1-stimulated pSMAD2 (Figure 5P) greatly increased the anti-proliferative effect of JQ1 or GS-626510 in shTRIM33 cells, yet alone had no effect on cell growth (Figures 5Q
25 and 5R). As with silencing of TβRII expression, chemical inhibition of TβRI also sensitized shCTRL cells to BETi. Interestingly, sensitization of shTRIM33 cells to BETi by treatment with LY2157299 was not accompanied by down regulation of MYC (Figure 5S). Thus, results with both TβRII knockdown and small molecule inhibitors of TβRI strongly suggest that TRIM33 promotes sensitivity to BETi at least in part
30 through attenuation of TGFβ signaling.

In order to determine whether enhanced TGFβ signaling is sufficient to induce resistance to BETi, the consequences of over-expressing TβRII were examined. Robust TGFβ1-induced SMAD2 phosphorylation was detected in TβRII-overexpressing cells,

but not in the empty vector control cells (Figure 5T). However, this was insufficient to confer resistance to either JQ1 or GS-626510 (Figure 5U).

TβRII over-expression also failed to protect MYC levels from downregulation by BETi treatment, even in the presence of exogenously added TGFβ1 (Figure 5V).
5 Taken together, these results suggest that TRIM33 knockdown confers resistance to BETi through combined independent effects on MYC transcription and TGFβ signaling.

The recent discovery of small molecule BET bromodomain inhibitors and the demonstration of their potent anti-proliferative activity in hematological and solid
10 tumors highlights the potential of BETi as anti-cancer agents. Acquisition of drug resistance is a recurring limitation to targeted anti-cancer therapies. Described herein, pooled shRNA screening was used to identify genes, whose silencing protects RKO colon cancer cells from two chemically distinct BETi: the originally characterized BET inhibitor, JQ1, and a newly developed inhibitor GS-626510.
15 The top hit from the screen was TRIM33, with its close family member, TRIM24, also being identified. These data suggest that loss of TRIM33 confers resistance to BETi, and this was confirmed in both short and long-term growth assays. Mechanistically, loss of TRIM33 reduces BETi-mediated down-regulation of MYC and sensitizes cells to TGFβ signaling. Notably, inhibition of TGFβ signaling re-sensitizes
20 TRIM33 knockdown cells to BETi, suggesting that combining TGFβ inhibitors with BETi may have therapeutic benefit.

Multiple studies have pointed to the oncogenic transcription factor MYC as a target of BETi in both hematopoietic and solid tumor cell lines. As shown previously for JQ1 treatment, both BETi employed in the study strongly decreased MYC mRNA
25 and protein levels in RKO colorectal cancer cells, and potently inhibited cell growth. Previously it was shown that ectopic expression of MYC partly protected a multiple myeloma cell line from the growth inhibitory effects of JQ1, affirming MYC suppression to be a major mechanism underlying growth suppression by BETi.

By contrast, it was reported that in lung adenocarcinoma cell lines, JQ1
30 suppressed growth by downregulating the transcription factor FOSL1 rather than MYC, suggesting that alternative mechanisms may underlie the activity of BETi in solid tumors.

It was observed that MYC overexpression in RKO cells attenuated the efficacy of BETi. In addition, RNAseq analysis showed no reduction in FOSL1 transcript level

upon BETi treatment of RKO cells. These observations support a central role for MYC as a key transcriptional target for BET bromodomains in colorectal cancer.

To identify genes whose loss conferred resistance to BETi, a pooled shRNA screen was performed with a library targeting genes annotated as protein kinases. It was found that loss of TRIM33 conferred resistance to either JQ1 or GS-626510 treatment, indicating that TRIM33 is required, in at least some cell types, for cells to be fully sensitive to BETi. In such cells, TRIM33 appears to promote downregulation of MYC by BETi.

Classically TRIM33, TRIM24 and TRIM28 act as potent transcriptional co-repressors when recruited to the promoters of target genes, and consistent with this mechanism, it was found that TRIM33 associates with the MYC promoter. Notably this association is enhanced by BETi, possibly due to direct competition between BRD4 and TRIM33 for binding at these sites.

Transcriptional modulation of MYC by TRIM33 could involve its E3 ligase activity, for example by triggering ubiquitin-mediated degradation of factors co-associated with promoter or enhancer regions. Attempts were made to test this model using TRIM33 mutants with impaired E3 ligase activity. Mutant TRIM33, while unable to restore JQ1 sensitivity in shTRIM33 cells, was also very poorly expressed, making it unclear whether its ligase activity was essential.

While this study was underway, several other groups reported alternative mechanisms of BETi resistance in other cancer lines. While the details of the specific adaptive pathways vary across cell types, a common feature of BETi resistance appears to be reactivation of BRD4-dependent target genes. Most of these reported models of resistance involve the emergence of mechanisms to drive MYC expression in the presence of BETi.

For example, up-regulation of the transcription factor GLI2 contributes to acquired BETi-resistance in pancreatic cancer cells (Kumar K, *et al.* (2015). *Sci Rep* 5:9489) by driving MYC expression, and in models of acute myeloid leukemia (AML), increased WNT signaling apparently bypasses BET bromodomain-mediated transcription to maintain MYC expression through utilization of a cryptic enhancer region.

The data described herein show that loss of TRIM33 partially protects MYC levels after BETi treatment, but loss of TRIM33 was not found to affect β-catenin

levels or localization in RKO cells. Furthermore, as judged by RNAseq analysis, TRIM33 knockdown did not induce GLI2 in RKO cells.

Thus, while TRIM33 knockdown apparently confers BETi-resistance at least in part by preventing MYC downregulation, the pathways involved are distinct from those previously characterized. In cell lines where BETi function independently of MYC, reported mechanisms of resistance likewise appear to involve maintaining expression of BRD4-target genes. For example, triple negative breast cancer cells can acquire BETi-resistance through BRD4 hyperphosphorylation, which drives expression of target genes through interactions with the mediator complex in a manner independent of the acetylated lysine binding pocket of its bromodomains. As with each of these described mechanisms of resistance, sparing of critical target genes appears to be an important component of BETi resistance caused by loss of TRIM33.

While multiple studies have addressed adaptive responses to BETi and mechanisms of acquired resistance, much less is understood about factors controlling intrinsic susceptibility of tumors to BETi. Mutations in PIK3CA appear to confer intrinsic resistance to BETi in breast cancer cell lines, yet the molecular basis for this phenomenon is currently unknown. Across a panel of cell lines tested, no correlation between the level of TRIM33 protein expression and sensitivity to BETi was found, suggesting that TRIM33 status is not predictive of intrinsic resistance.

A short isoform of BRD4 was recently shown to be an inhibitor of DNA damage response signaling by influencing chromatin structure independently of its role as a transcriptional activator. Resistance to BETi could theoretically arise by reduction of DNA damage signaling, bypassing growth arrest. However, it was found that TRIM33 knockdown did not alter DNA damage signaling as assessed by γH2AX staining, suggesting that an alternative resistance pathway must be involved.

Consistent with prior reports implicating TRIM33 in TGFβ signaling, loss of TRIM33 sensitized cells to TGFβ. However, in contrast to previous studies suggesting that TRIM33 acts as an E3 ubiquitin ligase for SMAD4, loss of TRIM33 strongly enhanced SMAD2 phosphorylation independently of SMAD4 and was associated with increased expression of TβRII. TRIM33 may therefore act as a direct modulator of TβRII gene transcription.

Importantly, downregulation of TGFβ signaling, either by silencing TβRII expression or with a small molecule inhibitor of TβRI, sensitized TRIM33 knockdown cells to BETi. Notably, while overexpressing TβRII was sufficient to sensitize cells to

TGFβ1, it did not prevent BETi-mediated suppression of MYC levels or cell growth. Thus, while promoting TGFβ signaling cannot explain all of the effects of TRIM33 knockdown on BETi sensitivity, inhibition of TGFβ signaling was sufficient to sensitize cells to BETi.

5 How increased TGFβ signaling contributes to BETi resistance is unclear, but it is noteworthy that in non-small cell lung cancer cell lines, knockdown of mediator complex component MED12 confers resistance to multiple kinase inhibitors through a transcription-independent mechanism that results in stabilization of TβRII. Likewise, knockdown of the transcription factor, SOX10, in melanoma cell lines induces BRAF
10 inhibitor resistance by induction of TβRII and TGFβ signaling, ultimately resulting in increased receptor tyrosine kinase expression. In both of these contexts, TGFβ-induced resistance to targeted therapies is associated with enhanced signaling through the ERK MAP kinase pathway.

Notably, in addition to up-regulated Wnt signaling, BETi-resistance in AML
15 was also associated with up-regulated TGFβ-dependent gene expression. These observations are consistent with the finding that potentiated TGFβ signaling contributes to shTRIM33-mediated BETi resistance and suggests that TGFβ inhibitors may be valuable in combination with BETi in a range of malignancies. The ability of TGFβ inhibitors to potentiate the effect of BETi and to function in the setting of TRIM33 loss
20 provides a potential clinical strategy to overcome or delay acquired resistance.

Other Embodiments

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or
25 subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this
30 invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of increasing anti-tumor sensitivity to a bromodomain and extraterminal domain protein inhibitor (BETi) in a cell in a subject, the method comprising administering a composition comprising a TGFβ pathway inhibitor and a BETi to a subject in need thereof, wherein the TGFβ pathway inhibitor sensitizes the cell to the BETi.

2. A method of treating a tumor comprising administering to a subject in need thereof a composition comprising a TGFβ pathway inhibitor and a bromodomain and extraterminal domain protein inhibitor (BETi), wherein the TGFβ pathway inhibitor sensitizes the tumor to the BETi.

3. A method of increasing anti-tumor sensitivity to a bromodomain and extraterminal domain protein inhibitor (BETi) of a cell in a subject, the method comprising administering a composition comprising a BETi and one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33 to a subject in need thereof, wherein the TRIM33 sensitizes the cell to the BETi.

4. A method of treating a tumor comprising administering to a subject in need thereof a composition comprising a bromodomain and extraterminal domain protein inhibitor (BETi) and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein TRIM33 sensitizes the tumor to the BETi.

5. The method of any one of claims 1 or 2, wherein the TGFβ pathway inhibitor is selected from the group consisting of a small molecule inhibitor, an inhibitory nucleic acid, neutralizing antibody, and an antagonist.

6. The method of claim 5, wherein the small molecule inhibitor is selected from the group consisting of SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299, R268712, D4476, SB525334, GW788388, TEW-7197, and any combination thereof.

7. The method of claim 5, wherein the antagonist is selected from the group consisting of HtrA1, decorin, biglycan, fibromodulin, lumican, endoglin, somatostatin, follistatin, RAP-1332, pirfenidone (5-methyl-1-phenyl-2(1H)-pyridone), soluble ectodomains of TGFβ receptor type II (RII) or betaglycan (BG), and any combination thereof.

8. The method of claim 5, wherein the neutralizing antibody specifically binds one selected from the group consisting of TGFβ and a TGFβ receptor.

9. The method of any one of claims 3 or 4, wherein the nucleic acid encoding TRIM33 is an expression vector comprising a TRIM33 gene.

10. The method of claim 9, wherein the vector is a viral vector.

11. The method of any one of claims 1-4, wherein the BETi is selected from the group consisting of JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, TEN-010, and any combination thereof.

12. The method of any one of claims 2 or 4, wherein the tumor is a cancer selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer.

13. The method of any one of claims 1-4, wherein the subject has a cancer.

14. The method of claim 13, wherein the cancer is selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, and lung cancer.

15. The method of any one of claims 1 or 3, wherein the cell is BETi resistant.

16. The method of any one of claims 2 or 4, wherein the tumor is BETi resistant.

17. A composition comprising a TGFβ pathway inhibitor and a bromodomain and extraterminal domain protein inhibitor (BETi), wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

18. A composition comprising a BETi and at least one selected from the group consisting of TRIM33 or a fragment thereof, and a nucleic acid encoding TRIM33, wherein the TGFβ pathway inhibitor sensitizes cells to the BETi.

19. The composition of claim 17, wherein the TGFβ pathway inhibitor is selected from the group consisting of a small molecule inhibitor, an inhibitory nucleic acid, a neutralizing antibody, and an antagonist.

20. The composition of claim 19, wherein the small molecule inhibitor is selected from the group consisting of SB431542, A83-01, RepSox, SB208, SB505124, LY364947, LY2157299, R268712, D4476, SB525334, GW788388, TEW-7197, and any combination thereof.

21. The composition of claim 19, wherein the neutralizing antibody specifically binds one selected from the group consisting of TGFβ and a TGFβ receptor.

22. The composition of claim 19, wherein the antagonist is selected from the group consisting of HtrA1, decorin, biglycan, fibromodulin, lumican, endoglin, somatostatin, follistatin, RAP-1332, pirfenidone (5-methyl-1-phenyl-2(1H)-pyridone), soluble ectodomains of TGFβ receptor type II (RII) or betaglycan (BG), a neutralizing antibody against TGFβ or a TGFβ receptor, and any combination thereof.

23. The composition of claim 18, wherein the nucleic acid encoding TRIM33 is a vector comprising a TRIM33 gene.

24. The composition of claim 23, wherein the vector is a viral vector.

25. The composition of any one of claims 17 or 18, wherein the BETi is selected from the group consisting of JQ1, GS-626510, GS-5829, BMS 986158, RVX2135, CPI203, CPI-0610, ABBV-075, BAY1238097, INCB054329, FT-1101, PFI-1, I-BET151, ZEN-3365, I-BET762, OTX015, TEN-010, and any combination thereof.

26. A pharmaceutical composition comprising the composition of any one of claims 17 or 18 and a pharmaceutically acceptable carrier.

27. Use of the composition of claim 26 in the manufacture of a medicament for the treatment of a tumor or a cancer in a subject.

Figure 1A

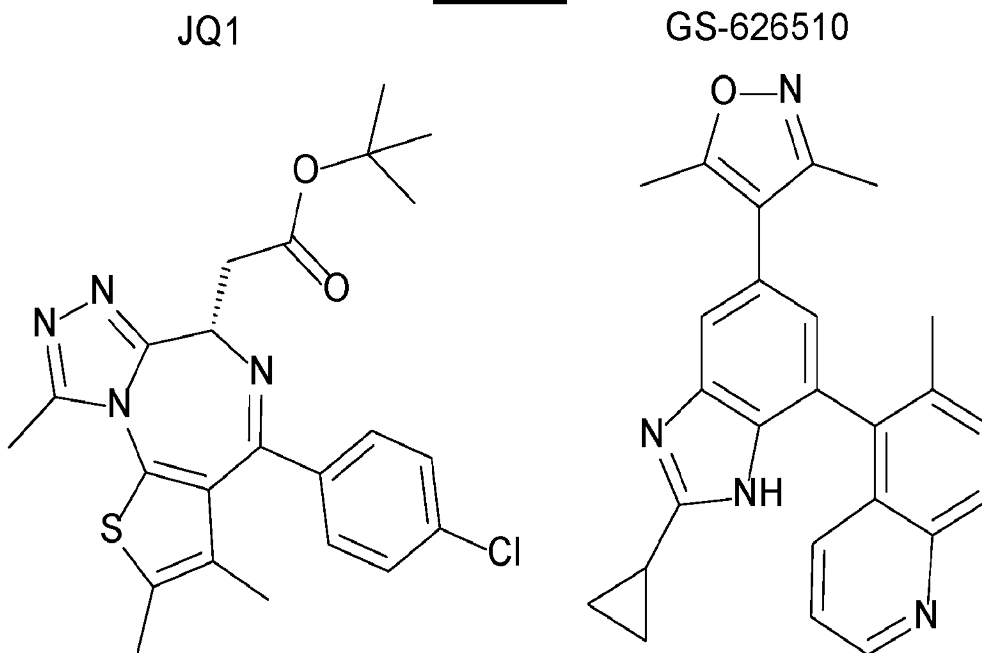


Figure 1B

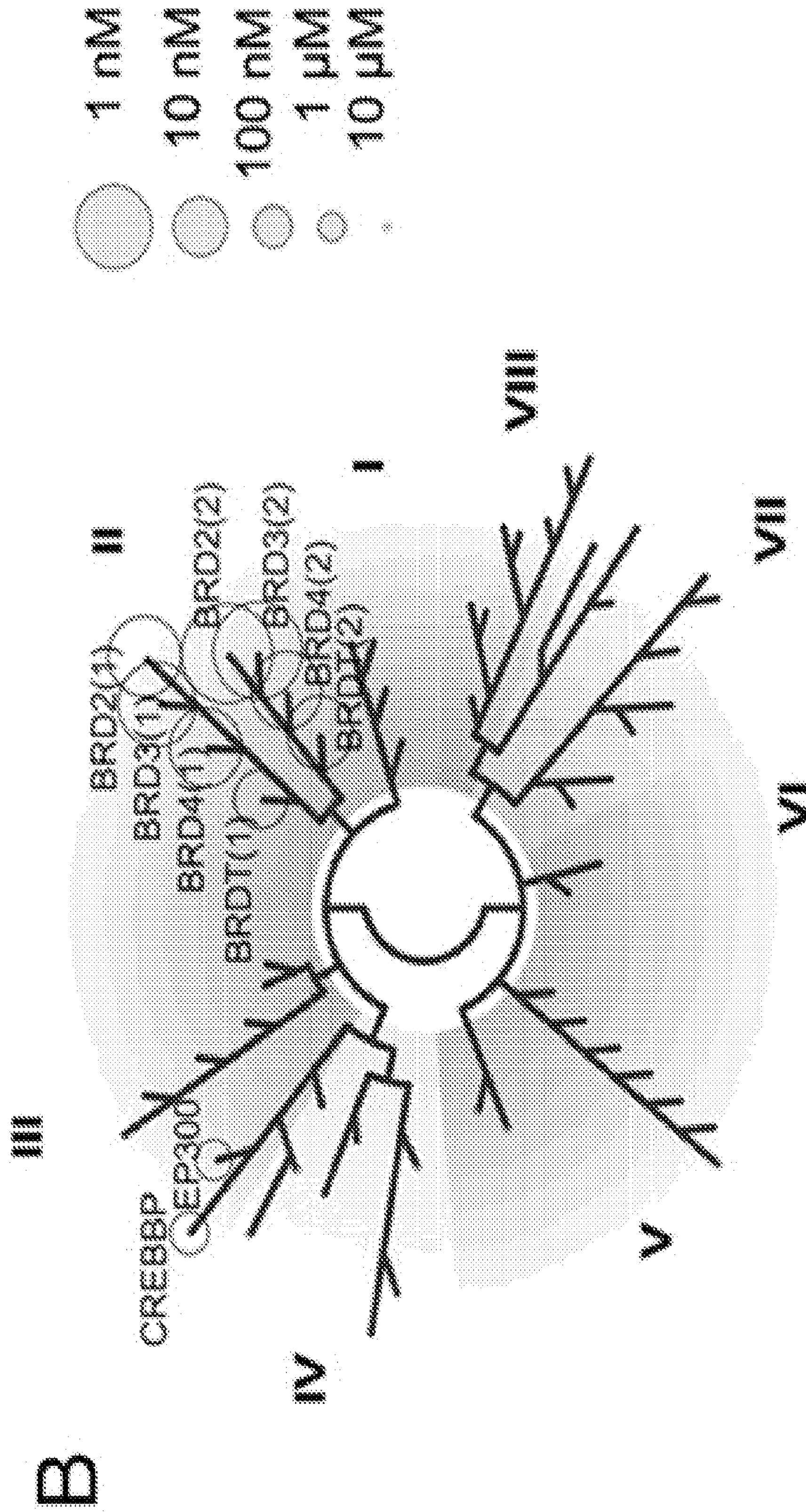
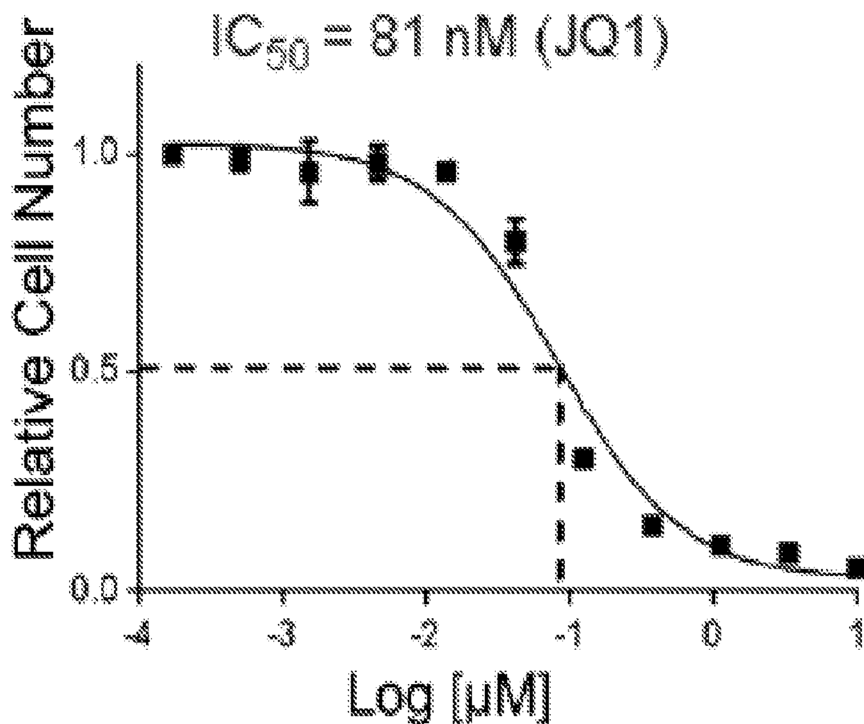


Figure 1C



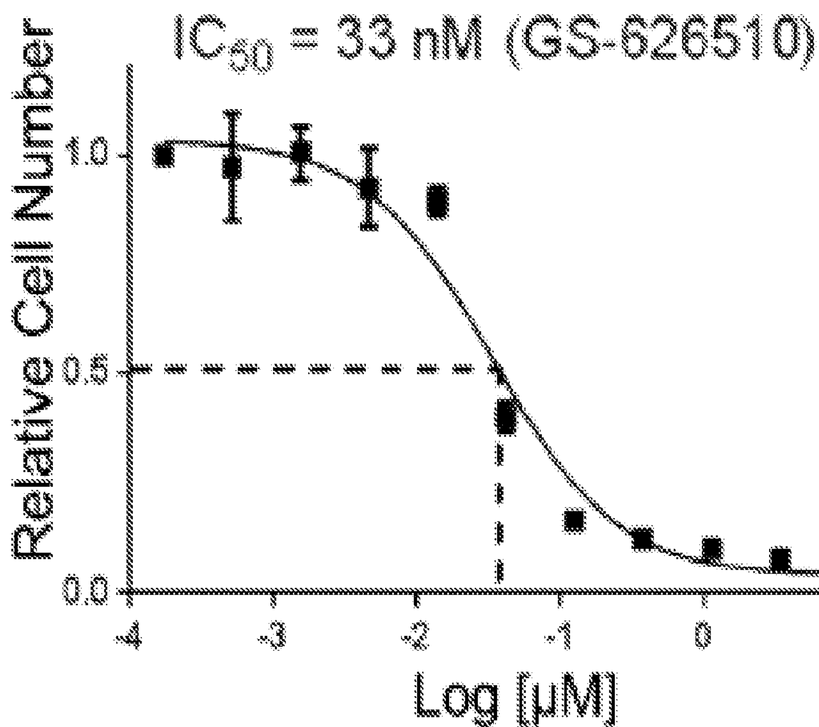


Figure 1D

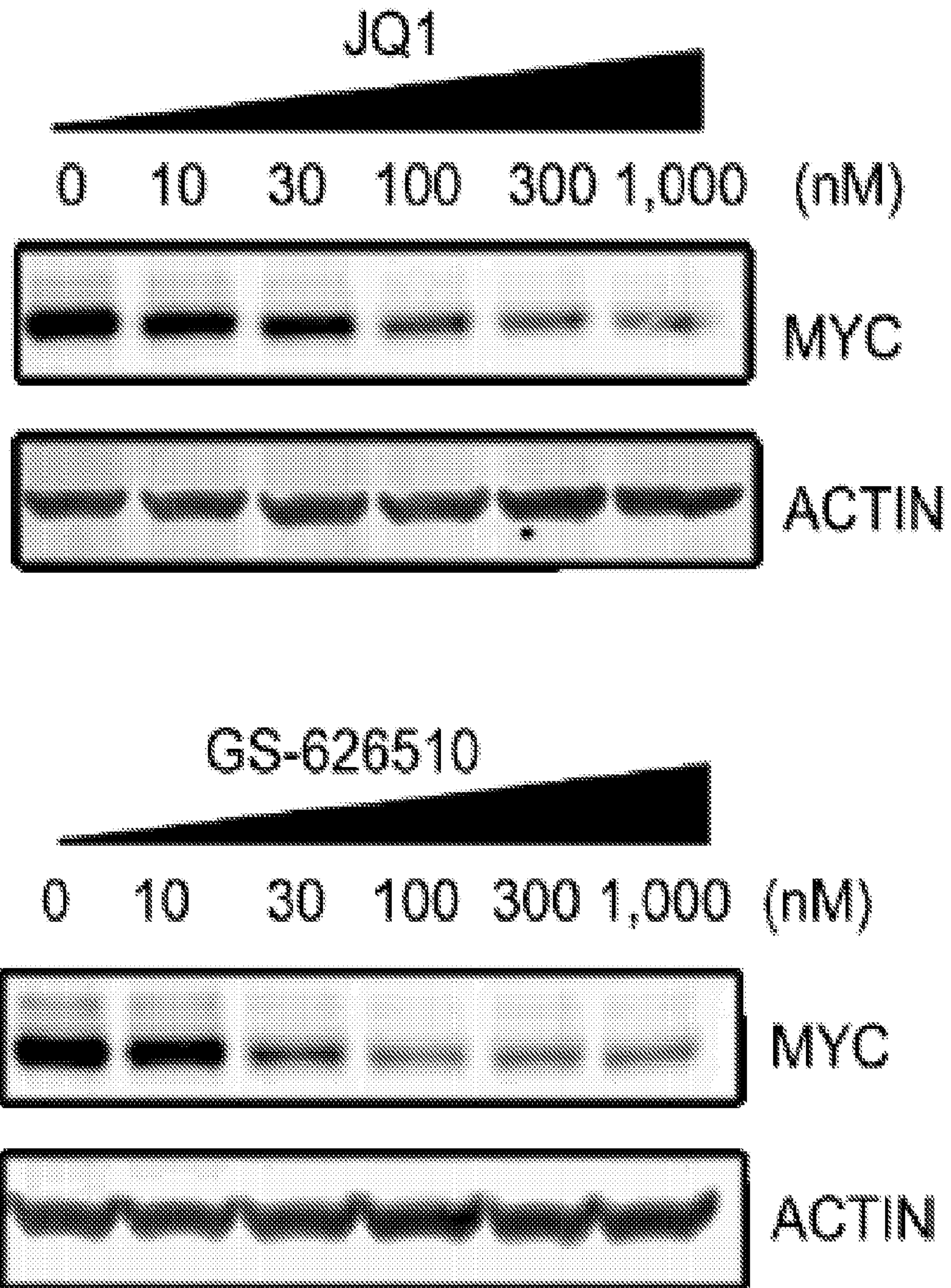


Figure 1E

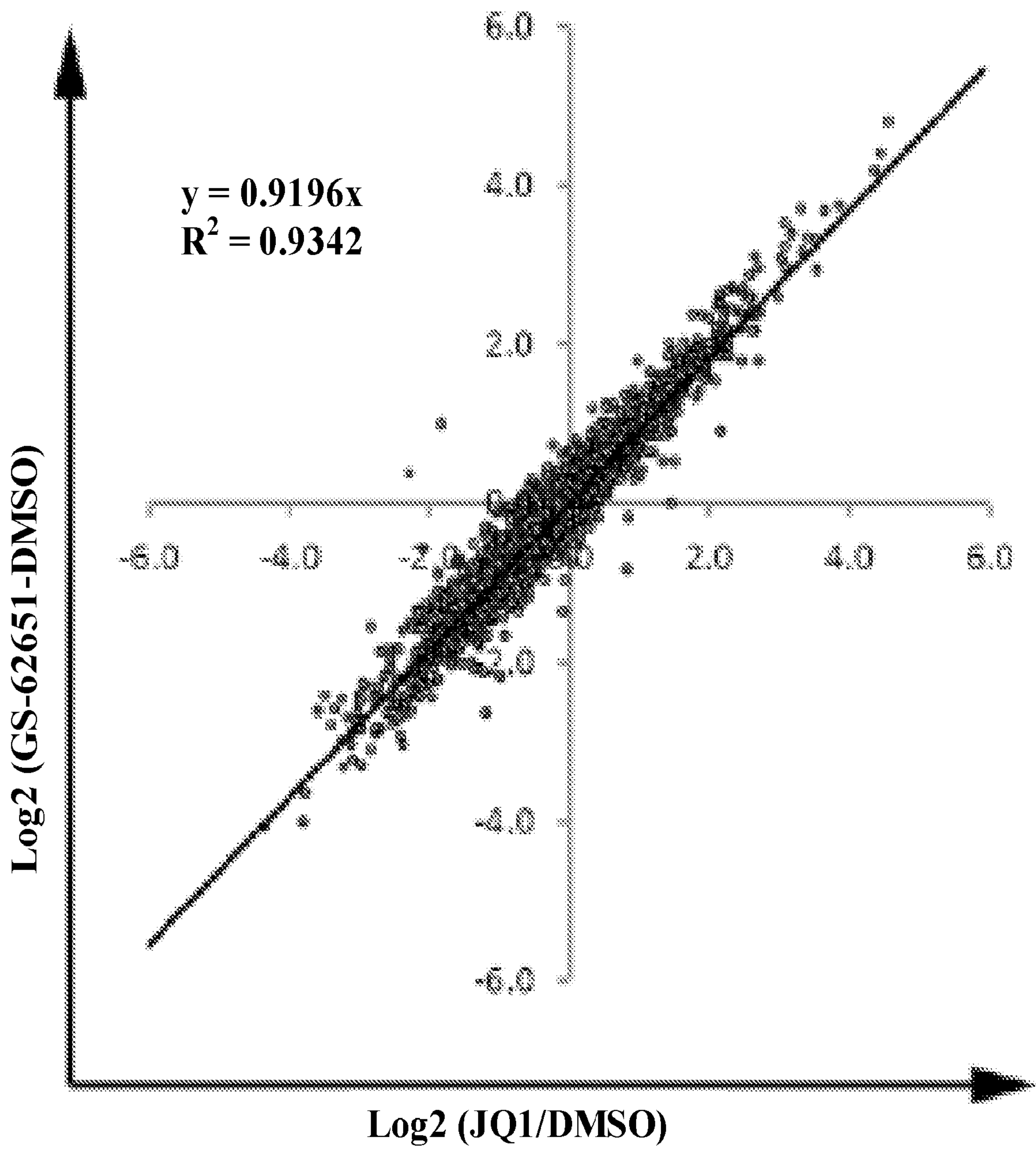


Figure 1E (cont.d)

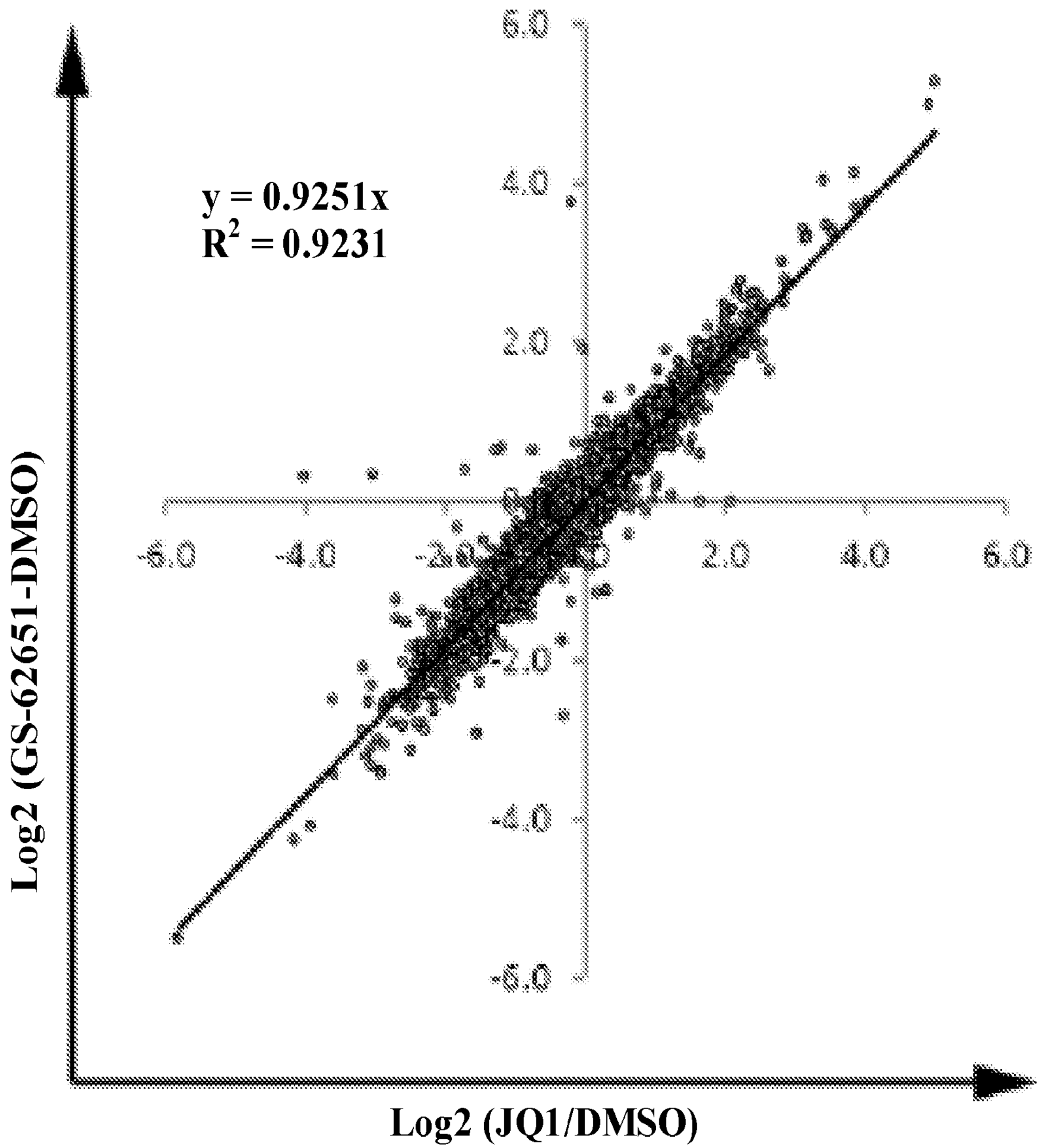


Figure 1F

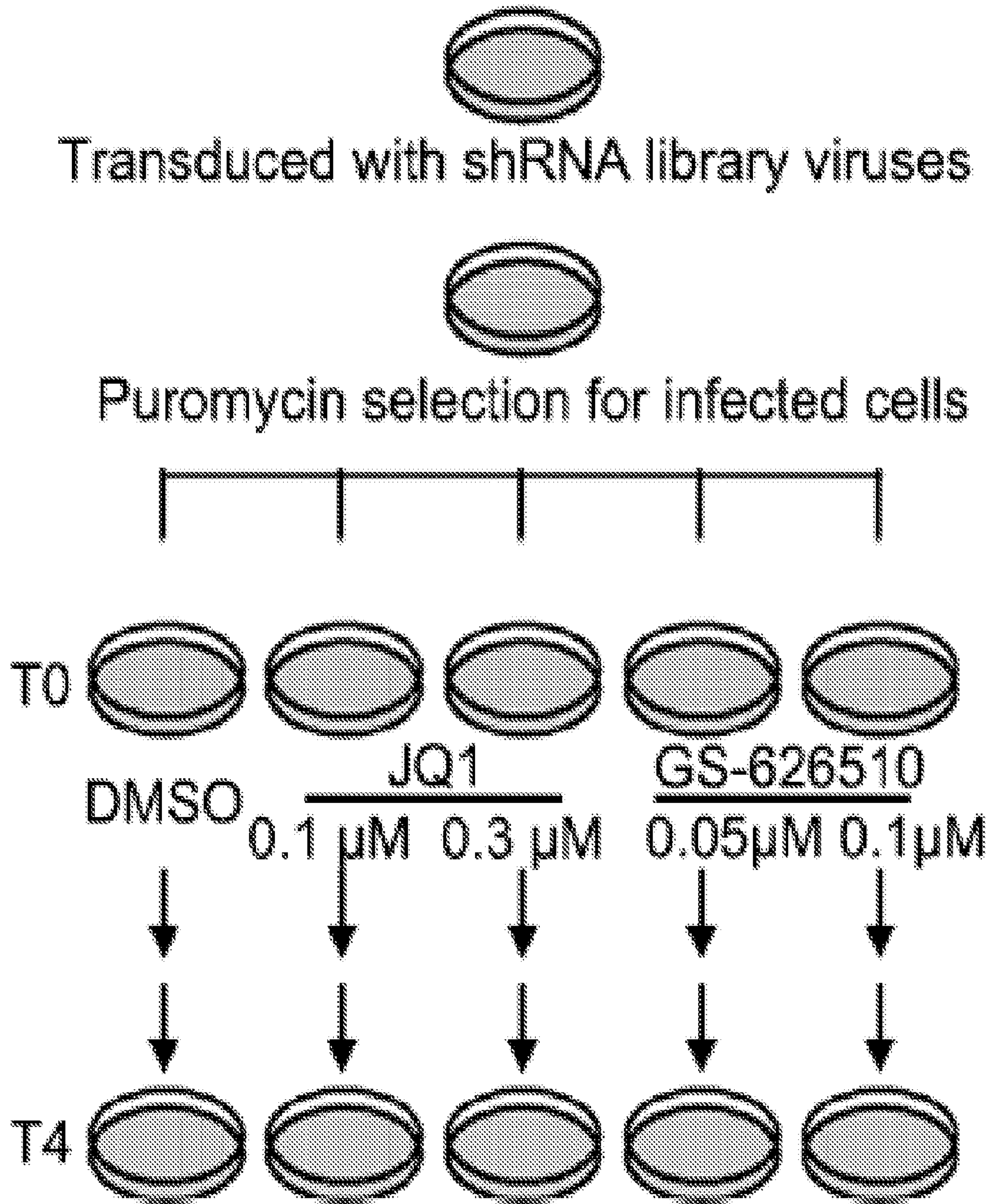


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Figure 1G

Rank	RIGER Analysis				
	DMSO	JQ1 0.1 μM	JQ1 0.3 μM	GS510 0.05 μM	GS510 0.1 μM
1	TNIK	TRIM33	TRIM33	TRIM33	TRIM33
2	PSKH1	TRIB3	DYRK1A	MAP3K5	BTK
3	CSK	BTK	MLKL	TRIM24	TRIM24
4	EPHA8	TRIM24	RNASEL	BTK	CDK13
5	MAP3K13	MARK3	TYK2	TRIB3	SLK
6	WNK2	DYRK1A	TRIM24	CAMKV	STK4
7	SLK	FLT4	GRK4	MAP3K3	SIK1
8	CHUK	EIF2AK4	BTK	ROS1	MAP3K3
9	STK36	PKMYT1	MYLK2	MAP3K1	CAMKK1
10	MAP3K10	STK38L	MAPK1	ADCK4	GRK5

Rank	RIGER Analysis				
	DMSO	JQ1 0.1 μM	JQ1 0.3 μM	GS510 0.05 μM	GS510 0.1 μM
1	PRKACB	TRIM33	TRIM33	TRIM24	DYRK1A
2	NRK	TRIM24	MAPK1	FAM20C	TRIM33
3	TESK2	FAM20C	CDC7	TRIM33	CDK13
4	MAST4	MAP3K3	EPHB6	MAP3K3	TRIM24
5	TAOK1	BTK	CAMK1D	EIF2AK4	NEK4
6	MAP3K13	ALK	MAP2K6	DYRK1A	MAPK8
7	TEX14	DYRK1A	DAPK3	TNNI3K	LIMK1
8	CAMKK2	NEK4	EIF2AK1	NEK4	PLK2
9	CDK10	EIF2AK4	CDK8	PTK7	CDK8
10	MAP3K11	CSNK1D	ULK3	CDK13	CDK10

Figure 1H

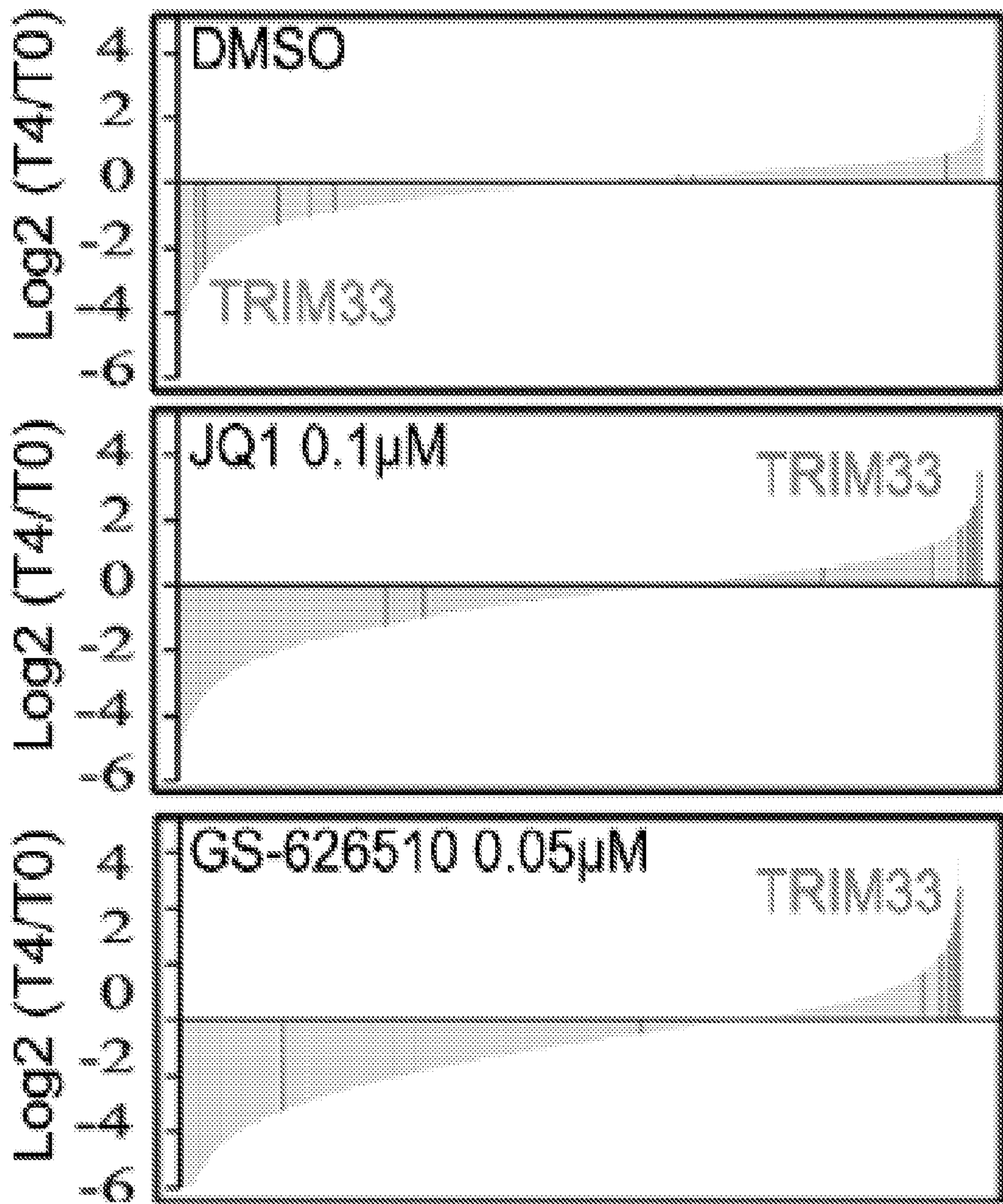
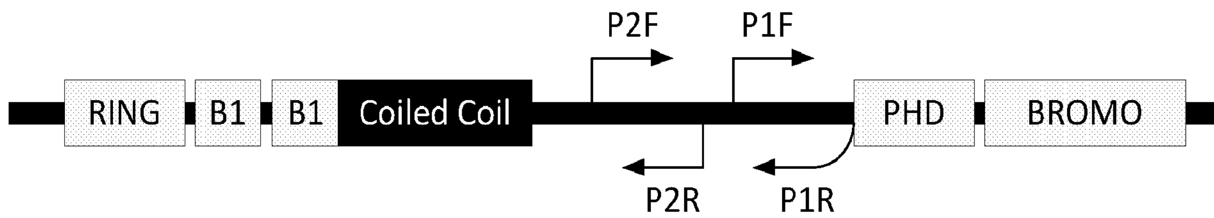
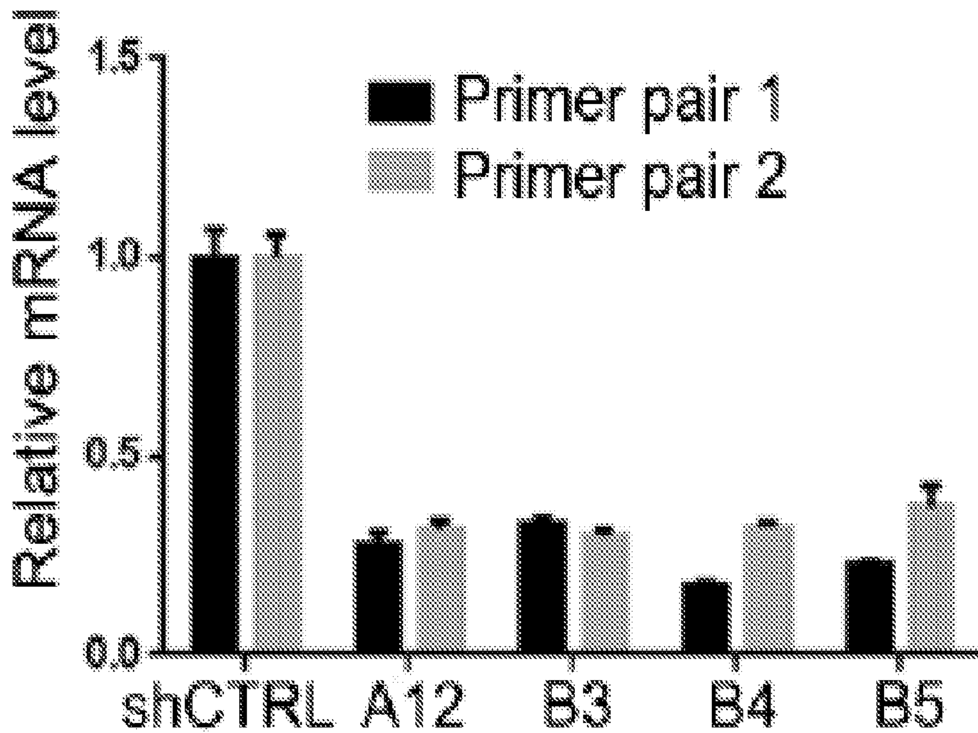


Figure 2A





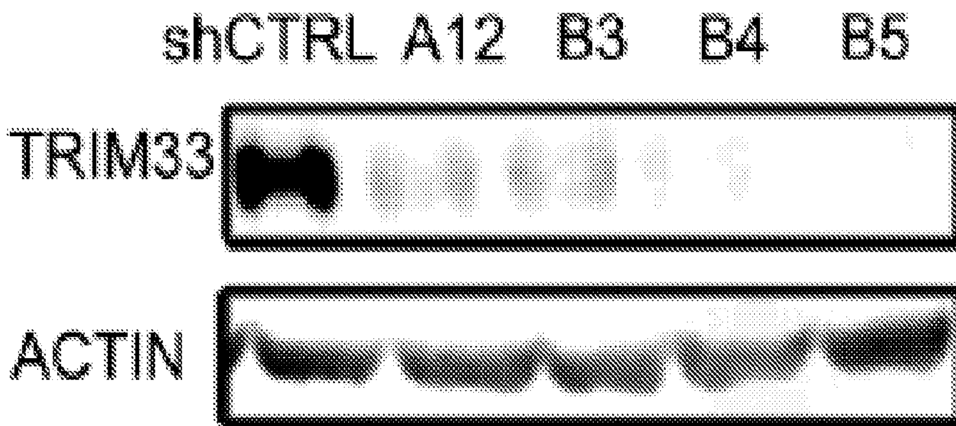


Figure 2B

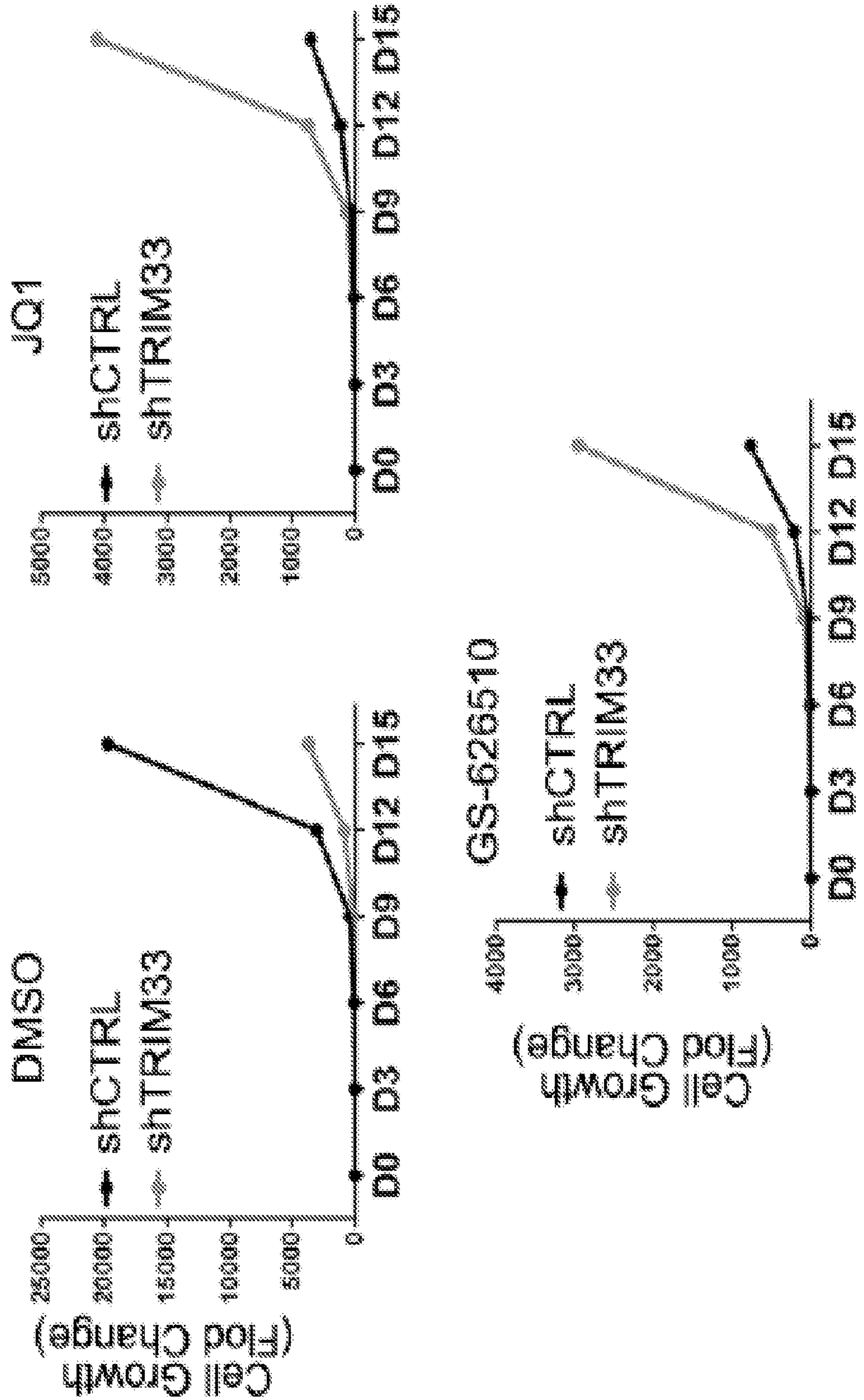


Figure 2C

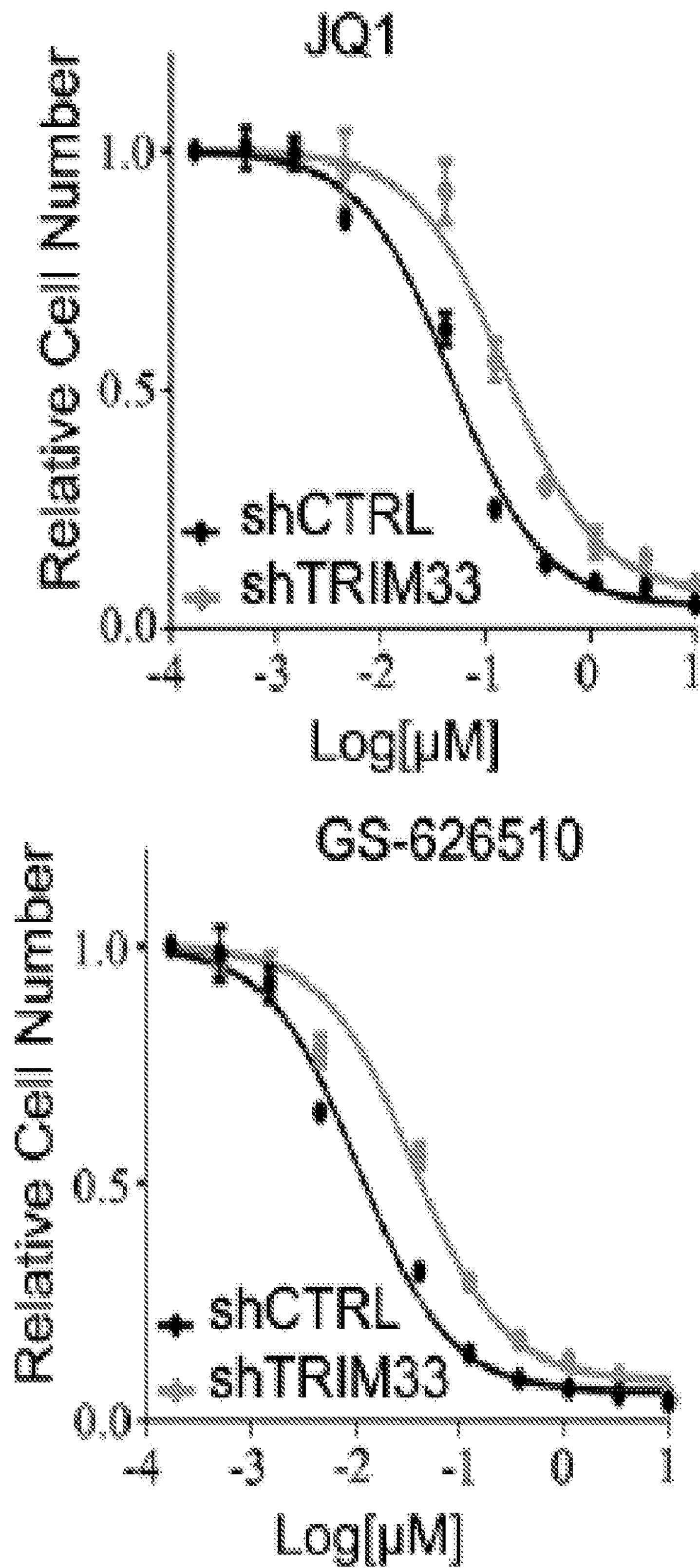
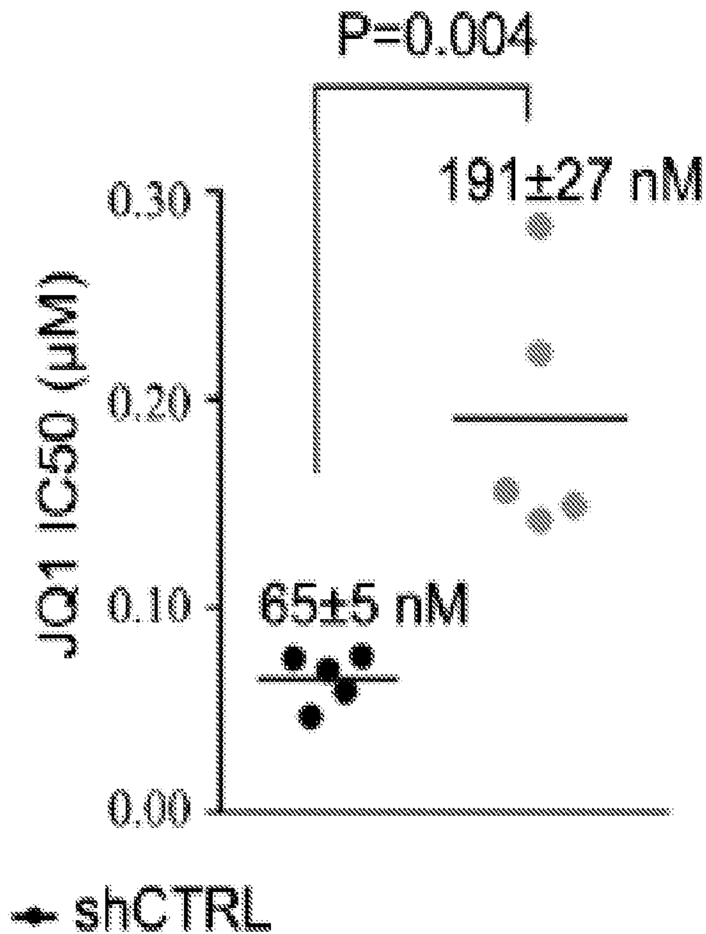


Figure 2D



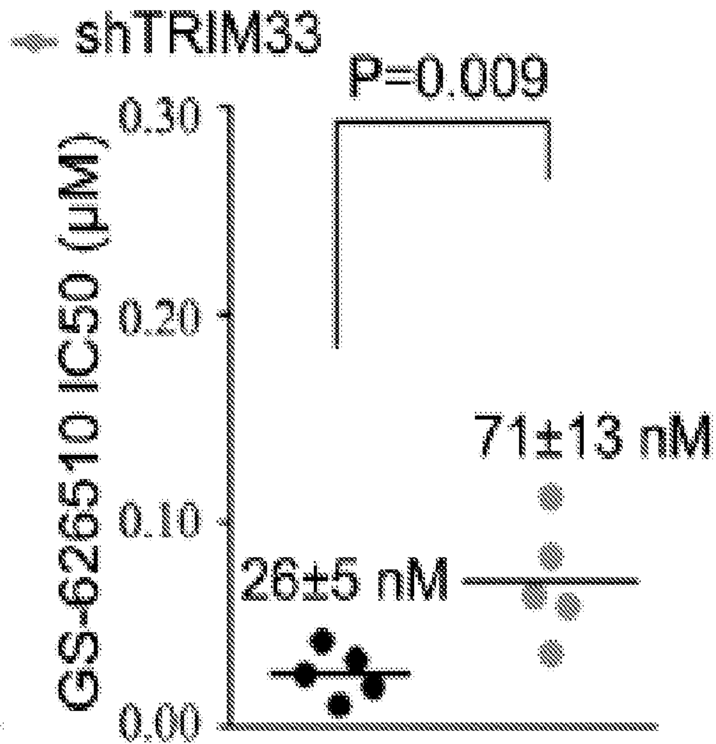


Figure 2E

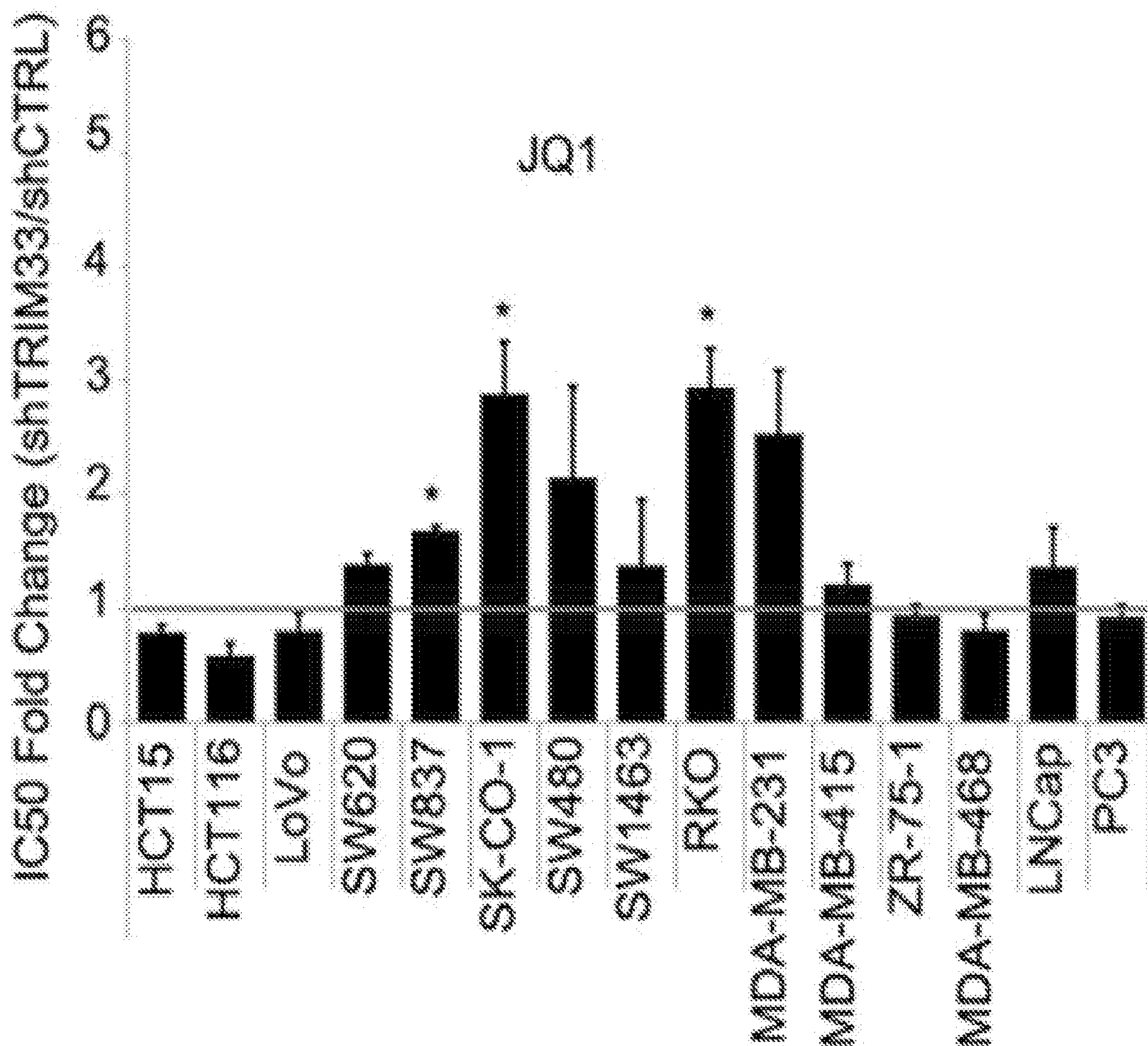


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Figure 2E (cont.d)

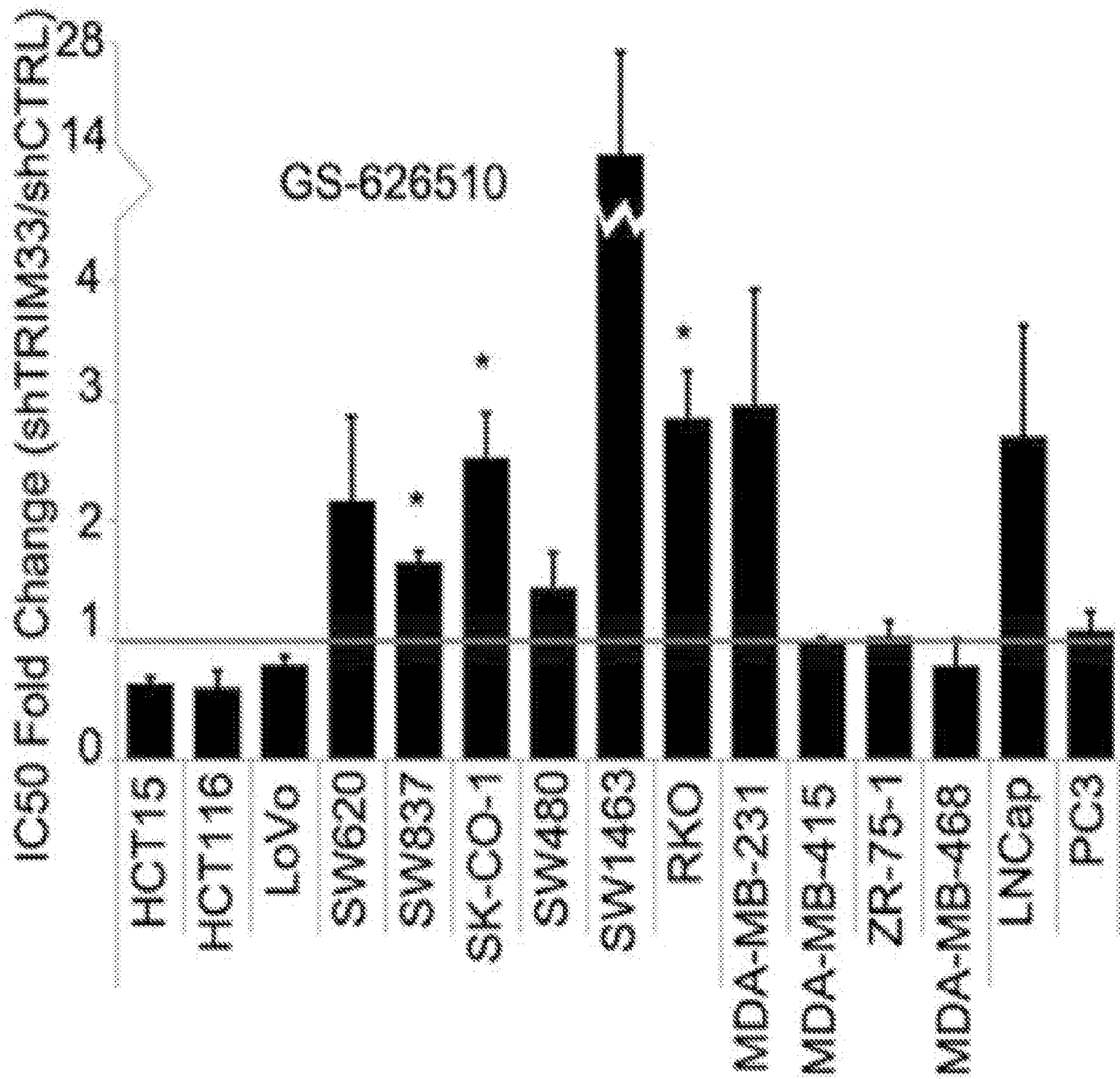
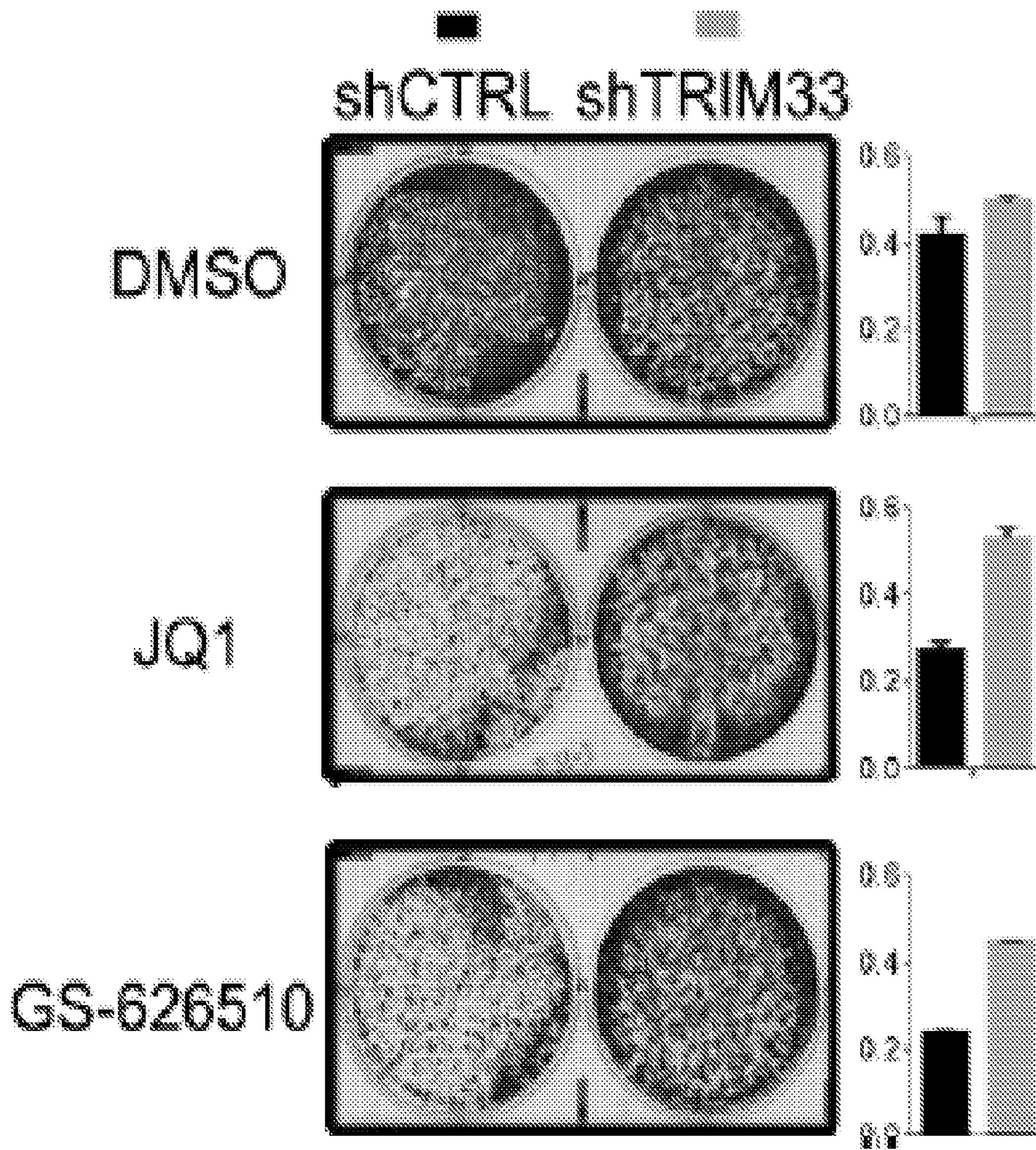
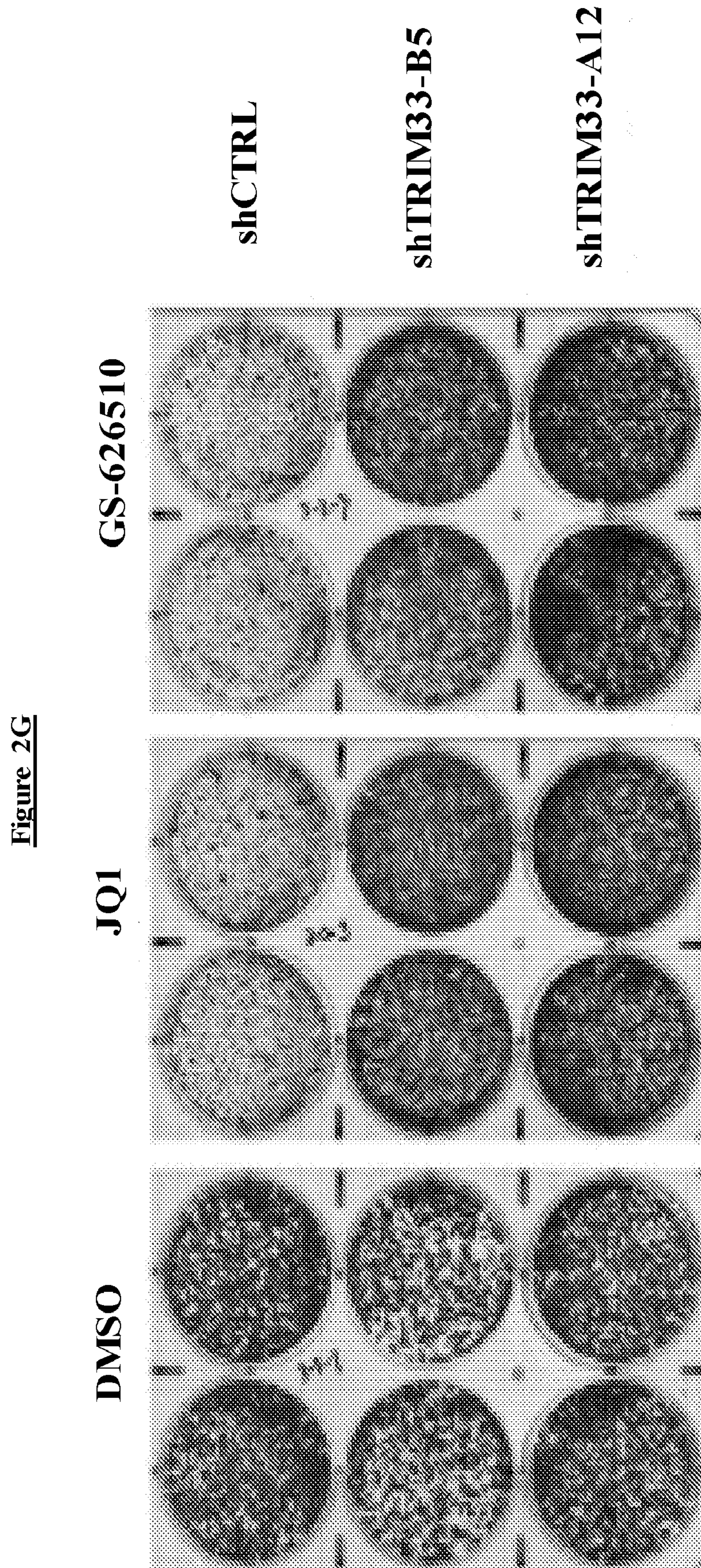


Figure 2F





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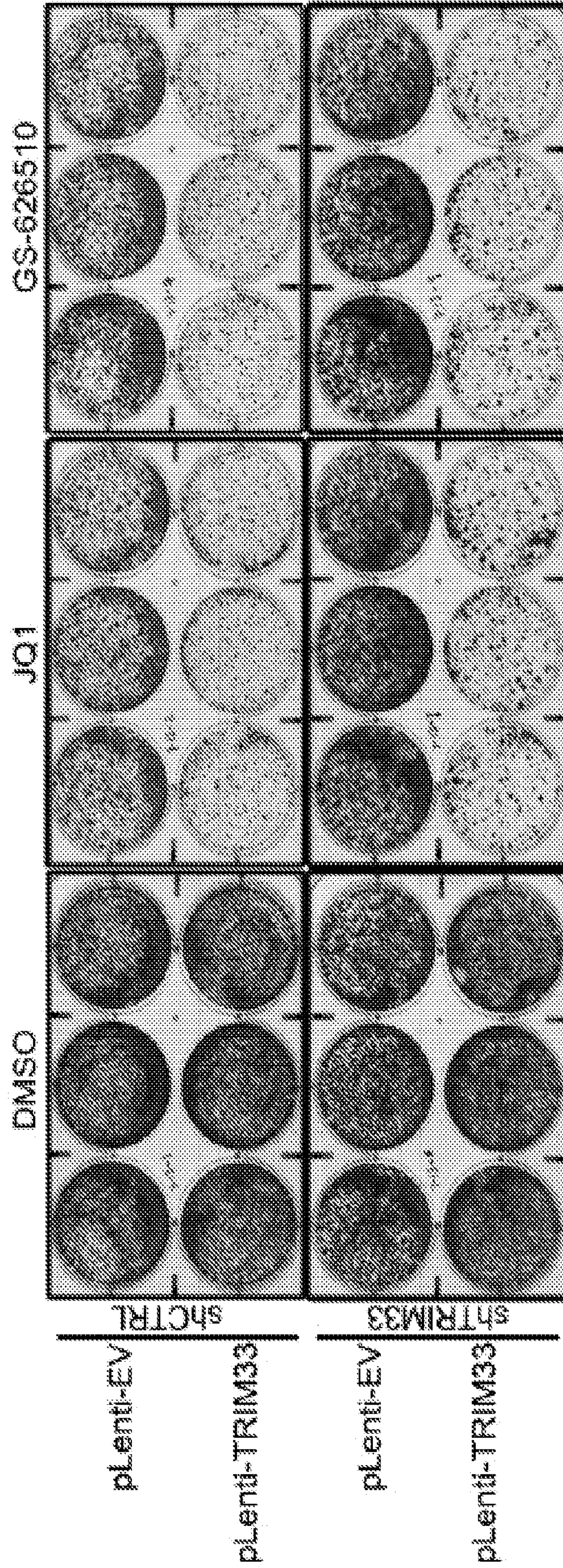


Figure 2H

Figure 2I

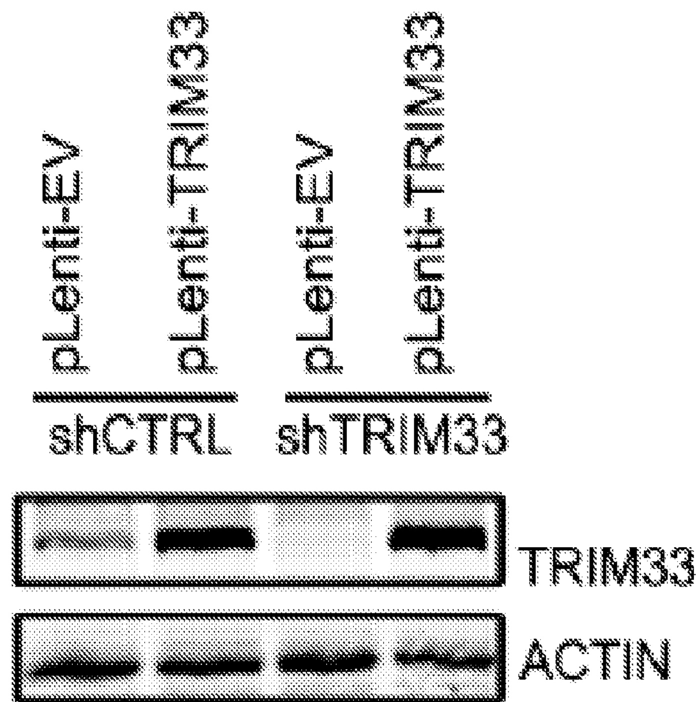
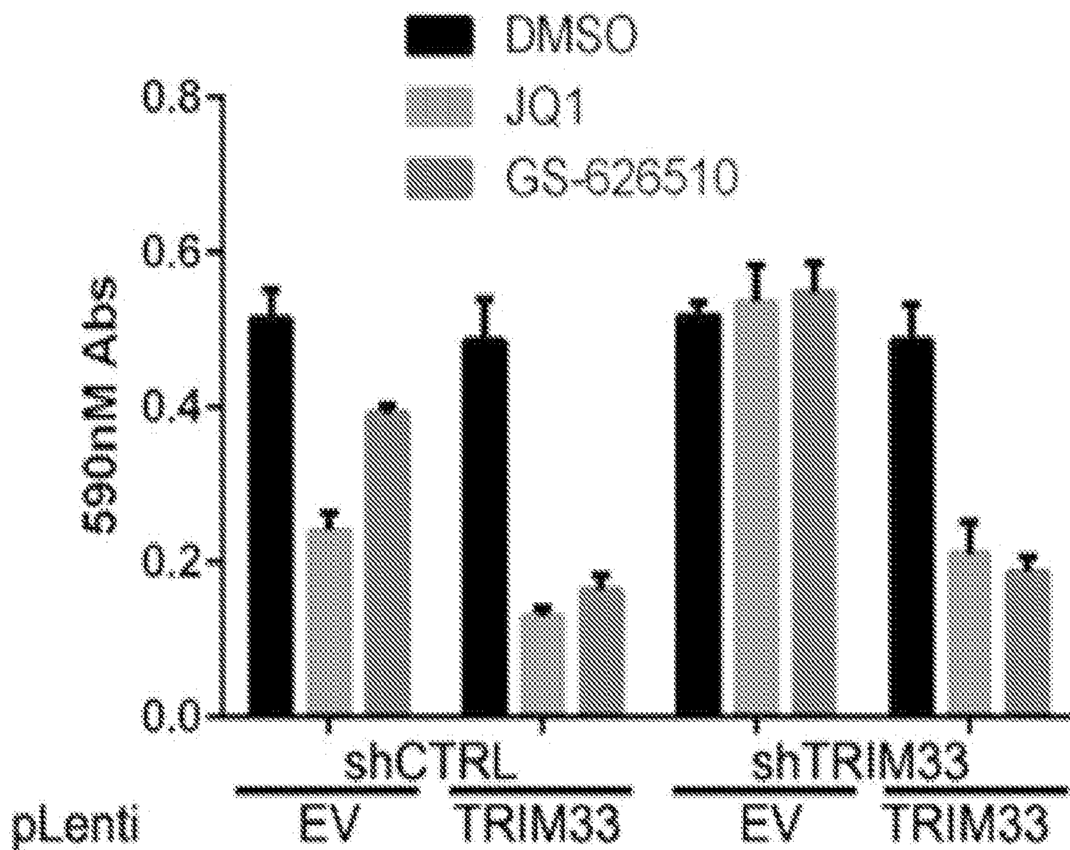


Figure 2J



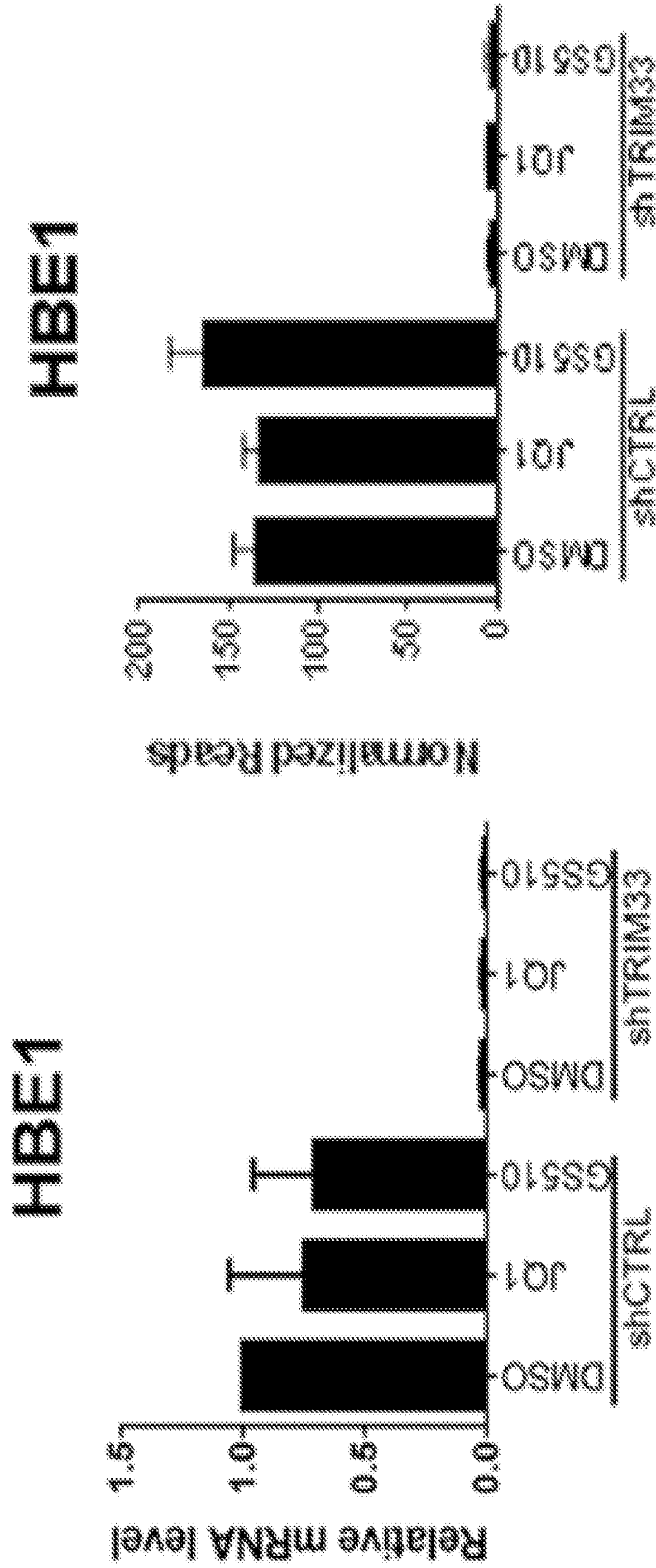
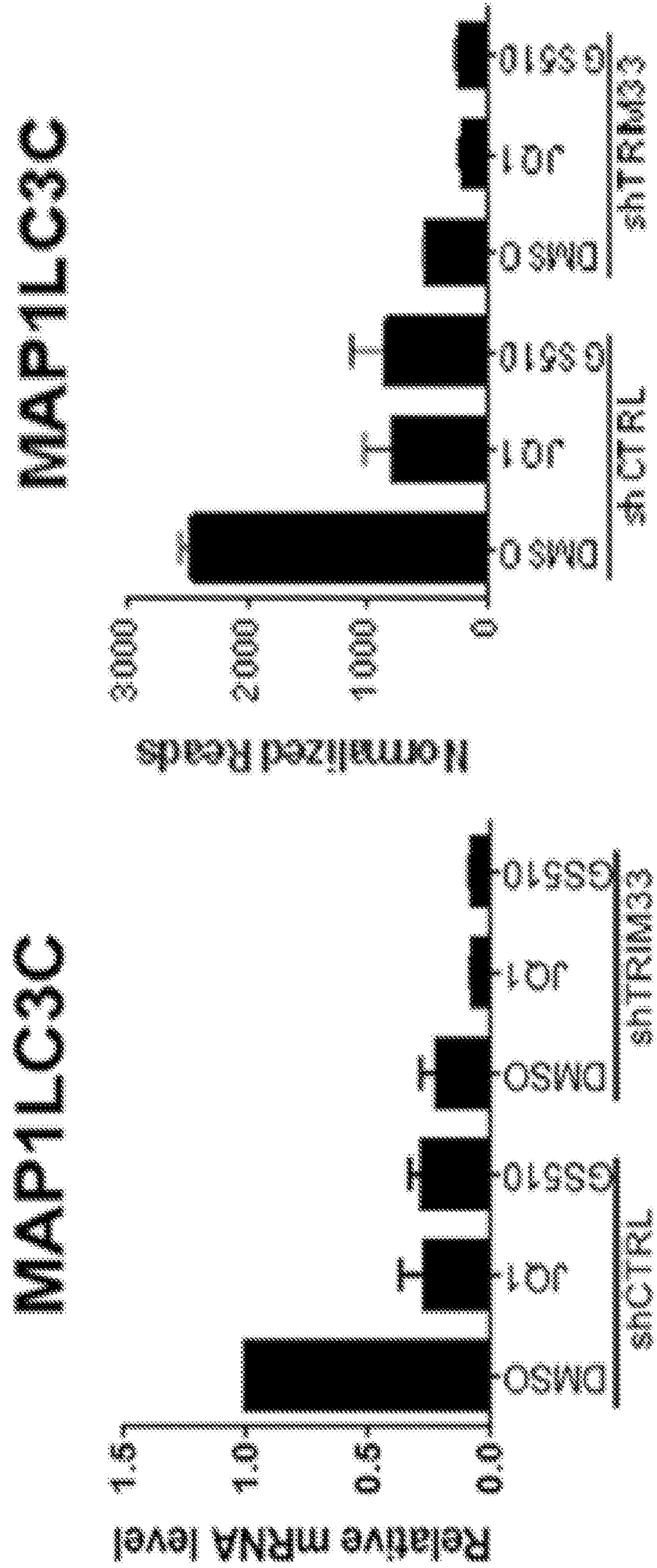


Figure 3A

Figure 3A continued



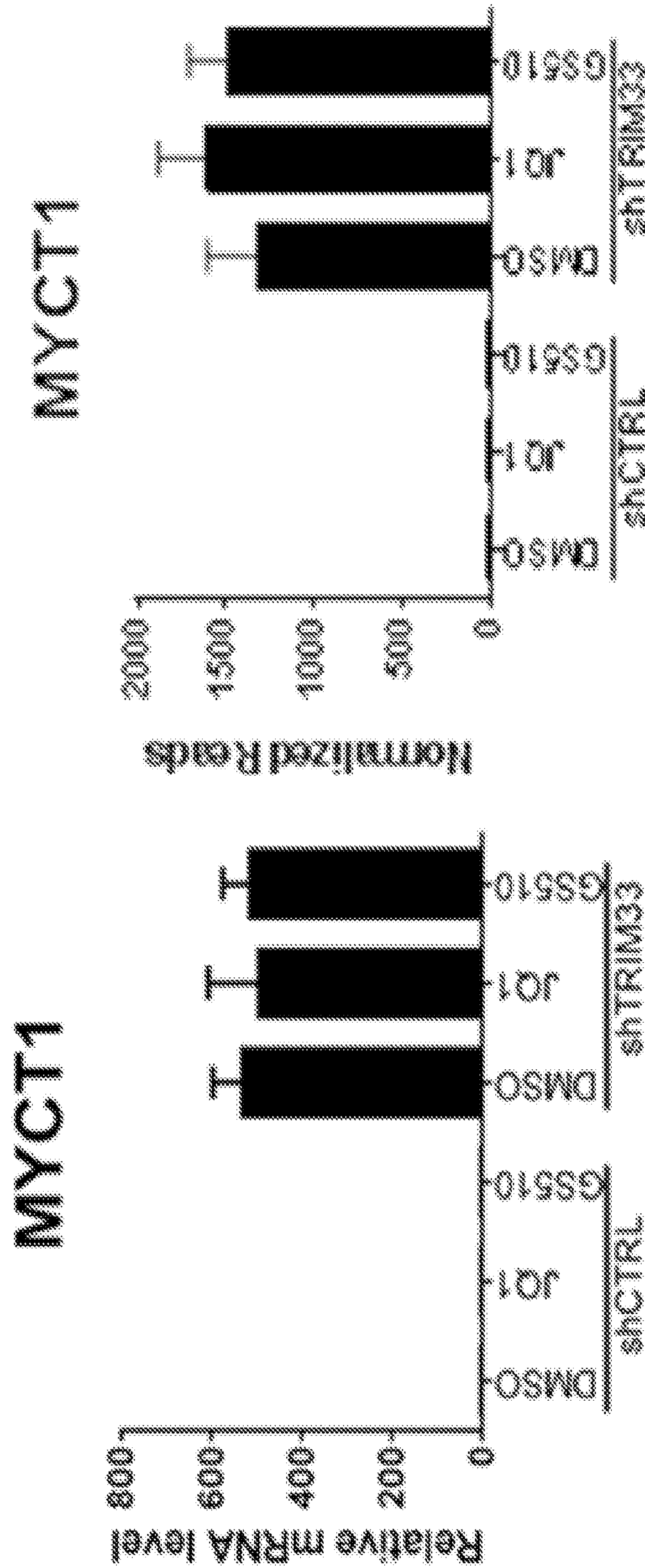


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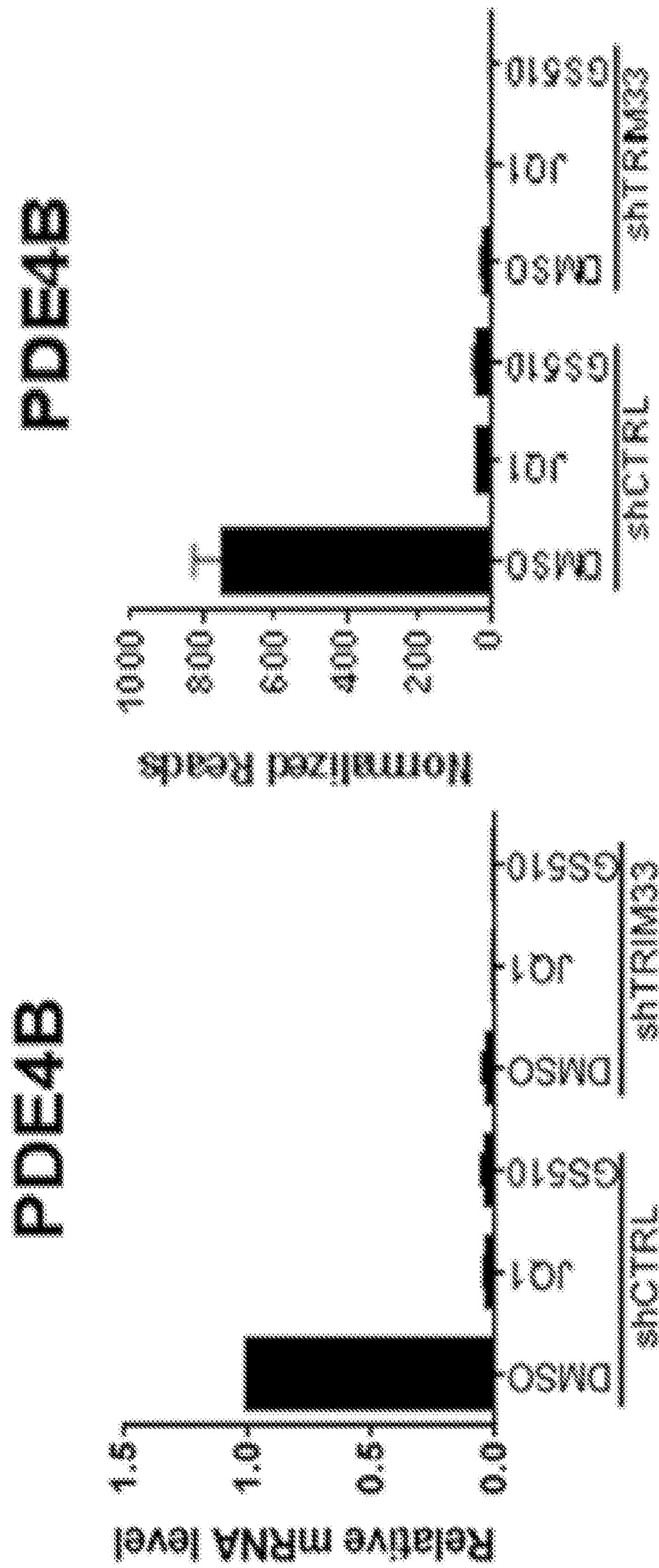
Figure 3B



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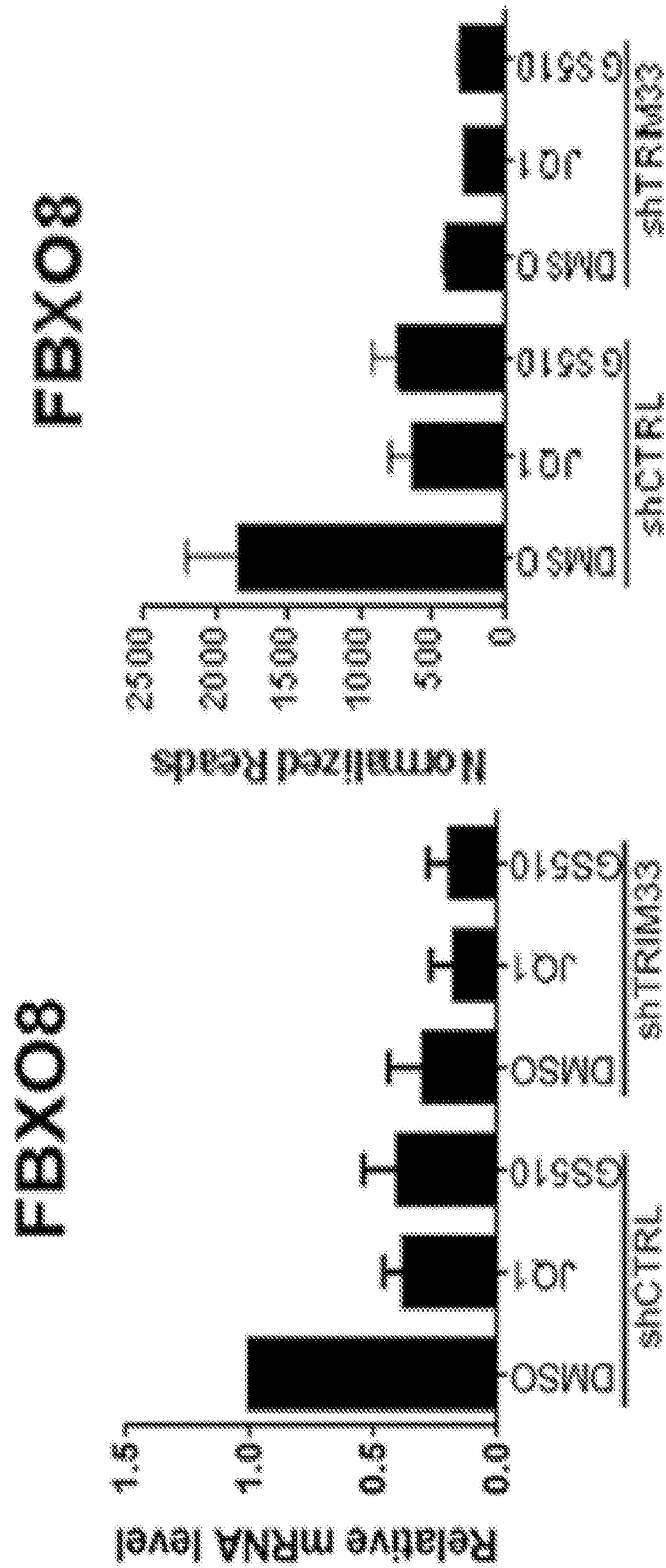

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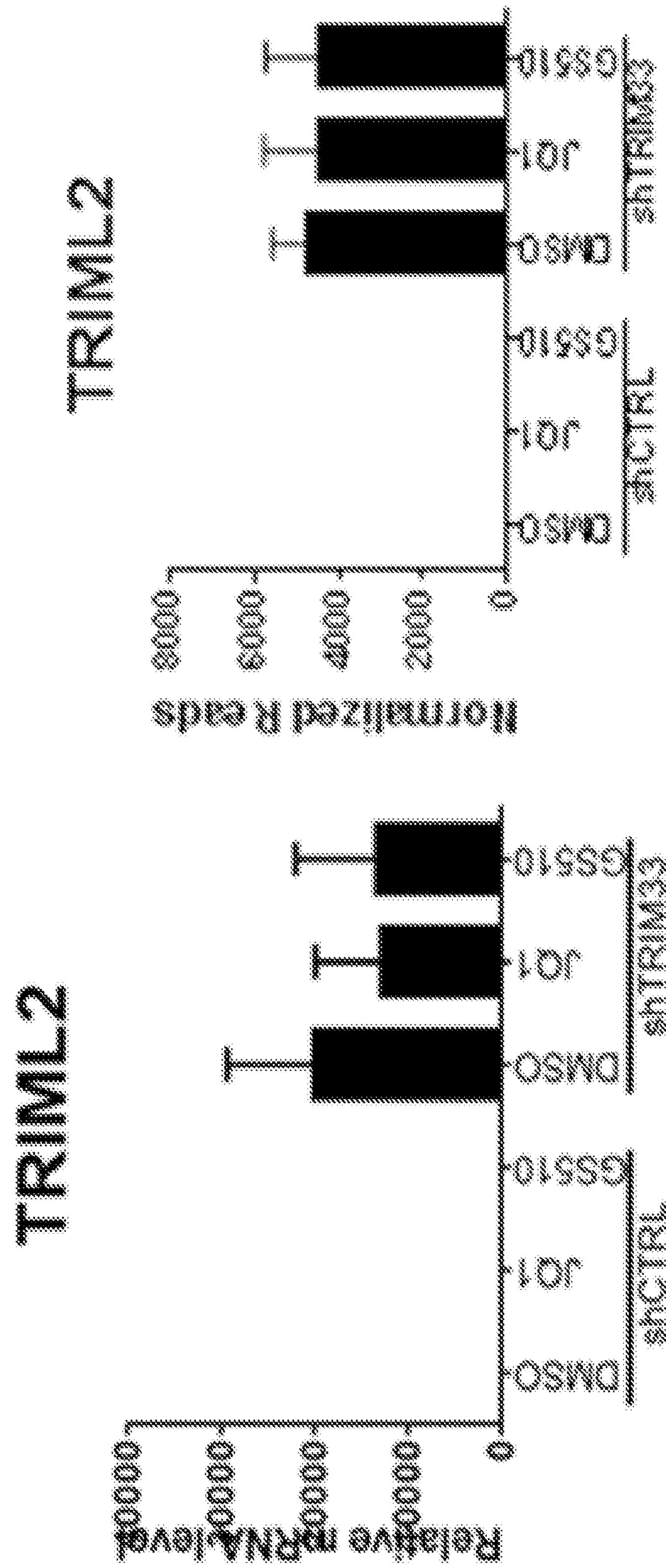

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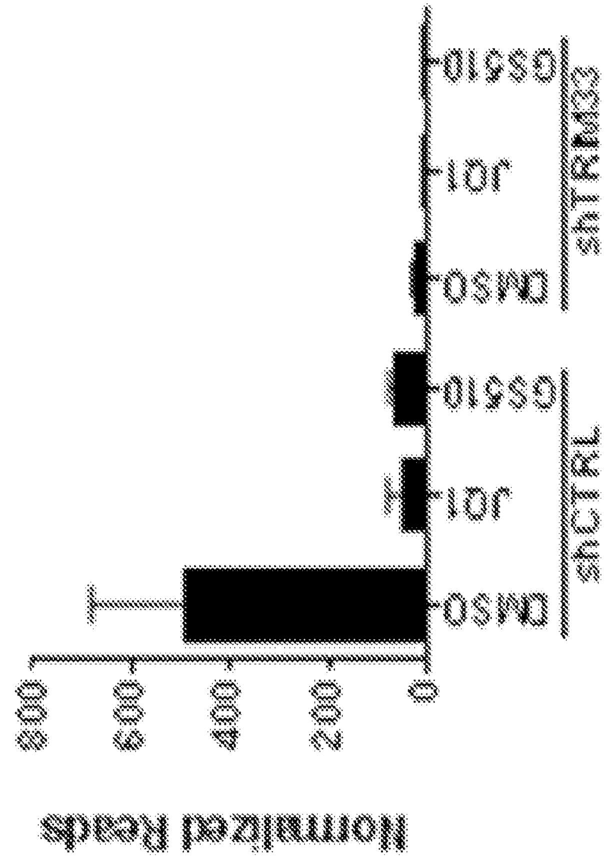
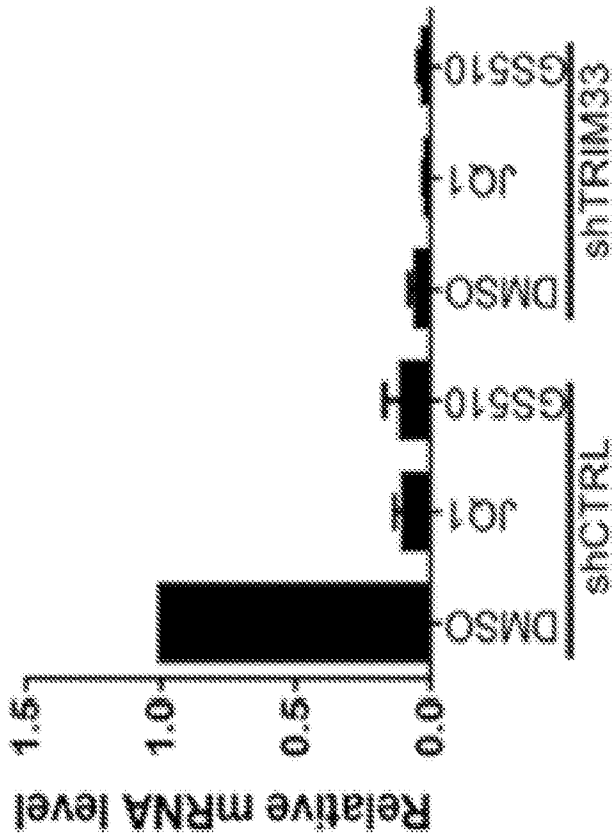
Figure 3C





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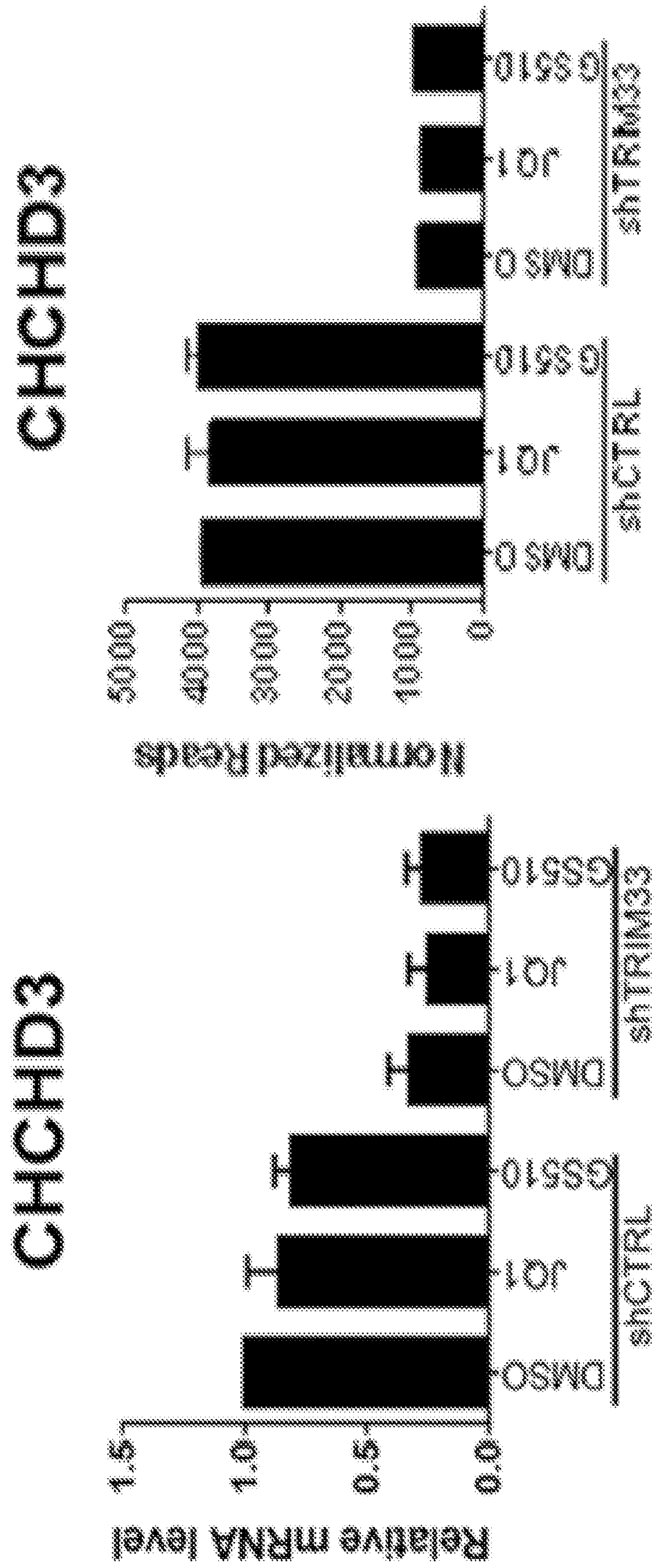

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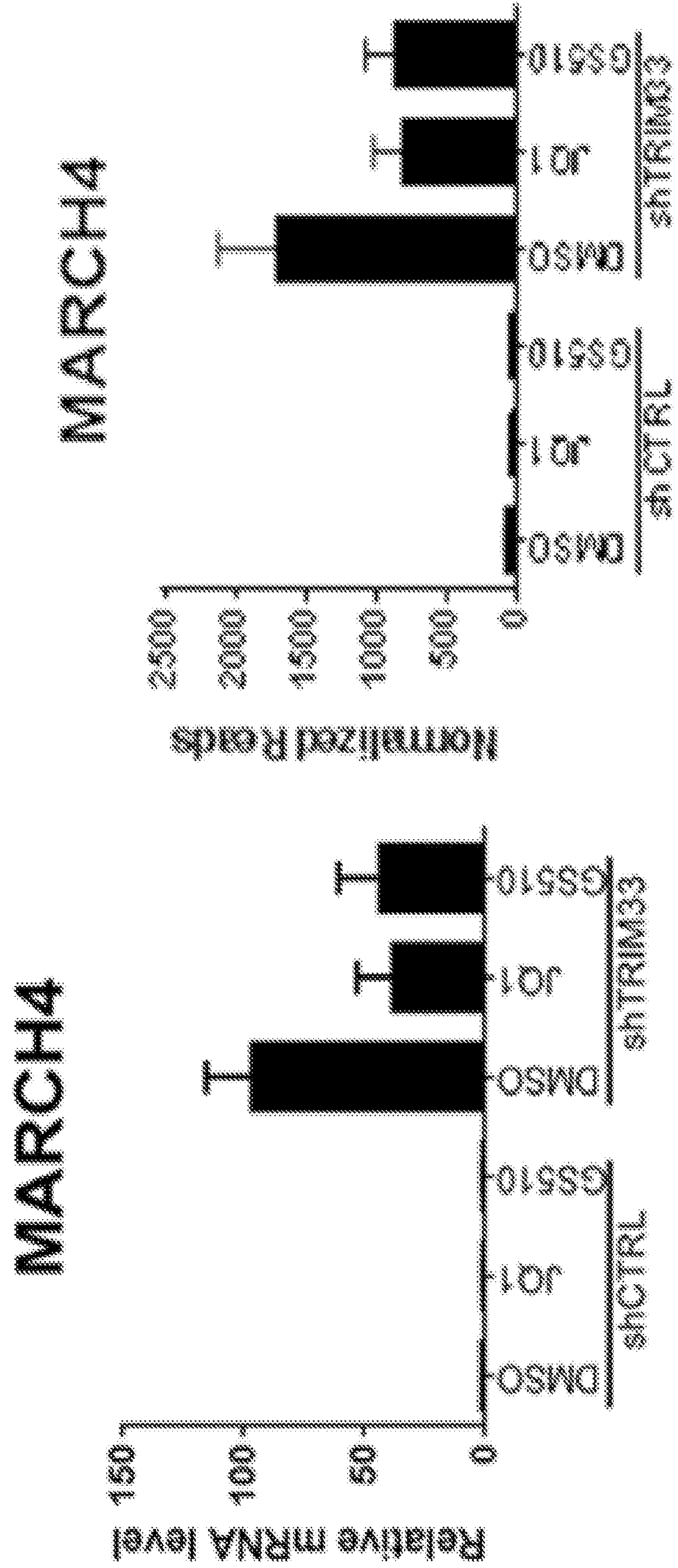
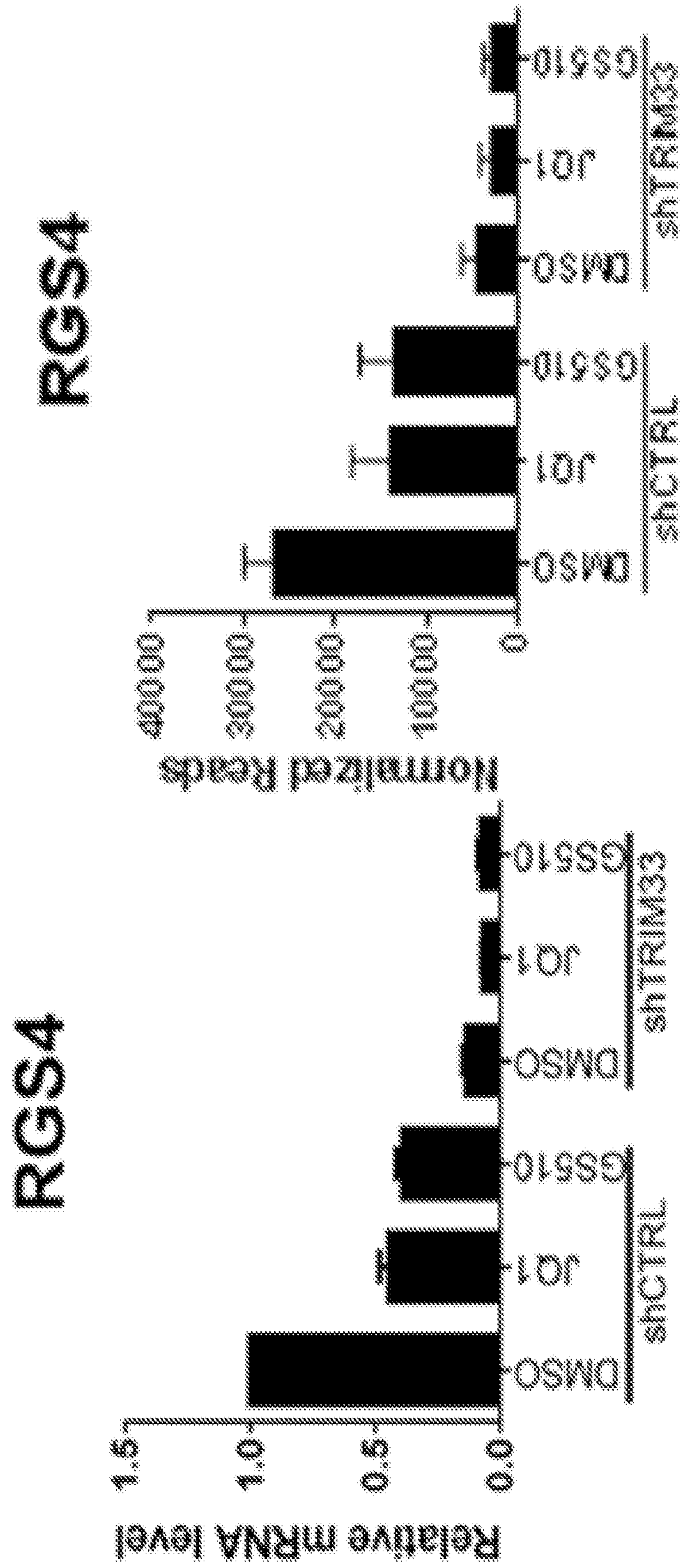


Figure 3D



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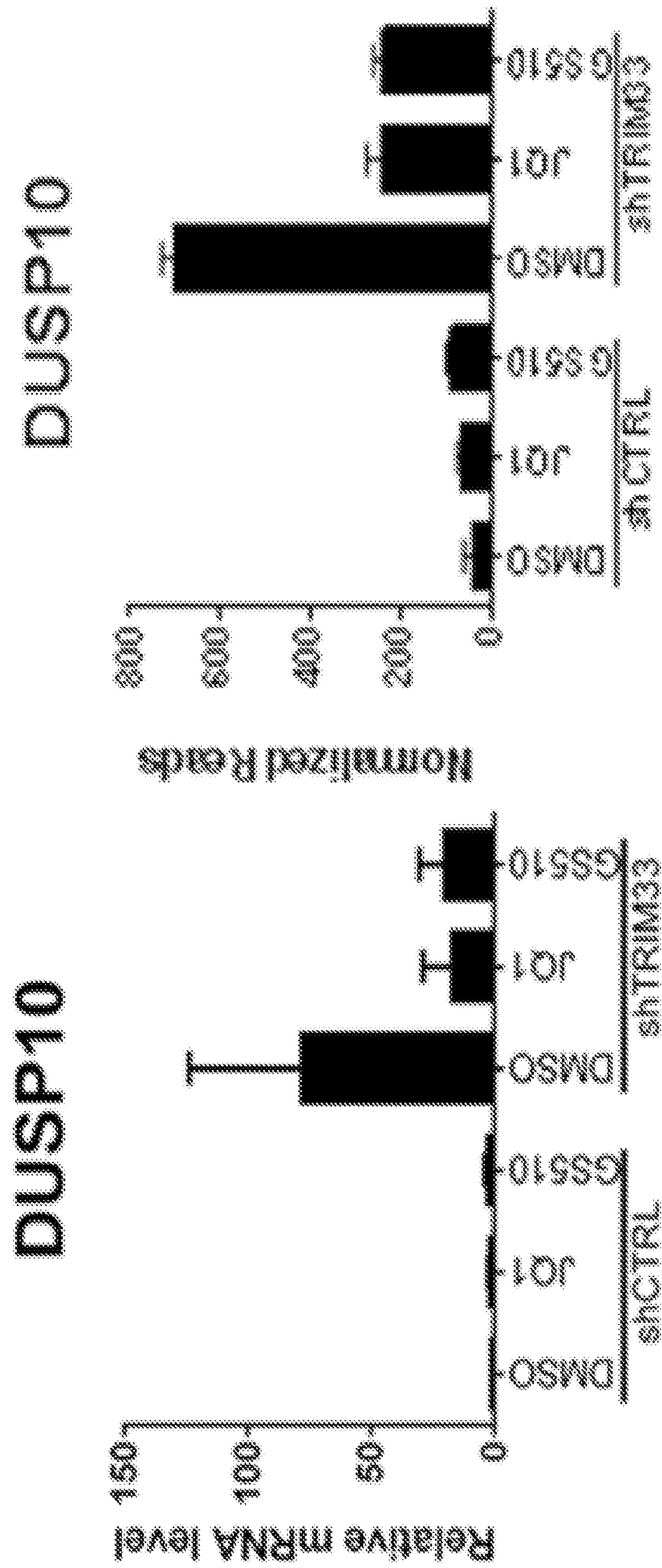

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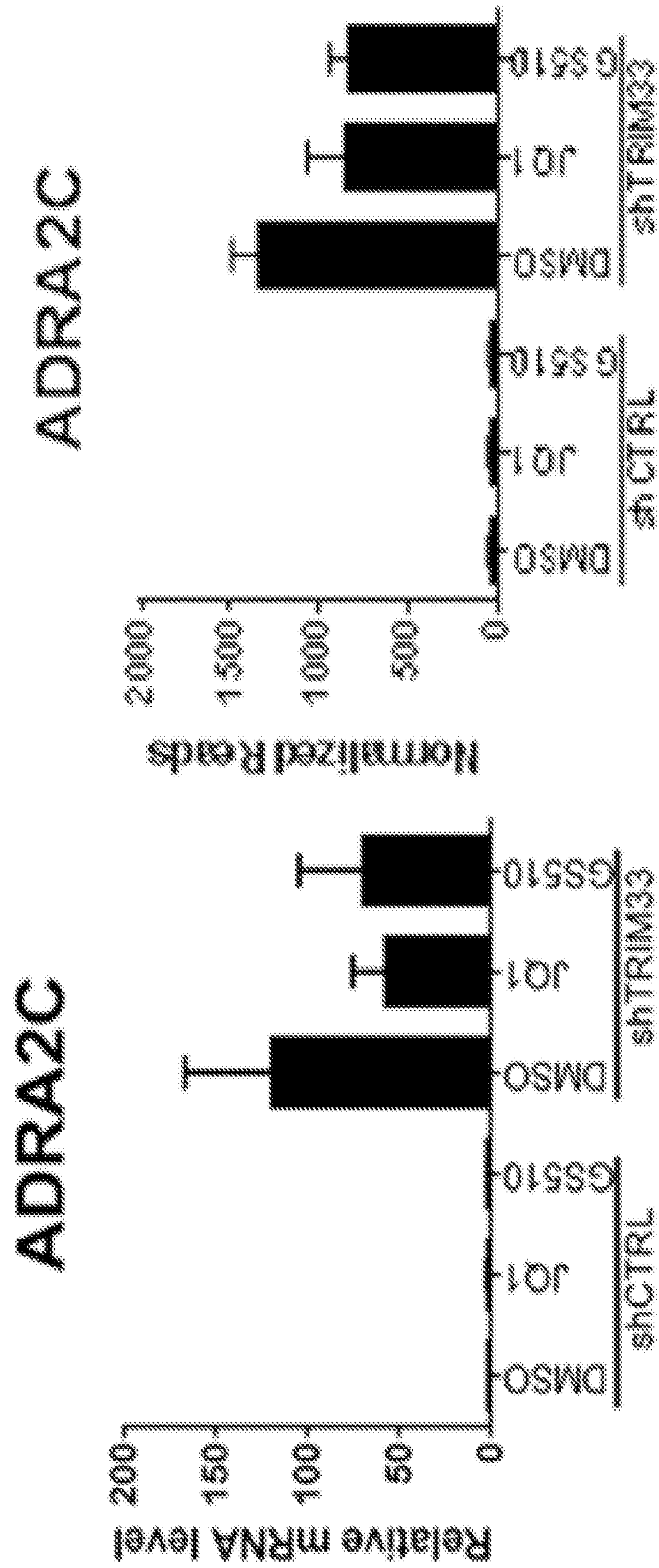
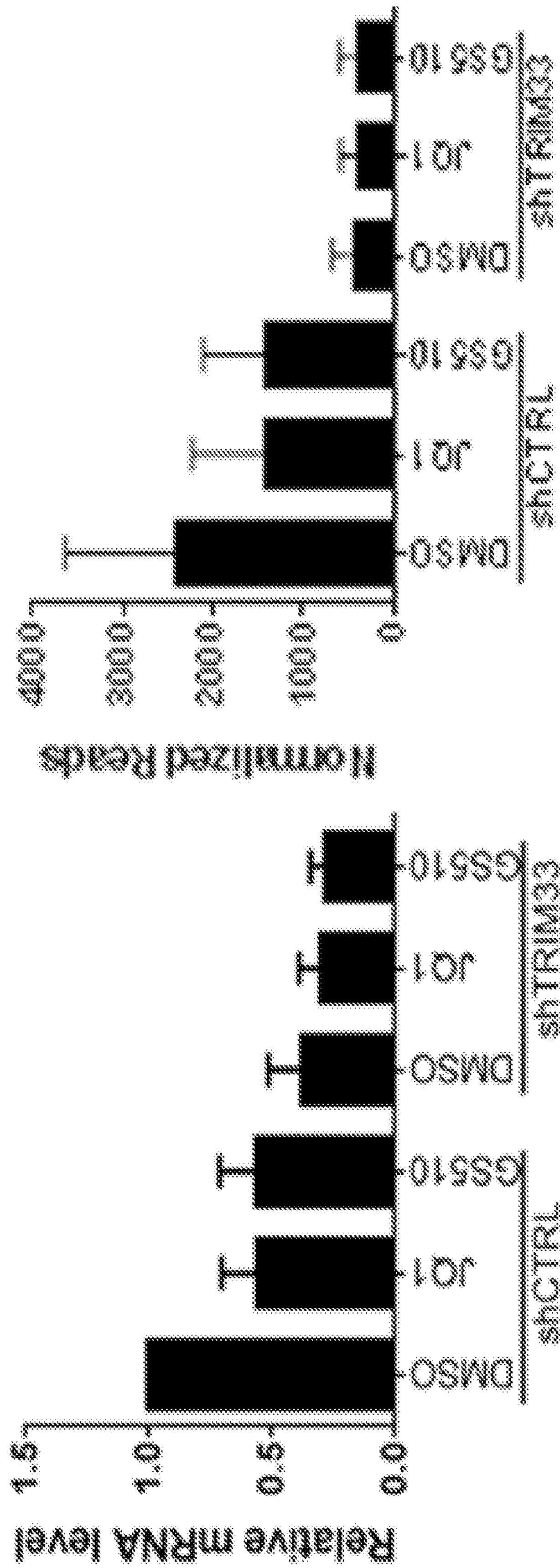


Figure 3E



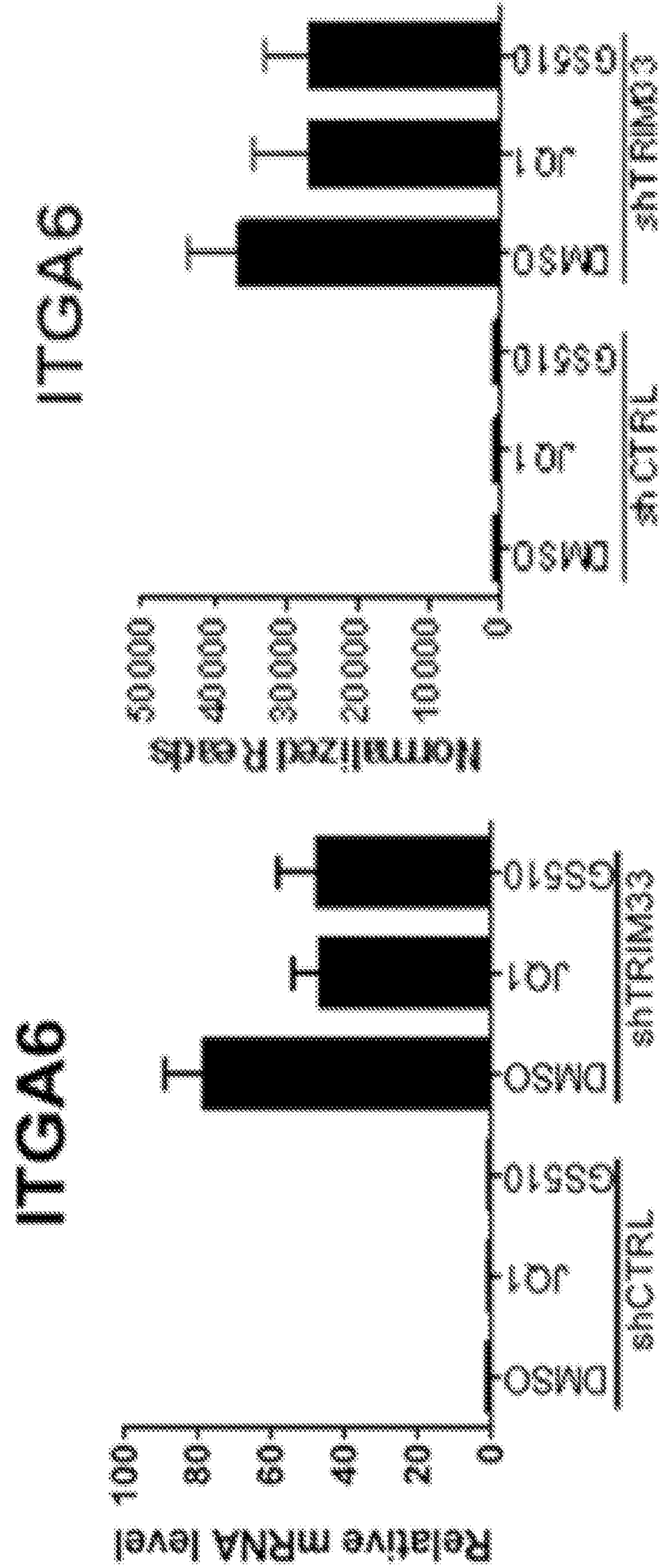


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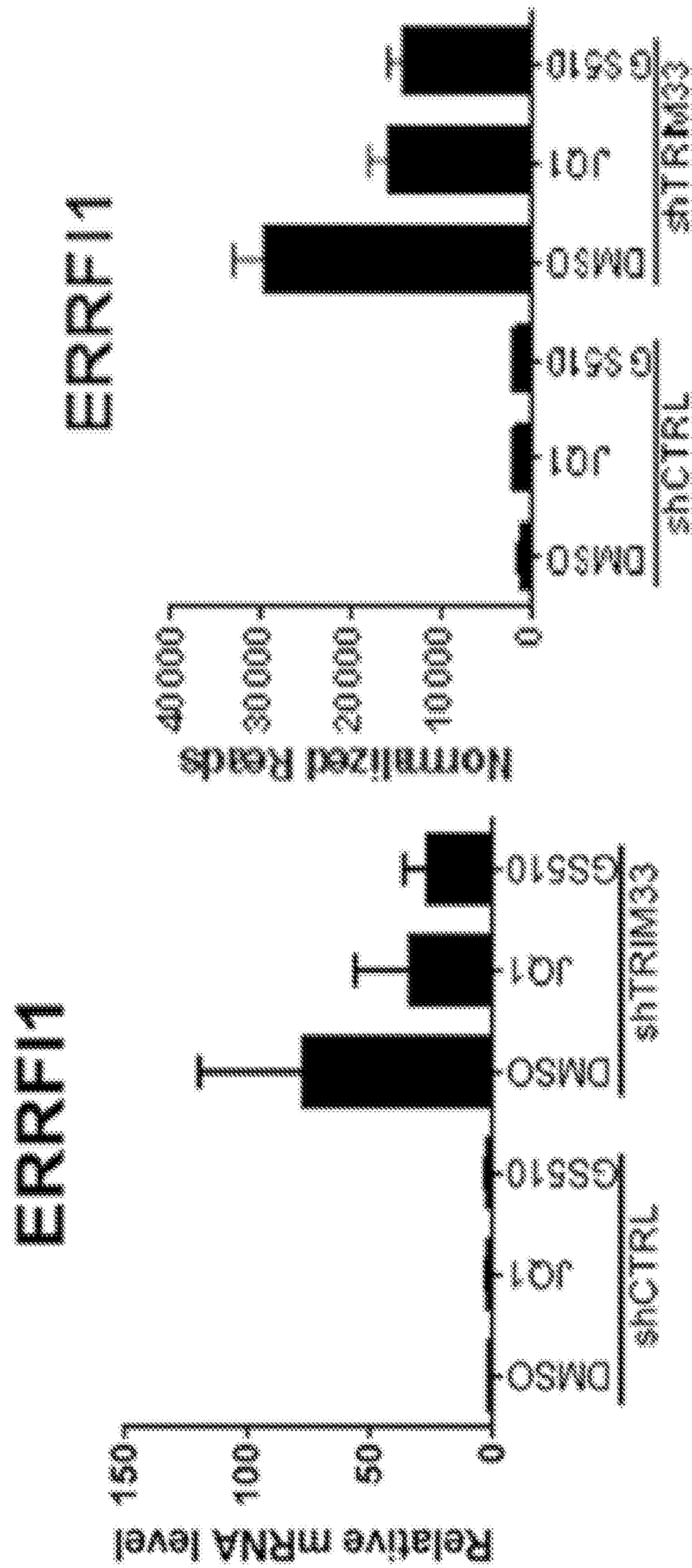
Figure 3E continued



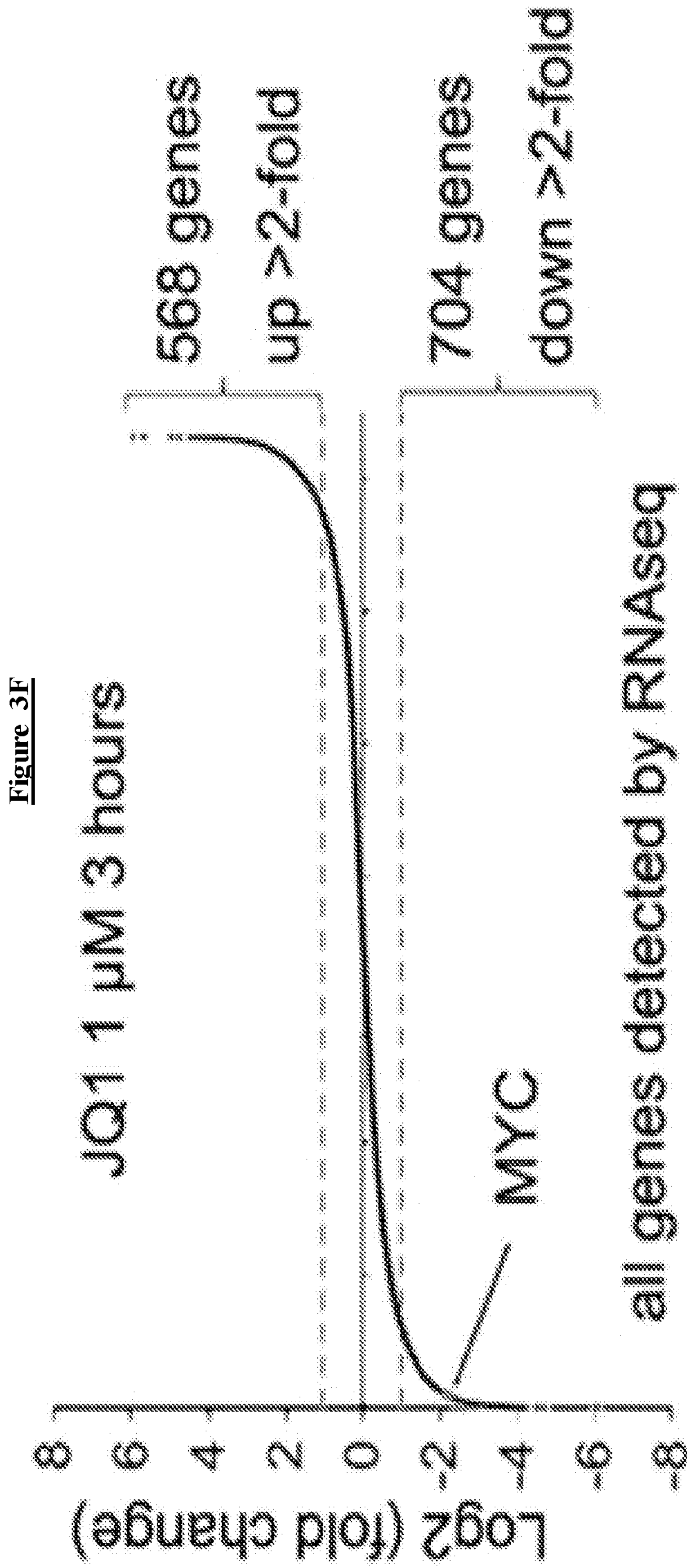


Figure 3F

Figure 3G

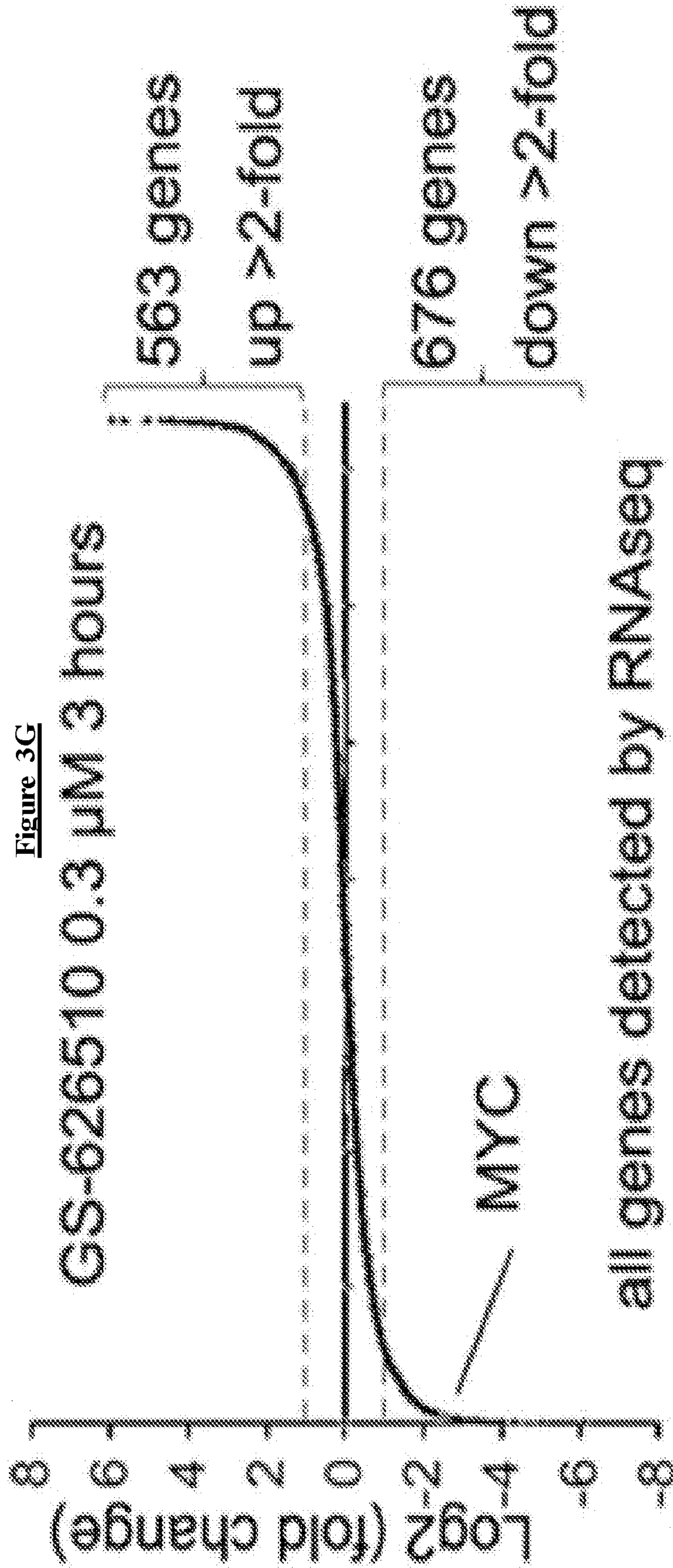


Figure 3H

Motif	Transcription factor	Description
GGGAGGRR	MAZ	MYC-associated zinc finger protein
AACTTT	N/A	Does not match any known transcription factor
CAGGTG	TCF3	Transcription factor 3
TGGAAA	NFAT	Nuclear factor of activated T cells
TTGTTT	FOXO4	Forkhead box O4
CTTTGT	LEF1	Lymphoid enhancer-binding factor 1
TGANTCA	JUN	JUN proto-oncogene
CACGTG	MYC	V-myc myelocytomatosis viral oncogene homolog
RYTTCCTG	ETS2	V-etx erythroblastosis virus E26 oncogene homolog2
TGTTTGY	FOXA1	Forkhead box A1

Motif	Genes in overlap	Genes in Gene Set	Fraction of Gene Set	p-value	FDR q-value
GGGAGGRR	141	2274	0.0620	3.09 E-26	2.58 E-23
AACTTT	125	1890	0.0661	1.02 E-25	4.27 E-23
CAGGTG	140	2485	0.0563	3.79 E-22	1.06 E-19
TGGAAA	117	1896	0.0617	1.22 E-21	2.54 E-19
TTGTTT	118	2061	0.0573	2.93 E-19	4.90 E-17
CTTTGT	111	1972	0.0563	1.27 E-17	1.76 E-15
TGANTCA	78	1121	0.0696	1.47 E-17	1.76 E-15
CACGTG	73	1032	0.707	7.00 E-17	7.32 E-15
RYTTCCTG	74	1085	0.0682	2.97 E-16	2.76 E-14
TGTTTGY	56	738	0.0759	1.71 E-14	1.43 E-12

Figure 3I

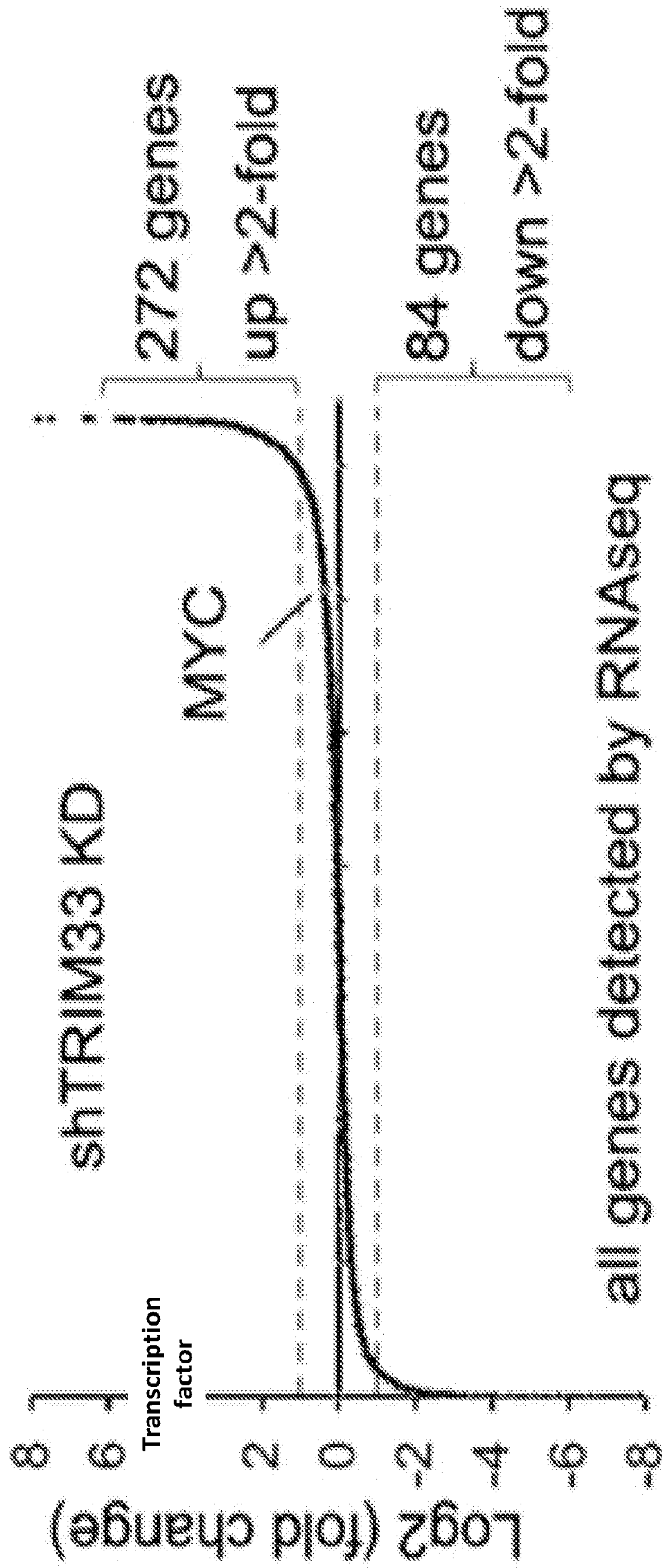


Figure 3J

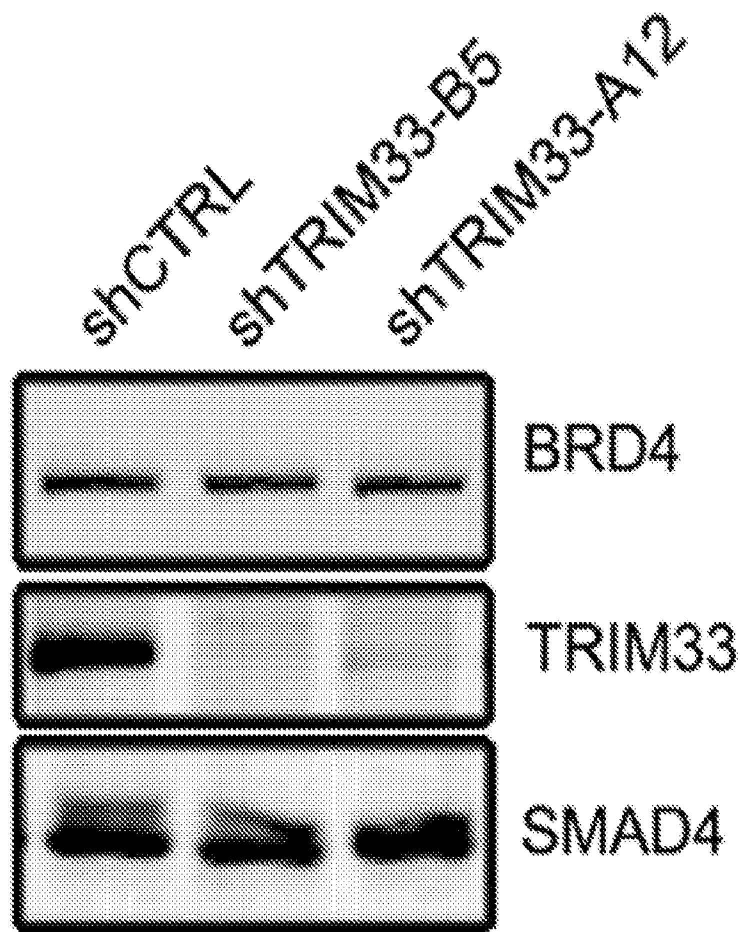


Figure 4A

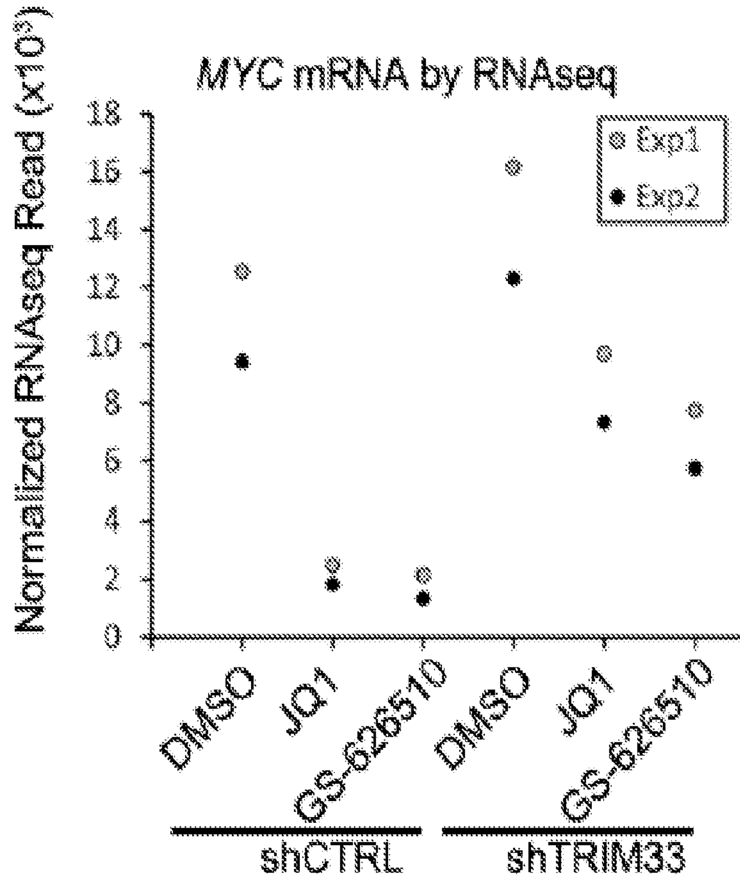


Figure 4B

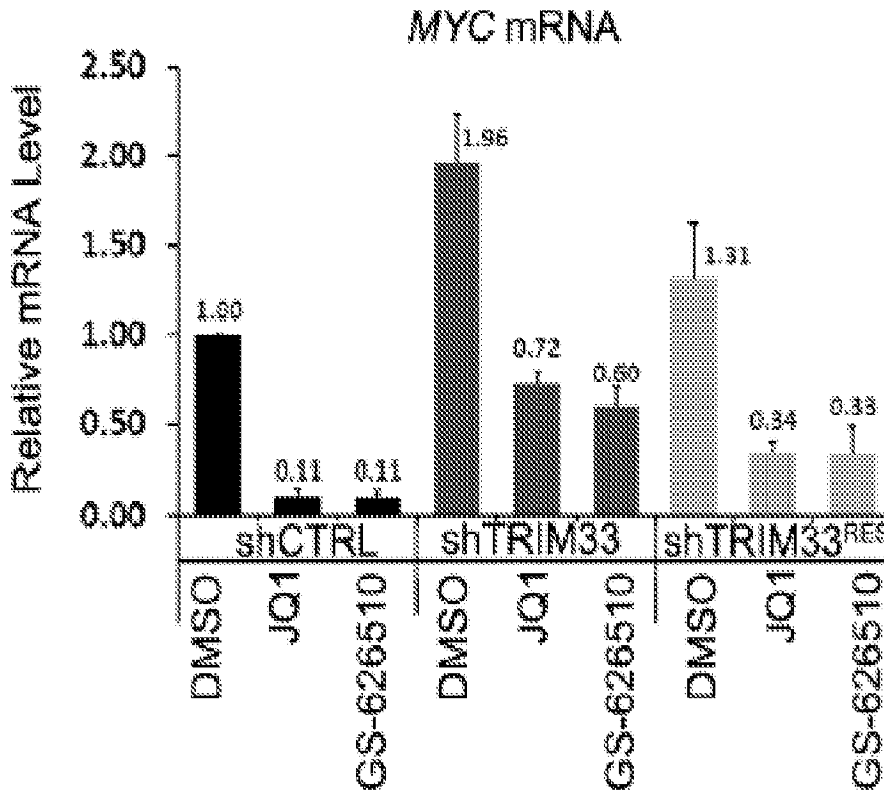


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Figure 4C

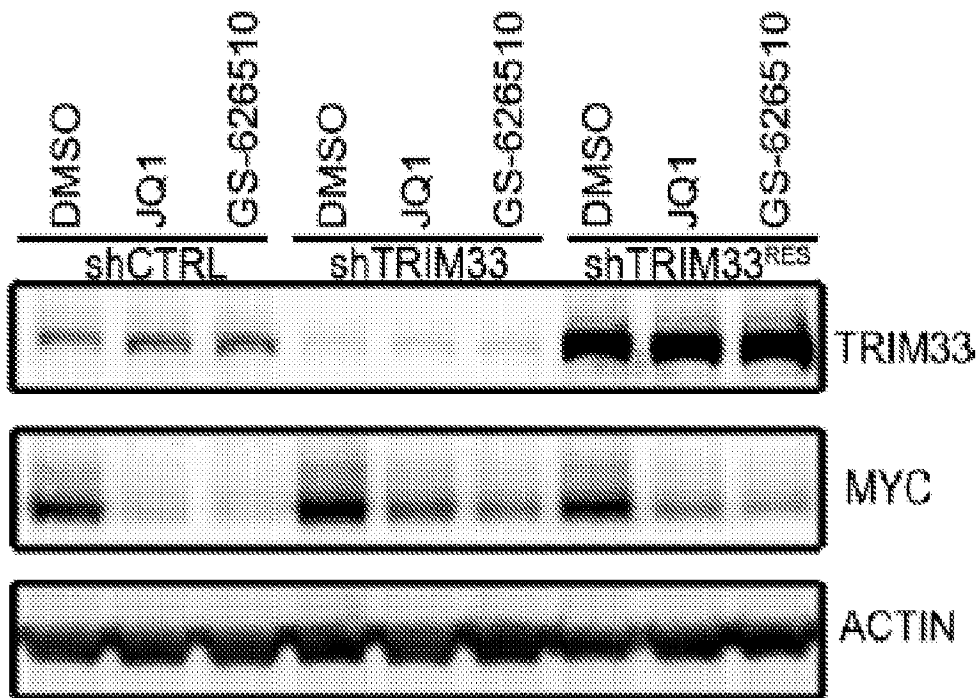

Figure 4D

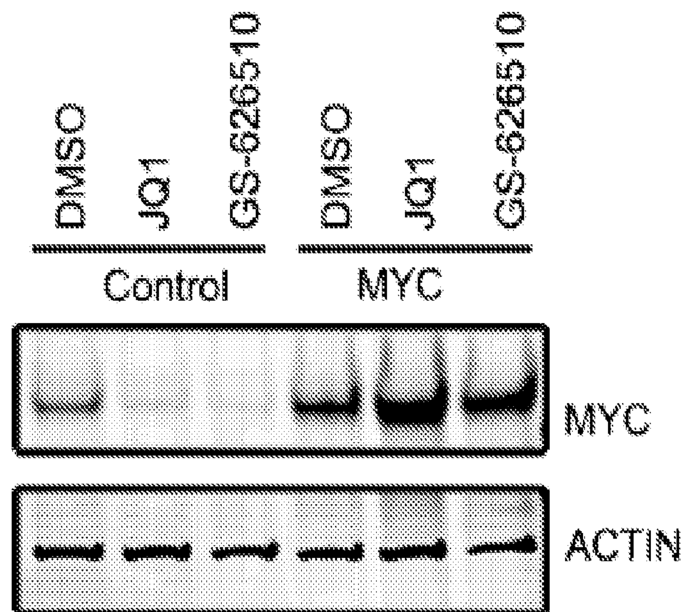

Figure 4E

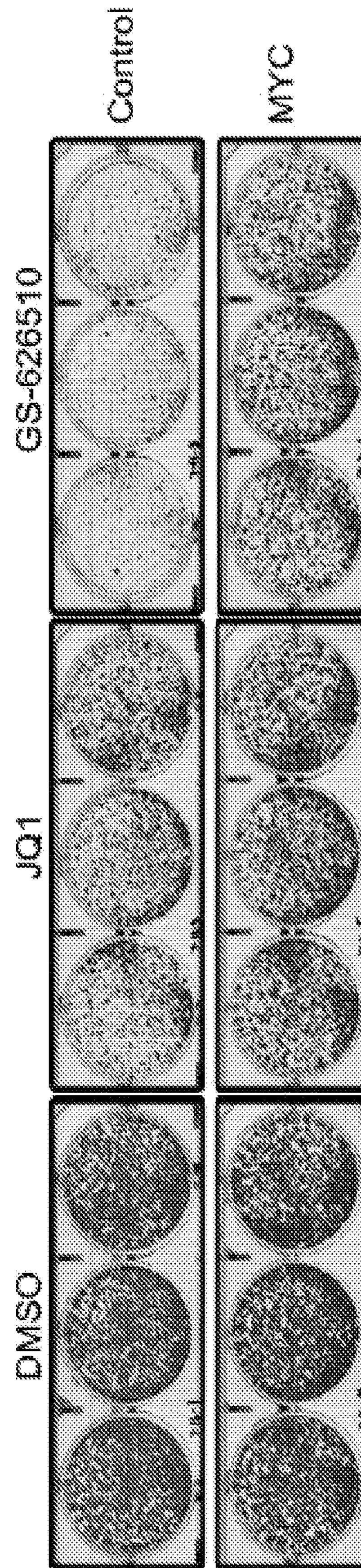

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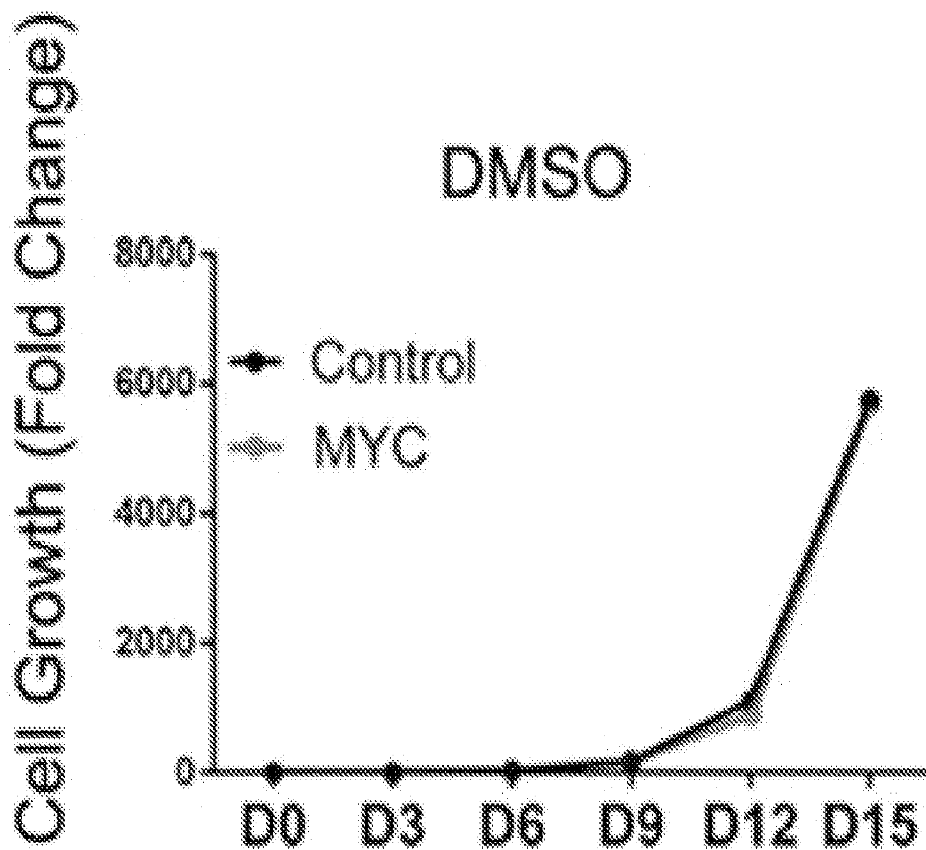
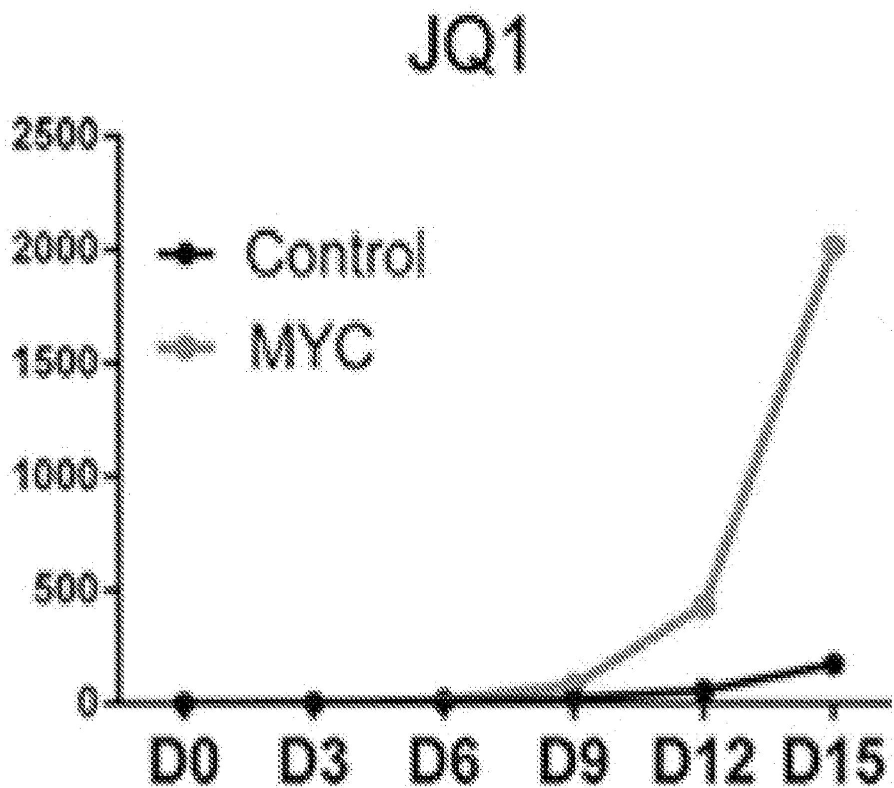
Figure 4F





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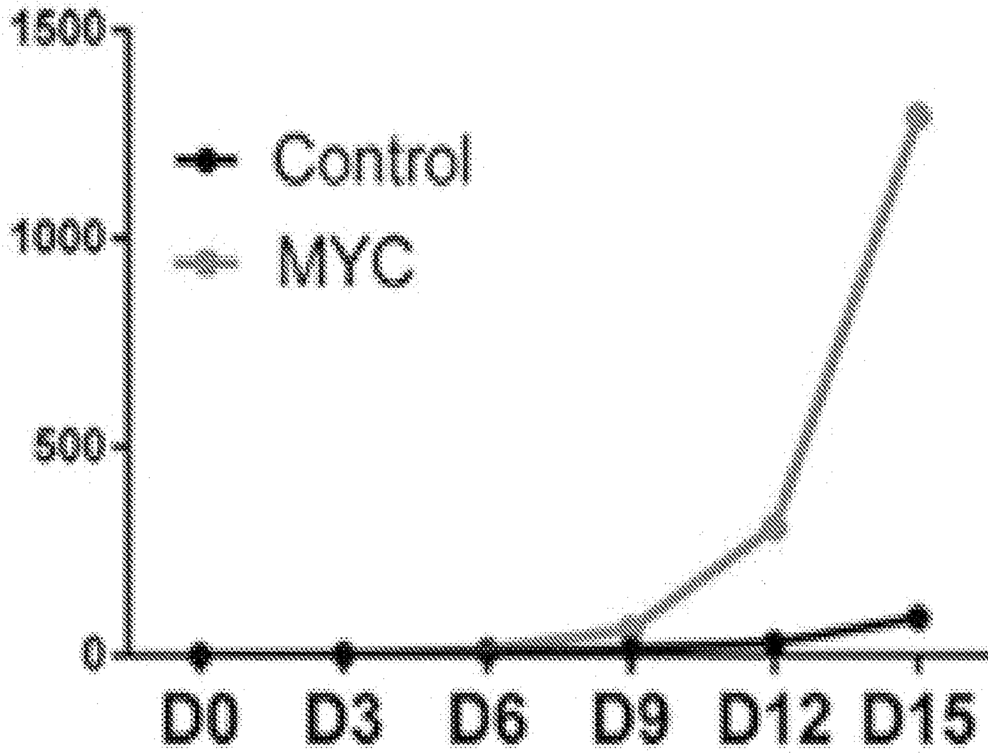

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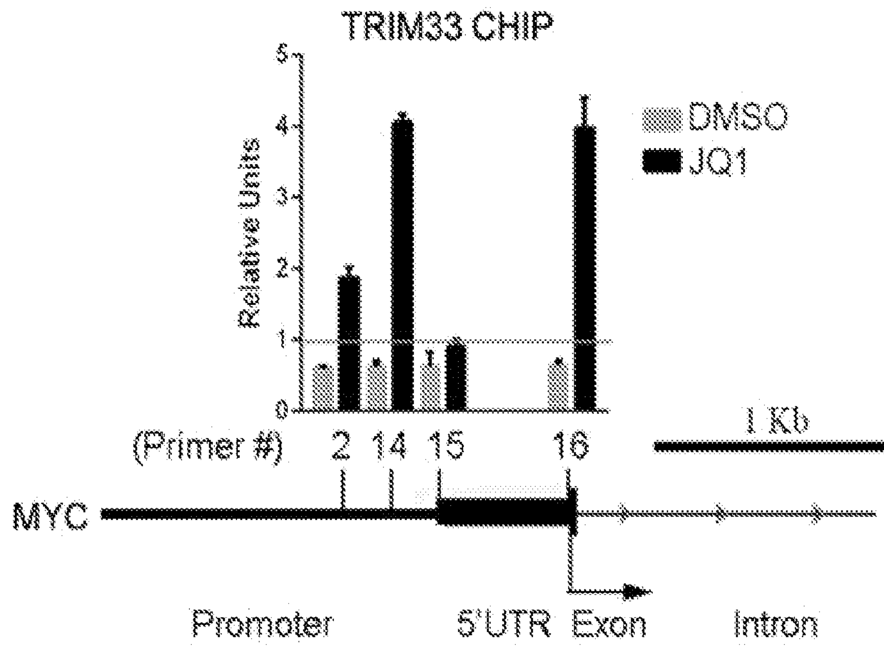


Figure 4H

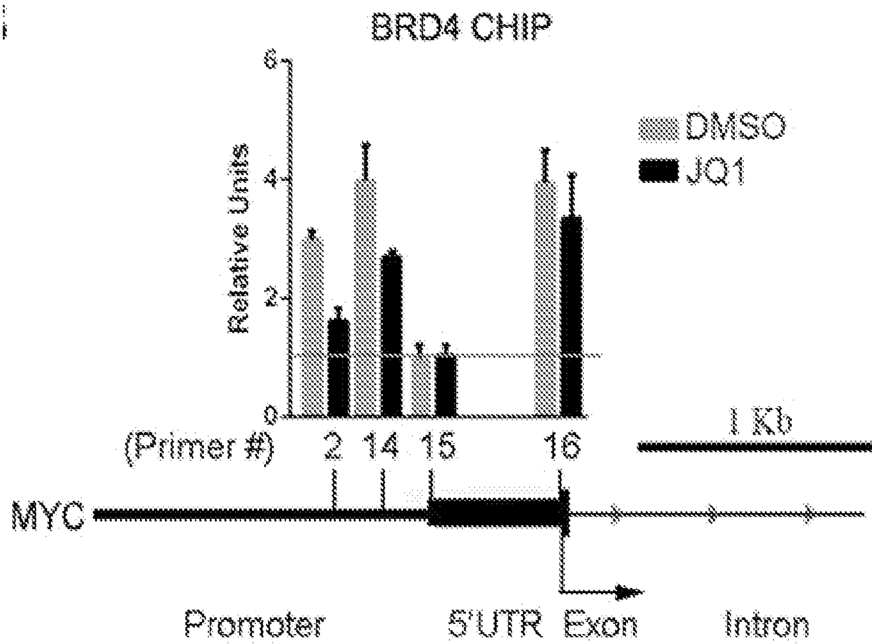
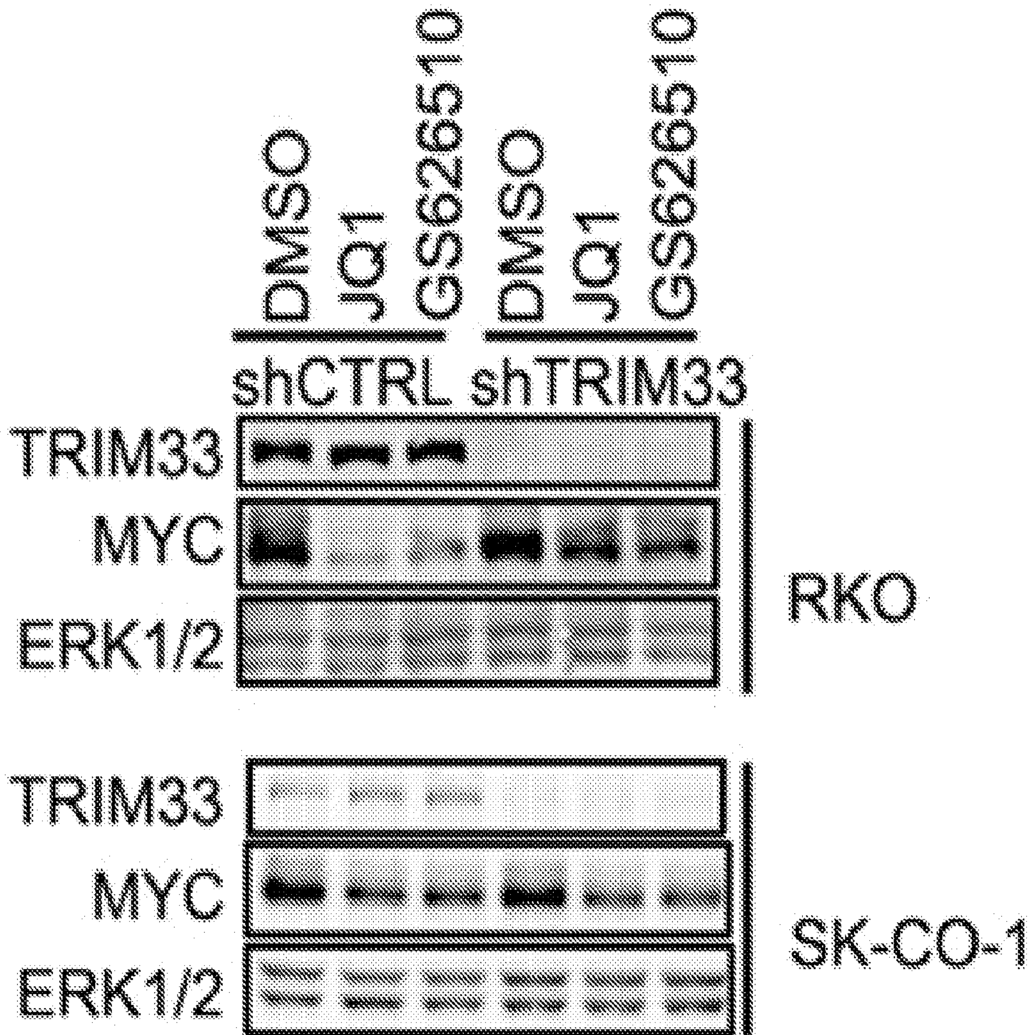


Figure 5A



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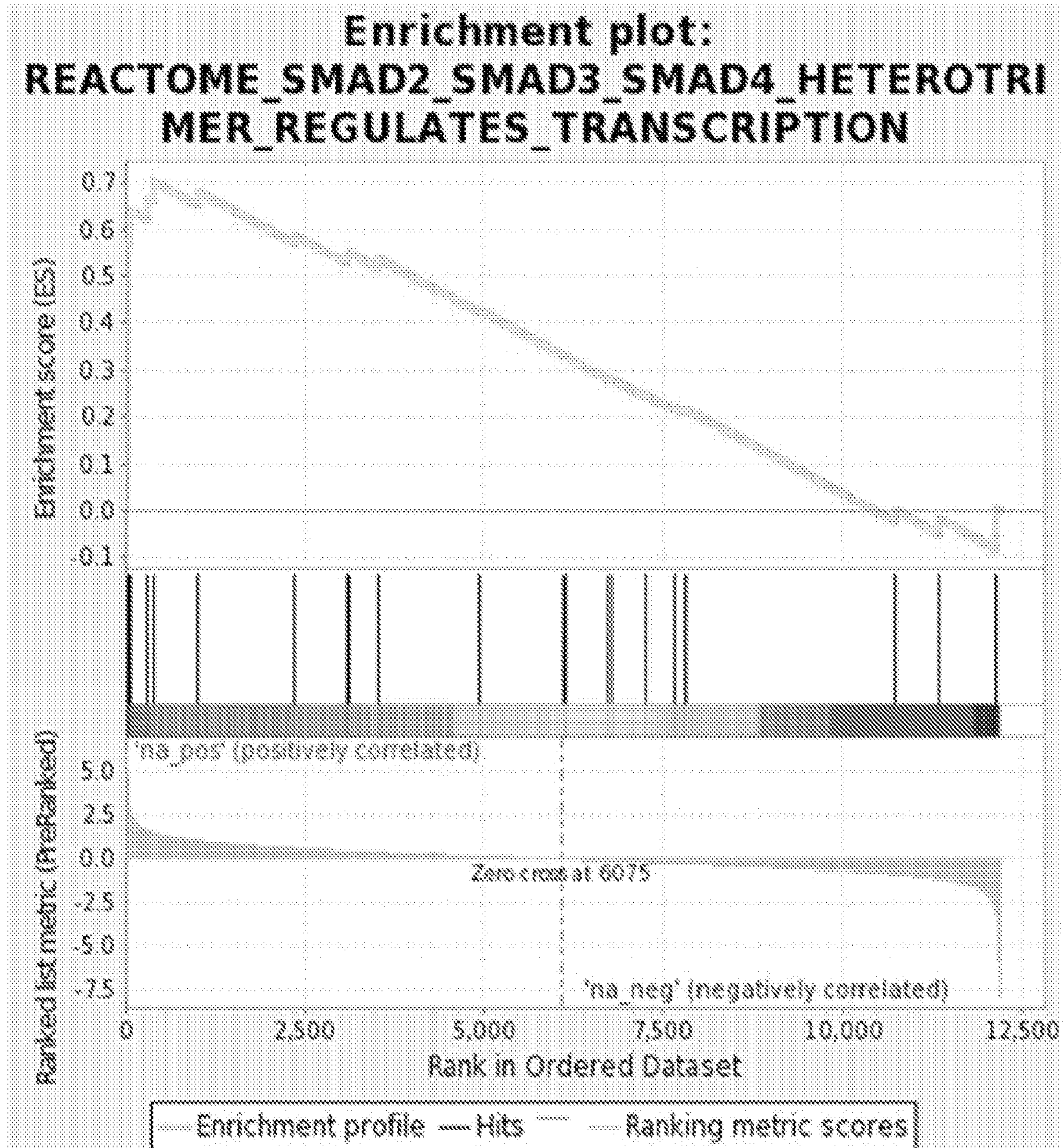

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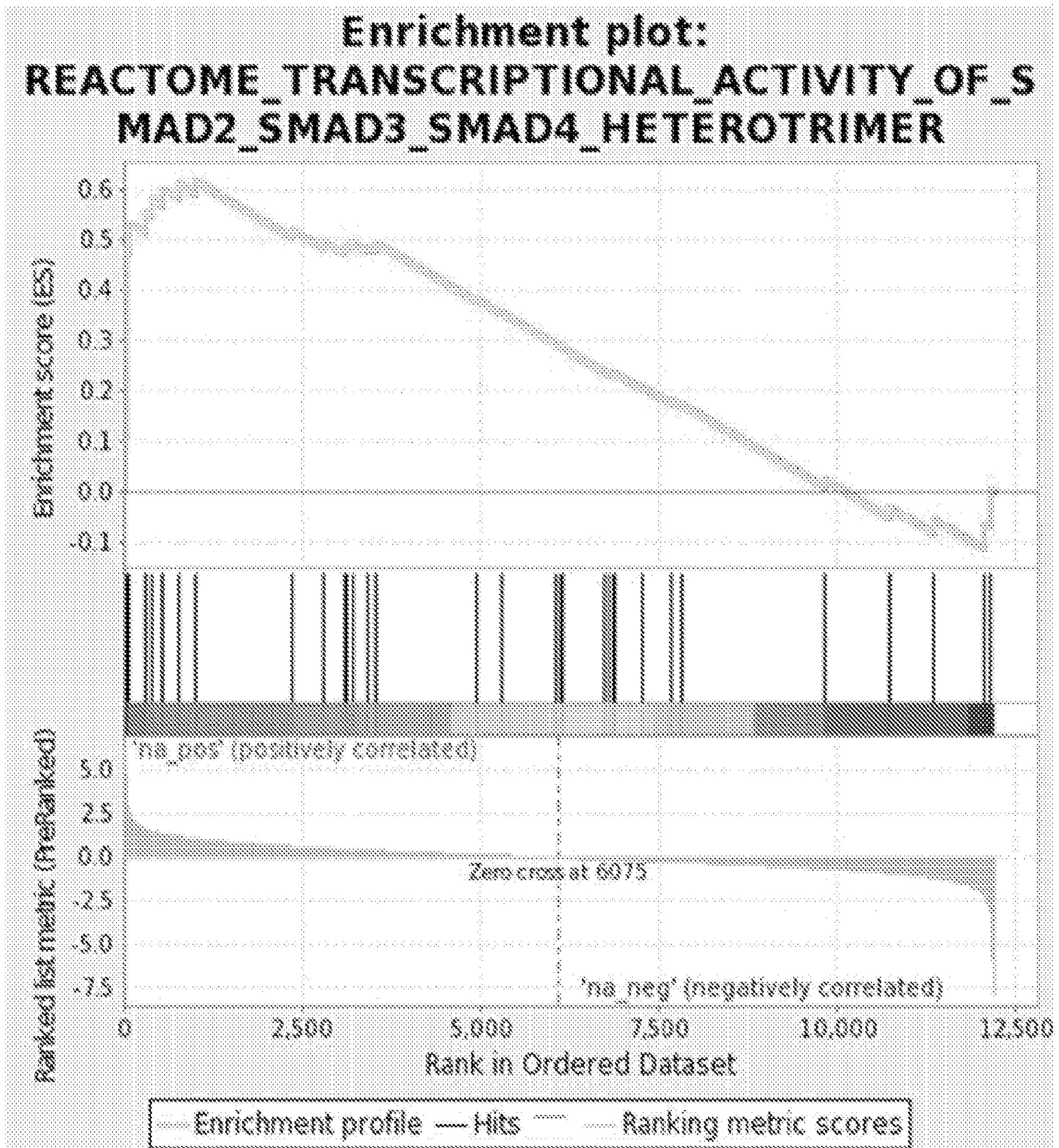


Figure 5D

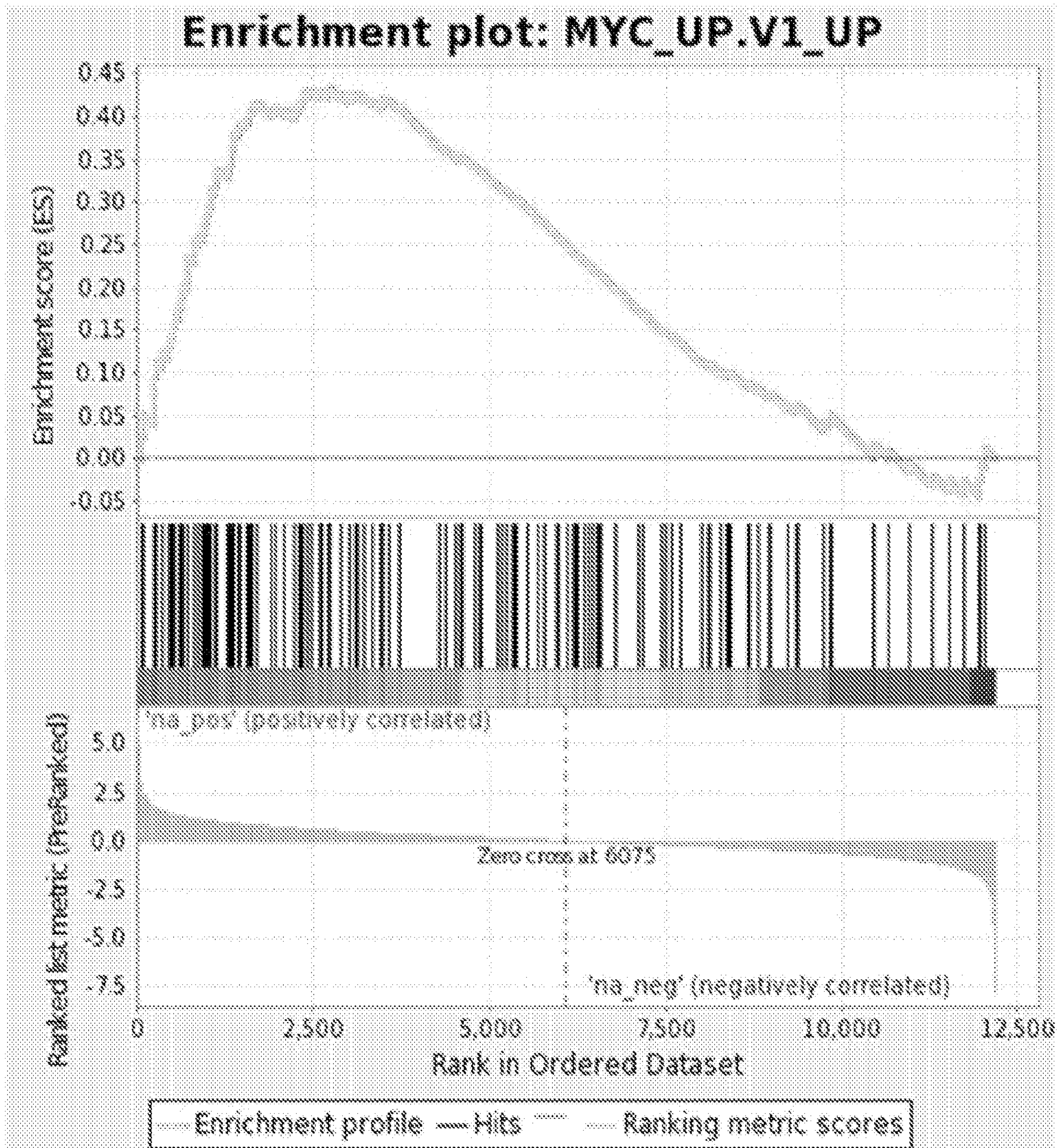
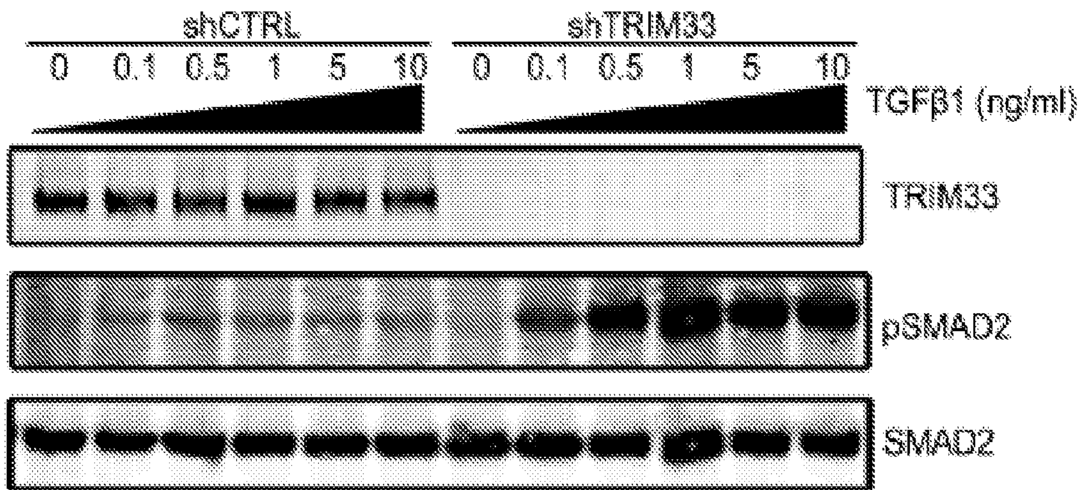


Figure 5E

Name	Size	NES	FDR q-val
REACTOME_SMAD2_SMAD3_SMAD4_HETEROTRIMER_REGULATES_TRASCIPTION	25	2.215	0.006
REACTOME_TRANSCIPTIONAL_ACTIVITY_OF_SMAD2_SMAD3_SMAD4_HETEROTRIMER	36	2.121	0.017
MANALO_HYPOXIA_DN	283	2.016	0.060
GSE19825_CD24LOW_VS_IL2RA_HIGH_DAY3_EFF_CD8_TCELL_DN	186	1.973	0.086
MYC_UP.V1_UP	141	1.919	0.139
LIEN_BREAST_CARCINOMA_METAPLASTIC_VS_DUCTAL_UP	49	1.916	0.120
GSE30962_PRIMARY_VS_SECONDARY_CHRONIC_LCMV_INF_CD8_TCELL_UP	170	1.909	0.113
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_GREEN_DN	22	1.908	0.100
BROWNE_HCMV_INFECTION_12HR_DN	67	1.906	0.091
REACTOME_PPARA_ACTIVATES_GENE_EXPRESSION	85	1.898	0.090
PID_CD40_PATHWAY	26	1.877	0.107
PID_SMAD2_3NUCLEARPATHWAY	66	1.876	0.099
GSE30962_ACUTE_VS_CHRONIC_LCMV_PRIMARY_INF_CD8_TCELL_DN	153	1.873	0.096
GUTIERREZ_CHRONIC_LYMPHOCYTIC_LEUKEMIA_DN	34	1.860	0.103
TSENG_ADIPOGENIC_POTENTIAL_UP	19	1.833	0.132

Figure 5F



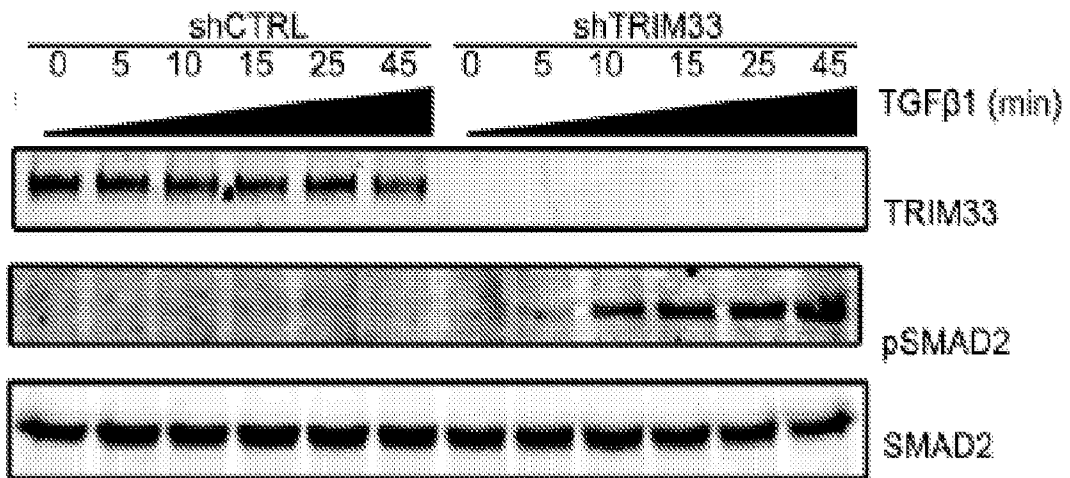


Figure 5G

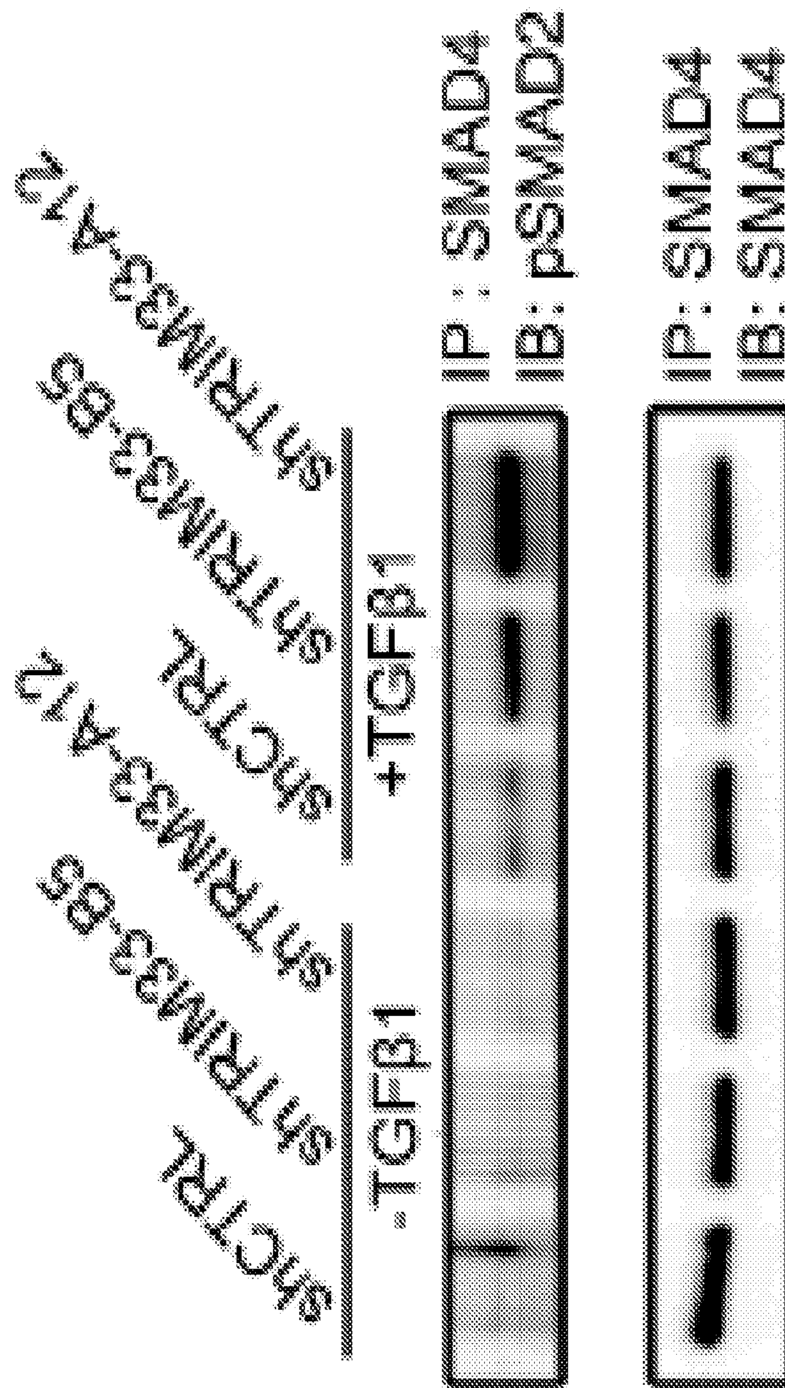


Figure 5H

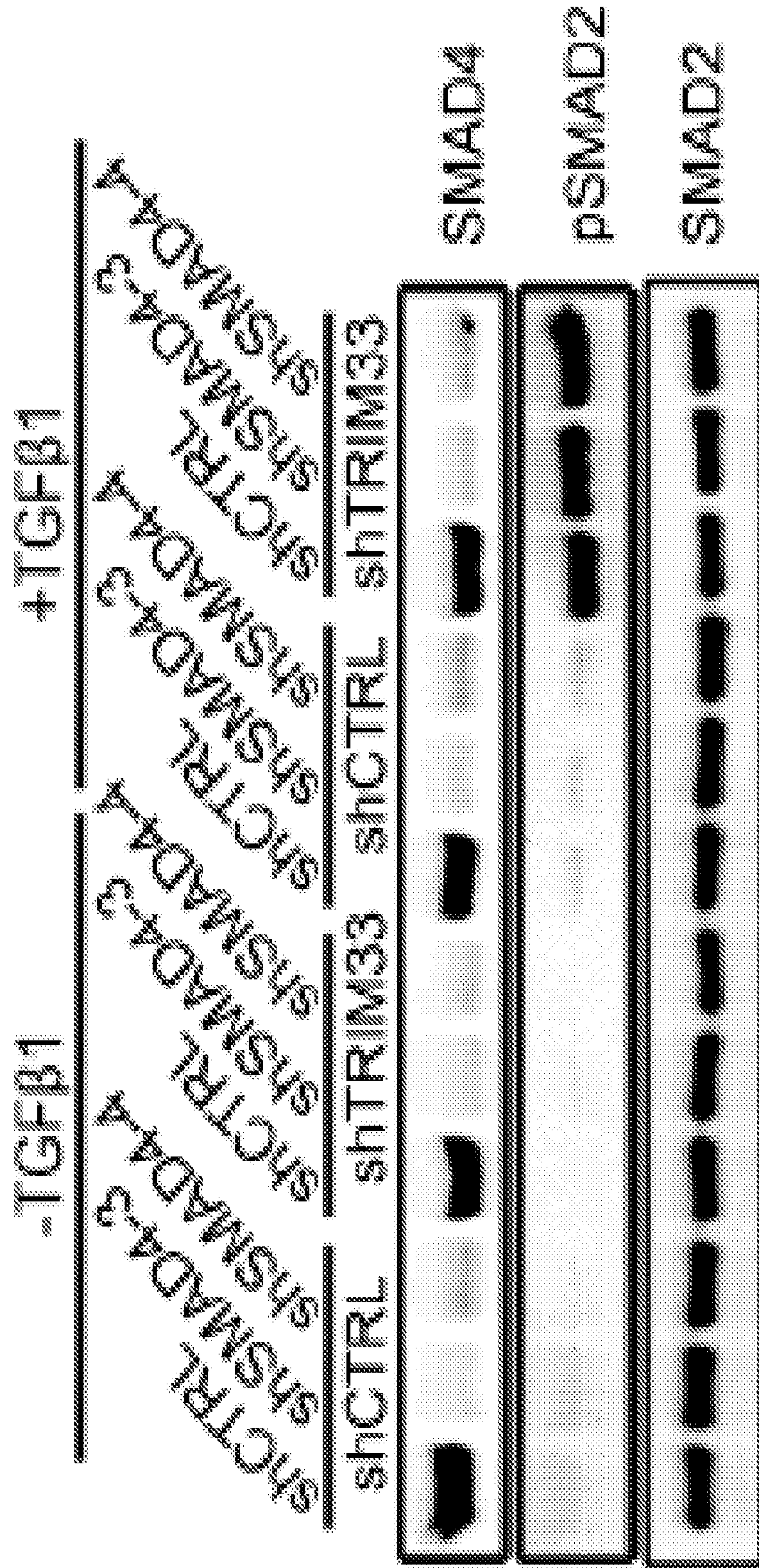


Figure 5I

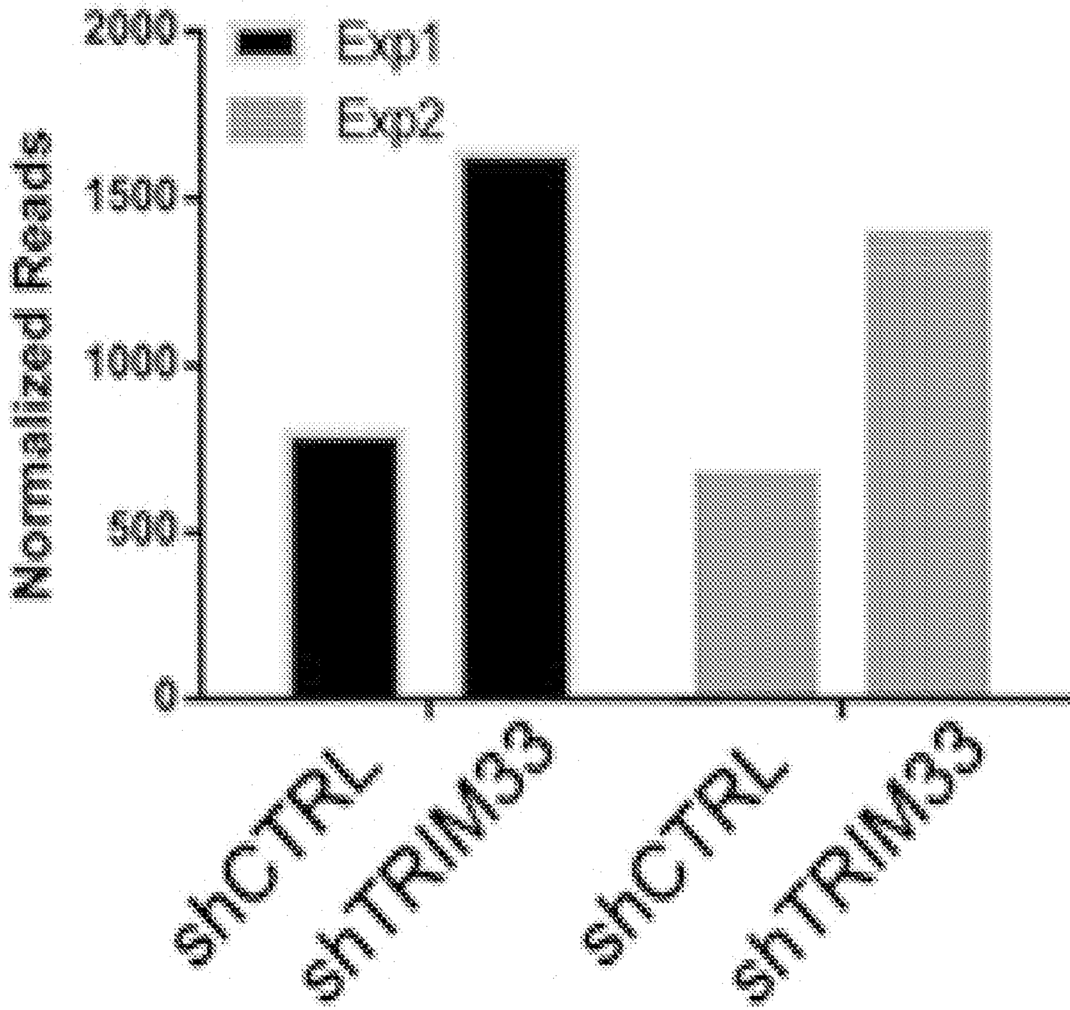


Figure 5J

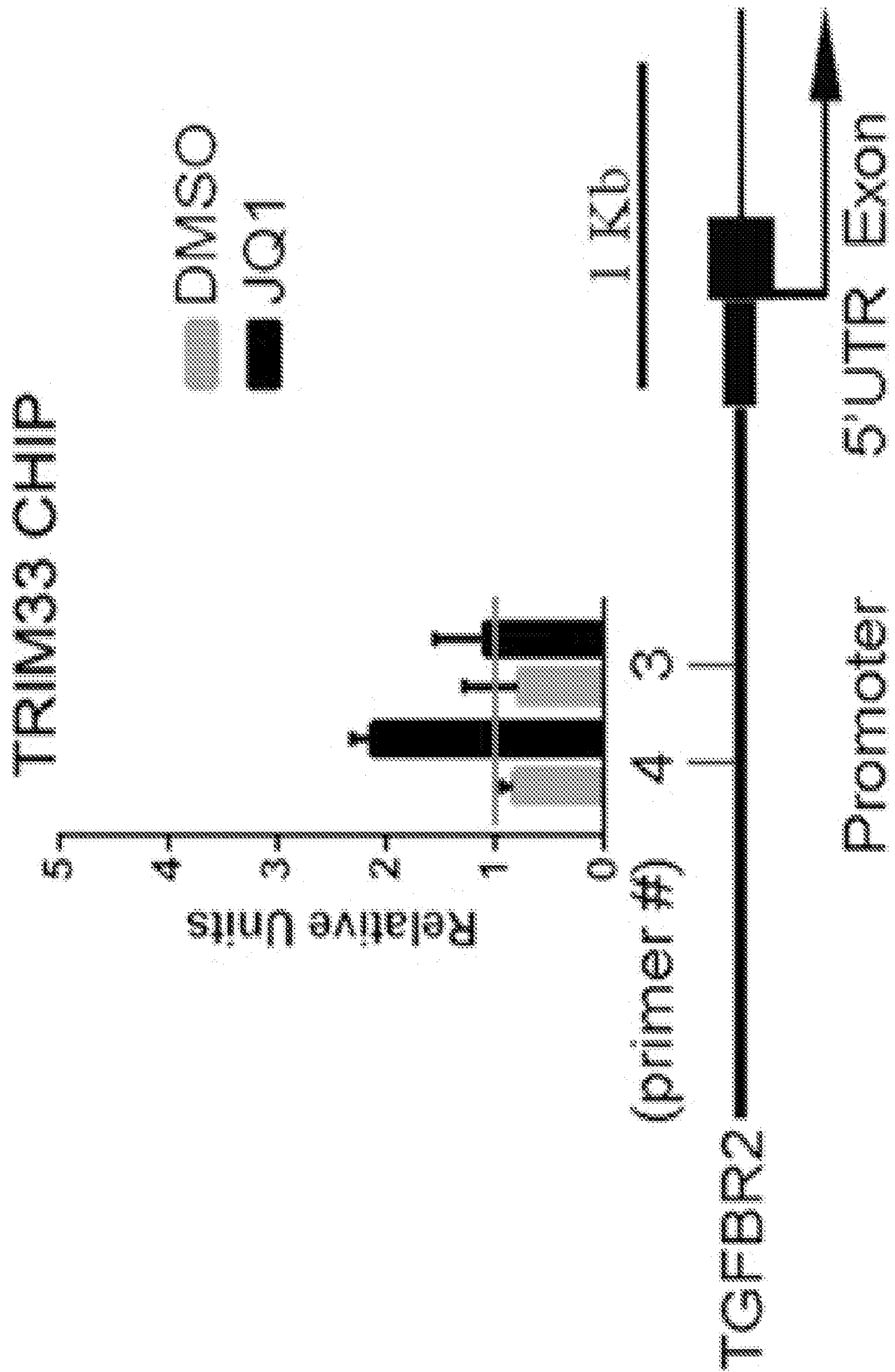
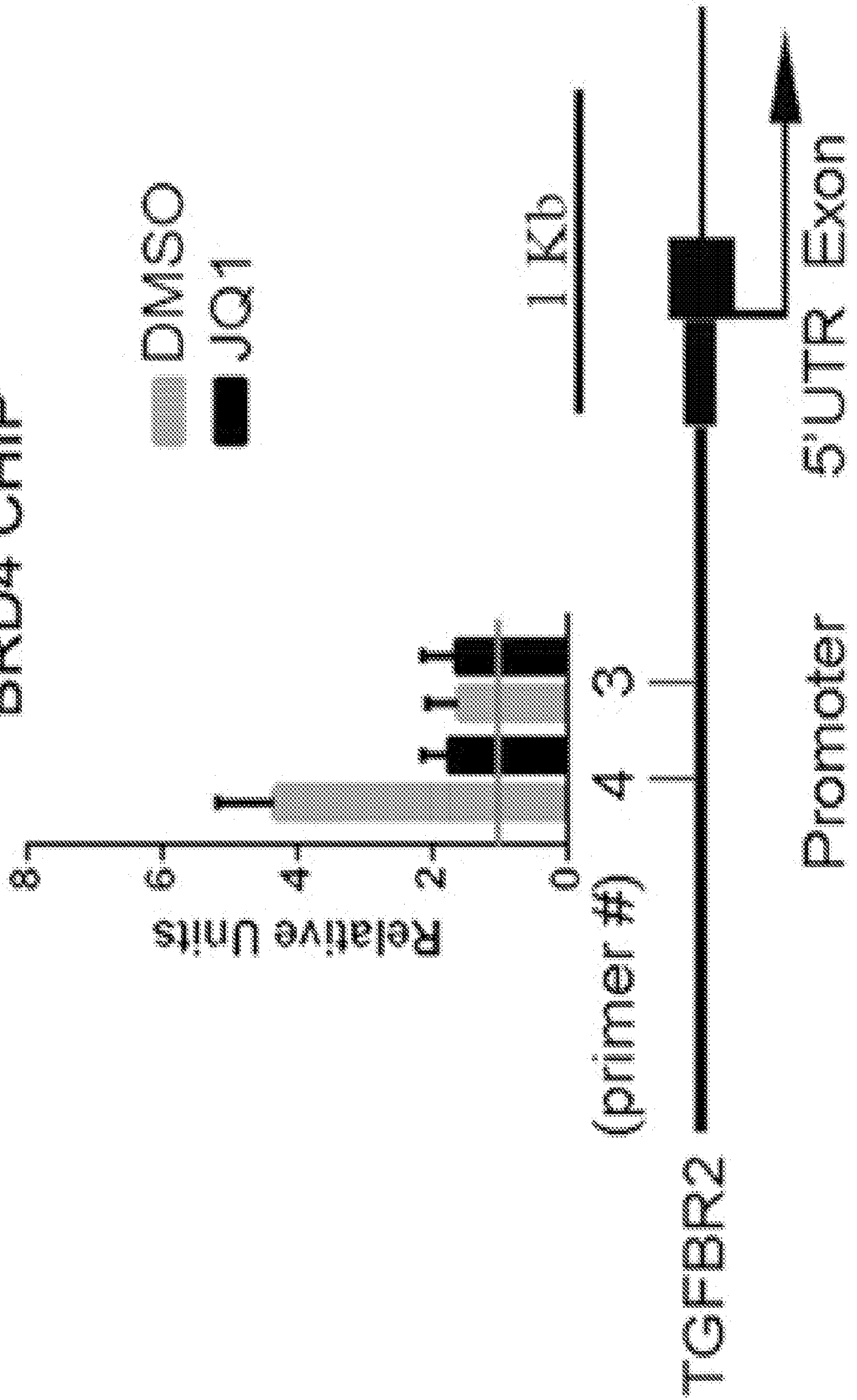


Figure 5K



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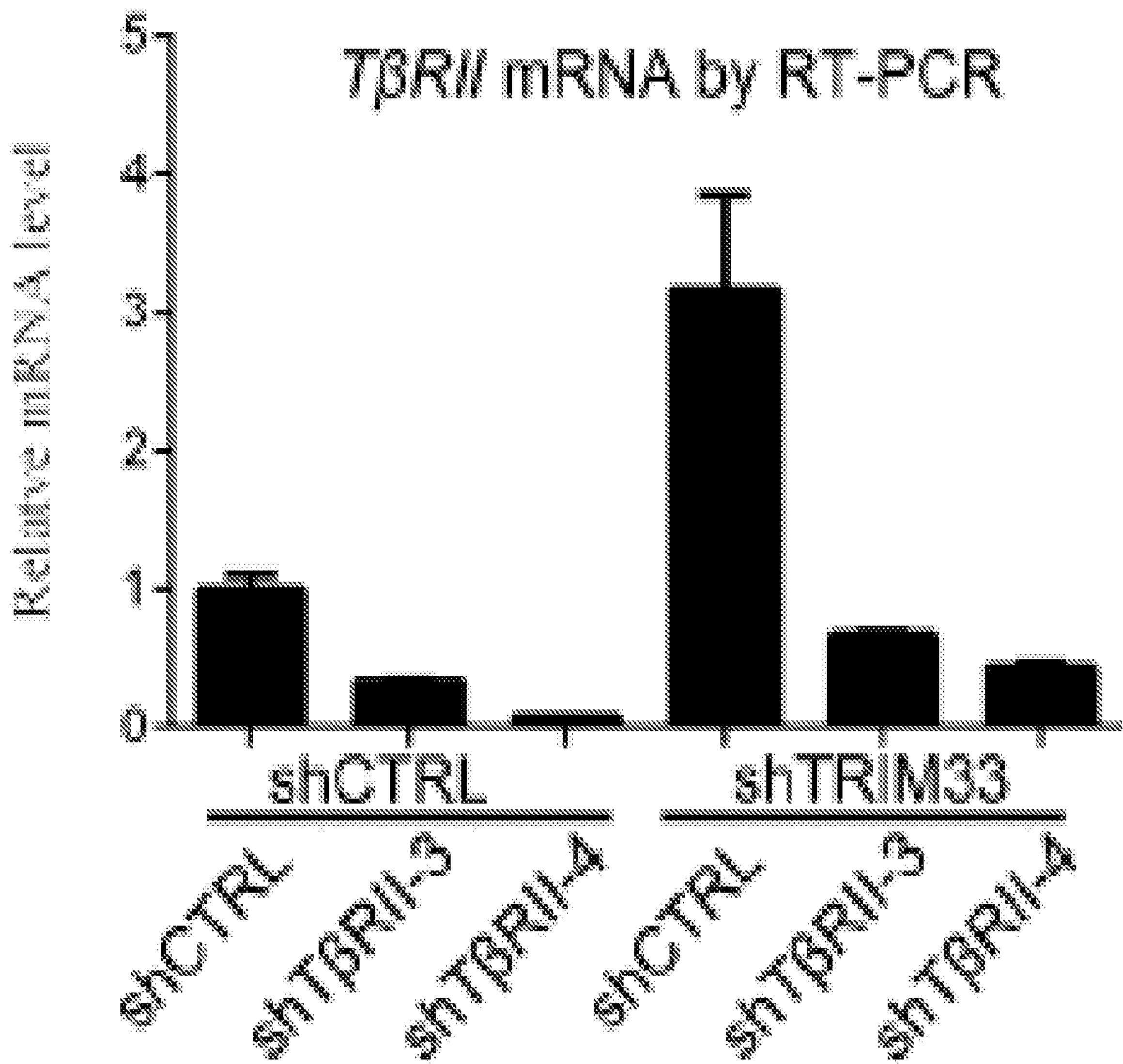

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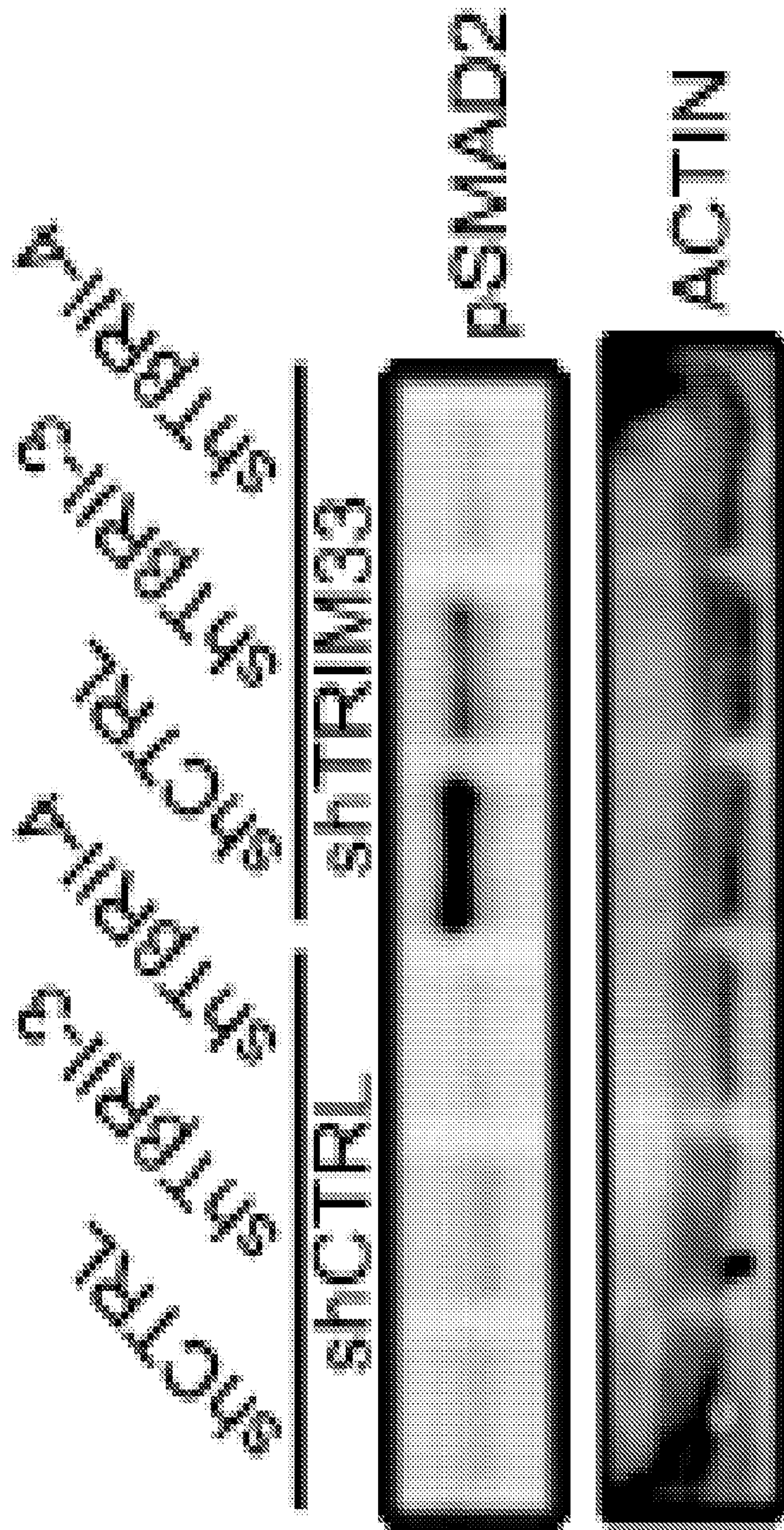

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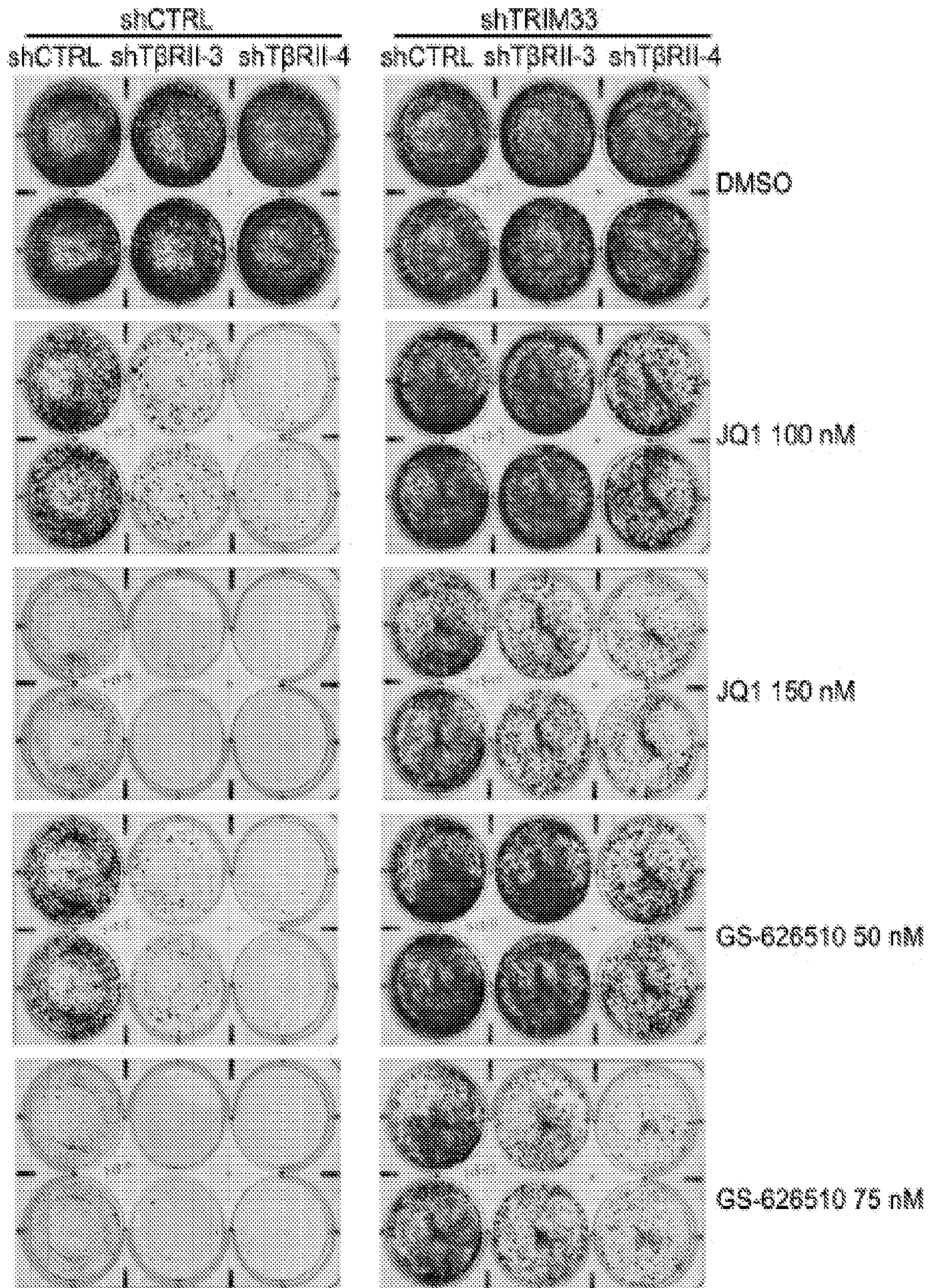
Figure 5N



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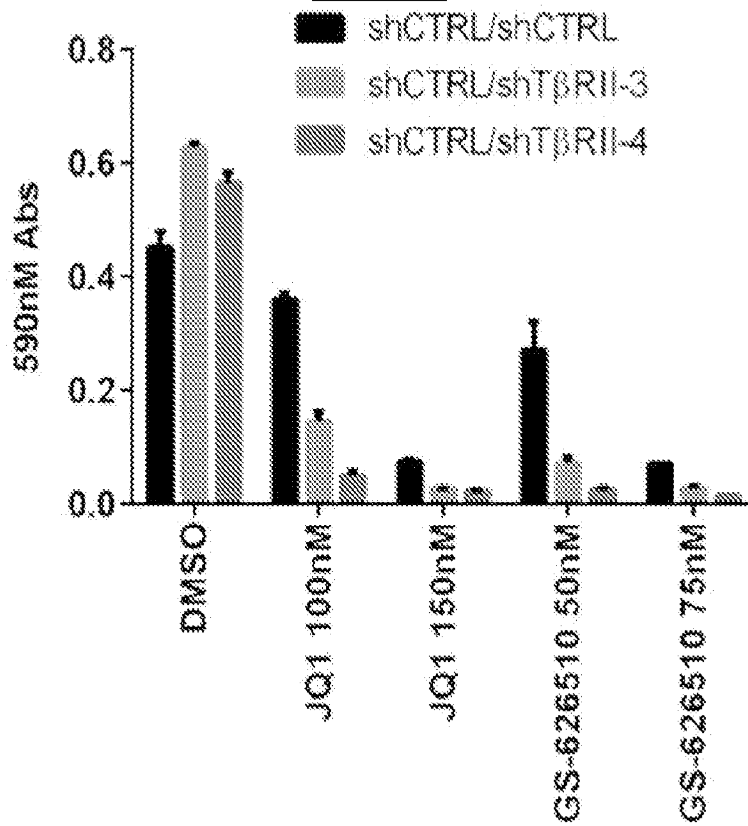
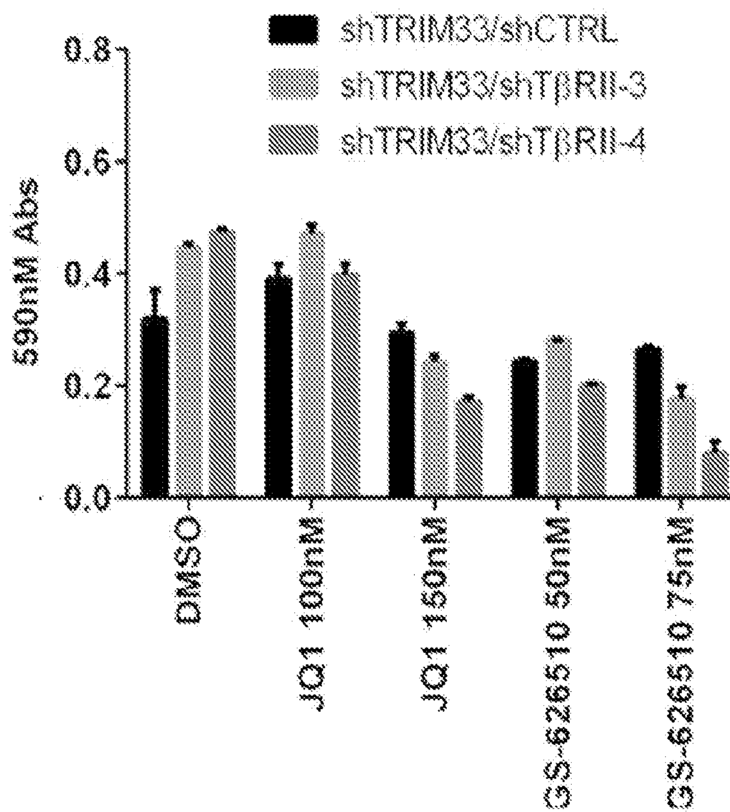




SUBSTITUTE SHEET (RULE 26)

Figure 5P

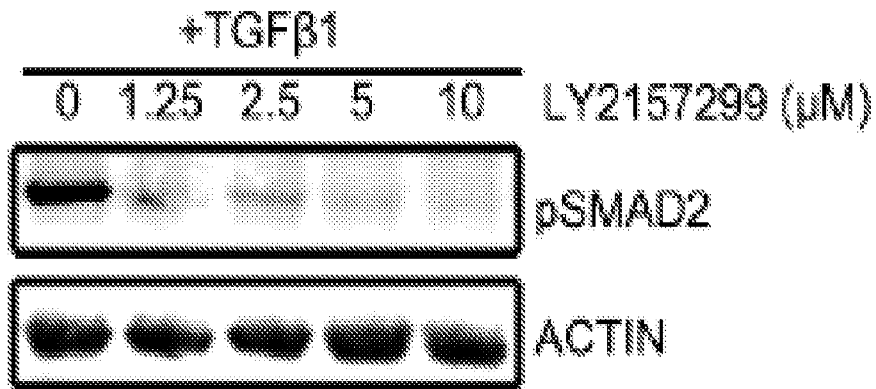
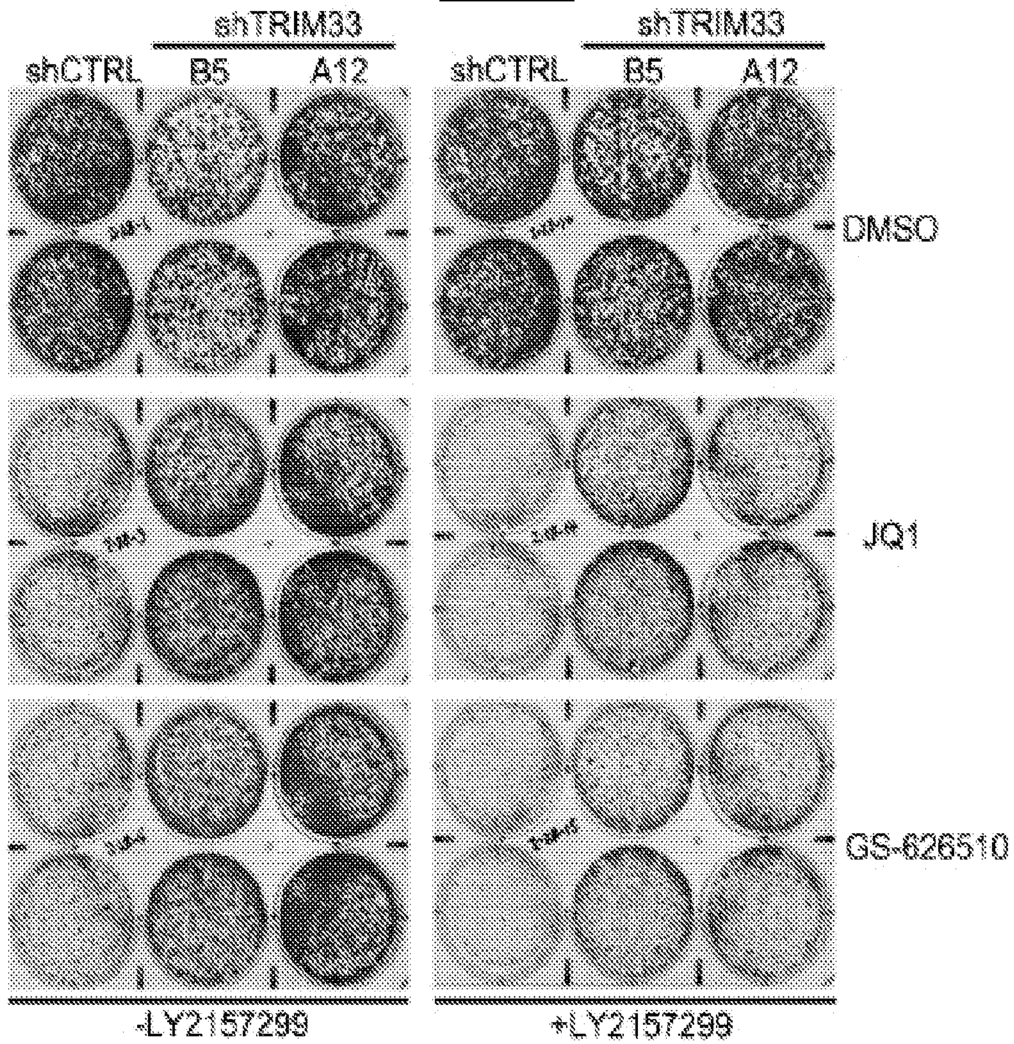


Figure 5Q



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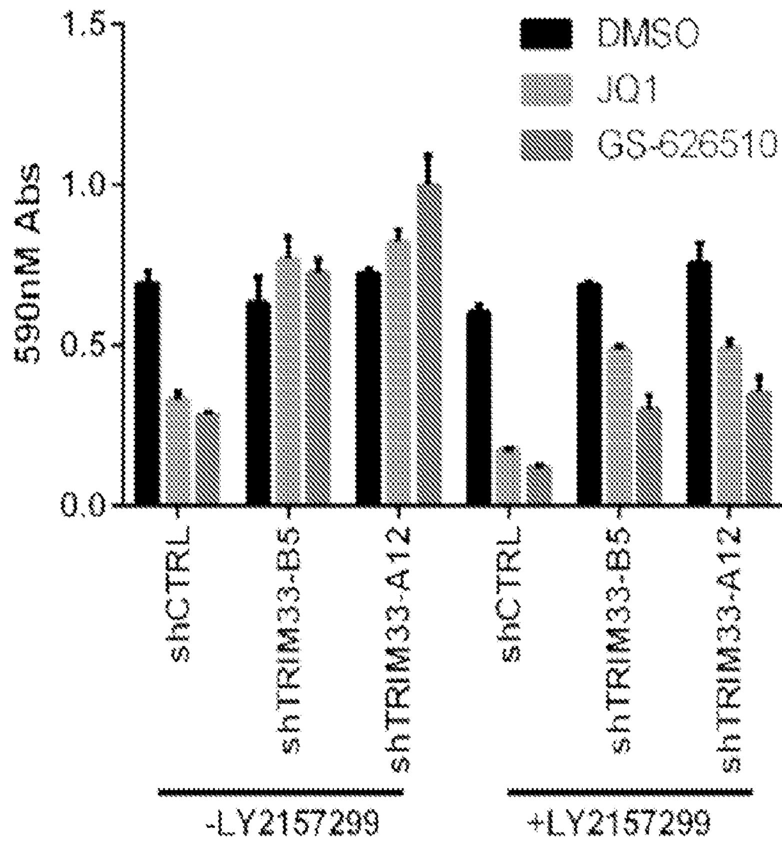

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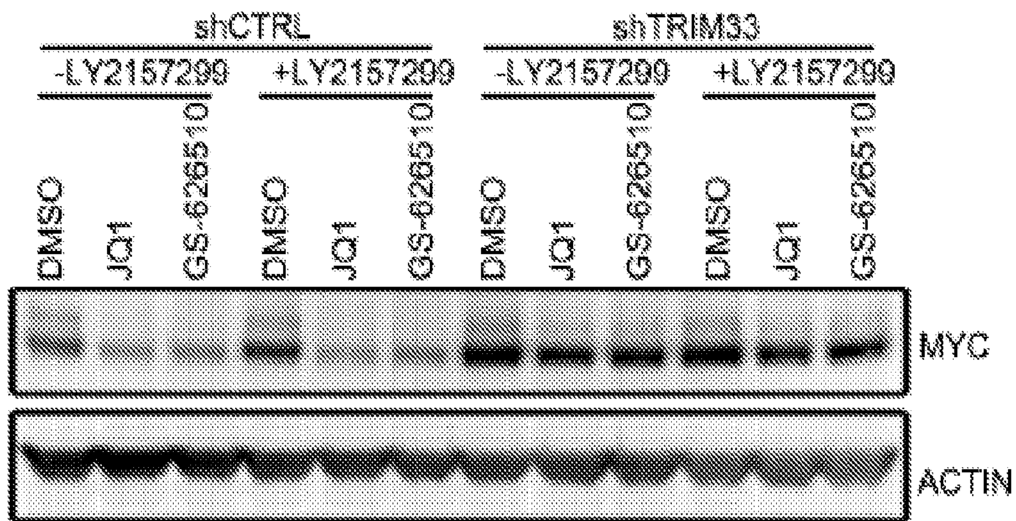

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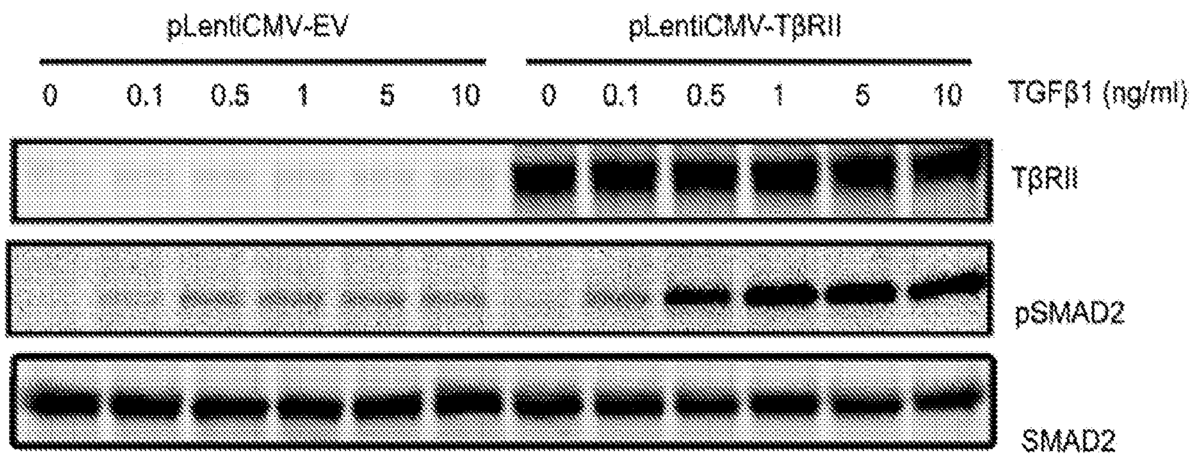

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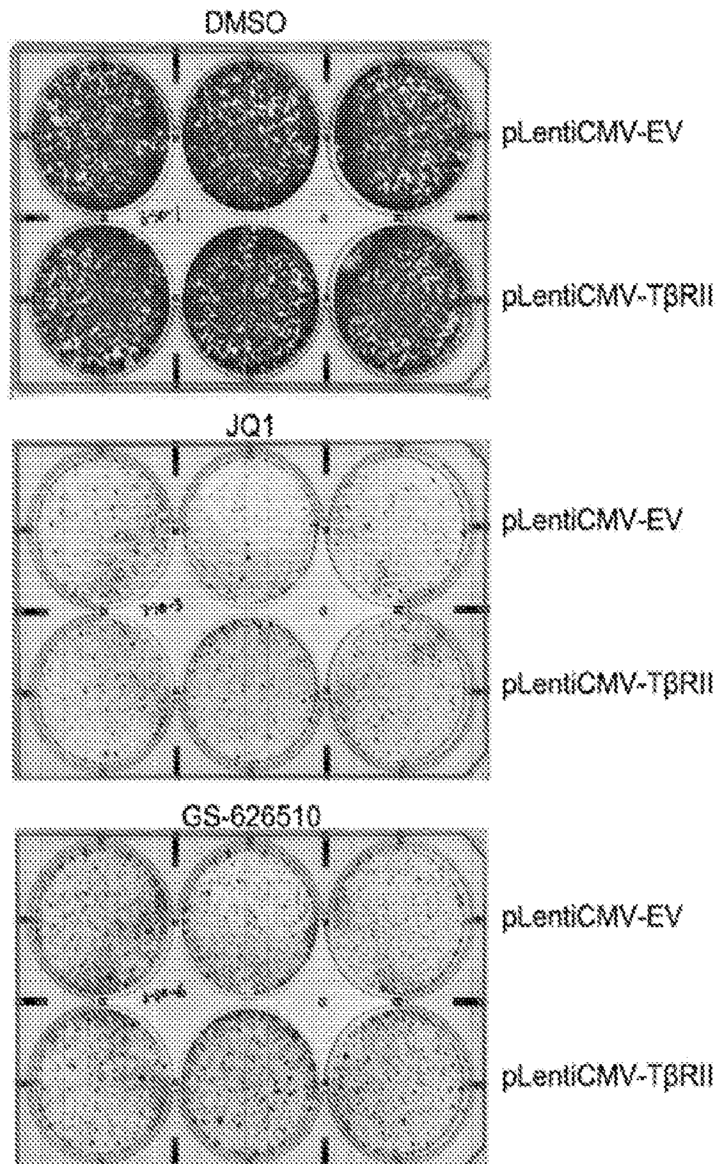

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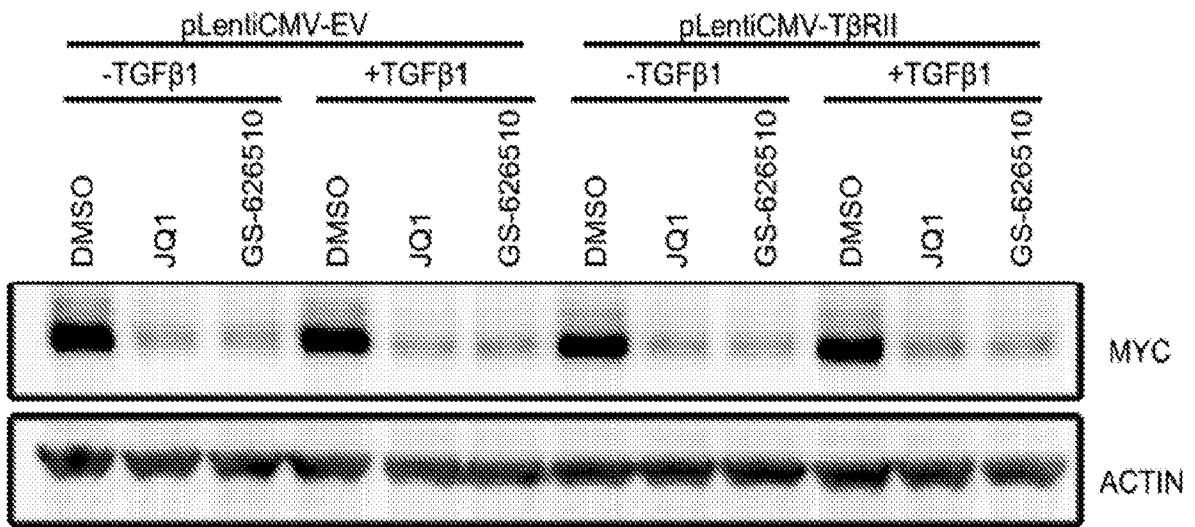

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/038669

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 41/02; A61K 31/437; A61K 31/4439; A61K 31/5377; A61K 38/00; A61K 39/395 (2017.01)

CPC - A61B 18/02; A61B 2018/00577; A61K 31/5377; A61K 45/06; C07D 471/04; G01N 33/5008 (2017.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/44A; 435/340; 514/234.5; 514/249 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0174138 A1 (THE BROAD INSTITUTE, INC. et al) 25 June 2015 (25.06.2015) entire document	1, 2, 5-8, 11-17, 19-22, 25-27
Y	LU et al. "Drug Delivery Using Nanoparticles for Cancer Stem-Like Cell Targeting," Frontiers in Pharmacology, 12 April 2016 (12.04.2016), Vol 7, Art. 84, Pgs. 1-12. entire document	1, 2, 4-8, 11-27
Y	FONG et al. "BET Inhibitor Resistance Emerges From Leukaemia Stem Cells," Nature, 24 September 2015 (24.09.2015), Vol. 525, No. 7570, Pgs. 538-542. entire document	3, 9, 10
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2017	22 SEP 2017

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/038669

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: