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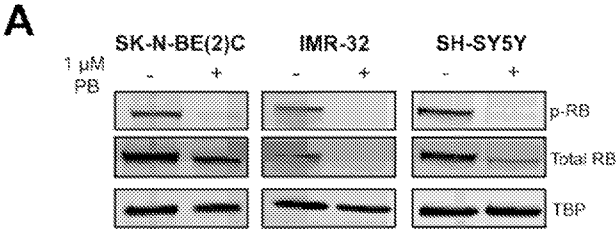
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(54) Title: TREATMENT OF NEUROBLASTOMA

Figure 1 A



(57) **Abstract:** The present invention relates to CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells and associated methods.

Treatment of Neuroblastoma

Field of Invention

- 5 The present invention relates to treatment of ADRN-type neuroblastoma and associated methods.

Background

- 10 Neuroblastoma is the most common extracranial solid tumour in infants, accounting for 15% of paediatric cancer deaths. In normal development, sympathoadrenal precursor cells derived from the neural crest differentiate into cell types including sympathetic neurons, adrenal chromaffin cells, and Schwann cells. In neuroblastoma these sympathetic precursor cells fail to differentiate and are locked into an immature state that drives tumour growth^{1,2}. However, a subset of
15 tumours can undergo spontaneous remission linked to tumour cell differentiation³⁻⁵. Neuroblastoma therefore presents a unique opportunity whereby differentiation therapies that reactivate normal developmental processes provide a promising therapeutic approach². The promise of differentiation therapies is exemplified by paediatric acute pro-myelocytic leukaemia where introduction of all-trans retinoic acid, a known differentiation-inducing agent, into
20 treatment regimens has dramatically improved complete remission rates^{6,7}. While retinoid treatments (namely 13-cis retinoic acid) are currently part of the treatment regimen for high-risk neuroblastoma, this treatment has varied efficacy in patients and is limited to maintenance therapy for minimal residual disease following aggressive chemo/radiotherapy⁸⁻¹¹. Novel, kinder therapies are therefore needed to treat this devastating childhood cancer and reactivating a
25 latent ability to undergo differentiation is a promising potential approach^{12,13}.

Summary of the Invention

- 30 The present invention provides a therapy for use in the treatment of neuroblastoma, in particular adrenergic-type neuroblastoma.

- While neuroblastomas have a low mutational burden, they have a strong epigenetic component; their pro-proliferative state and lineage identity is driven by a core regulatory circuitry (CRC) of transcription factors, themselves driven by clusters of enhancers often referred to as 'super-enhancers'^{14,15}. Neuroblastomas have been found to contain two cell types, namely adrenergic (ADRN) and mesenchymal (MES), that reflect different developmental stages of the sympathoadrenergic lineage¹⁵; and where each cell type is maintained by a distinct core regulatory circuitry and set of super-enhancers. While ADRN- and MES-type cells can spontaneously interconvert¹⁶, ADRN cells are the most tumorigenic^{15,17}. ADRN tumours,

encompassing both MYCN-amplified and non-amplified disease, are stalled in a noradrenergic sympathetic neuronal precursor state maintained by CRC transcription factors including ASCL1, PHOX2B, GATA3 and HAND2. If the correct cues can be identified, these tumours may be driven to re-enter a post-mitotic differentiated state.

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Therapeutically tipping the balance from proliferation to differentiation is likely to require: i) driving cell cycle exit, via downregulation of cell cycle genes and upregulation of CDK inhibitors, ii) driving upregulation of differentiation genes and, iii) resetting of the CRC gene network in favour of a stable differentiated state. Many ADRN neuroblastoma cell lines have shown neuronal differentiation *in vitro* in response to treatment with all-trans retinoic acid (herein referred to as RA, an active metabolite of 13-cis RA)^{18,19}, with RA recently shown to reset the CRC network in NMyC-driven neuroblastoma cells^{20,21}. Previous studies have also indicated that lengthening of the cell cycle in neuronal precursors promotes differentiation²²⁻²⁴. In addition, several preclinical studies suggest CDK inhibitors as promising treatments in neuroblastoma on the basis of their anti-proliferative functions^{8,25-28}. While clinical application of first generation pan-CDK inhibitors has generally proven unsuccessful in cancer therapeutic applications due to low specificity and off-target effects, several specific CDK inhibitors have now been developed²⁹. Palbociclib (PB), a CDK4/6 inhibitor (Ibrance®, Pfizer; PD-0332991) approved for front-line treatment of HR-positive and HER2-negative breast cancers in combination with endocrine therapy³⁰, has previously been shown to induce G1 arrest in neuroblastoma^{26,27,31}. Extensive overexpression of cyclin D-CDK4/6 components in neuroblastoma make this a particularly attractive therapeutic target³².

Herein the present inventors investigate CDK4/6 inhibitors as a clinically relevant therapy for neuroblastoma. The present inventors have demonstrated herein that CDK4/6 inhibitors reset the global transcriptional and epigenetic landscapes of ADRN-type neuroblastoma cells, resulting in a dual phenotypic endpoint that strongly favours neuronal differentiation whilst simultaneously decreasing proliferation. The inventors confirm CDK4/6 inhibitors are an effective agent resulting in significant survival benefit *in vivo* in mice. Additional features of an enduring post-mitotic differentiated state can be achieved in neuroblastoma cells by combining PB and RA, which act to significantly reset the oncogenic CRC in favour of differentiation. As such the present invention relates to a CDK4/6 inhibitor as a combinatorial therapy with retinoic acid, that could significantly improve neuroblastoma patient outcomes by re-engaging an anti-tumorigenic differentiation programme.

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An aspect of the invention relates to a CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.

An aspect of the invention relates to a CDK4/6 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises;

- screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
- 5 identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities,
- administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.

- 10 An aspect of the invention relates to a method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4/6 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic(ADRN)-type neuroblastoma cells.

- An aspect of the invention relates to a method of treating neuroblastoma in a subject, comprising;
- screening a biological sample obtained from said subject for expression of one or more
- 15 of MYCN, ALK and segmental chromosomal abnormalities,
- identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities,
- administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.

- 20 An aspect of the invention relates to a composition comprising;
- a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
- retinoic acid or a pharmaceutically acceptable salt thereof,,
- for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.

- 25 An aspect of the invention relates to a composition comprising;
- a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
- retinoic acid or a pharmaceutically acceptable salt thereof, and
- a pharmaceutically acceptable excipient.

- 30 An aspect of the invention relates to the use of a CDK4/6 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic cells.

- 35 An aspect of the invention relates to an *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising;
- contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4/6 inhibitor in combination with retinoic acid.

An aspect of the invention relates to an *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4/6 inhibitor in combination with retinoic acid comprising;

- 5 screening a biological sample obtained from a subject with neuroblastoma, for expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities, identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities, selecting said subject for therapy.

An aspect of the invention relates to a kit for the treatment of neuroblastoma comprising;

- 10 a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and retinoic acid or a pharmaceutically acceptable salt thereof, wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

An aspect of the invention relates to a method of prognosis or therapy monitoring comprising;

- 15 providing a biological sample obtained from a subject with neuroblastoma who has received treatment, screening said biological sample for one or more of the following features: increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1, changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or
20 MEIS1, or decreased expression of ASCL1 and/or HAND1.

An aspect of the invention relates to a method of prognosis or therapy monitoring comprising;

- 25 providing a biological sample obtained from a subject with neuroblastoma who has received treatment, screening said biological sample for alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, TrkA (NTRK1), and/or serum LDH level.

An aspect of the invention relates to a kit comprising a plurality of binding agents, wherein each
30 of said binding agents specifically binds to a distinct biomarker protein that is selectively upregulated or downregulated in neuroblastoma, wherein said biomarker proteins are selected from the group consisting of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, TrkA (NTRK1), and/or serum LDH.

35 **Figures**

Figure 1: Palbociclib drives cell cycle exit and differentiation in adrenergic neuroblastoma cells

(A.) Western blot analysis of phospho-RB and total RB protein levels in SK-N-BE(2)C, IMR-32 and SH-SY5Y cells, untreated and treated with 1 μ M palbociclib for 24h. TBP was used as a housekeeping loading control. (B-D) Representative fluorescent images of EdU incorporation following a 24h pulse in (B) SK-N-BE(2)C, (C) IMR-32 and (D) SH-SY5Y cells (pulse begun day 4 of 5 day treatment with vehicle (DMSO) or palbociclib (1 μ M)). Scale bar: 100 μ m. Analysis of % cells with EdU incorporation. n=3, mean $\pm$ SEM. ** $p \leq 0.01$, one-tailed paired t-test. DAPI nuclear counterstain (blue). (E-G) Crystal violet staining of (E) SK-N-BE(2)C, (F) IMR-32 and (G) SH-SY5Y cells treated with palbociclib or DMSO vehicle control for 5 days. Representative of n=3 experiments. Right-hand images show representative phase-contrast images prior to fixation. Arrows indicate examples of neurite extension. Scale bars: 2 mm and 100 μ m. (H-J) Immunocytochemistry analysis of TUBB3 (green) expression in (H) SK-N-BE(2)C, (I) IMR-32 and (J) SH-SY5Y and cells following 5 or 11 days of palbociclib treatment. Scale bar: 100 μ m. DAPI nuclear counterstain (blue).

Figure 2: Palbociclib drives transcriptional changes associated with differentiation of neuroblastoma

(A) Heatmap of all genes differentially expressed across the three cell lines for five biological replicates of control, 24h PB and 5/7 days PB samples. The z-score scaling for each gene has been applied internally per cell line. Clusters were assigned using k-means clustering on the scaled data from all cell lines. The number of genes in each cluster is indicated.

(B) Gene ontology analysis for cellular components using clusterProfiler conducted for each cluster of genes in (A). Terms are ordered by the adjusted p-value, the 10 most significant terms are shown.

Figure 3: Palbociclib rewires the epigenetic landscape to support differentiation.

(A) Heatmaps show the CPM normalised H3K27ac signal (average of five replicates) $\pm$ 2.5kb around the centre of H3K27ac broad peak loci. Differential H3K27ac marks between PB and control were determined using DiffBind and are grouped as increased ($\log_2FC > 0.5$ & $p_{adj} < 0.05$), no significant change ($p_{adj} > 0.5$) and decreased ($\log_2FC < -0.5$, $p_{adj} < 0.05$). Data shown for three cell lines: SK-N-BE(2)C in pink, IMR-32 in orange; and SH-SY5Y in blue. The number of increased, unchanged and decreased H3K27ac marks in each cell line are indicated at the side of each heatmap.

(B) Venn diagram shows the crossover of genes proximal (assigned using ChIPseeker) to H3K27ac marks that increase with PB treatment across cell lines, area is proportional to group size. The 10 most significant biological process gene ontology terms associated with the overlapping 1346 genes are shown.

(C) Venn diagram shows the crossover of genes proximal (assigned using ChIPseeker) to H3K27ac marks that decrease with PB treatment across cell lines, area is proportional to group size. The 10 most significant biological process gene ontology terms associated with the overlapping 518 genes are shown.

(D) Profiles of average normalised H3K27ac coverage (control in grey, PB in colour) across scaled super-enhancer regions. The super-enhancers are grouped for each cell line by how the total H3K27ac signal in the region changes with PB treatment: increased, sustained or decreased. The number of super-enhancers in each group are indicated.

- 5 (E) Gene ontology analysis of genes associated with super-enhancers that increase, maintain or decrease in H3K27ac signal after PB treatment in SK-N-BE(2)C, IMR-32 and SH-SY5Y cells. The top ten terms for each group are shown, there were no significant terms for IMR-32 decreased SEs.

Figure 4: Palbociclib inhibits tumour growth in *in vivo* mouse models of neuroblastoma

- 10 (A) Waterfall plot documenting relative changes in tumour volume at Day 7 in the Th-MYCN GEM model with vehicle or palbociclib at the indicated doses. Each line on the graph represents one mouse.

(B) Representative MRI sections of mice at day 1 and day 7 of treatment with vehicle or palbociclib (40 mg/kg). The white lines indicate the tumour circumference.

- 15 (C) Graph of relative body weight vs days elapsed after palbociclib treatment starts (n=4)

(D) Kaplan Meier survival curve of mice treated with palbociclib versus control untreated mice (n=4 for each condition). Log-rank (Mantel-Cox) test, $p = 0.0266$

(E) Graph of tumour size percentage relative to day 0 vs days after treatment starts (n=6 for each condition, control vehicle, or PB treatment (40 mg/kg)). Mixed two-way ANOVA, $p = 0.0064$.

- 20 (F) Graph of relative body weight vs days elapsed after palbociclib treatment starts (n=6).

Figure 5: Palbociclib and retinoic acid additively inhibit proliferation of neuroblastoma cells

- (A) CRC gene expression in RNA-seq data in three neuroblastoma cell lines. Data shown as fold enrichment of 5/7d PB treatment compared to control. Error bars reflect transformed log2 fold change standard error values from DESeq2 output. * indicates $p_{\text{adj}} < 0.05$ and $\log_2\text{FC} > 0.5$ or < -0.5 in DESeq2 output.

(B) Immunocytochemistry analysis of Ki67 (red) and TUBB3 (green) expression in untreated SK-N-BE(2)C cells and cells maintained in 1 μM PB. Representative of n=3 independent experiments. Scale bar: 100 μm .

- 30 (C) Crystal violet staining to visualise morphology of untreated SK-N-BE(2)C cells and cells maintained in 1 μM PB. Scale bar: 100 μm .

(D) (Left) Representative fluorescent images of EdU incorporation following a 24h pulse in untreated SK-N-BE(2)C cells and cells maintained in 1 μM PB. Scale bar: 100 μm . (Right) Analysis of % EdU positive cells. Mean $\pm$ SEM. n=3 for PB, where three independent 'cultures expanded in PB' were compared to the untreated control.

- 35 (E) Confluence analysis of untreated SK-N-BE(2)C cells and cells maintained in 1 μM PB. Mean $\pm$ SD. For untreated control n=6 technical replicates. For PB, n=3 where three independent 'cultures expanded in PB' were compared to the untreated control (each with n=6 technical replicates).

(F) (Upper) Crystal violet staining of SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days. Representative of n=4 experiments. Scale bar: 500 μ m. (Lower) Representative phase-contrast and crystal violet staining images SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days. Scale bars: 100 μ m. Data related for Figure 1E.

(G) Daily cell counts of SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 3 days. Total cell number shown as a percentage of cell number at Day 0 for each condition (n=4).

(H) Immunocytochemistry analysis of Ki67 (red) and TUBB3 (green) expression in SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days. Representative of n=4 biological replicates. Scale bar: 100 μ m.

(I) Representative fluorescent images of EdU incorporation following a 24h pulse in SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days (pulse began on day 4). Scale bar: 100 μ m.

(J) Quantification of % EdU positive cells following 24h pulse in SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days. n=4 biological replicates, Mean $\pm$ SEM. * $P \leq 0.05$; ** $P \leq 0.01$, repeated measures one-way ANOVA with Tukey's multiple comparison test. Data related to Figure 1B.

(K) Quantification of luminescence (RLU) from CellTiter-Glo[®] cell viability assay. SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days. n=4 biological replicates, Mean $\pm$ SEM. * $P \leq 0.05$; ** $P \leq 0.01$, repeated measures one-way ANOVA with Tukey's multiple comparison test.

Figure 6: PB+RA facilitate genome-wide changes favouring reduced proliferation and enhanced differentiation

(A) CRC gene expression in the RNA-seq data. RARA, RARB, SOX4 and MEIS1 constitute the retino-sympathetic CRC, all other genes are a part of the adrenergic CRC. Data shown as fold enrichment of treatment compared to DMSO control. Error bars reflect transformed log2 fold change standard error values from DESeq2 output. * indicates p.adj < 0.05 and log2FC > 0.5 or < -0.5 in DESeq2 output.

(B) Tracks show normalised H3K27ac signal (average of four biological replicates) at ASCL1 and HAND1 genes after treatment with DMSO, RA, PB or PB+RA.

(C) Heatmap of genes significantly differentially expressed in at least one comparison. A z-score has been applied to CPM normalised RNA-seq counts. Clusters were assigned using k-means clustering on the scaled data.

(D) Gene ontology analysis of cellular components for each of the five clusters identified in (C). The top 10 gene ontology terms for each group are shown.

(E) Significant differences in H3K27ac marks between conditions were determined using DiffBind and grouped based on how they change with RA, PB and PB+RA treatment. The boxplots show the log2 fold change in H3K27ac for these groups.

(F) Gene ontology analysis of cellular components for key upregulated H3K27ac mark groups shown in (D). The most proximal gene (within 100kb) was assigned to each H3K27ac mark. The top 10 gene ontology terms for each group are shown.

Figure 7: Dual PB+RA treatment promotes a transcriptional signature favouring patient survival and differentiation of tumour spheroids.

(A) Volcano plots show the change in gene expression in PB+RA compared to the control.

Genes with expression levels significantly associated with differential survival in neuroblastoma (determined using the R2 Genomics platform) are highlighted. Genes with high levels being correlated with poor survival are highlighted in orange, genes with low levels

being correlated with poor survival are highlighted in green.

(B) Transmission electron microscopy images of SK-N-BE(2)C cells treated with DMSO vehicle control, PB, RA or PB+RA for 5 days (representative of n=2 independent experiments, scale bars: 1 μ m). Higher magnification images for PB+RA condition show dense-core granules of 100-150 nm diameter and mitochondria within neurites, with examples marked by black arrows, scale bar = 500 nm.

(C) Percentage SK-N-BE(2)C spheroid area at Day 7 of treatment compared to Day 0. n=3, with n=12 spheroids per replicate, each represented by a single data point. *, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.001$; and ****, $P \leq 0.0001$, repeated-measures one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparison test.

(D) Representative phase-contrast images of SK-N-BE(2)C spheroids at Day 0 and Day 7 of treatment with DMSO (vehicle), PB, RA or PB+RA, at the same concentrations used throughout the manuscript. Scale bar = 400 μ m.

(E) Immunofluorescence images of tumour spheroids stained for TUBB3 (green) at Day 7 of treatment. Scale shown for each individual image. Higher magnification images shown with scale bars = 100 μ m.

(F) qRT-PCR analysis of *Ki67*, *E2F2* and *E2F8* expression levels in SK-N-BE(2)C spheroids treated with DMSO vehicle control, PB, RA or PB+RA for 7 days (~30 spheroids pooled, n=3). Mean +/- 95% CI. *, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.001$; and ****, $P \leq 0.0001$, repeated-measures one-way ANOVA with Tukey's multiple comparison test.

(G) qRT-PCR analysis of *STMN4* and *STMN2* expression levels in SK-N-BE(2)C spheroids treated with DMSO vehicle control, PB, RA or PB+RA for 7 days (~30 spheroids pooled, n=3). Mean +/- 95% CI. *, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.001$; and ****, $P \leq 0.0001$, repeated-measures one-way ANOVA with Tukey's multiple comparison test.

Figure 8. IC50 determination using cell confluency analysis from Incucyte® Live Imaging System. SK-N-BE(2)C cells were treated with a range of Abemaciclib or Ribociclib concentrations for 5 days. Calculated from n=3 biological replicates, with 4-6 technical replicates per dosage.

Figure 9. CDK4/6 inhibition enhances retinoic acid-induced differentiation in adherent SK-N-BE(2)C cells.

A) Representative phase-contrast images of SK-N-BE(2)C cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. Representative of $n = 3$ biological replicates. Scale bar: 50 μm .

B) Crystal violet staining of SK-N-BE(2)C cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. Representative of $n = 3$ biological replicates. Scale bar: 1 mm.

C) Immunocytochemistry analysis of TUBB3 (green) expression in SK-N-BE(2)C cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. Scale bar: 100 μm . DAPI nuclear counterstain (blue). Representative of $n = 3$ biological replicates.

D) Left: representative fluorescent images of EdU incorporation following a 24-h pulse in untreated SK-N-BE(2)C cells and cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. Scale bars: 100 μm . Right: analysis of % EdU-positive cells. Mean $\pm$ SD. $n = 2$ biological replicates, each in technical triplicate. $*p \leq 0.05$; $**p \leq 0.01$, $***p \leq 0.001$ one-way ANOVA with Tukey's multiple comparison test.

E) Confluence analysis SK-N-BE(2)C cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. Mean $\pm$ SD, $n=3$ biological replicates (each with $n=3$ technical replicates). Confluency presented as fold change of day 0 reading, where day 0 reading = 1. $*p \leq 0.05$; $**p \leq 0.01$, $***p \leq 0.001$ one-way ANOVA with Tukey's multiple comparison test at Day 5 timepoint.

F) qRT-PCR analysis of *STMN4*, *E2F8*, *PLK1* and *FOXM1* expression levels in SK-N-BE(2)C cells treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 5 days. $n = 3$ biological replicates. Mean $\pm$ 95% CI. $*p \leq 0.05$; $**p \leq 0.01$, $***p \leq 0.001$; and $****p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test. Selected comparisons shown for visibility.

Figure 10. CDK4/6 inhibition enhances retinoic acid-induced differentiation in 3D SK-N-BE(2)C spheroids.

A) Representative phase-contrast images of SK-N-BE(2)C spheroids at days 0 and 7 of treatment with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control, at the same concentrations used throughout the manuscript. Scale bar: 500 μm .

B) Percentage SK-N-BE(2)C spheroid area at day 7 of treatment compared with day 0. $n = 3$ biological replicates, with $n = 12$ spheroids per replicate, each represented by a single data point. $*p \leq 0.05$; $**p \leq 0.01$, $***p \leq 0.001$; and $****p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test.

C) Immunofluorescence images of tumour spheroids stained for TUBB3 (green) at day 7 of treatment. Scale shown for each individual image. Higher magnification images shown with scale bar: 100 μ m.

5 D) qRT-PCR analysis of *STMN4*, *E2F8*, *PLK1* and *FOXM1* expression levels in SK-N-BE(2)C spheroids treated with palbociclib, abemaciclib, ribociclib, RA or a combination of PB/ABE/RIBO and RA, or DMSO vehicle control for 7 days (~30 spheroids pooled, n = 3 biological replicates). Mean $\pm$ 95% confidence interval (CI). * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$; and **** $p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test.

10 Detailed Description of the Invention

The embodiments of the invention will now be further described. In the following passages, different embodiments are described. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary.

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Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, pathology, oncology, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Green and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012).

20

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Therapy

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The present invention relates to a CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.

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The present invention relates to a method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4/6 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.

Neuroblastoma is the most common extracranial solid tumour in infants, arising from developmentally stalled neural crest-derived cells. Neuroblastomas comprise two cells types firstly the adrenergic (ADRN)- type and the mesenchymal (MES)-type. ADRN-type neuroblastoma cells are the most tumorigenic. The present invention relates to the treatment of

subjects with neuroblastoma wherein said neuroblastoma comprises ADRN-type neuroblastoma cells. In an embodiment the treatment of neuroblastoma may comprise a step of identifying the subject as having neuroblastoma comprising ADRN-type neuroblastoma cells.

5 In an embodiment the ADRN-type neuroblastoma cells are characterised by one or more of expression of MYCN, ALK, NTRK and/or the presence of segmental chromosomal abnormalities. MYCN (ENSG00000134323, uniprot P04198) is the gene that encodes that N-myc proto-oncogene, amplification of MYCN is associated with a number of tumours including neuroblastoma. ALK (ENSG00000171094, uniprot Q9UM73) is the gene which encodes ALK
10 receptor tyrosine kinase which plays an important role in the development of the brain and exerts its effects on specific neurons in the nervous system. ALK has been found to be rearranged, mutated, or amplified in a series of tumours including anaplastic large cell lymphomas, neuroblastoma, and non-small cell lung cancer. NTRK1 (TrkA) (ENSG00000198400, uniprot P04629) is a gene which encodes neurotrophic receptor tyrosine kinase 1. Characterisation of
15 neuroblastoma cells may comprise screening for expression of MYCN, NTRK and/or ALK, wherein expression above a certain threshold is indicative of ADRN-type neuroblastoma. Characterisation of neuroblastoma cells may comprise screening for specific mutations or aberrations within MYCN, NTRK and/or ALK, wherein mutations or aberrations are indicative of ADRN-type neuroblastoma. Characterisation of neuroblastoma cells may comprise identifying
20 the presence of segmental chromosomal abnormalities, such as deletions of chromosome arms 1p (1p del) and 11q (11q del) and/or gains in chromosome 17q (17q gain). In some embodiments characterisation of neuroblastoma cells comprises identifying the presence of one or more of deletions of chromosome arms 1p (1p del) and 11q (11q del) and/or gains in chromosome 17q (17q gain).

25 In an embodiment the ADRN-type neuroblastoma cells are characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2. ASCL1 (ENSG00000139352, uniprot P50553) is a gene which encodes Achaete-scute homolog 1. PHOX2B (ENSG00000109132, uniprot Q99453) is a gene which encodes Paired-like homeobox 2b. GATA3
30 (ENSG00000107485, uniprot P23771) is a gene which encodes GATA3 transcription factor. HAND2 is a gene which encodes heart- and neural crest derivatives-expressed protein 2. In an embodiment the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2 wherein the neuroblastoma cells have already been characterised by one or more of MYCN, ALK and segmental chromosomal
35 abnormalities. Various combinations of the markers disclosed herein may be used to characterise ADRN-type neuroblastoma cells for example one or more of MYCN, ALK, segmental chromosomal abnormalities, ASCL1, PHOX2B, GATA3, HAND2 or any combination thereof may be used to characterise ADRN-type neuroblastoma cells.

ADRN-type neuroblastoma may be characterised by certain markers as discussed as such the method of therapy may involve a step of identifying whether a subject has ADRN-type neuroblastoma. As such an aspect of the present invention relates to a CDK4/6 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises;

- 5 screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK, NTRK and/or the presence of segmental chromosomal abnormalities, identifying said subject as having high risk neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.

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In an embodiment, the step of identifying whether a subject has high risk neuroblastoma may comprise screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK, NTRK, segmental chromosomal abnormalities, ASCL1, PHOX2B, GATA3, HAND2, or any combination thereof. In an embodiment, the step of identifying whether a subject
15 has high risk neuroblastoma may comprise screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK, NTRK, segmental chromosomal abnormalities, or any combination thereof. In an embodiment the step of identifying whether a subject has high risk neuroblastoma may comprise screening a biological sample obtained from a subject for expression of one or more ASCL1, PHOX2B, GATA3, HAND2, or any combination
20 thereof,

An aspect of the present invention relates to a method of treating neuroblastoma in a subject, comprising;

- 25 screening a biological sample obtained from said subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, identifying said subject as having high risk neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.

- 30 The method of treating neuroblastoma may comprise a step of identifying the type of neuroblastoma that a subject has e.g., whether the neuroblastoma is high risk, intermediate risk, ADRN-type etc. The step of identifying the type of neuroblastoma may comprise obtaining a biological sample from said subject in order to screen said sample. The biological sample may be tissue, saliva, urine, blood including whole blood and plasma. In order to identify the type of
35 neuroblastoma present, a biological sample will be screened for expression of one or more of MYCN, NTRK ALK and segmental chromosomal abnormalities. Further markers or features may also be used to identify the type of neuroblastoma such as expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.

In an embodiment identifying ADRN-type neuroblastoma comprises screening for expression of MYCN, NTRK and/or ALK, wherein expression above a certain threshold is indicative of ADRN-type neuroblastoma. Identification of a high-risk or ADRN-type neuroblastoma may comprise screening for specific mutations or aberrations within MYCN, NTRK and/or ALK, wherein
5 mutations or aberrations are indicative of ADRN-type neuroblastoma. Identification of a high-risk or ADRN-type neuroblastoma may comprise identifying the presence of segmental chromosomal abnormalities, such as deletions of chromosome arms 1p (1p del) and 11q (11q del) and gains in chromosome 17q (17q gain).

- 10 An aspect of the present invention relates to use of a CDK4/6 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

The term “ADRN-type neuroblastoma” as used herein may refer to high risk ADRN-type
15 neuroblastomas and/or intermediate risk ADRN-type neuroblastomas. ADRN-type neuroblastomas are generally considered to have poor prognosis. In an embodiment the neuroblastoma is selected from high risk MYCN-driven neuroblastoma, MYCN-amplified neuroblastoma, non-amplified neuroblastoma, ADRN-type high risk tumours, ADRN-type intermediate risk tumours, MYCN-amplified ADRN-type high risk tumours, MYCN-amplified
20 ADRN-type intermediate risk tumours, ALK-mutated ADRN-type high risk tumours, ALK-mutated ADRN-type intermediate risk tumours. In an embodiment high risk neuroblastoma includes ADRN-type neuroblastoma.

The term “high risk neuroblastoma” as used herein may refer to high risk ADRN-type
25 neuroblastoma, high risk MYCN-driven neuroblastoma, MYCN-amplified neuroblastoma, non-amplified neuroblastoma, ADRN-type high risk tumours, MYCN-amplified ADRN-type high risk tumours, ALK-mutated ADRN-type high risk tumours. In an embodiment high risk neuroblastoma includes high risk ADRN-type neuroblastoma.

30 In an embodiment the neuroblastoma may be relapsed or refractory disease, such as primary refractory or relapsed high risk-neuroblastoma, or from minimal residual disease in high-risk neuroblastoma. Both relapsed and refractory neuroblastomas are generally considered high risk disease. The term “relapsed neuroblastoma” refers to neuroblastoma that has returned during or after initial therapy. The term “refractory neuroblastoma” refers to neuroblastoma that does not
35 respond to initial treatment. The initial therapy may include treatment with chemotherapy, surgery, ‘high-dose’ chemotherapy, stem cell rescue, radiotherapy, immunotherapy therapy and/or retinoic acid therapy.

Where a subject is identified as having relapsed or refractory neuroblastoma they may be selected for treatment with a CDK4/6 inhibitor in combination with retinoic acid. In an embodiment there is provided a CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma is relapsed or refractory neuroblastoma. In an embodiment there is provided a CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises ADRN-type neuroblastoma cells and the neuroblastoma is relapsed or refractory neuroblastoma.

In an embodiment the subject may be selected for therapy with a CDK4/6 inhibitor in combination with retinoic acid, wherein the subject has been unresponsive or shown a low response to treatment with retinoic acid alone.

In some embodiments additional symptoms may indicate high risk and/or ADRN-type neuroblastoma. For example, in some cases neuroblastomas can be secretory and release adrenergic hormones leading to elevated catecholamines, in some cases sympathetic symptoms such as diarrhoea, flushing, tachycardia, which are indicative of ADRN-type neuroblastoma. Therefore, in some embodiments high risk or ADRN-type neuroblastoma is characterised by one or more of elevated catecholamines, diarrhoea, flushing, tachycardia, in combination with expression of one or more of MYCN, ALK and segmental chromosomal abnormalities. Other factors may also be used to identify high risk subject, for example the age of the subject may be a risk factor. Subjects older than 12 months to 18 months may be classed as high risk subjects. As such the age of the subject may be a criteria for selecting a subject for therapy according to the present invention.

In an embodiment the method of treating neuroblastoma comprises a step of identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities.

In an embodiment the CDK4/6 inhibitor is for use in the treatment of neuroblastoma in combination with retinoic acid, wherein the neuroblastoma has been shown to be unresponsive or have low response to retinoic acid alone. In an embodiment the neuroblastoma is refractory and the neuroblastoma has been shown to be unresponsive or have low response to initial therapy selected from one or more of chemotherapy, surgery, 'high-dose' chemotherapy, stem cell rescue, radiotherapy, immunotherapy therapy and/or retinoic acid therapy.

CDK4/6 inhibitor for use in the present invention are compounds which target cyclin dependent kinases 4 and/or 6 (CDK4/6). CDK4/6 are found both in healthy cells and cancer cells, wherein

they control how quickly cells grow and divide. CDK4/6 inhibitors which are suitable for use in the present invention include any compound, including small molecules or biologics, that targets and inhibits CDK4 and/or CDK6. The CDK4/6 inhibitors which may be used in the present invention may target CDK4 and/or CDK6. The CDK4/6 inhibitor may have a stronger effect, or
5 potency, on one of CDK4 or CDK6. In some embodiments the CDK4/6 inhibitor has a stronger effect, or potency, on CDK4 than CDK6. Examples of CDK4/6 inhibitors which exert a stronger effect, or potency on CDK4 than CDK6 include but are not limited to abemaciclib and ribociclib. In some embodiments the CDK4/6 inhibitor has a similar effect, or potency, on CDK4 and CDK6. Examples of CDK4/6 inhibitors which exert a similar effect, or potency, on CDK4 and CDK6
10 include but are not limited to palbociclib. Compounds which inhibit CDK4 and/or CDK6 can be identified using standard methods known to the skilled person. Examples of CDK4/6 inhibitors which are suitable for the present invention include but are not limited to palbociclib (also known as PD-033299, Ibrance), ribociclib (also known as LEE011, Kisqali and Kryxana), abemaciclib (LY2835219, Verzenio), and/or analogs thereof. The CDK4/6 inhibitor may comprise one of
15 palbociclib, ribociclib or abemaciclib or a combination thereof. In an embodiment the CDK4/6 inhibitor is palbociclib or an analog thereof. In an embodiment the CDK4/6 inhibitor is ribociclib or an analog thereof. In an embodiment the CDK4/6 inhibitor is abemaciclib or an analog thereof.

In an embodiment the CDK4/6 inhibitor is a selective CDK4/6 inhibitor, a selective CDK4/6
20 inhibitor refers to an inhibitor that specifically targets CDK4 and CDK6 i.e., the inhibitor does not target one of the other cyclin dependent kinases, for example CDK 1, 2, 3, 5, 7, or 9. In an embodiment the CDK4/6 inhibitor is a non-selective CDK4/6 inhibitor, a non-selective CDK4/6 inhibitor refers to an inhibitor that targets CDK4 and/or CDK6 but may also target one of the other cyclin dependent kinases, for example CDK 1, 2, 3, 5, 7, or 9. Abemaciclib may be an
25 example of a non-selective CDK 4/6 inhibitor.

The present invention also relates to the use of a CDK4 inhibitor in any of the aspects or embodiments set out herein. In particular the invention relates to a CDK4 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4 inhibitor is administered in combination with
30 retinoic acid and wherein said neuroblastoma comprises ADRN-type neuroblastoma cells. The invention relates to a method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises ADRN-type neuroblastoma cells. In an embodiment the CDK4 inhibitor is a selective CDK4 inhibitor, a selective CDK4 inhibitor refers to an inhibitor that specifically targets CDK4
35 i.e., the inhibitor does not target one of the other cyclin dependent kinases, for example CDK 1, 2, 3, 5, 6, 7 or 9. In an embodiment the CDK4 inhibitor is a non-selective CDK4 inhibitor, a non-selective CDK4 inhibitor refers to an inhibitor that targets CDK4 but may also target one of the other cyclin dependent kinases, for example CDK 1, 2, 3, 5, 6, 7, or 9.

In the present invention, the CDK4/6 inhibitor may be administered at a dose of between 0.1 to 200 mg/kg, 0.1 to 150 mg/kg, 0.1 to 100 mg/kg, 0.1 to 80 mg/kg, 0.1 to 60 mg/kg, 0.1 to 40 mg/kg, 0.1 to 20 mg/kg, 1 to 100 mg/kg, 1 to 80 mg/kg, 1 to 60 mg/kg, 1 to 40 mg/kg, 5 to 100 mg/kg, 5 to 80 mg/kg, 5 to 60 mg/kg, 5 to 40 mg/kg, preferably 20 to 60mg/kg, more preferably approximately 40 mg/kg. In some embodiments the CDK4/6 inhibitor may be administered once daily. In some embodiments the CDK4/6 inhibitor may be administered twice daily. In some embodiments where the CDK4/6 inhibitor is selected from palbociclib or ribociclib, the CDK4/6 inhibitor may be administered once daily. In some embodiments where the CDK4/6 inhibitor is abemaciclib, the CDK4/6 inhibitor may be administered twice daily. The skilled person will be able to determine a suitable dose regimen for the therapeutics based on which therapeutic is used and the intended patient, for example there may be differences in the dosage strategy depending on formulation of the therapeutic.

The present inventors have shown herein that it may be advantageous to administer the CDK4/6 inhibitor at a dose that inhibits cell division i.e. a dose that is cytostatic. In an embodiment the CDK4/6 inhibitor may be administered at a dose that is cytostatic for example the dose is sufficient to inhibit cell division. In some embodiments it may be advantageous to use a dose of the CDK4/6 inhibitor that is not sufficient to induce cell death i.e. a dose that that is not cytotoxic. In some embodiments the CDK4/6 inhibitor is administered at a dose that is cytostatic but not cytotoxic. The skilled person will be able to determine a suitable cytostatic dose using standard methods known in the art. For example a cell based assay could be used to determine a dose that is sufficient to inhibit cell division.

The CDK4/6 inhibitor may have an IC₅₀ for CDK4 that is the same or different to the IC₅₀ for CDK6. In an embodiment the CDK4/6 inhibitor has an IC₅₀ for CDK4 in the range of 1 to 50 nM, 1 to 40 nM, 1 to 30 nM, 1 to 20 nM, 3 to 18 nM, 4 to 17 nM, 5 to 16 nM, 6 to 15 nM, 7 to 14 nM, 8 to 13 nM or 9 to 12 nM, wherein the IC₅₀ is determined in a cell free assay. The IC₅₀ for CDK4 may be approximately 11nM for example between 5 and 15 nM. In an embodiment the CDK4/6 inhibitor has an IC₅₀ for CDK6 in the range of 1 to 50 nM, 1 to 40 nM, 1 to 30 nM, 2 to 29 nM, 3 to 28 nM, 4 to 27nM, 5 to 26 nM, 6 to 25 nM, 7 to 24 nM, 8 to 23 nM, 9 to 22 nM, 10 to 21 nM, 11 to 20 nM, 12 to 19 nM, 13 to 18 nM, 14 to 17 nM wherein the IC₅₀ is determined in a cell free assay. The IC₅₀ for CDK6 may be approximately 16 nM for example between 5 and 25 nM.

In an embodiment the CDK4/6 inhibitor may have an IC₅₀ for CDK4 and/or CDK6 in the range of 0.1 to 10μM wherein the IC₅₀ is determined *in vitro* in a cell based assay, for example the cell based assay described herein in Example 1 wherein the IC₅₀ is determined in terms of cell viability and/or cell proliferation. The CDK4/6 inhibitor may have an IC₅₀ for CDK4 and/or CDK6 between 0.2 to 9 μM, 0.3 to 8 μM, 0.4 to 7 μM, 0.5 to 6 μM, 0.6 to 5 μM, 0.7 to 4 μM, 0.8 to 3 μM, 0.9 to 2 μM. The CDK4/6 inhibitor may have an IC₅₀ for CDK4 and/or CDK6 of

approximately 1 μM , for example 0.5 to 5 μM wherein the IC_{50} is determined *in vitro* in a cell based assay. In an embodiment the CDK4/6 inhibitor may be palbociclib or an analog thereof and have a IC_{50} for CDK4 and/or CDK6 in the range of 0.1 to 10 μM wherein the IC_{50} is determined *in vitro* in a cell based assay, for example the cell based assay described herein in

5 Example 1 wherein the IC_{50} is determined in terms of cell viability and/or cell proliferation.

In an embodiment the CDK4/6 inhibitor may have an IC_{50} for CDK4 and/or CDK6 in the range of 150 to 450 nM wherein the IC_{50} is determined *in vitro* in a cell based assay wherein the IC_{50} is determined in terms of cell viability and/or cell proliferation. The CDK4/6 inhibitor may have

10 an IC_{50} in the range of 150 to 450 nM, 175 to 425 nM, 200 to 400 nM, 225 to 375 nM, 250 to 350 nM, 275 to 325 nM, The CDK4/6 inhibitor may have an IC_{50} for CDK4 and/or CDK6 of approximately 307 nM, for example between 250 to 350 nM wherein the IC_{50} is determined *in vitro* in a cell based assay. In an embodiment the CDK4/6 inhibitor may be Riboociclib or an analog thereof and have a IC_{50} for CDK4 and/or CDK6 in the range of 150 to 450 nM wherein

15 the IC_{50} is determined *in vitro* in a cell based assay, wherein the IC_{50} is determined in terms of cell viability and/or cell proliferation.

Retinoic acid is a derivative of vitamin A it has been shown to have an effect on the cell growth and differentiation in neuroblastoma. The present invention combines the use of CDK4/6

20 inhibitor with retinoic acid for a more effective therapy, wherein a synergistic or additive effect is observed in terms of cell differentiation and reduction of tumour growth. The retinoic acid that is used in the present invention may be selected from cis-retinoic acid or trans-retinoic acid. The term "cis-retinoic acid" includes 13-cis-retinoic acid (isotretinoin). The term "trans-retinoic acid" includes all-trans-retinoic acid (ATRA). The term "retinoic acid" as used herein may also refer to

25 other retinoid derivatives including fenretinide (N - (4-hydroxyphenyl)retinamide; 4-HPR), a synthetic retinoid derivative. In a preferred embodiment the retinoic acid is 13-cis-retinoic acid.

In an embodiment the retinoic acid is administered at a dose of 10 to 500 $\text{mg}/\text{m}^2/\text{day}$, 10 to 450 $\text{mg}/\text{m}^2/\text{day}$, 10 to 400 $\text{mg}/\text{m}^2/\text{day}$, 10 to 350 $\text{mg}/\text{m}^2/\text{day}$, 10 to 300 $\text{mg}/\text{m}^2/\text{day}$, 10 to 250

30 $\text{mg}/\text{m}^2/\text{day}$, 50 to 500 $\text{mg}/\text{m}^2/\text{day}$, 50 to 450 $\text{mg}/\text{m}^2/\text{day}$, 50 to 400 $\text{mg}/\text{m}^2/\text{day}$, 50 to 350 $\text{mg}/\text{m}^2/\text{day}$, 50 to 300 $\text{mg}/\text{m}^2/\text{day}$, 50 to 250 $\text{mg}/\text{m}^2/\text{day}$, 100 to 500 $\text{mg}/\text{m}^2/\text{day}$, 100 to 400 $\text{mg}/\text{m}^2/\text{day}$, 100 to 300 $\text{mg}/\text{m}^2/\text{day}$ or 100 to 200 $\text{mg}/\text{m}^2/\text{day}$.

The present invention relates to the combination of a CDK4/6 inhibitor and retinoic acid in the

35 treatment of neuroblastoma. The term combination, as used herein refers to wherein the CDK4/6 inhibitor is administered simultaneously, sequentially or separately with the retinoic acid. Simultaneous indicates that the CDK4/6 inhibitor and retinoic acid are administered at the same time. Sequentially indicates that the CDK4/6 inhibitor and retinoic acid are administered one after another. Separately indicates that the CDK4/6 inhibitor and retinoic acid are administered

at separate intervals, wherein there is a time period in between the administrations either of between 1 minute to 14 days, but wherein the CDK4/6 inhibitor and retinoic acid are administered within the same dosage cycle or treatment cycle. In an embodiment the treatment cycle is between 7 and 42 days, 14 and 42 days, 21 and 42 days, approximate 35 days. The

5 CDK4/6 inhibitor and retinoic acid may be administered simultaneously at any point within the treatment cycle. The CDK4/6 inhibitor and retinoic acid may be administered sequentially at any point within the treatment cycle. The CDK4/6 inhibitor and retinoic acid may be administered separately at any point within the treatment cycle. In an embodiment, where separate administration is used CDK4/6 inhibitor and retinoic acid are administered 1 minute to 42 days

10 apart, 1 minute to 38 days, 1 minute to 35 days, 1 minute to 30 days, 1 minute to 25 days, 1 minute to 20 days, 1 minute to 15 days, 1 minute to 11 days, 1 minute to 10 days, 1 minute to 7 days, 1 minute to 5 days, 1 minute to 4 days, 1 minute to 3 days, 1 minute to 2 days, 1 minute to 1 day apart. In an embodiment, where separate administration is used CDK4/6 inhibitor and retinoic acid are administered 1 minute to 24 hours, 1 minute to 22 hours, 1 minute to 20 hours,

15 1 minute to 18 hours, 1 minute to 16 hours, 1 minute to 14 hours, 1 minute to 12 hours, 1 minute to 10 hours, 1 minute to 8 hours, 1 minute to 6 hours, 1 minute to 4 hours, 1 minute to 2 hours, 1 minute to 1 hours apart. Accordingly, the invention relates to a CDK4/6 inhibitor and retinoic acid for use in combination, either simultaneously, sequentially or separately, in the treatment of neuroblastoma, wherein said neuroblastoma comprises ADRN-type neuroblastoma

20 cells. Accordingly, the invention also relates to a method of treatment for neuroblastoma comprising administering to the subject a CDK4/6 inhibitor in combination, either simultaneously, sequentially or separately, with retinoic acid, wherein said neuroblastoma comprises ADRN-type neuroblastoma cells.

25 In an embodiment the combination of the CDK4/6 inhibitor and retinoic acid results in an additive effect, or a more than additive effect for example a synergistic effect. A combination therapy is defined as affording an "additive effect", "synergistic effect" or a "synergistic treatment" if the effect is therapeutically superior, as measured by, for example, the extent of the response (e.g. apoptosis or cell viability), the response rate, the time to disease progression or the survival

30 period, to that achievable on dosing one or other of the components of the combination therapy at its conventional dose. For example, the effect of the combination therapy is additive if the effect is therapeutically superior to the effect achievable with said CDK4/6 inhibitor and said retinoic acid alone. For example, the effect of the combination treatment may be synergistic if the effect of the combination treatment is greater than the effect of the individual treatments added together. Further, the effect of the combination is beneficial (e.g. additive or synergistic)

35 if a beneficial effect is obtained in a group of subjects that does not respond (or responds poorly) to one of the therapies alone. In addition, the effect of the combination treatment is defined as affording a benefit (e.g. additive or synergistic effect) if one of the components is dosed at its conventional dose and the other component is dosed at a reduced dose and the therapeutic

effect, as measured by, for example, the extent of the response, the response rate, the time to disease progression or the survival period, is equivalent to or better than that achievable on dosing conventional amounts of either one of the components of the combination treatment.

- 5 In an embodiment the combination of the isolated binding molecule described herein and a checkpoint inhibitor results in an improved or synergistic effect in one or more of the following:
- (a) upregulating cell differentiation markers;
 - (b) downregulating cell proliferation markers;
 - (c) increasing neuronal differentiation of ADRN-type neuronal cells;
 - 10 (d) inducing epigenetic changes which promote differentiation;
 - (e) increasing neurite formation;
 - (f) decreasing expression of proliferative genes including but not limited to Ki67, E2F2, E2F8, PLK1 and/or FOXM1;
 - (g) increasing expression of retino-sympathetic CRC genes including but not limited to
 - 15 RARA, RARB, SOX4 and MEIS1;
 - (h) increasing expression of genes associated with the neuronal cell body as well as extracellular matrix cellular components;
 - (i) reduction in tumour size as measured in vitro or in vivo, for example measured in vitro by a tumour spheroid model,
 - 20 (j) induction of differentiation as measured in vitro by a tumour spheroid model, and/or
 - (k) reduction in tumour size as measured in vitro or in vivo in a patient derived xenograft model.

The therapy of the present invention comprises a CDK4/6 inhibitor and retinoic acid which may
 25 be used in combination with a further anti-cancer therapy. The further anti-cancer therapy is selected from chemotherapy, radiotherapy, surgery, immunotherapy, hormone therapy myeloablative therapy (MAT) and/or autologous stem cell rescue (ASCR).

In an embodiment the CDK4/6 inhibitor and retinoic acid of the present invention is used in
 30 combination with chemotherapy. The chemotherapy agent may be selected from one or more of the following agents cyclophosphamide, cisplatin, carboplatin, vincristine, doxorubicin (adriamycin), etoposide, topotecan, melphalan, busulfan, thiotepa, or a combination thereof.

In an embodiment the CDK4/6 inhibitor and retinoic acid of the present invention is used in
 35 combination with immunotherapy, wherein the immunotherapy comprises treatment with an anti-GD2 antibody. Suitable anti-GD2 antibodies include ch14.18/CHO or ch14.18/SP2/0. ch14.18/CHO is also known as Dinutuximab beta. ch14.18/SP2/0 is also known as Dinutuximab. In embodiments the immunotherapy may be administered in combination with a retinoid, for example cis-retinoic acid.. In embodiments where immunotherapy is used, cytokine treatment

may also be administered. The term "cytokines" as used herein refers to proteins, peptides, or glycoproteins which act as hormonal regulators or signalling molecules at nanomolar to picomolar concentrations and help in cell signalling. In an embodiment, the one or more cytokines are selected from immunomodulating agents, such as e.g. interleukins and/or interferons. In an embodiment, the one or more cytokines are selected from the group consisting of IL-2, GM-CSF, Granulocyte colony-stimulating factor (G-CSF), IL-12, and/or IL-15. In an embodiment cytokine treatment comprises treatment with IL-2, GM-CSF. In an embodiment where immunotherapy is used the immunotherapy may be provided as a fusion protein comprising an antibody and a cytokine. In one embodiment the fusion protein comprises an anti-GD-2 antibody and IL-2. In an embodiment the immunotherapy may be administered as a continuous or discontinuous infusion.

Where a further anticancer therapy is used the further anticancer therapy may be administered, prior to, simultaneously with or after administration of the CDK4/6 inhibitor and retinoic acid. In an embodiment the subject has received one or more anti-cancer agents prior to the administration of the CDK4/6 inhibitor.

An aspect of the present invention relates to a composition comprising;
a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.

As will be appreciated by the skilled person, the terms "treating", "treats" and "treatment" include both preventative and curative treatment of a condition, disease or disorder. These terms also include slowing, interrupting, controlling or stopping the progression of a condition, disease or disorder and preventing, curing, slowing, interrupting, controlling or stopping the symptoms of a condition, disease or disorder. As used herein, "treat", "treating" or "treatment" means inhibiting or relieving a disease or disease. For example, treatment can include a postponement of development of the symptoms associated with a disease or disease, and/or a reduction in the severity of such symptoms that will, or are expected, to develop with said disease. The terms include ameliorating existing symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result is being conferred on at least some of the mammals, e.g., human patients, being treated. Many medical treatments are effective for some, but not all, patients that undergo the treatment. In an embodiment the therapy treats a subject by enhancing differentiation of cells and/or reducing proliferation of cells.

The term "subject" or "patient" refers to an animal or human which is the object of treatment, observation, or experiment.

5 The therapeutically effective amount of the CDK4/6 inhibitor and the retinoic acid or a pharmaceutical composition of one or both of the CDK4/6 inhibitor and the retinoic acid may be administered orally, topically, by inhalation, insufflation or parenterally. As the skilled person will appreciate, the CDK4/6 inhibitor and retinoic acid, may be formulated, for example in a pharmaceutical composition as described herein. The CDK4/6 inhibitor and retinoic acid may be formulated for a particular administration route. For example, formulations suitable for oral
10 administration include tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs. Suitable formulations for topical use include, for example, creams, ointments, gels, or aqueous or oily solutions or suspensions. Suitable formulations for inhalation include, for example, as a fine powder or a liquid aerosol. Suitable formulations for administration by insufflation include, for example, a fine powder.
15 Suitable formulations for parenteral administration include, for example, a sterile aqueous or oily solution for intravenous, subcutaneous, intramuscular or intramuscular dosing or as a suppository for rectal dosing.

As will be appreciated by the skilled person, the therapeutically effective amount of the CDK4/6
20 inhibitor and the retinoic acid or pharmaceutical composition as described herein, will necessarily vary depending on the subject to be treated, the route of administration and the nature and severity of the disease to be treated.

Pharmaceutical Composition

25 An aspect of the invention relates to a composition comprising;
a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof, and
a pharmaceutically acceptable excipient.

30 In an embodiment the CDK4/6 inhibitor is formulated together with the retinoic acid for simultaneous administration.

35 The pharmaceutical compositions described herein may comprise the CDK4/6 inhibitor and/or retinoic acid. The pharmaceutical compositions described herein can be administered by any convenient route, including but not limited to oral, topical, parenteral, sublingual, rectal, vaginal, ocular, intranasal, pulmonary, intradermal, intravitreal, intratumoural, intramuscular, intraperitoneal, intravenous, subcutaneous, intracerebral, transdermal, transmucosal, by inhalation, or topical, particularly to the ears, nose, eyes, or skin or by inhalation. In another

embodiment, delivery is of the nucleic acid encoding the drug, e.g. a nucleic acid encoding the molecule of the invention is delivered.

5 Parenteral administration includes, for example, intravenous, intramuscular, intraarterial, intraperitoneal, intranasal, rectal, intravesical, intradermal, topical, intra-articular or subcutaneous administration. In an embodiment, the compositions are administered parenterally.

10 The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The term "carrier" refers to a diluent, adjuvant or excipient, with which a pharmaceutical composition of the present invention is administered. Such pharmaceutical carriers can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The carriers can be saline, gum acacia, gelatin, starch paste, talc, 15 keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents can be used. In one embodiment, when administered to an animal, the polypeptide of the present invention or compositions and pharmaceutically acceptable carriers are sterile. Water is a preferred carrier when the drug antibody conjugates of the present invention are administered intravenously. Saline solutions and aqueous dextrose and glycerol 20 solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH 25 buffering agents.

The pharmaceutical composition can be in the form of a liquid, e.g., a solution, syrup, solution, emulsion or suspension. The liquid can be useful for oral administration or for delivery by injection, infusion (e.g., IV infusion) or sub-cutaneous.

30

When intended for oral administration, the composition can be in solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

35 As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more of the following can be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrans, disintegrating agents such

as alginic acid, sodium alginate, corn starch and the like; lubricants such as magnesium stearate; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent. When the composition is in the form of a capsule (e. g. a gelatin capsule), it can contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrin or a fatty oil.

When intended for oral administration, a composition can comprise one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition for administration by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent can also be included.

Compositions can take the form of one or more dosage units.

In specific embodiments, it can be desirable to administer the composition locally to the area in need of treatment, or by intravenous injection or infusion.

The amount of the CD4/6 inhibitor and retinoic acid or pharmaceutical composition described herein that is effective/active in the treatment of a particular disease or condition will depend on the nature of the disease or condition and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness of the disease or disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. Factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account.

Methods

An aspect of the invention relates to an *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising;

contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4/6 inhibitor in combination with retinoic acid.

The biological sample which is contacted may be selected from tissue, saliva, urine, blood including whole blood and plasma. The biological sample may be obtained from a subject having or suspected of having high risk neuroblastoma. The biological sample may be obtained from a subject having or suspected of having ADRN-type neuroblastoma.

An aspect of the invention relates to an *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4/6 inhibitor in combination with retinoic acid comprising;

5 screening a biological sample obtained from a subject with neuroblastoma, for expression of one or more of MYCN, NTRK, ALK and segmental chromosomal abnormalities, identifying said subject as having high risk neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, selecting said subject for therapy.

10 Subjects which are suitable for therapy with a CDK4/6 inhibitor in combination with retinoic acid include subjects having high-risk or ADRN-type neuroblastoma. The step of identifying a subject as having high risk or ADRN-type neuroblastoma may comprise;

Screening for expression of MYCN, NTRK and/or ALK, wherein expression above a certain threshold is indicative of ADRN-type neuroblastoma, screening for specific mutations or aberrations within MYCN and/or ALK, wherein mutations or aberrations are indicative of ADRN-type neuroblastoma, and/or screening for the presence of segmental chromosomal abnormalities.

20 The step of identifying a subject as having high risk or ADRN-type neuroblastoma may comprise further screening of said biological sample for expression of ASCL1, PHOX2B, GATA3 and/or HAND2.

An aspect of the invention relates to a method of prognosis or therapy monitoring comprising; providing a biological sample obtained from a subject with neuroblastoma who has received treatment, screening said biological sample for one or more of the following features:

25 increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1, changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or MEIS1, or decreased expression of ASCL1 and/or HAND1.

30 RARA (ENSG00000131759, uniprot P10276) is a gene that encodes retinoic acid receptor alpha. RARB (ENSG00000077092, uniprot P10826) is a gene that encodes retinoic acid receptor beta. SOX4 (ENSG00000124766, uniprot Q06945) is a gene that encodes the SOX4 transcription factor. MEIS1 (ENSG00000143995, uniprot O00470) is a gene that encodes homeobox protein MEIS1. ASCL1 (ENSG00000139352, uniprot P50553) is a gene which encodes Achaete-scute homolog 1. HAND1 (ENSG00000113196, uniprot O96004) is a gene which encodes heart and neural crest-derived protein-1. The term "H3K27 ac deposition" refers to the acetylation present on lysine 27 of histone H3.

The method of prognosis or therapy monitoring may comprise a step of screening a biological sample for circulating tumour DNA (ctDNA). ctDNA levels may be used as an indication of treatment response. For example a decrease in the level of ctDNA may indicate a reduction in tumour size or positive response to therapy. ctDNA levels may be assessed from plasma, blood or any other suitable biological sample.

In some embodiments the method of prognosis or therapy monitoring further comprises a step of comparing said biological sample with a biological sample obtained from said patient prior to therapy. The sample obtained prior to therapy may be screened for one or more of the following features:

increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1, changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or MEIS1, or decreased expression of ASCL1 and/or HAND1, the results from the sample obtained prior to therapy may provide a reference value to compare the results from the biological sample obtained after the subject has received therapy.

An aspect of the invention relates to a method of prognosis or therapy monitoring comprising; providing a biological sample obtained from a subject with neuroblastoma who has received treatment, screening said biological sample for alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1, and/or serum lactate dehydrogenase level.

Ki67 (ENSG00000148773, uniprot P46013) is a gene that encodes proliferation marker Ki67. E2F1 (ENSG00000101412, uniprot Q01094), E2F2 (ENSG00000007968, Q14209) and E2F8 (ENSG00000129173, uniprot A0AVK6) are genes which encode E2F1, E2F2 and E2F8 transcription factors respectively. STMN4 (ENSG00000015592, uniprot Q9H169) and STMN2 (ENSG00000104435, uniprot Q93045) are genes which encode Stathmin 4 and Stathmin 2 respectively. NTRK1 (TrkA) (ENSG00000198400, uniprot P04629) is a gene which encodes neurotrophic receptor tyrosine kinase 1.

In some embodiments of the method of prognosis or therapy monitoring, the biological sample is also screened for alteration in the expression of PLK1 and/or FOXM1. PLK1 is a gene that encodes a serine/threonine-protein kinase, also known as polo-like kinase 1. FOXM1 is a gene that encodes forkhead box protein M1 a transcription factor which plays a key role in the cell cycle progression. The biological sample may be screened for alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1, PLK1, FOXM1 and/or serum lactate dehydrogenase level.

In some embodiments the method of prognosis or therapy monitoring further comprises a step of comparing said biological sample with a biological sample obtained from said patient prior to therapy. The sample obtained from said patient prior to therapy may be screened for altered expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum LDH level. The results from the sample obtained prior to therapy may provide a reference value to compare the results from the biological sample obtained after the subject has received therapy. The sample obtained from said patient prior to therapy may be screened for increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1. The sample obtained from said patient prior to therapy may be screened for changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or MEIS1. The sample obtained from said patient prior to therapy may be screened for decreased expression of ASCL1 and/or HAND1.

The results from the sample obtained prior to therapy may provide a reference value to compare the results from the biological sample obtained after the subject has received therapy. As such, a biological sample obtained from the subject after the subject has received therapy may be screened for any one of the features listed above in order to determine whether the therapy is effective

An aspect of the invention relates to a method of prognosis or therapy monitoring comprising; providing a biological sample obtained from a subject with neuroblastoma who has received treatment, screening said biological sample for one or more of the following characteristics; alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum lactate dehydrogenase level, increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1, changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or MEIS1, decreased expression of ASCL1 and/or HAND1 and/or any combination of the aforementioned features.

In an embodiment the downregulation of one or more of Ki67, E2F1, E2F2 and/or E2F8 is indicative of survival or response to therapy. In an embodiment the upregulation of one or more of NTRK1 (TrkA), STMN4, STMN2 and/or serum LDH level is indicative of survival or response to therapy.

Where serum LDH level is screened for in methods of the invention this may refer to identifying the amount of the protein LDH (lactate dehydrogenase) present in a subject's serum, methods to determine LDH level are known in the art.

The term “response to therapy” may refer to wherein a certain therapeutic effect is achieved. In some embodiments, the therapeutic effect may be an increase in immune response to the tumor, as determined, for example, by an increase in immune system biomarkers (e.g. blood parameters, such as lymphocyte counts and/or NK cell numbers; and/or cytokines). In some
5 embodiments, the therapeutic effect may be a reduction in tumour markers (e.g. catecholamines). In some embodiments, the therapeutic effect may be determined by methods such as metaiodobenzylguanidine scintigraphy (mIBG), magnetic resonance imaging (MRI) or X-ray computed tomography (CT), and/or bone marrow histology (assessed by aspirate or trephine biopsy), and/or CDC assays and/or WBTs. In some embodiments “response to therapy”
10 may refer to a complete response characterised by one or more of Complete disappearance of all measurable and evaluable disease, no new lesions, no disease-related symptoms, and/or no evidence of evaluable disease, including e.g. normalization of markers and/or other abnormal lab values. In some embodiments “response to therapy” may refer to a partial response characterised by one or more of no progression of evaluable disease, no new lesions.

15 As will be apparent to the skilled person the methods described herein may be performed *in vivo*, *in vitro* or *ex vivo* as appropriate.

20 The biological sample which is used in the methods of the present invention may be selected from tissue, saliva, urine, blood including whole blood and plasma

Kits

25 An aspect of the invention relates to a kit for the treatment of neuroblastoma comprising;
 a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
 retinoic acid or a pharmaceutically acceptable salt thereof,
 wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

30 The kit may comprise the CDK4/6 inhibitor and the retinoic acid formulated separately as distinct compositions. The kit may comprise the CDK4/6 inhibitor and the retinoic acid formulated together as a single composition. The kit may comprise the CDK4/6 inhibitor formulated in a pharmaceutical composition as described herein. The kit may comprise the retinoic acid formulated in a pharmaceutical composition as described herein. The CDK4/6 inhibitor and/or the retinoic acid may be formulated for a specific administration route. The kit may further
35 comprise instructions for use. The kit may further comprise a further anti-cancer treatment for example an immunotherapy such as Dinutuximab.

As disclosed herein the invention relates to methods of prognosis and therapy monitoring wherein a biological sample is screened for alterations in the expression of specific markers. As

such the invention also relates to a kit for use in one of these methods. According to the invention the kit comprises a plurality of binding agents, wherein each of said binding agents specifically binds to a biomarker protein selected from the group consisting of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, TrkA (NTRK1), and/or serum LDH. Additional binding agents may be provided which specifically bind to PLK1 and/or FOXM1. The biomarker protein selected from the group consisting of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, TrkA (NTRK1), PLK1, FOXM1 and/or serum LDH is either selectively upregulated or downregulated in neuroblastoma, wherein the difference in the expression level is indicative of survival or response to therapy.

- 10 The kit may comprise binding agents comprising antibodies and/or nucleic acids. The kit may comprise binding agents suitable for use in a sandwich hybridisation assay, a competitive hybridisation assay, a ligand binding assay, a hybrid ligand-binding assay, a dual-ligation hybridisation assay, a hybridisation-ligation assay, multiplex ligand binding assay. The kit may comprise one or more further components required to allow detection of one or more of the
- 15 biomarkers selected from Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), PLK1, FOXM1 and/or serum LDH. The further components may be selected from but not limited to; suitable buffers, excipients, diluents.

- 20 Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. While the foregoing disclosure provides a general description of the subject matter encompassed within the scope of the present disclosure, including methods, as well as the best mode thereof, of making and using this disclosure, the following examples are provided to further enable those skilled in the art to practice this disclosure. However, those skilled in the art will
- 25 appreciate that the specifics of these examples should not be read as limiting on the invention, the scope of which should be apprehended from the claims and equivalents thereof appended to this disclosure. Various further aspects and embodiments of the present disclosure will be apparent to those skilled in the art in view of the present disclosure.

- 30 All documents mentioned in this specification are incorporated herein by reference in their entirety, including references to gene accession numbers, scientific publications and references to patent publications.

- 35 "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein. Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

The term “comprising” or “comprises” where used herein means including the component(s) specified but not to the exclusion of the presence of other components. The term “consisting essentially of” or “consists essentially of” means including the components specified but
5 excluding other components except for materials present as impurities, unavoidable materials present as a result of processes used to provide the components and the like.

The term “consisting of” or “consists of” means including the components specified but excluding other components.

10 Whenever appropriate, depending upon the context, the use of the term “comprises” or “comprising” may also be taken to include the meaning “consists essentially of” or “consisting essentially of”, and also may also be taken to include the meaning “consists of” or “consisting of”.

15 The optional features set out herein may be used either individually or in combination with each other where appropriate and particularly in the combinations as set out in the accompanying claims. The optional features for each aspect or exemplary embodiment of the invention, as set out herein are also applicable to all other aspects or exemplary embodiments of the invention,
20 where appropriate. In other words, the skilled person reading this specification should consider the optional features for each aspect or exemplary embodiment of the invention as interchangeable and combinable between different aspects and exemplary embodiments.

25 It should be understood that while the use of words such as “preferable”, “preferably”, “preferred” or “more preferred” in the description suggest that a feature so described may be desirable, it may nevertheless not be necessary and embodiments lacking such a feature may be contemplated as within the scope of the invention as defined in the appended claims. In relation to the claims, it is intended that when words such as “a,” “an,” or “at least one,” are used to preface a feature there is no intention to limit the claim to only one such feature unless
30 specifically stated to the contrary in the claim.

The invention is further described in the following non-limiting embodiments.

Numbered Embodiments

35 1. A CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.

2. The CDK4/6 inhibitor for use according to embodiment 1, wherein the ADRN-type neuroblastoma cells are characterised by expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities.
3. The CDK4/6 inhibitor for use according to embodiments 1 or 2, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.
4. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the neuroblastoma is selected from high risk MYCN-driven neuroblastoma, MYCN-amplified neuroblastoma, non-amplified neuroblastoma, ADRN-type high risk tumours, ADRN-type intermediate risk tumours, MYCN-amplified ADRN-type high risk tumours, MYCN-amplified ADRN-type intermediate risk tumours, ALK-mutated ADRN-type high risk tumours, ALK-mutated ADRN-type intermediate risk tumours.
5. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the CDK4/6 inhibitor is selected from palbociclib, ribociclib and/or abemaciclib.
6. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the CDK4/6 inhibitor is administered at a dose of between 0.1 to 200 mg/kg, 1 to 100 mg/kg, 5 to 80 mg/kg, preferably 20 to 60mg/kg, more preferably approximately 40 mg/kg.
7. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the retinoic acid is cis-retinoic acid or trans-retinoic acid .
8. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the retinoic acid is administered at a dose of 1 to 500 mg/m²/day, 10 to 400 mg/m²/day, 20 to 300 mg/m²/day, 50 to 200 mg/m²/day.
9. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the CDK4/6 inhibitor is administered simultaneously, sequentially or separately with the retinoic acid.
10. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the CDK4/6 inhibitor and retinoic acid are used in combination with a further anti-cancer therapy.
11. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the further anti-cancer therapy is selected from chemotherapy, radiotherapy, surgery, immunotherapy, hormone therapy myeloablative therapy (MAT) and/or autologous stem cell rescue (ASCR).
12. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the subject received one or more anti-cancer agents prior to the administration of the CDK4/6 inhibitor.
13. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the neuroblastoma is identified as ADRN-type neuroblastoma.
14. The CDK4/6 inhibitor for use according to any preceding embodiments, wherein the CDK4/6 inhibitor is used at a cytostatic dose.
15. A CDK4/6 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises;

- screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
5 administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.
16. A method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4/6 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic(ADRN)-type neuroblastoma cells.
17. A method of treating neuroblastoma in a subject, comprising;
10 screening a biological sample obtained from said subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.
- 15 18. The method of embodiments 15 to 17, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.
19. A composition comprising;
a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
20 for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.
20. A composition comprising;
a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof, and
25 a pharmaceutically acceptable excipient.
21. Use of a CDK4/6 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic cells.
22. An *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising;
30 contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4/6 inhibitor in combination with retinoic acid.
23. An *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4/6 inhibitor in combination with retinoic acid comprising;
screening a biological sample obtained from a subject with neuroblastoma, for expression
35 of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
selecting said subject for therapy.
24. A kit for the treatment of neuroblastoma comprising;

a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

25. A method of prognosis or therapy monitoring comprising;
5 providing a biological sample obtained from a subject with neuroblastoma who has received treatment,
screening said biological sample for one or more of the following features:
increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1,
changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or
10 MEIS1, or
decreased expression of ASCL1 and/or HAND1.
26. A method of prognosis or therapy monitoring comprising;
providing a biological sample obtained from a subject with neuroblastoma who has received treatment,
15 screening said biological sample for alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum LDH level .
27. The method of any of embodiments 25 to 26, further comprising a step of comparing said biological sample with a biological sample obtained from said patient prior to therapy.
28. The method of any of embodiments 26 to 27, wherein the downregulation of Ki67, E2F1,
20 E2F2 and/or E2F8 is indicative of survival or response to therapy.
29. The method of any of embodiments 26 to 28, wherein the upregulation of NTRK1 (TrkA), STMN4, STMN2 and/or serum LDH level is indicative of survival or response to therapy.
30. A kit comprising a plurality of binding agents, wherein each of said binding agents specifically binds to a distinct biomarker protein that is selectively upregulated or downregulated
25 in neuroblastoma, wherein said biomarker proteins are selected from the group consisting of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum LDH.
31. A CDK4 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises ADRN-type neuroblastoma cells.
- 30 32. The CDK4 inhibitor for use according to embodiment 31, wherein the ADRN-type neuroblastoma cells are characterised by expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities.
33. The CDK4 inhibitor for use according to embodiments 31 or 32, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B,
35 GATA3 and/or HAND2.
34. The CDK4 inhibitor for use according to any one of embodiments 31 to 33, wherein the neuroblastoma is selected from high risk MYCN-driven neuroblastoma, MYCN-amplified neuroblastoma, non-amplified neuroblastoma, ADRN-type high risk tumours, ADRN-type intermediate risk tumours, MYCN-amplified ADRN-type high risk tumours, MYCN-amplified

ADRN-type intermediate risk tumours, ALK-mutated ADRN-type high risk tumours, ALK-mutated ADRN-type intermediate risk tumours.

35. The CDK4 inhibitor for use according to any one of embodiments 31 to 34, wherein the CDK4 inhibitor is selected from palbociclib, ribociclib and/or abemaciclib.
- 5 36. The CDK4 inhibitor for use according to any one of embodiments 31 to 35, wherein the CDK4 inhibitor is administered at a dose of between 0.1 to 200 mg/kg, 1 to 100 mg/kg, 5 to 80 mg/kg, preferably 20 to 60mg/kg, more preferably approximately 40 mg/kg.
37. The CDK4 inhibitor for use according to any one of embodiments 31 to 36, wherein the retinoic acid is cis-retinoic acid or trans-retinoic acid.
- 10 38. The CDK4 inhibitor for use according to any one of embodiments 31 to 37, wherein the retinoic acid is administered at a dose of 1 to 500 mg/m²/day, 10 to 400 mg/m²/day, 20 to 300 mg/m²/day, 50 to 200 mg/m²/day.
39. The CDK4 inhibitor for use according to any one of embodiments 31 to 38, wherein the CDK4/6 inhibitor is administered simultaneously, sequentially or separately with the retinoic acid.
- 15 40. The CDK4 inhibitor for use according to any one of embodiments 31 to 39, wherein the CDK4/6 inhibitor and retinoic acid are used in combination with a further anti-cancer therapy.
41. The CDK4 inhibitor for use according to any one of embodiments 31 to 40, wherein the further anti-cancer therapy is selected from chemotherapy, radiotherapy, surgery, immunotherapy, hormone therapy myeloablative therapy (MAT) and/or autologous stem cell
- 20 rescue (ASCR).
42. The CDK4 inhibitor for use according to any one of embodiments 31 to 41, wherein the subject received one or more anti-cancer agents prior to the administration of the CDK4 inhibitor.
43. The CDK4 inhibitor for use according to any one of embodiments 31 to 42, wherein the neuroblastoma is identified as ADRN-type neuroblastoma.
- 25 44. The CDK4 inhibitor for use according to any one of embodiments 31 to 43, wherein the CDK4/6 inhibitor is used at a cytostatic dose.
45. A CDK4 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises;
- 30 screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
- identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
- administering to said subject a CDK4 inhibitor in combination with retinoic acid.
46. A method of treating neuroblastoma in a subject, comprising administering to the subject
- 35 a CDK4 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic(ADRN)-type neuroblastoma cells.
47. A method of treating neuroblastoma in a subject, comprising;
- screening a biological sample obtained from said subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,

identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, administering to said subject a CDK4 inhibitor in combination with retinoic acid.

48. The method of any one of embodiments 45 to 47, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.

49. A composition comprising;
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.

50. A composition comprising;
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof, and
a pharmaceutically acceptable excipient.

51. Use of a CDK4 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic cells.

52. An *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising;
contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4 inhibitor in combination with retinoic acid.

53. An *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4 inhibitor in combination with retinoic acid comprising;
screening a biological sample obtained from a subject with neuroblastoma, for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
selecting said subject for therapy.

54. A kit for the treatment of neuroblastoma comprising;
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

The invention is further illustrated in the following non-limiting examples.

35 Features of the use of the present invention are described in further detail in relation to the abovementioned aspects of the invention, in the examples below.

The described and illustrated examples are to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the scope of the inventions as defined in the claims are desired to be protected.

5

EXAMPLES

Introduction

Neuroblastoma is the most common extracranial solid tumour in infants, arising from developmentally stalled neural crest-derived cells. Driving tumour differentiation is a promising therapeutic approach for this devastating disease. Here, we show the CDK4/6 inhibitor palbociclib not only inhibits proliferation but induces extensive neuronal differentiation of adrenergic neuroblastoma cells. Palbociclib-mediated differentiation is manifested by extensive phenotypic and transcriptional changes, accompanied by the establishment of a new epigenetic programme driving expression of mature neuronal features. *In vivo* palbociclib significantly inhibits tumour growth in mouse neuroblastoma models. Furthermore, dual treatment with retinoic acid resets the oncogenic adrenergic core regulatory circuit of neuroblastoma cells, further suppresses proliferation, and can enhance differentiation, altering gene expression in ways that significantly correlate with improved patient survival. We therefore identify palbociclib as a novel therapeutic approach to dramatically enhance neuroblastoma differentiation efficacy that could be used in combination with retinoic acid to improve patient outcome.

Example 1: Palbociclib inhibits proliferation and promotes differentiation in adrenergic neuroblastoma cells

Given the known links between cell cycle regulation in division and differentiation in the developing nervous system and in neuroblastoma^{23,32-34}, we first investigated the effects of the CDK4/6 inhibitor palbociclib (PB) on ADRN-type neuroblastoma cell lines SK-N-BE(2)C, IMR-32 and SH-SY5Y. Our aim is to find a therapy that will reactivate the latent differentiation potential of neuroblastoma cells in a variety of genetic backgrounds. We therefore chose cell lines that cover a mixture of backgrounds that are all CDK4/6 wild-type (WT)³⁵ with a range of CCND1 expression levels²⁶: SK-N-BE(2)C – 1p loss, TP53 mutant, MYCN amplified³⁶; IMR-32 - MYCN amplified, MEIS1 amplified, 1p loss^{37,38}; and SH-SY5Y - ALK mutant F1174L, 1q segment gain, 17q gain^{37,39}. Cells were treated with 1µM PB, a standard dosage used in cellular studies^{40,41} that is similar to the IC50s of all three lines. The CyclinD-CDK4/6 complex phosphorylates RB^{42,43}, a tumour suppressor that blocks the G1 to S phase transition, so reduced RB phosphorylation is an indicator of CDK4/6 inhibition. As expected, in all cell lines 24h PB treatment resulted in hypo-phosphorylation of RB (Figure 1A). We also see a reduction in the total RB level, as has been observed previously with CDK4/6 knockdown in neuroblastoma cell lines⁴⁴. Consistently, 5 days of PB treatment resulted in a significant decrease in proliferation

compared to the vehicle control, as shown by EdU incorporation and crystal violet staining (Figure 1B-G). Interestingly, we also observed coincident neuronal differentiation features upon PB treatment, namely neurite outgrowth and cell clustering (Figure 1E-G). Live-cell imaging shows a direct change in morphology during PB treatment, with negligible cell death. Automated
5 segmentation of neurite outgrowth using live-cell NeuroTrack analysis confirmed an increase in neurite length and branch points with PB treatment compared to the DMSO control in all cell lines. In contrast, the MES-type SH-EP and GIMEN neuroblastoma cell lines displayed decreased proliferation but no features of neuronal differentiation upon PB treatment, suggesting that the pro-differentiation effect is limited to ADRN-type neuroblastoma models.
10 Immunocytochemistry analysis of SK-N-BE(2)C, IMR-32 and SH-SY5Y cells following 5 or 11 days PB treatment confirmed neurite outgrowth accompanied by upregulation of the classical neuronal marker β -tubulin (TUBB3) (Figure 1H-J). These data demonstrate that PB not only drives cell cycle exit as expected, but also induces neuronal differentiation of ADRN-type neuroblastoma cells.

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Example 2: Palbociclib activates a transcriptional programme of neuronal differentiation

We next sought to characterise how PB alters the transcriptional landscape of neuroblastoma cells. SK-N-BE(2)C, IMR-32 and SH-SY5Y cell lines were treated with PB for short (24hr) or
20 longer (5-7 days) periods and RNA collected for RNA-seq. PB efficacy was confirmed by a strong downregulation of E2F target genes (directly regulated by pRB) at the 24hr time point, providing support for the antiproliferative effects of PB. For each cell line, differential expression analysis was conducted using DESeq2 to identify rapidly and more slowly responding genes⁴⁵. Clustering of these genes after normalisation within each cell line revealed six sets of genes that differed
25 in response to PB; importantly the PB response of each gene cluster is similar across cell lines (Figure 2A). Gene ontology analysis on genes within these clusters showed early downregulation of cell cycle-related genes and progressive downregulation of the spliceosome with later downregulation of mitochondria components (Figure 2A,B); this is consistent with the decreased proliferation we observed and perhaps reflective of changes in mitochondrial
30 dynamics known to occur as cells differentiate⁴⁶. Early upregulation of plasma membrane and ion channel complexes was also observed, followed later by upregulation of synapse and axon components indicative of differentiation (Figure 2A,B). Together these data show a robust reprogramming of the transcriptome by PB towards reduced proliferation and induction of neuronal differentiation in all three neuroblastoma cell lines.

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Example 3: Palbociclib rewires the epigenetic landscape consistent with differentiation

Given the strong epigenetic component known to drive neuroblastoma tumours^{15,47,48} we investigated the impact of PB treatment on the epigenetic landscape of neuroblastoma cells. We

conducted H3K27ac ChIP-seq (a marker of active enhancers⁴⁹) in SK-N-BE(2)C, IMR-32 and SH-SY5Y cell lines after longer PB treatment (5-7 days), when extensive morphological differentiation was observed. DiffBind⁵⁰ was used to determine regions with significantly altered deposition of H3K27ac. This analysis revealed extensive changes in H3K27ac marked regions following PB treatment in all three cell lines (Figure 3A). Common to at least two of three cell lines are 5641 increased sites of H3K27ac deposition, 2561 decreased sites and 2830 sites with sustained H3K27ac deposition before and after treatment. Differential H3K27ac marks were more likely to be distal from promoter regions and potentially associated with enhancer regions (data not shown). To understand how these epigenetic changes may link to gene expression, the most proximal gene (max distance 100kb) to the H3K27ac broad peak was assigned as a putative regulatory target. Gene ontology analysis showed that PB treatment resulted in a consistent increase in H3K27ac deposition proximal to genes associated with neuronal development biological processes (Figure 3B), consistent with the changes observed by RNA-seq (Figure 2). Genes proximal to reduced H3K27ac marks are associated with alternative developmental routes including gland, kidney epithelium and mesenchyme development (Figure 3C).

Changes in cell identity are governed by critical lineage-defining genes that are typically regulated by clusters of enhancers with high H3K27ac signal¹⁵, termed super-enhancers (SEs). To interrogate the effect of PB treatment on SE regions, H3K27ac broad peaks were stitched into clusters and a consistent normalised H3K27ac signal threshold used to identify super-enhancers, enabling comparisons between cell lines and conditions. Super-enhancers were grouped as either increased, sustained, or decreased based on the change in total H3K27ac signal in the SE between the PB and control condition in each cell line (Figure 3D); specific examples of SE regions with a consistently increased, maintained or decreased H3K27ac signal across the three cell lines were identified. Gene ontology analysis showed that genes within 100kb of SEs that have increased H3K27ac deposition upon PB treatment are related to neuron-to-neuron synapses, axons, dense-core granules and, in the case of SH-SY5Y cells, contractile fibres (Figure 3E). Interestingly, sustained SEs that are present before and after PB treatment are linked to genes associated with plasma membrane complexes, ion channel complexes and dendrite development (Figure 3E), perhaps reflecting the state of ADRN-type neuroblastoma cells that may already be partially primed to differentiate. Together, these H3K27ac broad-peak and super-enhancer analyses demonstrate that PB treatment restructures the epigenetic landscape of neuroblastoma cells to favour neuronal differentiation and axonogenesis as well as restrict alternative developmental pathways.

Example 4: Palbociclib inhibits tumour growth in in vivo mouse models of neuroblastoma

We surmised that the dual anti-proliferative and pro-differentiation effects of PB we observed in vitro could provide a novel therapeutic opportunity. To investigate this new insight in a more clinically relevant in vivo setting, we used the extensively characterised Th-MYCN genetically engineered mouse (GEM), known to mirror many clinical features of high-risk MYCN-driven neuroblastoma^{51,52}, in which MYCN is expressed under the control of the tyrosine hydroxylase (Th) promoter. We optimised the dosage of PB to stabilise tumour growth; MRI scans and tumour growth measurements show 40 mg/kg PB is sufficient to achieve this (Figure 4A,B) and monitoring mice weights show this dosage is well tolerated (Figure 4C). Importantly, we found that PB treatment at this dosage has a significant survival benefit (Figure 4D). To strengthen our in vivo findings, we next tested PB in immunocompromised mice xenografted with human neuroblastoma cells IMR-32. PB treatment was found to also effectively restrain tumour growth in this model (Figure 4E,F). Together this demonstrates that PB is able to restrain neuroblastoma growth in vivo and is an effective and tolerable cytostatic agent.

Example 5: Palbociclib and retinoic acid additively inhibit proliferation of neuroblastoma cells

Retinoic acid is already used clinically as a differentiating agent in maintenance therapy. Neuronal differentiation of neuroblastoma cells is likely to require both lengthening or arresting the cell cycle in G1 and resetting of the oncogenic core regulatory circuit (CRC)^{20,23,53–55}. Indeed previous evidence shows that retinoic acid (RA) can reset the ADRN CRC network in neuroblastoma cells and promote elements of differentiation^{19–21}. Our RNA-seq data revealed that PB does not consistently impact the expression levels of ADRN CRC transcription factors⁴⁷ (Figure 5A). Similarly, we did not observe robust changes to the oncogenic CRC-associated super-enhancer landscape (as determined by H3K27ac ChIP-seq) after PB treatment. In keeping with this, investigation of extended PB treatment (>17 days) revealed that, while all three cell lines displayed neuronal features, cells were nevertheless capable of expansion. As an example, SK-N-BE(2)C cells maintained in PB continued to divide slowly as indicated by persistent Ki67 expression, despite exhibiting neurite extension (Figure 5B,C). Proliferation was decreased compared to untreated cells, as shown by EdU incorporation, with a 30% decrease in positivity, and confluency analysis, with a doubling of confluency in ~80 h compared to 24 h for untreated cells (Figure 5D,E). These data suggest that while PB treatment alone significantly re-engages the differentiation programme in neuroblastoma cells, complete cell cycle arrest is not achieved and therefore, a combinatorial treatment may provide further benefit.

We therefore hypothesised that PB and RA together may further enhance the acquisition of a post-mitotic differentiated state. To investigate this, we treated SK-N-BE(2)C cells with PB (1 μ M), RA (10 μ M) or PB+RA in combination. Crystal violet staining following 5 days of treatment demonstrated that, while RA alone induced limited signs of morphological neuronal differentiation, both PB alone and PB+RA in combination induced extensive neurite formation

(Figure 5F). Daily cell counts before and during treatment showed that, for all treatments, cell numbers increase or remain constant over time, indicating a lack of cell death (Figure 5G). Live-cell imaging also shows a direct change in morphology during PB and PB+RA treatment, with negligible cell death. Ki67 immunocytochemistry, EdU incorporation analyses and CellTiter-Glo® cell viability assays all indicated a significant decrease in cycling cells in PB+RA-treated cells compared to PB alone (Figure 5H-K), with EdU positivity falling from ~40% to ~18%. Similar results were obtained in IMR-32 and SH-SY5Y cells, with decreased proliferation in PB+RA-treated cells compared to PB alone as assessed by EdU incorporation and decreased expression of the established proliferative gene Ki67, as well as E2F2 and E2F8. PB and PB+RA also increased expression of neuronal markers STMN4 and STMN2, in fitting with live-cell imaging showing morphological changes. These results demonstrate PB+RA further inhibits cell cycling compared to PB alone, indicating a potential benefit in combining these treatments.

Example 6: PB+RA facilitate genome-wide changes favouring reduced proliferation and enhanced differentiation

To further characterise the response of neuroblastoma cells to PB+RA, RNA-seq analysis and H3K27ac ChIP-seq were conducted in SK-N-BE(2)C cells treated with PB, RA or PB+RA for 5 days. First, we focused on expression of the oncogenic CRC genes. In line with our previous findings (Figure 5A), we observed that PB alone generally did not significantly change the RNA expression level of oncogenic CRC genes, whereas treatment with RA alone or with PB+RA led to more substantial changes in expression of many CRC-associated factors, including activating expression of the retino-sympathetic CRC genes RARA, RARB, SOX4 and MEIS1 (Figure 6A)²⁰, and we saw significant changes in H3K27ac deposition around these genes. We also observed downregulation in RNA expression and the surrounding H3K27ac marks of the bHLH transcription factors ASCL1 and HAND1 that are usually strongly expressed in proliferating neuroblasts (Figure 6B)⁵⁵. These data suggest that combining PB and RA has a much greater effect on CRC gene expression than PB alone.

Next, we examined the impact of the combination of PB+RA on gene expression more widely. k-means clustering was conducted on genes with significant differential expression in at least one comparison of conditions; this identified five gene clusters in terms of their responsiveness to PB, RA or PB+RA treatment. Downregulated genes formed one distinct cluster and generally showed a modest downregulation by RA alone, a greater response to PB alone, and an even stronger downregulation with the combined PB+RA treatment (cluster 1, Figure 6C). This cluster of genes was associated with cellular components involved in cell cycle progression (Figure 6D), consistent with the greater reduction in proliferation we observed with PB+RA (Figure 5F-K).

Genes upregulated in response to PB and/or RA could be separated into four distinct clusters. Genes that are upregulated only in response to RA are generally associated with cell-cell junctions and the extracellular matrix (cluster 2, Figure 6C,D), while genes upregulated only after PB treatment include those associated with postsynaptic membrane components (cluster 3, Figure 6C,D). Importantly, we observed a cluster of genes that were more substantially upregulated only after combined PB+RA treatment (cluster 4, Figure 6C) and these genes were associated with the neuronal cell body as well as extracellular matrix cellular components (Figure 6D), consistent with enhanced differentiation. In addition, another subset of genes was upregulated by PB and further upregulated by combined PB+RA treatment (cluster 5, Figure 6C); these were associated with synaptic vesicles, synaptic membranes and other components associated with neuronal development (Figure 6D).

We also compared how PB and RA, both independently and working together, restructure the epigenetic landscape by using H3K27ac as a marker of active enhancers. Significantly differential H3K27ac marks between conditions were determined using DiffBind⁵⁰; this was then used to group H3K27ac marks by how they change following PB, RA and combined PB+RA treatment (Figure 6E). This revealed a range of similar, additive, synergistic, PB-specific, RA-specific and antagonistic differences in H3K27ac mark regulation (Figure 6E). Of note, for the regions where this mark significantly changed in all three conditions (Figure 6E groups 1 and 21), the extent of change is greatest in the PB+RA combination, suggesting the drugs have an additive impact on H3K27ac deposition at these sites (Figure 6D). Assessing the location of these H3K27ac marks in relation to transcription start sites showed marks decreased in the presence of PB or PB+RA had a greater tendency to be located proximal to promoter regions and increased marks were more likely located in more distal regions.

To understand further the changes in regulation after PB and RA treatment, we focused in on the regions in Figure 6E that have increased H3K27ac signal after PB+RA treatment and assigned the most proximal gene (within 100kb). Gene ontology analysis showed the regions with increased H3K27ac in all individual conditions, but that showed the most increase in the combined PB+RA treatment (Figure 6E, group 21) relate to ion channels, synapses and axons (Figure 6F). In addition to these components, H3K27ac increases that are only found with RA or PB+RA also relate to sarcomere components, while H3K27ac increases that are only found with PB or PB+RA are associated with neuronal dense core vesicles (Figure 6F). Together these data highlight that both PB and RA alter the epigenetic landscape surrounding genes associated with neuronal differentiation, and via additive and drug-specific regulation may enhance these changes when used in combination.

Example 7: Dual PB+RA treatment promotes a transcriptional signature favouring patient survival

We next sought to assess how transcriptional changes that occur in response to PB+RA may point to potential benefits of such a treatment to patient survival. Using data from the R2 Genomics platform (<http://r2.amc.nl>) we identified two gene sets that positively and negatively correlate significantly with poor patient survival in neuroblastoma. We then looked at how these gene sets change in expression after combined PB+RA treatment of SK-N-BE(2)C cells compared to the vehicle-treated control. Strikingly, genes where high expression is usually associated with poor patient survival were strongly downregulated by PB+RA, while genes where low expression is associated with poor survival were significantly upregulated (Figure 7A). Thus, PB+RA is able to drive a transcriptional programme that is likely to be highly beneficial for patients.

Example 8: Treatment with PB+RA drives ultrastructural features of mature neuronal differentiation

To determine if the transcriptomic signatures of mature neuronal differentiation observed in cells treated with PB+RA would be reflected as complex structural and functional changes associated with post-mitotic neurons, we then examined ultrastructural changes of treated SK-N-BE(2)C cells by transmission electron microscopy. This analysis revealed striking ultrastructural features that are present after 5 days PB+RA treatment consistent with hallmarks of neuronal cells, including filaments, microtubules and mitochondria within neurites, as well as prominent dense-core granules localised at cell extremities (Figure 7B).

Example 9: PB+RA additively reduces growth and induces differentiation of tumour spheroids

Finally, to test our results in a more complex, clinically-relevant culture system, we investigated the effects of PB and RA on tumour spheroids. Spheroids were cultured in a 96 well format and treated upon reaching 200-400 µm in diameter, a size appropriate for drug screening studies⁵⁶. We observed that spheroid growth was inhibited by PB and PB+RA treatment for all three ADRN cell lines compared to the DMSO control (Figure 7C,D). Immunostaining analysis of these spheroids shows a change in cell morphology, with neurite extension, and upregulation in expression of the neuronal marker TUBB3 with PB and PB+RA treatment (Figure 7E). Fittingly, qRT-PCR analysis showed a downregulation in proliferative markers Ki67, E2F2 and E2F8 and an upregulation in neuronal differentiation markers STMN4 and STMN2 with PB or PB+RA (Figure 7F,G). In SK-N-BE(2)C spheroids, RA alone had a limited effect, while PB+RA reduced spheroid size, reduced proliferative marker expression and increased differentiation marker expression more than PB alone. Interestingly, while PB+RA dramatically reduces proliferation and enhances differentiation compared to RA alone in a consistent manner, enhanced differentiation by PB+RA compared to PB alone was variable across lines. Together our findings

suggest addition of PB to RA as a combinatorial treatment, or use of PB alone in tumours displaying low RA responsiveness, could provide significant clinical benefit in improving the efficacy of neuroblastoma differentiation therapy.

5 Example 10: All CDK4/6 inhibitors reduce proliferation and induce differentiation of SK-N-BE(2)C neuroblastoma cells

Neuroblastoma is the most common extracranial tumour in infants, accounting for approximately 15% of paediatric cancer deaths. These tumours are unique in that a subset, namely stage 4S,
10 undergo spontaneous regression driven by differentiation. Differentiation therapy, where cancer cells are re-routed back down their correct developmental pathway, is therefore a promising therapeutic avenue. We have shown that the CDK4/6 inhibitor palbociclib induces not only decreased proliferation but enhanced neuronal differentiation of neuroblastoma cells in vitro, and reduced tumour growth in mouse models of neuroblastoma. When combined with retinoic
15 acid in vitro, already used clinically for maintenance therapy, this differentiation is enhanced.

Here, we investigate the ability of three CDK4/6 inhibitors: palbociclib (PB), abemaciclib (ABE) and ribociclib (RIBO), to induce differentiation of the relapsed, high-risk NMYC-amplified neuroblastoma cell line SK-N-BE(2)C, with and without retinoic acid (RA). We find that CDK4/6
20 inhibitors display a class effect in reducing proliferation and inducing neuronal differentiation together with retinoic acid, both in 2D and 3D.

We first set out to compare the effects of the three FDA-approved CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib on ADRN-type neuroblastoma cells SK-N-BE(2)C. SK-N-BE(2)C cells
25 are derived from a relapsed tumour that is MYCN-amplified and therefore representative of high-risk disease. Previously, a dosage of 1 μ M palbociclib was used, a standard dosage used in cellular studies that is similar to the IC₅₀. We therefore ascertained the IC₅₀ of ribociclib and abemaciclib in SK-N-BE(2)C cells (2 μ M and 0.2 μ M, respectively) for comparison to palbociclib at 1 μ M (Figure 8). After 5 days of treatment with each CDK4/6 inhibitor, we observed a reduction
30 in proliferation compared to the DMSO vehicle control, as shown by imaging and crystal violet analysis (Fig 9A,B). Previously, we observed neuronal differentiation co-incident with reduced proliferation upon PB treatment, visible as neurite outgrowth. We also observed this phenomenon upon treatment of SK-N-BE(2)C cells with any of the three CDK4/6 inhibitors. Live imaging shows a change of cell morphology during the 5-day treatment, accompanied by
35 negligible cell death. The resulting morphology is also visible by immunocytochemistry for the classical neuronal marker β -tubulin (TUBB3), whose expression increases upon CDK4/6 inhibition (Fig 9C). Together these data show that all three CDK4/6 inhibitors are capable of reducing proliferation and inducing differentiation of the neuroblastoma cell line SK-N-BE(2)C in vitro, without extensive cell death.

Example 11: CDK4/6 inhibition enhances retinoic acid-induced differentiation in adherent SK-N-BE(2)C cells

- 5 Retinoic acid has been found to epigenetically reset the core regulatory circuit of ADRN-type neuroblastoma cells. We found that dual treatment with retinoic acid and palbociclib further suppressed proliferation and enhanced differentiation compared to either drug alone. We therefore next sought to determine if retinoic acid enhances differentiation in combination with palbociclib specifically, or with any CDK4/6 inhibitor. For all CDK4/6 inhibitors, CDK4/6 inhibition
- 10 or dual treatment with retinoic acid showed a greater decrease in proliferation compared to RA alone (Fig 9 A, B, D, E) as shown by crystal violet staining, EdU analysis and live-imaging confluency analyses. The decrease in proliferation by any CDK4/6 inhibitor was consistently enhanced by combinatorial treatment with retinoic acid.
- 15 Upon treatment with either CDK4/6 inhibitor alone, or in combination with RA, ICC showed an increase in TUBB3 expression and neurite extension (compared to DMSO or RA treatment) (Fig 9C). qRT-PCR analysis revealed a greater increase in expression of the differentiation marker STMN4, and a greater decrease in expression of the proliferative markers E2F8, PLK1 and FOXM1, upon treatment of cells with each CDK4/6 inhibitor plus RA, compared to treatment with
- 20 each CDK4/6 inhibitor alone (Fig 9F).

Example 12: CDK4/6 inhibitors enhance retinoic acid-induced differentiation in 3D SK-N-BE(2)C spheroids

- 25 Finally, we wanted to assess the effect of CDK4/6 inhibition on 3D tumour spheroids, a more complex, clinically relevant culture system. Spheroids were treated upon reaching 200-400 um in diameter, a size appropriate for drug screening studies, for 7 days. We observed a decrease in spheroid growth upon treatment with any CDK4/6 inhibitor compared to DMSO, enhanced by addition of retinoic acid (Fig 10 A,B). Immunostaining of spheroids for the neuronal marker
- 30 TUBB3 revealed a change in morphology and increase in neurite extension with CDK4/6 inhibitor and CDK4/6i+RA treatment (Fig 10C). Finally, qRT-PCR analysis revealed a consistent pattern of greater increase in STMN4 expression, and a greater decrease in E2F8, PLK1 and FOXM1 expression, upon treatment of spheroids with each CDK4/6 inhibitor plus RA, compared to treatment with each CDK4/6 inhibitor alone (Fig 10D). In summary, we find that CDK4/6
- 35 inhibitors display a class effect in reducing proliferation and inducing neuronal differentiation together with retinoic acid, both in 2D and 3D.

References

1. Cheung, N.-K.V., and Dyer, M.A. (2013). Neuroblastoma: developmental biology, cancer genomics and immunotherapy. *Nat Rev Cancer* 13, 397–411. 10.1038/nrc3526.
2. Zeineldin, M., Patel, A.G., and Dyer, M.A. (2022). Neuroblastoma: When differentiation goes awry. *Neuron* 110, 2916–2928. 10.1016/j.neuron.2022.07.012.
- 5 3. Brodeur, G.M. (2018). Spontaneous regression of neuroblastoma. *Cell Tissue Res* 372, 277–286. 10.1007/s00441-017-2761-2.
4. Liu, Q., Wang, Z., Jiang, Y., Shao, F., Ma, Y., Zhu, M., Luo, Q., Bi, Y., Cao, L., Peng, L., et al. (2022). Single-cell landscape analysis reveals distinct regression trajectories and novel prognostic biomarkers in primary neuroblastoma. *Genes & Diseases*, S2352304222000149.
- 10 10.1016/j.gendis.2021.12.020.
5. Radha, G., and Lopus, M. (2021). The spontaneous remission of cancer: Current insights and therapeutic significance. *Translational Oncology* 14, 101166. 10.1016/j.tranon.2021.101166.
6. Chen, Z., Wang, Z., Pang, J.C., Yu, Y., Bieerkehazhi, S., Lu, J., Hu, T., Zhao, Y., Xu, X., Zhang, H., et al. (2016). Multiple CDK inhibitor dinaciclib suppresses neuroblastoma growth via inhibiting CDK2 and CDK9 activity. *Sci Rep* 6, 29090. 10.1038/srep29090.
- 15 7. de Thé, H., and Chen, Z. (2010). Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. *Nat Rev Cancer* 10, 775–783. 10.1038/nrc2943.
8. Bayeva, N., Coll, E., and Piskareva, O. (2021). Differentiating Neuroblastoma: A Systematic Review of the Retinoic Acid, Its Derivatives, and Synergistic Interactions. *JPM* 11, 211. 10.3390/jpm11030211.
- 20 9. Matthay, K.K., Villablanca, J.G., Seeger, R.C., Stram, D.O., Harris, R.E., Ramsay, N.K., Swift, P., Shimada, H., Black, C.T., Brodeur, G.M., et al. (1999). Treatment of High-Risk Neuroblastoma with Intensive Chemotherapy, Radiotherapy, Autologous Bone Marrow Transplantation, and 13- cis -Retinoic Acid. *N Engl J Med* 341, 1165–1173. 10.1056/NEJM199910143411601.
- 25 10. Peinemann, F., van Dalen, E.C., Enk, H., and Berthold, F. (2017). Retinoic acid postconsolidation therapy for high-risk neuroblastoma patients treated with autologous haematopoietic stem cell transplantation. *Cochrane Database of Systematic Reviews* 2017. 10.1002/14651858.CD010685.pub3.
- 30 11. Reynolds, C.P., Matthay, K.K., Villablanca, J.G., and Maurer, B.J. (2003). Retinoid therapy of high-risk neuroblastoma. *Cancer Letters* 197, 185–192. 10.1016/S0304-3835(03)00108-3.
12. Cohen, L.E., Gordon, J.H., Popovsky, E.Y., Gunawardene, S., Duffey-Lind, E., Lehmann, L.E., and Diller, L.R. (2014). Late effects in children treated with intensive multimodal therapy for high-risk neuroblastoma: High incidence of endocrine and growth problems. *Bone Marrow Transplant* 49, 502–508. 10.1038/bmt.2013.218.
- 35 13. Friedman, D., and Henderson, T. (2018). Late Effects and Survivorship Issues in Patients with Neuroblastoma. *Children* 5, 107. 10.3390/children5080107.

14. Boeva, V., Louis-Brennetot, C., Peltier, A., Durand, S., Pierre-Eugène, C., Raynal, V., Etchevers, H.C., Thomas, S., Lermine, A., Daudigeos-Dubus, E., et al. (2017). Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries. *Nat Genet* 49, 1408–1413. 10.1038/ng.3921.
- 5 15. van Groningen, T., Koster, J., Valentijn, L.J., Zwijnenburg, D.A., Akogul, N., Hasselt, N.E., Broekmans, M., Haneveld, F., Nowakowska, N.E., Bras, J., et al. (2017). Neuroblastoma is composed of two super-enhancer-associated differentiation states. *Nat Genet* 49, 1261–1266. 10.1038/ng.3899.
- 10 16. van Groningen, T., Akogul, N., Westerhout, E.M., Chan, A., Hasselt, N.E., Zwijnenburg, D.A., Broekmans, M., Stroeken, P., Haneveld, F., Hooijer, G.K.J., et al. (2019). A NOTCH feed-forward loop drives reprogramming from adrenergic to mesenchymal state in neuroblastoma. *Nat Commun* 10, 1530. 10.1038/s41467-019-09470-w.
- 15 17. Lecca, M.C., Joonker, M.C., Abdul, U.K., Küçükosmanoglu, A., van Wieringen, W., and Westerman, B.A. (2018). Adrenergic to mesenchymal fate switching of neuroblastoma occurs spontaneously in vivo resulting in differential tumorigenic potential. *J. Mol. Clin. Med.* 1. 10.31083/j.jmcm.2018.04.4221.
18. Sidell, N. (1982). Retinoic Acid-Induced Growth Inhibition and Morphologic Differentiation of Human Neuroblastoma Cells In Vitro. *JNCI: Journal of the National Cancer Institute.* 10.1093/jnci/68.4.589.
- 20 19. Sidell, N., Altman, A., Haussler, M.R., and Seeger, R.C. (1983). Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Experimental Cell Research* 148, 21–30. 10.1016/0014-4827(83)90184-2.
20. Zimmerman, M.W., Durbin, A.D., He, S., Oppel, F., Shi, H., Tao, T., Li, Z., Berezovskaya, A., Liu, Y., Zhang, J., et al. (2021). Retinoic acid rewires the adrenergic core regulatory circuitry of childhood neuroblastoma. *SCIENCE ADVANCES* 7, eabe0834.
- 25 21. Gomez, R.L., Woods, L.M., Ramachandran, R., Abou Tayoun, A.N., Philpott, A., and Ali, F.R. (2022). Super-enhancer associated core regulatory circuits mediate susceptibility to retinoic acid in neuroblastoma cells. *Front. Cell Dev. Biol.* 10, 943924. 10.3389/fcell.2022.943924.
22. Cremisi, F., Philpott, A., and Ohnuma, S. (2003). Cell cycle and cell fate interactions in neural development. *Current Opinion in Neurobiology* 13, 26–33. 10.1016/S0959-4388(03)00005-9.
- 30 23. Hardwick, L.J.A., Ali, F.R., Azzarelli, R., and Philpott, A. (2015). Cell cycle regulation of proliferation versus differentiation in the central nervous system. *Cell Tissue Res* 359, 187–200. 10.1007/s00441-014-1895-8.
- 35 24. Vernon, A.E., Devine, C., and Philpott, A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in *Xenopus*. *Development* 130, 85–92. 10.1242/dev.00193.
25. Poon, E., Liang, T., Jamin, Y., Walz, S., Kwok, C., Hakkert, A., Barker, K., Urban, Z., Thway, K., Zeid, R., et al. (2020). Orally bioavailable CDK9/2 inhibitor shows mechanism-based

- therapeutic potential in MYCN-driven neuroblastoma. *Journal of Clinical Investigation* 130, 5875–5892. 10.1172/JCI134132.
26. Rihani, A., Vandesompele, J., Speleman, F., and Van Maerken, T. (2015). Inhibition of CDK4/6 as a novel therapeutic option for neuroblastoma. *Cancer Cell Int* 15, 76. 10.1186/s12935-015-0224-y.
27. Swadi, R.R., Sampat, K., Herrmann, A., Losty, P.D., See, V., and Moss, D.J. (2019). CDK inhibitors reduce cell proliferation and reverse hypoxia-induced metastasis of neuroblastoma tumours in a chick embryo model. *Sci Rep* 9, 9136. 10.1038/s41598-019-45571-8.
28. Wood, A.C., Krytska, K., Ryles, H.T., Infarinato, N.R., Sano, R., Hansel, T.D., Hart, L.S., King, F.J., Smith, T.R., Ainscow, E., et al. (2017). Dual ALK and CDK4/6 Inhibition Demonstrates Synergy against Neuroblastoma. *Clinical Cancer Research* 23, 2856–2868. 10.1158/1078-0432.CCR-16-1114.
29. Zhang, M., Zhang, L., Hei, R., Li, X., Cai, H., Wu, X., and Zheng, Q. (2021). CDK inhibitors in cancer therapy, an overview of recent development. *Am J Cancer Res* 11, 1913–1935.
30. Rugo, H.S., Finn, R.S., Diéras, V., Ettl, J., Lipatov, O., Joy, A.A., Harbeck, N., Castrellon, A., Iyer, S., Lu, D.R., et al. (2019). Palbociclib plus letrozole as first-line therapy in estrogen receptor-positive/human epidermal growth factor receptor 2-negative advanced breast cancer with extended follow-up. *Breast Cancer Res Treat* 174, 719–729. 10.1007/s10549-018-05125-4.
31. Morikawa, A., and Henry, N.L. (2015). Palbociclib for the Treatment of Estrogen Receptor-Positive, HER2-Negative Metastatic Breast Cancer. *Clinical Cancer Research* 21, 3591–3596. 10.1158/1078-0432.CCR-15-0390.
32. Molenaar, J.J., Ebus, M.E., Koster, J., Sluis, P. van, van Noesel, C.J.M., Versteeg, R., and Caron, H.N. (2008). Cyclin D1 and CDK4 Activity Contribute to the Undifferentiated Phenotype in Neuroblastoma. *Cancer Research* 68, 2599–2609. 10.1158/0008-5472.CAN-07-5032.
33. Calegari, F. (2005). Selective Lengthening of the Cell Cycle in the Neurogenic Subpopulation of Neural Progenitor Cells during Mouse Brain Development. *Journal of Neuroscience* 25, 6533–6538. 10.1523/JNEUROSCI.0778-05.2005.
34. Calegari, F., and Huttner, W.B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. *Journal of Cell Science* 116, 4947–4955. 10.1242/jcs.00825.
35. Wood, A.C., Krytska, K., Ryles, H.T., Infarinato, N.R., Sano, R., Hansel, T.D., Hart, L.S., King, F.J., Smith, T.R., Ainscow, E., et al. (2017). Dual ALK and CDK4/6 Inhibition Demonstrates Synergy against Neuroblastoma. *Clinical Cancer Research* 23, 2856–2868. 10.1158/1078-0432.CCR-16-1114.
36. Upton, K., Modi, A., Patel, K., Kendersky, N.M., Conkrite, K.L., Sussman, R.T., Way, G.P., Adams, R.N., Sacks, G.I., Fortina, P., et al. (2020). Epigenomic profiling of neuroblastoma cell lines. *Sci Data* 7, 116. 10.1038/s41597-020-0458-y.

37. Wang, H.Q., Halilovic, E., Li, X., Liang, J., Cao, Y., Rakiec, D.P., Ruddy, D.A., Jeay, S., Wuerthner, J.U., Timple, N., et al. (2017). Combined ALK and MDM2 inhibition increases antitumor activity and overcomes resistance in human ALK mutant neuroblastoma cell lines and xenograft models. *eLife* 6, e17137. 10.7554/eLife.17137.
- 5 38. Jones, T.A., Flomen, R.H., Senger, G., and Sheer, D. (2000). The homeobox gene MEIS1 is amplified in IMR-32 and highly expressed in other neuroblastoma cell lines. *European Journal of Cancer*.
39. Campos Cogo, S., Gradowski Farias da Costa do Nascimento, T., de Almeida Brehm Pinhatti, F., de França Junior, N., Santos Rodrigues, B., Cavalli, L.R., and Elifio-Esposito, S.
- 10 (2020). An overview of neuroblastoma cell lineage phenotypes and in vitro models. *Exp Biol Med (Maywood)* 245, 1637–1647. 10.1177/1535370220949237.
40. Jove, M., Spencer, J.A., Hubbard, M.E., Holden, E.C., O'Dea, R.D., Brook, B.S., Phillips, R.M., Smye, S.W., Loadman, P.M., and Twelves, C.J. (2019). Cellular Uptake and Efflux of Palbociclib In Vitro in Single Cell and Spheroid Models. *J Pharmacol Exp Ther* 370, 242–251.
- 15 10.1124/jpet.119.256693.
41. Llanos, S., Megias, D., Blanco-Aparicio, C., Hernández-Encinas, E., Rovira, M., Pietrocola, F., and Serrano, M. (2019). Lysosomal trapping of palbociclib and its functional implications. *Oncogene* 38, 3886–3902. 10.1038/s41388-019-0695-8.
42. Topacio, B.R., Zatulovskiy, E., Cristea, S., Xie, S., Tambo, C.S., Rubin, S.M., Sage, J.,
- 20 Kõivomägi, M., and Skotheim, J.M. (2019). Cyclin D-Cdk4,6 Drives Cell-Cycle Progression via the Retinoblastoma Protein's C-Terminal Helix. *Molecular Cell* 74, 758-770.e4. 10.1016/j.molcel.2019.03.020.
43. Connell-Crowley, L., Harper, J.W., and Goodrich, D.W. (1997). Cyclin D1/Cdk4 Regulates Retinoblastoma Protein- mediated Cell Cycle Arrest by Site-specific Phosphorylation. *Molecular*
- 25 *Biology of the Cell* 8.
44. Rader, J., Russell, M.R., Hart, L.S., Nakazawa, M.S., Belcastro, L.T., Martinez, D., Li, Y., Carpenter, E.L., Attiyeh, E.F., Diskin, S.J., et al. (2013). Dual CDK4/CDK6 Inhibition Induces Cell-Cycle Arrest and Senescence in Neuroblastoma. *Clinical Cancer Research* 19, 6173–6182. 10.1158/1078-0432.CCR-13-1675.
- 30 45. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550. 10.1186/s13059-014-0550-8.
46. Seo, B., Yoon, S., and Do, J. (2018). Mitochondrial Dynamics in Stem Cells and Differentiation. *IJMS* 19, 3893. 10.3390/ijms19123893.
47. Durbin, A.D., Zimmerman, M.W., Dharia, N.V., Abraham, B.J., Iniguez, A.B., Weichert-Leahey, N., He, S., Krill-Burger, J.M., Root, D.E., Vazquez, F., et al. (2018). Selective gene
- 35 dependencies in MYCN-amplified neuroblastoma include the core transcriptional regulatory circuitry. *Nat Genet* 50, 1240–1246. 10.1038/s41588-018-0191-z.

48. Gartlgruber, M., Sharma, A.K., Quintero, A., Dreidax, D., Jansky, S., Park, Y.-G., Kreth, S., Meder, J., Doncevic, D., Saary, P., et al. (2021). Super enhancers define regulatory subtypes and cell identity in neuroblastoma. *Nat Cancer* 2, 114–128. 10.1038/s43018-020-00145-w.
49. Creighton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J.,
5 Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21931–21936. 10.1073/pnas.1016071107.
50. Stark, R., and Brown, G. (2022). DiffBind: Differential binding analysis of ChIP-Seq peak data. 75.
- 10 51. Chesler, L., and Weiss, W.A. (2011). Genetically engineered murine models – Contribution to our understanding of the genetics, molecular pathology and therapeutic targeting of neuroblastoma. *Seminars in Cancer Biology* 21, 245–255. 10.1016/j.semcancer.2011.09.011.
52. Weiss, W.A. (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. *The EMBO Journal* 16, 2985–2995. 10.1093/emboj/16.11.2985.
- 15 53. Alleboina, S., Aljouada, N., Miller, M., and Freeman, K.W. (2021). Therapeutically targeting oncogenic CRCs facilitates induced differentiation of NB by RA and the BET bromodomain inhibitor. *Molecular Therapy - Oncolytics* 23, 181–191. 10.1016/j.omto.2021.09.004.
54. Hardwick, L.J.A., and Philpott, A. (2014). Nervous decision-making: to divide or differentiate. *Trends in Genetics* 30, 254–261. 10.1016/j.tig.2014.04.001.
- 20 55. Jahangiri, L., Pucci, P., Ishola, T., Pereira, J., Cavanagh, M.L., and Turner, S.D. (2021). Deep analysis of neuroblastoma core regulatory circuitries using online databases and integrated bioinformatics shows their pan-cancer roles as prognostic predictors. *Discov Onc* 12, 56. 10.1007/s12672-021-00452-3.
56. Vinci, M., Gowan, S., Boxall, F., Patterson, L., Zimmermann, M., Court, W., Lomas, C.,
25 Mendiola, M., Hardisson, D., and Eccles, S.A. (2012). Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol* 10, 29. 10.1186/1741-7007-10-29.
57. Wagner, V., and Gil, J. (2020). Senescence as a therapeutically relevant response to CDK4/6 inhibitors. *Oncogene* 39, 5165–5176. 10.1038/s41388-020-1354-9.
- 30 58. Rawson, C., Loo, D., Helmrich, A., Ernst, T., Natsuno, T., Merrill, G., and Barnes, D. (1991). Serum inhibition of proliferation of serum-free mouse embryo cells. *Experimental Cell Research* 192, 271–277. 10.1016/0014-4827(91)90186-X.
59. Georger, B., Bourdeaut, F., DuBois, S.G., Fischer, M., Geller, J.I., Gottardo, N.G., Marabelle, A., Pearson, A.D.J., Modak, S., Cash, T., et al. (2017). A Phase I Study of the CDK4/6
35 Inhibitor Ribociclib (LEE011) in Pediatric Patients with Malignant Rhabdoid Tumors, Neuroblastoma, and Other Solid Tumors. *Clinical Cancer Research* 23, 2433–2441. 10.1158/1078-0432.CCR-16-2898.

60. Tripathy, D., Bardia, A., and Sellers, W.R. (2017). Ribociclib (LEE011): Mechanism of Action and Clinical Impact of This Selective Cyclin-Dependent Kinase 4/6 Inhibitor in Various Solid Tumors. *Clin Cancer Res* 23, 3251–3262. 10.1158/1078-0432.CCR-16-3157.
61. Wick, M.R. (2000). Neuroendocrine Neoplasia. *American Journal of Clinical Pathology* 5 113, 331–335. 10.1309/ETJ3-QBUK-13QD-J8FP.
62. Kunz, P.L., Reidy-Lagunes, D., Anthony, L.B., Bertino, E.M., Brendtro, K., Chan, J.A., Chen, H., Jensen, R.T., Kim, M.K., Klimstra, D.S., et al. (2013). Consensus Guidelines for the Management and Treatment of Neuroendocrine Tumors. *Pancreas* 42, 557–577. 10.1097/MPA.0b013e31828e34a4.
- 10 63. Navalkele, P., O'Dorisio, M.S., O'Dorisio, T.M., Zamba, G.K.D., and Lynch, C.F. (2011). Incidence, survival, and prevalence of neuroendocrine tumors versus neuroblastoma in children and young adults: Nine standard SEER registries, 1975-2006: Neuroendocrine Tumors in Children, SEER Data. *Pediatr. Blood Cancer* 56, 50–57. 10.1002/pbc.22559.
64. de Thé, H. (2018). Differentiation therapy revisited. *Nat Rev Cancer* 18, 117–127. 15 10.1038/nrc.2017.103.
65. Zhu, A., Ibrahim, J.G., and Love, M.I. (2019). Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics* 35, 2084–2092. 10.1093/bioinformatics/bty895.
66. Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. 10.1093/bioinformatics/btq033. 20
67. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9, 357–359. 10.1038/nmeth.1923.
68. Park, S., Ahn, E.S., and Kim, Y. (2015). Neuroblastoma SH-SY5Y cell-derived exosomes stimulate dendrite-like outgrowths and modify the differentiation of A375 melanoma cells: 25 Neuroblastoma SH-SY5Y cell-derived exosomes. *Cell Biol Int* 39, 379–387. 10.1002/cbin.10401.
69. Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The Innovation* 2, 100141. 10.1016/j.xinn.2021.100141.
- 30 70. Gu, Z. (2022). Complex heatmap visualization. *iMeta* 1. 10.1002/imt2.43.
71. Ramírez, F., Ryan, D.P., Grüning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., Heyne, S., Dündar, F., and Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* 44, W160–W165. 10.1093/nar/gkw257.
72. Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose 35 program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. 10.1093/bioinformatics/btt656.
73. Thorvaldsdottir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in Bioinformatics* 14, 178–192. 10.1093/bib/bbs017.

74. Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., et al. (2008). Model-based Analysis of ChIP-Seq (MACS). *Genome Biol* 9, R137. 10.1186/gb-2008-9-9-r137.
75. Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: summarize
5 analysis results for multiple tools and samples in a single report. *Bioinformatics* 32, 3047–3048. 10.1093/bioinformatics/btw354.
76. Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes. *Cell* 153, 307–319. 10.1016/j.cell.2013.03.035.
- 10 77. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. 10.1093/bioinformatics/btp352.
78. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P.,
15 Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. 10.1093/bioinformatics/bts635.
79. Zerbino, D.R., Johnson, N., Juettemann, T., Wilder, S.P., and Flicek, P. (2014). WiggleTools: parallel processing of large collections of genome-wide datasets for visualization and statistical analysis. *Bioinformatics* 30, 1008–1009. 10.1093/bioinformatics/btt737.
- 20 80. Schmidt, D., Wilson, M.D., Spyrou, C., Brown, G.D., Hadfield, J., and Odom, D.T. (2009). ChIP-seq: Using high-throughput sequencing to discover protein–DNA interactions. *Methods* 48, 240–248. 10.1016/j.ymeth.2009.03.001.
81. Yu, G., Wang, L.-G., and He, Q.-Y. (2015). ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31, 2382–2383.
25 10.1093/bioinformatics/btv145.

CLAIMS

1. A CDK4/6 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4/6 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.
2. The CDK4/6 inhibitor for use according to claim 1, wherein the ADRN-type neuroblastoma cells are characterised by expression of one or more of MYCN, ALK, NTRK and segmental chromosomal abnormalities.
3. The CDK4/6 inhibitor for use according to claims 1 or 2, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.
4. The CDK4/6 inhibitor for use according to any preceding claim, wherein the neuroblastoma is selected from high risk MYCN-driven neuroblastoma, MYCN-amplified neuroblastoma, non-amplified neuroblastoma, ADRN-type high risk tumours, ADRN-type intermediate risk tumours, MYCN-amplified ADRN-type high risk tumours, MYCN-amplified ADRN-type intermediate risk tumours, ALK-mutated ADRN-type high risk tumours, ALK-mutated ADRN-type intermediate risk tumours.
5. The CDK4/6 inhibitor for use according to any preceding claim, wherein the CDK4/6 inhibitor is selected from palbociclib, ribociclib and/or abemaciclib.
6. The CDK4/6 inhibitor for use according to any preceding claim, wherein the CDK4/6 inhibitor is administered at a dose of between 0.1 to 200 mg/kg, 1 to 100 mg/kg, 5 to 80 mg/kg, preferably 20 to 60mg/kg, more preferably approximately 40 mg/kg.
7. The CDK4/6 inhibitor for use according to any preceding claim, wherein the retinoic acid is cis-retinoic acid or trans-retinoic acid .
8. The CDK4/6 inhibitor for use according to any preceding claim, wherein the retinoic acid is administered at a dose of 1 to 500 mg/m²/day, 10 to 400 mg/m²/day, 20 to 300 mg/m²/day, 50 to 200 mg/m²/day.
9. The CDK4/6 inhibitor for use according to any preceding claim, wherein the CDK4/6 inhibitor is administered simultaneously, sequentially or separately with the retinoic acid.
10. The CDK4/6 inhibitor for use according to any preceding claim, wherein the CDK4/6 inhibitor and retinoic acid are used in combination with a further anti-cancer therapy.
11. The CDK4/6 inhibitor for use according to any preceding claim, wherein the further anti-cancer therapy is selected from chemotherapy, radiotherapy, surgery, immunotherapy, hormone therapy myeloablative therapy (MAT) and/or autologous stem cell rescue (ASCR)..
12. The CDK4/6 inhibitor for use according to any preceding claim, wherein the subject received one or more anti-cancer agents prior to the administration of the CDK4/6 inhibitor.
13. The CDK4/6 inhibitor for use according to any preceding claim, wherein the neuroblastoma is identified as ADRN-type neuroblastoma.

14. The CDK4/6 inhibitor for use according to any preceding claim, wherein the CDK4/6 inhibitor is used at a cytostatic dose.
15. A CDK4/6 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises:
 - screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
 - identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
 - administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.
16. A method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4/6 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic(ADRN)-type neuroblastoma cells.
17. A method of treating neuroblastoma in a subject, comprising:
 - screening a biological sample obtained from said subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
 - identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
 - administering to said subject a CDK4/6 inhibitor in combination with retinoic acid.
18. The method of claims 15 to 17, wherein the ADRN-type neuroblastoma cells are further characterised by expression of one or more of ASCL1, PHOX2B, GATA3 and/or HAND2.
19. A composition comprising:
 - a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
 - retinoic acid or a pharmaceutically acceptable salt thereof,for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.
20. A composition comprising:
 - a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
 - retinoic acid or a pharmaceutically acceptable salt thereof, and
 - a pharmaceutically acceptable excipient.
21. Use of a CDK4/6 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic cells.
22. An *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising:
 - contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4/6 inhibitor in combination with retinoic acid.
23. An *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4/6 inhibitor in combination with retinoic acid comprising:
 - screening a biological sample obtained from a subject with neuroblastoma, for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,

identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities, selecting said subject for therapy.

24. A kit for the treatment of neuroblastoma comprising:
a CDK4/6 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.
25. A method of prognosis or therapy monitoring comprising:
providing a biological sample obtained from a subject with neuroblastoma who has received treatment,
screening said biological sample for one or more of the following features:
increased expression of one or more of RARA, RARB, SOX4 and/or MEIS1,
changes in H3K27ac deposition around one or more of RARA, RARB, SOX4 and/or MEIS1, or
decreased expression of ASCL1 and/or HAND1.
26. A method of prognosis or therapy monitoring comprising:
providing a biological sample obtained from a subject with neuroblastoma who has received treatment,
screening said biological sample for alteration in the expression of one or more of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum LDH level .
27. The method of any of claims 25 to 26, further comprising a step of comparing said biological sample with a biological sample obtained from said patient prior to therapy.
28. The method of any of claims 26 to 27, wherein the downregulation of Ki67, E2F1, E2F2 and/or E2F8 is indicative of survival or response to therapy.
29. The method of any of claims 26 to 28, wherein the upregulation of NTRK1 (TrkA), STMN4, STMN2 and/or serum LDH level is indicative of survival or response to therapy.
30. A kit comprising a plurality of binding agents, wherein each of said binding agents specifically binds to a distinct biomarker protein that is selectively upregulated or downregulated in neuroblastoma, wherein said biomarker proteins are selected from the group consisting of Ki67, E2F1, E2F2, E2F8, STMN4, STMN2, NTRK1 (TrkA), and/or serum LDH.
31. A CDK4 inhibitor for use in the treatment of neuroblastoma, wherein the CDK4 inhibitor is administered in combination with retinoic acid and wherein said neuroblastoma comprises adrenergic (ADRN)-type neuroblastoma cells.
32. A CDK4 inhibitor for use in a method of treating of neuroblastoma wherein the method comprises:
screening a biological sample obtained from a subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,

administering to said subject a CDK4 inhibitor in combination with retinoic acid.

33. A method of treating neuroblastoma in a subject, comprising administering to the subject a CDK4 inhibitor in combination with retinoic acid, wherein said neuroblastoma comprises adrenergic(ADRN)-type neuroblastoma cells.
34. A method of treating neuroblastoma in a subject, comprising:
screening a biological sample obtained from said subject for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
administering to said subject a CDK4 inhibitor in combination with retinoic acid.
35. A composition comprising:
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
for use in the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic-type cells.
36. A composition comprising:
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof, and
a pharmaceutically acceptable excipient.
37. Use of a CDK4 inhibitor and retinoic acid for the manufacture of a medicament for the treatment of neuroblastoma, wherein the neuroblastoma comprises adrenergic cells.
38. An *in vitro*, *in vivo* or *ex vivo* method of stimulating cell differentiation of ADRN-type neuroblastoma cells comprising:
contacting a biological sample comprising an ADRN-type neuroblastoma cell with a CDK4 inhibitor in combination with retinoic acid.
39. An *in vitro* or *ex vivo* method of identifying a subject as suitable for therapy with a CDK4 inhibitor in combination with retinoic acid comprising:
screening a biological sample obtained from a subject with neuroblastoma, for expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
identifying said subject as having ADRN-type neuroblastoma based on the expression of one or more of MYCN, ALK and segmental chromosomal abnormalities,
selecting said subject for therapy.
40. A kit for the treatment of neuroblastoma comprising:
a CDK4 inhibitor or a pharmaceutically acceptable salt thereof, and
retinoic acid or a pharmaceutically acceptable salt thereof,
wherein the neuroblastoma comprises ADRN-type neuroblastoma cells.

Figure 1 A-C

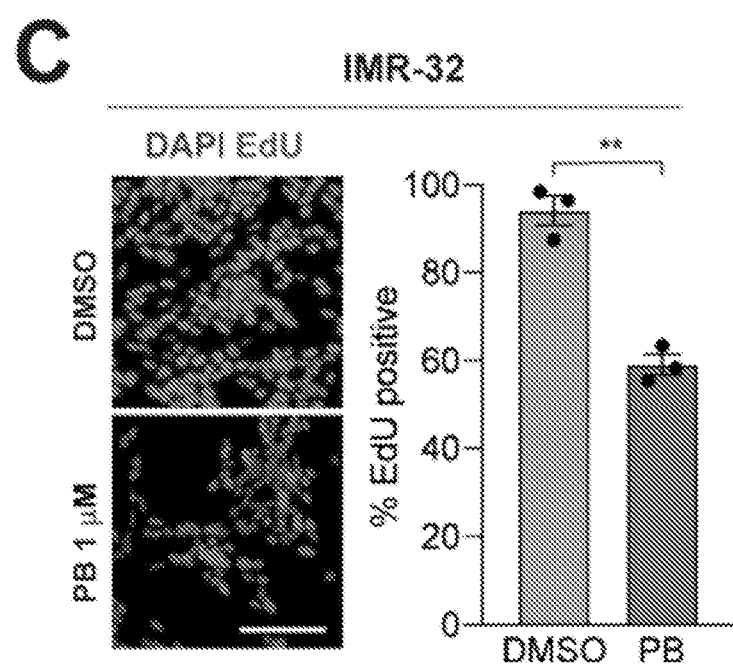
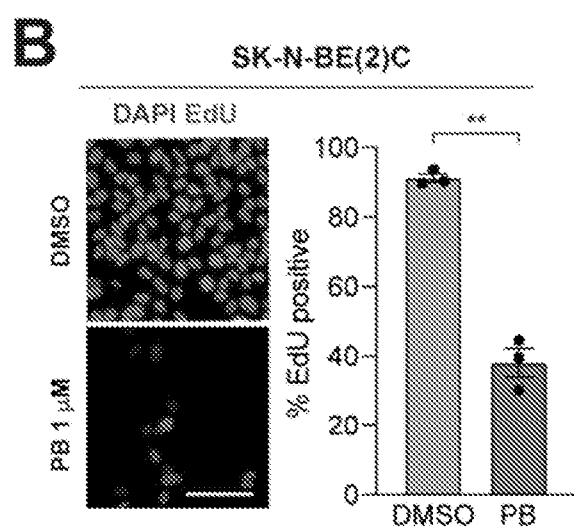
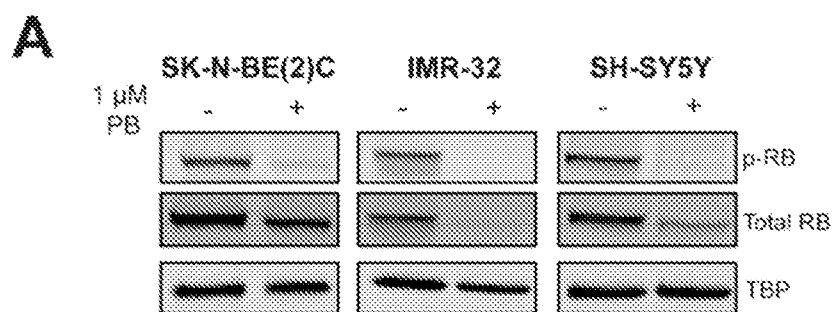


Figure 1 D-E

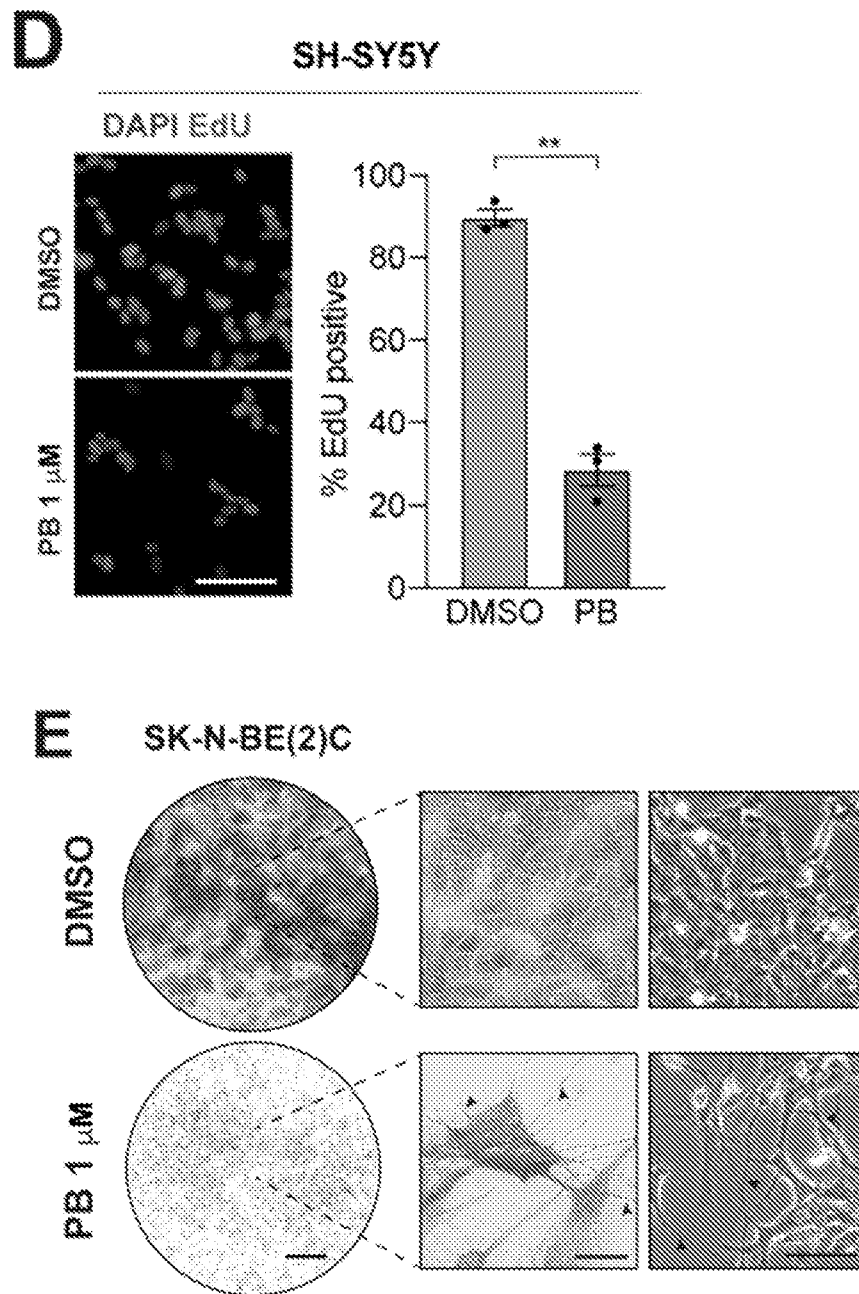


Figure 1 F-G

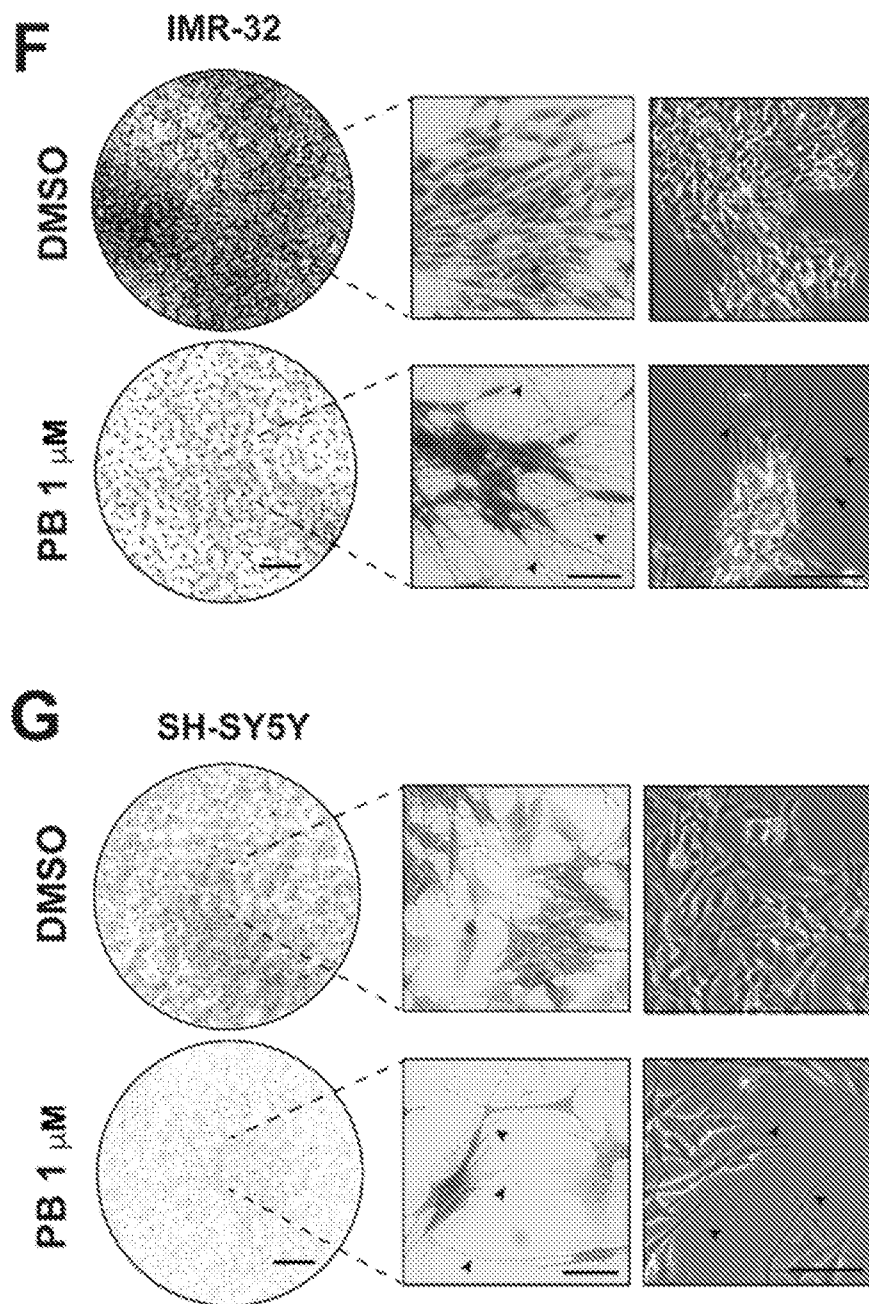


Figure 1 H-J

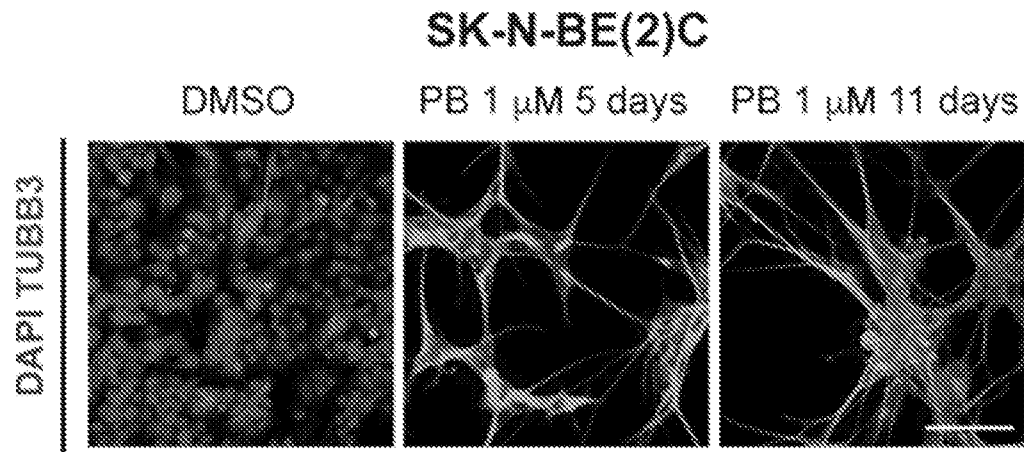
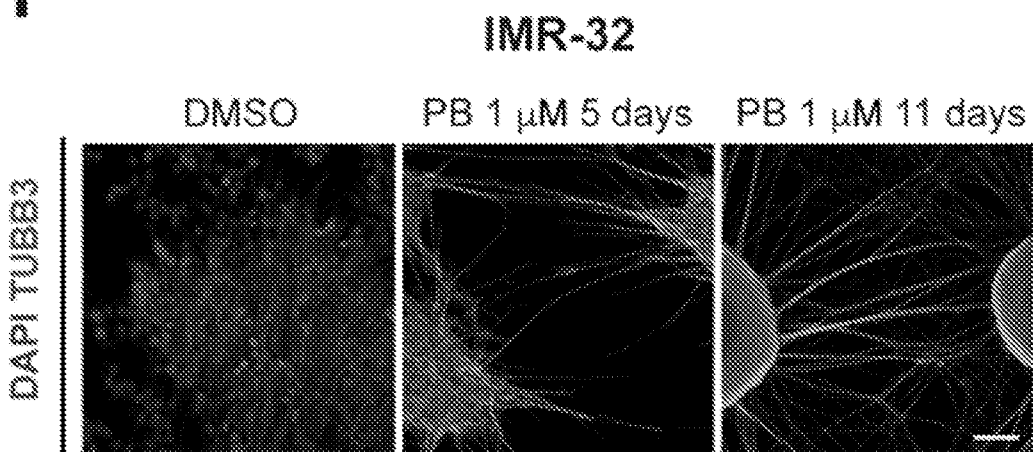
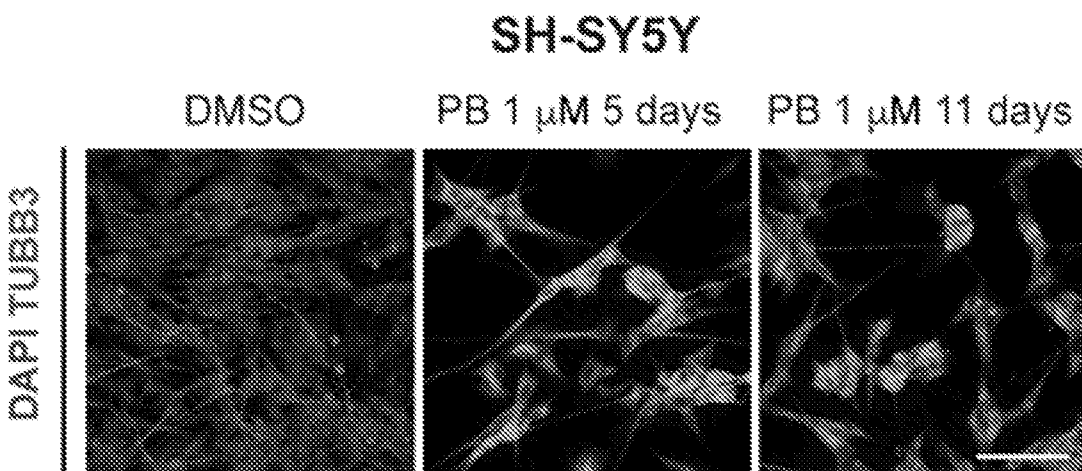
H**I****J**

Figure 2 A

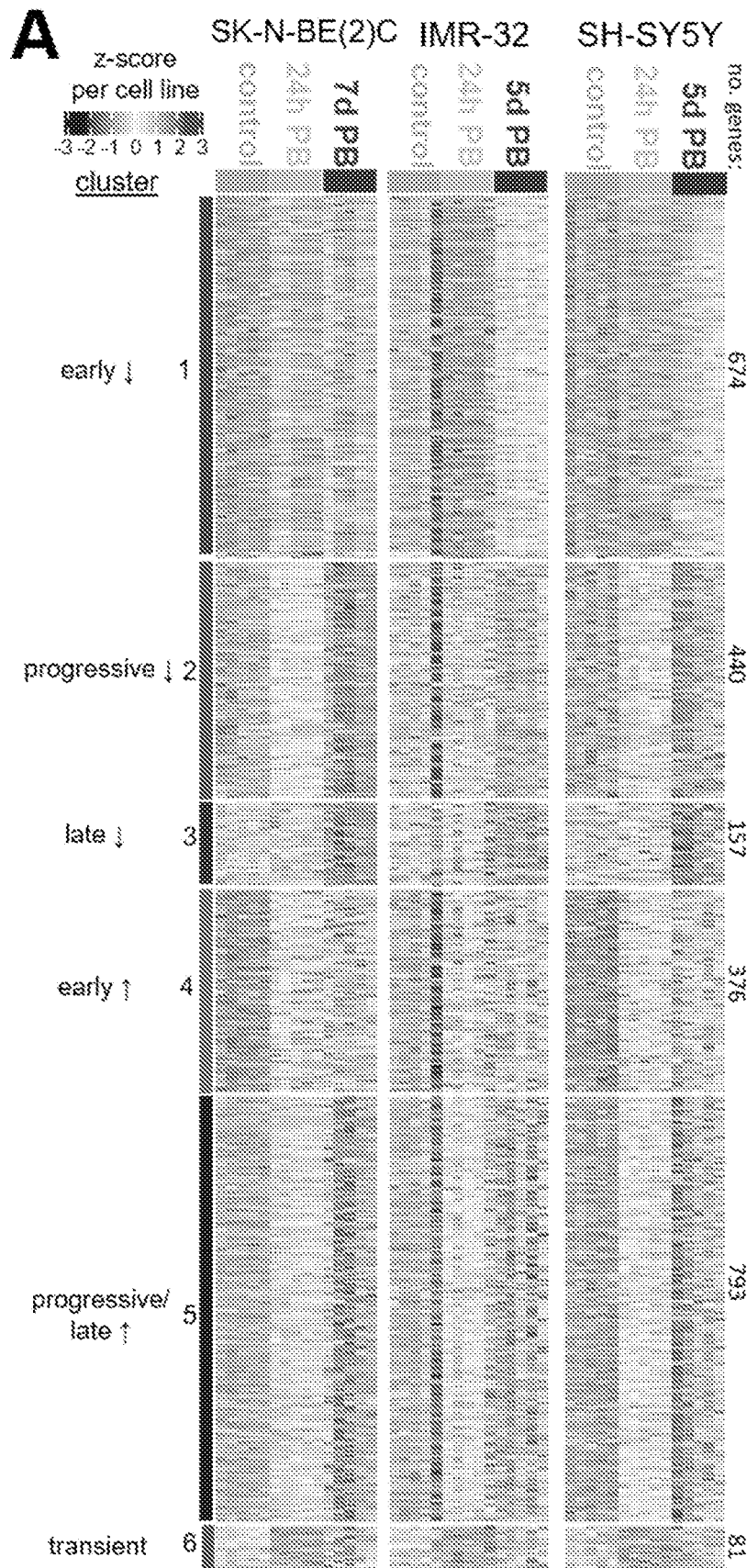


Figure 2B

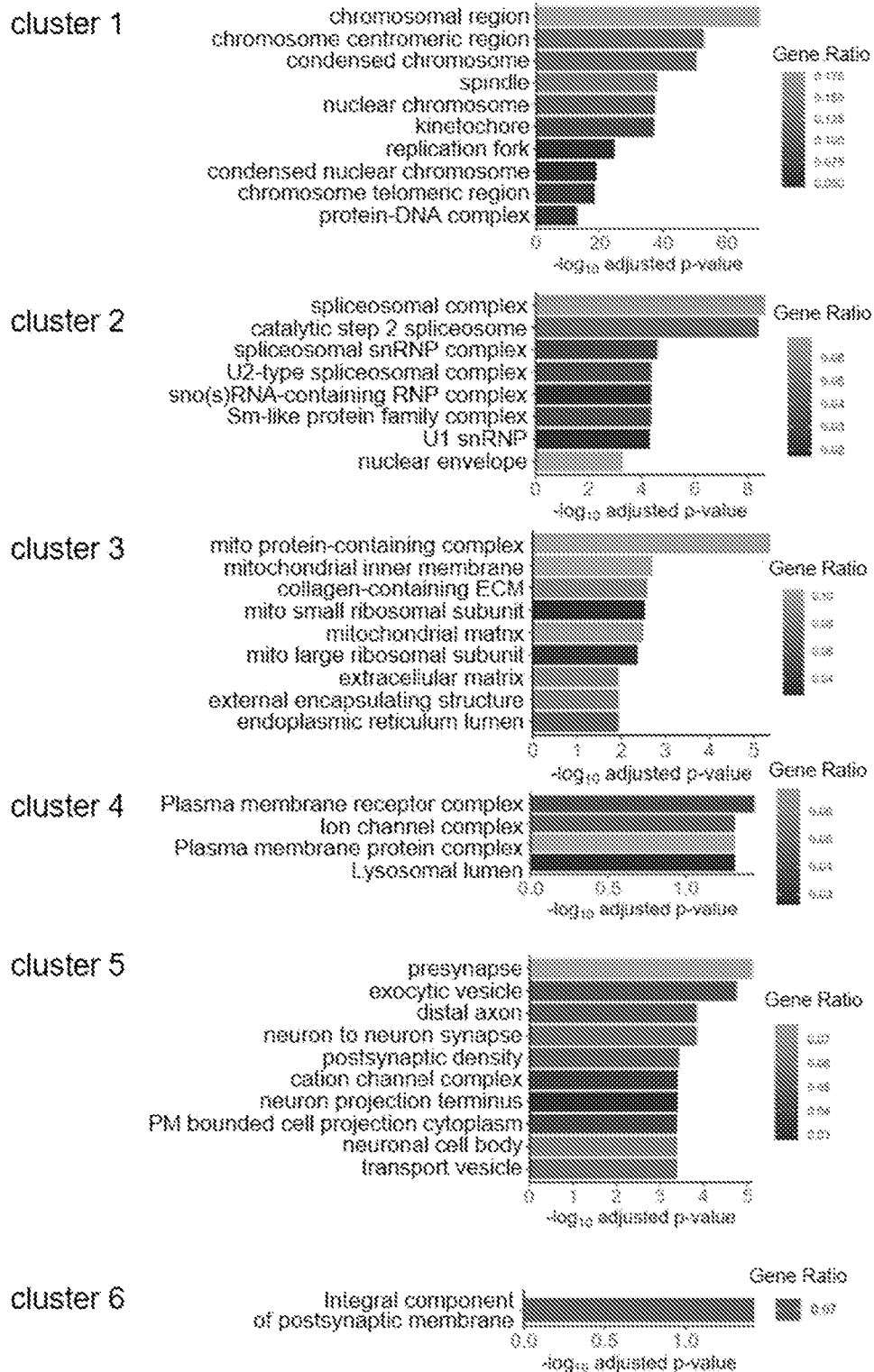
B**GO: Cellular Component**

Figure 3A

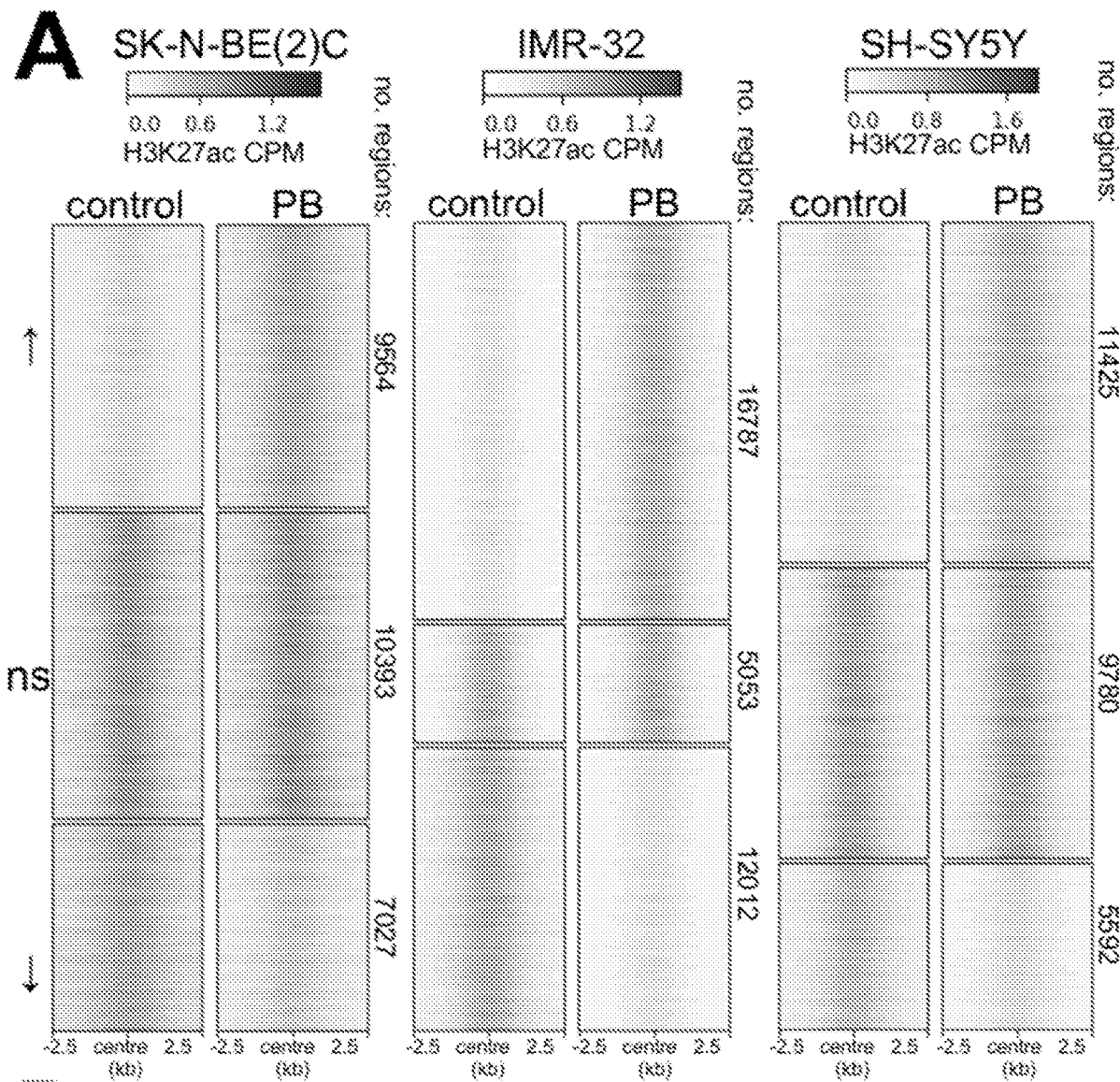


Figure 3 B-C

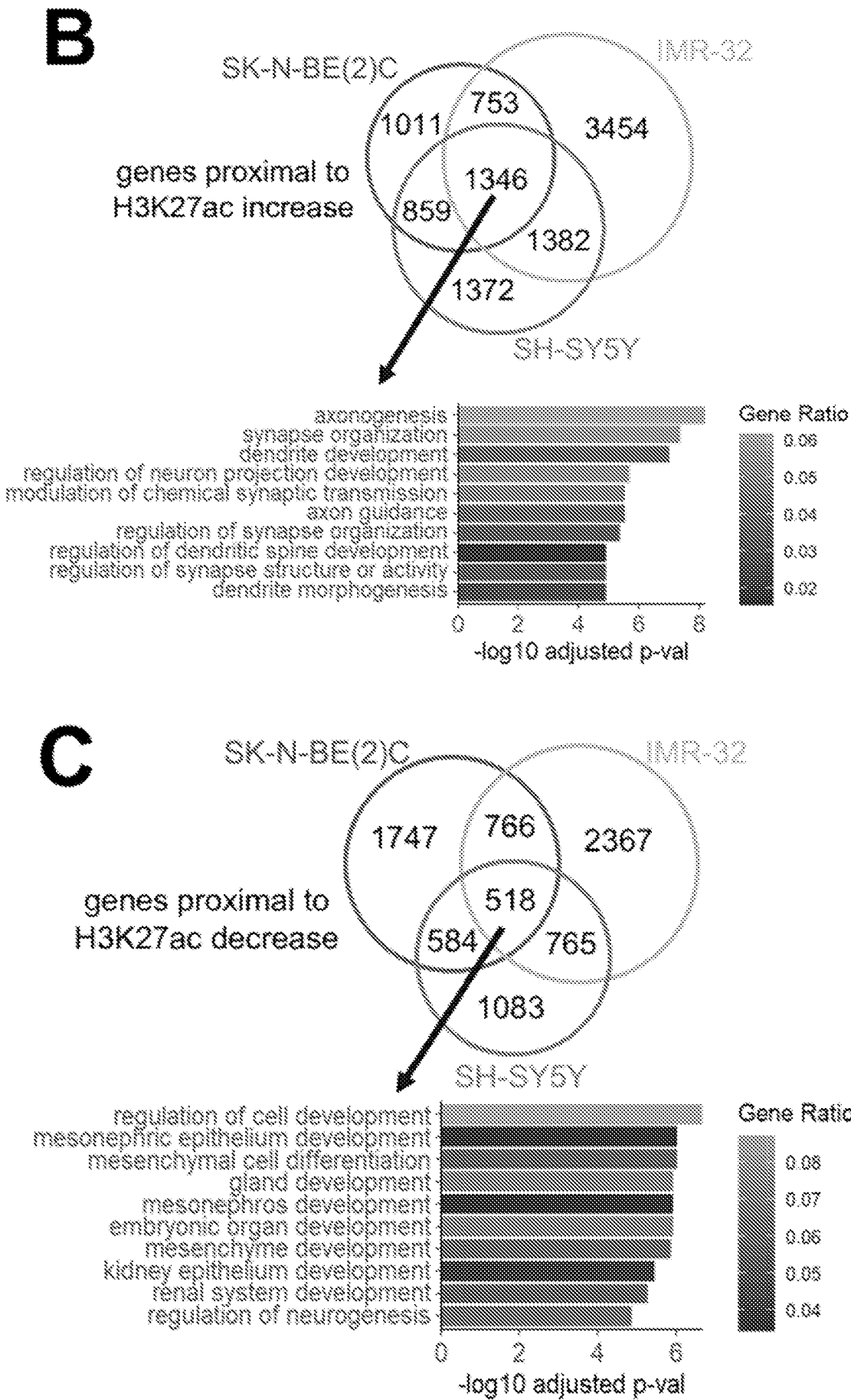


Figure 3D

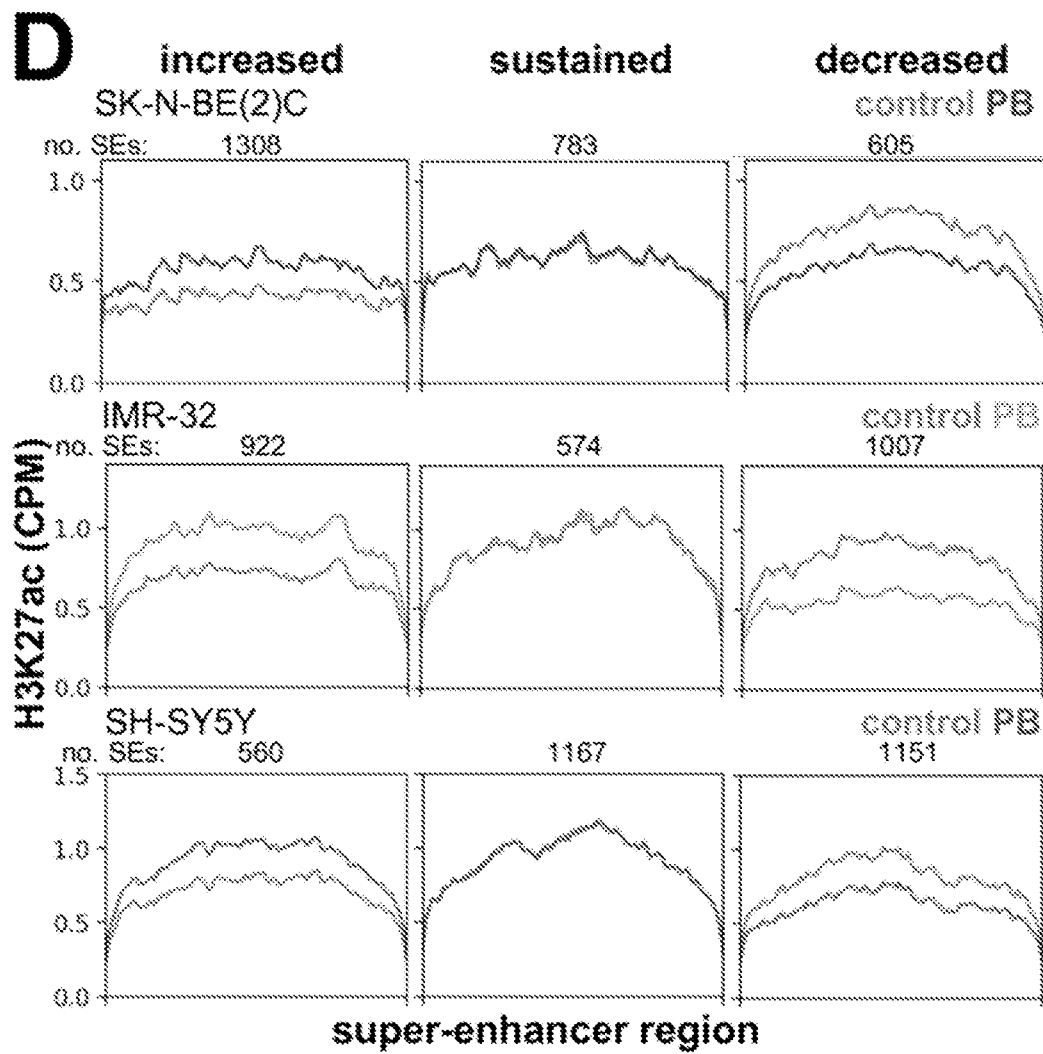


Figure 3E



Figure 4 A-F

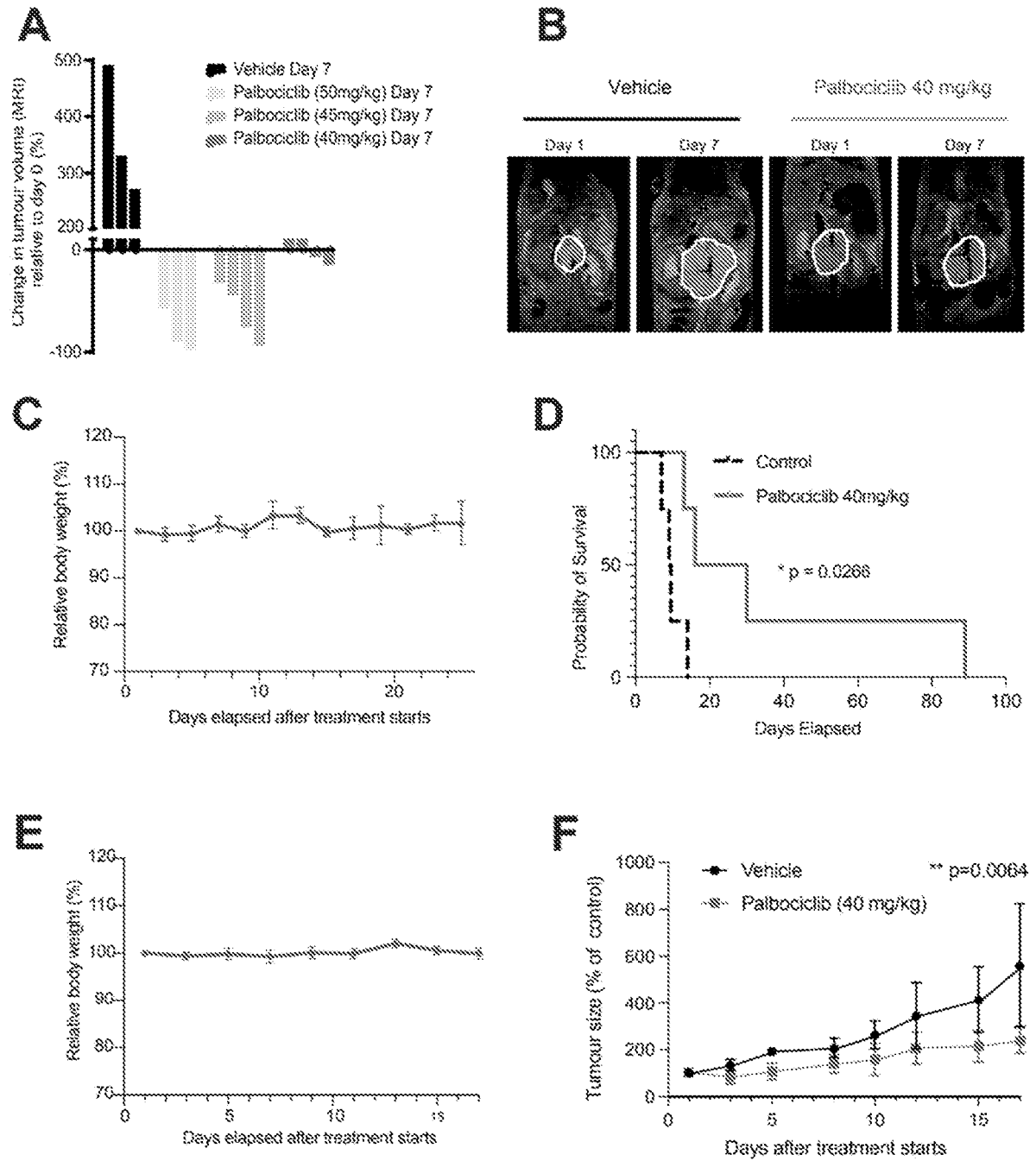


Figure 5 A-D

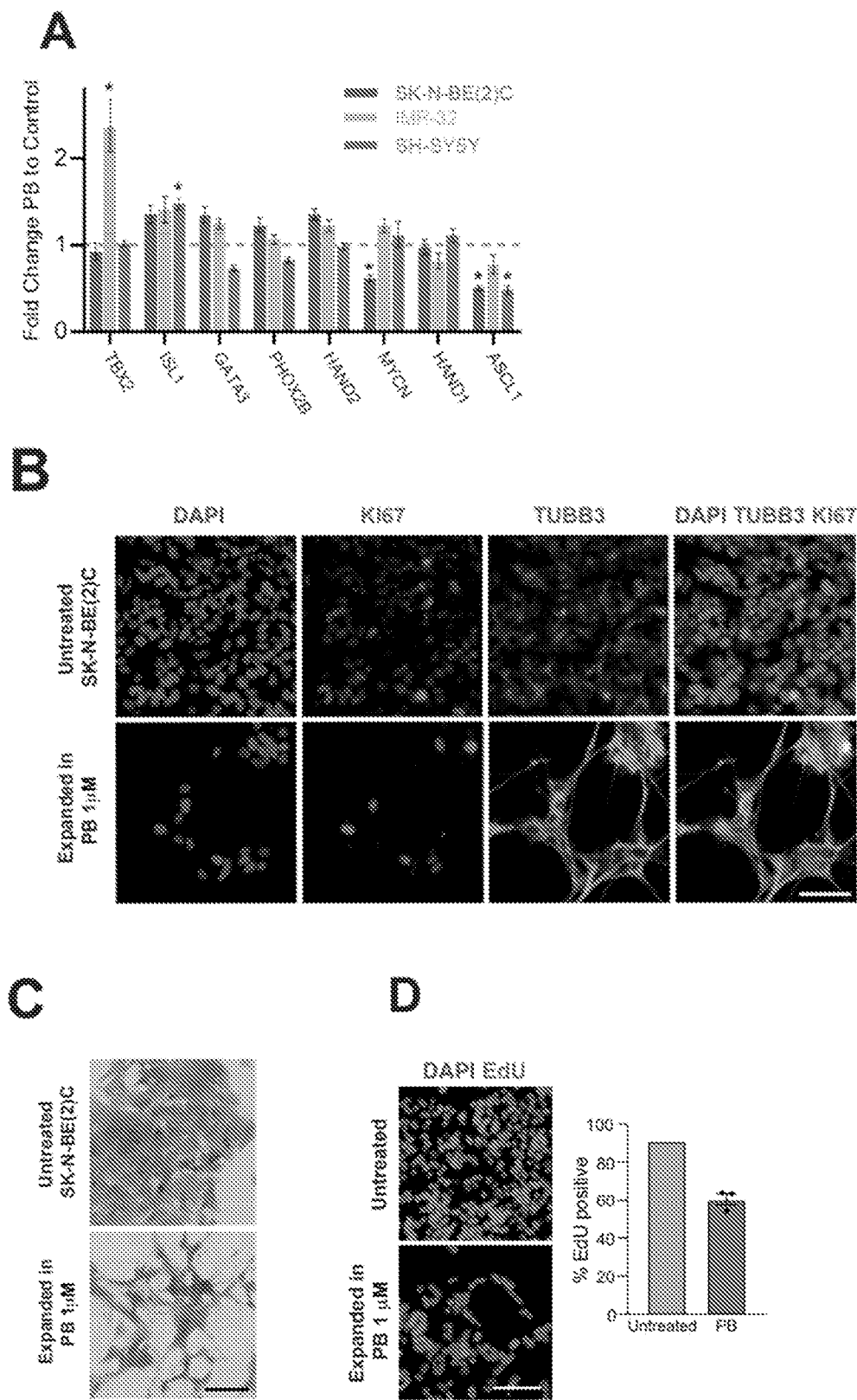


Figure 5 E-G

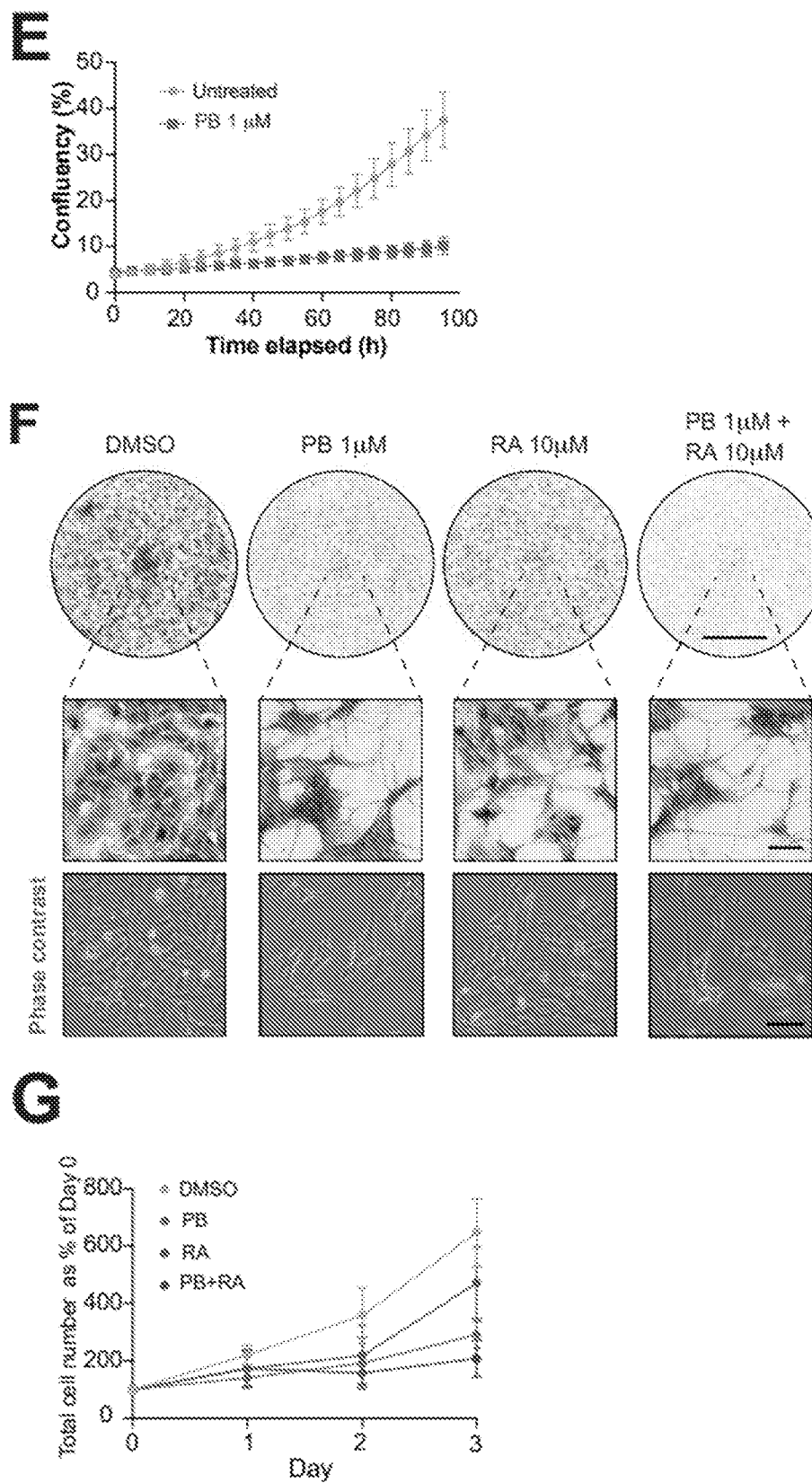


Figure 5H

H

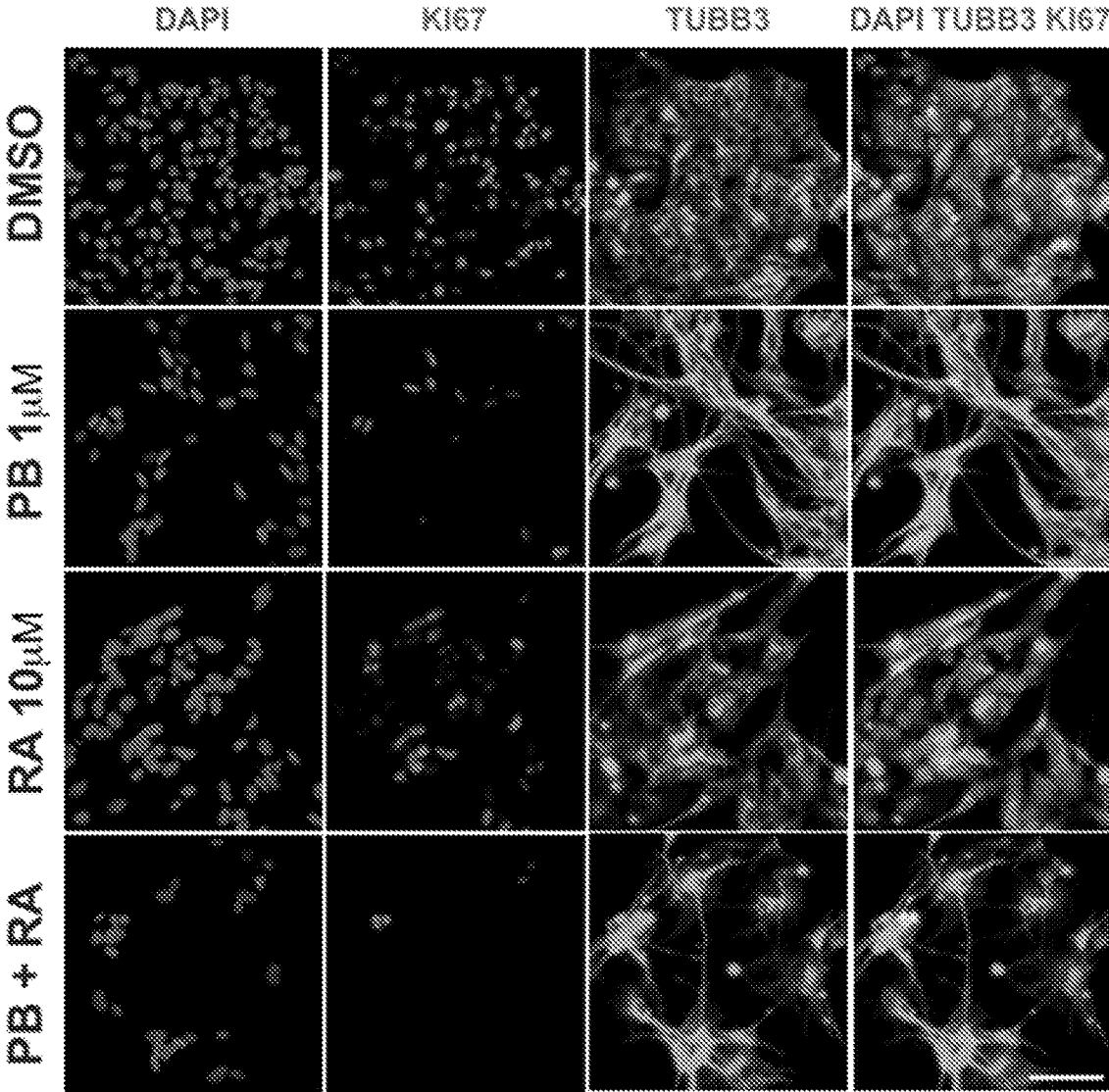


Figure 5 I-K

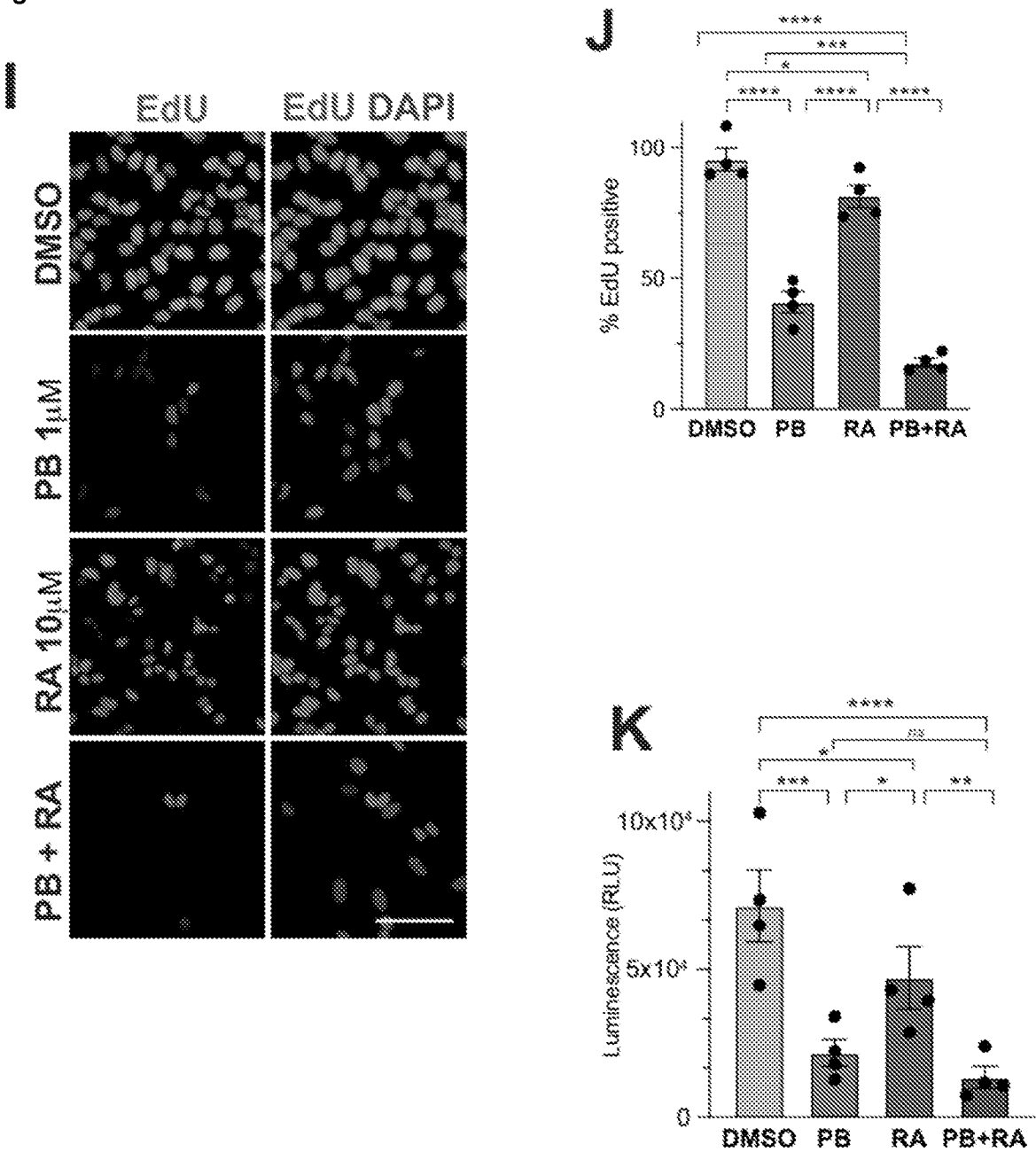


Figure 6 A-C

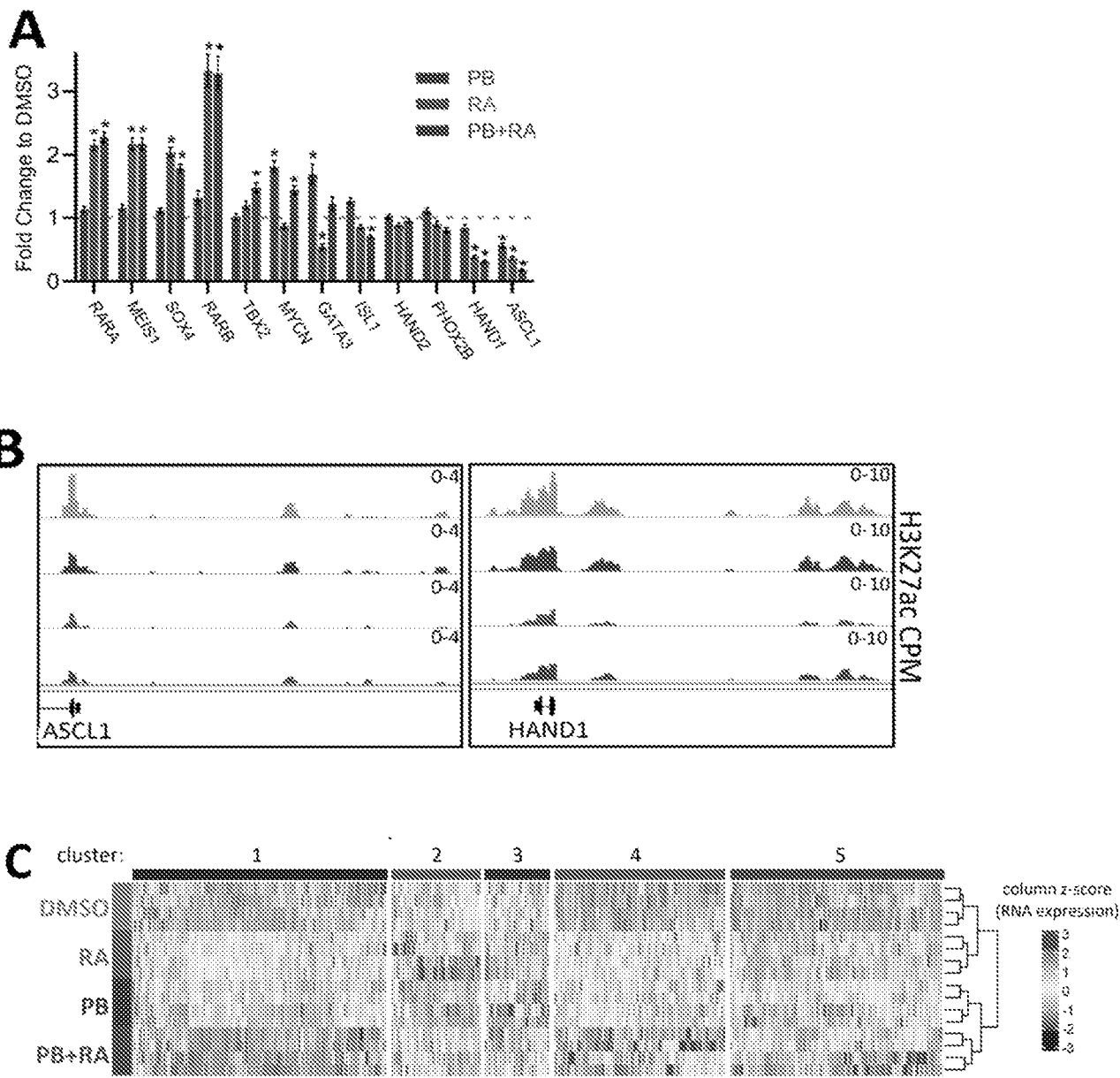


Figure 6 D-F

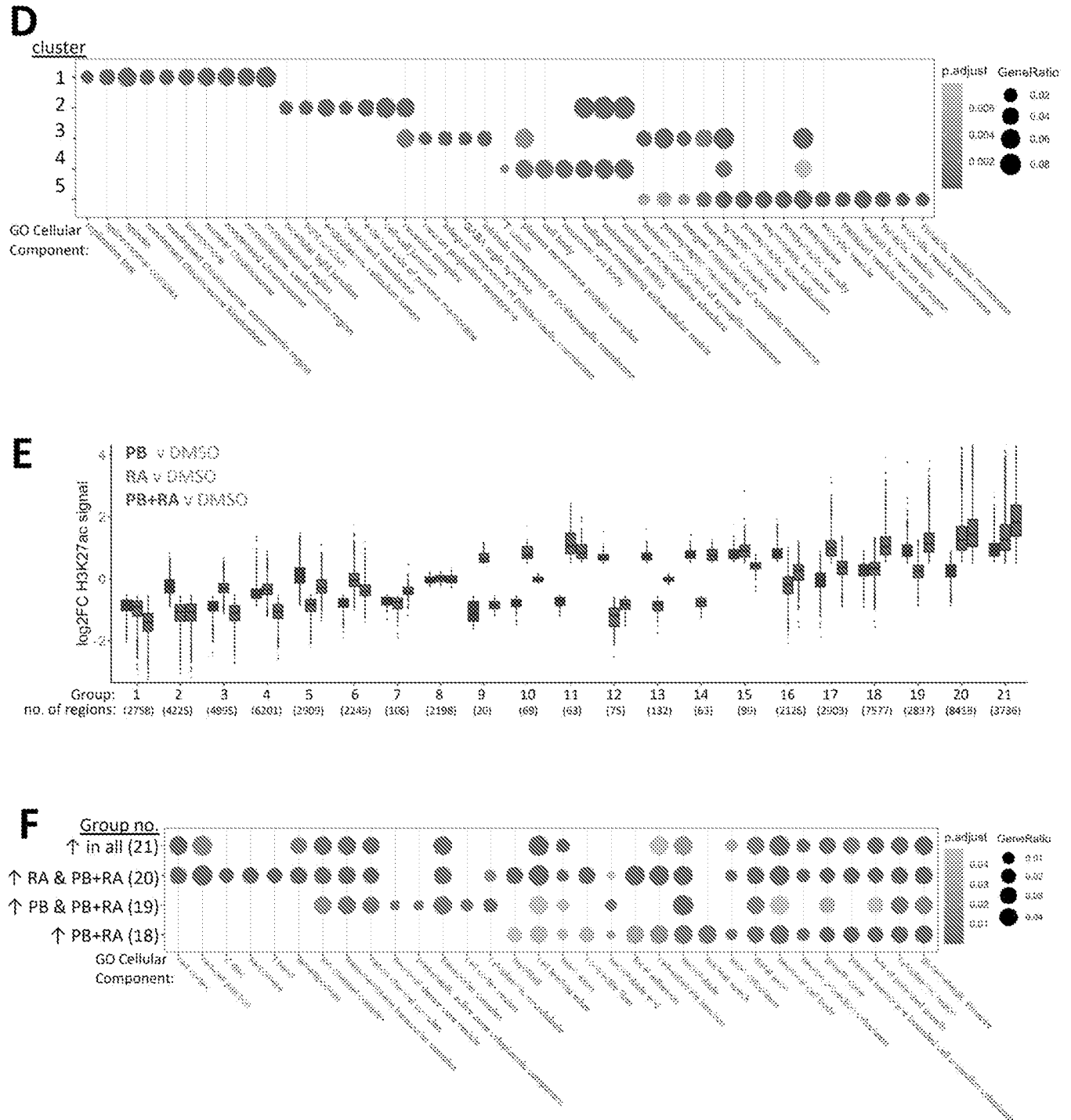


Figure 7 A-B

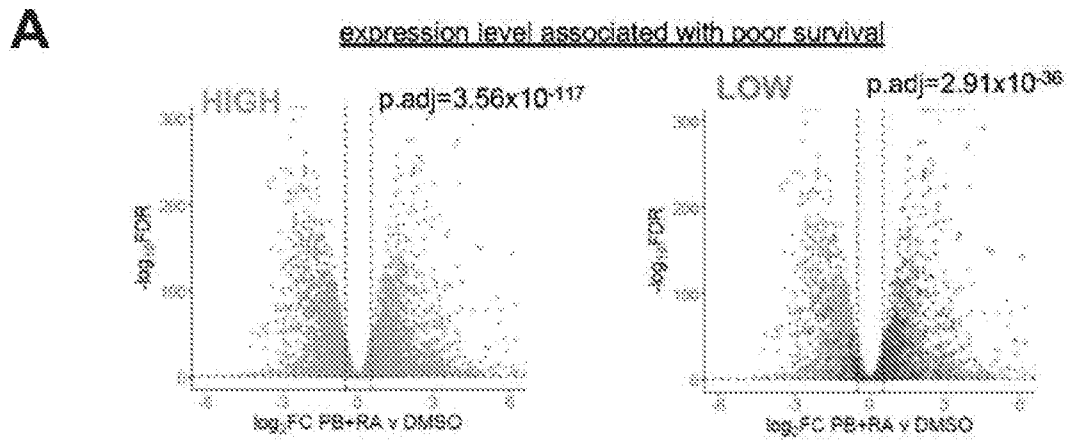
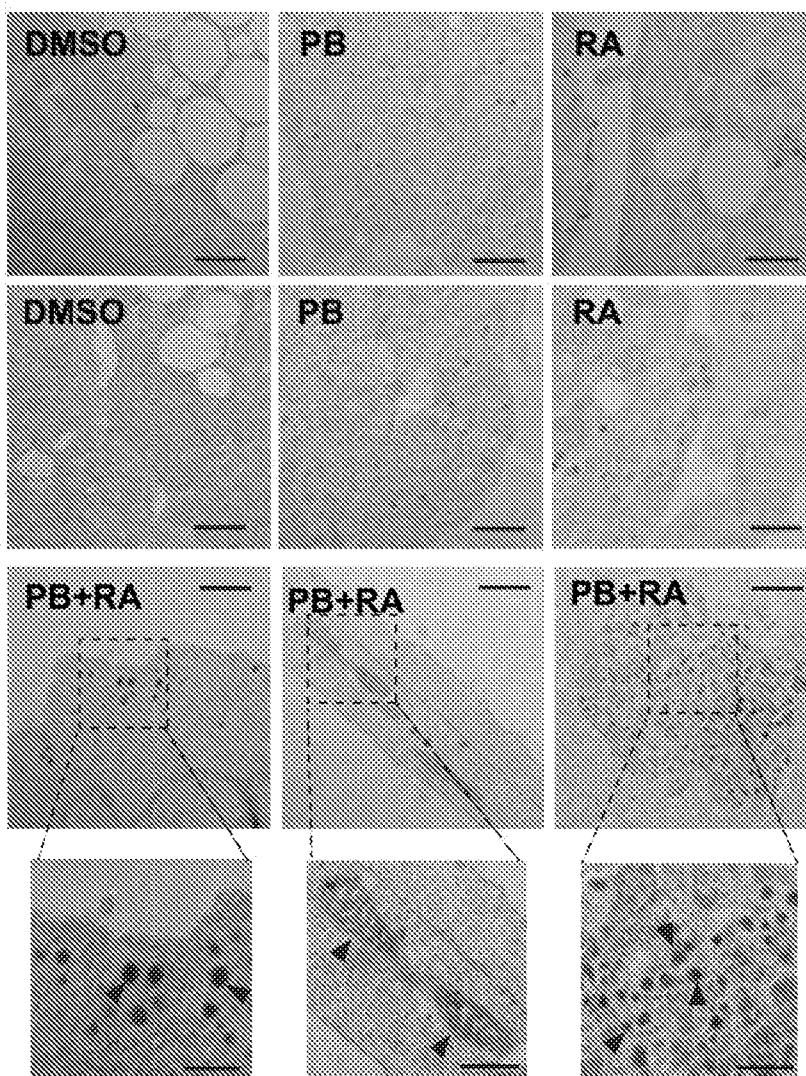
**B**

Figure 7 C-D

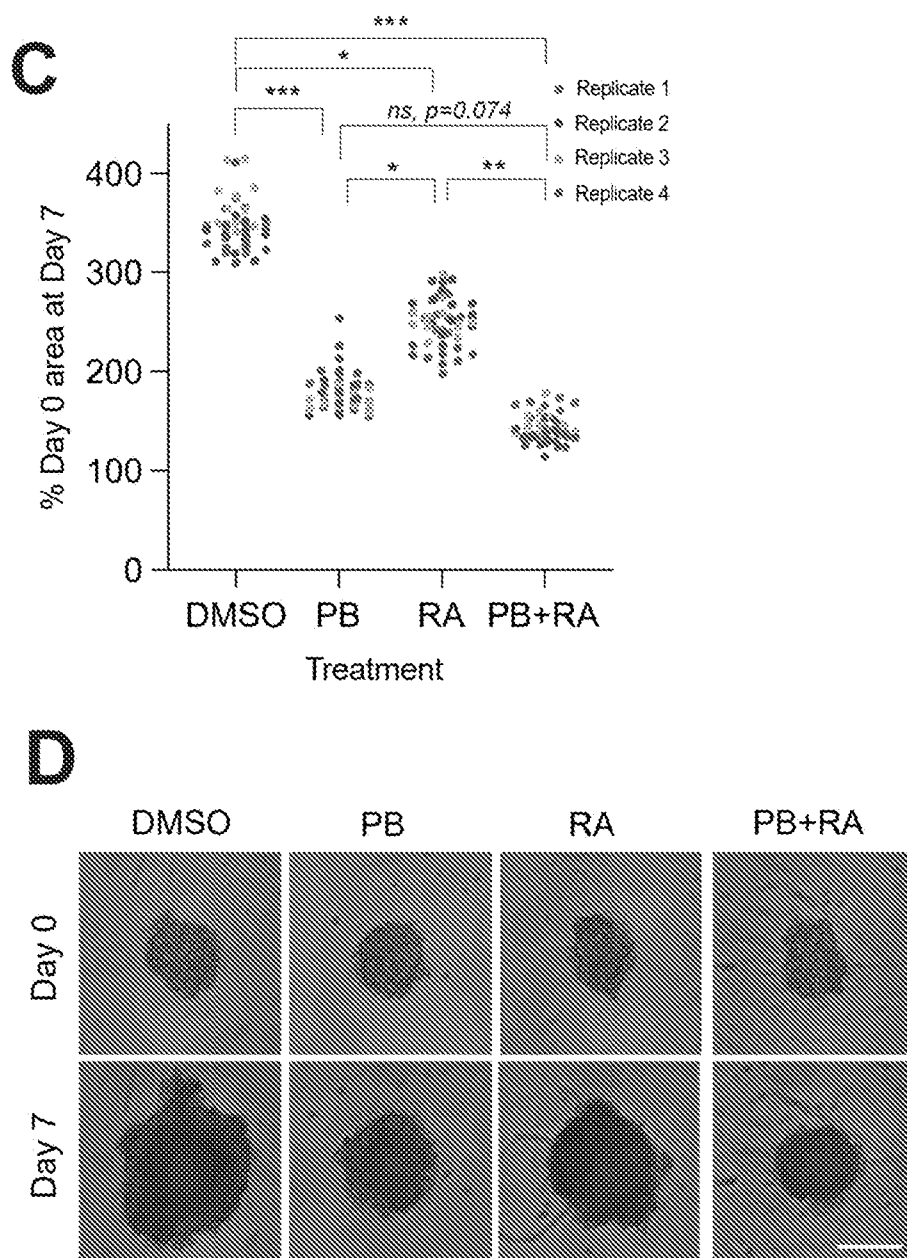


Figure 7 E-G

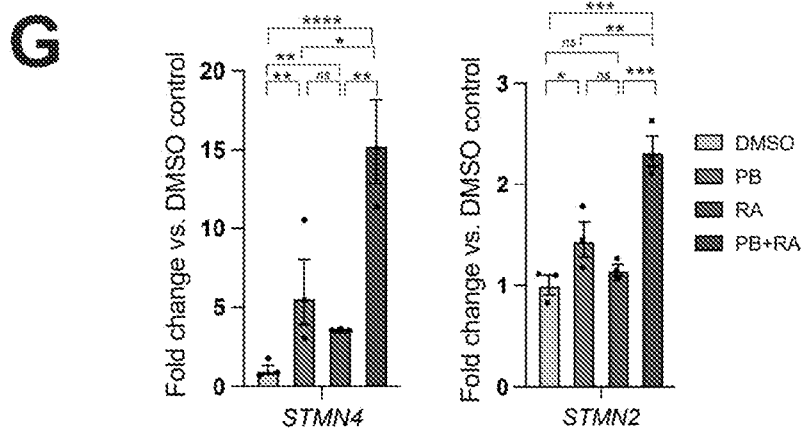
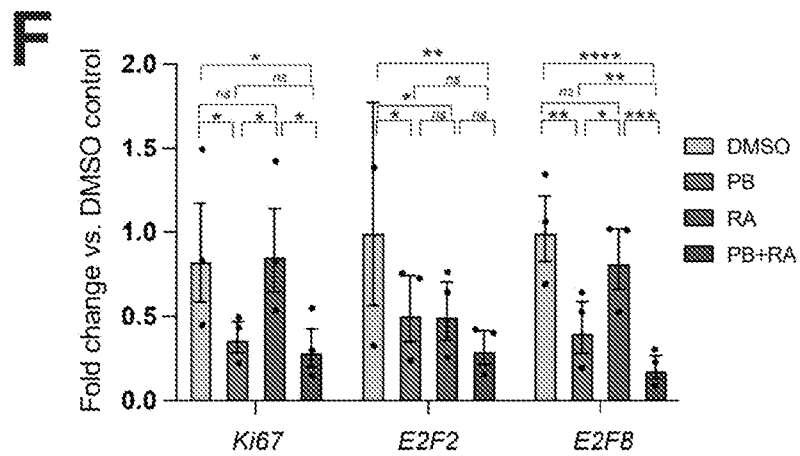
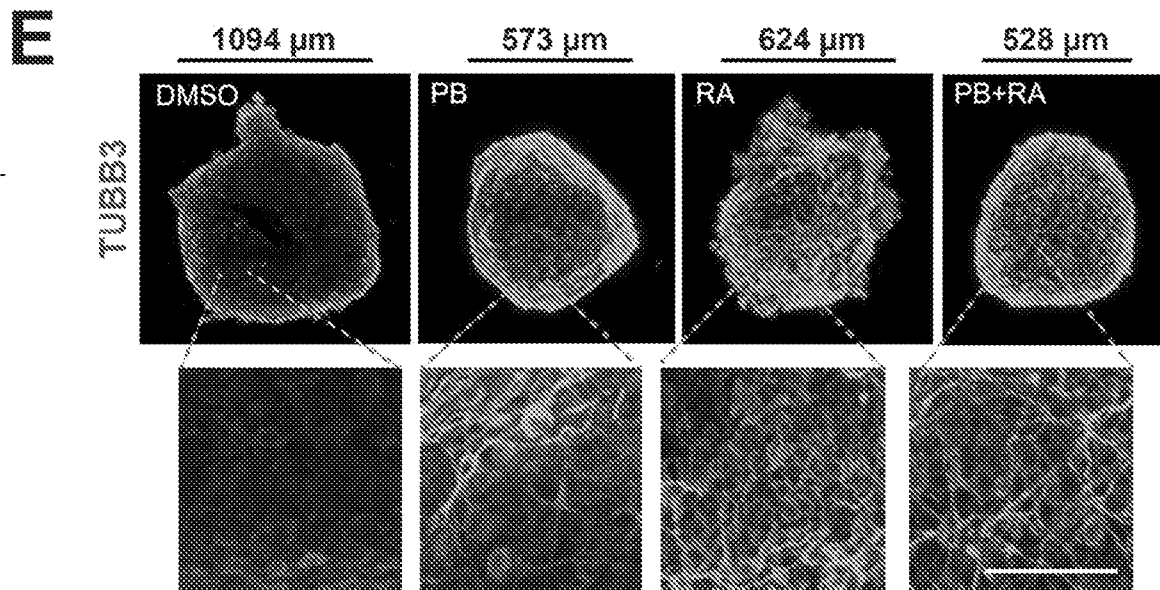


Figure 8

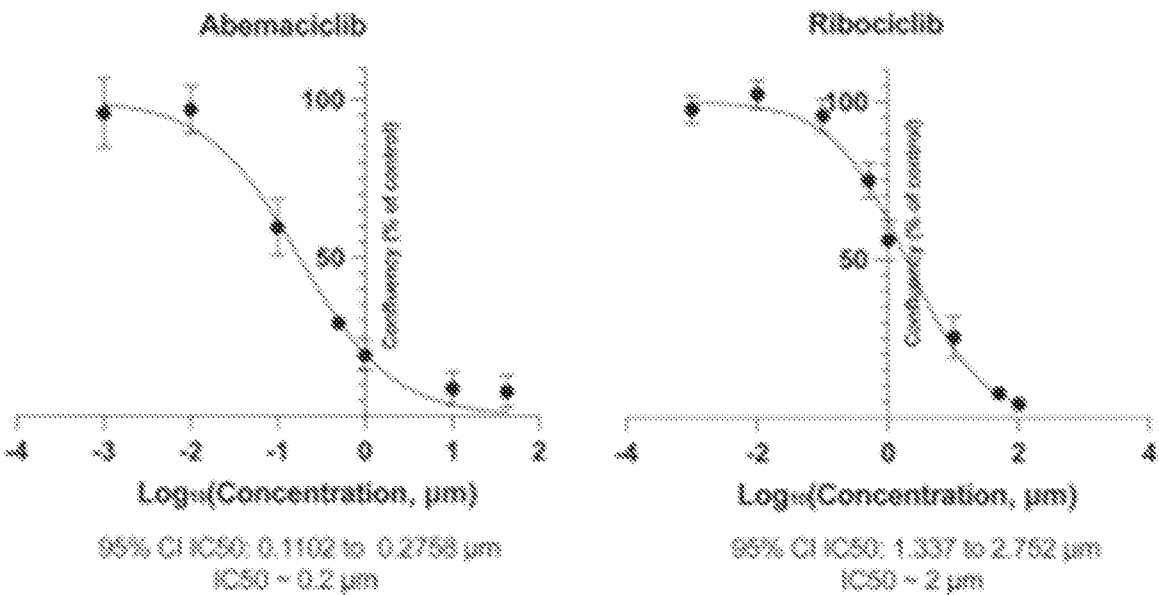


Figure 9 A-B

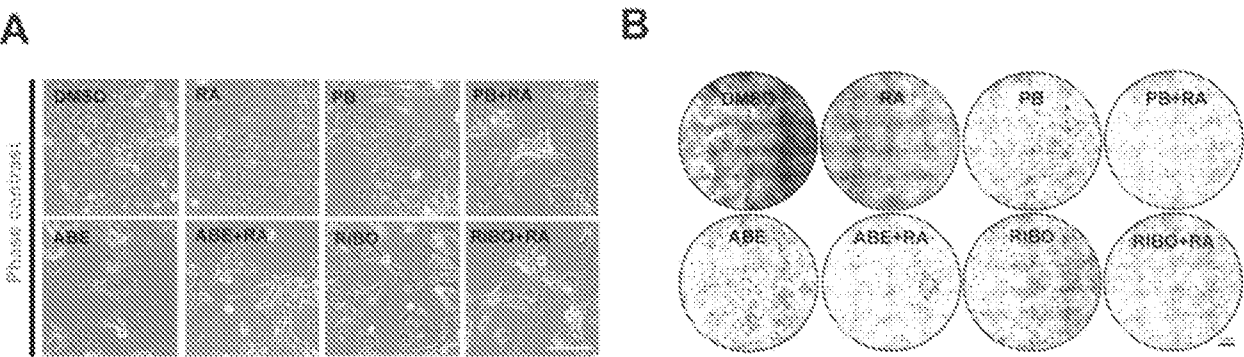
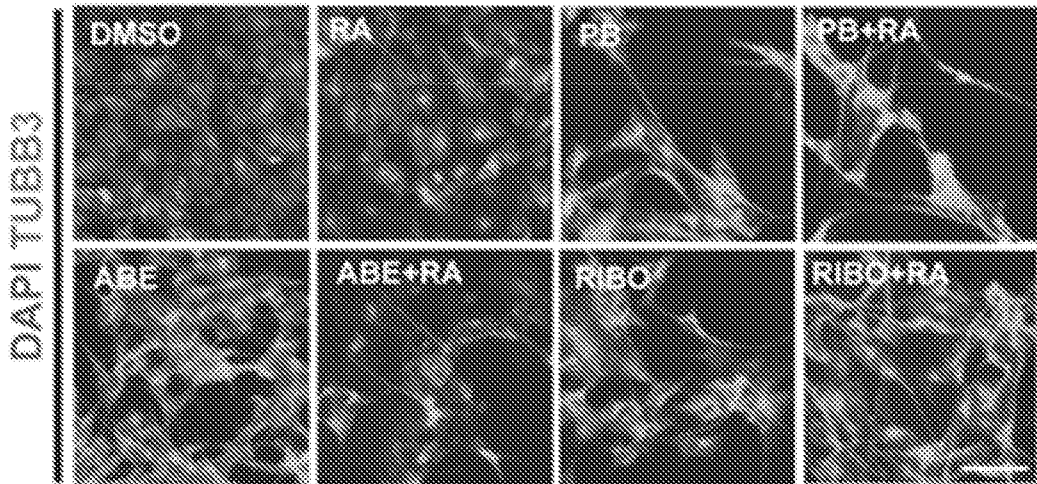


Figure 9 C-D

C



D

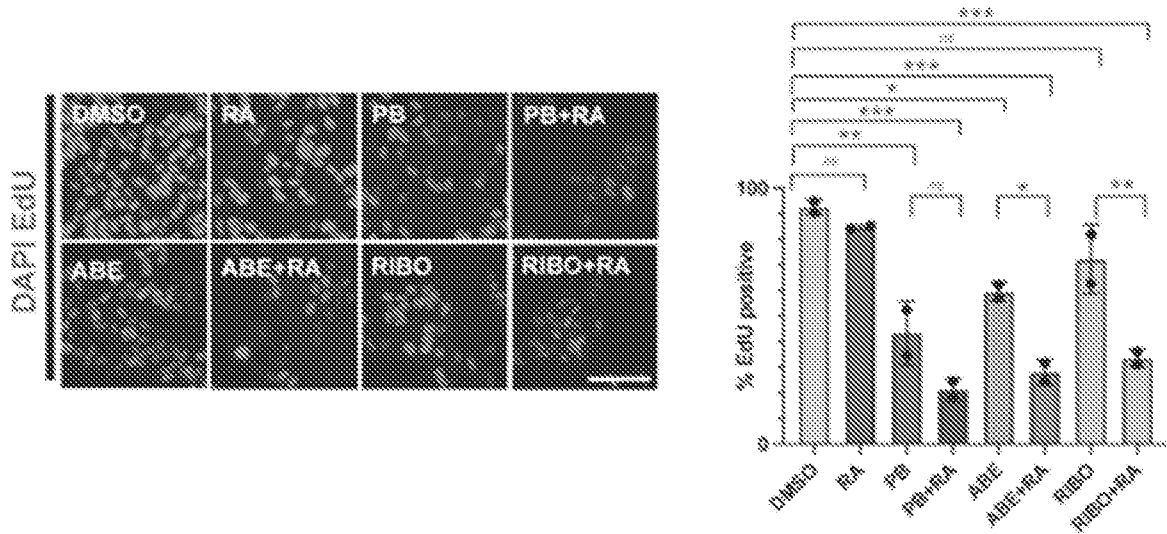
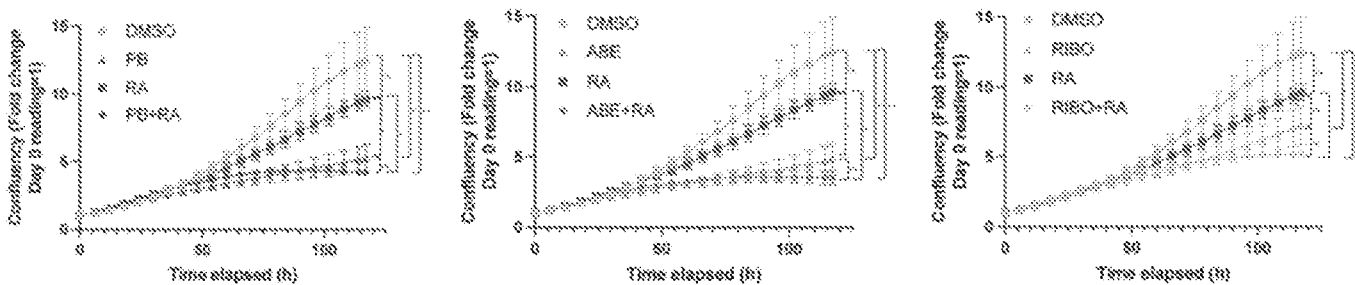


Figure 9 E-F

E



F

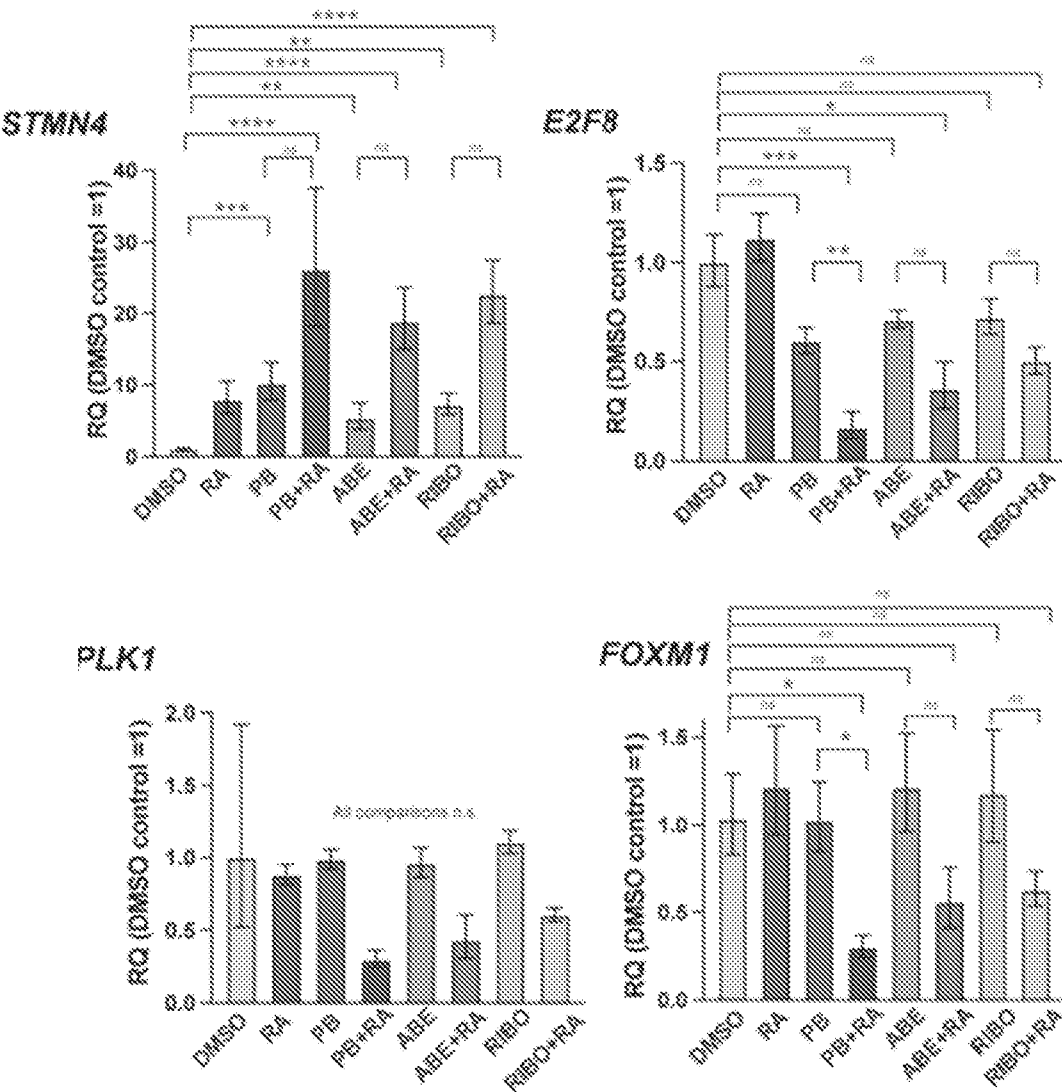


Figure 10 A-C

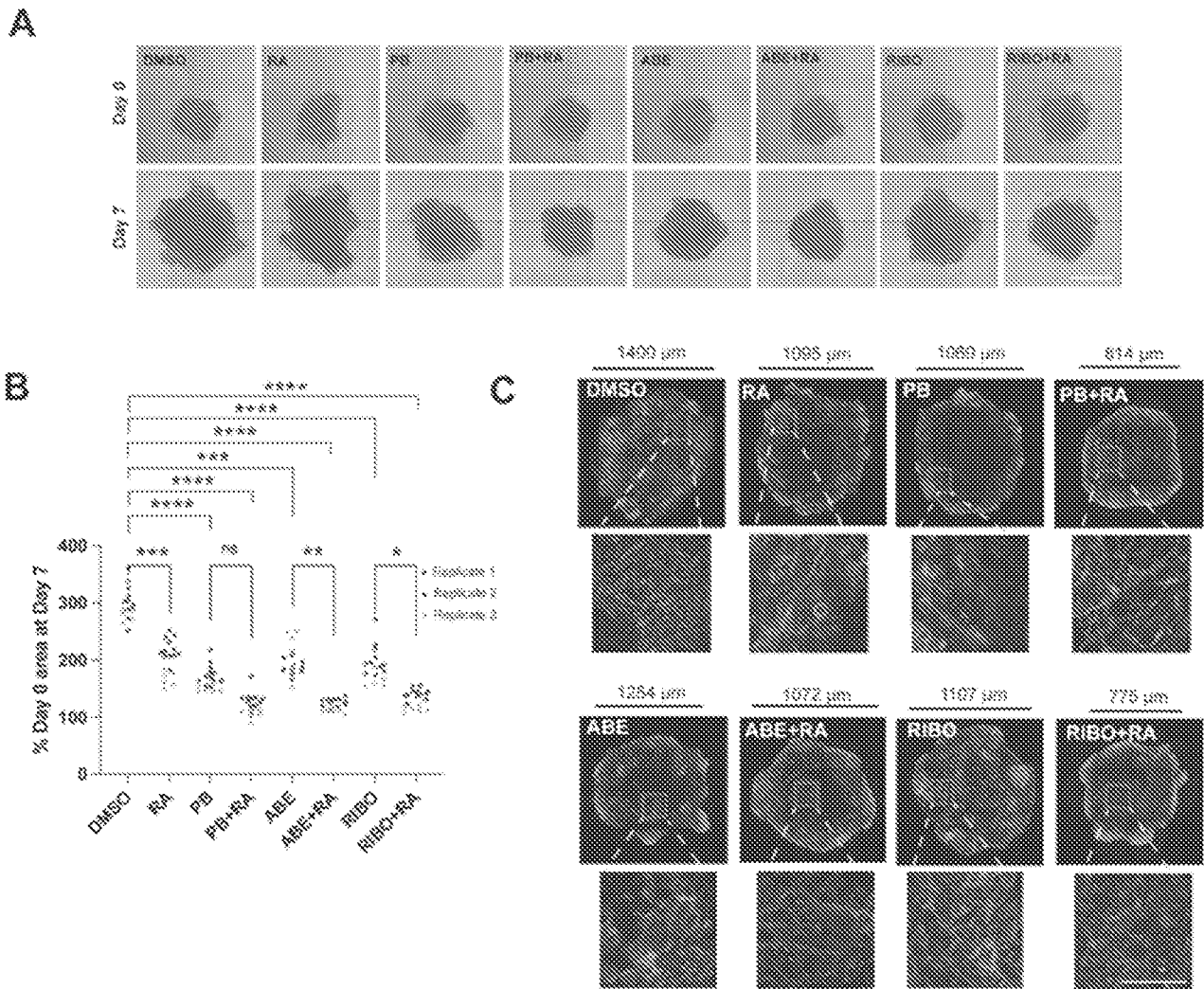
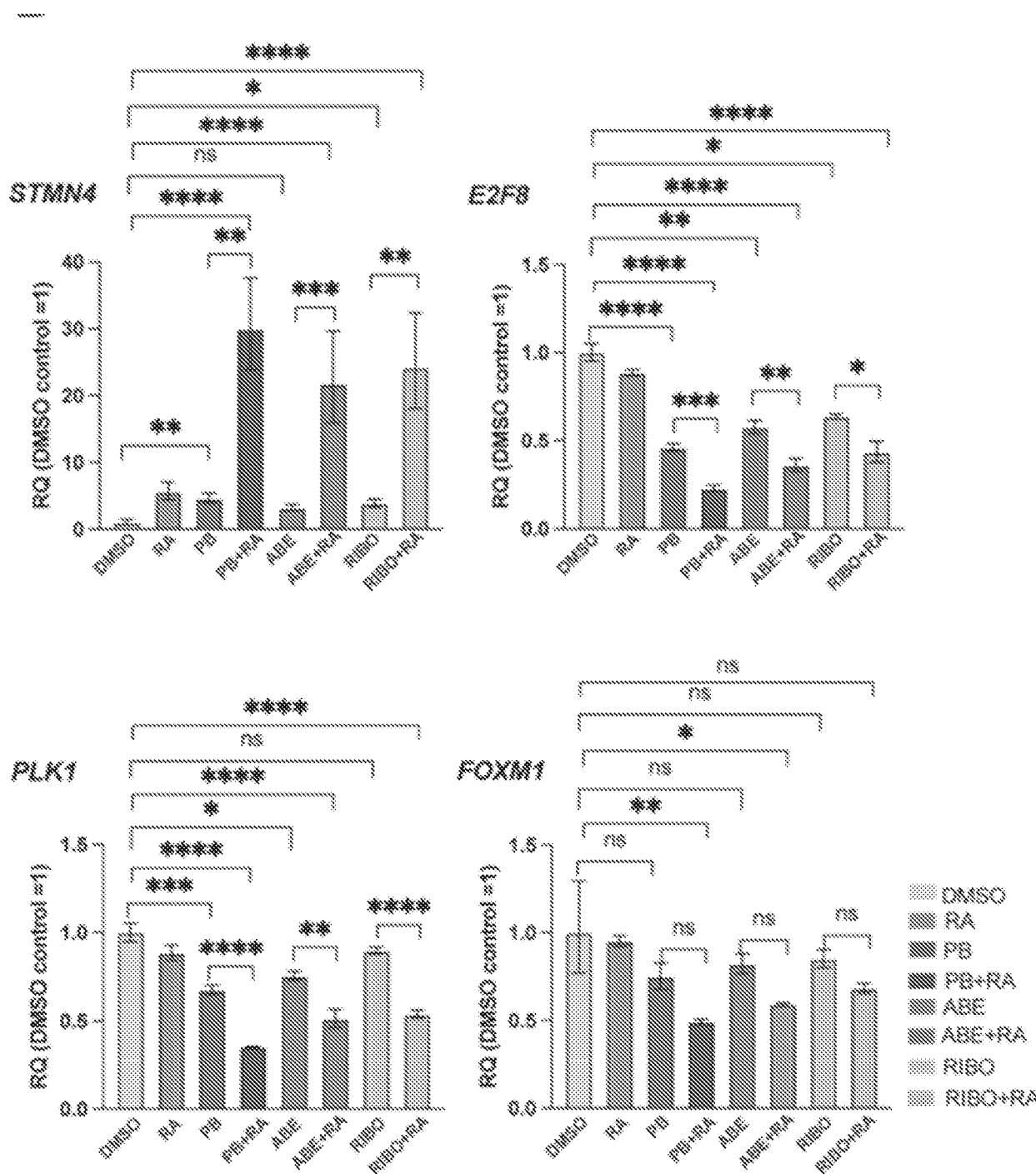


Figure 10D

D



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2024/052026

A. CLASSIFICATION OF SUBJECT MATTER		
INV. A61K31/203 A61K31/506 A61K31/519 A61K45/06 A61P35/00 G01N33/00		
ADD. 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) A61K G01N A61P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO - Internal		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/037527 A1 (CAMBRIDGE ENTPR LTD [GB]) 13 March 2014 (2014-03-13)	1-5,9, 13,16, 19-22,24
Y	page 50, paragraph 3 - page 51, paragraph 1; claims 1,9,11,16,20,21,39 -----	1-24, 31-40
X	CN 114 432 316 A (SECOND AUXILIARY HOSPITAL OF ANHUI MEDICAL UNIV) 6 May 2022 (2022-05-06) example 1 ----- -/-	20,24
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 25 October 2024		Date of mailing of the international search report 09/01/2025
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Allnutt, Sarah

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2024/052026

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JIN ZEGAO ET AL: "Development of differentiation modulators and targeted agents for treating neuroblastoma", EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, ELSEVIER MASSON, AMSTERDAM, NL, vol. 207, 8 September 2020 (2020-09-08), XP086336007, ISSN: 0223-5234, DOI: 10.1016/J.EJMECH.2020.112818 [retrieved on 2020-09-08] the whole document -----	1-24, 31-40
Y	JULIEANN RADER: "Dual CDK4/CDK6 Inhibition Induces Cell -Cycle Arrest and Secescence in Neuroblastoma", CLIN CANCER RES, 15 November 2013 (2013-11-15), pages 6173-6182, XP055764167, abstract -----	31-40
Y	MARTINEZ-MONLEON ANGELA ET AL: "Amplification of CDK4 and MDM2: a detailed study of a high-risk neuroblastoma subgroup", SCIENTIFIC REPORTS, vol. 12, no. 1, 20 July 2022 (2022-07-20), XP093218112, US ISSN: 2045-2322, DOI: 10.1038/s41598-022-16455-1 Retrieved from the Internet: URL:https://www.nature.com/articles/s41598-022-16455-1.pdf> abstract -----	31-40
X,P	FERGUSON KIRSTY M ET AL: "Palbociclib releases the latent differentiation capacity of neuroblastoma cells", DEVELOPMENTAL CELL, CELL PRESS, US, vol. 58, no. 19, 20 September 2023 (2023-09-20), page 1967, XP087414053, ISSN: 1534-5807, DOI: 10.1016/J.DEVCEL.2023.08.028 [retrieved on 2023-09-20] the whole document -----	1-24, 31-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2024/052026

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:
1 - 24, 31 - 40

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-24, 31-40

Treatment of neuroblastoma comprising the use of a retinoic acid in combination with a CDK4/6 or CD4K inhibitor.

2. claims: 25-30

Diagnosis or assessment of neuroblastoma comprising screening for biomarkers

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2024/052026

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014037527 A1	13-03-2014	NONE	
CN 114432316 A	06-05-2022	NONE	