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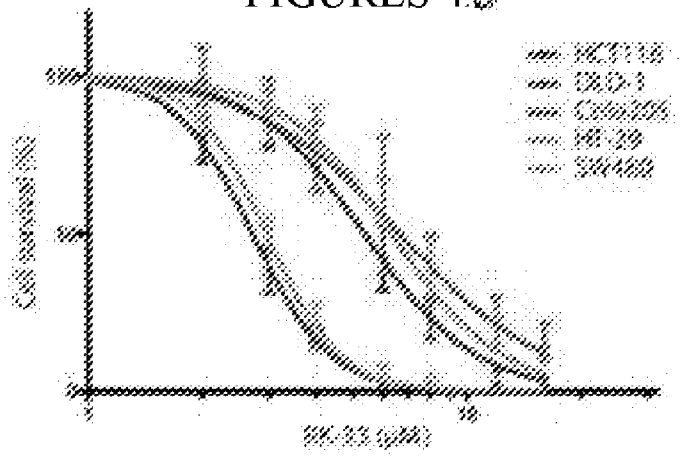
(54) Title: DEAD BOX RNA HELICASE DDX3 AS A THERAPEUTIC TARGET IN COLORECTAL CANCER AND METHODS OF TREATMENT THEREOF

FIGURES 4B



(57) Abstract: The present invention provides methods for down regulation of DDX3 in colorectal cancer cells by administration of an effective amount of a DDX3 inhibitor such as RK-33 or a salt, solvate, or derivative thereof. The present invention also provides uses of the DDX3 inhibitor such as RK-33 for treatment of colorectal disease in a subject.

# DEAD BOX RNA HELICASE DDX3 AS A THERAPEUTIC TARGET IN COLORECTAL CANCER AND METHODS OF TREATMENT THEREOF

## REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of U.S. Provisional Patent Application No. 62/134,963, filed on March 18, 2015, and is hereby incorporated by reference for all purposes as if fully set forth herein.

## INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

**[0002]** The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 10, 2016, is named P13469-02_ST25.txt and is 2,214 bytes in size.

## BACKGROUND OF THE INVENTION

**[0003]** Although significant advancements have been made in the prevention and treatment of colorectal cancer, this disease still ranks third on the list of causes of cancer related deaths in the United States, which underlines the need for development of new targeted therapies in this field. On a genomic level colorectal cancer is frequently characterized by loss of the tumor suppressor gene p53 and activation of the RAS-RAF signaling pathway, alterations that are common in a multitude of solid tumors. In addition, activation of the Wnt/β-catenin signaling pathway is prevailing and more specific to the colorectal cancer setting, where genetic aberrations in this pathway are found in over 90 percent of cases. The most common alteration is inactivation of the *APC* gene (>70%). Activating mutations in *CTNNB1*, the gene encoding for β-catenin, are less prevalent (5-10%). Thus, identifying druggable targets in this pathway would be beneficial for optimizing colorectal cancer treatment.

**[0004]** Over 85% of colorectal cancers are driven by aberrations in the Wnt-signaling pathway. Thus, identifying druggable targets in this pathway can be beneficial for optimizing colorectal cancer treatment. Within this context, a member of the RNA helicase gene family, DDX3, has been identified to exhibit oncogenic properties in breast and lung carcinomas as

well as medulloblastomas. Notably, recent studies have identified DDX3 as a multilevel activator of Wnt-signaling in both normal and transformed cells without activating mutations in the Wnt signaling pathway.

## SUMMARY OF THE INVENTION

**[0005]** In accordance with the inventive methods, we evaluated whether DDX3 also plays a role in the constitutionally activated Wnt-signaling that drives colorectal cancer and therefore could be a potential therapeutic target in this cancer type.

**[0006]** In accordance with an embodiment, the present invention provides a method for inhibiting DDX3 signaling in a colorectal cancer cell or population of cells comprising contacting the cell or population of cells with an effective amount of RK-33.

**[0007]** In accordance with an embodiment, the present invention provides method for treating colorectal cancer in a subject comprising administering to the subject an effective amount a pharmaceutical composition comprising RK-33 or a salt, solvate or derivative thereof, and a pharmaceutically acceptable carrier.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0008]** Figures 1A-1C: DDX3 dependency in colorectal cancer cell lines. A. Immunoblots of DDX3 expression in colorectal cancer cell lines HCT116 and HT29 before and after inhibition of DDX3 with 50 nM siDDX3. B. Proliferation of Colorectal cancer cell lines after knockdown of DDX3, measured by daily MTS assays. C. Cell cycle analysis after knockdown of DDX3. All experiments were performed three independent times, graphs represent mean ± SD, $*p < 0.05$

**[0009]** Figures 1A-2H: DDX3 is overexpressed in patients with colorectal cancer. DDX3 is overexpressed in 39% of patients. Low DDX3 expression in normal colon epithelium (A. & C.). High DDX3 expression in colorectal adenocarcinoma cells of the same patients (B. & D.) 54.2% of patients have similar levels of DDX3 expression in the normal mucosa (E.) and corresponding invasive cancer (F.). Only 6.8% of patients have decreased DDX3 in the invasive tumor (H.), when compared to adjacent normal mucosa (G.). 40 x magnification, scale bar indicates 25 μm.

**[0010]** Figures 3A-3D: High DDX3 expression is associated with nuclear β-catenin in colorectal cancer samples. Low DDX3 expression (A.) is associated with strong expression of β-catenin on the membranes and absence of β-catenin in the nuclei (B.) High DDX3 expression (C.) is associated with increased β-catenin expression in the cytoplasm and the nucleus (D.). 40 x magnification, scale bar indicates 25 μm.

**[0011]** Figures 4A-4I: RK-33 sensitivity in colorectal cancer cell lines. A. Immunoblot showing the relative DDX3 expression in adherent colorectal cancer cell lines. B. MTS assay showing cytotoxicity of RK-33 in different colorectal cancer cell lines. C. Immunoblot showing the relative DDX3 expression in patient-derived 3D cultures. D. Cytotoxicity assay showing the sensitivity of patient-derived 3D cultures of colorectal cancer. E. Example of cytotoxicity assay with RK-33 in CRC29 3D cultures. The DRAQ5 positive (red) areas are used to determine the outline of the spheroids. The Calcein AM (green) intensity within this area is used as a measure for living cells. F. Cell cycle analysis after DDX3 inhibition with increasing concentrations RK-33 in HCT116 and HT29. G. Immunoblots of DDX3 expression in colorectal cancer cell lines SW480 and DLD-1 before and after inhibition of DDX3 with 50 nM siDDX3. H. Proliferation of Colorectal cancer cell lines SW480 and DLD-1 after knockdown of DDX3, measured by daily MTS assays. I. Cell cycle analysis after knockdown of DDX3 in SW480 and DLD-1. All experiments were performed three independent times, graphs represent mean ± SD, * $p < 0.05$, ** $p < 0.01$.

**[0012]** Figures 5A-5F: DDX3 dependency in different colorectal cancer genetic subtypes. A. Immunoblot showing p53 and DDX3 expression in HCT116 with and without p53. B. MTS assay showing the relative cytotoxicity after RK-33 treatment in HCT116 with and without p53. C. Cell Cycle analysis of HCT116-p53-/- cells after DDX3 knockdown with 50 nM siDDX3. D. Immunoblots of DDX3 expression in HCT116 p53-/- before and after inhibition of DDX3 with 50 nM siDDX3. E. Relative sensitivity to RK-33 in parental HCT116 (CTNNB1Δ45/wt) and HCT116 with either the mutant CTNNB1 allele (CTNNB1-/wt) or the wild-type allele deleted (CTNNB1Δ45/-). F. Immunoblot showing the DDX3 expression in HCT116 with different β-catenin variants. All experiments were performed three independent times, graphs represent mean ± SD, *$p < 0.05$.

**[0013]** Figures 6A-6D: DDX3 inhibition results in reduced Wnt signaling activity. A. and B. TCF4-reporter assays after knockdown of DDX3 with 50 nM siDDX3 (A.) and inhibition of DDX3 with RK-33 (B.) in DDX3-dependent colorectal cancer cell lines HCT116 and HT29. C. and D. Relative mRNA expression of TCF4-target genes after knockdown of

DDX3 with 50 nM siDDX3 (C.) or DDX3 inhibition with RK-33 (D.). All experiments were performed three independent times, graphs represent mean ± SD, * $p < 0.05$, ** $p < 0.01$.

**[0014]** Figures 7A-7B: DDX5 and DDX17 expression after treatment with RK-33. A. DDX5 expression before and after DDX3 inhibition with RK-33. B. DDX17 expression before and after DDX3 inhibition with RK-33.

## DETAILED DESCRIPTION OF THE INVENTION

**[0015]** Previously, the present inventors demonstrated that DDX3 is overexpressed in breast and lung cancers and that targeting DDX3 by RK-33 promotes cell death (Oncogene. 2008; 27(28):3912-3922; EMBO molecular medicine. 2015)). This requirement for DDX3 can in part be explained by its involvement in Wnt signaling, as was shown previously by the inventors group and others (Science 2013; 339(6126):1436-1441). As the majority of colorectal cancers is driven by mutations in the Wnt-signaling pathway, we explored the possible contribution of DDX3 to Wnt-associated colorectal cancer oncogenesis. In accordance with the present invention, the inventors now show that DDX3 is overexpressed in 39% of colorectal cancers and that inhibition of DDX3 results in reduced Wnt signaling and a G1 arrest, making DDX3 an attractive therapeutic target in these cancers.

**[0016]** The clinical relevance of the development of Wnt signaling inhibitors which work in a constitutively activated setting is tremendous, since mutations in the Wnt-signaling pathway are not only the first genetic alterations in the adenoma-carcinoma sequence, but advanced colorectal cancers with mutations in *APC* or *CTNNB1* remain dependent on upstream Wnt signaling activity (Oncogene. 2005; 24(18):3054-3058; Nature Comm. 2013; 4:2610). Especially colorectal cancer stem cells rely on Wnt signaling, and their inhibition can therefore specifically inhibit the resistant tumor initiating cell population. The potential of these inventive methods is also reflected by the cytotoxic effect of RK-33 on 3D cultures of colorectal cancer stem cells described herein.

**[0017]** Colorectal cancer drug development is currently limited by a lack of pathway specific targets, potential redundancy of pathway components and toxicity (Nature reviews Cancer 2013; 13(1):11-26). The present inventive methods show that DDX3 is an integral component of Wnt signaling, and targeting DDX3 by RK-33 is a therapeutic option in cancer with activating mutations in Wnt-signaling.

**[0018]** In accordance with an embodiment, the present inventive methods show that administration of RK-33 resulted in DDX3 inhibition and in a reduction of proliferation in several colorectal cell lines and all of the colorectal cancer cell lines used in this study were susceptible to RK-33. This finding argues for the reliance of colorectal cancer cells on DDX3 for their survival and argues against a tumor suppressive role in this particular setting. Interestingly, within our cohort of colorectal cancer cell lines, we observed differential sensitivity to RK-33, indicating that other genetic factors may contribute to oncogenic addiction to DDX3 in neoplastic cells.

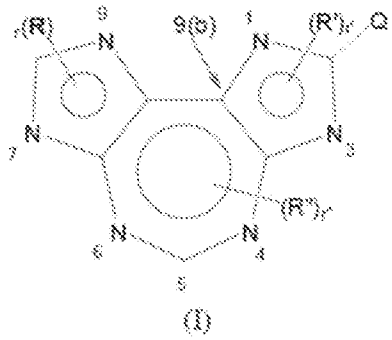
**[0019]** To determine if DDX3 is expressed in colorectal cancers, the inventors immunohistochemically stained a cohort of 303 Dutch and German colorectal cancer patients. 40.4% of these tumors were found to overexpress DDX3 in comparison to the surrounding normal tissue. DDX3 expression was found predominantly in the cytoplasm and occasionally in the nucleus. High cytoplasmic DDX3 expression correlated with nuclear β-catenin expression, a marker of activated Wnt-signaling. The presence of nuclear DDX3 expression correlated with shorter overall survival (HR = 2.38, 95% CI 1.45-3.93, $p < 0.001$). Functionally, these findings were validated in vitro and found that inhibition of DDX3 with siRNA resulted in reduced proliferation and a G1-arrest in the HCT116 and HT29 colorectal cancer cell lines. This finding further supports the potential oncogenic role of DDX3 in colorectal cancer.

**[0020]** With respect to targeting DDX3, a small molecule inhibitor of DDX3 was developed, and referred to as RK-33. RK-33 is designed to bind to the ATP-binding site of DDX3 and abrogate its functional activity. As proof of principle, it was demonstrated that RK-33 binds preferentially to DDX3 and not to DDX5 and DDX17, other members of the RNA helicase family. Moreover, RK-33 inhibited the helicase activity in an in vitro assay. Furthermore, treatment of colorectal cancer cell lines and patient derived 3D- tumor cell cultures indicated that RK-33 inhibits growth and promotes cell death with $IC_{50}$ values ranging from 2.5 to 8 μM.

**[0021]** To further elucidate the mechanism of RK-33, we studied if inhibition of DDX3 with RK-33 could cause inhibition of Wnt-signaling in colorectal cancer cell lines. Treatment with RK-33 indeed resulted in reduced TCF-reporter activity and lowered the mRNA expression levels of the Wnt-signaling downstream target genes AXIN-2, C-MYC, CCND1 and BIRC5A.

**[0022]** Thus, in accordance with an embodiment, the present invention provides the compound RK-33 and derivatives thereof, as a novel chemotherapeutic agent for the treatment of colorectal cancer in a subject.

**[0023]** RK-33 is a member of a novel class of fused diimidazodiazepines which are provided in PCT/US2009/005273 which discloses a compound of Formula (I):



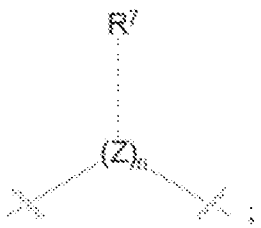
(I)

or pharmaceutically acceptable salts and prodrugs thereof,

wherein:

R, R', and R" are each independently a hydrogen, hydroxyl; substituted or unsubstituted: cyclic or acyclic alkyl group, cyclic or acyclic alkenyl group, cyclic or acyclic alkynyl group, aryl group, alkylaryl group, arylalkyl group, benzyl group, cyclic and acyclic heteroalkyl group, heteroaryl group; -C(O)R3; -C(S)R3; -S(O)R3; -S(O)2R3; -C(O)NR3R4; -C(S)NR3R4; C(S)YR3; -C(O)YR3; -β-D-ribosyl; -α-D-ribosyl; -β-Lribosyl; -α-L-ribosyl; 2'-deoxy- β -D-ribosyl; 2'-deoxy- β -L-ribosyl; 2'-deoxy- α -Dribosyl; 2'-deoxy- α -L-ribosyl; or ribose or deoxyribose sugars substituted with one or more halogens;

R, R', and R" can also form a ring with one or more C, S, O, N atoms such that, for example, R and R' together include:



$R^7$ is a hydrogen; hydroxyl; substituted and unsubstituted: cyclic and acyclic alkyl group, group, alkenyl group, alkynyl group, aryl group, aryloxy group, alkylary group, aryalkyl group, heteroaryl group, heterocycloalkyl group; -C(O)alkyl; -C(O)alkenyl; -C(O)alkynyl; -

C(O)aryl; -C(O)benzyl; -C(O)NR3R4; -C(S)alkyl; -C(S)alkenyl; -C(S)alkynyl; -C(S)aryl; -C(S)benzyl; -C(S)NR3R4; -C(O)N R3R4; -C(S)YR3; -C(O)YR3;

wherein

- - - - - Q is =O, =NH, or =S;

Y is O or S;

Z is CH, N, P, or C;

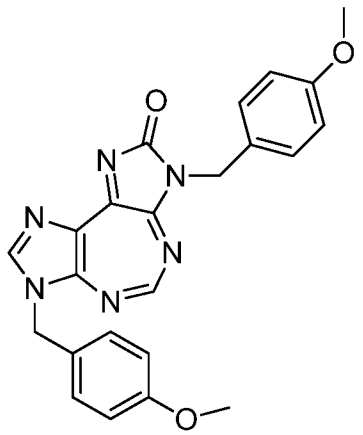
------- is a single bond or double bond; wherein if ------ is a double bond, R2 or R7 is independently O, S, or NH;

n is 1, 2, 3, or 4;

$R^3$ and $R^4$ are independently a hydrogen; hydroxyl; substituted or unsubstituted: cyclic or acyclic alkyl group, cyclic or acyclic alkenyl group, cyclic or acyclic alkynyl group, aryl group, alkylary group, aryalkyl group, heteroaryl group, heterocycloalkyl group; and

r, r', and r" are each independently an integer from 1 to 3.

**[0024]** The structure of RK-33 is provided below.



**RK-33**

**[0025]** In yet another embodiment, the present invention provides a novel small molecule, RK-33 that fits into the ATP binding domain of DDX3 and induces cell death in cancer cells. Thus, the present invention further provides a method of decreasing DDX3 functions in colorectal cancer cells induces cell, while having little or no toxicity in animals.

**[0026]** In accordance with an embodiment, the present invention provides a pharmaceutical composition comprising the compound RK-33, or a salt, solvate, stereoisomer, or derivative thereof, and a pharmaceutically acceptable carrier, for use as a

DDX3 inhibitor in a mammalian cell or population of cells, more preferably for use as an inhibitor of colorectal cancer in a subject suffering therefrom.

**[0027]** Accordingly, included within the compounds and derivatives of the present invention are the tautomeric forms of the disclosed compounds, isomeric forms including enantiomers, stereoisomers, and diastereoisomers, and the pharmaceutically-acceptable salts thereof. The term "pharmaceutically acceptable salts" embraces salts commonly used to form alkali metal salts and to form addition salts of free acids or free bases, such as those used to improve water solubility. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, sulphuric acid and phosphoric acid, and such organic acids as maleic acid, succinic acid and citric acid. Other pharmaceutically acceptable salts include salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, or with organic bases, such as dicyclohexylamine. Suitable pharmaceutically acceptable salts of the compounds of the present invention include, for example, acid addition salts which may, for example, be formed by mixing a solution of the compound according to the invention with a solution of a pharmaceutically acceptable acid, such as hydrochloric acid, sulphuric acid, methanesulphonic acid, fumaric acid, maleic acid, succinic acid, acetic acid, benzoic acid, oxalic acid, citric acid, tartaric acid, carbonic acid or phosphoric acid. All of these salts may be prepared by conventional means by reacting, for example, the appropriate acid or base with the corresponding compounds of the present invention.

**[0028]** Salts formed from free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

**[0029]** For use in medicines, the salts of the compounds of the present invention should be pharmaceutically acceptable salts. Other salts may, however, be useful in the preparation of the compounds according to the invention or of their pharmaceutically acceptable salts.

**[0030]** In addition, embodiments of the invention include hydrates of the compounds of the present invention. The term "hydrate" includes but is not limited to hemihydrate, monohydrate, dihydrate, trihydrate and the like. Hydrates of the compounds of the present invention may be prepared by contacting the compounds with water under suitable conditions to produce the hydrate of choice.

**[0031]** Embodiments of the invention also include a process for preparing pharmaceutical products comprising the compounds. The term "pharmaceutical product" means a composition suitable for pharmaceutical use (pharmaceutical composition), as defined herein. Pharmaceutical compositions formulated for particular applications comprising the compounds of the present invention are also part of this invention, and are to be considered an embodiment thereof.

**[0032]** As used herein, the term "treat," as well as words stemming therefrom, includes preventative as well as disorder remitative treatment. The terms "reduce," "suppress," "prevent," and "inhibit," as well as words stemming therefrom, have their commonly understood meaning of lessening or decreasing. These words do not necessarily imply 100% or complete treatment, reduction, suppression, or inhibition.

**[0033]** With respect to pharmaceutical compositions described herein, the pharmaceutically acceptable carrier can be any of those conventionally used, and is limited only by physico-chemical considerations, such as solubility and lack of reactivity with the active compound(s), and by the route of administration. The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those skilled in the art and are readily available to the public. Examples of the pharmaceutically acceptable carriers include soluble carriers such as known buffers which can be physiologically acceptable (e.g., phosphate buffer) as well as solid compositions such as solid-state carriers or latex beads. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the active agent(s), and one which has little or no detrimental side effects or toxicity under the conditions of use.

**[0034]** The carriers or diluents used herein may be solid carriers or diluents for solid formulations, liquid carriers or diluents for liquid formulations, or mixtures thereof.

**[0035]** Solid carriers or diluents include, but are not limited to, gums, starches (e.g., corn starch, pregelatinized starch), sugars (e.g., lactose, mannitol, sucrose, dextrose), cellulosic materials (e.g., microcrystalline cellulose), acrylates (e.g., polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof.

**[0036]** For liquid formulations, pharmaceutically acceptable carriers may be, for example, aqueous or non-aqueous solutions, suspensions, emulsions or oils. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include, for example, water, alcoholic/aqueous solutions, cyclodextrins, emulsions or suspensions, including saline and buffered media.

**[0037]** Examples of oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, mineral oil, olive oil, sunflower oil, fish-liver oil, sesame oil, cottonseed oil, corn oil, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include, for example, oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

**[0038]** Parenteral vehicles (for subcutaneous, intravenous, intraarterial, or intramuscular injection) include, for example, sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Formulations suitable for parenteral administration include, for example, aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

**[0039]** Intravenous vehicles include, for example, fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycols or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

**[0040]** In addition, in an embodiment, the compounds of the present invention may further comprise, for example, binders (e.g., acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g., cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (e.g., Tris-HCl, acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g. sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., cremophor, glycerol, polyethylene glycerol, benzlkonium chloride, benzyl benzoate, cyclodextrins, sorbitan esters, stearic acids), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g., hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents (e.g., carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweetners (e.g., aspartame, citric acid), preservatives (e.g., thimerosal, benzyl alcohol, parabens), lubricants (e.g., stearic

acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g., colloidal silicon dioxide), plasticizers (e.g., diethyl phthalate, triethyl citrate), emulsifiers (e.g., carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g., ethyl cellulose, acrylates, polymethacrylates), and/or adjuvants.

**[0041]** The choice of carrier will be determined, in part, by the particular compound, as well as by the particular method used to administer the compound. Accordingly, there are a variety of suitable formulations of the pharmaceutical composition of the invention. The following formulations for parenteral, subcutaneous, intravenous, intramuscular, intraarterial, intrathecal and interperitoneal administration are exemplary, and are in no way limiting. More than one route can be used to administer the compounds, and in certain instances, a particular route can provide a more immediate and more effective response than another route.

**[0042]** Suitable soaps for use in parenteral formulations include, for example, fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include, for example, (a) cationic detergents such as, for example, dimethyl dialkyl ammonium halides, and alkyl pyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepolypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl-β-aminopropionates, and 2-alkyl-imidazoline quaternary ammonium salts, and (e) mixtures thereof.

**[0043]** The parenteral formulations will typically contain from about 0.5% to about 25% by weight of the compounds in solution. Preservatives and buffers may be used. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants, for example, having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations will typically range from about 5% to about 15% by weight. Suitable surfactants include, for example, polyethylene glycol sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

**[0044]** The parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials, and can be stored in a freeze-dried (lyophilized)

condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets.

**[0045]** Injectable formulations are in accordance with the invention. The requirements for effective pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art (see, e.g., *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Trissel, 15th ed., pages 622-630 (2009)).

**[0046]** In accordance with another embodiment, the present invention provides a method of treating cancer in a subject comprising a) administering to the subject an effective amount of the pharmaceutical composition comprising compound RK-33, or a salt, solvate, stereoisomer, or derivative thereof, and a pharmaceutically acceptable carrier, in one or more doses, and b) administering ionizing radiation to the subject in proximity to the location of the cancer in the subject.

**[0047]** In an embodiment, the term "administering" means that the compounds of the present invention are introduced into a subject, preferably a subject receiving treatment for a proliferative disease, and the compounds are allowed to come in contact with the one or more disease related cells or population of cells *in vivo*.

**[0048]** As defined herein, in another embodiment, the term "contacting" means that the one or more compounds of the present invention are introduced into a sample having at least one cancer cell and appropriate enzymes or reagents, in a test tube, flask, tissue culture, chip, array, plate, microplate, capillary, or the like, and incubated at a temperature and time sufficient to permit binding and uptake of the at least one compound to the cancer cell. Methods for contacting the samples with the compounds, and other specific binding components are known to those skilled in the art, and may be selected depending on the type of assay protocol to be run. Incubation methods are also standard and are known to those skilled in the art.

**[0049]** Radiation therapy, radio-immunotherapy or pre-targeted radioimmunotherapy are used for the treatment of diseases of oncological nature. “Radiotherapy”, or radiation therapy, means the treatment of cancer and other diseases with ionizing radiation. Ionizing radiation deposits energy that injures or destroys cells in the area being treated (the target tissue) by damaging their genetic material, making it impossible for these cells to continue to grow. Radiotherapy may be used to treat localized solid tumors, such as cancers of the skin,

tongue, larynx, brain, breast, lung or uterine cervix. It can also be used to treat leukemia and lymphoma, i.e. cancers of the blood-forming cells and lymphatic system, respectively. One type of radiation therapy commonly used involves photons, e.g. X-rays. Depending on the amount of energy they possess, the rays can be used to destroy cancer cells on the surface of or deeper in the body. The higher the energy of the x-ray beam, the deeper the x-rays can go into the target tissue. Linear accelerators and betatrons are machines that produce x-rays of increasingly greater energy. The use of machines to focus radiation (such as x-rays) on a cancer site is called external beam radiotherapy. Gamma rays are another form of photons used in radiotherapy. Gamma rays are produced spontaneously as certain elements (such as radium, uranium, and cobalt 60) release radiation as they decompose, or decay. Another technique for delivering radiation to cancer cells is to place radioactive implants directly in a tumor or body cavity. This is called internal radiotherapy. Brachytherapy, interstitial irradiation, and intracavitary irradiation are types of internal radiotherapy. In this treatment, the radiation dose is concentrated in a small area, and the patient stays in the hospital for a few days. Internal radiotherapy is frequently used for cancers of the tongue, uterus, and cervix. A further technique is intra-operative irradiation, in which a large dose of external radiation is directed at the tumor and surrounding tissue during surgery. Another approach is particle beam radiation therapy. This type of therapy differs from photon radiotherapy in that it involves the use of fast-moving subatomic particles to treat localized cancers. Some particles (neutrons, pions, and heavy ions) deposit more energy along the path they take through tissue than do x-rays or gamma rays, thus causing more damage to the cells they hit. This type of radiation is often referred to as high linear energy transfer (high LET) radiation. Radio-sensitizers make the tumor cells more likely to be damaged, and radio-protectors protect normal tissues from the effects of radiation.

**[0050]** In a further embodiment, the pharmaceutical composition used in the method can include RK-33 and additional therapeutic agent or agents.

**[0051]** Suitable agents for use as additional therapeutic agent or agents include anti-neoplastic agents, such as androgen inhibitors, antimetabolites, cytotoxic agents, and immunomodulators. More specifically, anti-neoplastic agents can include alkylating agents, nitrogen mustard alkylating agents, nitrosourea alkylating agents, antimetabolites, purine analog antimetabolites, pyrimidine analog antimetabolites, hormonal antineoplastics, natural antineoplastics, antibiotic natural antineoplastics, and vinca alkaloid natural antineoplastics alkylating antineoplastic agents, such as carboplatin and cisplatin; nitrosourea alkylating

antineoplastic agents, such as carmustine (BCNU); antimetabolite antineoplastic agents, such as methotrexate; pyrimidine analog antineoplastic agents, such as fluorouracil (5-FU) and gemcitabine; hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as aldesleukin, interleukin-2, docetaxel, etoposide, interferon; paclitaxel, other taxane derivatives, and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, and mitomycin; and vinca alkaloid natural antineoplastics, such as vinblastine and vincristine.

**[0052]** As used herein, the term "subject" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

**[0053]** The term “chemotherapeutic agent” as well as words stemming therefrom, as used herein, generally includes pharmaceutically or therapeutically active compounds that work by interfering with DNA synthesis or function in cancer cells. Based on their chemical action at a cellular level, chemotherapeutic agents can be classified as cell-cycle specific agents (effective during certain phases of cell cycle) and cell-cycle nonspecific agents (effective during all phases of cell cycle). Without being limited to any particular example, examples of chemotherapeutic agents can include alkylating agents, angiogenesis inhibitors, aromatase inhibitors, antimetabolites, anthracyclines, antitumor antibiotics, monoclonal antibodies, platinums, topoisomerase inhibitors, checkpoint inhibitors and plant alkaloids.

**[0054]** In a further embodiment, the compositions and methods of the present invention can be used in combination with one or more additional therapeutically active agents which are known to be capable of treating conditions or diseases discussed above. For example, the compositions of the present invention could be used in combination with one or more known therapeutically active agents, to treat a proliferative disease. Non-limiting examples of other therapeutically active agents that can be readily combined in a pharmaceutical composition with the compositions and methods of the present invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid

molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

**[0055]** The term "modulate," as used herein means that the expression of DDX3, or level of RNA molecule or equivalent RNA molecules encoding one or more DDX3 protein or protein subunits, or activity of DDX3 protein or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

**[0056]** Typically, an attending physician will decide the dosage of the composition with which to treat each individual subject, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, compound to be administered, route of administration, and the severity of the condition being treated. By way of example, and not intending to limit the invention, the dose of the compositions of the present invention, and the additional therapeutic agents, if any, can be about 0.001 to about 1000 mg/kg body weight of the subject being treated, from about 0.01 to about 100 mg/kg body weight, from about 0.1 mg/kg to about 10 mg/kg, and from about 0.5 mg to about 5 mg/kg body weight. In another embodiment, the dose of the compositions of the present invention can be at a concentration from about 1 nM to about 100 mM, preferably from about 1 μM to about 50 mM, more preferably from about 100 μM to about 5 mM. In some embodiments the dose of the compositions of the present invention can be at a concentration of about 100 nM, 300 nM, 500 nM, 1 μM, 3 μM, 5 μM, 10 μM, 30 μM, 50 μM, 100 μM, 300 μM to about 1 mM.

**[0057]** In accordance with a further embodiment, the present invention provides a method for screening for agents which modulate the expression of DDX3 in a cancer cell or population of cancer cells comprising: administering a candidate agent to the CT and/or CRT transgenic mouse, wherein the presence or absence of an effect upon the expression of DDX3 in the cancer cell or population of cells in said mouse is indicative of the modulating activity of the candidate agent.

## EXAMPLES

**[0058]** Cell lines.

**[0059]** HCT116, HT29, Colo205, SW480 and DLD-1 were a kind gift of Professor Fred Bunz (Johns Hopkins University, Baltimore, MD, USA). The HCT116 p53 (Science. 1998; 282(5393):1497-1501) and β-catenin (PNAS USA 2002; 99(12):8265-8270) knockout cell

lines were kindly provided by Professor Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). All adherent colorectal cancer cell lines were grown in McCoy's5A supplemented with 10% fetal bovine serum. All cell lines were routinely tested for mycoplasma contamination by a PCR kit (30-1012K, ATCC, Manassas, VA, USA).

**[0060]** The colosphere cultures CR9, CRC29, CRC47 and L145 were a kind gift from Professor Onno Kranenburg (Utrecht University, Utrecht, The Netherlands). These cell lines were established from tumor specimens of primary colorectal cancers (CR-9, CRC29, CRC47) and colorectal cancer liver metastases (L145). A detailed description of how these colospheres were isolated and maintained has been provided previously (Gastroenterology. 2011; 141(1):269-278). Next generation sequencing was performed to assess the mutation status of 50 commonly mutated genes in these spheroids using the AmpliSeq Cancer Hotpot Panel v2 (LifeTechnologies, Carlsbad, CA, USA) on an Ion PGM platform. Publicly available mutation data of the adherent colorectal cancer cell lines were accessed through the canSAR platform (Nucleic acids research. 2012; 40(Database issue):D947-956).

**[0061]** DDX3 knockdown cell lines were generated by transfecting cells with jetPrime transfection reagent (Polyplus, New York, NY, USA) and 50 nM sicontrol (non-targeting pool) or siDDX3 sequences (ON-*TARGETplus,* Dharmacon, Lafayette, CO, USA).

**[0062]** Immunoblotting.

**[0063]** All cells were harvested at 50-70% confluency. For DDX3 knockdown experiments cells were harvested 72 hours after transfection. For RK-33 experiments cells were harvested after 24 hours exposure to the drug or vehicle control. For whole cellular protein extracts cells were lysed in SDS-extraction buffer (100 nM Tris-HCl, 2% SDS, 12% glycerol, 10 mM EDTA, pH 6.7) and sonicated on ice. 30 µg protein was loaded on 10% SDS-PAGE gels. After gel-electrophoresis proteins were transferred onto PVDF membranes, blocked with 5% milk and probed overnight with primary antibodies against DDX3 (1:1000, mAb AO196) (J. General Vrology, 2010; 91(Pt 1):122-132) and Actin (1:10000, A5441, Sigma-Aldrich), DDX5 (1:1000, pab204, EMD Millipore, Billarica, MA, USA), DDX17 (1:1000, Bethyl, Montgomery, TX, USA) and p53 (1:1000, DO-1, Santa-Cruz Biotechnology, Dallas, TX, USA), and followed by appropriate secondary antibodies. The blots were developed with clarity western ECL (Bio-Rad, Hercules, CA, USA) and imaged with G:BOX Chemi XR5 (Syngene, Frederick, MD, USA).

**[0064]** Proliferation assay and cytotoxicity assays.

**[0065]** For the proliferation assays 4-10 x $10^4$ cells were plated in a 24-well plate. The following day the cells were transfected with siDDX3 or sicontrol as described earlier. 48 hours after transfection 2-5 x $10^3$ cells were plated per well in a 96-well plate. The amount of viable cells per well was estimated every 24 hours by an MTS-assay (CellTiter 96 Aqueous One Solution, Promega, Madison, WI, USA). For this, the cells were incubated with MTS reagent for 2 hours, after which absorbance was measured at 490 nm with a Victor$^3$V plate reader (PerkinElmer, Waltham, MA, USA).

**[0066]** For the cytotoxicity assays with adherent colorectal cancer cell lines 2-5 x $10^3$ cells were plated per well in a 96-well plate. The following day RK-33 was added. DMSO was added as a vehicle control. Read out occurred after 72 hours of drug exposure with an MTS assay.

**[0067]** The cytotoxicity assays on colosphere cultures have been described extensively elsewhere (FEBS open bio. 2015; 5:85-90). Briefly, 80-100 spheroids were plated per well in a 96-well plate with RK-33 or DMSO. After 72 hours of drug exposure the total cell population was labelled with DRAQ5™ (Abcam, Cambridge, UK) and live cells were labelled with Calcein Green AM (LifeTechnologies, Carlsbad, CA, USA). Fluorescence was measured using a Cellomics Arrayscan VTI HCS Reader (Thermo Fisher Scientific, MA, USA).The percentage of dead cells was calculated by normalizing the levels of intensity to and expressed as a relative percentage of the plate-averaged vehicle treated control.

Cell cycle analysis.

[0068] Cell cycle analysis was performed as was described previously (Oncogene. 2012; 31(27):3223-3234). In short, for siDDX3 experiments 5-15 x $10^4$ cells were plated per well in a 6-well plate. The following day cells were transfected with sicontrol or siDDX3 and incubated for 72 hours. For experiments with RK-33 4-7.5 x $10^5$ cells were plated in a 6-well plate. The following day cells were incubated for 24 hours with RK-33. Subsequently, cells were harvested and fixed in 70% ethanol overnight at -20□C. Fixed cells were incubated in DNA staining solution (5µg/ml propidium iodide, 0.5mg/ml RNAse A) for 1 hour. Cell cycle acquisition was performed on a FACScan I or FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Statistical significance was assessed with a student's t-test.

**[0069]** TCF-reporter assays.

**[0070]** Transcriptional activity of TCF4 was measured using the dual luciferase assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. For this, cells

were transfected with 500 ng TOP-FLASH or FOP-FLASH constructs (Cell. 2002; 111(2):241-250) and 50 ng phRL Renilla constructs as transfection controls, using jetPrime transfection reagent (Polyplus, New York, NY, USA). Luminescence was measured using a luminometer (Berthold Sirius, Oak Ridge, TN, USA).

**[0071]** For the experiments with RK-33 3-4 x $10^4$ HCT116 and HT29 cells were plated in a 24-well plate. After 24 hours the cells were transfected with the TOP/FOP constructs. 7 hours after transfection 2.5 μM RK-33 or DMSO was added for 12-24 hours after which the cells were lysed. For the DDX3 knockdown experiments 12.5-15 x $10^3$ HCT116 and HT29 cells were plated in a 24-well plate. In the evening of the following day the cells were transfected with 50 nM siDDX3 as described earlier. The following morning the cells were transfected with the TOP/FOP constructs and incubated for another 48 hours after which the cells were lysed for the luciferase assay. Relative TCF4-promotor activity was calculated by normalizing TOP-FLASH and FOP-FLASH readings for Renilla luciferase readings and dividing normalized TOP-FLASH readings by normalized FOP-FLASH readings. Statistical significance was evaluated by a paired t-test.

**[0072]** Quantitative reverse transcriptase polymerase chain reaction.

**[0073]** HCT116 and HT29 cells were harvested after 12-24 hour exposure to RK-33 or 72 hours after transfection with 50 nM siDDX3. RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA, USA) and cDNA was manufactured using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), followed by qPCR using SYBR green (Bio-Rad, Hercules, CA, USA) on an CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA). Amplification of 36B4, a housekeeping gene, was used for normalizing gene expression values. Primer sequences: *DDX3* F 5'-GGAGGAAGTACAGCCAGCAAAG-3' (SEQ ID NO: 1), *DDX3* R 5'-CTGCCAATGCCATCGTAATCACTC-3' (SEQ ID NO: 2), *AXIN2* F 5'-TCAAGTGCAAACTTTCGCCAACC-3' (SEQ ID NO: 3), *AXIN2* R 5'-TAGCCAGAACCTATGTGATAAGG-3' (SEQ ID NO: 4), c-*MYC* F 5′-CGTCTCCACACATCAGCACAA-3′ (SEQ ID NO: 5), c-*MYC* R 5′-CACTGTCCAACTTGACCCTCTTG-3′ (SEQ ID NO: 6), *CCND1* F 5′-GGCGGAGGAGAACAAACAGA-3′ (SEQ ID NO: 7), *CCND1* R 5′-TGGCACAGAGGGCAACGA-3′ (SEQ ID NO: 8), *BIRC5A* F 5′-CCACCGCATCTCTACATTCA-3′ (SEQ ID NO: 9), *BIRC5A* R 5′-TATGTTCCTCTATGGGGTCG-3′ (SEQ ID NO: 10). Fold changes in mRNA expression

were calculated using the $2^{-\Delta\Delta CT}$ method. Statistical significance was calculated by performing a paired student's t-test on the ΔCT values (BMC bioinformatics. 2006; 7:85)

**[0074]** Patient samples.

**[0075]** A tissue microarray (TMA's) with samples from 72 colorectal cancer patients from the Academic Medical Center, Amsterdam was kindly provided by professor Johan Offerhaus (University Medical Center Utrecht). This TMA also included one punch of surrounding normal mucosa per patient. The construction of this TMA has been reported in detail elsewhere (Gastroenterology. 2008; 134(5):1332-1341). An additional TMA with 292 colorectal cancer samples from Paderborn, Germany was provided by prof. Horst Bürger. As we used archival leftover pathology material and our study does not affect the included patients, no ethical approval is required according to Dutch legislation. Anonymous or coded use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospitals (BMJ. 2002; 325(7365):648-651).

**[0076]** Immunohistochemistry.

**[0077]** 4 μm sections were cut, mounted on SuperFrost slides (Menzel&Glaeser, Brunswick, Germany), deparaffinized in xylene and rehydrated in decreasing ethanol dilutions. For DDX3 staining, endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide buffer for 15 minutes and was followed by antigen retrieval by boiling for 20 minutes in 10mM citrate buffer (pH 6.0). Slides were subsequently incubated in a humidified chamber for 1 hour with anti-DDX3 (1:1000, pAb r647) (J. Gen. Virol. 2010; 91(Pt 1):122-132). After washing with PBS, slides were incubated with poly-HRP-anti-mouse/rabbit/rat IgG (Brightvision, Immunologic, Duiven, The Netherlands) as a secondary antibody for 30 minutes at room temperature. Peroxidase activity was developed with diaminobenzidine and hydrogen peroxide substrate solution for 10 minutes. The slides were lightly counterstained with haematoxylin and mounted. Positive controls (tonsil) were used throughout. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

**[0078]** β-catenin staining was performed automatically with the Leica BOND RX (Leica Microsystems, Rijswijk, The Netherlands). Antigens were retrieved with Epitope Retrieval Solution 2. The primary antibody against β-catenin (clone 17C2, Novocastra, Eindhoven, The Netherlands) was used in a 1:20 concentration.

**[0079]** Scoring was performed by consensus of two observers (M.H.v.V., P.v.D.). Intensity of cytoplasmic DDX3 expression was scored semi-quantitatively as being absent,

low, moderate or strong. The TMAs included multiple cores per patient; the highest score was used for further analysis. Cases with absent to moderate scores were classified as having low DDX3 expression and evaluated against cases with strong expression. Intratumoral DDX3 expression was compared to that of the surrounding normal tissue in case the latter was available for comparison.

**[0080]** β-catenin expression was scored separately for each subcellular compartment. Membrane expression was scored as complete, partial or lost. Cytoplasmic expression was scored as normal or overexpressed. The percentage of positive nuclei was scored. A cut-off of lower or higher than 10% was used for analysis.

**[0081]** Clinicopathological characteristics were compared between DDX3 low and high expressing tumors. Discrete variables were compared by $\chi 2$ or Fisher's exact test and odds ratios (OR) were calculated with 95% confidence intervals (95% CI). Statistical analyses were performed using SPSS version 20.0.

## EXAMPLE 1

**[0082]** DDX3 inhibition results in growth inhibition in colorectal cancer cell lines.

**[0083]** To assess DDX3 dependency, siRNA was used to knock down DDX3 expression in the colorectal cell lines HCT116 and HT29 (Figure 1A). DDX3 knockdown resulted in a reduction of cell proliferation in both cell lines (Figure 1B). To evaluate whether the reduction of viable cells was the result of reduced proliferation or increased cell death, cell cycle analysis was performed by flow cytometry on these cell lines after treatment with siDDX3. As seen in figure 1C, cell cycle analysis indicated a clear G1 arrest in HCT116 cells with a 15.8% increase in G1-phase ($p = 0.02$) and a 17.0% decrease of cells in S-phase ($p = 0.01$). In addition, a slight decrease in S-phase was observed in HT29 (3.4%; $p = 0.05$). These results indicate that these colorectal cell lines are dependent on DDX3 for cell cycle progression.

## EXAMPLE 2

**[0084]** DDX3 expression in colorectal cancer patient samples.

**[0085]** To evaluate whether DDX3 is also expressed in colorectal cancers, 303 colorectal cancer specimens were immunohistochemically stained for DDX3 (Figure 2). High

cytoplasmic DDX3 expression was present in 124 samples (40.9%). Corresponding normal mucosa was available for 59 cases. Intratumoral expression was higher in 23 patients (39.0%; Figure 2A-D), similar in 32 patients (54.2%; Figure 2E-F) and lower in 4 patients (6.8%; Figure 2G-H), when compared to the surrounding morphologically normal mucosa.

**[0086]** Next, we compared DDX3 expression to other known clinicopathological characteristics (Table 1). Within this cohort of samples, DDX3 expression did not correlate with any of the other clinicopathological variables.

**[0087]** Table 1. Baseline characteristics of colorectal cancer patients with low and high DDX3 expression. P-values are determined by a chi-square test unless otherwise indicated: * Fisher's exact test.

| | | Total | | Low DDX3 | | High DDX3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % | *P*-value | RR | 95% CI |
| Total | | 303 | 100.0 | 179 | 59.1% | 124 | 40.9% | | | |
| Sex | Male | 169 | 55.8% | 102 | 57.0% | 67 | 54.0% | 0.61 | 1.08 | 0.84–1.39 |
| | Female | 134 | 44.2% | 77 | 43.0% | 57 | 46.0% | | | |
| TNM stage | 1 | 22 | 9.6% | 13 | 9.7% | 9 | 9.4% | 0.73 | | |
| | 2 | 98 | 42.6% | 54 | 40.3% | 44 | 45.8% | | | |
| | 3 | 80 | 34.8% | 47 | 35.1% | 33 | 34.4% | | | |
| | 4 | 30 | 13.0% | 20 | 14.9% | 10 | 10.4% | | | |
| Differentiation grade | Well | 16 | 5.3% | 8 | 4.5% | 8 | 6.5% | 0.62 | | |
| | Moderate | 228 | 75.7% | 134 | 75.3% | 94 | 76.4% | | | |
| | Poor | 57 | 18.9% | 36 | 20.2% | 21 | 17.1% | | | |
| Site of origin | Rectum | 95 | 31.4% | 56 | 31.3% | 39 | 31.5% | 0.98 | 1.00 | 0.85–1.16 |
| | Colon | 208 | 68.6% | 123 | 68.7% | 85 | 68.5% | | | |
| Tumor size | <40 mm | 49 | 22.0% | 24 | 19.0% | 25 | 25.8% | 0.44 | | |
| | 40–60 mm | 120 | 53.8% | 69 | 54.8% | 51 | 52.6% | | | |
| | >60 mm | 54 | 24.2% | 33 | 26.2% | 21 | 21.6% | | | |
| Age at time of diagnosis | <65 years | 82 | 27.1% | 54 | 30.2% | 28 | 22.6% | 0.25 | | |
| | 65–80 years | 167 | 55.1% | 92 | 51.4% | 75 | 60.5% | | | |
| | >80 years | 54 | 17.8% | 33 | 18.4% | 21 | 16.9% | | | |
| Surgical margins | negative | 230 | 99.1% | 135 | 100.0% | 95 | 97.9% | 0.17 | | |

| | | Total | | Low DDX3 | | High DDX3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % | *P*-value | RR | 95% CI |
| | positive | 2 | 0.9% | 0 | 0.0% | 2 | 2.1% | | | |

EXAMPLE 3

**[0088]** High DDX3 expression correlates with nuclear β-catenin.

Given the activating role of DDX3 in Wnt signaling in other settings, we wanted to determine whether high DDX3 expression is associated with activated Wnt signaling in colorectal cancer patient samples as reflected by an increased cytoplasmic and nuclear β-catenin pool (Figure 3). We separately scored the membranous, cytoplasmic and nuclear localization of β-catenin in tumors with low and high DDX3 expression, which is shown in Table 2. Nuclear β-catenin expression was significantly more prevalent in the DDX3 high group (59.3%) when compared to the DDX3 low group (33.5%; RR = 1.77; 95% CI = 1.36-2.31; p =2.47 x $10^{-5}$), indicating a connection between DDX3 levels and nuclear β-catenin accumulation. In addition, a trend was observed for more frequent overexpression of cytoplasmic β-catenin in DDX3 high tumors (73% vs 63%; RR = 1.16; 95% CI = 0.99-1.36; p =0.08), which often coincides with nuclear β-catenin expression as shown in figure 3D.

**[0089]** Table 2. Subcellular localization of β-catenin in colorectal cancer samples with low and high DDX3. P-values calculated by a chi-square test.

| | | Total | | Low DDX3 | | High DDX3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % | *P*-value | RR | 95% CI |
| Membranous β-catenin | complete expression | 231 | 83.4% | 136 | 83.4% | 95 | 83.3% | 0.82 | | |
| | partial expression | 29 | 10.5% | 16 | 9.8% | 13 | 11.4% | | | |
| | loss of expression | 17 | 6.1% | 11 | 6.7% | 6 | 5.3% | | | |
| Cytoplasmic β-catenin | normal expression | 91 | 32.9% | 60 | 37.0% | 31 | 27.0% | 0.08 | 1.16 | 0.99–1.36 |
| | overexpression | 186 | 67.1% | 102 | 63.0% | 84 | 73.0% | | | |
| Nuclear β-catenin | <10% | 153 | 55.8% | 107 | 66.5% | 46 | 40.7% | $2.47 \times 10^{-5}$ | 1.77 | 1.36–2.31 |
| | >10% | 121 | 44.2% | 54 | 33.5% | 67 | 59.3% | | | |

## EXAMPLE 4

**[0090]** Sensitivity of colorectal cancers to RK-33, a small molecule inhibitor of DDX3.

**[0091]** Considering the fact that DDX3 is overexpressed in colorectal cancers, we evaluated the *in vitro* sensitivity of colorectal cancer cells to DDX3 inhibition by RK-33, a small molecule inhibitor of DDX3. Five colorectal cancer cell lines (HCT116, HT29, DLD-1, SW480 and Colo205) were treated with RK-33 and cell viability was assessed by an MTS assay (Figures 4A-B). All cell lines had an $IC_{50}$ value in the low micromolar range (3-7 μM). To assess whether RK-33 also showed cytotoxicity in 3D cultures, we expanded our panel with four patient-derived colorectal cancer spheroid cell lines. Spheroid viability after RK-33 exposure was evaluated with an arrayscan (Figure 4C-E). The spheroids displayed comparable sensitivity to RK-33 as the adherent cell lines ($IC_{50}$ value range 3-9 μM). Treatment with RK-33 resulted in a G1 arrest in a dose-dependent manner in both HCT116 and HT29 (Figure 4F). An increase in the percentage of apoptotic cells could also be observed in HCT116 after DDX3 inhibition, but not in HT29 (data not shown). The differences in cell cycle distribution were more profound than the increase in apoptotic cells, indicating that the primary effect of DDX3 inhibition is a G1 arrest, which ultimately can result in apoptosis.

**[0092]** Both adherent cell lines and spheroids could be separated into two groups; a sensitive group of cell lines with an $IC_{50}$ value <3 μM (HCT116, CRC29, HT29) and a group with a 2-3 fold higher $IC_{50}$ value ranging from 5-9 μM (CR9, DLD-1, CRC47, SW480, Colo205, L145). Next, we assessed the functional role of DDX3 in the cell lines that were less sensitive to RK-33, by knocking down DDX3 with siDDX3 (Figure 4G). Unlike the RK-33 sensitive cell lines HCT116 and HT29, proliferation in DLD-1 and SW480 was not affected by DDX3 knockdown (Figure 4H) and only a moderate drop in S-phase was observed in SW480 (3.7%; $p = 0.02$). No effect on cell cycle was seen in DLD-1 (Figure 4I).

## EXAMPLE 5

**[0093]** Molecular predictors of DDX3 dependency.

**[0094]** Centered on our finding of differential sensitivity to RK-33, we hypothesized that sensitivity to RK-33 in colorectal cancer cells may also be associated with other genomic drivers of cellular transformation. Almost all colorectal cell lines and spheroids expressed

DDX3 protein (Figure 4A&C), but no direct correlation between RK-33 sensitivity and DDX3 expression levels was observed. Next, we assessed the most commonly occurring mutations in our cell line panel by next generation sequencing (Table 3). Interestingly, we found that two of the three RK-33 sensitive cell lines had wild-type *APC* and *TP53,* whereas the less sensitive group had mutations in these genes.

**[0095]** Table 3. The genomic background of colorectal cancer derived adherent cell lines and 3D cultures and their relative sensitivity to RK-33. Mutational status was derived from:*publicly available data in the CanSAR database †next-generation sequencing. - no mutation detected.

| Cell line | RK-33 Sensitivity | | Mutations | | | | MSI status |
|---|---|---|---|---|---|---|---|
| | IC50 (μM) | 95% CI | *APC* Truncation | *TP53* | *RAS/RAF* | *Others* | |
| HCT116* | 2.71 | 2.64–2.80 | – | – | *KRAS* | *PIK3CA, CTNNB1* | MSI |
| CRC29† | 2.89 | 2.66–3.14 | – | – | *KRAS, BRAF* | *SMAD4, STK11* | MSI |
| HT29* | 2.96 | 2.84–3.08 | E853X, T1556fsX3 | R273H | *BRAF* | *PIK3CA, SMAD4* | MSS |
| CR9† | 4.97 | 4.51–5.48 | R1114X, S1503E | R213X, V157A | – | *SMAD4, FBXW7, ERBB2* | |
| DLD-1* | 5.36 | 5.15–5.59 | I1417X | S241F | *KRAS* | | MSI |
| CRC47† | 5.54 | 5.07–6.05 | R1450X | P75LfsX48 | – | *PIK3CA* | MSI |
| SW480* | 6.32 | 5.60–7.15 | Q1338X | R273H, P309S | *KRAS* | *SMAD4* | MSS |
| Colo205* | 6.65 | 6.00–7.37 | T1556fsX3 | Y103fsX8 | *BRAF* | | MSS |
| L145† | 8.77 | 7.63–10.08 | S1436LfsX34 | R110P | *KRAS* | | MSS |

**[0096]** To further determine if *TP53* mutations in cell lines alters sensitivity to RK-33, we compared RK-33 sensitivity in isogenic cell lines with wild-type *TP53* (HCT116-p53$^{+/+}$) and without *TP53* (HCT116-p53$^{-/-}$; Figure 5A). Both cell lines were equally sensitive to DDX3 inhibition with RK-33 ($IC_{50}$ 2.52 *vs* 2.58 μM; Figure 5B). In addition, similar to the parental cell line that expresses p53 (Figure 1C), knockdown of DDX3 resulted in a G1 arrest (13.9% increase; $p = 0.04$) and a decrease of cells in S-phase (4.8%; $p = 0.11$; Figure 5C-D). This indicates that within our experimental setting the sensitivity of RK-33 is independent of p53 status.

## EXAMPLE 6

**[0097]** RK-33 sensitivity in relation to different mutations in the Wnt-signaling pathway.

**[0098]** Although DDX3 is thought to play a role in Wnt signaling, cells that harbor an *APC* mutation were less sensitive to RK-33 than cells with wild-type *APC*. Since *CTNNB1* and *DDX3X* mutations co-occur in Wnt-type medulloblastomas, we hypothesized that DDX3 dependency may be higher in cells with other genetic aberrations in the Wnt-signaling pathway, like mutations in the gene encoding β-catenin. We used HCT116 cells with either the wild-type allele deleted (HCT116 *CTNNB1*$^{\Delta 45/-}$) or the mutant β-catenin allele deleted (HCT116 *CTNNB1*$^{-/wt}$) to study the contribution of each allele to RK-33 sensitivity. Interestingly, we found that cells with mutant β-catenin were slightly more sensitive ($IC_{50}$ 2.68 μM) than those with only a wild-type allele ($IC_{50}$ 3.71 μM) and that DDX3 expression is slightly higher in HCT116 β-catenin$^{\Delta 45/-}$ cells (Figure 5D-E). These results indicate that *APC* wild-type colorectal cancers harboring an activating *CTNNB1* mutation may be more sensitive to RK-33 treatment.

## EXAMPLE 7

**[0099]** Inhibition of DDX3 results in reduced Wnt signaling.

**[0100]** To evaluate whether the observed proliferation inhibition is the result of interference with oncogenic Wnt signaling, we tested whether DDX3 inhibition causes a reduction in TCF4-promoter activity with a reporter assay. Knockdown of DDX3 resulted in a significant decrease in Wnt signaling in HCT116 (42%, $p = 0.001$) and a small decrease in HT29 (17%, $p = 0.23$; Figure 6A). RK-33 treatment resulted in an even greater inhibition of TCF4-reporter activity of 74% in HCT116 ($p = 0.0008$) and of 44% in HT29 ($p = 0.03$; Figure 6B). To validate whether the reduced TCF4-reporter activity also resulted in reduced mRNA expression of TCF4-regulated genes, we quantified transcript expression for c-*MYC*, *AXIN2*, *CCND1* and *BIRC5A*. As seen in Figure 6C and supplementary table 1, DDX3 knockdown resulted in reduced expression of *AXIN2*, *CCND1* and *BIRC5A* in HCT116. Similarly, a decrease was observed in *CCND1*, c-*MYC* and *BIRC5A* expression in HT29. RK-33 treatment also significantly reduced the amount of transcripts of c-*MYC*, *AXIN2*, *CCND1* and *BIRC5A* in HCT116 (Figure 6D). Again this result was slightly less profound in HT29, where RK-33 caused a reduction in *AXIN2*, *CCND1* and *BIRC5A*. Overall, inhibition of

DDX3 results in decreased Wnt signaling in HCT116 and to a lesser extent in HT29, which corresponds to their relative dependence on DDX3 for cell cycle progression.

## EXAMPLE 8

**[0101]** RK-33 treatment reduces DDX5 protein levels.

**[0102]** Since DDX5 and DDX17 are known mediators of Wnt signaling in colorectal cancer and DDX3 and DDX5 have been found to interact, we evaluated whether RK-33 treatment also influences DDX5 and DDX17 protein levels. Although we found earlier that RK-33 does not bind directly to DDX5, exposure to RK-33 resulted in decreased DDX5 protein levels, but did not affect DDX17 expression (Figure 7). This indicates that the observed reduction in Wnt signaling could be either as a direct result of decreased DDX3 levels, of the consequentially lowered DDX5 expression, or of a combination of both mechanisms.

**[0103]** All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

**[0104]** The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

**[0105]** Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Claims:

1. Use of a pharmaceutical composition comprising an effective amount RK-33 or a salt, solvate or derivative thereof, and a pharmaceutically acceptable carrier for treating colorectal cancer in a subject in need thereof.

2. The use of claim 1, wherein the compositions further comprises at least one additional chemotherapeutic agent.

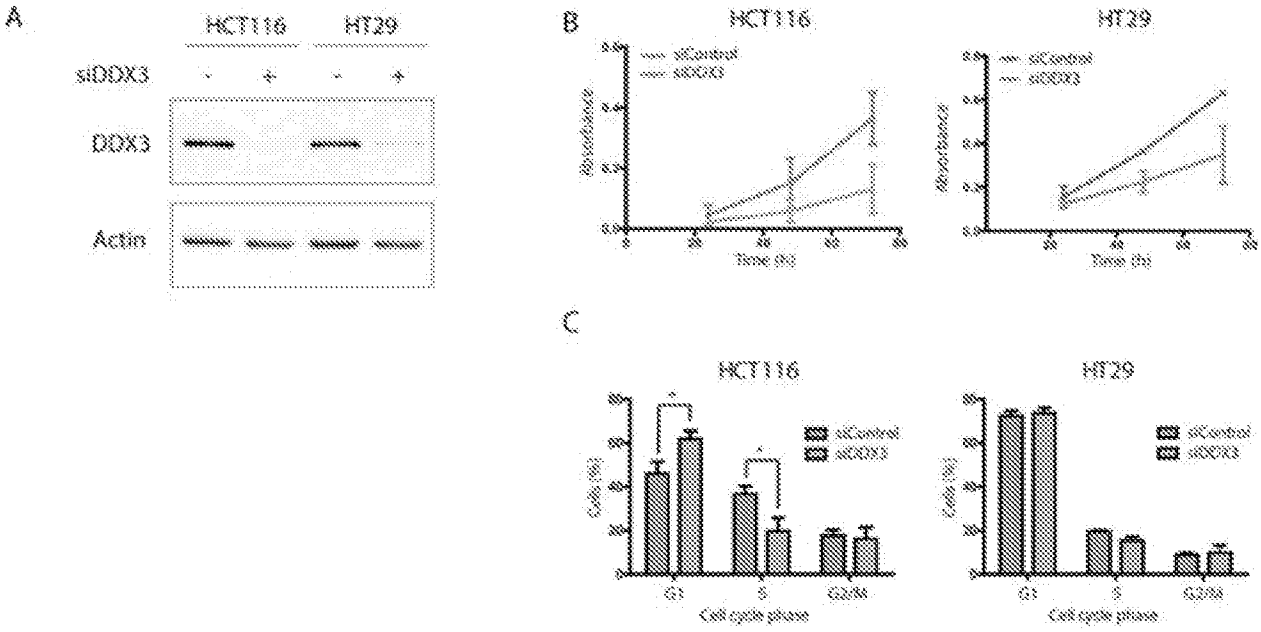
3. The use of either claims 1 or 2, wherein the subject is undergoing ionizing radiation therapy.

4. The use of either claims 1 or 2, wherein the systemic dose of RK-33 in the composition is at a concentration of between 100 nM to 1 mM.

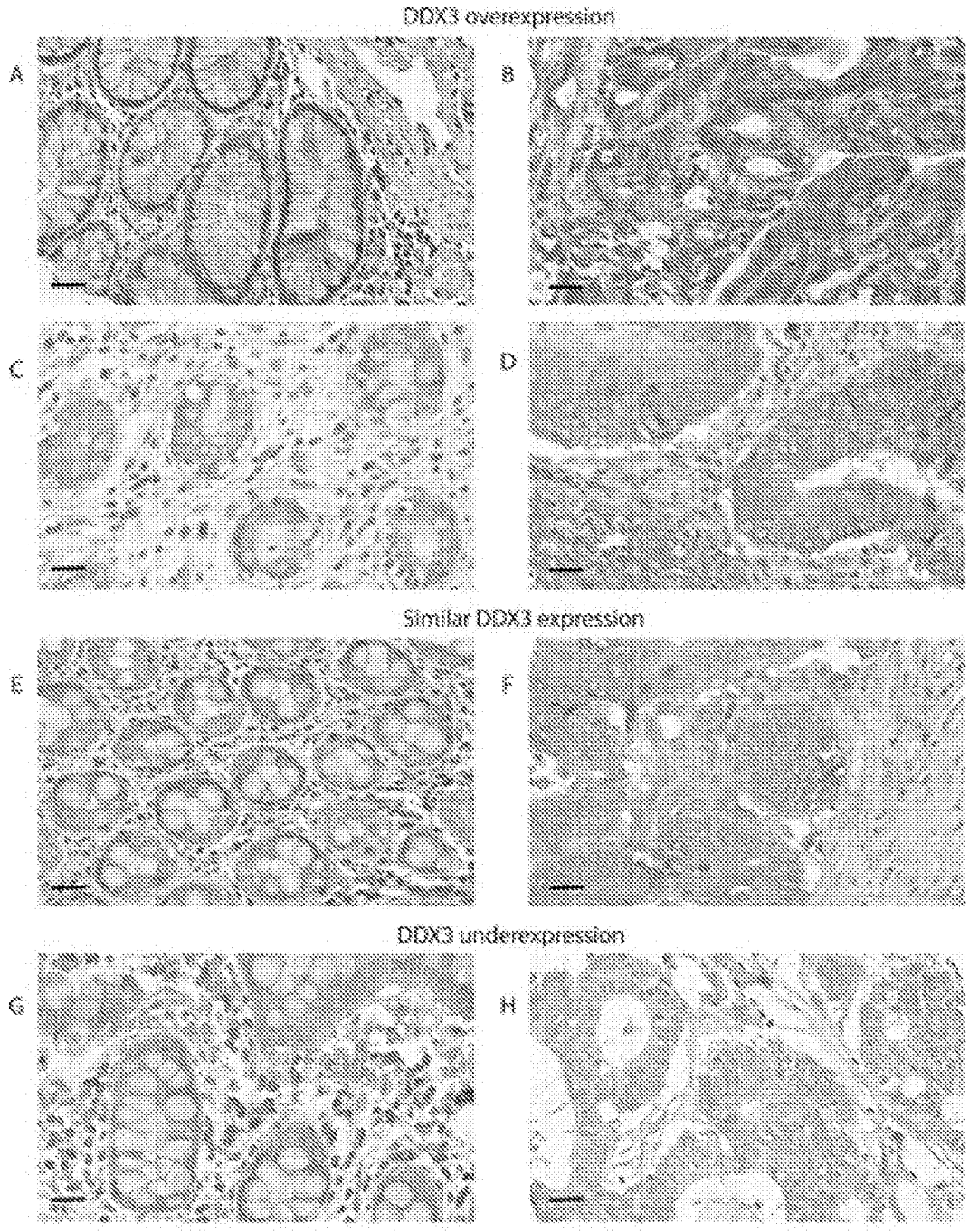
5. Use of a composition comprising RK-33 for inhibiting DDX3 signaling in a colorectal cancer cell or population of cells comprising contacting the cell or population of cells with an effective amount of RK-33.

6. The use of claim 5, wherein the concentration of RK-33 is between 100 nM to 1 mM.

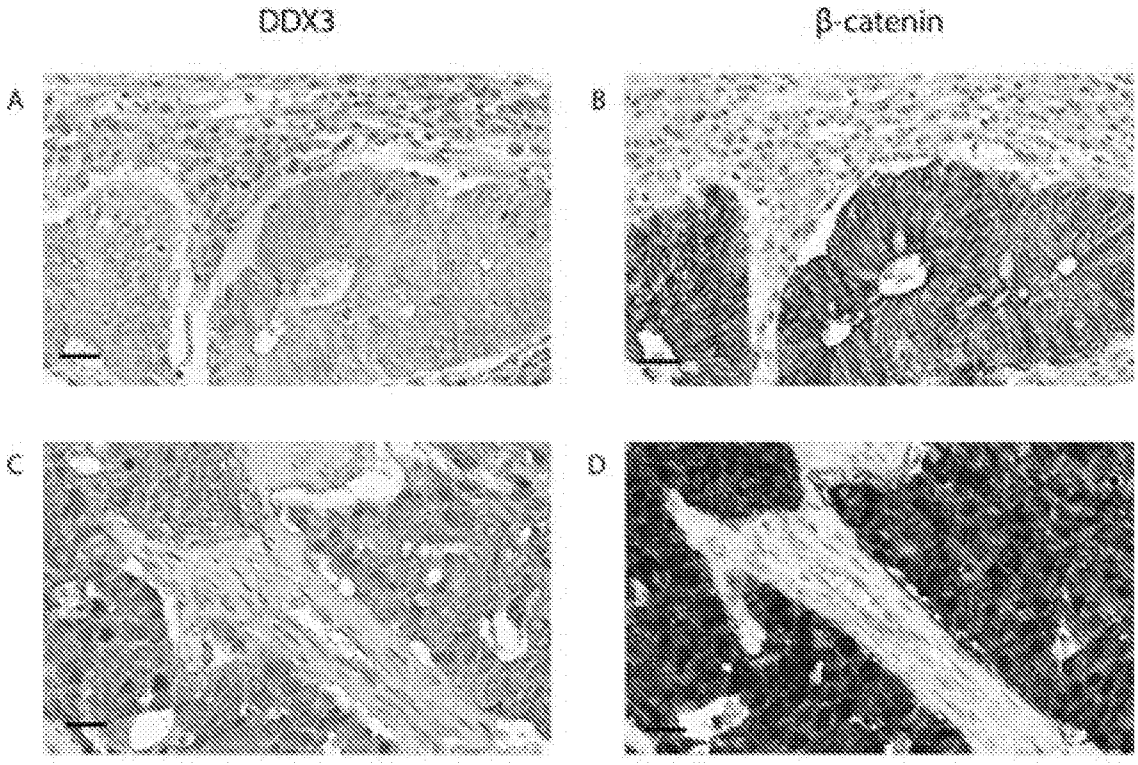
FIGURES 1A-1C

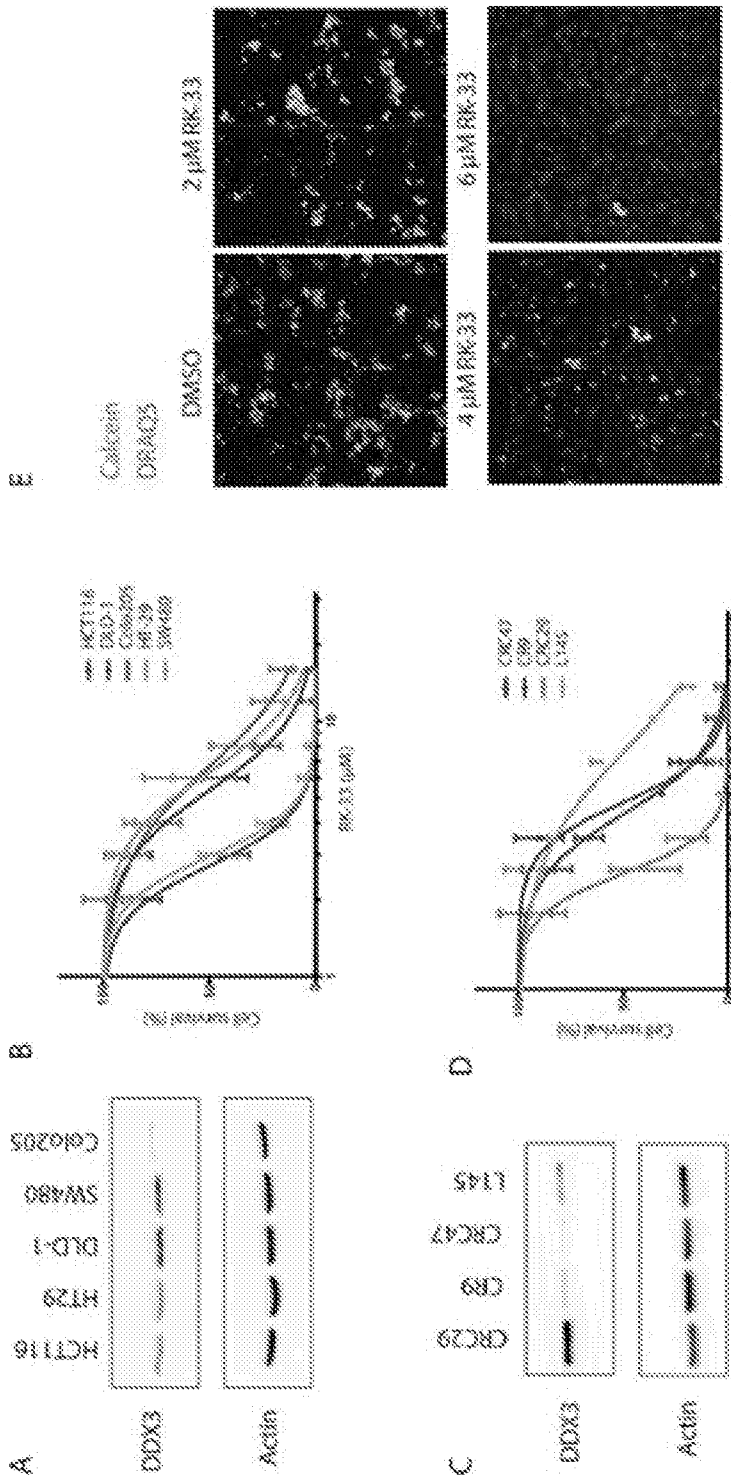


FIGURES 2A-2H



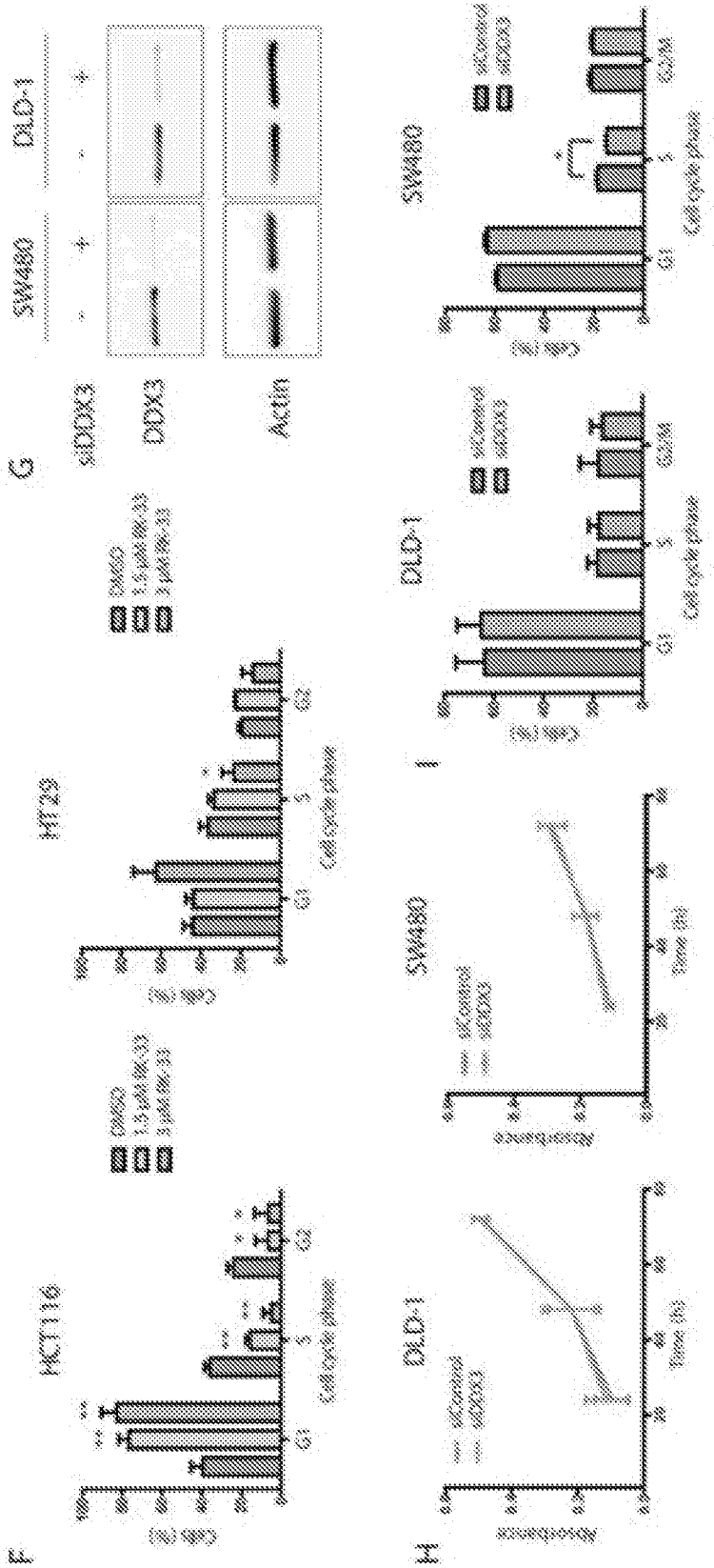
FIGURES 3A-3D



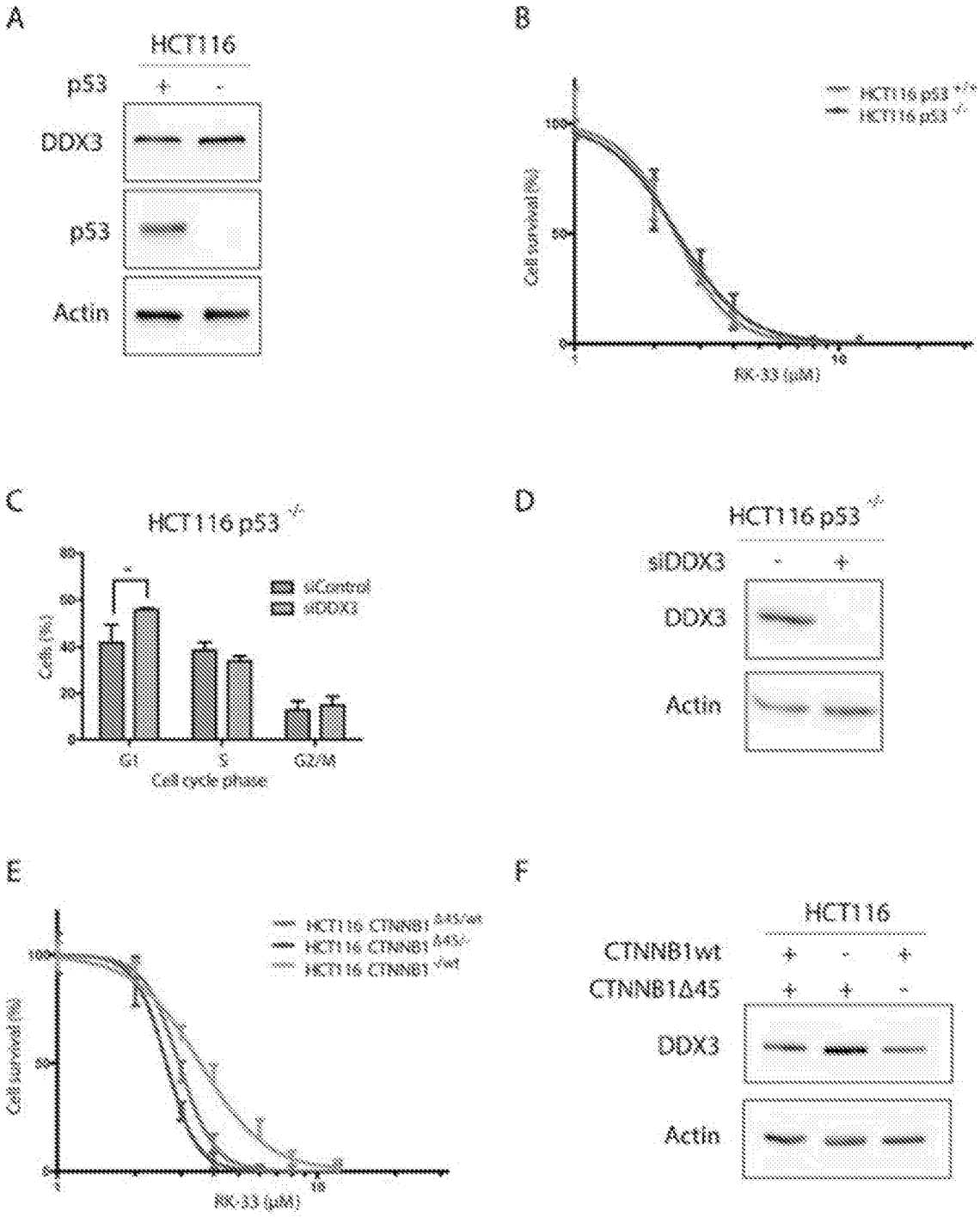



FIGURES 4A-4E

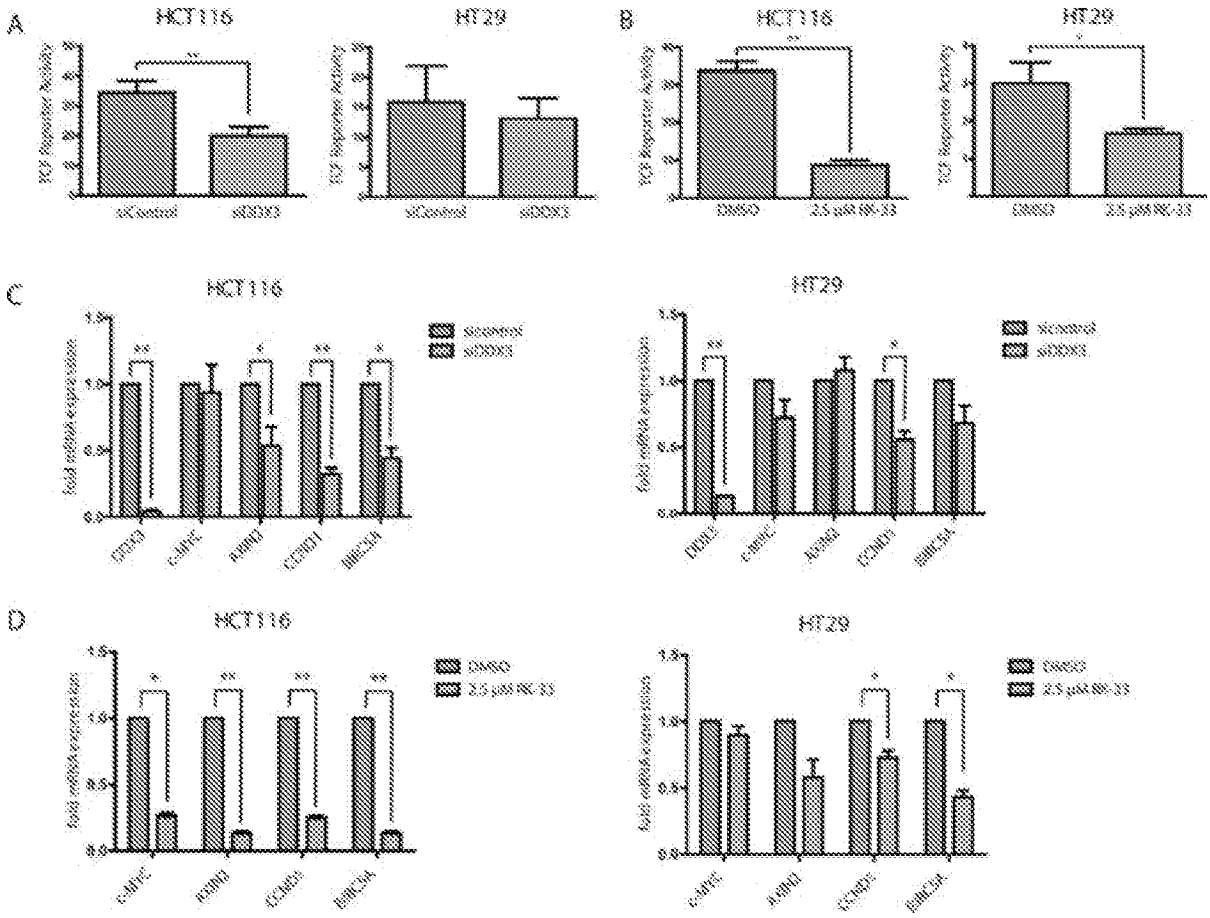
FIGURES 4F-4I



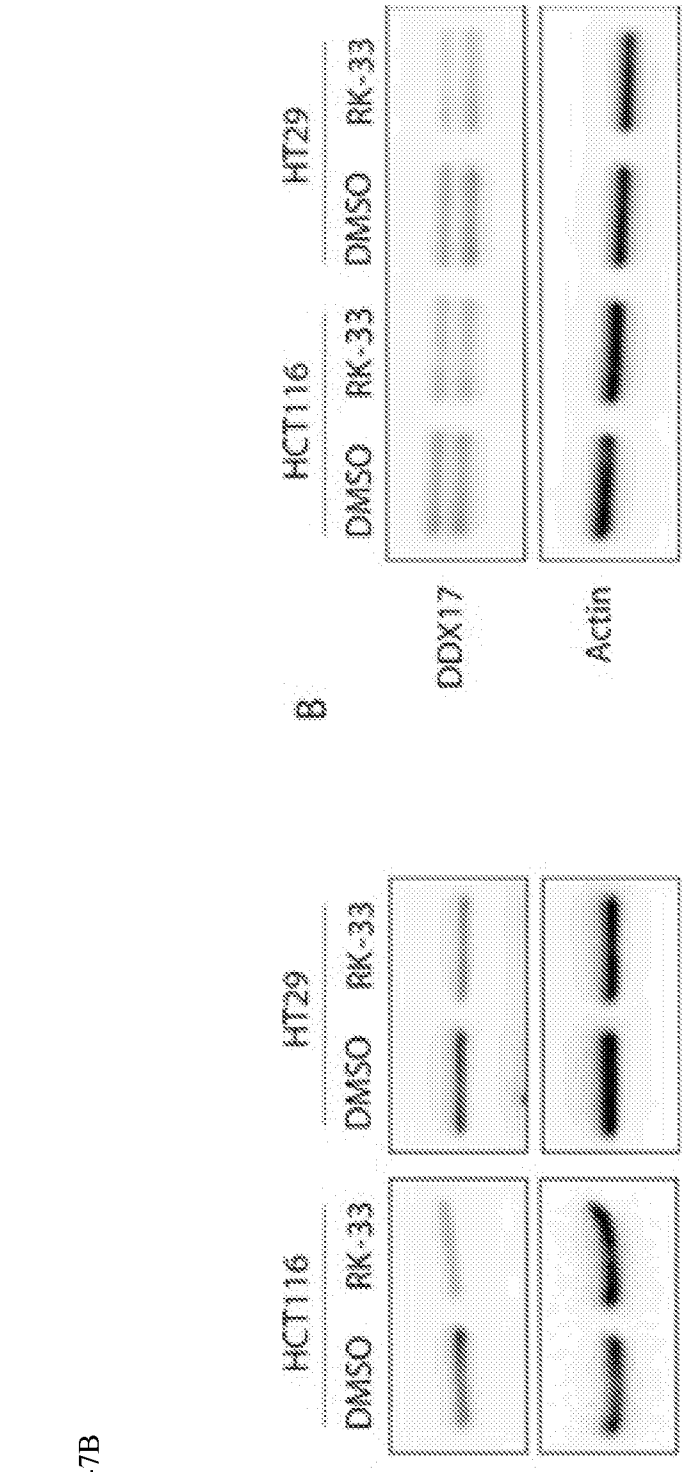
FIGURES 5A-5F



FIGURES 6A-6D



FIGURES 7A-7B



**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2016/022475**

**A. CLASSIFICATION OF SUBJECT MATTER**

**A61K 31/5517(2006.01)i, A61P 35/00(2006.01)i**

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 31/5517; C07D 487/14; G01N 33/68; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(Kipo internal) & STN (Registry, CAplus) & Google & Keywords: RK-33, cancer, DDX-3

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 August 2016 (25.08.2016) | **26 August 2016 (26.08.2016)** |


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**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2016/022475**

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| PX | HEERMA VAN VOSS, M. R. et al., "Identification of the DEAD box RNA helicase DDX3 as a therapeutic target in colorectal cancer", Oncotarget, [Epub.] 01 August 2015, Vol. 6, No. 29, pp. 28312-28326 See abstract; and figure 4. | 1-6 |

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Information on patent family members

International application No.

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