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(54) **Title:** PHOSPHORYLATION OF HISTONE H3 AT THREONINE 11 - A NOVEL EPIGENETIC MARK FOR TRANSCRIPTIONAL REGULATION

(57) **Abstract:** The present invention relates to a process for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells under histone-phosphorylating conditions, said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK1 thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said at least one androgen receptor-regulated mechanism in said mammalian cells.

Phosphorylation of Histone H3 at Threonine 11 – A novel Epigenetic Mark for Transcriptional Regulation

The present invention relates to a process for controlling androgen receptor-regulated mechanisms in mammalian cells under histone H3 at threonine 11-(H3T11-) phosphorylating conditions. Furthermore, the invention relates to a use of inhibitors having specificity for at least one protein kinase C-related kinase (PRK) for controlling androgen receptor-regulated mechanisms in mammalian cells.

Posttranslational modifications of histones such as methylation, acetylation and phoshorylation regulate chromatin structure and gene expression [1]. Threonine and serine residues are phosphorylated by specific kinases that stay under the control of signalling pathways. A phosphorylation of histone H3 at threonine 11 (H3T11) has not been linked to transcriptional regulation. The protein kinase C-related kinase 1 (PRK1) [2] has been shown to phosphorylate H3T11 upon ligand-dependent recruitment to androgen receptor (AR) target genes. H3T11 phosphorylation is an early event that precedes demethylation of mono-, di-, and trimethyl histone H3 at lysine 9 by JMJD2C and lysine specific demethylase 1 (LSD1). PRK1 is pivotal to AR function, since PRK1 knockdown by RNAi or PRK1 inhibition by treatment with Ro318220 impedes AR-dependent gene expression. Blocking PRK1 function abrogates androgen-induced phosphorylation of H3T11, but also blocks, in consequence, demethylation of mono-, di-, and trimethyl H3K9 as well as acetylation of histone H3 at lysines 9 and 14 (H3K9 and H3K14). Moreover, the presence of serine 5-phosphorylated RNA polymerase II is no longer observed at AR target promoters. Thus, phosphorylation of H3T11 by PRK1 establishes a novel epigenetic mark for transcriptional activation, identifying PRK1 as a gatekeeper of AR-regulated gene expression. This pathway is of utmost importance since knockdown of PRK1 in prostate cancer cells inhibits androgen-induced transcriptional activation and tumor cell prolif-

eration. Thus, our data suggest that specific gene regulation requires the assembly and coordinate action of kinases and demethylases. Furthermore, regulation of PRK1 activity alone or in combination with LSD1 and JMJD2C might be a promising therapeutic strategy to control AR activity in prostate cancer. Importantly, high PRK1 levels positively correlate with high Gleason scores of prostate carcinomas, allowing the present invention to be used in scoring prostate carcinomas.

The N-terminal tails of histones are subject to a plethora of posttranslational modifications such as acetylation, phosphorylation, and methylation by specific chromatin-modifying enzymes [1]. During gene expression, these modifications influence chromatin structure to facilitate the assembly of the RNA polymerase II transcription machinery [1, 3]. Androgen receptor (AR)-dependent gene expression is characterized by epigenetic changes such as removal of repressive methyl marks from lysine 9 of histone H3 (H3K9) [4, 5] and acetylation of lysines 9 and 14 of histone H3 (H3K9/K14) [6]. However, little is known about the upstream regulators that govern these epigenetic modifications. Since protein kinase C-related kinase 1 (PRK1) controls AR-dependent gene expression [2], we asked whether PRK1 signalling regulates epigenetic events at AR target genes.

Hence, it was an object of the present invention to identify further modulators of the AR-regulated gene expression and/or androgen-induced cell proliferation, particularly in mammalian cells.

Moreover, it was an object of the present invention to provide further modulators of histone modification, in particular of histone phosphorylation, methylation and acetylation.

Another object of the invention was to provide processes for controlling at least one androgen receptor-regulated mechanism in mammalian cells and for controlling the transcriptional AR activation induced by different routes.

A further object of the invention was to provide a new process for the prevention and/or treatment of prostate cancer.

Another object of the invention was to provide for the use of one or more than one inhibitor for the medicament manufacture, particularly for manufacturing a medicament for preventing and/or treating cancer, particularly prostate cancer.

Furthermore, it was an object of the invention to provide a means for scoring prostate cancers, particularly an assay system.

Finally, it was an object of the invention to provide an assay system for inhibitors having specificity for at least one PRK capable of blocking AR-induced prostate carcinoma cell proliferation.

The present invention relates to a process for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells under histone H3 at threonine 11- (H3T11-) phosphorylating conditions, said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said at least one androgen receptor-regulated mechanism in said mammalian cells.

Preferred embodiments of the invention are claimed in the dependent claims 2 to 5.

The invention also relates to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells.

Preferred embodiments of such use are claimed in claims 7 to 9.

The invention also relates to a process for controlling the androgen dependent gene expression induced by a phosphorylation of histone H3 at threonine 11 (H3T11) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression; and/or to a process for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression; and/or to a process for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression; and/or to a process for controlling the androgen dependent gene expression induced by a transition from the pre-initiation to the initiation complex which is characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at

least one PRK and optionally blocking said androgen dependent gene expression.

Preferred embodiments of said processes are claimed in the dependent claims 14 to 18.

Additionally, the invention relates to a use of at least inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a phosphorylation of histone H3 at threonine 11 (H3T11) in the presence of at least one protein kinase C-related kinase (PRK); and/or to a use of at least inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK); and/or to a use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least protein kinase C-related kinase (PRK); and/or to a use of at least inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a transition from the pre-initiation to the initiation complex which is characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK).

Preferred uses are claimed in dependent claims 23 to 25.

The invention also relates to a process for the prevention and/or treatment of prostate cancer, said process comprising administering, to a plurality of mam-

malian cells in need thereof, including prostate cancer cells in need thereof, at least one inhibitor with speficicity for at least one protein kinase C-related kinase, thereby modulating, preferably down-regulating, the phosphorylation of histone H3 at threonine 11 (H3T11) by said at least one PRK and/or modulating, preferably down-regulating, the demethylation of histone H3 at lysine 9 (H3K9) by at least one histone demethylase, preferably by lysine specific demethylase (LSD1) and/or by at least one JMJD, specifically by JMJD2C, each alone or both in combination, and/or modulating, preferably down-regulating, the acetylation of histone H3 at lysine 9 (H3K9) and/or of histone 3 at lysine 14 (H3K14) by at least one acetylase, and/or modulating, preferably down-regulating, the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) by CDK7.

The invention relates, too, to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for the prevention and/or treatment of prostate cancer.

The invention also relates to the use of at least one antibody with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a composition capable of scoring prostate carcinomas.

In addition, the invention relates to an assay system for screening inhibitors having specificity for at least one PRK capable of blocking AR-induced prostate carcinoma cell proliferation, said assay system comprising at least one PRK, an (optionally labelled) substrate [which, as the case may be, may also be a substrate with an antibody suitable for a detection], a phosphate-delivering component (as, for example, ATP) and suitable auxiliary substances as, for example one or more buffers and one or more pH-adjusting compound(s), etc..

Finally, the invention also relates to an assay system for scoring prostate carcinomas in a tissue sample, said assay system comprising a reagent for detecting the presence of PRK1. In a preferred embodiment of the assay system, said reagent is selected from the group consisting of an antibody against PRK1 and PCR primers.

The invention is further in detail described by referring to the annexed Figures; however, the Figures are intended to show exemplarily preferred embodiments of the invention, only. Hence, the invention is not restricted neither by the subsequent description of the preferred embodiments not by the reference to the Figures nor by the Figures itself.

Figure Legends

Figure 1: PRK1 controls AR-dependent gene expression and associates with chromatin. LNCaP cells were cultivated in the presence or absence of the AR agonist R1881. miRNA-mediated PRK1 knockdown (**a**) or the PRK1 inhibitor Ro318220 (**b**) reduce expression of the endogenous *PSA* and *KLK2* genes (**a**, left panel, **b**). Western blot analysis (**a**, right panel) verified the specific miRNA-mediated knockdown of PRK1. Bars represent mean +SD (n>4). ChIP and Re-ChIP (**c**) using the indicated antibodies demonstrate androgen-dependent association of PRK1 at promoters of AR-regulated genes. The precipitated chromatin was amplified by PCR using primers flanking the AREs in the promoter region of the *PSA* and *KLK2* genes, or the promoters of the unrelated *GAPDH* and *U6* genes.

Figure 2: PRK1 phosphorylates histone H3 at threonine 11 (H3T11). Bacterially expressed GST and GST-H3 fragments (**a**, **b**) or nucleosomes from HeLa cells (**c**) were incubated for the indicated time with active PRK1 or the kinase dead mutant PRK1 K644E in the presence or absence of the inhibitor Ro318220. Coomassie blue staining shows the amounts of GST fusion proteins used (**a**

and **b**, lower panels). Western blots were decorated with the indicated antibodies (**c**). LNCaP cells (**d**, **e**) were cultivated in the presence or absence of the AR agonist R1881, transfected with stealth RNAi, and subjected to ChIP with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking AREs in the promoter region of the *PSA* and *KLK2* genes. Western blot analysis (**d**, right panel) verified the specific siRNA-mediated knockdown of PRK1.

Figure 3: PRK1 controls epigenetic modifications of histone H3 and AR-dependent gene expression. For ChIP (**a**, **b**, **f**, **g**) and transient transfections (**c**, **d**, **e**), cells were cultivated in the presence or absence of the AR agonist R1881 and the inhibitor Ro318220 as indicated. LNCaP cells were transfected with stealth RNAi (**a, f**). ChIP analyses were performed with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking AREs in the promoter region of the *PSA* and *KLK2* genes. For transient transfections, CV1 (**c**, **e**) or 293 (**d**) cells were co-transfected with AR expression plasmid and AR-dependent reporters. Bars represent mean +SD (n>4).

Figure 4: PRK1 levels positively correlate with the malignancy of prostate cancer and control tumour cell proliferation. The correlation of high PRK1 expression with high Gleason score in a panel of 111 human prostate carcinomas is highly significant: $r=0.499$, $p<0.001$. Normal prostate specimens (n=20) are included as a control (**a**). In LNCaP cells, miRNA-mediated PRK1 knockdown severely reduces R1881-induced cell proliferation. Bars represent mean +SD (n>4) (**b**).

Supplementary Figure S1: PRK1 controls AR-dependent gene expression. LNCaP cells were cultivated in the presence or absence of the AR agonist R1881. miRNA-mediated PRK1 knockdown (**a**) or the inhibitor Ro318220 (**b**) severely reduce AR-dependent reporter activity. Bars represent mean +SD (n>4).

Supplementary Figure S2: In 293 cell lysates, the presence of PRK1 proteins used for the kinase assay was verified by Western blot analysis using an α-myc (**a**) or an α-flag (**b**) antibody.

Supplementary Figure S3: The α-phosphoH3T11 antibodies used for Western blot analysis (**a**) and for ChIP assays (**b**) specifically recognize H3 phosphoT11. 1 µg of the indicated peptide was spotted onto nitrocellulose (Protran BA 79, Schleicher & Schuell). The H3 1-20 peptide was obtained from Peptides & Elephants. The H3 5-13 phosphoS10 and H3 5-13 phosphoT11 peptides were obtained from Abcam. Western blots were decorated as indicated. Controls show equal amounts of Ponceau red stained peptides (**a**, **b**, lower panels).

Supplementary Figure S4 AR, PRK1 K644E, JMJD2C, and LSD1 do not influence the transcriptional activity of the TK-LUC control reporter. CV1 (**a**, **c**) or 293 (**b**) cells were co-transfected with expression plasmids and the TK-LUC reporter in the presence or absence of R1881 and Ro318220, as indicated. Bars represent mean +SD (n>4).

The invention is further described in detail by referring to the Figures.

By the processes of the invention, at least one androgen receptor-regulated (AR-regulated) mechanism in mammalian cells is controlled under histone-phosphorylating conditions. Such AR-regulated mechanisms are numerous in the mammalian body (which is the main, but not exclusive target of the present invention, since all processes of the invention are considered as occurring either *in vivo* or *in vitro*) and are well-known to a person skilled in the present field. Preferred (although not exclusive) embodiments of AR-regulated mechanisms in mammalian cells are all physiological processes controlled by the androgen receptor (AR), such as processes selected from the group consisting of the androgen receptor-controlled gene expression and the androgen-induced

cell proliferation and the androgen-induced function of the prostate and the androgen-induced build-up of muscles and the androgen-induced build-up of the bones, preferably the androgen-induced control of the bone density, and the androgen-induced fertility and the androgen-induced hair growth of a mammal.

In this connection, the term "mammal" has the meaning of covering all animals (including humans) which nourish the progeny by lactation. The invention is not restricted to humans and includes other mammals as, for example, cattle, horses, monkeys, dogs, cats, rabbits etc..

The term "under histone-phosphorylating conditions", as used in the specification and claims, means that the mammalian cells under observation are kept in a state where modifications of histones by condensation reactions at certain residues with phosphorus-containing residues, particularly with phosphate residues, can be carried out under biologically acceptable conditions or conditions comparable to biological conditions and optionally in the presence of suitable catalysing enzymes. Preferably, the phosphorylation occurs at histone H3 at threonine 11 (H3T11).

The process of the invention for controlling at least one androgen receptor-regulated mechanism in mammalian cells under histone-phosphorylating conditions at histone H3 at threonine 11 (H3T11) comprises the step of allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK. There may be used one inhibitor, or there may be used two or several inhibitors. The use of one inhibitor is preferred in accordance with the invention.

In a preferred embodiment of the invention, the specificity of the inhibitor or inhibitors used towards the at least one protein kinase C-related kinase (PRK) is high. In particularly preferred embodiments of the invention, the at least one inhibitor is a highly specific PRK inhibitor and, more preferably has a specificity

of < 100 nM. Particularly preferred embodiments of the invention relate to at least one inhibitor or exactly one inhibitor having a specificity of, for example 10 nM.

In more preferred embodiments, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of RNAis, antibodies, other peptides and dominant negative mutants of PRKs. Most preferably, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of miRNA, siRNA, micro-RNA, shRNA, anti-PRK1 antibodies and aptamers (i. e. small peptides attaching to the protein and inactivating it; the length of such aptamers may be about 10 peptides, without restricting this term to said length), as well as chemical compounds known to a skilled person to inhibit at least one PRK or several PRKs.

As already mentioned above, the process of the invention for controlling at least one AR-regulated process, in mammalian cells under histone-phosphorylating conditions at H3T11 may be performed *in vitro* or *in vivo*. By said process, the activity of said at least one PRK, preferably the activity of exactly one PRK, is modulated. The term "modulation", as used in the present description and in the claims, means any change in the activity of the enzyme, either accelerating or decelerating. In preferred embodiments of the invention, the PRK activity is modulated in the sense of a down-regulation, i. e. in the sense of a deceleration of the kinase reaction whereby, in specific and preferred cases, the PRK-catalysed (at least one) androgen receptor-regulated mechanism in the mammalian cell is blocked.

The at least one inhibitor to act on said at least one PRK may act, in the process of the invention, on any PRK known to a skilled person to be suitable for the desired purposes. In preferred embodiments of the invention, the PRK is se-

lected from the group consisting of PRK1, PRK2 and PKNß and, most preferably, is PRK1.

The invention is also directed to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells.

The term "medicament" is understood in the present description and claims to mean pharmaceutically effective agents or compositions (the latter comprising, in addition to the pharmaceutically effective agent, additional effective agents and/or auxiliary substances as, for example, fillers, solvents, coatings and other well known auxiliary substances) having a preventing or therapeutic effect on at least a part (e. g. a cell or a group of cells) of the mammalian body, as well as substances and compositions which may be used for diagnostic or other medically helpful purposes, for example (in the present case) for the scoring and evaluation of certain cells (or of their health status). One example is the scoring of prostate carcinoma cells.

With respect to the term "controlling at least one AR-regulated mechanism in a mammalian cell", reference is made to the above explanation which is included here by reference.

In the use of the invention, said at least one inhibitor being specific for at least one protein kinase C-related kinase is selected from the group consisting of PRK1, PRK2, and PKNß and most preferably is PRK1.

In a specifically preferred embodiment of the invention, the use of the at least one inhibitor having specificity, preferably high specificity, for at least one PRK is a use for the manufacture of a medicament for preventing and/or treating prostate cancer.

In accordance with the present invention, there is also provided a process for controlling the androgen dependent gene expression induced by a phosphorylation of histone H3 at threonine 11 (H3T11) in the presence of at least one protein kinase C-related kinase (PRK). Said process comprises the step of allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

Also in accordance with the present invention, there is provided a process for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK). Said process comprises the step of allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

Also in accordance with the present invention, there is provided a process for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least one protein kinase C-related kinase (PRK). Said process comprises the step of allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

Also in accordance with the present invention, there is also provided a process for controlling the androgen dependent gene expression induced by a transition from the pre-initiation to the initiation complex which is characterized by a phos-

phorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK). Said process comprises the step of allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

In all of the above processes, there may be used one inhibitor, or there may be used two or several inhibitors. The use of one inhibitor is preferred in accordance with the invention.

In a preferred embodiment of the invention, the specificity of the inhibitor or inhibitors used towards the at least one protein kinase C-related kinase (PRK) is high. In particularly preferred embodiments of the invention, the at least one inhibitor is a highly specific PRK inhibitor and, more preferably has a specificity of < 100 nM. Particularly preferred embodiments of the invention relate to at least one inhibitor or exactly one inhibitor having a specificity of, for example 10 nM.

In more preferred embodiments, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of RNAis, antibodies, other peptides and dominant negative mutants of PRKs. Most preferably, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of miRNA, siRNA, micro-RNA, shRNA, anti-PRK1 antibodies and aptamers, as well as chemical compounds known to a skilled person to inhibit at least one PRK or several PRKs.

As already mentioned above, the process of the invention for controlling the transcriptional AR activation in mammalian cells under histone H3 at threonine

T 11- (H3T11-) phosphorylating conditions may be performed *in vitro* or *in vivo*. By said process, the activity of said at least one PRK, preferably the activity of exactly one PRK, is modulated.

The term "modulation", as used in the present description and in the claims, means any change in the activity of the enzyme, either accelerating or decelerating. In preferred embodiments of the invention, the PRK activity is modulated in the sense of a down-regulation, i. e. in the sense of a deceleration of the kinase reaction whereby, in specific and preferred cases, the PRK-catalysed (at least one) androgen receptor-regulated mechanism in the mammalian cell is blocked.

The at least one inhibitor to act on said at least one PRK may act, in the process of the invention, on any PRK known to a skilled person to be suitable for the desired purposes. In preferred embodiments of the invention, the PRK is selected from the group consisting of PRK1, PRK2 and PKNß.

According to the invention, it is preferred that said modulation, preferably said down-regulation, of a PRK activity, for example of PRK1 activity, results into a modulation, preferably into an inhibition, of the H3T11 phosphorylation, and/or into a modulation, preferably into an inhibition, of the H3K9 demethylation, preferably of the trimethyl-H3K9 and/or of the dimethyl-H3K9 and/or of the monomethyl-H3K9, and/or into a modulation, preferably into an inhibition, of the H3K9 and/or H3K14 acetylation, and/or a modulation, preferably an inhibition, of the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II). All processes, i. e. phosphorylation, demethylation, acetylation and RNA polymerase transition, result into the same route, i. e. an activation of the process.

In a further preferred embodiment of the present invention, said modulation, preferably down-regulation, of a PRK activity results into a modulation, prefera-

bly an inhibition, of H3K9 demethylation by at least one histone demethylase, preferably by LSD1 and by at least one JMJD, specifically by JMJD2C, each alone or both in combination.

There may be used one histone demethylase or there may be used two or more histone demethylases. Most preferably, there is used one histone demethylase. Histone demethylases are known to a skilled person, and most of them are suitable for the purposes of the invention. Most preferably and advantageously, the histone demethylase is lysine specific demethylase (LSD1) [5] .

There may be used one JMJD, or there may be used two or several JMJD's; most preferably, the invention uses one JMJD. Several of them are known to a skilled person which may be suitable, e. g. JMJD2A, JMJD2B, JMJD2C etc.. In accordance with the invention, there is used JMJD2C, which was recognized recently [4] to demethylate trimethyl-H3K9 to dimethyl-H3K9; in contrast, LSD1 demethylates dimethyl-H3K9 to monomethyl-H3K9 and monomethyl-H3K9 to unmethylated H3K9.

In preferred embodiments of the invention, LSD1 and JMJD2C may be used each alone or both in combination.

Together with the above embodiment or alternatively to the above embodiment, said down-regulation of a PRK activity results into a modulation, preferably an inhibition, of the H3K9 acetylation and/or H3K14 acetylation by acetylases. There may be used generally known acetylases, and in accordance with the invention, acetylases as, for example, P300/CBP or TIP60 are preferred.

The above processes for controlling the androgen dependent gene expression induced by a phosphorylation of H3T11 in the presence of at least one PRK and/or for controlling the androgen dependent gene expression induced by a demethylation of H3K9 in the presence of at least one PRK and/or for control-

ling the androgen dependent gene expression induced by an acetylation of H3K9 and/or H3K14 in the presence of at least one PRK and/or for the androgen dependent gene expression induced by the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one PRK may be performed *in vivo* or *in vitro*. In further preferred embodiments of the invention, said at least one PRK, more preferably said one PRK, is selected from PRK1, PRK2 or PKNß and, utmost preferred, is PRK1.

The invention also relates to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a phosphorylation of H3T11 in the presence of at least one protein kinase C-related kinase (PRK) and/or relates to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK) and/or relates to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least protein kinase C-related kinase (PRK) and/or relates to the use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK). Also in connection to the above uses, the preferred embodiments of the invention are the same as mentioned above, and reference is made to the above detailed explanations.

Particularly preferred is the use of said at least one inhibitor, more preferably of said one inhibitor, with specificity for at least one PRK for the manufacture of a medicament for preventing and/or treating prostate cancer.

Additionally, the invention relates to a process for the prevention and/or treatment of prostate cancer, said process comprising administering, to one or a to plurality of mammalian cell(s) in need thereof, including prostate cancer cells in need thereof, at least one inhibitor with speficicity for at least one protein kinase C-related kinase (PRK). There may be administered one inhibitor, or there may be administered two or several inhibitors. Preferred is the administration of one inhibitor.

By such an administration, there is modulated, preferably there is down-regulated, the phosphorylation of histone H3 at threonine 11 (H3T11) by said at least one PRK and/or there is modulated, preferably there is down-regulated, the demethylation of histone H3 at lysine 9 (H3K9) by at least one histone demethylase, preferably by lysine specific demethylase (LSD1) and/or by at least one JMJD, and specifically by JMJD2C, each alone or both in combination, and/or there is modulated, preferably there is down-regulated, the acetylation of histone H3 at lysine 9 (H3K9) and/or of histone 3 at lysine 14 (H3K14) by at least one acetylase and/or there is modulated, preferably there is down-regulated, the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II).

One inhibitor with specificity, preferably with high specificity, towards at least one PRK may be used, or two or several inhibitors may be used. The use of one inhibitor is preferred in accordance with the invention.

In a preferred embodiment of the invention, the specificity of the inhibitor or inhibitors used towards the at least one protein kinase C-related kinase (PRK) is high. In particularly preferred embodiments of the invention, the at least one inhibitor is a highly specific PRK inhibitor and, more preferably has a specificity of < 100 nM. Particularly preferred embodiments of the invention relate to at least one inhibitor or exactly one inhibitor having a specificity of, for example 10 nM.

In more preferred embodiments, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of RNAis, antibodies, other peptides and dominant negative mutants of PRKs. Most preferably, said at least one inhibitor or the one inhibitor with high specificity towards at least one PRK is selected from the group consisting of miRNA, siRNA, micro-RNA, shRNA, anti-PRK1 antibodies and aptamers, as well as al chemical compounds known to a skilled person to be inhibitors of at least one PRK, specifically of PRK1.

As already mentioned above, the process of the invention for preventing and/or treating cancer in mammalian cells and particularly prostate cancer may be performed *in vitro* or *in vivo*. By said process, the activity of said at least one PRK, preferably the activity of exactly one PRK, is modulated.

The invention also relates to the use of at least one inhibitor with specificity, preferably with high specificity, for at least one protein kinase C-related kinase (PRK), more preferably for exactly one PRK, for the manufacture of a medicament for the prevention and/or treatment of prostate cancer.

The medicaments addressed above may be medicaments for any desirable administration route, for example the oral, enteral, intramuscular, intravenous, parenteral, and other known administration routes. The medicaments may be in any form suitable for the desired administration route, e. g. in the form of tab-

lets, lozenges, dragees, solutions, suspensions and other known administration or dosage forms, including those containing two components for simultaneous administration and/or effect or for simultaneous administration for successive (including sustained) effect or for successive administration for simultaneous or successive (including sustained) effect.

The invention also relates to the use of at least one antibody with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a composition, for example an assay system, capable of scoring prostate carcinomas. Such an assay system is considered to be capable to supplement, increase the reliability of and, in future, replace the state of the art Gleason scoring system used for scoring a prostate cancer tissue differentiation and malignancy grade.

The corresponding assay system comprises as the minimum components at least one PRK-specific antibody, the substrate (which usually is the tissue sample to be investigated physiologically) as well as auxiliary agents as, for example, buffers and pH value-adjusting agents, said auxiliary agents being well known to a skilled person and being at his disposition in accordance with the specific assay to be performed.

The invention also relates to an assay for screening inhibitors having specificity for at least one PRK capable of blocking AR-induced prostate carcinoma cell proliferation, said assay comprising one or more than one of the following (optionally consecutive) steps:

- conducting kinase assays for screening for specific PRK inhibitors, particularly for specific PRK1 inhibitors; an example of such kinase assays is described below, and the results are presented in Figures 2a, 2b, and 2c;

- conducting chromatin immunoprecipitation tests (chip) in order to learn whether the inhibitors identified in the previous step modulate, down-regulate or even block (inhibit) a PRK-mediated (particularly PRK1-mediated) phosphorylation at histone H3 at threonine 11 (H3T11) at androgen receptor-regulated (AR-regulated) target genes; the results of such a test are exemplarily shown in Figure 2e;

- verifying the inhibition of androgen receptor (AR) transcriptional activity by the PRK inhibitors (specifically PRK1 inhibitors) identified in the previous steps; examples of this step are shown in Figures 3c and 3d as well as in the Supplementary Figure S1b;

- conducting a test of PRK inhibitors, specifically the PRK1 inhibitors, identified in the previous steps for a modulation, preferably a down-regulation or even a blocking of androgen-induced tumour cell proliferation inhibitor; examples of such a test are shown in Figure 4b.

By such an assay comprising a sequence of tests, inhibitors having specificity for at least one PRK, specifically PRK1, capable of blocking AR-induced prostate carcinoma cell proliferation could easily be identified, and such tests could suitably be performed as an animal model.

The invention is now in detail further described in connection to the experiments performed by the inventors.

To initiate our study, we analysed the effect of PRK1 knockdown on the expression of endogenous AR target genes. LNCaP prostate tumour cells were transduced with lentiviruses expressing miRNAs directed against PRK1, which results in an efficient and specific down-regulation of endogenous PRK1 (Fig. 1a, right panel).

Quantitative RT-PCR analyses demonstrate that the reduction of PRK1 levels strongly impairs androgen-induced expression of endogenous AR target genes such as Prostate Specific Antigen (PSA) or Kallikrein 2 (KLK2) (Fig. 1a, left panel). In addition, treatment with the PRK1 inhibitor Ro318220 [2] severely impedes androgen-induced expression of AR target genes, showing that the kinase activity of PRK1 is essential for AR function (Fig. 1b). Similarly, miRNA-mediated knockdown of PRK1 or treatment with Ro318220 results in a strong decrease in ligand-induced expression of various AR-dependent reporters (Supplementary Fig S1a, b).

To investigate whether PRK1 associates with chromatin *in vivo*, LNCaP cells were subjected to chromatin immunoprecipitation (ChIP) in the presence or absence of the AR agonist R1881. PRK1 associates with the androgen response elements (AREs) located in the promoters of the *PSA* and *KLK2* genes in a ligand-dependent manner (Fig. 1c, left panel). Recruitment of PRK1 to chromatin is specific since DNA corresponding to the promoters of the unrelated *GAPDH* and *U6* genes is not enriched.

To show that PRK1 and AR are present in the same complex on the *PSA* and *KLK2* promoters, R1881-treated LNCaP cells were subjected to sequential chromatin immunoprecipitation (Re-ChIP), first with an α-AR and then with an α-PRK1 antibody. Importantly, the ARE-containing regions are specifically enriched, demonstrating that PRK1 and AR form a complex on chromatin in a ligand-dependent manner (Fig. 1c, right panel).

To understand how association of PRK1 and AR with chromatin results in increased gene expression, we tested whether PRK1 directly phosphorylates the N-terminal tail of histone H3. Myc-PRK1 and the flag-tagged kinase dead mutant PRK1 K644E [2] were immunoprecipitated from 293 cell lysates with an α-myc or an α-flag antibody, respectively (Supplementary Fig. S2a, b), and incubated with bacterially expressed and purified GST-H3 1-44 or GST control pro-

tein. GST-H3 1-44 is phosphorylated by PRK1, but not by PRK1 K644E (Fig. 2a). The GST control protein is not phosphorylated, thus demonstrating specificity. Furthermore, addition of Ro318220 completely blocks the phosphorylation of GST-H3 1-44 by PRK1 (Fig. 2a).

Deletion mapping revealed that only the fragment of histone H3 spanning amino acid residues 1 to 15 (H3 1-15), but not H3 16-30 or H3 29-44, is phosphorylated by purified recombinant PRK1 (Fig 2b). More importantly, mutation of threonine 11 to alanine in H3 1-15 (H3 1-15 T11A) abolishes phosphorylation, demonstrating that PRK1 targets histone H3 at threonine 11 (H3T11) (Fig. 2b). In addition, we incubated nucleosomes purified from HeLa cells with recombinant PRK1 in the presence or absence of Ro318220. Western blot analysis, performed with an α-phosphoH3T11 specific antibody (Supplementary Fig. S3a) demonstrates that PRK1 phosphorylates nucleosomes at H3T11 (Fig. 2c). This phosphorylation is blocked by Ro318220 (Fig. 2c).

To determine whether PRK1 controls phosphorylation of H3T11 at promoters of AR-regulated genes *in vivo*, LNCaP cells were first transfected with either an unrelated control siRNA or a siRNA directed against PRK1, in the presence or absence of R1881, and then subjected to ChIP. Addition of ligand results in phosphorylation of H3T11 at the AREs of the *PSA* and *KLK2* promoters (Fig. 2d, left panel). Androgen-induced phosphorylation at H3T11 is PRK1-dependent since it is blocked by knockdown of PRK1. PRK depletion is specific and does not affect the levels of endogenous AR (Fig. 2d, right panel). To corroborate that androgen-induced phosphorylation of H3T11 is executed by PRK1, LNCaP cells were cultivated in the presence or absence of Ro318220 and subjected to ChIP. As expected, Ro318220 efficiently blocks ligand-induced phosphorylation of H3T11 (Fig. 2e).

Taken together, these data demonstrate that PRK1 phosphorylates H3T11. Importantly, the phosphorylation of H3T11 associates with AR-dependent gene

expression, thus introducing phosphorylated H3T11 as a novel epigenetic mark for transcriptional activation.

Since ligand-dependent expression of AR target genes demands removal of repressive methyl marks from H3K9 [4, 5] and acetylation of histone H3K9/K14 [6], we analysed whether PRK1 controls changes in these epigenetic marks. Therefore, LNCaP cells cultivated in the presence or absence of R1881 were transfected with either an unrelated control siRNA or a siRNA directed against PRK1 and subjected to ChIP. Ligand-induced demethylation of tri-, di-, and monomethyl H3K9 at the AREs of the *PSA* and *KLK2* promoters is severely impaired by PRK1 knockdown (Fig. 3a). Furthermore, ligand-induced acetylation of H3K9/K14 is also blocked (Fig. 3a). Similarly, inhibition of PRK1 activity by Ro318220 results in loss of demethylation of H3K9 and acetylation of H3K9/K14 (Fig. 3b), providing evidence that the kinase activity of PRK1 is pivotal in controlling these epigenetic alterations at AR target genes.

As previously shown, JMJD2C [4] and LSD1 [5] remove repressive methyl marks from H3K9 during AR-dependent transcription. Since PRK1 controls demethylation of H3K9, we investigated the interplay between PRK1 and the demethylases during gene expression in transient transfections. Co-expression of AR with either JMJD2C (Fig. 3c and ref [4]) or LSD1 (Fig. 3d and ref [5]) results in a strong ligand-dependent activation of the PSA-LUC or MMTV-LUC reporters. Co-activation by the demethylases is abrogated by PRK1 K644E, acting as a dominant negative mutant, or by treatment with Ro318220. To examine the effect of PRK1 K644E on co-operative stimulation of AR activity by JMJD2C and LSD1, we expressed both demethylases in limited amounts, which alone do not activate AR, but together induce a strong AR superactivation [4]. As shown in Figure 3e, PRK1 K644E blocks co-operative stimulation of AR activity. The control reporter TK-LUC is not affected by PRK1 (Supplementary Fig. S4a-c). Collectively, these data demonstrate that PRK1 signalling controls transcriptional activation of AR by the demethylases JMJD2C and LSD1.

Initiation of transcription requires transition from the pre-initiation to the initiation complex, which is characterized by phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-P CTD pol II) by the CDK7 component of TFIIH [3]. To determine whether depletion or inhibition of PRK1 interfered with the formation of the transcriptional initiation complex at AR-regulated promoters, we performed ChIP using an antibody that specifically recognizes S5-P CTD pol II. Importantly, knockdown of PRK1 or treatment of cells with Ro318220 results in the loss of S5-P CTD pol II at the promoters of *PSA* and *KLK2*. In contrast, recruitment of RNA polymerase II is not affected, as shown by ChIP using an antibody directed against the N-terminal domain of RNA polymerase II (α-NTD pol II) (Fig. 3f, g).

Taken together, these data show that PRK1 not only controls changes in epigenetic marks on histone H3, but also regulates the transition from pre-initiation to initiation complex.

To unravel the physiological importance of PRK1, we investigated the levels of PRK1 *in vivo* by immunostaining a panel of 20 normal human prostates and 111 prostate carcinomas on tissue microarrays. Quantification of immunoreactivity by scoring staining intensity and percentage of positive carcinoma cells [7] reveals that high PRK1 expression significantly correlates with high Gleason scores and indicates aggressive biology of the tumours (Fig. 4a).

Furthermore, to examine whether PRK1 regulates tumour cell proliferation, we monitored androgen-dependent cell growth by quantifying proliferation of pLenti6-miRNA-PRK1-infected LNCaP cells. When compared to cells expressing an unrelated control miRNA, androgen-induced proliferation of LNCaP cells is dramatically reduced by PRK1 knockdown (Fig. 4b), thus underlining the importance of PRK1 in the control of AR-dependent tumour cell growth.

In summary, we demonstrate that phosphorylated H3T11 is a novel epigenetic mark for transcriptional regulation. Phosphorylation of H3T11 is executed by PRK1 in an androgen-dependent manner. By controlling subsequent steps of gene activation such as demethylation of tri-, di-, and monomethyl H3K9, acetylation of H3K9/K14, and the presence of S5-P CTD pol II at target promoters, PRK1 functions as a gatekeeper of AR-regulated gene expression. Of importance is our observation that inhibitors such as Ro318220 control the kinase activity of PRK1 and thereby regulate AR. Thus, specific modulation of PRK1 activity is a promising therapeutic strategy in the treatment of prostate cancer, where AR is pivotal to the control of tumour cell proliferation.

Examples

Plasmids

The following plasmids were described previously: pSG5-AR, pCMX-flag, pCMV-flag-PRK1 K644E, pcDNA3-myc-ΔNPRK1, TK-LUC, MMTV-LUC, Probasin-LUC, and PSA-LUC [2]; pCMX-flag-JMJD2C [4], pCMX-flag-LSD1 [5], GST-H3 1-44 [8].

To construct pLenti6-miRNA1-PRK1, pLenti6-miRNA2-PRK1, pGW-miRNA1-PRK1, and pGW-miRNA2-PRK1, the DNA corresponding to miRNA1-PRK1 (5'-TGCTGATTGCTGTAGGTCTGGATCATGTTTTGGCCACTG ACTGACATGATCCACCTACAAT-3' (Sequence Protocol: 1) and 5'-CCTGATTGCTGTAGGTGGATCATGTCAGTCAGTGGCCAAAACATGA TCCAGACCTACAGCAATC-3') (Sequence Protocol: 2) and miRNA2-PRK1 (5'-TGCTGTTACTGTCCTGCAACATCTGCGTTTTGGCCA CTGACTGACGCAGATGTCAGGACAGTAA-3' (Sequence Protocol: 3) and (5'-CCTGTTACTGTCCTGACATCTGCGTCAGTCAGTGGCCAAAACGCA GATGTTGCAGGACAGTAAC-3') (Sequence Protocol: 4) was cloned into pLenti6/V5-DEST and pcDNA-6.2-GW-EmGFP according to the manufacturer's instructions (Invitrogen). To construct GST-H3 1-15, GST-H3 1-15 T11A, GST-

H3 16-30, and GST-H3 29-44, the corresponding cDNA fragments were cloned into pGEX4T1. Cloning details can be obtained upon request.

Cell culture and transfection

CV1 and LNCaP cells were cultured and transfected as described [2]. The following amounts were transfected per well: 500 ng of MMTV-LUC, Probasin-LUC, or PSA-LUC; 25 ng of AR expression plasmid; 200 ng (Fig. 3e) or 400 ng (Fig. 3c, d) expression plasmids of LSD1 or JMJD2C, 150 ng PRK1 K644E, 1000 ng expression plasmid of miRNA-control, miRNA1-PRK1, or miRNA2-PRK1 (Supplementary Fig. S1a). Cells were cultivated for 18 hours in the presence or absence of 1 x 10^{-10} M R1881 (Sigma), 2.5 x 10^{-6} M (Fig. 3d) or 4.5 x 10^{-6} M (Fig. 3c) Ro318220 (Roche) as indicated. Luciferase activity was assayed as described [2]. All experiments were repeated at least four times in duplicate.

Generation of PRK1 antibody

The polyclonal rabbit-α-PRK1 antibody was generated according to standard procedures.

Chromatin Immunoprecipitation

ChIP and Re-ChIP experiments were performed as described [5, 9]. LNCaP cells were cultured for 45 min (Fig. 1c, 2d, 2e) or 210 min (Fig. 3a, b, f, g) in the presence or absence of 1 x 10^{-8} M R1881 as indicated. Ro318220 (1 x 10^{-5} M) was added to the LNCaP cells (Fig. 3b, g) 60 min before addition of R1881. Three days before harvesting, LNCaP cells were transfected with stealth RNAi (ctr: 5'-GAACAUGAUCCAGACCUACAGCAAU-3' (Sequence Protocol: 5); PRK1: 5'-GAAAGUCCUAGAUCCACACGCAAAU-3' (Sequence Protocol: 6); Invitrogen) following the manufacturer's instructions. Immunoprecipitation was performed with specific antibodies (α-monoMeH3K9, α-diMeH3K9, α-triMeH3K9, α-acetyl-H3K9/K14, α-H3, α-AR (Upstate Biotechnology), α-S5-P CTD pol II, α-phosphoH3T11 (Abcam), α-NTD pol II (N-20, Santa Cruz), α-LSD1 [5], α-JMJD2C [4], and α-PRK1) on protein A-Sepharose 4B (GE-

Healthcare). For PCR, 1-5 µl out of 50 µl DNA extract was used. PCR primers for ARE I+II (*PSA* -459/-121), ARE III (*PSA* -4288/-3922), *KLK2* (-343/-90), *GAPDH*, and *U6* were described previously [5].

Western blot analysis

Experiments were performed as described [2]. Western blots were decorated as indicated.

Cell proliferation assay

Experiments were performed as described [5]. pLenti6-miRNA-control, pLenti6-miRNA1-PRK1, and pLenti6-miRNA2-PRK1 were used to produce recombinant lentiviruses to infect LNCaP cells as described [10]. The infected cells were cultured for 72 hours in medium containing 10% double-stripped FCS. 1 x 10^4 cells were plated in a 96-well plate in the presence or absence of 1 x 10^{-9} M R1881. The cell proliferation Elisa BrdU Colorimetric Assay (Roche) was performed according to the manufacturer's instructions. The figure shows the percentage increase of proliferation in the presence versus absence of R1881. The experiments were performed in quintuplicate.

Quantitative RT-PCR and statistical analysis

Quantitative RT-PCR and statistical analysis were performed as described [5]. The primers for *GAPDH*, *PSA*, and *KLK2* were described previously [5].

***In vitro* kinase assay**

The kinase assays were performed as described [11]. 10 µg GST-tagged H3 proteins or 1 µg of nucleosomes purified from HeLa cells [12] were incubated with immunoprecipitated PRK1 proteins (Fig. 2a) or 1 µg purified recombinant PRK1 (Fig. 2b, c; ProQinase GmbH) for 0 to 10 min (Fig. 2c) or 20 min (Fig. 2a, b) at 30°C in kinase buffer containing 20 mM Tris-HCl pH 7.5, 20 µM ATP, 8 mM $MgCl_2$, and 5 µCi (γ-^{32}P) ATP. The reaction mixture was analysed by SDS-

PAGE followed by autoradiography or Western blotting using antibodies as indicated.

Statistical analysis of tissue microarrays

Clinical data of patients and procedures for generating the tissue microarrays were described previously[7]. Statistical analysis was performed with the Mann-Whitney U-Test using the SPSS 12.0 program (SPSS Inc.) and by calculating the two-tailed Spearman Rank correlation coefficient. The number of cases (n) analysed per Gleason score (Gs) were: Gs 3 (n=5); Gs 4 (n=12); Gs 5 (n=11); Gs 6 (n=25); Gs 7 (n=16); Gs 8 (n=23), Gs 9 (n=10); Gs 10 (n=9). Normal prostate specimen (n=20).

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Claims

1. A process for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells under histone H3 at threonine 11- (H3T11-) phosphorylating conditions, said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said at least one androgen receptor-regulated mechanism in said mammalian cells.

2. The process according to claim 1, wherein the at least one androgen receptor-regulated mechanism is at least one selected from the group consisting of the androgen receptor-controlled gene expression and the androgen-induced cell proliferation and the androgen-induced function of the prostate and the androgen-induced build-up of muscles and the androgen-induced build-up of the bone backbone, preferably the androgen-induced control of the bone density, and the androgen-induced fertility and the androgen-induced hair growth of a mammal.

3. The process according to claim 1 or claim 2, wherein the at least one inhibitor is a highly specific PRK inhibitor, preferably wherein said at least one inhibitor an inhibitor having a specificity of < 100 nM, more preferably wherein said at least one inhibitor is selected from the group consisting of RNAi's, antibodies, other peptides and dominant negative mutants of PRK's, most preferably wherein said at least one inhibitor is selected from the group consisting of miRNA, siRNA, micro-RNA, shRNA, anti-PRK1 antibodies and aptamers, as well as al chemical compounds known to a skilled person to be inhibitors of at least one PRK, specifically of PRK1.

4. The process according to any of the claims 1 to 3, said process being performed in vitro or in vivo.

5. The process according to any of claims 1 to 4, wherein said at least one PRK is selected from the group consisting of PRK1, PRK2, and PKNß.

6. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling at least one androgen receptor- (AR-) regulated mechanism in mammalian cells.

7. Use according to claim 6, wherein the at least one androgen receptor-regulated mechanism is selected from the group consisting of the androgen receptor-controlled gene expression and the androgen-induced cell proliferation and the androgen-induced function of the prostate and the androgen-induced build-up of muscles and the androgen-induced build-up of the bone backbone, preferably the androgen-induced control of the bone density, and the androgen-induced fertility and the androgen-induced hair growth of a mammal.

8. Use according to claim 6 or claim 7, said at least one inhibitor being specific for at least one protein kinase C-related kinase selected from the group consisting of PRK1, PRK2, and PKNß.

9. Use according to any of the claims 6 to 8 for the manufacture of a medicament for preventing and/or treating prostate cancer.

10. A process for controlling the androgen dependent gene expression induced by a phosphorylation of histone H3 at threonine 11 (H3T11) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at

least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

11. A process for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

12. A process for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulating, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

13. A process for controlling the androgen dependent gene expression induced by a transition from the pre-initiation to the initiation complex characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK), said process comprising allowing at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) to act on said at least one PRK, thereby modulat-

ing, preferably down-regulating, the activity of said at least one PRK and optionally blocking said androgen dependent gene expression.

14. The process according to any of the claims 10 to 13, wherein said modulation, preferably down-regulation, of a PRK activity results into a modulation, preferably into an inhibition, of the H3T11 phosphorylation and/or into a modulation, preferably into an inhibition of the H3K9 demethylation, preferably of the trimethyl-H3K9 and/or of the dimethyl-H3K9 and/or of the monomethyl-H3K9, and/or into a modulation, preferably into an inhibition, of the H3K9 and/or H3K14 acetylation.

15. The process according to any of the claims 10 to 14, wherein said modulation, preferably down-regulation, of a PRK activity results into a modulation, preferably an inhibition, of H3T11 phosphorylation by at least one PRK and/or wherein said modulation, preferably down-regulation, of a PRK activity results into a modulation, preferably an inhibition, of H3K9 demethylation by at least one histone demethylase and by at least one JMJD, each alone or both in combination, preferably by lysine specific demethylase (LSD1) and by JMJD2C, each alone or both in combination, and/or wherein said down-regulation of a PRK activity results into a modulation, preferably an inhibition, of the H3K9 acetylation and/or H3K14 acetylation by acetylases, preferably P300/CBP or TIP60, and/or wherein said down-regulation of a PRK activity results into a modulation, preferably an inhibition, of the transition from the pre-initiation to the initiation complex characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) by CDK7.

16. The process according to any of claims 10 to 15, said process being performed in vitro or in vivo.

17. The process according to any of claims 10 to 16, wherein said at least one PRK is selected from PRK1, PRK2, and PKNß.

18. The process according to any of claims 10 to 17, wherein the at least one inhibitor is a highly specific PRK inhibitor, preferably wherein said at least one inhibitor an inhibitor having a specificity of < 100 nM, more preferably wherein said at least one inhibitor is selected from RNAi's, antibodies, other peptides and dominant negative mutants of PRK's, most preferably wherein said at least one inhibitor is selected from miRNA, siRNA, micro-RNA, shRNA, anti-PRK1 antibodies and aptamers, as well as all chemical compounds known to a skilled person to be inhibitors of at least one PRK, specifically of PRK1.

19. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a phosphorylation of histone H3 at threonine 11 (H3T11) in the presence of at least one protein kinase C-related kinase (PRK).

20. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a demethylation of histone H3 at lysine 9 (H3K9) in the presence of at least one protein kinase C-related kinase (PRK).

21. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by an acetylation of histone H3 at lysine 9 (H3K9) and/or histone H3 at lysine 14 (H3K14) in the presence of at least protein kinase C-related kinase (PRK).

22. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for controlling the androgen dependent gene expression induced by a transition from the pre-initiation to the initiation complex which is characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) in the presence of at least one protein kinase C-related kinase (PRK).

23. The use according to any of the claims 19 to 22, wherein the androgen dependent gene expression control induced by a H3T11 phosphorylation is a H3T11 phosphorylation by PRK and/or wherein the androgen dependent gene expression induced by a H3K9 demethylation is a H3K9 demethylation by histone demethylases and by JMJD's, each alone or both in combination, preferably by lysine specific demethylase (LSD1) and by JMJD2C, each alone or both in combination, and/or wherein the androgen dependent gene expression induced by a H3K9 and/or H3K14 acetylation is a H3K9 and/or H3K14 acetylation by acetylases, and/or wherein said down-regulation of a PRK activity results into a modulation, preferably an inhibition, of the transition from the pre-initiation to the initiation complex characterized by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) by CDK7.

24. The use according to any of the claims 19 to 23, wherein said at least one inhibitor is specific for at least one protein kinase C-related kinase selected from PRK1, PRK2, and PKNß.

25. The use according to any of the claims 19 to 24 for the manufacture of a medicament for preventing and/or treating prostate cancer.

26. A process for the prevention and/or treatment of prostate cancer, said process comprising administering, to one or to a plurality of mammalian

cell(s) in need thereof, including prostate cancer cells in need thereof, at least one inhibitor with speficicity for at least one protein kinase C-related kinase (PRK), thereby modulating, preferably down-regulating, the phosphorylation of histone H3 at threonine 11 (H3T11) by said at least one PRK; and/or modulating, preferably down-regulating, the demethylation of histone H3 at lysine 9 (H3K9) by at least one histone demethylase, preferably by lysine specific demethylase (LSD1) and/or by at least one JMJD, specifically by JMJD2C, each alone or both in combination; and/or modulating, preferably down-regulating, the acetylation of histone H3 at lysine 9 (H3K9) and/or of histone 3 at lysine 14 (H3K14) by at least one acetylase and/or modulating, preferably down-regulating, the transition from the pre-initiation to the initiation complex by a phosphorylation of RNA polymerase II at serine 5 in the C-terminal repeat domain (S5-pCDTpol II) by CDK7.

27. Use of at least one inhibitor with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a medicament for the prevention and/or treatment of prostate cancer.

28. Use of at least one antibody with specificity for at least one protein kinase C-related kinase (PRK) for the manufacture of a composition capable of scoring prostate carcinomas.

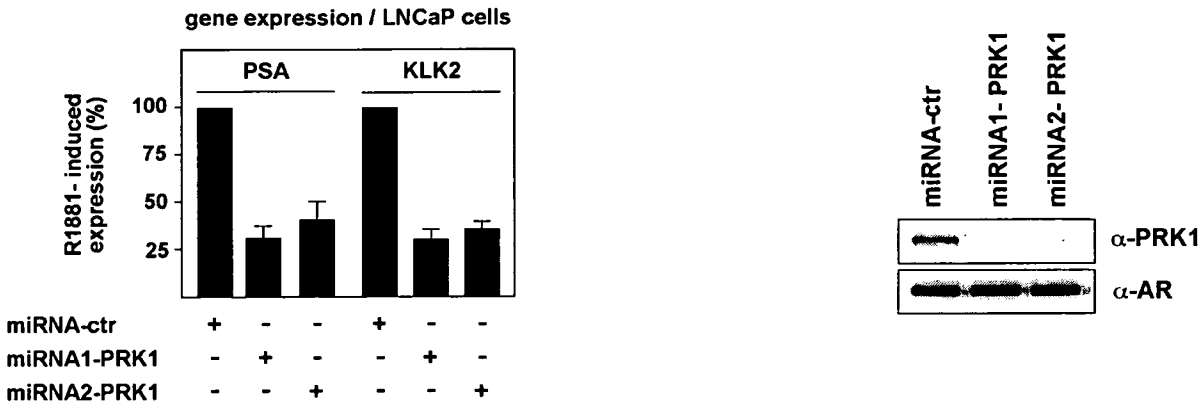
29. Assay system for screening inhibitors having specificity for at least one PRK capable of blocking AR-induced prostate carcinoma cell proliferation, said assay system comprising at least one PRK, an (optionally labelled) substrate [which, as the case may be, may also be a substrate with an antibody suitable for a detection], a phosphate-delivering component (as, for example, ATP) and suitable auxiliary substances as, for example one or more buffers and one or more pH-adjusting compound(s).

30. Assay system for scoring prostate carcinomas in a tissue sample, said assay system comprising a reagent for detecting the presence of PRK1.

31. The assay system of claim 30, wherein said reagent is selected from the group consisting of an antibody against PRK1 and PCR primers.

Schuele_Figure 1

a



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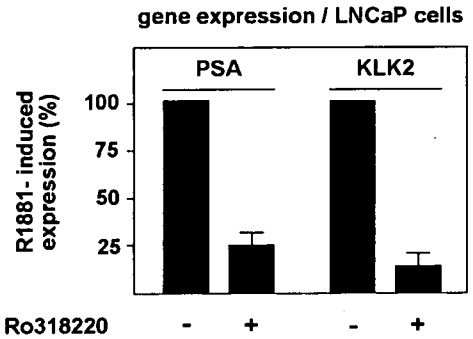

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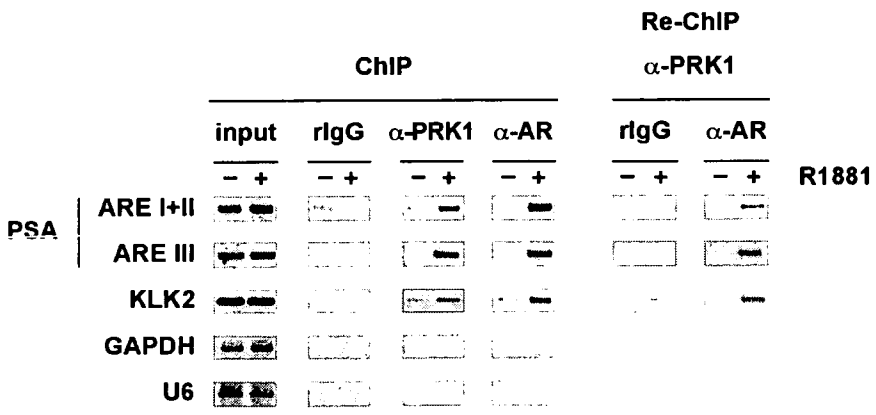
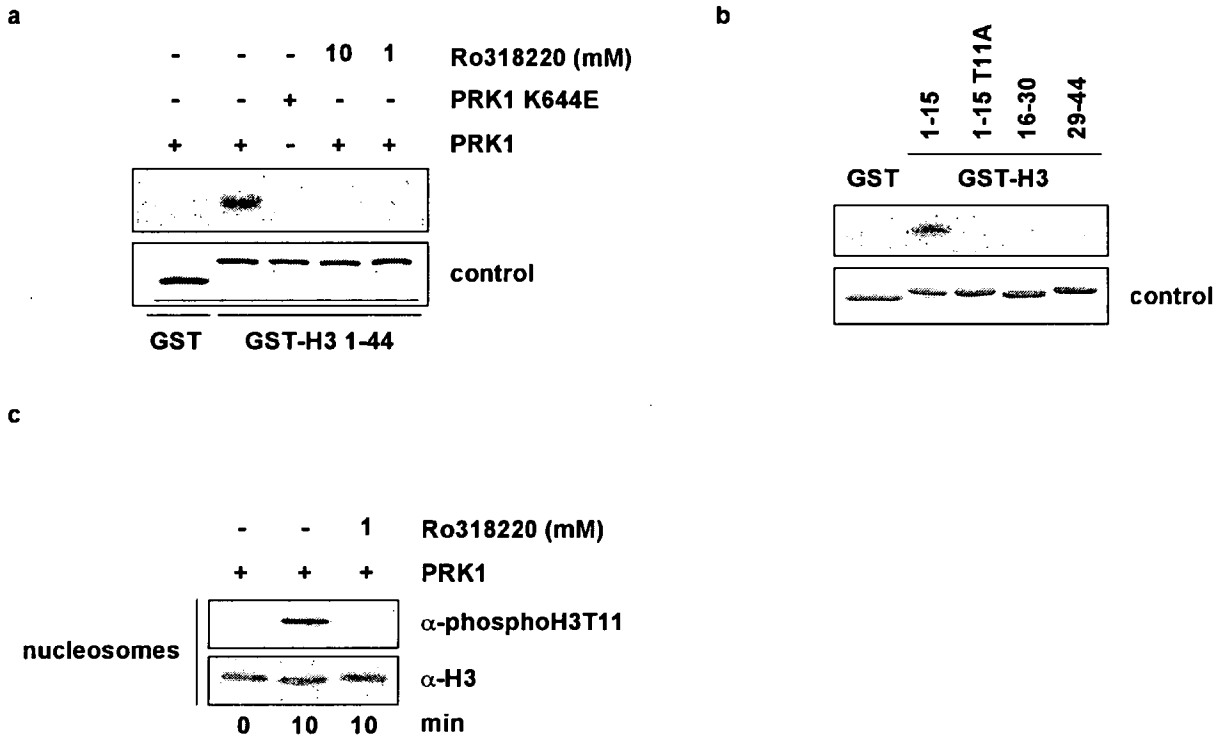
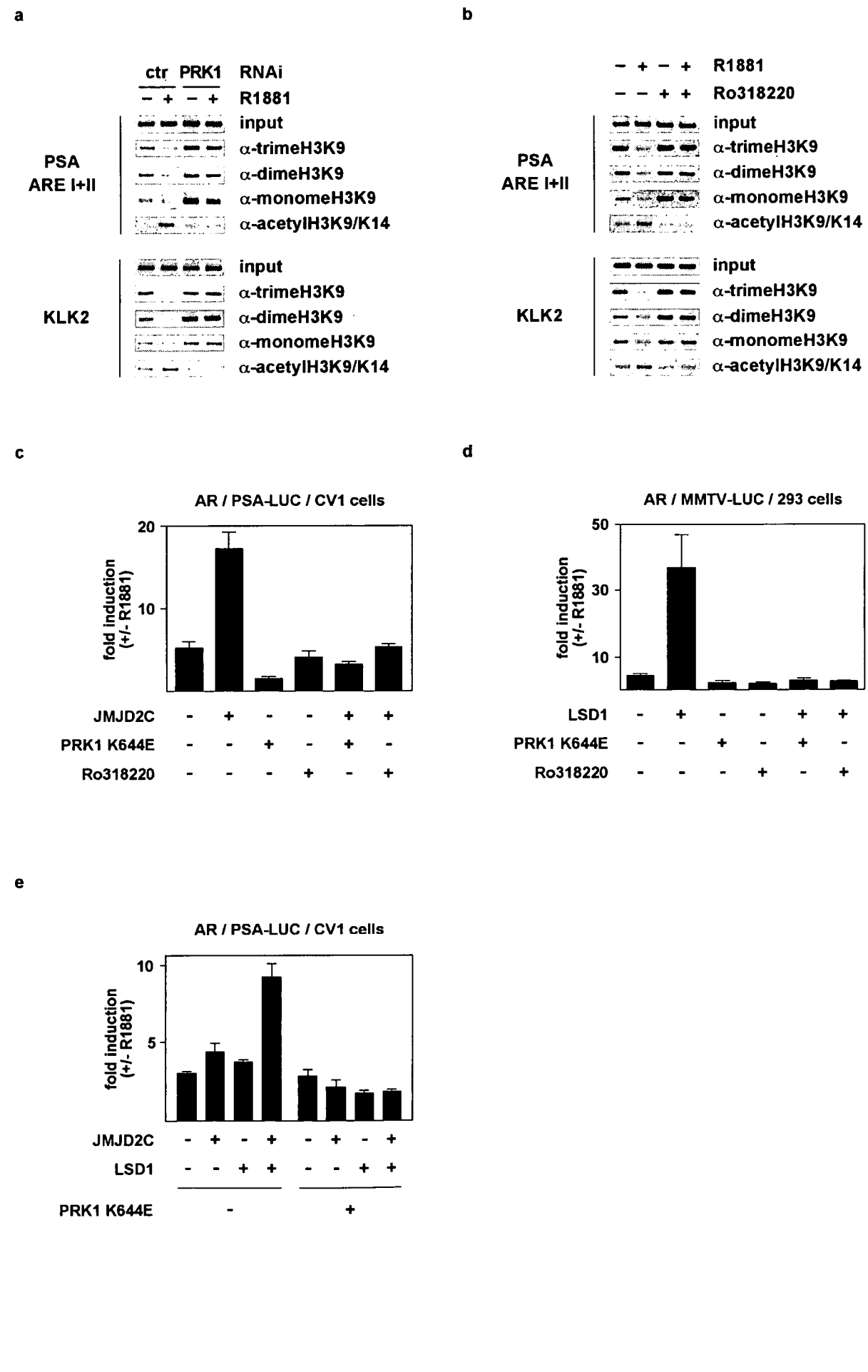


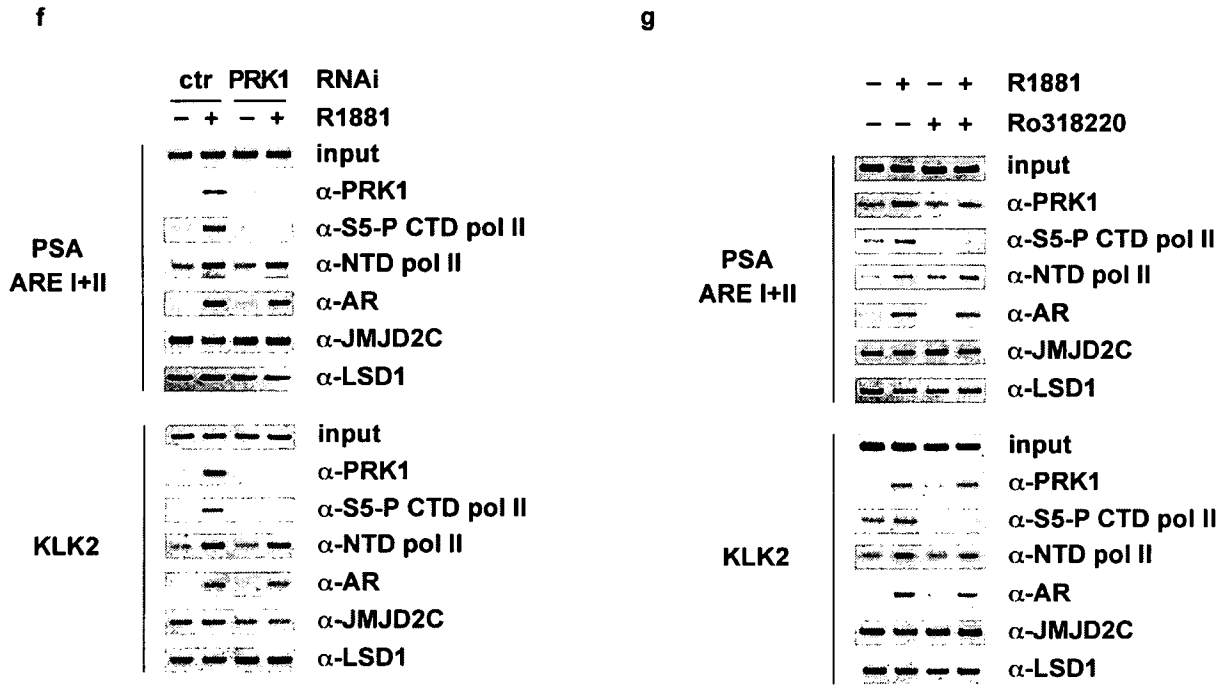
Schuele_Figure 2



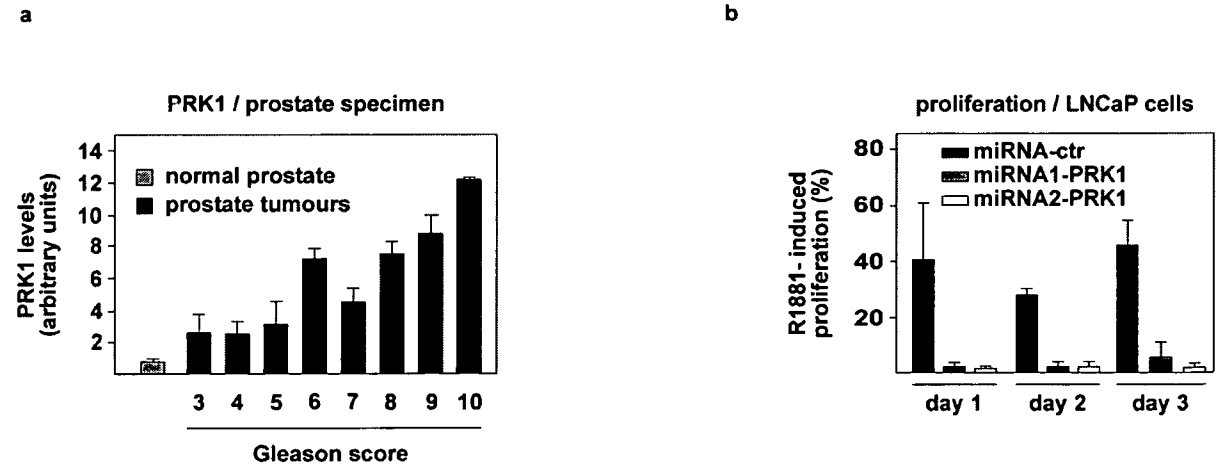
Schuele _Figure 3



Schuele_Figure 3

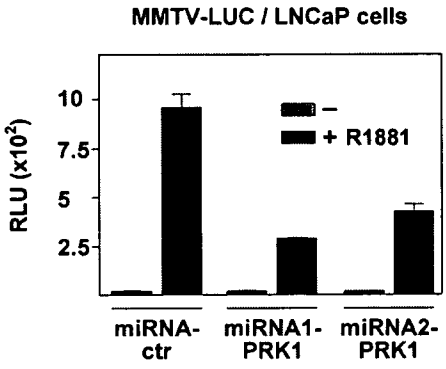


Schuele_Figure 4



Schuele_Supplementary Figure S1

a



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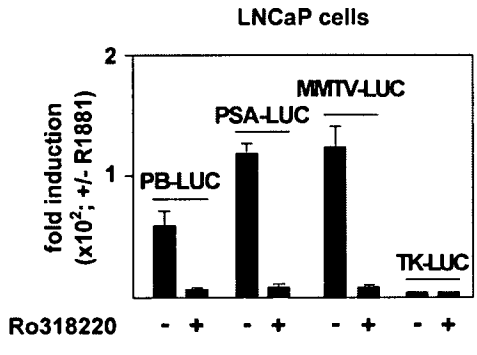

Schuele_Supplementary Figure S2

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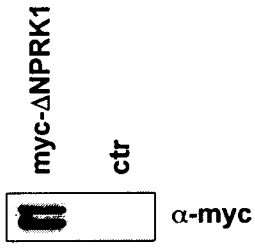

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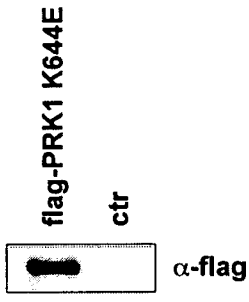
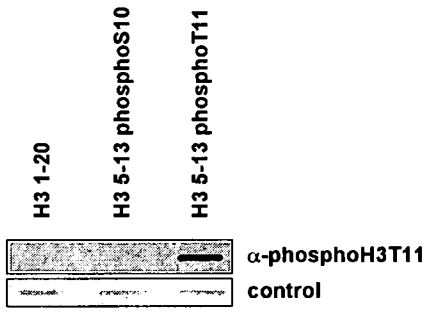


Schuele_Supplementary Figure S3

a



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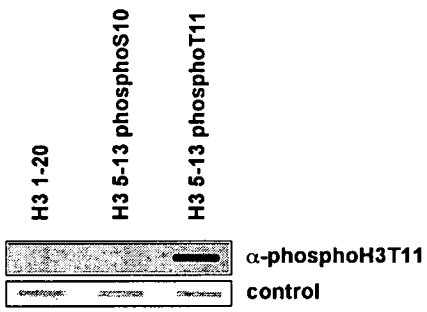

Schuele_Supplementary Figure S4

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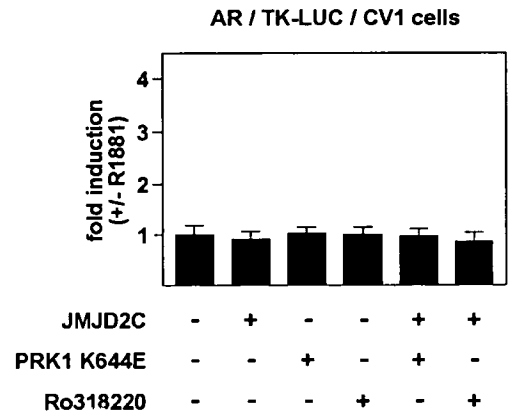

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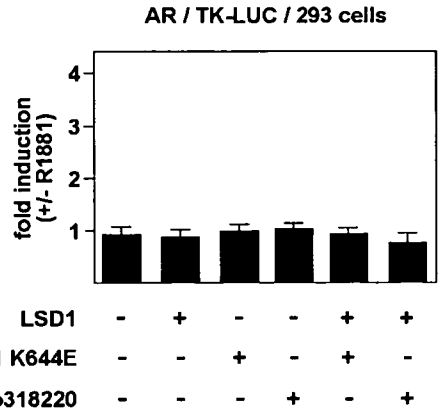

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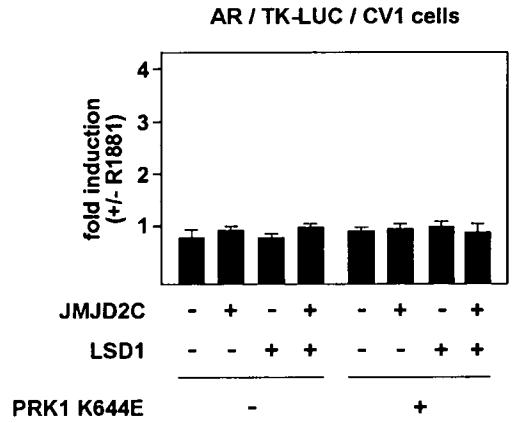

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/004201

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 G01N33/68 G01N33/74 C12N9/12 C07K14/47
C07K14/72 C12Q1/48 A61K38/00
ADD. A61P35/00 A61P13/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K G01N A61K

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	METZGER E ET AL: "A NOVEL INDUCIBLE TRANSACTIVATION DOMAIN IN THE ANDROGEN RECEPTOR: IMPLICATIONS FOR PRK IN PROSTATE CANCER" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 22, no. 2, 15 January 2003 (2003-01-15), pages 270-280, XP001182138 ISSN: 0261-4189 cited in the application the whole document ----- -/--	1-31

[X] Further documents are listed in the continuation of Box C.

[X] 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 document 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	Date of mailing of the international search report
9 September 2008	23/09/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Madruga, Jaime

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/004201

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHU YIMIN ET AL: "Signaling via a novel integral plasma membrane pool of a serine/threonine protein kinase PRK1 in mammalian cells." THE FASEB JOURNAL : OFFICIAL PUBLICATION OF THE FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY NOV 2004, vol. 18, no. 14, November 2004 (2004-11), pages 1722-1724, XP002462580 ISSN: 1530-6860 the whole document	1-31
X	EP 1 574 857 A (UNIVERSITAETSKLINIKUM FREIBURG [DE]) 14 September 2005 (2005-09-14) the whole document	1-31
A	PREUSS UTE ET AL: "Novel mitosis-specific phosphorylation of histone H3 at Thr11 mediated by Dlk/ZIP kinase." NUCLEIC ACIDS RESEARCH, vol. 31, no. 3, 1 February 2003 (2003-02-01), pages 878-885, XP002462581 ISSN: 0305-1048 the whole document	
A	NOWAK S J ET AL: "Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation" TRENDS IN GENETICS, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, NL, vol. 20, no. 4, April 2004 (2004-04), pages 214-220, XP004497332 ISSN: 0168-9525 the whole document	
A	WISSMANN MELANIE ET AL: "Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression" NATURE CELL BIOLOGY, MACMILLAN PUBLISHERS, GB, vol. 9, no. 3, March 2007 (2007-03), pages 347-358, XP002440811 ISSN: 1465-7392 cited in the application the whole document	

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/004201

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	METZGER ET AL: "Histone demethylation and androgen-dependent transcription" CURRENT OPINION IN GENETICS & DEVELOPMENT, CURRENT BIOLOGY LTD, XX, vol. 16, no. 5, October 2006 (2006-10), pages 513-517, XP005649903 ISSN: 0959-437X the whole document	
A	KANG Z ET AL: "COREGULATOR RECRUITMENT AND HISTONE MODIFICATIONS IN TRANSCRIPTIONAL REGULATION BY THE ANDROGEN RECEPTOR" MOLECULAR ENDOCRINOLOGY, BALTIMORE, MD, US, vol. 18, no. 11, November 2004 (2004-11), pages 2633-2648, XP009045419 ISSN: 0888-8809 the whole document	
A	JIA LI ET AL: "Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells" MOLECULAR AND CELLULAR BIOLOGY, vol. 26, no. 19, October 2006 (2006-10), pages 7331-7341, XP002462582 ISSN: 0270-7306 the whole document	
P,X	METZGER ERIC ET AL: "Phosphorylation of histone H3 at threonine 11 establishes a novel chromatin mark for transcriptional regulationx" NATURE CELL BIOLOGY, vol. 10, no. 1, January 2008 (2008-01), page 53, XP002494894 ISSN: 1465-7392 the whole document	1-31
P,X	-& CROCE L D ET AL: "Thrilling transcription through threonine phosphorylation" NATURE CELL BIOLOGY 200801 GB, vol. 10, no. 1, January 2008 (2008-01), pages 5-6, XP002494895 ISSN: 1465-7392 the whole document	1-31
T	SHIMADA M ET AL: "Checkpoints meet the transcription at a novel histone milestone (H3-T11)" CELL CYCLE 20080601 US, vol. 7, no. 11, 1 June 2008 (2008-06-01), pages 1555-1559, XP009105471 ISSN: 1538-4101 1551-4005 the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2008/004201

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. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-5, 10-18 and 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] 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:

see FURTHER INFORMATION sheet PCT/ISA/210

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:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable 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 reportcovers 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.:

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

Continuation of Box II.1

Although claims 1-5, 10-18 and 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.: -

Present claims 1-31 relate to a compound which has a given desired property or effect, namelyits ability to inhibit PRK. However, the description does not provide support and disclosure in the sense of Article 6 and 5 PCT for any such compound having the said property or effect and there is no common general knowledge of this kind available to the person skilled in the art. This non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search of the claim (PCT Guidelines 9.19 and 9.20).

The search of claims 1-31 was consequently restricted to the specifically disclosed compounds having the desired property or effect, see description, and to the broad concept of a compound having the desired property or effect.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/004201

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1574857 A	14-09-2005	CA 2559304 A1	13-10-2005
		WO 2005095957 A1	13-10-2005
		US 2007196882 A1	23-08-2007