

Method of Identifying a Subject for Therapy

Technical Field

The present invention relates, in general terms, to biomarkers and their use in medical treatment. Disclosed herein are methods of treatment and methods of identifying subjects for such treatment based on levels of pirin and/or iron in samples from the subject.

Background

Iron plays a central role in the control of various cellular activities, including the regulation of pathways like Wnt signalling and NF κ B signalling. Dysregulation of these pathways has been linked to carcinogenesis, particularly to the development of colorectal cancer (CRC). Human mutations leading to hereditary hemochromatosis (HH), which causes abnormally high dietary iron uptake, are also linked to significantly higher rates of CRC. Despite compelling evidence that increased heme iron and consumption of red meat has direct correlation with developing CRC, the mechanistic basis for that connection is still unclear. Nonetheless, it is generally recognised that iron plays an important role in cancer cell metabolism.

Iron supplementation is the standard of care for colorectal cancer (CRC) patients with anaemia due to excessive loss of blood in faeces. However, increased iron consumption risks prolonging or exacerbating disease in these patients. There is no method available to identify CRC patients with anaemia who may be contraindicated for iron supplementation therapy.

Iron chelators, such as deferasirox, were originally developed to reduce chronic iron overload in patients who are receiving long-term blood transfusions for conditions such as β -thalassemia and other chronic anaemias. Iron chelators have recently been shown to have some anti-tumor activity in cell culture experiments and *in vivo* studies, although their efficacy in patients is not well studied. There is also no method available to detect cancer patients who may respond to iron chelation therapy.

It would be desirable to overcome or ameliorate at least one of the above-described problems, or at least to provide a useful alternative.

Summary

Disclosed herein is method of identifying a subject who is likely to be responsive to treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

Disclosed herein is a method of predicting the prognosis of a subject following treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates the prognosis of the subject following treatment with the iron chelator.

Disclosed herein is a method of treating a subject, the method comprising a) detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject found likely to be responsive to treatment with an iron chelator.

Disclosed herein is a method of treating a subject, the method comprising a) selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject when the subject is found likely to be responsive to treatment with the iron chelator.

Disclosed herein is a method of selecting a subject for treatment with iron supplementation, the method comprising selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of

pirin and/or iron as compared to a reference indicates that the subject is likely to be unsuitable for treatment with the iron supplementation.

Disclosed herein is a method of identifying a subject who is unsuitable for treatment with iron supplementation, the method comprising detecting an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation.

Disclosed herein is a method of treating cancer in a subject, the method comprising administering an effective amount of a pirin inhibitor to the subject.

Disclosed herein is a method of identifying a subject who is likely to be responsive to treatment with a pirin inhibitor, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with the pirin inhibitor.

Disclosed herein is a method of sensitizing a subject to treatment with a pirin inhibitor, the method comprising administering an iron chelator to the subject.

Disclosed herein is a method of identifying a cancer cell that has high telomerase activity, the method comprising detecting pirin in the cancer cell, wherein an increased level of pirin as compared to a reference indicates that the cancer cell is likely to have high telomerase activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Iron level correlates with telomerase activity in CRC. A) Schematic of the tissue collection and follow-up assays used. B) Telomerase activity as measured by Telomerase Repeated Amplification Protocol (TRAP) assay from tumor and adjacent normal tissues (n=47). Tumors were segregated as telomerase low and telomerase high. The values were derived from a minimum of 5 replicates for each sample. C) Fold change of iron level (Fe^{3+}) measured using iron assay kit for each tumor and adjacent

normal tissue pairs (n=47). Tumors were segregated as iron low and iron high. The values were derived from a minimum of 3 replicates for each sample. ** p-value < 0.05

D) Representative images showing the iron staining and H&E staining for tumor and normal sections from 2 patient samples. The iron-stained region in tumor sections is marked by white dotted lines. E) Chart illustrating the number of High and low iron staining and/or telomerase activity in the patient's samples analyzed from panel B and C. Significance p-value = 0.0005 (Fisher exact test). F) Schematic representation of the engineering of the novel NanoLuc reporter cell line used for small molecule screening. G) Graph showing the activators and inhibitors hits obtained from HTS screen using small molecule library targeting epigenetic modifiers. The luciferase activity was measured as a readout. Shown in particular is a hit called SP2509 which was followed further.

Figure 2. Pirin is the target of SP2509 and regulates hTERT. A) Schematic of the ITDRF method used to identify the molecular target of SP2509. B) Graph showing proportion of pirin bound and stabilized by SP2509 in the soluble fraction of cellular lysate as measured by mass spectrometry. The cell lysates were incubated with DMSO or SP2509 for 5 minutes before denaturation by heat and samples preparation for LC/MS. C) Western blot showing the level of pirin and its stabilization at higher temperature by SP2509 (top) and CCT251236 (bottom). D) qPCR-based mRNA expression (top) and telomerase activity (bottom) in HCT116 and COLO205 cells treated with increasing doses of CCT251236. *** p-value < 0.001. E) Telomerase activity measured by TRAP assay in HCT116 cells 48 hours after pirin knockdown using 2 different siRNAs. *** p-value < 0.001. F) Telomerase activity measured by TRAP assay in HCT116 cells 48 hours after SP2509 treatment and pirin over expression. G) List of primary patient-derived cell lines (PDCs) indicating the site of the tumor, genomic alteration and CMS status. H) qPCR-based mRNA quantification of hTERT in PDCs treated with SP2509 or DMSO for 48 hours. * p-value < 0.05 ** p-value < 0.005 *** p-value < 0.0001. I) Image of PDX tumors after the termination of treatment in NSG mice groups injected with SP2509 or DMSO. J) Graph showing tumor volume over the study period of 2-weeks in NSG mice groups injected with SP2509 or DMSO (twice weekly). K) qPCR-based mRNA quantification of hTERT in PDXs treated with SP2509 or DMSO for a week.

Figure 3. Pirin activates *hTERT* transcription in a Fe³⁺ iron-dependent manner. A) Graph showing the level of iron in HCT116 cells after treatment with SP2509. Fe (II) is the reduced form of iron, Fe (III) is the oxidized form of iron. Error bars are obtained from standard deviation from 3 independent biological replicates. B-C) qPCR-based mRNA expression of *hTERT* (B) and telomerase activity by TRAP assay (C) from HCT116 cells treated with SP2509 or DFX for 48 hours. D-E) Telomerase activity by TRAP assay (D) and cell growth as measured by CCK8 assay (E) from HCT116 cells treated with PBS or rTF for 72 hours. F) qPCR-based mRNA expression analysis for Pirin gene from PDXs with the differential expression level of Pirin. Gene expression was normalized to beta-actin expression from each sample. G) Image of PDX tumors developed in NSG mice. PDX with low (0266^{Pirin Low}) and high (0186^{Pirin High}) Pirin expression in NSG mice were treated with iron chelator DFX or PBS for 3-weeks (twice weekly). H) Graph showing tumor volume over the study period of 3-weeks in NSG mice groups injected with DFX or PBS (twice weekly). I) Telomerase activity measured by TRAP assay in PDXs treated with DFX or PBS for 3 weeks in NSG mice. Tumors were harvested after study termination and a TRAP assay was performed. Error bars indicate the standard deviation from 5 biological replicates for each group. J) Image showing the iron stain by PB in PDX tumors (0186^{Pirin High}, 0266^{Pirin Low}) developed in NSG mice treated with PBS or DFX for 3 weeks. Iron-stained regions in tumor sections are marked by red arrows and magnified (bottom). K) Graph showing the level of iron (Fe (III)) in PDX tumors (0186^{Pirin High}, 0266^{Pirin Low}) developed in NSG mice treated with PBS or DFX for 3 weeks. Error bar indicates the standard deviation from 5 biological replicates for each group. * p-value <0.05, ** p-value <0.005, *** p-value <0.0001.

Figure 4. Iron and pirin levels display a significant clinical correlation in CRC. A) Western blot showing the level of pirin in normal (N) and tumor (T) sections of CRC patients samples. HSP90 was used as a loading control. B) Schematic illustrating the construction of tissue microarray and staining for iron and pirin in a large cohort of CRC patients involving 245 matched normal and tumor samples. C-D) Representative images of 2 patient samples (tumor and adjacent normal) stained for iron by PB staining (C) and pirin by immunohistochemistry (IHC) (D). The iron-stained region in tumor sections is marked by a red circle. E) Pie chart showing the percentage of tumor (left) and adjacent normal (right) with high and low staining of iron and pirin by PB staining

and IHC, respectively. F) Table showing the distribution of CMS types among the patient cohort selected for the staining of iron and pirin by PB staining and IHC, respectively, in the tumor and normal sections. G) Graph showing the percentage distribution of CMS types among the patient cohort with positive and negative staining of iron and pirin. H) Telomerase activity measured by TRAP assay in patients-derived organoids (PDO) representing different CMS types. PDOs were treated with SP2509 or DMSO for 48 hours. Tumors were harvested after study termination and a TRAP assay was performed. Error bars indicate the standard deviation from 3 biological replicates. * p-value <0.05, ** p-value <0.005, *** p-value <0.0001

Figure 5. Pirin and SP2509 inhibit *hTERT* expression through Sp1. A) Venn-diagram showing the Differentially Expressed Gene set (DEGs) from HCT116 cells treated with SP2509 (HCT116^{SP2509}) or with Pirin knockdown (HCT116^{PIR-KD}). The common DEGs are highlighted by a black box. B) Gene ontology analysis of common DEGs obtained from HCT116 cells treated with SP2509 or with Pirin knockdown. C) Transcription factor motif analysis from down-regulated genes in DEGs obtained from HCT116 cells with Pirin knockdown. D) Western blot image showing the level of Sp1 protein in HCT116 cells treated with SP2509 or with pirin knockdown (with 2 different siRNAs). E) Western blot image showing the level of Sp1 protein in HCT116 cells after overexpression of pirin. HSP90 was used as a loading control. F) ChIP-qPCR analysis of Sp1 occupancy on *hTERT* proximal promoter from HCT116 cells treated with SP2509 or with pirin knockdown. The samples were normalized to the input and IgG was used as a control. G) Western blot image showing the level of ubiquitination of Sp1 using Pan ubiquitin antibody (left) and K48 ubiquitin-specific antibody (right) in HCT116 cells stably overexpressing Pirin. H) Heat-map showing differential expression of ubiquitin ligases in HCT116 cells treated with SP2509 or with Pirin knockdown. The experiments involving pirin knockdown are transient with 2 different siRNAs.

Figure 6. Sp1 ubiquitination by Fbxw7 regulates *hTERT* reactivation. A) Western blot image showing the interaction of FBXW7 with Sp1 in HCT116 cells transfected with Fbxw7-plvx, SP1-HA, and Pirin-FLAG for Fbxw7, Sp1, and Pirin, respectively. Fbxw7 and Sp1 overexpression led to specific pulldown of Fbxw7 in IP fraction whereas Pirin overexpression led to the loss of this interaction between Fbxw7 and Sp1 in HCT116 cells. Total ubiquitination of Sp1 increased during its interaction with Fbxw7

and was reduced when cells overexpressed Pirin. Data are representative of multiple repeats with similar results. B) Image from Phospho imager showing the binding of various transcription factors on FBXW7 promoter with (+) and without (-) Pirin overexpression in HCT116 cells using EMSA. Ectopic expression of Pirin reduced the abundance of NFAT-1 and PU-1 on the proximal promoter of FBXW7. Data are representative of 2 repeats with similar results. C) Western blot image showing the level of Sp1 with and without Fbxw7 and Pirin overexpression in HCT116 cells. The level of Sp1 decreased upon Fbxw7 overexpression and was rescued by pirin overexpression. Data are representative of multiple repeats with similar results. D) ChIP-qPCR analysis of NFAT-1 occupancy on *FBXW7* proximal promoter from HCT116 cells treated with SP2509 (HCT116^{SP2509}) or with pirin knockdown (HCT116^{PIR-KD}) and compared to HCT116 control cells (HCT116^{CTRL}). The samples were normalized to the input and IgG was used as a control. E) Telomerase activity measured by TRAP assay in FBXW7 knockout (KO) cells of HCT116 and DLD-1. The KO cells were sorted and selected after CRISPR genome editing. *** p-value <0.0001. F) Model of *hTERT* reactivation by pirin. Pirin binds iron (sourced from red meat or iron overload due to genetic conditions like hereditary hemochromatosis) to become active and this complex reduces NFAT-1 occupancy on the FBXW7 promoter. Since NFAT-1 is an activator of FBXW7 promoter, this led to reduced synthesis of Fbxw7 protein and increased steady-state levels of Sp1. Consequently, Sp1 multimers activate *hTERT* transcription leading to reconstitution and activation of telomerase activity in cancer cells, leading to transformation.

Figure 7. Telomerase activity was measured by TRAP assay of freshly resected tissues samples from CRC patients who showed no significant change (A) or a significant increase (B) in tumor tissues compared to adjacent normal tissues.

Figure 8. Iron assay kit quantification of iron levels in freshly resected tissues samples from CRC patients who show no significant changes (A) or a significant increase (B) in tumor tissues compared to adjacent normal tissues.

Figure 9. (A) Plot showing the cut-off of luminescent signal used to select positive clones for the endogenous tagging of luciferase in the *hTERT* gene. The clones were

obtained from the HCT116 cell line using CRISPR genome editing. (B) Schematic representation of engineered novel NanoLuc reporter locus highlighting the PCR primers designed to check for proper insertion. (C) qPCR-based gene expression quantification of MYC, hTERT, and luciferase genes in the NanoLuc reporter cell line after knockdown of MYC for indicated time points. ** $p < 0.05$. (D) Image showing relative luciferase activity measured in NanoLuc reporter cell line after treatment with top inhibitor hits obtained by screening for 48 hours. Luciferase activity was measured using Nano-Glo luciferase reagent.

Figure 10. (A) Venn diagram comparing candidate hits from ITDRF of two biological replicate experiments in which cell lysates were treated with either DMSO or SP2509 and subjected to ITDRF. Pirin was identified in both replicates. (B) Cell viability assay performed on HCT116 cells with and without Pirin overexpression. Cells were treated with either DMSO or SP2509 for 48 hours, and viability was measured using the CCK8 assay. (C) Image from colony formation assay of HCT116 cell line treated with varying concentrations of CCT251236, which was added after HCT116 cells were seeded at 10,000 cells per well for 48 hours. Colony formation and growth were measured by crystal violet staining after 1 week. (D) qPCR-based gene expression quantification of *hTERT* gene in CRC and non-CRC cell lines treated with DMSO or CCT251236 for 48 hours. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. paired *t*-test.

Figure 11. (A-B) Quantification of transient LSD1 gene knockdown in HCT116, DLD-1, and HT29 cell lines treated with siRNA for 48 hours, as shown by qPCR (A) and western blotting (B). Beta-actin was used as a normalization control. (C) qPCR-based gene expression quantification of *hTERT* gene in HCT116, DLD-1, and HT29 cell lines after transient knockdown of LSD1. (D-E) Quantification of stable LSD1 gene knockdown in HCT116, DLD-1, and HT29 cell lines, as shown by qPCR (D) and western blotting (E). Beta-actin was used as a normalization control. (F) qPCR-based gene expression quantification of *hTERT* gene in HCT116, DLD-1, and HT29 cell lines after stable knockdown of LSD1. (G) qPCR-based gene expression quantification of *hTERT* gene in HCT116 cell line treated for 48 hours with different concentrations of indicated LSD1 inhibitors. (H) TRAP assay measurement of telomerase activity in HCT116 cells treated with SP2509 or MST-312 (known telomerase inhibitor) for 48 hours. * $p < 0.05$, *** $p < 0.005$.

Figure 12. (A) Schematic representation of iron-displacement assay. Pirin plasmid was purified from bacterial overexpression system using Ni-NTA beads and checked for purity by SDS-PAGE. 100 mM of purified Pirin protein was incubated with 200 mM FeSO_4 solution overnight at 4°C for iron reconstitution and again purified using Ni-NTA column to remove any excess FeSO_4 . The eluted fractions were run on SDS-PAGE and concentrated using Centricon. The iron reconstituted Pirin was incubated with either SP2509 or DMSO (control) at equimolar concentrations overnight at 4°C . The amount of iron released (free iron) in the control and SP2509-treated samples was then quantified using the iron assay kit by measuring the absorbance at 593 nm in a multi-plate reader. (B) Coomassie-stained gel image showing purity of Pirin after bacterial overexpression and reconstitution with iron. Arrow indicates Pirin protein molecular size. Lane 1-2 flow through; 4-9 elution fractions; 1-7 (right) elution fraction after protein reconstitution (C) Quantification of free iron (Total, Fe^{2+} , and Fe^{3+}) in the reaction after incubation of Pirin with DMSO or SP2509 at equimolar concentrations. SP2509 displaced the iron bound to Pirin, leading to a higher quantity of iron in the reaction.

Figure 13. (A-B) qPCR-based gene expression quantification of *hTERT* gene in various colorectal cancer (CRC) (A) and non-CRC cell lines (B) treated with DMSO or SP2509 for 48 hours at indicated concentrations. * $p < 0.05$, ** $p < 0.01$. (C) qPCR-based gene expression quantification of *hTERT* gene in isogenic cell lines carrying either wild type (WT) or C250T promoter mutant *hTERT* gene. (D) Image from colony formation assay of HCT116 cell line treated with varying concentrations of SP2509 as indicated. The drug was added after HCT116 cells were seeded at 10,000 cells per well and incubated for 48 hours. Colony formation and growth were measured by crystal violet staining after 1 week. (E) Flow cytometry analysis of HCT116 cells after treatment with increasing concentrations of SP2509 as shown. Cells were stained with 7-AAD and annexin V to measure early and late apoptosis, respectively. Q3 indicates the increase in early apoptotic cells with the increase in SP2509 concentration. Data is representative of three experiments with similar results. (F) Western blot stained for markers of apoptosis. HCT116 cells were treated with SP2509 at the indicated concentrations for 48 hours and harvested for cell lysate. HSP90 was used as a loading control. (G) Histogram showing analysis of HCT116 cell cycle. Cells were treated with SP2509 for

48 hours, and the proportion of cells in the different stages was analyzed by Propidium iodide staining using FACS.

Figure 14. (A) qPCR-based gene expression quantification of *hTERT* gene in COLO205 and HCT116 cells after treatment with SP2509 at different time points as indicated. (B) Cell viability was measured by CCK8 assay of COLO205 and HCT116 cells after treatment with SP2509 for 9 and 24 hours respectively. (C) Western blot stained for apoptosis markers from HCT116 cells treated with SP2509 at indicated concentrations for 24 and 36 hours. HSP90 was used as a loading control. (D-E) Measurement of apoptosis by Caspase-Glo 3/7 assay in HCT116 (D) and COLO205 (E) cells after 24 and 9 hours of SP2509 treatment, respectively. Doxorubicin was used as positive control, *** $p < 0.005$. (F-G) ChIP-qPCR analysis of histone marks (H3K4me3, H3K9Ac) and POL II occupancy of *hTERT* proximal promoter from HCT116 cells (F) and COLO205 cells (G) treated with SP2509 or DMSO for 24 hours. Samples were normalized to the input, and IgG was used as a control. (H) Image of xenograft tumors developed in NSG mice. HCT116-derived xenografts in NSG mice were treated with SP2509 or DMSO for 3 weeks (twice weekly) at 30 mg/kg body weight. (I) Graph showing tumor volume over 3-week study period in NSG mice injected with SP2509 or DMSO (twice weekly). (J) *hTERT* gene expression was measured by qPCR of harvested xenograft tumors in NSG mice treated with SP2509 or DMSO for 3 weeks. Error bars indicate standard deviation of five biological replicates for each group. (K) qPCR-based expression quantification of *hTERT* gene in various patient-derived (primary) cell lines. Expression was normalized to beta-actin levels. (L) Graph showing bodyweight of NSG mouse groups implanted with PDX tumors and injected with SP2509 or DMSO (twice weekly over a study period of 3 weeks. (M-N) Telomerase activity measured by TRAP (M) and qPCR-based analysis of *hTERT* gene expression (N) of hES cells (H9) treated with SP2509 or DMSO for 48 hours. MST-312 was used as a positive control to measure telomerase inhibition.

Figure 15. (A) Quantification of iron (Total, Fe^{2+} , and Fe^{3+}) in HCT116 cells after treatment with DMSO or deferasirox (DFX) for 48 hours. Error bars were obtained from at least two biological replicates. ** $p < 0.01$. (B) qPCR-based expression quantification of iron metabolism-related genes in HCT116 cells after treatment with DMSO, SP2509, or DFX for 48 hours. *** $p < 0.005$. (C) Image of HCT116-derived xenograft tumors

with stable Pirin knockdown (HCT116^{PIR KD}) or without knockdown (HCT116^{CTRL}) grown in NSG mice treated with iron chelator DFX or PBS for 3 weeks (twice weekly). (D) Graph showing tumor volume over the 3-week study period of NSG mice injected with DFX or PBS (twice weekly). (E) Telomerase activity measured by TRAP assay of xenografts in NSG mice treated with DFX or PBS for 3 weeks. Tumors were harvested after study termination, and TRAP assay was performed. Error bars indicate standard deviation of five biological replicates for each group. ** $p < 0.01$, *** $p < 0.005$. (F) qPCR-based expression quantification of *hTERT* gene in patient-derived organoids treated with either DMSO or SP2509 for 48 hours. Organoids represent different consensus molecular subtypes (CMS). ** $p < 0.01$, *** $p < 0.005$.

Figure 16. (A) Heatmap showing differentially expressed genes (DEG) from RNA-seq data. HCT116 cells were treated with DMSO or SP2509 or transfected with siRNA against pirin. Cells were harvested for RNA isolation 48 hours post-treatment. (B) Motif enrichment analysis of DEG of HCT116 cells treated with siRNA against pirin compared to that of control HCT116 cells. Red box highlights SP1 motif enrichment in down-regulated gene set. (C) qPCR-based expression quantification of pirin gene in HCT116 cells with stable overexpression of pirin. (D) qPCR-based expression quantification of SP1 gene in HCT116 cells treated with siRNA against pirin or SP2509. Cells were harvested after 48 hours of treatment. (E) Volcano plot showing DEGs from HCT116 cells treated with SP2509 (upper panel) or siRNA against pirin (lower panel). *Fbxw7* gene is highlighted in red while *RNF4* is in black. (F) qPCR-based expression quantification of *Fbxw7* gene in HCT116 cells treated with siRNA against pirin, SP2509, or cells with stable pirin overexpression. Cells were harvested after 48 hours of treatment. (G) Table showing list of E3 ubiquitin ligases upregulated in HCT116 cells after treatment with siRNA against pirin or SP2509. The list was obtained from RNA-seq data, and the scores indicate target prediction for SP1 ubiquitination.

Figure 17. (A) Western blot image showing ubiquitination of Sp1 in HCT116 cells transfected with *Fbxw7*-plvx, SP1-HA, and Pirin-FLAG for *Fbxw7*, Sp1, and Pirin, respectively. Anti-HA was used to pulldown SP1. Pirin overexpression led to a significant decrease in ubiquitination of Sp1 by FBXW7. Data are representative of multiple repeat experiments with similar results. (B) qPCR-based gene expression quantification of *NFAT-1* (top) and *Fbxw7* (bottom) in HCT116 cells after transient

knockdown of *NFAT-1* using two different siRNAs. Cells were harvested after 48 hours of knockdown. (C) Image from Phospho imager showing binding of SP1 consensus motif to *hTERT* gene promoter in the presence of SP2509 (at indicated concentration) or with Pirin overexpression in HCT116 cells using EMSA. Ectopic expression of pirin increased the abundance of SP1 bound to the proximal promoter of the *hTERT* gene. Data are representative of two repeat experiments with similar results. (D) qPCR-based gene expression quantification of *Fbxw7* in HCT116 cells after treatment with SP2509 or DFX for 48 hours. *** $p < 0.005$.

Detailed Description

The present specification teaches methods of treatment and methods of identifying subjects for such treatment based on levels of pirin and/or iron in samples from the subject.

Disclosed herein a method of identifying a subject who is likely to be responsive to treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

Without being bound by theory, the inventors show that iron plays a role in re-activation of telomerase in cells. Re-activation of telomerase activity is a hallmark of close to 90% of human cancers. One pathway linking iron and telomerase activation is controlled by pirin, an iron binding protein which was previously shown to activate NF κ B target genes. Pirin regulates telomerase activity and tumour growth *in vivo* in an iron-dependent manner. In some tumours, there is a significant correlation of high iron and pirin levels with high telomerase activity. Further, clinical samples from patients with colorectal cancer (CRC) of the consensus molecular subgroup 3 (CMS3) subtype show elevated iron and pirin levels. These observations show that iron and/or pirin levels may be relevant clinical biomarkers for selecting particular treatment courses or predicting treatment outcomes for certain groups of cancer patients. On one hand, patients with cancers that exhibit high levels of iron and/or pirin may benefit from iron chelation therapy, which may reduce telomerase activity and inhibit cancer proliferation. On the

other hand, iron supplementation may be contraindicated in cancer patients with anemia if the cancer is of a high-pirin and/or high-iron subtype.

In one embodiment, an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation. In one embodiment, an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation and likely to be responsive to treatment with an iron chelator.

Pirin herein refers to a protein or RNA product of the *PIR* gene, including any polymorphic or splice variants thereof. Pirin protein is a member of the cupin superfamily of proteins. It is a non-heme Fe^{2+} -binding nuclear protein with a putative role in transcription factor regulation.

In some embodiments the iron to be detected is the ferrous (i.e., Fe^{2+}) and/or ferric (i.e., Fe^{3+}) forms of iron. The iron may be protein-bound or non-protein-bound. Iron detection may involve detecting any one of or a combination of these forms of iron, e.g., protein-bound and non-protein-bound Fe^{2+} . Total iron refers to the total quantity of all of these forms of iron, i.e., protein-bound and non-protein-bound Fe^{2+} and Fe^{3+} .

The terms “detecting”, “determining”, “measuring”, “evaluating”, “assessing” and “assaying” are used interchangeably herein to refer to any form of measurement, and include determining if an element is present or not. These terms include both quantitative and/or qualitative determinations. Assessing may be relative or absolute. More than one method may be used to detect pirin and/or iron to allow cross-validation of findings.

In some embodiments, the method comprises detecting pirin protein or mRNA in the sample. Protein or mRNA detection may be performed using any of a wide variety of well-known methods for detecting expression of a transcribed nucleic acid or protein. Non-limiting examples of such methods include immunological methods for detecting nuclear proteins, protein purification methods, protein function or activity assays, mass spectrometry, nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods.

In one embodiment, pirin protein is detected using an antibody (e.g., a radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled antibody), an antibody derivative (e.g., an antibody conjugated with a substrate or with the protein or ligand of a protein-ligand pair like biotin-streptavidin), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically with pirin protein or a fragment thereof, including pirin which has undergone either all or a portion of post-translational modifications to which it is normally subjected in a cell (e.g., glycosylation, phosphorylation, methylation etc.). This detection may be performed as part of *in vitro* protein assay techniques well-known in the art, e.g., enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence staining. Alternatively, the known method of surface plasmon resonance (SPR) may be used to detect the interaction between pirin and anti-pirin antibodies or antibody fragments, and used to quantify the amount of pirin present in a sample.

Non-immunological methods may also be used to detect pirin protein. For instance, labelled aptamers (e.g., a radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled aptamer) may be used for pirin binding and detection. An aptamer refers to a nucleic acid that has a specific binding affinity for a target molecule. A suitable aptamer can be identified using any known method, including but not limited to the SELEX process. Once identified, an aptamer can be prepared or synthesized in accordance with any known method, including chemical synthesis methods and enzymatic synthesis methods. Other binding agents for detecting pirin may include, e.g., small molecules, lectins, ligand-binding receptors, affibodies, nanobodies, ankyrins, alternative antibody scaffolds (e.g. diabodies), imprinted polymers, avimers, peptidomimetics, peptoids, peptide nucleic acids, threose nucleic acids, synthetic receptors, and modifications and fragments of these.

Suitable fluorophores for labelling a pirin-binding agent for pirin detection include rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, allophycocyanin, PBXL-3, Qdot 605, Lissamine, phycoerythrin, Texas Red, and other such compounds. The fluorophore may also include an AlexaFluor molecule, e.g., AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680, or AlexaFluor 700.

A chemiluminescent label may also be used to label a pirin binding agent for pirin detection. Suitable chemiluminescent compounds include any of oxalyl chloride, Rodamin 6G, Ru(bipy)₃²⁺, TMAE (tetrakis(dimethylamino) ethylene), Pyrogallol (1,2,3- trihydroxybenzene), Lucigenin, peroxyoxalates, aryl oxalates, acridinium esters, dioxetanes, and others.

Suitable enzymes for labelling a pirin binding agent for pirin detection include, e.g., luciferases, luciferin, malate dehydrogenase, urease, horseradish peroxidase (HRP), alkaline phosphatase, beta-galactosidase, glucoamylase, lysozyme, glucose oxidase, galactose oxidase, glucose-6-phosphate dehydrogenase, uricase, xanthine oxidase, lactoperoxidase, microperoxidase, and the like.

Pirin may also be quantified, via its proteotypic peptides, by known mass spectrometry techniques, non-limiting examples of which include: electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/(MS)_n, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), tandem time-of-flight (TOF/TOF) technology, atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-(MS)_n, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-(MS)_n, quadrupole mass spectrometry, Fourier transform mass spectrometry (FTMS), quantitative mass spectrometry, and ion trap mass spectrometry. Any of these techniques may be combined with selected reaction monitoring (SRM) to produce more targeted quantitative measurements for pirin.

In another embodiment, pirin is detected by preparing mRNA or cDNA (i.e., a transcribed polynucleotide) from cells in a sample, and by hybridizing the mRNA/cDNA with a reference polynucleotide which is a complement of a pirin nucleic acid, or a fragment thereof. The reference polynucleotide may contain a label, e.g., a chromophore or fluorophore. The reference polynucleotide may be complementary to or homologous with at least a portion (e.g. at least 7, 10, 15, 20, 25, 30, 40, 50, 100, 500, or more nucleotide residues) of a pirin nucleic acid. Any known RNA isolation

technique that does not select against the isolation of mRNA can be utilized for the purification of mRNA from a sample. cDNA may be prepared from an mRNA preparation using reverse transcription polymerase chain reaction (RT-PCR). Prepared cDNA can be further amplified using any of a variety of polymerase chain reaction (PCR) methods prior to hybridization with the reference polynucleotide.

Alternatively, pirin mRNA can be detected using reverse transcription quantitative PCR (RT-PCR followed with qPCR). RT-PCR is used to create a cDNA from the mRNA. The cDNA may be used in a qPCR assay to produce fluorescence as the DNA amplification process progresses. By comparison to a standard curve, qPCR can produce an absolute measurement such as number of copies of pirin mRNA per cell.

Binding agents for pirin protein or mRNA (e.g., an antibody or polynucleotide) may be immobilized on a solid support, e.g., a microtiter plate or a microarray device, for high-throughput detection of pirin from a plurality of samples.

Total iron in a sample may be detected using various hematological and biochemical indices known in the art. Specific iron measurements include hemoglobin (Hgb), mean cell volume (MCV), hematocrit (Hct), erythrocyte protoporphyrin, plasma iron, transferrin, transferrin saturation levels (TSAT), serum ferritin (SF), soluble transferrin receptors (sTfR) and red-cell distribution width (RDW).

Total iron in samples may also be detected and quantified using various instrumental methods, including but not limited to atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS) and electron paramagnetic resonance spectroscopy. Alternatively, chromogenic or fluorescent probes may be used with more conventional spectroscopy instruments for iron detection. Examples of chromogenic chelators that form colored complexes with Fe^{2+} include 1,10-phenanthroline and its derivatives bathophenanthroline (BP) and bathophenanthroline disulfonic acid disodium salt (BPS), ferrozine and its analog ferene-S, and nitroso-PSAP. Perl's Prussian Blue is a widely available colored stain for identifying Fe^{3+} in tissue samples. A variety of fluorescent probes for binding Fe^{2+} and Fe^{3+} have also been described, e.g., in Hirayama et al., *J Clin Biochem Nut* 60(1):39-48 (2017).

It may be necessary to reduce Fe^{3+} to Fe^{2+} to permit reaction with an iron-binding agent. Suitable reducing agents for this purpose include but are not limited to L-ascorbic acid, thioglycolic acid, hydroxylamine hydrochloride, hydroquinone, hydrosulfite, sodium sulfite, hydrazine sulfate, metabisulfite (pyrosulfite) and the like.

In one embodiment, pirin or iron is detected using histological or cytological methods. The method used may depend on the type of sample. For example, histology may be used for solid or semi-solid tissue samples, whereas cytology is more suitable for fluid samples. Sample preparation techniques for histology or cytology are known in the art, and generally involve sample fixation, immobilization on a solid substrate (e.g., a microscope slide) and staining with one or more pirin- or iron-binding agents. The binding agents may be labelled (e.g., radio-labelled, chromophore-labelled, or fluorophore-labelled binding agents) or may themselves serve as a label (e.g., the binding agent may be a chromophore or fluorophore). Sample preparation for histology may also include tissue embedding and sectioning before staining. Sample preparation for histology and cytology may further include steps of sample dehydration and permeabilisation before staining. A wide range of dyes may be used to differentially highlight and contrast cellular, sub-cellular, and tissue features or morphological structures, e.g. hematoxylin to stain nuclei and Orange G-6 and Eosin Azure to stain the cytoplasm.

Regardless of the stains or processing used, the final evaluation of histological or cytological samples is made by some type of microscopy to permit a visual detection of pirin and/or iron. Exemplary microscopic methods include brightfield, phase contrast, fluorescence, and differential interference contrast.

In one embodiment, the sample for the methods herein is a cell or tissue sample (such as a biopsy sample) from a healthy or diseased tissue. The sample may be from a solid biopsy (e.g. from a tumor) or a liquid biopsy (e.g. a blood, serum or plasma sample). The sample may also include materials derived from a cell or tissue culture, e.g., a culture from a biopsy. Any suitable methods for obtaining a biological sample can be employed; exemplary methods include, e.g., phlebotomy, swab (e.g., buccal swab), fine needle aspiration and forceps biopsy. The sample may be pooled from multiple aliquots. The sample may also be subjected to a variety of well-known post-collection preparative

and storage techniques (e.g., nucleic acid and/or protein extraction, fixation, storage, freezing, ultrafiltration, concentration, evaporation, centrifugation, etc.) prior to pirin or iron detection.

The terms “subject”, “patient”, “host” or “individual”, used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, any member of the subphylum Chordata including primates (e.g., humans, monkeys and apes, and includes species of monkeys such as from the genus *Macaca* (e.g., cynomolgus monkeys such as *Macaca fascicularis*, and/or rhesus monkeys (*Macaca mulatta*)) and baboon (*Papio ursinus*), as well as marmosets (species from the genus *Callithrix*), squirrel monkeys (species from the genus *Saimiri*) and tamarins (species from the genus *Saguinus*), as well as species of apes such as chimpanzees (*Pan troglodytes*)), rodents (e.g., mice rats, guinea pigs), lagomorphs (e.g., rabbits, hares), bovines (e.g., cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., pigs), equines (e.g., horses), canines (e.g., dogs), felines (e.g., cats), avians (e.g., chickens, turkeys, ducks, geese, companion birds such as canaries, budgerigars etc.), marine mammals (e.g., dolphins, whales), reptiles (snakes, frogs, lizards etc.), and fish. In one embodiment, the subject is a human subject.

In one embodiment, the subject is suffering from cancer. In one embodiment the cancer is characterized as having high telomerase activity.

Telomerase herein refers to a protein or RNA product of the *TERT* gene, including any polymorphic or splice variants thereof. Telomerase expression may be quantified using any of the variety of methods described for detecting pirin levels, e.g. using a labelled antibody that binds to telomerase protein or a fragment thereof, or measuring levels of telomerase mRNA using quantitative RT-PCR.

The method as defined herein may comprise detecting telomerase activity in the sample. In one embodiment, an increased level of telomerase activity as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator. In one embodiment, an increased level of telomerase activity as compared to a reference

indicates that the subject is unsuitable for treatment with iron supplementation. In one embodiment, the telomerase activity is measured using a Telomerase Repeated Amplification Protocol (TRAP) assay.

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized in part by unregulated cell growth. As used herein, the term “cancer” refers to non-metastatic and metastatic cancers, including early stage and late-stage cancers. By “non-metastatic” is meant a cancer that remains at the primary site and has not penetrated into the lymphatic or blood vessel system or to tissues other than the primary site. The term “metastatic cancer” refers to cancer that has spread or is capable of spreading from one part of the body to another. Generally, a non-metastatic cancer is any cancer that is a Stage 0, I, or II cancer, and occasionally a Stage III cancer. A metastatic cancer, on the other hand, is usually a Stage IV cancer.

The term “cancer” includes but is not limited to, breast cancer, large intestinal cancer, lung cancer, small cell lung cancer, gastric (stomach) cancer, liver cancer, blood cancer, bone cancer, pancreatic cancer, skin cancer, head and/or neck cancer, cutaneous or intraocular melanoma, uterine sarcoma, ovarian cancer, rectal or colorectal cancer, anal cancer, colon cancer, fallopian tube carcinoma, endometrial carcinoma, cervical cancer, vulval cancer, squamous cell carcinoma, vaginal carcinoma, Hodgkin's disease, non-Hodgkin's lymphoma, esophageal cancer, small intestine cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue tumor, urethral cancer, penile cancer, prostate cancer, chronic or acute leukaemia, lymphocytic lymphoma, bladder cancer, kidney cancer, ureter cancer, renal cell carcinoma, renal pelvic carcinoma, CNS tumor, glioma, astrocytoma, glioblastoma multiforme, primary CNS lymphoma, bone marrow tumor, brain stem nerve gliomas, pituitary adenoma, uveal melanoma (also known as intraocular melanoma), testicular cancer, oral cancer, pharyngeal cancer or a combination thereof.

In one embodiment, the subject is suffering from colorectal cancer (CRC). In one embodiment the colorectal cancer is of a consensus molecular subgroup 1 (CMS1) or consensus molecular subgroup 3 (CMS3) subtype. In one embodiment, the CRC is of a CMS3 subtype.

The four CMS subtypes of colorectal cancer are described in Guinney *et al.*, *Nature Med* 21(11):1350-1356 (2015), and classification is based on the expression profile of particular genes in CRC cancer cells. CMS1 is the immunogenic subtype, enriched for *BRAF* mutations and characterized by microsatellite instability; CMS2 has epithelial characteristics with marked WNT and MYC signaling and high chromosomal instability; CMS3 also has epithelial features but less chromosomal instability, is enriched for *KRAS* mutations, and presents with evident metabolic dysregulation; and CMS4 is the mesenchymal subtype with prominent transforming growth factor β (TGF- β) activation, stromal invasion, angiogenesis, and an inflammatory, immunosuppressive phenotype.

In one embodiment, the subject is suffering from hemochromatosis. Generally, the various types of hemochromatosis are characterized by increased intestinal absorption of iron, which leads to deposition of iron in the body, i.e., in multiple organs such as liver, pancreas, heart and other organs. Excess iron deposition, if left untreated, causes tissue damage and fibrosis with irreversible damage to various organs, e.g., endocrine dysfunction or hepatic cirrhosis. Hemochromatosis may be treated using phlebotomy (removal of blood) or iron chelation therapy.

For the methods herein, pirin or iron levels in a sample are compared to levels of the same biomarkers in a reference to predict the course of a disease or the outcome of a treatment. In one embodiment the reference is a healthy (i.e., non-diseased) sample of the same tissue type from the same subject or from a different subject. In another embodiment the reference is a healthy sample from a different tissue type from the same subject. In yet another embodiment, the reference is a healthy cell from the same sample. The reference may also be population-average levels for the biomarkers in healthy cells or tissues, if such information becomes available as a result of routine performance of the methods described herein. Alternatively, the reference may be a healthy sample from the same subject obtained before the suspected onset of a disease, or an archived healthy sample from the subject.

An increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator. As used herein, the term “increase” or “increased” with reference to a biomarker such as pirin or iron may

refer to a statistically significant and measurable increase in the biomarker as compared to a reference. The increase may be an increase of at least about 10%, or an increase of at least about 20%, or an increase of at least about 30%, or an increase of at least about 40%, or an increase of at least about 50%. The terms “decreased” or “decreased” with reference to a biomarker are to be construed accordingly. A decrease may be a decrease of at least about 10%, at least about 20%, at least about 30%, at least about 40%, or at least about 50%.

In one embodiment, a lack of increase or a decrease in the level of pirin and/or iron as compared to the reference indicates that the subject is unlikely to be responsive to treatment with an iron chelator.

In some embodiments the method comprises administering an effective amount of an iron chelator to the subject found likely to be responsive to treatment with the iron chelator. In iron chelation therapy, an iron chelator binds free or “labile” iron in the blood and organs, which allows for removal of excess iron from the body. The iron chelator may be any iron chelator that is known in the art. In one embodiment the iron chelator is deferasirox, deferoxamine, or deferiprone.

In some embodiments the method further comprises administering an effective amount of a pirin inhibitor to the subject found likely to be responsive to treatment with the iron chelator.

The term “administering” refers to contacting, applying, injecting, transfusing or providing a drug as referred to herein to a subject.

By “effective amount”, in the context of treating or preventing a condition is meant the administration of an amount of an agent or composition to an individual in need of such treatment or prophylaxis, either in a single dose or as part of a series, that is effective for the prevention of incurring a symptom, holding in check such symptoms, and/or treating existing symptoms, of that condition. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the formulation of the composition, the assessment of

the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Treatment response herein may be a decrease in telomerase expression or telomerase activity and/or a reduction in cancer cell growth.

Cancer cell growth may be assayed, e.g., by measuring changes in cell number, DNA synthesis, cellular metabolism and/or level of cell proliferation markers, all of which are methods well known in the art. DNA synthesis may be quantified, for instance, by labelling cells with thymidine analogs like 5-bromo-2'-deoxyuridine (BrdU), 5-iodo-2'-deoxyuridine (IdU) or 5-chloro-2'-deoxyuridine (CldU), and detecting these analogs using labelled analog-specific antibodies. Cell metabolism may be detected, for instance, using tetrazolium salts like MTT, XTT and WST-1. These salts form a dye when present in a metabolically active environment. Cell proliferation may also be measured by detecting the levels of specific nuclear proteins that are expressed in proliferating cells but absent from non-proliferating cells. Non-limiting examples of such proliferation markers include Ki67, PCNA and MCM-2. Antibodies targeting these protein markers are widely available for use in cell proliferation immunoassays. Alternatively, cancer cell growth may be quantified by measuring changes in tumor size.

The terms “treating”, “treatment” and the like also include relieving, reducing, alleviating, ameliorating or otherwise inhibiting the effects of a subject's condition for at least a period of time. It is also to be understood that terms “treating”, “treatment” and the like do not imply that the condition, or a symptom thereof, is permanently relieved, reduced, alleviated, ameliorated or otherwise inhibited and therefore also encompasses the temporary relief, reduction, alleviation, amelioration or otherwise inhibition of the condition, or of a symptom thereof.

In one embodiment, there is provided a method of identifying a cancer subtype in a subject, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator. The cancer subtype may be one that is associated with increased level of pirin and/or iron as compared to a reference. The cancer subtype may be one that is likely to be

responsive to treatment with an iron chelator. The cancer subtype may be one that is unsuitable for treatment with iron supplementation.

The specification teaches a method of predicting the prognosis of a subject towards treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference provides an indication of whether the subject is likely to be responsive towards treatment with the iron chelator.

Disclosed herein is a method of predicting the prognosis of a subject following treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference provides an indication of the prognosis of the subject following treatment with the iron chelator.

The prognosis herein may be relief, reduction or alleviation of one or more symptoms in the subject. For a subject suffering from cancer, the prognosis may also be a decrease in telomerase expression or telomerase activity and/or a reduction in cancer cell growth.

Disclosed herein is a method of treating a subject, the method comprising a) detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject found likely to be responsive to treatment with an iron chelator.

In one embodiment, there is provided an iron chelator for use in the treatment of a subject found likely to be responsive to treatment with an iron chelator, wherein the subject is identified by detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

In one embodiment, there is provided the use of an iron chelator in the manufacture of a medicament for the treatment of a subject found likely to be responsive to treatment

with an iron chelator, wherein the subject is identified by detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

Disclosed herein is a method of treating a subject, the method comprising a) selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject when the subject is found likely to be responsive to treatment with the iron chelator.

In one embodiment, there is provided an iron chelator for use in the treatment of a subject, wherein the subject is selected by detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

In one embodiment, there is provided the use of an iron chelator in the manufacture of a medicament for the treatment of a subject, wherein the subject is selected by detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.

Also disclosed herein is a method of selecting a subject for treatment with iron supplementation, the method comprising a) selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be unsuitable for treatment with the iron supplementation.

The method may further comprise selecting a subject based on a decreased or unchanged level of pirin and/or iron, wherein a decreased or unchanged level of pirin and/or iron indicates that the subject is suitable for treatment with iron supplementation.

Disclosed herein is a method of identifying a subject who is unsuitable for treatment with iron supplementation, the method comprising detecting an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation. In one embodiment, the method further comprises treating the subject found unsuitable for treatment with iron supplementation with an iron chelator.

In one embodiment the subject is suffering from cancer. In one embodiment the subject is suffering from both cancer and anaemia. Without being bound by theory, subjects with cancer of a high-iron and/or high-pirin variety who are also anaemic may be contraindicated for iron supplementation due to the risk of iron supplementation exacerbating cancer cell proliferation through increased pirin and telomerase activity.

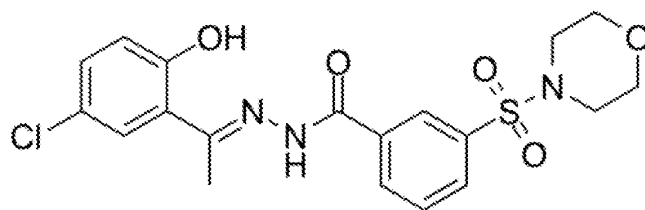
In one embodiment, there is provided a method of identifying a subject who is suitable for treatment with iron supplementation, the method comprising detecting a decreased or unchanged level of pirin and/or iron in a sample obtained from the subject, wherein a decreased or unchanged level of pirin and/or iron as compared to a reference indicates that the subject is suitable for treatment with iron supplementation. The method may further comprise treating the subject with iron supplementation.

Disclosed herein is a method of treating cancer in a subject, the method comprising administering an effective amount of a pirin inhibitor to the subject.

In one embodiment, there is provided a pirin inhibitor for use in treating cancer in a subject.

In one embodiment, there is provided the use of a pirin inhibitor in the manufacture of a medicament for the treatment of cancer in a subject.

In one embodiment, the pirin inhibitor is SP2509, or a derivative thereof.



SP2509

A “derivative” is a compound that is derived from a parent compound by a chemical reaction or by substitution of one atom or group of atoms in the parent compound with another atom or group of atoms. Accordingly, SP2509 or a derivative thereof can refer to SP2509 which has various pendant groups attached to it at various locations of the macrocycle, and can also refer to modifications within the backbone of the macrocycle.

Other pirin inhibitors include but are not limited to compounds that reduce the expression or activity of pirin protein, or promote the degradation of pirin mRNA or protein. For example, CCT367766 is a heterobifunctional compound which targets pirin protein for ubiquitinylation and degradation. Pirin inhibitors may also include triphenyl Compound A (TPh A) and the compounds CCG-1423 and CCG-203971 and their derivatives as described by Lisabeth *et al.* (*ACS Pharmacol Transl Sci.* 2(2): 92–100 (2019)).

In one embodiment, the pirin inhibitor is administered with an iron chelator. In one embodiment, the pirin inhibitor is administered with deferasirox, deferoxamine, or deferiprone.

The compounds (including iron chelators or pirin inhibitors) as defined herein can be administered to a subject as a pharmaceutically acceptable salt thereof. Suitable pharmaceutically acceptable salts include, but are not limited to salts of pharmaceutically acceptable inorganic acids such as hydrochloric, sulphuric, phosphoric, nitric, carbonic, boric, sulfamic, and hydrobromic acids, or salts of pharmaceutically acceptable organic acids such as acetic, propionic, butyric, tartaric, maleic, hydroxymaleic, fumaric, maleic, citric, lactic, mucic, gluconic, benzoic, succinic, oxalic, phenylacetic, methanesulphonic, toluenesulphonic,

benezenesulphonic, salicyclic sulphanilic, aspartic, glutamic, edetic, stearic, palmitic, oleic, lauric, pantothenic, tannic, ascorbic and valeric acids.

Base salts include, but are not limited to, those formed with pharmaceutically acceptable cations, such as sodium, potassium, lithium, calcium, magnesium, ammonium and alkylammonium. In particular, the present invention includes within its scope cationic salts e.g., sodium or potassium salts, or alkyl esters (e.g., methyl, ethyl) of the phosphate group.

Basic nitrogen-containing groups may be quarternised with such agents as lower alkyl halide, such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl and diethyl sulfate; and others.

The compounds or a salt, solvate, stereoisomer or prodrug thereof may be in crystalline form either as the free compound or as a solvate (e.g., hydrate) and it is intended that both forms are within the scope of the present invention. Methods of solvation are generally known within the art.

It will be appreciated that any compound that is a prodrug of the compound is also within the scope and spirit of the invention. Thus the compounds can be administered to a subject in the form of a pharmaceutically acceptable pro-drug. The term "pro-drug" is used in its broadest sense and encompasses those derivatives that are converted *in vivo* to the compound of the invention. Such derivatives would readily occur to those skilled in the art. Other texts which generally describe prodrugs (and the preparation thereof) include: *Design of Prodrugs*, 1985, H. Bundgaard (Elsevier); *The Practice of Medicinal Chemistry*, 1996, Camille G. Wermuth *et al.*, Chapter 31 (Academic Press); and *A Textbook of Drug Design and Development*, 1991, Bundgaard *et al.*, Chapter 5, (Harwood Academic Publishers). For example, amine moieties can be quarternised to improve its solubility.

The compounds, or a pharmaceutically acceptable salt, solvate or prodrug thereof is administered to the patient in a therapeutically effective amount. As used herein, a therapeutically effective amount is intended to include at least partially attaining the

desired effect, or delaying the onset of, or inhibiting the progression of, or halting or reversing altogether the onset or progression of cancer.

Suitable dosage amounts and dosing regimens can be determined by the attending physician and may depend on the severity of the condition as well as the general age, health and weight of the patient to be treated.

The compounds may be administered in a single dose or a series of doses. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a composition, preferably as a pharmaceutical composition. The formulation of such compositions is well known to those skilled in the art. The composition may contain any suitable carriers, diluents or excipients. These include all conventional solvents, dispersion media, fillers, solid carriers, coatings, antifungal and antibacterial agents, dermal penetration agents, surfactants, isotonic and absorption agents and the like. It will be understood that the compositions of the invention may also include other supplementary physiologically active agents.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the patient. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Carriers can include, for example, water, saline (e.g., normal saline (NS), phosphate-buffered saline (PBS), balanced saline solution (BSS)), sodium lactate Ringer's solution, dextrose, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances, such as wetting or emulsifying agents, buffers, and the like can be added. Proper fluidity can be

maintained, for example, by using a coating such as lecithin, by maintaining the required particle size in the case of dispersion and by using surfactants.

Administration of the compound, composition or combinations of the invention or the pharmaceutically acceptable salts thereof can be via any of the accepted modes of administration for agents that serve similar utilities including, but not limited to, orally, subcutaneously, intravenously, intranasally, topically, transdermally, intraperitoneally, intramuscularly, intrapulmonarily, vaginally, rectally, or intraocularly.

The compound, composition or combinations of the invention may be suitable for intravenous administration. The compound, composition or combinations of the invention may also be suitable for subcutaneous administration. Alternatively, the compound, composition or combinations of the invention may be injected directly to a site of a disease in the subject, e.g., to a tumor site.

The compound, composition or combinations of the invention may be suitable for oral administration and may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste. In another embodiment, the compounds or a pharmaceutically acceptable salt, solvate or prodrug is orally administrable.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying

proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

The compound, composition or combinations of the invention may be suitable for topical administration in the mouth including lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles comprising the active ingredient in an inert basis such as gelatine and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

The compound, composition or combinations of the invention may be suitable for topical administration to the skin may comprise the compounds dissolved or suspended in any suitable carrier or base and may be in the form of lotions, gel, creams, pastes, ointments and the like. Suitable carriers include mineral oil, propylene glycol, polyoxyethylene, polyoxypropylene, emulsifying wax, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. Transdermal patches may also be used to administer the compounds of the invention.

The compound, composition or combination of the invention may be suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the compound, composition or combination isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compound, composition or combination may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage composition or combinations are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the active ingredients particularly mentioned above, the composition or combination of this invention may include other agents conventional in the art having regard to the type of composition or combination in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include cornstarch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

Disclosed herein is a method of identifying a subject who is likely to be responsive to treatment with a pirin inhibitor, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with the pirin inhibitor.

Disclosed herein is a method of sensitizing a subject to treatment with a pirin inhibitor, the method comprising administering an iron chelator to the subject.

The terms “sensitize” and “sensitizing”, as used herein, refer to making, through the administration of a first agent (e.g., an iron chelator), a subject more susceptible, or more responsive, to the biological effects (e.g., inhibition of pirin activity) of a second agent. The sensitizing effect of a first agent on a subject can be measured as the difference in the intended biological effect (e.g., inhibition of pirin activity) observed upon the administration of a second agent with and without administration of the first agent.

Disclosed herein is a method of identifying a cancer cell that has high telomerase activity, the method comprising detecting pirin in the cancer cell, wherein an increased level of pirin as compared to a reference indicates that the cancer cell is likely to have high telomerase activity. Cancers with high telomerase activity may include colorectal cancer and glioblastoma, but are not limited to those examples.

Provided herein is a kit for detecting pirin and/or iron in a sample obtained from a subject. The kit may comprise one or more nucleic acid molecules (e.g., one or more primers (such as SEQ ID NOs: 17 and 18) or probes) or one or more polypeptides (e.g., an antigen-binding molecule) for detecting pirin in a sample. The one or more nucleic acid molecules or polypeptides may be detectably labelled (e.g., with a fluorophore). The kit may comprise one or more reagents for detecting iron in the sample. For example, the kit may comprise potassium ferrocyanide and optionally hydrochloric acid and/or a reducing agent. The kit may further comprise one or more nucleic acid molecules (such as SEQ ID NOs: 7 and 8) or one or more polypeptides for detecting telomerase. The kit may further comprise instructions for use in detecting pirin, telomerase and/or iron in the sample.

As used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (or).

As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or

information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Unless otherwise defined, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

EXAMPLES

Methods

Cell Culture

Colorectal cancer cell lines, HCT116, DLD-1, COLO205, LoVo, CaCo-2, HT29, SW620, SW480, HCT15; Glioblastoma cell line, U-251 cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, heat-inactivated), 1X non-essential amino acids (NEAA) (GIBCO) and Normocin (InvivoGen) in a 37°C incubator under 5% CO₂ condition. Cells were cultivated in 100mm petri dishes or T75 flasks with 6 mL or 8 mL growth media, respectively. Cells were washed with pre-warmed Phosphate Buffer Saline (PBS), and were detached using 0.05% trypsin for 5 minutes. Trypsin was then inactivated by mixing with serum-containing growth medium in at least 1:1 ratio, followed by centrifugation at 1,000 rpm for 5 minutes. After removing the medium,

cells were dissolved in fresh medium and were re-seeded. Growth medium was refreshed every 2-3 days. H9 (WA09) human embryonic stem cells (hESCs) were cultured in StemMACS™ iPS-Brew XF medium containing supplement in 6-well plates coated with Matrigel (Corning) in a 37°C incubator under 5% CO₂ condition. Cells were dissociated by scrapping followed by centrifugation at 1,000 rpm for 5 minutes. Cells were finally seeded into new wells coated with Matrigel and StemMACS™ iPS-Brew XF medium containing 10 µM ROCK Inhibitor, Y-27632, (STEMCELL Technologies). Patient-derived primary colorectal cancer cell lines were derived in the laboratory of Ramanuj DasGupta (manuscript in preparation). Primary colorectal cancer cell lines were grown in 6-well tissue-culture treated plates (Falcon) precoated with Coating Matrix Kit (Gibco), and were maintained in DMEM/F12 medium (Gibco) supplemented with penicillin-streptomycin (Gibco), B27 (without vitamin A, Gibco), 20ng/mL EGF (Gibco), and 10ng/mL bFGF (Gibco).

Construction of CRISPR-Cas9 Related Plasmids

The pSpCas9(BB)-2A-GFP (PX458) backbone plasmid was used to clone single guide RNA (sgRNAs). Multiple sgRNAs were designed using an online tool (<http://crispr.mit.edu/>) to find the most efficient sgRNA targeting the TERT promoter. Sequences of sgRNAs cloned into PX458 are as follows:

NLuc-TERT-sgRNA01: 5'-CCCCAGGCGCCGCACGAACG-3' (SEQ ID NO: 1)
NLuc-TERT-sgRNA02: 5'-G GGGAGCGCGCGGCATCGCG-3' (SEQ ID NO: 2)
NLuc-TERT-sgRNA03: 5'-CGGGGAGCG CGCGGCATCGC-3' (SEQ ID NO: 3)
NLuc-TERT-sgRNA04: 5'-AGCGCGCGGCATCGCGG GGG-3' (SEQ ID NO: 4)

Briefly, sense and antisense of sgRNA sequences were phosphorylated and annealed in ligation buffer containing T4 polynucleotide kinase using the following parameters: 37°C for 30 minutes; 95°C for 5 minutes; ramp down to 25°C at 5°C minutes⁻¹. The plasmid was digested using fast-digest BbsI (BpiI) (Thermo Scientific) enzyme, and was ligated with sgRNAs using T4 ligase (Invitrogen) in Tango buffer containing 10 mM DTT and 10 mM ATP. The reaction was kept at 37°C for 5 minutes; 21°C for 5 minutes for 6 cycles for ligation. Next, PlasmidSafe exonuclease (VWR) was added into

the reaction mixture to remove unligated plasmids, and the reaction was incubated at 37°C for 1 hour, followed by 70°C for 30 minutes.

pUC19 plasmids were used as a repair template of homologous recombination for CRISPR-Cas9 and sgRNA efficiency test, respectively. The DNA sequence of NanoLuc enzyme was amplified from pNL1.1 (Promega), left and right homology arms were amplified from the genomic DNA of HCT116. Amplified PCR products were assembled via second-round PCR. PCR reactions were carried out using Q5 high-fidelity DNA polymerase (NEB) in Q5 reaction buffer containing GC enhancer, 0.5 µM forward and reverse primers, DMSO, and dNTP at 98°C for 2 minutes, 30 cycles of [98°C for 30 sec, 72°C for 4 min], followed by 72°C for 7 minutes. The fully assembled fragment was then separated and extracted from a 0.7% agarose gel. The fragment was purified by PCR Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Next, the pUC19 plasmids and fragments were digested using HindIII and SalI (NEB). After digestion, plasmids and fragments were purified using a PCR purification kit (Qiagen), followed by ligation with T4 ligase in ligase buffer at 16°C overnight.

For transformation, 5 µL of each ligation reaction was mixed with 50 µL of competent TOP10 bacterial cells and were incubated on ice for 30 minutes. After 45 seconds of heat shock in a 42°C water bath, the reaction tubes were immediately incubated on ice for 2-3 min. Next, 1 mL of LB broth was added into each tube and left at 37°C for recovery. After 1 hour, the reaction mixture was plated on LB agar containing 100 µg/mL Ampicillin and was incubated at 37°C overnight. On the next day, bacteria colonies were picked and cultured in LB broth at 37°C overnight. Plasmids were isolated using FavorPrep plasmid DNA extraction kit mini (Favorgen) and were validated using Sanger sequencing [2 µL of plasmid, 1 µL of sequencing primer (10 µM), and 4 µL of Big Dye enzyme in 10 µL of reaction volume]. Sequencing PCR was then performed at 95°C for 3 minutes, 35 cycles of [95°C for 30 seconds, 50°C for 10 seconds, 60°C for 4 minutes], followed by 10°C for 30 minutes. Chromatograms were analyzed using ApE (version 2.0.55) software.

Genome Editing-Insertion of Luciferase reporter Gene

Approximately 2×10^5 cells were seeded in a 6-well plate. On the next day, the culture medium was replaced with 2 mL of OPTI-MEM (GIBCO), and cells were co-transfected with 2 μ g of PX458 plasmid targeting the TERT promoter and 2 μ g of repair template containing the nanoluciferase reporter gene, using Xtremegene 9 (Roche). GFP-positive cells were sorted into 96-well plates (1 cell/well) by a cell sorter (FACS) (MoFlo, Beckman) 48 hours after transfection. Every week, 100 μ L of culture medium was added to each well after cell sorting. After 3-4 weeks, a quarter of cells were transferred to a 384-well plate, while three-quarters of cells were transferred to a 24-well plate. Luciferase activity of clones in the 384-well plate was measured using NanoGlo (Promega). Genomic DNA was isolated from cells harboring luciferase activity in the 24-well plate. Briefly, cells were washed with PBS and cell pellets were dissolved in 200 μ L of tail lysis buffer [50 mM Tris pH 8.0, 100 mM NaCl, 100 mM EDTA, 1% SDS supplemented with 10 μ L Proteinase K (20 mg/mL)] and then incubated at 55°C overnight. DNA was precipitated by adding 200 μ L isopropanol followed by centrifugation at 10,000 rpm for 3 minutes. The supernatant was removed and DNA was washed with 500 μ L of 70% ethanol, followed by centrifugation at 10,000 rpm for 3 minutes. The pellet was air-dried for 1 hour at room temperature. Genomic DNA was dissolved in 100 μ L of nuclease-free ultrapure water (GIBCO) before quantification using Nanodrop (ThermoFisher). Clones were genotyped using region-specific primers. PCR reactions were performed at 98°C for 30 seconds, 30 cycles of [98°C for 10 sec, 60°C for 30 sec, 72°C for 4 min], followed by 72°C for 7 minutes.

High-Throughput Compound Screening

Approximately 2.5×10^3 HCT116-NLuc (H17) reporter cells were seeded into a white 384-well plate (Perkin Elmer) and spun down at 1,000 rpm for 1 minute followed by incubation in a 37°C incubator under 5% CO₂ condition. After 18-24 hours, cells were treated with about 200 compounds from both Selleckchem (cat# L1900) and Enzo Screen-well epigenetic inhibitor (cat# BML-2836) libraries at a final concentration of 1 μ M in 50 μ L for 2 days. DMSO percentage in the medium was kept at 1% (v/v) across all drug concentrations and control. Cell counting kit-8 (CCK8) (Sigma) was added 2 hours before the end of the experiment. After 2 hours, 30 μ L of medium containing CCK8 was transferred into a clear bottom 384-well plate for CCK8 assay, and measurement at 450 nm was performed. The rest of the medium in the white 384-well

plate was aspirated. Nano-Glo luciferase reagent was prepared by diluting the substrate 50 times in Nano-Glo buffer (2X). The 2X reagent was diluted to 1X by PBS, and 25 µl of 1X Nano-Glo luciferase reagent was added to cells directly. After 10 minutes of shaking the plate to ensure mixing, luminescence was measured with 1,000 ms integration time. Luciferase activity was calculated using the following formula:

$$\text{Luciferase Activity (\%)} = \frac{\text{Compound Luminescence}}{\text{DMSO Luminescence}} \times 100$$

Western Blotting

Cells were collected using a cell scraper on ice followed by centrifugation at 1,000 rpm for 5 minutes at 4°C, and then cells were washed once with cold PBS and centrifuged. After removal of PBS, cell pellets were incubated with Totex buffer (20 mM HEPES, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA) supplemented with 50 mM NaF, 0.3 mM Na₃VO₄ and protease inhibitor cocktail (Roche) for 30 minutes on ice followed by centrifugation (13,000 rpm for 20 minutes at 4°C) for isolation of total protein. The supernatant was transferred into fresh tubes and stored at -80°C. The supernatant containing protein was mixed with 4X SDS sample loading dye containing 10% β-mercaptoethanol and was heated at 95°C for 5 minutes. Proteins were resolved using a 4-12% gradient SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) followed by transfer onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electrophoretic transfer at 100 mA overnight. PVDF membranes were incubated in blocking buffer (either 5% non-fat milk or 5% BSA in PBS-Tween 20) at room temperature for 1 hour. Membranes were probed with an antibody at 4°C overnight. On the next day, membranes were washed three times for 10 minutes with washing buffer (PBS-Tween 20) and were incubated with secondary rabbit or mouse-HRP conjugated antibodies (1:5000 dilution; Santa Cruz) at room temperature for 1 hour. Membranes were washed four times with washing buffer before incubating with Western Bright ECL HRP or Sirius HRP substrate (Advansta) to detect proteins of interest. The signal was acquired using ChemDoc (Biorad) or captured on X-ray films (KODAK) and developed using a KODAK X-ray film developer. The band intensities were quantified using Image J software (v1.52h, USA) for densitometry analysis.

RNA Isolation and Real-Time Quantitative-PCR (qPCR)

Cells were incubated with TRIZOL (Invitrogen) at room temperature for 10 min for isolation of total RNA. Next, 200 μ l of chloroform was added and evenly mixed several times before centrifugation at 14,000 x g at 4°C for 15 minutes. The aqueous phase containing total RNA (400 μ L) was transferred into a new tube and mixed with 400 μ l 70% ethanol before loading into RNA spin columns (MN) and centrifugation at 11,000 x g for 30 seconds at 4°C. Washing was performed twice using 600 μ l of RA3 buffer first, and then 250 μ l of the same buffer. Finally, RNA was eluted from columns in 50 μ l of RNase free water. RNA concentration was measured with Nanodrop. Reverse transcription to cDNA was performed from 1 μ g of RNA using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher) at 25°C for 10 min, at 50°C for 1 hour, then at 80°C for 20 min. cDNA samples were diluted with DNase free water and 5 ng of cDNA was used for qPCR experiments. SsoAdvanced Universal SYBR Green Mix (Bio-Rad), Luna Universal qPCR Master Mix (NEB), and gene specific primers at 0.5 μ M final concentration, were used for each reaction. Primers used are shown in Table 1.

Table 1

Primer	Forward	Reverse
ACTB (β -ACTIN)	GCCAACCGCGAGAAGATG (SEQ ID NO: 5)	CCATCACGATGCCAGTGGT A (SEQ ID NO: 6)
TERT	CCAAGTTCCTGCACTGGCTG A (SEQ ID NO: 7)	TTCCCGATGCTGCCTGAC (SEQ ID NO: 8)
GABPA	GGACGGGTCTAGGTGAGAC A (SEQ ID NO: 9)	TGGCTGGAGTATTTCAAAG GAT (SEQ ID NO: 10)
MYC	GCTGCTTAGACGCTGGATTT (SEQ ID NO: 11)	CACCGAGTCGTAGTCGAGG T (SEQ ID NO: 12)
VIMENTIN	ACACCCTGCAATCTTTCAGA CA (SEQ ID NO: 13)	GATTCCACTTTGCGTTCAAG GT (SEQ ID NO: 14)
KDM1A (LSD1)	AGCGTCATGGTCTTATCAA (SEQ ID NO: 15)	GAAATGTGGCAACTCGTC (SEQ ID NO: 16)
PIR (PIRIN)	GGATGGTGTGACAGTTGCT G (SEQ ID NO: 17)	ATCATCGGGCCCAATATAC A (SEQ ID NO: 18)

Protein purification and iron assay

The His-tagged Pirin plasmid (pQStrep2-PIR; Addgene #31570) was transformed into BL21 DE3 cells and purified using Ni-NTA beads. The purified protein was pooled and concentrated by Centricon (Millipore). 100mM of the purified Pirin protein was incubated with 200mM FeSO₄ solution overnight at 4°C for iron reconstitution. The reconstituted Pirin protein was again purified using Ni-NTA column to remove any excess FeSO₄. The eluted fractions were run on SDS-PAGE and further concentrated using Centricon. The iron reconstituted Pirin was incubated either with SP2509 or DMSO (control) in equimolar concentration overnight at 4°C. The amount of iron released (free iron) in the control and SP2509 treated samples was then quantified using the iron assay kit (Sigma, Catalog Number MAK025) following the manufacturer's protocol. The absorbance at 593nm was measured using the Tecan multi-plate reader.

Drug Target Identification through Isothermal Dose-Response Fingerprint (ITDRF)

COLO205 colorectal cancer cells were collected using a cell scraper and centrifuged at 1,200 rpm for 4 min at 4°C. Then, cells were washed with cold PBS and centrifuged. The cell pellet was resuspended in cold lysis buffer (4x10⁸ cells/mL of lysis buffer comprising 50 mM HEPES, pH 7.5, 5 mM β-Glycerophosphate, 10 mM MgCl₂, 0.4% NP-40) with 0.1 mM Na₃VO₄ and protease inhibitor cocktail. The resuspended pellet was frozen and thawed three times and then passed through a syringe (30 gauge needle) several times. After that, the lysate was centrifuged at full speed for 20 minutes at 4°C. The supernatant was transferred into a new 1.5 mL tube. The protein-compound (SP2509) mixture was prepared by mixing 250 µg of proteins in 50 µl of lysis buffer containing different concentrations of the compound. The mixtures were incubated for 5 min at room temperature followed by heating to 54°C for 3 min and rapid cooling. Samples were spun down at 14,000 rpm for 20 minutes at 4°C, and the supernatant was transferred into a new 1.5 mL tube. 50 µg of samples were used for detergent (NP-40) removal. 50 µg of the sample was mixed with the same volume of 2X SDS Lysis buffer (10% SDS, 100 mM Triethylammonium bicarbonate, TEAB). Proteins were reduced by adding TCEP (Tris(2-carboxyethyl) phosphine hydrochloride) at a final concentration of 20 mM and heated at 95°C for 10 minutes followed by alkylation wherein

chloroacetamide (CAA) at a final concentration of 55 mM was added. Samples were incubated in the dark for 30 minutes, centrifuged at 13,000 x g for 8 minutes at 4°C and then transferred into a new tube. Phosphoric acid was added at a final concentration of 1.2% into samples to adjust pH. S-Trap binding buffer (90% methanol, 100 mM TEAB) was added to samples and loaded into S-Trap columns for centrifugation at 4,000 x g for 3 minutes. Columns were washed six times with 150 µl of S-Trap Binding Buffer. Next, a digestion buffer containing 2.5 µg of Trypsin in 50 mM TEAB was applied at 37°C for 4 hours. Peptides were eluted with 40 µl of 50 mM TEAB, and then 40 µl of 0.2% formic acid followed by 35 µl of 50% acetonitrile containing 0.2% formic acid. Three elutes were pooled.

Caspase-3/7 Activity Assay

COLO205 or HCT116 colorectal cancer cells (1×10^4 cells/well) were seeded in a white opaque 96-well plate one day before treatment. Cells were treated with SP2509 or Doxorubicin for 9 hours. After treatment, 100 µL of Caspase-Glo 3/7 reagent (Promega) was added to each 100 µl of medium containing cells. The plate was shaken for 1 minute, then incubated at room temperature for 30 minutes before measurement with a luminometer (USA).

Chromatin Immunoprecipitation (ChIP) qPCR

ChIP was performed on HCT116 and COLO205 cell lines as described previously (Akincilar *et al*, *Cancer Discov* 6: 1276-1291 (2016)), using the following antibodies (3 µg per ChIP): anti- H3K4Me3 (Millipore; 04-745); anti-H3K9Ac (Millipore; 07-352); anti-RNA POL II (Millipore; 05-623); anti-Sp1 (ActiveMotif; #39058); and nonspecific IgG (Santa Cruz). After elution, ChIP eluate was used for ChIP-qPCR with the following primers targeting the TSS regions of the TERT gene:

TSS hTERT-Forward: GCGGCGCGAGTTTCAG (SEQ ID NO: 19)

TSS hTERT-Reverse: AGCACCTCGCGGTAGTGG (SEQ ID NO: 20)

TRAP Assay

Cells (2×10^5) were plated 24 hours before compound treatment or siRNA transfection. The cells were collected at specified time points and lysed in $1\times$ CHAPS buffer containing a Protease inhibitor cocktail. The assay was performed in a qPCR setup with dNTPs and TS primers at 25°C for 30 minutes. Subsequently, qPCR was conducted using ACX primers for 40 cycles of 95°C for 5 seconds, 50°C for 6 seconds, and 72°C for 10 seconds. All samples were performed in triplicates.

RNA-seq library preparation

The RNA-sequencing library was prepared from two replicates of the HCT116 cells under different treatment conditions using the NEBNext Ultra II Directional RNA library preparation kit (NEB) according to the manufacturer's instructions.

RNA-seq analysis

Raw reads were filtered using FastQC v0.11.8 and Trimmomatic v0.321. Processed reads were aligned using HISAT2 v2.1 to the human genome (hg38) by Tophat2. Differential gene expression was performed by edgeR v3.26.4 package with default parameters. Genes with expression fold change ≥ 2 and false discovery rate (FDR) < 0.05 were defined as DEGs. Differentially expressed transcription factors were extracted from identified DEGs using the JASPAR 2020 database as a reference. Promoters of downregulated genes were analyzed for enriched motifs using HOMER v4.11. Gene ontology analysis was performed using clusterProfiler v3.12.0.

FACS

Cells were seeded in a 6-well plate one day before treatment with compounds. After treatment, cells were trypsinized and centrifuged to remove trypsin followed by washing with PBS. Cells were subsequently washed with 1X Annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) followed by centrifugation to remove the buffer. For apoptosis analysis, cells were incubated with 7-AAD (BD) in 1X Annexin V binding buffer at room temperature for 20 minutes in the dark. After incubation, cells were washed and centrifuged. Next, cells were incubated with Annexin V (BD) in 1X Annexin V binding buffer at room temperature for 20 minutes in the dark.

After incubation, flow cytometry was performed using the BD LSR II cytometer (BD biosciences). For cell cycle analysis, washed cells were vortexed and fixed in cold 70% ethanol added dropwise, and then rotated in the cold room for 30 minutes. After rotating cells in the cold room, they were washed three times with PBS/EDTA before centrifugation at 750 x g at 4°C for 8 minutes. Then, cells were resuspended in PBS/EDTA containing 100 µg/mL RNase and 50 µg/mL PI (Invitrogen), followed by incubation at 37°C for 30 minutes. After incubation, flow cytometry was performed using the BD LSR II machine and all analyses were performed using FlowJo (BD biosciences, USA).

Iron Assay

For the iron assay, tissues were made into a cell suspension using gentleMACS™ Dissociator (Miltenyi Biotec, Germany). The cells were lysed in the iron lysis buffer provided in the kit (Abcam, USA). The level of total and Fe²⁺ iron was measured as specified in the protocol. Fe³⁺ level was calculated by subtracting Fe²⁺ from total iron.

Perl's Iron staining:

To stain tissue section for Iron Fe³⁺, Iron Stain Kit (Prussian Blue stain) (Abcam, USA) was used. Briefly, the tissues sections from FFPE blocks were deparaffinized and rehydrate. The slides were then incubated for 3 min in iron stain solution (mix of potassium ferrocyanide and hydrochloric acid) followed by rinsing in distilled water. Then sections were counter-stained with nuclear fast red for 5 min and rinsed with distilled water. The slides were dehydrated, dried, and mounted.

Human subjects and Ethics statement

This study received ethics approval from the Human Ethics Committee of the Institute of Molecular and Cell Biology (IRB reference number #2021-198). Written consents were obtained for freshly resected tissue samples to be used in the research study from patients. The sample were obtained from National Centre for Cancer Singapore according to the institute's policy. According to French legislation no written consents are necessary for observational retrospective studies involving tissue microarray

sections. All data were analyzed anonymously. Each patient was identified with a patient deidentified number. Patients were aware that their data were stored in a specific database but were not informed that these data were used for research purposes. This procedure has been disclosed to the Ethics Committee that, in accordance with national legislation, approved it.

Animal studies

HCT116, CRC1489 primary cells (1×10^6 cells per site), or PDXs (0186^{Pirin High} or 0266^{Pirin Low}) were implanted subcutaneously into the right flanks of 5 – 7 weeks old NSG (Jackson Laboratory) mice. When tumors reached a volume of 60 – 100 mm³, the animals were randomly divided into either the treatment or control group. Mice in the treatment group were administered with compound SP2509 at 30 mg/kg once every 3 days via intraperitoneal injection. Mice in the control group were administered with the diluent [10% DMSO, 40% PEG 300 (Sigma Aldrich, cat# 90878), and 5% Tween-80 (Sigma Aldrich, cat# P4780) in saline at a final concentration of 3 mg/mL] in the absence of compound. SP2509 (cat# HY-12635) for *in vivo* studies was purchased from MedChemExpress. For the DFX study, the PDX was equally cut and re-implanted into NSG mice. After the tumor reached 60-100 mm³, animals were randomly administered with DFX [30mg/kg, oral, twice a week] or PBS. Tumor sizes were measured by calipers once every 3 days and tumor volumes were estimated using the following formula: Tumor volume = $1/2(\text{length} \times \text{width}^2)$.

Mice were euthanized when tumors in the control group reached 2,000 mm³. Tumors were harvested at the indicated time points for determining the effects of SP2509 or DFX on TERT expression. Tumors were minced into small pieces and a representative portion was homogenized in TRIzol (Invitrogen, cat# 15596018) using gentleMACS M Tubes (Miltenyi Biotec, cat# 130-093-236) and program RNA_01 on the gentleMACS dissociator machine. RNA was extracted using NucleoSpin RNA columns (Macherey Nagel, cat# 740955) according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed using the SuperScript IV VILO master mix (Invitrogen, cat# 11756500). Ten ng of cDNA was used for each qPCR reaction using a PowerUp SYBR green qPCR kit (Applied Biosystems, cat# A25918) and QuantStudio 7 Flex real-time

PCR system (Thermofisher). Sequences of the qPCR primers used are as follows:

TERT_F	CCAAGTTCCTGGACTGGCTGA	(SEQ ID NO: 21)
TERT_R	TTCCCGATGCTGCCTGAC	(SEQ ID NO: 22)
GAPDH_F	ACCCAGAAGACTGTGGATGG	(SEQ ID NO: 23)
GAPDH_R	TCTAGACGGCAGGTCAGGTC	(SEQ ID NO: 24)

Statistical analysis

For xenograft studies, two-way ANOVA tests were carried out to compare SP2509-treated tumor volumes for HCT116 and CRC1489 primary xenograft tumors with their respective controls. Two-way ANOVA test was also carried out between control and SP2509 treated mice with HCT116 and CRC1489 primary xenograft tumors respectively. *, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$.

For TERT expression in xenograft tumors, two-tailed unpaired t-tests were carried out to compare SP2509-treated tumors harvested 13 days after the start of treatment (D13) with the control. *, $P < 0.05$.

Example 1

It was discovered that iron (Fe^{3+}) is an activator of telomerase in CRC cells. Treatment of cells with iron chelator, Deferasirox (DFX), reduced telomerase activity accompanied by a significant decrease in Fe^{3+} level (Fig. 3A). Furthermore, when cells were treated with recombinant transferrin (an iron transporter), telomerase activity and cell growth were enhanced in a pirin dependent manner (Fig. 3D, 3E). This result indicated that pirin is an intracellular iron sensor and it activates telomerase activity in cells. Additional *in vivo* patient-derived xenograft (PDX) models showed that pirin inhibition combined with iron chelation reduces tumour growth accompanied by telomerase inhibition (Fig. 3G, 3I). Immunohistochemistry (IHC) staining for pirin and Prussian blue (PB) staining for Fe^{3+} was performed on patient tissue sections using tissue microarray. We found a significant proportion of tumours with high level of pirin and Fe^{3+} compared to normal tissues (Fig. 4C, 4D). Grouping of these tumours by consensus molecular subtype (CMS) indicated CMS3 subtype with significantly higher

percentage of high pirin+iron (Fe^{3+}) patients (Fig. 5). Telomerase activity from freshly resected samples showed significant association of high pirin+iron (Fe^{3+}) with high telomerase activity (Fig. 6). It was also observed that there was an enrichment for CMS3 subtype in this patient cohort. These observations illustrated that high pirin+iron (Fe^{3+}) could be a specific marker to identify patient subgroup (CMS3) for iron therapy which is standard of care for CRC patients with anaemic condition. Importantly, Fe supplementation may be detrimental in this subgroup and identifying such patients may be relevant for clinical care.

Example 2

Iron levels correlate with hTERT activity in CRC

Epidemiological studies have linked dietary iron intake with the risk of colorectal cancers. Also, increased heme iron and consumption of red meat correlate with an elevated risk of developing CRC. However, any molecular mechanism which explains this link remains elusive. Since *hTERT* reactivation is a rate-limiting step in transformation, we examined a correlation between iron and hTERT mediated telomerase activity using 47 fresh surgical samples. The use of freshly resected samples was essential for this analysis since hTERT enzymatic activity can be measured only in fresh samples. In tumor and adjacent normal sections, telomerase activity was measured using telomere repeat amplification protocol (TRAP) assay. Simultaneously, free iron levels were measured using an iron assay kit staining of tissue sections (Fig. 1A) and using Prussian Blue (PB). When compared to adjacent normal tissues, 12 of the 47 samples displayed high telomerase activity specifically in tumor sections (Fig. 1B and Fig. 7A, 7B). Quantification of iron in these 47 samples showed 10 of them to have high levels of iron (Fig. 1C and Fig. 8A, 8B). Strikingly, 9 of the tumors with high iron also had high telomerase activity (Fig. 1B, 1C). These results were validated by PB staining for iron levels (Fig. 1D) in tissue sections prepared from these tumors. Therefore, out of the 12 tumors with high telomerase activity, 9 had high iron levels (Fig. 1C). While these results indicated a statistically significant correlation between high telomerase activity and high iron levels (Fig. 1E, Fisher exact test $P=0.0001$), they, however, do not provide a mechanistic link between iron and *hTERT* regulation.

Identification of SP2509 as a regulator of hTERT activity and deconvolution of its target: Pirin

Since *hTERT* is tightly regulated at the transcriptional level, to understand if iron could regulate *hTERT* reactivation and hence its activity, a small molecule screen was set up to identify compounds that regulate *hTERT* transcription and aimed to identify the molecular targets of the compounds which may link *hTERT* activity to cognate target proteins regulating iron metabolism or activity. Given that *hTERT* promoter is a weak promoter and hTERT antibodies do not detect the few molecules of endogenous hTERT, for this screen, a novel reporter cell line was engineered with the Nanoluc gene under endogenous *hTERT* promoter in CRC cells (Fig. 1F). Multiple clones were screened, and positive clones were further validated by PCR, Sanger sequencing, and by knockdown of known *hTERT* transcriptional regulators (e.g. *myc*) (Fig. 9A-9C). A marked reduction in endogenous *hTERT* expression and endogenous *hTERT* promoter-driven NanoLuc activity was found within 24 hours (Fig. 9C) highlighting the sensitivity and robustness of the high throughput screening assay (HTS) employed. This method has been successful in the identification of genetic drivers of mutant *hTERT* promoters. Using this established reporter system, HTS was performed against several annotated small molecule libraries. As *hTERT* is well characterized to be regulated by various epigenetic modifications, a library of epigenetic modulators was also employed (Fig. 1G). Top hits from these screens, were analyzed for their interaction with known iron regulating pathways. SP2509 a potent inhibitor of *hTERT* transcription was selected for further validation (Fig. 1F and Fig. 9D) since a combination of isothermal-dose response fingerprint (ITDRF) and mass spectrometry (MS) methods (Fig. 2A) showed that SP2509 specifically bound to a protein called pirin, which consistently showed stabilization even at low doses of SP2509 in the whole cell lysate (Fig. 2B and Fig. 10A). Pirin is a transcriptional co-factor that is known to be regulated by iron-binding. These results were further confirmed by immunoblotting (Fig. 2C, upper panel). Importantly, CCT251236, a known modulator of pirin, also stabilized its levels in a temperature-dependent manner (Fig. 2C, lower panel), and it also reduced *hTERT* expression (Fig. 2D, top panel) and telomerase activity (Fig. 2D, bottom panel) in both HCT116 cells and COLO205 cells. Genetic depletion of pirin showed a significant reduction in telomerase activity much like the treatment of its agonists SP2509 and CCT251236 (Fig. 2E). Pirin overexpression rescued the effect of telomerase inhibition

and cell viability by SP2509 (Fig. 2F, 10B). Finally, CCT251236 treatment also reduced colony formation and showed some specificity towards CRC cell lines (Fig. 10C, 10D). In the literature, SP2509 is reported as a compound that targets a demethylase called LSD1. Surprisingly, LSD1 was not seen as a hit in ITDRF with SP2509. Furthermore, neither genetic depletion of LSD1 nor the treatment with other LSD1 inhibitors in the library showed any effect on *hTERT* expression (Fig. 11A-11G). But SP2509 treatment reproducibly and significantly reduced *hTERT* mRNA or telomerase activity in a dose-dependent manner (Fig. 11H). Together these results unequivocally identify pirin as the *bona fide* target of SP2509. These results also implicate that SP2509 could be inhibiting *hTERT* transcription by inhibiting pirin activity and that the pirin could be a link between iron and *hTERT* reactivation. Confirming this it was that found competitive displacement of iron by SP2509 in purified recombinant irin after reconstitution with FeSO_4 (Fig. 12A-C). The released iron (free form) is significantly higher in the SP2509 treated sample compared to DMSO treated sample (Fig. 12C). Since iron binding to the pirin regulates its activity, the presence of SP2509 interferes with this process and inhibits the formation of active pirin in cells.

SP2509 specifically reduces CRC growth through *hTERT* reduction

Next, the specificity of SP2509 in regulating *hTERT* expression and function was explored using a broad range of CRC and non-CRC cells. SP2509 reduced *hTERT* expression in a dose-dependent manner in CRC lines with HCT116 and COLO205 being the most sensitive cell lines (Fig. 13A). Non-CRC cell lines did not show any impact of SP2509 on *hTERT* levels (Fig. 13B). Reactivation of *hTERT* in 19% of all human cancers occurs via mutations in its promoter, now recognized as the third most genetic alteration in human cancers. Surprisingly, the use of cells isogenic for *hTERT* promoter showed that SP2509 significantly inhibited wild type (WT), but not mutant (C250T) promoter (Fig. 13C). Consequent to inhibition of *hTERT* activity, even low doses of SP2509 could inhibit colony formation (Fig. 13D). FACS analysis of early (Annexin V) and late (7-AAD, 7-aminoactinomycin D) apoptotic markers indicated a dose-dependent increase in early apoptotic cells 48 hours after treatment, while the proportion of late apoptotic cells remained constant (Fig. 13E). In addition, increased levels of apoptotic markers such as cleaved PARP, Caspase-3, and phosphorylated p53 were observed when cells were treated with SP2509 for 48 hours (Fig. 13F). SP2509

treatment caused a decrease in the percentage of G0/G1 cells and an increase in the percentage of dead cells (Fig. 13G). Cell growth and *hTERT* expression are known to be interdependent. To dissect if SP2509 treatment causes loss of hTERT as a cause or consequence of cell growth loss, HCT116 and COLO205 cells were treated with SP2509 followed by cell death assay and *hTERT* expression analysis at different time points. Immediate inhibition of *hTERT* expression ($\geq 50\%$) at 24 hours and 9 hrs in HCT116 and COLO205 cells respectively was seen upon SP2509 treatment (Fig. 14A). However, cell viability was unaffected by SP2509 treatment at these time points in both cell lines (Fig. 14B). Also, treatment for 24 to 36 hours did not trigger the classical hallmarks of apoptosis; namely cleavage of PARP and Caspase-3 (Fig. 14C). Besides, no significant activation of caspase-3/7 activity was observed during short-term SP2509 treatment (Fig. 14D, 14E). Finally, two common histone modifications at the *hTERT* promoter, H3 Lysine 4 trimethylation (H3K4me3) and H3 Lysine 9 acetylation (H3K9ac), along with the binding of RNA polymerase II (RNA Pol. II) decreased upon SP2509 treatment in HCT116 (24 hrs) and COLO205 (9 hrs) cells (Fig. 14F, 14G). The *in vivo* effect of SP2509 was analyzed using xenografts. Mice treated with SP2509 (30mg/kg, IP, twice a week) or DMSO for 2 weeks showed that SP2509 effectively reduced tumor growth at 2 weeks, concomitantly reducing *hTERT* expression as early as 7 days after treatment, without any gross changes in body weight (Fig. 14H-14J).

Next, the effect of SP2509 was assessed in human primary patient-derived CRC with distinct consensus molecular subtypes (CMS) with varying degrees of *hTERT* expression (Fig. 2G, 14K). Among 11 lines tested, 7 of them showed a significant reduction of *hTERT* upon SP2509 treatment (Fig. 2H). Surprisingly, most of the cells ($n=6$) in which SP2509 reduced *hTERT* expression were of CMS3 subtype (Fig. 2G). CRC 1489 (derived from a CMS3 tumor; MSS with a BRAF V600E mutation) with an intermediate response to SP2509 was chosen for *in vivo* studies. A significant reduction of tumor growth at 2 weeks by SP2509 treatment (Fig. 2I) was observed in this PDX without any gross body weight changes (Fig. 2J, 14L). Also, on day 7, *hTERT* showed a significant reduction in expression in SP2509 treated PDXs (Fig. 2K). One main reason for the lack of hTERT inhibitors in the clinic owes to the vulnerability of stem cells to hTERT-targeted enzymatic inhibitors since hTERT-dependent telomerase activity is essential for their self-renewal. Surprisingly, treatment of even the highest dose of SP2509 in H9 human embryonic stem cells (hESC) had no significant effect on

the expression of *hTERT* as well as telomerase activity (Fig. 14M, 14N). On the contrary, a known inhibitor of telomerase reverse transcriptase activity, MST-312, significantly impacted telomerase activity in these cells (Fig. 14N). Overall, these results suggest that SP2509 displays a high degree of selectivity in its effect in CRC cells and it mainly works by reducing telomerase activity due to the reduction of *hTERT* transcription possibly via Pirin.

Pirin, the target of SP2509 directly regulates hTERT activity in an iron-dependent manner

Pirin is a non-heme iron-binding protein and a redox sensor of iron in cells. Since increased iron intake and storage have a direct correlation with the risk of CRC, the role of SP2509 in regulating cellular signaling in response to iron levels was investigated. Cells treated with SP2509 showed a significant reduction in the total and Fe^{3+} (ferric-oxidized) form of iron (Fig. 3A). Strikingly, treatment of cells with a known iron chelator namely deferoxamine (DFX) also revealed a significant reduction of iron levels (Fig. 15A) and significantly reduced *hTERT* expression and telomerase activity, much like treatment with SP2509 (Fig. 3B, 3C). Strengthening this observation, iron supplementation assays in cultured cells showed that when supplemented, recombinant transferrin (rTF), a high-affinity iron binder and transporter protein, caused a significant increase in telomerase activity and a corresponding increase in cell growth in wild-type cells (HCT116^{CTRL}), and this effect was abolished in the stable pirin knocked down cells (HCT116^{PIR-KD}) (Fig. 3D, 3E), indicating that pirin is a sensor of iron levels and its role in telomerase activation depends on its binding to iron. On the other hand, cells treated with SP2509 and DFX showed significantly reduced expression of iron metabolism genes, supporting a mechanism of how these molecules decrease the iron load in cells (Fig. 15B). These results indicate that iron levels play a crucial role in controlling *hTERT* expression and telomerase activity in these cells. Xenografts of HCT116 cells (HCT116^{CTRL} and HCT116^{PIR-KD}) and patient-derived xenografts (PDXs) with either high (0186^{Pirin High}) or low Pirin (0266^{Pirin Low}) (Fig. 3F) showed a significant effect of iron chelation (using DFX) on tumor growth in high Pirin PDXs where tumor growth was strongly inhibited (Fig. 3G, 3H) along with a consistent decrease in telomerase activity and *hTERT* levels (Fig. 3I). Additionally, iron levels were assessed through Prussian blue iron staining in both PDXs (high and low) (Fig.

3J). Quantitative iron assays indicated higher levels of iron in pirin high PDX compared to pirin low PDXs (Fig. 3K). DFX treatment in both high and low pirin PDXs led to significant loss of iron levels indicating that the effect of tumor growth inhibition specifically in high pirin PDX is not due to selective loss of iron in high pirin PDXs (Fig. 3K). Similar results were obtained from HCT116 xenografts studies (Fig. 15C-15E). Pirin protein levels were then assessed by western blot in previously described resected tumor and adjacent normal samples (47 pairs) where telomerase activity was found to correlate with iron levels. Interestingly, a significant number of telomerase high tumors showed elevated levels of pirin (Fig. 4A). These results strengthened the notion that the observed association of iron and pirin with telomerase activity in tumors (Fig. 1B) has functional relevance, and prompted the examination of these relationships in a larger cohort of clinical samples. Towards this, a tissue microarray (TMA) consisting of resected samples of tumor and adjacent normal from 2 cohorts of CRC patients was created. In total, 245 patient tumor tissue sections along with their corresponding adjacent normal tissues were used to construct the TMAs. These TMAs were probed for Iron and pirin levels by Prussian blue (PB) stain and immunohistochemistry (IHC), respectively (Fig. 4B). Among these tissue sections, 80 patients had tumor-specific staining for iron while 16 adjacent normal tissues from patients displayed staining for iron. Representative images of 2 tumor samples positively stained for iron are shown (Fig. 4C). When compared to the normal tissues, elevated pirin staining was found in all tumors sections which stained positive for iron. Representative images of 2 tumor samples stained for Pirin IHC are shown (Fig. 4D). Compared to 6.5% of normal tissues, nearly 32.7% of tumors display co-staining of iron and pirin (Fig. 4E). Interestingly, CMS3 tumors displayed the most significant ($p < 0.01$) co-enrichment of iron & pirin (Fig. 4F, 4G). This finding also supports our previous observation that SP2509, which works through Pirin specificity shows effects in CMS3 subtype (Fig. 2G). Patients-derived organoids (PDO) representing CMS1, CMS2, CMS3, and CMS4 subtypes reconfirmed that SP2509 significantly inhibits *hTERT* in CMS3 and to a lesser degree in CMS1 PDOs (Fig. 4H, 15F). Overall, these results illustrate that iron and pirin together contribute to *hTERT* activation and SP2509 potentially disrupts this by reducing the level of (active) iron-bound and hence active pirin in cells. Clinical data showing enrichment of iron and Pirin in CMS3 subtype indicates that these tumors might be responsive to SP2509 treatment and suggests that the development of such a small

molecule could be a therapeutic way of targeting hTERT activity specifically CRCs, without toxicity to stem cells.

Molecular mechanism of Pirin mediated regulation of *hTERT* in CRC.

To unravel the mechanistic basis of Pirin-mediated transcriptional regulation of *hTERT*, RNA-seq was performed from control (HCT116^{CTRL}) cells upon SP2509 treatment (HCT116^{SP2509}) or Pirin knock down (HCT116^{PIR-KD}). A comparison of the differentially expressed gene (DEG) between HCT116^{CTRL} and HCT116^{SP2509} or HCT116^{PIR-KD} cells indicated 1104 common genes between SP2509-treated and HCT116^{PIR-KD} conditions (Fig. 5A). 485 genes were upregulated while 1028 genes were down-regulated in DEG (Fig. 16A). Gene ontology (GO) analysis of the upregulated genes showed an overwhelming number of GO terms related to the activated proteasome degradation pathway (Fig. 5B). Transcription factor (TF) motif analysis identified Sp1 motif enrichment among the downregulated genes in HCT116^{PIR-KD} cells (Fig. 5C). Surprisingly, 38% of the downregulated genes from HCT116^{PIR-KD} cells shared Sp1 binding motif(s) (Fig. 16B). Shared activation of proteasomal components in both HCT116^{SP2509} and HCT116^{PIR-KD} conditions and downregulation of Sp1 target genes in HCT116^{PIR-KD} led to the investigation of Sp1 protein level by western in HCT116^{CTRL} cells upon SP2509 or Pirin KD. Interestingly, in both conditions, Sp1 levels were significantly reduced (Fig. 5D). Contrarily, ectopic expression of Pirin led to increased Sp1 levels (Fig. 5E, 16C), suggesting that pirin is a regulator of Sp1. In line with these results, ChIP-qPCR on *hTERT* promoter revealed significantly reduced Sp1 occupancy in HCT116^{PIR-KD} or HCT116^{SP2509} cells (Fig. 5F). Reduction of Sp1 levels by SP2509 or pirin KD is seen only at the protein level since its mRNA remained unaltered in these conditions (Fig. 16D) suggesting a post-translational regulation by activated proteasome components as seen in the GO terms. To elucidate the mechanistic basis of these observations, the ubiquitination of Sp1 was analyzed using antibodies specific for Lys48 or Lys63 chains of ubiquitin (Ub). A significant reduction of Lys48 linked Ub chains was apparent in cells with ectopic expression of Pirin (Fig. 5G). Ubiquitination of Sp1 by RNF4- E3 ubiquitin ligase has been reported previously. However, our RNA-seq data revealed no significant changes in RNF4 levels (Fig. 16E). Surprisingly, FBXW7, an E3 ligase was significantly upregulated in both HCT116^{PIR-KD} and HCT116^{SP2509} cells both in RNA-seq (Fig. 5H) and validated by qPCR (Fig. 16F). FBXW7 also was

predicted to be among the top hits when E3 ligases targeting Sp1 protein were predicted *in silico* using an E3 ligase target prediction (Fig. 16G). Indeed, immunoprecipitation experiments showed biochemical interaction of FBXW7 with Sp1 (Fig. 6A, lane 5, 6 and Fig. 17A). Ectopic expression of FBXW7 expression decreased Sp1 levels (Fig. 6B, lane 2 vs lane 3), while ectopic expression of Pirin rescued the effect of FBXW7 mediated destabilization of SP1 (Fig. 6B, lane 3 vs lane 4). Since pirin regulates FBXW7 transcriptionally, its influence on the transcription factor binding on the FBXW7 promoter was investigated. Gel shift assay (EMSA) assays showed reduced binding of NFAT-1 in FBW7 promoter upon pirin overexpression (Fig. 6C, compare lanes 17 & 18), suggesting that Pirin competes with NFAT-1 as a mechanism to repress FBXW7. Indeed, knocking down NFAT-1 reduced the expression of FBXW7 (Fig. 17B). In contrast, SP2509 treatment or pirin knockdown causes increased NFAT-1 occupancy on the FBXW7 promoter (Fig. 6D). Corroborating our findings, SP2509 treatment reduced Sp1 occupancy on the consensus sequence while Pirin overexpression led to increased Sp1 binding on its consensus sequences (Fig 17C). It is well-established that there are multiple Sp1 binding sites in *hTERT* promoter and the binding of SP1 to these sites together regulates *hTERT* transcription. A parallel observation that both SP2509 and DFX treatment increased FBXW7 expression further strengthens our findings (Fig 17D). Finally, to elucidate if FBXW7 controls telomerase activity, a genomic knockout of FBXW7 was created in HCT116 cells and DLD-1 cells and it was found that there was a significant increase in the telomerase activity (Fig. 6E). Taken together these results suggest that modulation of Sp1 levels is the primary mechanism via which pirin regulates *hTERT* transcription. It is also concluded that this regulation occurs when pirin binds to iron and that SP2509 represents a small molecule that can functionally modulate pirin and its target *hTERT* through stabilizing Sp1 protein level (Fig. 6F).

CLAIMS

1. A method of identifying a subject who is likely to be responsive to treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator.
2. The method of claim 1, wherein the method comprises detecting pirin protein or mRNA.
3. The method of claim 1 or 2, wherein the method comprises detecting Fe^{2+} and/or Fe^{3+} .
4. The method of any one of claims 1 to 3, wherein the iron chelator is deferasirox, deferoxamine, or deferiprone.
5. The method of any one of claims 1 to 4, wherein the subject is suffering from cancer or hemochromatosis.
6. The method of claim 5, wherein the cancer is one that is characterized as having high telomerase activity.
7. The method of claim 6, wherein the method comprises detecting telomerase activity in the sample.
8. The method of any one of claims 5 to 7, wherein the cancer is colorectal cancer.
9. The method of claim 8, wherein the colorectal cancer is of a consensus molecular subgroup 3 (CMS3) subtype.
10. The method of any one of claims 1 to 9, wherein the sample is a cancer sample.
11. The method of any one of claims 1 to 10, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation.

12. The method of any one of claims 1 to 11, wherein the method comprises administering an effective amount of an iron chelator to the subject found likely to be responsive to treatment with the iron chelator.
13. The method of any one of claims 1 to 12, wherein the method further comprises administering an effective amount of a pirin inhibitor to the subject found likely to be responsive to treatment with the iron chelator.
14. A method of predicting the prognosis of a subject following treatment with an iron chelator, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference provides an indication of the prognosis of the subject following treatment with the iron chelator.
15. A method of treating a subject, the method comprising a) detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject found likely to be responsive to treatment with an iron chelator.
16. A method of treating a subject, the method comprising a) selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with an iron chelator; and b) administering an effective amount of an iron chelator to the subject when the subject is found likely to be responsive to treatment with the iron chelator.
17. A method of selecting a subject for treatment with iron supplementation, the method comprising selecting a subject based on an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be unsuitable for treatment with the iron supplementation.

18. The method of claim 17, wherein the subject is suffering from cancer.
19. The method of claim 17 or 18, wherein the subject is suffering from anaemia.
20. A method of identifying a subject who is unsuitable for treatment with iron supplementation, the method comprising detecting an increased level of pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is unsuitable for treatment with iron supplementation.
21. The method of claim 20, wherein the method further comprises treating the subject found unsuitable for treatment with iron supplementation with an iron chelator.
22. A method of treating cancer in a subject, the method comprising administering an effective amount of a pirin inhibitor to the subject.
23. The method of claim 22, wherein the pirin inhibitor is SP2509 or a derivative thereof.
24. The method of claim 22 or 23, wherein the pirin inhibitor is administered with an iron chelator.
25. The method of claim 24, wherein the iron chelator is deferasirox, deferoxamine, or deferiprone.
26. A method of identifying a subject who is likely to be responsive to treatment with a pirin inhibitor, the method comprising detecting pirin and/or iron in a sample obtained from the subject, wherein an increased level of pirin and/or iron as compared to a reference indicates that the subject is likely to be responsive to treatment with the pirin inhibitor.
27. A method of sensitizing a subject to treatment with a pirin inhibitor, the method comprising administering an iron chelator to the subject.

28. A method of identifying a cancer cell that has high telomerase activity, the method comprising detecting pirin in the cancer cell, wherein an increased level of pirin as compared to a reference indicates that the cancer cell is likely to have high telomerase activity.