Glucocorticoid receptor triggers a reversible drug-tolerant dormancy state with acquired therapeutic vulnerabilities in lung cancer

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The glucocorticoid receptor (GR) regulates gene expression, governing aspects of homeostasis, but is also involved in cancer. Pharmacological GR activation is frequently used to alleviate therapy-related side-effects. While prior studies have shown GR activation might also have anti-proliferative action on tumours, the underpinnings of glucocorticoid action and its direct effectors in non-lymphoid solid cancers remain elusive. Here, we study the mechanisms of glucocorticoid response, focusing on lung cancer. We show that GR activation induces reversible cancer cell dormancy characterised by anticancer drug tolerance, and activation of growth factor survival signalling accompanied by vulnerability to inhibitors. GR-induced dormancy is dependent on a single GR-target gene, CDKN1C, regulated through chromatin looping of a GR-occupied upstream distal enhancer in a SWI/SNF-dependent fashion. These insights illustrate the importance of GR signalling in non-lymphoid solid cancer biology, particularly in lung cancer, and warrant caution for use of glucocorticoids in treatment of anticancer therapy related side-effects.
The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily and a ligand-activated transcription factor. This multidomain protein exerts its function through chromatin binding and communication with the transcription machinery, ultimately modulating the expression of a large number of genes, across diverse cell types. As a homeostatic regulator, GR has an imperative role in neuroendocrine integration, circadian rhythm, immune system control and glucose metabolism. The action of this transcription factor extends beyond general physiology as its impact can be seen in various disease types, including cancer.

While pharmacological agonists of the GR (e.g., prednisone and dexamethasone) have been intensively used as therapeutics in the treatment of lymphoid cancers, for non-lymphoid solid (i.e., non-haematologic) cancer patients they are utilised solely as an adjuvant treatment to alleviate symptoms caused by anticancer therapy. However, studies on in vitro and in vivo models of numerous non-lymphoid solid cancer types (e.g., prostate, lung and breast cancer) have shown that glucocorticoids (GCs) decrease cancer incidence and reduce the growth of cancer.

In addition, in aged mouse haploinsufficiency models, GR loss predisposes tumour development across multiple organ systems. Despite these observations, a precise mode-of-action through which GCs affect non-lymphoid solid cancers remains unclear.

Herein, we elucidate the molecular mechanisms by which GR activation blocks cell proliferation in non-lymphoid solid cancers with the primary focus on lung cancer. We demonstrate that GR activation induces cancer cell dormancy, accompanied by a diminished response to a large array of anticancer drugs, activation of growth factor survival signalling (IGF-1R) and acquisition of vulnerability to IGF-1R inhibitors in cell lines and xenograft models. Furthermore, we reveal that this phenotype is dependent on GR-mediated regulation of CDKN1C (which encodes for p57) in a SWI/SNF-dependent fashion through long-range genomic regulation of an upstream distal enhancer. Ultimately, using transcriptomics and chromatin accessibility data of clinical samples, we show that this mode of regulation occurs in multiple human non-lymphoid solid cancer types.

**Results**

**Stress hormone receptor activation leads to cell dormancy.** In order to study the phenotypic and genotypic consequences of GR activation, five non-small cell lung cancer models (Supplementary Fig. 1a) were selected based on their steroid hormone receptor expression profiles. Expression of GR was confirmed by western blot analysis (Fig. 1a), demonstrating comparable expression levels across five cell lines. The GC treatment (specific treatment information per experiment can be found in Supplementary Table 1) of A549, H2122 and H1944 led to a significant reduction in proliferation rate as observed in live-cell tracking experiments using SIR-DNA (Fig. 1b). Conversely, growth rates of H1975 and H460 cell lines were unaffected by GC therapy (Fig. 1b). The propidium iodide-staining and subsequent flow cytometry analysis revealed that the drop in proliferation rate was underlined by a reduction in the S phase and an increase in the G0/G1 phase of the cell cycle (Supplementary Fig. 1b). Treatment with GCs did not induce apoptosis, as demonstrated by the absence of cleaved PARP detected by means of western blot analysis (Supplementary Fig. 1c).

In agreement with the observed growth arrest upon GC treatment, a high degree of protein dephosphorylation was observed (Fig. 1c), most of which were involved in direct regulation of transcription and cell cycle as evidenced by gene-set analysis (Fig. 1d). This was accompanied by a strong, significant downregulation of E2F targets (Hallmark gene sets; M5925) on the whole-proteome level (Fig. 1e). Conversely, the phospho-proteomes of the H1975 and H460, cell lines that are not growth-arrested by GCs, were not significantly altered by GC treatment (Supplementary Fig. 1d).

As lack of cleaved PARP suggested that growth arrest does not involve apoptosis (Supplementary Fig. 1c), we inspected whether GC treatment led to the acquisition of senescence. Firstly, we observed a significant (FDR q value: A549 = 0.007; H2122 = 0.019; H1944 = 0.05) enrichment score for senescence gene-set (Fridman Senescence Signature, M9143) on whole-proteome level (Fig. 1f) in GC-treated cells compared to the vehicle-treated conditions. In agreement with this, we detected positive staining for senescence-associated β-galactosidase upon GC stimulation in A549, H2122 and H1944, but not H1975 and H460 (Fig. 1g, h). However, cell cycle exit was neither accompanied by changes in p53 protein expression levels (Supplementary Fig. 1c) nor activation of the p53 pathway as shown by gene-set enrichment analysis of RNA sequencing and full proteome datasets (Supplementary Fig. 1e). Upon ligand withdrawal, the growth inhibition was lost and cells restarted proliferating (Supplementary Fig. 1f). In addition, a decrease was observed in the overall metabolic activity/capacity, as evidenced by a significant decrease in oxygen consumption rate (reflecting mitochondrial respiration; Fig. 1i and S1g), and extracellular acidification rate (reflecting glycolytic output; Fig. 1j).

Furthermore, we investigated the gene signatures of cell cycle and senescence in human lung adenocarcinoma tumours stratified on the basis of GR activity (calculated as Z score of 253 genes associated with GR activation; 25% split). In support of our experimental findings, The Cancer Genome Atlas (TCGA)-based analysis revealed that human lung tumours with high GR activity have higher expression level of senescence-associated genes, and lower expression of cell cycle-related genes in comparison to tumours with low GR activity (Supplementary Fig. 1h, i). Importantly, Kaplan–Meier survival analysis was performed on data from 1529 lung cancer patients, of whom the majority did not receive (neo)adjuvant therapy before and after surgery. The patients were divided into three groups based on transcriptomics-derived GR activity and the Kaplan–Meier analysis demonstrated that patients with high GR activity have a more favourable outcome based on overall survival (Supplementary Fig. 1j, l) and recurrence-free survival (Supplementary Fig. 1k, m) probabilities than patients with intermediate or low levels of GR activity.

Taken together, we conclude that GCs induce a transition to a dormant, reversible cellular state. Importantly, the induction of growth arrest by stress hormone receptor activation extends to other non-lymphoid solid cancer types, as this was also observed in primary patient-derived and pre-established models of mesothelioma; a cancer type derived from cells of mesodermal lineage (Supplementary Fig. 2).

**Glucocorticoid-induced cell dormancy is characterised by anticancer drug tolerance and activation of IGF-1R survival signalling.** To further characterise the GC-induced cell dormancy and the underlying molecular pathways that support survival, we performed a drug screen (2277 compounds from diverse sub-libraries) in the H1944 cell line. The cells were cultured in the presence or absence of GCs for 2 days, then divided over different arms of the screen (Supplementary Fig. 3a)—(1) vehicle arm, (2) GC pre-treated arm in which GC treatment was added before and continued throughout the screen and (3) a GC-co-treatment arm in which GCs were added at the same time as the library compounds. For all arms, the library compounds were used at two concentrations (Supplementary Fig. 3a–c). The analysis demonstrated that patients with high GR activity have a more favourable outcome based on overall survival (Supplementary Fig. 1j, l) and recurrence-free survival (Supplementary Fig. 1k, m) probabilities than patients with intermediate or low levels of GR activity.

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different concentrations—1 μM and 5 μM. After 6 days of exposure to the library drugs, cell viability was assessed using a CellTiter-Blue assay (Supplementary Fig. 3a), and GC arms were compared to the vehicle arm.

Firstly, GCs decreased the sensitivity to numerous drugs in both GC pre-treatment and co-treatment arms (Fig. 2a and Supplementary Data 1). Using a selected array of drugs based on the first screen, we have found that GCs significantly reduced the effectiveness of these drugs in another lung cancer model, the H2122 cell line (Fig. 2b). By means of Compound Set Enrichment Analysis within the CSgator, a comprehensive analytic tool for setwise interpretation of compounds\textsuperscript{23}, we reveal that compounds with reduced effectiveness after/during GC treatment were predominantly threonine protease, kinase, guanylate cyclase and structural protein inhibitors (Fig. 2c). Importantly, we show that among these are various drugs clinically approved for the treatment of lung cancer, including vinorelbine tartrate, dabrafenib, trametinib and docetaxel (Supplementary Fig. 3b). Secondly, the GR activation also increased sensitivity to nine inhibitors. More specifically, all identified compounds with a significant degree of drug response enhancement (Padj < 0.05 and difference $<-0.3$) on GR-treatment were classified as IGF pathway inhibitors (Fig. 2a and Supplementary Fig. 3c). We have successfully validated these findings across the three cell lines (A549, H2122 and H1944) using a logarithmic range of concentrations and drug stock obtained from a different supplier
Fig. 2 Cell dormancy phenotype is accompanied by drug tolerance and viability maintenance via growth factor signalling activation. a Scatter plot showing viability differences of vehicle and glucocorticoid pre-treated or co-treated screen arms with either 1 μM and 5 μM of the screening drugs. Compounds having significant differential viability are depicted in green (Padj < 0.05). Adjusted P values (Padj) were determined by two-sided t test with multiple testing correction (Benjamini–Hochberg method). b Normalised viability of H2122 cell lines in response to compounds found to diminish viability (<0.7) of vehicle arm of H1944 cell lines in the first screen at 5 μM concentration. P values were determined by two-sided Welch’s t test. c Compound Set Enrichment Analysis data computed using CSgator software of drugs with reduced efficacy upon GC treatment. Random controls of the same size were generated to compute background enrichment (n = 20). P values (FDR-adjusted) were determined by CSgator software. d PhosphoPath pathway analysis of phosphoproteomics data depicting signalling changes in glucocorticoid-treated cells (AS49, H2122 and H1944) (n = 3). P values were determined by PhosphoPath software. e Representative images showing immunohistochemical stainings of xenograft cancer samples obtained after treatment of NOD-SCID-γ mice with either vehicle (Veh) or glucocorticoids (GC) (n = 4). Primary antibody was omitted as staining control. Scale bar, 100 μm. f Normalised tumour growth in xenograft models of H1944 cells in NOD-SCID-γ mice treated with either vehicle (Veh = blue), linsitinib (Lin = green), glucocorticoids (GC = red), or combination (GC + Lin = purple). Arrows indicate when treatment was started. Mean values ± SEM depicted (Dexa + Lin n = 4, Dexa n = 6, Veh n = 5, Lin n = 6 animals). P values were determined by mixed-model ANOVA (Tukey’s multiple comparison test).

(Supplementary Fig. 3d). The modest response to GC/IGF-1R inhibitor combination in H2122 may be explained by the pre-existing dependency of this cell line on IGF-1R signalling in absence of GCs, as suggested by the data available on The Cancer Dependency Map portal24 (Supplementary Fig. 3e). In conjunction with induced vulnerability to IGF-1R inhibitors, phospho-pathway analysis of the three cell lines revealed significant changes in the protein phosphorylation status of insulin signalling (including IGF-1R protein) and the related downstream pathways25,26, confirming the implied increase in their activity upon GR activation (Fig. 2d).

Treatment of xenograft animals with GCs elicited a pronounced effect on the transcriptome of the engrafted H1944 tumours (Supplementary Fig. 3f). Activation of GR increased gene expression of its target genes, including GILZ1, FKBP5 and PERI (Supplementary Fig. 3g). Interestingly, we observed that metastasis-associated genes MYCN, ID4 and VCAM1 were significantly downregulated by GC treatment (Supplementary Fig. 3g). Furthermore, we confirmed our previous findings from in vitro experiments, showing that GR activation leads to a significant enrichment of the GR activity signature and Fridman Senescence signature, and significant downregulation of genes involved in E2F signalling (Supplementary Fig. 3h). In agreement with this, the treatment of animals bearing H1944 xenograft tumours with GCs led to a decrease in Ki67 immunostaining and retinoblastoma (Rb) phosphorylation (Fig. 2e; no changes in total Rb levels (Supplementary Fig. 3i)), without induction of p21 expression and apoptosis (Supplementary Fig. 3i), as seen by the absence of cleaved Caspase-3 signal. Importantly, activation of GR promoted phosphorylation of IGF-1R in xenograft tumours (Fig. 2e). In line with this, GC-induced IGF-1R inhibitor vulnerability was tested in vivo, in NOD-SCID-γ xenograft models of the H1944 cell line. Treatment with GCs led to stable tumour growth arrest in comparison to the vehicle-treated mice.
The addition of IGFR-1 inhibitor linisitinib to treatment schedule of these mice led to a sharp and sustained decrease in tumour size (Fig. 2f). We successfully validated these findings in an A549 cells xenograft model in NOD-SCID-γ mice (Supplementary Fig. 4a, b). In animals bearing A549 xenografts, we demonstrated that the combination of two different IGFR-1 inhibitors (linisitinib and GSK1838705A) with GCs had a significant effect on tumour size in comparison to the GC- monotherapy arm (Supplementary Fig. 4a, b).

To investigate if GR directly contributes to the modulation of IGF-1R signalling, we explored the GC-treatment time-course RNA sequencing dataset in A549 cell line27. In the insulin signalling gene set (M18155), exclusively FOXO1, IRS2 and PYGB (glycogen phosphorylase; not part of canonical IGF-1R signalling28) were stably upregulated by GCs (Supplementary Fig. 4c). We focused on inspecting the GR regulation of two genes directly involved in the IGF-1R pathway—FOXO129 and IRS230. For this, we made use of GR CHIP sequencing and Hi-C time-course data from A549 cells27. GR chromatin binding to several enhancers (Supplementary Fig. 4d, e) containing the FOXO1 and IRS2 gene loci was observed (Supplementary Fig. 4f, g, left). In addition to binding of GR to these enhancers, induction of a single enhancer–promoter loop containing two GR-binding sites in the loop anchor for the FOXO1 gene was observed (Supplementary Fig. 4f, right). As for IRS2, a complex web of six enhancer–promoter loops was detected, containing nine GR-binding sites (Supplementary Fig. 4g, right).

Collectively, these data show that activation of GR with GCs induces broad tolerance to anticancer drugs and that viability of GC-induced dormant cells is maintained via engagement of the IGFR-1 signalling pathway.

**Cell cycle inhibitor p57 is necessary for glucocorticoid-induced cell dormancy.** In order to identify the driver of GC-induced cell dormancy, we performed RNA sequencing in the A549, H2122 and H1944 cell lines treated with vehicle or GCs for 8 h. Comparison of GC-induced transcriptional modulation across the cell lines revealed a high degree of similarity (Fig. 3a). A focused analysis on genes differentially expressed upon GR activation (−2 ≤ log2 fold ≥ 2 and Padj ≤ 0.01) revealed 65 genes shared between the three cell lines (Fig. 3b); with only one being a cell cycle regulator; CDKN1C (which encodes for p57). In addition, this gene was found upregulated in the H2795 mesothelioma cell line which was growth-arrested by GCs, but not in two GC-resistant mesothelioma models (Supplementary Fig. 5a).

Expression of p57 was analysed by immunofluorescence and western blot. GC-dependent induction of p57 and its nuclear localisation were found exclusively in the dormant condition (A549, H2122 and H1944), while not detected in the GC-unresponsive H1975 and H460 models (Fig. 3c and S5b). Furthermore, in line with its well-described cell cycle inhibitory function31 rapid immunoprecipitation of endogenous proteins (RIME)32 in H2122 cells demonstrated that p57 interacts with various CDKs (CDK 1, 2, 4 and 6) as well as other cell cycle-related proteins such as CCNB1, not previously reported as p57 interacting protein (Fig. 3d and Supplementary Data 2). Importantly, upregulation of CDKN1C mRNA (Fig. 3e) preceded the transcriptional downregulation of various cell cycle genes (including CCND3 and CCNE2) which was observed after 4 h of GC treatment (Fig. 3f). Cumulatively, these data suggest that p57 may be involved in initiation of dormancy upon GR activation.

To address whether p57 is required for GR-induced cell cycle exit, we performed CRISPR-Cas9-mediated disruption of the CDKN1C gene in the A549, H2122 and H1944 cell lines. While GR nuclear translocation following GC treatment was not affected, induction of p57 expression in a polyclonal CDKN1C knockout (p57-KO) population was greatly diminished (Fig. 3g and Supplementary Fig. 5c). To inspect whether GCs are still able to induce cell dormancy in p57-KO cells, live-cell imaging of SiR-DNA-stained cells with and without GCs was performed, and the number of cells undergoing mitosis in the first 60 h of treatment quantified. In agreement with our hypothesis, the genetic disruption of the CDKN1C gene was sufficient to diminish cell dormancy induction by GCs (Fig. 3h).

In the absence of GCs, no statistically significant differences in mRNA expression were detected between the p57-WT and p57-KO H2122 cells (Supplementary Fig. 5e, f). While CRISPR-Cas9-mediated disruption of p57 did not alter transcriptional modulation of active GR-associated genes (Fig. 3i) and Supplementary Fig. 5g), it diminished downregulation of genes involved in cell cycle (Supplementary Fig. 5g–i). In conjunction with this, the changes in gene-set enrichment analysis of E2F targets (Fig. 3j) and cell cycle-related genes (Fig. 3k) typically induced by GCs were not observed in the p57-KO model, confirming the hypothesis that p57 upregulation is necessary for the growth-arrest phenotype.

Taken together, our data show that direct GR-mediated upregulation of a single gene (CDKN1C) is required to initiate growth arrest in human lung cancer cell line models.

**Glucocorticoid receptor regulates CDKN1C expression through a previously uncharacterised distal enhancer.** The regulation of CDKN1C by enhancers has been under debate and the precise enhancers controlling its expression in human cells remain unknown33,34. To address if CDKN1C upregulation is directly dependent on GC-mediated activation of GR instead of an off-target effect of the ligand (e.g., activation of mineralocorticoid receptor), we generated GR knockout (GR-KO) H2122 cell lines. In H2122 GR-WT cells, nuclear localisation of GR and a concomitant expression of p57 was observed upon GC treatment, while in the polyclonal GR-KO cell population no signal for GR nor p57 was detected (Supplementary Fig. 6a).

Therefore, we sought to establish direct regulation of the CDKN1C gene by GR and to elaborate on the mechanism using ChIP sequencing. We observed chromatin binding of GR at three different sites (Enhancers 1, 2 and 3) located within the topologically associating domain (TAD) region containing a large part of KCNQ1 and the entire CDKN1C gene, flanked by CTCF sites as determined by Hi-C and ChIP-sequencing analysis (Fig. 4a). Interestingly, GR chromatin binding, as established by ChIP sequencing, was not detected at the CDKN1C promoter, contrasting a prior electrophoretic mobility shift assay (EMSA)-based study35. This discrepancy may potentially be explained by the absence of chromatin context in EMSA experiments. The active enhancer-associated factors36, histone acetyltransferase p300 and H3K27Ac chromatin mark, were most pronounced at Enhancer 1 (Fig. 4b). In addition, cohesin (SMC3/Rad21) recruitment, known to be crucial for enhancer–promoter contacts, was observed at Enhancer 1 and the CDKN1C promoter (Fig. 4b). The intra-TAD localisation, GR binding, p300
recruitment, strong H3K27Ac signal and cohesin localisation, all suggest that Enhancer 1 (hereinafter referred to as CERES (CDKN1C Enhancer Regulated by Steroids)) is the main regulatory element through which GR regulates CDKN1C gene expression, while Enhancers 2 and 3 could potentially serve as auxiliary enhancers.

To establish whether these particular enhancers and the CDKN1C locus are in proximity to one another in 3D genome space, we performed 4C-seq experiments\(^{37}\). The unbiased interaction analyses from the viewpoint of the CDKN1C promoter revealed that GC treatment enhanced the interaction with two distal regions within the KCNO1 gene (a and b) (Fig. 4c). Regions a and b coincided with locations of CERES and enhancers 2/3, respectively. To unequivocally show that signal originating from the region a is driven by close proximity of CDKN1C promoter and CERES, we performed the reciprocal 4C-seq experiment from the CERES viewpoint. In A549, H2122 and H1944, we observed a statistically significant enhancement of the contact between this enhancer and CDKN1C promoter by GCs (Fig. 4d). Conversely, this enhancement was absent in two GC-unresponsive models of lung cancer, H1975 and H460 cell lines (Supplementary Fig. 6b).

To investigate the contribution of individual enhancers to the GR-induced CDKN1C gene upregulation, we performed CRISPR-Cas9 experiments to excise the individual enhancer elements
from the genome. Using pairs of guide RNAs, we excised either the CERES, E2 or E3 enhancer. In addition, we excised the Cdkn1c gene and Abcb1 promoter as positive and negative controls, respectively. Upon excision of the Cdkn1c gene and the CERES enhancer in a polyclonal cell population, we observed a significant decrease in Cdkn1c upregulation, (Fig. 4e) and rescue from growth arrest (Fig. 4f) in comparison to the negative control condition. This was not observed in the E2 and E3 deletion experiments, where induction of Cdkn1c (Fig. 4e) and the degree of growth arrest (Fig. 4f) were comparable to the ones of the control cell lines. These experiments show that the CERES enhancer is required for a robust upregulation of Cdkn1c by GR and therefore transition to a dormant state.

Collectively, we have discovered a GR-driven enhancer that regulates Cdkn1c gene through long-distance chromatin interactions, thereby controlling cell dormancy entry.

SWI/SNF complex is an integral part of a proficient GR transcriptional machinery controlling the expression of Cdkn1c. To gain more insight into the mechanism of GR regulation of Cdkn1c, we compared cell lines in which GR was able to induce dormancy (A549, H2122 and H1944) to the ones in which it cannot (H1975 and H460). Across all the cell lines used, GR was able to readily translocate to the nucleus in response to GCs (Fig. 5a) and effectively bind thousands of sites in the genome (Fig. 5b), as demonstrated by immunofluorescence and ChIP-sequencing experiments, respectively. In contrast to that, GR-driven gene expression changes were observed in the GC-growth-arrested cell lines (A549, H2122 and H1944), while this was strongly attenuated in the GC-unresponsive cell lines H1975 and H460 (Fig. 5c). As co-regulator recruitment is imperative for transcriptional modulation, we subsequently investigated the molecular composition of the GR transcriptional complex by performing RIME32. In the dormancy-induced cell lines, GR was able to successfully recruit numerous proteins to its complex, including Ncoa1 and Nrip1 (Fig. 5d and Supplementary Data 3), previously reported to be critical for GR-driven transcriptional regulation38. Despite the ability to bind chromatin (Fig. 5b), GR was unable to stably recruit coregulators in H1975 and H460 (Fig. 5d and Table S4). To unravel the composition of the active chromatin-bound GR complex, we performed a statistical comparison of GR-active (A549/H2122/H1944) and GR-inactive (H1975/H460) cell lines (Supplementary Data 4). While GR itself was detected at comparable levels (Fig. 5e), pathway enrichment analysis (Gene Ontology gene-sets) revealed that an active-GR interactome is composed out of four major parts that include the nuclear transcription factor complex (nominal P value = 0.018), SWI/SNF complex (nominal P value < 0.001), mediator complex (nominal P value = 0.056), and the RNA polymerase II complex (nominal P value = 0.001) (Fig. 5f).

The SWI/SNF chromatin remodelling complex was of particular interest, as transcriptional downregulation of its members has been associated with GC-resistance in human acute lymphoblastic leukaemia39,40. Firstly, we interrogated whether GR activity is affected in human lung tumours bearing deleterious mutations in the members of the SWI/SNF complex. For this, we made use of a GR activity score (explained above) and the lung adenocarcinoma dataset from TCGA (91/877 tumours harboured SWI/SNF mutations; Smarcb1 18.09%; Smarcc2 9.52%, Smarcd2 6.66%, Smarcd3 1.90%, Arid1a 29.52%, Arid2 31.42%, Smarce1 2.85%). The GR activity score was significantly lower in the tumours bearing SWI/SNF mutations (Fig. 5g), suggesting that these may influence GR activity. Using publicly available data of GC-growth-arrested cervical cancer cell line model (HeLa) that upregulate Cdkn1c upon GC treatment (Supplementary Fig. 7a–c), we observed binding of GR and multiple SWI/SNF members to the CERES enhancer (Supplementary Fig. 7d), suggesting that this mechanism is active in other cancer types. To experimentally test if a causal relationship between SWI/SNF complex and GR activity exists, we performed short hairpin (shRNA) mediated knockdown (at least two shRNAs per target) of each SWI/SNF complex member in the H2122 cell line. Efficient knockdown for all eight SWI/SNF components was confirmed using RT-qPCR (Fig. 5h, left). Following this, we treated the knockdown models with GCs and performed RT-qPCR analysis for Cdkn1c and housekeeping reference genes. Interestingly, while knockdown of Arid1a, Smarce1, Smarca2 and Smarcb1 had a negative impact on GR-mediated Cdkn1c expression, loss of Smarcc2 and Smarcd2 further boosted of GR-induced upregulation of Cdkn1c (Fig. 5h, middle). The knockdown of Arid2 and Smarcd3 had no impact on Cdkn1c upregulation (Fig. 5h, middle). To confirm these findings on protein level, we performed an immunofluorescence staining of p57 in GC-treated condition and quantified the percentage of cells expressing the protein and the intensity of the signal in over 10,000 cells per knockdown model. Taking both metrics into account, we confirmed that the effects observed on
transcription level are also seen on protein level (Fig. 5h, right), further strengthening our conclusions that GR gene regulation and dormancy induction are under the direct control of SWI/SNF complex functionality and composition.

With this, we have shown that the SWI/SNF remodelling complex forms an essential part of the GR transcriptional machinery, necessary for the regulation of p57, which is required to drive cells into dormancy.

Accessibility of CERES in human cancer samples is associated with GR-dependent CDKN1C expression and activity. To investigate the clinical validity of the GR-driven CDKN1C enhancer identified in this study, we explored transcriptomics and chromatin accessibility (Assay for Transposase-Accessible Chromatin (ATAC) sequencing) datasets of the TCGA cohort (Fig. 6a). We observed that samples with high chromatin accessibility of CERES have high expression of CDKN1C, accompanied
by low expression of genes involved in cancer cell proliferation (MKI67 and PCNA) and aggressiveness (FOXM1) (Fig. 6b). Conversely, samples with low chromatin accessibility of CERES have low expression of CDKN1C, and high expression of MKI67, PCNA and FOXM1 (Fig. 6b). The observed correlation of chromatin accessibility of CERES and CDKN1C expression is significantly higher than the level of correlation seen for any of the 306 enhancers found in the genomic vicinity of CDKN1C, which do not correlate with its levels (Fig. 6c). In addition, CERES accessibility does not correlate with the expression of any of the four neighbouring genes proximal to the CDKN1C locus (Fig. 6d). Furthermore, correlation between CERES accessibility and CDKN1C expression was found to be dependent on GR mRNA levels and increased in a step-wise manner with the removal of tumour samples with the lowest GR expression levels (Fig. 6e). This was not the case for the correlation of accessibility of 306 surrounding enhancers with CDKN1C levels (Fig. 6e).

These data support our in vitro findings, suggesting the relevance of GR-mediated regulation of CDKN1C by CERES in clinical samples of human non-lymphoid solid cancers.

Discussion
Pharmacological activation of the GR is a proven, effective treatment strategy for lymphoid cancers, including acute lymphoblastic leukaemia, chronic lymphocytic leukaemia and multiple myeloma. On the other hand, in non-lymphoid solid cancers, GR agonists are commonly prescribed to alleviate the side effects of treatment. However, several lines of evidence point towards a direct effect of GR activation on cancer cell behaviour, including invasion, apoptosis resistance and growth. For example, using in vitro and mouse models of non-small cell lung cancer, it has been shown that GC treatment diminishes cancer incidence and growth in the context of lung cancer development. Despite these observations, little is known about the mode of GR action and its direct effectors in non-lymphoid solid cancer types. Therefore, using a multidisciplinary approach we studied the molecular mechanisms of stress hormone receptor action in non-lymphoid solid cancers, focusing on lung cancer (Fig. 7).

We have found that lung cancer cells react to GCs by transitioning to a dormant state accompanied by activation of IGF-1R survival signalling. While this is the first report of cell dormancy induction by stress hormone receptor activation in cancer, it was previously suggested that GCs may induce cell cycle exit in tenocytes, thymic epithelial cells, and neural stem cells, potentially contributing to long-term degenerative changes in tendon tissue, development of neural disorders and T cell-mediated autoimmune diseases, respectively. It is highly likely, however, that cellular and molecular modes of dormancy activation may differ between target tissues and/or pathologies due to the cell-type-specific nature of GR action.

Stress hormone-induced cell dormancy is driven by p57 and is characterised by both attributes independently associated with either senescence (senescence-associated β-galactosidase positivity and enrichment of a senescence-related gene signature) or quiescence (reversible state, a silent metabolic profile, lack of p53 response and p16/p21 upregulation, and activation of growth factor signalling (IGF-1R)) according to the recent guidelines by the International Cell Senescence Association. The initiating driver of the phenotype reported in this manuscript is p57, a cell cycle inhibitor, known to have additional mechanisms in comparison to the other family members that drive quiescence (p27) and senescence (p16/p21). Recent evidence exists of p57 being able to initiate both senescence and quiescence in human primary tissue models depending on environmental cues.

In relation to this, it could be hypothesised that the composite phenotype we observed (in part senescence, in part quiescence) is caused by the altered action of p57 in cancer. In contrast to prior studies on cancer models, the cell cycle exit and dormant state reported in this manuscript is induced by a physiological ligand found in circulation, to which all cancers are exposed, and is not caused by pharmacologically induced DNA damage or inhibition of cell cycle machinery. Of particular interest is the GC-induced tolerance to various anticancer drugs. While the decrease in sensitivity to selected chemotherapeutics after GC treatment has been observed previously, we have unbiasedly profiled a large number of compounds to show that this generally applies to various drugs. Our findings complement previous reports in raising concern about the widespread use of GCs in the management of anticancer therapy side effects and warrant clinical caution and investigation. The viability in this GC-induced multidrug-tolerant state is maintained via engagement of IGF-1R signalling. As this signalling plays a significant role in maintaining cell survival, the induced activity of this pathway might provide key survival mechanisms, potentially yielding a therapeutic opportunity, as demonstrated by a significant reduction in viability upon its inhibition. It was previously observed that IGF-1R activity is necessary for cell viability maintenance of cancer cells subpopulations following lethal drug exposure. In addition, activity of this signalling is needed for entry and exit from quiescence in a nutrient-depletion pancreatic cancer in vitro model. In conjunction with this, it could be hypothesised that IGF-1R activation in accordance with the circulating levels of GCs and the circadian rhythm may also enable cancer cells to readily react to
daily changes in the environment, allowing them to rapidly switch between a proliferating state and dormancy.

It is well documented that GR activation modulates the expression of a large number of genes throughout the genome. However, direct causality in linking particular genes to phenotypes is still understudied with a limited number of examples described to date. Our findings demonstrate that a single GR-target gene (p57) drives the induction of the reported dormancy phenotype, as discussed above.

The most complex member of the Cip/Kip family, p57, is a key protein involved in the development, organ morphogenesis, and tumour suppression. This protein exerts its function through interaction with and direct inhibition of various cyclin-dependent kinases, which in turn leads to dephosphorylation of cell cycle proteins (including retinoblastoma), inactivation of E2F and cell cycle exit. While the molecular mechanisms behind p57-induced cell cycle exit are known, the regulation of this key cell cycle inhibitor by transcription factors is proposed to be complex, cell-type specific, and not entirely understood.

We show that GR directly regulates CDKN1C gene expression, through induction of chromatin looping initiated by binding to CERES, a distal enhancer located in the KCNQ1 gene, in a SWI/SNF chromatin remodelling complex-dependent fashion.

Precise regulation of CDKN1C is imperative for embryogenesis, differentiation, as well as tumour suppression. In relation to its diverse roles, CDKN1C expression is dynamically linked to
Fig. 5 SWI/SNF complex fine-tunes expression of CDKN1C. a Representative immunofluorescence images showing expression and localisation of GR (green), treated with glucocorticoids (GC) or control (Veh), using DAPI as nuclear staining (blue) (n = 3). Scale bar, 10 μm. b Heatmap of ChIP–sequencing signal around peak midpoint for all sites detected across the genome (top), and average signal of GR ChIP–seq experiments across all sites called over input control (bottom), for untreated (Veh) and glucocorticoid-treated (GC) cells. c Scatter plot depicting differential gene expression changes upon GC treatment in RNA sequencing. Genes significantly (Padj ≤ 0.01) up- or downregulated by GCs are depicted in red (n = 2). Adjusted P values were determined by DESeq2 (Wald test P values corrected for multiple testing using Benjamini and Hochberg method). d Scatter plot depicting enrichment over IgG control in a GR-RIME experiment. Proteins considered to be recruited by GR are 2.5 LFQ enriched over IgG (dotted line) and significant (−log(P value) >2; red) (n = 3). P values were determined by two-sided t test. e Volcano plot depicting differentially enriched interactors in GR-RIME experiments between three cell lines with active and two cell lines with inactive GR (n = 3). P values were determined by two-sided t test. f GSEA enrichment profiles for RNA polymerase II transcription factor complex (M17103; blue). Nuclear transcription factor complex (M17532; purple), SWI/SNF complex (M17713; red) and mediator complex (M17759; green) gene sets based on A549/H2122/H1944 and H1975/H460 comparison GR-RIME dataset (n = 3). Nominal P values were determined by GSEA software. g Box plot depicting GR activity (z score of 253 genes) in SWI/SNF WT (WT; n = 786) and mutant (n = 91) human lung adenocarcinoma tumours. The central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The notch displays a confidence interval around the median. The maximum whisker lengths are specified as 1.5 times the interquartile range and outliers are depicted as filled circles. P values were determined by Wilcoxon rank-sum test with continuity correction. h (left) Normalised mRNA expression level relative to shControl for SMARCD3, ARID2, SMARCC2, SMARCC1, SMARCA2, ARID1A and ARID1B, in cell lines with shRNA targeting respective genes. Mean values with ±SEM depicted. ≥2 shRNAs per gene in biological duplicates (n = 2). h (middle) Normalised (relative to untreated condition) CDKN1C mRNA expression level of GC-treated shSMARCD3, shARID2, shSMARCD2, shSMARCC2, shSMARCC1, shSMARCA2, shSMARCE1 and shARID1A H2122 cell lines. Mean values with ±SEM depicted. ≥2 shRNAs per gene in biological duplicates (n = 2). h (right) Protein expression index (number of positive cells * average signal intensity) depicting quantified expression of p57 in immunofluorescence experiments using shSMARCD3, shARID2, shSMARCD2, shSMARCC2, shSMARCC1, shSMARCA2, shSMARCE1 and shARID1A H2122 cell lines. Mean values with ±SEM depicted. ≥2 shRNAs per gene in biological duplicates (n = 2), ≥10,000 cells quantified.

Fig. 6 CDKN1C mRNA expression is linked to chromatin accessibility of CERES and GR levels in clinical samples. a Biopsies of human primary non-lymphoid solid cancers were subjected to whole-transcriptome and chromatin accessibility analysis by the TCGA (n = 404). b (top) Representative samples showing chromatin accessibility signal at CERES enhancer. b (bottom) Expression of CDKN1C, MKI67, PCNA, and FOXM1 sorted based on CERES accessibility. Survival events (alive = white, dead = green), SWI/SNF mutation status (WT = white, mutated = green), and cancer types are displayed below. c Histogram depicting correlation value counts for CDKN1C expression levels and 306 up- and downstream enhancers in its genomic vicinity. Red line shows the correlation of CERES accessibility with CDKN1C expression. d Histograms showing correlation of four proximal genes with the accessibility of 306 up- and downstream enhancers surrounding CDKN1C. Red lines show correlations of these genes with CERES accessibility. e Line plot showing Spearman’s correlation of accessibility of CERES (red) or 306 (blue) surrounding enhancers with CDKN1C mRNA levels, and NR3C1 mRNA levels (green).

In this study, we describe a GR-driven distal enhancer (>100 kb upstream of CDKN1C, within an intron of KCNQ1 gene) of which the physical interaction with the CDKN1C promoter is enhanced by GR activation. Importantly, we functionally probed this enhancer

numerous homeostatic cues and the circadian rhythm in various tissue types84. Prior research has suggested that CDKN1C is regulated in a complex combinatorial fashion, through an imprinting control region and unidentified enhancers located in the KCNQ1 gene13,34.
using CRISPR-Cas9-based deletion to demonstrate that this enhancer is critically involved in GR-driven CDKN1C expression. We also presented evidence that GR-mediated regulation of CDKN1C by the CERES enhancer occurs in clinical samples, highlighting the relevance of this regulation in tumour development and progression. Strikingly, a deletion in the KCNQ1 gene spanning the discovered genetic element CERES was detected and causally linked to Beckwith–Wiedemann Syndrome and Silver–Russell Syndrome, both known to be consequences of CDKN1C loss/downregulation85,86, suggesting a physiological role of the discovered enhancer in maintaining expression of this important cell cycle regulator.

Our GR transcriptional complex analysis gives insight into how this factor operates in cancer. We show that upon chromatin binding, GR recruits a proficient transcriptional complex composed of coregulators, SWI/SNF chromatin remodelers, mediator subunits and the RNA polymerase machinery. Specifically, as SWI/SNF members have been related to GC-resistance in clinical models of acute lymphoblastic leukaemia, we experimentally...
probe and demonstrate that p57 regulation by GR is SWI/SNF-dependent. The members of the SWI/SNF family have been found to be involved in gene regulation57–59, mostly through activating transcription, however, they may also directly repress gene expression56. In terms of GR biology, prior work has defined SWI/SNF, especially the BRG1 subunit (SMARC4) as a critical component promoting transcriptional activity of this nuclear receptor91–94. Our findings, albeit limited to the key driver of cell dormancy, show that the role of SWI/SNF members in GR biology is more complex than originally thought. Specifically, while some SWI/SNF subunits seem to be involved in the positive regulation of CDKN1C, others seem to repress GR transcription for this gene. To our knowledge, this is the first report showing that particular subunits of SWI/SNF complex antagonise GR transcriptional modulation. Overall our findings suggest that the specific composition of the SWI/SNF complex may play a role in adjusting the level of transcriptional output of GR at the CDKN1C locus.

We speculate that the role of GR to induce cellular dormancy in cancer may be reminiscent of its signalling in normal tissue. In agreement with this, activation of GR has previously been linked to cell differentiation and lineage selection59,96. As tumours are exposed to GCs produced by the adrenal gland and released into the circulation, this dormant state might be a feature of various early-stage human tumours, supported by the observation that GR expression is lower in various cancer types (including lung, breast and prostate) as compared to normal tissue and that it may serve as a tumour suppressor14. Furthermore, cell dormancy has been shown to be under circadian control in various tissues and stem cells97,98. In relation to that, the well-known day–night rhythmic behaviour of GC levels may also suggest that cell dormancy, as well as chemotherapeutic response in cancer, is subjected to circadian rhythm through intratumoral GR activity.

We conclude that SWI/SNF-dependent expression of CDKN1C, facilitated by looping of a specific GR-bound distal enhancer CERES, induces a reversible dormant state in which cells become tolerant to a large array of anticancer drugs and acquire IGF-1R signalling dependency.

Methods

**Cell lines.** A549, H2122, H1944, H1975 and H460 cells were obtained from Rene Bernard’s lab (Netherlands Cancer Institute, Netherlands). HEK293T cells were obtained from American Type Culture Collection (ATCC). The human mesothelioma cell lines M28 and VAM1 were provided by Courtney Broadus (University of California, USA), while the NCI-H2795 (H2795) was obtained from Umut Dmertmert (Sanger Institute, UK). The primary human mesothelioma cell lines PV130913, PV041214, PV200218, PV150318, PV240418, PV180518 and PV250518 were generated by Laurel Schunselaar (Netherlands Cancer Institute, Netherlands).

A549 and H2795 cell lines were maintained in DMEM/F12 (1:1) (X) + Glutamax (Life Technologies), while HEK293T, M28, PV130913, PV041214, PV200218, PV150318, PV240418, PV180518 and PV250518 were generated by Laurel Schunselaar (Netherlands Cancer Institute, Netherlands).

**RNA sequencing.** Cells were serum-starved for 3 days before they were treated with hydrocortisone (2.75 µM) for 8 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. The quality and quantity of the total RNA were assessed by the 2100 Bioanalyzer using a NanoChip (Agilent, USA). Total RNA samples having an RNA integrity number (RIN) above 8 were subjected to library generation.

**ChIP-sequencing.** Chromatin immunoprecipitations were performed as previously described104. Nuclear lysates were incubated with 7.5 µl of GR antibody (De62L, Cell Signalling Technology) pre-bound to 50 µl of protein A beads per sample. Immunoprecipitated DNA was processed for library preparation (0801-0303, KAPA biosytems kit). Samples were sequenced using an Illumina HiSeq2500 genome assembler (65 bp reads, single end), and aligned to the Human Reference Genome (hg19, February 2009). Reads were filtered based on MAQp quality (samtools v1.8); quality ≥20 and duplicate reads were removed (Picard Mark-Dups v2.18). Peak calling over input control was performed using MACS2 (v2.1) peak caller. MACS2 was run with the default parameters. Genome browser snapshots, heatmaps and density plots were generated using Easeq (http://easeq.net)105.

**Western blot.** Cells were lysed in 2x Laemmli buffer (120 µl Tris, 2% glycerol, 4% SDS). Total protein content was quantified by BCA assay (23227, Thermo Fisher Scientific). Cell lysates containing equal amounts of protein were analysed by SDS-PAGE, after protein transfer, nitrocellulose membranes were incubated with antibodies against GR (H204, Cell Signalling Technology) pre-bound to 50 µl of protein A beads per sample. Immunodetected bands were visualized by ECL using LumiGlo Luminol (Fisher Scientific). Band intensity was quantified using ImageJ software.

**Seahorse.** Cellular respiration was measured using a Seahorse XF24 Bioanalyser (Seahorse Biosciences). A549, H2122, and H1944 cells were all seeded at 75,000 cells per well to XFe24 cell culture microplates (102340-100, Seahorse Biosciences) and cultured overnight before the analysis. The analysis was performed according to the manufacturer’s instructions in DMEM (D5000, Sigma Aldrich) supplemented with 10 mM d-glucose and 4 mM t-glutamine for the oxygen consumption rate (OCR) experiments. For OCR measurements, the following reagents that selectively inhibit mitochondrial function were added: oligomycin (1 µM; an ATP synthase (complex V) inhibitor), FCCP (0.4 µM; a mitochondrial complex III inhibitor) and antimycin A (1 µM; a mitochondrial complex I inhibitor). Results were normalised to DNA content using nanodrop quantification.

**Senescence-associated β-galactosidase assay.** Cytochemical staining for senescence-associated β-galactosidase was performed as described before107. Cells were cultured and subsequently fixed by fresh methanol for 5 min without hydrocortisone (2.75 µM) was added for 2 days. After incubation, cells were washed twice with PBS and fixed with 3.7% formaldehyde for 5 min. Following that, cells were stained for β-galactosidase activity using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Sigma) or vehicle, and harvested at the indicated timepoint. Specific conditions, treatment duration and compounds used can be found in Table S1.
were washed with PBS before they were incubated with X-gal staining solution (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate buffer, 5 mM potassium ferrocyanide, 2 mM potassium ferricyanide; 150 mM NaCl; 150 mM NaF) overnight at 37 °C. X-gal is an artificial substrate of the β-galactosidase enzyme and is used to detect senescence-associated β-galactosidase. The next day, cells were washed with PBS and imaged with a Zeiss Axiovert S100 inverted microscope (Zeiss, Germany).

**Cell cycle analysis with flow cytometry.** Cells were serum-starved for at least 3 days and then treated with propidium iodide (2.75 μM) for 2 h. Cells were then washed with PBS and centrifuged at 800×g for 5 min. The pellets were resuspended in cold PBS and centrifuged two more times. The pellets were then resuspended in PBS and stored at 4 °C till flow cytometric analyses. Flow cytometric measurements were performed on LSRFortessa SORP 2 (BD Biosciences) and cell cycle distribution analysed with FlowJo Software (FlowJo LLC).

**Annexin V/propidium iodide apoptosis assay with flow cytometry.** Annexin V/propidium iodide apoptosis assays were performed as described previously.4 Cells were serum-starved for at least 3 days. Following that, cells were either left untreated (FLPS) or treated with HC at a concentration of 2.75 μM for 6 days. As a positive control, cells were treated with 30 μM cisplatin to induce apoptosis. Cells were harvested and centrifuged for 10 min at 335g at 4 °C. The pellet was resuspended in cold PBS and fixed with 80% ethanol overnight at 4 °C. The next day, the pellets were resuspended in Annexin V binding buffer and Annexin V was added according to the manufacturer’s recommendations. Samples were incubated for 15 min at room temperature in the dark. Propidium iodide (10 μg/mL) was added for staining. After that, cells were analysed with FITC and PI fluorescence using a FACSCalibur flow cytometer (Thermo Fisher Scientific, USA) and cell populations analysed with FlowJo Software (FlowJo LLC, USA).

**Xenografts.** The H1944 or A549 cells were trypsinised and resuspended in PBS at a density of six (H1944) or two (A549) million cells/50 μl and mixed with an equal volume of BME (#3533-005-02, Sigma-Aldrich) NOD-scid-γ (H1944) or non-scid-γ (NSG) mice (5-7 weeks old) were inoculated with six million cells per animal subcutaneously into one of the flanks. Once the tumour size reached between 100 and 300 mm³, mice were inoculated with 4 mg/kg dexamethasone (D2915-100MG, Sigma-Aldrich; dissolved in water), 25 mg/kg 14N (HY-10191, MedChemExpress; dissolved in 25 mM tartaric acid), 25 mg/kg 14N (HY-10191, MedChemExpress; dissolved in 25 mM tartaric acid) at 4 °C. The mice were monitored by calliper measurements every 2 days. Mice were kept under standard conditions (HY-10191, MedChemExpress; dissolved in 25 mM tartaric acid), 25 mg/kg 14N (HY-10191, MedChemExpress; dissolved in 25 mM tartaric acid) at 4 °C, where they were maintained for 2 days. Subsequently, the cells were harvested and centrifuged for 4 min at 335g at 4 °C. The pellets were resuspended in PBS and stored at 4 °C till flow cytometric analyses. Flow cytometric measurements were performed on an Attune NxT Flow Cytometer (Thermo Fisher Scientific, USA) and cell populations analysed with FlowJo Software (FlowJo LLC, USA).

**Immunofluorescence and quantification.** After hormone deprivation cells were treated with 2.5 μM hydrocortisone or left untreated for 8 h. Cells were washed and fixed in 2% paraformaldehyde for 10 min at room temperature. Subsequently, cells were permeabilized in 0.5% Triton X-100 for 10 min. Blocking of nonspecific binding was performed with 5% BSA, 0.1% NaN₃ in PBS. Cells were then incubated with primary antibodies: Goat anti-GFP (1:50), goat anti-RFP (1:50) for 60 min at 37 °C and washed three times with cold PBS. Following that, cells were incubated with secondary antibodies: Alexa Fluor 488 (A11001, Thermo Fisher Scientific) (1:1000) and goat anti-rabbit IgG (A11008, Thermo Fisher Scientific) (1:1000) for 30 min at room temperature. Following that, samples were incubated with secondary antibodies: Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A11001, Thermo Fisher Scientific) (1:1000) and goat anti-rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21244, Thermo Fisher Scientific) (1:1000). Finally, samples were counterstained with 4',6-diamidino-2-phenylinodole (DAPI) and analysed by either laser confocal microscopy (SP5, Leica) or screening fluorescent microscope (TIRF, Leica).

*For single-cell analysis, images were analysed in FIJI,*110 p57-positive cells were manually selected in a fully automatic, unbiased manner, with a custom-made ImageJ macro script. For every image, the DAPI channel was used to segment cell nuclei into ROIs as follows. After rolling ball background subtraction (40-micron radius) and a median filter (1.5-micron radius), local thresholding was applied (Mean method, 8-micron radius, with four times the standard deviation of the background as the threshold). Cells were then separated by a distance transform watershed operation to separate touching nuclei. The mean p57 signal was then measured inside the obtained ROIs. Cells were considered to be positive (negative) if this mean value was higher (lower) than a certain threshold, determined using untreated control samples. The resulting images with filled ROIs were overlaid with the original data for visual inspection.

**Drug screen.** Before the start of the screen, H1944 cells were cultured in medium without or with glucocorticoids (hydrocortisone, 2.75 μM) for 2 days. Using the Multidrop Combi (Thermo Fisher Scientific), untreated H1944 cells were seeded into 384-well plates either at low (1000 cells) or high (2500 cells) confluence, while the pre-treated H1944 cells were seeded at high (4500 cells) confluence. After 24 h, the NCI compound collection of purchased drugs (Selleck GPCR (236 drugs), epigenetic inhibitors (111 drugs), apoptosis inhibitors (23 drugs), phosphatase inhibitors (18 drugs), epigenetic inhibitors (160 drugs), LOPAC (1280 drugs) and NCI oncology (114 drugs)) was added. This library was stored and handled as recommended by the manufacturer. Compounds from the master plate were diluted in daughter plates containing complete RPMI-1640 medium, using the MICROLAB STAR liquid handling workstation (Hamilton). From the daughter plates, the diluted compounds were transferred into 384-well assay plates, in triplicate, with final concentrations of 1 μM and 5 μM. In addition, positive (1 μM Phenylarsine oxide) and negative (0.1% DMSO) controls were added alternately to wells in column 2 and 23 of each assay plate. After 6 days, viability was measured using a CellTiter-Blue assay (G8081/2, Promega) followed by a direct transfer, or, if required, scored in a fully automatic, unbiased manner, with a custom-made ImageJ macro script. For every image, the DAPI channel was used to segment cell nuclei into ROIs as follows. After rolling ball background subtraction (40-micron radius) and a median filter (1.5-micron radius), local thresholding was applied (Mean method, 8-micron radius, with four times the standard deviation of the background as the threshold). Cells were then separated by a distance transform watershed operation to separate touching nuclei. The mean p57 signal was then measured inside the obtained ROIs. Cells were considered to be positive (negative) if this mean value was higher (lower) than a certain threshold, determined using untreated control samples. The resulting images with filled ROIs were overlaid with the original data for visual inspection.

**Phosphoproteomic analysis.** After hormone deprivation, cells were treated with 2.75 μM hydrocortisone or left untreated (Yeh) for 48 h. For protein digestion, subtract the values were eluted from the analytical column in a 90-min linear gradient.

Raw data were analysed by Proteome Discoverer (PD) (v. 2.3.0.523, Thermo Scientific) using standard settings. MS/MS data were searched against the Swissprot database (release 2018_06) using Mascott (v. 2.6.1, Matrix Science, UK) with Homo sapiens as taxonomy filter (20,381 entries) for the GR-RIME experiment, whereas Sequest HT was used for the p57-RIME experiment. The maximum allowed precursor mass error was set to 1% and as an additional filter Mascot peptide ion score >20 or Sequest HT XCorr>1 was set. The PD output file containing the abundances was loaded into Perseus (version 1.6.1.3) [02] LFQ intensities were Log2-transformed and the proteins were filtered for at least 66% valid values. Missing values were replaced by imputation based on the standard settings of Perseus, i.e., a normal distribution using a width of 0.3 and a downshift of 1.8. Differentially expressed proteins were determined using a t-test. The comparison between the pooled cell lines of the GR-RIME experiment was IgG corrected.
frozen cell pellets were lysed in boiling Guanidine (GuHCl) lysis buffer as previously described112. Protein concentration was determined with a Pierce Coomassie Plus protein assay kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Aliquots corresponding to 1.1 mg of protein were digested with Lys-C (Wako) for 2 h at 37 °C, enzyme/substrate ratio 1:100. The mixture was then diluted to 2 M GuHCl and digested overnight at 37 °C with trypsin (Sigma-Aldrich) in enzyme/substrate ratio 1:100. Digestion was quenched by the addition of TFA (final concentration 1%), after which the peptides were desalted on a Sep-Pak C18 cartridge (Waters, Massachusetts, USA). From the eluates, aliquots were collected for proteome analysis, the remainder being reserved for phosphoproteome analysis. Samples were vacuum dried and stored at −80 °C until LC-MS/MS analysis or phosphopeptide enrichment.

Phosphorylated peptides were enriched from 1 mg of total peptides using High-Select Fe-NTA Phosphopeptide Enrichment Kit (Thermo Scientific), according to the manufacturer’s instructions, with the exception that the dried eluates were reconstituted in 15 μl of 2% formic acid. Peptide mixtures were analysed by nanolC-MS/MS on an Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer equipped with an EASY-NLC 1200 system (Thermo Scientific). Samples were directly loaded onto the analytical column (ReproSil-Pur 120 C18-AQ, 1.9 μm, 75 μm × 500 mm, packed in-house). Solvent A was 0.1% formic acid/water and solvent B was 0.1% formic acid/80% acetonitrile. Solvent A was slowly filtered at the elutional column at a constant flow rate of 250 nl/min. For single-run proteome analysis, a 4-h gradient was employed containing a linear increase from 7 to 30% solvent B, followed by a 15-min wash, whereas for single-run phosphoproteome analysis, a 2-h linear gradient (from 4 to 22% solvent B, followed by a 15-min wash) was used. Protein data analysis was performed using PD (v. 2.3.0.523, Thermo Scientific) using standard settings. MS/MS data were searched against the human Swissprot database (20,417 entries, release 2019_02) with the Gene Expression Omnibus (GEO) and Proteomics Identification (PRIDE) databases, under accession numbers GSE195946 and FXD021924, respectively. Public datasets used in this study were available from GEO or ENCODE, archived under the following codes: U01HG007900, GSE24397 and GSE49591. All the other data are available within the article and its Supplementary Information. Source data are provided with this paper.

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GR ChIP-seq and Hi-C time-course analysis. Chromatin loops spanning either FOXO1 or IRS2 loci in A549 previously identified using Hi-C were analysed. Dynamic loops showing a significant increase or decrease in chromatin interaction frequency after 1, 4, 8 and 12 h of dexamethasone exposure were reported. GR and c-Jun binding sites identified using ChIP-seq and chromatin interaction counts were fitted into generalised linear models (GLMs) using edgeR and a likelihood ratio test was performed to identify significant hits (FDR ≤0.05) as previously described. Log2 fold-change values were calculated for each dexamethasone timepoint over the absence of dexamethasone.

Immunohistochemistry. Immunocytochemistry xenograft tumour samples were performed by an optimised protocol previously reported125,126 using the following primary antibodies: Ki67 (ab155580, Abcam), phospho-Rb (Ser780, #9307, Cell Signalling), phospho-IGF-1R (11161, ab39398, Abcam), total Rb (ab181616, Abcam), p21 (sc-6246, Santa Cruz), and cleaved caspase-3 (#9661, Cell Signalling).

Statistical analysis. Statistical analysis was performed using Prism (GraphPad, San Diego, CA). Normality was tested using D’Agostino–Pearson and Shapiro–Wilks test. Technique-specific statistical tests are described within their corresponding method subsection.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All genomic and mass spectrometry data generated in this study have been deposited in the Gene Expression Omnibus (GEO) and Proteomics Identification (PRIDE) databases, under accession numbers GSE195946 and FXD021924, respectively. Public datasets used in this study were available from GEO or ENCODE, archived under the following codes: U01HG007900, GSE24397 and GSE49591. All the other data are available within the article and its Supplementary Information. Source data are provided with this paper.

4C analysis. 4C was performed as previously described115 with minor modifications116. 4C libraries were sequenced on a MiSeq and analysed with a custom 4C mapping pipeline (https://github.com/deWitLab/4C_mapping). 4C ligation data were mapped to hg19. Normalisation and downstream analysis were done using peakCtools. The genome and GC-treated conditions were compared using the Wilcoxon test for the following genetic locations: region a (chr11: 2773921–2812270), region b (chr11: 2830667–2882981) and CDKN1C gene (chr11: 2893641–2926016). Primer sequences are listed in Supplementary Table 2.

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Author contributions

S.P. and W.Z. conceived the project outline and coordinated the project. S.P. designed and implemented the project. W.Z. was responsible for project funding and supervision. N.M.P. and A.G.Z. have contributed equally to this work. S.P. and K.M. performed RNA and ChIP-sequencing experiments. S.P., K.S. and A.H. performed flow cytometry experiments. A.H. generated all the data on mesothelioma cell lines. S.P. and K.M. evaluated the immunohistochemistry data. S.P., K.S. and F.A. generated and performed experiments on knockout/knockdown models. S.P., M.B., D.D., L.H. and M.A. performed mass spectrometry experiments and analysis. A.G.M. performed live-cell imaging experiments and analysis. S.P., K.S., H.T., T.S. and E.J.D. performed 4C

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experiments and analysis. A.B. and T.R. analysed GR ChIP and Hi-C time-course data. S.P., J.S. and W.F. performed and analysed seahorse experiments. S.P., K.S., B.M., C.L. and R.B. designed, executed and analysed the drug screen. S.P., M.D.W., J.J. and K.E.dV. designed and analysed xenograft experiments. S.P. and B.vdB. performed image analysis. S.P. performed computational analysis of the majority of the data. S.P., T.C. and B.G. performed computational analysis of TCGA and publically available clinical data. S.P., K.S., I.M.P., A.G.M., R.M. and W.Z. discussed and interpreted the data. S.P. designed and created all the schematics found in the figures. S.P. wrote the manuscript, with input from all authors.

**Competing interests**

S.P., K.S. and W.Z. have been designated as inventors in the European patent application #19205735.4 – 1112 filed by Stichting Het Nederlands Kanker Instituut-Antoni Van Leeuwenhoek Ziekenhuis. This patent application is currently pending. The patent application covers the discoveries presented in Fig. 2, S3 and S4. The remaining authors declare no competing interests.

**Additional information**

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