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Early View

Original article

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Reptin drives tumour progression and resistance to chemotherapy in non-small cell lung cancer

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Summary:

The AAA+ ATPase Reptin confers tumour progression, resistance to chemotherapy and poor outcome in NSCLC patients.

Abstract

While targeted non-small cell lung cancer (NSCLC) therapies improved outcome of defined disease subtypes, prognosis of most of the patients remains poor. We found the AAA+ ATPase Reptin to be highly expressed in the vast majority of 278 NSCLC tumour samples. Thus, the objective of the study was to assess the role of Reptin in NSCLC.

Survival analyses of 1,145 NSCLC patients revealed that high RNA expression levels of Reptin are associated with adverse outcome. Knock down of Reptin in human NSCLC cells impaired growth *ex vivo* and eliminated engraftment in a xenograft model. We uncovered direct interaction of Reptin with histone deacetylase 1 (HDAC1), as the critical mechanism driving NSCLC tumour progression. Pharmacological disruption of Reptin/HDAC1 complex resulted in substantial decrease of NSCLC cell proliferation and induced significant sensitization to cisplatin.

In conclusion, our results identify Reptin as a novel independent prognostic factor and as a key regulator mediating proliferation and clonal growth of human NSCLC cells *ex vivo* and *in vivo*. We unveil a Reptin/HDAC1 protein complex whose pharmacological disruption sensitizes NSCLC cells to cisplatin, suggesting this approach for application in clinical trials.

Introduction

The identification of various oncogenic driver mutations [1] as well as evidence of high expression of distinct membrane proteins modulating the immune system have enabled the development of powerful new targeted NSCLC therapies [2-4]. In spite of these breakthrough discoveries, responses to most of the standard NSCLC therapies are still poor and/or short-lived. Until today, lung cancer represents the leading cause of cancer mortality worldwide, and prognosis especially of NSCLC patients with advanced or metastatic stage of the disease remains poor [5]. Identification of parameters predicting early metastasis or dismal prognosis of the patients may help to select patients requiring alternative treatment strategies. Moreover, unraveling the molecular mechanisms underlying carcinogenesis and tumour progression is needed to design more specific and efficacious substances targeting NSCLC.

NSCLC is largely determined by transcriptional deregulation based on a broad variety of genetic and epigenetic events. Alterations of the transcriptional programs result in cellular transformation and/or tumour progression and maintenance [6]. In the last few years, the AAA+ ATPase family member Reptin has been implicated in various physiological processes including transcriptional regulation, remodeling of chromatin and repair of DNA damage [7, 8], mechanisms that are frequently deteriorated in NSCLC cells. Thus, we sought to determine the role of Reptin in lung cancer and as a potential novel target for future NSCLC therapies.

Material and methods

Lung cancer Tissue Microarray (TMA)

Tumour material and clinical follow up data was available from 321 NSCLC patients from the Thoracic Departments in Ostercappeln, Germany (study collective I; n = 265 NSCLC tissue samples) and Mainz, Germany (study collective II; n = 56 NSCLC tissue samples) (median age: 66 years) that underwent curative resection. Clinical follow up information and approval of the study by the Ethical committees of Muenster and Mainz were obtained for the collection of paraffin embedded tissue samples for biomarker testing. Due to the retrospective, anonym character of the analysis, written patient consent was not required. Clinical TNM staging (including clinical examination, CT scans, sonography, endoscopy, MRI, bone scan) was based on IUCC/AJCC recommendations. In terms of definite tumour staging, pathological exploration was carried out post-surgically. Primary pulmonary lesions were pathologically classified based on the WHO 2004 guidelines; 149 specimens were classified as squamous cell carcinoma, 125 as adenocarcinoma and 47 as large cell carcinoma. Patients with stage IV, neoadjuvant treatment, R1 or R2 resection status or with non-specified tumour histology (e.g. NSCLC not otherwise specified) were excluded from our study. Thus, 278 patient samples remained for analysis. Regular follow-up was performed for all patients, including systemic re-staging after 3, 6, 12, 18, 24, 36, 48 etc. months or earlier, if clinically required. Survival time was either computed from the date of histological diagnosis to death or to the date of last contact. Baseline information of the NSCLC population is shown in Supplementary Table S1. TMAs were generated from formalin-fixed, paraffin-embedded tissue specimens (FFPE) and as described previously [9]. The following primary antibody was applied: Anti-human Reptin antibody (BD Bioscience, ab No. 612482, clone

42/TIP49b). Immunoreaction was visualized with a biotinylated secondary antibody (LSAB/AP, #K5005 Dako) including the red chromogen and according to the manufacturer´s instructions. Immunohistochemical assessment was performed applying a quantitative H-score as a continuous value, essentially based on staining intensity according to parameters proposed by Harvey et al. [10], as well as percentage of stained cells. Establishing a reference framework for the analysis, prototypical examples of four different staining intensities (0, none; 1, weak, 2, moderate; and 3, high) were assembled through an initial screen of the stained set of samples (supplementary methods). Based on the reference framework, staining intensity of each tumor sample was determined, integrating proportional information through the assignment of heterogeneously staining tumors to the higher intensity category if at least 10% of the tumor cells fulfilled its criteria. Four investigators (J.-H.M., W.H., G.K., M.F.A) independently analyzed TMA slides. Samples with discordant assessment results were re-evaluated and a consensus was reached. H-score was calculated via multiplication of intensity x % of stained cells. Optimal cut-off for overall survival was assessed using maximally selected log-rank statistics in R version 3.4.3. Association of clinico-pathological parameters with Reptin expression was tested using two-sided Fisher's exact test. Univariate overall survival analysis was performed using the Kaplan-Meier method and log rank tests. A multivariable Cox proportional hazards model was fitted using a forward step-wise variable selection (inclusion criteria: p-value of the likelihood ratio test ≤0.05) to identify independent prognostic factors for overall survival. Patients with missing values in the cofactors were excluded from the analysis. All statistical tests were performed as exploratory analyses on a local significance level of 0.05. SPSS (SPSS Statistics, Version 22.0 released 2013, IBM Corp., Armonk, NY) was used for all statistical analyses.

Plasmids, antibodies and primers

Human Reptin shRNAs were a kind gift from C. Abraham; sequences were: Reptin TRCN0000051563, pLKO shRNA #1, CCGTGATGTAACAAGGATTGA, and TRCN0000051564, pLKO shRNA #2, CGAGAAAGACACGAAGCAGAT [11]. Sequences of sgRNAs targeting Reptin are provided in supplementary figure S2e).

HDAC1-pcDNA3 plasmid was a kind gift from K. Glaser. We used the following antibodies in this study: Flag M2 (A8592, Sigma-Aldrich), Reptin antibody (anti-TIP49b, BD 612482), anti-β-actin (A5441, Sigma-Aldrich), anti-CK7 (SP52) rabbit monoclonal (Ventana Medical Systems, 790-4462), Opti view DAB IHC Detection kit (Ventana Medical Sytsems), Goat anti mouse (115-036-062), Goat anti rabbit (111-036 -045), secondary antibodies, (Dianova).

Cell Culture and Reagents

Human NSCLC cell lines A549, LUDLU 1 and HOP62 were obtained from Sigma-Aldrich and Charles River Laboratories respectively.

A549 and HOP62 cells were maintained in D10 medium (DMEM, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin). LUDLU 1 cell line was maintained in R10 medium (RPMI, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin). DMEM: D5796, RPMI: R8758, P/S: P0781-100ML, (Sigma-Aldrich). FCS (S0115, Biochrom), Trypsin (BE02-007E, Lonza). INT (p-Iodonitrotetrazolium Violet, I8377, Sigma-Aldrich), entinostat (MS-275, sc-279455, SantaCruz Biotechnologies), cisplatin (PZN 06559665,TEVA), DMSO (20385.01, Serva).

Lentiviral Transduction, colony formation and proliferation assays

Lentiviral transduction was performed on NSCLC cells, adding 6 ml of D10 containing virus and 6 µg/ml polybrene (107689, Sigma, Saint Louis, USA). Transduced cells were selected with appropriate antibiotics.

For induction of human Reptin knock down, cells were transduced with pLKO.1 puromycin plasmids containing short hairpin RNAs (shRNAs) targeting human Reptin. Scrambled sequences were used as controls (sequences are available upon request). After antibiotic selection, cells were plated into methylcellulose medium containing specific drug treatment. Colonies were scored after 7-10 days of culture and labeled with INT o/n.

Proliferation assays were performed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (G54301, Promega).

Immunoprecipitation and Immunoblots Analysis

For generic immunoprecipitation, cells were lysed in NP-40 lysis buffer (0,5% NP-40, 50mM Tris-base pH 7.5, 5mM EDTA, 150 mM NaCl, 0.2 µM DTT, 10% Glycerol, protease inhibitors) for 1 hour at 4 °C. Protein concentration was measured by the PerkinElmer VictorX3 spectrophotometer using the Bio-Rad protein assay reagent (Pierce). Same amounts of total protein were incubated with the respective antibody o/n, precipitated with protein A/G Dynal beads (Invitrogen) at 4 °C for 1 h and then washed with 0.5% NP-40 washing buffer. Eluted proteins were resolved by SDS–PAGE. Membranes were probed with antibodies described above. For immunoblotting, samples were lysed with RIPA buffer (150 mmol/L NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mmol/L Tris·HCl, pH 8.0). Equal amount of proteins were resolved in NuPAGE Novex 4-12% Bis-Tris Gel 1.0 mm, 10 well (NP0321Box, Invitrogen). PVDF membranes (MerkMillipore) were blotted with

indicated antibodies and detected by Western Lightning ECL reagent (Perkin Elmer). Density quantification of the bands was analysed using Image J software.

Mouse Xenograft

NOD.Cg-*Prkdcscid IL2rgtmWjl*/Sz (NSG) mice were used for transplantation experiments. Mice were injected intravenously with 1 x 10^6 test cells. All experimental procedures were approved by the "Landesamt für Natur, Umwelt und Verbraucherschutz NRW" (GEZ 84-02.04.2014.A508) and performed as conform to the regulations.

Flow Cytometric Analysis of Human Cell Lines

Immunophenotypic analysis was performed by flow cytometric analysis using fluorochrome-conjugated monoclonal antibodies, PE anti-human CD326 (EpCAM) (Mouse IgG2b, BD 324205) and PE Mouse IgG1k (Isotype control, BD 555749).
Staining was generally performed in SM (PBS; 0.2% FCS) and incubated on ice for 30 min, washed twice and resuspended in SM before analysis using a BD LSR II system (Becton Dickinson Inc.).

Results

Reptin is overexpressed in the vast majority of NSCLC tumour tissues.

To assess the role of Reptin in NSCLC, we analysed protein expression in tissue samples of 49 NSCLC patients by Western Blot and of 278 NSCLC patients by immunohistochemistry. Western Blot analyses showed that Reptin is significantly overexpressed in the vast majority of NSCLC patients comparing Reptin expression levels in tumour tissues versus adjacent normal lung tissues (figures 1a and 1b). Reptin expression was significantly higher in tumour tissues of both, patients with

pulmonary squamous cell carcinoma (SCC) as well as non-SCC. We found Reptin to be expressed higher in the tumour than in the adjacent normal tissue in 92% of SCC patients (n=26) and 65% of non-SCC patients (n=23) (figures 1a and 1b). In immunohistochemistry (IHC), tumour cells displayed a nuclear staining pattern for Reptin in both, non-SCC as well as SCC lung cancer tumours (figures 1c and 1d).

Reptin confers poor prognosis in NSCLC patients.

We wondered whether overexpression of Reptin impacts prognosis of NSCLC patients and performed IHC on tissue microarrays (TMA) with samples of 278 NSCLC patients, including 150 patients with pulmonary non-SCC and 128 patients with SCC (supplementary Table S1).

Tumour tissues with H-score ≤ 160 defined patients with low Reptin expression whereas, those with H-score > 160 defined patients with high Reptin expression. Patients with low expression of Reptin showed significant overall survival, ($p=0.002$), (figure 2a). Analyzing survival data of 1,145 NSCLC patients from a publicly available dataset [12] as validation cohort, we confirmed significantly decreased overall survival ($p<0.0001$) of patients with high mRNA expression levels of Reptin as compared to those with low ones (dichotomized at median) (figure 2b). Among 596 patients with recorded clinical progression data, time to progression was also significantly reduced for patients with high expression of Reptin ($p=0.00065$) (figure 2b). Multivariate analyses using the same database confirmed Reptin as a risk factor for poor overall and progression-free survival independent of histology and disease stage ($p<0.0001$ and $p=0.0089$ respectively).

Patients with highly differentiated NSCLC tumours (G1-2) are known to have improved long-term outcome [13]. Accordingly, in our cohort of NSCLC tumour samples analyzed by immunohistochemistry, we were able to confirm a strong trend towards better overall survival for this patient group (G1-2) (n=102) as compared to

the group with less differentiated (G3-4) (n=170) tumours (p=0.063)–(supplementary figure S1a). Patients with highly differentiated tumours showing high expression of Reptin had significantly worsened overall survival as compared to patients from the same subgroup with low expression of Reptin ($p < 0.001$) (figure 2c). Among patients with poorly differentiated tumours this difference was not seen (figure 2c and supplementary figure S1b and S1c). Significantly improved overall survival among patients with highly differentiated NSCLC showing low expression of Reptin was confirmed for non-SCC patients ($p < 0.001$). SCC patients with G1-G2 tumours with high Reptin expression represented a much smaller subgroup in our cohort and yet, showed a strong trend towards shorter overall survival (p=0.086) (figure 2d). Thus, survival analyses revealed a significant prognostic impact of high expression of Reptin in NSCLC patients.

Knock down of Reptin impairs growth of human NSCLC cells *ex vivo* and blocks their engraftment *in vivo*

In order to assess the role of Reptin in growth and proliferation of NSCLC cells, we performed lentiviral transduction with two validated shRNAs targeting human Reptin [11] in A549 and HOP62 human adenocarcinoma (AC) cells as well as in LUDLU 1 human pulmonary SCC cells. Stable knock down of Reptin was achieved in all cell lines as compared to scrambled controls (figure 3a). Suppression of Reptin significantly impaired clonal expansion of all cell lines as assessed by colony numbers after plating in methylcellulose (figure 3b). Moreover, all three cell lines showed a significant reduction of proliferation upon knock down of Reptin (figure 3c). To exclude shRNA mediated off target effects we designed sgRNAs targeting different exons of human Reptin (supplementary figure S2e) and cloned them into a lentiviral CRISPR-Cas9 vector. Again, a stable knock down of Reptin in A549, HOP62 and LUDLU 1 cells was achieved via lentiviral transduction of our constructs

(supplementary figure S2a and data not shown). In both, pulmonary AC as well as SCC cells, dependence of colony formation capacity and proliferation of Reptin expression levels was confirmed (supplementary figure S2b-S2d and data not shown).

Knock down of Reptin in A549 cells was also able to block engraftment in 5 out of 6 NSG mice, whereas all mice transplanted with the same cells transduced with a scrambled control developed metastatic lung cancer (n=6) within 70-110 days (figure 3d). Organs of diseased mice showed heavy infiltration by human NSCLC cells as assessed via flow cytometric detection of strong expression of human epithelial cell adhesion molecule (EpCAM), CD326, in cells isolated from metastatic lesions (figure 3d). Furthermore, histological analyses confirmed infiltration of murine organs by human invasive adenocarcinoma with strong expression of human CK7 (supplementary figure S3a and S3b). Thus, growth of human NSCLC cells *ex vivo* and engraftment *in vivo* substantially depend on the presence of Reptin.

Pharmacological disruption of a Reptin/HDAC1 complex in NSCLC cells significantly reduces clonal growth.

We were able to confirm direct interaction of Reptin with HDAC1, one of its suspected partners for transcriptional repression, in human HEK-293 cells (supplementary figure S4) [14]. In pulmonary AC expression of HDAC1 correlates with poor outcome of the patients [15]. To evaluate whether Reptin and HDAC1 also associate in NSCLC cells we performed further co-immunoprecipitation experiments. Indeed, we were able to detect direct interaction of Reptin with HDAC1 in all three lung cancer cell lines tested (A549, HOP62, LUDLU 1). Interestingly, exposing NSCLC cells to MS-275 (entinostat), a class 1 HDAC inhibitor with special affinity to HDAC1, this protein-protein interaction was significantly reduced in both, pulmonary AC as well as SCC cells (figure 4a). As a next step, we treated A549, HOP62 as well

as LUDLU 1 cells with different concentrations of MS-275. Both, proliferation as well as colony formation capacity of all three cell lines (A549, HOP62, LUDLU 1), were significantly reduced upon increasing exposure to MS-275 (figure 4b-4d). Thus, pharmacological inhibition of the Reptin/HDAC1 protein complex might be a promising new strategy for future therapeutic approaches in NSCLC therapies.

Disruption of a Reptin/HDAC1 complex enhances sensitivity of NSCLC cells to cisplatin.

Until today platinum-doublet chemotherapy remains the backbone for most of the NSCLC patients in (neo) adjuvant as well as palliative care settings. Unfortunately, the majority of NSCLC patients either shows upfront platinum-resistance or will develop relapse or disease progression after initial treatment response. Thus, we tempted to assess if combining cisplatin with MS-275 can enhance the therapeutic effect of the chemotherapy. Strikingly, exposure of pulmonary AC as well as SCC cells even to low doses of MS-275 profoundly increased the effect of cisplatin on these cells. Whereas low doses of cisplatin induced a mild to moderate reduction of both, colony formation capacity as well as proliferation of A549, HOP62 and LUDLU 1 cells, addition of low doses of MS-275 significantly enhanced its effect (figure 5a-c). To confirm the role of Reptin in this complex for sensitization of lung cancer cells to treatment with cisplatin we exposed human NSCLC cells with shRNA mediated knock down of Reptin to low doses of cisplatin. Again, effect of the chemotherapy was significantly more profound as compared to scrambled controls (figure 5d). Thus, targeting the Reptin/HDAC1 complex might be a valid option to improve the effect of future platinum-based chemotherapy regimens in NSCLC patients.

Discussion

During the past few years all three pillars of NSCLC management, prevention, diagnostics and therapy, have made significant progress [2, 16]. However, with over 1.5 million deaths per year lung cancer remains one of the major causes of cancer deaths worldwide [5]. Diagnosis at advanced stage of the disease, early relapses and/or metastasis, as well as poor responses of patients with metastatic lung cancer to the therapies available are the major hurdles for successful lung cancer treatment. Recent clinical trials gave evidence that identification and targeting of molecular alterations in NSCLC can broaden physicians´ repertoire for the treatment of lung cancer patients substantially [2, 4]. However, none of the substances available is expected to achieve cure of the majority of NSCLC patients diagnosed with advanced stage of the disease. Thus, unveiling the key mechanisms of deregulation of the transcriptional and epigenetic programs driving lung cancer remains the main goal in lung cancer research.

We reveal a novel function of the AAA+ ATPase Reptin as a key player in NSCLC. We were able to show that Reptin is highly expressed in the vast majority of NSCLC tumours irrespective of their histological subtype and that high expression levels of Reptin are associated with adverse prognosis of affected patients. Moreover, the comparably poor prognosis of NSCLC patients with well-differentiated tumours and high expression of Reptin suggests this protein to be a predictor of early metastasis in this subgroup. We provide several lines of evidence that Reptin functions as a critical regulator of NSCLC cell proliferation and clonal growth. Importantly, knock down of Reptin in NSCLC cells significantly impairs their capacity to induce metastatic lung cancer in a murine xenograft model.

Thus, our data present Reptin as a potential new molecular marker for the prediction of prognosis of NSCLC patients as well as a future therapeutic target for NSCLC

treatment.

Furthermore, our data move Reptin into the focus as novel target for lung cancer therapies. However, direct targeting of Reptin is not yet applicable. Thus, targeting key interaction partners of Reptin could be a reasonable alternative approach to impair signalling pathways directed by Reptin. The fact that Reptin can be part of different multiprotein complexes implicates its function in a variety of cellular processes including those relevant for oncogenesis and tumour progression. A Reptin chromatin remodelling complex directing transcriptional regulation in coordinated action with beta-catenin has been shown to down-regulate *KAI1* [17]. However, knockdown of Reptin in NSCLC cells did not affect expression levels of *KAI1* (data not shown). Thus, our data implicate a novel pathway driven via Reptin/HDAC1 interaction specifically in NSCLC. Interestingly, high expression of HDAC1 confers poor prognosis in AC lung cancer patients [15]. In our study, we were able to show that in NSCLC cells Reptin recruits HDAC1 into a protein complex, and that this complex can be disrupted upon exposure to MS-275, a potent HDAC1 inhibitor, resulting in substantial suppression of growth of lung cancer cells.

HDACs largely contribute to oncogenesis and cancer progression [18]. Therefore, inhibition of HDACs has become highly attractive for targeted cancer therapies. Multiple clinical trials using HDAC inhibitors have been concluded and are currently under way to assess the potential of these drugs in various types of cancer, and the first HDAC inhibitors have recently been approved for several malignant diseases. However, single agent HDAC inhibition may not be feasible as a therapeutic approach for NSCLC treatment, since various clinical trials including some for NSCLC have largely failed to show breakthrough effects using HDAC inhibitors as a monotherapy [19], and high doses of HDAC inhibitors confer considerable toxicity to patients [20]. On the other hand, results of various clinical trials suggested that

combination treatments including HDAC inhibition are far more promising for oncological treatment strategies [19]. Thus, in our study we exposed pulmonary AC and SCC cells to low doses of MS-275 combined with cisplatin. MS-275 significantly enhanced response of the cells to cisplatin showing substantial effect even at cisplatin concentrations without relevant efficacy as a single agent.

Since the majority of patients with locally advanced or metastatic NSCLC do not harbor oncogenic drivers directly tractable via specific inhibitors, they still receive platinum based double chemotherapies in specific neoadjuvant, adjuvant, as well as palliative treatment settings. Furthermore, many patients initially treated with molecular therapies will eventually require chemotherapy at a later stage of their course of therapy. Substantial toxicity as well as limited response rates and durations after these cytotoxic regimens urge the need for new substances improving treatment response and toxicity profiles. Previous lung cancer trials with combination treatments including HDAC inhibitors failed to significantly improve patient outcome in most of the subgroups. However, the more specific and selective HDAC inhibitor MS-275 should still be considered as a promising drug for NSCLC patients, even more since its effect appears more profound on transformed than on normal cells [21]. It may just need to be applied together with the right partner. The mechanisms of action of HDACs in NSCLC need to be precisely understood and their inhibition performed as targeted as possible. Here, as a proof of principle study, we are able to show that combined treatment of cisplatin and entinostat could be used successfully to enhance response of human NSCLC cells to platinum based therapy significantly. In order to unveil the spectrum of cellular mechanisms affected by this combined treatment, future proteomic and epigenetic analyses, focusing on Reptin/HDAC1 interactions, might help to understand the dynamics of signaling pathways driven by this complex and its inhibition in NSCLC cells.

In conclusion, our results describe Reptin as a novel regulator mediating proliferation and clonal growth of human NSCLC cells *ex vivo* and *in vivo*. We show that high expression of Reptin confers poor prognosis in lung cancer patients and unveil a Reptin/HDAC1 protein complex whose pharmacological disruption sensitizes NSCLC cells to cisplatin. These results shed light into NSCLC oncogenesis and may help to identify patients at higher risk for early disease progression or relapse and those that are less likely to respond satisfactorily to platinum-based chemotherapies. Ultimately, our study may pave the avenue towards new therapeutic strategies combining HDAC1 inhibitors with current standard therapy regimens.

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References

1. Heist RS, Engelman JA. SnapShot: non-small cell lung cancer. *Cancer Cell* 2012; 21: 448.e2.

2. Hirsch FR, Herbst RS, Gandara DR. EGFR tyrosine kinase inhibitors in squamous cell lung cancer. *Lancet Oncol.* 2015; 16: 872–873.

3. Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou S-HI, Dezube BJ, Jänne PA, Costa DB, Varella-Garcia M, Kim W-H, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N. Engl. J. Med.* 2010; 363: 1693–1703.

4. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, Gottfried M, Peled N, Tafreshi A, Cuffe S, O'Brien M, Rao S, Hotta K, Leiby MA, Lubiniecki GM, Shentu Y, Rangwala R, Brahmer JR, KEYNOTE-024 Investigators. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* 2016.

5. Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang X-S, Bannon F, Ahn JV, Johnson CJ, Bonaventure A, Marcos-Gragera R, Stiller C, Azevedo e Silva G, Chen W-Q, Ogunbiyi OJ, Rachet B, Soeberg MJ, You H, Matsuda T, Bielska-Lasota M, Storm H, Tucker TC, Coleman MP, CONCORD Working Group. Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* 2015; 385: 977–1010.

6. Swanton C, Govindan R. Clinical Implications of Genomic Discoveries in Lung Cancer. *N. Engl. J. Med.* 2016; 374: 1864–1873.

7. Matias PM, Baek SH, Bandeiras TM, Dutta A, Houry WA, Llorca O, Rosenbaum J. The AAA+ proteins Pontin and Reptin enter adult age: from understanding their basic biology to the identification of selective inhibitors. *Front Mol Biosci* 2015; 2: 17.

8. Gallant P. Control of transcription by Pontin and Reptin. *Trends in Cell Biology* 2007; 17: 187–192.

9. Schmidt LH, Kümmel A, Görlich D, Mohr M, Bröckling S, Mikesch J-H, Grünewald I, Marra A, Schultheis AM, Wardelmann E, Müller-Tidow C, Spieker T, Schliemann C, Berdel WE, Wiewrodt R, Hartmann W. PD-1 and PD-L1 Expression in NSCLC Indicate a Favorable Prognosis in Defined Subgroups. *PLoS ONE* 2015; 10: e0136023.

10. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J. Clin. Oncol.* 1999; 17: 1474–1481.

11. Gallant-Behm CL, Ramsey MR, Bensard CL, Nojek I, Tran J, Liu M, Ellisen LW, Espinosa JM. ΔNp63α represses anti-proliferative genes via H2A.Z

deposition. *Genes & Development* 2012; 26: 2325–2336.

12. Győrffy B, Surowiak P, Budczies J, Lánczky A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *PLoS ONE* 2013; 8: e82241.

13. Pilotto S, Sperduti I, Novello S, Peretti U, Milella M, Facciolo F, Vari S, Leuzzi G, Vavalà T, Marchetti A, Mucilli F, Crinò L, Puma F, Kinspergher S, Santo A, Carbognin L, Brunelli M, Chilosi M, Scarpa A, Tortora G, Bria E. Risk Stratification Model for Resected Squamous-Cell Lung Cancer Patients According to Clinical and Pathological Factors. *J Thorac Oncol* 2015; 10: 1341–1348.

14. Rashid S, Pilecka I, Torun A, Olchowik M, Bielinska B, Miaczynska M. Endosomal adaptor proteins APPL1 and APPL2 are novel activators of beta-catenin/TCF-mediated transcription. *Journal of Biological Chemistry* 2009; 284: 18115–18128.

15. Minamiya Y, Ono T, Saito H, Takahashi N, Ito M, Mitsui M, Motoyama S, Ogawa J. Expression of histone deacetylase 1 correlates with a poor prognosis in patients with adenocarcinoma of the lung. *Lung Cancer* 2011; 74: 300–304.

16. Reck M, Krzakowski M, Chmielowska E, Sebastian M, Hadler D, Fox T, Wang Q, Greenberg J, Beckman RA, Pawel von J. A randomized, double-blind, placebo-controlled phase 2 study of tigatuzumab (CS-1008) in combination with carboplatin/paclitaxel in patients with chemotherapy-naïve metastatic/unresectable non-small cell lung cancer. *Lung Cancer* 2013; 82: 441–448.

17. Kim JH, Kim B, Cai L, Choi HJ, Ohgi KA, Tran C, Chen C, Chung CH, Huber O, Rose DW, Sawyers CL, Rosenfeld MG, Baek SH. Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. *Nature* 2005; 434: 921–926.

18. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov* 2014; 13: 673–691.

19. New M, Olzscha H, La Thangue NB. HDAC inhibitor-based therapies: can we interpret the code? *Mol Oncol* 2012; 6: 637–656.

20. Subramanian S, Bates SE, Wright JJ, Espinoza-Delgado I, Piekarz RL. Clinical Toxicities of Histone Deacetylase Inhibitors. *Pharmaceuticals (Basel)* 2010; 3: 2751–2767.

21. Ungerstedt JS, Sowa Y, Xu W-S, Shao Y, Dokmanovic M, Perez G, Ngo L, Holmgren A, Jiang X, Marks PA. Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102: 673–678.

Figures

FIGURE 1: Reptin is highly expressed in the majority of human NSCLC.

(a and b) Immunoblot analyses and quantification of Reptin expression in tumour tissue versus adjacent normal lung tissue of patients with pulmonary non-SCC (a) and SCC (b). Error bars represent standard deviations (SD) of at least three independent experiments. (c and d) Immunohistochemical staining of Reptin in non-SCC (c) and SCC (d) NSCLC tumour tissues.

FIGURE 2: Reptin confers poor prognosis in NSCLC patients.

(a-d) Prognostic values of Reptin expression in NSCLC patients. Overall survival of all patients (a), overall (left) and progression-free (right) survival of NSCLC patients in a larger patient cohort from publicly available dataset (b), overall survival of NSCLC patients with highly differentiated (G1-2) (left) and poorly differentiated (G3-4) tumours (right) (c), in non-SCC patients (d) with G1-2 tumours (left), and SCC patients with G1-2 tumours (right) respectively. Unpaired T-test was used to determine statistical significance in the immunoblots bar charts.

FIGURE 3: Knock down of Reptin suppresses clonal expansion and proliferation of human NSCLC cells ex vivo and impairs engraftment in a xenograft model.

a) Knock down of Reptin in human pulmonary AC (A549 and HOP62) and SCC (LUDLU 1) cells via validated shRNAs #1 and #2 as compared to scrambled controls, assessed via immunoblotting. b) and c) Colony numbers of A549, HOP62 and LUDLU 1 lung cancer cells in methylcellulose upon knock down of Reptin and as compared to scrambled controls (b) and proliferation of these cells as assessed by MTS-assay (c). Error bars represent SD of at least three independent experiments. d) Left, disease-free survival of NSG mice injected with A549 NSCLC cells with shRNA mediated knock down of Reptin as compared to scramble control group. Log-rank

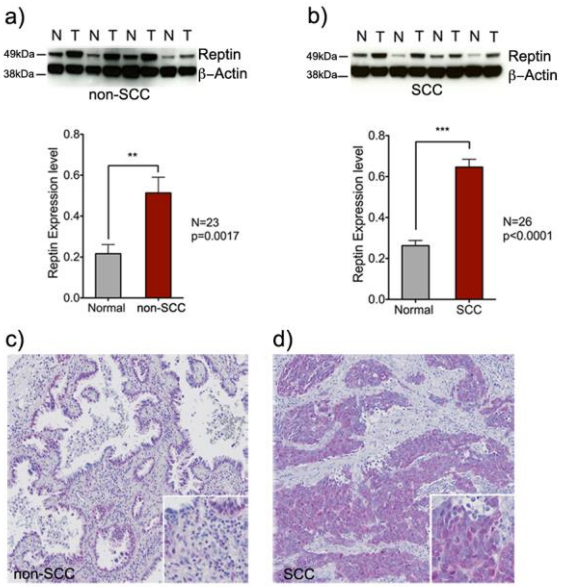
(Mantel-Cox) Test was used to compare survival curves. P-values < 0.05 were considered statistically significant. Right, representative flow cytometry analysis after staining cells from metastatic tissues from mice of control group with human anti-CD-326-PE.

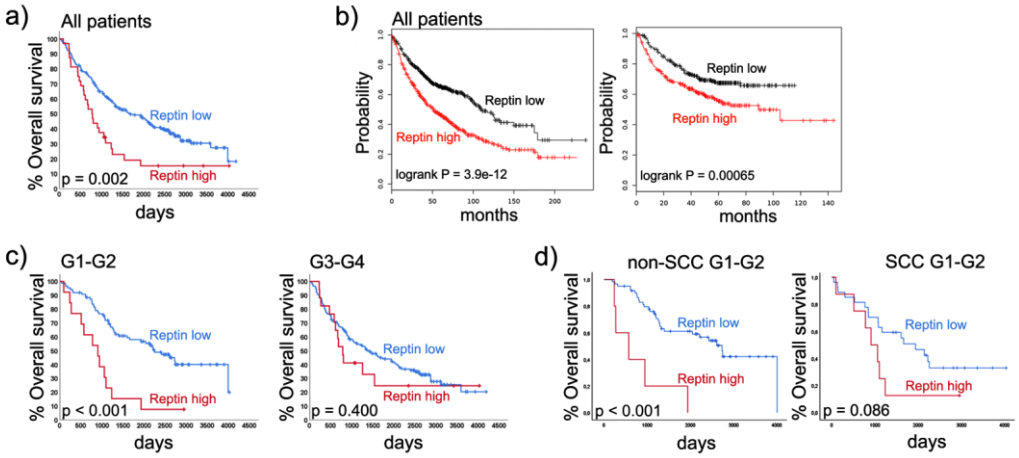
FIGURE 4: Pharmacological disruption of Reptin/HDAC1 complex significantly suppresses colony formation and proliferation of human NSCLC cells.

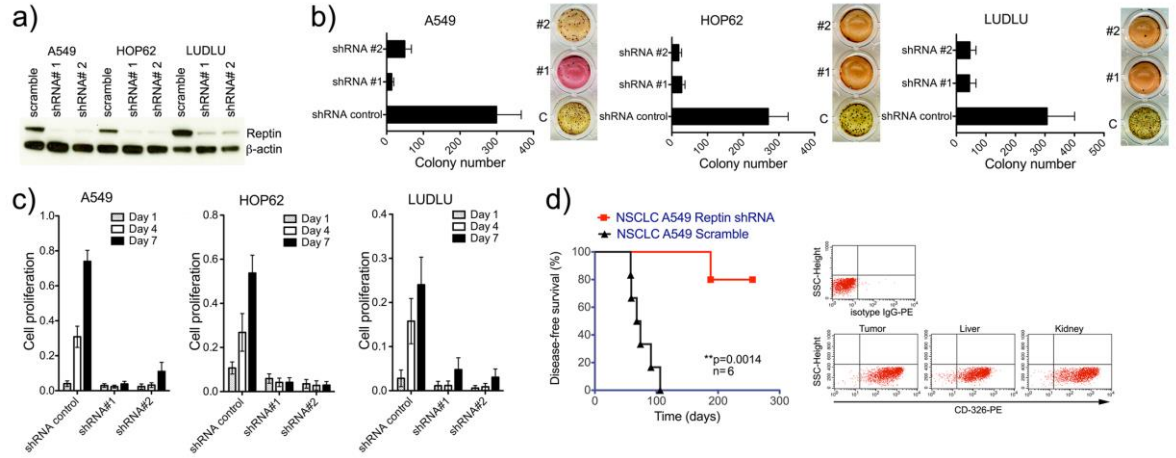
a) Co-immunoprecipitation (CoIP) analyses of human A549, HOP62 and LUDLU 1 cells in the presence or absence of Flag-tagged HDAC1, and cultured in the presence or absence of MS-275 at the indicated concentration. b)-d), Proliferation assays (b), colony formation assays (c) and INT staining (d) of the three indicated human NSCLC cell lines in the presence or absence of MS-275. Colonies were counted after 7 days. One-way Anova test was used to determine statistical significance for all bar charts representing proliferation and colony formation assays of cells treated with MS-275.

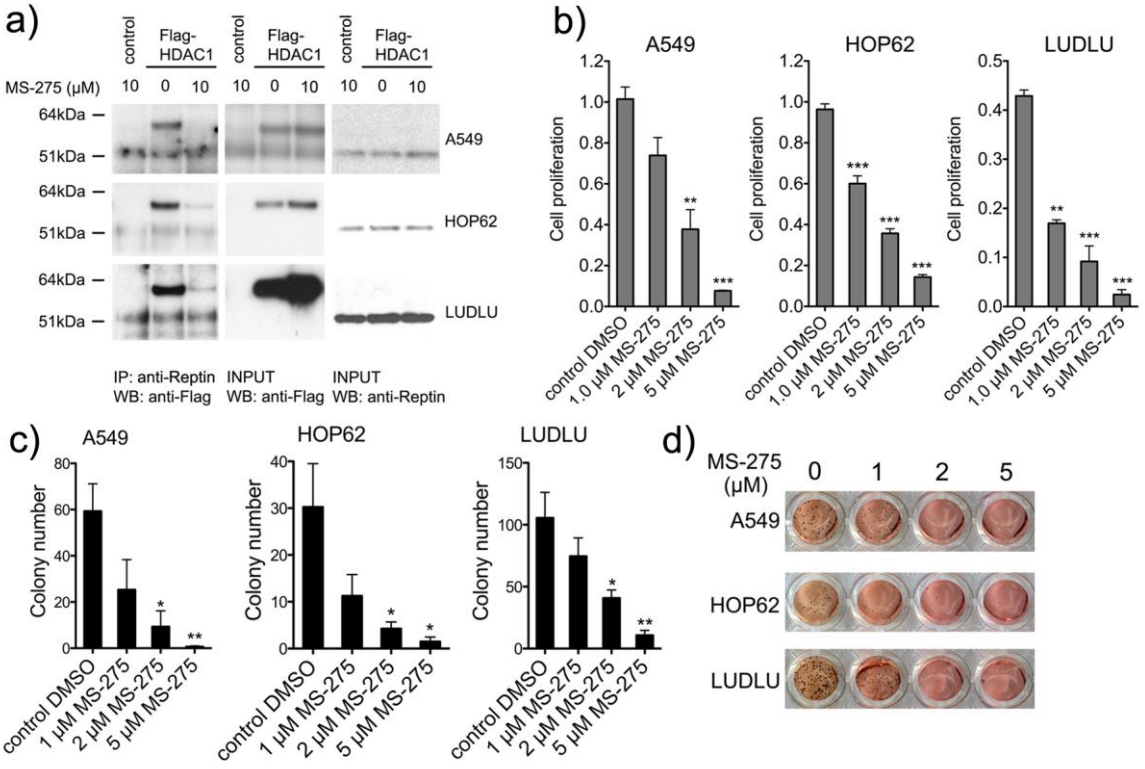
FIGURE 5: Disruption of Reptin/HDAC1 protein complex by MS-275 sensitizes NSCLC cells to cisplatin.

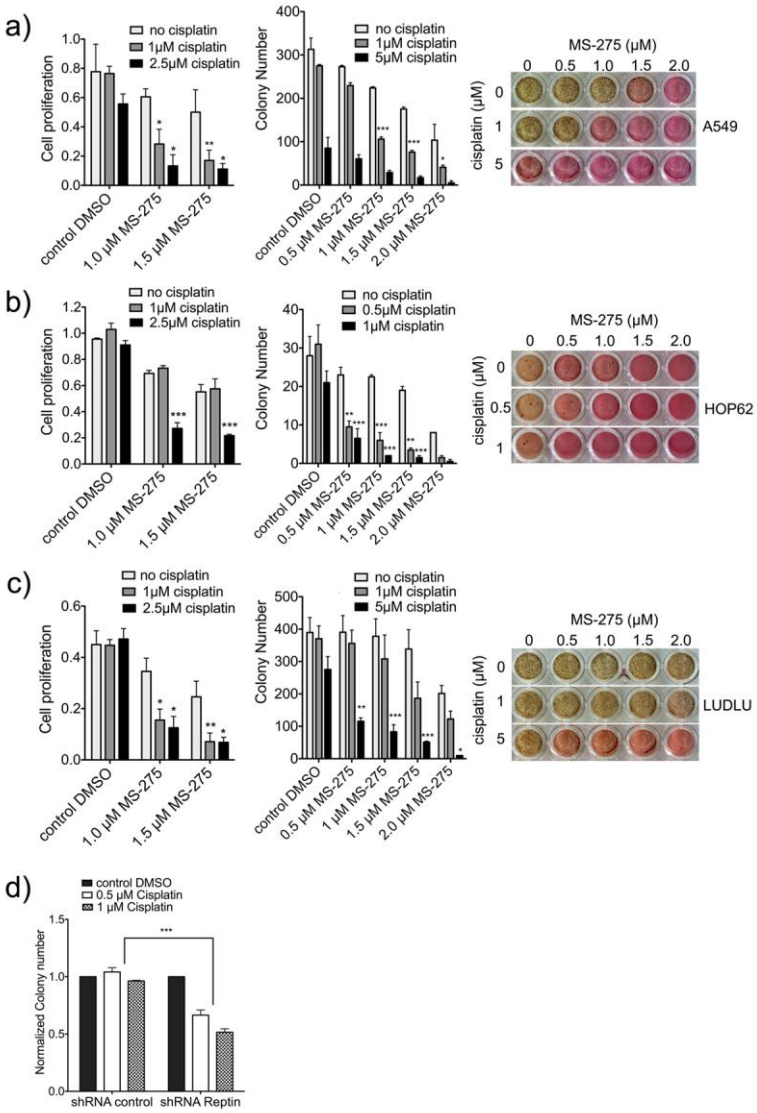
a-c), Combined treatment of human NSCLC cells of the indicated cell lines using cisplatin and MS-275 at the indicated concentrations: proliferation assays, colony formation assays and INT staining of A549 (a), HOP62 (b), LUDLU 1 (c) are displayed. Two-way Anova test was used to determine statistical significance for all bar charts for proliferation and colony formation assays of cells under combination treatment. d) Knock down of Reptin sensitizes human NSCLC to treatment with cisplatin. shRNA mediated knock down of Reptin enhances effect of Cisplatin treatment on clonal expansion of human A549 NSCLC cells *ex vivo* as compared to scramble control. Error bars represent SD of at least three independent experiments.


a)
N T N T N T N T
49kDa
38kDa
Reptin
β–Actin
non-SCC
Reptin Expression level
**
N=23
p=0.0017
Normal
non-SCC
b)
N T N T N T N T
49kDa
38kDa
Reptin
β–Actin
SCC
Reptin Expression level
***
N=26
p<0.0001
Normal
SCC
c)
non-SCC
d)
SCC

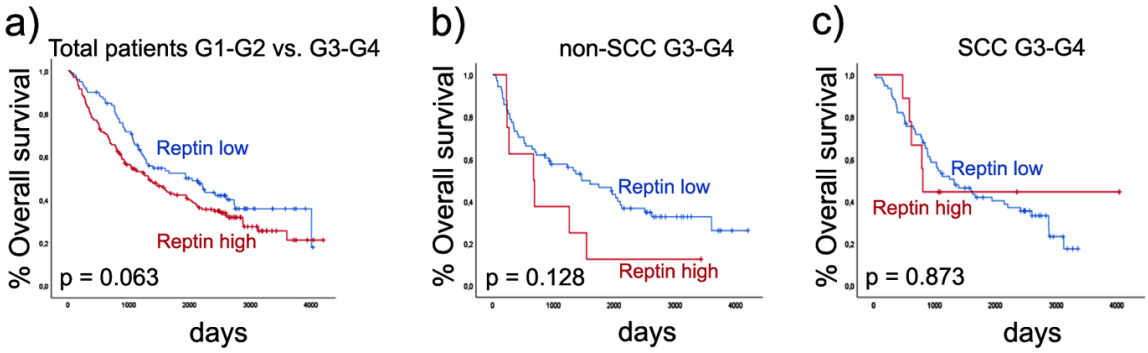

a)
All patients
% Overall survival
Reptin low
Reptin high
p = 0.002
days
b)
All patients
Probability
Reptin low
Reptin high
logrank P = 3.9e-12
months
Probability
Reptin low
Reptin high
logrank P = 0.00065
months
c)
G1-G2
% Overall survival
Reptin low
Reptin high
p < 0.001
days
G3-G4
% Overall survival
Reptin low
Reptin high
p = 0.400
days
d)
non-SCC G1-G2
% Overall survival
Reptin low
Reptin high
p < 0.001
days
SCC G1-G2
% Overall survival
Reptin low
Reptin high
p = 0.086
days


a)
A549
HOP62
LUDLU
scramble
shRNA# 1
shRNA# 2
Reptin
β-actin
b)
A549
HOP62
LUDLU
shRNA #2
shRNA #1
shRNA control
Colony number
#2
#1
C
c)
A549
HOP62
LUDLU
Day 1
Day 4
Day 7
Cell proliferation
shRNA control
shRNA#1
shRNA#2
d)
NSCLC A549 Reptin shRNA
NSCLC A549 Scramble
Disease-free survival (%)
**p=0.0014
n=6
Time (days)
SSC-Height
isotype IgG-PE
Tumor
Liver
Kidney
CD-326-PE


a)
control
Flag-HDAC1
MS-275 (μM)
10 0 10
64kDa
51kDa
A549
HOP62
LUDLU
IP: anti-Reptin
WB: anti-Flag
INPUT
WB: anti-Flag
INPUT
WB: anti-Reptin
b)
A549
HOP62
LUDLU
Cell proliferation
control DMSO
1.0 μM MS-275
2 μM MS-275
5 μM MS-275
c)
A549
HOP62
LUDLU
Colony number
d)
MS-275 (μM)
0 1 2 5
A549
HOP62
LUDLU

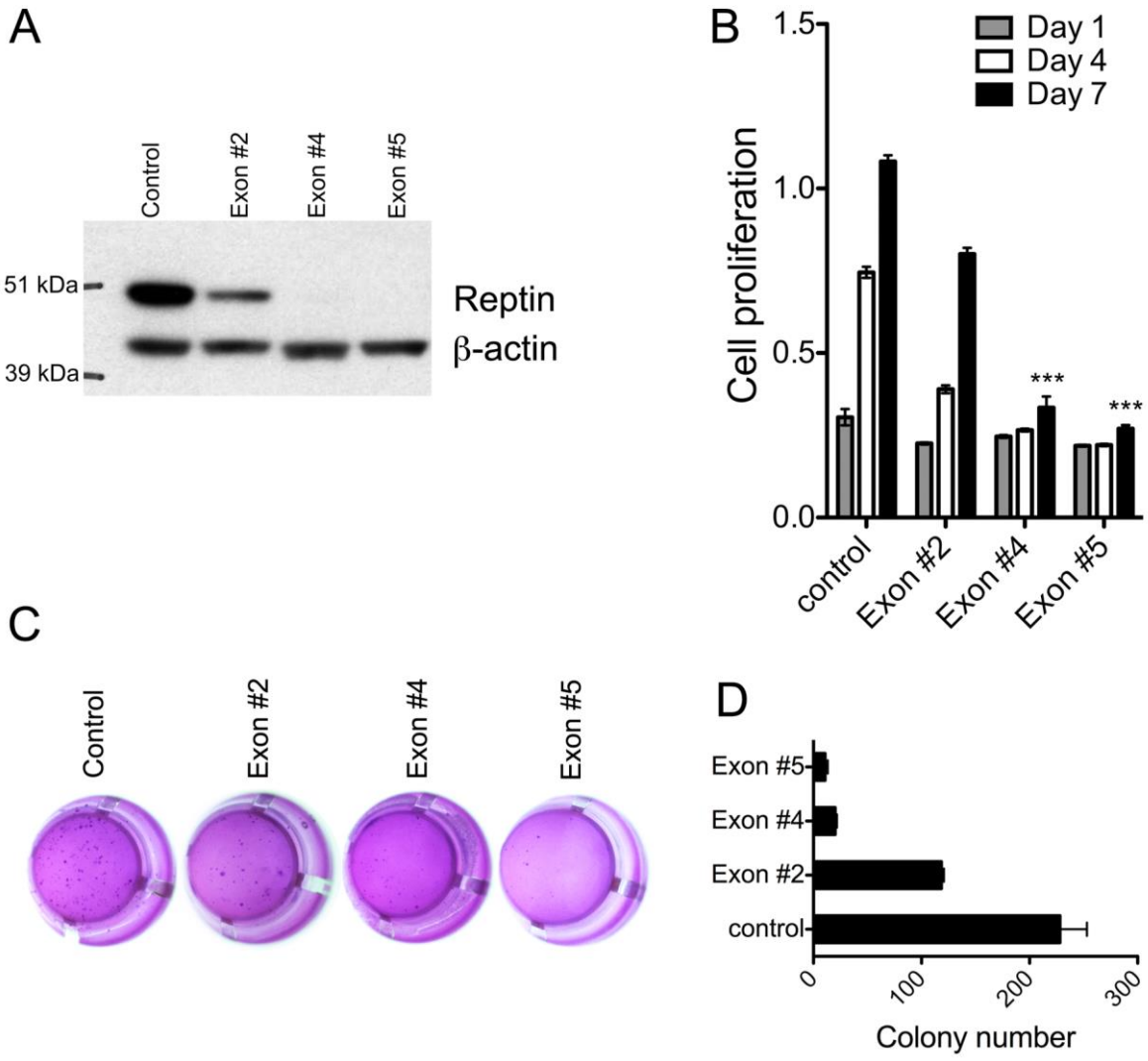

a)
no cisplatin
1μM cisplatin
2.5μM cisplatin
Cell proliferation
control DMSO
1.0 μM MS-275
1.5 μM MS-275
no cisplatin
1μM cisplatin
5μM cisplatin
Colony Number
control DMSO
0.5 μM MS-275
1 μM MS-275
1.5 μM MS-275
2.0 μM MS-275
MS-275 (μM)
0 0.5 1.0 1.5 2.0
cisplatin (μM)
0
1
5
A549
b)
no cisplatin
1μM cisplatin
2.5μM cisplatin
Cell proliferation
control DMSO
1.0 μM MS-275
1.5 μM MS-275
no cisplatin
0.5μM cisplatin
1μM cisplatin
Colony Number
control DMSO
0.5 μM MS-275
1 μM MS-275
1.5 μM MS-275
2.0 μM MS-275
MS-275 (μM)
0 0.5 1.0 1.5 2.0
cisplatin (μM)
0
0.5
1
HOP62
c)
no cisplatin
1μM cisplatin
2.5μM cisplatin
Cell proliferation
control DMSO
1.0 μM MS-275
1.5 μM MS-275
no cisplatin
1μM cisplatin
5μM cisplatin
Colony Number
control DMSO
0.5 μM MS-275
1 μM MS-275
1.5 μM MS-275
2.0 μM MS-275
MS-275 (μM)
0 0.5 1.0 1.5 2.0
cisplatin (μM)
0
1
5
LUDLU
d)
control DMSO
0.5 μM Cisplatin
1 μM Cisplatin
Normalized Colony number
shRNA control
shRNA Reptin

Supplementary Fig. S1



Supplementary Fig. S1: Prognostic values of tumor grade and expression of Reptin in NSCLC patients. **S1A**, overall survival of NSCLC patients with highly differentiated (G1-2) as compared to poorly differentiated (G3-4) tumors, **S1B and S1C,** overall survival of non-SCC (**S1B**) and SCC (**S1C**) NSCLC patients with G3-4 tumors and with high (red) versus low (blue) expression of Reptin. Unpaired T-test was used to determine statistical significance in the immunoblots bar charts.

Figure S2

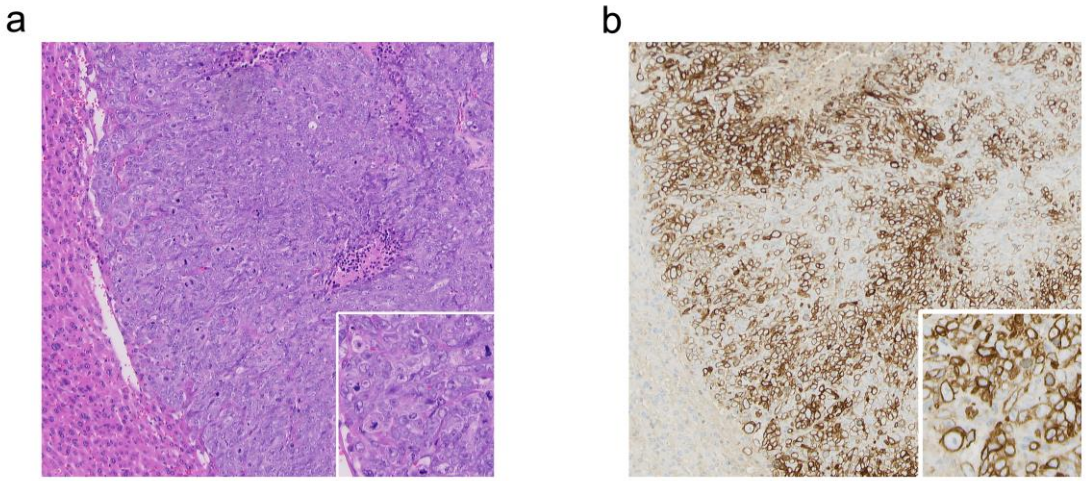


E

sgRNA	Sequence 1	Sequence 2
REPTIN sgRNA Exon 2	CACCGCTTGTTACATCACGGATCTC	AAACGAGATCCGTGATGTAACAAGC
REPTIN sgRNA Exon 4	CACCGAGCAATAAGGACTGCCCGAC	AAACGTCGGGCAGTCCTTATTGCTC
REPTIN sgRNA Exon 5	CACCGTGCGAACGCCGATGGACCGC	AAACGCGGTCCATCGGCGTTCGCAC

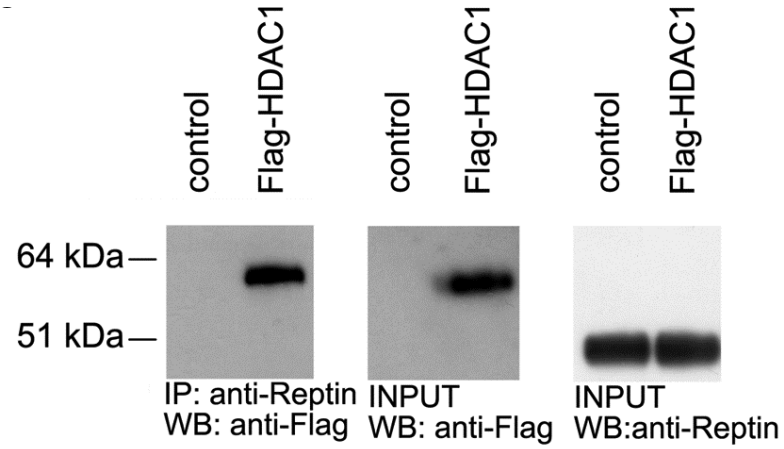
Supplementary Fig. S2: Knock down of Reptin suppresses clonal expansion and proliferation of human NSCLC cells *ex vivo*. **S2A,** efficacious CRISPR-Cas9 system mediated knock down/knock out of Reptin in human non-SCC A549 NSCLC cells using 3 different sgRNAs targeting the indicated exons and as compared to scramble control as assessed via immunoblotting. Total amount of protein was measured by β-actin. **S2B-S2D,** knock down of Reptin induces significant reduction of proliferation **(S2B)** and colony numbers **(S2C, S2D)** of human A549 lung cancer cells as assessed via MTS-assay and colony formation assay in methylcellulose respectively and as compared to scramble controls. Error bars represent SD of three independent experiments. **S2E**, sequences of small guide (sg) RNAs used for CRIPSR-Cas9 system mediated knock down of Reptin in human NSCLC cells.

Figure S3

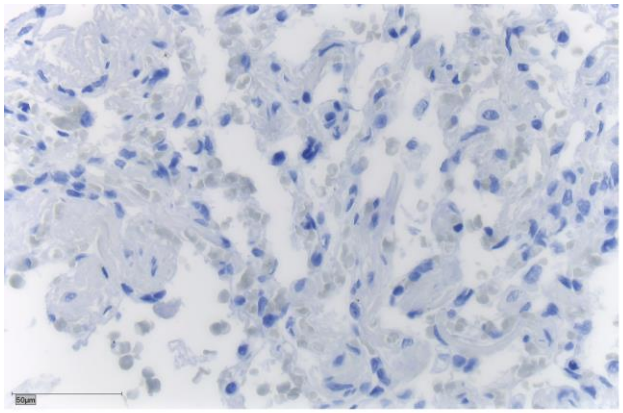


Supplementary Fig. S3: H&E staining and immunohistochemistry analysis showing infiltration of metastatic tissues from mice from control group with cells of human adenocarcinoma (**S3a**) with strong expression of human CK7 (**S3b**).

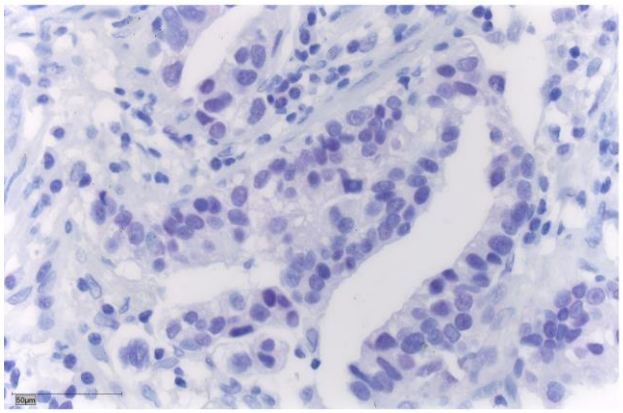
Figure S4



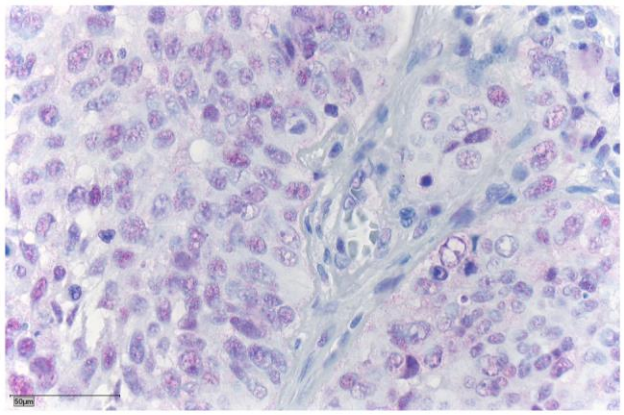
Supplementary Fig. S4: Reptin associates with HDAC1 in HEK-293 cells in a protein complex. Representative co-immunoprecipitation (CoIP) analysis of human HEK-293 cells in the presence or absence of Flag-tagged HDAC1 and according loading controls (input).



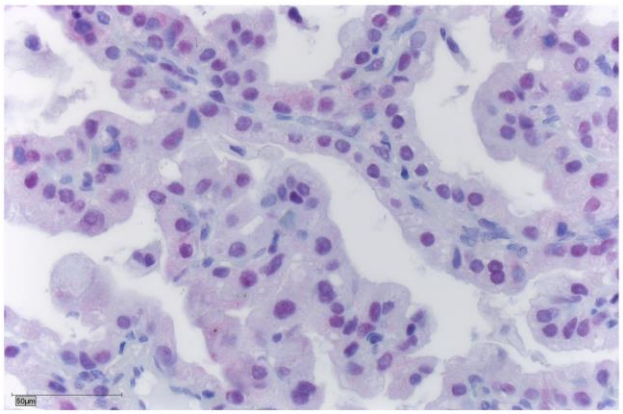
score 0



score 1



score 2



score 3

Supplementary methods: Semi-quantitative immunohistochemical assessment was performed applying a modified H-score essentially based on the parameters proposed by Harvey et al. (1999). Establishing a reference framework for the analysis, prototypical examples of four different staining intensities (0, none; 1, weak, 2, moderate; and 3, high). Examples representing each of the four staining intensities are displayed above.

Parameter	(n=278)	% of non-missing values
Median age (years)	66	
Male Sex	215	77
Performance status		
ECOG 0	48	17
ECOG I	203	73
ECOG >II	15	5
Tumor stage		
Stage I	192	69
Stage II	59	21
Stage III	27	10
Tumor size		
pT1	86	31
pT2	170	61
pT3	13	5
pT4	7	2
Tumor histology		
Squamous cell carcinoma	128	46
Adenocarcinoma	108	39
Large cell carcinoma	42	15
Tumor grading		
G1-G2	102	37
G3-G4	170	61
Reptin H-score in NSCLC		
H-score ≤ 160	32	12
H-score > 160	246	88

Supplementary Table S1:

Baseline characteristics of the study population (n = 278). For 12, 6 and 2 patients respectively data for ECOG status, tumour grading and tumour size was not available.