1 USP28 enables oncogenic transformation of respiratory cells and its inhibition
2 potentiates molecular therapy targeting mutant EGFR, BRAF and PI3K.

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Abstract

Oncogenic transformation of lung epithelial cells is a multi-step process, frequently starting with the inactivation of tumor suppressors and subsequent activating mutations in proto-oncogenes, such as members of the PI3K or MAPK family. Cells undergoing transformation have to adjust to changes, such as metabolic requirements. This is achieved, in part, by modulating the protein abundance of transcription factors, which manifest these adjustments. Here, we report that the deubiquitylase USP28 enables oncogenic reprogramming by regulating the protein abundance of proto-oncogenes, such as c-JUN, c-MYC, NOTCH and ∆NP63, at early stages of malignant transformation. USP28 is increased in cancer compared to normal cells due to a feed-forward loop, driven by increased amounts of oncogenic transcription factors, such as c-MYC and c-JUN. Irrespective of oncogenic driver, interference with USP28 abundance or activity suppresses growth and survival of transformed lung cells. Furthermore, inhibition of USP28 via a small molecule inhibitor reset the proteome of transformed cells towards a 'pre-malignant' state, and its inhibition cooperated with clinically established compounds used to target $EGFR^{L858R}$, $BRAF^{V600E}$ or $PI3K^{H1047R}$ driven tumor cells. Targeting USP28 protein abundance already at an early stage via inhibition of its activity therefore is a feasible strategy for the treatment of early stage lung tumours and the observed synergism with current standard of care inhibitors holds the potential for improved targeting of established tumors.

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70

71 **Introduction**
72
73 In the past decade, with the advent of targeted therapy, great advancements towards
74 the treatment of progressed Non-Small Cell Lung Cancer (NSCLC) in distinct patient
75 cohorts were achieved(1), while patients with early disease do not benefit from these
76 new treatments(2, 3). For this cohort, the curative treatment, still today, is the
77 surgical resection of a lung lobe. This is a severe procedure, inflicting major damage,
78 requires an extended recovery time and can result in therapy induced mortality(4, 5).
79 Furthermore, therapy failure in late stage tumours by establishment of treatment
80 escape mechanisms is a common observation in NSCLC, significantly affecting
81 patient survival(6, 7). Overall, survival rates have only marginally improved and most
82 patients still succumb to the disease(1).
83
84 Therefore, targeting of common essential pathways and exploiting tumour intrinsic
85 vulnerabilities holds the potential to not only improve current treatment for late stage,
86 but also for early stage patients. One central cellular component tumour cells alter
87 during oncogenic transformation is the ubiquitin proteasome system (UPS)(8, 9). The
88 dysregulation of the UPS is a prerequisite for tumor cells to tolerate increased
89 proliferation, metabolic changes, immune evasion and proteostatic stress
90 management(10). All these processes are 'hallmarks of cancer' and therefore
91 significantly contribute to disease progression, therapy failure and shorted survival.
92 Therefore, cancer cells, when compared to non-transformed cells, are dependent on
93 the ubiquitin system(11, 12). As a consequence, tumour cells develop exploitable
94 dependencies towards the expression and abundance of discreet members of the
95 UPS[12].
96
97 Despite the prominent involvement of the UPS in cancer, our understanding of how
98 tumour cells alter the UPS system very early in transformation is rather limited(12).
99 The identification of essential and druggable key-players within this class of enzymes
100 has the potential to hold novel therapeutic strategies. Deubiquitinating enzymes are
101 such a therapeutically promising class of enzymes, as individual members can be
102 targeted by small molecule inhibitors(13-15).
103
104 In this study, we report that the deubiquitylase USP28 presents a UPS enzyme,
105 which is commonly upregulated during early stages of oncogenic transformation in
106 lung cancer. Irrespective of oncogenic driver, tumour cells upregulate USP28, which
107 stabilizes proto-oncogenes, such as c-MYC, c-JUN or NOTCH. Tumour cells are
108 addicted to USP28 to allow oncogenic transformation and its inhibition via the small
109 molecule inhibitor AZ1(16) partially reverts the oncogenic transformation. Finally,
110 combining USP28 targeting with targeted therapy against commonly found
111 oncogenic drivers potentiates treatment responses, at least *in cellulo*, indicating that
112 the UPS system, exemplified by USP28, is a promising target structure for lung
113 cancer.
114

115

Materials and Methods

116

117

Cell lines

118

119

120 Human basal bronchial epithelial BEAS-2B cells were originally transformed with
121 SV40-large-T-antigen (Reddel et al., 1988). The cell line BEAS-2B was a kind gift of
122 Prof. Marco A. Calzado Canales (Universidad de Córdoba, Hospital Reina Sofia,
123 Córdoba, Spain). BEAS-2B Oncogenic cells were generated upon retroviral infection
124 of BEAS-2B DIF with the next plasmids: EGFR (addgene number: #11011), EGFR
125 L858R (addgene number: #11012), pBabe puro HA PIK3CA (addgene number:
126 #12522), pBabe puro HA PIK3CA H1047R (addgene number: #12524), pBabe puro
127 HA PIK3CA E545K (addgene number: #12525), pBabe puro HRAS G12D (HRAS
128 G12D was cloned into pBabe puro in our lab) and pBabe puro BRAF V600E
129 (addgene number: #15269). The plasmids EGFR and EGFR L858R were a gift from
130 Matthew Meyerson (Greulich H, Chen TH et al. 2005). pBabe puro HA PIK3CA
131 H1047R, HA PIK3CA E545K, HA PIK3CA were a gift from Jean Zhao (Zhao JJ, Liu
132 Z, Wang L et al. 2005). pBabe Puro BRAF V600E was a gift from William Hah
133 (Boehm et al Cell. 2007) For virus production HEK293-T cells were used. Cell lines
134 used in this publication are listed in the supplementary table called: Consumables
135 and resources.

136

Tissue culture reagents and drugs.

137

138

139 Cells were plated on Greiner dishes and incubated at 37 °C, 95 % relative humidity
140 and 5 % CO_2 in a cell incubator for optimal growth conditions. DIF BEAS-2B,
141 oncogenic BEAS-2B and HEK-293T cells were cultured in DMEM (Gibco)
142 supplemented with 10% fetal bovine serum (FCS)/ 1% Pen-Strep. UD cells were
143 cultured in LHC-9 (Gibco) supplemented with 1% Pen/Strep. To cultivate UD BEAS-
144 2B cells, the dishes were pre-coated with pre-coating solution composed by: 0.03%
145 Collagen (in 0.1 M acetic acid), 0.01% Fibronectin and 0.001% BSA. UD cells were
146 supplemented with 10% FCS to induce pre-oncogenic differentiation. Cells were
147 routinely tested for mycoplasma via PCR.The reagents and drugs were dissolved in
148 Dimethyl sulfoxide (DMSO). AZ1, Gefitinib, Buparlisib and Vemurafenib were
149 purchased from Selleckchem. Drugs and reagents are listed in the supplementary
150 table called: Consumables and resources.

151

AAV, Retrovirus and Lentivirus production and purification

152

153

154 Adeno-associated viruses (AAVs) were generated and packaged in HEK293-T cells
155 seeded in 15-cm cell culture dishes (60-70% confluence). Cells were transfected
156 with the plasmid of interest (10 μg), pHelper (15 μg) and pAAV-DJ (10 μg) using PEI
157 in ratio 2:1 (70 μg). After 96 hours, AAV Virus isolation from cells was performed as
158 previously described (17). For Retrovirus production, HEK293 cells (70%

159 confluence) were transfected with the babe plasmid of interest (15 μg), pUMVC (10
160 μg) and VSV-G (10 μg) using PEI (70 μg). After 96 H, the medium containing retrovirus
161 was filtered (0.45 μM) and stored at -80°C. For Lentivirus production, HEK293 cells
162 (70% confluence) were transfected with the plasmid of interest (15 μg), pPAX (10
163 μg) and pPMD2 (10 μg) using PEI (70 μg). After 96 H, the medium containing
164 lentivirus was filtered (0.45 μM) and stored at -80°C.
165
166 **In vitro DNA transfection and infection**
167
168 DNA transfection was performed exposing 60% confluence BEAS-2B cells plated in
169 a 6-well cell culture dish to a mix of 2.5μg plasmid of interest, 200μl DMEM free
170 serum and 5μl PEI (1:2 ratio). Upon 6h incubation at 37°C, 5% CO_2 and 95%
171 relative humidity, the medium was removed and substituted by DMEM (Gibco)
172 supplemented with 10% FCS/ 1% Pen-Strep. For viral infection, 10 MOI (multiplicity
173 of infection) of Retroviruses (LVs) were added to normal medium of the cells in the
174 presence of polybrene (5μg/ml). Cells exposed to the viruses were incubated at
175 37°C, 5% CO_2 and 95% relative humidity for 4 days. The infected cells were
176 identified and selected by exposure to 2.5μg/ml Puromycin for 72h.
177
178 **RT-PCR**
179
180 RNA was isolated with Peq GOLD Trifast (Peqlab), as indicated in the
181 manufacturer's instructions. RNA was reverse transcribed into cDNA using random
182 hexanucleotide primers and M-MLV enzyme (Promega). Quantitative RT-PCR was
183 performed with SYBR Green mix (ABgene) on the instrument ´´Step One Realtime
184 Cycler´´(ABgene) The RT-PCR program employed in this research is the following:
185 95°C for 15 min., 40x [95°C for 15 sec., 60°C for 20 sec. and 72°C for 15 sec.], 95°C
186 for 15 sec. and 60°C for 60 sec. Relative expression was generally calculated with
187 ΔΔCt relative quantification method. Melt curve was performed for all primers. For
188 visualization purposes, Excel (Microsoft) and Affinity Designer were used as
189 bioinformatic tools. Primers used for this publication are listed in the supplementary
190 table called: Consumables and resources.
191
192 **Plasmids, sgRNA and shRNA Design**
193
194 sgRNAs were designed using the CRISPR online tool: https://zlab.bio/guide-design-
195 resources). shRNAs were designed using SPLASH-algorithm:
196 http://splashrna.mskcc.org/) or RNAi Consortium/Broad Institute:
197 www.broadinstitute.org/rnai-consortium/rnai-consortium-shrna-library.
198 Oligonucleotides used in this publication are listed in the supplementary table called:
199 Consumables and resources.
200
201 **Operetta analysis, Immunofluorescence, cell viability, Bliss synergy and GI50**
202

203 Number of cells was quantified using Operetta High-Content Imaging System
204 (PerkinElmer) (number of DAPI positive cells) or Invitrogen Countess II FL (number
205 of cells after trypsinization) upon indicated treatments. For the Operetta High-
206 Content Imaging System, cells were seeded in 384-well plates at equal density and
207 exposed to indicated treatments. Then, cells were fixed using 4% PFA for 10
208 minutes and then, permealized using 0,5% Triton x100 in PBS for 5 minutes. For IF,
209 primary antibodies (1/100) were incubated ON at 4°C, followed by subsequent
210 incubation with the secondary antibody (1/300) for 1 hour at room temperature. After
211 antibody exposure, samples were washed twice with PBS. Before quantification cells
212 were stained with DAPI (ThermoFischer). For the quantification of dead cells, 1ug/ml
213 PI was added to the cell medium of live cells for 20 minutes upon indicated
214 treatments. For quantification of dead cells, 1ug/ml PI (Sigma Aldrich) was added to
215 the cell medium of live cells for 20 minutes upon indicated treatments. For
216 quantification of proliferative cells, samples were subjected to KI67 (Santa cruz ab:
217 sc-23900; 1/100) staining by IF before imaging. Number of dead, proliferative and
218 total cells were determined counting the number of positive nucleus for PI, KI67 or
219 DAPI with the Harmony Software (Perkin Elmer). Bliss synergy was calculated using
220 the total number of cells upon indicated treatments. For calculation of synergy,
221 Combenefit software was previously described (Di Veroli GY et al 2016). $GI50_{50}$ was
222 generated using the online tool: www.aatbio.com. For Crystal violet cell viability, cells
223 were stained with 0.5% Crystal violet and analyzed using ImageJ software (staining
224 intensity is between 0 to 255). For visualization purposes, Excel (Microsoft) and
225 Affinity Designer were used as bioinformatic tools. Antibodies used in this publication
226 are listed in the supplementary table called: Consumables and resources.
227
228 **Immunoblot**
229
230 Cells were lysed in RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM
231 Na^2EDTA, 1mM EGTA, 1% NP-40 and 1% sodium deoxycholate), supplemented
232 with proteinase inhibitor (1/100) via sonication with Branson Sonifier 250 (duty cycle
233 at 20% and output control set on level 2; 10 sonication / 1 minute cycles per sample).
234 50μg protein was boiled in 5x Laemmli buffer (312.5mM Tris-HCl pH 6.8, 500 mM
235 DTT, 0.0001% Bromphenol blue, 10% SDS and 50% Glycerol) for 5 min and
236 separated on 10% Tris-gels in Running buffer (1.25M Tris base, 1.25M glycine and
237 1% SDS). After separation, protein was transferred to Polyvinylidene difluoride
238 membranes (Immobilon-FL) in Transfer Buffer (25mM Tris base, 192mM glycine and
239 20% methanol). Membrane was exposed to blocking buffer (0.1% casein, 0.2xPBS
240 and 0.1% Tween20) for 45-60 min at room temperature (RT). Then, membranes
241 were incubated with primary antibody (1/1000) in a buffer composed by 0.1% casein,
242 0.2x PBS and 0.1% Tween20) for 6h at room temperature (RT). Membrane was
243 incubated with indicated secondary Antibody (1/10000) in a buffer composed by
244 0.1% casein, 0.2x PBS, 0.1% Tween20 and 0.01% SDS for 1h at RT. Membranes
245 were recorded in Odyssey® CLx Imaging System, and analysed using Image Studio

246 software (Licor Sciences). Antibodies used in this publication are listed in the
247 supplementary table called: Consumables and resources.

248

249 **Ubiquitin Suicide Probe/Warhead DUB activity assays**

250

251 Cells were resuspended in HR-buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl2, 250
252 mM Sucrose, 0,1 % NP-40), supplemented with Protease-Inhibitor. Lysis was
253 performed by three freeze-thaw cycles. 25 µg of cell lysate were transferred to a new
254 eppedornf tube and 3 µL of a 1:1:1 mixture of Ub-VME, Ub-VS, Ub-PA suicide-
255 probes (UbiQ) resuspended in 50 mM NaOAc, 5 % DMSO were added to the
256 mixture. In order to adjust the pH, 50 mM NaOH was added. Then, samples were
257 mixed and incubated for 1 hour at 37°C shaking. After addition of Laemmli-buffer,
258 samples were boiled for 5 min and applied to immunoblot was performed.

259

260 **Human lung cancer samples**

261

262 Human samples were obtained from Pathology Department Córdoba (Spain),
263 Pathology Department University Hospital Würzburg (Germany) and U.S. Biomax
264 (lung microarray slides; slide LC2083). Informed consent was obtained from all
265 patients. Experiments were in agreement to the principles set out in the WMA
266 Declaration of Helsinki and the Department of Health and Human Services Belmont
267 Report. Samples are approved under ethical approval license decret 439/2010
268 (Hospital Universitario Reina Sofía) and Ethics approval 17/01/2006 (University
269 Hospital Würzburg).

270

271 **Analysis of human publicly available datasets**

272

273 Oncoprints were generated using cBioportal online tool. Briefly, Oncoprints
274 generates graphical representations of genomic alterations, somatic mutations, copy
275 number alterations and mRNA expression changes. Graphical representations of
276 somatic mutations were performed using the online tool Cbioportal. TCGA data was
277 used for the different analysis. Correlation analysis and USP28 expression in
278 different subtypes of ADC and SCC lung tumors were obtained using GEPIA's
279 software (Tang Z. et al. 2017). For GEPIA gene expression. the differential analysis
280 was based on: "TCGA tumors vs (TCGA normal)", whereas the expression data
281 were log2(TPM+1) transformed and the log2FC was defined as median(tumor) –
282 median(normal). p-values were calculated with a one-way ANOVA comparing tumor
283 with normal tissue.

284

285 The online tool KMplot (Nagy et al. 2018) was used to analyze different types of
286 survivals and generate Kaplan-meier curves based on gene expression data from
287 microarrays obtained from GEO, caBIG and TCGA. Using the KM plotter online tool,
288 patients were split using the option 'Auto select best cutoff' in high or low USP28
289 gene expression groups. P values for log rank tests of the Kaplan–Meier curves

290   were calculated with the online tool KM plotter. Depmap (version 2020) was used to
291   analyze and visualize Pearson correlation between the genetic expression of USP28
292   and BRAF/AKT2 in cancer cell lines. p-value and linear regression was calculated by
293   the online tool depmap. https://depmap.org/

294

295   Survival of patients with KRAS, EGFR, PIK3CA and BRAF mutated tumors was
296   analysed using the online tool UCSC Xena (Goldman et al. 2020). USP28 gene
297   expression of lung cancer samples (including KRAS, EGFR, PIK3CA and BRAF
298   mutated samples) were obtained from TCGA dataset. Gene expression was
299   downloaded as log2 (norm_count+1). Samples were divided in two groups
300   (High/Low USP28) based on USP28 expression. Expression of USP28 was defined
301   as high when the respective expression levels were higher than the median
302   expression levels of the analysed dataset. Expression of USP28 was defined as low
303   when the respective expression levels were lower than the median expression levels
304   of the analysed dataset. Box plots using TCGA and GTEx data were generated
305   using the online tool BoxPlotR (Spitzer M. et al. 2014) In box plots, the centre line
306   reflects the median and the upper and lower box limits indicate the first and third
307   quartiles. Whiskers extend 1.5× the IQR. For BoxplotR, the data previously
308   download from UCSC Xena was used to generate the graphics, p-values were
309   calculated using two-tailed t-test. Softwares used for this publication are listed in the
310   supplementary table called: Consumables and resources.

311

312   **Animal Experiments and histology.**

313

314   All *in vivo* experiments were approved by the Regierung Unterfranken and the ethics
315   committee under the license numbers 2532-2-362, 2532-2-367, 2532-2-374 and
316   2532-2-1003. All animals are housed in standard cages in pathogen-free facilities on
317   a 12h light/dark cycle with *ad libitum* access to food and water. FELASA2014
318   guidelines were followed for animal maintenance. Veterinarians supervise the
319   welfare of the animals every day. In presence of pain, stress or suffering, mice were
320   immediately euthanized by cervical dislocation upon Isoflurane anesthesia. The
321   mouse strains used for this publication are listed in the supplementary table called:
322   Consumables and resources.

323

324   Adult mice were anesthetized with Isoflurane and intratracheally intubated with 50 µl
325   AAV virus (3 × 10^7 PFU) as previously decribed (17). Animals were sacrificed by
326   cervical dislocation and lungs were fixed using 5% NBF. For IHC and H&E, slides
327   were de-paraffinized and rehydrated following the previously reported protocol (17).
328   Briefly, IHC slides were subjected to epitope retrieval and blocked in 3% BSA at RT
329   for 1h. Antibody manufacturer instructions were followed for every antibody. But in
330   general, primary antibodies (diluted in 1% BSA) were incubated ON at 4°C followed
331   by three washes with PBS and the subsequent incubation with the DAB secondary
332   antibody for 1 hour at RT. Then, slides were washed twice with 1xPBS for 5 min and
333   stained with the DAB staining solution in 1xPBS. Upon DAB staining, slides were

334   counteracted with hematoxylin and washed three times with 1x PBS for 5 min.
335   Slides were mounted with 200 µl of Mowiol® 40-88 covered up by a glass coverslip.
336   IHC slides were recorded using Pannoramic DESK scanner or using
337   FSX100 microscopy system (Olympus) and analysed using Case Viewer software
338   (3DHISTECH), QuPath software and ImageJ. IF samples were recorded using
339   FSX100 microscopy system (Olympus). Antibodies used in this publication are listed
340   in the supplementary table called: Consumables and resources.
341

Organotypic lung tumor slice cultures ex vivo
343

344   Lung tumors developed upon endotracheal transplantation of KPL cells as previously
345   described (17) or WT lung tissue from WT C57BL6/J-Rosa26 Sor-CAGG-Cas9-
346   IRES-eGFP animals were explanted and sectioned in slices using the vibratome. Ex
347   vivo slices were relocated in cell culture dishes and maintained in standard cell
348   culture medium (DMEM, 10% FCS) and conditions (37°C, 5% CO2 and 95% relative
349   humidity).
350

Sample preparation for mass spectrometry
352

353   The sample preparation was performed as described previously. In brief, lysates
354   were precipitated by methanol/chloroform and proteins resuspended in 8 M Urea/10
355   mM EPPS pH 8.2. Concentration of proteins was determined by Bradford assay and
356   100 µg of protein per samples was used for digestion. For digestion, the samples
357   were diluted to 1 M Urea with 10mM EPPS pH 8.2 and incubated overnight with 1:50
358   LysC (Wako Chemicals) and 1:100 Sequencing grade trypsin (Promega). Digests
359   were acidified using TFA and tryptic peptides were purified by tC18 SepPak (50 mg,
360   Waters). 10 µg peptides per sample were TMT labelled and the mixing was
361   normalized after a single injection measurement by LC-MS/MS to equimolar ratios
362   for each channel. A bridge channel was prepared by pooling 3 µg from all 24
363   samples which were TMT-labeled together and split into two 10 µg samples for each
364   plex, 130 µg of pooled peptides were dried for High pH Reversed-phase
365   fractionation.
366

High pH reversed-phase fractionation
368

369   Labeled peptide samples were pooled, fractionated into 8 fractions using the High
370   pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher Scientific 84868)
371   according to the manufacturer protocol and dried. Additionally, for label free single
372   shots, 10 µg of peptide is cleaned up with Empore C18 stage tipping and dried right
373   away for shooting.
374

LC-MS³ proteomics
376

377 All mass spectrometry data was acquired in centroid mode on an Orbitrap Fusion
378 Lumos mass spectrometer hyphenated to an easy-nLC 1200 nano HPLC system
379 using a nanoFlex ion source (ThermoFisher Scientific) applying a spray voltage of
380 2.6 kV with the transfer tube heated to 300°C and a funnel RF of 30%. Internal mass
381 calibration was enabled (lock mass 445.12003 m/z). Peptides were separated on a
382 self-made, 32 cm long, 75µm ID fused-silica column, packed in house with 1.9 µm
383 C18 particles (ReproSil-Pur, Dr. Maisch) and heated to 50°C using an integrated
384 column oven (Sonation). HPLC solvents consisted of 0.1% Formic acid in water
385 (Buffer A) and 0.1% Formic acid, 80% acetonitrile in water (Buffer B).

386 For total proteome analysis, a synchronous precursor selection (SPS) multi-notch
387 MS3 method was used in order to minimize ratio compression as previously
388 described (McAlister et al., 2014). Individual peptide fractions were eluted by a non-
389 linear gradient from 3 to 60% B over 150 minutes followed by a step-wise increase to
390 95% B in 6 minutes which was held for another 9 minutes. Full scan MS spectra
391 (350-1400 m/z) were acquired with a resolution of 120,000 at m/z 200, maximum
392 injection time of 100 ms and AGC target value of 4×10^5. The most intense
393 precursors with a charge state between 2 and 6 per full scan were selected for
394 fragmentation within 3 s cycle time and isolated with a quadrupole isolation window
395 of 0.7 Th. MS2 scans were performed in the Ion trap (Turbo) using a maximum
396 injection time of 50ms, AGC target value of 15×10^4 and fragmented using CID with
397 a normalized collision energy (NCE) of 35%. SPS-MS3 scans for quantification were
398 performed on the 10 most intense MS2 fragment ions with an isolation window of 1.2
399 Th (MS) and 2 m/z (MS2). Ions were fragmented using HCD with an NCE of 65%
400 and analyzed in the Orbitrap with a resolution of 50,000 at m/z 200, scan range of
401 110-500 m/z, AGC target value of 1.5×10^5 and a maximum injection time of 150ms.
402 Repeated sequencing of already acquired precursors was limited by setting a
403 dynamic exclusion of 60 seconds and 7 ppm and advanced peak determination was
404 deactivated.

405 **Proteomics analysis**

406 Proteomics raw files were processed using proteome discoverer 2.2 (ThermoFisher).
407 Spectra were recalibrated using the Homo sapiens SwissProt database (2020-03-12)
408 and TMTpro (+304.207 Da) as static modification at N-terminus and Lysines,
409 together with Carbamidomethyl at cysteine residues. Spectra were searched against
410 human database and common contaminants using Sequest HT with oxidation (M) as
411 dynamic modification together with methionine-loss + acetylation and acetylation at
412 the protein terminus. TMTpro (N-term, K) and carbamidomethyl were set as fixed
413 modifications. Quantifications of spectra were rejected if average S/N values were
414 below 5 across all channels and/or isolation interference exceeded 50%. Protein
415 abundances were calculated by summing all peptide quantifications for each protein.
416 Two mixing two plexes, a bridge channel was used additionally. Internal reference
417 scaling (IRS) normalization was performed to obtain proteomics data set across two
418 plexes.
419

420 Reactome analysis was performed with PANTHER using the "Statistical
421 overrepresentation test" tool with default settings. For Reactome analysis and Violin
422 plots, the common proteins significantly dysregulated in EGFR L858R, BRAF V600E
423 and PIK3CA H1047R BEAS-2B respect to DIF. BEAS-2B cells were selected.
424 Proteins were considered significantly downregulated when p-value<0.05. Z-score
425 heatmap visualization was performed using Morpheus (Broad Institute). The
426 maximum and minimal Z-score per row used in heatmaps was calculated using
427 Morpheus (Broad Institute). Volcano plots were generating using the software
428 Instant Clue (Nolte et al. 2018). Venn Diagrams were performed using the online
429 tool: http://bioinformatics.psb.ugent.be/webtools/Venn/. PCA analysis was performed
430 using the online tool CLUSTVIS (Tauno Metsalu and Jaak Vilo 2015). Violin plots
431 were generated using the online tool BoxPlotR (Spitzer M. et al. 2014). For
432 visualization purposes, Excel (Microsoft) and Affinity Designer were used as
433 bioinformatic tools.
434

435 **Data and Software availablitiy**
436 Proteomic data is available at PRIDE.
437

438 **Contact for reagent and resource sharing**

439 Further information and requests for resources and reagents should be directed to
440 and will be fulfilled by the Lead Contact, Markus E. Diefenbacher
441 (markus.diefenbacher@uni-wuerzburg.de).
442

443 **Results**
444

445 ***USP28 is expressed in human 'cell-of-origin' for NSCLC and upregulated***
446 ***irrespective of lung tumour subtype***
447

448 Previous work has identified the 'cell-of-origin' for NSCLC, in particular
449 adenocarcinoma (BADJ (SPC$^+$/CC10$^+$) and AT2 (SPC$^+$) pulmonary cells)(18, 19),
450 while squamous cell carcinomas, one of the most mutated tumour entities,
451 demonstrated a rather high degree of plasticity and flexibility regarding the required
452 cell of origin(20). Here, the genetic driver combination was the determining
453 factor(21).
454

455 We previously observed that genetic loss of USP28 affected tumor burden in a
456 murine *in vivo* NSCLC model(22). To investigate whether this could be attributed to
457 the expression of USP28 in the stem cell/cell of origin compartment of NSCLC, we
458 performed immunohistochemistry against USP28 in human lung tissue samples.
459 High USP28 expression was detectable in the tracheal basal cells (triangular-like
460 shape), BADJ and AT2 cells, the 'cells-of-origin' for NSCLC (Figure 1A and 1B).
461 Next, we were intrigued if USP28 expression is altered relative to tumour type and/or
462 grade. To address this question, we analysed publicly available gene expression

463 datasets of NSCLC patients (Figure 1C). *USP28* was found to be upregulated
464 already at an early stage in lung cancer, when compared to wild type tissue (Figure
465 1C). Furthermore, *USP28* upregulation is a common feature of tumour cells,
466 irrespective of histological or molecular tumour subtype (Figure 1C, S1A and S1B).
467 This was further validated by immunostaining of non-transformed versus tumour
468 samples from NSCLC patients, where a significant increase in USP28 protein
469 abundance was detected already at low grade stages for lung ADC and SCC (Figure
470 1D and 1E).
471
472 Analysing survival data and correlating USP28 expression to tumour stages it
473 became clear that, especially at early stages, USP28^{high} expressing tumours
474 significantly correlated with an overall shortened survival (Figure 1F), and this
475 observation was independent of tumour subtype (Figure 1G).
476
477 These data indicated that USP28 is upregulated upon oncogenic transformation and
478 that upregulation appears to be an early event in the tumorigenesis of lung and holds
479 prognostic value.
480
481 ***USP28 is expressed in murine lung stem cells and required to establish***
482 ***oncogenic transformation in vivo.***
483
484 To investigate that USP28 is required for NSCLC induction and its upregulation
485 occurs at early stages, we utilised CRISPR/Cas9 genetic engineering mouse models
486 of NSCLC(22, 23).
487
488 To this end, we analysed the expression pattern of Usp28 by immunohistochemistry
489 in wild-type, non-transformed lungs (Figure 2A). We observed that USP28 showed a
490 comparable expression to human samples (Figure 2A and Figure 1B). Overall Usp28
491 expression was elevated in putative stem cells, when compared to
492 surrounding/neighbouring differentiated cells (Figure 2A).
493
494 Next, we wondered if the transcriptional upregulation of USP28 already occurs at
495 point of transformation. To address this question, we used an *ex vivo* organotypic
496 lung slice culture model. Here, a non-transformed lung from a C53BL6/J-*Rosa26*$^{Sor-}$
497 $^{CAGG-Cas9-IRES-eGFP}$ mouse(24) was sectioned into 100µm thick sections by using a
498 vibratome and cultured in standard medium. 24 hours post sectioning, slices were
499 infected using an adeno-associated virus (AAV) expressing the fluorescent protein
500 mCherry as infection marker, together with sgRNA to delete *Trp53* and mutate
501 endogenous *Kras* to *Kras*^{G12D} (KP, Figure 2B)(22, 23). As a control vector we used
502 an mCherry expressing AAV. 7 days post infection, the lung slices were harvested
503 and mRNA expression of Usp28 analysed using quantitative PCR (Figure 2B). Here,
504 *Usp28* expression was significant increased at an early transformative state, when
505 compared to control virus infected tissue samples.
506

507 Next, we analysed the expression of Usp28 and its substrates c-Myc, c-Jun and
508 Notch1 at various grades in murine primary tumours, generated by intratracheal
509 infection with a KP encoding AAV (Figure 2C). Mice were infected at around 8
510 weeks of age and sacrificed 12 weeks post infection. Tumour grade and type were
511 assessed using histopathological means (H&E, Ttf-1). Already in low grade primary
512 tumours Usp28 and its substrates were significantly upregulated when compared to
513 adjacent, non-transformed lung epithelial tissue (Figure 2D). The increase of Usp28
514 and the oncogenic transcription factors persisted in higher grade tumours, as seen
515 by immunohistochemistry (Figure 2D), thereby confirming the observation made in
516 patients (Figure 1C, 1D, 1E, S1A and S1B).

517

518 Next, we wondered if the upregulation of Usp28 at an early stage is independent of
519 the oncogenic driver (Figure S2A and S2B). For that purpose, we generated murine
520 primary NSCLC using BrafV600E as oncogenic driver. BRAF is genetically altered in
521 28% of SCC and 25% of ADC lung tumour samples (Figure S2A). To analyse early
522 stage tumours, mice were sacrificed 4 weeks post infection (Figure S2B). Tumour
523 grade and type were determined using H&E and immunohistologic stainings against
524 Ttf1 and Pcna. Already in early stage lung primary tumours, Usp28 was over-
525 expressed when compared to non-transformed tissue (Figure S2C).

526

527 Previously we reported that loss of Usp28 affected the induction of lung squamous
528 cancer, and its genetic loss affected overall tumour burden (22). To investigate if loss
529 of Usp28 affects tumour induction at an early stage or just reduces proliferation of
530 transformed cells, next, we infected constitutive Cas9 expressing mice with an AAV
531 virus containing either an sgRNA to delete *Trp53* and mutate endogenous *Kras* to
532 *Kras*^{G12D} (KP, Figure 2E) or a virus harbouring additional two sgRNA targeting
533 endogenous Usp28 (KPU, Figure 2E). Mice were sacrificed 4 weeks post infection
534 and while in KP infected animals' tumour lesions were detectable, in KPU however,
535 no lesions could be observed (Figure 2F).

536

537 These data suggest that USP28 is expressed in tumour initiating cells and is
538 required during early stages of lung cancer transformation independent of tumour
539 subtype or oncogenic driver.

540

541 ***USP28 is increased during early transformation in human BEAS-2B***
542 ***differentiation assay.***

543

544 As it appears that transcriptional upregulation and increased protein abundance of
545 USP28 is a required early event in oncogenic transformation, we used a human cell
546 line system to recapitulate these early events. The immortalised human tracheal cell
547 line BEAS-2B retains the ability to grow as a progenitor like cell, but in a cell-density
548 dependent fashion or under the exposure of fetal calf serum (FCS) can terminally
549 differentiate into a squamous like, pre-oncogenic and highly proliferative cell (Figure
550 3A)(25, 26). Indeed, culturing these cells under progenitor specific culturing

551 conditions maintained the cells in a 'spindle-like' shape as previously reported
552 (Figure 3B; undifferentiated BEAS-2B; BEAS-2B^{UD}). Upon FCS exposure,
553 BEAS2B^{UD} cells start to alter their morphology and resemble a squamous phenotype
554 (Figure 3B and 3C; differentiated BEAS-2B; BEAS-2B^{DIF})(25). Furthermore, USP28
555 protein abundance was increased in BEAS-2B^{DIF} when compared BEAS-2B^{UD} to by
556 immunofluorescence and immunoblotting against endogenous USP28 (Figure 3B
557 and 3C). Not only is USP28 upregulated, but the amount of enzymatically active
558 USP28 was increased as well, indicated by the amount of USP28 bound to a
559 ubiquitin suicide probe/warhead (Figure 3D). Upon pre-oncogenic differentiation, the
560 known USP28 target ΔNp63 increased in protein abundance, as shown by
561 immunoblotting comparing BEAS-2B^{DIF} with BEAS-2B^{UD} (Figure 3B). To investigate if
562 the observed differentiation and the consecutive increase in oncoprotein abundance
563 is USP28 dependent, we exposed BEAS-2B^{UD} to FCS (Figure 3E). Upon serum
564 pulse cells start to differentiate, visualised by brightfield microscopy, and increase
565 the expression of USP28 and its target ΔNp63 (Figure 3E). In order to block USP28,
566 we made use of the published USP28 inhibitor AZ-1(16, 22). Addition of AZ-1
567 resulted in the degradation of USP28 and reduction in ΔNp63 and cells maintained
568 an undifferentiated morphology (Figure 3E).
569
570 These data demonstrate that USP28 is upregulated during pre-malignant
571 transformation and this leads to an increase of USP28 target oncoproteins.
572
573 ***Oncogenic transformation of BEAS-2B^{DIF} via EGFR-PI3K-MAPK pathway***
574 ***upregulate USP28 and accelerates tumour cell growth***
575
576 During the oncogenic transformation process, cells acquire additional alterations,
577 which are the prerequisite to establish a tumour(27). Using public datasets we
578 identified that USP28 expression strongly correlates to the expression of common
579 driver mutations found in NSCLC, encompassing either amplification or mutation of
580 BRAF, EGFR, PI3K and RAS (Figure 4A and 4B, S3A and S3B). HRAS was the
581 exemption in RAS family, as no correlation with USP28 was observed in ADC,
582 however NRAS and KRAS positively correlated with USP28 in ADC human samples
583 (Figure 4B, S3A and S3B). To test this observation, we *in vitro* retroviral transformed
584 BEAS-2B^{DIF} with various oncogenes (BEAS-2B^{ONC}; Figure S3C) and investigated the
585 effects on USP28 abundance by immunoblotting and RT-PCR. As a control, we used
586 a virus only encoding Puromycin resistance (Figure S3D and S3E). EGFR WT,
587 EGFRL858R, HRASG12D, BRAFV600E, PIK3CA WT, PIK3CAE545A and
588 PIK3CAH1047R expression was confirmed by immunoblotting and RT-PCR (Figure
589 S3D and S3E). While USP28 was detectable in control transformed cells, the
590 expression of oncogenes further increased USP28 levels and mRNA (Figure 4C and
591 4D). Not only was the DUB increased, but also the USP28 target protein ΔNp63,
592 along with KRT14, a known ΔNp63 transcriptional target gene (Figure 4C and
593 4D)(28). It is noteworthy, that the overexpression of wild type PIK3CA/p110 had little
594 to no effect on overall protein increase (Figure 4C and 4D). Here, only the mutant

595 variants E545K and H1047R led to an increase in USP28 and ΔNp63 (Figure 4C and
596 4D). More studies are required to elucidate the differences between functional
597 mutations and amplification of PIK3CA.
598
599 Previous studies reported that USP28 is a direct target of c-JUN and c-MYC (Figure
600 S4A)(29, 30). Since both proto-oncogenes exert an important role during oncogenic
601 transformation and are increased upon EGFR-PI3K-MAPK mediated oncogenic
602 transformation(31), we wondered if the regulation of USP28 in lung cancer depends
603 on these transcription factors as well. Transient transfection of c-MYC and c-JUN
604 increased USP28 protein abundance compared to control plasmid transfection in
605 BEAS-2B cells (Figure S4B). Furthermore, BEAS-2B^{DIF} cells showed higher protein
606 abundance for USP28 and its known substrates c-MYC, c-JUN and NOTCH1 than
607 BEAS-2B^{UD} (Figure S4C) and oncogenic transformation of BEAS-2B^{DIF} further
608 increased USP28, c-MYC, c-JUN and NOTCH1 protein abundance (Figure S4C).
609
610 In summary, c-MYC, c-JUN and NOTCH1 are downstream targets of the PI3K-
611 MAPK pathways, and establish a feed forward loop in oncogenic transformed cells,
612 contributing to their increased abundance (Figure S4D). Analysing public datasets
613 revealed that gene expression of the PI3K-MAPK downstream effectors, AKT2 and
614 BRAF, positively correlated with USP28 in lung cancer cell lines and in various
615 tumour entities (Figure S4E). Notably, melanoma and tumours arising in liver, eye
616 and bone showed weak correlation between USP28 and AKT2-BRAF, and for
617 thyroid cancer we observed a negative correlation. This tumour type presents a
618 tumour entity with overall better prognosis and survival(32).
619
620 Next, we wondered if oncogenic transformation of BEAS-2B^{DIF} alters cellular growth
621 responses. To investigate potential effects, we compared growth rates of BEAS-
622 2B^{UD}, BEAS-2B^{DIF} and BEAS-2B^{ONC} for 4 days (Figure 3E). While the differentiation
623 already enhanced proliferation and is a consequence of enriched proto-oncogene
624 abundance (Figure S4C), upon overexpression of oncogenes, irrespective of
625 oncogenic driver, all generated cell lines demonstrated a significant increase in
626 proliferation, except overexpression of wild type PIK3CA/p110 (Figure 4E).
627
628 This observation indicates that USP28 is a downstream target of the PI3K-MAPK
629 pathway (Figure 4F) and is contributing to cellular transformation by stabilising proto-
630 oncogenes (Figure S4D).
631
632 ***Malignant transformation renders tumour cells dependent on USP28***
633
634 As USP28 was upregulated in an oncogene dependent fashion, we wondered if the
635 increase in USP28 contributes to the pro-proliferative phenotype. The inducible
636 overexpression of murine USP28 was sufficient to increase the proliferation of
637 BEAS-2B^{DIF} to a comparable extent than PIK3CA mutant cells (Figure 5A).
638 Conversely, using two independent shRNA sequences to target USP28 expression,

639 irrespective of oncogenic driver, loss of USP28 impaired the proliferation of
640 transformed cells (Figure 5B). This was further confirmed using the KRAS G12S
641 mutant lung cancer cell line A549 (Figure S5A). Depletion of USP28 by two
642 independent shRNA sequences strongly reduced the overall abundance of MYC as
643 well the proliferation marker PCNA (Figure S5B). As a consequence, A549
644 proliferation was significantly reduced (Figure S5C). Next, we wondered if the small
645 molecule inhibitor AZ1, which impairs USP25/28 enzymatic activity, affects
646 proliferation of oncogenic transformed BEAS-2B. Furthermore, we wanted to
647 address if the inhibitor shows selectivity towards specific oncogenic drivers. To this
648 end, BEAS-2B^{DIF} and BEAS-2B^{ONC} cells were grown in the presence of increasing
649 concentration of AZ1, followed by calculating the half maximal inhibitory
650 concentration (IC$_{50}$) by assessing cell numbers. It was revealed that EGFRL858R
651 and BRAFV600E transduced BEAS-2B^{ONC} cells tolerated ~16µM AZ1, while cells
652 transformed by PIK3CAH1047R required ~20µM (Figure S5D). Given the rather
653 comparable IC$_{50}$ concentrations, we treated BEAS-2B^{DIF} and BEAS-2B^{ONC} cells with
654 15 µM AZ1 for 24 hours, followed by immunoblotting against USP28 and its
655 substrates NOTCH1, c-MYC and c-JUN (Figure 5C). Upon exposure to AZ1, all cell
656 lines showed a marked reduction in the protein abundance of USP28 and its target
657 substrates (Figure 5C and 3E). Next, we investigated if AZ1 impairs cell proliferation
658 or increases cell death in a dose dependent fashion, and if AZ1 affects all cells or is
659 limited to oncogenic transformed cells (Figure 5D and 5E). Cells were grown in the
660 presence of increasing concentrations of AZ1 for 72 hours, followed by *in vivo*
661 exposure to propidium iodide (PI) as a marker for dead cells (Figure 5E) and
662 immune- fluorescent staining of the proliferative marker Ki67 (Figure 5D). While the
663 non-oncogenic BEAS-2B^{DIF} showed weak reduction in cell proliferation and mild
664 increase in nuclear PI positive cells, BEAS-2B^{ONC} cells, irrespective of oncogenic
665 driver, significantly reduced cell proliferation in an AZ1 concentration dependent
666 fashion (Figure 5D). Additionally, when BEAS-2B^{ONC} cells were exposed to AZ1
667 concentrations reaching or exciding the oncogene corresponding IC$_{50}$, PI
668 incorporation was significantly enriched (Figure 5D and 5E). Surprisingly,
669 BRAFV600E transformed BEAS-2B demonstrated a high degree of sensitivity
670 towards AZ1 (Figure 5E), as it was previously reported that, in melanoma, loss of
671 USP28 was required to induce oncogenic transformation and resistance.
672
673 Overall, our data demonstrated that USP28 is required to maintain tumour cell
674 proliferation and survival *in cellulo,* hence, tumour cells become addicted to USP28.
675
676 ***Inhibition of USP28 via AZ1 'resets' the proteome of oncogenic transduced***
677 ***cells towards a 'non-oncogenic' state and induces pro-apoptotic signatures***
678
679 To gather further insights into how targeting of USP28 via AZ1 affects oncogenic
680 transduced BEAS-2B cells, we conducted mass spectrometric analysis and
681 compared the proteome of control and oncogenic transduced cells upon exposure to
682 AZ1 (Figure S6A). Principal component analysis identified that oncogenic

683 transduction resulted in distinct changes of the proteome (Figure 6A and Figure
684 S6B). Not only were the proteomes of BEAS-2B^{onc} different when compared to non-
685 oncogenic cells, but the oncogenic driver used established distinct proteomic
686 patterns (Figure 6A). Upon exposure to AZ1, the proteomes of BEAS-2B^{onc},
687 however, significantly changed and clustered with the expression of non-oncogenic
688 BEAS-2B (Figure 6A and S6C). Analysing the proteome of the three BEAS-2B^{onc} cell
689 lines post AZ1 exposure revealed that a set of proteins dysregulated during
690 oncogenic transformation was commonly affected in an AZ1 dependent fashion; 45
691 proteins were decreased and 29 proteins commonly increased in BEAS-2B^{onc}
692 (Figure 6B, 6C and S6D). Proteins upregulated in EGFRL858R transduced BEAS-2B
693 were repressed upon exposure to AZ1, while proteins decreased during the course
694 of oncogenic transformation enriched upon blockage of USP28 activity (Figure 6C,
695 6D and S6D). Addition of 15 µM AZ1 for 72 hours, however, not only reduced the
696 abundance of proto-oncogenes, but re-shaped global protein abundance, closer
697 resembling non-oncogenic BEAS-2B (Figure 6C and 6D). Inhibition of USP28 via
698 AZ1 in oncogenic transduced BEAS-2B significantly repressed the abundance of
699 proteins involved in negative regulation of the ubiquitin proteasome system,
700 decreased RTK/growth factor signalling and vesicle transport, while oncogenic
701 BEAS-2B upregulated proteins involved in differentiation, immune signalling,
702 apoptosis and necrosis (Figure 6E).

703

704 Hence, acute inhibition of USP28 via AZ1 decreases the amount of proto-
705 oncogenes, thereby affecting global protein abundance. USP28 is required to
706 accommodate oncogenic transformation and to suppress anti-proliferative and pro-
707 apoptotic signatures.

708

709 ***USP28 inhibition potentiates targeted molecular therapy***

710

711 To investigate if USP28 is a putative prognostic marker for NSCLC, we analysed
712 public available datasets and observed that USP28 expression significantly
713 correlates with progression free survival (PFS, p=4.6e-05), Figure 7A and S7A). In
714 the Post Progression Survival cohort, elevated expression of USP28 directly
715 correlated with shortened survival (overall and Stage I, S7A and S7B). These data
716 suggest that USP28 levels strongly determine the survival of the NSCLC patients in
717 response to therapy.

718

719 Since NSCLC is a genetically very heterogeneous tumour type, next, we wondered if
720 patient survival depended on the combination of oncogenic driver and the expression
721 of USP28 (Figure 7B and S7C). Indeed, the analysis of public available data leads to
722 the suggestion that, irrespective of oncogenic driver, an increased expression of
723 USP28 significantly shortens survival for tumours driven by mutations in the
724 oncogenes EGFR (Δ574 days), PIK3CA (Δ177 days) or BRAF (Δ922 days), while in
725 tumours driven by mutations in genes of the RAS family, USP28 had very little effect

726 on patient survival (Δ58 days, Figure 7B). These data suggest that USP28 is a
727 suitable prognostic marker and oncogene in NSCLC.
728 As cancer panel sequencing is implemented in the clinics and pathway-specific
729 inhibitors available, we were wondering if disruption of the oncogenic pathways
730 would directly affect USP28 and hence, the abundance of its downstream effectors.
731 Analysis of publicly available data regarding putative drug sensitivity of tumour cells
732 in direct correlation to USP28 expression scored the PIK3-, EGFR- and MAPK
733 pathway as top hits (Figure 7C), directly confirmed our experimental data regarding
734 USP28 expression relative to oncogenic drivers. Since several potent pathway
735 inhibitors are readily available (Figure 7D), we wondered if targeted therapy would
736 synergize with targeted inhibition of USP28 via AZ1. To this end, we exposed our
737 BEAS-2B^{ONC} cell lines to either a selective inhibitor (EGFRL858R= Gefitinib;
738 PIK3CAH1047R= Buparlisib; BRAFV600E= Vemurafenib), AZ1, or a combination
739 thereof for 24 hours, followed by immunoblotting against USP28 and its substrates
740 (Figure 7E). Selective pathway interference was evaluated by immunoblotting
741 (Figure S7D). Monotherapy via selective pathway inhibitors affected downstream
742 signalling cascades, reduced the abundance of USP28, led to a reduction in the
743 protein levels of NOTCH1, c-MYC and c-JUN and reduced cell viability (Figure 7E
744 and S7E). Similar effects were observed by administering AZ1; treated cells showed
745 a reduction in USP28 abundance, along with reduced protein levels of its substrates
746 and decreased viability (Figure 7E and S7E). Combinatorial treatment, however,
747 significantly reduced the amount of USP28 and diminished the abundance of
748 NOTCH1, cMYC and cJUN (Figure 7E). Not only does co-treatment with AZ1
749 sensitize BEAS-2B^{ONC} cells to targeted therapy, but it synergises with Gefitinib,
750 Bupalislib and Vemurafenib, as seen by viability assays in BEAS-2B^{ONC} cells (Figure
751 7F). The synergistic effect of AZ1 and targeted therapy does not only stem from
752 impairment of tumour cell proliferation, but also leads to onset of cell death, as seen
753 by Ki67 expression and PI incorporation experiments of BEAS-2B^{ONC} cells cultivated
754 for 72 hours in the presence of the single compounds or combinations thereof at
755 synergistic concentrations (AZ1: 7.5 μM, Gefitinib: 7.5 μM; Buparlisib: 0.5 μM;
756 Vemurafenib: 7.5 μM; Figure 7G, H and S7E). Here, exposure to 7.5 μM AZ1 did not
757 affect proliferation nor induced cell death (Figure 7G and H). While monotherapy with
758 targeted inhibitors led to a reduction in proliferation, it did not result in cell death, as
759 seen by loss of Ki67 expression and the absence of PI incorporation (Figure 7G and
760 H). The combinatorial treatment, however, resulted in a significant increase in PI
761 positive cells, while maintaining a proliferative block.
762
763 USP28 therefore, functions as a signal amplifier downstream of various oncogenic
764 signalling cascades and is required to allow oncogenic transformation. Its inhibition
765 potentiates targeted therapy by impairing the protein stability of oncogenic
766 transcription factors, required to maintain tumour cell proliferation, and presents a
767 promising target for drug development.
768
769 **Discussion**

770

771 Oncogenic transformation of somatic cells is a multistage process frequently starting
772 with the inactivation of tumor suppressors and subsequent gain of activating
773 mutations in oncogenic drivers, such as members of the PI3K or MAPK family.
774 These changes result in the increased abundance of proto-oncogenes, such as c-
775 MYC[33], JUN[34] or NOTCH, driving cell proliferation, dedifferentiation, metabolic
776 changes, DNA damage control, immune evasion and proteostatic stress
777 management, the 'hallmarks of cancer'(23).

778

779 Cells undergoing transformation partially counter this intrinsic stress by re-adjusting
780 the UPS. USP28 is involved in the control of a plethora of biological processes. It is
781 involved in the regulation of cell proliferation and differentiation via its ability to
782 regulate the abundance of the proto-oncogenes, such as c-MYC, c-JUN or NOTCH
783 (Figure 8A) (29). It is involved in transcriptional control via regulating the abundance
784 of the histone modifier LSD1/KDM1A(35) and it is part of the DNA damage
785 machinery, where it interacts with ATM(36), CLSPN(37), or TP53BP1(38).

786

787 Overall, USP28 functions as a proto-oncogene and contributes to establishing the
788 hallmarks of cancer in cells undergoing oncogenic transformation, at least in
789 lung(22). Here, USP28 is expressed in the stem cell niche and elevated abundance
790 detected in 'cells or origin'. Its upregulation is an early event, as already low grade
791 tumours showed enhanced abundance for this particular DUB, and its expression
792 coincided with shortened overall survival. In a cellular multi-stage transformation
793 model, we were able to recapitulate the stepwise increase in USP28 abundance and
794 enhanced activity, which occurred independent to the oncogenic driver present.
795 USP28 is a transcriptional target of its own substrates(29), and via a feed forward
796 loop, increased in cancer compared to normal. Irrespective of oncogenic driver,
797 interference with USP28 abundance or activity suppressed tumour cell growth and
798 survival of transformed lung cells *in vitro* and *in vivo*. Inhibition of USP28 via the
799 small molecule inhibitor AZ1 restored the proteome of oncogenic transformed BEAS-
800 2B cells towards a pre-malignant state, demonstrating that interference with USP28
801 abundance and activity has a far-reaching biological impact. USP28 is a downstream
802 target of RTK signaling cascades and required to establish oncogenic transformation
803 by its ability to control the abundance of proto-oncogenes. The inhibition of the DUB
804 not only repressed tumour cell growth but led to a pro-apoptotic phenotype,
805 predominantly in oncogenic transformed cells, while control cells were not affected.
806 Here, the cell cycle was affected, leading to a reduction in proliferation.

807

808 This is in stark contrast to two recent reports. In a small cohort of melanoma
809 patients, where tumours are predominantly driven by mutant BRAF(V600E), USP28
810 is genetically lost(39). These USP28 mutant patients present enhanced MAPK
811 signaling via hyperstabilisation of RAF family members and resistance to BRAF
812 inhibitors. Here, loss of USP28 presents a negative survival marker. The second
813 study identified that, in melanoma cells, USP28 is cleaved by caspase 8 to overcome

814 G2/M cell cycle arrest in a TP53 dependent fashion(40). Loss of USP28 in tumour
815 cells is favored as it results in TP53 protein destabilization, thereby establishing a
816 switch of cell fate, from apoptosis towards mitosis. Hence, USP28 functions as a
817 tumour suppressor in melanoma(41). In line with these reports, we could not detect a
818 correlation between USP28 and BRAF expression in human skin cancer samples.
819 This could be indicating that this tumour entity indeed does not rely on the DUB and
820 hence, alternative mechanisms deviating from our observation in lung and SCC, are
821 possible.
822
823 In lung, and as previously reported for squamous cell carcinoma(22), targeting
824 USP28 presents a suitable lever for therapeutic engagement, as one could propose
825 that at least in lung, tumour cells become addicted to USP28. In line with this
826 hypothesis, we indeed did observe that loss of USP28 reduced oncogenic cell
827 proliferation and its genetic loss impaired tumour onset *in vivo*. The inhibition of
828 USP28, via a small molecule inhibitor AZ1, induced pro-apoptotic signaling in cells
829 expressing potent oncogenic driver mutants. In lung, the expression of USP28
830 directly correlated with shortened patient survival, irrespective of oncogenic driver.
831 As USP28 functions as an 'amplifier' downstream of RTKs, its inhibition cooperated
832 with personalized targeted therapy against specific oncogenic driver mutations
833 (Figure 8B).
834
835 Overall, our data suggest that targeting USP28 protein abundance and activity
836 already at an early stage, therefore, is a promising strategy for the treatment of lung
837 tumours in combination to personalized targeted therapy.
838

839 **Acknowledgements**

845

846 **Conflict of Interest:**

847 The authors declare no potential conflicts of interest.

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997   **Figure Legends**
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999   **Figure 1: *USP28 is expressed in human 'cell-of-origin' for NSCLC and**
1000   **upregulated irrespective of lung tumour subtype***
1001   A) Schematic representation of the cellular composition of the tracheal, bronchio-
1002   alveolar and alveolar compartment. Highlighted are the respective tissue
1003   residing stem cells. Trachea = basal cells, Bronchio-alveolar duct junction =
1004   BADJ cells, Alveolar compartment = AT2 cells.
1005   B) Immuno-histochemical staining of endogenous USP28 in patient lung
1006   resected material. Shown are representative images of alveolar, bronchial and
1007   tracheal sections.
1008   C) Expression of USP28 in non-transformed and NSCLC Adenocarcinoma
1009   (ADC) and Squamous Cell Carcinoma (SCC) samples, relative to tumour
1010   stage (T1-T4). Public available data. p-values were calculated using two-tailed
1011   T-test statistical analysis. Plot was generated using the online tool
1012   www.gepia2.cancer-pku.cn.
1013   D) Immuno-histochemical staining of USP28 on human NSCLC tissue micro
1014   arrays of ADC and SCC origin, ranging from Grade 1 to 3. Where applicable,
1015   non-transformed adjacent tissue was included. Shown are representative
1016   images per tumour type.
1017   E) Immuno-histochemical staining of USP28 on human lung cancer tissue micro
1018   arrays of various lung cancer subtypes. Where applicable, non-transformed
1019   adjacent tissue was included. lung resected material. Shown are
1020   representative images per tumour type.

1021   F) Kaplan-Meier plots of NSCLC patient Overall Survival (OS), relative to USP28
1022   expression, at Stage 1 (p=<0.0005), Stage 2 (p=0.02) to Stage 3 (p=0.55).
1023   Data was generated using the online tool www.kmplot.com.
1024   G) Kaplan-Meier plots of NSCLC ADC and SCC patient Overall Survival (OS),
1025   relative to USP28 expression, at Stage 1 (ADC p=0.0063; SCC p=0,00057).
1026   Data was generated using the online tool www.kmplot.com.
1027
1028 See also Supplementary Figure S1.
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1030 Figure 2: **USP28 is expressed in murine lung stem cells and required to**
1031 **establish oncogenic transformation in vivo.**
1032   A) Immuno-histochemical staining of endogenous Usp28 in murine lung wild type
1033   tissue. Shown are representative images of alveolar, bronchial, bronchio-
1034   alveolar duct junction and tracheal sections. Red boxes indicate highlighted
1035   areas.
1036   B) *Ex vivo* onset of oncogenic transduction by CRISPR mediated gene editing
1037   and deletion of *Trp53* and mutation of *KRas* to *KRas*^{G12D} (KP) upon AAV
1038   infection of organotypic lung slice cultures. Lung slice cultures were generated
1039   from *C57Bl6/J-Rosa26*$^{Sor-CAGG-Cas9-IRES-eGFP}$ mice and AAV encodes mCherry
1040   as marker. Fluorescent images of lung slice cultures post infection with AAV.
1041   GFP = lung; RFP = AAV infected lung epithelial cells. Tissue sections were
1042   harvested and subjected to RNA isolation, followed by RT-PCR analysis of
1043   *Usp28* mRNA expression in control and KP infected slices. n= 3 sections
1044   each. p-values were calculated using two-tailed T-test statistical analysis.
1045   C) Schematic representation of *in vivo* CRISPR gene editing to delete *Trp53* and
1046   mutate *KRas* to *KRas*^{G12D} (KP) upon intratracheal administering of AAV.
1047   Animals are sacrificed 12 weeks post infection.
1048   D) Representative images of immuno-histochemical staining against endogenous
1049   Usp28, Nkx2-1/Ttf-1, c-Jun, Notch1 and c-Myc in murine lungs infected with
1050   either control virus (WT) or KP. Shown are representative tumours spanning
1051   grade 1 to 3, with a low and high magnification of individual tumour areas. n=3
1052   E) Schematic representation of *in vivo* CRISPR gene editing to delete *Trp53*,
1053   mutate *KRas* to *KRas*^{G12D} (KP) or co-delete *Usp28* (KPU) upon intratracheal
1054   administering of AAV. Animals are sacrificed 4 weeks post infection. n=6.
1055   F) Representative H&E images KP and KPU 4 weeks post infection.
1056   Quantification of tumour burden per animal. n=6. p-values were calculated
1057   using two-tailed T-test statistical analysis.
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1059 See also Supplementary Figure S2.
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1061 Figure 3: **USP28 is increased during early transformation in human BEAS-2B**
1062 **differentiation assay**

A) Schematic model of the trans-differentiation by culturing stem cell like, undifferentiated BEAS-2B (BEAS-2B^{UND}) in the presence of 10% fetal calf serum to induce squamous like differentiation, BEAS-2B^{DIF}.

B) Representative brightfield-images of BEAS-2B prior and post serum induced trans-differentiation (72 hours in the presence of 10% FCS, UND to DIF). Immunoblot against endogenous USP28 and ΔNP63 of BEAS-2B^{UND} and BEAS-2B^{DIF}. ACTIN served as loading control. Representative blot of n=3.

C) Immunofluorescence of endogenous USP28 prior and post differentiation. USP28 in green, ACTIN in red, DAPI as nuclear counterstain.

D) Immunoblot against endogenous USP28 in the absence or presence of a Ubiquitin suicide probe (warhead, WH) to assess USP28 enzymatic activity in undifferentiated and differentiated BEAS-2B cells. Upon binding to the activity probe, a shift in molecular weight is observed, indicative of enzymatic activity (see arrows). ACTIN served as loading control. Representative immunoblot of n=3.

E) Representative brightfield-images of BEAS-2B prior and post culture in serum induced trans-differentiation conditions, in the presence or absence of the USP28 inhibitor AZ1 (15µM, 72 hours). Immunoblot against endogenous USP28 and ΔNP63 of BEAS-2B^{UND} exposed to either 10% FCS or 10% FCS and 15µM AZ1. ACTIN served as loading control. Representative blot of n=3.

Figure 4: *Oncogenic transformation of BEAS-2B^{DIF} via EGFR-PI3K-MAPK pathway upregulate USP28 and accelerates tumour cell growth*

A) Frequently occurring genetic alteration in recurring oncogenic drivers found in NSCLC (ADC and SCC). Oncoprints generated with the online tool www.cbioportal.org.

B) mRNA expression Spearman´s correlation between USP28 and EGFR, HRAS, BRAF or PIK3CA in NSCLC (SCC and ADC). Correlation and p-value generated with the online tool GEPIA www.gepia2.cancer-pku.cn/ .

C) Immunoblot against endogenous USP28 and the oncogenic transcription factor ΔNP63 either BEAS-2B^{DIF} or BEAS-2B^{DIF} upon retroviral transduction to express the indicated oncogenes EGFR (wild type (WT) and L858R), HRAS (G12D), BRAF (V600E) and PIK3CA (wild type (WT), E545K and H1047R), respectively. ACTIN served as loading control. Representative immunoblot of n=3.

D) RT-PCR of USP28, the SCC transcription factor ΔNP63 and its target Cytokeratin 14 (KRT14) in BEAS-2B^{UND}, BEAS-2B^{DIF} or the various BEAS-2B^{ONC} as presented in C). Shown are Log2fold change expression data, relative to ACTIN and normalized to the respective expression in BEAS-2B^{UND} and standard deviation. Shown are mean values and standard deviation of n=3

E) Relative cell numbers and assessment of growth capacity of BEAS-2B^{UND}, BEAS-2B^{DIF} or the various BEAS-2B^{ONC} over a total of 4 days. Cell numbers were analyzed every day. Shown are mean values and standard deviation.

1107   n=3 experiments. p-values were calculated using two-tailed T-test statistical
1108   analysis.

1109   F) Schematic model of the various stages of oncogenic transformation, as
1110   recapitulated by the trans-differentiation from BEAS-2B^{UND} to BEAS-2B^{DIF},
1111   and from BEAS-2B^{DIF} to BEAS-2B^{ONC} (oncogenic transformed BEAS-2B^{DIF}).
1112   The observed increases recapitulate the increase in USP28 protein
1113   abundance as seen in human NSCLC samples.

1114

1115 See also Supplementary Figure S3 and S4.

1116

1117 Figure 5: ***Malignant transformation renders tumour cells dependent on USP28***

1118   A) Immunoblot showing protein abundance of USP28 in BEAS-2B^{DIF} upon
1119   lentiviral transduction with either a control or a doxycycline inducible
1120   overexpression of murine Usp28. BEAS-2B^{DIF} control and mUsp28 cells were
1121   cultured in the presence of 1µg/ml doxycycline for 72 hours prior to
1122   immunoblotting. ACTIN served as loading control. For growth analysis, cells
1123   were pre-cultured for 72 hours in the presence of 1µg/ml doxycycline,
1124   followed by re-seeding and counting of cells at day 1, day 3 and day 6. Shown
1125   are mean values and standard deviation of n=3. p-values were calculated
1126   using two-tailed T-test statistical analysis.

1127   B) Immunoblot showing protein abundance of USP28 in oncogenic transduced
1128   BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and BRAFV600E) upon
1129   lentiviral transduction with either a control or two individual constitutive shRNA
1130   targeting USP28. ACTIN served as loading control. For growth analysis, cells
1131   were seeded at equal cell density and counted at day 1, day 2 and day 4.
1132   Shown are mean values and standard deviation of n=3.

1133   C) Immunoblots against endogenous USP28 and its substrates NOTCH1, c-MYC
1134   and c-JUN in either BEAS-2B^{DIF} or oncogenic transduced BEAS-2B^{ONC}
1135   (EGFRL858R, PIK3CA L1047R and BRAFV600E) upon exposure to either
1136   DMSO or 15µM AZ1 for 24 hours. VINCULIN served as loading control.

1137   D) Immunofluorescence of Ki-67 expression in BEAS-2B^{DIF} or oncogenic
1138   transduced BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and BRAFV600E)
1139   cultured in the presence of increasing concentrations of AZ1 (0 (DMSO),
1140   7.5µM, 15 µM, 30 µM) for 72 hours to assess effects in cell proliferation.
1141   Shown are representative cells. Quantification of Ki-67 expression in 30-45
1142   20x fields from independent wells per condition. DAPI served as nuclear
1143   marker. Shown are mean values and standard deviation. p-values were
1144   calculated using two-tailed T-test statistical analysis.

1145   E) Immunofluorescence of propidium iodide (PI) incorporation in BEAS-2B^{DIF} or
1146   oncogenic transduced BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and
1147   BRAFV600E) cultured in the presence of increasing concentrations of AZ1 (0
1148   (DMSO), 7.5µM, 15 µM, 30 µM) for 72 hours to assess effects on cell survival
1149   and apoptosis. Shown are representative cells. Quantification of PI positive

1150   cells in 30-45 20x fields from independent wells per condition. DAPI served as
1151   nuclear marker. Shown are mean values and standard deviation. p-values
1152   were calculated using two-tailed T-test statistical analysis.

1153

1154 See also Supplementary Figure S5.

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1156 Figure 6: *Inhibition of USP28 via AZ1 'resets' the proteome of oncogenic*
1157 *transduced cells towards a 'non-oncogenic' state and induces pro-apoptotic*
1158 *signatures*

1159 A) Principal Component Analysis (PCA) of the whole proteome of BEAS-2B^{DIF}
1160   and BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R), treated with either 15 µM
1161   AZ1 for 72 hours or exposed to control solvent (DMSO). N=3 samples.
1162   Analysis generated with Clustvis online tool (https://biit.cs.ut.ee/clustvis).

1163 B) Venn diagram illustrating proteins changed upon exposure of BEAS-2B^{ONC}
1164   (EGFRL858R, PIK3CA L1047R) to 15 µM AZ1 for 72 hours. Highlighted are
1165   numbers of proteins dysregulated during oncogenic transformation (BEAS-2B
1166   → BEAS-2B^{ONC}) and decreased or increased upon AZ1 exposure. Discreet or
1167   common deregulated protein numbers are indicated within the corresponding
1168   overlapping graphs. Common, oncogenic driver independent number of
1169   deregulated proteins are highlighted in the center. Analysis of n=3 samples
1170   per oncogenic driver.

1171 C) Heatmap of proteins identified in B) for BEAS-2B^{DIF} ,BEAS-2B^{EGFRL858R} and
1172   BEAS-2B^{EGFRL858R} treated with 15 µM AZ1 for 72 hours. Shown are n=3
1173   experiments and data presented as Z score values. Red= high Z-score
1174   protein abundance, blue = low Z-score protein abundance.

1175 D) Violin plots illustrating changes of BEAS-2B^{DIF} and BEAS-2B^{ONC} pre- and
1176   post-treatment with 15 µM AZ1 for 72 hours for decreased or increased
1177   proteins identified in B). p-values were calculated using two-tailed T-test
1178   statistical analysis.For Violin plot, white circles show the medians; box limits
1179   indicate the 25th and 75th percentiles as determined by R software; whiskers
1180   extend 1.5 times the interquartile range from the 25th and 75th percentiles;
1181   polygons represent density estimates of data and extend to extreme values.
1182   Violin plot were generated with BoxplotR. http://shiny.chemgrid.org/boxplotr/

1183 E) GO biological process significantly reduced or increased in BEAS-2B^{ONC} upon
1184   15 µM AZ1 for 72 hours. The analysis was performed with the proteins
1185   identified in B). The analysis was performed using the online tool Panther.
1186   http://www.pantherdb.org

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1188 See also Supplementary Figure S6.

1189

1190 Figure 7: *USP28 inhibition potentiates targeted molecular therapy*

1191

A) Kaplan Meier plot of Progression free survival (PFS) of NSCLC patients relative to USP28 mRNA expression. n=596 samples, p=<0.0005. Data generated with the online tool www.kmplot.com.

B) Analysis of public available datasets analysing USP28 mRNA expression and Overall Survival (OS) in patients with mutations in the oncogenic driver EGFR, RAS, PIK3CA or BRAF. Samples were divided in two groups based on USP28 mRNA expression: High USP28 (higher than the median USP28 expression) and Low USP28 (lower than the median USP28 expression). Survival days was determined for both groups. Data was obtained from the online tool https://xena.ucsc.edu/. In box plots, the centre line reflects the median and the upper and lower box limits indicate the first and third quartiles. Whiskers extend 1.5× the IQR.

C) Pearson correlation between sensitivity of EGFR-PI3K-MAPK inhibitors and USP28 expression in human NSCLC cell lines. Data was generated with the online tool Cancer Therapeutics Response Portal V2 (https://portals.broadinstitute.org/ctrp/). In plos, the lower line reflects the median and the upper box limit indicates the first quartile. Whiskers extend 1.5× the IQR.

D) Schematic representation of EGFR-PI3K-MAPK pathway analysed in this study and potential pathway interference opportunities by EGFR-PI3K-MAPK and AZ1 inhibitors. Green = EGFR-PI3K-MAPK inhibitors. Violet = AZ1.

E) Immunoblots of BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and BRAFV600E) cultured in the presence of either control solvent (DMSO), pathway specific inhibitors (EGFR: 20 µM Gefitinib; PIK3CA: 1µM Buparsilib; BRAF: 20 µM Vemurafenib), 15 µM AZ1 or a combination thereof for 24 hours.

F) BLISS synergism score of BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and BRAFV600E) cultured in the presence of either control solvent (DMSO), pathway specific inhibitors (EGFR: Gefitinib; PIK3CA: Buparsilib; BRAF: Vemurafenib), AZ1 and combination thereof for 72 hours at indicated concentrations. Shown are representative DAPI images of cells 72 hours post culture in the presence of DMSO, single treatment with 7.5 µM AZ1, 7.5 µM Gefitinib, 0.5µM Buparlisib, 7.5µM Vemurafenib or combination of AZ1 with the respective personalized molecular therapy for 72 hours. Synergism was calculated by cell quantification of 30-45 20x fields from independent wells in control (DMSO) and the indicated treated conditions.

G) Immunofluorescence of Ki-67 expression in oncogenic transduced BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and BRAFV600E), cultured in the presence of DMSO, single treatment with 7.5 µM AZ1, 7.5 µM Gefitinib, 0.5µM Buparlisib, 7.5µM Vemurafenib or combination of AZ1 with the respective pathway inhibitor for 72 hours, to assess effects in cell proliferation. Shown are representative cells. Quantification of Ki-67 expression in 30 to 45 20x fields from different wells in control (DMSO) and treated conditions. DAPI

1235 served as nuclear marker. Shown are mean values and standard deviation.
1236 p-values were calculated using two-tailed T-test statistical analysis.
1237 H) Immunofluorescence of propidium iodide (PI) in vivo incorporation of
1238 oncogenic transduced BEAS-2B^{ONC} (EGFRL858R, PIK3CA L1047R and
1239 BRAFV600E), cultured in the presence of DMSO, single treatment with 7.5
1240 µM AZ1, 7.5 µM Gefitinib, 0.5µM Buparlisib, 7.5µM Vemurafenib or
1241 combination of AZ1 with the respective pathway inhibitor for 72 hours, to
1242 assess effects in cell proliferation. Shown are representative cells.
1243 Quantification of PI positive cells in 30 to 45 20x fields from different wells in
1244 control (DMSO) and treated conditions. DAPI served as nuclear marker.
1245 Shown are mean values and standard deviation. p-values were calculated
1246 using two-tailed T-test statistical analysis.
1247
1248 See also Supplementary Figure S7.
1249
1250 Figure 8: **USP28 enables oncogenic reprograming of respiratory cells during**
1251 **early transformation and its inhibition potentiates targeted molecular therapy.**
1252
1253 A) Schematic representation of the mechanism presented in this manuscript.
1254 Oncogenic transformation via EGFR-PI3K-MAPK pathway increases USP28
1255 transcription via c-MYC/ c-JUN. USP28 stabilizes the oncoproteins c-MYC/ c-
1256 JUN stablishing a direct feedback loop.
1257 B) Schematic representation of the synergy between EGFR-PI3K-MAPK
1258 targeted molecular therapies and AZ1 presented in this manuscript.
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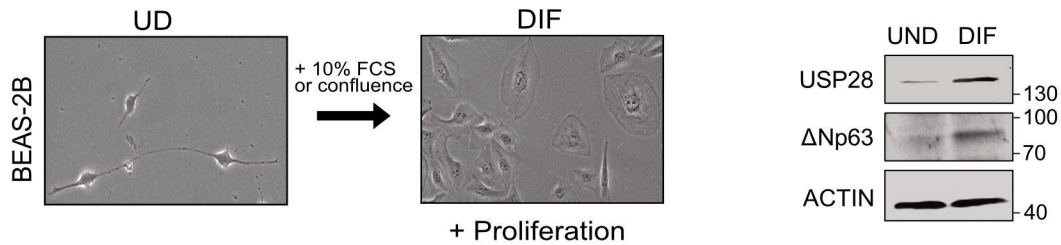1272
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Prieto-Garcia et al. Figure 1

A

Alveole | BADJ | Bronchiole | Trachea
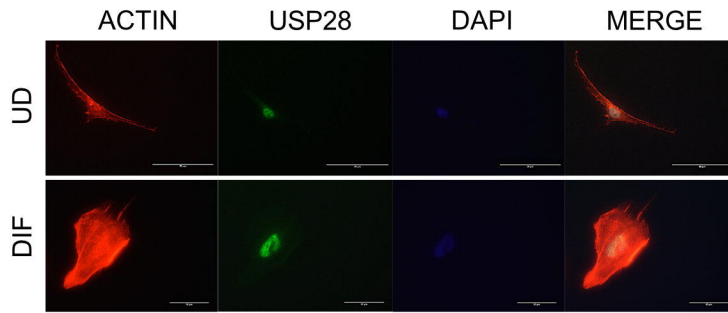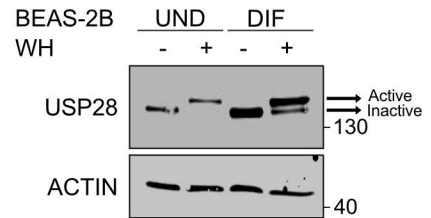
murine Usp28

B

mCherry
KRas^{G12D}:Trp53$^\Delta$

WT Lung (GFP+ mice)

GFP | RFP

mCherry

KRas^{G12D} Trp53

USP28

0.0031

Rel. mRNA

mCherry | KRas^{G12D} Trp53$^\Delta$

C

CRISPR/Cas9 tumour model

+ AAV i.t.

Rosa^{26} SpCas9 IRES GFP

K-Ras / K-Ras / K-Ras^{G12D} — KRas^{G12D}

Trp53 — Trp53$^\Delta$

intratracheal AAV administration — analysis

birth — 8 weeks — 20 weeks

D

KP	WT		Early T Stage		Medium T Stage		Late T Stage	
	10X	40X	10X	40X	10X	40X	10X	40X
Usp28								
Ttf1								
c-Jun								
Notch1								
c-Myc								

E

CRISPR/Cas9 tumour model

+ AAV i.t.

Rosa^{26} SpCas9 IRES GFP

K-Ras / K-Ras / K-Ras^{G12D}

Trp53

KRas^{G12D}

Trp53$^\Delta$

intratracheal AAV administration — analysis

birth — 8 weeks — 12 weeks

F

H&E

KRas^{G12D}:Trp53$^\Delta$

Usp28^{WT}

Usp28$^\Delta$

0.035

Number of Tumours

USP28 WT | USP28 KO

Prieto-Garcia et al. Figure 2

Prieto-Garcia et al. Figure 3

Prieto-Garcia et al. Figure 4

Prieto-Garcia et al. Figure 5

A. Similarity PC analysis (full proteome)
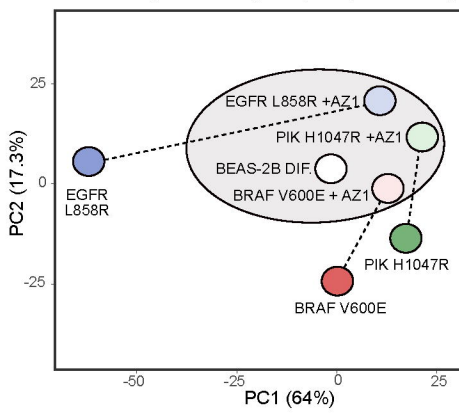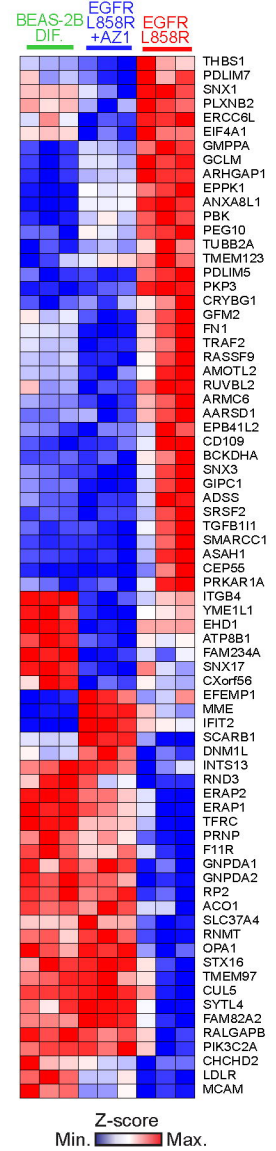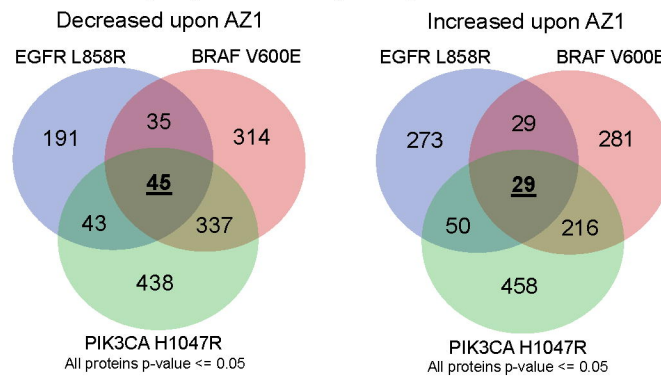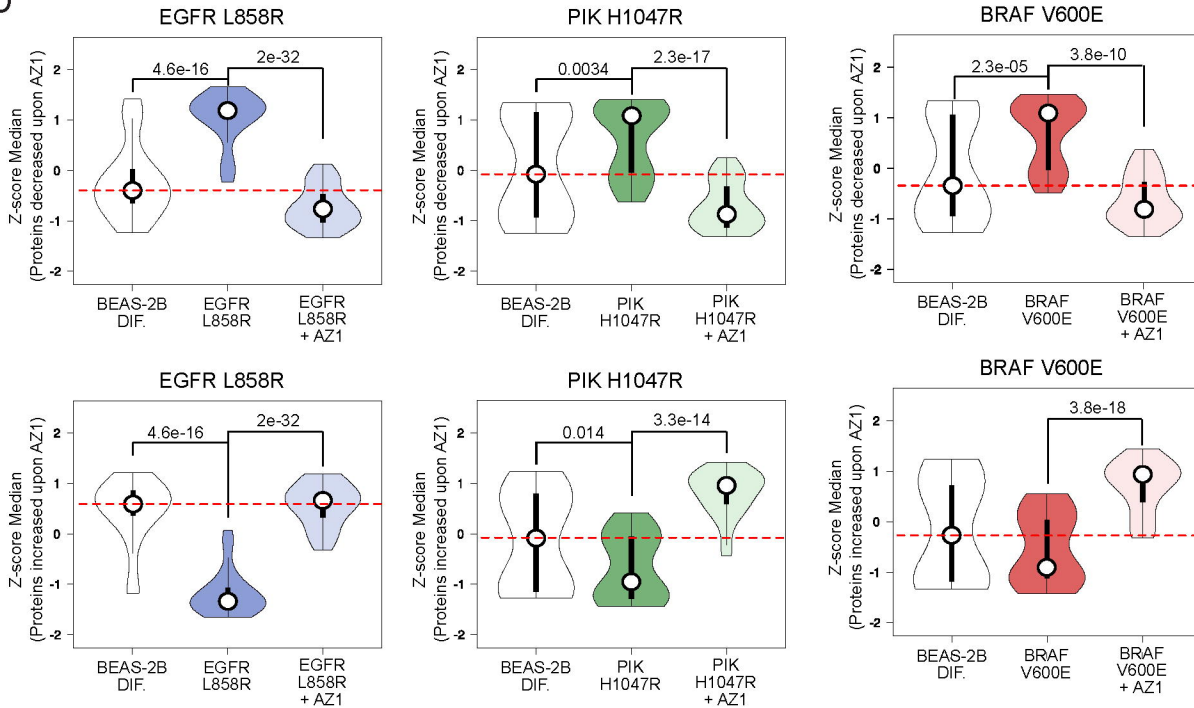
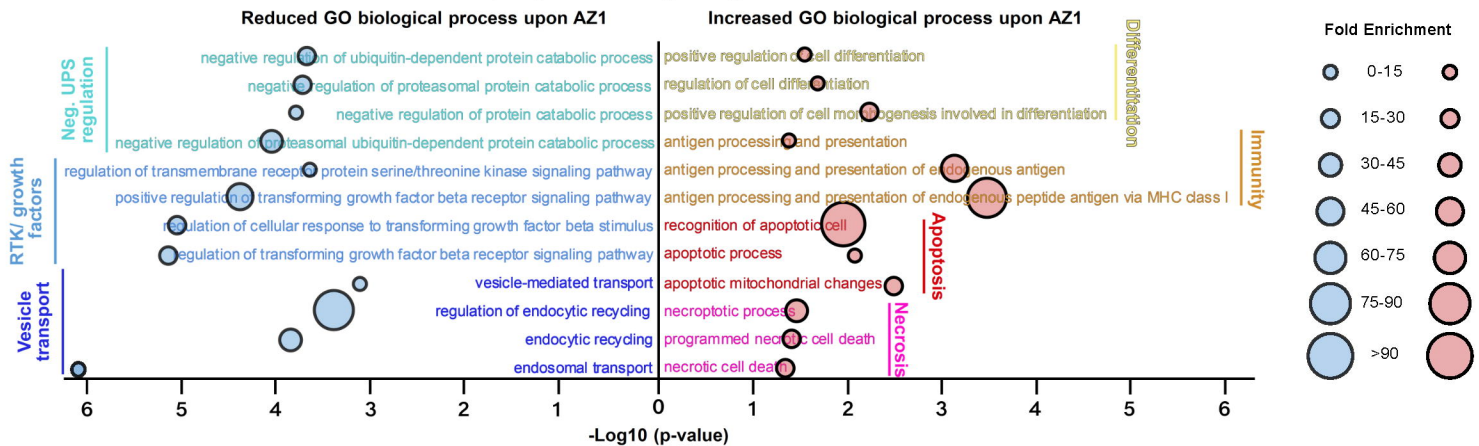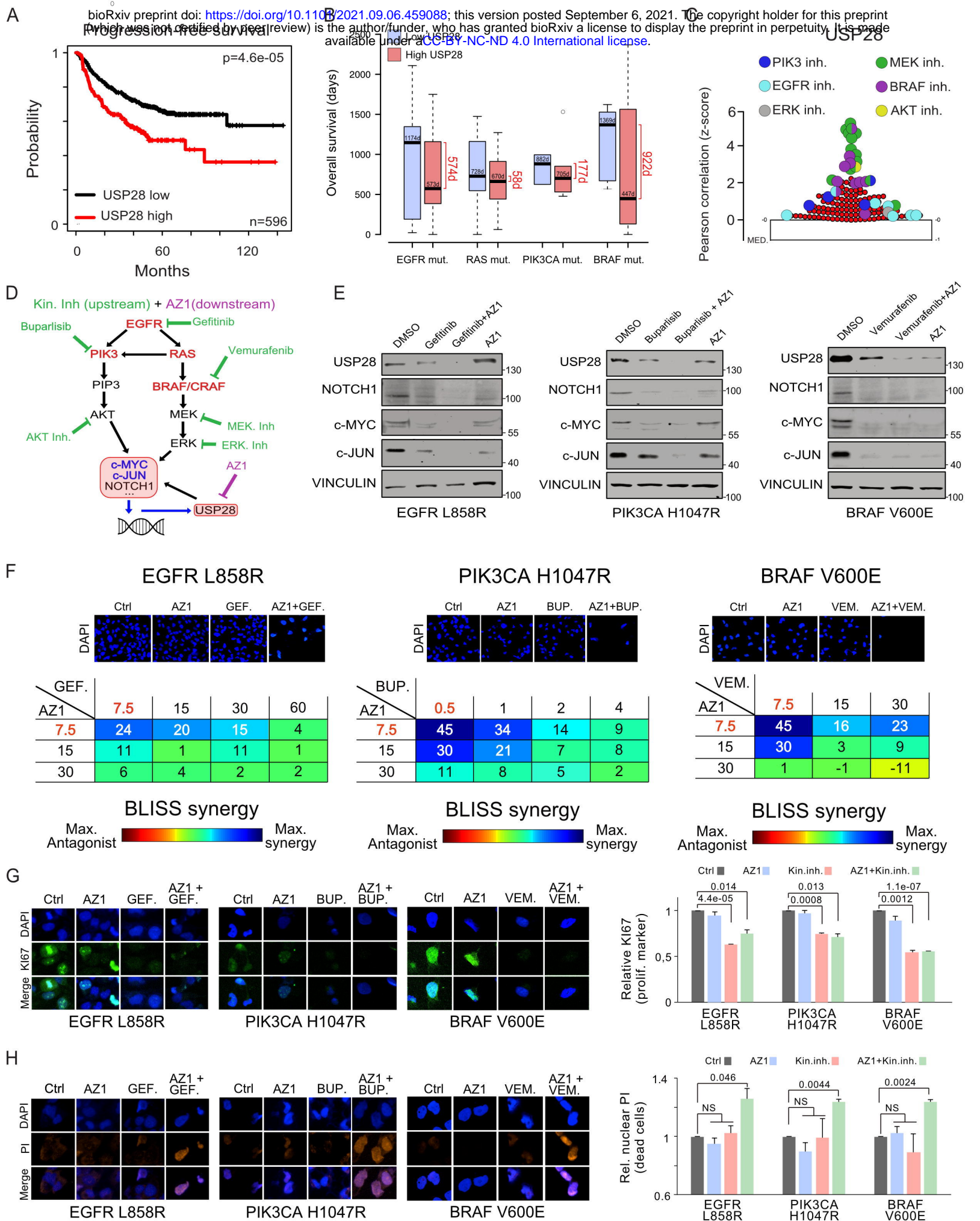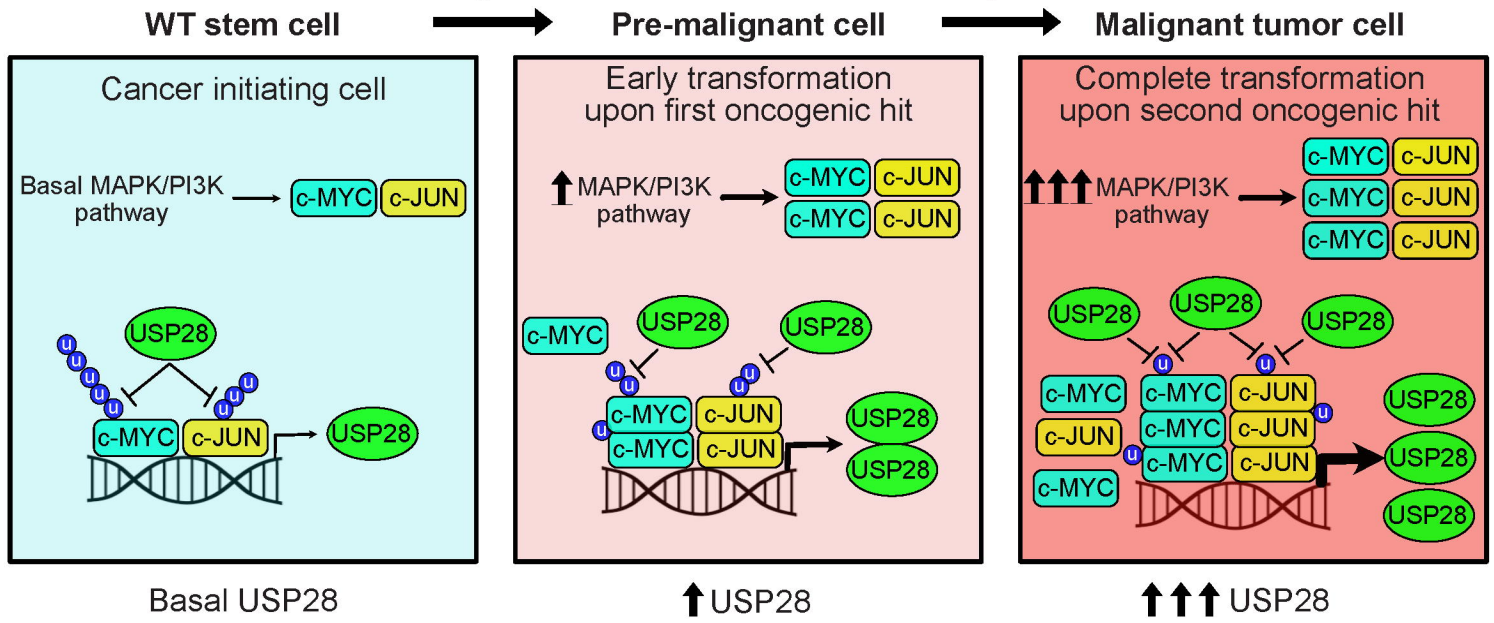B. Proteins dysregulated during oncogenic transformation

C.

D.

E. Proteins dysregulated during oncogenic transformation

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