

1 **TITLE: YAP/TAZ and EZH2 synergize to impair tumor suppressor**
2 **activity of TGFBR2 in non-small cell lung cancer.**

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18 **Running Title.** YAP/TAZ and EZH2 co-repress oncosuppressor genes in NSCLC

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29

30 **Abstract**

31 Lung cancer is the leading cause of cancer-related deaths, worldwide. Non–small cell lung
32 cancer (NSCLC) is the most prevalent lung cancer subtype. YAP and TAZ have been
33 implicated in lung cancer by acting as transcriptional co-activators of oncogenes or as
34 transcriptional co-repressors of tumor suppressor genes. Previously we reported that YAP
35 and TAZ regulate microRNAs expression in NSCLC. Among the set of regulated miRNAs,
36 the oncogenic miR-25, 93, and 106b, clustering within the MCM7 gene were selected for
37 further studies. We firstly identified Transforming Growth Factor- β (TGF- β) Receptor 2
38 (TGFB2), a member of the TGF- β signaling, as a target of the miRNA cluster, which
39 exhibited prognostic value because of its tumor suppressor activity. We found that
40 YAP/TAZ-mediated repression of TGFB2 occurs both: post-transcriptionally through the
41 miR-106b-25 cluster and transcriptionally by engaging the EZH2 epigenetic repressor that
42 we reported here as a novel target gene of YAP/TAZ. Furthermore, we document that
43 YAP/TAZ and EZH2 cooperate in lung tumorigenesis by transcriptionally repressing a
44 specific subset of tumor suppressor genes, including TGFB2. Our findings point to
45 YAP/TAZ and EZH2 as potential therapeutic targets for NSCLC treatment.

46 **Keywords.** Hippo pathway, PRC2, NSCLC, tumor suppressors, tazemetostat

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48

49 **1. Introduction**

50 Lung cancer continues to be the leading cause of cancer deaths, worldwide (1). Current
51 treatments that are based on surgery, radiation, chemotherapy, laser, and photodynamic

52 therapy result in the modest increase of the overall survival of the patients. The Non-Small
53 Cell Lung Cancer (NSCLC) still remains one of the most aggressive subtypes of lung
54 cancer with the lowest survival rate (2). The development of targeted therapies and, more
55 recently immunotherapy, have improved the overall outcome for lung cancer patients (3)
56 (4). However, ultimately most of the patients either suffer adverse side effects of
57 therapeutic interventions or develop resistance. Consequently then, these therapies are
58 interrupted or terminated. Combinatorial treatments are intensely investigated now
59 because they allow the use of lower doses of drugs, reducing side effects, and they could
60 likely bypass the mechanisms of resistance. In the context of lung cancer, YAP and TAZ,
61 the downstream effectors of the Hippo tumor suppressor pathway promote cell
62 proliferation, survival, migration, invasiveness, and immune escape, which *in vivo* results
63 in tumor development, progression and resistance to therapies (reviewed in (5)). This
64 makes YAP and TAZ attractive therapeutic targets in lung cancer. By deciphering
65 signaling mechanisms that are orchestrated by YAP and TAZ, we should get a better
66 insight into the molecular pathology of lung cancer in general.
67 Previously, we found that YAP/TAZ, and its preferred TEAD1 transcription factor, regulate
68 the expression of the oncogenic cluster of miR-25, 93, and 106b, which is located within
69 the transcript of the Mini-Chromosome Maintenance 7 (MCM7) gene (6). The regulation of
70 miRNAs and long non-coding RNAs, in addition to that of coding genes, further expands
71 the plethora of oncogenic mechanisms that can be orchestrated by YAP/TAZ. Recently we
72 described the post-transcriptional inhibition of the p21 tumor suppressor gene as one of
73 the mechanisms that underlie NSCLC. In the present work, we focused on TGFBR2, a
74 novel potential target of the miR-106b-25 cluster. The TGFBR2 gene encodes for the
75 Transforming Growth Factor- β (TGF- β) receptor 2, a member of the TGF- β signaling,
76 which is involved in embryonic development, tissue homeostasis, and tumorigenesis. The
77 TGFBR2 signals through the regulation of cell growth, differentiation, apoptosis, invasion,

78 angiogenesis, and immune response (7). Upon ligand binding, the TGFBR2 forms a
79 hetero-tetrameric complex with TGFBR1, which phosphorylates and activates receptor-
80 regulated SMADs (Small, Mothers Against Decapentaplegic), namely SMAD2 and
81 SMAD3. In turn, activated SMAD2 and SMAD3 associate with SMAD4, translocate into
82 the nucleus, and engage transcription factors and co-activators to regulate gene
83 expression program that is cell-context dependent (8). Generally, in cancer, the TGF- β
84 pathway can be either: pro-oncogenic, inducing epithelial-mesenchymal transition (EMT),
85 migration, invasion, and metastasis, or tumor-suppressive, by inducing growth arrest,
86 apoptosis, and prevention of immortalization. The signaling outcome, however, is always
87 tumor- and cancer stage-dependent (9).

88 Here, we show that TGFBR2 acts as a *bona fide* tumor suppressor and therefore it could
89 be of prognostic value for NSCLC, especially at the early stages of the disease. YAP/TAZ
90 signal to maintain low expression levels of TGFBR2 in NSCLC through at least two
91 molecular mechanisms: post-transcriptionally (mediated by miR-106b-25 cluster) and
92 transcriptionally (mediated by the epigenetic repressor Enhancer of Zeste Homologue 2,
93 EZH2). We also find that YAP/TAZ/EZH2 cooperate in the repression of the TGFBR2
94 gene, as well as other tumor suppressor genes. Therefore, the combinatorial targeting of
95 YAP/TAZ and EZH2 may represent a novel therapeutic strategy for controlling NSCLC.

96

97 **2. Materials and Methods**

98 **Cell culture and transfection**

99 Human H1299, H1975, A459 and U293 cells were purchased from the American Type
100 Culture Collection (ATCC, Manassas, VA) and routinely tested by PCR for mycoplasma
101 contamination by using the following primers: Myco_fw1: 5'-
102 ACACCATGGGAGCTGGTAAT-3', Myco_rev1: 5'-CTTCATCGACTTTCAG

103 ACCCAAGGCA-3'. A459 cells were grown in RPMI medium (Invitrogen, Carlsbad,CA)
104 supplemented with 10% fetal bovine serum and Pen/Strep antibiotic at 37°C in a balanced
105 air humidified incubator with 5% CO₂. Lipofectamine RNAimax (Invitrogen) was used in
106 accordance with the manufacturer's instruction for transfection with siRNAs, LNA and
107 miRNA mimics. siRNAs were used at the final amount of 300 pmol in 60 mm dish. List of
108 siRNA used for functional in vitro experiments is given in Supplementary Materials and
109 Methods. LNA inhibitors for miR-25, 93 and 106b (Exiqon, Vedbk,Denmark) were used at
110 a final amount of 150 pmol in 60 mm dish. For mature miR-25, 93 and 106b
111 overexpression, we used mirVana™ miRNA Mimic Negative Control #1 (Ambion) or hsa-
112 miR-25-3p, hsa-miR-93-5p, or hsa-miR-106b-3p mirVana™ miRNA Mimic (Ambion) at
113 final concentration of 5 nM. Plasmids were transfected with Lipofectamine 2000
114 (Invitrogen) in accordance with the manufacturer's instruction at a final concentration of 1
115 µg in a 60 mm dish. Cells were collected 48-72h post transfection for subsequent
116 analyses.

117

118 **Plasmids**

119 The plasmid for EZH2 overexpression was obtained by cloning the EZH2 cDNA into
120 pCDNA3 backbone with a myc-tag and was a kind gift from the laboratory of Dr. Daniela
121 Palacios (Santa Lucia Foundation, Rome Italy). The plasmid for TGFBR2 3'UTR luciferase
122 assay (Psi-check2-TGFBR2) was a kind gift from the laboratory of Dr. Stefen Wiemann
123 (German Cancer Research Center (DKFZ), Heidelberg, Germany).

124

125 **Stable transfection**

126 H1299 and H1975 cells were transfected using Lipofectamine 2000 with a pCDNA3-myc-
127 EZH2 plasmid. 24 h after transfection, cells were diluted at 20–30% confluency and fresh
128 medium with 1ng/µg G418 was added for a selection of stably transfected cells every 3–4

129 days. Cells were selected for 15-20 days and then they were grown in fresh medium
130 containing 1 ng/μl G418, tested for correct EZH2 overexpression and expanded. For all
131 experiments, cells were maintained in fresh medium containing 0,5 μg/μl G418.

132

133 **Clonogenic assay**

134 Cells were transfected as indicated above and 48h-72h later they were detached and
135 seeded at 500–1000 cells/well into 6-well or 12-well dishes. Fresh medium was added
136 every 4 days. After 7–14 days, colonies were stained with crystal violet and counted.

137

138 **Pharmacological treatment and Chemical reagents**

139 Dasatinib and Tazemetostat (EPZ-6438) were obtained from Selleck Chemicals (Houston,
140 TX); To determine the IC50 of these drugs, lung adenocarcinoma cells were seeded in
141 triplicate at a density of 1,000 cells/well. The following day, cells were treated with the
142 drugs at increasing concentrations, and ATP lite assay (Promega) was performed after 72
143 hours of treatment. The dual drug studies (Dasatinib plus Tazemetostat) were performed
144 in a similar manner with the doses indicated in the figures.

145 For colony assay upon treatment with Dasatinib and Tazemetostat, cells were grown for 9
146 days with or without Tazemetostat (added every 3-4 days) at different indicated doses,
147 then they were seeded in triplicate at a density of 1,000 cells/well in a six-well multiwell.
148 Every 3-4 days, fresh medium was added with or without dasatinib and Tazemetostat,
149 alone or in combination, at the indicated doses. Colonies were stained with crystal violet
150 and counted after 10–14 days.

151 **FACS cell cycle analysis**

152 For cell cycle analysis, cells were collected 48-72h after interference, fixed in 70% ethanol
153 and stored at -20°C (up to weeks). Fixed cells were treated with RNase at 1 mg/ml final
154 concentration for 30 min at 37°C or overnight at 4°C before adding 5 mg/ml PI and
155 analyzed with Guava EasyCyte 8HT flow cytometer equipped with Guava Soft 2.1
156 (Millipore).

157

158 **Protein extracts and Western blot analysis**

159 For the preparation of whole-cell lysates, cells were lysed in buffer with 50 mM Tris-HCl
160 pH 7.6, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 and fresh protease inhibitors. Extracts
161 were sonicated for 10 + 15s at 80% amplitude and centrifuged at 12 000~ rpm for 10 min
162 to remove cell debris. For preparation of nuclear and cytoplasmic extracts, cells were lysed
163 in a Cytoplasmic Extract (CE) buffer (10mM Tris-Cl pH 7.5, 60mM KCl, 1mM EDTA,
164 0,075% NP40, proteinase inhibitors) for 3 minutes on ice. The lysate was then centrifuged
165 at 1500 rpm at 4°C for 4 minutes. The supernatant (Cytoplasmic Extract) was collected
166 into a fresh tube. The pellet was washed three times in cold CE buffer without NP40 and
167 lysed in Nuclear Extract (NE) buffer (20mM Tris-Cl pH 8.0, 420mM NaCl, 1,5 mM MgCl₂,
168 0,2 mM EDTA, proteinase inhibitors) for 10 minutes and sonicated. CE and NE extracts
169 were then centrifuged at max speed for 10 minutes to pellet any residual nuclei. Protein
170 concentrations were determined by colorimetric assay (Bio-Rad). Antibodies used for
171 Western Blotting are listed in the Supplementary Materials and Methods.

172

173 **MiRNA and transcript analysis**

174 Total RNA was extracted using TRIzol (Ambion) according to the manufacturer's
175 recommendations. For miR analysis, 30 ng RNA was retrotranscribed using the TaqMan
176 microRNA Reverse Transcription Kit (Applied Biosystem) and Real-time PCR of miR

177 expression was carried out in a final volume of 10 μ l using TaqMan MicroRNA Assays
178 (Applied Biosystems) and normalized on RNU48 and RNU49 as endogenous controls. We
179 have chosen RNU48 and RNU49 because they were not modulated in our experimental
180 conditions. TaqMan probes for miRNAs and RNU were purchased from Applied
181 Biosystems. For gene transcript analysis, 1 μ g RNA was retrotranscribed using M-MLV
182 reverse transcriptase (Invitrogen) following the manufacturer instructions. Real time PCR
183 was performed into a final volume of 10 μ l using Sybr Green PCR master mix, and
184 normalized on GAPDH. All the real-time PCR assays were performed by using an Applied
185 Biosystems® 7500 fast or *StepOne* Real-Time PCR Instruments. Each analysis was
186 performed at least on three independent biological replicates. List of primers used for Real
187 time PCR is given in Supplementary Materials and Methods.

188

189 **Chromatin Immunoprecipitation (ChIP)**

190 ChIP-qPCR was performed as described previously (6) and the method is given in
191 Supplementary Materials and Methods.

192

193 **Luciferase assay**

194 Luciferase assay was performed as described previously (6) and the protocol is given in
195 Supplementary Materials and Methods.

196

197 **Analysis of Differentially expressed genes**

198 Deregulation of genes in different set of patient samples was assessed by two tailed
199 student's t test, and a false discovery rate procedure was performed to take into account
200 multiple comparisons. The significance level was set to 5%. Analyses were performed by
201 Matlab (The MathWorks Inc.). Association between pairs of genes was evaluated by
202 calculating Pearson's R correlation coefficient.

203

204 **Curves of OS and DFS**

205 Curves of overall survival or disease-free survival in TCGA patients were evaluated by
206 Kaplan–Meier method. Curves of patients with high and low signals were considered to
207 establish statistical significance by using the logrank test. Analyses were performed by
208 Matlab (The MathWorks Inc.). Disease-free survival includes both progression free survival
209 and recurrence free survival.

210

211 See Supplementary Material and Methods for the list of antibodies used for western
212 blotting, sequence of siRNA used for interference experiments and sequence of primers
213 used for transcript analyses and for Chlp.

214

215 **3. Results**

216 **TGFBR2 acts as a tumor suppressor in lung cancer and is a direct target of miR-** 217 **106b-25 cluster**

218 To dissect mechanistically how MCM7 and its ‘hosted’ miR-106b-25 cluster elicit their
219 oncogenic activities in lung cancer, we analyzed lung cancer genome atlas (TCGA),
220 searching for transcripts that are inversely correlated to the miR cluster and therefore
221 predict potential therapeutic targets for lung cancer. Indeed, we found a number of
222 transcripts that code for proteins that signal in cancer pathways (6). From among them, we
223 elected to focus on TGFBR2 that was inversely correlated with MCM7 in a robust way and
224 hosted miRNAs with R Spearman ranging from -0,23 for miR-25 to -0,52 for MCM7 (**Fig.**
225 **1A**, Supplementary Fig. S1A). In cancer, the TGF- β pathway can be either pro-oncogenic
226 or tumor-suppressive, depending on both the kind of tumor and its stage (9). Using the
227 ‘FireBrowse Gene Expression’ computer platform (<http://firebrowse.org>) we analyzed
228 transcript expression data of different cancer types of patient samples deposited in the

229 TCGA dataset. We found that TGFBR2 expression is lower in tumor tissues when
230 compared to non-tumor controls in several cancers, including Lung Adenocarcinoma
231 (LUAD), Lung Squamous cell Carcinoma (LUSC), the two main types of NSCLC (**Fig. 1B-**
232 **C**, Supplementary Fig. S1B). Interestingly, in contrast to most of the analyzed tumor types,
233 pancreatic adenocarcinomas and glioblastoma tissues exhibited higher levels of TGFBR2
234 expression, compared to non-tumoral controls. This data is in line with the notion of dual
235 activity of TGFBR2: being either oncogenic or tumor-suppressive, depending on the
236 cancer type (Supplementary Fig. S1B).

237 Based on the median level of TGFBR2 transcript (10) the stratification of lung cancer
238 patients from TCGA was performed. The patients were categorized into either a high- or
239 low-expressing group. The data revealed that patients with a lower expression of TGFBR2
240 exhibited a shorter OS (overall survival) than those with a higher level of TGFBR2. This
241 was even more evident in LUAD than in LUSC patients (**Fig. 1D-E**). Stratification of
242 LUAD patients based on the stage of the tumor indicated that the association of low
243 TGFBR2 expression with shorter OS was stronger in early-stage (stage I) patients than in
244 those in advanced stages (stages II and III) (**Fig. 1F-H**, Supplementary Fig. S1C) (9).

245 At the cellular level, we found that either the depletion of MCM7 transcript or the
246 transfection of the locked-nucleic-acid-based-construct (LNA) that suppressed the
247 endogenous miR-25/93/106b cluster led to the increase in the levels of the TGFBR2
248 transcript and its protein in two representatives NSCLC cell lines: H1299 and H1975 (Fig.
249 **1I**, Supplementary Fig. S1D). Conversely, ectopic expression of synthetic miRNA mimics
250 reduced the expression of TGFBR2, when compared to control cells (**Fig. 1J**).

251 Importantly, we demonstrated the direct binding of miR-93 and miR-106b to the TGFBR2-
252 3'UTR through luciferase reporter assays. H1299 and H1975 cells were co-transfected
253 with miRNAs mimics and the psiCHECK2 plasmid that contained the TGFBR2-3'UTR
254 downstream of the luciferase gene. The cells exhibited reduced luciferase activity when

255 compared to cells co-transfected with the same vector and control mimic (**Fig. 1J**). This
256 effect was not observed in cells transfected with an empty vector or when the TGFBR2-
257 3'UTR was mutated in the cognate sequence recognized by miR-93 and miR-106b (**Fig.**
258 **1J**, Supplementary Fig. S1E). Collectively, these findings document the tumor-suppressive
259 activity of TGFBR2 and its post-transcriptional regulation by the oncogenic miR-
260 25/93/106b cluster in cell culture models of lung cancer.

261

262 **YAP and TAZ depletion affects the tumor suppressor TGF- β signaling**

263 We previously showed that YAP and TAZ transcriptionally regulate MCM7 and the miRs
264 that are located within the MCM7 gene (6). Herein, MCM7 and its hosted miRs are shown
265 as negative regulators of TGFBR2 (**Fig. 1** and Supplementary Fig. S1A, D). Thus we
266 sought to investigate whether the depletion of YAP/TAZ was able to affect the level of
267 TGFBR2. We found that YAP/TAZ depletion led to the up-regulation of both TGFBR2
268 transcript and protein as a consequence of reduced expression of MCM7 and its miR
269 cluster, as well (**Fig. 2A-B**, Supplementary Fig. S1F-G). Reassuringly, the ectopic
270 expression of miR25, 93, and 106b upon YAP/TAZ depletion partially rescued this effect
271 as well as the ability of H1299 and H1975 cells to form colonies (**Fig. 2C-D**,
272 Supplementary Fig. S1H-K). The increased expression of TGFBR2 in siYAP/TAZ cells
273 correlated with an increase of total SMAD2 and SMAD3 in the cytoplasm and of pSMAD3
274 in the nucleus (**Fig. 2E-F**, Supplementary Fig. S2A-B). TGF- β -induced tumor suppressor
275 response caused a cell accumulation at the G1/S transition that paired with increased
276 expression of the p21 and p15 tumor suppressors and reduced expression of the CDK
277 activator cdc25A, respectively. This occurred through p53-independent mechanisms
278 involving SMADs and Sp1 (11) (12) (13). In agreement with these items of evidence, a
279 depletion of SMAD2/3/4 partially rescued the colony-forming potential of siYAP/TAZ cells
280 (**Fig. 2G**, Supplementary Fig. S2C-G) with a partial rescue of p21 repression and cdc25A

281 expression (**Fig. 2F**, Supplementary Fig. S2B). These results suggest that the signaling by
282 TGFBR2 tumor suppressor that is orchestrated by the YAP/TAZ/MCM7 axis does involve
283 SMADs.

284

285 **EZH2 mediates YAP transcriptional repression of TGFBR2**

286 Our findings document that the increase of the TGFBR2 transcript is higher upon YAP/TAZ
287 interference (**Fig. 2B**, Supplementary Fig. S1G) than upon miRNA depletion
288 (Supplementary Fig. S1D). This suggests that YAP/TAZ may repress TGFBR2 through
289 additional mechanisms. We noticed that the Enhancer of Zeste Homologue 2 (EZH2), the
290 enzymatic component of the Polycomb Repressive Complex 2 (PRC2), was previously
291 found as an epigenetic repressor of TGFBR2 in lung cancer (14). EZH2 is overexpressed
292 in diverse cancer types, among them is lung cancer (Fig. 3a, Additional file 3: Figure S3a)
293 in which EZH2 contributes to the increase in tumor growth, metastatic potential and
294 therefore results in poor outcome and resistance to therapies. This occurs through the
295 aberrant repression of tumor-suppressor genes, which is mediated by the enzymatic three-
296 methylation of Lysine 27 of histone H3 (H3K27me3) (15). Here we found that EZH2
297 depletion reduced the colony-forming potential and affected the expression of cell cycle-
298 related genes in both H1299 and H1975 lung cancer cell lines (**Fig. 3B-C**, Supplementary
299 Fig. S3B). An inverse correlation between EZH2 and TGFBR2 transcripts was found in the
300 TCGA lung cancer dataset (**Fig. 3D**). Interestingly, we observed that EZH2 depletion
301 released the expression of TGFBR2 transcript in NSCLC cells (**Fig. 3E**, Supplementary
302 Fig. S3C). It has been previously reported that YAP and TAZ depletion affected
303 PRC2/EZH2 signature genes in melanoma cells (16). Furthermore, YAP favored the
304 recruitment of the PRC2 complex onto the promoter of the GDF15 gene in breast cancer
305 cells, whose transcriptional repression promoted metastasis (17). Moreover, YAP was
306 shown to regulate the E2F1 transcription factor (18,19), and to cooperate with E2F1 in the

307 regulation of many cell cycle-related genes (20). E2F1, in turn, was shown to regulate
308 EZH2 expression through the binding onto its promoter (21). Therefore, we asked whether
309 YAP and TAZ could regulate either EZH2 expression or cooperate with EZH2 in NSCLC.
310 Interestingly, both EZH2 and YAP/TAZ depletion promoted the expression of two direct
311 EZH2 target genes (22,23) such as PUMA and p16 in lung cancer cell lines (**Fig. 3C**,
312 Supplementary Fig. S3D). Moreover, ectopic expression of EZH2 in lung cancer cells
313 concomitantly depleted of YAP and TAZ proteins partially rescued their colony-forming
314 ability and also partially reversed the expression of TGFBR2 (**Fig. 3F-I**). Notably, we found
315 that YAP/TAZ interference in H1299 and H1975 cells reduced both EZH2 transcript and
316 protein expression (**Fig. 4A**, Supplementary Fig. S3E, right panel). The other PRC2
317 complex components, EED and SUZ12, were not affected at the transcriptional level but
318 were reduced at the protein level (Supplementary Fig. S3F-G). This might be due to the
319 reduced stability of the PRC2 complex upon reduced expression of EZH2. This effect was
320 more evident for SUZ12 (Supplementary Fig. S3F). In line with these observations, lower
321 levels of SUZ12 protein were also observed upon EZH2 interference (Supplementary Fig.
322 S3H-I). In sum, these findings indicate that YAP and TAZ are regulating the abundance of
323 EZH2 in lung cancer cell lines.

324

325 **YAP/TAZ/TEAD is recruited onto EZH2 promoter**

326 Using the “LASAGNA Search” software we analyzed genomic sequences for the TEAD-
327 binding sites in the EZH2 promoter. We found four potential binding sites in proximity of
328 the transcription start site (TSS) of the EZH2 promoter (P1 and P2, P3, P4 **Fig. 4B**). The
329 analysis of “UCSC Genome Browser” indicated that P3 and P4 sites could be occupied by
330 E2F1 and TEAD4 transcription factors (Supplementary Fig. S4A, red boxes). Chromatin
331 Immunoprecipitation (CHIP) assays revealed that YAP, TAZ, and TEAD were recruited
332 onto the EZH2 promoter regions in H1299 cells (**Fig. 4C**, Supplementary Fig. S4B). As

333 expected, no specific enrichment was detected upon YAP, TAZ, or TEAD depletion. Also,
334 in a control assay, we did not observe any complexes on an arbitrary intronic region that
335 did not contain TEAD-binding sites (**Fig. 4B-C**, Supplementary Figs. S3E, S4A, green
336 box). Moreover, the acetylation of histone H4, a mark of active transcription, was strongly
337 reduced at the TSS upon YAP/TAZ/TEAD interference (**Fig. 4D**, Supplementary Fig. S3E).
338 These items of evidence confirm that YAP, TAZ, and TEAD regulate EZH2 in NSCLC at
339 the level of transcription. Collectively, our findings suggest that YAP/TAZ and the PRC2
340 complex cooperate to inhibit the expression of TGFBR2 through transcriptional
341 mechanisms (**Fig. 4E**).

342

343 **YAP/TAZ and EZH2 co-repress tumor suppressor genes in NSCLC**

344 We performed single and combined depletion of YAP/TAZ and EZH2 in H1299 and H1975
345 lung cancer cell lines. Combined interference was more effective than single interference
346 in the inhibition of cell cycle progression, as measured through the expression of the cell
347 cycle regulator cdc25A (**Fig. 5A**, Supplementary Fig. S4C) and through cell cycle profile
348 (**Fig. 5B**, Supplementary Fig. S4D). In addition, YAP/TAZ and EZH2 depletion
349 synergistically affected colony formation of lung cancer cell lines (**Fig. 5C**, Supplementary
350 Fig. S4E).

351 To understand whether co-repression of tumor-suppressor genes as for TGFBR2 could be
352 a broad oncogenic activity mediated by YAP/TAZ/TEAD/EZH2, we searched the published
353 literature for other genes that could be potentially repressed by both YAP/TAZ/TEAD and
354 EZH2 in NSCLC or in other cell lines or models (**Table 1**). All of these genes were found to
355 be associated with tumor suppression or cell differentiation and their aberrant repression
356 was associated with tumorigenesis or stemness (6,24-30).

357

358 Using the “Firebrowse” (<http://firebrowse.org>) search platform, we found that genes listed

359 in **Table 1** were down-regulated in tumors compared to non-tumoral tissues, both in lung
360 adenocarcinomas and squamous cell carcinomas (**Fig. 5D**). Furthermore, by employing
361 “Cistrome Data Browser” (<http://cistrome.org>), we revealed that YAP and TEAD4 factors
362 as well as the H3K27me3 histone methylation signature occupied and were mapped,
363 respectively, on the regulatory elements of these genes in lung cancer cell lines as well as
364 in the IMR90 lung foetal cell line (Supplementary Fig. S5A-F). Interestingly, CHIP assays
365 showed that the depletion of YAP/TAZ and EZH2 reduced the enrichment of H3K27me3
366 on these targets (**Fig. 5E**). These results were robust and statistically significant upon
367 EZH2 depletion, while a general trend was seen upon YAP/TAZ interference (**Fig. 5E**).
368 Interestingly, the enrichments in the H3K27Ac histone signature showed an opposite trend
369 when compared to the H3K27me3 histone signature upon either YAP/TAZ or EZH2
370 depletion. These results revealed a common regulatory switch from repression
371 (methylation) towards activation (acetylation) of the targeted loci (**Fig. 5F**). This effect was
372 specific and was not observed for the U2 snRNA gene (RNU2), a constitutively transcribed
373 locus that was used as a control (**Fig. 5E-F**). The total level of H3 was not affected in all of
374 the analyzed targets (Supplementary Fig. S5G). Accordingly, the de-repression of the
375 analyzed transcripts (**Table1**) was seen upon both YAP/TAZ and EZH2 depletion (**Fig. 5G**,
376 Supplementary Fig. S5H).

377 Collectively, these findings indicate that co-repression of tumor suppressor genes by the
378 concerted action of YAP/TAZ and EZH2 might play a broad role in lung tumorigenesis.

379

380 **Pharmacological targeting of YAP/TAZ and EZH2 affects synergistically lung cancer**
381 **cell survival.**

382 We treated both H1299 and H1975 lung cancer cell lines with Dasatinib and Tazemostat
383 (EPZ-6438) to pharmacologically inhibit YAP/TAZ (34) and EZH2 (35) respectively.
384 Dasatinib treatment affected the transcriptional activity of YAP and TAZ in a dose-

385 dependent manner. This was shown by a reduced expression of their well-known targets,
386 such as CTGF and ANKRD1 (36) as well as the newly characterized target EZH2.
387 However, TGFBR2 and p21 transcripts were de-repressed (Supplementary Fig. S6A, B).
388 In addition, the expression of the cell cycle regulators cdc25A and c-myc was diminished,
389 indicating a delayed cell cycle progression (Supplementary Fig. S6A, B). Tazemetostat
390 treatment inhibited the enzymatic activity of EZH2 as shown by the reduction of
391 H3K27me3 signatures globally, and it de-repressed PRC2 target genes in a dose and
392 time-dependent manner (Supplementary Fig. S7A, B). This effect was more pronounced in
393 H1299 than in H1975 cells (Supplementary Fig. S7A). In fact, H1975 were more resistant
394 to Tazemetostat, as shown by the IC50 value measured through the “ATPlite” assay
395 (Supplementary Fig. S8A, B). Some genes showed a more pronounced de-repression at
396 longer time points, probably because of the slow kinetics of H3K27me3 turnover, as was
397 shown previously (Supplementary Fig. S7A, B) (37). Functionally, the combination therapy
398 with Dasatinib and Tazemetostat acted synergistically in affecting the colony formation and
399 cell growth of H1299, H1975, as well as of A549 lung cancer cell line that was previously
400 shown to be resistant to Dasatinib (38) (39) (**Fig. 6A-E**, Supplementary Fig. S8C-E). This
401 effect was more evident in H1299, as observed at a very low dose of Tazemetostat (2 μ M)
402 and with low doses of Dasatinib (0,025 μ M) (**Fig. 6A-B**, Supplementary Fig. S8C). In the
403 resistant A549 cells, increasing doses of Dasatinib up to 0,1 μ M in the absence of
404 Tazemetostat did not reduce the number, but only the size of the colonies (**Fig. 6E**, lower
405 panel, Supplementary Fig. S8E, left panel). However, the addition of Tazemetostat (2 μ M)
406 sensitized cells to Dasatinib and reduced both the number and size of the colonies (**Fig.**
407 **6E**, Supplementary Fig. S8E). While low amounts of Dasatinib alone, or Tazemetostat
408 (2 μ M) alone, did not cause de-repression of TGFBR2, p21 or SMAD7 transcripts, the
409 combination of the two drugs de-repressed these transcripts (to different extend, though)
410 in both H1299 and H1975 cell lines (Supplementary Fig. S9A, B). In summary, these

411 findings may provide a basis for testing the combination of Dasatinib and Tazemetostat as
412 a therapeutic modality for the treatment of NSCLC patients (**Fig. 6F**).

413

414 **4. Discussion**

415 We report here that YAP and TAZ elicit, at least in part, their oncogenic roles in NSCLC
416 through the negative regulation of the tumor-suppressor “arm” of the TGF- β signaling
417 pathway. It has been previously reported that YAP and TAZ crosstalk with the TGF- β
418 pathway either by cooperating with its pro-tumorigenic signaling (i.e., induction of EMT)
419 (40) or antagonizing its pro-apoptotic/tumor-suppressor activity. YAP prevents TGF- β 1-
420 mediated apoptosis without affecting EMT in normal mouse mammary epithelial cells (41).
421 Similarly, YAP and TAZ promote TGF- β -induced EMT by inhibiting the TGF- β tumor
422 suppression activity in breast cancer (42). Herein, we also show an antagonism between
423 YAP/TAZ-oncogenic and TGF- β /SMAD-tumor-suppressive activities in NSCLC cells
424 through the inhibition of TGFBR2. This inhibition is elicited through the aberrant activation
425 of two oncogenic loci: EZH2 that represses TGFBR2 transcriptionally (14) and MCM7 that
426 harbors the oncogenic cluster of miR106b-25. That mRNA cluster represses TGFBR2 at
427 the post-transcriptional level. Clinically, lower expression of TGFBR2 in tumor tissues of
428 lung cancer patients associates with poorer prognosis. This appears even more evident in
429 the early stages of the disease.

430

431 Interestingly, the cooperation between YAP/TAZ and EZH2 to maintain the expression of
432 tumor-suppressor proteins at relatively low levels applies also to the direct targets of
433 EZH2. YAP and TAZ are extensively studied for their role as transcriptional co-activators,
434 while their role as transcriptional co-repressors is still emerging but has not been fully
435 clarified (32,43-45). Our findings help to dissect mechanistically the YAP/TAZ-mediated
436 transcriptional repression of a subset of tumor-suppressor genes through the cooperation

437 with EZH2. This was previously shown for the GDF15 gene in breast cancer cell lines (17),
438 but we extend these findings also to other genes in NSCLC. The subset of common
439 YAP/TAZ/PRC2 targets might be different with regard to cell and tissue contexts.
440 Moreover, we did not find any physical interaction between YAP and EZH2 in our
441 experimental model (data not shown). Future work will be aimed at looking for potential
442 partners or protein complexes that may help the recruitment of YAP and EZH2 on the
443 same genomic targets and mediate the formation of a repressive chromatin conformation.
444 The NuRD complex due to its previously reported recruitment onto some YAP/TAZ
445 repressed targets might be a strong candidate (32,44,45). The H3K27 de-acetylation
446 mediated by the NuRD complex was shown in turn to recruit the PRC2 complex onto
447 bivalent genes, in which both acetylation (associated with transcriptional activation) and
448 methylation (associated with repression) of H3K27 are present. The balance between the
449 two modifications determines the transcriptional state of the target genes (46). In
450 agreement with this hypothesis, the depletion of YAP and TAZ reduces the enrichment of
451 H3K27me3 and increases H3K27Ac at the YAP/TAZ/EZH2 co-repressed loci suggesting
452 that YAP and TAZ may facilitate EZH2 mediated H3K27 methylation.

453 We also confirmed the transcriptional and functional synergism between YAP/TAZ and
454 EZH2 in NSCLC through their combined inhibition upon pharmacological treatment with
455 Dasatinib and Tazemetostat (EPZ-6438). Both are FDA approved drugs targeting YAP
456 and EZH2 activity, respectively (34) (35). Tazemetostat has been tested in early phase
457 trials for rhabdoid tumors, B-Cell Non-Hodgkin lymphoma, and has been recently
458 approved for the treatment of epithelioid sarcoma (47). These tumors are characterized by
459 overexpression or hyperactivation of EZH2 (48-50). However, in other cancer types,
460 Tazemetostat was not as effective as a single therapeutic agent, suggesting the need to
461 test the drug in combination with other anticancer agents (49). In NSCLC, Tazemetostat is
462 currently evaluated in a clinical trial that involves multiple immunotherapy-based treatment

463 combinations, both as the first-line and the second-line therapy of patients with confirmed
464 metastases (ClinicalTrials.gov Identifier: NCT03337698). Dasatinib has failed in NSCLC
465 clinical trials as single-agent due to its relatively high toxicity and it is currently used in the
466 early phase trials in combination with EGFR inhibitors or immunotherapy
467 (ClinicalTrials.gov Identifier: NCT02954523, NCT02750514). In our model (NSCLC cell
468 lines), we observed a synergistic effect of Dasatinib and Tazemetostat treatment in the
469 inhibition of cell proliferation and in the de-repression of tumor-suppression genes co-
470 repressed by the combinatorial activity of YAP and EZH2.

471

472 **Conclusions**

473 In sum, our findings suggest that the newly characterized YAP/TAZ/EZH2 oncogenic axis
474 may represent a potential therapeutic target in lung cancer. More precisely, our results
475 provide a rationale for a two-pronged strategy for inhibiting both YAP/TAZ as well as EZH2
476 for an efficient therapeutic outcome, because of their functional synergy.

477 **5. Acknowledgements**

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479 the pCDNA3-EZH2 construct. We thank Dr. Stefen Wiemann (German Cancer Research
480 Center (DKFZ), Heidelberg, Germany) for kindly sharing the plasmid for TGFBR2 3'UTR
481 luciferase assay (Psi-check2-TGFBR2).

482

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Fig. 1

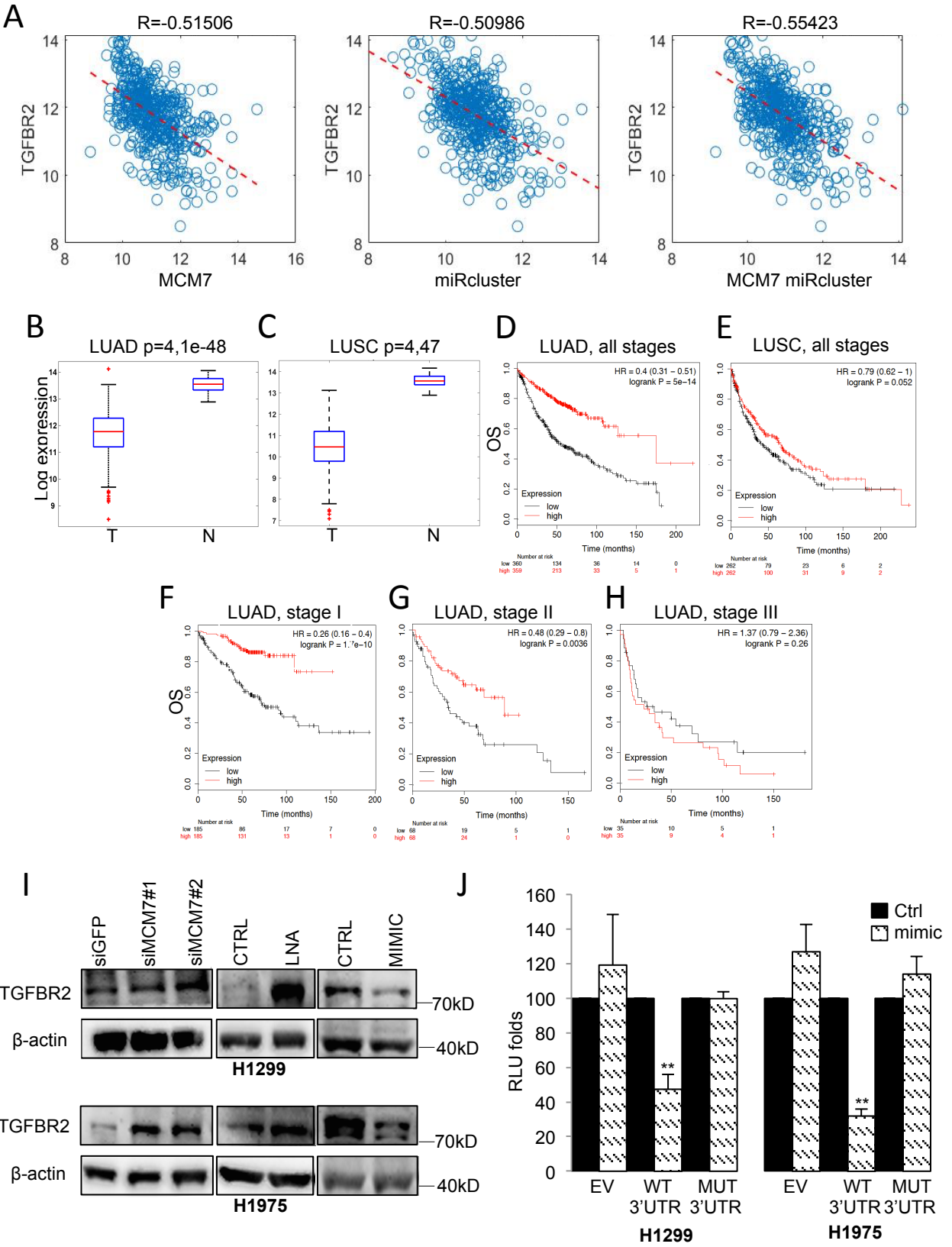


Fig. 1 TGFBR2 is a direct target of the oncogenic miR-106b-25 cluster and exhibits prognostic value in NSCLC. **A**, Dot plots showing the correlation between TGFBR2 transcript and MCM7 (left panel), TGFBR2 and the miR106b-25 cluster (mid panel), and between TGFBR2 and MCM7/miR cluster together (right panel) in Lung Adenocarcinoma (LUAD) patients from the TCGA. **B-C**, Boxplot showing the expression of TGFBR2 transcript in tumoral (T) compared to non-tumoral (N) tissues in lung adenocarcinoma (LUAD) patients (**B**) and lung squamous cell carcinoma (LUSC) (**C**) from the TCGA dataset. Number of LUAD samples: 510T, 58N. Number of LUSC samples: 501T, 51N. **D-H**, Kaplan-Meier (KM) survival of lung adenocarcinoma (**D**) and squamous cell carcinoma patients (**E**), and KM of adenocarcinoma patients stratified for tumor stage (**F-H**), with high or low expression of TGFBR2. The number of patients is indicated below the plots. **I**, Western blot analysis for TGFBR2 expression upon depletion of MCM7 (left panels), upon either transfection with LNA inhibitors (mid panels) or mimics (right panels) for miR-25, 93, and 106b in H1975 cells (upper panels) and H1299 (lower panels). β -actin expression was used for equal protein loading. **J**, Luciferase assay of the psiCHECK2-TGFBR2-3'UTR reporter wild type or mutated in the seed sequence recognized by miR-93 and 106b in H1299 transiently co-transfected either with control mimic or mimic for miR-93 and miR-106b. Data are compared to signals obtained from cells co-transfected with the same mimic and psiCHECK2 Empty Vector as a control. Firefly luciferase was used to normalize the Renilla luciferase. All the experiments have been performed in triplicate. Data are presented as mean \pm SEM. Two-tailed t-test analysis was applied to calculate the P values. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$

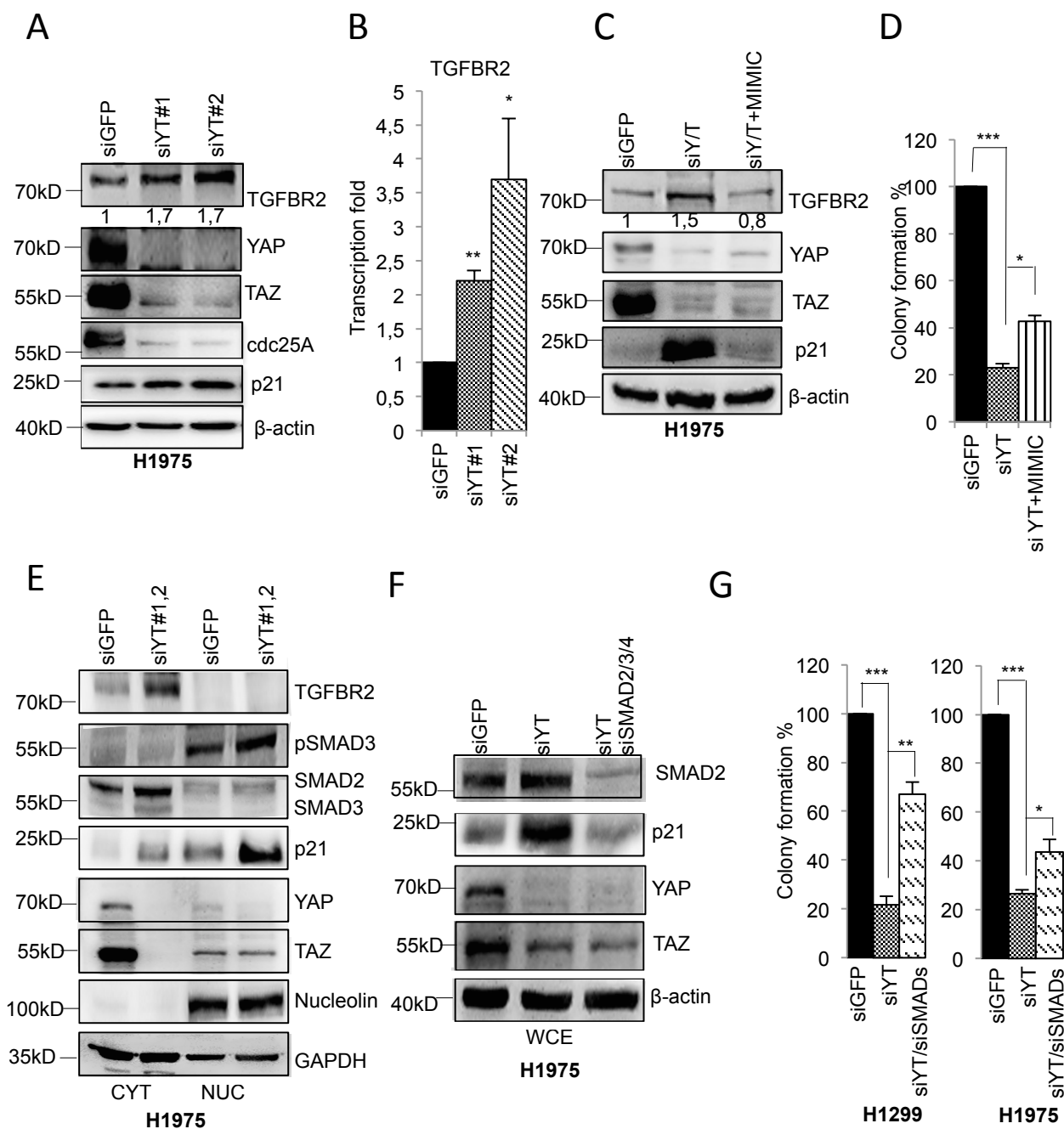
Fig. 2

Fig. 2 YAP/TAZ control TGFBR2 expression acting upstream to the miR cluster. **A-B**, Western blot analysis for expression of the indicated proteins normalized to β -actin (**A**) and quantification by real time-PCR of TGFBR2 transcript normalized to GAPDH (**B**) in H1975 cells depleted for YAP and TAZ proteins compared to control cells. The experiments have been performed in triplicate. **C**, Western blot analysis of the indicated proteins, normalized to β -actin in H1975 upon the interference of YAP and TAZ with or without mimic miR-93 and 106b. **D**, Quantification of colony formation of H1975 cells upon YAP/TAZ interference with or without concomitant transfection with mimic miR-93 and 106b, compared to controls. Data are presented as mean \pm SEM. Two-tailed t-test analysis was applied to calculate the P values. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$. **E**, Western blot analysis of nucleo-cytoplasmic extracts from H1975 cells showing the abundance of the indicated proteins upon YAP/TAZ interference compared to siGFP control cells. Nucleolin and β -actin were used as a nuclear and cytoplasmic loading control, respectively. **F**, Western blot analysis of whole-cell extracts (WCE) of the indicated proteins normalized to β -actin in H1975 cells upon the interference of YAP and TAZ with two different combinations of alternative siRNA. **G**, Quantification of colony formation in H1299 and H1975 cells upon depletion of YAP/TAZ with or without interfering with SMAD2/3/4 expression compared to control cells.

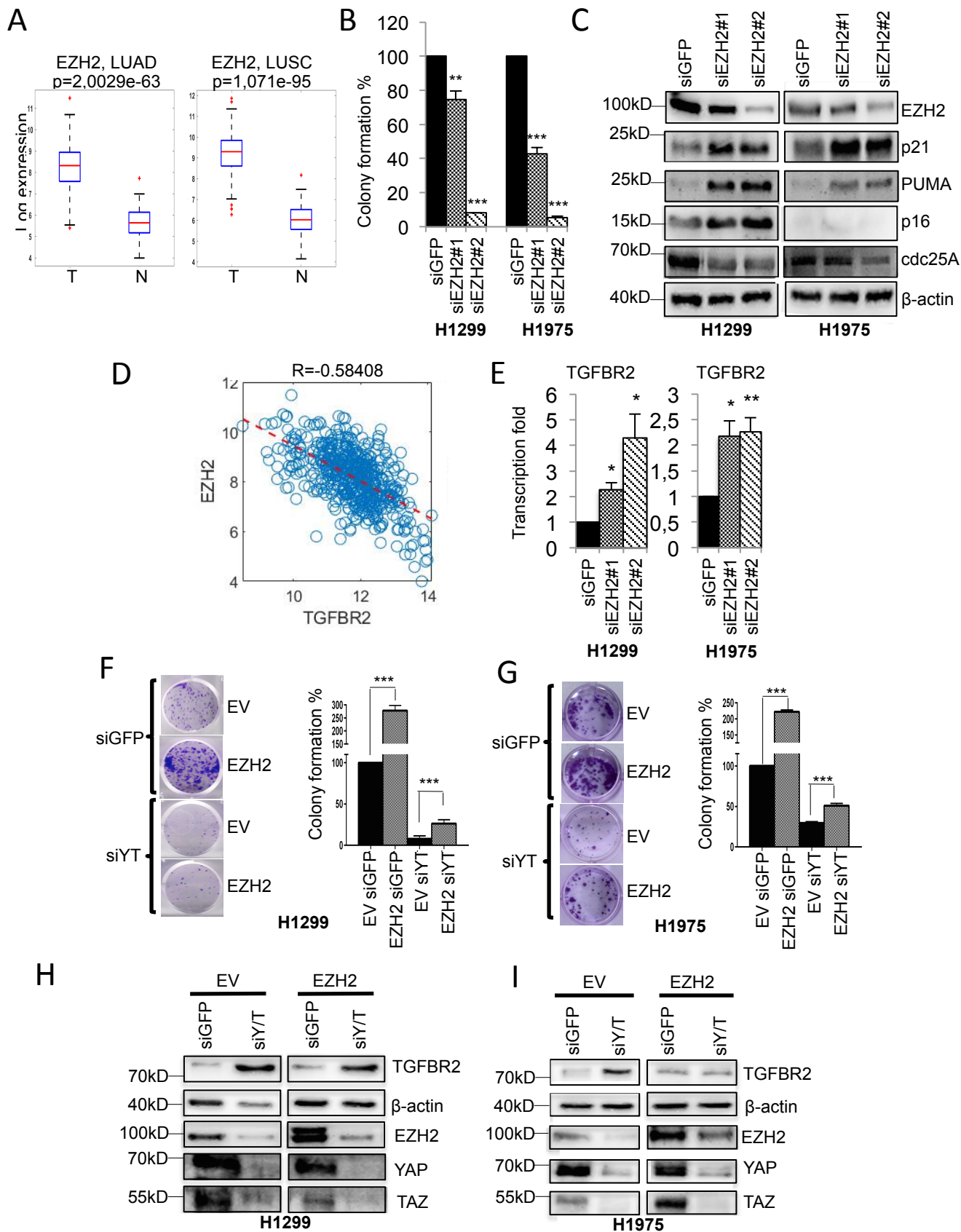
Fig. 3

Fig. 3 YAP and TAZ inhibit transcriptionally TGFBR2 expression through the oncogenic EZH2 repressor. **A**, Boxplot of the abundance of EZH2 in tumoral (T) compared to non-tumoral (N) tissues in lung adenocarcinoma patients (LUAD, left) and lung squamous cell carcinoma (LUSC, right) from the TCGA. The number of LUAD samples: 510T, 58N. The number of LUSC samples: 501T, 51N. **B**, Quantification of colony formation in H1299 (left) and H1975 (right) upon EZH2 depletion compared to control cells. **C**, Western blot analysis of the indicated proteins normalized to β -actin in H1299 (left) and H1975 cells (right) upon depletion of EZH2 protein. **D**, Dot plot showing the correlation between TGFBR2 and EZH2 transcripts in lung adenocarcinoma patients. **E**, RT-PCR quantification of TGFBR2 transcript upon depletion of EZH2 in H1299 (left) and H1975 (right). **F-G**, Representative images of cell colony formation assay (left panels) and quantification of relative colony formation (right panels) in H1299 (**F**) and H1975 (**G**) treated with siRNA against YAP/TAZ with or without concomitant overexpression of EZH2 compared to cells transfected with siGFP and empty vector as a control. Data are presented as mean \pm SEM of three technical replicates of one representative experiment. Two-tailed t-test analysis was applied to calculate the p values. The experiment has been performed in triplicate. **H-I**, Western blot analysis of the indicated proteins normalized to β -actin in H1299 (**H**) and H1975 (**I**).

Fig. 4

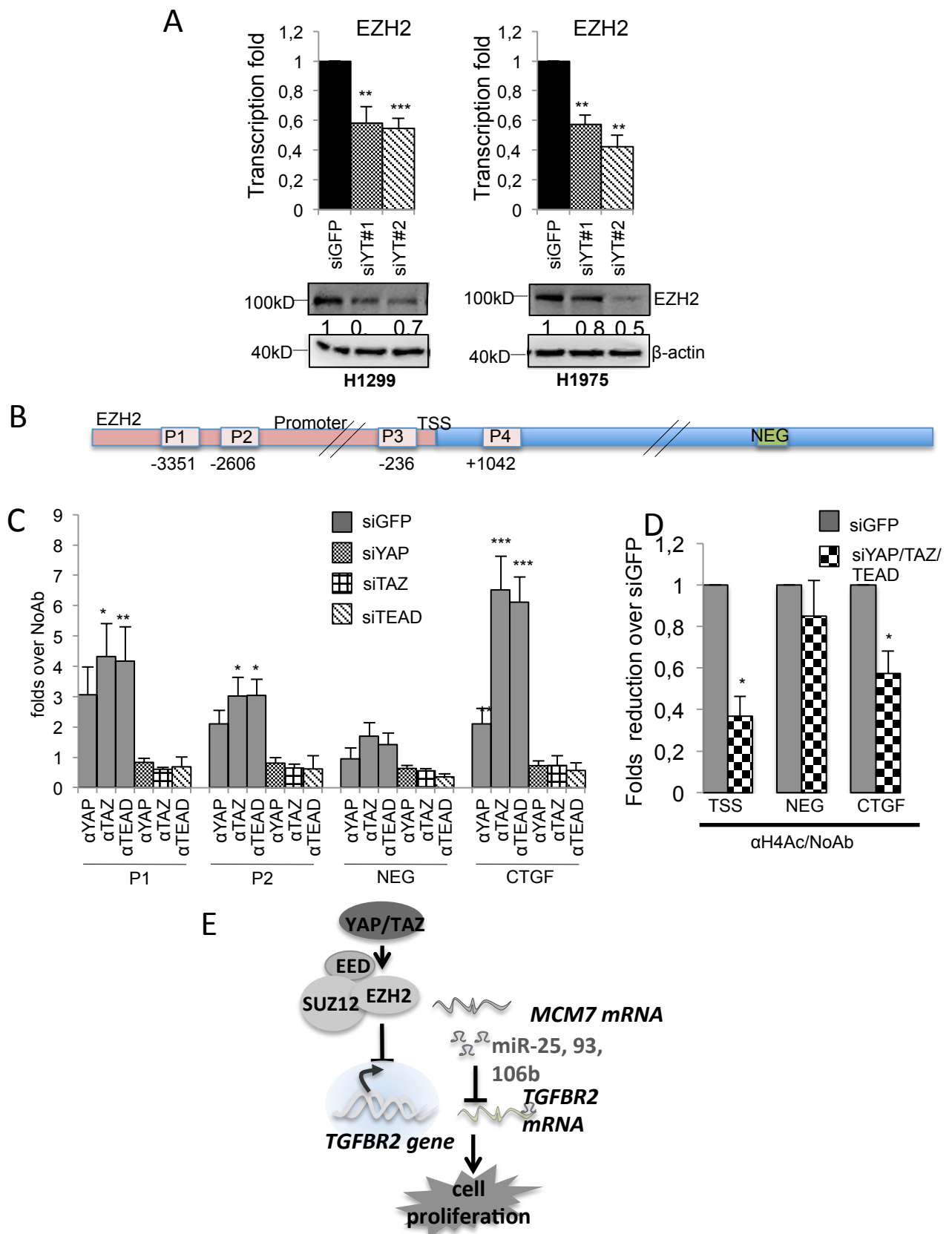


Fig. 4 YAP/TAZ regulates EZH2 expression.

A, RT-PCR quantification (upper panel) and western blot analysis (lower panel) of EZH2 transcript and protein, respectively, in H1299 (left) and H1975 cells (right) upon YAP and TAZ interference with two combinations of alternative siRNAs. **B**, Schematic representation of the EZH2 locus and the regions containing the putative consensus for TEAD (P1, P2, P3, P4) with their relative position respect to TSS, and with the region used as a negative control of YAP/TAZ/TEAD binding (NEG). **C**, Fold enrichment of YAP, TAZ, and TEAD1 proteins onto the indicated sites of EZH2 locus in H1299 cells depleted for YAP, TAZ, and TEAD1 compared to control cells. CTGF promoter was used as a positive control while an intronic region of the EZH2 locus was used as a negative control. Data are presented as mean \pm SEM of at least three biological replicates. For each antibody, fold enrichment was calculated over no antibody control. **D**, Chromatin Immunoprecipitation analysis of the abundance of H4Ac onto the indicated genomic sites (TSS, EZH2 NEG, and CTGF) in cells simultaneously depleted for YAP/TAZ/TEAD1 compared to siGFP as control. Fold enrichment was calculated over no antibody control and then normalized to the siGFP control that was adjusted to 1. The experiments were performed in triplicate. Two-tailed t-test analysis was applied to calculate the P values. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$. **E**, Working model indicating that YAP and TAZ downregulate TGFBR2 expression through miR-25,93 and 106b (post-transcriptionally) and the transcriptional repressor EZH2 (transcriptionally) in NSCLC.

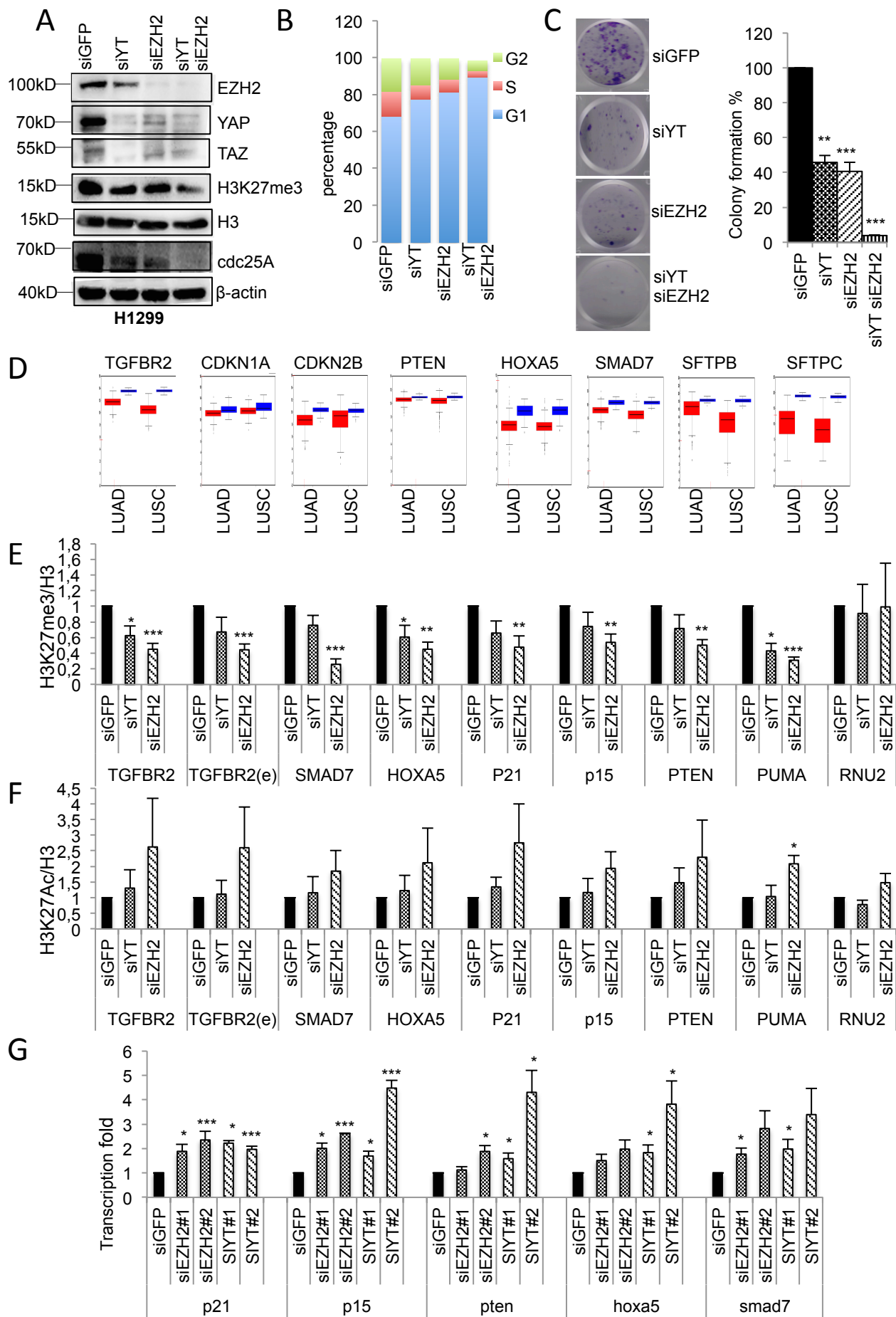
Fig. 5

Fig. 5 YAP/TAZ and EZH2 synergistically affect the cell growth of lung cancer cell lines. **A**, Western blot analysis of the indicated proteins in H1299 cells upon depletion of YAP/TAZ and EZH2, either alone or in combination, compared to control cells. **B-C**, Cells (%) in G1, S, and G2 phases (**b**) and colony quantification (**c**) of H1299 cells, upon depletion of YAP/TAZ or EZH2, alone or in combination, respect to control cells. **D**, Boxplots representing the expression profile of the indicated transcripts in LUng Adeno Carcinoma (LUAD) and Lung Squamous cell Carcinoma (LUSC) from patient samples of TCGA dataset as obtained from the FireBrowse Gene Expression Viewer. **E-F**, Chromatin Immunoprecipitation fold enrichment of H3K27me/H3 (**E**) and H3K27Ac/H3 (**F**) on the indicated loci. TGFBR2(e) indicates the enhancer region shown in figure S5a. For each antibody, fold enrichment was calculated over no antibody control and normalized to the siGFP control that was adjusted to 1. Experiments were performed in triplicate. Two-tailed t-test analysis was applied to calculate the P values. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$. (G) Real-time PCR analysis of the indicated transcripts in H1299 cells upon depletion of either YAP/TAZ or EZH2 proteins.

Fig. 6

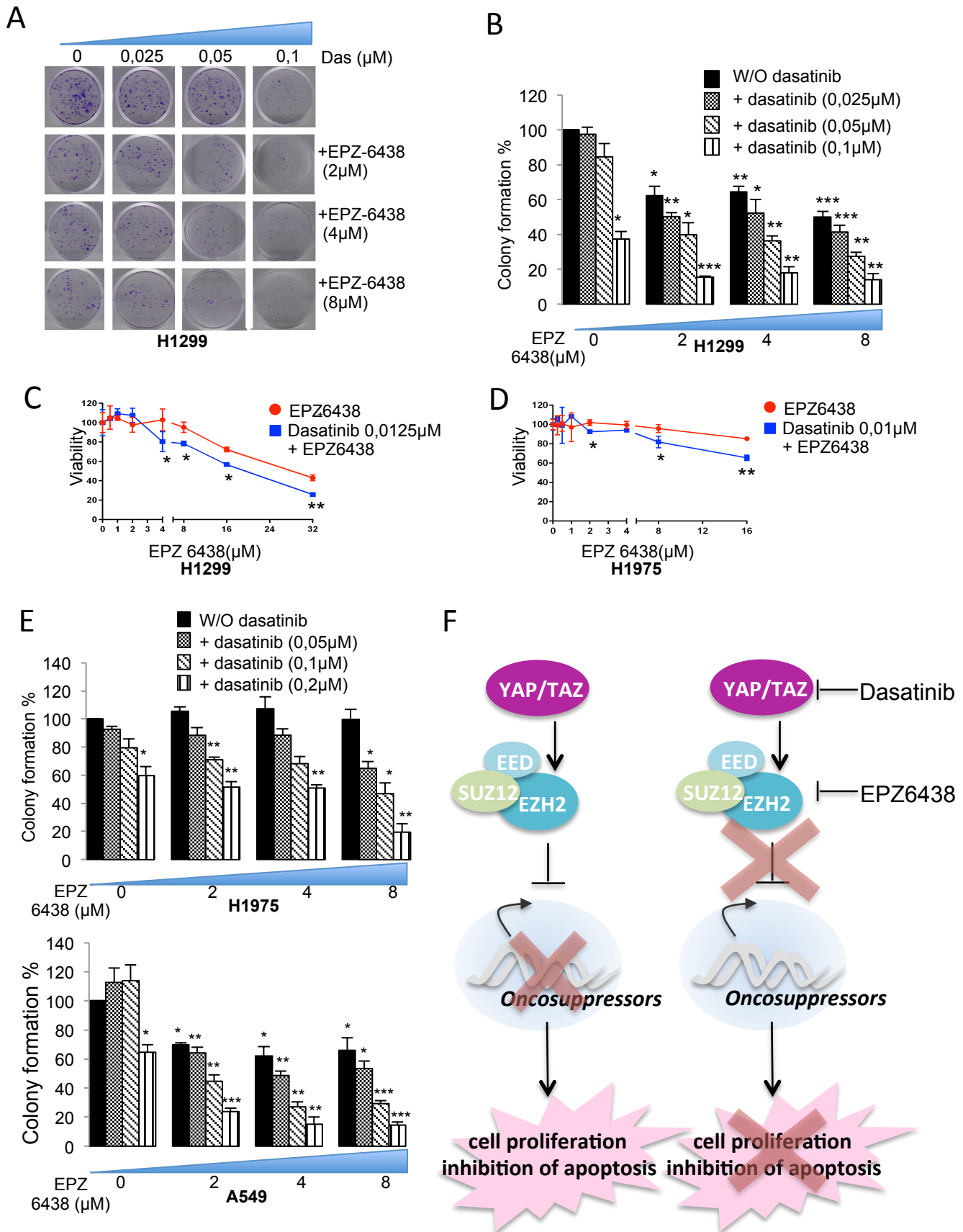


Fig. 6 Low doses of Dasatinib with Tazemetostat synergistically affect cell proliferation. **A-B**, Representative images (**A**) and quantification (**B**) of colony formation upon treatment with different combinations of dasatinib and Tazemetostat (EPZ-6438) at the indicated doses in the H1299 cell line. **C-D**, Viability of H1299 cells (**C**) and H1975 cells (**D**) as measured with ATPlite assay after 72h treatment with a fixed dose of dasatinib and growing doses of Tazemetostat. **E**, Quantification of colony formation upon treatment with different combinations of Dasatinib and Tazemetostat in H1975 (upper panel) and A549 cells (lower panel). **F**, Schematic model of the oncogenic role of the YAP/TAZ/EZH2 axis which contribute to the aberrant proliferation of lung cancer cell lines. Pharmacological inhibition of YAP and EZH2 impairs the repression of tumor suppressor genes thereby reducing cell proliferation of lung cancer cell lines.

Table1. Genes repressed by YAP and EZH2 IN NSCLC.

Gene	Repressed by EZH2	Repressed by YAP	Cell type/ tissue	REF
CDKN1A	✓	✓	NSCLC	(31)
			NSCLC	(24)
CDKN2B	✓		NSCLC	(25)
PTEN	✓	✓	NSCLC	(26)
			MCF10A	(27)
HOXA5	✓	✓	NSCLC	(28)
			MCF10A	(32)
SMAD7	✓	✓	Renal fibroblasts	(29)
			Dermal fibroblasts	(33)
SFTPB		✓	Lung of MST1/2-- mice	(30)
SFTPC		✓	Lung of MST1/2-- mice	(30)

List of genes found in the published literature to be repressed by either YAP/TAZ and EZH2.