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Netrin-1 and UNC5B Cooperate with Integrins to Mediate YAP-Driven Cytostasis

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Running Title: Mechanism of YAP-driven cytostasis

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Abstract

1
2 **Opposite expression and pro- or anti-cancer function of YAP and its paralog TAZ/WWTR1 stratify**
3 **cancers into binary YAP^{on} and YAP^{off} classes. These transcriptional coactivators are oncogenic in YAP^{on}**
4 **cancers. In contrast, YAP/TAZ are silenced epigenetically along with their integrin and extracellular**
5 **matrix adhesion target genes in neural and neuroendocrine YAP^{off} cancers (e.g., small cell lung cancer,**
6 **retinoblastoma). Forced YAP/TAZ expression induces these targets, causing cytostasis in part through**
7 **Integrin- α V/ β 5, independent of the integrin-binding RGD ligand. Other effectors of this anti-cancer**
8 **YAP function are unknown. Here, using CRISPR screens, we link the Netrin receptor UNC5B to YAP-**
9 **induced cytostasis in YAP^{off} cancers. Forced YAP expression induces UNC5B through TEAD DNA-**
10 **binding partners, as either TEAD1/4-loss or a YAP mutation that disrupts TEAD-binding (S94A) blocks**
11 **whereas a TEAD-activator fusion (TEAD(DBD)-VP64) promotes UNC5B induction. Ectopic YAP**
12 **expression also upregulates UNC5B relatives and their netrin ligands in YAP^{off} cancers. Netrins are**
13 **considered pro-tumorigenic, but knockout and peptide/decoy-receptor blocking assays reveal that in**
14 **YAP^{off} cancers UNC5B and Netrin-1 can cooperate with integrin- α V/ β 5 to mediate YAP-induced**
15 **cytostasis. These data pinpoint an unsuspected Netrin-1/UNC5B/integrin- α V/ β 5 axis as a critical**
16 **effector of YAP tumor suppressor activity.**

17 18 **Significance Statement**

19 Netrins are widely perceived as pro-cancer proteins but here we uncover an anti-cancer function for
20 Netrin-1 and its receptor UNC5B.

21 Introduction

22 Heterogeneity, plasticity, and clonal evolution drive tumor complexity, hindering successful diagnosis
23 and treatment. Overcoming these hurdles is critical to improve cancer treatment. Identifying
24 overarching principles of cancer biology that span tumor type and defining the molecular underpinnings
25 of these pan-cancer rules can pinpoint broadly relevant therapeutics. We demonstrated that all cancers
26 can be stratified into binary YAP^{on} and YAP^{off} classes with distinct genetic and therapeutic vulnerabilities
27 based on opposite expression and function of YAP and its paralog TAZ/WWTR1 (1).

28 YAP and TAZ are transcriptional coactivators that are downstream targets of the Hippo signaling
29 pathway. In YAP^{on} cancers, YAP/TAZ are well-known oncogenes that are recruited to distal enhancers by
30 TEAD-family DNA-binding proteins where they cooperate with AP1 family transcription factors to induce
31 cell cycle genes (2). In contrast, in YAP^{off} cancers YAP and TAZ are epigenetically silenced along with > 80
32 of their adhesion target genes, including integrins and extracellular matrix (ECM) proteins, such as
33 collagens, fibronectin and laminins, which are reactivated upon forced expression of YAP (1). Discovered
34 through principal component analysis, we previously termed this binary-cancer-defining set as PC1⁺
35 genes, but also now refer to them as YAP^{Ad} genes (for YAP adhesion targets). YAP^{off} cancers consist of all
36 leukemias and lymphomas, all neuroendocrine and many neural cancers. In this context, YAP/TAZ are
37 tumor suppressors, contrasting their oncogenic function in YAP^{on} cancers (1,3,4). In liquid
38 (hematopoietic) YAP^{off} cancers, such as multiple myeloma, YAP and TAZ can induce apoptosis through
39 various mechanisms (5–7). Alternatively, in solid neural and neuroendocrine YAP^{off} cancers, such as
40 retinoblastoma, small cell lung cancer (SCLC), small cell neuroendocrine prostate cancer, and Merkel cell
41 carcinoma, among others, ectopic YAP/TAZ cooperate with TEAD-family proteins to induce cytostasis
42 (1,3). In addition, YAP silencing is also critical to permit metastasis of SCLC (8). Several YAP^{off} cancers
43 arise from cells-of-origin that intrinsically silence YAP/TAZ (1), and consistent with that observation, YAP

44 can cooperates with NOTCH and REST to antagonize neuroendocrine lineage genesis during
45 development and repair (9).

46 The mechanism by which ectopic YAP/TAZ drive cytostasis in YAP^{off} cancers is incompletely understood.
47 In retinoblastoma and SCLC it requires YAP-induction of the Integrin- α V/ β 5 axis (1). Humans possess 18
48 α -chain and eight β -chain integrin members that can generate at least 24 different integrin α / β
49 heterodimers (10,11). While some integrins are broadly expressed, others are more tissue restricted,
50 such as β 2-containing integrins expressed by leukocytes (11,12). Integrins bind various cell surface,
51 secreted and ECM ligands to mediate processes such as cell-cell interactions, adhesion, cellular
52 migration, and extravasation (10). Interaction of integrins with ECM proteins plays a central role in
53 controlling cellular adhesion, which often involves binding of α V- or β 1-containing integrins to RGD
54 (arginine-glycine-aspartate) or related motifs in ECM proteins such as fibronectin and vitronectin (11).
55 Interestingly, we found that, consistent with the silencing of YAP^{Ad} genes, all YAP^{off} cancers grow as
56 non/semi-adherent cultures, contrasting YAP^{on} cancers that grow as adherent cultures (1). Furthermore,
57 forced YAP expression induced RGD-dependent adhesion of YAP^{off} cells (1). However, whereas Integrin-
58 α V/ β 5 blocking antibodies attenuated YAP-induced cytostasis, they did not consistently block YAP-
59 induced adhesion (1). Additionally, RGD peptides did not affect YAP-induced cytostasis, but did disrupt
60 YAP-induced adhesion (1). Thus, Integrin α V/ β 5 causes cytostasis independent of YAP-induced adhesion
61 or Integrin-RGD interactions. However, other components that facilitate cytostasis are unknown. Here,
62 we employ CRISPR screens to expose new effectors of YAP-induced cytostasis in YAP^{off} cancers.

63 **Materials and Methods**

64 **Cell culture**

65 Y79 (RRID:CVCL_1893) and WERI-RB1 (RRID: CVCL_1792) retinoblastoma lines were already present in
66 the Bremner lab and were cultured in RPMI-1640 supplemented with 10% FBS. NCI-H209 (RRID:
67 CVCL_1525) SCLC cells were obtained from Dr. Susan Cole (Queen's University) and were cultured in
68 RPMI-1640 with 7.5% FBS, while NCI-H2171 (RRID: CVCL_1536) SCLC cells were obtained from ATCC and
69 were cultured in HITES media (DMEM:F12 supplemented with 0.005 mg/ml insulin, 0.01 mg/ml
70 transferrin, 10 nM hydrocortisone, 10 nM β -estradiol, 30 nM sodium selenite, 4.5 mM (final
71 concentration) L-glutamine and 5% FBS). Lenti-X 293 cells (used to generate lentiviruses) were
72 purchased from Clontech/Takara and were cultured in DMEM with 10% FBS. Cells were maintained at
73 37°C and 5% CO₂. All lines were routinely confirmed negative for mycoplasma (at least every six months)
74 using the eMyco PLUS Mycoplasma PCR Detection Kit. Retinoblastoma and SCLC lines were validated by
75 short tandem repeat (STR) analysis performed at The Centre for Applied Genomics at SickKids Hospital
76 (Toronto, ON). Cells were maintained in culture for a maximum of three months before fresh aliquots
77 were thawed and used.

78 **Lentivirus production**

79 YAP and TEAD4(DBD)-VP64 vectors have been described previously (1,13). Our YAP expression vectors
80 are available from Addgene (Cat# 174168-174175; RRID:Addgene_174168 to RRID:Addgene_174175).
81 Retinoblastoma lines used the PGKp series (Addgene, cat# 174172-174175), while SCLC lines used the
82 EFSp series (Addgene cat# 174168-174171). Lentiviruses were produced using Lenti-X 293 cells, as we
83 detailed previously (13).

84 **CRISPR screen to identify YAP effectors**

85 The CRISPR screen was previously detailed (1) and is outlined in **Fig. 1a**. Briefly, a pooled CRISPR sgRNA
86 library was constructed by cloning sgRNA sequences (4/gene plus 50 non-targeting controls) into the
87 LentiCRISPR v2 lentiviral backbone (Addgene plasmid #52961; RRID:Addgene_52961). The sgRNA
88 sequences were previously published (1). Lentivirus was then generated and used to transduce Y79 cells
89 at a multiplicity of infection (MOI) of 0.3. After selection for transduced cells using puromycin, cells were
90 collected 10 days after transduction. A portion of the cells was harvested and genomic DNA extracted
91 (Qiagen DNEasy Blood and Tissue Kit) as an input sample. The remainder of the cells were transduced
92 with either a YAP-expressing lentivirus or empty vector control. Five days later, YAP and GFP expression
93 were confirmed using western blotting and/or flow cytometry and then cells were cultured for an
94 additional 10 days (total of 15 days after YAP expression and 25 days after initial transduction with the
95 CRISPR library). At this time, cells were harvested and genomic DNA isolated. The screen was performed
96 in biological quadruplicate. sgRNA sequences were then PCR amplified from genomic DNA and indexed
97 using Illumina i5 and i7 sequences. These libraries were then subjected to deep sequencing on an
98 Illumina NextSeq 500 (Illumina NextSeq 500/550 Hi Output Kit v2.5 with 22 dark cycles and 26 light
99 cycles). The resulting FASTQ files were converted to real-time base call (.bcl) files using Illumina
100 bcl2fastq2 conversion software v2.17, and then sequencing reads mapped to the sgRNA library. Read
101 counts for each sgRNA in each sample were normalized to total reads for that sample and sgRNAs with
102 low read counts (<20) were excluded. For each biological replicate, counts for each sgRNA in YAP-
103 expressing cells (day 25) were compared to both the Empty (day 25) and Input (day 10) samples to
104 generate an enrichment or depletion ratio, then the median ratio between the replicates was calculated
105 for each sgRNA. To prioritize possible YAP effectors, we focused on genes with ≥ 2 sgRNAs that were
106 enriched ≥ 1.5 -fold in YAP-expressing/Empty vector cells, and that were also enriched in YAP-
107 expressing/Input cells. To identify possible synergistic hits (e.g. genes that increase YAP activity), we

108 focused on genes with ≥ 2 sgRNAs that were depleted ≥ 1.5 -fold in YAP-expressing/Empty vector cells, as
109 well as in YAP-expressing/Input cells.

110 **Cell growth rescue experiments**

111 To generate pooled Y79 knockout lines and appropriate controls, Y79 cells were transduced with two
112 separate control sgRNAs (sgControl; sgControl #1 – GACCGAACGATCTCGCGTA; sgControl #2 –
113 CGCTTCCGCGCCCGTTCAA) or two sgRNAs targeting either TEAD1 (sgTEAD1-3415 –
114 GGCCGGGAATGATTCAAACA; sgTEAD1-3418 – ACATGGTGGATAGATAGCCA) or UNC5B (sgUNC5B-3692 –
115 CCAGAACGACCACGTCACAC; sgUNC5B-3693 – ATACCCTAGCGATTTCCGCC), and then selected in
116 puromycin (2 $\mu\text{g/ml}$), similar to the outline of the CRISPR screen (**Fig. 1a**). sgRNA sequences were cloned
117 into the LentiCRISPR v2 vector. Approximately 10-12 days after the pooled lines were generated, cells
118 were then transduced with YAP or Empty vector control (GFP only) lentiviruses so that $>90\%$ of cells
119 were transduced as determined by YAP and/or GFP flow cytometry. Five days after viral transduction
120 (peak of YAP expression), a portion of cells were harvested for western blot and flow cytometry to
121 assess YAP and GFP expression. The remainder of cells were plated and then counted 10 days later
122 (15 days after YAP virus transduction). The ratio of YAP-expressing to Empty vector cells was then
123 calculated for each sgRNA and then TEAD1 or UNC5B knockout lines were compared to control sgRNA
124 expressing cells.

125 For netrin blocking experiments, cells were transduced with YAP-expressing or control (Empty vector)
126 lentiviruses. The following day, cells were either left untreated or were treated with the indicated netrin
127 blocking agent (anti-Netrin 1 (NET1-H-mAb) (14,15) or netrin trapping reagent (ectodomain UNC5-Fc
128 (16)) at 10 or 20 $\mu\text{g/ml}$ (retinoblastoma lines) or 20 $\mu\text{g/ml}$ (SCLC lines). Fresh blocking agent was added
129 to the cells every 3-4 days and cells counted 15 days after initial transduction. Integrin blocking
130 experiments were performed similarly, except the Integrin- $\alpha\text{V}/\beta 5$ blocking antibody (Sant Cruz Biotech,
131 sc-81632; RRID:AB_1123634) was used at 2.5 $\mu\text{g/ml}$.

132 **Western blotting, flow cytometry and RT-qPCR**

133 Our western blotting and flow cytometry protocols have been thoroughly described elsewhere (13). The
134 following antibodies were used: YAP/TAZ (Santa Cruz Biotech, sc-101199; RRID: AB_1131430), GFP
135 (Santa Cruz Biotech, sc-9996; RRID: AB_627695), TUBULIN (Santa Cruz Biotech, sc-32293; RRID:
136 AB_628412), TEAD1 (BD Biosciences, 610923; RRID: AB_398238) and UNC5B (Cell Signaling Technology,
137 13851; RRID:AB_2798330). RNA extraction and RT-qPCR protocols have been described previously (1).

138 **Data Availability Statement.**

139 All data are provided in the main and supplementary figures and tables. Queries can be sent to R.B.

140 Results

141 A CRISPR screen to identify YAP effectors

142 To identify key mediators of YAP tumor suppressor activity, we performed a targeted CRISPR screen in
143 Y79 retinoblastoma cells (**Fig. 1a**) (1). The library consisted of ~4 sgRNAs/gene targeting ~950 genes
144 including YAP targets from retinoblastoma and SCLC cell lines/PC1+ genes, non-targeting sgRNAs and
145 controls, including sgRNAs targeting YAP, TEADs or Hippo pathway components (**Supplementary Table**
146 **S1**). Y79 cells were transduced with a pooled lentiviral library and 10 days later “input” cells were
147 harvested. Remaining cells were then transduced with either a YAP-expressing or control (empty)
148 vector. Both vectors co-expressed GFP to track transduced cells. After five days, YAP/GFP expression
149 was confirmed by western blotting and/or flow cytometry (**Fig. 1b**) and then cells were cultured an
150 additional 10 days. At endpoint (day 25), empty vector (“Empty”) and YAP-expressing (“YAP”) cells were
151 harvested and subjected to deep sequencing along with Input (day 10) samples. We then compared
152 YAP-expressing cells to Empty and Input cells (**Fig. 1c**). We first defined possible YAP effectors as genes
153 with at least two sgRNAs enriched in YAP-expressing (day 25) compared to Empty (day 25) cells (**Fig. 1c-**
154 **ii**). In addition, we prioritized genes from the latter list that also had at least one sgRNA enriched in YAP-
155 expressing (day 25) vs. Input (day 10) cells (**Fig. 1c-ii**). Negative regulators of YAP (synergistic hits) would
156 be depleted in YAP-expressing cells compared to Empty-vector or Input cells (**Fig. 1c-iii**).

157 We anticipated that sgRNAs targeting YAP and TEADs would rescue YAP-induced cytostasis and would
158 thus score as hits in the screen. Indeed, YAP sgRNAs targeting ectopic YAP rescued cell number, as did
159 TEAD1 or TEAD4 sgRNAs despite potential redundancy with other TEAD family members (**Fig. 2a**,
160 **Supplementary Figure S1a**). Validation assays confirmed that TEAD1 contributed to YAP-induced
161 cytostasis (**Fig. 2b**). Further validating our approach, sgRNAs targeting known YAP inhibitors exacerbated
162 YAP-induced cytostasis. These included AMOTL2, KIRREL and NF2, which negatively regulate YAP by

163 activating the LATS kinases or directly sequestering YAP in the cytoplasm (**Fig. 2a, Supplementary Figure**
164 **S1a**) (17–20). All four MORC2 sgRNAs also enhanced YAP-induced cytostasis (**Supplementary Figure**
165 **S1a**). We did not pursue this novel genetic interaction further here as our focus was to identify effectors
166 of YAP activity. As a repressor in neural cells (21) MORC2 may help silence cytotstatic adhesion/ECM
167 genes in YAP^{off} cancers. Antagonizing the effects of forced YAP expression in YAP^{off} cancer contrasts the
168 role of MORC2 in hepatocarcinoma, a YAP^{on} cancer, where it represses NF2 and KIBRA to promote YAP
169 activity (22,23).

170 Having identified multiple anticipated hits that validate the screen, we next focused on possible
171 effectors of YAP tumor suppressor activity that were also induced following forced YAP expression in
172 YAP^{off} cancers. We identified four such hits including integrin β 5 (ITGB5), which we validated previously
173 (1); the Ras family GTPase, RAB25; TGF β -induced factor homeobox 2 (TGIF2); and unc-5 netrin receptor
174 B (UNC5B) (**Fig. 2a, Supplementary Figure S1a**). Follow-up experiments did not validate RAB25, but
175 knockout of the homeodomain transcription factor TGIF2 did ameliorate YAP-mediated cytostasis
176 (**Supplementary Figure S1b**). TGIF2 was included in the screen because it is a YAP- induced target gene
177 in SHP77 SCLC cells (1), but it was not induced by YAP in Y79 retinoblastoma cells (**Supplementary Figure**
178 **S1c**). RNA-Seq data from several other YAP^{off} cell lines (1) demonstrated that *TGIF2* is also not YAP-
179 induced in these cancers. Nevertheless, whether YAP-responsive or expressed constitutively, our data
180 indicate that this homeobox protein is an important component of YAP-induced cytostasis. Among 51
181 randomly chosen targets we included in the CRISPR library that were expressed but not YAP-targets in
182 SHP77 and Y79 cells, three of four SAP30 sgRNAs ameliorated YAP-driven cytostasis (**Supplementary**
183 **Table S1**). SAP30 is best known as a core component of the SIN3 repressor complex, and can also
184 functions as a coactivator (24). As our focus was on YAP-induced genes we did not pursue this hit
185 further, but it is noteworthy that SAP30 and TGIF2 interact (25). A genome-wide screen is needed to
186 define the full complement of YAP cytostasis effectors whose expression is YAP-independent. Finally,

187 validation studies with two independent sgRNAs confirmed that knocking out the YAP-inducible target
188 UNC5B partially rescued YAP-mediated cytostasis (**Fig. 2c**). As we saw before for ITGB5 (1), UNC5B
189 knockout did not affect expression of ectopic YAP or induction of YAP target genes (**Fig. 2c-d**), thus it is a
190 downstream effector and not an upstream regulator of YAP. Hereafter, we focused on the UNC5
191 pathway.

192 **YAP regulates expression of netrins and UNC5 proteins in YAP^{off} cancers**

193 Next, we examined the extent to which UNC5B is induced following forced YAP expression and/or
194 constitutively expressed in YAP^{off} cancers, and whether induction is dependent on TEAD. For this we
195 first used a lentiviral expression system that generates levels of YAP or YAP mutants that resemble
196 endogenous YAP levels in YAP^{on} cancers (1,13). As predicted, wild type (wt) or constitutively active YAP
197 (YAP^{5SA}), but not the TEAD-binding mutant (YAP^{S94A}), induced UNC5B in Y79 cells (**Fig. 3a**). The TEAD4
198 DNA-binding domain fused to a VP64 transcriptional activation domain (TEAD4(DBD)-VP64)
199 recapitulates cytostasis induced by forced YAP expression in YAP^{off} cells (1). Consistent with the latter,
200 TEAD4(DBD)-VP64 induced UNC5B in Y79 cells (**Fig. 3b**). YAP, YAP^{5SA} and TEAD4(DBD)-VP64, but not
201 controls, also induced UNC5B in WERI-RB1 retinoblastoma cells (**Fig. 3c, d**). Moreover, YAP or YAP^{5SA},
202 but not YAP^{S94A} induced UNC5B in DU4475 YAP^{off} breast neuroendocrine cancer cells (**Fig. 3e**). However,
203 YAP did not induce UNC5B in several SCLC lines, and modestly downregulated UNC5B in some cases (**Fig.**
204 **3f**). Whether slight downregulation of UNC5B is a direct effect of YAP or a feedback mechanism to
205 mitigate YAP-driven cytostasis remains to be determined. Interestingly, UNC5B was already highly
206 expressed in many SCLC (NCI-H69, NCI-N417, NCI-H82) and some other YAP^{off} cell lines
207 (retinoblastoma/RB1021, neuroendocrine prostate/NCI-H660) relative to YAP^{on} lines (**Fig. 3g**), which
208 may explain why YAP does not further increase expression in some contexts. These data suggest that
209 intrinsically high as well as YAP-induced expression of UNC5B may facilitate YAP-driven cytostasis.

210 UNC5B is one of several UNC5 family members (UNC5A-D) that bind to Netrin-1 and 3 (26). Netrin-1 is a
211 secreted protein initially described as a neuronal navigation cue, which was more recently propose to
212 promote tumor progression in multiple human cancers (27,28). Recent work has also implicated Netrin-
213 3 in promoting SCLC and neuroblastoma (29). Other netrin family members such as Netrin-4 and Netrin-
214 G1 and G2 are more divergent and do not interact with UNC5B. We asked if YAP induces other UNC5
215 and/or NTN members in YAP^{off} cancers. Examination of RNA-Seq data from retinoblastoma and SCLC (1)
216 revealed up-regulation of multiple UNC5 and NTN members following forced YAP expression, including
217 UNC5B/C/D and NTN1, but not NTN3 (**Fig. 3h**). Thus, YAP can induce several UNC5 receptors and their
218 ligand NTN1 in YAP^{off} cancers.

219 **Blocking Netrin-UNC5B Signaling Rescues YAP-Induced Cytostasis**

220 The data above reveals that forced YAP expression induces UNC5 and Netrin-1 in YAP^{off} cells, and UNC5B
221 expression is intrinsically high in some YAP^{off} contexts. Thus, we studied the extent to which Netrin-
222 UNC5B signaling is required for YAP-induced cytostasis across various YAP^{off} cancers. We utilized two
223 strategies to neutralize netrin, including a blocking antibody (NET1-H-mAb) (14,15) or a Netrin-1
224 trapping reagent (ectodomain UNC5-Fc) (16). Both strategies produced a dose-dependent rescue of
225 YAP-induced cytostasis in Y79 cells (**Fig. 4a**), confirming UNC5B knockout data (**Fig. 2a, c**). Indeed,
226 blocking Netrin-1 (**Fig. 4a**) rescued growth to a similar extent as UNC5B knockout (**Fig. 2c**). Blocking
227 Netrin-1 also ameliorated YAP-induced cytostasis in WERI-RB1 retinoblastoma cells (**Fig. 4b**). As noted
228 above, forced YAP expression did not increase already high UNC5B levels in SCLC lines such as NCI-
229 H2171 and NCI-H209, but YAP induced Netrin-1 in both contexts (**Fig. 3h**), and both the Netrin-1
230 antibody and trapping agents alleviated YAP-induced cytostasis in NCI-H2171 and NCI-H209 SCLC cells
231 (**Fig. 4c, d**). Netrin blockade did not affect expression of ectopic YAP or induction of YAP target genes,
232 thus Netrin-1, like UNC5 (this work) and ITGB5 (1), act downstream of YAP (**Supplementary Figure S2**).

233 Together, these data reveal that UNC5-NTN1 signaling is a vital effector of YAP-induced cytotostasis in
234 YAP^{off} neural and neuroendocrine cancers.

235 Blocking the Integrin- α V/ β 5 dimer, either via gene deletion or with blocking antibodies, ameliorates
236 YAP-induced cytotostasis in YAP^{off} cancers (1), similar to the effects of inhibiting Netrin-1/UNC5 activity
237 (**Fig. 2c, 4a-d**). Integrins and the netrin-UNC5 pathway co-operate in several settings (30–33), thus we
238 asked if they similarly co-operate to mediate YAP-driven cytotostasis. If these YAP effectors regulate
239 separate pathways to induce cytotostasis, targeting both would be additive or synergistic. However, if they
240 function in the same pathway, targeting both effectors should have the same effect as blocking either.
241 Thus, we treated wild type or *UNC5B* null Y79 cells with an Integrin- α V/ β 5 blocking antibody. As seen
242 before (1), inhibiting Integrin- α V/ β 5 in wild type Y79 cells mitigated YAP-induced cytotostasis (**Fig. 4e**).
243 Importantly, the magnitude of rescue with the Integrin- α V/ β 5 blocking antibody was identical in *UNC5B*
244 null cells (**Fig. 4e**). These data suggest that netrin-UNC5 and Integrin- α V/ β 5 cooperate in the same
245 pathway to mediate YAP cytotostatic activity in YAP^{off} cancers (**Fig. 4f**).

246 Discussion

247 A positive association between netrin and cancer progression is well-established (27). As a ligand for its
248 cognate death receptors, over expression of netrin, either by the cancer cells or neighboring cells such
249 as cancer associated fibroblasts, promotes survival and stemness (34–38). Netrin can also promote
250 endothelial cell survival, angiogenesis and vascular mimicry (39–43), and supports cancer cell migration
251 and invasion (44–48). Moreover, a recent first-in-class anti-netrin therapeutic showed efficacy in a
252 clinical trial for endometrial cancer, impeding both cell survival and epithelial mesenchyme transition
253 (49). Indeed, it is well-established that netrin is oncogenic in various cancers, such as melanoma,
254 pancreatic ductal carcinoma, hepatocellular carcinoma, prostate carcinoma, and endometrial cancer
255 (44–49), and all of these are YAP^{on} cancers (1). In that context, YAP is tumorigenic, contrasting its tumor
256 suppressor function in YAP^{off} cancers that consist of all neuroendocrine and haematopoietic cancers as
257 well as several neural cancers (1). The potent anti-proliferative effect explains why YAP and its paralog
258 WWTR1/TAZ are silenced in YAP^{off} cancers. Here, a targeted CRISPR/Cas9 screen identified the UNC5B
259 receptor as a key mediator of YAP-induced cytostasis in YAP^{off} cancers. Forced YAP expression induced
260 the expression of UNC5B and the related UNC5 family receptors, UNC5C and D, as well as their ligand
261 NTN1/Netrin-1 in YAP^{off} cancers. In contrast, mining transcriptome data from eight YAP^{on} lines with YAP
262 or YAP/TAZ knockdown (1) revealed that YAP/TAZ do not upregulate these genes in that context
263 (**Supplementary Figure S3**), suggesting that YAP-induction of UNC5-family/NTN1 genes is YAP^{off}-cancer-
264 specific. Induction of UNC5-family and NTN1 in YAP^{off} cancers depended on YAP/TEAD binding as
265 YAP^{S94A}, which cannot bind TEADs, failed to induce UNC5B, and a TEAD-DBD-VP64 fusion mimicked the
266 effects of YAP. Whether induction of UNC5-members and/or NTN1 by YAP/TEAD is direct or indirect
267 remains to be determined. TEAD4 ChIP-Seq data in these same YAP^{off} lines (1) did not reveal TEAD4
268 binding at the promoter of these genes, but YAP/TEAD primarily regulate genes via distal enhancers (50–

269 54), so it is possible that YAP/TEAD directly induce the expression of UNC5-family members and NTN1
270 via distant enhancers. Using either a netrin blocking antibody or a Netrin-1 trapping reagent we
271 demonstrated that netrin signaling is a key anti-cancer effector of ectopic YAP across multiple YAP^{off}
272 contexts. In this work and a prior study (1), we focused on deducing how YAP initiates cytostasis, thus
273 we specifically tested whether blocking UNC5-NTN or Integrin signaling at the onset of forced YAP
274 expression rescues cytostasis. In future studies it will be interesting to add blocking antibodies after
275 cytostasis is established to deduce whether the same factors also maintain YAP-driven cytostasis. Our
276 genetic and antibody-blocking strategies revealed that UNC5/netrin signaling co-operates with the
277 Integrin- α V/ β 5 pathway to mediate the cytostatic effects of forced YAP expression in YAP^{off} cancers. It is
278 noteworthy that forced YAP expression in YAP^{off} cancer induces multiple ECM components, including
279 direct targets of netrin/integrin complexes such as collagens and laminins (1,31). Thus, our work
280 provides a coherent framework within which to understand why YAP is cytostatic in YAP^{off} cancers.

281 Our results linking Integrin α V/ β 5 (ref (1)) to the Netrin-1/UNC5 axis (this work) are consistent
282 with functional netrin/integrin interactions observed in other settings. For example, Netrin-1 activates
283 Integrin β 1 to drive migration and metastasis of neuroblastoma and, of particular note, both proteins
284 are in a complex together (30). Moreover, α 6/ β 4 integrin mediates pancreatic epithelial cell adhesion to
285 netrin-1 (31), and netrin also binds α 3 β 1 integrin to regulate interneuron migration in the cortex (32).
286 We envisage, therefore, that the ability of Netrin-1, UNC5, and Integrin- α V/ β 5 to arrest growth in YAP^{off}
287 cancers likely involves their physical interaction, although that remains to be demonstrated formally. To
288 our knowledge, our data provides the first example of netrin/integrin cooperation to inhibit cancer cell
289 growth. There are indeed scant examples of netrin suppressing cancer. In one other case - pancreatic
290 ductal adenocarcinoma (PDAC), a YAP^{on} cancer (1) - netrin suppresses 3D tumor growth in xenograft
291 models (55). In that YAP^{on} context netrin suppresses the expression of oncogenic integrin β 4 indicating

292 netrin/integrin antagonism, which stands in stark contrast to YAP^{off} cancers where our results reveal that
293 Netrin-1 and integrins cooperate to inhibit proliferation.

294 While YAP, TAZ, and ITGB5 are down-regulated in YAP^{off} cancers (1), the UNC5 family show
295 varying levels depending on the paralog. Our results reveal that even where UNC5B is constitutively
296 expressed, it is required for YAP-driven cytotaxis. Another hit in our screen, the homeobox protein
297 TGIF2, was constitutively expressed in most tested YAP^{off} cancer lines, but our genetic studies suggest it
298 is also key for YAP-mediated growth-arrest. TGIF2 promotes a variety of YAP^{on} cancers, such as ovarian
299 and cervical cancer (56,57). It has been studied much less in YAP^{off} cancers, although the paralog TGIF1
300 suppresses acute myeloid leukemia consistent with our results in neural/neuroendocrine YAP^{off} cancers
301 (58). TGIF2 is connected to TEAD/YAP in other contexts, as this homeobox protein can convert
302 hepatocytes to pancreatic progenitors which involves induction of TEAD2 (59), and TEAD/YAP induce
303 factors that promote pancreatic development (60). Of note, constitutively expressed SAP30 was also a
304 hit in our screen, which can interact with TGIF2 (25). Overall, whether the distinct YAP effectors that
305 cooperate to promote cytotaxis in YAP^{off} cancers are YAP-induced or constitutive is context dependent,
306 but both groups are essential. In contexts where constitutively expressed netrin and/or UNC5-proteins
307 contribute to cytotaxis, it is likely they are cooperating with other YAP-induced targets, such as
308 additional UNC5 members and Integrin- β 5.

309 There are limitations of our study. Ectopic YAP potently arrests growth of SCLC and
310 retinoblastoma cells *in vivo* and *in vitro* (1), and while we demonstrated an anti-proliferative role for
311 netrin, UNC5 and integrins *in vitro*, further work is required to test this effect *in vivo*. Also, we
312 functionally assessed the anti-cancer netrin/UNC5/Integrin cooperation in neuroendocrine and neural
313 cancers, and although we also observed YAP-induced expression of UNC5B in neuroendocrine breast
314 cancer cells, additional functional analyses are required to test whether the cytotstatic effect extends to
315 this and other YAP^{off} cancers, particularly the large hematopoietic class (1). Forced YAP expression

316 suppresses multiple neuroendocrine and neural cancers in a TEAD-dependent fashion (1,3), although
317 suppression of multiple myeloma by TAZ is TEAD-independent (6). In addition, our work does not show
318 the extent to which different members of the netrin and UNC5 family are utilized to mediate context-
319 specific anti-proliferative effects of YAP. Nevertheless, this study does reveal that ectopic YAP up-
320 regulates UNC5 family members to distinct extents in YAP^{off} cancers. While YAP induced UNC5 and NTN
321 mRNAs in multiple YAP^{off} cancer lines, and we confirmed that YAP or TEAD-VP64 induced UNC5B protein
322 across multiple lines, we were unable to confirm whether other UNC5 proteins or NTN1 protein were
323 induced in lines where their mRNAs were up-regulated. We tested a goat polyclonal antibody to UNC5C
324 (R&D Systems, cat# AF1005), which was published to work (61), but in our hands the antibody detected
325 many bands, perhaps reflecting lot variation typical of polyclonal antibodies. We also tested a published
326 NTN1 antibody (Abcam cat# ab126729) but did not detect protein from suspension cultures perhaps
327 because secreted NTN1 is released in suspension cultures (which are YAP^{off} lines), and while we did
328 detect an induced band at the appropriate size using cells adhered to polyD-lysine, there were also
329 numerous background bands. Despite these technical hurdles in detecting all the proteins by Western,
330 the evidence that they mediate YAP-induced cytostasis in YAP^{off} cancers is compelling. Thus, UNC5B
331 protein-induction was detected in multiple lines, UNC5B-knockout countered YAP-induced cytostasis in
332 Y79 retinoblastoma cells, and both a Netrin blocking antibody and a Netrin trapping reagent (UNC5-Fc)
333 also had this anti-cytostatic effect across multiple YAP^{off} cancer cell lines. We also did not interrogate
334 rigorously the role of other netrins in YAP-mediated cytostasis. Consistent with prior work showing that
335 Netrin-3 promotes SCLC (29), we found that Netrin-1 but not Netrin-3 was induced following forced YAP
336 expression in this cancer. However, it is unclear how Netrin-3 would affect SCLC cells expressing YAP.
337 We also observed Netrin-4 induction, but as this ligand does not bind the UNC5 receptor it was not
338 pursued further. Finally, the signals downstream of the Netrin-1/UNC5B/Integrin- α V/ β 5 that cause
339 cytostasis remain to be deduced. However, of note, although netrins contain an RGD motif, interaction

340 with integrins occurs through a distinct 25 amino acid peptide (31), providing a logical hypothesis as to
341 why YAP-induced cytostasis in YAP^{off} cancers is RGD-independent (1).

342 In summary, our work illuminates how YAP promotes cytostasis in YAP^{off} cancers. It also exposes
343 a unique example of how Netrin-1 can cooperate with integrins to inhibit rather than promote cancer
344 cell growth, underscoring the striking differences between binary YAP^{off} / YAP^{on} cancer classes.

345

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502

503 Figure Legends

504 **Figure 1. Outline of CRISPR screen to identify YAP effectors.** **A.** Schematic of the CRISPR screen
 505 highlighting timepoints for viral transduction and sample collection. **B.** Western blot (left)
 506 showing expression of ectopic YAP in Y79 cells from three replicates of the CRISPR screen. YAP
 507 expression is quantified relative to YAP^{on} NCI-H661 cells. Flow cytometry plot (right) for GFP
 508 demonstrating that ~99% of cells are transduced with the Empty or YAP expression vector. n =
 509 4. **C.** Criteria used to define hits from the CRISPR screen as (i) having no effect on YAP activity;
 510 (ii) being a possible YAP effector; (iii) being a possible YAP inhibitor. YAP-expressing cells (at day
 511 25) were compared to either Input (day 10) or Empty (day 25) cells. sgRNAs that were enriched
 512 in YAP-expressing compared to Empty or Input samples represent possible YAP effectors, while
 513 sgRNAs depleted in YAP-expressing cells represented possible YAP inhibitors.

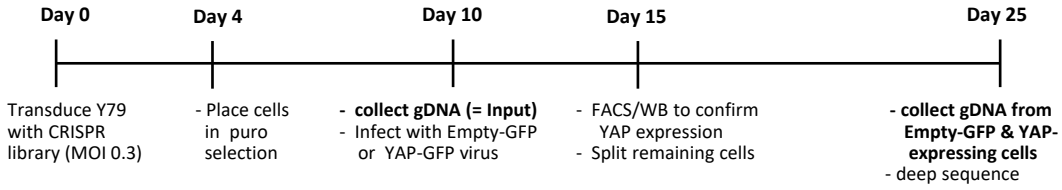
514 **Figure 2. A CRISPR screen identifies UNC5B as a YAP effector.** **A.** Z-scores comparing YAP-
 515 expressing (day 25) to Empty (day 25) cells for each sgRNA in the CRISPR screen. Select genes
 516 are indicated. n = 4. **B.** TEAD1 knockout rescues YAP-induced cytostasis in Y79 cells (left).
 517 Western blot showing TEAD1 knockout efficiency and expression of ectopic YAP (right). * p <
 518 0.05 compared to sgControl cells, n = 3. **C.** UNC5B knockout rescues YAP-induced cytostasis in
 519 Y79 cells (left). Western blot showing UNC5B knockout efficiency and expression of ectopic YAP
 520 (right). *** p < 0.001 compared to sgControl cells, n = 4. **D.** RT-qPCR for YAP target genes
 521 (*AJUBA*, *AMOTL2*, *CYR61* and *AHNAK*) or a control gene (*RER1*) in control or UNC5B knockout
 522 Y79 cells +/- ectopic YAP expression. n = 3.

523 **Figure 3. YAP induces Netrin and UNC5 family members in YAP^{off} cancers.** **A-F.** UNC5B
 524 Western blots from YAP^{off} cells ectopically expressing YAP or YAP mutants (A, C, E, F) or a TEAD4
 525 DNA-binding-domain (DBD)-VP64 fusion protein or controls (B, D). Cell lines and tumor types
 526 are indicated in each panel. n = 3. **G.** UNC5B Western blot from YAP^{off} and YAP^{on} cell lines. n = 2.
 527 **H.** Heatmap showing the effect of ectopic YAP on the expression of UNC5A-D or Netrins (NTN1,
 528 2 and 4) in YAP^{off} cells. * FDR < 0.05; N = not detected. RNA expression levels mined from RNA-
 529 Seq data in (1).

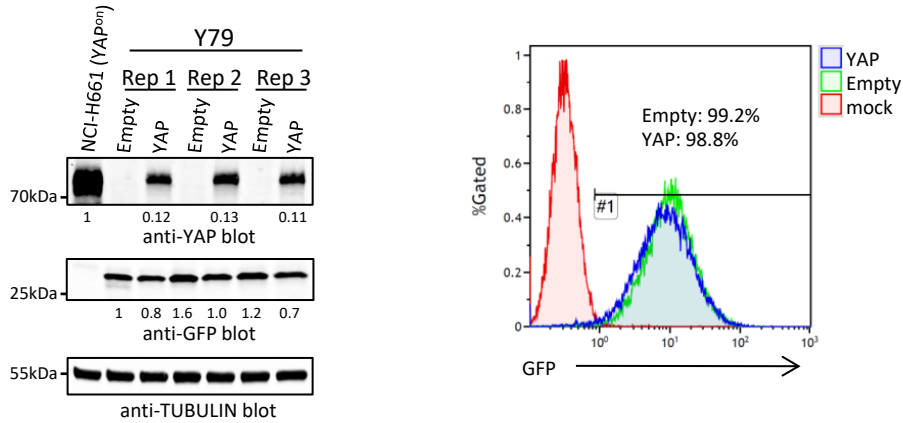
530 **Figure 4. A Netrin-UNC5-ITGAV/B5 pathway mediates YAP-induced cytostasis.** **A-D.** A netrin
 531 blocking antibody (α -NTN) or trapping reagent (UNC5-Fc) alleviates YAP-induced cytostasis in
 532 YAP^{off} cell lines. Cell lines and tumor types are indicated in each panel. Y79 cells expressed wild
 533 type YAP, while WERI-RB1, NCI-H209 and NCI-H2171 expressed YAP^{55A}. * p < 0.05, ** p < 0.01,
 534 *** p < 0.001 compared to untreated (untr) cells; n = 3-5 (panel A-B) or 3 (panels C-D). **E.**
 535 Rescue of YAP-induced cytostasis in control or UNC5B knockout Y79 cells treated with an
 536 Integrin- α V/ β 5 blocking antibody. * p < 0.05 compared to untreated sgControl cells; n = 3. **F.**
 537 Summary of the mechanism of YAP-induced cytostasis in YAP^{off} cancers.

Fig 1: Outline of CRISPR screen to identify YAP effectors

A.



B.



C.

sgRNA classification:

Comparison
YAP day 25/Empty day 25:
YAP day 25/Input day 10:

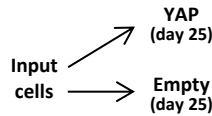
i. No effect on YAP activity

No change
No change



ii. Possible YAP effector

Enriched
Enriched



iii. Possible YAP inhibitor

Depleted
Depleted

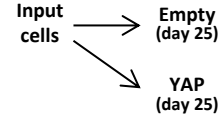


Fig 2: A CRISPR screen identifies UNC5B as a YAP effector

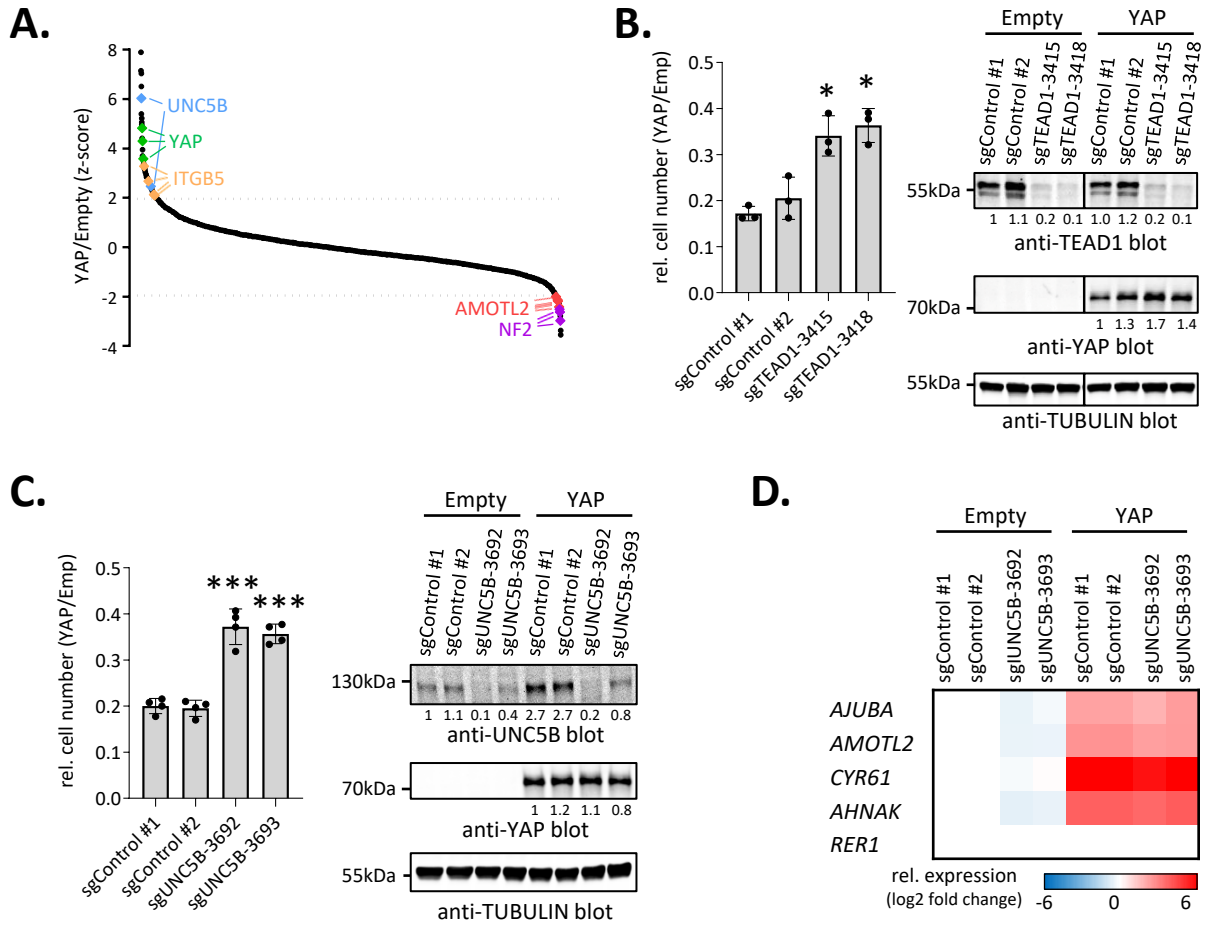


Fig 3 YAP induces Netrin and UNC5 family members in YAP^{off} cancers

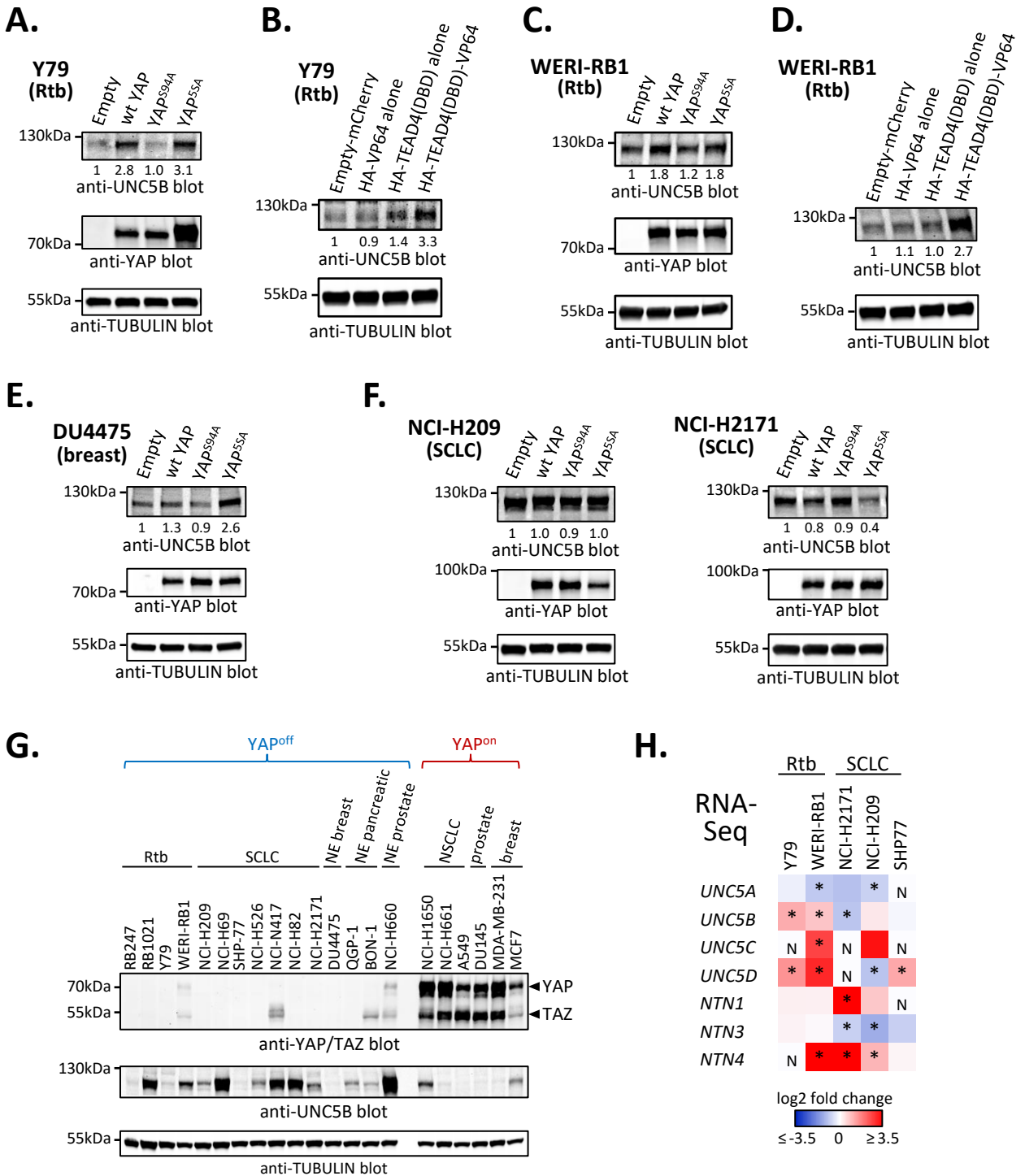


Fig 4: A Netrin-UNC5-ITGAV/B5 pathway mediates YAP-induced cytotostasis

