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1 **UBR5 is co-amplified with MYC in breast tumors and encodes**
2 **an ubiquitin ligase that limits MYC-dependent apoptosis**

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35

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39

40

41 **Abstract**

42

43 For maximal oncogenic activity, cellular MYC protein levels need to be tightly
44 controlled so that they do not induce apoptosis. Here, we show how ubiquitin
45 ligase UBR5 functions as a molecular rheostat to prevent excess
46 accumulation of MYC protein. UBR5 ubiquitinates MYC, and its effects on
47 MYC protein stability are independent of FBXW7. Silencing of endogenous
48 UBR5 induced MYC protein expression and regulated MYC target genes.
49 Consistent with the tumor suppressor function of UBR5 (Hyd) in Drosophila,
50 Hyd suppressed dMyc-dependent overgrowth of wing imaginal discs. In
51 contrast, in cancer cells UBR5 suppressed MYC-dependent priming to
52 therapy-induced apoptosis. Of direct cancer relevance, MYC and UBR5
53 genes were co-amplified in MYC-driven human cancers. Functionally, UBR5
54 suppressed MYC-mediated apoptosis in p53-mutant breast cancer cells with
55 UBR5/MYC co-amplification. Further, single-cell immunofluorescence analysis
56 demonstrated reciprocal expression of UBR5 and MYC in human basal-type
57 breast cancer tissues. In summary, UBR5 is a novel MYC ubiquitin ligase and
58 an endogenous rheostat for MYC activity. In MYC amplified, and p53-mutant
59 breast cancer cells, UBR5 has an important role in suppressing MYC-
60 mediated apoptosis priming and in protection from drug-induced apoptosis.

61

62 **Significance:**

63 Findings identify UBR5 as a novel MYC regulator, the inactivation of which
64 could be very important for understanding of MYC dysregulation on cancer
65 cells.

66

67 **Introduction**

68

69 Emerging notion of overall poor concordance between gene amplifications
70 and corresponding protein expression levels in cancer (1) highlights the need
71 for better understanding of how protein expression levels are regulated at
72 post-translational levels. The transcription factor MYC regulates numerous
73 physiological and pathological processes. Critically, distinct protein expression
74 levels dictate MYC's biological output *in vitro* and *in vivo* (2-5). MYC protein
75 levels are tightly regulated post-translationally by phosphorylation, and by
76 ubiquitin-mediated degradation (6-10). Especially in cancers, MYC is a classic
77 example of an oncoprotein, whose overexpression at protein level do not
78 match neither with the extent of mRNA overexpression, nor with the frequency
79 of genetic amplifications (7,11).

80

81 In addition to cell culture and mouse models (3,4,8,12,13), the role of MYC
82 has been widely studied in *Drosophila*, where the conserved homolog of
83 MYC, dMyc regulates tissue growth and animal size (14,15). In particular,
84 overexpression of dMyc induces growth of cells of the imaginal discs, which
85 are the organs giving rise to wings in adult fly (14). Importantly, dMYC can
86 complement mammalian MYC *in vivo* (13) implying high degree of functional
87 conservation. In humans, MYC is a master regulator of malignant growth.
88 However, MYC protein levels exceeding the optimal proliferation promoting
89 levels primes both normal and cancer cells to apoptosis (2,3,5,12). The
90 principle of MYC-mediated apoptosis priming was originally revealed by
91 findings showing that highly overexpressed transgenic MYC initiates both
92 proliferation and apoptosis programs in pancreatic islet cells. However, MYC

93 overexpression was capable to drive tumourigenesis only in the context of
94 efficient apoptosis suppression (12). More recently, the *in vivo* importance of
95 MYC expression levels in defining the balance between proliferation and
96 apoptosis was validated by allelic series of inducible MYC expression (3). In
97 addition to spontaneous apoptosis, high MYC levels prime tumour cells to
98 apoptosis induction by drugs that interfere with replication and cell division,
99 such as topoisomerase inhibitors or taxanes (2,16,17). Indeed, MYC is an
100 important determinant of *in vivo* cell survival both during development, and in
101 response to cancer therapy (4). However, how MYC protein levels are
102 controlled endogenously to maintain an optimal MYC balance is incompletely
103 understood.

104

105 UBR5 (Ubiquitin protein ligase E3 component n-recognin 5) is an evolutionary
106 conserved E3 ubiquitin ligase that destabilize proteins with N-terminal
107 recognition sequences exposed by proteolytic cleavage (18,19). Recently
108 UBR5 has been shown to target substrates also through other recognition
109 mechanisms than N-terminal recognition (20). UBR5 is essential for
110 mammalian development (21), and has been linked to both pro-tumourigenic
111 and tumor-suppressor activities (19,21-23). However, the mechanism by
112 which UBR5 would promote tumor growth are unclear. It is also unclear how
113 UBR5 may have pro-tumorigenic role in human cancer cells (19,22-24),
114 whereas it has tumor suppressor activity in *Drosophila* (25,26).

115

116 Here, we have identified UBR5 as an FBXW7-independent ubiquitin ligase
117 and rheostat for MYC. Consistent with tumor suppressive activity of UBR5 in

118 *Drosophila*, loss of UBR5 induced overgrowth of *Drosophila* wing imaginal
119 disc epithelium in a dMyc-dependent manner. On the contrary, UBR5-
120 mediated MYC suppression was found to protect the cancer cells from
121 apoptosis priming. Further, our data reveal genetic co-amplification of *UBR5*
122 and *MYC* in several solid human cancers; and demonstrate functional
123 relevance of reciprocal protein level regulation between these two cancer
124 genes in defining the apoptosis sensitivity of p53-mutant breast cancer cells.

125

126

127 **Materials and methods**

128

129

130 **Cell culture and transfection**

131 HCC38, HCC1937 and HCC1395 cell lines were obtained from American
132 Type Culture Collection (ATCC). Osteosarcoma-MYC-off cell line(27) was a
133 generous gift from Professor Dean Felsher (Stanford University). HeLa,
134 U2OS, T98G, HEK293, Osteosarcoma-MYC-off, MDA-MB-468 and MDA-MB-
135 231 cell lines were cultured in DMEM (Sigma). HCC38, HCC1937 and IMR-32
136 cell lines were cultured in RPMI (ATCC-modified vision, Thermo Fisher
137 Scientific). MCF10A cells were cultured as described previously (8). The cell
138 lines were authenticated by ATCC, European Collection of Authenticated Cell
139 Cultures (ECACC) or at Institut für Rechtsmedizin, Universität Würzburg. All
140 the cell lines were tested negative for Mycoplasma during the period of this
141 study. Drosophila S2 cells were cultured in Schneider's Drosophila Medium
142 (Thermo). All growth mediums were supplemented with 10% heat-inactivated
143 FBS (Gibco), 2 mmol/L L-glutamine, and penicillin (50 units/mL)/streptomycin
144 (50 mg/mL).

145 GFP-UBR5, GFP-UBR5-ΔHECT, Flag-UBR5, and Flag-UBR5-ΔHECT were
146 kind gifts from Darren Saunders & Charles Watts(28,29). V5-MYC, V5-
147 MYC^{T58A} and have been described previously (10). Flag-MYC plasmids and
148 HA-MYC (1-262) were from Prof. Bruno Amati. HA-MYC-WT and HA-MYC
149 mutants were kind gifts from Prof. William P. Tansey. HA-ubiquitin was a kind
150 gift from Prof. Lea Sistonen. Plasmids were transfected with Lipofectamine®
151 2000 Transfection Reagent (Thermo Fisher Scientific) according to the
152 manufacturer's instructions. After 48 hours transfection, cell lysate was
153 collected. Small interfering RNA (siRNA) transfections were performed with

154 Oligofectamine™ Transfection Reagent (Thermo Fisher Scientific) following to
155 the manufacturer's protocol. Three days after transfections, cells were
156 harvested for analysis. siRNA target sequences are in supplementary table 1.
157 For double-stranded RNA (dsRNA) mediated RNAi in Drosophila S2 cells, the
158 DNA fragment of hyd was amplified with primers flanked by T7 promoter, and
159 dsRNA was produced using the TranscriptAid T7 High yield Transcription Kit
160 (Thermo). To knock down Hyd, S2 cells were cultured with 5 ug/ml dsRNA for
161 120h.

162

163

164 **Immunoblotting and immunoprecipitation**

165 The following are antibodies used for western blot: UBR5 (sc-515494, Santa
166 cruz), MYC (ab32072, Abcam), cleaved PARP (ab32064, Abcam), BIM (2933,
167 Cell signaling), Vinculin (sc-25336, Santa cruz), GAPDH (5G4-6C5, HyTest
168 Ltd), HA (3724, Cell signaling), Flag (F3165, Sigma), GFP (sc-9996, Santa
169 cruz), GST (sc-138, Santa cruz), V5 (R960-25, Invitrogen), Histone H3
170 (ab1791, Abcam) and Lys48-Specific antibody (05-1307, Millipore).
171 Secondary antibodies are from Dako (P0447 and P0399). Densitometric
172 analysis of the blots was performed using ImageJ. For immunoprecipitation,
173 the cells were lysed in IP buffer (150mM NaCl, 1% NP-40, 50mM Tris pH 8.0)
174 supplemented with protease and phosphatase inhibitors. Cell lysates were
175 centrifuged and immunoprecipitated with the indicated antibodies for 2 h at 4
176 °C, following by adding Protein A-Sepharose or protein G-Sepharose beads
177 (Sigma) overnight. The beads were washed with IP buffer, boiled in SDS
178 sample buffer and analysed by immunoblotting. MYC (N-262, sc-764), and V5
179 agarose (A7345, Sigma) were used for immunoprecipitation.

180 To immunoprecipitate dMyc, lysates were incubated 2 hours with anti-dMyc
181 beads. Anti-dMyc beads were made from rabbit anti-dMyc antibody (Santa
182 Cruz) and 50% protein A Sepharose beads (Amersham). Immunoprecipitates
183 were washed five times with NP-40 lysis buffer, boiled in 2X SDS sample
184 buffer, and resolved on 8% SDS-PAGE for analysing via Western blotting.
185 Immunoprecipitated Myc was detected using mouse anti-Myc antibody (a kind
186 gift from Dr. Peter Gallant).

187

188 **Ubiquitination assays**

189 For ubiquitination assay in denaturating condition, HEK293 cells transfected
190 with relevant plasmids were lysed in a buffer containing 6 M guanidinium-HCl,
191 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl (pH 8), 5 mM imidazole and
192 10 mM β-mercaptoethanol (β-ME). The lysates were incubated with Ni-NTA
193 agarose beads (Qiagen) at 4 °C overnight. The beads were washed once with
194 a buffer containing 6 M guanidinium-HCl, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄,
195 0.01 M Tris-HCl (pH 8) and 10 mM β-ME, and twice with a buffer containing
196 8 M urea, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl (pH 8), 10 mM β-
197 ME and 0.1% Triton X-100. His-Ub-conjugated proteins were eluted using a
198 buffer containing 200 mM imidazole, 0.15 M Tris (pH 6.7), 30% glycerol,
199 0.72 M β-ME and 5% SDS. *In vitro* ubiquitination assays were performed in
200 reactions containing 2 µg ubiquitin (Boston Biochem), 2 mM ATP, 80 ng
201 hUBE1 (Boston Biochem), 2 µg UbcH5c (Boston Biochem), recombinant
202 GST-MYC and GFP-UBR5 purified from HEK293 cells, in a buffer with
203 800 mM Tris-HCl (pH 7.5), 200 mM MgCl₂ and 12 mM DTT. The reactions
204 were incubated at 37 °C for 90 minutes, followed by separation in Mini-

205 PROTEAN TGX precast gels (Biorad). The samples were analyzed by
206 Western blotting using GFP and GST antibodies.

207

208 **Immunofluorescence staining**

209 Cells plated on chambered coverslip (80826, Ibidi) were transfected with
210 scrambled siRNA or UBR5 siRNA. After 72 hours transfection, the cells were
211 fixed with 4% paraformaldehyde 15 minutes under room temperature, and
212 then cells were permeabilized with 0.5% Triton X-100 in PBS on ice for 5
213 minutes. Next, the cells were blocked by 10% normal goat serum (ab7481,
214 Abcam) diluted in PBS for 30 minutes, and followed by incubating the primary
215 antibodies anti-UBR5 (sc-515494, Santa cruz) and anti-MYC(ab32072,
216 Abcam) overnight at 4°C. Subsequently, cells were washed with PBS and
217 incubated with secondary antibodies, Alex Fluor 594 goat anti-Mouse IgG (A-
218 11005, Invitrogen) and Alex Fluor 488 goat anti-rabbit IgG (A-11008,
219 Invitrogen) for 1 hour under room temperature. After secondary antibody
220 incubation, the cells were washed with PBS and nuclei were stained with
221 DAPI (D1306, Invitrogen) in PBS at RT for 10 min. Images were acquired with
222 confocal microscope (LSM780, Carl Zeiss).

223

224 ***Drosophila* genetics**

225 Fly stocks used in this study are: Myc RNAi (VDRC 2947), hyd mutant allele
226 K3.5(30) and MARCM82b (a kind gift from Dr. Osamu Shimmi). To induce
227 MARCM clones in developing wing discs(31), larvae were heat-shocked for
228 one hour at 37°C at 72 hours after egg laying. Wing imaginal discs were
229 dissected from late third instar larvae, fixed in 4% formaldehyde for 30 min,

230 after washing in PBT (0.3% Triton X 100 in PBS), samples were mounted with
231 Vectashield Mounting Medium with DAPI (Mediq), and imaged using a Zeiss
232 LSM 700 microscope. Clone roundness was analyzed as described earlier
233 (32). Volume per cell and pH3 positive cells were counted using Imaris
234 software. To induce MARCM clones in larvae fat body, larvae were heat-
235 shocked for one hour at 37°C at 24 hours after egg laying. Fat bodies were
236 dissected from third instar larvae, fixed in 4% formaldehyde for 30 min, and
237 washed in PBT (0.3% Triton X 100 in PBS). After blocking in 5% BSA in PBT
238 for 3 hours at RT, primary antibody (anti-FBL, 1:500, Abcam) was incubated
239 at 4 C o/n. Primary antibody were washed with PBT and secondary antibody
240 (anti-mouse Alexa fluor 647, Life Technologies) was incubated for 4 hours at
241 RT. After three washes, samples were mounted with Vectashield Mounting
242 Medium with DAPI (Mediq), and imaged using a Zeiss LSM 700 microscope.

243 Full genotypes in Fig 3d:

244 yw, hsFLP/+; Tub-G4, UAS-mCD8 GFP/+; FRT82b, Tub-Gal80/FRT82,
245 hydK3.5

246 Full genotypes in Fig 3e and f:

247 Ctrl: yw, hsFLP/+; Tub-G4, UAS-mCD8 GFP/+; FRT82b, Tub-Gal80/FRT82b

248 Myc RNAi: yw, hsFLP/+; Tub-G4, UAS-mCD8 GFP/Myc RNAi; FRT82b, Tub-
249 Gal80/FRT82b

250 hyd mutant: yw, hsFLP/+; Tub-G4, UAS-mCD8 GFP/+; FRT82b, Tub-
251 Gal80/FRT82, hyd K3.5

252 hyd mutant, Myc RNAi: yw, hsFLP/+; Tub-G4, UAS-mCD8 GFP/Myc RNAi;
253 FRT82b, Tub-Gal80/ FRT82b, hyd K3.5

254 **Breast cancer IHC and IF analysis**

255 Tissue material was treated according to standard histology practice, i.e. fixed
256 in buffered formalin (pH 7.0) and embedded into paraffin blocks. Tissue
257 microarrays (TMAs) were prepared by collecting two 1.5 mm diameter tissue
258 cores from the representative tumor area, defined by an experienced breast
259 cancer pathologist (PK), of each breast cancer patient. Both
260 immunohistochemistry (IHC) and double immunofluorescence (IF) were
261 performed on sections cut at 3.5 µm. For MYC (ab32072, Abcam, Y69), IHC
262 was performed with Lab Vision Autostainer 480 (Thermo-Fisher Scientific,
263 Fremont, CA, USA) and detected with PowerVision+ polymer kit
264 (DPVB+110HRP; Immunovision Technologies, Vision Biosystems, Norwell,
265 MA, USA) according to standard protocol with diaminobenzidine as
266 chromogen. Before staining, tissue sections were deparaffinized and treated
267 twice for 7 min each in Target Retrieval Solution, pH 9 (S2367, Dako,
268 Glostrup, Denmark) in a microwave oven for antigen retrieval. MYC antibody
269 was applied at a dilution of 1:250. An automated immunostaining machine
270 Discovery XT (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ,
271 USA) was used for UBR5 (sc-515494, Santa Cruz Biotechnology) IHC and
272 UBR5/MYC double IF. Deparaffinization, epitope retrieval (standard option,
273 Cell Conditioning 1 reagent, 950-124, Roche/Ventana) and primary antibody
274 incubation (40 min at 37° C, dilution 1:500 for UBR5-IHC, and 1:1000 and
275 1:50 for UBR5 and MYC, respectively, for double-IF) were done on the
276 platform. OmniMap HRP (760-4310, Roche/Ventana) and ChromoMap DAB
277 Kit (760-159, Roche/Ventana) were applied for detection of UBR5 IHC. For
278 double IF, OmniMap HRP (Roche/Ventana, 760-4310 and 760-4311 for anti-

279 mouse and anti-rabbit, respectively) together with Rhodamine and FAM
280 fluorescent substrates (Roche/Ventana, 760-233 and 760-243, respectively)
281 were used. Finally, IF slides were mounted applying ProLong® Gold antifade
282 reagent with DAPI (P36935, Molecular Probes by LifeTechnologies). Two
283 slides were stained as controls for double IF. One, by replacing UBR5 primary
284 antibody, and the other, by replacing MYC primary antibody with antibody
285 diluent.

286

287 **Results**

288

289 **UBR5 suppress MYC protein expression**

290 We conducted a screen for ubiquitin ligases that regulate MYC protein levels
291 using a library of siRNAs targeting 591 ubiquitin ligases (Fig. 1A). To focus on
292 ubiquitin ligases that would not regulate MYC stability via the important MYC
293 ubiquitin ligase FBXW7 (9,10,34), the siRNA library was screened against
294 U2OS cells stably expressing MYCT58A mutant (threonine 58 mutated to
295 alanine) that is resistant to FBXW7-mediated destabilization (9,10). 48 hours
296 after siRNA transfection, cells were treated for 3.5 hours with cycloheximide to
297 emphasize the impact of protein stability, and immunofluorescence (IF)
298 detection of MYC was thereafter used as a read-out in a high-content
299 imaging-based assay (Fig. 1A). Among a small group of siRNAs consistently
300 affecting MYCT58A levels (Table S2), the HECT-domain containing E3 ligase
301 UBR5 (alias EDD) was the only E3 ligase affecting only protein levels but not
302 mRNA levels (Fig. S1A), and was therefore selected for further validation
303 experiments.

304

305 Physical association between endogenous UBR5 and MYC in HeLa cell
306 nuclei was confirmed by proximity ligation analysis (PLA) (Fig. 1B). The
307 specificity of the PLA reaction was confirmed by staining with PLA secondary
308 antibodies alone (Fig. S1B), and by a clear decrease of positive MYC-UBR5
309 PLA signals (Fig. 1B), and of UBR5 immunofluorescence signal (Fig. S1C, D)
310 by siRNA treatments. Whereas MYC and UBR5 siRNA treatments exclusively

311 decreased the number of nuclear PLA signals, they did not affect cytoplasmic
312 signals (Fig. 1B).

313

314 To confirm FBXW7-independent function of UBR5, we studied whether MYC
315 binding to UBR5 is affected by MYC T58A mutation, that abolishes
316 recognition by FBXW7 (7,9,35). As a result, GFP-UBR5 associated equally
317 efficiently with both forms of MYC (Fig. 1C). Lack of interaction between
318 overexpressed GFP and MYC was controlled separately (Fig. S1E).
319 Additionally, UBR5 overexpression reduced the levels of MYC T58A and WT
320 MYC to a similar degree (Fig. 1D). Importantly, mutant UBR5 lacking the
321 ubiquitin ligase HECT domain did not reduce the MYC levels (Fig. 1D).

322

323 By using different MYC fragments (36, 37), we delineated aminoacids
324 181-189 of MYC as the minimal candidate region for MYC-UBR5 association
325 (Fig. 1E and S1F). Importantly, the MYC mutant deficient in UBR5 binding
326 (d127-189) repeatedly showed higher protein expression than either WT
327 MYC, or the N-terminal MYC mutants (Fig. 1E and Fig. S1G), and was
328 resistant to inhibition by UBR5 overexpression (Fig. S1G).

329

330 The effects of UBR5 depletion on endogenous MYC protein levels in HeLa
331 cells was confirmed by immunoblot analysis using six independent UBR5
332 siRNA sequences (Fig. 1F). However, MYC did not regulate UBR5 mRNA or
333 protein expression (Fig. S1H, I). In addition to HeLa cells, endogenous MYC
334 protein induction by UBR5 inhibition was confirmed in T98G glioblastoma, and
335 MDA-MB-231 breast cancer cells, and in immortalized MCF-10A mammary

336 epithelial cells (Fig. 1G). In addition, we performed RNA-sequencing analysis
337 of HeLa cells depleted of MYC or UBR5 (Fig. S1I). By gene set enrichment
338 analysis (GSEA), we found that UBR5 depletion induces expression of same
339 MYC target signature that is suppressed by MYC depletion (Fig. 1H). A
340 heatmap of the individual genes regulated to opposite direction with UBR5 or
341 MYC siRNA is shown in figure 1I, and the genes are listed in supplementary
342 table 3.

343

344 **UBR5 is a MYC ubiquitin ligase**

345

346 UBR5 depletion induced a robust increase in the levels of MYC protein but its
347 impact on the MYC mRNA levels was minimal (Fig. 2A). Furthermore, UBR5
348 depletion significantly increased MYC protein expression when ectopic MYC
349 mRNA expression was induced by gradual removal of doxycycline from
350 mouse OS-Tet-Off-MYC cells (27), further supporting a regulation at post-
351 translational levels (Fig. 2B). Consistent with UBR5 regulating MYC protein
352 stability, UBR5 depletion stabilized MYC in a cycloheximide chase experiment
353 in HeLa (Fig. 2C and S2A), and in U2OS cells (Fig. S2B, C). Regarding other
354 MYC family members, endogenous MYCN was also stabilized by UBR5
355 siRNA in IMR-32 neuroblastoma cells (Fig. S2D, E). Further validating the
356 UBR5 degron on MYC, MYC d129-189 mutant was insensitive to UBR5-
357 mediated destabilization (Fig. S2F, G). Notably, depletion of either FBXW7 or
358 UBR5 increased endogenous MYC stability, and co-depletion of both ubiquitin
359 ligases further increased MYC stability (Fig. 2D and S2H, I). Additive effects
360 of UBR5 and FBXW7 inhibition on MYC stability were confirmed by Eilers

361 laboratory by using independent UBR5 and FBXW7 siRNAs (Fig. S2J). These
362 results clearly support independent roles for UBR5 and FBXW7 in MYC
363 protein regulation.

364

365 Consistent with ubiquitination-mediated regulation of MYC by UBR5, MYC
366 inhibition by overexpression of the wild-type UBR5 was rescued by co-
367 treatment with proteasome inhibitor MG132 (Fig. 2E, F). Moreover, in
368 ubiquitination assays, only overexpression of the wild-type UBR5, but not the
369 HECT mutant, induced ubiquitination of V5-MYC (Fig. 2G). Reciprocally,
370 siRNA-mediated depletion of UBR5 potently inhibited MYC ubiquitination (Fig.
371 2H). Moreover, we found that UBR5-induced ubiquitination of MYC is at least
372 in part via K48-linked ubiquitin chains (Fig. 2I), which are mainly responsible
373 for proteasomal degradation of proteins. Additionally, increased covalent
374 attachment of ubiquitin to MYC in UBR5 overexpressing cells was confirmed
375 by performing the His-ubiquitin pull-down under denaturing conditions (Fig.
376 S2K). Finally, by using purified UBR5 and MYC proteins, we demonstrate that
377 UBR5 can *in vitro* conjugate ubiquitin to the 1-262 fragment of recombinant
378 MYC, spanning the UBR5 degron (Fig. 2J).

379

380 Together, these data identify UBR5 as a novel MYC ubiquitin ligase that
381 regulates MYC stability independently of FBXW7.

382

383 **UBR5 controls *in vivo* tissue growth in dMyc-dependent manner in**
384 ***Drosophila***

385

386 To explore the possible functional conservation, and the role of the UBR5-
387 MYC axis in normal tissue homeostasis *in vivo*, we utilized a *Drosophila*
388 *melanogaster* model. The *Drosophila* UBR5 ortholog Hyd (Hyperplastic discs)
389 was originally identified as a putative tumor suppressor (19,22,26,38). RNAi-
390 mediated depletion of Hyd in *Drosophila* S2 cells (Fig. S3A) led to elevated
391 levels of *Drosophila* MYC (dMyc) protein (Fig. 3A), whereas the mRNA levels
392 of dMyc did not correlate with dMyc protein induction (Fig. 3B). Importantly,
393 the levels of Hyd RNA reduction by RNAi was directly reflected in a
394 comparable 2-fold induction of dMyc protein levels (Fig. S3A and 3A). Similar
395 to regulation of MYC target genes by UBR5 in human cells (Fig. 1H,I), Hyd
396 depletion induced mRNA expression of two dMyc targets eIF6 and Nop5 (Fig.
397 3C and S3B), and protein expression of Fibrillarin (Fig. 3D,E), which is a well-
398 established MYC target in both human and *Drosophila*.

399 The *Drosophila* models allows the use of somatic recombination to generate
400 clones of mutant tissue in an otherwise heterozygous background. Clones of
401 *hyd*^{K3.5} mutant cells generated during mid larval development (72 h after egg
402 laying) showed a phenotype clearly visible in the adult wings (Fig. S3C).
403 Wings with *hyd* mutant clones were irregular, lacking the normal flat wing
404 morphology, and displaying uneven wing margins and veins (Fig. 3F and
405 S3C, see arrows). Notably, this irregular wing phenotype was strongly
406 suppressed by simultaneous knockdown of dMyc in the mutant clones (Fig.
407 3F and S3C).

408 The use of the MARCM system to generate mutant clones allowed us to GFP
409 label, and to visualize the tissue morphology of the wing imaginal discs, the
410 larval wing precursors (31). Control and Myc RNAi clones appeared

411 morphologically normal with GFP positive clones having a normal but irregular
412 shape (Fig. 3G, H). In contrast, *hyd* mutant clones had a round morphology
413 with clusters of cells growing out from the epithelial plane (Fig. 3G, H), which
414 was confirmed by quantification of the clone roundness (Fig. 3H). The
415 roundness phenotype in wing clones has been earlier validated to mark
416 activated Ras-MAPK-MYC signalling (32). Most importantly, simultaneous
417 RNAi-mediated knockdown of dMyc suppressed the round morphology, thus
418 rescuing the phenotype of the *hyd* mutant clones, which was further confirmed
419 by quantification (Fig. 3H).

420 To dissect the relevance of cell size and proliferation (15) in dMyc-dependent
421 regulation of *Drosophila* tissue growth by Hyd, we first analyzed cell size in
422 *hyd* mutant clones. *hyd* mutant cells were significantly larger than controls and
423 this phenotype was fully suppressed by simultaneous knockdown of dMyc
424 (Fig. 3I). We also observed higher percentage of phospho-histone H3, a
425 marker commonly used as a proxy for cell proliferation, in the *hyd* mutant
426 clones, but this phenotype was independent of dMyc expression (Fig. S3D).
427 Thus, we conclude that the increased cell size, and roundness (established
428 mark of activated MYC signaling (32)), but not proliferation, are dependent on
429 dMyc in *hyd* mutant *Drosophila* tissues.

430

431 These results demonstrate that in *Drosophila* suppression of dMyc protein
432 expression is a critical part of growth control by the ortholog of UBR5 *in vivo*.

433

434 **UBR5 suppresses MYC-mediated apoptosis priming in cancer cells**

435

436 Proliferation and apoptosis are known to be governed by different levels of
437 MYC (2-5,12,17,39,40). In HeLa cells, UBR5 depletion decreased colony
438 growth (Fig. 4A), suggesting that MYC levels may have reached the levels
439 that prime them to apoptosis. This was confirmed by the increased PARP
440 cleavage, and by the induction of Caspase-3/7 activity in at least a fraction of
441 UBR5-depleted cells (Fig. 4B, C). To evaluate the balance between MYC-
442 regulated proliferation and apoptosis, we titrated UBR5 siRNA and studied the
443 dose-dependent correlation between MYC levels, and induction of cleaved-
444 PARP or inhibition of a directly MYC-regulated cell cycle inhibitor p21 (41).
445 Interestingly, while p21 suppression was almost maximal already with 10 nM
446 of UBR siRNA (Fig. 4D, E), cleaved-PARP continued to increase with higher
447 UBR5 siRNA concentrations (Fig. 4D, E). These results indicate that both
448 proliferation and apoptosis priming are initiated upon increased MYC
449 expression, and that loss of cells in colony growth assay (Fig. 4A) is a result
450 of MYC expression exceeding the apoptosis-inducing levels. Importantly,
451 apoptosis priming by UBR5 inhibition was highly dependent on MYC
452 induction. First, both PARP cleavage, and caspase-3/7 activation were fully
453 rescued by concurrent MYC depletion (Fig. 4F, G). Second, UBR5 depletion
454 was unable to induce PARP cleavage in OS-Tet-Off-MYC cells with maximal
455 doxycycline-elicited MYC suppression (Fig. 4H).

456

457 Mechanistically MYC overexpression can induce apoptosis either via ARF-
458 MDM2-p53 dependent pathway (2,42,43), or by engaging pro-apoptotic BH3
459 protein BIM in a p53-independent manner (40,41,44). Supportive of a BIM-

460 dependent mechanism, we found that co-depletion of BIM abolished
461 apoptosis induction in UBR5-depleted cells (Fig. 4I). Moreover, BIM induction
462 by UBR5 siRNA was abolished by co-depletion of MYC (Fig. 4J). On the other
463 hand, cells depleted of p53 displayed equally potent colony growth reduction,
464 and PARP cleavage by UBR5 depletion than control cells (Fig S4A, B).
465 Further, endogenous inactivating p53 mutations did not prevent MYC-
466 dependent PARP cleavage, or BIM induction, in UBR5 siRNA transfected
467 breast cancer cells (Fig. S4C).

468

469 MYC-dependent apoptosis priming is particularly relevant for increased
470 sensitivity of cancer cells to antimitotic agents such as taxanes (17), and to
471 drugs that interfere with high replication activity, such as topoisomerase I
472 inhibitors (camptothecins) (16). Accordingly, growth inhibition in UBR5
473 depleted cells was greatly potentiated by treatment of cells with either taxol or
474 camptothecin, at doses that alone do not induce massive loss of cells (Fig 4K,
475 S4D). Importantly, MYC expression was essential for hypersensitivity of
476 UBR5 depleted cells to both taxol and camptothecin (Fig. 4K, L) since drug
477 responses were indistinguishable between the control cells, and cells treated
478 with UBR5+MYC siRNA in a concentration-dependent manner (Fig. S4E).

479

480 Collectively these results demonstrate that UBR5 suppresses MYC-mediated
481 but p53-independent apoptosis priming in cancer cells.

482

483 **Co-amplification of *UBR5* and *MYC* protects breast cancer cells from**
484 **MYC-mediated priming to drug-induced apoptosis**

485

486 The results above suggest that expression of UBR5 might provide a selective
487 advantage for cancer cells by suppressing MYC-mediated apoptosis priming.
488 The cancer types in which this biology is particularly relevant would be
489 expected to exhibit co-expression of *Ubr5* and *Myc* at mRNA level, but
490 UBR5^{high}/MYC^{low} status at protein level (see cartoon Fig. 5A). Across 672 cell
491 lines and over 20 different cancer types (45), a weak but statistically
492 significant correlation between *Ubr5* and *Myc* mRNA expression levels was
493 detected (Pearson correlation 0.21, p<0.01; Fig. S5A). Significant positive
494 correlation between *Myc* and *Ubr5* mRNA expression was observed in four
495 cancer types, ovary, lymphoid, breast and pancreas (Fig. S5A). Importantly,
496 MYC has been closely linked to progression of all these four cancer types,
497 and existing data indicate a growth-promoting role of UBR5 in at least
498 pancreatic, ovary, and breast cancer (22,23,46,47).

499

500 We noted that both *UBR5* and *MYC* genes are located in the long arm of
501 chromosome 8, suggesting a model where *UBR5* co-amplification with *MYC*
502 might provide means to control excessive MYC protein levels, and thereby
503 protect cells from apoptosis priming (Fig. 5A). Examination of TCGA cancer
504 patient amplification data for *UBR5* and *MYC* in the four cancer types with an
505 evidence for mRNA co-expression (Fig. S5A), revealed that the percentage of
506 patient samples with single amplification of either *UBR5* or *MYC* ranged from
507 2 % in lymphoid cancers, to 44 % in ovary cancers (Table 1). Interestingly, co-
508 amplification of *UBR5* and *MYC* appeared in 65% of breast, 42% of ovary,
509 and 39% of pancreas cancers. On the other hand amplification of *UBR5* alone

510 was a very rare event in any of these cancer types. Breast cancers were the
511 only cancer type in which the percentage of cancers with co-amplification
512 exceeded the percentage of cancers with *MYC* amplification alone (Table 1),
513 indicating that *UBR5* co-amplification, and *UBR5* protein expression, may
514 provide a particular benefit for breast cancer cells with *MYC* amplification.

515
516 To assess functional relevance of *UBR5* in co-amplified breast cancer cells,
517 we correlated breast cancer cell line *UBR5* essentiality index (zGARP
518 score)(48), with *MYC/UBR5* gene copy numbers in these same cells. Notably,
519 we observed a statistically significant correlation between the zGARP score
520 and *MYC/UBR5* gene copy numbers in cell lines with zGARP score < -1.5
521 (Fig. 5B). Next, we tested the xenograft growth potential of *UBR5*-depleted
522 HCC38 cells. Transient siRNA transfection of HCC38 cells resulted in at least
523 10 days suppression of *UBR5* protein expression validating the approach for
524 xenograft experiment (Fig. S5B). Remarkably, *UBR5* depletion from HCC38
525 even with transient siRNA transfection was sufficient to almost entirely inhibit
526 tumor growth in mice (Fig. 5C). *In culture*, we observed that *UBR5* depletion
527 in both HCC38 and HCC1937 cells did induce *MYC* protein levels and *MYC*-
528 dependent apoptosis priming (as observed by PARP cleavage) (Fig. 5D, and
529 S5C). As HCC38 harbors p53 R273L mutation in the DNA-binding domain,
530 these results further strengthen the p53-independence of apoptosis priming
531 induced by loss of *UBR5*.

532
533 Patients with co-amplification of *MYC* and *UBR5* had significantly poorer
534 overall survival than patients without either of the genes amplified (Fig. S5D).
535 However, as survival of patients with *MYC* amplification alone did not

536 statistically differ from survival of patients with co-amplification, we conclude
537 that potential relevance of UBR5 mediated MYC suppression in co-amplified
538 breast cancers could rather associate with the resistance to therapy-induced
539 apoptosis. Fully supporting the hypothesis, UBR5 depletion from HCC38 cells
540 with *MYC/UBR5* co-amplification resulted in modest MYC-dependent
541 inhibition of colony growth, but led to a very potent MYC-dependent
542 sensitisation of cell killing to both FDA approved topoisomerase I inhibitors,
543 Irinotecan and Topotecan (Fig. 5E; the full concentration-dependent dose-
544 response curves are show in Figure S5E,F). We also confirmed a significant
545 MYC-dependent priming to Taxol-induced cell killing by UBR5 inhibition in
546 HCC38 cells (Fig. 5F).

547

548 Together these results identify co-amplification of *MYC* and *UBR5* in large
549 fraction of MYC-dependent solid cancer types. Results further indicate that
550 *UBR5* co-amplification with *MYC* might be relevant to suppress drug-induced
551 apoptosis in p53-mutant breast cancer cells.

552

553 **UBR5 dominates MYC protein levels in individual breast cancer cells *in***
554 ***vivo***

555

556 Finally, we searched for *in vivo* evidence that UBR5 suppresses MYC protein
557 expression in human breast cancer tissue. Consistently with published results
558 (49), *MYC* amplification was most frequent in basal-type breast cancers, and
559 this was also the subtype where co-amplification was most pronounced (Fig.
560 S6A). Based on this information, we used tissue microarray of 345 samples

561 from 197 human basal-type breast cancers to examine correlation between
562 UBR5 and MYC protein expression *in vivo*. The IHC staining was optimized
563 such that intensities from 0 to +++ were reliably observed with both UBR5 and
564 MYC antibodies (Fig. S6B). Consistently with the hypothesis of UBR5
565 dominating MYC expression, the percentage of staining positive cells (+, ++,
566 or +++) was greater for UBR5 than for MYC in 78% of the samples (Fig. S6C).
567 This finding was re-confirmed by using an independent set of human breast
568 cancer samples ($n = 74$), and a different staining protocol in Klefström
569 laboratory (Fig. S6D).

570

571 When tumor samples were examined by using adjacent 3 μM tissue sections,
572 we observed an apparent dichotomy of UBR5 and MYC protein levels in
573 individual basal type breast cancer cells (Fig. S6E; regions 1 and 2).
574 Importantly, while UBR5 and MYC positivity is almost exclusively confined to
575 tumor cells (Fig. S6E, F), we could identify some immune-like cells that also
576 show very clear inverse correlation between UBR5 and MYC expression (Fig.
577 S6E). These results further emphasize the general importance of our results
578 across different cell types.

579

580 To quantitatively validate these observations, we developed a double
581 immunofluorescence (IF) staining protocol. Antibody specificity was
582 demonstrated by siRNA-mediated depletion (Fig. 1B and S1D), and by
583 secondary antibody only control staining (Fig. S6G). Further, the double-IF
584 was optimized so that maximal staining intensities were practically
585 indistinguishable with both UBR5 and MYC antibodies (Fig. S6H). Thus,

586 individual cells could be categorized into MYC^{high}/UBR5^{low} (green),
587 UBR5^{high}/MYC^{low} (red) and MYC^{high}/UBR5^{high} (yellow) phenotypes (Fig. 6A).
588 Staining of TMA cores revealed that basal-type breast cancers could be
589 broadly divided into either UBR5 dominant or MYC dominant, or to cancers
590 that showed both UBR5 or MYC dominant cells (Fig. 6B). Supporting the IHC
591 results, UBR5 was overall more highly expressed than MYC across all
592 quantified 18 667 cells (Fig. 6C). Pairwise comparison of UBR5 and MYC
593 intensities in all quantified individual cells demonstrated that in 64% of cells
594 UBR5 was more highly expressed than MYC, whereas 27% of cells were
595 MYC dominant (Fig. 6D). Fully consistent with a dichotomy of UBR5 and MYC
596 expression (Fig. S6E), only 9% of the cells displayed equal UBR5 and MYC
597 protein expression intensities (yellow) (Fig. 6D).

598

599 To demonstrate statistical significance of UBR5 dominance over MYC at
600 single cell level, we performed a pairwise density distribution analysis of
601 UBR5 dominance from all 18 667 individual cells. To this end, MYC intensity
602 in each individual cell was subtracted from UBR5 intensity from the same cell,
603 and distribution of the resulting pairwise intensity difference was blotted on a
604 scale that corresponded to actual IF intensity levels (Fig. 6E, S6H). The null
605 hypothesis was that UBR5 and MYC expression would have been equal, and
606 thus pairwise UBR5-MYC intensity difference would have centered around
607 zero. The results however show that cells with either UBR5 dominance (UBR5
608 intensity-MYC intensity > 0), or MYC dominance (UBR5 intensity-MYC
609 intensity < 0), constitute two different populations (Fig. 6E). Moreover, the
610 median of UBR5 intensity-MYC intensity was strongly positive, and the

611 density of UBR5 dominant cells is significantly higher than with MYC dominant
612 cells. Thereby these results confirm that UBR5 dominates MYC protein
613 expression in a majority of individual basal type breast cancer cells *in vivo*.
614 This conclusion was further supported by regression analysis between UBR5
615 and MYC IF intensities in individual cells that was clearly indicative of
616 negative correlation between these proteins at the single cell level (Fig. 6F).
617 Interestingly, all cells having lower than approximately 30% of maximal UBR5
618 intensity had strong MYC expression (Fig. 6F), indicating that other
619 mechanisms are not capable of effectively suppressing MYC in basal type
620 breast cancers in the absence of UBR5. Based on our other results (Fig. 4K,I,
621 5E,F, and S5E,F), these UBR5^{low}/MYC^{high} cells would constitute the Irinotecan
622 and Topotecan sensitive cell population *in vivo*.

623

624 This first single cell analysis of protein expression of MYC and its ubiquitin
625 ligase demonstrate that UBR5 and MYC are exclusive for high level protein
626 expression in most of the basal type breast cancer cells *in vivo*. As high UBR5
627 expression dominated high MYC expression in most of the cells, these results
628 provide *in vivo* relevance for the role of UBR5 in negative regulation of MYC.

629 **Discussion**

630
631 UBR5 regulates various cellular processes (19,28,50,51), but the role for
632 UBR5 in both inhibiting and promoting organismal growth has been enigmatic
633 (19,22,23,25,46,50,52). The observed overgrowth in *Drosophila* epithelial
634 tissue in *hyd* mutant flies via dMyc is fully consistent with the reported growth
635 suppressor role of Hyd (19,25). On the other hand, UBR5-mediated
636 suppression of MYC-induced apoptosis observed in cancer cell lines is well in
637 accordance with previously reported oncogenic activities of UBR5 (22,23). In
638 support of *in vivo* relevance of these observations, we demonstrate both that
639 UBR5 suppresses dMyc activity in wing development (Fig. 3), and that in most
640 of the basal type breast tumor cells UBR5 dominates MYC protein levels at
641 the single cell level (Fig. 6C-F, S6E). Directly supportive of oncogenic role of
642 UBR5 in human cancer cells *in vivo*, UBR5 depleted basal-like breast cancer
643 cells with *UBR5/MYC* amplification failed to grow as xenograft tumors (Fig.
644 5C). Collectively, we conclude that UBR5 finetunes MYC protein expression
645 to the levels that balance cell growth and survival (Fig. 6G).

646

647 Although the conclusions that lower levels of MYC would be more beneficial
648 for the tumor are somewhat counter intuitive, they are fully in accordance with
649 previous results using transgenic MYC mouse models. These studies have
650 demonstrated the importance of MYC protein levels in controlling the delicate
651 balance between proliferation and apoptosis (2,3,12). Whereas these
652 previous studies convincingly demonstrated that the dose of MYC protein is
653 critical to sustain maximal fitness of the cell, identification of endogenous
654 candidate rheostat proteins such as UBR5, that define MYC levels *in vivo*,

655 may help in future to better understand how the balance between MYC-
656 mediated regulation of cell growth and apoptosis is defined by cell intrinsic
657 mechanisms. Notably, in addition to demonstrating both the *in vitro* and the *in*
658 *vivo* relevance of UBR5-MYC axis, we also show that opposite to some of the
659 other UBR5 functions that are strictly restricted to certain cell types (21,52),
660 we failed to identify a cell type in which UBR5 inhibition would have not
661 increased MYC protein expression. Therefore, we anticipate these findings to
662 have wide-reaching implications in different biological systems.

663

664 Co-amplification with *MYC* introduces UBR5 as so far unique MYC ubiquitin
665 ligase. Based on amplification frequencies of *UBR5* and *MYC*, it is clear that
666 *UBR5* amplification alone does not provide significant benefit for breast
667 cancer cells. However, also *MYC* amplification without *UBR5* amplification
668 was only seen in 28% of the samples with any amplifications, whereas co-
669 amplification was observed in 70% of all cases in which *MYC* was amplified
670 (Table 1). Together with results that *MYC/UBR5* co-amplification frequency
671 correlates with essentiality of UBR5 for breast cancer cell survival (Fig.5B),
672 and with UBR5-mediated protection of *MYC/UBR5* co-amplified breast cancer
673 cells drug-induced apoptosis (Fig. 5E,F), we postulate that co-amplification of
674 *UBR5* gives a survival benefit to *MYC* amplified cells for example under drug-
675 induced stress conditions. An additional discovery with high cancer relevance
676 was p53-independence of MYC-mediated apoptosis in UBR5 inhibited cells.
677 As cancer cells with combination of *MYC*-amplification and p53-mutation
678 constitutes the most malignant cell population in many cancers, identification
679 of a ubiquitin ligase regulating MYC-mediated apoptosis in this type of cells

680 constitutes a major advancement with profound future potential. Intriguingly,
681 opposite to breast cancers, *UBR5* amplifications were not observed in
682 lymphoid cancers. The discrepancy between solid and lymphoid cancers in
683 regards to their *UBR5* status is illustrated also by the fact that frequent *UBR5*
684 mutations (which are predicted to disrupt E3 ligase activity) are observed in
685 mantle cell lymphoma (24). Thereby, it is possible that whereas *UBR5*
686 promotes oncogenesis in solid cancers by decreasing MYC-mediated
687 apoptosis, in hematological cancers *UBR5* functions as a tumor suppressor,
688 and this activity is lost by frequent loss-of-function mutations.

689

690 Even though the study did not directly address potential role of *UBR5* as a
691 cancer therapy target, our results proposes for a possible strategy to unleash
692 MYC's pro-apoptotic activity for cancer therapy. Based on our data, we
693 envision that *UBR5* inhibition could harmonize the tumor cells to more
694 uniformly express MYC protein at the levels that would predispose the cells to
695 cell killing by cancer drugs. Optimally this could prevent re-appearance of
696 those cancer cell populations that were protected from the initial drug-induced
697 apoptosis due to their low MYC expression. In addition to the presented data,
698 this model is supported by recent demonstration of MYC as a major *in vivo*
699 determinant of taxane response (17), and the data that pretreatment
700 mitochondrial priming correlates with clinical response to cytotoxic
701 chemotherapy in cancer (53). Unfortunately, testing of this hypothesis is
702 currently unfeasible because no small molecule inhibitors for *UBR5* are
703 available. Another potential clinical application of the data is use of breast

704 cancer tissue UBR5^{low}/MYC^{high} IHC status for patient stratification to
705 Topoisomerase I inhibitor therapies.

706

707 In summary, this study characterizes UBR5 as a novel MYC ubiquitin ligase
708 controlling MYC activity in development of epithelial tissue *in vivo*, and in p53-
709 mutant cancer cells. Collectively the results provide a novel molecular
710 explanation for both the tumor suppressive, as well as tumor promoting roles
711 of UBR5. Co-amplification of *UBR5* with *MYC* indicates for a concept that at
712 certain circumstances it is beneficial for cancer cells to gain strong oncogenic
713 activity, but simultaneously assure that this activity is kept in leash by co-
714 amplification of the natural suppressor mechanism.

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736

737

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- 896
- 897

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901 **Table 1 Distribution of amplification rates of MYC and UBR5 in TCGA patient samples in the**
 902 **indicated cancer types**

	Breast	Pancreas	Ovary	Lymphoid
Total number of sample	1080	183	579	48
No. of samples with either MYC and/or UBR5 amplifications (% of total)	247 (22.8%)	23 (12.5%)	254 (43.8%)	1 (2%)
Cancers with MYC amplifications only (% of amplifications)	28%	61%	54%	1%
Cancer with UBR5 amplifications only (% of amplifications)	7%	0%	4%	0%
Cancers with MYC/UBR5 co-amplifications (% of amplifications)	65%	39%	42%	0%

903

904

905 **Figure legends:**

906

907 **Fig.1 UBR5 negatively regulates MYC protein levels.** **A**, High-content
 908 imaging-based screen was performed to identify E3 ubiquitin ligases that
 909 regulate MYCT58A. An example of immunofluorescence staining of MYC and
 910 a secondary antibody control only is shown. **B**, Proximity ligation assay
 911 analysis of the UBR5 and MYC association in HeLa cells transfected with
 912 scrambled (Scr), MYC siRNA or UBR5 siRNA. Shown is a quantification of the
 913 average number of UBR5-MYC PLA signals per cell in cytoplasm and
 914 nucleus. **C**, UBR5, wild-type MYC or MYCT58A mutants were overexpressed
 915 in HEK293 cells by transient transfection. Cell lysates were
 916 immunoprecipitated with GFP or V5 agarose and interaction was detected
 917 with relevant antibodies. The numbers show fold change of relevant proteins.
 918 Shown is a representative experiment; n=3. **D**, HEK293 cells were co-

919 transfected with empty vector, GFP-tagged UBR5, UBR5 mutant, V5-tagged
920 MYC and MYCT58A mutant plasmids. Shown is a representative experiment;
921 n=2. **E**, UBR5, wild-type MYC or MYC mutants were overexpressed in
922 HEK293 cells by transient transfection. Cell lysates were immunoprecipitated
923 with HA antibody and interaction was detected with GFP antibody. Shown is a
924 representative experiment; n=3. **F**, Hela cells were transfected with scrambled
925 (Scr) or 6 different UBR5 siRNAs. After 72 hours, cell lysates were collected
926 to detect MYC expression by western blot. **G**, Effect of UBR5 siRNA on MYC
927 in T98G, MDA-MB-231 and MCF10A cells. **H**, Gene set enrichment analysis
928 (GSEA) of RNA-seq data generated in Hela cells transfected with Scr, UBR5
929 or MYC siRNAs. NES, normalized enrichment score. **I**, Heatmap illustrating
930 the expression level changes (>2 folds by siUBR5) of genes co-regulated by
931 UBR5 and MYC in (H).

932

933 **Fig.2 Validation of UBR5 as a MYC ubiquitin ligase.** **A**, MYC mRNA and
934 protein expression in Hela cells transfected with scrambled (Scr) or UBR5
935 (5#) siRNA. Error bars represent SD (for mRNA expression, n=3; for protein
936 expression, n=14), ***, P < 0.001, ****, P < 0.0001 by student's t-test. **B**,
937 Analysis of MYC expression in mouse osteosarcoma-MYC-off cells
938 transfected with scrambled (Scr) or UBR5 siRNA. **C**, Hela cells transfected
939 with scrambled (Scr) or UBR5 (5#) siRNA for 72 hours were treated with
940 cycloheximide (CHX, 60 μ g/ml) for indicated time points. For Western blot see
941 Fig S2A. Error bars mean \pm SD. *, P < 0.05 by student's t-test (n=3). **D**, Hela
942 cells transfected with indicated siRNAs for 72 hours were treated with
943 cycloheximide (CHX, 60 μ g/ml) for different time points. For Western blot see

944 Fig S2I. Error bars mean \pm SD. *, P < 0.05 by student's t-test (n=3). **E**,
945 HEK293 cells were co-transfected with GFP-tagged wild type UBR5, UBR5-
946 HECT domain mutant, and V5-tagged MYC plasmid. After 48 hours
947 transfection, the cells were treated with MG132 (20 μ M) 6 hours. SE and LE
948 represent short and long exposure time. **F**, Quantification of MYC expression
949 from (E). Error bars show SD from 4 independent experiments. *, P < 0.05 by
950 student's t-test. **G,H,I** HEK293 (G) or HeLa (H,I) cells were co-transfected with
951 indicated plasmids. After 48 hours transfection, the cells were treated with
952 MG132 (20 μ M) for 6 hours. Immunoprecipitation was performed with anti-V5
953 agarose. Ubiquitination of MYC was detected by HA antibody (G,H) or
954 ubiquitin K48 specific antibody. **J**, Recombinant GST-MYC (1-262) and GFP-
955 UBR5 purified from HEK293 cells were incubated in *in vitro*-ubiquitination
956 reactions. Samples were analyzed by western blotting using GFP and GST
957 antibodies. **G-J**, Shown is a representative experiment; n=2-3.

958

959 **Fig. 3 Loss of UBR5 ortholog Hyd drives wing imaginal disc overgrowth**
960 **in Myc-dependent manner.** **A**, Immunoblot showing immunoprecipitated
961 dMyc levels in S2 cells transfected with Scr or Hyd dsRNA. Kinesin was used
962 as a control for the input. Shown is a representative blot of two experiments
963 with similar results. **B**, Quantitative RT-PCR analysis of dMyc mRNA
964 expression in control and Hyd depleted S2 cells. RP49 was used as a
965 reference gene. Error bar shows SD. n=3. **C**, eIF6 mRNA expression in
966 control and Hyd depleted S2 cells. Error bar shows SD. n=3. *, P < 0.05 by
967 student's t-test. **D**, Loss of Hyd in GFP-labelled fat body mutant clones leads
968 to increase of Fibrillarin expression. **E**, Quantification of (D). The Fibrillarin

969 level of an adjacent GFP negative cell was used as control. n=10, **, P <
970 0.01 by student's t-test. **F**, Induction of *hyd* mutant clones leads to uneven
971 wing morphology, which is suppressed by simultaneous knockdown of Myc
972 within the clones. Arrows in magnified image show uneven wing margins and
973 veins. **G**, Loss of Hyd in GFP-labelled mutant clones leads to MYC-dependent
974 overgrowth indicated by roundness, and loss of normal epithelial morphology.
975 Scale bar means 10 μ m. **H**, Representative images show clone morphology in
976 wing imaginal discs in control and *hyd* mutant clones. Scale bar means 10
977 μ m. Quantification of the clone roundness was shown. Error bar shows SD.
978 n=30. **, P <0.01 by student's t-test. **I**, Loss of Hyd in GFP-labelled wing disc
979 mutant clones leads to increased cell volume. Simultaneous knockdown of
980 dMyc suppresses this phenotype. n=3, *, P < 0.05 by student's t-test.

981

982 **Fig. 4 UBR5 suppresses MYC-mediated apoptosis in cancer cells. A,**
983 Hela cells transfected with three UBR5 siRNAs were cultured 7 days for
984 colony growth assay. Error bars show SD. n=3. **, P <0.01 by student's t-test.
985 **B**, Hela cells were transfected with two UBR5 siRNAs for 72 hours. Total cell
986 lysates were subjected to immunoblotting with indicated antibodies. **C**,
987 Caspase 3/7 activity was examined in UBR5 depleted Hela cells. Error bars
988 show SD for 7 technical repeats with similar results. **, P <0.01 by student's t-
989 test. **D**, Hela cells were transfected with different concentrations of UBR5
990 siRNA for 72 hours. Cell lysates were analyzed by western blot with indicated
991 antibodies. **E**, Quantification of MYC, cleaved-PARP and p21 expression from
992 (D). Error bars show SD from 2 independent experiments. **F**, Hela cells were
993 transfected with two UBR5 siRNAs, or with combination of UBR5 siRNA and

994 MYC siRNA for 72 hours. Cell lysates were collected for western blot with
995 indicated antibodies. **G**, Caspase 3/7 activity was examined in Hela cells
996 transfected with indicated siRNAs. Error bars show SD of 2 independent
997 experiments. *, P < 0.05 by student's t-test. **H**, The osteosarcoma-MYC-off
998 cells treated with 20ng/ml doxycycline were transfected with scrambled (Scr)
999 or UBR5 siRNA and cultured in medium with 20ng/ml doxycycline. After 48
1000 hours, medium was changed with or without 20ng/ml doxycycline for culture
1001 another 48 hours. **I**, Caspase 3/7 activity was examined in Hela cells
1002 transfected with indicated siRNAs. Shown is mean ± SD of 2 independent
1003 experiments. *, P < 0.05 by the student's t- test. **J**, Hela cells were transfected
1004 with Scr, UBR5 siRNAs, combination of UBR5 and MYC siRNA, and
1005 combination of UBR5 and BIM siRNAs. **K**, Colony growth assay were
1006 performed in Hela cells transfected with Scr, siUBR5 or combination of UBR5
1007 and MYC siRNAs followed by 24 hours treatment of Taxol (2nM) or CPT
1008 (20nM). After 7 days, cells were fixed and stained. **L**, Quantification of drug
1009 response fold changes relative to Scr siRNA in (K). Error bars show SEM of 4
1010 independent experiments. *, P < 0.05 by student's t-test.

1011

1012 **Fig. 5 UBR5 and MYC are co-amplified in human breast cancer. A,**
1013 Schematic figure of *UBR5* and *MYC* gene locus in the long arm of
1014 chromosome 8 and indicated relationship between their expression at mRNA
1015 and protein levels. Whereas mRNA expression is predicted to correlate in co-
1016 amplified samples (upward arrows), UBR5 is predicted to suppress MYC
1017 protein expression in these samples (opposite direction arrows). **B**, Scatter
1018 plot showing the relationship between *UBR5/MYC* co-amplification and *UBR5*

1019 gene essentiality in the breast cancer cell lines. The minimum of *UBR5* and
1020 *MYC* amplification levels were correlated against the zGARP scores, a
1021 measure of *UBR5* essentiality in a shRNA dropout screen. **C**, The volume of
1022 tumors isolated from mice on the final day of the experiment (day 28) (n=8).
1023 **, P < 0.01 by student's t-test. **D**, *UBR5/MYC* co-amplified HCC1937 or
1024 HCC38 cells were transfected with indicated siRNAs. **E**, Colony growth assay
1025 of siRNA transfected HCC38 cells was performed following 24 hours
1026 treatment with Irinotecan (80nM) or Topotecan (40nM). Error bars show SEM
1027 of 2 independent experiments. **F**, Colony growth assay of siRNA transfected
1028 HCC38 cells was performed following 24 hours treatment with Taxol (8nm).
1029 Error bars show SEM of 3 independent experiments. *, P < 0.05, **, P < 0.01
1030 by student's t-test.

1031

1032 **Fig. 6 UBR5 controls MYC protein expression at single cell level in basal**
1033 **type breast cancer *in vivo*. A**, Intensity of MYC and UBR5 protein from dual
1034 immunofluorescence staining was used to categorize individual cells in breast
1035 cancer tissues into either MYC or UBR5 dominant. **B**, Dual
1036 immunofluorescent staining of UBR5 and MYC in TMA of human basal-type
1037 breast cancer. **C**, Quantification of UBR5 and MYC staining at single cell level
1038 from 18 667 cells by software FIJI. Error bars mean SD. ****, P < 0.0001. **D**,
1039 Pie-chart analysis of distribution of cells based on whether either MYC (green)
1040 or UBR5 (red) was expressed at higher level. Cells in which there was < 10%
1041 intensity difference between UBR5 and MYC were classified as having equal
1042 protein expression (yellow). **E**, Pairwise density distribution analysis of 18 667
1043 individual cells reveals bimodal distribution of UBR5 or MYC dominant cells.

1044 **F**, Regression analysis between UBR5 and MYC IF intensities in individual
1045 cells (n=18 667). **G**, Schematic presentation of UBR5-mediated control of
1046 MYC levels critical for proliferation and apoptosis regulation. MYC protein
1047 levels accumulate differently between UBR5 high and low expressing cells. In
1048 UBR5 low expressing cells (blue area), MYC protein accumulates efficiently
1049 due to high protein stability, and high MYC protein expressing cells exceed
1050 the apoptosis priming levels. In UBR5 high expressing cells (green area),
1051 UBR5 restrains MYC protein accumulation to the levels that maximally
1052 support proliferation, but do not exceed the levels that would result in
1053 apoptosis priming even with maximal c-Myc mRNA transcription rates.

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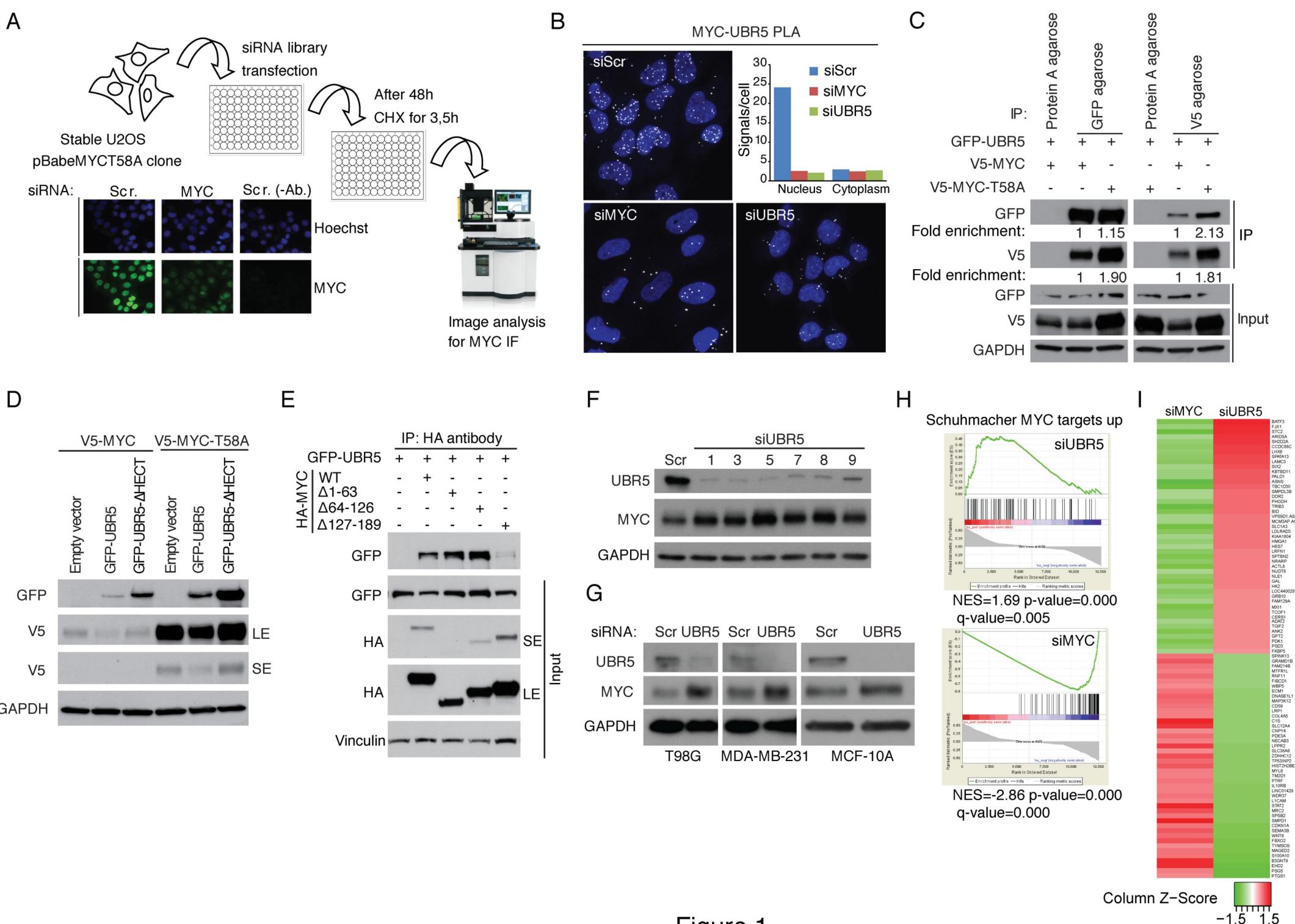
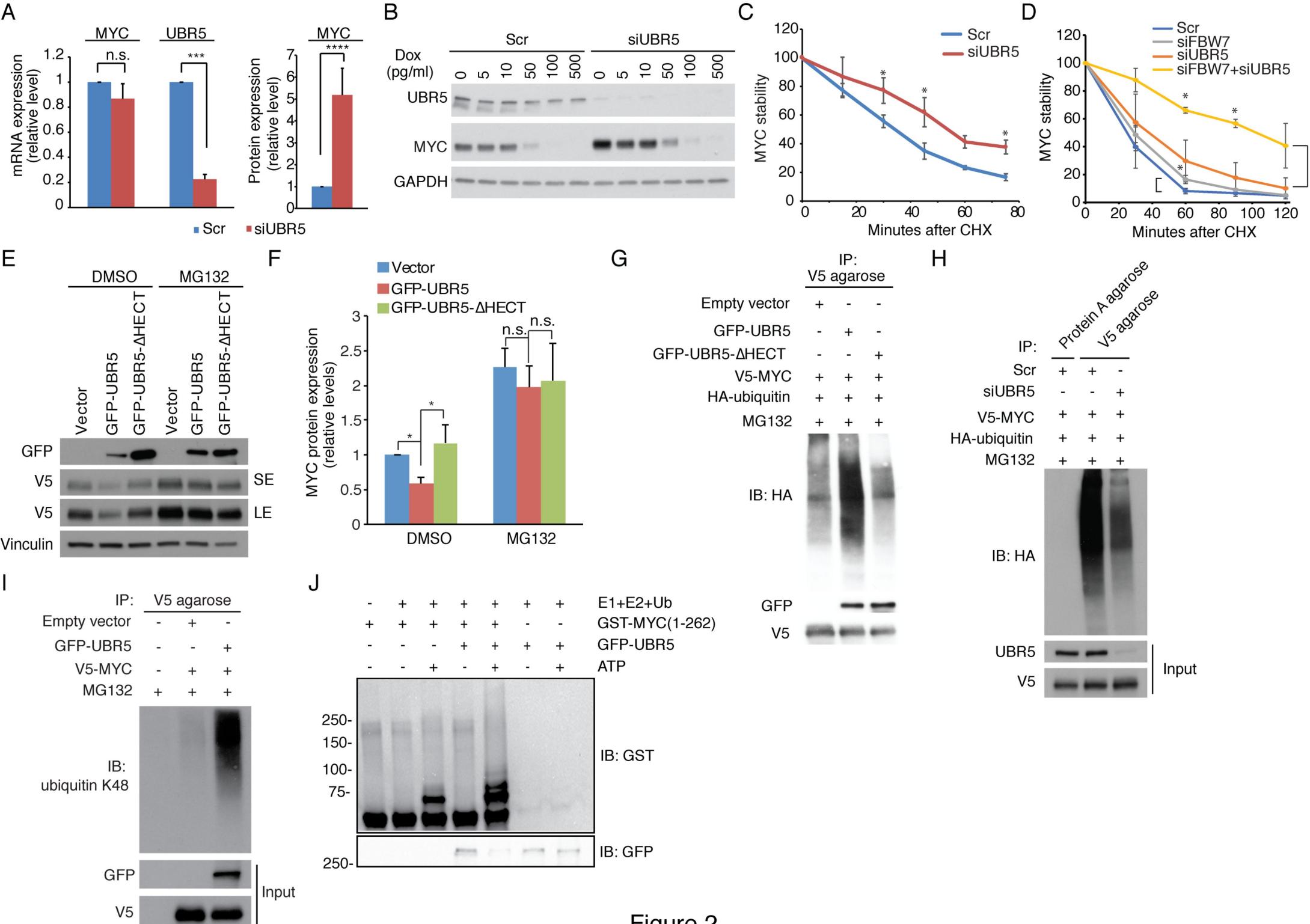


Figure 1



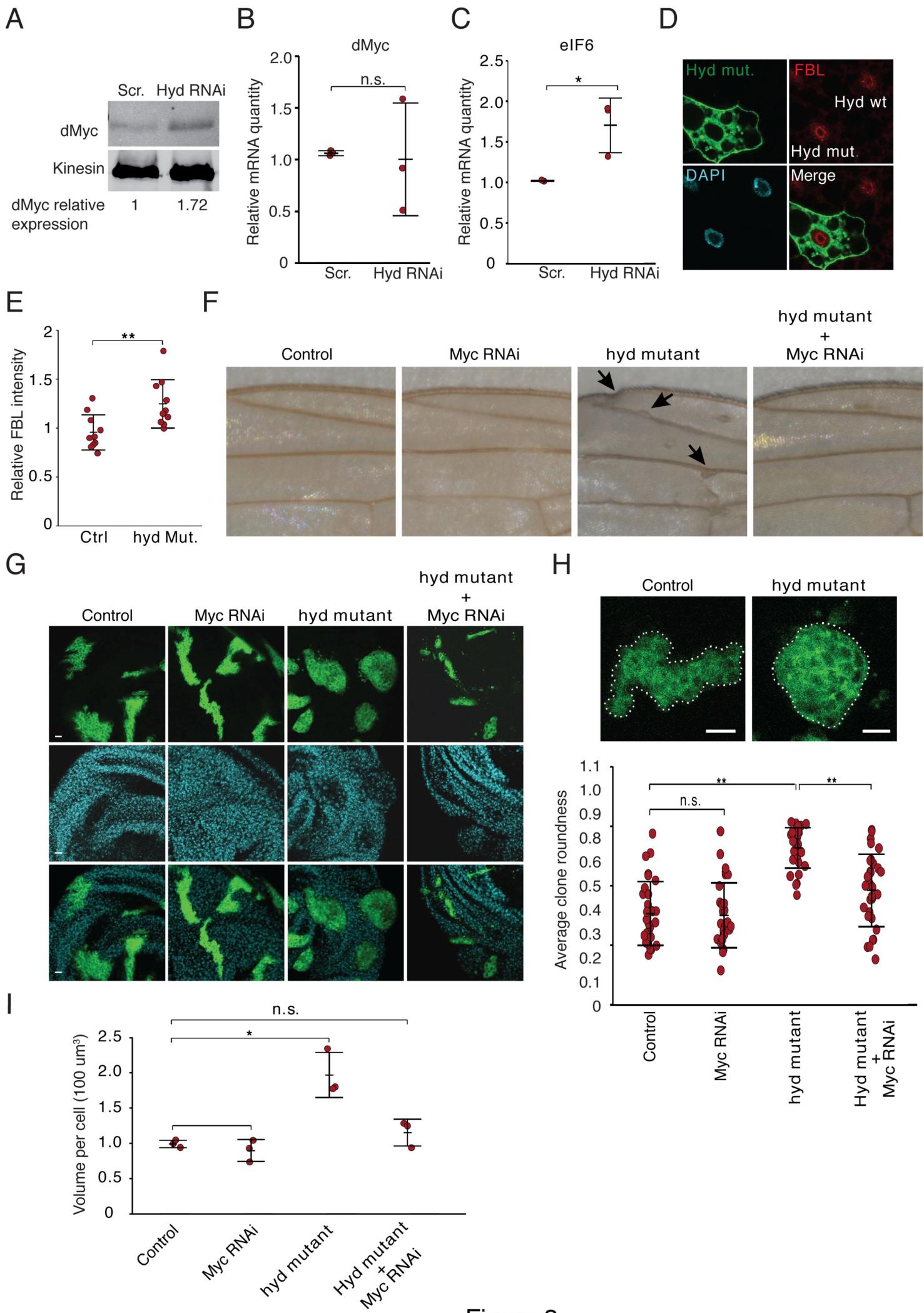


Figure 3

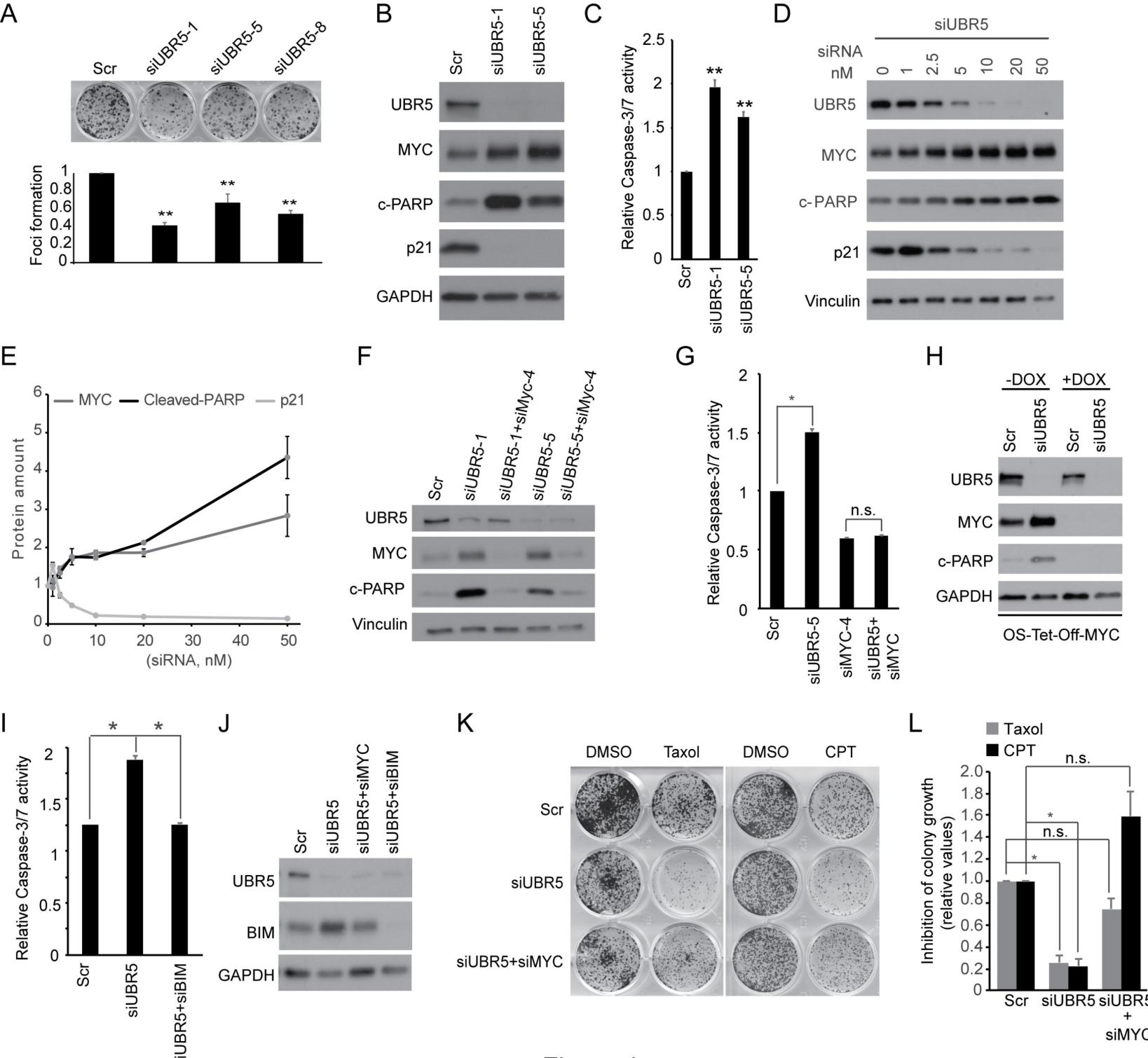


Figure 4

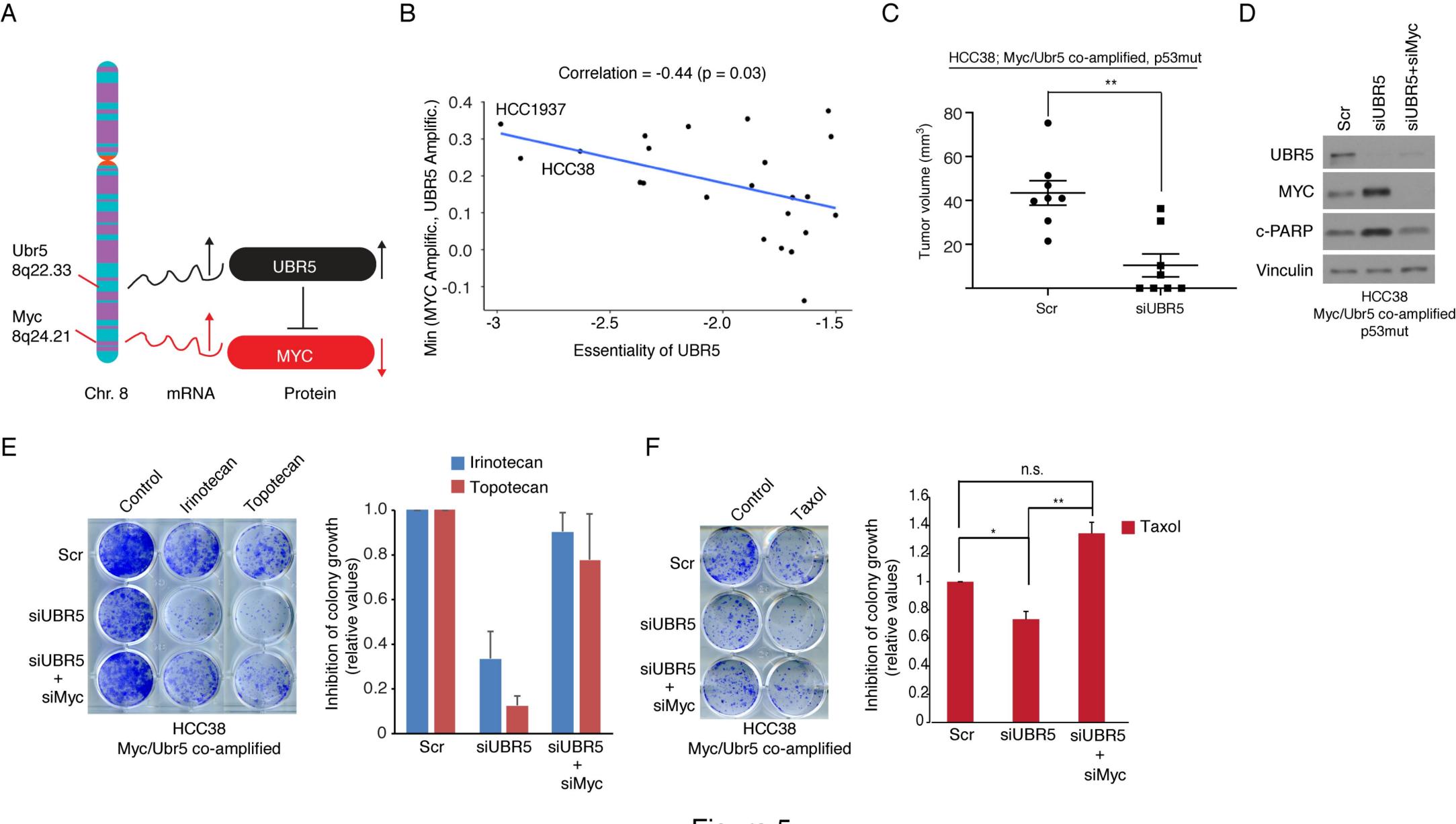
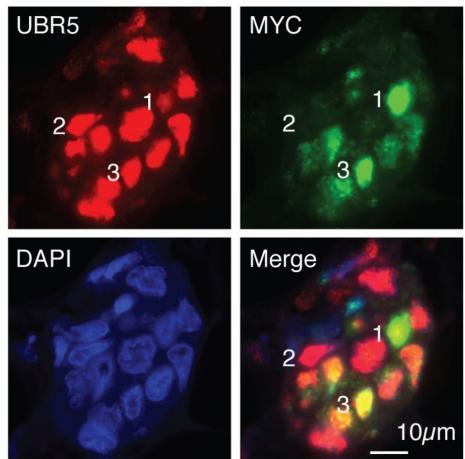


Figure 5

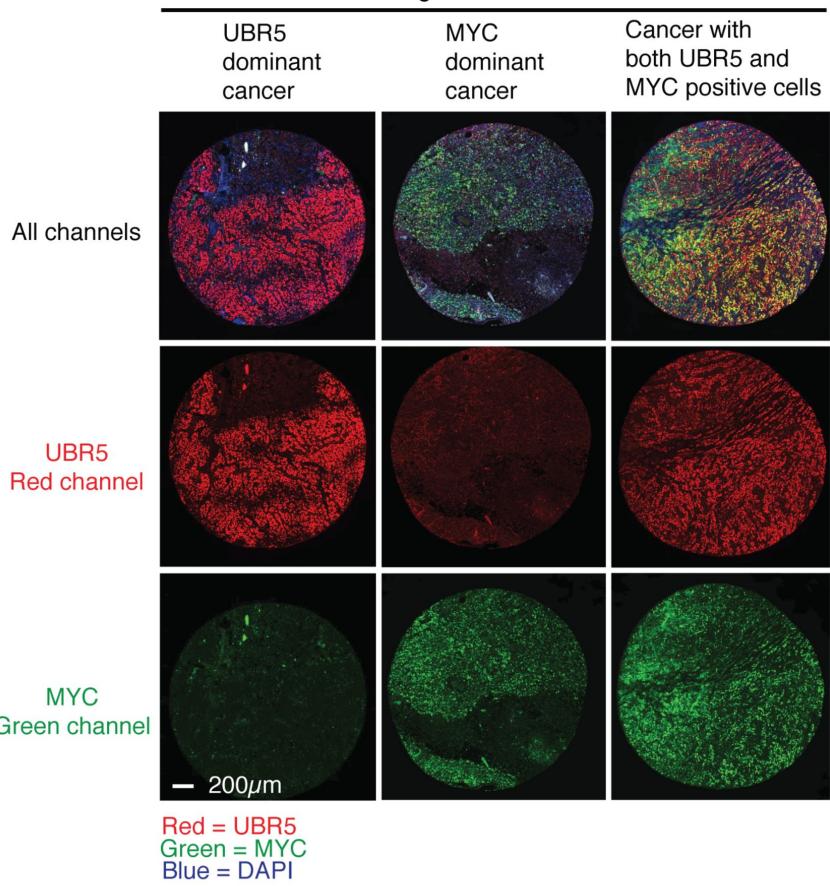
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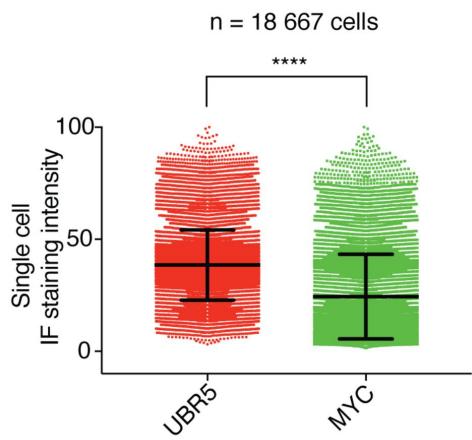
1 MYC dominant cell
2 UBR5 dominant cell
3 Equal UBR5 and MYC expression

B

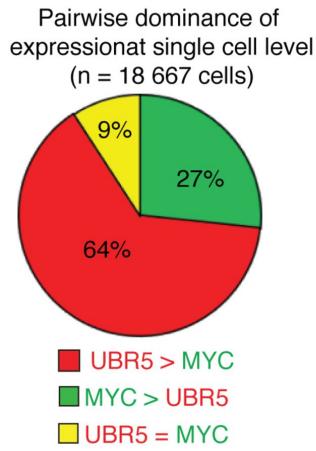
IF co-staining with UBR5 and MYC ab.



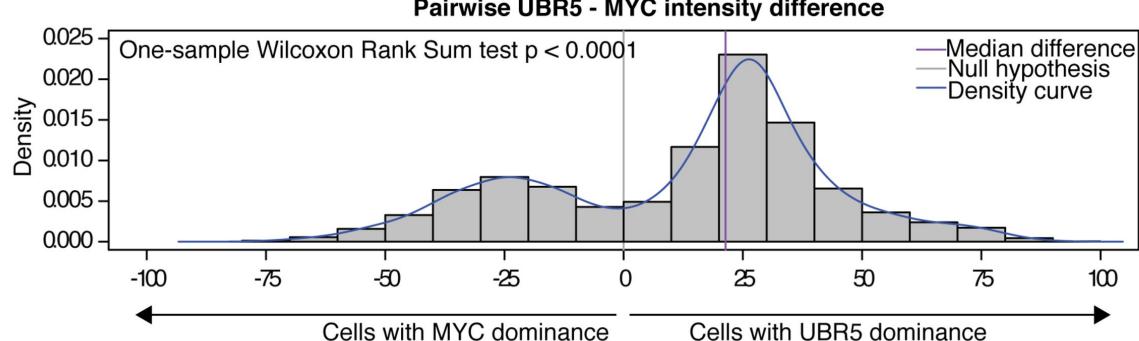
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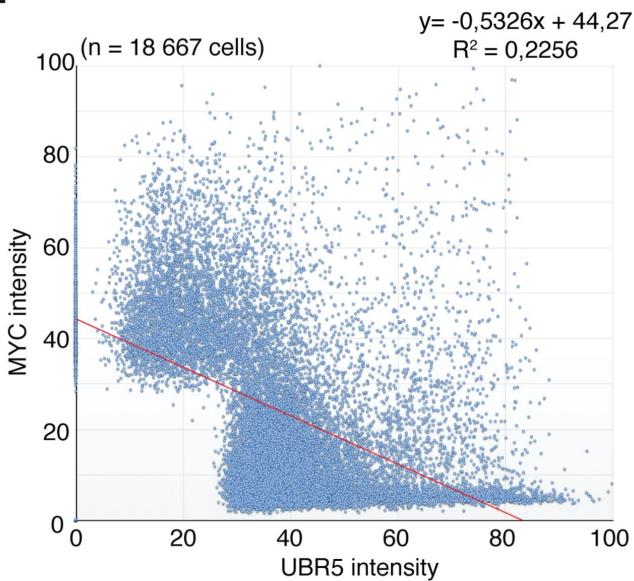
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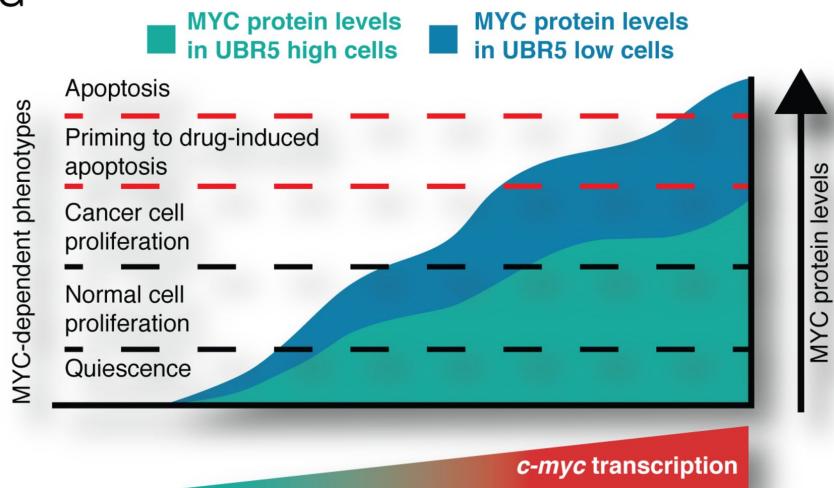
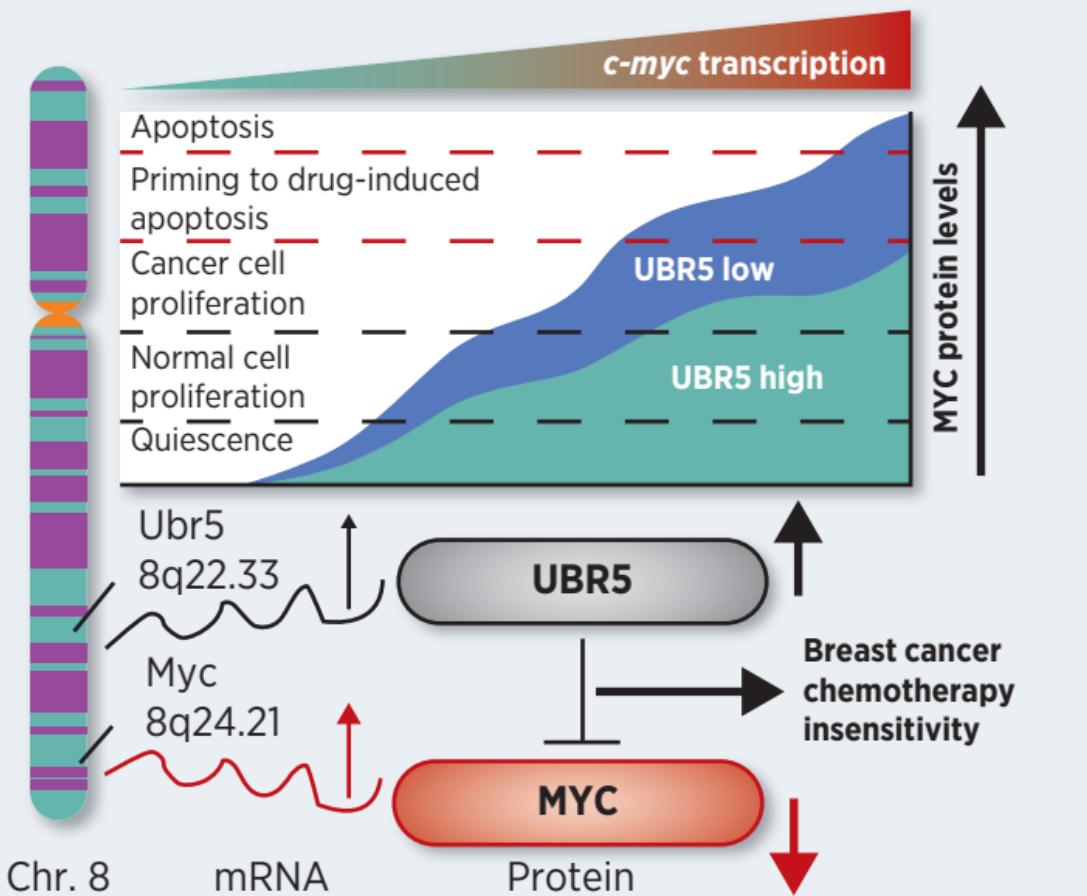


Figure 6



Co-amplification of *UBR5* with *MYC* is a cancer cell intrinsic mechanism to dampen *MYC*-mediated apoptosis in response to chemotherapy.