dNTP metabolism links mechanical cues and YAP/TAZ to cell growth and oncogene-induced senescence

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Abstract

YAP/TAZ, downstream transducers of the Hippo pathway, are powerful regulators of cancer growth. How these factors control proliferation remains poorly defined. Here, we found that YAP/TAZ directly regulate expression of key enzymes involved in deoxynucleotide biosynthesis and maintain dNTP precursor pools in human cancer cells. Regulation of deoxynucleotide metabolism is required for YAP-induced cell growth and underlies the resistance of YAP-addicted cells to chemotherapeutics targeting dNTP synthesis. During RAS-induced senescence, YAP/TAZ bypass RAS-mediated inhibition of nucleotide metabolism and control senescence. Endogenous YAP/TAZ targets and signatures are inhibited by RAS/MEK1 during senescence, and depletion of YAP/TAZ is sufficient to cause senescence-associated phenotypes, suggesting a role for YAP/TAZ in suppression of senescence. Finally, mechanical cues, such as ECM stiffness and cell geometry, regulate senescence in a YAP-dependent manner. This study indicates that YAP/TAZ couples cell proliferation with a metabolism suited for DNA replication and facilitates escape from oncogene-induced senescence. We speculate that this activity might be relevant during the initial phases of tumour progression or during experimental stem cell reprogramming induced by YAP.

Keywords mechanotransduction; nucleotide metabolism; oncogene-induced senescence; YAP/TAZ

Subject Categories Cancer; Metabolism

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Introduction

YAP (yes-associated protein 1) and TAZ (transcriptional coactivator with PDZ-binding motif, also known as WWTR1) are sibling transcriptional coactivators that shuttle between the cytoplasm and the nucleus where they regulate gene transcription by interacting with TEAD transcription factors (Piccolo *et al.*, 2014). YAP and TAZ have

been identified as the downstream transducers of the Hippo pathway, key regulator of tissue proliferation and organ size across animals (Halder & Johnson, 2011). More recently, several reports unveiled multiple regulatory inputs that can influence YAP and TAZ activity, including cell geometry and the mechanical properties of the extracellular matrix, RHO activity, cell–cell junctions, growth-factor signalling and metabolic pathways (Piccolo *et al.*, 2014; Santinon *et al.*, 2015; Dupont, 2016). This led to the notion that YAP and TAZ integrate different cellular and extracellular cues into a coherent biological response.

One key biological effect of YAP and TAZ is the regulation of cell proliferation, and this is associated with the ability of YAP and TAZ to promote self-renewal of stem cells (Camargo *et al.*, 2007; Cordenonsi *et al.*, 2011; Panciera *et al.*, 2016). *In vivo*, this activity appears dispensable in homeostatic conditions of adult tissues, but is fundamental for embryonic tissue proliferation, when tissues need to regenerate after damage, or when cells undergo neoplastic transformation (Azzolin *et al.*, 2014; Zanconato *et al.*, 2015; Moya & Halder, 2016). This makes YAP and TAZ ideal tumour-specific targets to develop new approaches to disable cancer cell growth and tumour-seeding ability. In this light, understanding what are the genetic programmes activated by YAP and TAZ might provide important information for treatment and new molecular targets to harm YAP-addicted tumour cells (Zanconato *et al.*, 2016).

In this study, we found that YAP and TAZ control the transcription of a set of enzymes involved in deoxynucleotide biosynthesis, such that YAP and TAZ activity impacts dNTP metabolism and the availability of dNTP precursors for DNA replication. Regulation of nucleotide metabolism is required for YAP-induced breast cancer cell growth and underlies the resistance of YAP-addicted breast cancer cells to chemotherapeutics targeting dNTP synthesis. Moreover, prompted by the notion that dNTP regulation is a crucial step regulating Ras-induced senescence (Aird *et al.*, 2013; Mannava *et al.*, 2013), we found that YAP is sufficient to overcome Ras-induced inhibition of nucleotide metabolism and senescence and that endogenous YAP/TAZ activity is inhibited by Ras during the early phase of oncogene-induced senescence. As such, depletion of YAP/TAZ or their inhibition by

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upstream inputs, including mechanical cues, induces senescence-associated phenotypes in primary cells.

Results

YAP and TAZ regulate deoxynucleotide metabolism in cancer cells

To gain insights into how YAP and TAZ regulate cell proliferation, we performed gene list enrichment analysis on genes regulated by YAP/TAZ in MDA-MB-231 cells (Enzo *et al*, 2015). Many pathways linked to cell-cycle regulation were significantly enriched among the genes downregulated in cells depleted of YAP and TAZ, including genes involved in G1/S and G2/M transition, DNA repair and DNA replication, in line with recent findings (Zanconato *et al*, 2015; Jang *et al*, 2017). Interestingly, we also found a very significant enrichment in several key and rate-limiting enzymes involved in deoxynucleotide (dNTP) synthesis, including ribonucleotide reductase (RNR) subunits *RRM1* and *RRM2*, thymidylate synthetase (*TYMS*), deoxythymidylate kinase (*DTYMK*) and thymidine kinase (*TK1*) (Fig EV1A and B), that are key to ensure progression of cells through the S-phase.

We validated this observation by qPCR (Figs 1A and EV1C) and surveyed published microarrays, which indicated a coherent and general regulation of dNTP metabolic enzymes by YAP/TAZ in multiple cancer cell lines (Li *et al*, 2014; Mori *et al*, 2014), in *MST1/2* knockout mice (Fitamant *et al*, 2015) and in livers overexpressing YAP (Yimlamai *et al*, 2014; Fig EV1D). In line with these data, inhibition of YAP/TAZ activity by established upstream inputs (Hamaratoglu *et al*, 2006; Dupont *et al*, 2011; Kim *et al*, 2013) led to a coherent inhibition of these metabolic enzymes (Fig EV1E). Moreover, sustaining YAP activity in conditions where endogenous YAP/TAZ are inhibited, such as growth in the absence of cell–substrate adhesions (Zhao *et al*, 2012), induced expression of these genes (Figs 1B and EV1F).

We next asked whether regulation of dNTP metabolism genes by YAP/TAZ was secondary to regulation of the cell cycle. For this, we sorted MDA-MB-231 cells in the different phases of the cell cycle based on the FUCCI cell-cycle indicators (Sakaue-Sawano *et al*, 2008): as shown in Figs 1C, and EV1G and H, knockdown of YAP/TAZ regulates dNTP genes independently of the cell cycle, suggesting a direct transcriptional event. We thus queried recent genome-wide chromatin immunoprecipitation experiments (Zanconato *et al*, 2015) and found evidence for a TEAD-dependent enhancer in at least three of these genes, which we then validated (Fig 1D). Importantly, YAP/TAZ not only regulate dNTP metabolism gene

expression, but are quantitatively required to sustain the synthesis of all four dNTP (Fig 1E). Collectively, this indicates that YAP/TAZ directly regulate deoxynucleotide metabolism.

Regulation of nucleotide metabolism genes is relevant for the ability of Myc and mutant-p53 oncogenes to promote cell proliferation (Hsieh *et al*, 2015; Kollareddy *et al*, 2015). We promoted cell growth in soft agar by stable expression of activated YAP 5SA and challenged this by RNA interference for selected dNTP metabolism enzymes regulated by YAP/TAZ. As shown in Figs 2A and B, and EV2A and B, *RRM2* and *DTYMK* are required for YAP-induced cancer cell growth. Similar results were obtained when endogenous YAP/TAZ activity and proliferation were promoted by stiffening the extracellular matrix (Aragona *et al*, 2013; Fig 2C). Drugs targeting dNTP synthesis and the activity of *RRM2* are often used as chemotherapeutics to treat tumours, including breast cancer; resistance to these drugs can be observed in some cases, and this has been linked to overexpression of *RRM1/2* subunits (Vander Heiden, 2011; Aye *et al*, 2015). We thus compared the sensitivity to gemcitabine treatment of breast cancer cells in conditions of low YAP activity (soft agar) and of increased YAP activity (by expression of YAP 5SA), and found that YAP promotes gemcitabine resistance (Fig 2D). In line with this result, we found evidence that YAP/TAZ target genes are higher in breast cancer cells displaying reduced sensitivity to gemcitabine in the NCI60 cell line database (Fig 2E).

Collectively, data gathered so far indicate that nucleotide metabolism is one of the important genetic programmes sustained by YAP/TAZ in cancer cells to promote proliferation, and this might have relevance for the treatment of YAP-driven tumours.

YAP overcomes blockade of dNTP metabolism during oncogene-induced senescence

Oncogene-induced senescence (OIS) is observed in primary cells and tissues upon activation of oncogenes such as Ras (Campisi, 2013). OIS entails growth arrest together with the emergence of other hallmark phenotypes, including the expression of acidic beta-galactosidase activity (senescence-activated beta-gal or SA β gal), nuclear enlargement with downregulation of LaminB1 expression and the formation of senescence-associated heterochromatin foci (SAHF), and expression of secreted cytokines (senescence-activated secretory phenotype or SASP). OIS is considered a tumour-suppressor mechanism that opposes cell transformation (Prieur & Peeper, 2008; Campisi, 2013). Recent evidence indicates that deoxynucleotide metabolism plays an important role during

Figure 1. YAP/TAZ regulate the expression of key genes involved in dNTP metabolism in breast cancer cells.

- A qPCR for nucleotide metabolism genes on MDA-MB-231 transfected with control siRNA (siCo.) or YAP/TAZ siRNA (siYT). Here and throughout the figures, qPCR data are relative to *GAPDH* levels; mean expression levels in control cells were set to 1, and all other samples are expressed relative to this. $n = 6$.
 - B MDA-MB-231 cells infected with control vector (–) or with YAP 5SA were seeded either on tissue-culture plastic (attached) or on top of agarose (suspended). *P*-value relative to control attached cells. $n = 4$.
 - C qPCR on selected dNTP genes in the different phases of the cell cycle, as obtained by sorting MDA-MB-231 cells expressing Fucci cell cycle indicators. Red NOT Green: G1 phase; Red AND Green: early S; Green NOT Red: S/G2/M. $n = 4$.
 - D Chromatin immunoprecipitation from MDA-MB-231 cells treated with vehicle or with latrunculin A (LatA, 650 nM), leading to YAP/TAZ inhibition, for 6 h. Amplification of *Haemoglobin beta* (*HBB*) serves as negative control; for each promoter, values are relative to IgG IP, arbitrarily set to 1. $^{\circ}P < 0.08$ (two tailed Student's *t*-test). $n = 4$.
 - E Steady-state dNTP pools measured in MDA-MB-231 cells transfected with the indicated siRNAs and expressing siRNA-insensitive YAP as indicated. $n = 4$.
- Data information: Data are mean and SD with $^{\circ}P < 0.05$ (two-tailed Student's *t*-test).

Figure 1.

oncogene-induced senescence: Ras activation inhibits the expression of key dNTP enzymes, leading to replication stress and in turn inducing the senescent response (Aird *et al*, 2013; Mannava *et al*, 2013; Wiley & Campisi, 2016). Interestingly, these enzymes are the same that we found regulated by YAP and TAZ in cancer cells.

To test whether YAP could intersect OIS, we first checked that dNTP enzymes were effectively regulated by YAP and TAZ also in WI38 human primary lung fibroblasts, an established cellular system for senescence (Fig EV3A and B). Then, we transduced oncogenic Ras (H-Ras G12V) to initiate the senescence response, alone or with activated YAP-5SA, and found that YAP was sufficient to reactivate dNTP enzyme expression (Fig 3A and B). This occurs independently of Myc (Fig EV3C and D), even if YAP can intersect Myc activity (Crocì *et al*, 2017), and if Myc is a known regulator of dNTP metabolism (Hsieh *et al*, 2015). Coherently, YAP also rescued dNTP biosynthesis, indicating an effective rescue of deoxynucleotide metabolism (Fig 3C).

We then looked at phenotypes induced downstream of blockade of nucleotide metabolism (Aird *et al*, 2013) such as proliferation (Fig 4A–C), LaminB1 expression (Fig 5A), SAHF formation (Fig 5B and C), SA β gal (Fig 5D and E) and expression of SASP genes (Fig 5F), and found that all these phenotypes were remarkably rescued by YAP. Similar results were observed in IMR90 human fibroblasts and in HPNE (Chang *et al*, 2014) ductal pancreatic cells (Fig EV4B–D). Of note, a mutated version of YAP unable to drive transcription (S94A) was unable to counteract Ras-induced senescence phenotypes (see Figs 4B, and 5B and C). Importantly, knock-down of RRM2 reinstalled senescent phenotypes in cells co-expressing Ras and YAP (Fig 5G and H). Collectively, these data suggest that YAP overcomes Ras-induced senescent phenotypes by sustaining dNTP metabolism.

Ras inhibits endogenous YAP/TAZ targets in cells undergoing senescence

Prompted by our findings, we also tested whether Ras inhibits endogenous YAP/TAZ activity. We first surveyed available microarray experiments performed on fibroblasts expressing activated Ras and found a significant enrichment of YAP/TAZ-induced targets among the genes inhibited by Ras (Figs 6A, and EV5A and B); conversely, we found the targets inhibited by YAP/TAZ enriched among the genes induced by Ras (Fig EV5A and B). As a control, signatures containing SASP genes (Campisi & d'Adda di Fagagna, 2007; Rajagopalan & Long, 2012; Kansara *et al*, 2013; Moiseeva *et al*, 2013) were also enriched among the genes induced by Ras (Figs 6A, and EV5A and B). Interestingly, similar evidence was

obtained from data in primary human melanocytes expressing BRAF (V600E; Pawlikowski *et al*, 2013; Fig EV5C). We then directly monitored the expression of a set of genes broadly regulated by YAP/TAZ (see below, Fig EV6A), including *CTGF*, *CYR61*, *ANKRD1* and the newly validated *CRIM1*, and found that Ras inhibited their expression (Fig 6B). Importantly, this regulation, as well as regulation of dNTP metabolism genes (see below, Fig 6E and G), was already evident at early time-points following Ras expression, before the induction of growth inhibition, SAHF and SA β gal (Fig EV5D–F). This makes unlikely the possibility that regulation of YAP/TAZ targets, including dNTP metabolism, is a secondary effect of senescence establishment. Further supporting the view that YAP/TAZ are regulated before growth arrest, expression of YAP/TAZ target genes was not inhibited upon forced expression of the CDK inhibitor p16/CDKN2A (Fig EV5G and H).

Senescence is a tumour-suppressive mechanism during skin tumorigenesis induced by TPA/DMBA treatment, where Ras is mutated (Fujiki *et al*, 1989) and where the transition from papillomas to carcinomas often entails inactivation of the *p16/CDKN2A* locus (McCreery *et al*, 2015). Taking advantage of available gene expression data from single mouse skin papillomas (McCreery *et al*, 2015), we classified them according to high vs. low levels of SASP as a proxy for Ras-induced senescence, and monitored the levels of YAP/TAZ target genes in these two groups. As shown in Fig 6C, papillomas displaying higher levels of SASP genes expressed significantly lower levels of YAP/TAZ target genes, in line with their inhibition during OIS observed *in vitro*.

To gain further insights into YAP/TAZ inhibition by Ras, we explored at early time-points which Ras-induced signalling pathway (i.e., p38, MAPK and JNK) was more specifically involved. For this, we overexpressed the activated forms of MEK1 (MAPK), MEK3 (p38) and MEK4 (JNK), some of which are sufficient to induce senescence (Lin *et al*, 1998; Wang *et al*, 2002), and monitored YAP/TAZ target genes. MEK1 was much stronger at inhibiting YAP/TAZ activity compared to the other MEKs (Fig 6D), and this correlated with senescence induction. In line, we observed a significant enrichment of YAP/TAZ target genes among the genes inhibited by MEK1 in IMR90 fibroblasts by GSEA (Fig EV5K). As a control, we also monitored selected markers for dNTP metabolism, proliferation and SASP (Figs 6E, and EV5I and J). Importantly, when we challenged Ras-mediated effects with small molecule inhibitors of the MEK1/2 or p38 pathways, we found that MEK inhibition was sufficient to rescue YAP/TAZ target gene expression (Fig 6F and G). These data, together with previous observations, suggest that YAP/TAZ inhibition, proximal to Ras/MEK activation, is required for induction of senescence.

Figure 2. YAP-induced growth requires key deoxynucleotide metabolism genes.

- A Depletion of DTYMK or RRM2 in MDA-MB-231 cells impairs YAP-induced colony-forming ability in soft agar. The graph quantifies the number of colonies growing on a defined surface area, equal for all experimental conditions. $n = 4$. Data are mean and SD with $*P < 0.05$ (two-tailed Student's *t*-test).
- B Representative pictures of colonies quantified in (A). Pictures indicate that YAP 5SA increases not only the number of colonies but also their size, while siRRM2 and siDTYMK act in the opposite way. Scale bar: 80 μ m.
- C Representative pictures of MCF10A-MII cells transfected with the indicated siRNA and embedded in a soft matrix (Matrigel) or in a stiff matrix (Matrigel plus CollagenI), the latter sustaining growth through YAP/TAZ (Aragona *et al*, 2013). Scale bar: 200 μ m. $n = 4$.
- D Control and YAP 5SA-expressing MDA-MB-231 cells in soft agar were treated with the indicated doses of gemcitabine. $n = 6$.
- E Levels of YAP/TAZ target genes in breast cancer cell lines displaying resistance to gemcitabine as compared to sensitive cell lines, based on NCI60 database (see Materials and Methods). Boxes: 25th to 75th percentile; whiskers: smallest to largest value. $**P < 0.005$ (unpaired two-tailed *t*-test).

Figure 2.

Figure 3. YAP overcomes Ras-induced inhibition of dNTP metabolism in primary human fibroblasts.

A qPCR for dNTP metabolism genes in WI38 cells infected with Ras or Ras together with YAP 5SA. Control cells (–) were infected with an empty virus. Here and throughout Figs 4 and 5, cells were harvested 6 days after starting selection of infected cells with puromycin. *n* = 6.

B Western blot analysis of WI38 cell extracts infected as in (A). GAPDH is the loading control. Two independent experiments.

C Steady-state size of dNTP pools in WI38 cells infected as indicated in (A). *n* = 4.

Data information: Data are mean and SD with **P* < 0.05 (two-tailed Student's *t*-test).

YAP/TAZ requirement unveils a connection between mechanical cues and senescence

We next asked whether YAP/TAZ inhibition might be also sufficient to recapitulate at least some senescent phenotypes. We knocked

down YAP and monitored a panel of senescence markers including proliferation (Fig 7A and B), SASP (Fig 7C), SA β gal expression (Fig 7D) and SAHF formation (Fig 7E), and found them all regulated in WI38 cells. Of note, this happened in the absence of increased MAPK phosphorylation (Fig EV6C), in keeping with YAP/TAZ

Figure 4. YAP overcomes Ras-induced growth arrest.

A Expression of active YAP 5SA overcomes regulation of cell-cycle genes by Ras in WI38 cells (6 days). $n = 4$.
 B EdU incorporation in WI38 cells expressing the indicated retroviral transgenes. YAP 5SA S94A-mutant is unable to bind TEAD co-factors and to activate transcription. Representative results of a single experiment (> 350 cells per condition); $n = 4$.
 C Representative EdU stainings and nuclear counterstains (DAPI) of WI38 cells quantified in (B). Scale bar: 80 μm .

Data information: Data are mean and SD with $*P < 0.05$ (two-tailed Student's t -test).

Figure 5. YAP overcomes Ras-induced senescent phenotypes.

A Western blot analysis of WI38 cells expressing the indicated retroviral transgenes (here and throughout the figure, 6 days). GAPDH serves as loading control. Two independent experiments.
 B SAHF (senescence-associated heterochromatin foci) formation WI38 cells expressing the indicated retroviral transgenes. See Fig EV4A for further controls on Ras. Representative results of a single experiment (> 100 cells per condition); $n = 3$.
 C Representative pictures of DAPI staining to visualize SAHF quantified in (B). Scale bar: 15 μm .
 D Induction of SA β gal (senescence-associated β -galactosidase) activity in WI38 cells. Representative results of a single experiment (> 100 cells per condition); $n = 3$.
 E Representative pictures of SA β gal staining quantified in (D). Scale bar: 20 μm .
 F qPCR for SASP (senescence-associated secretory phenotype) marker genes in WI38 cells. $n = 6$.
 G Quantification of proliferating WI38 cells as measured by EdU incorporation. Control and RRM2 siRNAs were transfected 1 day after starting selection. Representative results of a single experiment (> 100 cells per condition); $n = 4$.
 H WI38 cells were processed as in (G) but stained for SAHF. Representative results of a single experiment (> 100 cells per condition); $n = 3$.

Data information: Data are mean and SD with $*P < 0.05$ (two-tailed Student's t -test).

Figure 5.

Figure 6.

Figure 6. Ras and MEK1 inhibit YAP/TAZ transcriptional activity during induction of senescence.

- A Representative gene set enrichment analysis for YAP/TAZ target genes and SASP genes (Rajagopalan) in published microarrays of primary cells undergoing Ras-induced senescence (FDR q -value < 0.05). See Fig EV5A–C for complete results.
- B Ras activation inhibits YAP/TAZ target genes in WI38 cells expressing the indicated retroviral transgenes for 6 days. *CTGF*, *CYR61* and *ANKRD1* are known YAP/TAZ target genes; *CRIM1* is another widely regulated target (Enzo *et al*, 2015; Kim *et al*, 2015; Noguchi *et al*, 2017). $n = 6$.
- C Average levels of a YAP/TAZ target gene signature in mouse skin papillomas with mutated Ras and classified according to high or low levels of the indicated SASP gene signatures (see Materials and Methods). Boxes: 25th to 75th percentile; whiskers: smallest to largest value. Closed circles indicate expression values in the single papillomas ($n = 27$).
- D qPCR for endogenous YAP/TAZ target genes in WI38 cells expressing active forms of Ras, MEK1, MEK3 or MEK4 for 2 days. This indicates early inhibition of YAP/TAZ. $n = 4$.
- E qPCR on WI38 cells infected as in (D) for dNTP metabolism genes (orange), of the *CCNA2* proliferation marker, and of SASP genes (purple). $n = 4$.
- F, G qPCR on WI38 cells expressing active Ras and treated with 10 μ M U0126 MEK1/2 inhibitor (2 days). $n = 4$.
- Data information: (B, D–G) Data are mean and SD with $*P < 0.05$ (two-tailed Student's t -test). (C) $*P < 0.05$ unpaired two-tailed Student's t -test.

acting downstream of Ras. Moreover, YAP/TAZ downregulation was associated with the emergence of senescence-associated phenotypes also in transformed MDA-MB-231 cells (Fig EV6D and E).

YAP and TAZ activity can be regulated, among other inputs, by mechanical cues (Dupont *et al*, 2011; Dupont, 2016); we thus asked whether mechanical conditions inducing YAP/TAZ inhibition would also induce senescence phenotypes. We first addressed this hypothesis by seeding cells on soft fibronectin-coated acrylamide hydrogels (Elastic modulus ≈ 0.7 KPa); analysis of dNTP genes, of SASP and SA β gal expression, indicated that a soft ECM induces senescence (Figs 7F–I, and EV6F and G). To provide independent evidence, we monitored cells seeded on micropatterned fibronectin-coated islands of limiting size (Dupont *et al*, 2011; Wada *et al*, 2011), and found that also a small cell geometry induces SA β gal, in addition to growth arrest, in WI38 fibroblasts (Figs 7J, and EV6H and I). Importantly, as in the case of Ras shown above, this critically depends on YAP/TAZ inhibition (Fig 7K). As a control, YAP/TAZ was unable to rescue senescent phenotypes induced by the DNA-damaging agent doxorubicin (Fig EV6J and K). Collectively, these results indicate that YAP inhibition is instrumental to induce cell senescence downstream of mechanical cues and that senescence is a novel mechano-regulated phenotype.

Discussion

It is well established that YAP/TAZ powerfully regulate cell proliferation; however, what is the genetic programme underlying this

activity is only starting to emerge, and likely entails regulation of several processes in parallel (Zanconato *et al*, 2015; Jang *et al*, 2017). Here, we focused on the observation that YAP/TAZ sustain the expression of a series of key nucleotide metabolism genes across several actively proliferating cell lines, which results in profound regulation of dNTP steady-state levels. A similar connection has been proposed in the zebrafish liver, although in that case YAP regulates glutamine metabolism to indirectly sustain deoxynucleotide synthesis (Cox *et al*, 2016). Our data instead indicate that YAP directly regulates, in combination with TEAD factors, at least some key players in dNTP metabolism. This is required for their growth-inducing activity when cells are placed in suspension, or promoted by stiffening of the ECM microenvironment. Moreover, elevation of dNTP metabolism genes by YAP correlates with increased resistance to a chemotherapeutic acting specifically on dNTP metabolism. Thus, YAP not only regulates G1/S transition, but also directly supports a steady supply of dNTPs for DNA replication.

Prompted by these findings, and given the previously established role for dNTP metabolism in oncogene-induced senescence, we also found that YAP, by sustaining dNTP production and likely other genetic programmes, prevents induction of growth arrest and overcomes Ras-induced senescence in human primary cells. Furthermore, we serendipitously found that Ras activation entails the inhibition of endogenous YAP/TAZ activity, and that depletion of YAP/TAZ is sufficient to induce senescent phenotypes, indicating that regulation of YAP/TAZ is part of the chain of events leading to senescence, at least *in vitro*. This preferentially occurs upon activation of MEK1, which

Figure 7. YAP inhibition by ECM mechanical cues is sufficient to induce senescent phenotypes.

- A, B siRNA knockdown of YAP/TAZ induces growth arrest in WI38 cells as assayed by expression of cell-cycle genes at 2 days (A), $n = 4$, and EdU incorporation at 5 days (B), representative results of a single experiment (> 600 cells per condition); $n = 4$. See Fig EV6A for efficiency of siYT on its direct targets.
- C qPCR for SASP markers on WI38 cells transfected as in (A) at 2 days. $n = 4$.
- D Induction of SA β gal activity at 5 days in WI38 cells. Representative result of a single experiment (> 160 cells per condition); $n = 4$. Scale bar of the representative pictures: 20 μ m.
- E SAHF formation at 5 days in WI38 cells. Representative result of a single experiment (> 160 cells per condition); $n = 4$. Scale bar of the representative DAPI stainings: 10 μ m.
- F–H qPCR for dNTP metabolism genes (A) proliferation markers (B) and SASP markers (C) on WI38 cells plated on tissue-culture plastic (stiff) or on fibronectin-coated hydrogels (soft, elastic modulus $E \approx 0.7$ KPa). $n = 4$.
- I WI38 cells plated on tissue-culture plastic (stiff) or on fibronectin-coated hydrogels (soft, elastic modulus $E \approx 0.7$ KPa) and stained for SA β gal activity. On the right: representative pictures of the cells. Note the rounded cell shape of cells on the soft hydrogel. $n = 3$. Scale bar: 40 μ m.
- J WI38 cells were plated on fibronectin-coated glass coverslips (Unpatt.) or on fibronectin-coated small islands of 2,025 μ m² (medium-small) or 1,024 μ m² (small) and stained for SA β gal activity. Representative result of a single experiment (> 100 cells per condition); $n = 2$. On the right: representative pictures of the cells. Scale bar: 30 μ m.
- K WI38 cells were infected with control vector (–) or YAP 5SA-encoding retrovirus and plated on 2,025- μ m² fibronectin-coated small islands. Cells were stained for SA β gal activity and scored for strong, weak or negative SA β gal activity. Pictures show representative SA β gal stainings. Scale bar: 30 μ m.
- Data information: Data are mean and SD with $*P < 0.05$ (two-tailed Student's t -test).

Figure 7.

we found required and sufficient to inhibit YAP/TAZ targets during OIS. Finally, in keeping with a proximal inhibition downstream of Ras, we observed early inhibition of YAP/TAZ targets.

The link between YAP/TAZ and OIS might be relevant in the context of skin tumorigenesis, as we found that papillomas harbouring Ras activation and displaying higher levels of SASP genes, that we used as a proxy for senescence, also display lower levels of YAP/TAZ target genes. In more advanced tumours, YAP/TAZ cooperate with Ras, such that they can even substitute for Ras addiction in well-formed tumours; in this case, YAP/TAZ and Ras converge on regulation of proliferation-associated genes (Kapoor *et al*, 2014; Shao *et al*, 2014; Zhang *et al*, 2014). This suggests the interesting possibility that the opposing effects of Ras during tumorigenesis, that is, senescence (early on) and cell growth (later during tumour development), entail opposing effects of Ras on YAP/TAZ. One potential mechanism underlying the effects of Ras on YAP/TAZ might be the differential regulation of AP1 (i.e., Jun/Fos) factors: AP1 dimers containing c-Jun promote tumorigenesis and cooperate with YAP/TAZ to support proliferation, while JunB inhibits tumorigenesis and induces p16/CDKN2A, a hallmark of senescence (Passequé & Wagner, 2000; Das *et al*, 2007; Jin *et al*, 2011; Zanconato *et al*, 2015). It will be thus interesting to test the possibility that JunB interferes with YAP/TAZ activity.

Further extending our results, we found that a mechanical microenvironment promoting YAP/TAZ inhibition also promotes primary cell senescence. On one side, this expands the array of mechanoresponses available to study these processes; more in general, it reinforces the notion that mechanical cues, by regulating YAP/TAZ, can set the threshold for the appearance of multiple cellular phenotypes. Given the close link between YAP/TAZ activity, *in vitro* stem cell renewal and reprogramming of differentiated cells into their corresponding progenitors (Ramos and Camargo, 2012; Nowell *et al*, 2016; Panciera *et al*, 2016), it is tempting to speculate that the anti-senescence activity of YAP/TAZ (and of stiff ECM substrata) here described might be useful to more efficiently drive cell reprogramming (Utikal *et al*, 2009; Soria-Valles *et al*, 2015; Hayashi *et al*, 2016). Finally, this might also contribute to the ability of YAP/TAZ and of ECM stiffness to support the growth of primary stem cells and organoids *ex vivo* (Gilbert *et al*, 2010; Gjorevski *et al*, 2016).

Materials and Methods

Plasmids and reagents

pBABEpuro RasG12V (HRas) was Addgene 9051 from Dr. Hahn. pBABEpuro-YAP (wild-type or 5SA-mutant, siRNA resistant) were as in Aragona *et al* (2013). The other retroviral constructs were generated by standard subcloning of IRES-GFP, IRES-YAP 5SA or IRES-YAP 5SA S94A in pBABEpuro RasG12V. pBABEpuro empty vector was used as control retroviral transduction. pBABEpuro MEK1Q56P, pBABEpuro MEK3E and pBABEpuro MEK4E were a kind gift from Peiqing Sun. Lentiviral CSII-CMV-Ras-IRES-GFP-IRES2-Bsd and CSII-CMV-Ras-IRES-YAP 5SA-IRES2-Bsd were generated by standard subcloning. CSII-CMV-MCS-IRES2-Bsd empty vector was used as control. pXJ40-HA-Merlin/NF2 (S518A) was

Addgene 19700 from Dr. Giancotti. All plasmids were sequence-verified prior to use.

Latrunculin A and forskolin were from Sigma; gemcitabine and U0126 were from Santa Cruz Biotechnology. All these molecules were dissolved as recommended and controlled by using equal concentrations of vehicle alone.

Cell culture, transfections and retroviral transductions

MDA-MB-231 (STR verified) breast cancer cells were cultured in DMEM/F12 with 10% FBS and 2 mM glutamine; MCF10AT1K (also known as MII, a gift from Dr. Santner) in DMEM/F12 with 5% horse serum, 2 mM glutamine, insulin, cholera toxin, hydrocortisone and EGF (Debnath *et al*, 2003); low-passage WI38 human fibroblasts, purchased from Public Health England Culture Collections (ECACC catalog 90020107), and low-passage IMR90 (Dr. Rampazzo) in MEM supplemented with 10% FBS and 2 mM glutamine. HPNE cells were cultured as in Chang *et al* (2014). WI38 and IMR90 were expanded and cultured in low oxygen conditions (5%) to avoid replicative senescence; for infection or transfection, cells were moved to a standard incubator with normal oxygen conditions until harvesting. All the experiments on WI38 fibroblasts were concluded before 30 population doublings.

siRNAs were transfected by using Lipofectamine RNAi MAX (Life Technologies). siRNAs were dsRNA oligos with overhanging dTdT (Life Technologies), based on FlexiTube GeneSolution siRNA sets (Qiagen). Targeted sense sequences of the mRNA are as follows: siYAP: CUGGUCAGAGAUACUUCUU; siTAZ: AGGUACUCCUCAA UCACA; siRRM2 #1: GAGGAGAGAGUAAGAGAAA, siRRM2 #2: AG ACAGACUUAUGCUGGAA; siDTYMK #1: CAGUUGGAAUUCUCCUAA; siDTYMK #2: CCGGAAAGAUCAACUGAAA; siDTYMK #3: GAU GGUGGAUGCUUCCAAA; siMyc: GGUCAGAGUCUGGAUCCACC. Control siRNA was Qiagen AllStars Negative Control siRNA. DNA was transfected by using Transit-LT1 (MirusBio). For retroviral production, HEK293gp cells were transiently transfected with retroviral vector and pMD2 env (packaging vector). 48 h after transfection, virus particles were filtered (0.45 µm) and used diluted with polybrene to infect cells. Starting 48 h after infection, cells were selected by puromycin for 2 days and then released in fresh medium. Cells were collected for RNA extraction, proliferation and other OIS analyses 2 or 6 days after the beginning of the selection.

Quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen), DNase step included, according to the manufacturer's procedures. cDNA was synthesized using M-MLV Reverse Transcriptase (Life Technologies) and oligo-dT primers. Quantitative PCR was carried out with FastStart SYBR Green Master Mix (Roche) on a QuantStudio 6 real-time thermal cycler. *GAPDH* expression levels were used as internal control to normalize gene expression data between samples. Sequences of primers are provided as Table EV1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation analyses were performed as previously described (Enzo *et al*, 2015). Sequences of primers and of the peaks are provided in as Tables EV1 and EV2.

Soft agar and 3D cell culture

For soft agar, we embedded MDA-MB-231 cells in growth medium containing 0.3% low melting agarose layered on a pre-gelled 0.6% agarose bed. When solidified, each well was filled with growth medium and the medium was renewed twice per week. Four weeks later, images were acquired to count colonies. For soft agar assay on MDA-MB-231 transfected with siRNA, we trypsinized cells 1 day after siRNA transfection and plated 10,000 cells for each condition in 6-well plates in duplicates; for soft agar assay on MDA-MB-231 cells treated with gemcitabine, 500 cells were embedded in 96-well in triplicates and drug treatments were serially diluted in medium. Cells were embedded in soft agar 24 h after siRNA transfection. The graphs quantify the number of colonies growing on a defined surface area, equal for all experimental conditions. To monitor gene expression in these conditions, MDA-MB-231 cells were plated in six wells on top of 0.3% low melting agarose and collected after 72 h for RNA extraction.

For 3D cell culture, 500 MCF10A-MII cells were embedded in growth factor-reduced Matrigel (BD Biosciences) (soft matrix) or 200 MCF10A-MII cells in a mixture of growth factor-reduced Matrigel and CollagenI (Trevigen Culturex 3D Culture Matrix Rat CollagenI; stiff matrix). CollagenI solution was neutralized on ice with 0.1 M NaOH in PBS to bring the pH to 7.5. A solution of 60% CollagenI and 40% Matrigel was then mixed on ice to obtain stiff matrix. Cells were then embedded in stiff or soft matrixes as in Aragona *et al* (2013). We used less cells in the stiffer matrix to compensate for increased cell growth. Seven days later, images of the 3D cell cultures were acquired.

EdU, SA β gal and SAHF detection

Cells were plated on top of 13-mm-diameter fibronectin-coated glass coverslips for 48 h or 72 h before fixation. Cells were incubated for 1 h with EdU before fixation with 3.7% formaldehyde and then analysed according to manufacturer's instructions (Life Technologies). SA β gal staining was performed exactly as described in Dimri *et al* (1995). SAHF were visualized by DAPI staining on fixed cells at high magnification (63 $\times$ objective).

Antibodies, Western blotting and immunofluorescence

Antibodies were YAP/TAZ (sc101199), Ras (Hras sc-520), Lamin B1 (sc-6216), p-ERK (sc-7383), RRM2 (sc-10844), CCNA2 (sc-596) and GAPDH (Millipore MAB374). Western blotting was performed as in Dupont *et al* (2009) and immunofluorescence as in Aragona *et al* (2013).

Determination of intracellular dNTP pools

dNTP pool extraction and size determination were carried out as in Ferraro *et al* (2010); Rampazzo *et al* (2010).

Microfabrications

Fibronectin-coated micropatterned glass slides were purchased from Cytoo SA (PADO-1 custom mask, available to all users upon

request); the sizes of the square island were as in Dupont *et al* (2011). For each slide, 60,000 WI38 cells were plated in a 35-mm-diameter dish, washed after 2 h to remove unattached cells and processed for EdU or SA β gal staining after 48 h. For fibronectin-coated hydrogels, 150,000 WI38 cells were seeded in drop on top of hydrogels and the dishes containing hydrogels were filled with medium after overnight incubation. Hydrogels were harvested for RNA extraction or fixed for EdU or SA β gal staining 48 h after plating. Fibronectin-coated hydrogels were produced according to standard procedures as in Dupont *et al*, 2011).

Analysis of published microarray data

YAP/TAZ target genes in MDA-MB-231 cells were defined as in Enzo *et al* (2015), by selecting genes concordantly downregulated upon transfection of two different siRNA mixes targeting YAP and TAZ (at least twofold and with a *q*-value < 0.05 for each siRNA mix), as compared to cells transfected with a non-targeting control siRNA.

Gene list enrichment analysis was performed using ENRICHR (<http://amp.pharm.mssm.edu/Enrichr/>; Kuleshov *et al*, 2016) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) 2016 Database for annotation. KEGG pathways were considered significantly enriched based on adjusted *P*-value < 0.05.

For YAP/TAZ regulated nucleotide metabolism genes in other cell lines, we checked for genes significantly downregulated (in experiments entailing YAP/TAZ knockdown) or upregulated (in experiments entailing YAP overexpression) (*P*-value < 0.05 after Benjamini and Hochberg adjustment) using GEO2R differential expression analysis (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) directly on the GEO database. The following datasets were used, by selecting the appropriate samples: GSE49384 (Mori *et al*, 2014), GSE56157 (Li *et al*, 2014), GSE65665 (Fitamant *et al*, 2015) and GSE55560 (Yimlamai *et al*, 2014).

Drug response data for breast cancer cell lines have been retrieved from the NCI60 DTP portal GI₅₀ Data (Sept 2014). Briefly, GI₅₀ Data file contains a matrix of GI₅₀ values for 50,816 molecules tested on 115 cancer cell lines. For any compound, GI₅₀ values are computed as minus the log₁₀ of IC₅₀, that is, the drug concentration necessary to inhibit 50% growth of treated cells relative to untreated controls. Prior to analysis, for any compound, GI₅₀ been transformed back to IC₅₀ and then the IC₅₀ has been normalized dividing the IC₅₀ of any cell line by the mean IC₅₀ across all 115 cell lines. The normalized IC₅₀ (in log₂ scale) of a compound has been then used to define drug sensitivity of a cell line in term of (i) sensitive if the normalized log₂ IC₅₀ was lower than two standard deviations of the distribution of all log₂ IC₅₀ in the given cell line, and (ii) resistant otherwise (Taccioli *et al*, 2015). Gene expression data of breast cancer cell lines from the NCI-60 panel were downloaded from GSE5720 and GSE32474.

For GSEA of YAP/TAZ target genes, we used signatures previously published in Enzo *et al* (2015); Zanconato *et al* (2015). SASP signatures were derived from Moiseeva *et al* (2013); Rajagopalan and Long (2012); Kansara *et al* (2013).

Gene expression data for OIS were as follows: GSE59522 and GSE60652 for Ras activation in IMR90 primary human fibroblasts (Young *et al*, 2009; Takebayashi *et al*, 2015); GSE2487 for MEK activation in IMR90 primary human fibroblasts (Collado *et al*, 2005);

GSE46801 for BRAF activation in primary human melanocytes (Pawlikowski *et al*, 2013).

Gene expression data for TPA/DMBA-induced mouse papillomas were from GSE63967 (McCreery *et al*, 2015). Mouse skin papillomas were classified as SASP signature “low” if the combined signature score, obtained summarizing the standardized expression levels of the signature genes into a score with zero mean (Adorno *et al*, 2009), was negative and as SASP signature “high” if the combined score was positive. SASP and YAP/TAZ signatures were converted into mouse gene symbols using the Human and Mouse Homology report file of the Mouse Genome Informatics database (http://www.informatics.jax.org/downloads/reports/HMD_HumanPhenotype.rpt).

All microarray data analyses have been performed in R version 3.3.2.

Statistical analyses

Unless otherwise indicated, data are shown as mean plus standard deviations. Analysis was performed by applying unpaired two-tailed Student's *t*-test between the indicated samples (see Figures); if not explicitly indicated, the bars with the * are compared to the respective control bars.

Every experiment was repeated independently; for each experiment, at least two biological replicates were processed.

Data availability

Data obtained during this study are available within the article and from the corresponding author upon reasonable request. All microarray data are available: GSE49384, GSE56157, GSE65665, GSE55560, GSE5720, GSE32474, GSE59522, GSE60652, GSE2487, GSE46801, GSE63967, GSE59232.

Expanded View for this article is available online.

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Author contributions

GS and IB performed and analysed experiments, with help from PR and AP. EF and CR performed dNTP quantifications. SB performed bioinformatics analysis. GS and SD wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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