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Extracellular matrix stiffening promotes a super-competitive behavior in mutant p53 pre-neoplastic cells

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To my parents

Carla and Danilo,

for their continuous support.

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ABSTRACT

Tumors develop through genetic and epigenetic changes that alter cell proliferation, and selection of reprogrammed cells that adapt to the changing micro-environmental conditions, including physical constraints, nutrient fluctuation, hypoxia and oxidative stress. In healthy tissues, normal cells are able to recognize and restrain the expansion of cells bearing cancer-driving mutations, through "cell competition", an evolutionarily conserved process by which fitter cells outcompete less fit cells. Nonetheless, during aging, clones of cells bearing cancer-driving mutations accumulate in many tissues, and some of these clones may evolve into malignancy. Interestingly, certain conditions associated with aging, such as inflammation and stiffening of the extracellular matrix (ECM), have been shown to alter the process of cell competition and thus favor tissue colonization by cells bearing cancer-driving mutations. This suggests that tissue micro-environmental cues could play a key role in the regulation of cell competition and thus in the formation of pre-malignant cell clones. However, the underlying mechanisms are still poorly understood, and their knowledge may provide opportunities to interfere with tumor initiation and evolution.

TP53 is one of the most frequently mutated genes in cancer, with a high prevalence of oncogenic missense mutations (mutp53), and mutp53 pre-malignant cell clones have been shown to accumulate during aging in different tissues, including esophagus, skin and breast. Notably, work in our laboratory showed that, in cancer cells, micro-environmental cues, including mechanical stress caused by ECM stiffening, play a key role in stabilization/activation of mutp53, and activated mutp53 rewires cell transcriptome and proteome, thus fostering cell proliferation, metastasis and chemoresistance.

Based on this evidence, we posit that in mutp53 pre-malignant cells, mechanical stimuli from the ECM could promote mutp53 stabilization/activity and thus a transcriptional program that allows pre-malignant cells to outcompete normal neighbors.

To study the impact of ECM stiffening on the competition between mutp53 pre-malignant and normal cells, we generated ad-hoc cellular models, consisting in human mammary epithelial cell lines expressing either wild-type p53 and H2B-mCherry (wt/Ch) fluorescent protein, or expressing missense mutant HA::p53^{R175H} and GFP (mutp53/GFP), under the control of a doxycycline-inducible promoter. These cell lines were used to establish cell competition assays by co-culturing on substrates in the range of either physiological stiffness typical of mammary tissue (1 kPa), supra-physiological stiffness (4 kPa), pathological stiffness typical of mammary

tumors (8 and 50 kPa), or hyperstiff (plastic). The results of these assays showed that ECM stiffening promoted a competitive growth advantage of mutp53/GFP over wt/Ch cells, and the latter were eliminated by cell death.

We investigated the underlying mechanisms, by isolating the mutp53/GFP and wt/Ch cells from co-culture on stiff substrate, and analyzing genes differentially expressed, by transcriptomics. We found that, in mutp53/GFP cells outcompeting wt/Ch neighboring cells, ECM stiffness led to activation of the YAP transcription co-factor, key regulator of mechano-signaling, and YAP bound mutp53. This resulted in the activation of a mup53/YAP transcriptional program, associated with increased mutp53/GFP cell proliferation. We also investigated pathways specifically upregulated in co-cultured vs mono-cultured wt/Ch cells and found that wt/Ch outcompeted by mutp53/GFP cells displayed activation of wild-type p53 and ferroptotic cell death.

Our results suggest that, upon ECM stiffening, mutp53 cells hijack the process of cell competition, thus becoming “super-competitors” that expand at the expense of surrounding normal cells. We further provide evidence that this process could depend on an ECM stiffness-stimulated mup53/YAP transcriptional gene expression program. Interfering with this program may thus tilt competition in favor of normal cells and suppress tumor initiation.

1.INTRODUCTION

1.1. Role of cell-cell competitive interactions in tumor initiation and evolution.

Cancer is currently the second largest mortality disease in the world, preceded only by cardiovascular diseases, according to estimates from the World Health Organization (WHO) in 2020 (www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death). Tumors develop through genetic and epigenetic changes that modify fundamental cellular programs of proliferation and differentiation, followed by selection of reprogrammed cells that best adapt to microenvironmental conditions (Gerstung et al. 2020). These comprise a variety of suboptimal, unfavorable, or challenging conditions, including hypoxia, oxidative stress, nutrient fluctuation and physical constraints, and even different types of treatments, including chemo and targeted therapies, to which cancers are or become resistant (Grasmann, Mondal, and Leithner 2021) (**Figure 1)**.

Given the remarkable adaptive potential of tumor cells, it is increasingly apparent that evolutionarily informed approaches are necessary to circumvent and forestall disease progression.

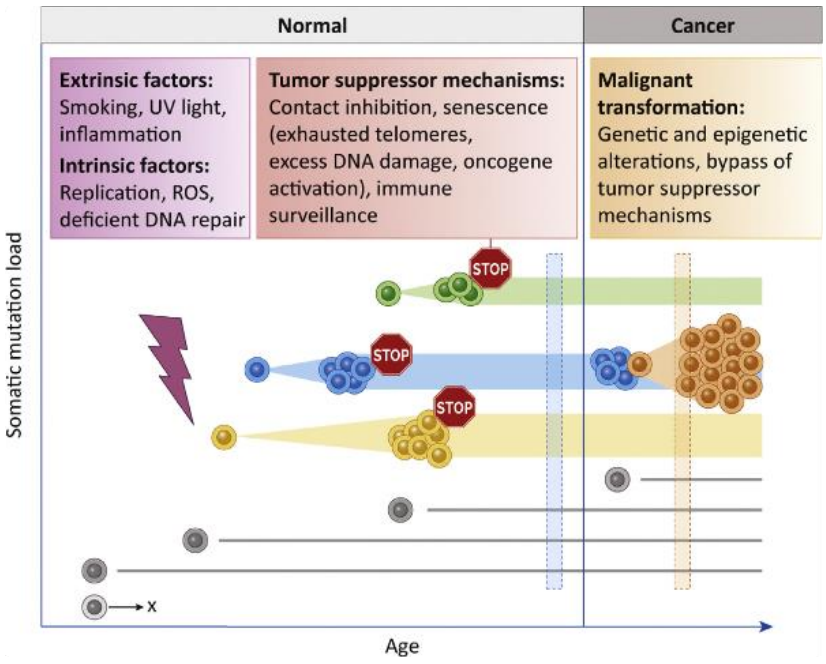

Figure 1. DNA mutations accumulate throughout life due to DNA replication, environmental and lifestyle factors.

Mutations might be neutral (grey lines), deleterious (X), or might confer a proliferative advantage and the cell clonally expands (green, blue, yellow). With age, tumor suppressor mechanisms are bypassed by additional mutations and/or by their progressive decline. Thus, in some clones additional rounds of mutation, selection and clonal expansion enable cells to become malignant. A mid-age biopsy (blue rectangle) might carry cancer-associated mutations from pre-malignant clones. In a cancer biopsy (orange rectangle), malignant clones might be identifiable due larger size compared with the background clone (adapted Kendy, Zhang, and Risques 2019).

1.1.1 Early events in tumorigenesis

Cancer is considered as a disease of aging. The incidence curves for most common cancers are strikingly similar, rising exponentially after the age of 50 years, despite the large variance in the numbers of driver mutations evident in these cancers, and indeed older age is the main risk factor for cancer (Laconi, Marongiu, and DeGregori 2020) (**Figure 2**).

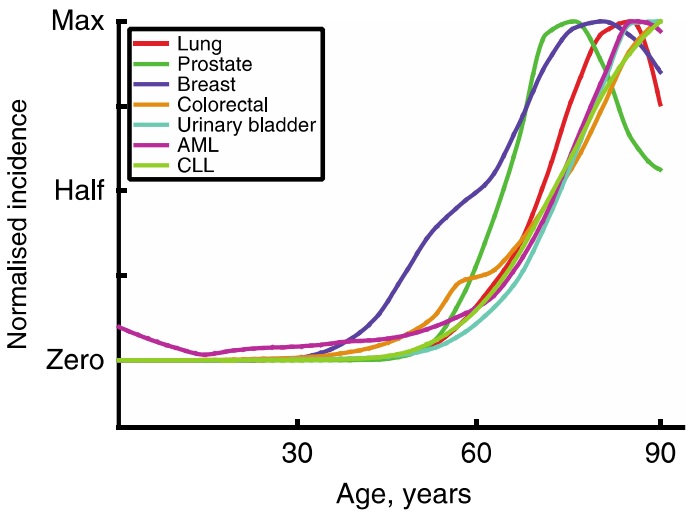

Figure 2. Age-dependent incidence for the most common cancers and leukemias.

The incidence of the five most common cancers (excluding skin cancers) and the two most common leukemia in the United States from 2012 to 2016. Data are from the National Cancer Institute (adapted from Laconi, Marongiu, and DeGregori 2020).

The process of tumorigenesis begins long before the growth of a clinically detectable lesion and even before the rise of any morphological markers of pre-malignancy. Yet, little is known about the early events occurring in the very first phases of cancer evolution, mainly because of the difficulty to detect and characterize an early pre-cancerous stage before the clinical diagnosis is established (Curtius, Wright, and Graham 2018). Outstanding questions including the nature of the cell of origin of cancer, when it emerges, what genetic events and what molecular

mechanisms are involved there, and how those events are influenced by the (micro)environment and extrinsic factors. Answering these questions is instrumental to achieve a better understanding of carcinogenesis, early diagnosis, prediction and even prevention of cancer.

Several hypotheses have attempted to explain the process of carcinogenesis and its link with aging. Although not necessarily mutually exclusive, they can be conceptually included into two broad categories: those focused on age-related alterations occurring in rare cells, putative precursors of pre-malignant, and/or neoplastic cells, and those placing more emphasis on age-dependent changes taking place in the bulk of the tissue, organ, or organismal context in which cancer originates (**Figure 3**).

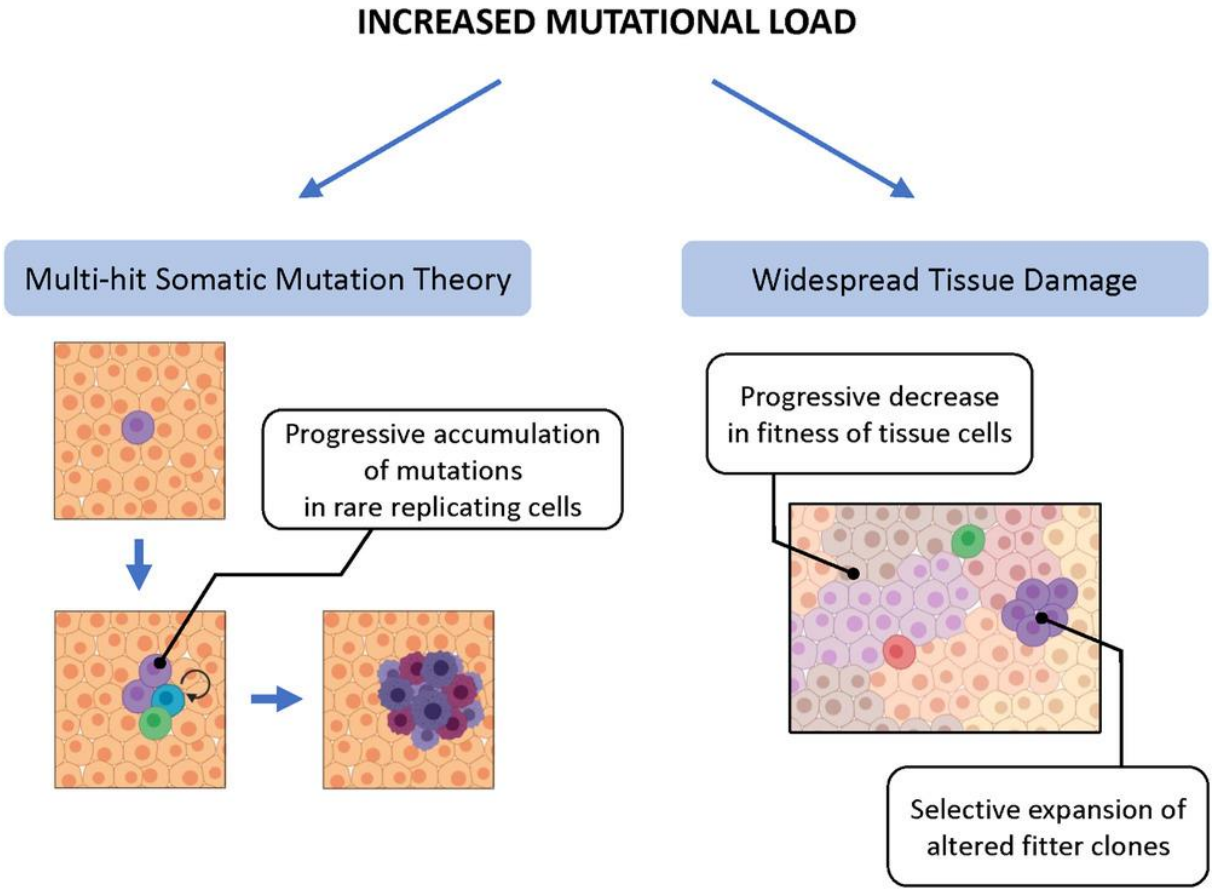

Figure 3. Possible links between aging and cancer.

Left: age-related alterations occurring in rare cells, putative precursors of preneoplastic, and/or neoplastic cells. Right: age-dependent changes taking place in the bulk of the tissue, organ, or organismal context in which cancer originates (adapted from Marongiu and Laconi 2020)**.**

Although there are certainly areas of overlapping between the two analytical approaches, placing emphasis on the role of tissue, organ, or organismal context in the pathogenesis of

neoplastic disease has represented a significant departure from the cell-centric view that has dominated cancer research for several decades.

There is now sufficient evidence to indicate that aging is associated with the emergence of a clonogenic and neoplastic-prone tissue landscape, which fuels early stages of cancer development and helps explaining the rise in cancer incidence and mortality in older individual (**Figure 4**).

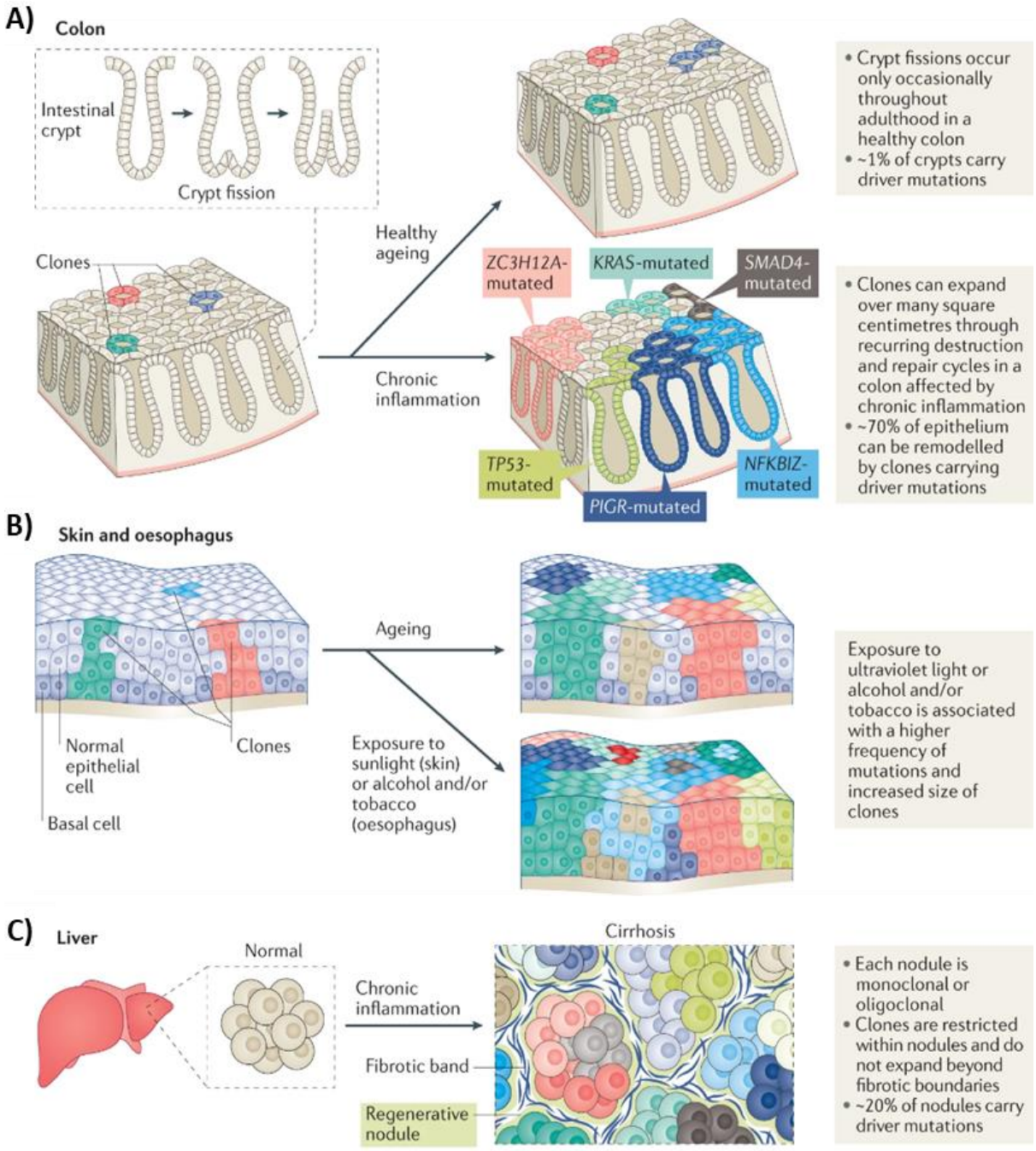

Figure 4. Aging-associated expansion of clones harboring cancer-driving mutations in normal and non-cancer diseased tissues (adapted from Kakiuchi and Ogawa 2021).

The understanding of clonal expansion in normal or non-cancer tissues has substantially advanced by a revolution in sequencing technologies that enabled high-throughput detection of somatic mutations. According to recent studies, every human tissue seems to accumulate somatic mutations throughout life at a rate that depends on the tissue type (estimated 13-44 mutations per genome per year), and the underlying mechanisms have been studied by assessing unique patterns of mutations, called mutational signatures (Alexandrov et al. 2013; 2020). Importantly, in the past decade, several high-throughput sequencing data revealed that, in humans, clones of phenotypically normal cells harboring cancer-associated mutations accumulate with age in healthy tissues, and also in tissues affected by non-cancer pathological conditions (**Table 1**). The best studied and characterized example is probably the age-dependent expansion of clones in hematopoiesis, which in most cases involves cells harboring somatic mutations in genes *known to be implicated in hematologic cancers,* such as *DNMT3A*, *ASXL1*, *TET2* and *TP53* (Genovese et al. 2014; Jaiswal et al. 2014). Age-dependent accumulation of clones of phenotypically normal cells carrying cancer-associated mutations has been recently found to be quite common also in many other healthy tissues, including esophagus, skin, lung, bladder, colon, breast and endometrium (Fowler et al. 2021; Cereser et al. 2023; R. Li et al. 2020; Martincorena and Campbell 2015; Martincorena et al. 2018; Nicholson et al. 2018; Yokoyama et al. 2019; Yoshida et al. 2020), and also in non-cancerous diseased tissues, such as chronically inflamed gastroenteric system (Kakiuchi and Ogawa 2021; Nanki et al. 2020; Stachler et al. 2018) and cirrhotic liver (Zhu et al. 2019). Even in these cases, the expanded clones were found to harbor somatic mutations in genes *implicated in the cancers occurring in the same tissues, such as TP53* (esophagus, skin, lung, bladder, colon, liver, breast), *FAT1* (esophagus, skin, lung), *NOTCH* (skin, esophagus, lung), *RAS* (skin, colon, endometrium) and *PI3KCA* (esophagus endometrium) (Fowler et al. 2021; Cereser et al. 2023; R. Li et al. 2020; Martincorena and Campbell 2015; Martincorena et al. 2018; Nicholson et al. 2018; Yokoyama et al. 2019; Yoshida et al. 2020).

Of note, the rate of appearance and growth of these clones has been found to be accelerated by exposure to environmental factors known to have carcinogenic potential, such as exposure of lung and esophageal epithelium to tobacco smoke, of skin to ultraviolet light, and of esophagus o alcohol consumption (Brunner et al. 2019; Yizhak et al. 2019; Yoshida et al. 2020; Lawson et al. 2020). Such clones play a role in the formation of a so-called cancerized field, defined as a large tissue area in which a normal cell population is replaced by a cancer-primed cell population that may or may not exhibit morphological change (for example, cancerized cells may look normal or could exhibit hyperplasia, metaplasia, or dysplasia). Over time, these fields

can develop into malignant tumors assuming additional genetic mutations, and indeed field cancerization (also known as field effect or field change) is now recognized to underlie the development of several types of cancer, including lung, colon, skin, prostate and bladder carcinoma (Curtius, Wright, and Graham 2018) .

Organ/disease	Sequencing methods	Driver genes[a]	Comments
Physiological state			
Blood (clonal haematopoiesis)	WES, targeted sequencing	*DNMT3A* > *TET2* > *ASXL1*, *JAK2*, *GNAS*, *GNB1*, *CBL*, *TP53*, *PPM1D*, *SF3B1*, *SRSF2*	Accelerating factors include ageing, smoking and genetic predisposition (non-Hispanics, SNPs), and microbiome *DNMT3A* and *PPM1D* mutations are more frequent in clonal haematopoiesis than myeloid malignancies
Skin	Targeted sequencing	*NOTCH1* > *NOTCH2* > *FAT1* > *TP53*, *NOTCH3*, *RBM10*	Ultraviolet is an accelerating factor *TP53* is an SCC-prone gene *NOTCH1* is a normal skin-prone gene
Oesophagus	WES, targeted sequencing	*NOTCH1* > *TP53* > *FAT1*, *NOTCH2*, *NOTCH3*, *KMT2D*, *ZFP36L2*, *PPM1D*, *PIK3CA*, *CHEK2*, *PAX9*, *ARID1A*, *CUL3*, *AJUBA*, *ARID2*, *TP63*, *NFE2L2*, *CCND1*	Accelerating factors include ageing, alcohol drinking and tobacco *TP53* is an ESCC-prone gene *NOTCH1*, *FAT1*, *NOTCH3*, *NOTCH2*, *ZFP36L2*, *CHEK2*, *PPM1D* and *PAX9* are normal oesophagus-prone genes
Nevus; skin melanocyte	Targeted sequencing, WES	*BRAF*, *NF1*, *RASA2*, *CBL*, *MAP2K1*, *NRAS*, *ARID2*, *CDKN2A*, *PTEN*, *PPP6C*, *DDX3X*	Almost all nevi carry $BRAF^{V600E}$ mutations Skin melanocytes frequently carry mutations in the MAPK pathway genes except for $BRAF^{V600E}$ *NRAS*, *TERT*, *CDKN2A*, *TP53* and *PTEN* are melanoma-prone genes
Bronchus	WGS	*NOTCH1* > *FAT1* > *TP53* > *ARID1A*, *ARID2*, *FAT1*, *CHEK2*, *PTEN*, *CHEK2*	Tobacco is an accelerating factor
Colon	WES, WGS	*AXIN2*, *STAG2*	Only ~1% of crypts acquire driver mutations 0.4% of colon crypts are replaced annually and clonal expansion is rarely observed
Endometrium	WGS, targeted sequencing	*PIK3CA* > *ARHGAP35* > *PIK3R1* > *FBXW7*, *ZFHX3*, *FOXA2*, *ERBB2*, *CHD4*, *KRAS*, *SPOP*, *PPP2R1A*, *ERBB3*	Almost all glands in older people acquire driver mutations Parity has a negative association with driver mutations *PTEN* is an endometrioid cancer-prone gene *TP53* is a serous cancer-prone gene
Urothelium	WGS, WES, targeted sequencing	*KMT2D* > *KDM6A* > *ARID1A* > *RBM10* > *EP300* > *STAG2*, *NOTCH2*, *CDKN2A*, *CREBBP*, *FOXQ1*, *RHOA*, *ERCC2*, *KLF5*, *ZFP36L1*, *ELF3*, *GNA13*, *PTEN*, *TP53*	The mutational burden and size of clones in subjects with bladder cancer are modestly increased compared with those without cancer Exposure to aristolochic acid accelerates clonal expansion *TERTp*, *TP53*, *FGFR3* and *PIK3CA* are bladder cancer-prone genes
Disease			
Ulcerative colitis	WES, targeted sequencing	*NFKBIZ* > *ARID1A* > *PIGR* > *ZC3H12A*, *KRAS* > *FBXW7*, *TRAF3IP2*, *HNRNPF*, *ARID1B*, *BCOR*, *BCORL1*, *ETV6*, *RNF43*, *TP53*	*TP53*, *KRAS*, *APC*, *RNF43*, *SMAD4* and *FBXW7* are colitis-associated cancer-prone genes *NFKBIZ* and *ZC3H12A* are non-cancer epithelium-prone genes
Liver cirrhosis	WGS, WES, targeted sequencing	*ALB*, *ACVR2A*, *ARID1A*, *ARID2*, *NCOR1*, *TP53*, *PKD1*, *KMT2D*	Only ~5% of expanded clones have driver mutations and the majority of expanded clones do not have driver mutations *TERTp* is a hepatocellular carcinoma-prone gene
Aplastic anaemia	WES	*PIGA*, *BCOR*, *BCORL1*, *DNMT3A*, *ASXL1*	*PIGA*, *BCOR* and *BCORL1* are genes associated with favourable outcome *DNMT3A* and *ASXL1* are genes associated with unfavourable outcome
Barrett's oesophagus	WGS, WES, targeted sequencing	*CDKN2A* > *ARID1A* > *TP53* > *ARID1B*, *APC*, *SMAD4*, *ERBB2*, *KMT2D*	*TP53* is an oesophageal adenocarcinoma-prone gene *CDKN2A* and *ARID1A* are Barrett's epithelia-prone genes
Stomach intestinal metaplasia	Targeted sequencing	*FBXW7*	*TP53*, *ARID1A* and *FBXW7* are gastric cancer-prone genes
Kidney	WGS	*H19* locus methylation	*H19* methylated clone expands during fetal nephrogenesis

ESCC, oesophageal squamous cell carcinoma; SCC, squamous cell carcinoma; WES, whole-exome sequencing; WGS, whole-genome sequencing. [a]We pr
rough estimation of relative frequencies of different mutations, instead of providing absolute frequencies, which are influenced by several factors such a
sensitivity to detect mutations.

Table 1. Clonal expansion in apparently normal tissues (adapted from Kakiuchi and Ogawa 2021)**.**

The processes that, in healthy tissues, underpin age-dependent selection and accumulation of phenotypically normal cell clones harboring cancer-associated mutations remain poorly understood. Possibilities include cell autonomous effects, such as increased cell division or decreased differentiation rates, and extrinsic effects due to competition between mutant and neighboring wild-type cells.

Recent studies in mouse models have shown that the patchwork of mutant clones in aged human esophageal epithelium, including the *TP53* and *NOTCH* genes, could be recapitulated by treatment of mice with mutagens found in tobacco smoke, and that, in these conditions, clone dynamics depend on competition between mutant and neighboring wild-type cells (Colom et al. 2020). This suggests that competitive interactions of (pre)-neoplastic cells with microenvironment are crucial to determine their growth.

1.1.2. Cell competition and its role in tumor suppression

Increasing evidence suggests that, in multicellular organisms, "cell competition" plays key role in the regulation of tissue development, homeostasis and disease, including initiation and evolution of tumors, and even neurodegeneration (Costa-Rodrigues, Couceiro, and Moreno 2021; Pelham, Nagane, and Madan 2020; Marongiu, Cheri, and Laconi 2021).

Cell competition is an adaptive process evolutionarily conserved from *Drosophila* to mammals, consisting in the sensing, active elimination, and replacement of less fit cells (also dubbed "losers") by neighboring fitter cells (also dubbed "winners"). This involves process short-range cell interactions that enable cells to compete for space and survival (Vishwakarma and Piddini 2020).

Fitness is not an absolute universal quality, instead it is heavily context dependent, and strongly influenced by different factors, including metabolic activity, mechanical and structural properties, aneuploidy, and cellular damage, but not others such as cell size and growth rate (Baker 2020; W. Kim and Jain 2020).

A key defining feature of cell competition is that elimination of loser cells is also context dependent, as they are viable on their own. This elimination can be executed in various ways, including apoptosis, senescence, engulfment, extrusion, autophagy, induction of differentiation, displacement of stem cell by oriented cell divisions (Baker 2020), necroptosis (Watanabe et al. 2018) and entosis (Sun et al. 2014), and can be compensated by proliferation (Costa-Rodrigues, Couceiro, and Moreno 2021) or even hypertrophy (Tamori and Deng 2013) of winner cells.

The process of cell competition was first described in the 1970s in *Drosophila melanogaster*, in a study focused on the function of genes later shown to encode ribosomal proteins (Morata

and Ripoll 1975). Homozygous loss-of-function mutations in these proteins lead to early organism lethality, while heterozygous individuals reach the adult stage and are fertile, though compared to wild-type individuals they grow more slowly and display shorter bristles, a phenotype from which these mutations were dubbed *Minute*. In contrast, in mosaic larval epithelia, heterozygous *Minute* cells surrounded by wild-type cells are progressively eliminated, through induction of apoptosis, and replaced by the growing wild-type neighbors.

After those pioneer observations, evidence accumulating in the last decade have shown that, during both development and ageing, cell competition acts physiologically as a surveillance mechanism that removes incipient suboptimal, damaged and potentially dangerous cells at the interface with fitter cells, thus maintaining the homeostasis and overall health of tissues and of the entire organism (Costa-Rodrigues, Couceiro, and Moreno 2021).

Evidence from mouse have shown that, during early mammal development, cell competition removes mis-specifying and tetraploid cells from the epiblast, thus maintaining the pluripotent stem/progenitor cell pool, before gastrulation and specification of the germline. This process has been shown to be driven by higher levels, in winners vs losers, of the transcription regulators YAP/TEAD and c-MYC, of p53-dependent mTOR signaling, or of ERK signaling (Bowling et al. 2018; Díaz-Díaz et al. 2017; Hashimoto and Sasaki 2019; Sancho et al. 2013; Clavería et al. 2013). Recently, it has been also shown that, during zebrafish embryogenesis, cell competition corrects noisy Wnt morphogen gradients, suggesting that it could act as a mechanism to achieve robust patterning (Akieda et al. 2019).

Furthermore, multiple experimental evidence obtained from *Drosophila* and mouse *in vivo* models, and from *ex-vivo* cells culture systems, have shown that, within healthy tissues, normal cells are able to eliminate incipient pre-malignant cells bearing tumor suppressor/oncogene mutations, indicating a tumor suppressive role of cell competition (**Figure 5**). In this regard, the initial discovery made in *Drosophila* showed that cells with a homozygous loss-of-function mutation in tumor suppressor genes that regulate cell polarity, such as *scribble*, overgrow in a homotypic environment, but are eliminated when surrounded by wild-type cells (Reviewed in Morata 2021). Later, in both *Drosophila* and mammalian models, it was shown that wild-type cells are able to recognize and outcompete also neighboring cells harboring oncogenic mutations, for example in p53, Ras, Src, and YAP (Chiba et al. 2016; Morata and Ballesteros-Arias 2015; Watanabe et al. 2018; Parker et al. 2021).

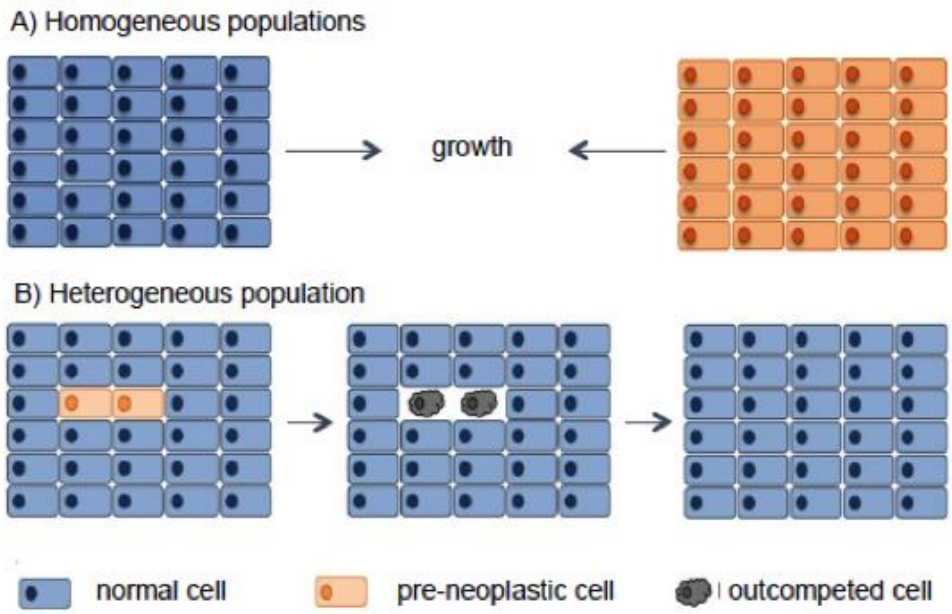

Figure 5. Tumor suppressive role of cell competition.

In healthy tissues, normal cells are able to outcompete and eliminate incipient pre-malignant cells, which would be otherwise able to grow as homogenous populations (adapted from Bowling, Lawlor, and Rodríguez 2019).

1.1.3. Super-competition: hijacking of cell competition in tumorigenesis

The documented colonization of different tissues by cells harboring cancer-associated driver mutations, during aging, upon exposure to environmental stressors, and in non-cancer disease conditions such as chronic inflammation, implies that in these contexts pre-malignant cells evade tumor-suppressive cell competition, and become themselves able to outcompete neighboring cells (Vishwakarma and Piddini 2020). Indeed, this is supported by accumulating experimental evidence suggesting that, in certain conditions, pre-malignant cells can hijack cell competition surveillance mechanisms, a process that has been dubbed "super-competition" (Abrams 2002; Moreno and Basler 2004) (**Figure 6**).

Pioneering evidence of this process showed that, in *Drosophila* growing epithelia, large clones of cells overexpressing Myc outcompeted wild-type neighbors (Moreno and Basler 2004). After these initial observations, further studies in *Drosophila* epithelia have suggested that pre-malignant (e.g. *RasV12* mutant) cells are able to outcompete wild-type neighbors upon cell crowding/compaction (Morata and Calleja 2020; Levayer, Dupont, and Moreno 2016; Moreno et al. 2019).

Moreover, pre-malignant cells harboring cancer-associated p53 missense mutations, have been shown to become able to outcompete wild-type neighboring cells upon chronic inflammation,

in the mouse intestinal epithelium (Vermeulen et al. 2013), and upon irradiation, in mouse esophagus (Fernandez-Antoran et al. 2019) and hematopoietic stem and progenitor cell (HSPC) compartment (Bondar and Medzhitov 2010). Chronic inflammation has been also shown to inhibit the elimination, and promote super-competition, of RasV12 expressing cells in mouse intestinal epithelium and pancreas (Sasaki et al. 2018).

Very recently, super-competition has been proposed to contribute to the expansion also of *APC*$^{-/-}$ cells in the intestinal epithelium, via multiple mechanisms, including downregulation of Hippo signaling in winners vs losers in *Drosophila* (Suijkerbuijk et al. 2016), secretion of the WNT antagonist NOTUM by winner cells, inducing differentiation of neighboring normal stem cells in mice (Flanagan et al. 2021).

In addition to its involvement in the expansion of pre-malignant cells in the early stages of tumorigenesis, super-competition has been recently shown to play a role in tumor invasion in *Drosophila* models (Eichenlaub, Cohen, and Herranz 2016) and in breast and colon tumor progression and metastasis in mouse models and human cancer (Madan et al. 2019).

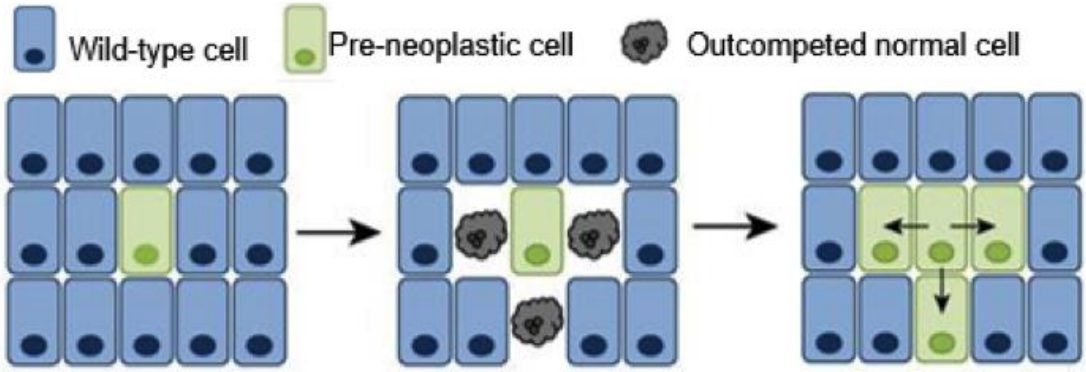

Figure 6. Hijacking of cell competition by (pre)-neoplastic cells.

In aged or non-cancer diseased tissues, pre-malignant cells evade tumor suppressive outcompetition by normal neighbors, survive and become super-competitors, which expand at the expense of surrounding cells (adapted from Bowling, Lawlor, and Rodríguez 2019).

2. Mechanisms of cell competition

Three main mechanisms through which differences between cells could lead to cell competition have been identified (**Figure 7**): i) specific receptor-ligand recognition of differences through cell-cell contact; ii) mechanical stresses generated by the differential growth of cell populations, affecting cell survival, proliferation and mobility; iii) unbalance of cell death, survival and differentiation signals, leading to a locally inhospitable environment for one cell population.

The first mechanism of cell competition was identified in 2010 in *Drosophila* by the group of Eduardo Moreno and colleagues (Rhiner et al. 2010). These authors generated, in fly epithelia, clones of cells overexpressing the Myc oncogene, which behaved as super-competitor, and analyzed the transcriptome of these mosaic tissues, identifying a group of genes selectively upregulated early in loser cells. Among these, they found that specific isoforms of the transmembrane protein Flower were expressed by loser cells and interacted with the Flower ubiquitous isoform expressed by winners. This cell contact-dependent molecular recognition mechanism has been recently found conserved from flies to humans, being involved in tumor progression and metastasis promoted by super-competition in mouse models and human cancer cells (Madan et al. 2019; Petrova et al. 2012). Another evolutionarily conserved receptor-ligand mechanism of cell competition has been recently described in cell culture and *Drosophila* models and suggested to be important for tumorigenesis in mammals. This mechanism involves Eph-ephrin signaling, which regulates cell repulsion and cell segregation, processes that prevent cell mixing and drive boundary formation during tissue development and maintenance. In the mouse pancreas, the binding of Ephrin proteins, exposed on the surface of normal epithelial cells, with the tyrosine kinase receptor EphA2, expressed in RASV12 mutant pre-malignant cells, promotes tumor suppressive cell competition, through apical extrusion of the pre-malignant cells from the epithelium (Porazinski et al. 2016; Hill et al. 2021). However, RASV12 mutant cells that are not in contact with ligand-expressing neighbors can escape cell competition, and eventually lead to tumor progression and metastasis (Hill et al. 2021). Of note, in the mouse lung epithelium, apical extrusion of RASV12 mutant cells has been also found to involve the interaction of the major histocompatibility complex class I (MHC-I) with the leukocyte immunoglobulin-like receptor B3 (LILRB3) expressed in normal neighbors (Ayukawa et al. 2021).

Mechanical regulation of cell competition has been observed in a range of biological contexts. For instance, in both cellular and in vivo mammalian models, acto-myosin contraction has been found to promote the extrusion of cells expressing the RASV12 or Src oncogenes, during tumor suppressive cell competition (Anton et al. 2018; Kajita et al. 2010; Porazinski et al. 2016; Kon et al. 2017; Ohoka et al. 2015). This process, also dubbed epithelial defense against cancer (EDAC), is regulated by Filamin (FLN), an Actin filament cross-linking protein (Kajita et al. 2014). In a manner dependent on Rho GTPase and Rho-associated kinase (ROCK), FLN accumulates in wild-type cells at the interface with oncogene-expressing cells, which are then extruded from the epithelium. ROCK has been also involved in mechanical competition driven by cell compaction in mammalian cell cultures. It has been shown that *scribble* defective

epithelial cells are hypersensitive to compaction, and transcriptomic analysis of these cells co-cultured with wild-type neighbors has revealed that this phenotype is due to increased levels and activity of the tumor suppressor p53 (Wagstaff et al. 2016). In these cells, compaction caused by cell crowding with co-cultured wild-type neighbors leads, via ROCK activation, to further p53 elevation, causing their apical extrusion and apoptosis. A similar mechanism has been recently described also in cells located at wound edges, which guide collective migration and healing, upon compaction caused by wound closure (Kozyrska et al. 2022). Compaction has been further found to regulate cell competition via the key mechano-transducer YAP (Price et al. 2021). In populations of human pluripotent stem cells (hPSCs) with different growth rates, in slower growing cells under compaction YAP is inhibited, and they are outcompeted by faster growing neighbors with high YAP activity, Of note, recent evidence suggests that the mechanical properties of the extracellular matrix (ECM), which are critical in tumor progression and in some conditions that favor development of pre-malignant lesions (e.g. fibrosis, Piersma, Hayward, and Weaver 2020), could impact on cell competition. In particular, during EDAC, ECM stiffening has been found to reduce Cdc42 RhoGTPase activity in wild-type cells, and thus FLN accumulation at the interface with RASV12 mutant cells, which are then retained in the epithelium (Pothapragada et al. 2022). This suggests that ECM stiffening could inhibit the tumor suppressive function of cell competition.

Regarding the role of cell death, survival and differentiation signals in the regulation of cell competition, multiple mechanisms based on secreted factors have been implicated, including TNFα (Yamamoto et al. 2017), Dpp/BMP (Moreno, Basler, and Morata 2002) and IL6-like cytokines (Kucinski et al. 2017; Kolahgar et al. 2015; Rodrigues et al. 2012). Moreover, secreted BMP ligands and WNT antagonists have been also recently found to play key roles in the regulation of cell competition during tumor initiation. These factors were identified by directly analyzing the transcriptomes of stem cells harboring tumor suppressor/oncogenic mutations and wild-type neighbors, competing in the mouse intestinal epithelium (Yum et al. 2021; Flanagan et al. 2021; van Neerven et al. 2021).

Evidence of crosstalk among the different types of cell competition mechanisms are emerging (Nagata et al. 2019; Parker et al. 2021; Porazinski et al. 2016; Ayukawa et al. 2021). For example, in wild-type cells involved in the EDAC process, ROCK activation has been found to be promoted through interaction of the LILRB3 receptor with MHC-I expressed in RASV12 mutant cells (Ayukawa et al. 2021). However, whether the different types of cell competition mechanisms might be reconducted to a unifying pathway is an open question.

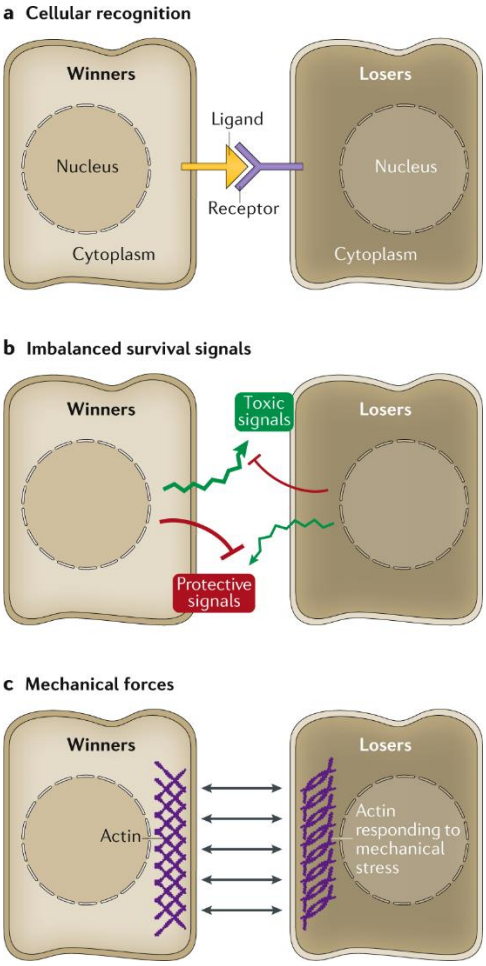

Figure 7. Interactions between cell populations leading to cell competition.

Cell competition could be mediated by several general mechanisms of cell interaction. **a**) Molecular recognition of distinct surface properties might occur at the interface between cell populations, triggering a response to eliminate one class of cell. A receptor is shown on the competed cells, recognizing the presence of cells that will eliminate them, but the reverse is also plausible. **b**) Non-autonomous toxic and protective signals that are balanced within homogeneous populations may become unbalanced at the boundary between non-homogeneous populations, leading to a local toxic signal. **c**) Where winners and losers grow at different rates, mechanical stress may result and have different consequences for each cell population (adapted from Baker 2020).

3. Role of mechanical cues in tissue homeostasis and cancer

Animal organs consist of various types of cells, including parenchymal and stromal cells such as fibroblasts, endothelial cells, and immune cells. These cells secrete soluble factors (e.g., growth factors and cytokines) and macromolecules that form the extracellular matrix (ECM), such as collagen and fibronectin. The dynamic and spatiotemporal interplay among all these components governs the physiological and pathological processes of tissues, including growth, homeostasis, and regeneration (Humphrey, Dufresne, and Schwartz 2014) .

All the cells of an organism are subjected to forces, including compressive, tensile, fluid shear

stress and hydrostatic pressure, which play key roles in tissue physiological and pathological processes (DuFort, Paszek, and Weaver 2011). Mechanical stimuli from the ECM activate integrins in cell-ECM adhesions, while forces transmitted through cell-cell adhesions are sensed by cadherins. These adhesive structures are linked with the cytoskeleton, which in turn interacts with enzymes, organelles and, through the LINC protein complex, with the nucleus, thus modulating their organization and function (Phuyal et al. 2023). Intra/extra-cellular forces also impact membranes tension, activating mechanosensitive ion channels (e.g., PIEZO1) (Nava et al. 2020). Ultimately, mechanical inputs trigger gene programs controlled by cytoplasmic/nuclear localization of mechanosensitive transcription (co)factors. These include MRTF and YAP/TAZ, key regulators of tissue growth/regeneration, and a growing list of oncogenes (e.g., missense mutant p53) and metabolic sensors (e.g., HIF1a, SREBPs, NRF2) (Saraswathibhatla, Indana, and Chaudhuri 2023; Bertolio et al. 2019) .

In the tumor microenvironment, cancer cells adaptively respond to a variety of stimuli, generated at the intracellular level and by changes in the extracellular compartment, including fluctuations of metabolites levels and modifications of physical traits. These comprise increased forces exerted by cells, ECM components (solid stress) and interstitial fluid, increased stiffness (up to tenfold that of normal tissues), and tissue disorganization (e.g., cell crowding/ misplacement, altered porosity) (Zahir et al. 2020; Nia, Munn, and Jain 2020; Mohammadi and Sahai 2018). Furthermore, cancer cells contribute to TME physical changes by overgrowing, invading surrounding tissues, and modifying ECM composition, organization, and stiffness. These modifications are induced by cancer cells both directly (e.g., secretion of ECM components and collagen crosslink) and indirectly by regulating the activity of stromal cells, such as cancer associated fibro- blasts (CAFs) and macrophages (Hayward, Muncie, and Weaver 2021).

4. The tumor suppressor p53 and its role in cell competition

Recent comprehensive analyses of multiple human samples have highlighted that, across normal tissues, somatic clonal expansion of cells harboring mutations in tumor suppressors/oncogenes is most frequently associated with mutations in the *TP53* gene (Yizhak et al. 2019), which encodes the tumor suppressor p53, and multiple experimental evidence are emerging in support of an important role of p53 in cell competition (Bondar and Medzhitov 2010; Kon et al. 2017; Fernandez-Antoran et al. 2019; Vermeulen et al. 2013; Murai et al. 2022).

4.1. The p53 pathway and its role in tumor suppression

p53 is a transcription factor that controls several gene expression programs and it is a central regulator of cellular stress responses, integrating signals from different sources, such as genotoxic, oxidative, metabolic and oncogenic stress, to determine cell fate according to the type, severity, and duration of the stress and to the cellular context (Lane and Levine 2010; Bieging, Mello, and Attardi 2014). The induction of a p53 response can help cells by supporting adaptation and survival or induce a permanent withdrawal of proliferative capacity through the induction of differentiation, senescence, or cell death (**Figure 8**). Through these functions, in multicellular organisms, p53 plays a key role in the suppression of neoplastic transformation. Indeed, in humans, *TP53* is the most commonly mutated gene in cancer, and *TP53* mutations are associated with adverse prognosis in many sporadic cancers (Kandoth et al. 2013). Furthermore, germline *TP53* missense mutations cause the Li-Fraumeni Syndrome (LFS), a rare familial cancer predisposition (Malkin et al. 1990). Moreover, the tumor suppressive role of p53 has been experimentally demonstrated in animal models (Boutelle and Attardi 2021).

The ability of p53 to integrate signals implies the existence of multiple levels of regulation of p53 functions, which include transcriptional, post-transcriptional and post-translational regulatory mechanisms. This diversity is echoed in the complexity of p53 gene and protein structure. In vertebrates, the p53 protein belongs to a family comprising two other members, p63, and p73, with overlapping and distinct functions. Most importantly, studies in knockout mice showed that all the three p53 family proteins play a role in the surveillance of genome fidelity in the germline, while, differently from p53, p63 and p73 function in epithelial differentiation and in neuronal development, respectively. p53 family proteins are encoded by three paralogue genes (in humans *TP53*, *TP63* and *TP73*) and function as homotetramers (though p63 and p73 can form also heterotetramers).

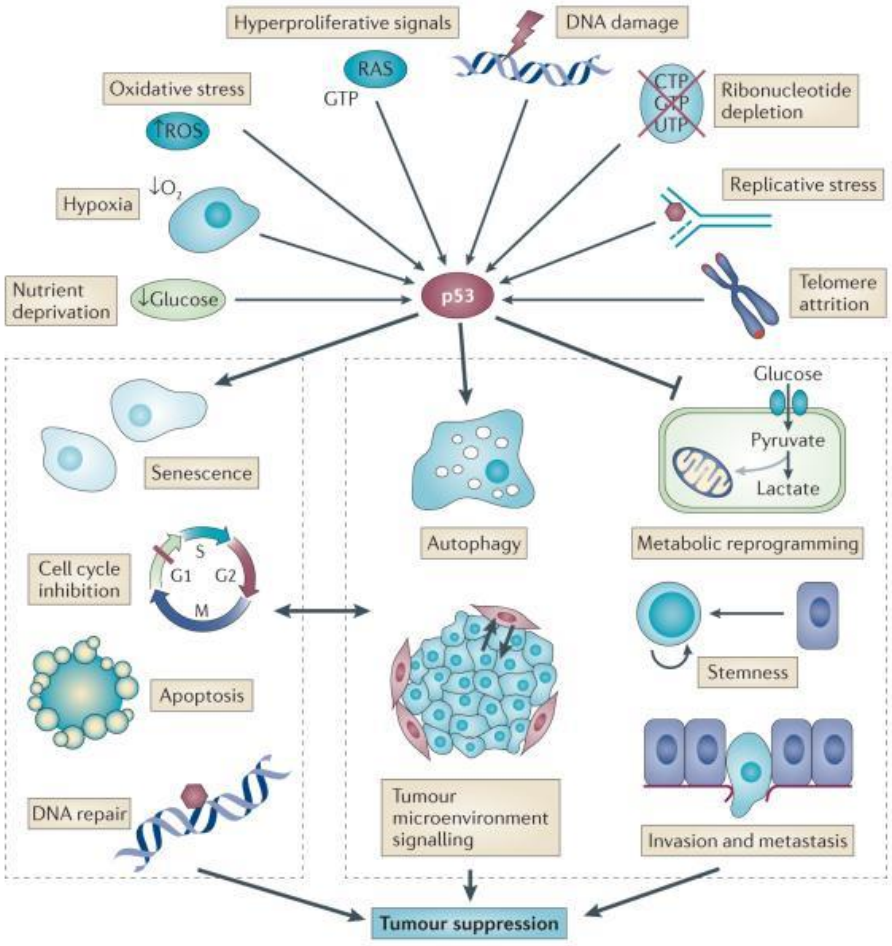

Figure 8. p53 acts as a node in the sensing of multiple signals activating a complex network of cellular responses (adapted from Bieging, Mello, and Attardi 2014)**.**

Their structure includes N-terminal transactivation domains (TAD), highly similar DNA binding domains (DBD), followed by oligomerization domains (OD) that regulate protein interactions, DNA binding and transcription (Belyi and Levine 2009). At the C-terminus, p63 and p73 have and additional domain (SAM, sterile alpha motif) that confers protein stability. p53 family genes have been identified in metazoans, and a recent bioinformatics study suggested their presence also in unicellular holozoans (Bartas et al. 2021). It is thought that these genes arose from a p63-like ancestor, and their functions diversified after duplications and rearrangements (Belyi and Levine 2009). Of note, several p53/p63/p73 protein isoforms, produced through alternative promoters, splicing sites and/or translation initiation site, have been identified, and this provides an additional layer of regulatory mechanism (Marcel et al. 2011).

Regulation of p53 function at the post-translational level has been shown to involve different types of modifications, including phosphorylation, ubiquitination, acetylation, sumoylation and

glycosylation, which can regulate p53 protein stability, subcellular localization and transcriptional activity (Dai and Gu 2010). In unstressed conditions, p53 is maintained at very low levels through proteasomal degradation mediated by the E3-ubiquitin ligase MDM2 and its cofactor MDMX. When cells are exposed to stresses, MDM2 dissociates from p53, which consequently increases its protein levels. Of note, p53 transcriptionally activates MDM2 itself, thus ensuring a negative feedback mechanism to control p53 response (Bieging, Mello, and Attardi 2014). Induction of p53 transcriptional activity leads to a transient or even permanent cell cycle arrest. Indeed, p53 controls the G1/S and G2/M cell cycle checkpoints through transcriptional activation of the cyclin-dependent kinase inhibitor CDKN1A/p21 and of growth arrest and DNA Damage inducible alpha (GADD45) and Stratifin1 (SFN), respectively (Mercer et al. 1994; Hermeking et al. 1997). This allows, for instance in case of DNA injuries, to avoid DNA damage propagation while triggering the activity of the DNA repair machineries, inducing genes involved in nucleotide and base excision repair, mismatch repair and recombination. However, when the damage is too severe and cannot be repaired, the prolonged increase of p21 upregulates the CDK inhibitor p16 and consequently activates RB transcriptional program, thus leading to senescence (Qian and Chen 2013). Moreover, p53 can activate the cell death program by transcriptionally inducing the expression of several pro-apoptotic proteins that belong to the BCL-2 family such as BAX, NOXA and PUMA, while repressing anti-apoptotic ones (Bieging, Mello, and Attardi 2014). In addition, p53 can promote apoptosis in a transcription-independent manner by inducing mitochondrial membrane permeabilization via interaction with BCL-XL and BCL-2 proteins (Giorgi et al. 2015; Mihara et al. 2003).

p53 also exerts its oncosuppressive functions by promoting autophagy, for example by modulating the expression of autophagy inducers (e.g. DRAM) or even inhibiting mTOR and the PI3K-AKT axis (Kenzelmann Broz and Attardi 2013). However, p53 can also limit or inhibit autophagy in a context-dependent manner (Shi, Norberg, and Vakifahmetoglu-Norberg 2021).

Regarding cellular response to oxidative stress, p53 maintains redox homeostasis by inducing the expression of antioxidant genes, however in case of severe stress p53 can promote pro-oxidant genes, thus exacerbating redox imbalance and promoting apoptosis (Liu, Chen, and St. Clair 2008).

4.2. Role of *TP53* mutations in cancer

Loss of tumor suppressive functions of wild-type p53 (wtp53) constitutes a fundamental prerequisite for cell transformation and tumor progression. Indeed, the primary outcome of *TP53* mutations, which occur in almost 50% of all cancers, is the loss of wtp53 functions, and in a large proportion of cancers in which *TP53* is not mutated, p53 is downregulated or inactivated through different mechanisms (Bouaoun et al. 2016).

Mutations in p53 have been found to occur at early and late stages of tumor progression in multiple cancers, including those of breast, prostate, head and neck, pancreas, brain, lung, ovary, colorectum, esophagus (Rivlin et al. 2011).

However, at odds with most tumor suppressors, most (about 75%) of *TP53* mutations are missense mutations that produce single amino acidic substitutions, mainly in the DBD (**Figure 9**). In particular, six residues in this domain are more frequently affected by substitution and for this reason they are defined "hotspots" (R175H, R273H, G245S, R248Q/W, R249S and R282W) (Freed-Pastor et al. 2012). Depending on whether the substitution impairs the function of a DNA binding residue (e.g. R248Q/W, R273H) or disrupts the local/global protein structure (e.g. R175H, R249S, G245S), p53 mutations have been categorized as "contact" or "structural/conformational" mutants, respectively (Alvarado-Ortiz et al. 2021).

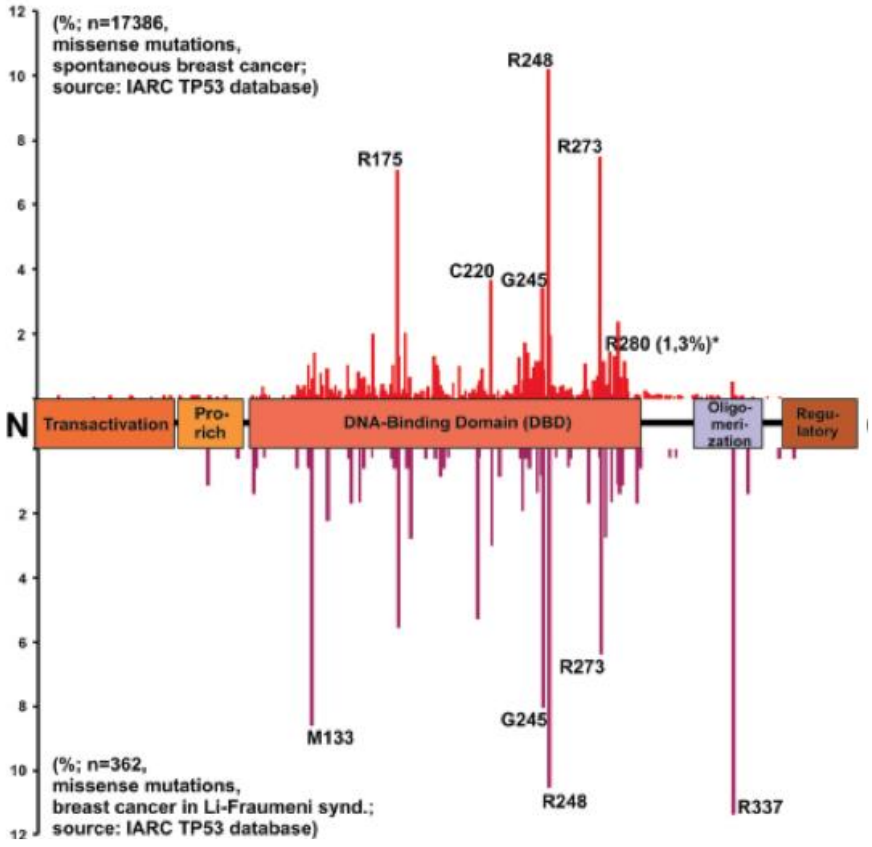

Figure 9. Frequency and structure of p53 missense alterations in breast cancer.

Human p53 domain structure with indicated frequency (percent bars) of missense changes in TP53 found in spontaneous (above) or Li–Fraumeni-associated (below) breast cancer. The five most frequently changed codons are indicated by numbers and residue names. The codon 280 marked with (*) is included due to the widespread use of the MDA-MB-231 cell line, bearing R280K p53, as a model for invasive breast cancer (Walerych et al. 2012).

p53 missense mutant proteins (hereafter referred to as "mutp53") lose the ability to activate canonical p53 target genes and to interact with canonical partners, and thus the oncosuppressive activities of wtp53 (**Figure 10**).

mutp53 also acquires a dominant negative effect over the wild-type counterpart, by forming heterotetramers with it (Mantovani, Collavin, and Del Sal 2019). Moreover, mutp53 can gain new oncogenic properties through a complex repertoire of interactions with transcription factors, enzymes and other proteins involved in a plethora of cellular processes (M. P. Kim and Lozano 2018). Consequently, reshaping cancer cell's transcriptome, proteome, and metabolic network, mutp53 acts as driver oncogene and promotes cancer progression, metastasis dissemination, and drug resistance.

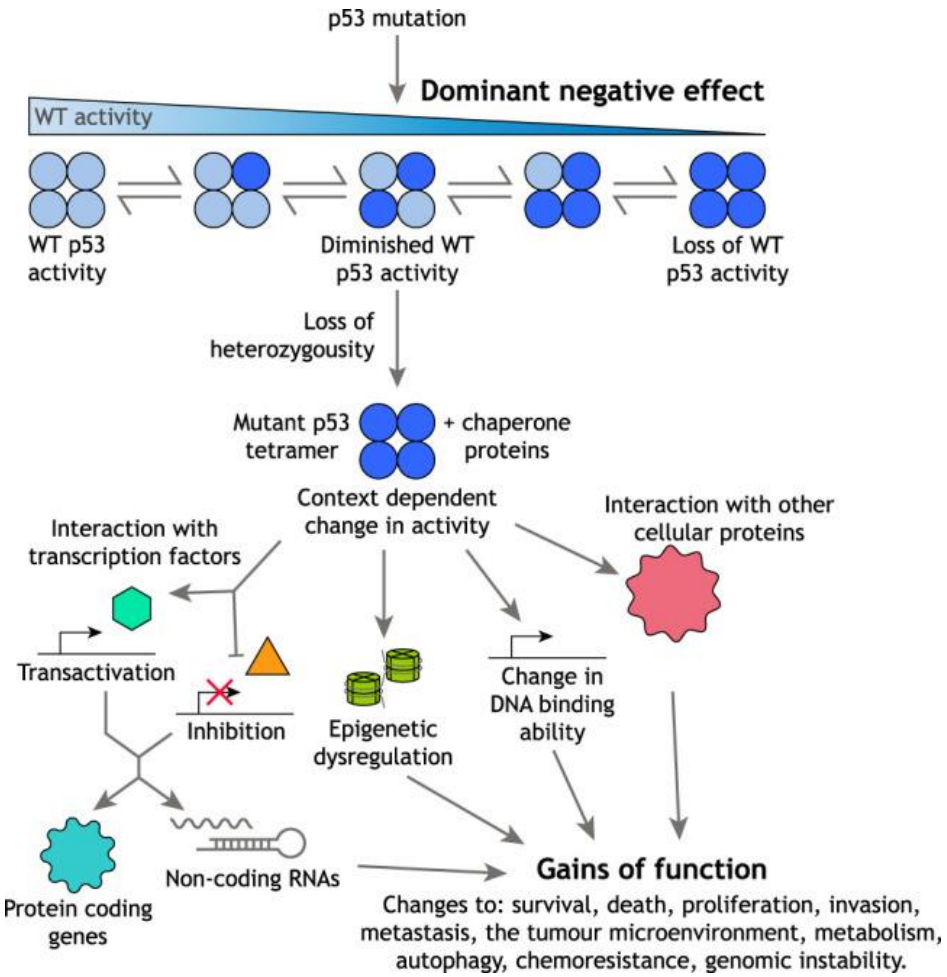

Figure 10. Mechanisms of mutant p53 action.

Mutant p53 mutation can form heterotetramers with wild-type (WT) p53. This dominant-negative effect reduces WT p53 activity and allow tumor development. However, many tumors lose the remaining WT p53. Mutant p53 is frequently stabilized in cancer cells, in part as a consequence of complex with chaperone proteins, allowing it to mediate a variety of new activities (gains of function) via a number of different mechanisms (adapted from Pilley, Rodriguez, and Vousden 2021).

Similarly to its wild-type counterpart, mutp53 becomes activated in response to several stresses and exerts its functions sustaining cancer cells survival in adverse conditions like in presence of DNA damages caused by high proliferation rate and oxidative stress that characterize malignant cells (Mantovani, Collavin, and Del Sal 2019).

4.3. Regulation of mutant p53 in cancer

Functions of p53, as well as of mutp53, strongly rely on its cellular levels, thus every condition that alters its expression and stabilization is critical for its activation and for cell transformation. p53 is subjected to a variety of post-translational modifications that importantly modulate its stabilization and its activation in response to stress signals (Gu and Zhu 2012). In tumor tissues, mutp53 stabilization is required for the execution of its pro-tumoral functions and, interestingly, mutp53 triggers positive feedback loops that feed its own accumulation (Mantovani, Collavin, and Del Sal 2019). This is achieved through the stable association of mutp53 with components of the HSP chaperone machinery that inhibit MDM2 activity towards mutp53 (Schulz-Heddergott and Moll 2018). Indeed, pharmacological inhibition of HSP90 or of its activators, elicits mutp53 degradation and impairment of its oncogenic functions both in vitro and in vivo (D. Li, Marchenko, and Moll 2011). In addition, mutp53 directly upregulates HSP90 expression by potentiating stabilization and activation of heat-shock factor-1 (HSF1), master transcription factor of heat-shock proteins, thus sustaining its own accumulation (D. Li et al. 2014).

Moreover, mutp53 stabilization is mediated by altered mechanical inputs displayed by tumor tissues. Indeed, our laboratory demonstrated that a dense and stiff ECM leads to mutp53 activation, in response to focal adhesion signaling and RhoA-dependent actin cytoskeleton remodeling (Ingallina et al. 2018). In agreement, accumulation of mutp53 within tumors is heterogeneous and locally influenced by tissue rigidity, being increased in fibrotic regions (Ingallina et al. 2018). Also in this case, mutp53 sustains its own stabilization through activation of several responses. Indeed, mutp53 promotes RhoA activation by inducing its positive regulators GEF-H1 and RhoGDI and by stimulating the mevalonate pathway (MVP) that drives prenylation and plasma membrane translocation of RhoA, an event that is required for its activation by mechanical inputs (Mizuarai, Yamanaka, and Kotani 2006; Ingallina et al. 2018).

Of note our laboratory has also recently demonstrated that mutp53 induces tumor cells secretion of pro-malignant soluble factors in the TME, potentiating ECM deposition and stiffening (Capaci et al. 2020). This indicates that mutp53 establishes a vicious cycle that leads to its accumulation in response to mechanical inputs.

Other extracellular environmental conditions, such as nutrient availability, importantly modulate mutp53 oncogenic activities. Indeed, it has been shown that glucose restriction induces mutp53 deacetylation and autophagy-mediated degradation. This results in reduced accumulation of mutp53 and impaired growth of tumor xenograft harboring mutp53 (Rodriguez et al. 2012). Of note, mutp53 increases glucose uptake in the cells by promoting translocation of GLUT1 transporters to the plasma membrane thus counteracting autophagy and enhancing its own protein levels (C. Zhang et al. 2013) (**Figure 11**).

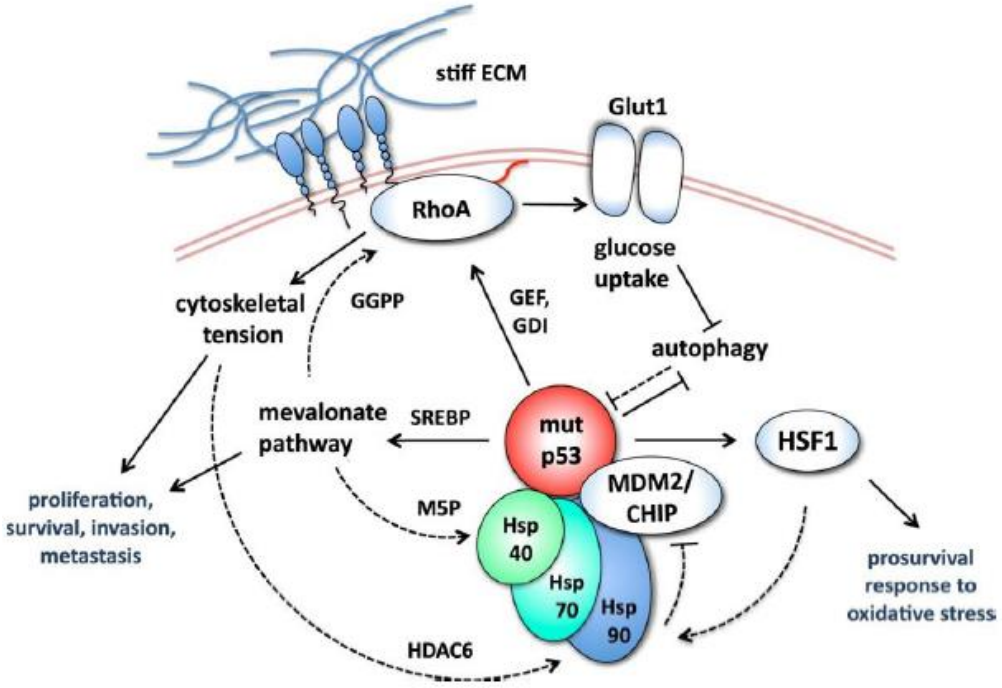

Figure 11. Molecular mechanisms of mutant p53 regulation in cancer cells.

HSP proteins determine mutp53 stabilization in cancer cells by inhibiting its ubiquitin ligase MDM2 and mutp53 in turn stimulates HSF1 thus increasing HSP90 transcription. mup53 increases GLUT1 membrane translocation and consequently glucose uptake, thus limiting its own autophagic degradation. Moreover, acting on SREBP, it promotes the MVP thus sustaining RhoA geranyl-geranylation, a step required for its stabilization downstream to a stiff ECM (adped from Mantovani, Collavin, and Del Sal 2019).

4.4. Role of p53 in cell competition

As mentioned above, p53 has been found to play a role in cell competition in different contexts, including development, wound healing, and tumorigenesis.

Several studies have shown that p53 activity can track with the elimination of loser cells during development. An unbiased search for genes that controlled cell competition in mice showed that cells lacking p53 were able to outcompete cells retaining wtp53 in the embryo (Dejosez et al. 2013). Also, transcriptomic analysis in mis-specifying and tetraploid cells emerging in the mouse epiblast, revealed that p53 upregulation determines a "loser" fate and induces apoptosis (Bowling et al. 2018; Horii et al. 2015). A similar role of p53 has been described, in chimeric mice, for the elimination of *Mdm2* and *Mdm4* haploinsufficient cells, which involved inhibition of proliferation rather than apoptosis induction (Zhang et al., 2017). A role of p53 in the elimination of loser cells has been also shown during the healing of epithelial wounds, in which p53 promotes the selective elimination of cells located at the wound edge that are under compaction (Kozyrska et al. 2022) and tumor suppressive cell competition, as in the case of scribble deficient epithelial cells subject to compaction by wild-type neighbors (Wagstaff et al. 2016).

On the other hand, p53 mutations have been found associated with loser or winner cell fate in a context-dependent manner. For example, in mouse intestinal organoids, overexpression of missense mutp53 (R175H or R273H) in sporadic cells leads to their basal extrusion and death by necroptosis (Watanabe et al. 2018). Indeed, also in the mouse esophagus, the growth of sporadic mutp53 clones has been shown to be constrained by competition for space and survival with neighboring cells (Murai et al. 2022). These results suggest that wild-type cells are able to recognize and eliminate mutp53 cells. In contrast, in certain conditions, mutp53 cells emerging in a tissue can survive and eventually outcompete neighbors, this was observed, for example, in mouse intestinal stem cells upon inflammation (Vermeulen et al. 2013), and in mouse HSPCs upon irradiation (Bondar and Medzhitov 2010). Recent studies in mice have suggested that the acquisition of a winner fate by mutp53 stem cells in irradiated esophagus could be due to a higher mutp53 stem cells division vs differentiation rate, as compared to wild-types neighboring stem cells (Fernandez-Antoran et al. 2019; Murai et al. 2022). Whether this or other mechanisms operate in other normal tissues in which p53 mutations frequently occur (Yizhak et al. 2019), and these mechanisms are regulated by (micro)environmental has still to be unveiled.

2. AIM OF THE THESIS

Tumors develop through genetic and epigenetic changes that alter cell proliferation, and selection of reprogrammed cells that adapt to the changing micro-environmental conditions, including physical constraints, nutrient fluctuation, hypoxia and oxidative stress. In healthy tissues, normal cells are able to recognize and restrain the expansion of cells bearing cancer-driving mutations, through "cell competition", an evolutionarily conserved process by which fitter cells outcompete less fit cells. Nonetheless, during aging, clones of cells bearing cancer-driving mutations accumulate in many tissues, and some of these clones may evolve into malignancy. Interestingly, certain conditions associated with aging, such as inflammation and stiffening of the extracellular matrix (ECM), have been shown to alter the process of cell competition and thus favor tissue colonization by cells bearing cancer-driving mutations. This suggests that tissue micro-environmental cues could play a key role in the regulation of cell competition and thus the formation of pre-malignant cell clones. However, the underlying mechanisms are still poorly understood, and their knowledge may provide opportunities to interfere with tumor initiation and evolution.

TP53 is one of the most frequently mutated genes in cancer, with a high prevalence of oncogenic missense mutations (mutp53), and mutp53 pre-malignant cell clones have been shown to accumulate during aging in different tissues, including esophagus, skin and breast. Notably, work in our laboratory showed that, in cancer cells, micro-environmental cues, including mechanical stress caused by ECM stiffening, play a key role in stabilization/activation of mutp53, and activated mutp53 rewires cell transcriptome and proteome, thus fostering cell proliferation, metastasis and chemoresistance.

Furthermore, it was shown that neoplastic scribble deficient cells surrounded by normal cells are outcompeted by mechanical extrusion from the epithelial layer, and this depends on wild-type p53 activation in scribble deficient cells. Instead, scribble/p53 double deficient cells are not outcompeted by normal cells, suggesting that p53 status plays a key role in determining the fitness of mechanically stressed neoplastic cells.

Based on this evidence, we posit that in mutp53 pre-malignant cells, mechanical stimuli from the ECM could promote mutp53 stabilization/activity and thus a transcriptional program that allows pre-malignant cells to outcompete normal neighbors.

To study the impact of ECM stiffening on the competition between mutp53 pre-malignant and normal cells, we sought to generate ad-hoc cellular models, consisting in human pre-malignant

cell lines expressing either wild-type or mutp53 and different fluorescent tracers. These lines could be used to establish cell competition assays, by co-cultures on substrates with increasing stiffness, and isolate the two competing cell populations to investigate pathways regulating cell competition, by transcriptomic analysis (**Figure 12**).

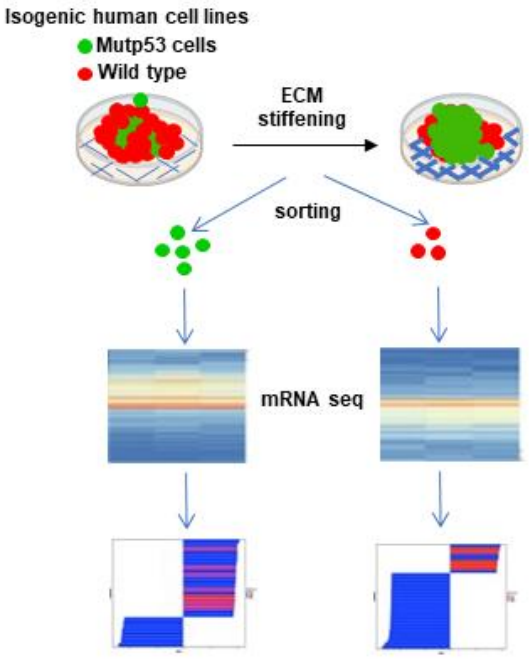

Figure 12. Experimental strategy aimed at identifying molecular pathways driving the expansion of mutp53 cells at expense of wild-type cells, on stiff substrates, through RNA sequencing analysis.

3. RESULTS

3.1. Generation of fluorescently traceable wild-type and mutp53-expressing pre-malignant mammary epithelial cell lines.

Previous work in our group has shown that ECM stiffening leads to mutp53 stabilization and activation in malignant cells, in particular in breast cancer cells (Tombari et al. 2023; Capaci et al. 2020; Ingallina et al. 2018). Hence, as a model to study the impact of ECM stiffness on mutp53-driven competition between pre-malignant and wild-type (wt) cells, we sought to utilize mammary epithelial cells. To this aim, we used MCF-10A cells, a line of immortalized, non-transformed, wild-type (wt) mammary epithelial cells, originally derived from an adult female donor (Soule et al. 1990). We generated a line expressing the endogenous wt p53 and a red fluorescent mCherry-tagged Histone 2B reporter (wt/H2B-mCherry line), by stable infection with a pBABE-H2B-mCherry retroviral construct. The H2B::mCherry protein was localized in the cell nucleus, as shown by immunofluorescence with an anti-RFP antibody (**Figure 13A**). As a model of pre-malignant cells, we generated an isogenic line expressing both mutp53 and wtp53. Recent work in mouse models has provided evidence that, in pre-malignant *TP53*$^{mut/+}$, spontaneous *TP53* loss-of-heterozygosity (LOH) could lead to extensive chromosomal instability (Murai et al. 2022). Hence, we established a line that could be maintained in culture in conditions in which the expression of mutp53 was not constitutive, thus avoiding potential *TP53* LOH, but could be switched ON and OFF. To this aim, we stably infected MCF-10A cells with a pCW57-tGFP-P2A-HA::p53^{R175H} lentiviral construct (mutp53/GFP line), expressing the tetracycline-controlled transactivator (rtTA) under the human phosphoglycerate promote (hPGK), and a bi-cistronic tGFP-2A-HA::p53^{R175H} transcript driven by the tetracycline responsive element (TRE). In these cells, the expression of N-terminal HA-tagged p53^{R175H} and turbo-Green Fluorescent Protein could be switched ON by supplementing the culture medium with the tetracycline analog doxycycline (**Figure 13B)**. tGFP was localized in both nucleus and cytoplasm, and HA::p53^{R175H} protein mainly in the nucleus, as shown by immunofluorescence with anti-turbo GFP and anti-HA antibodies (**Figure 13B, lower panels**). Nuclear HA::p53^{R175H} protein was also detected by immunofluorescence with the anti-p53 DO-1 antibody, which recognizes both wt and mutp53 (**Figure 13B, lower panels**). Furthermore, the induction of the HA::p53^{R175H} protein was demonstrated by western blot with an anti-HA antibody (**Figure 13C, upper panels**), and western blot with the anti-p53

DO-1 detected both HA::p53^{R175H} and endogenous wt p53 proteins, showing that HA::p53^{R175H} protein was expressed at higher levels (**Figure 13C, lower panels**).

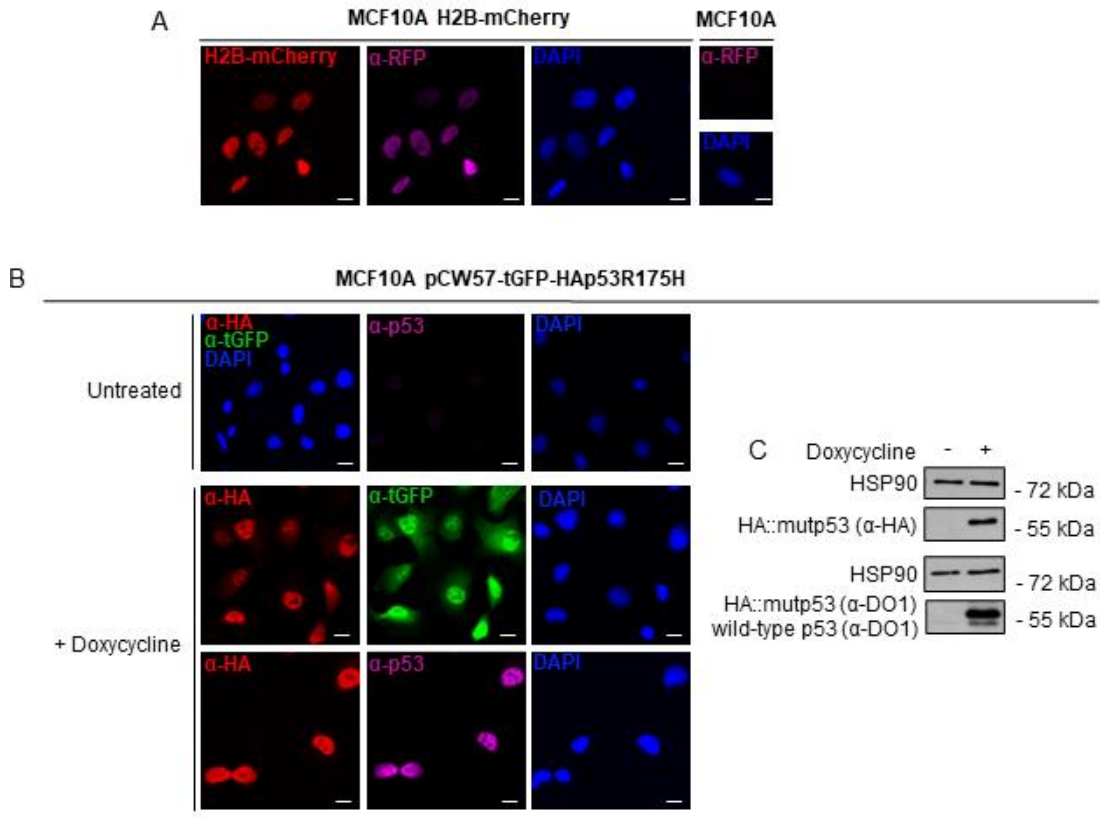

Figure 13. Generation of fluorescently labelled wild-type and mutp53-expressing pre-malignant mammary epithelial cell lines.

A) Immunofluorescence analysis of H2B-mCherry fluorescent protein (in magenta) using the anti-RFP antibody in human MCF-10A cells stably infected with the pBABE-H2B-mCherry retroviral construct (MCF-10A mCherry cells) and in non-infected MCF-10A cells. In red, the mCherry endogenous fluorescent signal is displayed. Nuclei are stained with DAPI. Scales bar, 15 µm. **B)** Immunofluorescence analysis of HA::p53^{R175H} (in red), turbo GFP (in green) and p53 (in magenta) proteins, using anti-HA tag, anti-tGFP and anti-p53 DO-1 antibodies, in human MCF-10A cells stably infected with pCW57-tGFP-P2A-HA::p53^{R175H} lentiviral construct (mutp53/GFP line), expressing the tetracycline-controlled transactivator (rtTA), under the human phosphoglycerate promoter (hPGK), and a bi-cistronic tGFP-P2A-HA::p53^{R175H} transcript driven by the tetracycline responsive element (TRE). Untreated cells are shown in the upper panels and cells treated with 1 µg/ml doxycycline for 24 hours, in the lower panels. The anti-p53 DO-1 antibody detects both the induced HA::p53^{R175H} and the endogenous wild-type p53. Nuclei are stained with DAPI. Scales bar, 15 µm. **C)** Western Blot analyses of HA::p53^{R175H} protein, using anti-HA and anti-p53 DO-1 antibodies in MCF-10A HA::p53^{R175H}, untreated or treated with 1 µg/ml doxycycline for 24 hours. The nontumor-derived polymorphism P72R causes a slight delayed migration in mutp53, compared with wild-type p53 (Capaci et al. 2020). HSP90 was used as loading control. The blot is representative of N=3 biological replicates.

We then verified the functionality of wt p53 in wt/H2B-mCherry cells, by assessing the activation of wt p53 in response to DNA damage, which typically leads to cell cycle arrest via transcriptional induction of the p53 target p21/CDKN1A. Treatment with the DNA-intercalating agent doxorubicin, which is known to inhibit Topoisomerase-II and thus DNA

replication, leading to induction of wt p53 response (Watanabe et al. 2018), caused an increase of wt p53 protein levels, associated with p21 induction, as shown by western blot (**Figure 14A,B**).

Next, we assessed whether, in mutp53/GFP cells, expression of $p53^{R175H}$, which is known to exert a dominant negative effect on wt p53, could inhibit the function of endogenous wt p53. To this aim, first we verified the functionality of endogenous wt p53 in mutp53/GFP cells cultured in doxycycline-free culture medium, in which endogenous wt p53, but not mutp53, was expressed. In these conditions, doxorubicin treatment caused an increase of the protein levels of endogenous wt p53, and also a strong induction of p21, as shown by western blot (**Figure 14A,B**). In doxycycline-supplemented medium, wt p53 protein levels were similar to those observed in doxorubicin-treated mutp53/GFP cells cultured in doxycycline-free medium, nonetheless p21 was not upregulated, as shown by western blot (**Figure 14A,B**). Doxorubicin treatment caused a further increase of endogenous wt p53 protein levels, however this did not lead to the induction of p21 (**Figure 14A-C**). We also verified that wt p53 response was not affected by doxycycline, showing that, in wt/H2B-mCherry cells cultured in doxycycline-supplemented medium, doxorubicin treatment caused the upregulation of wt p53 protein levels and strong induction of p21, as shown by western blot (**Figure 14A-C**).

These results demonstrated that, in pre-malignant cells, $p53^{R175H}$ could inhibit, through a dominant-negative effect, the wt p53 response to DNA damage.

In cancer cells, some missense mutant p53 proteins, including $p53^{R175H}$, have been demonstrated to have also a neomorphic function (GOF) and we sought to analyze this function in mutp53/GFP pre-malignant cells. p53 GOF mutants have been shown to act as transcriptional co-activators, promoting oncogenic gene programs (Mantovani, Collavin, and Del Sal 2019). In particular, multiple cancer cell lines expressing different p53 GOF mutants, including $p53^{R175H}$, were shown to directly target and upregulate the expression of proteasome genes (Walerych et al. 2016). Hence, as a benchmark of mutp53 GOF, we analyzed the mRNA levels of the proteasome gene *PSMB9*, in mutp53/GFP cells expressing $p53^{R175H}$, and as control, in wt/H2B-mCherry cells. Interestingly, in mutp53/GFP cells, $p53^{R175H}$ expression caused an upregulation of *PSMB9*, as compared with wt/H2B-mCherry cells (**Figure 14D**), suggesting that, in pre-malignant cells, $p53^{R175H}$ might exert GOF activity.

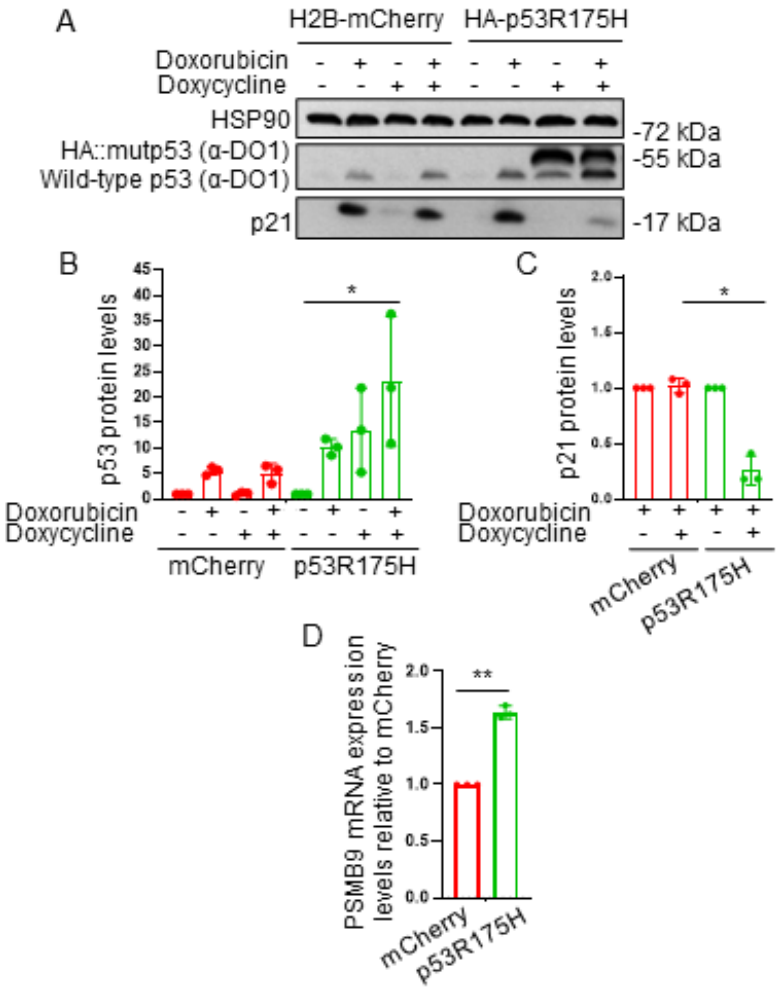

FIGURE 14. p53^{R175H} exerts dominant negative effects over wild-type p53 and induces proteasome genes in MCF-10A cells.

A) Western Blot analyses of wild-type p53, HA::p53^{R175H} (HA::mutp53) and p21 proteins using anti-p53 DO-1 and anti-p21 antibodies in MCF-10A mCherry cells and HA::p53^{R175H} cells not treated or treated with doxorubicin (250 ng/mL) for 12h in doxycycline free or supplemented-(1 µg/ml) medium. HSP90 was used as loading control. The blot is representative of N=3 biological replicates. **B)** Quantification of wild-type p53 protein levels in mCherry and HA::p53^{R175H} cells relative to untreated conditions. Values represent mean ± s.d. *P value <0.05, by two-tailed Student's t-test. **C)** Quantification of p21 protein levels in mCherry and HA::p53^{R175H} cells cultured in doxycycline and doxorubicin-supplemented medium, relative to doxorubicin treated condition. Values represent mean ± s.d. *P value <0.05, by two-tailed Student's t-test. **D)** RT-qPCR analysis of PSMB9 mRNA levels in MCF-10A mCherry and HA::p53^{R175H} cells, relative to mCherry cell line. GAPDH was used as reference for quantification. Values represent mean ± s.d. **P value <0.01, by paired two-tailed Student's t-test of N=3 biological replicates.

3.2. Assessing the impact of ECM stiffening in wild-type and mutp53 mammary epithelial cells mono-cultures.

In adult women, hormone fluctuations, during the menstrual cycle, pregnancy, lactation and menopause, lead to changes of the mammary gland structure and function, including its

mechanical properties. On average, healthy breast tissue displays a 1 kPa stiffness, which raises in physio-pathological conditions, such as fibrosis (2-4 kPa), and cancer (8-50 kPa). To assess the mechano-response of wt/H2B-mCherry and $p53^{R175H}$-expressing mutp53/GFP cells to ECM stiffness, we cultured these cells in 2D on 0.02 mg/mL type I collagen-coated polyacrylamide hydrogel substrates of physiological (1 kPa), supra-physiological (4 kPa), pathological (8 and 50 kPa) stiffness, and also on hyperstiff (10 GPa) plastic substrate, in doxycycline-supplemented medium. In these conditions, we assessed the activation of the key controller of cellular mechano-response, the transcription co-factor YAP, by analyzing its localization and target genes expression. In wt/H2B-mCherry cells, cultured on soft (1 kPa) substrate, YAP was uniformly distributed in nucleus and cytoplasm, and only a minimal percentage (%) of cells displayed a level of YAP in the nucleus higher than the cytoplasm, as shown by immunofluorescence with an anti-YAP antibody (**Figure 15A,C**). On stiff substrates (8 kPa, 50 kPa and plastic) this percentage was increased, indicating YAP enrichment in the nucleus (**Figure 15A,C**), and this was associated with an increase of the mRNA levels of the YAP targets *CTGF* and *Cyr61*, as shown by qPCR (**Figure 15D**). We also verified wt p53 expression, and found that, on soft substrate (1 kPa), wt p53 levels were slightly reduced, as compared with stiff substrates, as shown by western blot with the anti-p53 DO-1 antibody (**Figure 15F,G**).

Also in $p53^{R175H}$-expressing mutp53/GFP cells, substrate stiffening caused nuclear YAP enrichment, as shown by immunofluorescence with an anti-YAP antibody (**Figure 15B,C**), as well as *CTGF* and *Cyr61* mRNA levels upregulation, as shown by qPCR (**Figure 15E**). On soft substrate (1 kPa), the levels of HA::$p53^{R175H}$ protein were comparable to those detected on stiff substrates, while the levels of endogenous wt p53 were instead slightly higher, as shown by western blot with the anti-p53 DO-1 antibody, though the increase was not statistically significant (**Figure 15H,I**).

These results showed that, in both wt/H2B-mCherry cells and mutp53/GFP pre-malignant cells expressing $p53^{R175H}$, in response to ECM mechanical stimuli, a YAP-dependent transcriptional response is activated.

It has been shown that, in cancer cells, a mutp53/MVP/Rho axis activates YAP/TAZ, with consequent increased cell stemness and proliferation (Capaci et al. 2020; Ingallina et al. 2018; Sorrentino et al. 2014). Interestingly we observed that, in mutp53/GFP expressing $p53^{R175H}$ and cultured on stiff substrates, both YAP nuclear enrichment and *CTGF* mRNA upregulation were stronger than wt/H2B-mCherry cells (**Figure 15C-E**), suggesting that, also in pre-malignant cells, mutp53 may empower YAP activity**.**

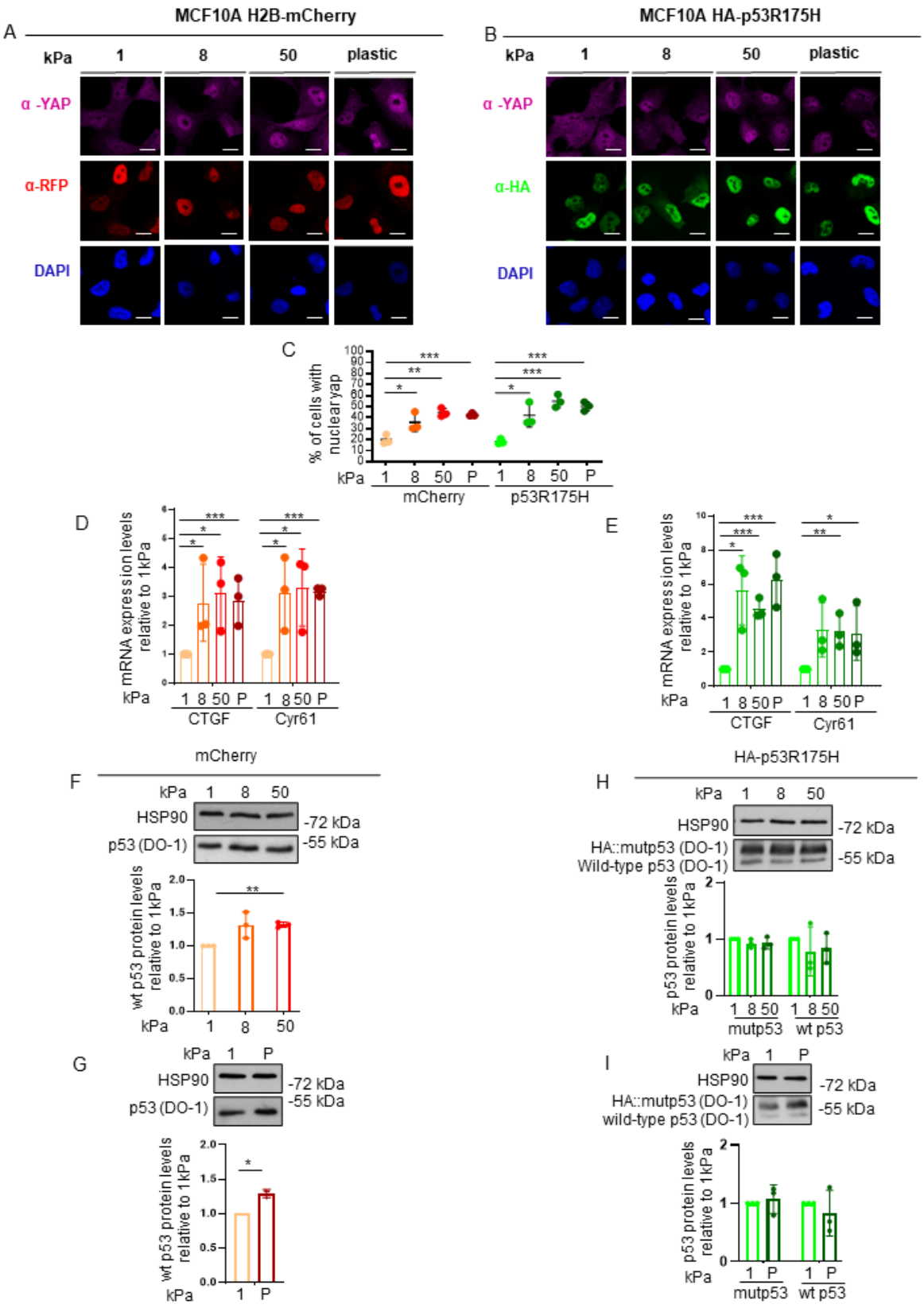

FIGURE 15. Analysis of the impact of ECM stiffening on mono-cultured mCherry and HA::p53^{R175H} MCF-10A cells.

A-B) Immunofluorescence analysis of YAP (in magenta) and mCherry (in red) proteins using the anti-YAP and anti-RFP antibody in MCF-10A mCherry cells **(A)** and of YAP and HA::p53^{R175H} (in green) proteins using anti-YAP and anti-HA antibody in MCF-10A HA::p53^{R175H} cells **(B)** cultured on collagen-coated 1,8,50 kPa stiffness hydrogel and plastic substrates. Cells were cultured in doxycycline-supplemented medium (1 µg/ml) for 48h. Nuclei are stained with DAPI. Scales bar, 15 µm. Panels are representative of N=3 biological replicates. **C)** Quantification of mCherry and HA::p53^{R175H} cells displaying nuclear YAP localization. 100-200 cells were counted in each condition. Values represent mean ± s.d. *P value <0.05, **P value <0.01 ***P value <0.001 by two-tailed Student's t-test of N=3 biological replicates. **D)** RT-qPCR analysis of the RNA levels of the YAP targets CTGF and Cyr61 in MCF-10A mCherry cells. H3 was used as reference for quantification. Values represent mean ± s.d. *P value <0.05, ***P value <0.001 by Student's t-test in N=3 biological replicates. **E)** RT-qPCR analysis of the RNA levels of the YAP targets CTGF and Cyr61 in MCF-10A HA::p53^{R175H} cells. GAPDH was used as reference for quantification. Values represent mean ± s.d. *P value <0.05, **P value <0.01, ***P value <0.001 by two-tailed Student's t-test of N=3 biological replicates. **F-G)** Western Blot analyses of wild-type p53 using anti-p53 DO-1 antibody in MCF-10A mCherry cells cultured on collagen-coated 1,8,50 kPa stiffness hydrogel substrates **(F)** and on 1 kPa and plastic substrates **(G)** for 48h in doxycycline-supplemented medium (1 µg/ml). HSP90 was used as loading control. The blots are representative of N=3 biological replicates. Quantification of wild-type p53 protein level is shown in the lower panel. Values represent mean ± s.d. *P value <0.05, **P value <0.01 by two-tailed Student's t-test in N=3 biological replicates. **H-I)** Western Blot analyses of wild-type p53 and HA::p53^{R175H} using anti-p53 DO-1 antibody in MCF-10A HA::p53^{R175H} cells cultured on collagen-coated 1,8,50 kPa stiffness hydrogel substrates **(H)** and on 1 kPa and plastic substrates **(I)** for 48h in doxycycline-supplemented medium (1 µg/ml). HSP90 was used as loading control. The blot is representative of N=3 biological replicates.

3.3. ECM stiffening promotes competitive growth advantage of mutp53 pre-malignant mammary epithelial cells over neighboring wt cells.

We used the wt/H2B-mCherry cell line and the mutp53/GFP cell line expressing p53^{R175H} to establish co-culture models, and thus to study the impact of ECM mechanical stimuli on the competition between mutp53 pre-malignant cells and wt neighboring cells. To this aim, first we seeded (day 0), for each line, 600 cells/mm^2 on physiological stiffness substrate (1 kPa), in 0.2 µg/ml Hoechst and 1 µg/ml doxycycline-supplemented culture medium and let them grow, replacing the culture medium every 48h (**Figure 16A**). The number of Hoechst-positive nuclei was quantified daily, by live imaging (**Figure 16A**). wt/H2B-mCherry cell reached their homeostatic density (1400 cells/mm^2) in about 7-8 days, with a cell doubling time (cdt), calculated between day 1 and day 6, of 3.1 days (**Figure 16A,B**). p53^{R175H}-expressing mutp53/GFP cells displayed a slightly lower cdt of 2.8 days, between day 1 and day 6, and in about 7-8 days, reached a higher homeostatic density (2400 cells/mm^2) (**Figure 16A,B**).

Then we assessed the impact of substrate stiffness on cell growth. For each line, we seeded 600 cells/mm^2 on 4 kPa, 8 kPa, 50 kPa stiffness substrates and plastic, in 0.2 µg/ml Hoechst and 1 µg/ml doxycycline-supplemented culture medium, and let them grow replacing the culture medium every 48h. The wt/H2B-mCherry cells reached their homeostatic density (1400 cells/mm^2) in about 7-8 days on the softer substrate 4 kPa (dt 2.9 days), and 6-7 days on 8 kPa (dt 2.7 days), 50 kPa (dt 2.4 days) stiffness substrates and plastic (dt 2.0 days) (**Figure 16B**).

The mutp53/GFP cell reached their homeostatic density (2400 cells/ mm^2) in about 7-8 days on the softer substrate 4 kPa (dt 2.9 days), and 6-7 days on 8 kPa (dt 2.5 days), 50 kPa (dt 2.0 days) stiffness substrates and plastic (dt 2.1 days) (**Figure 16B**). These results showed that ECM stiffening was associated with increasing growth rate, consistent with the observed ECM stiffening-dependent activation of YAP (**Figure 15A-E**), which is known to induce cell proliferation gene programs (Zanconato et al. 2015; Cordenonsi et al. 2011).

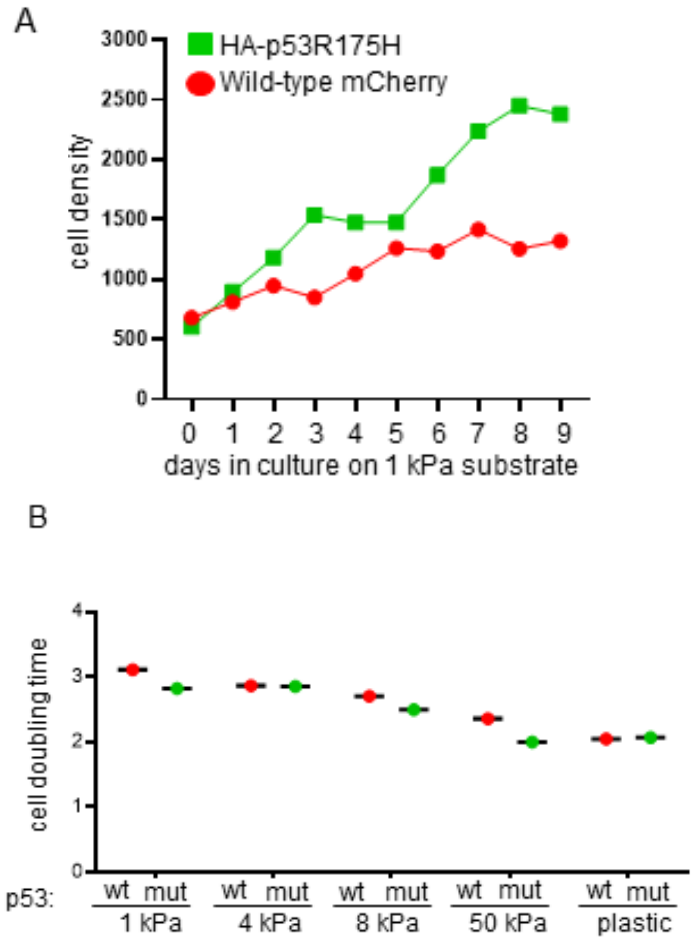

FIGURE 16. Calculation of the doubling time of MCF-10A mCherry and HA::p53^{R175H} cells on substrate with different stiffness.

A) Live imaging analysis showing the growth of mCherry and HA::p53^{R175H} cells cultured on collagen-coated 1 kPa stiffness hydrogel substrate. 600 cells/mm^2 were seeded at day 0 and let grow for 9 days in doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml)-supplemented medium, replaced every 48h. Growth quantification was performed by acquiring pictures every 24h and by counting at least 2000 Hoechst-positive cells. Each dot in the figure represents the mean value of the number of cells/mm^2 counted in N=3 fields. **B)** Cell doubling time of mCherry and HA::p53^{R175H}/GFP cells seeded with 600 cells/mm^2 density at day 0 on 1,4,8,50 kPa and plastic substrates. Each dot in the figure represents the mean of Hoechst-positive cells counted in N=2 fields.

Next, we sought to establish a cell competition assay in which wt/H2B-mCherry and $p53^{R175H}$-expressing mutp53/GFP pre-malignant cells were co-cultured, and we could assess whether the behavior of each cell population was modified by competitive interactions, as compared with mono-cultured wt/H2B-mCherry and $p53^{R175H}$-expressing mutp53/GFP pre-malignant cells, respectively. Our aim was to use this assay to assess the impact of ECM stiffness on the competition between $p53^{R175H}$-expressing mutp53/GFP cells and wt/H2B-mCherry cells. To this aim, we established mono-cultures of wt/H2B-mCherry and mutp53/GFP cells on the substrate with physiological stiffness (1 kPa), in 0.2 µg/ml Hoechst- and doxycycline-supplemented culture medium, replaced every 48h. Cells were seeded (day 0) at a density (530 cells/mm^2) such that, according to their dts (**Figure 17D**), both mono-cultures reached the homeostatic density in 7-8 days, as shown by live imaging daily quantification of mCherry and GFP cells (**Figure 17A,B**). In parallel, we co-seeded wt/H2B-mCherry and mutp53/GFP cells at a 2:1 ratio, with a total seeding density equal to mono-cultures (530 cells/mm^2). In these conditions, both cell types displayed a dt (calculated between day 1 and day 6), comparable to the respective mono-culture (**Figure 17D**). The same assay was run on 4 kPa, 8 kPa, 50 kPa stiffness substrates and plastic (**Figure 17C**). On each substrate, cells were seeded with densities (420 cells/mm^2, 370 cells/mm^2, 320 cells/mm^2, respectively) equal for wt/H2B-mCherry and mutp53/GFP cells mono-culture and 2:1 co-culture, such that, according to their dts, the homeostatic density was reached 7-8 days. On the 4 kPa stiffness substrate, the dts of co-cultured wt/H2B-mCherry and mutp53/GFP cells were comparable to the respective mono-culture (**Figure 17D**), similar to softer substrate (1 kPa). Interestingly, on stiffer substrates (8 kPa, 50 kPa and plastic), the dt was higher in co-cultured than mono-cultured wt/H2B-mCherry cells (**Figure 17D**). Conversely, the dt was lower in co-cultured than mono-cultured mutp53/GFP cells (**Figure 17D**).

The results of this assays showed that ECM stiffening promoted a competitive growth advantage of $p53^{R175H}$-expressing pre-malignant mammary epithelial cells over wt neighboring cells.

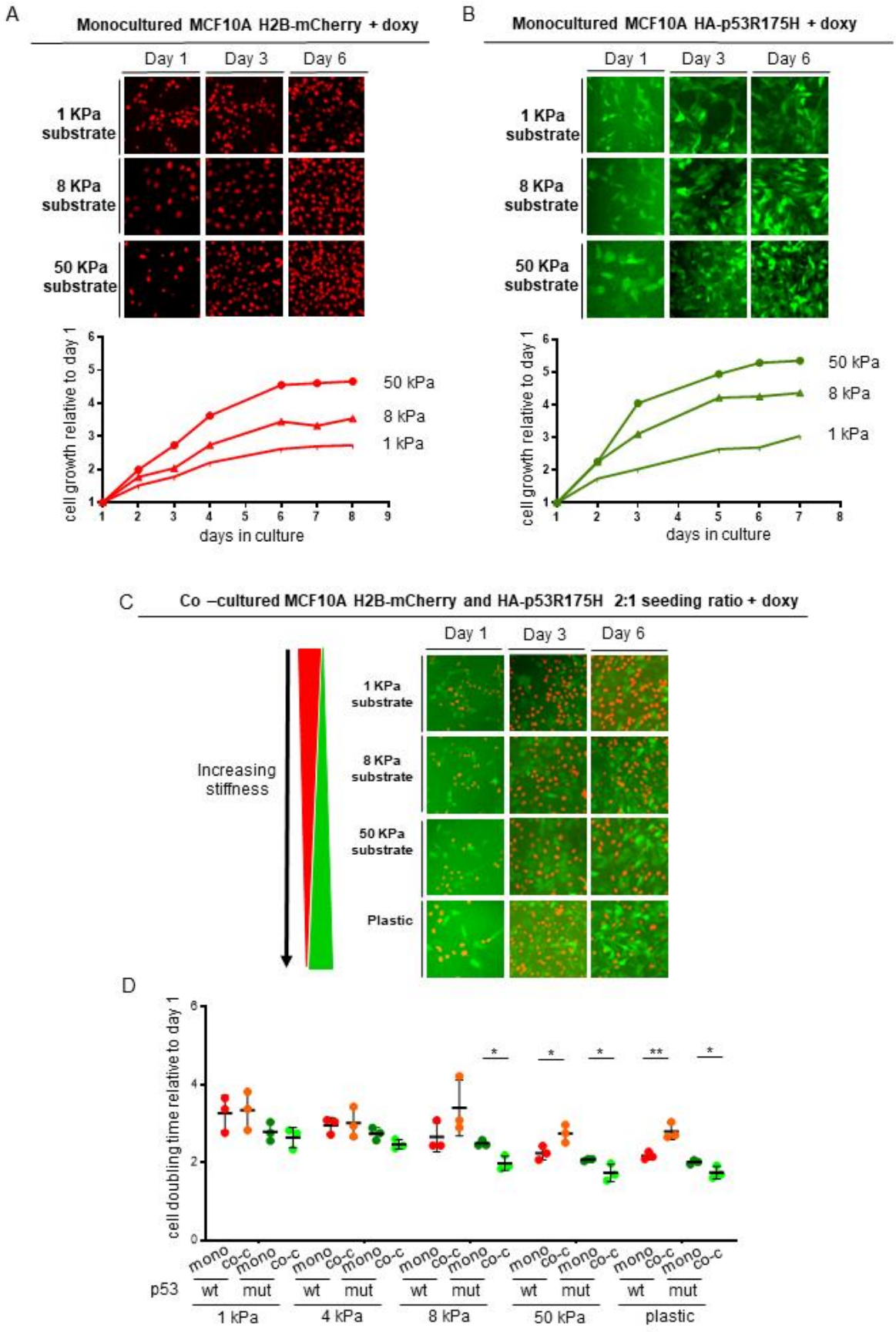

FIGURE 17. MCF-10A expressing HA::p53^{R175H} outcompete wild-type cells when co-cultured on stiff substrates.

A) Live imaging analysis of mCherry mono-cultures grown on collagen-coated 1,8,50 kPa stiffness hydrogel substrates after 1, 3 and 6 days in culture. Cells were cultured in doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml)-supplemented medium, replaced every 48h. Live imaging pictures were acquired every 24h. Quantification of Hoechst-positive cell growth relative to day 1 (lower panel) by counting at least 2000 cells. **B)** Live imaging analysis of HA::p53^{R175H}/GFP mono-cultures grown on collagen-coated 1,8,50 kPa stiffness hydrogel substrates after 1, 3 and 6 days in culture. Cells were cultured in doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml)-supplemented medium, replaced every 48h. Live imaging pictures were acquired every 24h. Quantification of Hoechst-positive cell growth relative to day 1 (lower panel) by counting at least 2000 cells. **C)** Live imaging pictures of mCherry and HA::p53^{R175H}/GFP cells during a cell competition assay performed on collagen-coated 1,4,8,50 kPa stiffness hydrogels and plastic substrates after 1, 3 and 6 days in culture. Cells were cultured in doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml)-supplemented medium, replaced every 48h. A schematic representation of the proportion of mCherry cells and HA::p53^{R175H}/GFP cells growing on substrates with increasing stiffness is shown on the left. **D)** Cell doubling time of mCherry and HA::p53^{R175H}/GFP cells during a cell competition assay on collagen-coated 1,4,8,50 kPa stiffness hydrogel and plastic substrates. Cells were cultured in doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml)-supplemented medium, replaced every 48h. Cell doubling time was calculated between day 1 and day 6 by counting at least 2000 cells. The values represent mean ± s.d. *P value ≤ 0.05, **P value <0.01, by Student's t-test in N=3 fields.

3.4. mutp53 pre-malignant winner cells co-cultured with wt cells on stiff substrate display increased proliferation.

To assess whether ECM stiffening promoted a competitive growth advantage of p53^{R175H}-expressing mutp53/GFP cells, over wt neighboring cells, by regulating cell proliferation, we performed the cell competition assay on plastic, quantifying the number of cells in S-phase, by BrdU incorporation. Cells were seeded (day 0) on plastic, in doxycycline-supplemented culture medium, replaced every 48h, and administered with a 4h pulse of BrdU during growth (day 4). In wt/H2B-mCherry cells co-cultured with mutp53/GFP cells, the dt was higher than mono-cultured wt/H2B-mCherry cells, however the percentage of BrdU-positive cells was comparable (**Figure 18A-C**). In contrast, this percentage was higher in co-cultured than mono-cultured mutp53/GFP cells (**Figure 18A,D).**

These results suggested that, on stiff substrate, p53^{R175H}-expressing mutp53/GFP pre-malignant cells, competed with wt neighboring cells, increased their proliferation, and acquired a winner phenotype.

3.5. Wt cells co-cultured with mutant p53 pre-malignant cells on stiff substrate undergo cell death.

We then assessed whether the growth rate reduction in co-cultured vs mono-cultured wt/H2B-mCherry cells on plastic could be due to cell death. To this aim, we performed the cell competition assay, quantifying the number of dying cells during growth (day 3), by TUNEL staining. In co-culture, the percentage of TUNEL-positive wt/H2B-mCherry cells was higher, as compared with mono-culture (**Figure 19A,B**). In contrast, in co-culture on soft substrate, a condition in which wt/H2B-mCherry cells were not outcompeted by mutp53/GFP cells, the percentage of TUNEL-positive wt/H2B-mCherry cells was comparable to mono-culture (**Figure 19C,D)**. These results suggested that substrate stiffening led wt cells to compete with $p53^{R175H}$-expressing pre-malignant cells, to acquire a loser phenotype, and undergo cell death.

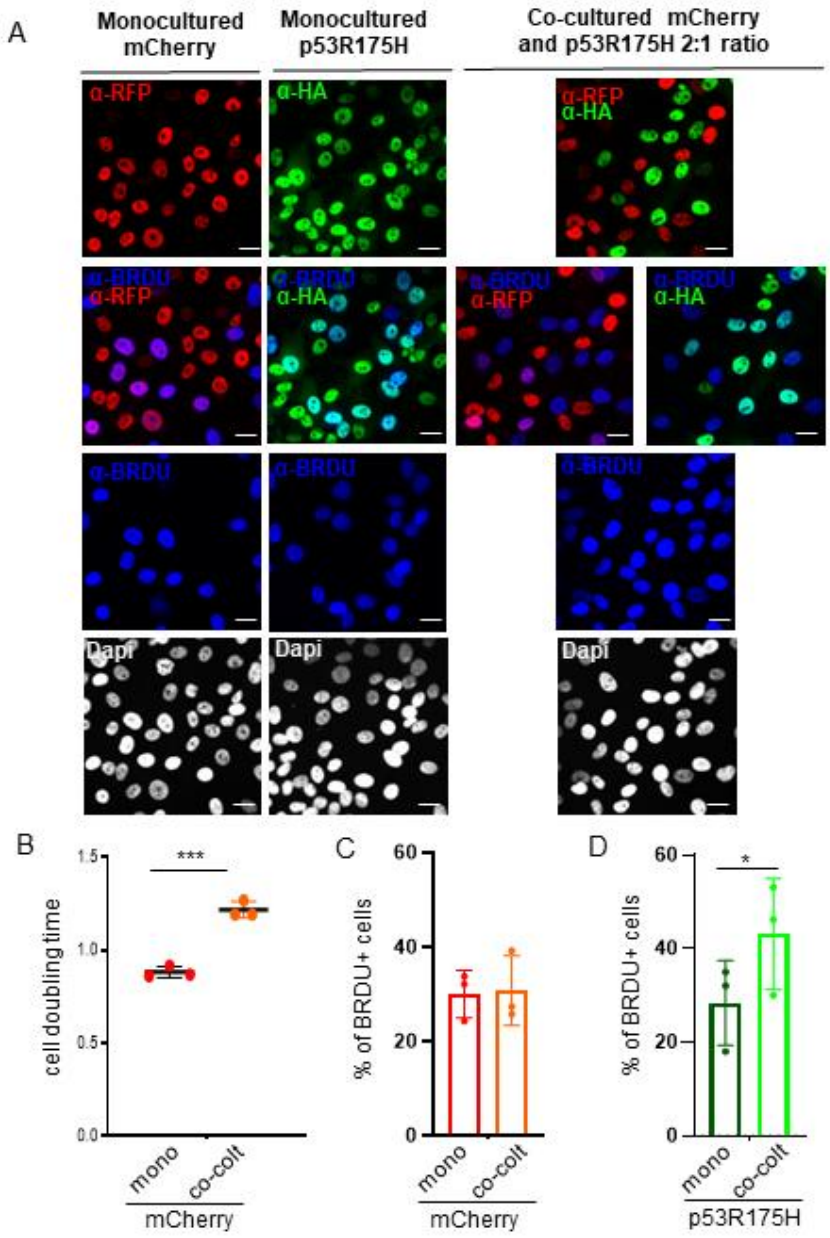

FIGURE 18. MCF-10A cells expressing HA::p53^{R175H} display higher rate of proliferation when co-cultured with mCherry cells on plastic substrate as compared with mono-culture.

A) Immunofluorescence analysis of BrdU (in blue) incorporated (after a 4h 20 μM pulse, at day 4) by mCherry and HA::p53^{R175H}/GFP expressing cells during a cell competition assay performed on plastic substrate. Cells were cultured in doxycycline-supplemented medium (1 μg/ml). mCherry (in red) and HA::p53^{R175H} (in green) proteins were detected by using anti-RFP and anti HA-tag antibodies. Nuclei are stained with DAPI (in grey). Scales bar, 15 μm. The panel is representative of N=3 biological replicates. **B)** Quantification of the doubling time of mono-cultured mCherry cells and mCherry co-cultured with HA::p53^{R175H} cells was calculated by counting mCherry cells in N=3 fields containing at least 300 cells. Values represent mean ± s.d. ***P value <0.001 by two-tailed Student's t-test of N=3 fields. **C)** Quantification of mono-cultured and co-cultured mCherry BrdU positive nuclei (in about 3000 cells). **D)** Quantification of mono-cultured and co-cultured HA::p53^{R175H}/GFP cells BrdU positive (in about 5000 cells). Values represent mean ± s.d. *P value <0.05, by paired two-tailed Student's t-test of N=3 fields.

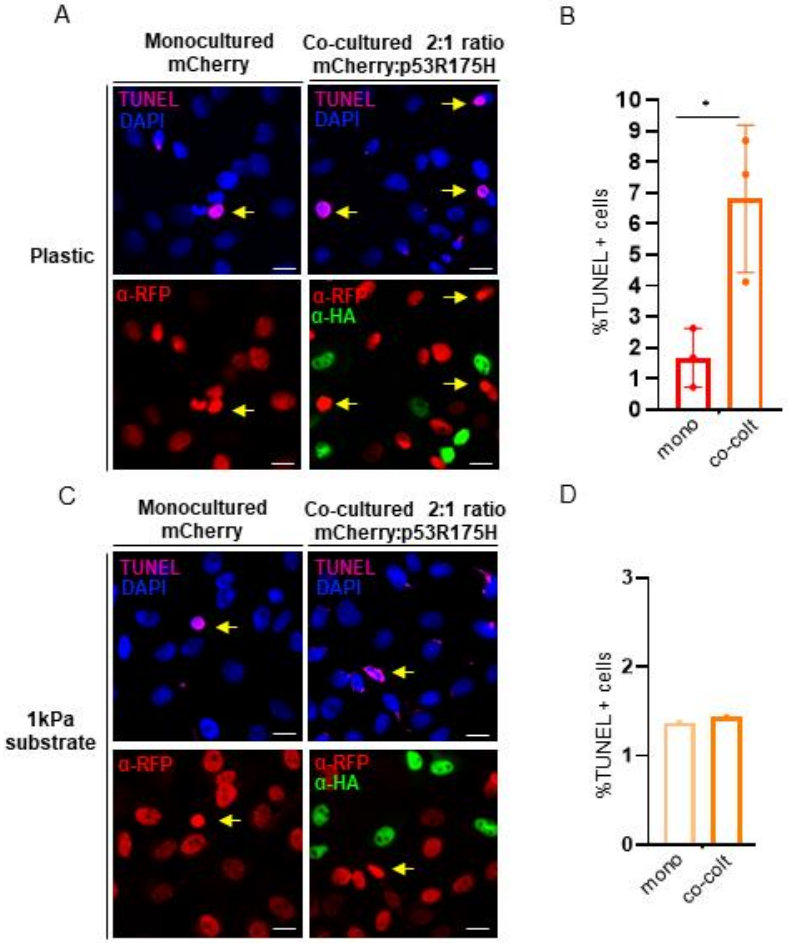

FIGURE 19. MCF-10A wt/H2B-mCherry cells co-cultured with HA::p53^{R175H}/GFP show higher percentage of cell death compared to mono-cultured MCF-10A wt/H2B-mCherry.

A) Tunel staining (in magenta) of mono-cultured and co-cultured wt/H2B-mCherry with HA::p53^{R175H}/GFP cells on plastic substrates. Cells were cultured in doxycycline-supplemented medium (1 μg/ml). mCherry (in red) and HA::p53^{R175H}/GFP proteins (in green) were detected by using anti-RFP and anti-HA antibody, respectively. Yellow arrows indicate mCherry cells positive for TUNEL. Nuclei are stained with DAPI. Scales bar, 15 μm. The images are representative of N=3 biological replicates. **B)** Quantification of mono-cultured mCherry and co-cultured mCherry TUNEL positive nuclei counted (about 600 cells were analyzed in each experiment). Values represent mean ± s.d. *P value <0.05, by two-tailed Student's t-test of N=3 biological replicates. **C**) Tunel staining (in magenta) of mono-cultured and co-cultured wt/H2B-mCherry with HA::p53^{R175H}/GFP cells on collagen-coated

1 kPa stiffness hydrogel substrate. Cells were cultured in doxycycline-supplemented medium (1 µg/ml). mCherry (in red) and HA::p53^{R175H}/GFP proteins (in green) were detected by using anti-RFP and anti-HA antibody, respectively. Yellow arrows indicate mCherry cells positive for TUNEL. Nuclei are stained with DAPI. Scales bar, 15 µm. The images are representative of N=1 experiment. **D**) Quantification of mono-cultured mCherry and co-cultured mCherry TUNEL positive nuclei counted (about 700 cells were analyzed in this experiment). Values are representative of N=1 replicate.

3.6. A mutp53/YAP cooperative gene program is activated in winner pre-malignant cells.

To assess whether, in pre-malignant cells on stiff substrate, the acquisition of winner phenotype could be associated with a mutp53 GOF program, we analyzed the transcriptome of mutp53/GFP and wt/H2B-mCherry competing cells. We ran N=3 replicate competition assays on plastic, in doxycycline-supplemented culture medium, replaced every 48h. For each replicate experiment, during cell growth (day 3) we FACS-sorted GFP- and mCherry-positive cells from mono- and co-cultures. Cells from all the replicate experiments were then used to perform RNA-seq. The gene expression data were first subjected to principal component analysis (PCA). mutp53/GFP and wt/H2B-mCherry cells displayed distinct clustering (**Figure 20A**). Furthermore, in each replicate experiment, both mutp53/GFP and wt/H2B-mCherry cells from the co-culture displayed a shift, as compared with the respective mono-culture (**Figure 20A**), showing that the acquisition of winner and loser phenotypes were associated with gene expression changes (**Figure 20B**).

Next, to assess whether a mutp53 GOF program was activated in winner pre-malignant cells, first we generated a mutp53 GOF gene signature from *TP53*^{R175H} knock-in MCF-10A pre-malignant cells (Redman-Rivera et al. 2021). This signature was composed of 254 genes, whose expression in *TP53*^{R175H} knock-in, but not in *TP53* null cells, displayed a statistically significant increase, as compared with wt cells (Redman-Rivera et al. 2021). Then we assessed the status of these genes in the transcriptomes of co-cultured mutp53/GFP and wt/H2B-mCherry cells. Interestingly, the signature was enriched in genes upregulated in mutp53/GFP winner cells, as shown by Gene Set Enrichment Analysis (GSEA) (**Figure 20C**), indicating the activation of a mutp53 GOF program.

To investigate the impact of this program in winner cells, we sought to identify the pathways displaying statistically significant upregulation in co-cultured mutp53/GFP vs wt/H2B-mCherry cells, by GSEA. Interestingly, this analysis highlighted, among the most upregulated pathways, the activation of YAP, as shown by the enrichment of the YAP gene signature from Cordenonsi et al. 2011 in genes upregulated in mutp53/GFP (**Figure 20D**).

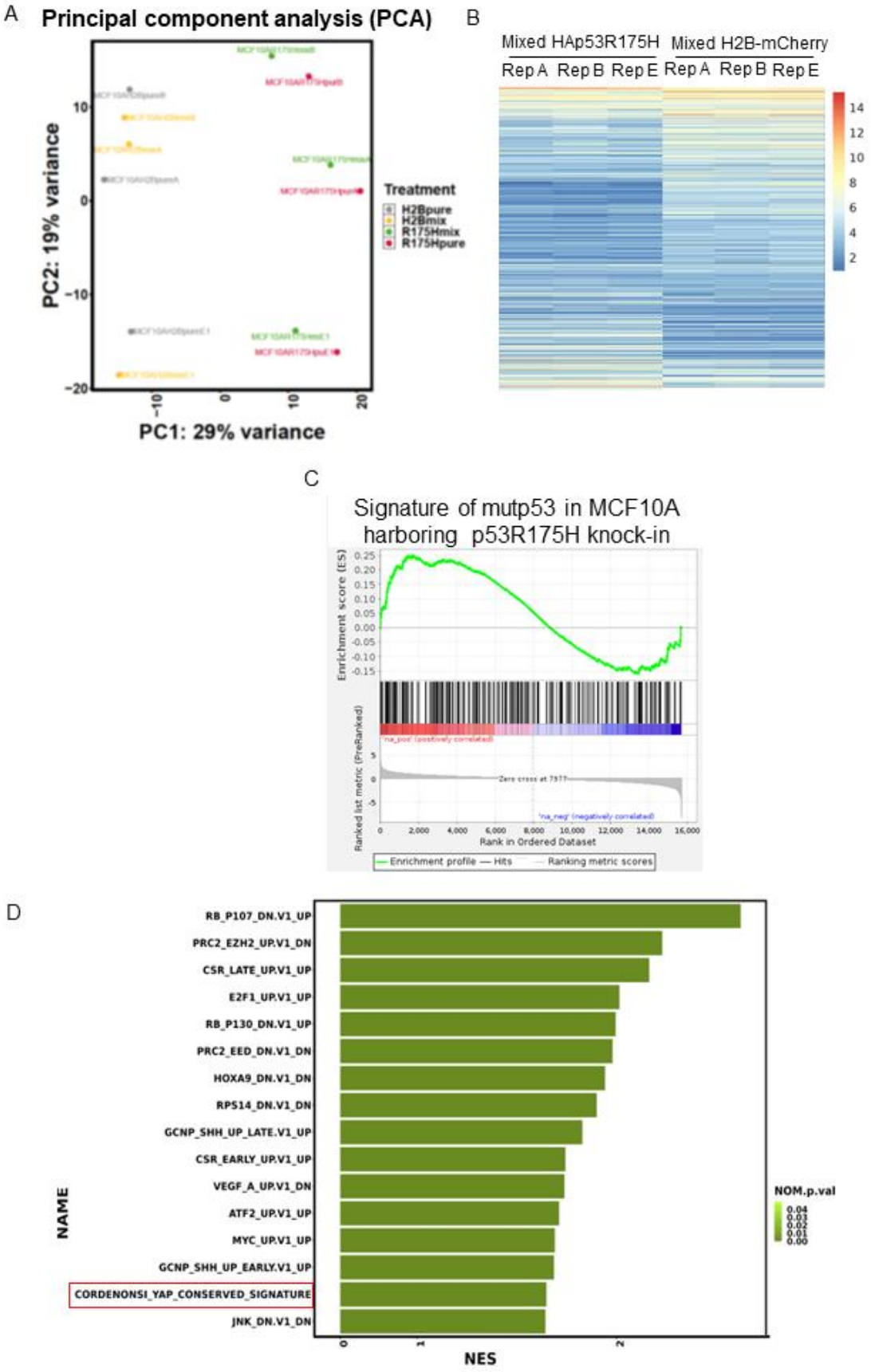

Figure 20. Transcriptomic analysis of competing mutp53/GFP and wt/H2B-mCherry cells.

A) Principal component analysis of pure and mixed wt/H2B-mCherry cells with mutp53/GFP. **B)** Gene expression

Heatmap of mixed mutp53/GFP cells and wt/H2B-mCherry cells (A, B, E replicates). **C)** mutp53/GFP cells display the upregulation of a mutp53 GOF transcriptional signature derived from *TP53*^{R175H} knock-in MCF-10A pre-malignant cells (by GSEA). **D)** mutp53/GFP cells display the upregulation of YAP gene signature from (Cordenonsi et al. 2011). NES, normalized enrichment score.

In breast cancer cells, YAP was previously shown to stimulate the transcriptional activity of p53 GOF mutants, including p53^{R175H} (Di Agostino et al. 2016). Mechanistically, YAP was shown to interact with mutp53, and through the binding to the NF-Y transcription factor, induced a set of target genes stimulating proliferation (Di Agostino et al. 2016). Hence, we reasoned that, in mutp53/GFP pre-malignant cells on high substrate stiffness, nuclear YAP could interact with mutp53 and stimulate its GOF transcriptional program. To test this hypothesis, we ran the competition assay with the wt/H2B-mCherry and the mutp53/GFP cell lines on collagen-coated hydrogels 1 kPa, 8 kPa, 50 kPa stiffness substrates and plastic, in doxycycline-supplemented medium (1 µg/ml), replaced every 48h. In the co-culture, during growth, at day 2 we analyzed YAP protein localization. On 1 kPa stiffness substrate, YAP was uniformly distributed in nucleus and cytoplasm, and only a minimal percentage (%) of cells displayed a level of YAP in the nucleus higher than the cytoplasm, as shown by immunofluorescence with an anti-YAP antibody (**Figure 21A**). This percentage was increased on the 8 kPa, and even higher on the 50 kPa stiffness substrate and plastic. A similar trend was observed in mutp53/GFP cells, and interestingly, on each substrate the percentage of cell with YAP nuclear enrichment was higher than wt/H2B-mCherry cells (**Figure 21A**). Then, we assessed whether, in the mutp53/GFP cells, YAP could interact with mutp53. To this aim, we performed a Proximity Ligation Assay (PLA) with anti-YAP and anti-p53 DO-1 antibodies, in mutp53/GFP cells cultured on soft (1 kPa) and stiff (plastic) substrates, in doxycycline-supplemented culture medium (1 µg/ml), and as control in wt/H2B-mCherry cells. On stiff substrate, all mutp53/GFP cells displayed YAP/mutp53 protein interaction, while this was detected only in a fraction of cells (%) on soft substrate (**Figure 21B**). In contrast, wt p53 did not interact with YAP, as shown by PLA in the wt/H2B-mCherry cells (**Figure 21B**).

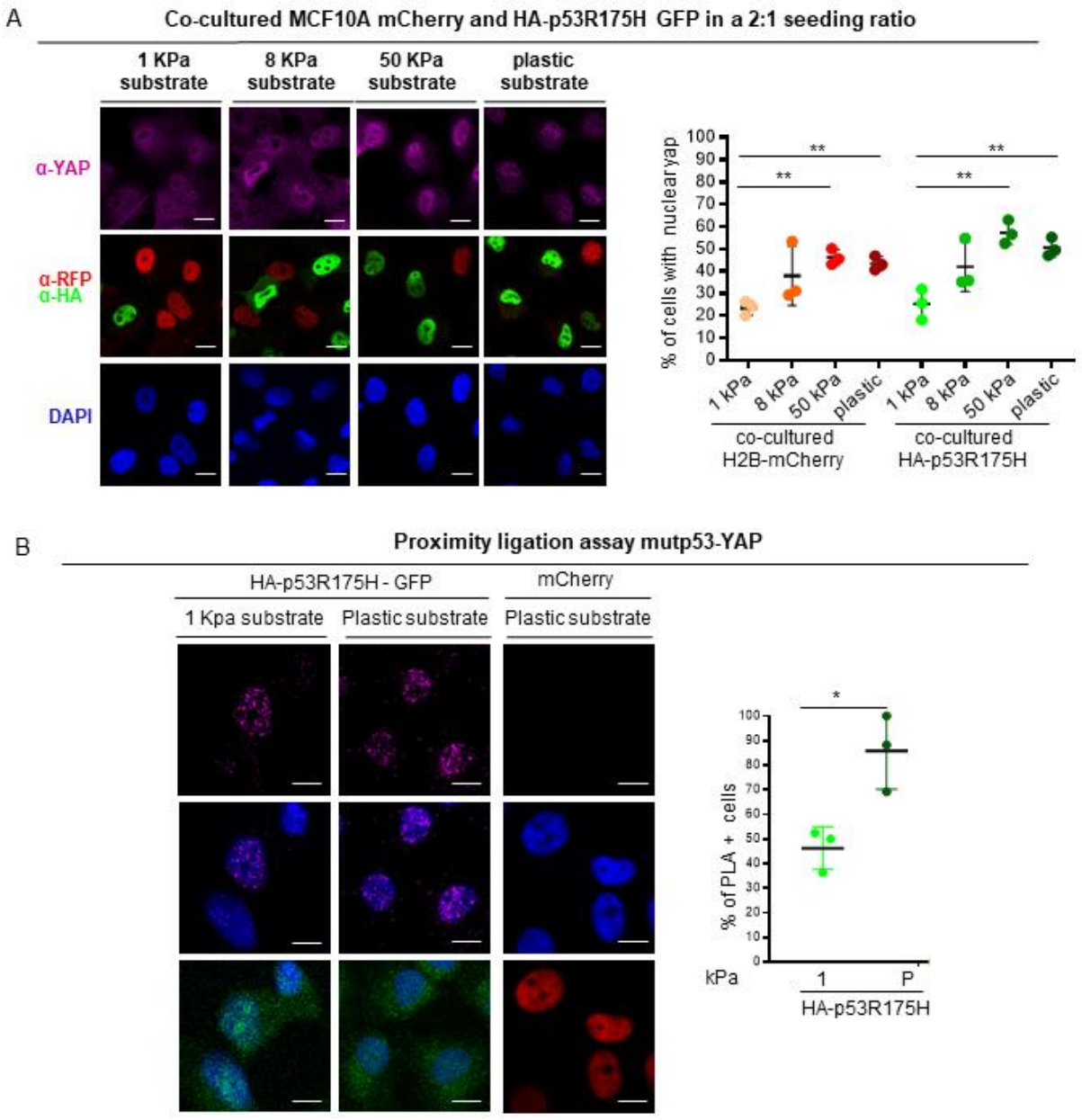

FIGURE 21. Substrate stiffening promotes the interaction between HA::p53^{R175H} and YAP proteins.

A) Immunofluorescence analysis of YAP protein (in magenta) using an anti-YAP antibody in co-cultured mCherry and HA::p53^{R175H} expressing cells (2:1 seeding ratio) on collagen-coated 1,8,50 kPa stiffness hydrogel and plastic substrates. Cells were cultured for 48h in doxycycline-supplemented medium (1 µg/ml). mCherry (in red) and HA::p5:3^{R175H} (in green) proteins were detected by using anti-RFP and anti-HA antibodies. Nuclei are stained with DAPI. Scales bar, 15 µm. The panel is representative of N=3 biological replicates. Quantification (right panel) of co-cultured mCherry and HA::p53^{R175H} cells with nuclear YAP localization (150-400 cells were analyzed in each condition). Values represent the mean ± s.d. **P value <0.01 by two-tailed Student's t- test of N=3 biological replicates. **B)** Proximity ligation assay (PLA) of HA::p53^{R175H} and YAP proteins by using anti-p53 DO-1 and anti-YAP antibodies in HA::p53^{R175H} cells cultured on collagen-coated 1 kPa stiffness substrate (left panel) and on plastic substrate (middle panel). PLA of wt p53 and YAP proteins by using anti-p53 DO-1 and anti-YAP antibodies in mCherry cells grown on plastic substrate (right panel). Cells were cultured for 48h in doxycycline-supplemented medium (1 µg/ml). Quantification (right panel) of HA::p53^{R175H} cell nuclei displaying mutp53-YAP interaction, as shown by PLA, on 1 kPa and on plastic substrates (100 to 250 cells were analyzed). Values represent mean ± s.d. *P value <0.05, by two-tailed Student's t-test of N=3 fields.

Next, we sought to identify direct target genes induced, in winner cells, by YAP and mutp53. To this aim, we generated a signature (which we dubbed mutp53 target genes) of mutp53 direct targets from available mutp53 ChIP-seq datasets (Walerych et al. 2016; Hammal et al. 2022), and assessed the status of these genes in the transcriptomes of co-cultured mutp53/GFP and wt/H2B-mCherry cells. The signature was enriched in genes upregulated in mutp53/GFP winner cells, as shown by GSEA (**Figure 22A**). Then we assessed whether, among mutp53 direct targets upregulated in mutp53/GFP winner cells, we could identify genes targeted also by YAP. For this, first we assessed the status of a YAP direct targets gene signature, from available YAP ChIP-seq experiments in mutp53 breast cancer cells (Zanconato et al. 2015). Also, this signature was enriched in genes upregulated in mutp53/GFP winner cells, as shown by GSEA (**Figure 22B**). Then, among these genes, we could identify a subset that was also targeted by mutp53 and upregulated in mutp53/GFP winner cells (**Table 2**). To assess the impact of this program in winner cells, we then inspected the pathways displaying statistically significant upregulation in co-cultured mutp53/GFP vs wt/H2B-mCherry cells, by GSEA. Interestingly, we found the upregulation of several pathways related to positive regulation of cell proliferation, including DNA replication and cell cycle (**Figure 22C**). This was consistent with the increased proliferation of mutp53/GFP winner cells (**Figure 18**). Of note, GSEA did not show upregulation of NF-Y targets, which were previously proposed to be co-regulated by mutp53 and YAP in cancer cells (Di Agostino et al. 2016). This suggests that, in pre-malignant winner cells, the mutp53/YAP program could involve different transcription factor(s).

Next, we investigated the mechanisms by which mutp53/GFP winner cells could outcompete wt cells. For both mutp53/GFP and wt/H2B-mCherry cells, we analyzed the genes differentially expressed in co-culture vs mono-culture conditions (**Figure 23A**), and identified the pathways specifically upregulated in mutp53/GFP winner cells, compared with wt/H2B-mCherry loser cells, as shown by GSEA NES difference. Then we assessed whether, among these pathways, we could identify signatures of competitive interactions. This analysis highlighted, among the most enriched pathways, the upregulation of signatures related to regulation of the expression of the plasma membrane proteins SLITs, and their ROBO receptors, and of SLIT/ROBO signaling (**Figure 23B**).

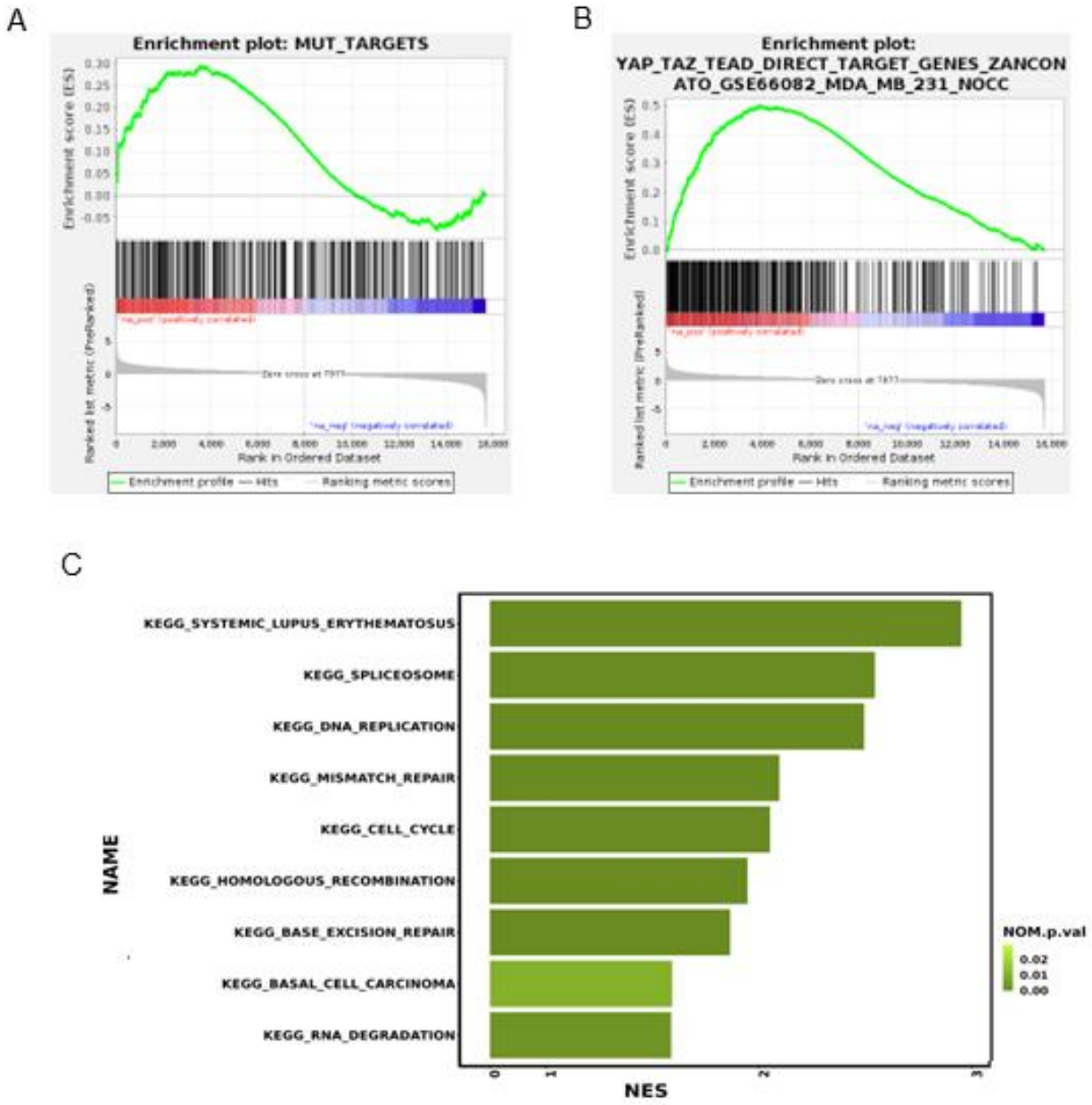

Figure 22. GESEA in co-cultured mutp53/GFP vs wt/H2B-mCherry.

Gene	Gene Name
FZD8	frizzled class receptor 8
BGN	biglycan
KCNJ2	potassium inwardly rectifying channel subfamily J member 2
LRRC15	leucine rich repeat containing 15
SPIN4	spindlin family member 4
DDT	D-dopachrome tautomerase
SPRY2	sprouty RTK signaling antagonist 2
NUF2	NUF2 component of NDC80 kinetochore complex
STAC2	SH3 and cysteine rich domain 2
UBE2S	ubiquitin conjugating enzyme E2 S
TMEM160	transmembrane protein 160
ATAD2	ATPase family AAA domain containing 2
FZD2	frizzled class receptor 2
WDR62	WD repeat domain 62
CACNB1	calcium voltage-gated channel auxiliary subunit beta 1
PATL1	PAT1 homolog 1, processing body mRNA decay factor
F3	coagulation factor III, tissue factor
MAP2K7	mitogen-activated protein kinase kinase 7
ILF3	interleukin enhancer binding factor 3
PRMT5	protein arginine methyltransferase 5
RAB33A	RAB33A, member RAS oncogene family
PARD6A	par-6 family cell polarity regulator alpha
MIR425	microRNA 425

Table 2. mutp53 and YAP targets upregulated in mutp53/GFP winner cells.

This pathway, known to be involved in the regulation of tissue homeostasis and also diseases, including cancer cell survival, growth and migration (Tong et al. 2019; Borrell et al. 2012), has been recently shown to mediate cell competition in different contexts (Vaughen and Igaki 2016; Stine et al. 2014). Interestingly, mutp53/GFP winner cells displayed among the gene upregulated in the SLIT\ROBO signature SLIT2 gene (**Figure 23C**), and our analysis of a YAP direct targets gene signature, from available YAP ChIP-seq experiments in mutp53 breast cancer cells (Zanconato et al. 2015), highlighted that SLIT2 was also a direct target of YAP. On the other hand, wt/H2B-mCherry loser cells expressed the ROBO1 receptor. These data suggest that SLIT/ROBO signaling could play a role in the competition between the two cell types.

Together, these results suggest that ECM stiffening promotes the activation of mutp53/YAP cooperative gene program that leads mutp53 pre-malignant cells to expand at the expense of wt neighboring cells.

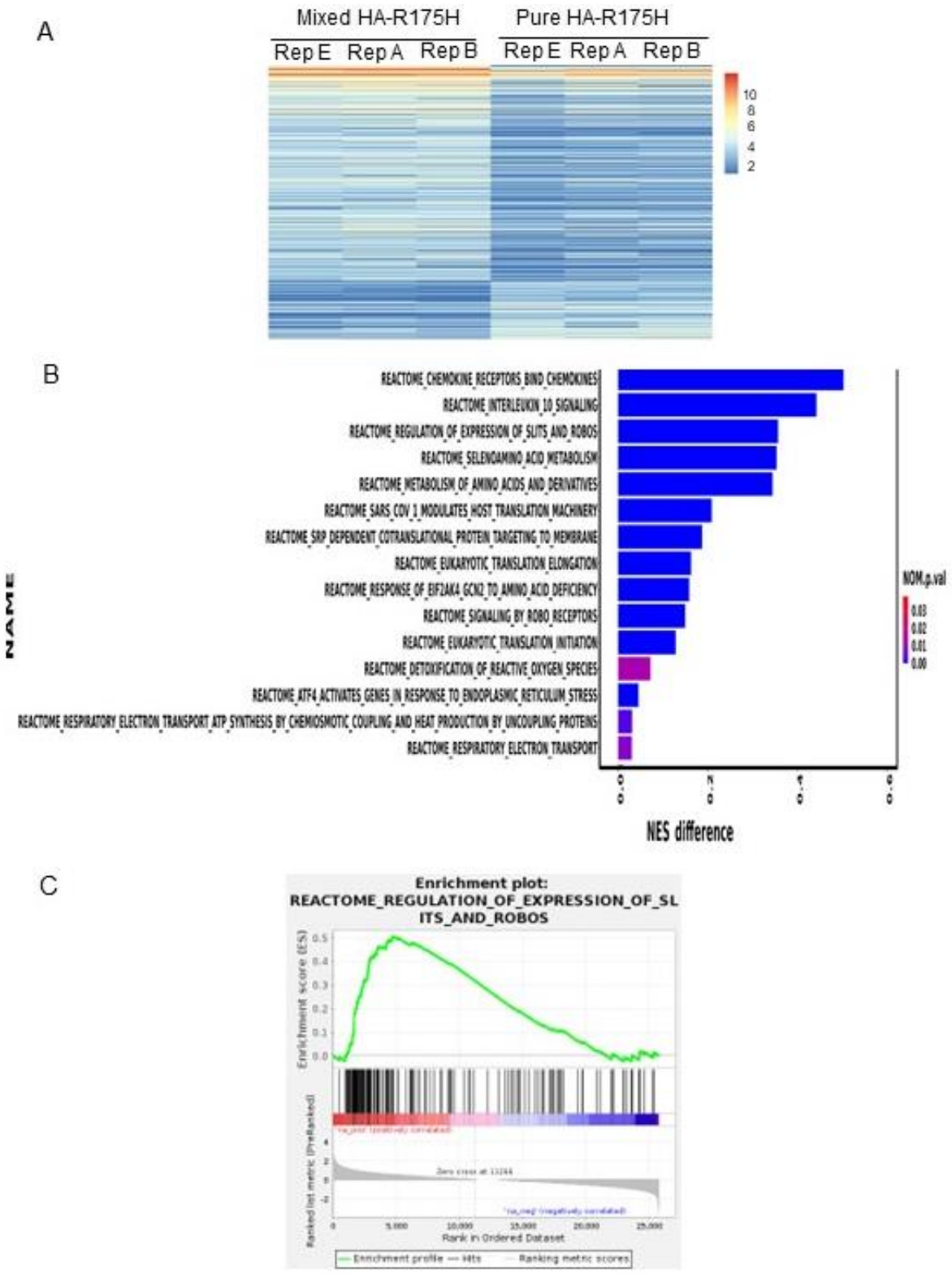

Figure 23. Heatmap (replicate E, A, B) and enrichment plots of MCF-10A mutp53/GFP mono-cultured vs co-cultured with wt/mCherry cells and GSEA of co-cultured MCF-10A mutp53/GFP vs wt/H2B-mCherry.

3.7. Loser wt cells outcompeted by mutp53 pre-malignant cells display signatures of wt p53 activation and ferroptosis.

To investigate the mechanisms by which wt/H2B-mCherry acquire a loser phenotype, we analyzed the genes differentially expressed in co-culture vs mono-culture conditions (**Figure 24A**), and identified the pathways upregulated in wt/H2B-mCherry loser, but not in mutp53/GFP winner cells, by GSEA. This analysis highlighted, among the most enriched pathways, the upregulation of signatures related to wt p53 activation (**Figure 24B-F**). Interestingly, we observed also upregulation of signatures of induction of cell death, and in particular ferroptosis (**Figure 24G**), a pathway that has been previously shown to be regulated by p53 (Jiang et al. 2015).

Then we also assessed whether the acquisition of loser phenotype by wt/H2B-mCherry cells was associated with an increase of wt p53 protein levels. To this aim, we ran the competition assay with the wt/H2B-mCherry and the mutp53/GFP cell lines, on stiff substrates (8 kPa, 50 kPa and plastic), and as control on soft (1 kPa) substrate, in doxycycline-supplemented culture medium, replaced every 48h. In all conditions, during growth (day 2), immunofluorescence with the DO-1 antibody detected the induction of mutp53 in mutp53/GFP cells. Also, on stiff substrates, nuclear wt p53 protein was detected in a fraction of wt/H2B-mCherry cells slightly higher in co-culture than in the mono-culture (**Figure 25A**). This fraction was very low in wt/H2B-mCherry co-cultured and mono-cultured on the soft substrate (**Figure 25A**).

Furthermore, during cell growth (day 3), GFP- and mCherry-positive cells from mono- and co-cultures were FACS-sorted and mutp53 and wt p53 protein levels assessed by western blot with the DO-1 antibody. In mutp53/GFP cells, both proteins were expressed at similar levels in co-culture, as compared with mono-culture, and also did not change on stiff (50 kPa) vs soft (1 kPa) substrate (**Figure 25C**).

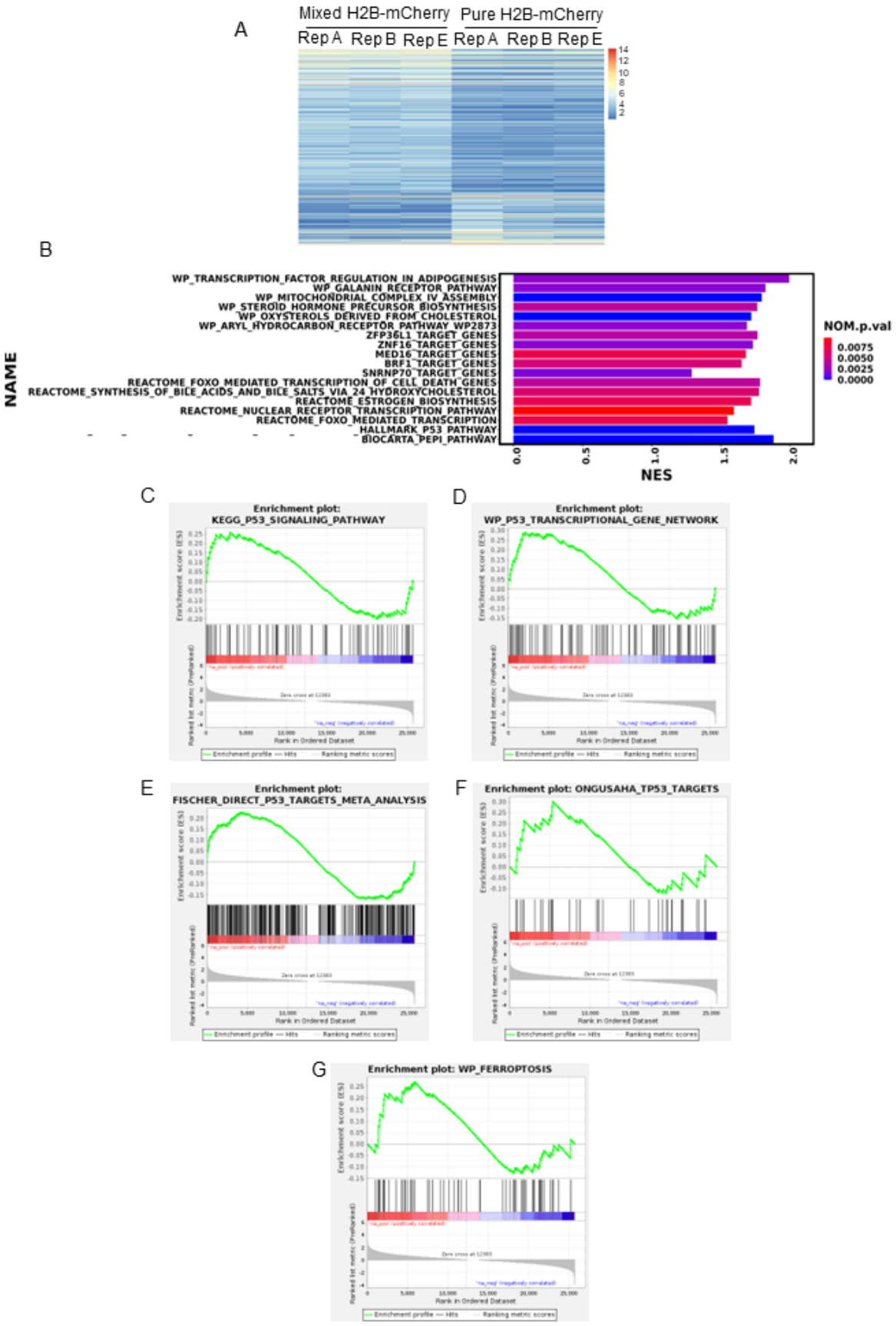

Figure 24. Heatmap and enrichment plots of MCF-10A mCherry cells mono-cultured and co-cultured with mutp53/GFP cells.

In wt/H2B-mCherry loser cells from the co-culture on stiff substrate (50 kPa), wt p53 protein levels displayed an increase, as compared with the respective mono-culture (**Figure 25B**). In contrast, on soft substrate, the wt p53 protein levels in co-cultured wt/H2B-mCherry cells were similar to the respective mono-culture.

Together, these results suggest that ECM leads mutp53 pre-malignant cells to outcompete wt neighboring cells by induction of wt p53 activity and ferroptotic cells death.

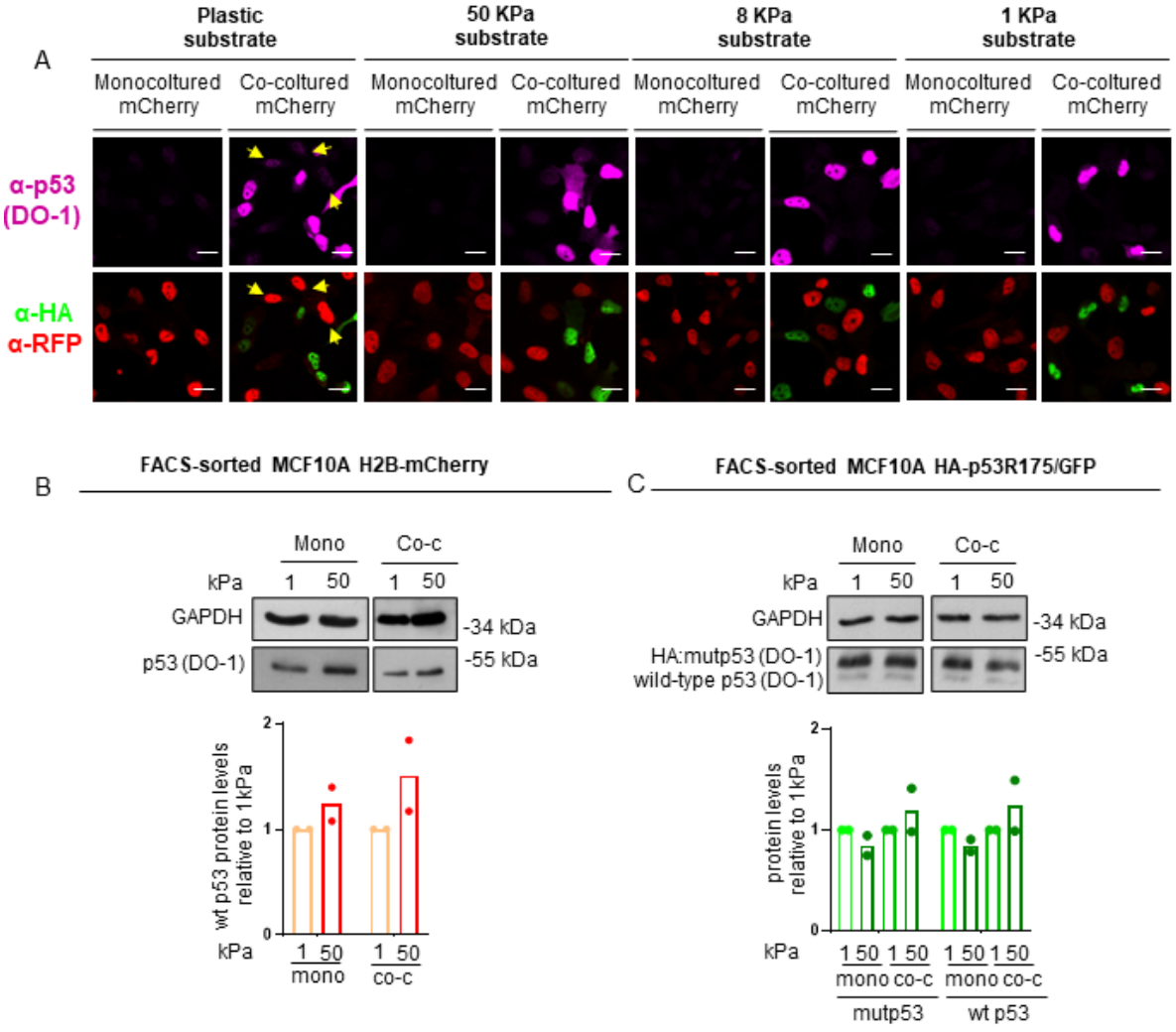

Figure 25. Analysis of wt p53 protein expression in mCherry and mutp53/GFP cells mono-cultured and co-cultured on soft and stiff substrates.

A) Immunofluorescence analysis of wild-type p53 protein (in magenta) using anti-p53 DO-1 antibody in mono-cultured mCherry cells and in mCherry cells co-cultured with HA::p53^{R175H} cells (2:1 seeding ratio) on plastic and on collagen-coated 1,8,50 kPa stiffness hydrogel substrates. Cells were cultured for 48h in doxycycline-supplemented medium (1 µg/ml). mCherry (in red) and HA::p53^{R175H} (in green) proteins were detected by using anti-RFP and anti HA-tag antibodies. Yellow arrows indicate wt p53 positive mCherry cells. Nuclei are stained with DAPI. Scales bar, 15 µm. **B**) Western Blot analysis of wild-type p53 using anti-p53 DO-1 antibody in mCherry cells mono-cultured and co-cultured with HA::p53^{R175H} cells (2:1 seeding ratio) grown on collagen-coated 1 kPa and 50 kPa stiffness hydrogel substrate. Cells were cultured for 96h in doxycycline-supplemented

medium (1 µg/ml), replaced each 48h and then isolated through FACS-sorting to perform Western blot. GAPDH was used as loading control. The blots are representative of N=2 biological replicates. **C**) Western blot analyses of wild-type p53 and HA::p53^{R175H} cells using anti-p53 DO-1 antibody in HA::p53^{R175H} cells mono-cultured and co-cultured with mCherry cells (2:1 seeding ratio in favor of mCherry) grown on collagen-coated 1 kPa and 50 kPa stiffness hydrogel substrate. Cells were cultured for 96h in doxycycline-supplemented medium (1 µg/ml) replaced each 48h and then isolated through FACS- sorting. HSP90 was used as loading control. The blots are representative of N=2 biological replicates.

4. DISCUSSION AND FUTURE DIRECTIONS

4.1. ECM stiffening promotes a super-competitive behavior in mutp53 pre-malignant mammary epithelial cells.

TP53 missense mutations are highly prevalent in many cancers. Importantly, mutp53 cell clones have been found also to accumulate with age in normal conditions, without any apparent histological alteration, with a relatively high frequency in some tissues, such as the esophageal and skin epithelia (Martincorena et al. 2018; Yizhak et al. 2019), and recently also in the mammary tissue (Cereser et al. 2023). Also, this accumulation was found to be promoted in certain conditions to increase somatic mutations rate and cancer risk, such as smoking (Martincorena et al. 2018).

Studies in mouse models have recently shown that, in different tissues, such as the esophageal, skin and intestinal epithelia, pre-malignant cells harboring p53 missense mutations compete for space and survival with wt neighboring cells (Murai et al. 2022; Murai et al. 2018; Fernandez-Antoran et al. 2019; Vermeulen et al. 2013). These studies have provided evidence that, in physiological normal conditions, wt cells are able to outcompete mutp53 cells (Watanabe et al. 2018). However, during aging (Murai et al. 2022) and in aging-associated physio-pathological conditions, such as tissue alterations caused by exposure to mutagens and inflammation, mutp53 could survive and expand (Murai et al. 2022; Murai et al. 2018; Fernandez-Antoran et al. 2019; Vermeulen et al. 2013).

Our results show that mutp53 pre-malignant mammary epithelial cells cultured on substrates with physiological stiffness (1 kPa) do not display a competitive growth advantage over wt neighboring cells. In contrast, on substrates with a stiffness typical of tissue with pathological alterations, such as fibrosis and cancer (8 kPa, 50 kPa and also hyper-stiff plastic), mutp53 cells outcompete wt neighboring cells. This suggests that mechanical stimuli generated by ECM stiffening, could be a key factor for pre-malignant mammary cell expansion. High ECM stiffness associated with fibrosis and inflammation has been shown to favor tumorigenesis in different tissues, including also the esophagus (Naranjo et al. 2020). Thus, our findings could be relevant for the expansion of pre-malignant cells in multiple contexts. In line with our observations, EDAC which was shown to lead to outcompetition of MDCK cells expressing different oncogenes (e.g., $p53^{R175H}$ and RASV12) (Watanabe et al. 2018), by wt neighbors, is impaired by substrate stiffening (Pothapragada et al. 2022). It will be important to further investigate the impact of mechanical, and also other challenges, on the competition of mutp53

pre-malignant with surrounding cells, using in vivo models in which appearance and evolution of mutp53 clones could be mimicked in a tissue context, and also in 3D ex-vivo models. To this aim, we will take advantage of mice in which conversion of wt p53 allele(s) in mutp53 missense mutant allele(s) (R172H and R245W, equivalent to human R175H and R248W) can be obtained in a controlled manner by tamoxifen-inducible CRE recombinase (derived from $p53^{WM}$ mice (Y. Zhang et al. 2018)). In these mice, we have introduced a Lox-membraneTomato-STOP-Lox-membraneGFP (mTmG) reporter, which allows to label all cells with Tomato, and upon CRE recombination, cells expressing mutp53 can thus traced with GFP. These mice will be used to generate wt/mutp53 cells mosaic organoids from different tissues (e.g. breast, esophagus), which will be subjected to micro-environmental challenges (e.g. mechanical stress by culturing in matrices with pathological stiffness), and wt/mutp53 cells competition phenotypes (proliferation, survival, death) can be assessed.

Our data also suggest that interfering with ECM stiffening, for example with inhibitors of collagen cross-linking, could dampen the competitive growth advantage of mutp53 pre-malignant cells.

4.2. mutp53 super-competitors display activation of a mutp53/YAP cooperative gene program.

Multiple evidence has shown that, in different contexts, such as embryonic development and tissue homeostasis, differences in p53 status and activation in cells, including loss and missense mutations, can trigger cell competition (Bowling et al. 2018; Bondar and Medzhitov 2010). Our results suggest that, in pre-malignant mammary cells, mutp53 could play a key role in the acquisition of a winner phenotype, through a GOF transcriptional program. These results were obtained by analyzing the transcriptome of an MCF-10A cell line expressing exogenous mutp53, GFP and endogenous $TP53^{+/+}$ alleles (mutp53/GFP), compared with co-cultured H2B-mCherry-expressing $TP53^{+/+}$ (wt/H2B-mCherry) MCF-10A cells. From this analysis, we identified mutp53 target genes upregulated in mutp53/GFP cells. In these cells mutp53 expression was controlled by an inducible promoter, and its induction led to expression of mutp53 protein at supraphysiological levels, higher than the endogenous promoter. However, our results were validated by verifying that genes upregulated by mutp53 in $TP53^{R175H/R175H}$ knock-in MCF-10A cells, in which mutp53 is expressed at physiological level (Redman-Rivera et al. 2021), were enriched in mutp53/GFP cells.

Mechanistically, our work provides evidence that ECM stiffening leads to activation of YAP,

which binds and could cooperate with mutp53 acting as a transcriptional co-factor. This leads to the activation of a mutp53 GOF transcriptional program. Our data show that this program includes some genes previously identified as mutp53 and YAP/TEAD targets. Hence, it will be interesting to assess the role of TEAD transcription factors in mutp53 winner cells, for instance analyzing whether these factors bind mutp53. The identified program may also involve yet-to-be identified transcription factors. Thus, it will be important to assess the landscape of mutp53/YAP direct targets by integrating our RNA-seq with (epi)genomic analyses, such as mutp53 and YAP ChIP-seq in winner vs loser cells.

The activation of TEAD targets could contribute to the increased cell proliferation, which was suggested by our BrdU incorporation analysis in winner cells. We also assessed how the mutp53/YAP gene program could drive the super-competitive behavior of mutp53 cells, which involves the interaction with wt neighboring cells, leading to elimination of these cells. Interestingly, in mutp53 winner cells, we found increased expression of SLIT2, and our GSEA also showed that this gene was targeted by YAP in mutp53 cells (Zanconato et al. 2015), and that SLIT/ROBO signaling was activated. Interestingly, a recent study has shown that, in the *Drosophila* wing epithelium, scribble-/- cells surrounded by wt cells displayed expression of the SLIT ligand receptor ROBO2. Conversely, wt cells expressed the SLIT2 ligand, which bound ROBOs, leading to the extrusion of scribble-/- cells from the epithelium. Notably, wt/H2B-mCherry loser cells displayed upregulation of the SLIT receptor ROBO1. It is then conceivable that SLIT2, upregulated in mutp53 winner cells, could bind ROBO1 in surrounding wt cells, and this could lead to the acquisition of wt cell loser phenotype. Hence, it will be interesting to assess whether interfering with this signalling pathway could modulate competition between wt and mutp53 pre-malignant cells.

Work from our lab and others has shown that mutp53 is stabilized by mechanical stimuli, including those generated by a stiff ECM, through RhoA-dependent acto-myosin contraction (Ingallina et al. 2017; Sorrentino et al. 2014). Our data are consistent with a model in which, in pre-malignant cells, ECM stiffening could promote both mutp53 stabilization and YAP activation, thus inducing a mutp53 GOF winner gene program. This model could be validated by assessing whether mechanical stimuli could lead to mutp53 stabilization in cellular models and tissues from $p53^{WM}$ mice, and whether this led to mutp53 program, also by in situ transcriptomic analyses at single cell level.

The 2D cell culture models that we established, and also ex vivo models from $p53^{WM}$ mice, could be suitable systems to assess whether interfering with mutp53/YAP interaction could

dampen the competitive growth advantage of mutp53 pre-malignant cells, and tilt the competition in favor of wt cells. In this regard, it will be important to map the mutp53 protein domain required for interaction with YAP. Of note, work from our group previously identified aptamer peptides that target different mutp53 protein domains (Lisek et al. 2018), and these molecules could be tested for their ability to bind the mutp53 protein domain required for interaction with YAP and thus inhibit the mutp53/YAP gene program. In addition, PLA which has been previously used for cell-based High Throughput Screening (HTS) (Kiehn and Car 2017), may be exploited to identify molecules that inhibit mutp53/YAP interaction, and thus mutp53 winner cell expansion, for instance by a repositioning screen of FDA-approved drugs. Clones with cancer-driving mutations have been found to accumulate in multiple normal tissues, however, only some of these clones eventually evolve into cancer. The availability of tools to identify and/or predict the evolution of these high-risk clones, could be relevant for prevention/early detection of tumorigenesis. In this regard, the mutp53/YAP target genes identified by our analysis represent a signature of mutp53 pre-malignant cells whose expansion is promoted by tissue microenvironmental alterations (i.e. ECM stiffening) know to increase the risk of tumorigenesis. It will be important to validate this signature using in vivo models and in available datasets of human (pre)-cancer tissue.

In addition, the GSEA showed that in the mutp53 winner cells, also some metabolic pathways were altered, for instance regulation of aminoacid metabolism, upregulation of mevalonate pathway and of mitochondrial translation (data not shown). Interestingly, work from our group recently showed that, in breast cancer cells, mutp53 GOF leads to upregulation of a serin-glycine synthesis and essential aminoacid uptake, favoring growth and malignancy (Tombari et al. 2023). Also, previous work from our lab showed that GOF mutp53, upon stabilization by mechanical stimuli, promotes mevalonate pathway activation, enhancing cancer cell stemness and growth (Sorrentino et al. 2014). Hence, it is conceivable that regulation of these metabolic pathways could also contribute to the acquisition of the winner phenotype in mutp53 pre-malignant cells. This is supported by recent evidence that, in pre-neoplastic cells, increase of mutp53 protein levels, as shown by traceable mutp53 fusion proteins, was associated with upregulation of serine synthesis (Yao et al. 2023).

4.3. wt cells interacting with mutp53 super-competitors are eliminated by cell death.

In different cell competition contexts, outcompeted cells have been shown to acquire a loser phenotype through various mechanism, including stem cell differentiation, senescence, extrusion from epithelia and death pathways (e.g., apoptosis, necroptosis, entosis). Our results suggest that mutp53 winner pre-malignant cells could induce ferroptosis in surrounding wt cells. It will be important to functionally validate the role of ferroptosis in wt/mutp53 cells competition. In this context, inhibition of this pathway may be sufficient to restrain mutp53 expansion.

In epithelial cell competition, loser cell death has been frequently found associated with extrusion from the epithelial layer. Interestingly, a recent study showed that, when cultured on substrate with physiological stiffness (around 1 kPa), mutant RASV12-expressing MDCK epithelial cells were outcompeted, undergoing extrusion, by wt neighboring cells (Pothapragada et al. 2022). In contrast, on substrate with pathological stiffness, RASV12-expressing cells were retained in the epithelial layer. These data further support our results showing that ECM stiffening could be a key factor in the regulation of cell competition. It would be interesting to assess whether extrusion contributes to the elimination of also wt/H2B-mCherry loser mammary cells outcompeted by mutp53 cells.

Furthermore, our results showed that wt/H2B-mCherry loser mammary cells also display activation of wt p53, which has been shown to induce cell death through multiple mechanisms, including apoptosis, necrosis and also ferroptosis (Aubrey et al. 2018; Jiang et al. 2015; Napoletano et al. 2017). This induction has been shown to drive the acquisition of loser cell phenotype through different mechanisms. For instance, by induction of senescence (Bondar and Medzhitov 2010) and also extrusion upon mechanical compaction due to cell crowding (Wagstaff et al. 2016; Kozyrska et al. 2022). It is thus conceivable that, in wt/H2B-mCherry loser mammary cells, wt p53 activation could result from compaction due to cell crowding caused by mutp53 proliferation. This could be assessed by time-lapse imaging of mutp53 cells competing with wt cells expressing wt p53 activation reporters.

5. MATERIAL AND METHODS

Cell cultures

MCF-10A were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/HAM'S F12 (1:1) supplemented with 5% Horse Serum (HS), 100 U/mL penicillin and 10 µg/mL streptomycin, 20 ng/ml recombinant human epidermal growth factor (EGF), 10 µg/ml recombinant human insulin, 500 ng/ml hydrocortisone. Mycoplasma free cultures were used, as assessed by myco plasma test.

Reagents and plasmids

Depending on the experiment, cells were treated with the following compounds at the concentration and for the time indicated in the figure legends: 1 µg/ml Doxycycline (Sigma, D9891); 250 ng/ml Doxorubicin (Sigma, D1515); Thermofisher (33342) 0.2 µg/ml.

The retroviral construct pBABE-mCherry was kindly provided by Dr. Giorgio Scita (IFOM Milan, Italy). The pCW57-tGFP-P2A-HA::p53^{R175H} lentiviral vector was previously generated in that lab, by cloning the HA tagged human *TP53* mutated sequence into the pCW57-GFP-P2A-MCS vector (Addgene #71783).

Lentiviral and retroviral infection

The MCF10-A cell line expressing the H2B-mCherry red fluorescent protein was obtained by transducing MCF-10A cells with the retroviral pBABE H2B-mCherry construct. For retroviral production we used Phoenix-GP cells transfected using PEI 2X (1 mg/ml) with 10 µg of the pBABE H2B-mCherry construct and 5 µg of PMD2G.

The MCF-10A cell line co-expressing HA-p53R175H and turbo GFP fluorescent protein was obtained by transducing MCF-10A cells with the lentiviral pCW57-tGFP-P2A-HA::p53^{R175H}. Lentiviral preparation was obtained using HEK-293T cells transfected using PEI 2X (1 mg/ml) with 10 µg of pCW57-tGFP-P2A-HA::p53^{R175H} and 3,5 ug of PMD2 and 7,5 ug of ps-PAX2 packaging vectors.

For both retro and lenti viral preparations, the supernatant containing viral particles was collected after 48-72h, then it was centrifuged 5 min at 500g and filtered through 0.45 µm low-

protein binding filter. Then, the virus-containing medium was added to MCF-10A cells for 24h and cells were then selected in puromycin-supplemented medium 1 µg/ml.

For each experiment, the transduced cell lines were preliminary cultured for 72h in medium supplemented with sublethal doses of puromycin 1 µg/ml.

Cell competition assays

Cell competition assays were performed on collagen-coated (0.02 mg/ml) hydrogels with 1, 4, 8, 50 kPa elastic moduli (Matrigen Softwell SW6-COL-1/4/8/50) and on plastic. MCF-10A stably infected with the retroviral pBABE-mCherry construct, or the lentiviral pCW57-tGFP-P2A-HA::p53^{R175H} construct, were cultured as mono-cultures or as co-cultures with a 2:1 seeding ratio in favor of mCherry cells and were seeded at day 0 at 530 cells/mm^2, 420 cells/mm^2, 370 cells/mm^2, 320 cells/mm^2 on collagen-coated 1, 4, 8, 50 kPa hydrogel and plastic substrates, respectively). Cells were cultured in Doxycycline (1 µg/ml) and Hoechst (0.2 µg/ml) supplemented medium, replaced every 48h. Every 24 hours pictures of N=3 fields per condition were acquired through live imaging analysis with Nikon Eclipse Ti microscope. The number of Hoechst positive, mCherry positive and HA::p53^{R175H}/GFP cells was quantified through Fiji software.

Protein extraction

Prior to protein extraction cells were trypsinized from plastic or from collagen-coated 1 kPa and 50 kPa stiffness hydrogels. After 96h from seeding, mCherry cells and HA::p53^{R175H}/GFP cells grown in co-culture were isolated by sorting (BD FACSAria II) using 488 wavelength excitation and with 610/20 nm and 530/30 nm wavelength emission, respectively.

Then, the collected cells were lysed in Lysis Buffer solution composed by NP40 1%, Tris-HCL pH=8 50 mM, NaCl 150 mM, EDTA 1mM with the addition of proteases and phosphatase inhibitors i.e. PMFS 1 mM (Sigma-Aldrich), NaF 5 mM (Sigma-Aldrich), Na3VO4 1 mM (Sigma-Aldrich), CLAP 10 µg/mL (Sigma-68 Aldrich). The amount of proteins in each sample was quantified with Bio-Rad Protein Assay Reagent (Bio-Rad). The samples were then sonicated in Laemmli Buffer and boiled at 95 °C for 5 min.

Western blot

Purified proteins were loaded and separated by SDS-PAGE. Stacking gel was composed by 5% acrylamide, 0.14% bisacrylamide, 0.125M Tris-HCl pH 6.9, 0.1% SDS. Running gel was composed by 12.5% or 17.5% acrylamide, 0.1% bisacrylamide, 0.374 M Tris-HCl pH 8.7, 0.1% SDS. Then proteins were transferred from gel to nitrocellulose membranes (Amersham) using a transfer buffer solution containing Tris 48 mM, glycine 39 mM, SDS 4.13 mM, 20% methanol. Membranes were incubated in Blotto-tween solution composed by 0.2% Tween-20 and not fat dry milk 5% in PBS. After incubation with primary antibodies, membranes were incubated with anti-mouse, or anti-rabbit, or anti-rat HRP-conjugated secondary antibodies (Sigma-Aldrich). Membranes were analyzed by chemiluminescence using Pierce ECLTM Western Blotting Substrate or Pierce ECLTM Plus Western Blotting Substrate.

Primary antibody used were the following: mouse-anti p53 DO-1 (Santa Cruz, sc-126) 1:1000 or 1:5000; rat anti-HA tag (Roche, 11867423001) 1:2000; rabbit anti-p21 (Santa Cruz, sc-397) 1:500; mouse anti-GAPDH (Santa Cruz, sc-32233) 1:2000; mouse anti-HSP90 (Santa Cruz, sc-13119) 1:10000.

Immunofluorescence

Immunofluorescence analyses were performed in cells cultured on Poly-L-lysine coated glass slides or on 0.02 mg/ml type I-collagen-coated slides on 1, 8, 50 kPa hydrogels (Matrigen softslip, SS12-COL-1/8/50-EA). Cells were washed twice in PBS and then fixed in 4% paraformaldehyde at room temperature (RT) for 15 minutes. Cells were then rinsed in PBS and permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at RT. After incubation with 3% Fetal Bovine Serum FBS (blocking solution) in PBS for 30 minutes at RT, cells were incubated with the specific primary antibody diluted in blocking solution at 4°C for 16 hours in a humidified chamber. Samples were then incubated with Alexa Fluor secondary antibodies conjugated with fluorescent dye in blocking solution at 37°C for 45 minutes. Nuclei were stained with DAPI (Sigma, D9542) at RT for 10 min, and slides were mounted with Prolong Gold fluorescence anti-fading reagent (Invitrogen, P36930).

Primary antibodies used were the following: mouse-anti p53 DO-1 (Santa Cruz, sc-126) 1:100; mouse anti turbo-GFP (Origene, TA150041) 1:100; rabbit anti-turbo-GFP (Invitrogen) 1:100;

rabbit anti-RFP (Invitrogen, R10367) 1:600; rat anti-HA tag (Roche, 11867423001) 1:200; mouse anti-YAP (Santa Cruz, sc-101199) 1:200.

Secondary antibodies (Life technologies) were used at 1:500 dilution and were the following: goat anti-mouse Alexa Fluor 488; donkey anti-mouse Alexa Fluor 647; goat anti-rabbit Alexa Fluor 488; goat anti-rabbit Alexa Fluor 568; donkey anti-rat Alexa fluor 488; donkey anti-rat Alexa fluor 594.

BrdU incorporation assay

1x10^4 MCF10-A cells were seeded on plastic 6-well plates, cultured in medium with 1 µg/ml doxycycline and changed each 48h. After 96h from seeding, medium was changed and the DNA precursor bromodeoxyuridine (BrdU) (20 µM) was added for 4h. Then cells were fixed in 4% paraformaldehyde at RT for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at RT followed by washes in PBS. After incubation with 3% Fetal Bovine Serum in PBS (blocking solution), cells were washed three times with NaOH 50 mM solution and washed again in PBS. Then, cells were subject to BrdU immunofluorescence. Cells were incubated with primary anti-BrdU antibody solution at 37°C for 1h and then they were incubated with secondary donkey anti-mouse Alexa fluor 647 (Life Technologies) antibody (1:500 dilution). Nuclei were stained with DAPI (Sigma, D9542) at RT for 10 min and slides were mounted with Prolong Gold fluorescence anti-fading reagent (Invitrogen, P36930).

Proximity ligation assay (PLA)

Proximity ligation assay (PLA) was performed using Duolink *in situ* PLA probe anti-Mouse and anti-Rabbit (Sigma Aldrich, DUO92004 and DUO92002) and the *in situ* detection reagent far red (DUO92013) according to the manufacturer's protocol. Nuclei were stained with DAPI (Sigma, D9542) at RT for 10 min and slides were mounted with Prolong Gold fluorescence anti-fading reagent (Invitrogen, P36930).

Primary antibodies were incubated 16h at 4°C in a humidified chamber and were the following: rabbit anti-YAP (Cell Signalling Technologies, 4912) 1:50 and mouse anti-p53 DO-1 (Santa Cruz, sc-126) 1:50.

TUNEL assay

Cells grown on plastic substrate or on collagen-coated (0.02 mg/ml)1 kPa stiffness hydrogels were fixed in 4% paraformaldehyde at RT for 15 minutes, washed in PBS and stained with Click-it Plus TUNEL assay with Alexa Fluor 647 dye (Invitrogen, C10619) according to the manufacturer's protocol. The standard protocol was implemented by performing a permeabilization step incubating cells in sodium citrate 100 mM, 0.25% Triton X-100 in PBS at 65°C for 30 minutes, followed by further cell permeabilization in 0.25% Triton X-100 PBS for 30 minutes. Nuclei were stained with DAPI (Sigma, D9542) at RT for 10 min and slides were mounted with Prolong Gold fluorescence anti-fading reagent (Invitrogen, P36930).

Image acquisition and analysis

Images of stained samples were acquired with a ZEISS LSM 880 confocal microscope while pictures of cells in live imaging were acquired with Nikon Eclipse Ti microscope. Images were analyzed by FIJI Software.

RNA extraction and qRT-PCR

For total RNA extraction cells were harvested in Tryzol lyses reagent (Qiagen)**.**
Using the iScript™ Advanced cDNA Synthesis Kit (Biorad, 172-5038) the extracted RNA was retrotranscribed to synthesize cDNA. Then, real-time quantitative PCR on the cDNA template was performed using the SsoAdvanced SYBR Green Supermix (BIORAD) reagent and a BIORAD CFX96 Touch TM Real-Time PCR Detection System thermocycler. Quantification was based on the 2-ΔΔCt method using *H3* or *GAPDH* as reference. Each experimental sample is the average of a technical duplicate. PCR primer sequences were the following:

Gene target	**Primer sequence**
Human PSMB9	FW: GGCGTTGTGATGGGTTCTGA
	REV: AGAGAGTGCACAGTAGATGCG
Human CTGF	FW: AGGAGTGGGTGTGTGACGA
	REV: CCAGGCAGTTGGCTCTAATC
Human Cyr61	FW: AGCCTCGCATCCTATACAACC
	REV: TTCTTTCACAA GGCGGCACTC

Human H3	FW: GTGAAGAAACCTCATCGTTACAGGCCTGGT
	REV: CTGCAAAGCACCAATAGCTGCACTCTGGAA
Human GAPDH	FW: GAGTCAACGGATTTGGTCGT
	REV: GACAAGCTTCCCGTTCTCAG

RNA-seq

MCF10-A stably infected with the retroviral pBABE H2B-mCherry or with the lentiviral pCW57-tGFP-P2A-HA::p53^{R175H} constructs were cultured as mono-cultures or as co-cultures with a 2:1 seeding ratio in favor of mCherry cells and were seeded at day 0 at the same total cell density (320 cells/mm^2) in doxycycline-supplemented medium, changed after 48h.

After 72h from seeding, cells were trypsinized and centrifuged for 5 minutes at 500g. Then cells were resuspended in PBS supplemented with 5 mM EDTA and filtered using 0.70µm filter. Then, mCherry positive cells and HA::p53^{R175H} /GFP positive cells were isolated by sorting (BD FACSAria II) using 488 wavelength excitation and with 610/20 nm and 530/30 nm wavelength emission, respectively.

During sorting, cells were collected in the MCF-10A medium and rinsed twice in PBS. Samples were lysed in Qiazol lysis reagent (Qiagen) and RNA was extracted.

The RNA quality was checked by using the Agilent RNA ScreenTape System on TapeStation 4150 (Agilent).

RNA libraries were obtained by TruSeq stranded total RNA with Ribo-Zero Gold library construction kit and were sequenced using Illumina NovaSeq 6000 for 150 bp paired-end sequencing.

RNA-seq data analysis

Reads quality was verified using fast QC (version 0.11.9; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were trimmed for adapters, polyA read-through, and low-quality tails (quality <Q20) using TRIMGALORE (version 0.6.7), resulting on average 7M trimmed reads per sample. Reads were subsequently aligned to the human reference genome (hg38) using STAR (version 2.7.10b) with default parameters and counted using htseq-count. Trimming and alignment were verified using MultiQC (version 1.10). Differential gene expression analysis was performed using the DESeq function and Principal Component Analysis was performed using the plotPCA method of the DESeq2 package (version 1.40.1). Over-representation analysis was performed using Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea/index.jsp, version 4.3.2) using the

statistical value derived from DESeq2 result table and gene sets of the Hallmark, Biocarta, KEGG, PID, Reactome, WikiPathways, microRNA and transcription factors targets, gene ontology (GO), oncogenic and immunologic gene set collections from the Broad Institute Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb). GSEA software was applied on expression data of cells transfected with pCW57-tGFP-P2A-HA::p53^{R175H} or with a pBABE-H2B-mCherry. Gene sets were considered significantly enriched at pvalue < 0.05 when using 1,000 permutations of gene sets. Gene expression heatmaps have been generated calculating the variance stabilizing transformation (VST) from raw expression values. Barplot and PCA plots has been realized using the R package ggplot2 (version 3.4.3), while the heatmaps using the R package pheatmap (version 1.0.12). Enrichment plots have been done by the GSEA software. All analyses were performed using R 4.3.1 and publicly available packages explicitly cited in the manuscript. No custom functions were written for the analysis.

Retrieving direct targets from REMAP2022 and BAER

The lists of direct targets of TP53 and YAP have been retrieved from REMAP2022 database (https://remap2022.univ-amu.fr/) and, subsequently divided depending on the mutational status of TP53 in each cell line into: breast cancer cell line with missense TP53, non-breast cancer cell line with missense TP53, breast cancer cell line with wt TP53, non-breast cancer cell line with wt TP53 and healthy tissue cell line. An additional list of TP53 direct targets have been retrieved from the Binding And Expression Resource (BAER) hub (https://orio.niehs.nih.gov/ucscview/nguyen/p53BAER/hub.txt).

Statistics

Experiments are representative of at three independent experiments, unless otherwise stated. Graph bars represent mean ± s.d. from at least n=3 biological replicates. Each blot and IF panel is a representative image of the experiments showing similar results. P values were determined using the following statistic tests as indicated in figure legends: two-tailed Student's t-test, with a 95% confidence threshold; One-way ANOVA (Fisher's LSD) ($\alpha<0.05$) Exact test p-value (FDR) ($\alpha<0.05$); Wilcoxon Rank Sum test (pval) and Bonferroni correction (padj) ($\alpha<0.05$); Wald test and Bonferroni correction (padj) ($\alpha<0.05$).

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