Kozyrska, K., Pilia, G., Vishwakarma, M., Wagstaff, L., Goschorska, M., Cirillo, S., Mohamad, S., Gallacher, K., Carazo Salas, R. E., & Piddini, E. (2022). p53 directs leader cell behavior, migration, and clearance during epithelial repair. *Science*, 375(6581), [eabl8876]. <https://doi.org/10.1126/science.abl8876>

Peer reviewed version

Link to published version (if available):
[10.1126/science.abl8876](https://doi.org/10.1126/science.abl8876)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via American Association for the Advancement of Science at <https://www.science.org/doi/10.1126/science.abl8876> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: <http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

p53 directs leader cell behavior, migration and clearance during epithelial repair

Kasia Kozyrska^{1,2,†‡}, Giulia Pilia^{1,†}, Medhavi Vishwakarma^{1,§,¶}, Laura Wagstaff^{2,§,#}, Maja Goschorska¹, Silvia Cirillo¹, Saad Mohamad¹, Kelli Gallacher^{1**}, Rafael E. Carazo Salas¹ and Eugenia Piddini^{1*}

¹ School of Cellular and Molecular Medicine, University of Bristol; Bristol, BS8 1TD , UK.

² The Wellcome Trust/Cancer Research UK Gurdon Institute and Zoology Department, University of Cambridge; Cambridge, CB2 1QN, UK.

[†] Joint First authors

[§] Equal contribution

[‡] Present address: Horizon Discovery Ltd.; Cambridge, CB25 9TL, UK

[¶] Present address: Centre for BioSystems Science and Engineering, Indian Institute of Science Bangalore, Karnataka, 560012, India

[#] Present address: School of Biological Sciences, University of East Anglia; Norwich, NR4 7UA, UK

^{**} Present address: Department of Biology and Biochemistry, University of Bath; Bath, BA2 7AY, UK

^{*} Correspondence to: eugenia.piddini@bristol.ac.uk

Abstract

Epithelial cells migrate across wounds to repair injured tissue. Leader cells at the front of migrating sheets often drive this process. However, it is unclear how leaders emerge from an apparently homogeneous epithelial cell population. Here, we characterized leaders emerging from epithelial monolayers in cell culture and found that they activated the stress sensor p53, which was sufficient to initiate leader cell behavior. p53 activated the cell cycle inhibitor p21^{WAF1/Cip1}, which in turn induced leader behavior via inhibition of cyclin-dependent kinase (CDK) activity. p53 also induced crowding hypersensitivity in leader cells such that, upon epithelial closure, they were eliminated by cell competition. Thus, mechanically-induced p53 directs emergence of a transient population of leader cells that drive migration and ensures their clearance upon epithelial repair.

One Sentence Summary

Injury promotes epithelial repair by inducing activation of p53 and p21^{WAF1/Cip1} in leader cells, which drive cell migration.

Main Text

When the integrity of an epithelial monolayer is compromised by injury or wounding, epithelial cells extend and migrate over the exposed area to seal the open space. In some epithelia, wound closure is driven by leader cells, which are located at the edge of the epithelial front and guide the collective migration of epithelial sheets (1-8). Follower cells trail behind as mechanically and physically coherent protrusions, advancing gap closure (3, 4, 9). Leader cells have a characteristic flattened morphology, distinct cytoskeletal properties and activate specific migratory pathways (2, 5, 10, 11), however they originate from the same epithelial cells as follower cells. How leader cells arise from a seemingly homogeneous population of epithelial cells is unclear. Studies have proposed that geometrical and mechanical cues at the wound edge may induce asymmetry and that this may be sufficient to instruct the leader cell fate (12-15). However, only a few cells at the wound edge become leaders suggesting that additional factors are involved.

p53 elevation induces leader cell behavior

Madin-Darby Canine Kidney (MDCK) epithelial cells are a well-characterized model for investigating leader cell migration and epithelial repair (2, 9, 10, 14, 15). While imaging colonies of wild-type MDCK cells, we observed the emergence of cells with a flattened morphology, resembling senescent cells (16) (Fig. 1A). Upon contact with colonies of cells with epithelial morphology, these flat cells led the colonies in directed migration (90% of cases, n= 112, Fig. 1A-C, S1A-B and Movie S1). Such collectively migrating finger-like structures have been previously observed in MDCK cells (10) and are reminiscent of leader-driven migration in scratched epithelial

monolayers (2, 9, 11). Because these cells appear in the absence of epithelial injury, we termed them 'spontaneous leaders'.

Leader cells have increased surface area, frequent binucleation and lower division rates compared to normal epithelial cells (2, 9, 11, 17). Spontaneous leaders shared these features -- they divided only in 2.5% of the cases observed (n=89) during the course of our movies (> 48 hours) and were often binucleated (Fig. S1C). To determine if binucleation is sufficient to induce leader status we treated MDCK cells with the myosin inhibitor blebbistatin, which prevents cytokinesis while allowing karyokinesis to proceed (18). To visualize blebbistatin-induced binucleated cells (BBCs), we treated cells expressing nuclear GFP with blebbistatin and co-cultured them with untreated, unlabeled wild-type cells. The resulting BBCs were morphologically similar to spontaneous leaders and led migration, recruiting unlabeled wild-type cells as followers (Fig. S1D).

The 'tetraploidy checkpoint' triggered upon cytokinesis failure induces cell cycle arrest in a p53-dependent manner (19). Indeed, p53 levels were elevated in BBCs (Fig. S1E-F). To determine p53 levels in spontaneous leaders, we used live imaging to identify and track spontaneous leaders (Fig. 1D, first three panels), and then located them following fixation. Immunostaining revealed that spontaneous leaders exhibited significantly higher p53 levels than neighboring non-leader cells (Fig. 1D, rightmost panel and Fig. 1E).

To determine if elevated p53 induces the leader cell fate, we treated wild-type MDCK cells expressing nuclear GFP with the DNA damaging agent Mitomycin C (MMC) (20), to stabilize p53 irreversibly (Fig. S1G). After extensive washing to prevent MMC carryover, we co-seeded MMC-treated, GFP-labelled cells with untreated, unlabeled wild-type cells (Fig. 2A). MMC treatment

was sufficient to induce the formation of migrating ‘fingers’ of untreated follower cells led by MMC-treated cells (Fig. 2B-D, S1H-I, and Movie S2), suggesting that p53 elevation is sufficient to trigger the leader phenotype.

Beside p53 activation, MMC causes extensive DNA damage response and reactive oxygen species activation (21, 22). To activate p53 more specifically and in the absence of DNA damage we used Nutlin-3, an Mdm2 inhibitor that stabilizes p53 (23), at doses that slow down but do not arrest cell proliferation (24) (Fig. S1J-K). We co-cultured GFP-labelled wild-type cells with *p53* knockout (*p53KO*) cells (24) in the presence of Nutlin-3, so that p53 would be elevated only in one population. Under these conditions, p53 activation induced a leader phenotype in wild-type cells, while *p53KO* cells behaved as followers (Fig. S1L-O). Thus, p53 elevation is sufficient to instruct the leader cell fate.

To test whether p53 is also required for leader cell migration, we generated MDCK cells that inducibly express the p53 peptide inhibitor GSE-22 (25) in tandem with GFP. GSE-22 expression reduced the frequency but did not abolish the formation of MMC-induced leaders (Fig. S2A-E). Next, we ablated p53 function by Crispr mutagenesis, generating *p53KO* clones, which we verified functionally (Fig. S2F-G) and by sequencing (Fig. S2H). Functional ablation of *p53* substantially reduced the incidence of leader behavior in the binucleated cell assay (Fig. S2I-J). It also inhibited strongly the emergence of leaders induced by MMC, which dropped from 80% in wild-type cells to just under 20% in *p53KO* cells (Fig. 2D-E, S1H and Movie S3). This could also be observed as a reduction in speed, displacement, distance and persistence of migration for wild-type cells in contact with *p53KO* MMC-treated cells, compared to those in contact with wild-type MMC-treated

cells (Fig. 2F-I, and Fig. S2K-M). Thus, whilst few cells behaving as leaders still remained, the great majority of cells did not display leader migration in the absence of p53, indicating that p53 is an important contributor to leader cell migration.

p21 promotes the emergence of leader cells downstream of p53

Spontaneous leader cells, BBCs, and MMC-treated cells are all cell cycle arrested, and Nutlin-3 treated leaders divide less frequently than untreated controls, suggesting that factors required for the leader phenotype could include p53 effectors involved in cell cycle arrest. The main p53 target gene involved in cell cycle arrest is cyclin-dependent kinase inhibitor 1A (CDKN1A), also known as p21^{WAF1/Cip1} (p21) (21, 26). p21 was indeed upregulated in spontaneous leaders (Fig. 3A-B). To determine whether p21 is necessary for leader fate we generated two *p21* knockout clones by Crispr mutagenesis and verified *p21* disruption functionally (Fig. S3A) and by sequencing (Fig. S3B). *p21KO* cells treated with MMC were significantly less likely to behave as leaders than wild-type cells (Fig. 3C-D, Fig S3C and Movie S4). This could also be observed as a reduction in speed, displacement, distance and persistence of migration for wild-type cells in contact with *p21KO* MMC-treated cells, compared to those in contact with wild-type MMC-treated cells (Fig. 3E-I and Fig. S3D-E).

To ask whether p21 is sufficient to induce leader cell behavior, we generated a clonal MDCK cell line inducibly over-expressing p21 (p21OE). p21 over-expression resulted in cell cycle arrest, as expected, but also caused cells to flatten and take on a leader-like morphology (Fig. S3F-G). Upon

contact with wild-type cells, p21OE cells behaved as leaders (Fig. 4A-C, Fig. S3H and Movie S5, left), indicating that p21 over-expression is sufficient for leader cell behavior. Thus, p53 activation induces leader cells by up-regulating p21.

p21 promotes the emergence of leader cells via CDK inhibition

Next, we wondered how p21 induces leader cell migration. Because p21 is an inhibitor of cyclin-dependent kinases (CDKs) activity (26), we asked whether CDK inhibition plays a role in leader cell migration. First, we generated a cell line inducibly over-expressing the CDK inhibitor p16 (27) and found that p16 over-expression also induced leader cell migration (Fig. 4D-E and Movie S5, right). Given that p21 and p16 are structurally unrelated (28), this strongly suggested that they induce leader cell fate by inhibiting CDKs, because it would be unlikely that they share additional common targets. Thus, we hypothesized that leader-follower behavior ensues when cells with low CDK activity (leaders) come into contact with cells with relatively high CDK activity (followers). To seek further validation of this hypothesis, we used small molecule inhibitors of CDK function. Accordingly, adding CDK inhibitors to migrating leader-followers pairs should stall leader cell migration, by turning followers into low-CDK leader-like cells. Indeed, addition of both CDK2 inhibitor K0386 and CDK1 inhibitor RO-3306 stalled spontaneous leader-driven migration (Fig. 4F-G and Movie S6). Thus, CDK inhibition instructs leader cell behavior. Furthermore, because

inhibition of cell cycle progression upon damage can be independent of p53 or p21, the findings may explain the incomplete inhibition of leader behavior upon p53 or p21 loss of function.

p53 and p21 induce leaders through Rac1, PI3K and ITG β 1 activation

Spontaneous leader cells are known to upregulate Ras-related C3 botulinum toxin substrate 1 (Rac1), integrin β 1 (ITG β 1), and phosphoinositide 3-kinase (PI3K), and require their activities for cell migration (10). We therefore tested whether these were also up-regulated and required in p53/p21-induced leaders. Both PI3K and ITG β 1 were up-regulated in leaders generated by MMC treatment (Fig. S4A-D) and by Nutlin-induced p53 elevation (Fig. S4E-J). Surface levels of ITG β 1 were also increased (Fig. S4K-L), suggesting that ITG β 1 elevation results in higher integrin activity at the plasma membrane. However, neither PI3K or ITG β 1 mRNA were elevated upon MMC-treatment (Fig. S4M-N), indicating that their elevation was because of post-transcriptional regulation. PI3K and ITG β 1 upregulation was partially mediated by p21, because *p21KO* cells showed reduced PI3K and ITG β 1 upregulation upon treatment with Nutlin (Fig. S4E-J). Induction of leaders by p21 over-expression was also sufficient to upregulate PI3K and ITG β 1 (Fig. S4O-R), which was also upregulated by addition of the CDK1 inhibitor RO-3306 (Fig. S4S-T). Notably, chemical inhibition of PI3K and Rac1 prevented the active migration of MMC-generated leaders (Fig. S5A-B and Movie S7), indicating that these pathways are also necessary for p53/p21-

mediated leader cell migration. Thus, p53 and p21 induce leader specification via elevation of the leader fate effectors PI3K and ITG β 1, likely through CDK inhibition.

Injury-induced p53 drives collective cell migration during epithelial repair

Because p53/p21-induced leaders share the same molecular mechanisms as spontaneous leaders to drive migration, we wondered whether p53, p21 and the resulting CDK inhibition also play a role in the migration of leaders driving epithelial repair. First, we asked about the cell cycle status of injury-induced leaders. We generated a cell line expressing the cell cycle reporter FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator (29)) and monitored cell cycle progression in leaders emerging upon epithelial scratch. We found that cells became leaders in any phase of the cell cycle (Fig. S6A). However, they spent more time in the cell cycle phase they were in, compared to non-leader cells (Fig. 5A). This indicates that leaders tend to experience cell cycle delay and therefore, likely, CDK inhibition. This was not a general feature of migrating cells, because migrating followers led by spontaneous leaders did not cycle slower than non-follower cells in the same culture (Fig. S6B). Thus, a slower cell cycle is not an obligate feature of cells engaging in migration. In addition, scratch-induced leaders showed high levels of both p53 (Fig. 5B-C) and p21 (Fig. S6C-D). Furthermore, leaders induced by barrier release (Fig. S6E) also

showed elevated p53 (Fig. S6F-G) and p21 (Fig. S6H-I), suggesting that the p53/p21 pathway is a general driver of leader cell migration in MDCK cells.

We next wondered how p53 becomes activated at the edge of injured epithelial monolayer and hypothesized that mechanical damage, inflicted when epithelia are scratched, could be triggering p53 elevation. To measure p53 activation in live and migrating epithelial sheets, we generated a cell line carrying a live fluorescent p53 reporter consisting of short lived NLS-GFP under the control of p21 promoter sequences that respond to p53 (Fig. S6J) (30). We found that scratching induced p53 activity along the scratched epithelial edge (Fig. 5D, dotted white strip, and E), relative to cells away from the scratch (Fig. 5D, cells within the yellow dotted line, and E). This was not accompanied by detectable DNA damage (Fig. S6K), suggesting that p53 was not activated by mechanically-induced DNA damage. Instead, p53 activation in edge cells was prevented by inhibition of p38 (Fig. 5F-G), a stress-related kinase which can activate p53 upon mechanical stress (24, 31). Thus, scratch-induced mechanical insult, via p38, elevates p53, which in turn instructs leader specification to drive epithelial gap closure.

We reasoned that if the p53/p21 pathway plays an active role during repair of injured MDCK monolayers, then inhibiting p53 should slow down closure of the gap, whereas activating p53 should accelerate epithelial repair. Inhibiting p53 function by GSE-22 overexpression was sufficient to reduce the migration speed of scratched monolayers (Fig. 5H-J). Ablating *p21* also resulted in slower migration of scratched epithelial sheets relative to wild-type control (Fig. 5K and Fig S6L-M). To test whether activating p53 can accelerate migration, we used laser-induced DNA damage (32) to elevate p53 specifically in the first row of cells at the edge of the migrating

front (Fig. 5L and Fig. S6N). DNA damage in edge cells was sufficient to accelerate cell migration, an effect that could be suppressed by p53 inhibition (Fig. 5M-N and Movie S8). Thus, p53 and its target gene p21 are the signals activated at the edge of damaged epithelia that instruct leader cell fate.

Leader cells are eliminated by cell competition upon epithelial repair

p53 elevation in MDCK cells causes mechanical cell-competition (24): cells with high p53 acquire hypersensitivity to cell crowding and behave as ‘mechanical losers’, undergoing cell extrusion and/or apoptosis when compacted by cells with low p53 (24). Because our data indicate that high p53 is a hallmark of scratch-induced leaders, we wondered whether these leaders might be eliminated upon closure of the epithelial gap, when compacted by follower cells. Both spontaneous leaders (Fig. 6A-B) and scratch-induced leaders (Fig. 6C-D and Movie S9) underwent extrusion or death at high rates upon compaction by followers (75.9% and 40%, respectively). This cell elimination was not mediated by p21. p21 ablation did not suppress compaction hypersensitivity in cells with high-p53 (Fig. 6E), neither did it rescue their loser status in competition experiments with p53 mutant cells (Fig. 6F and Movie S10). In addition, leaders induced by p21 overexpression did not behave as losers in competition experiments with wild-type cells (Fig. 6G), suggesting that p21 is not sufficient for mechanical loser status. Because overexpression of p21 generated leaders that are not mechanical losers, this allowed us to investigate the consequences of failure to clear leader cells upon epithelial gap closure. More than half of the p21 overexpressing cells (56.9% on average in three independent experiments, n=373) displayed an aberrant

morphology after the epithelium was repaired, compromising the regular cobble-stone like morphology of the epithelium (Fig. 6H).

Discussion

Understanding how leader cells emerge in damaged epithelia is important to understand the process driving wound healing and to identify interventions that could accelerate and improve wound repair. Our work reveals that p53 is a key cell determinant instructing leader cell fate (Fig. 6I-J). In damaged epithelia, injury itself causes the emergence of leader cells by mechanically inducing p53 elevation. p53 plays two critical roles in epithelial repair. It initiates leader-driven epithelial closure, as the juxtaposition of cells with high (leaders) and low (followers) p53 drives directional cell migration across the gap. Once the epithelium has been repaired, p53 induces leader cell clearance by cell competition, reinstating epithelial integrity.

Our work demonstrates that p53 induces leader fate through the transcriptional activation of p21 and that p21, in turn, induces leader fate via inhibition of CDK activity. Cell cycle delay is a feature common to both spontaneous and injury-induced leaders in our system. Non-proliferating cells at the front of collectively migrating cells have been reported in several *in vivo* contexts, such as during cardiomyocytes migration (17), in skin epithelia upon wounding (7, 8), in angiogenic sprouting (33), in the migration of often aneuploid cytotrophoblast cells (34) and in metastatic cancer cell migration promoted by radiotherapy-induced senescent cells (35). Our work suggests

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

that developmentally controlled or injury-induced p53, p21 or CDK inhibition may constitute a general mechanism to induce collective leader-driven cell migration.

Materials and Methods

Antibodies and drugs

For immunofluorescence we used: rabbit anti-p53 (1:750, 9382, RRID:AB_331476, Cell Signalling Technology), rabbit anti-p21 (1:200, sc-397, RRID:AB_632126, Santa Cruz Biotechnology), rat anti-ITG β 1 (1:500, AIIB2, RRID:AB_528306, DSHB), mouse anti-PI3K (1:200, 610045, RRID: AB_397460, BD Biosciences), rabbit anti- γ H2AX (Ser 139) (1:200, 9718, RRID:AB_561076, Cell Signalling Technology), rabbit anti-cleaved caspase-3 antibody (1:200, 9661, RRID:AB_2341188, Cell Signaling Technology), mouse anti-ZO-1 (1:500, 33-9100, RRID:AB_2533147, Thermo Fisher Scientific), Alexa Fluor conjugated secondary antibodies (1:1,000; Thermo Fisher Scientific).

Treatments/reagents used: Rac1 inhibitor Z62954982 (100 μ M in DMSO, 553512, Merck), PI3K inhibitor LY294002 (10 μ M in DMSO, L9908, Merck), CDK1 inhibitor RO-3306 (10 μ M in DMSO, SML0569, Sigma-Aldrich), CDK2 inhibitor K0386 (3 μ M in DMSO, S8100, Selleckchem), Nutlin-3 (concentration as specified, in DMSO, CAY10004372, Cambridge Bioscience), p38 inhibitor (10 μ M in DMSO SB202190, S7067, Calbiochem), Mitomycin C (7.5

µg/ml in water, M4287, Merck), doxycycline (1 µg/ml in water, D9891, Merck), puromycin (0.65 µg/ml in water, P9620, Merck), G418 (400 µg/ml, 10131035, Thermo Scientific), Hoechst 33342 (3 µg/ml in water, H3570, Thermo Fisher Scientific), DAPI (1 µg/ml in water, D3571, Thermo Fisher Scientific).

Cell culture and plasmids

MDCK cells were maintained in DMEM (21885, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; P30-3305, FBS Standard, South America, PAN Biotech) in a humidified incubator at 37°C and 5% CO₂. Wild-type MDCK cells were obtained from Yasuyuki Fujita (Kyoto University); MDCK wild-type GFP-NLS cells have been previously described (24).

MDCK *p53KO* GFP-NLS clones 6 and 14 were generated by infecting a MDCK *p53KO* pool (24) with lentiviral construct pGIPZ-turboGFP-NLS-Puro (24). Selection post infection was carried out in 0.65 µg/ml puromycin. The resulting GFP-NLS labelled pool was then plated at one cell per well in 96-well plates; clones were expanded and verified by immunofluorescence, sequencing, and Western blotting.

MDCK *p21KO* clones G5 and E9 were generated using Cas9 D10A CRISPR technology. sgRNAs against canine *CDKN1A* were designed manually following published methods (36). Target sequences used: 5'-CGGCAAGCCTTGCTGCCATG(AGG)-3' and 5'-TGGACAGCGAGCAGCTGCGC(CGG)-3'. sgRNAs were cloned individually into PX461

vectors (RRID:Addgene_48140 (36)) and co-transfected as a pair into wild-type MDCK cells using Lipofectamine 2000 (Thermo Fisher Scientific). The transfected cells were treated with 11 μ M Nutlin-3 for 6 days to enrich for non-growth-inhibited *p21KO* cells. The pool was then plated at one cell per well in 96-well plates; the resulting clones were expanded and verified by immunofluorescence and sequencing. For use in the competition experiment a *p21KO* GFP positive population was obtained by infecting the *p21KO* G5 clone with a lentiviral construct containing pGIPZ-GFP-NLS-Puro as described in (24) and selected using 0.65 μ g/ml puromycin.

To overexpress p21, p21 cDNA was amplified from a cDNA library obtained from MDCK *scribble* shRNA cells treated with tetracycline to induce *scribble* knockdown, as these cells have been shown to express high levels of p21 (24). The resulting PCR product was introduced into doxycycline-inducible pTRIPZ-RFP-NLS-Puro (24) with a P2A peptide bridging the two proteins to make pTRIPZ-p21-P2A-RFP-NLS-Puro. MDCK wild-type cells were infected with the final construct, selected using 0.65 μ g/ml puromycin, and then plated in 96-well plates to isolate single clones that were verified by immunofluorescence. For use in competition experiments, a p21OE GFP positive population was obtained by infecting a p21OE clone with a lentiviral construct containing pGIPZ-GFP-NLS-Puro, as described (24) and selecting GFP positive cells via FACS sorting.

To overexpress p16, the human p16 cDNA was amplified from pLenti CMV p16 Neo (w111-1) (RRID:Addgene_22260), using a modified forward primer to reintroduce the eight N-terminal amino acids missing when compared to the full-length human p16 sequence (transcript

ENST00000304494.9, Ensembl). The resulting PCR product was introduced into doxycycline-inducible pTRIPZ-RFP-NLS-Puro (24) with a P2A peptide bridging the two proteins to make pTRIPZ-p16-P2A-RFP-NLS-Puro. MDCK wild-type cells were infected with the final construct, and selected using 0.65 µg/ml puromycin to generate a pool population.

To overexpress the dominant negative GSE-22 peptide (25), amino acids 302-381 of canine p53 (homologous to the original rat sequence) were amplified from the MDCK cDNA library used in p21 cloning; a forward primer carrying an adaptor with three start ATG codons and a reverse primer containing three stop codons were used to account for all three reading frames, as was done in the original publication. The resulting PCR product was introduced into doxycycline-inducible pTRIPZ-GFP-NLS-Puro with a P2A peptide bridging the two proteins to make pTRIPZ-GSE-22-P2A-GFP-NLS-Puro. MDCK wild-type cells were infected with the final construct and selected using 0.65 µg/ml puromycin. Clones were selected by FACS, sorting cells with the top 5% GFP intensity on a 96- well plate. p53 inhibition in the clones was verified functionally, by western blotting, showing absence of p21 induction upon DNA damage.

The MDCK FUCCI cell line was generated by infecting cells with the ready-made IncuCyte® Cell Cycle Red/Green Lentivirus Reagent (Sartorius, 4779), according to manufacturer protocols and selected using 0.65 µg/ml puromycin to generate a pool population.

MDCK p21 reporter cell line was generated by transfecting a plasmid containing destabilized turbo GFP downstream of p21 promoter sequences in MDCK cells. The plasmid was generated

following a strategy similar to the one described in (30). In short, we obtained a plasmid carrying p21 promoter sequences, described in (30). We amplified the p21 sequences and turbo GFP from pTRIPZ GFP-NLS-Puro and cloned them upstream of a PEST destabilization signal in pcDNA3.3 d2eGFP (37) (RRID:Addgene_26821), after excision of CMV promoter, CMV enhancer, and eGFP from the backbone plasmid. Cells transfected with the resulting plasmid were selected using 400 $\mu\text{g/ml}$ G418 and clones were picked based on their brightness via FACS. A further round of selection was performed upon Nutlin-3 treatment to isolate clones with high post-treatment to pre-treatment signal ratio.

Mitomycin C (MMC) assays

On day 1, 2,500 untreated wild-type MDCK cells (labelled with GFP-NLS or unlabeled, as appropriate) per well were seeded in 24-well plates. On day 2, a second population of MDCK cells was trypsinized, and 1×10^6 of these were incubated in suspension in 5 ml complete media containing 7.5 $\mu\text{g/ml}$ MMC for 1 hour at 37°C and 5% CO₂. Cells were then washed at least three times in complete media (spinning down the cells after each wash) to reduce MMC carryover, and then 5,000 MMC-treated cells or 10,000 untreated control cells were plated on top of the pre-seeded untreated population. Live imaging began on day 3; samples were imaged every 2 hours, except for the CDK1 inhibitor experiment where imaging occurred every 45minutes. Where applicable, inhibitors were added after leader-follower behavior had emerged and imaging continued for 4 to 24 more hours.

Spontaneous leader assays

5,000 wild-type MDCK cells were seeded in a 24-well plate or, if immunofluorescence was to be performed at end of live imaging, in a gridded tissue culture plate (μ -Dish 35 mm Grid-500, 81166, ibidi). Approximately 48 hours later, cells with spontaneous leader morphology were selected and imaged every 2 hours. Where applicable, at the end of live imaging cells were fixed in 4% PFA (15713-S, Electron Microscopy Sciences) in PBS for 10 min at RT.

Scratch and barrier release assays

For the scratch assay 27,000-35,000 wild-type, p21*KO*, GSE-22 or FUCCI MDCK cells were seeded in the middle of a 'fence' (Aix Scientifics, <http://www.aix-scientifics.co.uk/en/fences.html>) placed in a 24-well or into a 96-well plate. The 'fence' was removed approximately 5 hours after seeding and the culture medium changed. Where indicated doxycycline-supplemented medium was used to induce GSE-22 expression. Approximately 20 hours later, a P1000 pipette tip was used to generate a scratch-wound through the middle of the now confluent monolayer and the culture medium was changed.

For the scratch assay with the p21 reporter cell line 80,000-100,000 cells were seeded per well on a glass bottom 24-well plate (Sensoplate™, 662892, Grenier Bio-One), previously coated with 20 μ g/ml fibronectin (F1141, Thermo Fisher Scientific) in PBS for 1 h at 37 °C. 16 hours after seeding, the p38 inhibitor was added where indicated and approximately 32 hours later the monolayer was scratched, as described above, and imaging was started.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

For the barrier release assay 25,000-28,000 wild-type MDCK cells were seeded in each chamber of a 2-wells silicone insert (ibidi, 80209). Culture medium was added outside of the chambers 5 hours after seeding and approximately 20 hours later the barrier was removed.

Imaging was started approximately 1 hour after scratching/removing the barrier and images were acquired every 1-2 hours. If immunofluorescence was to be performed after live imaging, cells were seeded on optically clear plates (μ -Plate 24 well, 82406, ibidi or CellCarrier-96, 6005550, PerkinElmer).

Laser photo-damage experiments

On day 1, 25,000-30,000 GSE-22 carrying cells per well were plated on an optically clear 96-well plate (CellCarrier-96, 6005550, PerkinElmer). Cells were left to adhere overnight and where indicated the culture medium was replaced with doxycycline-supplemented medium to induce GSE-22 expression. On day 2, the cell monolayer was scratched through the middle of each well using a P1000 tip and returned to the 37°C / 5% CO₂ incubator for approximately 30 minutes. Cells were then treated in the incubator with 3 μ g/ml Hoechst 33342 diluted in complete medium for 10 min and washed three times in complete medium. Irradiation along half of the wound edge was performed using a 20 $\times$ air objective on a Leica SP8 confocal microscope, which was set up to keep the samples at 37°C / 5% CO₂; the other half of the wound was not irradiated and served as control. Laser settings used: 40 frames at 100% 405 nm laser with the FRAP booster on. Samples

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

were then live imaged using a Nikon BioStation CT system every 2 hours for at least 24 hours. For γ H2AX stainings, samples were fixed and processed 2-4 hours after irradiation.

Blebbistatin-induced binucleated cell (BBC) generation

On day 1, 5,000 unlabeled wild-type MDCK cells were plated per gridded dish (μ -Dish 35 mm Grid-500, 81166, ibidi). Also on day 1, 12,000 GFP-NLS labelled WT MDCK cells were incubated with 37.5 μ M blebbistatin in DMSO (B0560, Merck) in a 12-well plate. 16 hours later, on day 2, cells were washed three times in PBS and left to recover for 4 hours in complete media. Cells were harvested by trypsinization and, after large clumps were removed using a 20 μ m filter (04-0042-2315, CellTrics), were plated on the gridded dishes containing unlabeled wild-type MDCK cells. Imaging was started on day 3; cells were imaged every 2-4 hours.

Real-time quantitative reverse-transcription PCR

On day 1, 400,000 MDCK cells per well were seeded in a 6-well plate and left to adhere overnight. On day 2, cells were incubated in complete medium containing 7.5 μ g/ml MMC for 1 hour at 37°C and 5% CO₂. After approximately 16 hours from treatment, cells were lysed and their RNA was extracted with the Quick-RNA Miniprep Kit (Zymo Research, R1054/R1055) according to the supplier instructions. cDNA was retrotranscribed using the SuperScript™ III Reverse Transcriptase kit (Thermo Fisher, 18080044) as recommended by the supplier. The qPCR was

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

performed in a StepOne™ Real-Time PCR System (Thermo Fisher) using the QuantiNova SYBR® Green PCR Kit (Quiagen, 208056).

The following primers were used: ITGB1 (5'GCGTTGCTGCTGATTTGGAA3' and 5'ATTTTCACCCGTGTCCCATT3'), PI3KCA (5'TGCTGAACCCTATTGGTG3' and 5'TACAGTCCAGAAGCTCCA3'), GAPDH (5'AGTCCATCTCCATCTTCCAG3' and 5'CGTCACGCCACATCTTCC 3').

The fold change in gene expression was calculated using the comparative Ct method, normalising to GAPDH.

Cell competition experiments

Competing mixed MDCK cultures were seeded on the outside of the barrier created by the insertion of 'fences' (Aix Scientifics, <http://www.aix-scientifics.co.uk/en/fences.html>) in a 24-well plate. 19,000 cells of mixed *p53KO:p21KO* GFP-NLS cells at a ratio of 8.5:1 or 8,000 cells of mixed WT:p21OE GFP-NLS cells at a ratio of 9:1 were used. 2,000 cells of a pure *p21KO* GFP-NLS or p21OE GFP-NLS population were seeded in the center of the 'fence' in the same well and served as control mono-culture. Approximately 5 hours after plating, 'fences' were removed and the culture medium replaced.

For the p21OE experiment, on day 2 p21 overexpression was induced with doxycycline and imaging was started on day 3. Images were acquired every 2 hours for a further 4 or 5 days. Fresh doxycycline-supplemented culture medium was supplied every second day. Live imaging of mixed and pure cultures not supplemented with doxycycline was performed as a control.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

For the *p21KO* experiment, on day 3 p53 elevation was induced with 12 μ M Nutlin-3 and imaging was started on day 4. Images were acquired every 2 hours for a further 3 or 4 days. Fresh Nutlin-3-supplemented culture medium was supplied every day, with the concentration of Nutlin-3 brought to 15 μ M from day 5 to compensate for the increased cell number. Time-lapse imaging of mixed and pure cultures treated with DMSO and of treated and untreated *p53KO*:WT GFP-NLS cultures was performed as a control.

Compaction experiments

Cells were pre-treated with 15 μ M Nutlin-3 or DMSO for 24 hours. 95,000-125,000 (high density) or 15,000-20,000 (low density) MDCK wild-type or *p21KO* cells were then seeded in each of the two compartments of a stretching device (24). In brief, the device is composed of a flexible PDMS membrane (Gel pak PF-60-X4, 150 μ m thickness, Teltek) held by a custom-made stretcher (University of Bristol) which, once stretched by 2 cm, provides a 57% stretch compared to the resting length. Two cell seeding chambers are created on the stretched membrane by attaching a silicone insert with two compartments (6.6 $\times$ 13 mm each) and the PDMS membrane is then coated with 25 μ g/ml fibronectin in PBS (F1141, Thermo Fisher Scientific) for 1 h at 37 $^{\circ}$ C. Low- and high-density cells were seeded side- by-side on the two chambers of the same membrane and were therefore processed in parallel, minimizing sample-to-sample variability during culture, compaction and staining.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

Once seeded, cells were left to adhere to the membrane for 24 hrs and then the stretching was released to induce compaction. 5 hours after release, cells were fixed and processed for anti-Caspase-3 immunofluorescence.

Immunofluorescence

Depending on the assay, cells were cultured on glass coverslips, optically clear tissue culture dishes or PDMS membrane. Cells were fixed in 4% PFA (15713-S, Electron Microscopy Sciences) in PBS for 10 min at RT, quenched for 10 min at RT in 50 mM in NH_4Cl in PBS, and permeabilized for 10 min at RT in 0.1% Triton X-100 in PBS. Samples were blocked for at least 30 min in 2% bovine serum albumin (BSA; A2153, Sigma) 2% FBS in PBS at RT. Both primary and secondary antibodies were diluted in blocking solution diluted 1:1 with PBS. Primary antibody incubations were either a minimum of 1 hour at RT or overnight at 4°C. Following 4 x 5 minutes washes in PBS, secondary antibodies were incubated for 1 hour at RT, then washed 4 x 5 minutes in PBS. Coverslips were mounted in FluorSave (345789, Merck). Optically clear dishes were either covered with FluorSave and sealed with a coverslip or imaged directly, submerged in PBS. For immunostaining against phosphorylated proteins the fixing solution was supplemented with PhosSTOP (1 tablet per 10ml, Sigma), all PBS-based solutions were substituted with TBS-based ones, and blocking solution was substituted with 5% BSA in TBS.

Imaging and image analysis

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

Imaging of fixed samples was done at RT on a Leica SP5 or SP8 confocal microscope using a 60x or 40x oil objective or on the PerkinElmer Opera LX system using a 20x water objective. Laser photo-damage experiments were done on a Leica SP8 confocal microscope set up to keep the samples at 37°C / 5% CO₂ during imaging. All images in the figures are presented as sum projection.

Mean intensities of nuclear p53 and p21 were measured using DAPI as a mask to segment the nuclear volumes in 3D using Volocity (PerkinElmer). Mean intensities per cell of ITGβ-1 and PI3K were measured in FIJI as the integrated density of fluorescence measured on an average intensity projection, multiplied by the number of Z slices and divided by the number of nuclei.

Live imaging was performed on a Nikon BioStation CT system at 37°C / 5% CO₂ using 10x or 4x air objectives with an imaging frequency of 0.25-4 hours for all experiments except for the one involving the p21 reporter cell line; media was changed every 2–3 days, unless otherwise stated. For the p21 reporter experiment, live imaging was performed on a Yokogawa Cell Voyager 7000S 37°C / 5% CO₂ using a 20x air objective with an imaging frequency of 2 hours.

Particle image velocimetry (PIV) was performed by using a Deep learning (DL)- PIV approach. In short, we used the DL-unsupervised optical flow method, described in (38). We trained the pre-trained network provided in (38) using 650 video images of MDCK cells in different settings. The network was then applied to a selected subset of movies of migrating MMC-induced leaders or spontaneous leaders/followers pairs imaged every 15 minutes.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

Manual cell tracking was done by marking coordinates of cell nuclei over time using MtrackJ plugin in ImageJ. Cells from untreated / MMC-treated or spontaneous leaders/followers pairs were tracked starting from 8 hours (four time points) before to 12 hours (six time points) after contact between cells was established, thereby making coordinate 0,0 a point when the two cells meet. Coordinates of cell tracks were analyzed in Microsoft Excel.

The speed of migration of leader/follower pairs was measured in FIJI by using a custom-made algorithm tracking the position of the colonies borders over time (see data availability statement). The resulting coordinates were analyzed in Microsoft Excel to calculate the speed of migration and the relative distance between prospective leaders and followers colonies. The time of contact between colonies is reported as zero in the graphs.

The fluorescence intensity of p21 reporter cells was quantified manually by selecting edge (first row at the migrating front) or bulk (from fifth row of the cell monolayer inwards) cells and quantifying the mean intensity of each nucleus with Volocity (PerkinElmer). Flat field correction was performed in each image before analysis.

The migration speed of scratched epithelial fronts was measured in FIJI by manually drawing freehand lines outlining the wound edge at the first and final time points. If any wound edges were not parallel to the y axis, images were adequately rotated. The median x position for each line was recorded to calculate the median x displacement in pixels, which was then divided by the duration of the experiment in hours to generate a speed value in pixels/h and then converted to $\mu\text{m/h}$. As density impacts the speed of migration, we selected only fields with comparable initial density. The initial cell density of the monolayer was obtained by segmenting cells in phase using a custom-made algorithm (see data availability statement).

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

All graphs were plotted using GraphPad Prism. Figures were made using Adobe Illustrator CS6.

Statistical analysis

Logistic regression analysis was performed using R and the reported p-values have been corrected using the false discovery rate method. All other statistical analyses were performed using GraphPad Prism. For the RT-qPCR experiments the p values were obtained using the Wilcoxon signed rank test, all the other p values were obtained using the Mann-Whitney U test.

Standard guidelines have been followed in providing sufficient methods information for this work, and a Materials Design Analysis Reporting checklist has been provided at submission.

References and Notes

1. P. Friedl, D. Gilmour, Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol.* **10**, 445–457 (2009).
2. M. Poujade *et al.*, Collective migration of an epithelial monolayer in response to a model wound. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 15988–15993 (2007).

3. S. Begnaud, T. Chen, D. Delacour, R.-M. Mège, B. Ladoux, Mechanics of epithelial tissues during gap closure. *Curr. Opin. Cell Biol.* **42**, 52–62 (2016).
4. V. Hakim, P. Silberzan, Collective cell migration: a physics perspective. *Rep Prog Phys.* **80**, 076601 (2017).
5. R. Mayor, S. Etienne-Manneville, The front and rear of collective cell migration. *Nat Rev Mol Cell Biol.* **17**, 97–109 (2016).
6. E. Theveneau, C. Linker, Leaders in collective migration: are front cells really endowed with a particular set of skills? *F1000Res.* **6**, 1899 (2017).
7. S. Park *et al.*, Tissue-scale coordination of cellular behaviour promotes epidermal wound repair in live mice. *Nat. Cell Biol.* **19**, 155–163 (2017).
8. M. Aragona *et al.*, Defining stem cell dynamics and migration during wound healing in mouse skin epidermis. *Nature Communications.* **8**, 14684–14 (2017).
9. M. Reffay *et al.*, Interplay of RhoA and mechanical forces in collective cell migration driven by leader cells. *Nat. Cell Biol.* **16**, 217–223 (2014).
10. N. Yamaguchi, T. Mizutani, K. Kawabata, H. Haga, Leader cells regulate collective cell migration via Rac activation in the downstream signaling of integrin β 1 and PI3K. *Sci Rep.* **5**, 7656 (2015).

11. T. Omelchenko, J. M. Vasiliev, I. M. Gelfand, H. H. Feder, E. M. Bonder, Rho-dependent formation of epithelial “leader” cells during wound healing. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 10788–10793 (2003).
12. S. Mark *et al.*, Physical model of the dynamic instability in an expanding cell culture. *Biophys. J.* **98**, 361–370 (2010).
13. S. Rausch *et al.*, Polarizing cytoskeletal tension to induce leader cell formation during collective cell migration. *Biointerphases.* **8**, 32 (2013).
14. A. Ravasio *et al.*, Gap geometry dictates epithelial closure efficiency. *Nature Communications.* **6**, 7683 (2015).
15. M. Vishwakarma *et al.*, Mechanical interactions among followers determine the emergence of leaders in migrating epithelial cell collectives. *Nature Communications.* **9**, 3469–12 (2018).
16. J. Campisi, Aging, cellular senescence, and cancer. *Annu. Rev. Physiol.* **75**, 685–705 (2013).
17. J. Cao *et al.*, Tension Creates an Endoreplication Wavefront that Leads Regeneration of Epicardial Tissue. *Dev. Cell.* **42**, 600–615.e4 (2017).
18. A. F. Straight *et al.*, Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science.* **299**, 1743–1747 (2003).

19. P. R. Andreassen, O. D. Lohez, F. B. Lacroix, R. L. Margolis, Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell.* **12**, 1315–1328 (2001).
20. M. M. Cohen, M. W. Shaw, Effects of Mitomycin C on human chromosomes. *J. Cell Biol.* **23**, 386–395 (1964).
21. J. S. Lanni, T. Jacks, Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Molecular and Cellular Biology.* **18**, 1055–1064 (1998).
22. J. Y. Park, Y. R. Seo, Enhancement of mitomycin C-induced apoptosis in Nrf2-deficient human colon cancer cells. *Mol. Cell. Toxicol.* **6**, 51–56 (2010).
23. L. T. Vassilev *et al.*, In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* **303**, 844–848 (2004).
24. L. Wagstaff *et al.*, Mechanical cell competition kills cells via induction of lethal p53 levels. *Nature Communications.* **7**, 11373 (2016).
25. V. S. Ossovskaya *et al.*, Use of genetic suppressor elements to dissect distinct biological effects of separate p53 domains. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10309–10314 (1996).
26. V. Dulić *et al.*, p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell.* **76**, 1013–1023 (1994).

27. M. Serrano, G. J. Hannon, D. Beach, A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. **366**, 704–707 (1993).
28. A. Besson, S. F. Dowdy, J. M. Roberts, CDK inhibitors: cell cycle regulators and beyond. *Dev. Cell*. **14**, 159–169 (2008).
29. A. Sakaue-Sawano *et al.*, Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. *Cell*. **132**, 487–498 (2008).
30. A. L. Paek, J. C. Liu, A. Loewer, W. C. Forrester, G. Lahav, Cell-to-Cell Variation in p53 Dynamics Leads to Fractional Killing. *Cell*. **165**, 631–642 (2016).
31. M. Hofmann *et al.*, Mechanical pressure-induced phosphorylation of p38 mitogen-activated protein kinase in epithelial cells via Src and protein kinase C. *Biochemical and Biophysical Research Communications*. **316**, 673–679 (2004).
32. C. Dinant, M. de Jager, J. Essers *et al.*, Activation of multiple DNA repair pathways by sub-nuclear damage induction methods. *J Cell Sci*. **120**, 2731–2740 (2007).
33. H. Gerhardt, *et al.*, VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology*. **161**, 1163–1177 (2003).
34. J. F. Weier *et al.*, Human cytotrophoblasts acquire aneuploidies as they differentiate to an invasive phenotype. *Developmental Biology*. **279**, 420–432 (2005).
35. M. Demaria *et al.*, Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discov*. **7**, 165–176 (2017).

36. F. A. Ran *et al.*, Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. **8**, 2281–2308 (2013).
37. L. Warren *et al.*, Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Stem Cell*. **7**, 618–630 (2010).
38. M. Zhang, M.D. Piggott, Unsupervised Learning of Particle Image Velocimetry. In: High Performance Computing. Lecture Notes in Computer Science *Springer, Cham*. **12321**, 102–115 (2020).

Acknowledgments: We thank Cristina Tommasi for input on the project, the Wolfson Bioimaging Facility for access to microscopes and for image analysis support (Stephen Cross). We thank our anonymous reviewers for constructive feedback and suggestions and Life Science Editors (LSE) for editorial assistance. Funding: This work was supported by a Cambridge Cancer Centre PhD studentship to MG, a Cancer Research UK Programme Grant to EP (A12460), a Cancer Research UK Programme Foundation Award to EP (Grant C38607/A26831) and a Royal Society University

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

Research fellowship to EP (UF0905080). EP is a Wellcome Trust Senior Research Fellow (205010/Z/, 16/Z).

Author contributions: EP conceived and led the project. EP, LW, GP and KK designed the experimental strategy. Using protocols developed by LW, MG performed the experiments on BBCs, except for anti-p53 immunostaining of wild-type BBCs (done by LW) and quantification of the leader behavior of *p53KO* BBCs (done by KK). MG generated GFP-labelled clonal *p53KO* lines. Experiments involving the p21 reporter cell line were performed by MV. The mechanical compression experiments were performed by SC. KG performed part of the FUCCI experiments and their analysis. KK performed all cloning and generated all cell lines except the FUCCI cell line and the GFP positive *p21KO* and p21OE cell lines, which were generated by GP. SM and RCS generated the custom-made algorithm for cell segmentation and count in phase. SM performed the PIV analyses. Manual and automated cell tracking analyses were performed by MV.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

All the other experiments and analyses were carried out by KK and GP. The manuscript was prepared by EP, KK and GP with input from MV.

Competing interests: Authors declare no competing interests; Data and materials availability: All data is available in the main text or the supplementary materials. The custom made algorithms employed for image analysis are available on GitHub (https://bit.ly/Kozyrska_Pilia_et_al2021).

Fig. 1. Spontaneous leaders elevate p53. (A) Movie stills of wild-type MDCK cultures showing a spontaneously emerging leader (arrow). (B-C) Displacement (B) and migration persistence (C) of spontaneous leaders before and after contact with follower colonies. (D) Movie stills of a migrating spontaneous leader (arrow), followed by p53 immunostaining (fourth panel) of the field indicated (dotted yellow lines). (E) Quantification of single-cell nuclear p53 intensity of spontaneous leaders and surrounding non-leaders. Black bars= median in (B-C) and (E); n= number of cells in (E). Data are from one representative repeat of three biological replicates in (E) or from selected movies of three biological replicates in (B-C). p values from Mann-Whitney U test in (B-C) and (E). **All figures:** White dashed line= initial contact point; black dashed line= final contact point; empty circles overlayed on bar charts indicate values obtained in each repetition; scale bars 100μm (movie sequences) and 50μm (confocal images); fluorescence values are expressed as arbitrary units.

Fig. 2. p53 instructs leader cell behavior. (A) Experimental design for co-culture of untreated and Mitomycin C (MMC)-treated cells. (B) Movie stills of co-cultures of MMC-treated GFP-positive wild-type cells and untreated unlabeled wild-type cells. (C) Movie stills from co-cultures of untreated unlabeled wild-type cells and untreated wild-type cells expressing nuclear GFP. (D) Percentage of MMC-treated wild-type or *p53KO* cells acting as leaders. (E) Movie stills of co-cultures of untreated unlabeled wild-type and MMC-treated GFP-positive *p53KO* cells. (F-G) Tracking of mean migration speed of and mean respective distance between pairs of untreated wild-type and MMC-treated wild-type cells (F) or *p53KO* cells (G). (H) Tracks of wild-type cells upon contact with MMC-treated wild-type (blue) or *p53KO* (grey) cells. (I) Persistence of migration of wild-type followers upon contact with MMC-treated wild-type or *p53KO* cells. Bar

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

chart shows mean values in (D). Black bars= median in (I) n= number of contacts in (D) and (F-I). Data are pooled from three biological replicates in (D) or from selected movies of three biological replicates in (F-I). Error bars= $\pm$ SEM in (D) and (F-G). p values from logistic regression in (D) and from Mann-Whitney U test in (I).

Fig. 3. The p53 target p21 drives leader cell migration. (A-B) Movie stills of a migrating spontaneous leader (arrow), subsequently immunostained for p21 (A, dotted yellow lines indicate the corresponding field) and corresponding fluorescence intensity quantification (B). (C) Movie stills of GFP-positive wild-type cells co-cultured with MMC-treated *p21KO* cells. (D) Percentage of MMC-treated wild-type or *p21KO* cells acting as leaders. (E-F) Tracking of mean migration speed of and mean respective distance between pairs of untreated wild-type cells and MMC-treated wild-type cells (E) or *p21KO* cells (F). (G) Tracks of wild-type cells migration upon contact with MMC-treated wild-type (blue) or *p21KO* cells (grey). (H-I) Persistence of migration (H) and displacement (I) of wild-type colonies upon contact with MMC-treated wild-type or *p21KO* cells. Bar charts show mean values in (D). Black bars= median in (B) and (H-I). Error bars= $\pm$ SEM in

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

(D-F). n= number of cells in (B), number of contacts in (D-F). Data from one representative repeat of three biological replicates in (B), pooled from three biological replicates in (D) or from selected movies of three biological replicates in (E-I). p values from Mann-Whitney U test in (B) and (H-I) and from logistic regression in (D). DAPI= 4',6-diamidino-2-phenylindole.

Fig. 4. CDK inhibition promotes leader cell specification. (A) Movie stills of co-cultures of GFP-positive wild-type cells and cells over-expressing p21 (p21OE). (B) Percentage of p21OE cells acting as leaders. (C) Tracking of mean migration speed of and mean respective distance between pairs of wild-type cells and p21OE cells. (D) Movie stills of co-cultures of GFP-positive wild-type cells and cells over-expressing p16 (p16OE). (E) Percentage of p16OE cells acting as leaders. (F-G) Percentage of spontaneous leaders that keep migrating upon addition of DMSO, CDK2 inhibitor K03861 (F) or CDK1 inhibitor RO-3306 (G). Bar charts show mean values in (B) and (E-G). Error bars= $\pm$ SEM in (B-C) and (E-G). n= number of contacts in (B-C) and (E), number of spontaneous leaders in (F-G). Data pooled from three biological replicates in (B) and (E-G) or from selected movies of three biological replicates in (C). p values from logistic regression in (B) and (E-G).

Fig. 5. Injury-induced p53 elevation drives collective migration in epithelial repair. (A) Quantification of cell cycle phase duration in FUCCI-reporter expressing scratch-induced leaders. (B) Movie stills showing emergence of scratch-induced leaders (arrow) from a monolayer of wild-type cells and p53 immunostaining of the indicated field (dotted lines). (C) Quantification of nuclear p53 intensity of scratch-induced leaders and surrounding non-leaders from experiments as in (B). (D-E) Movie still of cells expressing p21 promoter-driven nuclear GFP after scratching (D) and corresponding quantifications (E). Images are pseudo-colored to reflect signal intensity. Edge

cells are within white dotted lines; bulk cells are within yellow dotted lines. T indicates time from scratching. **(F-G)** Movie still of cells expressing p21 promoter-driven nuclear GFP after scratching as in **(D)**, but in the presence of p38 inhibitor SB202190, and corresponding quantifications **(G)**. **(H-J)** Movie stills of uninduced control **(H)** or cells expressing the p53 inhibitor GSE-22 **(I)** after scratch and corresponding quantifications of migration speed **(J)**. **(K)** Quantification of migration speed of wild-type or *p21KO* cells after scratch. **(L)** Experimental design to induce localized p53 activation at the edge of scratched monolayers. **(M-N)** Movie stills of control uninduced and not-irradiated (first panel), uninduced and irradiated (second panel) or GSE-22 expressing and irradiated (third panel) cells after scratching **(M)** and corresponding quantifications of migration speeds **(N)**. Black bars= median in **(A)**, **(C)**, **(E)** **(G)**, **(J-K)** and **(N)**; n= number of cells in **(C)**, number of movies in **(J-K)** and **(N)**. Data from one representative repeat of three biological replicates in **(A)**, **(J-K)** and **(N)**, or one representative movie of three biological replicates in **(E)** and **(G)**. p values from Mann-Whitney U test.

Fig. 6. Leader cells are eliminated upon epithelial repair. (A) Movie stills following the fate of a spontaneous leader (arrow). (B) Percentage of spontaneous leaders that are eliminated when surrounded by neighbors. (C) Movie stills of scratch-induced leaders (Cyan, yellow and magenta arrows point to different leaders over time), showing their elimination at gap closure. (D)

Percentage of leader or surrounding non-leader cells eliminated at gap closure. **(E)** Quantification of apoptotic events (cleaved Caspase-3-positive cells) in wild-type or *p21KO* Nutlin-3 treated cells grown at low- or high-density and following compaction. **(F)** Mean number of *p21KO* cells (percentage of initial number/field) in Nutlin-3 treated pure cultures (Monoculture) or confronted with *p53KO* cells (Co-culture). **(G)** Mean number of p21OE cells (percentage of initial number/field) in pure cultures (Monoculture) or confronted with wild-type cells (Co-culture). **(H)** Confocal imaging of ZO-1 immunostaining of a co-culture at confluency of p21OE and GFP positive wild-type cells. **(I-J)** Diagram and model summarizing the function of p53/p21 in epithelial repair. Epithelial scratching induces p53 and its target p21, which drives leader cell migration and epithelial closure via CDK inhibition. p53 (but not p21) elevation also induces leader cells to behave as mechanical losers, causing their elimination upon epithelial closure. Bar charts show mean values in (B) and (D). Black bars= median in (E). Error bars= $\pm$ SEM in (F-G). n= number of cells in (B), and (D), number of fields in (F-G). Data from one representative repeat of three biological replicates in (E) and (F-G), pooled from three biological replicates in (B) and (D) or from one selected field of three biological replicates in (H). p values from logistic regression in (D) and from Mann-Whitney U test in (E).

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

Supplementary Materials

Figs. S1 to S6

Movies S1-10

Supplementary Figures

Fig. S1. p53 promotes leader migration. (A) Movie stills and Particle Image Velocimetry (PIV) analysis of an emerging spontaneous leader in a wild-type MDCK culture. PIV is reported in comparison to 15 minutes before the timepoint of the presented image. Color hues in PIV show direction as per legend, their intensity correlates with speed of migration. Cells have little coordinated movement at the time of contact (TOC) but migrate collectively after engaging with the leader. (B) Cell tracks of spontaneous leaders (continuous line) and followers (dashed line) pairs (same color) upon contact. (C) Movie stills of a binucleated spontaneous leader emerging from a wild-type MDCK population expressing nuclear GFP. (D) BBCs, generated from wild-type cells expressing nuclear GFP, display leader behavior in contact with unlabeled wild-type cells. (E-F) Confocal image containing normal cells and BBCs immunostained with anti-p53 (E), showing elevated p53 levels in plurinucleated cells compared to mononucleated neighbors, as quantified in (F). (G) Confocal images of co-cultures of unlabeled wild-type cells and MMC-treated cells expressing nuclear GFP, immunostained against p53. Cyan arrows in the left panel indicate GFP positive cells. (H) Percentage of wild-type or *p53KO* cells not treated with MMC acting as leaders (control for Fig.2D). (I) Movie stills and PIV analysis of MMC-induced GFP-positive wild-type leaders contacting an untreated neighbour colony. PIV is reported in comparison to 15 minutes before the timepoint of the presented image. Color hues in PIV show direction as per legend, their intensity correlates with speed of migration. Cells migrate coordinately after engaging with the leader. TOC=Time of contact. (J-K) Wild-type cells expressing nuclear GFP still divide when treated with sub-lethal doses of Nutlin-3 (J), albeit at a slower rate than cells treated with the DMSO carrier alone (K). (L) Movie stills from co-cultures of unlabeled *p53KO* and GFP-positive wild-type cells in the presence of DMSO. (M) Movie stills from co-cultures of

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

unlabeled *p53KO* cells and wild-type cells expressing nuclear GFP, treated with Nutlin-3. (N) Percentage of wild-type cells acting as leaders to *p53KO* cells, when treated with Nutlin-3 or DMSO. (O) Tracking of mean migration speed of and mean respective distance between pairs of Nutlin-3 treated pairs of wild-type and *p53KO* cells. Bar charts show mean values in (H) and (N). Black bars= median in (F); n= number of cells in (F), number of contacts in (H) and (N-O). Data from one representative repeat in (F), pooled from three biological replicates in (H) and (N) or from selected movies of three biological replicates in (B) and (O). Error bars= $\pm$ SEM in (H) and (N-O). p values from Mann-Whitney U test in (F) and from logistic regression in (H) and (N). DAPI= 4',6-diamidino-2-phenylindole. **Here and throughout all figures:** White dashed line= initial contact point; black dashed line= final contact point; empty circles overlayed on bar charts indicate values obtained in each repetition; scale bars 100 μ m (movie sequences) and 50 μ m (confocal images); fluorescence values are expressed as arbitrary units.

Fig. S2. Inhibition or mutation of p53 impairs leader behavior. (A) Quantification of the frequency of MMC-treated cells acting as leaders when p53 is inhibited by GSE-22 expression. C5.4 and C5.5 are two different GSE-22 carrying clones. (B-E) Movie stills of MMC-treated uninduced control cells (B-C) or MMC-treated cells inducibly expressing GSE-22 (D-E). (F-G) Confocal images of p53 immunostaining. Blebbistatin elevates p53 in control GFP-positive wild-type cells (F) but not in GFP-positive *p53KO* clones (G, Clone C6 shown here). (H) Schematic representation of the canine *p53* exon 4 sequence, targeted for CRISPR/Cas9-mediated mutagenesis. Gene disruptions in *p53KO* clones C6 and C14 are indicated. The two clones behaved similarly in all experiments. (I) Quantification of the frequency of leader cells in wild-type or *p53KO* BBCs upon contact with wild-type cells. (J) Movie stills from co-cultures of *p53KO* BBCs expressing GFP with untreated wild-type unlabeled cells. (K) Cell tracks of pairs (same color) of wild-type GFP-positive cells treated with MMC (continuous line) and wild-type neighbors (dashed line) upon contact, showing coordinated migration. (L) Cell tracks of pairs (same color) of *p53KO* GFP-positive cells treated with MMC (continuous line) and wild-type neighbors (dashed line) upon contact, showing lack of coordinated migration. (M) Quantification of displacement of wild-type colonies upon contact with MMC-treated wild-type or *p53KO* cells. Bar charts show mean values in (A) and (I). Black bars= median in (M); n= number of contacts in (A) and (I); error bars= $\pm$ SEM in (A) and (I). Data pooled from three biological replicates in (A) and (I) or from selected movies of three biological replicates in (K-M). p values from logistic regression in (A) and (I) and from Mann-Whitney U test in (M). DAPI= 4',6-diamidino-2-phenylindole.

Fig. S3. p21 instructs leader behavior. (A) Confocal images of p21 immunostaining after 24 hours of Nutlin-3 treatment of wild-type or *p21KO* clones E9 and G5. (B) Schematic representation of the canine *p21* exon 2 sequence, which was targeted for CRISPR/Cas9-mediated mutagenesis.

Gene disruption in *p21KO* clones E9 and G5 are indicated. The two clones behaved similarly in all experiments. **(C)** Movie stills of co-cultures of MMC-treated unlabeled wild-type cells and untreated wild-type cells expressing nuclear GFP. Control experiment for Fig. 4A. **(D-E)** Cell tracks of pairs (same color) of MMC- treated (continuous line) wild-type (D) or *p21KO* (E) cells and wild-type GFP-positive neighbors (dashed line) upon contact. **(F-G)** Cells overexpressing p21 (p21OE) in a Doxycyclin (DOX) -inducible manner displayed normal morphology in the absence of Doxycyclin (F); upon Doxycyclin addition (G) cells stopped dividing and flattened. **(H)** Percentage of wild-type cells behaving as leaders when treated with or without Doxycyclin. Control for Fig 4B and E. Bar chart shows mean values in (H). Data from selected movies from three biological replicates in (D-E), from representative images of one of > 5 biological replicates in (F-G) and pooled from three biological replicates in (H). n= number of contacts in (H); error bars= $\pm$ SEM in (H). p value from logistic regression in (H).

Fig. S4. p53 promotes non-transcriptional elevation of leader cell markers via p21. (A-B)

Confocal images of PI3K immunostained co-cultures of unlabeled wild-type cells and MMC-treated wild-type cells expressing nuclear GFP (A) and relative quantification of average fluorescence intensity per cell (B). (C-D) Confocal images of ITG β 1 immunostained co-cultures of unlabeled wild-type cells and MMC-treated wild-type cells expressing nuclear GFP (C) and relative quantification of average fluorescence intensity per cell (D). (E-G) Confocal images of PI3K immunostained co-cultures of wild-type cells expressing nuclear GFP and *p21KO* unlabeled cells treated with DMSO alone (E) or with Nutlin-3 (F) and relative quantification of average fluorescence intensity per cell (G). (H-J) Confocal images of ITG β 1 immunostained co-cultures of wild-type cells expressing nuclear GFP and *p21KO* unlabeled cells treated with DMSO alone (H) or with Nutlin-3 (I) and relative quantification of average fluorescence intensity per cell (J). (K-L) Confocal images of surface ITG β 1 immunostained co-cultures of unlabeled wild-type cells and MMC-treated wild-type cells expressing nuclear GFP (K) and relative quantification of average fluorescence intensity per cell (L). (M-N) Quantitative PCR analysis of PI3K (M) or ITG β 1 (N) mRNA expression in wild-type cells with or without MMC treatment. (O-P) Confocal images of PI3K immunostained co-cultures of wild-type cells expressing nuclear GFP and cells inducibly overexpressing p21 (p21OE) (O) and relative quantification of average fluorescence intensity per cell (P). (Q-R) Confocal images of ITG β 1 immunostained co-cultures of wild-type cells expressing nuclear GFP and p21OE cells (Q) and relative quantification of average fluorescence intensity per cell (R). (S-T) Confocal images of surface ITG β 1 immunostained wild-type cells treated with DMSO or CDK1 inhibitor RO-3306 (S) and corresponding quantification of fluorescence intensity (T). Bar charts show mean values in (M-N). Black bars= median in (B),

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

(D), (G), (J), (L), (P), (R) and (T); n= number of cells in (B), (D), (G), (J), (L), (P), (R) and (T). Data from one representative repeat of three biological replicates in (B), (D), (G), (J), (L), (P), (R) and (T) or pooled from three biological replicates in (M-N). Error bars= $\pm$ SEM in (M-N). p values from Mann-Whitney U test in (B), (D), (G), (J), (L), (P), (R) and (T); from Wilcoxon signed rank test in (M-N).

Fig. S5. p53/p21-induced leader migration is dependent on PI3K and Rac1. (A) Movie stills of co-cultures of unlabeled wild-type cells and GFP-positive MMC-treated wild-type cells. DMSO (top panel), PI3K inhibitor LY294002 (middle panel), or Rac1 inhibitor Z62954982 (bottom panel) were added after 22 hours and migration was followed for a further 16 hours. White dashed line=

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.

initial contact point; yellow dashed line= contact point at addition of inhibitor; black dashed line= final contact point. **(B)** Tracking of mean migration speed of pairs of migrating MMC-treated leader cells and untreated unlabelled wild-type followers before and after PI3K or Rac1 inhibition or DMSO addition. Data from selected movies from three biological replicates in (B); n= number of contact in (B); error bars= $\pm$ SEM.

Fig. S6. p53 and p21 elevation drive collective migration in epithelial repair. (A) Movie still of FUCCI-expressing cells following scratching of the monolayer, showing leaders at different cell cycle phases (arrows). (B) Quantification of cell cycle duration in followers of spontaneous

leaders or non-migrating neighbours. **(C)** Movie stills showing emergence of leader cells (arrow) following a scratch (first two panels). Right panel shows p21 immunostaining of the field indicated (dotted lines). **(D)** Quantification of single-cell nuclear p21 intensity of scratch-induced leaders and surrounding non-leaders. **(E)** Experimental design of the barrier release migration experiment. **(F)** Movie stills (first two panels) showing migrating leaders (arrows) emerging following barrier release. Right panel shows a confocal image of the indicated field (dotted lines) following p53 immunostaining. **(G)** Quantification of single-cell nuclear p53 intensity of barrier release-induced leaders and surrounding non-leaders. **(H)** Movie stills (first two panels) showing a migrating leader (arrow) emerging following barrier release. Right panel shows a confocal image of the indicated field (dotted lines) following p21 immunostaining. **(I)** Quantification of single-cell nuclear p21 intensity of barrier release-induced leaders and surrounding non-leaders. **(J)** Schematic representation of the architecture of the p21 reporter construct. NLS= nuclear localisation sequence. PEST= protein destabilization motif rich in proline, glutamate, serine and threonine. **(K)** Quantification of single-cell nuclear γ H2AX signal in immunostaining of scratched epithelial monolayer cells at the edge or in the bulk of the monolayer. **(L-M)** Movie stills of wild-type (L) or *p21KO* (M) cells migrating after scratch wound (corresponding to the quantification in Fig. 3K). **(N)** (Left) Confocal image of a scratched monolayer of Hoechst-treated wild-type cells indicating the irradiated region (marked by a green line). Center and right panels are confocal images of a γ H2AX immunostaining of the dotted area 2 hours post-irradiation. Black bars= median in (B), (D), (G), (I) and (K); n= number of cells in (B), (D), (G), (I) and (K). Data from one representative repeat of three biological replicates in (B), (D), (G) and (I) or four biological replicates in (K). p values from Mann-Whitney U test. DAPI = 4',6-diamidino-2-phenylindole.

This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published as an Online Research article in Science, 375, (2022), doi: 10.1126/science.aabl8876.