Radiation exposure elicits a neutrophil-driven response in healthy lung tissue that enhances metastatic colonization

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Radiotherapy (RT) is received by approximately 60% of all cancer patients and remains one of the most successful non-surgical techniques to achieve local tumor control. In recent decades, technological advances and increased sophistication in imaging have significantly improved the accuracy, efficacy and tolerability of RT. However, radiation-induced damage in nontarget tissues can still occur, inducing a local injury. A previous study from our laboratory identified the presence of a tissue regeneration program within the metastatic environment of pulmonary metastases from breast cancer, generating the intriguing hypothesis that an injury could predispose the tissue for metastatic growth. In this study, we aimed to test whether acute lung injury triggered by radiation would set the stage for a perturbed tissue interaction with arriving cancer cells and foster metastasis. Both clinical and experimental studies have reported poor prognosis and increased metastasis associated with tumors occurring within a pre-irradiated site. This has been attributed to both the release of pro-migratory factors from the irradiated loci of tumor and increased myeloid cell mobilization induced by the primary tumor. These studies, however, have not addressed whether an injury to healthy tissues induced by off-target radiation impacts subsequent metastatic growth within that organ. A 1973 study reported that radiation exposure influenced lung integrity and the seeding of metastatic cells, while an earlier clinical study indeed suggested that lung metastases are increased after post-operative RT for breast cancer. Here, analysis of women with clinically and pathologically comparable breast tumors revealed a significantly higher incidence of ipsilateral pulmonary metastasis (occurring on the same side as the breast cancer) in women who received post-operative RT compared to surgery alone. Of course, the outdated RT technologies used in this study would have generated a large off-target radiation volume compared to more precise modern image-guided platforms. Nonetheless, the biology behind the metastatic risk increase is not fully understood. Uncovering these features may reveal important mechanistic connections between the processes of tissue injury and cancer.

In the present study, we set out to elucidate the injury response of healthy lung tissue to radiation exposure. We report that radiation can generate a profoundly pro-metastatic microenvironment. Importantly, we found that lung neutrophils are key components of this tumor promotion effect. Although neutrophils are vital for the host defense against pathogens, their infiltration and overzealous activation during acute inflammatory responses can exacerbate tissue damage. However, an important role in the repair and regeneration of damaged epithelium in certain contexts is also emerging. In cancer, neutrophils are well known to contribute to many aspects of tumor progression and metastasis, and a plethora of pro-tumorigenic activities have been defined. Here, we describe an unexpected activity of neutrophils in the irradiated tissue, which, by influencing the responses of lung resident cells, bridges their tissue-repair functions with their tumor-supportive activity. Mechanistically, we identified Notch as the mediator of the neutrophil-dependent lung epithelial response to radiation injury, which is central to this tumor-supportive function. This surprising phenomenon of neutrophil tissue-priming following radiation exposure may have implications for the tissue response to radiation in patients. It warrants clinical studies to investigate neutrophil-driven perturbations in cancer patients undergoing RT.

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Results
Radiation exposure in healthy lung tissue boosts metastasis.
To examine the effects of off-target radiation exposure on healthy lung tissue, we delivered a single 13 Gy dose of focused radiation specifically to the thoracic cavity of anaesthetized female BALB/c mice. We focused on the short-term tissue response to this acute lung injury, which in the long-term typically results in pneumonia (Fig. 1a). Mice were allowed to recover for 4 days, before being orthotopically injected with cancer cells into the mammary fat pad to induce primary tumors and test the onset of spontaneous metastasis (Fig. 1a). We first induced nonmetastatic 4T07 primary tumors, where cancer cells can disseminate but are unable to initiate metastatic growth. Strikingly, we found an abundance of metastases within the lungs of mice previously irradiated, while only rare metastatic foci were detected in control lungs (sham-irradiated) (Fig. 1b,c). Similarly, when lungs were harvested from irradiated mice harboring metastatic 4T1 tumors, we observed, at an early stage of tumor progression, a significant increase in metastatic burden compared to control mice while the primary tumor size was not affected (Extended Data Figs. 1a–c and 2a). We next confirmed our findings in an experimental metastasis model, in which metastases are induced via a single tail intravenous (i.v.) injection to drive cancer cell seeding in the lungs of mice that have received targeted lung irradiation seven days earlier (Fig. 1d). Consistently, a profound increase in metastasis was observed following irradiation in FVB mice who received an i.v. injection of primary cancer cells isolated from tumors developed in mammary tumor virus-promoter middle tumor-antigen (MMTV-PyMT) mice crossed with actin-green fluorescent protein (GFP) transgenic mice (Extended Data Fig. 1d,e). This pro-metastatic effect of radiation was not unique for breast cancer cells, as a strong induction in metastasis was also observed in immune-compromised mice that were inoculated with the human esophageal adenocarcinoma cell line Flo-1 following lung irradiation (Fig. 1e,f). Moreover, when injecting human non-small-cell lung cancer cell lines A549 and H460, we could only detect metastatic foci in pre-irradiated mice and not control

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animals (Extended Data Fig. 1f). Thus, pre-exposure of healthy lung tissue to radiation strongly supports the growth of cancer cells across multiple mouse genetic backgrounds and induces an aggressive metastatic phenotype in poorly metastatic cells.

Since cancer patients are typically treated with fractionated RT, in which low doses of radiation are delivered over consecutive days, we employed fractionated dosing in our experimental setting (Fig. 1g). As with single-dose RT, a profound enhancement of 4T1 cancer cell metastasis was observed in BALB/c mice that received doses of 3 × 4 Gy compared to control mice (Fig. 1h,i), with the effect more pronounced with an additional 4 Gy fraction. We calculated the biological effective dose (BED) in our different experimental settings, which measures the true biological dose delivered to a given tissue by a particular combination of dose-per-fraction and total dose, characterized by a specific $\alpha/\beta$ ratio$^{42}$. We used an $\alpha/\beta$ of 10 in BED calculations for this early radiation-induced reaction$^{42}$. Importantly, the lowest fractionated regime (3 × 4 Gy) delivered almost half the BED of a single high-dose 13 Gy treatment (BED 16.8 versus 29.9 Gy), yet still led to a strong boost in metastatic proficiency of the lung. Next, we used image-guided, focused radiation to specifically target the right lung lobe of BALB/c mice, whilst leaving the left lung, heart and trachea largely unexposed (Fig. 1j). Given that the right is the larger side of the murine lung, more metastases are found on this side in both control and irradiated animals. Therefore, we measured if irradiation of the right side further increased the right/left ratio of metastases (Fig. 1j). Notably, we observed a significant enrichment of 4T1 metastatic breast tumors in the lobe pre-exposed to either a medium (8 Gy) or high-dose of radiation (12 Gy) when compared to the nonirradiated control mice (Fig. 1k). No changes in metastasis were observed in the nonirradiated left side of the lung between the experimental groups (Extended Data Fig. 1g), excluding systemic priming from irradiated to nonirradiated tissue in this setting. Therefore, smaller irradiation volumes exert a similar effect to whole thoracic radiation and the influence of radiation-induced cardiac damage on metastatic growth in our system can be excluded.

Radiation induces neutrophil infiltration and activation. To probe how the tissue alterations induced by lung irradiation fueled subsequent metastatic growth, we analyzed lung tissue from BALB/c mice at day 7 following a single-dose of 13 Gy irradiation, in the absence of cancer cell seeding. At this early timepoint, no overt alterations in the lungs were observed histologically (Fig. 2a), although, as expected, we detected radiation-induced DNA damage and senescence (Extended Data Fig. 3a–d). When analyzing immune infiltration in the lung at this timepoint, we observed a marked neutrophil infiltration compared to other immune subsets in the irradiated lungs (Fig. 2b,c and Extended Data Fig. 2b). No significant alterations were detected within the adaptive immune compartment upon irradiation (Fig. 2c). This neutrophil increase was also reflected in the abundance of intratumoral neutrophil infiltration in the 4T1 metastatic lesions subsequently growing in pre-irradiated lungs compared to controls (Extended Data Fig. 3e). The pro-metastatic effect of radiation was maintained for murine cancer cells in immunodeficient mice, as we observed for human cell lines (Fig. 1e,f and Extended Data Figs. 1f and 3f).

Interestingly, we found that neutrophils accumulating in irradiated lungs at this early timepoint homogenously acquired a distinctive, hyper-segmented nuclear morphology, a hallmark feature of neutrophil activation (Fig. 2d,e). To further characterize this activated status, we performed mass-spectrometry based proteomic profiling of isolated lung and bone marrow neutrophils from irradiated and control mice (Fig. 2f). Striking differences were observed in the proteome of neutrophils harvested from irradiated lungs, while the proteome of bone marrow neutrophils isolated from distal femurs of the two groups were highly comparable (Extended Data Fig. 3g,h), although a level of radiation in the bone marrow of ribs cannot be excluded. Consistent with their hyper-segmented nuclear morphology (Fig. 2d,e), Metacore analysis of over-represented proteins revealed that neutrophils from irradiated lungs acquire a pro-inflammatory, activated phenotype with an increase in granule protein content (Fig. 2g,h and Extended Data Fig. 3i). Thus, radiation exposure in the lungs triggers a profound local activation of neutrophils. Recent evidence indicates that neutrophils adapt accordingly to tissue-derived signals and have a variable life time in mouse tissues, which is approximately 10.1 h in the lung$^{41}$. To test a direct effect of radiation exposure on neutrophil activation and their persistence in the tissue, we gave mice a pulse of 5-ethyl-2’-deoxyuridine (EdU) 1 h before irradiation and analyzed neutrophils from lungs and bone marrow (Extended Data Fig. 3j). Flow cytometry analysis revealed negligible EdU positivity seven days post-irradiation in neutrophils from both compartments, implying complete neutrophil turnover during this time (Extended Data Fig. 3k). We concluded that activated neutrophils observed seven days post-irradiation were not directly exposed to radiation, and that neutrophil recruitment and activation is likely triggered by the local lung environment.

Radiation-primed neutrophils support metastatic colonization. Pro-inflammatory neutrophils with hyper-segmented nuclei have previously been linked with anti-cancer activity, including in the context of RT$^{43,44}$. To test whether radiation-primed neutrophils alter the metastatic proficiency of irradiated lung tissue, we used daily injections of an anti-Ly6G antibody to deplete neutrophils from mice exposed to lung irradiation. 4T1-GFP$^+$ cancer cells were i.v. inoculated seven days post-irradiation (Fig. 3a). Strikingly, radiation-primed neutrophils strongly supported metastatic growth, as their depletion dramatically reduced the tumor burden in the lung (Fig. 3b–d).

Many factors are at play when lungs are injured by radiation. For instance, the integrity of the vasculature is compromised, which enhances extravasation. Indeed, cancer cells were increased 72 h after seeding in previously irradiated lungs, a timepoint when extravasation is complete; however, this was unaffected by the absence of neutrophils (Extended Data Fig. 4a–c). NETosis has been increasingly reported as a mechanism mediating neutrophil pro-metastatic functions via inducing extracellular matrix remodeling and directly increasing cancer cell growth$^{20–22}$. However, we did not detect NETosis in lung neutrophils at various timepoints following radiation exposure (Extended Data Fig. 4d).

We reasoned there might be various nonmutually exclusive mechanisms by which neutrophil recruitment and activation could favor metastatic growth in irradiated lungs$^{45}$. Radiation-primed neutrophils could boost the growth of cancer cells directly or by orchestrating a tumor-supportive tissue response. To test if neutrophil activation directly influences their interaction with cancer cells, we performed in vitro co-cultures using a porous three-dimensional (3D) Alvetex scaffold (Extended Data Fig. 4e). We reported previously that lung neutrophils support cancer cell growth in this co-culture system$^3$, but no further boost was observed when neutrophils were harvested from irradiated lungs (Extended Data Fig. 4f). In addition, we did not detect an increase in cancer cells EdU incorporation following 2D co-culture with radiation-primed lung neutrophils compared to control neutrophils (Extended Data Fig. 4g,h). These results suggest radiation-primed lung neutrophils do not offer an additional direct growth-promoting advantage to cancer cells compared to control neutrophils.

This prompted us to test the alternative hypothesis that radiation-primed neutrophils boosted metastasis by perturbing the lung microenvironment before the arrival of cancer cells. To determine this, we performed targeted lung irradiations in neutropenic G-csf-knockout mice that are unable to mobilize neutrophils from...
Fig. 2 | Radiation exposure induces the infiltration and local activation of lung neutrophils. a,b, Representative H&E images of lungs (a) and S100A9 immunostaining for neutrophils (b) from control and irradiated mice at day 7 (n = 6 mice per group, two independent experiments). Scale bars, 250 μm (a) and 100 μm (b). c, Immune cell frequencies in the lungs estimated by FACS, seven days post-irradiation (n = 7 mice per group, two independent experiments). Data are presented either as the frequency among live cells (for total CD45+ immune cells) or frequency among CD45+ cells. d,e, Representative immunofluorescent images (d) and quantification of nuclear segmentation (e) from sorted Ly6G+ lung neutrophils harvested from control and irradiated lungs at day 7 post-irradiation (n = 7 mice per group, two independent experiments). DAPI staining was performed on fixed cells plated on poly-lysine coverslips (n = 2 coverslips per mouse for each experiment). Each data point (n = 30 untreated, n = 32 irradiated) represents the average segmentation across all cells within the field of view. Scale bars: main panel, 100 μm; enlarged insets, 10 μm. f, Experimental setup for quantitative mass-spectrometry based proteomic analysis of Ly6G+ positive cells. Cells were isolated from the lungs and from bone marrow extracted from the femur of control and irradiated BALB/c mice at day 7 by MACS-sorting. g, Metacore pathway analysis of proteins upregulated by >2 fold in lung neutrophils from irradiated mice, compared to control mice (n = 3 mice per group). h, Granule protein upregulation in the lungs of irradiated versus untreated mice (n = 3 mice per group). Each dot represents an individual granule protein (n = 24), red dots depict an enrichment in irradiated mice, blue dots represent downregulation. All data represented as mean ± s.e.m. Statistical analysis by a two-tailed t-test with Welch’s correction for c and unpaired two-tailed t-test for e. Gating strategies for FACS analysis provided in Extended Data Fig. 2. FDR, false discovery rate; NS, not significant.

...the bone marrows. Irradiated G-csf-knockout mice were treated with rGCSF to permit neutrophil recruitment to the lungs (Fig. 3e), after which infiltrating neutrophils displayed a marked increase in nuclear segmentation, indicating an activated phenotype similar to wild-type mice (Fig. 3f,g). rGCSF treatment was stopped 48 h before i.v. injection with MMTV-PyMT primary mammary cancer cells, by which time the mice had returned to their original neutropenic state (Extended Data Fig. 5a,b). Importantly, long-term analysis...
revealed a significant increase in metastatic incidence in irradiated mice pretreated with rGCSF (Fig. 3h,i). Since the number of neutrophils was only increased before cancer cell seeding, this suggests that neutrophils may indirectly influence cancer cell growth in the irradiated lung by perturbing the tissue microenvironment.

**Neutrophils influence the epithelial response to radiation.** We next tested whether radiation-primed neutrophils alone were sufficient to drive a change in the healthy lung tissue microenvironment and influence metastatic receptiveness. We performed adoptive transfer of control or radiation-primed lung neutrophils into the lungs of naïve recipient mice via a single i.v. injection (Fig. 4a). After four days of lung ‘conditioning’, at which time turnover of transferred neutrophils had occurred,

4T1-GFP+ breast cancer cells were seeded to induce metastasis (Fig. 4a). Notably, mice whose lungs were conditioned with radiation-primed neutrophils had a marked increase in cancer cell growth compared with mice conditioned with control lung neutrophils (Fig. 4b–d). Thus, radiation-primed neutrophils can incite a tissue change in naïve lung tissue.

We then sought to uncover this neutrophil tissue-perturbation activity in the context of radiation exposure. We used flow cytometry to sort either epithelial (CD45−CD31−Ter119−EpCAM+) or mesenchymal (CD45−CD31−Ter119−EpCAM+) cells from con-
Fig. 4 | Radiation-primed neutrophils perturb the lung tissue environment. a, Experimental setup for the neutrophil adoptive transfer. Control or radiation-primed Ly6G+ lung neutrophils were MACS-sorted seven days following irradiation and intravenously injected into naïve recipient BALB/c mice. Mice were given an i.v. injection of 4T1-GFP+ cancer cells four days later and metastatic burden assessed after one week. b–d, Quantification of the entire lung (serial sectioning) (b) and representative images of GFP (c) and H&E stained lungs (d) (n = 6 untreated, n = 7 irradiated mice, two independent experiments). Metastatic foci are outlined and indicated with arrows. See methods for quantification details. Scale bars, 100 μm. e, Schematic depicting the experimental setup for bulk RNA sequencing. BALB/c mice were given daily injections of anti-Ly6G (α-Ly6G) to deplete neutrophils or a control IgG antibody, beginning the day before targeted lung irradiation. Flow cytometry was used to isolate CD45+ cells from untreated, irradiated and neutrophil-depleted irradiated mice seven days post-irradiation (n = 4 mice per group). f, PCA of Lin+EpcAM+ epithelial cell signatures following RNA-seq analysis of untreated, irradiated and neutrophil-depleted irradiated lung samples. Each dot represents an individual mouse, with oval enclosing the samples from each group to highlight their similarity in the PCA plot. g, Experimental setup for lung epithelial analysis. Lin+EpcAM+ lung epithelial cells harvested from untreated, irradiated and neutrophil-depleted irradiated BALB/c mice (seven days post-irradiation) were sorted by flow cytometry and co-cultured in Matrigel with MLg normal lung fibroblasts to generate lung organoids. h,i, Representative images (h) and quantification (i) of lung organoid co-cultures. Scale bars, 1,000 μm. Quantification of organoid number is shown as the percentage reduction in organoids compared to the control (untreated) group. Each dot represents an individual mouse, with the three independent experiments indicated by colored dots (n = 12 mice per group). Triplicate technical replicates were quantified for each mouse. Data represented as mean ± s.e.m. Statistical analysis by two-way ANOVA for b and nonparametric two-tailed Mann–Whitney test for i. Gating strategies for FACS provided in Extended Data Fig. 2.

trol and irradiated lungs in the presence or absence of neutrophils (Fig. 4e). As expected, RNA sequencing showed significant alterations in the transcriptome of both cell types seven days after irradiation, while a profound influence of neutrophils also emerged (Fig. 4f and Extended Data Fig. 6a). Particularly in the lung epithelium, the absence of neutrophils resulted in a completely different irradiated cell cluster in the principle component analysis (PCA) (Fig. 4f). To test if the neutrophil-mediated transcriptional response was reflected in a functional difference in the epithelial compartment, we performed a lung organoid assay12. This is the gold-standard assay whereby lung progenitors are challenged to survive and grow 3D organoid structures ex vivo. Lung epithelial cells were isolated from mice irradiated either in the presence or absence of neutrophils and co-cultured with lung fibroblasts in Matrigel (Fig. 4e,g). As expected, epithelial cells injured by radiation sharply reduced the overall number of organoids compared to non-injured cells13, yet strikingly epithelial cells irradiated in the absence of neutrophils were almost completely devoid of organoid forming ability (Fig. 4h,i). These data suggest that neutrophils are required to support lung epithelial fitness and progenitor function upon irradiation. To examine whether radiation-induced alterations in lung epithelial cells also functionally affects their interaction with cancer cells, we cultured them ex vivo on 3D Alveta scaffolds with MMTV-PyMT-GFP+ cancer cells (as in Extended Data Fig. 4e). After confirming that the survival of the epithelial cells on the scaffolds was not affected by irradiation, we found that the growth advantage provided by epithelial cells was almost entirely abolished when the irradiation occurred in the absence of neutrophils (Extended Data Fig. 6b–e).

These functional differences in irradiated epithelial cells from neutrophil-depleted mice prompted us to further examine the transcriptional signatures of the different epithelial cell clusters we had distinguished by PCA analysis (Fig. 4f). We identified a distinctive cluster of genes whose expression was specifically enriched upon
Fig. 5 | Notch is activated in the lung epithelium and enhances spontaneous metastasis. a, Heatmap of LinEpCAM+ lung epithelial cells from control (untreated, UT), irradiated (IR) and neutrophil-depleted irradiated mice (left) (hierarchically clustered samples in columns and genes in rows, n = 4 mice per group), and Metacore pathway analysis (right) of the genes triggered by irradiation, but influenced by neutrophils (gene set B, indicated by the arrow). b, Heatmap showing selected genes from gene set B from a (hierarchically clustered samples in columns and selected genes in rows). The colour scale in the heatmap represents high expression (red) to low expression (blue) values. c, Representative images for Hes1 immunostaining in lung tissue from untreated or irradiated mice at day 7. Positive cells are indicated by arrows in the enlarged inset. Scale bars, 100 μm (inset) (n = 4 mice per group, one experiment). d, Experiment setup for genetic Notch activation. PyMT/Notch mice (MMTV-PyMT/SPC-Cre-ERT2/Rosa26NICD-IRES-GFP) or PyMT/Control mice (MMTV-PyMT/SPC-Cre-ERT2) were administered tamoxifen by oral gavage (40 mg kg−1) over three consecutive days to drive Cre expression in lung alveolar cells. Lungs were harvested 14 days after the last tamoxifen dose and assessed for metastatic burden. e, Representative H&E stained lungs (e) and histology quantification of lung metastases (f) from PyMT/Control and PyMT/Notch mice (n = 6 PyMT/Control mice, n = 8 PyMT/Notch mice, spontaneous metastasis assessed over n = 6 independent tamoxifen administrations). Scale bars, 250 μm. g, Representative immunofluorescence images from DAPI (blue) and Hes1 (red) stained lung sections from PyMT/Control (left panel, n = 5) and PyMT/Notch (right, n = 7) mice harvested at experimental endpoint. Lung metastases are depicted with a dashed line. Scale bars, 50 μm. h, Enlargement from g showing Hes1 (red) and GFP (green) immunofluorescence staining in the PyMT/Notch metastatic lesion (n = 7 mice). Positive cells are indicated by arrows. Scale bars, 10 μm. All data represented as mean ± s.e.m. Statistical analysis by a two-tailed t-test with Welch’s correction for f. The violin plot displays the median, 25th and 75th percentiles as well as the density of data points. Dots represent individual mice. Histology quantification process outlined in Supplementary File 1. ROS, reactive oxygen species.
irradiation and boosted by the presence of neutrophils (gene set B, Fig. 5a, left). In the mesenchymal compartment, a neutrophil-driven response was also evident; however, this was less clear and the presence of neutrophils appeared to have a greater impact on genes downregulated upon irradiation (gene set A, Extended Data Fig. 6f). Metacore pathway analysis of the neutrophil-promoted genes in epithelial cells (gene set B, Fig. 5a, right) revealed an enrichment of Notch signaling, a critical regulator of stem cell proliferation and differentiation during lung development and tissue-repair, particularly in receptors Notch1, 3 and 4 and the ligandsDll1 and Dll4 (Fig. 5b). The Notch signature was validated by quantitative PCR with reverse transcription (RT–qPCR), whereby a neutrophil-dependent induction of the canonical target genes Hes1 and Hey1 was also evident, as well as by immunofluorescence on isolated irradiated lung epithelial cells, where a reduction of intracellular activated Notch was detected in the absence of neutrophils (Extended Data Fig. 7a,b). Interestingly, in line with previous evidence of neutrophil-dependent vascular repair, neutrophils also appeared to influence Notch genes in endothelial cells isolated from irradiated mice as shown by RT–qPCR (Extended Data Fig. 7c), although they were excluded during cell sorting for the RNA-seq analysis (Fig. 4e). Taken together, our findings suggest that the epithelial response to radiation injury and their subsequent interaction with cancer cells is markedly influenced by the presence of neutrophils.

**Radiation enhances Notch signaling and cancer cell stemness.** Given the enrichment in Notch signaling within the lung epithelium of irradiated mice (Fig. 5a–c), we explored whether genetic Notch activation in control mice, mimicking the activation observed downstream of radiation–primed neutrophils, could recapitulate the pro-metastatic effect of radiation. To test this, we forced the activation of Notch specifically in the lung epithelium of nonirradiated mice by crossing Rosa26\textsuperscript{CRE-IRE-STOP-EGFP}\textsuperscript{Sftpc-CreER} mice with Sftpc-CreER mice that express Cre recombinase in lung alveolar type II cells. The resulting progeny were then crossed with the mammary tumor model MMTV-PyMT. Following the onset of mammary tumors, we administered tamoxifen to drive constitutive expression of the Notch intracellular domain in the lung alveoli in the absence of radiation exposure (Fig. 5d and Extended Data Fig. 8a). As expected, forced activation of Notch in the lung had no impact on primary tumor growth (Extended Data Fig. 8b). In contrast, when we analyzed the lungs two weeks post-tamoxifen, we observed a striking enhancement of spontaneous metastasis in mice with activated Notch (PyMT/Notch) compared to control mice (Fig. 5e,f), indicating that cancer cells directly profit from enhanced Notch signals within the lung epithelium. Notch activation, marked by the expression of the target gene Hes1, was clearly observed in the epithelium adjacent to metastatic foci in PyMT/Notch lungs, but not in control mice (Fig. 5g). Importantly, Hes1\textsuperscript{+} alveolar epithelial cells co-expressing GFP were readily detectable within the metastatic lesions of PyMT/Notch mice and, within the metastatic environment, adjacent cancer cells also showed staining (Fig. 5h). This suggests a local induction of Notch signaling in metastatic cells. Indeed, there was a marked enrichment of Hes1 expression in the tumor cells themselves, compared to tumor cells from control mice (Extended Data Fig. 8c). This suggests that persistent Notch activation in epithelial cells within the metastatic microenvironment of the lung can foster the growth of arriving tumor cells. Of note, in this context, any potential influence of lung neutrophils would be independent from their radiation-primed activity.

The identification of Notch activation within the lung alveolar compartment of irradiated mice (Fig. 5a–c and Extended Data Fig. 7a,b), together with the evidence that these cells are part of the early metastatic niche, prompted us to assess whether Notch-activated alveolar cells could be detected in the metastatic niche of pre-irradiated lungs. To test this, we utilized Cherry-niche labeling 4T1 cancer cells, which are able to identify neighboring noncancerous cells by mCherry uptake\textsuperscript{27} (Fig. 6a). We isolated mCherry\textsuperscript{+} noncancerous lung niche cells (CD45\textsuperscript{–}GFP\textsuperscript{+}Cherry\textsuperscript{+}) and Cherry\textsuperscript{–} distant lung cells (CD45\textsuperscript{–}GFP\textsuperscript{–}Cherry\textsuperscript{–}) from pre-irradiated or control lungs harboring metastases (Extended Data Fig. 2c) and performed single-cell RNA sequencing. Uniform manifold approximation and projection (UMAP) analysis was used for the unbiased identification of epithelial, endothelial and mesenchymal cell clusters (Fig. 6b). We identified those clusters by the expression of EpCAM, CD31 (PECAM-1) and PDGFR\textbeta, respectively (Extended Data Fig. 8d). Excitingly, Notch signaling was enriched within the epithelial compartment of the metastatic niche (Cherry\textsuperscript{+}) compared to the distal lung epithelial cells (Cherry\textsuperscript{–}) of irradiated mice (Fig. 6c). Thus, the Notch-high environment we observed at day 7 (before cancer cell seeding) becomes positively selected within the metastatic niche of breast cancer cells 14 days after the radiation-induced lung injury. It should be noted that the endothelial compartment (which also displayed Notch activation upon irradiation, Extended Data Fig. 7c) also contributes to the Notch-high environment within the metastatic niche, although we did not detect an enrichment compared to the distal lung (Fig. 6c). These findings, together with the enrichment of Notch in metastatic cells surrounded by Notch-activated lung epithelium (Fig. 5d–h), prompted us to examine Notch signaling within cancer cells growing in pre-irradiated lungs in the presence or absence of neutrophils (from Fig. 3a). Importantly, the nuclear localization of the Notch downstream DNA-binding protein RBP and the expression of target gene Hes1 was strongly enriched in metastatic 4T1 cells growing in irradiated lungs compared to control tumors. This was almost entirely abrogated by depleting neutrophils (Fig. 6e and Extended Data Fig. 8e), as well as in irradiated G-csf-knockout mice (Fig. 3e and Extended Data Fig. 8f).

Notch signaling is critical for the regulation of stemness in cancer cells\textsuperscript{6}; therefore, we tested if metastases high in Notch activity also show an increase in stemness potential ex vivo. We isolated metastatic 4T1 breast cancer cells growing in the lungs of pre-irradiated (Notch\textsuperscript{high}) or control mice (Notch\textsuperscript{low}) and analyzed their proliferative capacity ex vivo. We isolated metastatic 4T1 cells growing in irradiated lungs compared to control mice, which was reduced in metastases in neutrophil-depleted lungs (in which Notch expression is lower) (Fig. 6i,j). Interestingly, Sox9 has also been reported as a direct target gene of Notch\textsuperscript{14}. Together, these data support the notion that Notch activation, which is part of the neutrophil-dependent radiation injury response, may be exploited by cancer cells seeding in the lung to enhance their stemness potential.

**Radiation-enhanced metastasis requires degranulation.** We have shown that radiation-activated neutrophils show a pro-inflammatory phenotype and an increase in granule proteins (Fig. 2g,h); therefore, we tested if this tissue-perturbation effect was mediated by their degranulation. We performed targeted lung irradiation in the presence of a degranulation inhibitor Nexinhibi\textsuperscript{20} to prevent neutrophil exocytosis (Fig. 7a). Strikingly, this led to the abrogation of radiation-mediated metastatic enhancement and the reduction of Notch activation and Sox9 expression in metastatic 4T1 cells (Fig. 7b–d). To test the contribution of a single granule protein, Neutrophil elastase (Ela2), in the neutrophil-mediated tissue response, we irradiated the lungs of Ela2-knockout mice and C57BL/6 littermates and intravenously injected E0771 breast...
Radiation exposure boosts Notch signaling and stemness in metastatic cells. a. Cherry-niche labeling tool. Irradiated BALB/c mice were intravenously injected with GFP⁺ 4T1-sLP-Cherry-labeling cancer cells seven days post-irradiation. One week later, GFP CD45⁻ Ter119⁻ mCherry⁺ (red) and GFP CD45⁺ Ter119⁺ mCherry⁻ (gray) were FACS-sorted from metastatic lungs, representing the labeled metastatic niche or distant lung cells, respectively. b. Combined UMAP plot of cells from the mCherry⁺ niche and mCherry⁻ distant lung (n = 10 mice, pooled). Clusters representing epithelial cells (EpCAM⁺), fibroblasts (Pdgfra/β+) and endothelial cells (Pecam1(CD31)⁺) are outlined. c. Notch signaling (Reactome) score in irradiated Cherry⁺ niche (red) and Cherry⁻ distant lung (blue) cells for the compartments in b, calculated in VISION (Methods). d,e. Quantification of RBPJ (d) and Hes1 (e) immuno staining in lung metastases from control (untreated), irradiated and neutrophil-depleted irradiated mice at day 14 (n = 7 mice per group, two independent experiments). Representative images in Extended Data Fig. 8e, quantification in methods. f. Cancer stemness assays. Metastatic lungs from control/irradiated BALB/c mice were harvested and the cell suspension plated to select for 4T1 metastatic cells (methods). The following day, equal numbers of cancer cells were plated in 2D or embedded in Matrigel. Colony/tumoroid formation was quantified after 7 days. g. Representative image of Giemsa-stained six-well plate after seeding 1,000 or 5,000 cancer cells from control/irradiated lungs (left) and quantification (right) (n = 10 mice per group, three independent experiments). Scale bars, 1 cm. h. Representative image (left) and tumor organoid quantification (right) (n = 7 mice per group, two independent experiments). Scale bars, 100 μm (main), 10 μm (enlarged inset). All data represented as mean ± s.e.m. Statistical analysis by two-tailed nonparametric Mann–Whitney test for g, one-way ANOVA for d, e and i, and unpaired two-tailed t-test for h. For box plots, boxes represent 25th/75th percentiles, the line represents the median and whiskers indicates the minimum/maximum values. Dots represent individual mice. FACS gating strategies in Extended Data Fig. 2. pos, positive.

cancer cells one week later (Fig. 7e). We observed a marked reduction in the metastatic proficiency of Ela2-knockout lungs as well as a dampening of Notch activation (Fig. 7f–h). Therefore, neutrophils strongly influence the response of lung epithelial cells to radiation injury, likely via the process of degranulation.

Finally, to confirm that Notch signaling activation in metastatic cells growing within irradiated lungs is responsible for enhancing their tumorigenicity, we treated control/irradiated mice with the γ-secretase inhibitor N-[N-(3,5-difluorphenacetyl)-L-alanyl]-S-ph enylglycine t-butyl ester (DAPT) from the time of metastatic seeding
A fundamental step in the metastatic cascade is the generation of a favorable metastatic niche\(^3\). Notch signaling, which is mediated by radiation-primed neutrophils influencing lung epithelial cell responses (Fig. 8e). We observed that neutrophils play a critical role particularly for the early response of lung tissue to breast cancer growth involves the activation of regeneration\(^2\). Therefore, in the present study, we probed whether the induction of a regenerative state in the tissue due to an injury would trigger a favorable environment for metastatic growth. RT remains one of the most effective means of achieving curative outcomes for cancer patients with localized disease. However, in many patients the frequent development of metastasis significantly limits the long-term treatment success. Currently, the biological responses of healthy tissues to radiation-induced injury, the key inflammatory components involved and the influence of this response on the metastatic proficiency of that organ are incompletely characterized.

To address these questions, we designed a preclinical study using targeted thoracic irradiation to generate an acute radiation injury to the lungs prior to the seeding of breast cancer cells. We found that radiation exposure to healthy lung tissue induced a hospitable environment for metastatic growth. Most importantly, we show a key function of neutrophils recruited to the injured lung, which locally activate and influence the response of resident lung cells. We observed that neutrophils play a critical role particularly for the lung epithelium and directly support the progenitor function of alveolar cells when tested ex vivo.

We found a neutrophil-dependent enhancement of stem cell signaling including Notch in lung epithelial cells. In the adult lung, Notch is required for the regeneration of several airway cell types after chemical or infection-induced injury, including basal cells\(^1\), club cells\(^4\), alveolar type I cells\(^4\) and a population of lineage-negative epithelial progenitor cells\(^6\). Moreover, Notch signaling in response to irradiation was reported to promote the survival of basal human...
Fig. 8 | Inhibition of Notch signaling attenuates the radiation-driven enhancement of metastatic growth in vivo. a, Schematic depicting Notch signaling inhibition in vivo. Non irradiated (UT) or irradiated (IR) BALB/c mice were administered the γ-secretase inhibitor DAPT (10 mg kg⁻¹) or a vehicle control daily, beginning at the time of 4T1 cancer cell injection and continuing until the experimental endpoint one week later. b, c, Representative immunostaining for Ki67 (b) and quantification (c) of the number of Ki67⁺ metastatic lesions in UT and IR mice treated with vehicle (veh) or DAPT (n = 8 mice per group, two independent experiments). The quantification of Ki67⁺ metastatic foci is depicted as fold change, relative to the non irradiated (UT) mice treated with vehicle. Ki67⁺ metastatic foci are indicated with a dashed line. Scale bars, 250 μm. d, Quantification of Hes1 immunostaining in metastatic lesions from b (n = 8 mice per group, two independent experiments) (Methods). All data represented as mean ± s.e.m. Statistical analysis by one-way ANOVA for e and a two-tailed t-test for d. e, Proposed model for radiation-enhanced lung metastasis. Radiation exposure in healthy lung tissue leads to excessive neutrophil accumulation and activation, inducing an array of tissue perturbations such as Notch activation within epithelial cells. Together these alterations foster a pro-tumorigenic milieu within the irradiated lung, fueling the subsequent growth of arriving metastatic cancer cells.

and murine airway stem cells. Here we identified an enrichment of Notch signaling within the metastatic niche of pre-irradiated lungs and demonstrate that the neutrophil presence results in Notch activation in metastatic cancer cells. Importantly, this induction of Notch signaling is a dominant effector enhancing tumorigensis. In many types of human cancer including breast and lung, the Notch pathway is required for cancer stem cell self-renewal. The transcription factor Sox9 is an essential regulator of self-renewal in cancer stem cells, with its overexpression tightly correlated with metastasis formation and poor survival in multiple cancers. Indeed, we observed the Notch⁺Sox9⁺ phenotype in breast cancer cells growing specifically within neutrophil-poor irradiated lungs, along with their profoundly enhanced tumorigenicity. This suggests the induction of a regenerative program promoting cancer stemness.

We also observed a neutrophil-dependent boost in Notch signaling in irradiated endothelial cells; therefore, we cannot exclude their involvement as an intermediary between the neutrophils and the epithelium. Indeed, neutrophils were shown to support lung vascular repair following whole-body irradiation. In addition, the neutrophil-mediated change in the response of mesenchymal cells could also be relevant for metastatic outcome. Future studies are required to resolve the role of radiation-primed neutrophils in the perturbation of other cellular components or determine if a similar activation occurs in other tissues exposed to radiation. Furthermore, since acute radiation injury can contribute to the onset of pneumonitis and fibrosis in the long term, our data encourages further investigation into the role of radiation-primed neutrophils in these chronic pathologies.

Taken together, our study shows that a high level of radiation exposure in healthy lung tissue has profound effects on the tissue microenvironment that inadvertently increases its metastatic potential. Our findings place radiation-primed neutrophils as a key modulator of these pro-metastatic alterations.

Nowadays, the technologies used to administer conformal RT are highly effective in limiting both the dose and volume of tissue within the radiation field, making the treatment much safer for patients. We acknowledge that in our experimental setting, the volume of exposed tissue is considerably larger than in the clinic. Nonetheless, given the striking effects of radiation-primed neutrophils observed in this study using preclinical models, together with the abundant evidence of their multiple pro-tumor functions, our work encourages closer attention to neutrophil responses in cancer patients receiving RT. Moreover, since the radiation-induced neutrophil activity is, at least in part, dependent on their degranulation, this study supports the interest in developing inhibitors of cancer cell exosome release for clinical use.

Methods
All experiments in this study were approved by the Francis Crick Institute and University College London ethical review committees and conducted according to UK Home Office regulations under the project license P83B37/83C and PPL70/9032, respectively. Further information on research design, statistics and
technical information is available in the Nature Research Reporting Summary linked to this article.

Statistics and reproducibility. All statistical analyses were performed using Prism (v.9.1.1, GraphPad Software). Graphic display was performed in Prism and illustrative figures created with Biorender.com. A Kolmogorov–Smirnov normality test was performed before any other statistical test. After, if any of the comparative groups failed normality (or the number too low to estimate normality), a nonparametric Mann–Whitney test was performed. When groups showed a normal difference in the variance, we used a t-test with Welch’s correction. When assessing statistics of three or more groups, we performed one-way analysis of variance (ANOVA) controlling for multiple comparison or nonparametric Kruskal–Wallis test. 3D co-arrays on scaffolds were assessed via two-way ANOVA.

Litter mice were randomized before radiation exposure into control or irradiation groups. Since in vivo treatments were performed in predetermined groups (control vs radiation), no further randomization could be performed. In vitro 2D/3D co-culture assays and organoid assays were blinded at quantification. For all histological analysis, quantification between different experimental groups was performed blinded. No statistical methods were used to predetermine sample sizes, but these are similar to those reported in our previous publications. All replicate/experiment numbers are clearly stated in the figure legends. No data were excluded.

Mouse strains. Breeding and all animal procedures were performed in accordance with UK Home Office regulations under project license PPL80/2531 and PPL70/9032. Female mice 6–12 weeks old were used in all experiments. Wild-type BALB/c/c, C57BL/6J, FVB/n, NSG and Nude mice from The Jackson Laboratory were provided by The Francis Crick Biological Research Facility. MMTV-PyMT mice on a FVB background were originally obtained from The Jackson Laboratory, actin-GFP mice and a total deficient FVB/n were originally a gift from Huelsken laboratory (EPFL, Lausanne, Switzerland) and bred at the Francis Crick Institute. G-csf−/− knockout mice were also a gift from the J. Huelsken laboratory and backcrossed to the FVB/n (N10) background. Stpc-CreER2R2 mice on a C57BL/6J background were a gift from J. Lee laboratory (MRC Cambridge Stem Cell Institute). Rosa26−/− mice on a mixed background were purchased from The Jackson Laboratory (stock 001859). Eμ-Cre knock-in mice were originally purchased from the European Mouse Mutant Archive. For experiments in which mice developed a primary tumor, the maximum tumor size did not exceed 1.5 cm, in compliance with project license PPL80/2531.

Whole lung targeted irradiation. Mice were anaesthetized with fentanyl (0.05 mg kg−1), midazolam (5 mg kg−1) and medetomidine (0.5 mg kg−1); then given a single 13 Gy dose of radiation (300 kV, 10 mA, 1 mm Cu filtration), targeted to the thoracic cavity. For fractionation experiments, 4 Gy was delivered on three or four consecutive days. Computed tomography (CT)-based guidance was not used, but beam targeting to the lungs was achieved using a 7 mm collimator (1 cm field diameter). The total field source distance was 20 cm. The machine was calibrated daily to achieve a consistent total dose. The dose was given at a rate of approximately 1.4–1.5 Gy min−1 each time, with a radiation time of approximately 8–9 min for 13 Gy and 2–3 min for 4 Gy. Probes confirmed radiation outside the collimator was negligible before the experiments. The anesthesia was reversed with midazolam (1 mg kg−1) and flumazenil (0.5 mg kg−1). Recovery was performed in warming chambers at 37 °C. Control mice received a sham-irradiation, whereby they were placed under anesthetic for the same length of time and recovered in warming chambers.

Targeted partial-lung irradiation. An Xstrahl Small Animal Radiation Research Platform S/N 525722 irradiator (225 kV X-ray tube, half value layer 0.847 mm Cu), with 0.1 mm integral Be filtration, was used for mouse treatments. Treatment was performed at University College London under project license PPL70/9032. Cone-beam CT was performed before each treatment to confirm target position. Each mouse was anaesthetized with isoflurane and positioned for optimal right lung targeting on a 3D printed bed. The bed was rotated between the X-ray source and a digital flat-panel detector. The images were obtained at 60 kVp and 0.8 mA with 1 mm Al filtration of an uncollimated primary beam. During rotation, 360 projections were acquired (approximately 1° increments for each projection, approximately 0.01 Gy total radiation dose). The cone-beam CT projections were rendered into a 3D image reconstruction, using the FDK algorithm with a voxel size from 0.05 mm to 0.5 mm.

Muriplan software was used to set radiodensity thresholds for different tissues and enable treatment planning, setting an isocenter to target the right lung individually and avoid the left lung, heart and trachea. Dose calculation was performed using a Monte Carlo simulation superposition-convolution dose algorithm specific to the lung and target clinically.

Mice received a single treatment of either 8 Gy or 12 Gy, delivered as one angled beam, targeted to maximize dose delivery to a single lung. The beam used was 220 kVp and 13 mA, filtered with 0.15 mm Cu, dose rate 2.37 Gy min−1 under reference conditions. The beam was collimated to match the volume treatment, with beam cross section up to 10 x 10 mm². The time calculated to deliver 8 Gy to individual mice varied between 222 and 229 s, and to deliver 12 Gy varied between 318 and 348 s.

Cell culture. All cell lines were provided by the Cell Services Unit of The Francis Crick Institute, where they were authenticated using Short-Tandem Repeat profiling and species-identification tests and confirmed to be mycoplasma-free. Cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Labtech) and 100 U ml−1 penicillin-streptomycine (Thermo Fisher Scientific). MMTV-PyMT cells were isolated from late stage carcinomas and cultured on collagen-coated dishes in MEM medium (MEM/F12 (Thermo Fisher Scientific)) with 2% FBS, 100 U ml−1 penicillin-streptomycine, 20 mg ml−1 EGF (Thermo Fisher Scientific) and 10 μg ml−1 insulin (Sigma-Aldrich) collagen solution. A375 cells were cultured in DMEM/F12 with 10% FBS and 1% penicillin-streptomycin containing 1% Glicercol (Biological Research Facility). NSG (200 μl HEPES in HBSS (Thermo Fisher Scientific). All cells were cultured at 37 °C and 5% CO2.

Cherry labeling tool. The Cherry labeling tool developed by our laboratory has been recently described in detail27. A soluble peptide and a modified transactivator of transcription (TAT) peptide were cloned upstream of the mCherry complementary DNA, under the control of a mouse PGK promoter (sLP-Cherry). The sLP-Cherry sequence was then cloned into a pRRL lentiviral backbone. 4T1 cancer cells were stably infected with LP-Cherry and pLentiGFP lentiviral particles and subsequently sorted by flow cytometry to isolate 4T1-s-GFP+ labeling cells.

Induction of metastasis. To induce spontaneous metastases, mice were given an orthotopic injection of 50 μl growth factor-reduced (GFR) Matrigel (BD Biosciences) containing 1 x 104 4T1 or 4T07 breast cancer cells in the left mammary fat pad, using a 29-gauge insulin syringe. The injections were performed under anesthesia (isoFlurane).

To induce experimental metastasis, 4T1 breast cancer cells (0.5 x 106), MMTV-PyMT primary mammary tumor cells (1 x 106), E0771 breast cancer cells (0.5 x 106) were resuspended in 100 μl PBS and i.v. injected into BALB/c, G-csf−/− knockout or C57BL/6J mice, respectively. For human cancer cell metastasis, Fl-1 esophageal cancer cells (1 x 105) were i.v. injected into NSG mice, and H460 and A549 lung cancer cells (1 x 106) were injected into BALB/c mice. All animals were monitored daily for unexpected clinical signs following the project license PPL80/2531 guidelines and the principles set out in the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research (UK). The rationale and process for histological quantification of lung metastatic burden is provided in Supplementary File 1.

In vivo treatments. For neoptolemus-depletion, rat anti-Ly-6G antibody (BioXcell, clone IA1, 12.5 μg per mouse) in PBS or rat IgG isotype control (Cell Services Unit, Francis Crick Institute) was administered daily to BALB/c female mice via intraperitoneal injection. Recombinant G-CSF protein (Novoprotein, C002, 5 μg per mouse in PBS) was administered subcutaneously to G-csf−/− knockout mice every other day for a total of four injections. For Notch inhibition, BALB/c female mice received daily intraperitoneal injections of the γ-secretase inhibitor DAPT dissolved in corn oil (Sigma, 10 mg per kg body weight) or a vehicle control. To inhibit neoptolemus degradation, BALB/c female mice given an intraperitoneal injection of Nelnihinho20 (30 mg kg−1) or a vehicle control three times per week for two weeks. For tamoxifen administration, tamoxifen (Sigma-Aldrich) was dissolved in corn oil in a 40 mg ml−1 stock solution. Three doses (0.2 mg per g body weight) were given via oral gavage over consecutive days; the mice were harvested two weeks after the final dose.

Tissue digestion for cell isolation or analysis. Lung tissue was minced manually with scissors and digested with Liberase TM and TH (Roche Diagnostics) and DNase I (Merck Sigma-Aldrich) in HBSS for 30 min at 37 °C in a shaker at 180 r.p.m. Samples were passed through a 100 μm filter and centrifuged at 1,250 r.p.m. for 10 min. The cell-pellet was incubated in Red Blood Cell Lysis buffer (Miltenyi Biotec) for 5 min at room temperature. After centrifugation, cells were washed with magnetic-activated cell sorting (MACS) buffer (0.5% BSA and 250 mM EDTA in PBS) and passed through a 20 μm strainer-capped tube to generate a single cell suspension. Antibody staining was then performed for cell isolation (flow cytometry or magnetic-bead based cell separation) or for flow cytometry analysis.

Fluorescence-activated cell sorting analysis and cell sorting. Mouse lung single-cell suspensions prepared as above were incubated with mouse FcR blocking Reagent (Miltenyi Biotec) for 10 min at 4 °C followed by incubation with fluorescently conjugated antibodies for 30 min at 4 °C (see Supplementary Table 1 for antibody list). Cells were washed twice with MACS buffer, then incubated with 4’,6-diamidino-2-phenylindole (DAPI) to stain dead cells. Flow cytometry analyses were performed on a BD LSR-Fortessa (BD Biosciences) and subsequently analyzed using FlowJo v.10.4.2. FlowJo, LCC 2006-2018. Cell sorting was performed on a BD Influx cell sorter (BD Biosciences) using the BD FACS Software v.1.2.0.142.
Lung organoid assay. Lung organoid co-culture assays have been previously described. Lung epithelial cells (EpCAM^+CD45^−CD31^−Ter119^+) from control BALB/c mice or irradiated mice underwent fluorescence-activated cell sorting (FACS) and were resuspended in 3D organoid media (DMEM/F12 with 10% FBS, 100 U ml^−1 penicillin-streptomycin and insulin/transferrin/selenium (Merck Sigma-Aldrich)). Cells were mixed with murine normal lung fibroblast (MLg) cells and resuspended in GFR Matrigel at a ratio of 1:1. Then 100 μl of this mixture was pipetted into a 24-well transwell insert with 0.4 μm pore (Corning). In each insert, 5,000 epithelial cells and 25,000 MLg cells were seeded. After incubating for 30 min at 37 °C, 500 μl organoid media was added to the lower chamber and media changed every other day. Bright-field images were acquired after 14 days using an EVOS microscope (Thermo Fisher Scientific) and quantified using Fiji v. 2.0.0-rc-69/1.52r, Imagej).

Cancer cell stemness assay. Control and irradiated lungs harboring 4T1 metastases were dissociated into a single-cell suspension as described above. Cells were plated overnight (>12 h) in DMEM with 10% FBS and 100 U ml^−1 penicillin-streptomycin. The following day, wells were washed 4x in PBS to remove nonadherent cells, trypsinized and counted. For all experiments, a metastasis-free normal lung was isolated by MACS-sorting. Cells were seeded on top of the cancer cells (50,000 cells per well). After a 12 h (1:1) in DMEM with 10% FBS and 100 U ml^−1 penicillin-streptomycin, 20 ng ml^−1 β2-galactosidase, Sigma-Aldrich), according to the manufacturer’s instructions. β2-galactosidase staining was performed on lung cryosections preserved in OCT medium. For quantification, the Li minimum cross entropy thresholding algorithm was performed on image stacks.

3D cell culture. GFP^+MMTV-PyMT cells were seeded (5,000 cells per well) in a collagen-coated Alvelox Scaffold 96-well plate (ReproCELL). The following day, lungs were harvested from control or irradiated BALB/c mice (seven days post-irradiation), and EpCAM^+ epithelial cells or Ly6G^+ neutrophils were isolated by MACS-sorting. Cells were seeded on top of the cancer cells (50,000 cells per well). The growth of GFP^+ cells was monitored using the SteREO Lumar.V12 stereomicroscope (Zeiss) and images quantified using ImageJ. For quantification, the Li minimum cross entropy thresholding algorithm was performed on image stacks.

EdU in vitro proliferation assay. GFP^+MMTV-PyMT cells were seeded at a density of 10,000 cells per well in collagen-coated six-well plates in MEM media. The following day, lungs were harvested from control or irradiated BALB/c mice (seven days post-irradiation), and EpCAM^+ epithelial cells or Ly6G^+ neutrophils were isolated by MACS-sorting and were pooled lysed in 900 μl with 50 mM TEAB. Reduction and alkylation was performed using dithiothreitol and iodoacetamide in 50 mM TEAB. Proteins were then precipitated overnight by adding six volumes of ice-cold acetone. The next day, proteins were resuspended in 25 μl of 50 mM TEAB. Proteins were digested by trypsin for 16 h at 37 °C (1:50 w:w). Peptides were then desalted using a C18 macrospin column and peptides were eluted with 80% acetonitrile.

In vivo image. Mice were treated with a single intraperitoneal injection of EdU dissolved in PBS (25 mg kg^−1 body weight). Mice received targeted lung irradiation 1 h later and were sacrificed one day post-irradiation. Ti2o gavage and bone marrow were harvested and EdU incorporation assessed in single-cell suspensions stained for Ly6G^−PE (BD Biosciences, clone 1A8) using the Click-iT Plus EdU Flow Cytometry Assay Kit. Data were acquired on a BD LSRII Fortessa and analyzed using FlowJo v.10.4.2.

Immunostaining quantification. To quantify HeLa, RBPJ and Sox9 expression in metastases, positive cells within each metastatic area were counted and normalized to tumor size by dividing the number of positive cells within each metastasis by the tumor area, as measured by ImageJ. A minimum of 20 images per lung were counted. The intensity of RBPJ staining was determined using ImageJ. Following color deconvolution, the mean gray value was measured specifically for the RBPJ signal and normalized to the maximum gray value of each image. The optical density was determined by the calculation OD = log(max gray value/mean gray value). The resulting optical density values were averaged across all images per sample, generating an average intensity of RBPJ staining per lung.

Senescence-associated β-galactosidase staining. Senescence-associated β-galactosidase staining was performed on lung cryosections preserved in OCT freezing medium, using the Senescence β-galactosidase staining kit (catalog no. 9860, Cell Signaling Technology), according to the manufacturer’s instructions. Cells were washed 3 times with 0.1% Triton X-100 in PBS and incubated with a blocking solution (1% BSA, 5% goat serum in PBS) for 1 h at room temperature. Sections were incubated overnight at 4 °C with primary antibodies (Supplementary Table 1). The following day, sections were incubated for 1 h at room temperature with an anti-activated Notch1 antibody (Abcam, ab89252) diluted in blocking solution followed by a 1 h incubation with a goat-anti-rabbit AlexaFluor 488 (1:500, Thermo Fisher Scientific) at room temperature. Coverslips were then mounted onto slides using Vectashield Mounting Medium with DAPI for imaging. Images were acquired using a Zeiss Upright710 confocal microscope with NIS-elements software (v.4.51, Nikon, Japan) and analyzed performed with ImageJ.

Immunofluorescent staining on coverslips. GFP^+ epithelial cells were MACS-sorted from control, irradiated and neutrophil-depleted irradiated lungs, plated on poly-l-lysine glass coverslips for 15 min at room temperature and fixed in 4% PFA in PBS for 10 min. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with a blocking solution (1% BSA, 5% goat serum in PBS) for 1 h at room temperature. Next, cells were incubated for 1 h at room temperature with an anti-activated Notch1 antibody (Abcam, ab89252) diluted in blocking solution followed by a 1 h incubation with a goat-anti-rabbit AlexaFluor 488 (1:500, Thermo Fisher Scientific) at room temperature. Coverslips were then mounted onto slides using Vectashield Mounting Medium with DAPI for imaging. Images were acquired using a Zeiss Upright710 confocal microscope, and quantified using the MeasureObjectIntENSITY function in CellProfiler. For analysis of neutrophil nuclear segmentation, Ly6G^+ neutrophils were MACS-sorted from lung suspensions and plated on poly-l-lysine coverslips as described above. After fixation, coverslips were mounted onto slides using Vectashield Mounting Medium with DAPI and images captured with a Zeiss Upright710 confocal microscope. Fiji v. 2.0.0-rc-69/1.52r, ImageJ) was used to analyze fluorescence images.

Senescence-associated β-galactosidase staining was performed on lung cryosections preserved in OCT freezing medium, using the Senescence β-galactosidase staining kit (catalog no. 9860, Cell Signaling Technology), according to the manufacturer’s instructions. Cells were washed 3 times with 0.1% Triton X-100 in PBS and incubated with a blocking solution (1% BSA, 5% goat serum in PBS) for 1 h at room temperature. Sections were washed three times with PBS and incubated for 48 h at 37 °C with the staining solution containing X-gal in N-N-dimethylformamide (pH 6.0). Sections were counterstained with Nuclear Fast Red (catalog no. H-3403, Vector), mounted in VectoMount AQ (catalog no. H-5301, Vector) and imaged on a Nikon Eclipse 90i microscope. For quantification, the area of SA-β-gal^+ regions per lung was measured using ImageJ.

Quantitative proteomics of neutrophils using tandem mass tags labeling. Ly6G^+ lung or bone marrow neutrophils were isolated by MACS. Cells were lysed by adding three cell pellet volumes of lysis buffer (8 M urea, 50 mM TEAB, protease inhibitors) followed by sonication. Insoluble material was removed by centrifugation at 16,000 g for 10 min at 4 °C. Following a bicinec acid assay for protein quantification, 25 μg of total protein was transferred into six labeled tubes and adjusted to a final volume of 25 μl with 50 mM TEAB. Reduction and alkylation was performed using dithiothreitol and iodoacetamide in 50 mM TEAB. Proteins were then precipitated overnight by adding six volumes of ice-cold acetone. The following day, proteins were resuspended in 25 μl of 50 mM TEAB and digested by trypsin for 16 h at 37 °C (1.50 w:w). Peptides were then desalted using a C18 macropin column (The Nest Group Inc) and re-solubilized in 50 mM TEAB and subsequently labeled using a 0.2 mg tandem mass tags 10-plex kit (Thermo Scientific). Peptide labeling efficiency was assessed using a QExactive orbitrap (Thermo Scientific) mass spectrometer to ensure that all samples displayed >98% labeling efficiency. Next, 5% hydroxyamine was added to each sample to quencher unreacted label and the samples were mixed. The 10-plex sample was desalted using a C18 macropin column and peptides were eluted with 80% acetonitrile.
and dried in a vacuum centrifuge. High pH reversed-phase fractionation (Pierce, UK) was employed with nine fraction collected and dried to completeness. Each fraction was analyzed using a Fusion Lumos Tribrid orbitrap mass spectrometer coupled to an Ultimate 3000 HPLC system. The sample was loaded onto a C18 trap column (Acclaim PepMap 100 75 μM×2 cm) then transferred onto a C18 reversed-phase column (PepMap RSLC; 50 cm length, 75 μm inner diameter). Peptides were eluted with a linear gradient of 2–25% Buffer B (75% acetonitrile, 20% water, 0.1% formic acid, 5% DMSO) at a flow rate of 273 ml min⁻¹ over 135 min followed by a linear gradient of 25–100% Buffer B in 1 min. MS1 instrument settings: spectra were acquired in the orbitrap at 120,000 resolution with scan range of 350–1,500 m/z; maximum injection time 50 ms; AGC target value 4E5; 30% RF lens setting; 90s dynamic exclusion. MS2 instrument settings: precursor ions with charge states z=2–7 were selected for mass spectrometry (MS)/MS higher-energy collision-induced dissociation fragmentation; 38% energy; acquired in the orbitrap at 60,000 resolution; maximum injection time 105 ms; AGC target value 1E5. All data were analyzed with MaxQuant software v.1.6.0.13. MS/MS spectra were searched against a UniProtKB Mus musculus database downloaded in July 2017 and a list of default contaminants. Reversed decoy sequences were used to estimate false discovery rates. Additional MaxQuant setting were: trypsin with two missed cleavages, carbamidomethylation of cysteine as a fixed modification, N-terminal protein acetylation and methionine oxidation as variable modifications. The MaxQuant output proteinGroups.txt file was imported to Perseus v.1.4.0.2. Proteins were filtered to common contaminants and decoy sequences. Intensity values were log 2 transformed and normalization for each channel was performed by median subtraction.

RT-qPCR. RNA was isolated from sorted lung populations using the RNeasy Mini kit (Qiagen), with an on-column DNAse treatment (Qiagen). Complementary DNA was synthesized with random primers, using the SuperScript III First Strand Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The PCR, data collection and data analysis was performed on a QuantStudio 3 Real-time PCR system (Thermo Fisher Scientific), using a PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase (gapdh) was used as an internal expression reference. Primer sequences are provided in Supplementary Table 2.

RNA sequencing sample preparation. Single-cell RNA sequencing. Single-cell suspensions of metastatic lungs from control or irradiated mice (n=10 per group, pooled) were prepared as described above. CD45⁻Ter119⁻ cells were sorted by flow cytometry following staining with anti-mouse CD45, Ter119 and DAPI. Library generation for 10x Genomics analysis was performed using the Chromium Single Cell 3’ Reagents Kits (10x Genomics), followed by sequencing on an HiSeq4000 (Illumina) to achieve an average of 50,000 reads per cell.

Bulk RNA sequencing. Lung CD45⁺CD31⁻Ter119⁺EpCAM⁺ and CD45⁺CD31⁻Ter119⁻EpCAM⁻ cells were sorted from control, irradiated or neutrophil-depleted irradiated mice seven days post-irradiation by flow cytometry. Total RNA was isolated using the mirNeasy Mini Kit (Qiagen, catalog no. 217084), according to the manufacturer's instructions. Library generation was performed using the KAPA RNA HyperPrep with RiboseErase (Roche), followed by sequencing on a HiSeq (Illumina), to achieve an average of 30 million reads per sample.

Bioinformatic analysis. Bulk RNA sequencing. Raw fast files were adapter trimmed using Cutadapt (v.2.9.1) and to trimmomatic release 68 with associated ensemble transcript definitions using STAR v.2.5.2a (ref. 4) wrapped by SREMP v.1.3.0.0 (ref. 3), which was used to calculate estimated read counts per gene. Where necessary, bam files were merged using samtools v.1.8 (ref. 4). Estimated counts from samples in the Epithelial and Mesenchymal groups were normalized separately, with normalization and differential expression of genes being called between group type using the R package DESeq2 v.1.12.3 (ref. 3). Genes with an adjusted P value less than or equal to 0.05 were said to be differentially expressed. Differentially expressed genes were further analyzed for their pathway enrichments using Metacore (v.21.3, https://portal.genego.com). Heatmaps were generated using the R package pheatmap v.1.0.8 (https://cran.r-project.org/web/packages/pheatmap/index.html). All analyses were performed using R v.3.3.1 (https://www.r-project.org/).

Single-cell RNA sequencing. Raw reads were first processes by the Cell Ranger v.2.1 pipeline, using STAR (v.2.5.1b) to align to the mm10 transcriptome, with raw reads being adapter trimmed using Cutadapt (v.2.9.1) and to trimmomatic release 86 with associated ensemble transcript definitions using STAR v.2.5.2a (ref. 4) wrapped by SREMP v.1.3.0.0 (ref. 3), which was used to calculate estimated read counts per gene. Where necessary, bam files were merged using samtools v.1.8 (ref. 4). Estimated counts from samples in the Epithelial and Mesenchymal groups were normalized separately, with normalization and differential expression of genes being called between group type using the R package DESeq2 v.1.12.3 (ref. 3). Genes with an adjusted P value less than or equal to 0.05 were said to be differentially expressed. Differentially expressed genes were further analyzed for their pathway enrichments using Metacore (v.21.3, https://portal.genego.com). Heatmaps were generated using the R package pheatmap v.1.0.8 (https://cran.r-project.org/web/packages/pheatmap/index.html). All analyses were performed using R v.3.3.1 (https://www.r-project.org/).
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Author contributions

E.N. designed and performed most of the experiments, analyzed and interpreted the data. V.L.B. managed colony breeding and generated experimental mice, supported the animal work and provided technical support and discussion. L.O. developed the sLp-Cherry labeling tool and provided valuable discussion. A.K and N.R. performed the single-cell RNA analysis. C.A.N.S. performed the γ-H2AX immunofluorescence and SA-β-gal staining. M.V. performed the injections and harvested mice for the image-guided focused irradiation. S.A. performed the neutrophil proteomics. R.C. planned and performed the image-guided, focused partial-lung irradiation. L.O., A.R., N.R. and V.L.B. critically reviewed the manuscript. I.M. designed and supervised the study and interpreted the data. I.M. and E.N. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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**Extended Data Fig. 1 | Radiation exposure in healthy lung tissue enhances metastasis.**

**a.** Representative H&E images of metastatic lungs from control (UT) and irradiated BALB/c mice orthotopically injected with 4T1 breast cancer cells to generate a primary tumour. The metastatic area is depicted with a dashed line (n = 4 mice per group, 2 independent experiments). Scale bar, 250 μm. **b,c,** FACS quantification of GFP+ tumour cells in the metastatic lung (b) and primary tumour volume (c) from control and irradiated mice (n = 4 mice per group, one experiment). **d,e,** Representative H&E images (d) and FACS quantification of GFP+ tumour cells (e) from metastatic lungs from control (UT) or irradiated FVB mice intravenously injected with GFP+ MMTV-PyMT primary mammary tumour cells at day 7 (n = 5 per group, one experiment). Scale bar, 100 μm. **f,** Table and representative immunostaining of human-specific Lamin A/C to detect human lung cancer cells growing in BALB/c Nude mice (n = 4 mice per group for each cell line tested). Mice were intravenously injected with H460 or A549 human NSCLC cells 7 days following targeted lung irradiation (or sham-irradiation for control mice) and lungs harvested 3 weeks later. The table depicts the number of mice in which h-Lamin A/C+ cancer cells were present in the lungs. No foci were detected in control mice from either experimental group. The representative images show an example of a Lamin A/C+ metastatic foci within an irradiated lung, for each cell line. The H460 metastatic lesion (left) is indicated with the arrow. Scale bar, 250 μm. **g,** Violin plot showing the number of metastatic foci in the non-irradiated left lung lobe from mice that received image-guided targeted radiation to the right lung (n = 5 for 8 Gy mice; n = 7 for 12 Gy mice), compared with control mice (n = 6). The violin plot displays the median, 25th and 75th percentiles as well as the density of data points. Dots represent individual mice. All data represented as mean ± s.e.m. Statistical analysis by non-parametric two-tailed Mann-Whitney test for (b), (c) and (e) and a one-way ANOVA with multiple comparisons for (g). UT, untreated. IR, irradiated. Gating strategies for FACS analysis provided in Extended Data Fig. 2. Histology quantification process outlined in Supplementary File 1.
Extended Data Fig. 2 | FACS gating strategy. a–c, Example of FACS gating strategy to determine the frequency of (a) GFP+ cancer cells or (b) Ly6G+ neutrophils in the lung tissue of control/irradiated mice, or (c) Lineage-/EpCAM+ epithelial cells in the irradiated metastatic niche (Cherry-labelled, left), or unlabelled distant lung (Cherry-negative, right). All samples were gated to exclude debris and doublets, followed by live cell discrimination by DAPI staining. All gates were set based on fluorescence-minus-one (FMO) controls, containing all antibodies minus the one of interest, to determine the background signal. Importantly, lung tissue displays a high level of autofluorescence, which needs to be considered when excluding dead cells (an FMO-DAPI is critical for this).
**Extended Data Fig. 3** | Radiation exposure induces lung perturbations including neutrophil infiltration and activation. a,b. Representative immunofluorescent images (a) and quantification (b) of phospho-Histone H2A.X (Ser139) (green) and DAPI (blue) stained lungs from control/irradiated mice, 7 days post-irradiation (n = 3 mice per group, one experiment). Scale bar, 10 μm. 6 fields of view were quantified per mouse. c,d. Representative images of senescence-associated β-galactosidase (SA-β-gal) staining on lung cryosections (c) and quantification (d) from control/irradiated mice, 7 days post-irradiation (n = 3 mice per group, one experiment). Scale bar, 25 μm. e. Quantification of immunostaining for S100A9+ neutrophils in metastases from control and irradiated lungs at day 14 (7 days post-i.v., Fig. 1d) (n = 6 mice per group, 2 independent experiments). The number of neutrophils within the metastatic area was normalised to tumour area. f. FACS quantification of GFP+ cancer cells in control/irradiated lungs from RAG1-ko mice at day 14 (7 days post-IV). n = 9 mice per group, two independent experiments, grey dots C57BL/6J and white dots FVB background. g,h. Volcano plots showing protein expression from irradiated versus control lung (g) and bone marrow (h) neutrophils. A selection of differentially expressed proteins in the lung are depicted in red, with the same proteins shown in bone marrow samples (n = 3 mice per group). i. Table of granule proteins within primary, secondary or tertiary granules with their fold change in irradiated lung neutrophils (IR) vs untreated control (UT). Nd = not detected. Highly enriched proteins are highlighted in red. j. Mice received an intraperitoneal EdU injection (25 mg/kg) 1h prior to lung irradiation. Lungs/bone marrow were harvested 1h or 7 days later, and EdU incorporation assessed by FACS. k. EdU incorporation in Ly6G+ neutrophils from bone marrow (left) and lungs (right) from control (UT) and irradiated (IR) mice (n = 2 control/irradiated mice at 2h, n = 3 control/irradiated mice at day 7). A representative of two independent experiments is shown (total for both experiments n = 5 control/irradiated mice at 2h, n = 6 control/irradiated mice at day 7). All data represented as mean ± s.e.m. Statistical analysis by two-tailed t-test with Welch’s correction for (d) and (e), two-tailed non-parametric Mann-Whitney test for (f) and (k) and one-sample t-test (for value different from 2) for (b). UT, untreated; IR, irradiated. FACS gating strategies provided in extended Data Fig. 2.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Radiation-primed neutrophil fuel metastatic growth independently of NETosis and extravasation. **a**, Experimental setup. Irradiated mice were given daily injections of an anti-Ly6G neutrophil depletion antibody or IgG control, beginning the day before an IV injection of 4T1-GFP+ cancer cells. Mice were collected 72 h post-i.v. **b,c**, Frequency of Ly6G+ neutrophils (b) and GFP+ cancer cells (c) among live cells by FACS (n = 7 mice per group, 2 independent experiments). **d**, Representative immunofluorescent images of myeloperoxidase (MPO, green), citrullinated histone-H3 (Cit-H3, red) and DAPI (blue) stained lungs at 2 h, 24 h and 7 days post-irradiation (n = 3 mice per group, each timepoint). The positive control represents lung tissue from a mouse infected intratracheally with *C.albicans* and harvested 24 h later. Scale bar, 100 μm. **e**, Schematic of 3D co-cultures. GFP+MMTV-PyMT cancer cells were seeded in Alvetex™ Scaffold 96-well plates with MACS-sorted Ly6G+ neutrophils from control or irradiated mice, harvested 7 days after irradiation. **f**, GFP signal quantification. Cancer cell growth on the scaffold is shown as the fold change compared to cancer cells alone (n = 3 mice, each with at least 3 technical replicates, see methods). **g**, Schematic of GFP+MMTV-PyMT cancer cell proliferation in 2D co-culture with MACS-sorted Ly6G+ lung neutrophils from control (UT) or irradiated mice, harvested 7 days after irradiation (n = 6 mice per group). Cells were treated with EdU (20 μM) and incorporation was assessed by FACS 6 h later. **h**, Quantification of EdU+ cells among GFP+ cancer cells. PyMT cancer cells co-cultured with neutrophils isolated from control (UT) or irradiated (IR) lungs (n = 6 mice per group) were compared to PyMT cells cultured alone (n = 2 replicates). All data represented as mean ± s.e.m. Statistical analysis by one-way ANOVA for (b) and (c), two-way ANOVA for (f) and an unpaired two-tailed t-test for (h). UT, untreated; IR, irradiated; NT, neutrophils. Gating strategies for FACS analysis provided in extended Data Fig. 2.
Extended Data Fig. 5 | Recombinant G-CSF treatment permits controlled neutrophil recruitment. a, Experimental setup for rG-CSF time course. Mice were given a subcutaneous injection of rGCSF every second day for a total of 4 doses, beginning the day before irradiations. On day 0, 2, 4, 6, 7 and 8, a blood sample was taken from each mouse to quantify Ly6G+ neutrophils. Mice were harvested at day 8. b, FACS analysis of Ly6G+CD11b+ neutrophils among CD45+ cells in the blood (n = 6 mice per group). The day of rGCSF injections is depicted in blue and indicated by an arrow. The day of irradiation (day 0), the day of cancer cell injection (day 7) and the day of lung seeding (day 8) are indicated. Each dot represents an individual mouse, treated with either rGCSF (blue dots) or PBS (black dots). IV, intravenous; rG-CSF, recombinant G-CSF; ko, knock-out.
Extended Data Fig. 6 | Primed neutrophils influence the lung epithelial cell response to radiation-induced injury. **a**, Principle Component Analysis (PCA) of Lin-EpCAM⁻ mesenchymal cell signatures following RNA-seq analysis of control, irradiated and neutrophil-depleted irradiated lungs. Each dot represents an individual mouse, ovals enclose samples from each group to highlight their similarity in the PCA plot (n = 4 mice per group). **b,c**, 3D co-culture of GFP/MMTV-PyMT⁺ cancer cells on Alvetex™ Scaffolds with EpCAM⁺ lung epithelial cells isolated from control (UT), irradiated (IR) and neutrophil-depleted irradiated (IR + α-Ly6G) mice, 7 days after irradiation with **(b)** showing GFP signal quantification at day 4, normalised to cancer cell growth alone and **(c)** displaying representative images of GFP intensity at day 4 (n = 9 mice total per group, 3 independent experiments). Each dot in (b) represents the average of n = 3 mice for an independent experiment, with at least 3 technical replicates quantified per mouse in each experiment. Scale bar, 400 μm. **d,e**, 3D culture of GFP-EpCAM⁺ epithelial cells to assess survival on the scaffold, with **(d)** showing representative images of GFP intensity on the scaffold at day 4 (n = 3 mice per group, at least 3 technical replicates per mouse) and **(e)** showing GFP signal quantification (n = 3 mice per group, at least 3 technical replicates per mouse). Each dot represents an individual mouse. GFP-EpCAM⁺ cells were sorted from the lungs of control, irradiated and neutrophil-depleted irradiated actin-GFP mice 7 days after irradiation, and seeded in Alvetex™ Scaffolds. **f**, Heatmap of Lin-EpCAM⁻ mesenchymal cells from control, irradiated and neutrophil-depleted irradiated lungs (hierarchically clustered samples in columns and genes in rows) (n = 4 mice per group). All data represented as mean ± s.e.m. Statistical analysis by one-way ANOVA for (b). UT, untreated; IR, irradiated; Ep, epithelial.
Extended Data Fig. 7 | Radiation-induced Notch signalling in the lung environment is boosted by the presence of neutrophils. a, Representative images and quantification of immunofluorescent staining for the Notch Intracellular Domain (NCID) in irradiated EpCAM+ lung epithelial cells that were MACS-sorted and seeded on coverslips. Cells were harvested at day 7 from irradiated mice that were treated daily with an anti-Ly6G neutrophil depletion antibody or an IgG control antibody. Cells were quantified using CellProfiler, each dot represents the intensity of nuclear NCID staining in an individual cell (n = 130 IgG cells, n = 205 α-Ly6G cells, see methods). n = 3 mice per group, 3 technical replicates quantified per mouse. Scale bar, 5 μm. b, Quantitative RT-PCR validation of differentially-expressed genes identified by RNA-sequencing in sorted Lin-epCAM+ lung epithelial cells. Lungs were harvested from an independent cohort of control, irradiated and neutrophil-depleted irradiated mice (n = 3 mice per group). c, Quantitative RT-PCR expression of Notch genes in sorted CD31+ lung endothelial cells from control, irradiated and neutrophil-depleted irradiated mice (n = 4 mice per group). Gapdh was used as a housekeeper gene for normalisation in (b) and (c). All data represented as mean ± s.e.m. Statistical analysis by unpaired two-tailed t-test for (a) and one-way ANOVA for (b) and (c). UT, untreated; IR, irradiated; Ep, epithelial.
Extended Data Fig. 8 | Radiation-exposure boosts Notch-signalling and stemness in metastatic cells. a, Quantitative RT-PCR analysis of Notch1 and target gene Hes1 in lineage-traced lung alveolar type 2 cells from SPC-Cre-ERT2 control mice and SPC-Cre-ERT2/Rosa26NICD-IRES-GFP Notch-activated mice. Mice were administered tamoxifen by oral gavage (40 mg/kg) over three consecutive days. Lungs were harvested 14 days after the last tamoxifen dose and GFP⁺ cells were sorted by flow cytometry (n = 3 control mice, n = 4 Notch mice). b, Primary tumour weight from PyMT/Control (n = 6) and PyMT/Notch mice (n = 8) harvested two weeks post-tamoxifen induction. c, Quantification and representative images of Hes1 immunostaining in lung metastases from PyMT/Control (n = 5) and PyMT/Notch mice (n = 7) mice (metastatic lungs harvested over n = 5 independent tamoxifen administrations, immunostaining quantification in methods). The enlarged inset shows nuclear localisation. Scale bar, 100 μm (main image), 10 μm (enlarged inset). d, Combined Uniform Manifold Approximation and Projection (UMAP) plot of cells from the mCherry⁺ niche and mCherry⁻ distant lung from irradiated mice (n = 10 mice, pooled). The expression level of EpCAM (distinguishing epithelial cells), Pdgfra and Pdgfrb (fibroblasts) and Pecam1 (endothelial cells) is indicated in blue. e, Representative immunostaining for RBPJ (top panel) and Hes1 (lower panel) in metastatic lungs from control, irradiated, and neutrophil-depleted irradiated mice harvested at day 14 (n = 7 mice per group, 2 independent experiments). Quantification shown in Fig. 6d,e. The enlarged inset shows nuclear localisation within tumour cells. Hes1⁺ cells are indicated by arrows. Scale bar, 100 μm (main image), 10 μm (enlarged inset). f, Representative immunostaining and quantification of RBPJ staining intensity in metastatic lungs from irradiated FVB mice pre-treated with rGCSF or PBS prior to injection of MMTV-PyMT cancer cells (Fig. 3e, n = 5 mice per group). Staining intensity within the metastatic area was measured using ImageJ (see methods). Scale bar, 100 μm. All data represented as mean ± s.e.m. Statistical analysis by non-parametric two-tailed Mann-Whitney test for (a), a two-tailed unpaired t-test for (b), (c) and (f). UT, untreated; IR, irradiated. The violin plot displays the median, 25th and 75th percentiles as well as the density of data points. Dots represent individual mice. Gating strategies for FACS sorting for (d) provided in Extended Data Fig. 2.
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Software and code

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Data collection Flow cytometry: samples were run on a BD Influx cell sorter (BD Biosciences) using the BD FACs Software 1.2.0.142.

Data analysis Statistics: analyses were performed using Prism software (version 9.1.1, GraphPad Software, USA). Fluorescence imaging: Fiji (version 2.0.0-rc69/1.52r, ImageJ) and Adobe Photoshop CC 2018 (version 21.0.1, Adobe, USA) were used to analyse fluorescence images. Immunohistochemistry: images were acquired using NIS-elements software (version 4.5.1, Nikon, Japan) and analysis performed with Fiji (version 2.0.0-rc69/1.52r, ImageJ). Flow cytometry: data analyses were carried out using Flowlo 10.4.2 (FlowJo, LLC 2006-2018, USA). Proteomics: all data were analysed with MaxQuant software version 1.6.0.13. Reversed decay sequences were used to estimate false discovery rates. The MaxQuant output proteinGroups.txt file was imported to Perseus version 1.4.0.2.

Bulk-RNA sequencing: Raw fastq files were adapter trimmed using CutAdapt 1.5 and mapped to GRCh38 release 86 with associated ensemble transcript definitions using STAR 2.5.2a wrapped by RSEM 1.3.0 which was used to calculate estimated read counts per gene. Where necessary, bam files were merged using samtools 1.8. Estimated counts were from samples in the epithelial and Mesenchymal groups were normalised separately, with normalisation and differential expression of genes being called between genotype group using the R package DESeq2 1.12.3. Differentially expressed genes were further analysed for pathway enrichments using Metacore (version 21.3, https://portal.genego.com). Heatmaps were generated using the R package heatmap 1.0.8. All analyses were performed using R 3.3.1.

Single-cell RNA-seqencing: the Cell Ranger v2.1.1 pipeline was used to process raw reads, using STAR (v2.5.3b) to align to the mm10 transcriptome, deconvolve reads to their cell of origin using the UMI tags and report cell-specific gene expression counts. All subsequent analyses were performed in R version 4.0.3 using the Seurat package (4.0.1). Single cell signature scores were calculated for niche and distal cells within each cell-type subset using the Vision package v.2.1.0/54.

See methods for further details.

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The bulk RNA sequencing datasets (GSE180823) and the single cell RNA sequencing datasets (GSE181306) are deposited in the Gene Expression Omnibus (GEO, NCBI) repository. The proteomic datasets are deposited in PRoteomics IDentiFications (PRIDE) repository (PXD027628). The UniProtKB Mus musculus database was accessed in July 2017 and used for analysis of the proteomic dataset. All data is publicly available.

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Life sciences study design

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| Sample size | Sample sizes were based on previous publications from our laboratory. In these previous experiments, the sufficient numbers of mice needed in each group to yield a two-sided statistical test was determined, with the potential to reject the null hypothesis with a power (1 - beta) of 80%, subject to alpha = 0.05. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded |
| Replication | Unless otherwise specified in the figure legends, experiments were reproduced in at least two independent experiments. Each independent experiment contained a minimum of 3 mice per group. For in vitro or ex vivo experiments, at least 3 technical replicates were performed in addition to biological replicates. All replicate/experiment numbers are clearly stated in the figure legends. |
| Randomization | Litter mice were randomized prior to radiation exposure into control or irradiation groups. For in vivo therapeutic treatments, treatments were performed in pre-determined groups (control vs. irradiation) so no further randomization could be performed. For in vitro/ex vivo experiments, where possible groups were randomized (such as the MMTV-PyMT cells growing on scaffolds). However, this was not possible with the majority of in vitro/ex vivo experiments, since they used cells isolated from pre-determined groups of mice (e.g. neutrophils isolated from control/irradiated mice). |
| Blinding | Experiments using in vitro 2D/3D co-culture scaffold assays and lung organoid assays were blinded at quantification. For imaging studies, investigators were not blinded during data collection, however all image analysis (H&E quantification of metastatic burden, immunohistochemistry/Immunofluorescence, SA-B-gal, neutrophil nuclear shape), quantification between the different experimental groups was always performed blinded. |

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| ☒ Human research participants | |
| ☒ Clinical data | |
| ☒ Dual use research of concern | |

Antibodies

Antibodies used | ANTIBODY_COMPANY_CATALOGUE_No_CLONAL_CLONE_DILUTION (Technique)
Antibodies used

CD45-BV421_Biolegend_103133_Rat monoclonal_(30-F11)_1:200 [FC]
CD45-APC_eBioscience_17-0451-83_Rat monoclonal_(30-F11)_1:200 [FC]
CD45-PerCP-Cy5.5_eBioscience_45-0453-82_Rat monoclonal_(30-F11)_1:200 [FC]
CD326(EPAC)-PE_eBioscience_12-579-T9-82_Rat monoclonal_(G8.8)_1:100 [FC/MACS]
CD326(EPAC)-APC_eBioscience_17-5791-81_Rat monoclonal_(G8.8)_1:100 [FC]
CD326(EPAC)-APC750ire_Biolegend_118208_Rat monoclonal_(G8.8)_1:200 [FC]
Ly6G-PE_8D_Bioscience_554161_Rat monoclonal_(1A8)_1:100 [FC/MACS]
Ly6G-V450_BD Bioscience_560603_Rat monoclonal_(1A8)_1:100 [FC]
TER-119-BV421_Biolegend_116233_Rat monoclonal_(TER-119)_1:200 [FC]
CD31_BV421_Biolegend_103423_Rat monoclonal_(390)_1:200 [FC]
F4/80-A647_eBioscience_MF48021_Rat monoclonal_(BM8)_1:100 [FC]
CD49b-APC_eBioscience_12-5971-82_Rat monoclonal_(D05)_1:100 [FC]
CD11c-PE_eBioscience_12-0114-82_Rat monoclonal_(N418)_1:100 [FC]
CD3-APC-eFluor780_eBioscience_47-0032-82_Rat monoclonal_(17A2)_1:100 [FC]
CD19-eFluor450_eBioscience_48-0193-82_Rat monoclonal_(eBio103)_1:100 [FC]
Ki67_Abcam_Ab16667_Rabbit monoclonal_(SP6)_1:300 [IF]
S100A9_Francis Crick Institute (in house)_2B10_1:25 [IH]
GFPI_Abcam_Ab6673_Goat polyclonal_1:300 [HC]
Sox9_Millipore_ab5535_Rabbit polyclonal_1:500 [HC]
Hes1_Cell signalling_11988_Rabbit monoclonal_(D6F2U)_1:350 [IH]
RBPI_Cell signalling_5313_Rabbit monoclonal_(D10A4)_1:2000 [HC]
Histone H2A_Xipser139_Sigma_05-636_Mouse monoclonal_(4B4W01)_1:300 [IF]
MPO_RD_O_A63667_Goat polyclonal_1:40 [IF]
Histone3_Cit_Abcam_Ab5103_Rabbit polyclonal_1:500 [IF]
Anti-Lamin A_Lamin F_Abcam_108595_Rabbit monoclonal_(EPR4100)_1:1000 [IHC]
Donkey anti-Rabbit S5S_THERMOFISHER_A31572_1:500 [IF]
Donkey anti-Goat 488_Thermofisher_A11055_1:500 [IF]
Goat anti-Rabbit IgG antibody (H+L), Biotinylated_Vector.laboratories_BA-1000_1:250 [IHC]
Rabbit anti-Goat IgG antibody (H+L), Biotinylated_Vector.laboratories_BA-5000_1:250 [IHC]
Rabbit anti-Rat IgG antibody (H+L), Biotinylated_Vector.laboratories_BA-4001_1:250 [IHC]
Horse anti-Mouse IgG antibody (H+L), Biotinylated_Vector.laboratories_BA-2000_1:250 [IHC]
All antibodies supplied in Supplementary File 3.

Validation

The antibodies used have been validated accordingly to manufacturer's instructions. Mouse lung cell suspensions were used to validate FACS antibodies. Mouse lung sections were used to validates the antibodies for IF or IHC staining.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

All human and mouse cell lines (4T1, 4T7, Mlg, E0771, Flo-1, H460, A549) were provided by the Cell Services Unit of The Francis Crick Institute.
For primary cells, MMTV-PyMT cells were isolated from growing MMTV-PyMT tumours (FVB background).

Authentication

Short Tandem Repeat (STR) was used to identify all cell lines used while SPIP was used to confirm the species of origin.

Mycoplasma contamination

All cells are routinely tested for Mycoplasma by the Cell Services Unit of The Francis Crick Institute. All cell lines used were confirmed mycoplasma-free.

Commonly misidentified lines

No commonly misidentified lines were used.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Mice used for this work were as follows: wild type Balb/c, FVB/n, C57BL/6J, NSG, Nude; MMTV-PyMT/actin-GFP, RAG1 knock out mice and Gcsf Ko mice on FVB background, Ela2 ko and Sfpcc-CreERT2 mice on C57Bl/6j background, Rosa26-NICD-IRES-GFP mice on a mixed background. All mice used were females between 6 and 12 weeks of age. All mice were bred in house at The Francis Crick Biological Research Facility, according to UK Home Office Regulations. Light cycles were 7am-7pm, the ambient temperature and humidity was maintained at 21 degrees and 50%, respectively.

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve samples collected from the fields.

Ethics oversight

All experiments in this study were approved by the Francis Crick Institute and University College London ethical review committees, and conducted according to UK Home Office regulations under the project license PPL002536 and PPL0079032.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Lung tissue was minced manually with scissors and then digested with a mixture of Liberase TM and TH (Roche Diagnostics, Switzerland) and DNase I (Merck Sigma-Aldrich, Germany) in HBSS solution for 30 min at 37°C in a shaker at 180 rpm. Samples were then passed through a 100 um filter and centrifuged at 1250 rpm for 10 min. The resulting cell pellet was incubated in Red Blood Cell Lysis buffer (Miltenyi Biotec, Germany) for 5 min at room temperature. After centrifugation, the cell pellet was washed with MACS buffer (0.5% BSA and 250 mM EDTA in PBS) and samples were passed through a 20 um strainer-capped flow cytometry tube to generate a single cell suspension. Antibody staining was then performed for cell isolation (flow cytometry or magnetic-bead based cell separation), or for flow cytometry analysis.

Instrument

Flow cytometry analyses were carried out on a BD LSR-Fortessa (BD Biosciences, USA). All cell-sorting experiments were carried out on a BD Influx cell sorter (BD Biosciences, USA).

Software

FlowJo 10.4.2 (FlowJo, LLC 2006-2018, USA) was used for analysis.

Cell population abundance

Purity check was routinely performed after each sorting. Cells were used when purity was above 85%.

Gating strategy

All samples were first gated to exclude cellular debris using SSC-A/FSC-A (by excluding cells on the x/y axis, an example is provided in Supplementary File 4). A diagonal gate using FSC-H/FSC-A was then used for doublet discrimination. Live cell discrimination was next determined by negative DAPI staining, with an FMO(fluorescence minus one)-DAPI sample used to define the live cell boundary. After dead cell exclusion, FMO controls were used to define positive and negatively stained cell boundaries for each antibody used. The gating strategies used to define GFP+ cancer cells, Ly6G+ neutrophils and Lin-EpCAM + epithelial cells from the metastatic niche/distant lung is provided in Supplementary File 4.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.