Ki-67 supports global gene expression programmes driving tumourigenesis

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Abstract

Ki-67 is a nuclear protein universally expressed in proliferating vertebrate cells (1), a characteristic that underlies its widespread use as a cancer biomarker. It organises heterochromatin (2,3) and the mitotic chromosome periphery (2,4–6), but it is dispensable for cell proliferation in vivo. It thus remains unclear whether Ki-67 confers any advantages to cancer cells. Here, using mouse models of breast and intestinal cancer, we show that Ki-67 ensures gene expression programmes required for both carcinogenesis and tumour immunogenicity. We find that germline disruption of Ki-67 protects mice against intestinal tumourigenesis, while in mammary carcinoma cells, ablation of Ki-67 slows tumour growth and inhibits metastasis, despite the failure of cells lacking Ki-67 to induce an effective anti-tumour immune response. Mechanistically, Ki-67 loss leads to widespread deregulation of gene expression, including genes involved in the epithelial-mesenchymal transition (EMT), immune responses and drug metabolism. This results in loss of mesenchymal and stem cell characteristics, downregulation of MHC class I expression and sensitisation to various drug classes. Our results suggest that, by structuring chromatin to allow global transcriptome changes, Ki-67 allows cancer cells to adapt to their environment. This is required for all stages of tumourigenesis and drug resistance, but, by maintaining tumour immunogenicity, it also confers a targetable vulnerability on cancer cells.

Results and discussion

Although Ki-67 is universally expressed in dividing cancer cells, cell proliferation is not affected by Ki-67 gene disruption in any cell type yet analysed in vitro, nor during mouse development. However, Ki-67 might still be important in carcinogenesis since it contributes to organisation of chromatin, which controls gene expression and thus cellular plasticity, a characteristic of cancer cells. We first tested the involvement of Ki-67 in tumour initiation using an in vitro model. We transduced our previously generated Mki67+/+ or TALEN-mutated Mki67−/− 3T3 fibroblasts with oncogenic mutant H-Ras (G12V), and assessed colony formation as an indicator of transformation. While H-Ras-transduced control 3T3 cells efficiently formed colonies, Mki67−/− 3T3 cells did not, indicating that Ki-67 expression is required for transformation (Extended Data Fig. 1). We next determined whether Ki-67 knockout affects tumour initiation in vivo. We used a mouse model of colon carcinogenesis induced chemically by azoxymethane / dextran sodium sulphate (AOM-DSS) treatment, and tested the effect of a germline TALEN-disruption of Ki-67 (Mki672ntΔ/2ntΔ) that we generated. As expected, AOM-DSS efficiently induced colon tumours within 16 weeks in wild-type and Mki672ntΔ/+ mice; however, no macroscopic lesions were observed in Mki672ntΔ/2ntΔ mice.
We confirmed that this was not due to an inability to induce inflammation, since DSS alone triggered similar inflammatory responses in mice of all genotypes (Extended Data Fig. 2). Furthermore, immune cell proliferation and differentiation in Mki67\textsuperscript{2nt\textasciitilde2nt\textasciitilde} mice appeared normal in response to haemolytic anaemia triggered by phenylhydrazine treatment (Extended Data Fig. 3). This suggests that Ki-67 is specifically required for initiation of tumourigenesis. To confirm this, we used a genetic model of intestinal tumourigenesis. We crossed Mki67\textsuperscript{2nt\textasciitilde2nt\textasciitilde} mice with Apc\textsuperscript{\textasciitilde\textasciitilde14/\textasciitilde14} mice, which rapidly develop tumours in the intestine due to loss of the second allele of the Apc tumour suppressor gene\textsuperscript{12}. While Apc\textsuperscript{\textasciitilde\textasciitilde14/\textasciitilde14} Mki67\textsuperscript{2nt\textasciitilde2nt\textasciitilde} mice formed multiple intestinal tumours as expected, tumour burden was strongly reduced in Apc\textsuperscript{\textasciitilde\textasciitilde14/\textasciitilde14} Mki67\textsuperscript{2nt\textasciitilde2nt\textasciitilde} mice (Fig. 1c,d). These results indicate that Ki-67 is specifically required for efficient initiation of tumourigenesis induced either by an oncogene, chemical mutagenesis or loss of a tumour suppressor.

We next asked whether Ki-67 is required for tumour growth and metastasis from already transformed cells. As a first approach, we knocked down Ki-67 by stable shRNA in a commonly used aggressive human cervical cancer cell line, HeLa S3, and grafted the resulting cells and an shRNA control line into opposing flanks of athymic nude mice. Ki-67 knockdown was maintained \textit{in vivo} (Extended Data Fig. 4a,b) and, as expected, this did not affect cell proliferation (as shown by unchanged PCNA and mitotic indices; Extended data Fig. 4c,d). However, tumour growth was severely slowed (Fig. 2a) and histopathological analysis showed vast areas of necrosis and apoptosis accompanied by disorganised vascular and lymphatic networks, indicating disrupted angiogenesis (Fig. 2b,c). To ensure that phenotypes of Ki-67 downregulation were not specific to the tumour type or knockdown approach, we generated a biallelic MKI67 gene knockout (KO) by CRISPR-Cas9 in human MDA-MB-231 triple negative breast cancer cells (Extended Data Fig. 4e,f). As expected, MKI67\textsuperscript{-} cells proliferated normally \textit{in vitro} (Extended Data Fig. 4g,h) but tumours from xenografts in nude mice grew much slower than controls, again showing normal cell proliferation but defective angiogenesis (Fig. 2d-f). Thus, in xenograft experiments, Ki-67 sustains tumour growth but not by controlling cell proliferation.

To determine effects of Ki-67 loss in a mouse model that more closely mimics human breast cancer, we used 4T1 mammary carcinoma cells. This cell line is derived from BALB/c mice, is highly tumourigenic, spontaneously metastasises to distant organs\textsuperscript{13,14}, and, when grafted in a syngeneic background, a biphasic tumour progression profile is observed due to an induced immune response\textsuperscript{15}. As expected, CRISPR-Cas9-mediated Mki67 knockout did not affect 4T1 cell proliferation rates (Extended Data Fig. 5a-d). We engrafted WT and Ki-67 knockout 4T1 cells orthotopically into mammary fat pads in both immune-proficient syngeneic
BALB/c mice and immunodeficient (athymic-nude and NOD/SCID) mice, the comparison of which should allow identification of the effects of the immune system. In immunodeficient mice, tumours from Mki67−/− 4T1 cells grew significantly slower than from Ki-67-expressing 4T1 cells (Fig. 3a-c). In immune-proficient BALB/c mice, control 4T1 tumours established quickly and, as expected, initially regressed before regrowing (Fig. 3d). However, in these mice, Mki67−/− 4T1 cells formed primary tumours as efficiently as controls, but no initial regression occurred, overriding the slower growth of knockout tumours and resulting in higher final tumour burden than controls (Fig. 3d). This suggested a defective immune response in mice bearing Ki-67 knockout tumours. We analysed this by immunophenotyping of spleens and lymph nodes. The fraction of dendritic cells, B-cells and T-cells (except for T-reg) was reduced in Mki67−/− 4T1 tumour-bearing mice, as a result of a dramatic increase in cells positive for CD11b (a common myeloid marker, expressed by monocytes, neutrophils, NK cells, granulocytes, macrophages and myeloid-derived suppressor cells (MDSC)) (Extended Data Fig. 6). In agreement, immunohistological analysis revealed increased infiltration of MDSC in knockout tumours (using the MDSC marker, GR1; Fig. 3e), possibly indicating an immunosuppressive environment that protects the tumours against immune-mediated cytotoxicity.

In immune-proficient mice, despite the impaired immune-response to Mki67−/− 4T1 cells and a consequently higher tumour burden, there were noticeably less metastases than from control 4T1 cells, although this failed to reach statistical significance (p = 0.13) (Fig 3f). However, the reduction in visible metastasis from Mki67−/− 4T1 cells was highly significant in immunodeficient mice (Fig. 3g) or in immune-proficient mice injected in the tail vein to circumvent the immune-targeting of tumour cells at the primary-site (Fig. 3h). We quantified micrometastases in nude mice by dissociating lung tissue after 2 weeks and growing cells in the presence of 6-thioguanine, to which 4T1 cells are resistant14. The number of metastatic cells from Ki-67 knockouts was reduced nearly 100-fold (Extended Data Fig. 7). These results highlight a trade-off for cancer cells, whereby Ki-67 expression sustains tumour growth and promotes metastasis, yet also confers efficient targeting by the immune system.

Since we previously found that Ki-67 knockout in mouse NIH3T3 cells or knockdown in human cancer cells does not alter cell proliferation nor induce cell death, but disrupts chromatin organisation2 and alters gene expression, we hypothesised that the latter might explain the requirements for Ki-67 in tumourigenesis. To explore this idea we first compared transcriptomes of WT and Mki67−/− NIH3T3 cells by RNA-seq. Consistent with roles for Ki-67 in maintaining global chromatin organisation, this revealed widespread changes in gene expression, with 2558 genes significantly deregulated in independent clones of Mki67−/− cells.
transcriptomes are remodelled in 4T1 cells lacking Ki-67. In these cancer cells, Mki67 knockout caused genome-scale gene expression alterations: 4979 genes were deregulated, of which 1239 and 585 genes were >2-fold down-regulated and up-regulated, respectively (Fig. 4a; Extended Data Fig. 8b; Supplementary Table 2). There was little overlap in the deregulated genes between Mki67−/− (epithelial) and NIH3T3 (mesenchymal) cells (Extended Data Fig. 8b; Supplementary Tables 1,2) suggesting that Ki-67 ensures gene expression programmes in different cell types by maintaining chromatin organisation rather than directly regulating specific genes. RNA-seq of early stage tumours from WT and Ki-67 mutant 4T1 cells grafted into nude mice showed that Ki-67-dependent transcriptome changes were preserved in vivo (Fig. 4b).

We performed pathway analysis of genes highly deregulated in Ki-67 knockout 4T1 cells to better understand the links between Ki-67-mediated gene expression control and tumourigenesis. We found upregulation of Notch targets and downregulation of genes involved in the inflammatory response, Wnt pathway and the EMT (Fig. 4c) which we validated by qRT-PCR (Extended Data Fig. 8c). Importantly, although the Notch pathway is oncogenic in T cell acute lymphoid leukaemia, it can act as a tumour suppressor in specific cellular contexts16, can block Wnt signaling17,18 (a driver of tumourigenesis, cell stemness and the EMT) and induce drug resistance19. We confirmed Notch pathway upregulation at the protein level (Fig. 4d). We further verified that Ki-67 knockout 4T1 cells had lost expression of the mesenchymal marker vimentin but upregulated E-cadherin, and had a more epithelial morphology (Fig. 4e). Downregulated genes were enriched in targets of nuclear factor erythroid 2-related factor 2 (NFE2L2), which upregulates genes in response to oxidative stress; interferon regulatory factor 8 (IRF8); polycomb-repression complex 2 (PRC2), which mediates H3K27me3 and is a well-characterised regulator of the EMT20,21; and the pluripotency factors Nanog and Sox2 (Extended Data Fig. 8d). We focused on PRC2 as we previously found10 that in human osteosarcoma U2OS cells, Ki-67 binds to the essential PRC2 component SUZ12. To test whether the repression of the EMT in Ki-67 knockout depends on PRC2, we disrupted Suz12 or Ezh2 in WT and Mki67−/− 4T1 cells using CRISPR-Cas9. This partially rescued expression of vimentin (Extended Data Fig. 8e,f). However, it did not restore tumour growth rates nor metastasis in vivo from Mki67−/− 4T1 cells, nor affect tumourigenicity of control cells, in nude mice (Extended Data Fig. 8g). Thus, the PRC2 complex activity is restrained by Ki-67, but does not in itself have either oncogenic or tumour suppressive activity in this system. As such, the reported PRC2 roles in cancer22,23 might not
apply generally, and a single chromatin regulation pathway cannot account for Ki-67 roles in supporting the EMT and tumourigenesis.

Like Ki-67, the EMT is required for metastasis in some mouse cancer models\(^{24,25}\). The EMT is also closely associated with a stem-like state\(^{26-28}\), as well as resistance to chemotherapeutic drugs\(^{29}\) and induction of immune responses\(^{30}\). We therefore next investigated the importance of Ki-67 for these three EMT-linked characteristics.

First, we analysed two indicators of tumour cell stemness: the ability to form spheroids in the absence of adhesion to a surface\(^{26,31}\), and aldehyde dehydrogenase activity\(^{32,33}\). Both parameters were strongly reduced in Ki-67 knockout 4T1 cells (Fig. 4f,g).

Second, we noticed that 26 genes involved in drug metabolism were downregulated in Ki-67 knockout 4T1 cells, while only one was upregulated, suggesting that Ki-67 expression might determine sensitivity to chemotherapeutic drugs (Extended Data Fig. 9a,b). To test this, we performed an automated gene-drug screen using the Prestwick chemical library, composed of 1283 FDA-approved small molecules, as well as salinomycin, a positive control found to target cancer stem cells\(^{34}\), and 6-thioguanine, which was originally used to isolate 4T1 cells\(^{13}\). Control 4T1 cells were sensitive to 102 drugs at 10µM concentration, while the two \(\text{Mki67}^{-/-}\) clones were sensitive to 99 and 98 respectively, with 82 hits common to the three cell lines (Extended Data Fig. 9c; Supplementary Data). This suggests that Ki-67 loss does not qualitatively alter the drug-sensitivity profiles. We next determined the IC\(_{50}\) of 10 common hits used in cancer therapy. Importantly, \(\text{Mki67}^{-/-}\) cells were markedly more sensitive to all the molecules tested (Fig. 4h; Extended Data Fig. 9d).

Third, genes directing mouse major histocompatibility complex (MHC) class I-mediated antigen presentation \(\text{Tap2}, \text{Psmb8}\) and \(\text{Psmb9}\) were downregulated in \(\text{Mki67}^{-/-}\) 4T1 cells (Extended Data Fig. 8c; Supplementary Table 2). Flow cytometry confirmed lower expression of the mouse MHC class I molecules H2D and H2K (Fig. 4i), providing an explanation for how Ki-67 knockout affects tumour recognition by the immune system. We obtained similar results in human \(\text{MKI67}^{-/-}\) MDA-MB-231 cells, implying that roles of Ki-67 in maintaining MHC expression are conserved across species (Extended Data Fig. 10).

The above results show that Ki-67 is not required for cancer cell proliferation in vivo, but supports coherent transcriptional programmes that are involved in tumour cell plasticity, drug resistance and induction of immune responses. Together, these likely account for the roles of Ki-67 in tumourigenesis revealed in this study (Fig. 4j). Clues to its biochemical mechanism of action are suggested by the fact that Ki-67 is an intrinsically disordered protein (IDP) and behaves as a surfactant for mitotic chromatin\(^{5}\). Since we previously found that heterochromatin is more dispersed in cells lacking Ki-67 (ref. 2), we propose that these
properties may also allow Ki-67 to regulate interphase chromatin. Due to their conformational flexibility, IDPs can act as hubs to bind many protein partners\(^3\(^\text{5}\)\), and we previously identified many chromatin regulators interacting with Ki-67 (ref. 2). However, disrupting one such interactor, the PRC2 complex, does not restore tumourigenic capacity to Ki-67 mutant cancer cells, consistent with the idea that Ki-67 may act more globally.

While it has long been thought that global changes in nuclear structure observed in cancer cells might contribute functionally to carcinogenesis\(^3\(^\text{6}\)\), mechanistic insight into this question has been lacking. We speculate that the alterations of nuclear organisation seen in cancer cells may reflect their increased cellular plasticity. By binding multiple chromatin regulators and organising the transcriptome at a genome scale, Ki-67 might contribute to both nuclear structuration and cellular plasticity, thereby conferring on cancer cells the ability to adapt to their environment.

References


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Competing interests

The authors declare that they have no competing interests.

Author Contributions


Data availability

All RNA-seq raw data will be deposited in GEO and will be publicly available prior to publication of the manuscript.
Figure 1: Germline disruption of Ki-67 protects mice against intestinal tumourigenesis.

a. IHC analysis of β-catenin expression in whole intestines recovered from each mouse group (Mki67+/+; Mki67+/2ntΔ and Mki672ntΔ/2ntΔ) at week-16 post AOM-DSS treatment. Scale bar, 2.5mm. Insets show accumulation of β-catenin in nuclei. Scale bar, 500μm. b. Quantification of the number (top) and area (bottom) of neoplastic lesions. Error bar, SEM (n= 5 Mki67+/+ mice; n= 4 Mki67+/2ntΔ mice; n= 9 Mki672ntΔ/2ntΔ mice). c. IHC staining of β-catenin in whole intestines from 6-7-month old Apc+/Δ14 Mki67+/+2ntΔ and Apc+/Δ14 Mki672ntΔ/2ntΔ mice. Scale bar, 5mm. Insets show accumulation of β-catenin in nuclei. Scale bar, 500μm. d. Quantification of the number (top) and total area (bottom) of neoplastic lesions. Error bars, SEM (n= 7 Apc+/Δ14 Mki67+/+2ntΔ mice; n= 6 Apc+/Δ14 Mki672ntΔ/2ntΔ mice).
Fig. 2

a) Tumour volume (mm$^3$) over time for shRNA CTRL and shRNA Ki-67 treatments in HeLa cells.

b) Histological section showing proliferation and necrosis areas.

c) Vascular and lymphatic network images for shRNA CTRL and shRNA Ki-67 treatments in MDA-MB-231 cells.

d) Tumour volume (mm$^3$) over weeks for CTRL and MDA-MB-231 $MKI67^{+/−}$ treatments.

*Significant difference (p < 0.05)

**Significant difference (p < 0.01)

***Significant difference (p < 0.001)

****Significant difference (p < 0.0001)
Figure 2: Ki-67 sustains tumour growth independently of cell proliferation. 

a-c. HeLa shRNA CTRL or shRNA Ki-67 were injected subcutaneously into 6-8-week old nude mice. a. Tumour burden (n=19 mice). b. (Top left) Hematoxylin and eosin (H&E)-stained tumour sections highlighting proliferation and necrotic areas. Scale bar, 3mm. (Top right) Percentage of necrotic area for each of 3 separate tumours from different mice per cell line, mean ± SEM. (Bottom left) Immunohistochemistry (IHC) staining for cleaved caspase 3. Scale bar, 1mm (250µm for insets). (Bottom right) Percentage of cleaved caspase 3-positive cells for tumours from different mice, mean ± SEM. c. H&E-stained tumour sections showing vascular (arrows; scale bar, 100µm) and lymphatic networks (arrows; scale bar, 400µm). While in CTRL tumours fibrovascular network is thin and regular, in shRNA Ki-67 tumours, the network is disorganised, thick and fibroed. The lymph vessels in shRNA Ki-67 tumours are dilated, characteristic for oedema and indicative of slow fluid flow. 

d-f. Xenografts of MDA-MB-231 CTRL or Ki-67 KO cells in mammary fat pads of nude mice. d. Tumour burden. Error bars, SEM (n=8 mice). e. IHC images of tumours stained for Ki-67 and PCNA. Scale bar, 100µm. f. IHC analysis of vascular and lymphatic networks (arrows). Left, H&E; scale bar, 400µm. Right, CD31 staining; scale bar, 800µm. Areas of necrosis are indicated. The defects in Ki-67 KO tumours are similar as those observed in shRNA Ki-67 tumours.
Figure 3: Metastasis and anti-tumour immune responses require Ki-67.

a. 4T1 CTRL or Mki67<sup>−/−</sup> orthotopic xenografts in NOD/SCID mice. Tumour growth was monitored for 3 weeks (left). Error bars, SEM (n=6 mice).
b. IHC staining for Ki-67 and PCNA in 4T1 CTRL or Mki67<sup>−/−</sup> tumours. Scale bar, 100μm.
c. 4T1 CTRL or Mki67<sup>−/−</sup> orthotopic xenografts in athymic nude mice. Error bars, SEM (n=8 mice).
d. Tumour growth of 4T1 CTRL or Mki67<sup>−/−</sup> orthotopic xenografts in immunocompetent BALB/c mice. Error bars, SEM (n=8 mice).
e. IHC analysis of 4T1 CTRL or Mki67<sup>−/−</sup> tumours (week-4 post-transplantation) stained for Gr-1, a myeloid-derived suppressor cell (MDSC) marker. Scale bar, 100μm.
f. Quantification of lung metastases in BALB/c mice in d. Error bars, SEM.
g. Quantification of lung metastases in nude mice in c. Error bars, SEM.
h. Bottom, representative images of lungs stained to visualise metastases (white nodules); scale bar, 10mm.

4T1 CTRL or Mki67<sup>−/−</sup> cells were injected via tail vein into immune-competent BALB/c mice. Representative images of stained lungs (day 21 post-injection), metastases are white. Scale bar, 10mm.
Figure 4: Ki-67 ablation deregulates gene expression programmes involved in cancer cell stemness, drug resistance and immunogenicity.

a,b. Dot plot analysis of differentially expressed genes (DEGs) in 4T1 Mki67+/− cells (a) and 4T1 Mki67−/−-derived tumours (b). Red dots: DEGs with adjusted p-value < 0.05; purple dots: genes whose log2 fold change, LFC > 1 or < -1, is statistically significant (adjusted p-value < 0.05); grey dots: not significant, NS. c. Gene Set Enrichment Analysis (GSEA) of highly down-regulated genes in 4T1 Mki67−/− cells. d. Immunoblot analysis of Hes1 expression in 4T1 CTRL and Mki67−/− cells. e. Immunoblot (top) and immunofluorescence (bottom; scale bar, 10μm) analysis of vimentin and E-cadherin expression in 4T1 CTRL and Mki67−/− cells. f. Mammosphere formation assay of 4T1 CTRL or Mki67−/− cells. Representative images (top; scale bar, 400 μm) and quantification (bottom; error bars, SEM, n=10) after 7 days. g. Aldehyde dehydrogenase 1 activity measured using a flow-cytometry assay in 4T1 CTRL and Mki67−/− cells. DAEB, inhibitor of ALDH was used as a negative control. Left, representative FACS images. Right, quantification of ALDH+ cells. Error bars, SEM (n=2 independent analyses). h. IC50 of 4T1 CTRL or Mki67−/− cells treated with indicated compounds. i. Flow-cytometry analysis of MHC class I expression. 4T1 CTRL and Mki67−/− cells were stained with anti-H2Dα-FITC and anti-H2Kα-PE, or control isotypes. Top, representative images. Bottom, quantification (n=1 of each clone). j. Model of Ki-67 mechanism in tumour progression and cancer cell dissemination (see text).
Extended Data Fig. 1: Ki-67 is required for cell transformation.
CTRL or TALEN-mutated Mki67\textsuperscript{−/−} 3T3 fibroblasts were transduced with either empty or H-Ras\textsuperscript{G12V}-expressing retroviruses. a, immunoblot analysis of the expression of the indicated proteins. Actin serves as loading control. b, Crystal violet staining of the colonies formed.
Extended Data Fig. 2: Mki67<sup>−/−</sup> mice have normal inflammatory response. 

a. Body weight (g) measurement in Mki67<sup>−/+2ntΔ</sup> and Mki67<sup>−/−2ntΔ</sup> mice during the AOM-DSS experiment. 
b. Assessment of inflammatory response in colon and mesenteric lymph nodes in Mki67<sup>−/+2ntΔ</sup> and Mki67<sup>−/−2ntΔ</sup> mice, treated or not with DSS (H&E staining; scale bar, 1mm).
Extended Data Fig. 3: *Mki67*2nt/2nt mice have normal response to haemolytic anaemia triggered by phenylhydrazine (PHZ) treatment.
Analysis of number and percentage of indicated populations of blood, bone marrow and spleen cells in *Mki67*+2nt/Δ and *Mki67*2nt/2nt mouse, treated or not with PHZ.
Extended Data Fig. 4: Ablation of Ki-67 does not affect cell proliferation. 
a-d. Characterisation of HeLa shRNA CTRL and shRNA Ki-67 xenografts. a. Left, IHC staining for Ki-67; necrotic areas are highlighted. Right, quantification of Ki-67-positive cells in tumours (n=3 mice). b. Relative Ki-67 mRNA expression levels in HeLa shRNA CTRL and shRNA Ki-67 parental cells and xenografts. c. H&E (top; arrows indicate mitotic cells) and PCNA (bottom) staining of HeLa shRNA CTRL and shRNA Ki-67 xenografts. d. Quantification of mitotic indices and PCNA-positive cells. 
e-h. Characterisation of MDA-MB-231 CTRL and MKI67−/− cells. e. Scheme of CRISPR-Cas9-mediated disruption of MKI67 gene (targeting exon 6 and resulting in a 2nt-deletion). f. Relative Ki-67 mRNA expression in parental MDA-MB-231 cells and three MKI67−/− clones. g. Immunoblotting for the indicated proteins of parental MDA-MB-231 cells and six MKI67−/− clones. h. Quantification of the number of CTRL and MKI67−/− MDA-MB-231 Edu-positive cells, pulsed with EdU for either 1h or 24h.
Extended Data Fig. 5: Knockout of Mki67 in 4T1 cells.

a. Scheme of CRISPR-Cas9-mediated disruption of Mki67 gene in 4T1 cells (targeting exon 3 and resulting in a 5nt-deletion).
b. Relative Ki-67 mRNA expression in parental 4T1 cells and three Mki67−/− clones. c. Immunoblotting for the indicated proteins of parental 4T1 cells and Mki67−/− clones. d. Quantification of the number of CTRL and Mki67−/− EdU-positive cells, pulsed with EdU for either 1h or 24h.
Extended Data Fig. 6: Ki-67 knockout induces an immunosuppressive tumour environment. Immunohistological analysis of spleens and lymph nodes (draining, DLN; and non-draining, non-DLN) performed in week 1 and 4 in mice xenografted with 4T1 CTRL and Mki67<sup>−/−</sup> cells (n=4).
Extended Data Fig. 7: Ki-67 knockout reduces metastatic potential.
Lung tissue from mice injected via tail vein with 4T1 CTRL or Mki67\(^{-/-}\) cells (2 per condition) was dissociated and resulting cells were maintained in the presence of 6-thioguanine, to select for 4T1 cells. Left, crystal violet staining of resulting colonies. Right, quantification of the number of 4T1 CTRL and Mki67\(^{-/-}\) colonies.
Extended Data Fig. 8: Ki-67 controls gene expression, in part through PRC2.

a. Dot plot analysis of differentially expressed genes (DEGs) in 3T3 Mki67−/− cells. Red dots: DEGs with adjusted p-value < 0.05; purple dots: genes whose log2 fold change, LFC >1 or <-1, is statistically significant (adjusted p-value < 0.05); grey dots: not significant, NS. b. Venn diagrams of DEGs in 3T3 and 4T1 Mki67−/− cells under condition of p-value < 0.05 (left), and p-value (LFC >1 or <-1) < 0.05 (right). c. Quantitative RT-PCR analysis of differentially expressed mesenchymal and epithelial markers in 4T1 Mki67−/− cells; fold change in expression ± SD is shown. d. Gene set enrichment performed over the ENCODE and ChEA Consensus transcription factors from ChIP-X gene sets. FDR, false discovery rate adjusted p-values. e. Western-blot analysis of Suz12 and Vimentin levels in 4T1 cells, CTRL, and single or double knockouts for Mki67, Suz12 and Ezh2. Actin serves as loading control. f. Immunofluorescence staining of Vimentin in 4T1 CTRL, Mki67−/−, Mki67−/−;Suz12−/− and Mki67−/−;Ezh2−/− cells. g. Tumour growth of 4T1 CTRL, Suz12−/−, Mki67−/−, and Mki67−/−;Suz12−/− xenografts over 3 weeks (n=3).
Ext. Fig. 9

(a) Hallmark: Xenobiotic Metabolism

(b) Upregulated

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(c) 4T1 CTRL

4T1 Mki67/−/− #1

4T1 Mki67/−/− #2
Extended Data Fig. 9: Ki-67 knockout sensitises cells to various drugs.

a. Xenobiotic metabolism is one of hallmarks found in pathway analysis of genes downregulated in Mki67\textsuperscript{−/−} cells. b. List of drug-metabolising enzymes with altered expression in Mki67\textsuperscript{−/−} cells. c. Results of the automated gene-drug screen using the Prestwick chemical library. ‘Hits’ were identified applying the Z-score statistical method and are located below the dashed red line. Drugs chosen for subsequent dose-response experiments are highlighted in colour for CTRL and Mki67\textsuperscript{−/−} clones. d. Dose-response curves of 4T1 CTRL or Mki67\textsuperscript{−/−} cells treated with indicated compounds and their corresponding IC\textsubscript{50}.
Extended Data Fig. 10. MHC class I expression requires Ki-67 in MDA-MB-231 cells. MDA-MB-231 CTRL and MKI67<sup>−/−</sup> cells were stained with anti-HLA-PE or control isotype. Top, representative images of FACS analysis. Bottom, quantification; median ± SD (n=2 independent experiments).
Methods

Cell lines and mice

4T1 cells were provided by Robert Hipskind (IGMM, Montpellier); MDA-MB-231 cells were obtained from SIRIC, Montpellier. NIH 3T3, 4T1, MDA-MB-231 and S3 HeLa cells were grown in Dulbecco modified Eagle medium (DMEM - high glucose, pyruvate, GlutaMAX – Gibco® Life LifeTechnologies) supplemented with 10% foetal bovine serum (SIGMA or DUTSCHER). Cells were grown under standard conditions at 37°C in humidified incubator containing 5% CO₂.

6-8 weeks-old female BALB/c (BALB/cOlaHsd), athymic nude (Hsd:Athymic Foxn1nu/Foxn1+), and NOD.SCID (NOD.CB17-Prkdcscid/NCrHsd) mice were purchased from Envigo. C57BL/6 ApcΔ14 mice were provided by Philippe Jay (IGF, Montpellier).

Animal studies

All animal experiments were performed in accordance with international ethics standards and were subjected to approval by the Animal Experimentation Ethics Committee of Languedoc Roussillon (n°APAFIS#8637-2017012311188975v3).

Antibodies and plasmids

Antibodies: Ki-67 (clone SP6; Abcam), cyclin A2 (6E6; Novocastra), PCNA (ab18197; Abcam), β-catenin (BD610154; BD-Bioscience), Ras (G12V Mutant Specific D2H12, #14412; CST), actin (A2066; Sigma), vimentin (D21H3, #5741; CST), E-Cadherin (24E10, #3195; CST), Suz12 (D39F6, #3737; CST), H3K27me3 (#39155, Active Motif), H2Dd-FITC (#553579; BD Biosciences), H2Kd-PE (SF1-1.1; BD Biosciences), HLA-PE (EMR8-5; BD Biosciences).

Lentiviral Vectors used: LentiCRISPRv2 (Addgene #52961), pMD2.G (Addgene #12259), psPAX2 (Addgene #12260). pHIV-Luc-ZsGreen (Addgene #39196) was used to generate bicistronic lentivirus that expresses both Luciferase and Zsgreen.

Retroviral vectors used: pBabe-puro (Addgene #1764), pBabe-puro H-RasG12V (Addgene #1768). gag/pol (retroviral packaging) and Maloney (envelope) were gift from Leatitia Linares (IRCM, Montpellier).

pUC57-U6-sgRNA (#51132, Addgene); Cas9-VP12 (#72247, Addgene), modified by adding T2A-GFP at the C-terminal end (gift from Edouard Bertrand, IGMM).

CRISPR/Cas9-mediated genome editing
The sgRNAs targeting mouse Mki67 exon 3 or human MKI67 exon 6 and non-targeting control sequences were previously designed\textsuperscript{37}, and cloning of the target sequence into the LentiCRISPRV2 lentiviral vector was conducted as described\textsuperscript{37}. Lentiviruses encoding the sgRNA targeting sequences were produced. Transduced cells (4T1 and MDA-MB-231) expressing CRISPR/Cas9 were selected using puromycin. Resistant cells were isolated and seeded as single cell clones in 96 well-plates.

For Suz12 and Ezh2 knockout, sgRNA targeting sequences\textsuperscript{37} were synthesised with BpiI sticky ends and cloned into the pUC57-U6-sgRNA vector. Cas9-VP12-T2A-GFP and sgRNA vectors were transfected into 4T1 WT and Ki-67\textsuperscript{-/-} #2 clone using Lipofectamine 2000 (ThermoFisher). 24h post-transfection, GFP-positive cells were sorted into 96-well plates by flow cytometry (FACS Aria, BD). After 10-12 days of culture, colonies were picked and screened (Cellomics, Thermo), using Suz12 or H3K9me3 antibodies. Positive knockout clones were confirmed by PCR, IF and western blotting.

AOM-DSS-induced colon carcinogenesis
Mice (Mki67\textsuperscript{+/+}; Mki67\textsuperscript{+/2nt\textbackslash}∆ & Mki67\textsuperscript{2nt\textbackslash}∆/2nt\textbackslash}∆) were given a single intraperitoneal injection of AOM (10 mg/kg in 0.9% saline; A5486, Sigma-Aldrich), and one week later, 2% Dextran Sodium Sulfate (DSS; MP Biomedicals) was administered in the drinking water for 7 consecutive days. Mice were sacrificed at week 16-post AOM-DSS treatment and colon tissues were removed.

Colons were flushed and fixed overnight in neutral buffered formalin (10%) before paraffin embedding. Briefly, 4µm thick sections were dewaxed in xylene and rehydrated in graded alcohol baths. Slides were incubated in 3% H\textsubscript{2}O\textsubscript{2} for 20 min and washed in PBS to quench endogenous peroxidase activity. Antigen retrieval was performed by boiling slides for 20 min in 10 mM sodium citrate buffer, pH 6.0. Nonspecific binding sites were blocked in blocking buffer (TBS, pH 7.4, 5% dried milk, 0.5% Triton X-100) for 60 min at RT. Sections were incubated with anti-β-catenin antibody diluted in blocking buffer overnight at 4°C. Envision+ (Dako) was used as a secondary reagent. Signals were developed with DAB (Sigma-Aldrich). After dehydration, sections were mounted in Pertex (Histolab) and imaged using the Nanozoomer-XR Digital slide Scanner C12000-01 (Hamamatsu).

DNA replication assay, EdU labelling
Cells were treated with 10 µM 5-ethyl-2'-deoxyuridine (EdU; LifeTechnologies) for the indicated time, harvested, washed once with cold PBS, resuspended in 300µL cold PBS and fixed with 700µL ice-cold 100% ethanol. Click reaction was performed according to the
manufacturer instructions (Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit; Invitrogen) and cells were analysed by flow-cytometry (BD FACSCanto II). FlowJo® software was used for analysis.

Mammosphere assay

The mammosphere formation assay was performed as previously described38. Briefly, 500 cells were plated per well in a low-adherence 96-well plate coated with poly-2-hydroxyethyl-methacrylate (poly-Hema). After 7 days in culture (37°C, 5% CO2), images of formed mammospheres were acquired and counted using automated high-content microscopy analysis (Cellomics, Thermo).

Aldehyde Dehydrogenase 1 (ALDH1) activity

ALDH1 enzymatic activity was determined using the ALDEFLUOR kit (Stem Cell Technologies) according to the manufacturer instructions. For each sample, half the cell/substrate mixture was treated with diethylaminobenzaldehyde (DEAB; ALDH inhibitor). ALDEFLUOR/DEAB treated cells were used to define negative gates. FACS was performed with ≥10⁵ cells.

Xenografts

Animals were housed in the animal facility of IGMM and were maintained in a pathogen-free environment and fed ad libitum. To generate primary tumours, 10⁶ cells (4T1) or 3x10⁶ cells (MDA-MB-231) of log-phase viable ‘mouse pathogen-free’ (Test: IMPACT1, Iddex) were implanted into the fourth inguinal mammary gland (in 50 µl PBS (4T1) or 200 µl PBS (MDA-MB-231)). Primary tumour volume was measured every week by electronic calliper using the formula “π/6*S² (Smaller radius)*L (Larger radius)”.

At the end of the experiment, following sacrifice, primary tumours were excised and fixed over-night in neutral buffered formalin (10%) before paraffin embedding (see above). IHC analysis of Ki-67 expression of the different tumour tissue sections was conducted as described above.
Visualisation of lung metastases

Dissected lungs were stained with 15% India Ink diluted in distilled water, and subsequently washed with 5 ml Fekete’s solution (40 ml glacial acid acetic, 80 ml formalin, 580 ml ethanol 100%, 200 ml water). A binocular microscope connected to a digital camera was used to visualise and count the metastatic nodules.

Automated drug library screen

We performed an initial screen of all 1,280 FDA-approved drugs in the Prestwick Chemical Library (Prestwick Chemical, Illkirch-Graffenstaden, France) at 10 μM in white clear-bottom 96-well plates. Briefly, we prepared seeding solutions at a density of 2 × 10^5 cells/mL in DMEM complete medium (10%FBS), and dispensed 100 μL into 96-well plates using a Multidrop Combi Dispenser (Thermo Scientific™). Next, we prepared 1x treatment solutions of the Prestwick compounds in DMEM complete medium at a concentration of 10 μM using a Tecan EVO200 robotic liquid handling system (Tecan Trading AG). We removed medium from the pre-seeded 96-well plates and we dispensed 100 μL of 1x treatment solutions using the Tecan robot. We incubated plates at 37°C, 5% CO₂ for 48 h in an automated incubator (Cytomat, Thermo Scientific™) associated with the Tecan robot. After treatment, cell survival was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation) in accordance with the manufacturer’s instructions. Luminescence signal was automatically measured with a Tecan Infinity F500 microplate reader (Tecan Trading AG). “Hits” were identified applying the Z-score statistical method (see statistical analysis section). The same method was used for subsequent dose-response experiments on selected “hits”.

Induction of anemia with PHZ

Anemia was induced in adult Mki67^{2ntL/2ntL} mice and littermate controls (Mki67^{+2ntL}) by injection of 40 mg/kg phenylhydrazine (PHZ) on days 0, 1, and 3. Animals were sacrificed on day 5 and blood, spleen and bone marrow were collected. Hematocrit was measured as previously described. Cell suspensions were generated and immunophenotyping performed by flow cytometry.

Immunophenotyping and flow cytometry analyses

Cells isolated from peripheral lymph nodes tumor (draining and non-draining for tumor-bearing mice) as well as spleen and bone marrow, were stained with Sytox blue or Live/dead...
fixable viability dye (Life Technologies and eBioscience, respectively) together with the appropriate conjugated anti-CD3, CD45, CD62L, CD4, CD8, CD44, CD25, F4/80, CD19, B220, NK1.1, Gr1, CD11b, CD11c, Ter119, CD71 and Foxp3 antibodies (eBioscience/ThermoFisher or Becton Dickinson, San Diego, CA). Intracellular staining for Foxp3 was performed using the eBioscience Fixation/Permeabilization kit.

Cells were analysed on a FACS Fortessa (BD Biosciences) flow cytometer. Data analyses were performed using FlowJo Mac v.10 (Tree Star) or DIVA (Becton Dickinson) software.

Prior to intracellular cytokine stainings, cells were activated with PMA (Sigma-Aldrich; 100 ng/ml)/ Ionomycin (Sigma-Aldrich; 1ug/ml) in the presence of brefeldin A (Sigma-Aldrich; 10ug/ml) for 3.5-4h at 37°C. Cell surface staining was performed, after which cells were fixed and permeabilised using a fixation/permeabilization kit (BD Biosciences or eBioscience) followed by intracellular cytokine staining.

**Antibodies used for immunophenotyping**

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Cell extracts and Western blotting

Frozen cell pellets (harvested by trypsinization, washed with cold PBS) were lysed directly in Laemmli buffer at 95°C. Protein concentrations were determined by BCA protein assay (Pierce Biotechnology). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% and 12.5% gels) and transferred to Immobilon membranes (Milipore) at 1.15 mA/cm² for 120 min with a semi-dry blotting apparatus. Membranes were blocked in TBS-T pH 7.6 (20 mM Tris, 140 mM NaCl, 0.1% Tween-20) containing non-fat dry milk (5%), incubated with the primary antibody for 2 hours at RT or over-night at 4°C, washed several times with TBS-T for a total of 45 minutes, incubated with secondary antibody at 1/5000 dilution for 1 hour at RT and washed several times in TBS-T. Signals were detected using Western Lightning Plus-ECL (PerkinElmer) and Amersham Hyperfilm (GE Healthcare).

qRT-PCR

For reverse transcription (cDNA synthesis), 500 ng of purified RNA in total volume of 10 µl, extracted by RNeasy Mini Kit (Qiagen), were mixed with 1 µl of 10 mM dNTPs mix (LifeTechnologies) and 1 µl of 50 µM random hexaprimers (NEB). Samples were incubated at 65°C for 5 minutes, transferred to ice, 5 µl 5x First Strand Buffer, 2 µl Li100 mM DTT and 1 µl RNasin RNase Inhibitor (Promega) were added and samples were incubated at 25°C for 10 minutes, 42°C for 2 minutes. 1 µl of SuperScript® III Reverse Transcriptase (LifeTechnologies) was added to each sample and incubated at 50°C for 60 minutes, 70°C for 15 minutes.

qPCR was performed using LightCycler® 480 SYBR Green I Master (Roche) and LightCycler® 480 qPCR machine. The reaction contained 5 ng of cDNA, 2 µl of 1 µM qPCR primer pair, 5 µl 2x Master Mix, and final volume made up to 10 µl with DNase free water. Primers used for both mouse (Mki67) and human (MKI67) Ki-67 in addition to the housekeeping genes are similar to those used by Sobecki et al., 2016. qPCR was conducted at 95°C for 10 min, and then 40 cycles of 95°C 20s, 58°C 20s and 72°C 20s. The specificity of the reaction was verified by melting curve analysis.

PCR primers used in this study.

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**Colony formation assay**

3T3 wild-type (WT) and two Ki-67 TALEN-mutant clones were transduced with either empty control or H-Ras<sup>G12V</sup> expressing retroviruses. After verification of Ras<sup>G12V</sup> expression, cells were seeded at 10<sup>5</sup> cells/well (6 well-plate) in triplicate and allowed to grow for 2-weeks.
(media were changed every 2 days). Cells were then fixed (4% formaldehyde) and stained with 0.5% (w/v) crystal violet to visualise the colonies formed.

**RNA sequencing library prep**

Total RNA was extracted using Trizol (Life Technologies) following manufacturer's instructions from wild type and two clones of 4T1 Ki-67 knockout cell lines. RNA integrity was analysed on Agilent 2100 bioanalyzer. For library preparation, cDNA synthesis was performed on rRNA-depleted samples using the TruSeq Stranded Total RNA Library Preparation (RS-122-2301). All sequencing libraries were prepared in two or three biological replicates. Indexed cDNA libraries were sequenced by MGX (Montpellier) on Illumina HiSeq2000 with a single 50 bp read and a 10 bp index read.

**Sequencing of cDNA libraries and data processing**

FastQC was used to perform quality control of the sequencing. Using the tool STAR 2.6.0a(2), all the reads that passed the quality control were aligned to the mouse reference genome (GRCm38.p6) and the counts per gene were quantified. The release 93 of the Ensembl mouse genome annotations were used for establishing the coordinates of each gene and their transcripts. Differential expression analysis was performed in R using the DESeq2(2) library embedded in an in-house script. After normalization of the read counts per gene, a negative binomial generalised linear model was fitted considering single factor design for assessing the differential expression between Mki67 knockout and wild-type samples. Gene set enrichment analysis of the 4T1 Mki67−/− cell line vs WT cell line was performed using the javaGSEA(3) desktop application with a log-fold-change pre-ranked gene list.

**Gene set enrichment for transcription factors.**

The gene set enrichment was performed over the ENCODE and ChEA Consensus TFs from ChIP-X gene set, which contains consensus target genes for transcriptions factors present in ENCODE and ChEA databases. The p value was computed with the Fisher Exact test and then adjusted for multiple hypothesis testing in order to obtain the FDR (false discovery rate) adjusted p-value.

**MHC class I expression analysis**

The MHC class I expression analysis was performed as previously described46. 4T1 CTRL and Mki67−/− cells were incubated with anti-H2Dd-FITC, anti-H2Kd-PE or control isotypes, and
MDA-MB-231 CTRL and Mki67\(^{+}\) cells were incubated with anti-HLA-PE or control isotype, in PBS/BSA 2% at 4°C for 20 min. After 2 washes in PBS/BSA 2%, cells were analysed by FACS.

**Statistical analysis**

Significant differences between the different experimental groups were tested using an unpaired two-tailed Student’s \( t \)-test or ANOVA in Prism 5 (GraphPad). For all analyses, \( p \) values < 0.05 (*), \( p \) values < 0.01 (**), \( p \) values < 0.001 (***) and \( p \) values < 0.0001 (****) were considered to indicate a statistically significant result.


