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Ki-67 regulates global gene expression and promotes sequential stages of carcinogenesis

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Running title: Ki-67 drives carcinogenesis

ABSTRACT

Ki-67 is a nuclear protein that is expressed in all proliferating vertebrate cells. Here, we demonstrate that, although Ki-67 is not required for cell proliferation, its genetic ablation inhibits each step of tumour initiation, growth and metastasis. Mice lacking Ki-67 are resistant to chemical or genetic induction of intestinal tumourigenesis. In established cancer cells, Ki-67 knockout causes global transcriptome remodelling that alters the epithelial-mesenchymal balance and suppresses stem cell characteristics. When grafted into mice, tumour growth is slowed and metastasis is abrogated, despite normal cell proliferation rates. Yet Ki-67 loss also downregulates MHC class I antigen presentation and, in the 4T1 syngeneic model of mammary carcinoma, leads to an immune-suppressive environment that prevents the early phase of tumour regression. Finally,

genes involved in xenobiotic metabolism are downregulated, and cells are sensitised to various drug classes. Our results suggest that Ki-67 enables transcriptional programmes required for cellular adaptation to the environment. This facilitates multiple steps of carcinogenesis and drug resistance, yet may render cancer cells more susceptible to anti-tumour immune responses.

SIGNIFICANCE STATEMENT

Ki-67 is a nuclear protein present in all proliferating vertebrate cells, and is widely used as a marker in clinical cancer histopathology. However, its cellular functions have remained largely mysterious, and whether it plays any roles in cancer was unknown. In this work we show genetically that Ki-67 is not required for cell proliferation in tumours but it is required for all stages of carcinogenesis. The effects on cell transformation, tumour growth, metastasis and drug sensitivity correlate with genome-scale changes in gene expression which modify cellular programmes implicated in cancer. Thus, Ki-67 expression is advantageous for cancer cells, but it comes with an Achilles heel: it makes them more visible to the immune system.

INTRODUCTION

Ki-67 is a nuclear protein expressed only in proliferating vertebrate cells, a property underlying its widespread use in oncology as a biomarker (1). Its expression is routinely assessed in histopathology to grade tumours; there are also indications for its use as a prognostic marker (2), although uncertainty over the relationship between Ki-67 index and prognosis remains. The cellular functions of Ki-67 are not well understood, and whether it is involved in tumourigenesis is unclear.

For a long time, Ki-67 was thought to be required for cell proliferation (3–8) and early work suggested that it promotes ribosomal RNA transcription (4, 9). However, recent genetic studies have shown that despite promoting formation of the perichromosomal layer of mitotic chromosomes (9–12), it is not required for cell proliferation (10–13). It is also dispensable for ribosomal RNA synthesis and processing, and mice lacking Ki-67 develop and age normally (11). In addition, Ki-67 is not overexpressed in cancers; rather, Ki-67 expression is controlled by cell cycle regulators, including CDKs, the activating subunit of the ubiquitin ligase APC/C-CDH1, which is required for destruction of mitotic cyclins, and the cell cycle transcription factor B-Myb (11, 14, 15). These recent studies raise the question of whether Ki-67 plays any role in tumourigenesis, which has not been addressed genetically.

Despite not being essential for cell proliferation, Ki-67 might be important in carcinogenesis for other reasons. We previously identified over 50 Ki-67-interacting proteins that are involved in

transcription and chromatin regulation. We also found that Ki-67 organises heterochromatin: in NIH3T3 cells with TALEN-mediated biallelic disruption of the Ki-67 gene, or human cancer cells with stable knockdown of Ki-67, repressive histone marks H3K9me3 and H4K20me3 are dispersed, the heterochromatin is less compact, and association between centromeres and nucleoli is disrupted. Conversely, overexpression of Ki-67 caused ectopic heterochromatin formation (11). Involvement of Ki-67 in chromatin organisation was corroborated by a study showing that Ki-67 is required to maintain heterochromatin marks at inactive X-chromosomes in non-transformed cells (16). Heterochromatin is a phenotypic marker of multiple cancers (17), suggesting that it might be involved in carcinogenesis. We thus tested whether and how Ki-67 is required for different steps of carcinogenesis.

RESULTS

Ki-67 is dispensable in cancer cell lines yet is rarely mutated in human cancers.

Ki-67 expression is widely used as a marker for cell proliferation in cancer, but whether it is important for carcinogenesis is unclear. We previously found no adverse effects on cell proliferation of either shRNA-mediated knockdown of Ki-67 in U2OS or HeLa cells, or TALEN-mediated disruption of the gene encoding Ki-67 in NIH/3T3 cells (11). To see whether this is true across different cancer types, we interrogated the Cancer Dependency Map project DepMap (<https://depmap.org/portal/>) (18) using the latest CRISPR (Avana 20Q1) and RNAi (Broad Institute) datasets. As controls, we compared Ki-67 with two proto-oncogenes that have roles in cell proliferation, *MYC* and *CCND1*, encoding c-Myc and cyclin D1, respectively, and with *PCNA*, encoding Proliferating Cell Nuclear Antigen, whose expression profile is similar to that of Ki-67.

Ki-67, PCNA and c-Myc were universally expressed, while cyclin D1 was expressed in most cell lines, though not all (Fig. 1A), possibly due to compensation by a different D-type cyclin. In CRISPR-Cas9 screens, PCNA was essential for proliferation of 775 of 777 cell lines tested, while 719 out of 726 lines required c-Myc, and 579 out of 726 required cyclin D1 (Fig. 1B). In contrast, Ki-67 knockout did not affect cell proliferation in 725 out of 739 cell lines (Fig. 1B), confirming that it is generally dispensable for cell proliferation in human cancer cells. Nevertheless, like *PCNA*, *MKI67* showed almost no copy number variations among the different cell lines, in contrast to the two cancer driver oncogenes *MYC* and *CCND1*, which were frequently amplified (Fig. 1C). The same is true in data from clinical samples of cancer patients in the cBioPortal database, which further

showed that only 5% of cancers presented mutations in *MKI67* (Fig. 1D). Almost all of these were mis-sense mutations, which might be passenger mutations.

Thus, even though Ki-67 is dispensable for cell proliferation in virtually all cancer cells, it is ubiquitously expressed at similar levels in all cancers, corroborating our previous findings that variability in Ki-67 expression is accounted for by its regulation through the cell cycle (14). Together, these observations suggest that Ki-67 may provide benefit to cancer cells. In contrast, its overexpression may be counter-selected in cancers, which would fit with our finding that increased levels of Ki-67 arrest cell proliferation (11).

The lack of overexpression or deletion of Ki-67 in cancers implies that correlating Ki-67 expression with patient survival is likely to simply reflect the impact of the fraction of proliferating cells. If so, then PCNA expression should give similar results. To address this question, we queried survival correlations in different cancer types with *MKI67* and *PCNA* mRNA levels in TCGA expression data using OncoLnc (19). For both genes, there was either no correlation (breast and colorectal cancer), a modest positive correlation (lung cancer), or a strong negative correlation (liver cancer and renal cancer) of expression levels with survival (SI Appendix Fig. S1). Thus, in several different cancers, despite the contrasting requirements for PCNA and Ki-67 for cell proliferation, the correlation of expression of each gene with survival is very similar.

Mice lacking Ki-67 are resistant to intestinal tumourigenesis.

The fact that Ki-67 is ubiquitously expressed in cancers but is not required for cell proliferation raises the question of whether it has functional roles in carcinogenesis. To see whether Ki-67 knockout affects initiation of tumourigenesis *in vivo*, we used a germline TALEN-disruption of Ki-67 (*Mki67*^{2ntΔ/2ntΔ}) that we generated (11). We first employed chemical induction of colon carcinogenesis by azoxymethane / dextran sodium sulphate (AOM-DSS) treatment (20) in WT and *Mki67*^{2ntΔ/2ntΔ} mice. We observed that DSS alone induced a similar decrease in body weight in control and mutated mice compared to the controls (SI Appendix Fig. S2A). Histopathological examination of colonic sections revealed a typical DSS-induced colitis in both genotypes (SI Appendix Fig. S2B), with increased numbers of immune cells in the lamina propria, moderate crypt cells damage and hyperplasia. As expected, AOM-DSS efficiently induced colon tumours within 16 weeks in both wild-type and *Mki67*^{2ntΔ/+} mice (indeed, heterozygous mice had bigger lesions; future studies will address effects of Ki-67 gene dosage on tumour growth). However, no macroscopic lesions were observed in *Mki67*^{2ntΔ/2ntΔ} mice (Fig. 2A, B). This suggests that Ki-67 is

specifically required for initiation of tumourigenesis. To confirm these findings, we used a genetic model of intestinal tumourigenesis. We crossed *Mki67*^{2ntΔ/2ntΔ} mice with *Apc*^{Δ14/+} mice, which rapidly develop tumours in the intestine due to loss of the second allele of the *Apc* tumour suppressor gene (21). While, as expected, *Apc*^{Δ14/+} *Mki67*^{2ntΔ/+} mice formed multiple colon tumours, tumour burden was strongly reduced in *Apc*^{Δ14/+} *Mki67*^{2ntΔ/2ntΔ} mice (Fig. 2C, D).

Thus, Ki-67 is required for efficient initiation of tumourigenesis induced by chemical mutagenesis or loss of a tumour suppressor *in vivo*, suggesting that it might be required for cell transformation. To test this, we transduced our previously generated *Mki67*^{+/+} or TALEN-mutated *Mki67*^{-/-} NIH/3T3 fibroblasts (11) with oncogenic mutant H-Ras (G12V), and evaluated colony formation as an indicator of transformation. While H-Ras^{G12V}-transduced control 3T3 cells efficiently formed colonies, *Mki67*^{-/-} 3T3 cells did not, despite having similar rates of cell proliferation (SI Appendix Fig. S3). This indicates that Ki-67 expression facilitates oncogene-induced transformation in these cells.

Loss of Ki-67 causes global transcriptome changes and deregulates pathways involved in cancer.

Since we previously found that knockdown of Ki-67 in cancer cells altered their chromatin organisation and affected gene expression (11), we hypothesised that the resistance of Ki-67 knockout cells to transformation might also result from gene expression changes. We first performed RNA-sequencing analysis of Ki-67 wild-type and knockout NIH/3T3 cells. This revealed surprisingly wide-ranging effects of Ki-67 loss on the transcriptome, with 2558 genes significantly deregulated in independent clones of *Mki67*^{-/-} cells ($q < 0.05$) (Fig. 3A; Dataset S1). This level of transcriptome alteration suggested a global effect on chromatin rather than a direct involvement of Ki-67 in controlling specific pathways or transcription factors, which is consistent with our previous finding that Ki-67 interacts with many general chromatin regulators in the U2OS cancer cell line (11). We therefore expected that Ki-67 knockout would also extensively affect the transcriptome of established cancer cells, with possible consequences for tumourigenicity. To investigate this, we used the syngeneic 4T1 mouse mammary carcinoma model, which is derived from BALB/c mice. This cell line mimics human triple-negative breast cancer, is highly invasive and spontaneously metastasises to distant organs (22, 23). As expected, 4T1 cell proliferation rates were unaffected by CRISPR-Cas9-mediated *Mki67* gene knockout (SI Appendix Fig. S4A-D). In 4T1 cells, *Mki67* knockout caused even more extensive gene expression alterations than in

NIH/3T3 cells: 4979 genes were deregulated, of which 1239 and 585 genes were >2-fold down-regulated and up-regulated, respectively (Fig. 3B, C; Dataset S2). There was little overlap in the deregulated genes between *Mki67*^{-/-} 4T1 (epithelial) and NIH/3T3 (mesenchymal) cells (Fig. 3C, Tables S1, S2) in accordance with our hypothesis that, by organising chromatin, Ki-67 enables global gene regulation in different cell types rather than directly controlling specific genes.

To see whether the global effect of Ki-67 knockout on gene expression is conserved across cancer cell types and species, we next disrupted the *MKI67* gene by CRISPR-Cas9 in human MDA-MB-231 triple negative breast cancer cells (SI Appendix Fig. S5A, B), which is a highly mesenchymal-like cell line due to an extensive epithelial-mesenchymal transition (EMT). As expected, *MKI67*^{-/-} MDA-MB-231 cells proliferated normally *in vitro* (SI Appendix Fig. S5C-D). Transcriptome analysis by RNA-seq showed that Ki-67 knockout in this cell line also caused genome-scale alterations in gene expression (Fig. 3D), with 9127 genes deregulated, 914 of which were up-or downregulated by a factor of >2 (Dataset S3).

We investigated whether the extensive transcriptome changes seen in cancer cells upon Ki-67 knockout affect pathways involved in tumourigenesis. In 4T1 cells, bioinformatic analysis of the most up- and downregulated genes revealed deregulation of various components of inflammation, apoptosis, p53, the EMT, estrogen response, K-Ras signalling and hypoxia (Fig. 3E). We also manually analysed transcriptome data and noticed upregulation of the Notch pathway, downregulation of the EMT, the Wnt pathway, antigen presentation, and aldehyde metabolism, which we validated by qRT-PCR (Fig. 3F). Downregulated genes were enriched in targets of nuclear factor erythroid 2-related factor 2 (NFE2L2), one of the major orchestrators of responses to oxidative stress; polycomb-repression complex 2 (PRC2), which mediates Histone H3 lysine-27 trimethylation (H3K27me3) and is a well-characterised regulator of the EMT (24, 25); the pluripotency factors Nanog and Sox2; and interferon regulatory factor 8 (IRF8) (Fig. 3G). All of these pathways have previously been implicated in tumourigenesis. In MDA-MB-231 cells, like 4T1 cells, pathway analysis also revealed genes involved in the EMT, inflammatory response, early estrogen response, K-RAS signalling and hypoxia, while a significant portion of the deregulated genes were under the control of PRC2 and ESR1 (estrogen receptor 1; Fig. 3H, I and Dataset S3). In summary, similar pathways involved in cancer are affected upon Ki-67 knockout in different cancer cell lines.

The prevalence of downregulation of gene expression in Ki-67 knockout cells, the enrichment in PRC2 targets among these genes, and our previous observations that Ki-67

associates with the essential PRC2 component SUZ12 (11), prompted us to ask whether loss of Ki-67 affects genome-wide distribution of H3K27me3. To answer this question, we performed chromatin immunoprecipitation with high-throughput sequencing (ChIP-Seq) on WT and Ki-67 knockout 4T1 cells. While there were no genome-wide changes in H3K27me3 distribution, a substantial subfraction of genes showed an increase in this mark (SI Appendix Fig. 6A). This was particularly evident for genes strongly repressed in Ki-67 knockout cells (Fig. 3J, left). To investigate correlations between changes in the levels of histone modifications and of gene expression, we assigned an average value of the repressive H3K9me3, as well as activatory H3K4me3 and H3K27ac reads across 10kb surrounding the transcription start site, and plotted the differences in these values between wild-type and Ki-67 knockout cells with gene expression changes. This showed a strong correlation between the change in gene expression and H3K27me3 and H3K4me3 (Figure 3K), while H3K27ac levels only correlated well with the most highly downregulated genes (SI Appendix Fig. S6B). Of 9 genes whose downregulated expression in Ki-67 knockout cells we confirmed by qRT-PCR, we only found an obvious increase of H3K27me3 on the EMT-promoting transcription factor *Twist1*. This correlated with downregulation of active promoter-associated H3K4me3 and H3K27ac (Fig. 3L). There was also a slight increase of H3K27me3 and reduction in H3K27ac at the Vimentin promoter (SI Appendix Fig. S6C). Taken together, these results suggest that Ki-67 loss generally increases PRC2-mediated repressive histone marks at downregulated genes, but most of the expression alterations resulting from Ki-67 knockout may not be directly due to modulation by PRC2. Instead, they are likely knock-on effects of altered expression of other transcriptional regulators that result in changes in active promoter-associated histone marks.

Ki-67 promotes stem cell characteristics and controls the EMT in mammary carcinoma.

We next investigated the biological consequences resulting from these global transcriptome alterations in cancer cells lacking Ki-67. Importantly, although the Notch pathway is oncogenic in T-cell acute lymphoid leukaemia, it can act as a tumour suppressor in specific cellular contexts (26), can block Wnt signalling (27, 28) (a driver of tumourigenesis, cell stemness and the EMT) and induce drug resistance (29). We confirmed Notch pathway upregulation at the protein level (Fig. 4A). While the EMT is closely associated with a stem-like state (30–32), the most stem-like states appear to show a hybrid expression of both epithelial and mesenchymal characteristics, with the most epithelial and mesenchymal cells losing stemness (33–35). We

found that 4T1 cells express both E-cadherin and vimentin, suggesting a highly stem-like state (Fig. 4B), but Ki-67 knockout 4T1 cells had reduced expression of the mesenchymal marker vimentin and upregulated E-cadherin (Fig. 4B, C). To see whether this translates to a loss of stem-like character, we analysed aldehyde dehydrogenase (ALDH) activity, which is a *bona fide* marker of stem and progenitor cells (36, 37). ALDH activity was strongly reduced in 4T1 Ki-67 knockout cells (Fig. 4D). Furthermore, the ability to form spheroids in the absence of adhesion to a surface, another characteristic of stem cells (30, 38) was also largely decreased (Fig. 4E).

To test whether repression of the EMT in Ki-67 knockouts depends on PRC2, we additionally disrupted PRC2 components *Suz12* or *Ezh2* in WT and *Mki67*^{-/-} 4T1 cells using CRISPR-Cas9 (SI Appendix Fig. S7A). This partially rescued vimentin expression, indicative of an increased ability to undergo an EMT (SI Appendix Fig. S7B), but did not restore it to levels observed in WT cells. This corroborates the results of the H3K27me3 ChIP-seq analysis described above, indicating that the influence of PRC2 activity on gene expression changes observed in Ki-67 knockout cells is limited.

Analysis of EMT markers in MDA-MB-231 cells showed that Ki-67 knockout cells retained a mesenchymal character, with vimentin but no E-cadherin staining (Fig. 4B, C), suggesting that they are far from a hybrid-like EMT state. Importantly, Ki-67 knockout cells had even higher vimentin expression, implying a further distance from the putative stem-like hybrid state. In agreement with this hypothesis, neither WT nor Ki-67 knockout MDA-MB-231 cells showed significant ALDH activity (SI Appendix Fig. S8A). Importantly, Ki-67 knockout cells were unable to form mammospheres, indicating a conserved requirement for Ki-67 in maintaining ability to seed formation of new cell colonies (SI Appendix Fig. S8B). As such, loss of Ki-67 reverses cells to a more epithelial or a more mesenchymal state, depending on the cell type of origin. This perturbation of the EMT correlates with a reduction of stem-like character in different cell lines.

Ki-67 expression promotes tumour growth.

To determine whether these phenotypic alterations affect the tumorigenicity of cancer cells, we first engrafted WT and Ki-67 knockout 4T1 cells orthotopically into mouse mammary fat pads. Since Ki-67 knockout caused alteration of inflammatory response genes (Fig. 3E), we initially used athymic-nude and NOD/SCID mice, allowing us to assess roles of Ki-67 in tumour growth and metastasis while minimising possible confounding effects of an altered immune response. 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 well preserved *in vivo* (Fig. 5A, Dataset S4), including down-regulation of mesenchymal genes, upregulation of epithelial genes and of the Notch pathway, which was validated by increased HES1 staining in tumours (SI Appendix Fig. S9A). Reduced vimentin staining of Ki-67-mutant tumours confirmed the loss of EMT *in vivo* (Fig. 5B). As assessed by PCNA and phospho-histone H3S10 staining, cell proliferation *in vivo* was unaffected by loss of Ki-67 (Fig. 5C, D). However, tumours from *Mki67*^{-/-} 4T1 cells grew significantly slower than from control cells in both types of immunodeficient mice (Fig. 5E, F). There were no apparent differences in apoptosis upon Ki-67 knockout in 4T1 cells *in vitro* (SI Appendix Fig. S9B), and, while there was significantly increased apoptosis in one of the two 4T1 Ki-67 knockout clones *in vivo* (SI Appendix Fig. S9C, D), both clones had similarly slowed tumour progression. Analysis of necrosis revealed variability between tumours and clones (SI Appendix Fig. S9E, F). As assessed by γ -H2A.X staining, we did not find evidence for increased DNA damage in Ki-67 knockout cells nor tumours (SI Appendix Fig. S9G, H). Thus, *in vivo* differences in tumour growth between WT and Ki-67 knockout tumours cannot be explained by either reduced cell proliferation or by increased DNA damage or cell death. It is theoretically possible that the slower tumour growth of Ki-67 knockouts might be explained by a marginally lower intrinsic cell proliferation rate that we did not detect when culturing WT and knockout cell lines individually, but might become visible over longer time-scales and when co-cultured with wild type cells. To test this, we performed competition experiments *in vitro* between WT and knockout 4T1 cells over 12 days. We found that the initial 50/50 ratio of wild-type and Ki-67 knockout 4T1 cells was maintained after two weeks of co-culture (SI Appendix Fig. S10A-C), ruling out this possible explanation.

Next, since Ki-67 4T1 knockout cells had downregulated expression of several angiogenic factors, including angiopoietin-1, both in cell culture and in tumours (Dataset S2, S4), we analysed angiogenesis in WT and Ki-67 knockout tumours by CD31-staining of endothelial cells. Indeed, we found that the mean blood vessel density was significantly reduced in tumours from both Ki-67 knockout clones in NOD/SCID mice (Fig. 5G, H). Tumours lacking Ki-67 also appeared more fibrotic than controls, as indicated by Sirius-red staining of collagen (Fig. 5I). However, blood vessel density was comparable between wild-type and Ki-67 knockout tumours in nude mice (Fig. 5J). Moreover, the distribution of vessels of different sizes was similar between all genotypes and mouse backgrounds (SI Appendix Fig. S9I). Therefore, while we cannot rule out a contribution of either reduced or altered angiogenesis to the slow growth of knockout tumours, this cannot explain the differences seen in all situations.

We also analysed tumours from xenografts of wild-type and Ki-67 mutant MDA-MB-231 cells in nude mice. We similarly observed reduced tumour growth rate despite normal cell proliferation (SI Appendix Fig. S11A-C). As with 4T1 cells, there was no detectable increase in apoptosis in MDA-MB-231 cells lacking Ki-67 *in vitro* (SI Appendix Fig. S9B). However, apoptosis, but not DNA damage (SI Appendix Fig. S11D), was significantly increased in Ki-67 knockout MDA-MB-231 tumours (SI Appendix Fig. S11E), necrosis was reduced (SI Appendix Fig. S11F), fibrosis was more apparent (SI Appendix Fig. S11G), while mean blood vessel density and distribution of vessels of different sizes was comparable with control tumours (SI Appendix Fig. S11H, I).

To assess the generality of these observations in a different tumour type, we stably knocked down Ki-67 by 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 *in vivo* (SI Appendix Fig. S12A, B). Again, tumour growth was severely impaired (SI Appendix Fig. S12C), despite unaffected cell proliferation (as shown by unchanged PCNA and mitotic indices; SI Appendix Fig. S12D, E), while there was increased necrosis and apoptosis (SI Appendix Fig. S12F, G). It is interesting to note that shRNA of Ki-67 was sufficient to induce strong phenotypes.

In conclusion, Ki-67 knockout or knockdown in several different established cancer cell lines consistently results in slower tumour growth upon grafts in mice despite unchanged cell proliferation, and often leads to non-cell autonomous increases in apoptosis or fibrosis, with necrosis and angiogenesis more variably affected. However, none of these plausible explanations for the reduced tumour growth is true in all experimental situations. These results show that effects of Ki-67 loss are wide-ranging and multifactorial.

Lastly, since many of the genes repressed in the absence of Ki-67 are under the control of PRC2 and concurrent knock-out of PRC2 genes partly restores the EMT to Ki-67 KO cells (SI Appendix Fig. S7), we tested whether the inactivation of PRC2 in cells lacking Ki-67 could restore tumour growth. We injected *Suz12*^{-/-}, *Mki67*^{-/-} and *Suz12*^{-/-} *Mki67*^{-/-} 4T1 cells orthotopically into nude mice, and found that *Suz12* knockout did not restore *Mki67*^{-/-} 4T1 tumour growth rates, nor affect tumourigenicity of control cells (SI Appendix Fig. S13). As such, PRC2 contributes to suppressing the EMT in the absence of Ki-67, but it does not in itself have either pro- or anti-tumour activity in this context, in contrast to other systems (25, 39). This result also suggests that Ki-67 roles in tumourigenesis depend on several chromatin regulatory pathways, consistent with it being a hub for interactions with multiple chromatin regulators.

Ki-67 promotes metastasis but enables efficient anti-tumour immune responses.

We observed that in orthotopic grafts, control 4T1 cells metastasised to the lungs in 4 weeks in nude mice, but metastases were largely absent at this point in mice bearing *Mki67*^{-/-} tumours (Fig. 6A). We tested whether this was due to differences in detachment from the primary tumour or ability to seed metastases. To do this, we quantified rates of metastasis formation of control and Ki-67 knockout 4T1 cells by injecting cells directly into the tail vein, then dissociating lung tissue after 3 weeks and growing cells in the presence of 6-thioguanine, to which 4T1 cells are resistant. The number of metastatic cells that formed colonies was reduced nearly 100-fold in Ki-67 knockouts (Fig. 6B). This points to an essential requirement for Ki-67 in seeding metastasis, in accordance with its apparent role in conferring stem-like characteristics. We could not test whether this reduced metastatic capacity was conserved in MDA-MB-231 cells since, in our experiments, neither Ki-67 knockout nor control MDA-MB-231 xenografts generated any visible lung metastases, consistent with their lack of stem-like character.

We next investigated how Ki-67 expression affects tumourigenesis and metastasis in the context of an intact immune system, by engrafting WT or *Mki67*^{-/-} 4T1 cells into immune-proficient BALB/c mice. As expected (40), control 4T1 tumours established quickly and initially regressed before regrowing (Fig. 6C). This initial regression has been attributed to a strong anti-tumour immune response (40). Surprisingly, no such initial regression occurred when *Mki67*^{-/-} 4T1 cells were engrafted (Fig. 6C), suggesting that Ki-67 knockout cells fail to trigger an efficient immune response. Nevertheless, despite the consequently higher tumour burden, there were either similar or less metastases than from control 4T1 cells (Fig. 6D), although differences failed to reach statistical significance ($p = 0.13$). We surmised that injection of 4T1 cells directly into the circulation via the tail vein should circumvent the immune-targeting of tumour cells at the primary-site, allowing direct assessment of the capacity of cells to establish a metastatic niche. Importantly, WT 4T1 cells again more efficiently colonised lungs in this setting than *Mki67*^{-/-} 4T1 cells (Fig. 6E), despite the higher overall tumour burden in the latter. Together, the results of these experiments underline the requirement for Ki-67 in seeding metastasis. They also suggest a defective immune response to Ki-67 knockout tumours. In agreement, immunohistological analysis revealed increased infiltration of Myeloid-Derived Suppressor Cells (41) in knockout tumours (using the MDSC marker, GR1; Fig. 6F), possibly indicating an immunosuppressive environment that protects the tumours against immune-mediated cytotoxicity. To investigate why Ki-67 knockout cells fail to induce an efficient anti-tumour immune response, we analysed the expression of the mouse major histocompatibility complex (MHC) class I, responsible for antigen presentation. Transcriptome

analysis showed downregulated expression of factors implicated in antigen processing and loading on MHC I: Tapasin, Tap1, Tap2, Psmb8 and Psmb9 in *Mki67*^{-/-} 4T1 cells, while tumours showed a further downregulation of Erp1 and B2M (Dataset S2, S4 and Fig. 3B, F). Flow cytometry revealed lower expression of MHC class I molecules H2D and H2K (Fig. 6G). MHC class I expression was also downregulated in *MKI67*^{-/-} MDA-MB-231 cells (SI Appendix Fig. S14), suggesting that roles of Ki-67 in maintaining MHC expression are conserved across species.

Cells lacking Ki-67 are sensitised to drugs.

The above results show that Ki-67 enables cell transformation, tumour growth and metastasis, yet also confers efficient targeting of tumours by the immune system. Finally, we asked whether Ki-67 would also influence drug responses, as, in addition to stem-like characteristics, the EMT has also been associated with resistance to chemotherapeutic drugs (42). 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 affect sensitivity to chemotherapeutic drugs (Fig. 7A, SI Appendix Fig. S15A). To test this, we performed an automated gene-drug screen using the Prestwick chemical library, composed of 1283 FDA-approved small molecules. We also included salinomycin, a positive control found to target cancer stem cells (43), and 6-thioguanine, which was originally used to isolate 4T1 cells (22). Control 4T1 cells were sensitive to 102 drugs at 10 μ M concentration, while the two *Mki67*^{-/-} clones were sensitive to 99 and 98 respectively, with 82 hits common to the three cell lines (SI Appendix Fig. S15B; Dataset S5). This suggests that Ki-67 loss does not qualitatively alter the drug-sensitivity profiles. We next determined the IC₅₀ of 10 hits commonly used in cancer therapy. Importantly, *Mki67*^{-/-} cells were markedly more sensitive to all the molecules tested (Fig. 7B). As such, by supporting expression of xenobiotic metabolism genes, Ki-67 provides cancer cells with a degree of protection against therapeutic drugs.

DISCUSSION

The above results show that Ki-67 is not required for cancer cell proliferation *in vitro* or *in vivo* in any cell type tested, but its expression critically influences all steps of tumourigenesis, including initiation, progression and metastasis, as well as immune responses and drug sensitivity. In both human and mouse mammary carcinoma cells, we present evidence that this is because Ki-67 sustains transcriptional programmes needed for tumour cells to adapt to their environment. This

is indicated by the failure to generate intestinal tumours in Ki-67-mutant mice, the reduced ability of some Ki-67 knockout cancer cells to induce angiogenesis, the presence of both epithelial and mesenchymal characteristics in Ki-67-positive epithelial cancer cells, which are altered in cells lacking Ki-67, the inability of the latter to colonise other tissues and give rise to metastases, their increased sensitivity to drugs, and their reduced interactions with the immune system. These phenotypes correlate well with alterations of expression of genes involved in the EMT, antigen presentation, drug metabolism, and other cancer-associated hallmarks.

Our data suggest that Ki-67 does not work by directly controlling expression of specific genes, since in Ki-67 knockout mouse fibroblast or epithelial cancer cells there are relatively few common deregulated genes. Instead, we find evidence that Ki-67 regulates general chromatin states that affect expression of genes in fundamental biological processes. This is supported by the fact that gene expression alterations are global, *i.e.* well within an order of magnitude of the number of genes in the genome, with around 2500 genes altered in non-transformed fibroblasts, nearly 5000 genes deregulated in Ki-67 knockout mouse mammary carcinoma cells, and over 9000 (*i.e.* the majority of expressed genes) in human mammary cancer cells. We compared changes in expression of genes involved in biological processes that are significantly enriched in Ki-67 knockout 3T3, 4T1, 4T1 tumour grafts and human MDA-MB-231 cells (SI Appendix Fig. S16). This clearly shows the similarity of affected pathways, and even genes, among these different knockouts, and highlights the fact that within common pathways different individual genes may be affected among different cell types. The similarity between the changes in different mammary carcinoma cells is apparent both *in vitro* and in tumours.

Our interpretation is also consistent with the extensive alterations in chromatin histone marks. With such wide-ranging transcriptome changes, it is almost impossible to attribute the loss of tumorigenicity to changes in expression of candidate genes, or even single regulatory mechanisms. Indeed, inactivation of the PRC2 complex did not restore tumorigenicity to Ki-67 knockout 4T1 cells. However, similar cancer-associated pathways are deregulated in 4T1 and MDA-MB-231 cells, suggesting general conservation of regulatory mechanisms.

A role in cancer-promoting transcriptional programmes provides a plausible explanation for the ubiquitous expression of Ki-67 across essentially all cancer types. Indeed, we show that Ki-67 is required for efficient tumour growth, independently of cell proliferation, in different human and mouse cancer cell types. Although to some observers it might appear paradoxical that tumour progression is reduced in Ki-67 knockouts without changes in cell proliferation rates, our

data suggest several possible mechanisms. We observed non-cell autonomous effects *in vivo*, including increases in apoptosis or fibrosis, to which altered angiogenesis could be a contributor. Other forms of cell death cannot be ruled out, but we did not find a consistent increase in necrosis. Additionally, it is possible that tumours might arise from a smaller number of initiating cells, due to a loss of stem-like character. In support of this, spheroid formation is decreased in both highly epithelial (4T1) and highly mesenchymal (MDA-MB-231) cancer cells. The fact that Ki-67 knockout reduces ability to seed metastases is consistent with this idea.

Whether or not Ki-67 expression maintains cancer stem cells (CSC) as such, is debatable. A recent study in the human colorectal cancer cell line DLD-1 showed that Ki-67 knockout reduces the number of cells expressing the antigen CD133, commonly assumed to be a marker of cancer stem cells (13). However, in intestinal crypts, CD133 expression is not specific to stem cells, and CD133-negative cancer cells were equally capable of sustaining tumourigenesis in a long-term serial transplantation model (44). Furthermore, the concept that CSCs are rare pre-existing populations of cells with hard-wired CSC properties, a model which emerged from xenograft experiments using sorted populations of hematopoietic stem cells, no longer appears valid, at least for solid tumours (45). Lineage tracing experiments in the intestine showed that tumours arise from stem cells which divide rapidly, make up around 10% of the cell in intestinal crypts, and can be regenerated from non-stem cells (45). A more recent concept is that epithelial cells are phenotypically plastic, in a manner dependent on EMT-inducing transcription factors (46), with no such fixed entity as CSC or non-CSC. Emerging evidence suggests that cells can reside in various phenotypic stages along the EMT-spectrum with concurrent expression of epithelial and mesenchymal traits, and that such hybrid states are important for carcinogenesis (33–35). Our data showing that ALDH activity, which is a *bona fide* marker for intestinal stem cells and breast and colon CSC (36, 37), is expressed by a significant fraction ($\approx 10\%$) of WT epithelial cancer cells but far fewer ($< 3\%$) Ki-67 knockout cells, are consistent with this model.

The EMT and stemness have previously been correlated with drug metabolism (42, 47). We find that, in cancer cells with a reversion of the EMT, Ki-67 loss leads to a reduced expression of genes encoding xenobiotic metabolism factors, which translates into increased sensitivity to all drugs tested in 4T1 cells. This is likely a conserved phenotype of Ki-67 loss, since Ki-67 knockout HeLa, DLD1 and MCF10A cells were also found to be more sensitive to all drugs tested (10, 13).

Previous studies of links between stem cell characteristics and carcinogenesis have focused on transcriptional states, converging on Myc-regulated targets as the strongest link

between stem cell and cancer cell transcriptional signatures (48). However, while in Ki-67 knockout NIH/3T3 cells Myc was slightly downregulated (Dataset S1), it was significantly upregulated in 4T1 cells (Dataset S2) ruling out the possibility that the reduced oncogenicity of these cells is simply due to loss of Myc, and again favouring a global effect of Ki-67 loss on the chromatin state.

Our hypothesis is consistent with our previous identification of many general chromatin regulators interacting with Ki-67, including components of PRC1 and PRC2, REST, NuRD, NIF1, NuA4, MLL, SET1, NoRC and NCOA6 complexes (11). Disrupting one such interactor, the PRC2 complex, partially, but not fully, rescued mesenchymal traits in Ki-67 mutant epithelial cancer cells, consistent with the idea that Ki-67 acts through multiple chromatin regulatory complexes. In accordance, loss of PRC2 did not restore tumourigenicity to *Mki67*^{-/-} 4T1 cells. Identifying the exact biochemical mechanisms by which Ki-67 affects the chromatin state and gene expression will require further studies. Ki-67 has no enzymatic activity and appears to be a largely intrinsically disordered protein, potentially providing hub-like properties for protein-protein interactions. How this confers the ability to adapt to the environment is not clear, but one can speculate that its binding of a large number of general transcription regulators is involved in maintenance of meta-stable states between different transcriptional programmes - in other words, transcriptional plasticity.

In conclusion, Ki-67, which is universally expressed in proliferating cells, enables multiple steps in carcinogenesis in different cancer types that require drastic changes in transcriptional programmes. Therapeutic targeting of Ki-67 itself will likely be challenging, since it is an intrinsically-disordered protein with no inherent enzyme activity. However, it could be of therapeutic benefit to inhibit its effectors in control of cellular adaptation to the environment. Alternatively, our results also suggest that Ki-67 confers an Achilles' heel to cancer cells, namely, their recognition by the immune-system. It will be interesting to see if non-proliferating cells, which do not express Ki-67, are consequently more resistant to immune-mediated killing. If this is the case, then, paradoxically, it might be advantageous to promote, rather than hinder, cell proliferation in order for immunotherapy to be optimally effective.

MATERIALS AND METHODS

Details of all materials and methods can be found in the *SI Appendix*. Materials include all animals, cell lines, antibodies, plasmids, PCR primers and reagents. Methods include CRISPR/Cas9-

mediated genome editing, AOM-DSS-mediated colon carcinogenesis, DNA replication assay, Mammosphere assay, Aldehyde Dehydrogenase (ALDH) activity assay, Xenografts, Visualisation of lung metastases, Cell extracts and Western-blotting, Histology and immunostaining, qRT-PCR, Colony formation assay, RNA sequencing library preparation and sequencing, Chromatin Immunoprecipitation and sequencing (ChIP-Seq), Sequence data processing, Gene set enrichment analysis, Automated drug library screen and Statistical analysis.

Data availability

All RNA-seq and ChIP-seq data have been deposited in GEO as a reference series, with the accession number GSE163114. This data in GEO will be made publicly available prior to publication of the manuscript.

SUPPORTING INFORMATION

Supporting information includes 16 figures and 5 Datasets.

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AUTHOR CONTRIBUTIONS

D.F. conceived and supervised the project. K.M., L.K., and D.F. designed experiments and interpreted the data. K.M., N.A., D.C., M.S., P.S., E.A., A.A., S.P., N.P., F.B., B.B., and C.H-K. performed experiments and interpreted the data. G.D. performed data analysis and interpretation. L.K. and D.F. wrote the manuscript, with contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Fig. 1. Ki-67 is dispensable for human cancer cells but is rarely mutated. (A) Expression levels of Ki-67 (MKI67), PCNA, cyclin D1 (CCND1) and c-Myc (MYC) in cell lines from the Cancer Cell Line Encyclopaedia. Values are $\log_2(\text{Transcripts Per Kilobase Million [TPM]}+1)$ of TPM-normalised RNA-seq data. (B) Relative copy number values for each gene from Sanger and Broad Institute whole exome sequencing data of cancer cell lines. (C) Dependency Mapper (DepMap) analysis of dependency of cancer cell lines on each gene, from genome-wide CRISPR (blue) and RNAi (violet) screens. Lower values of Gene Effect indicate higher likelihood of dependency in a given cell line. 0 corresponds to a non-essential gene, -1 is the median of all common essential genes. (D) cBioPortal mutation analysis of each gene in Pan-cancer TCGA data.

Fig. 2. Germline disruption of Ki-67 protects mice against intestinal tumourigenesis. (A, C) IHC staining of β -catenin in whole intestines from 6-7-month-old $Apc^{+/Δ14}Mki67^{+/2ntΔ}$ (A) and $Apc^{+/Δ14}Mki67^{2ntΔ/2ntΔ}$ (C) mice. Scale bar, 5mm. Insets show accumulation of β -catenin in nuclei. Scale bar, 500 μ m. (B, 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 $Apc^{+/Δ14}Mki67^{2ntΔ/2ntΔ}$ mice).

Fig. 3. Ki-67 ablation deregulates global gene expression programs in 4T1 cells. Dot plot analysis of differentially expressed genes (DEGs) in NIH/3T3 $Mki67^{-/-}$ cells (A), 4T1 $Mki67^{-/-}$ cells (B), and MDA-MB-231 $Mki67^{-/-}$ cells (D). Red dots: DEGs with p-value < 0.05; purple dots: \log_2 fold change, LFC >1 or <-1, p-value < 0.05; grey dots: not significant, NS. (C) Venn diagrams of DEGs in NIH/3T3 and 4T1 $Mki67^{-/-}$ cells under condition of p-value < 0.05 (top), and p-value (LFC >1 or <-1) < 0.05 (bottom). Gene set enrichment analysis of highly deregulated genes in 4T1 $Mki67^{-/-}$ cells (E) and MDA-MB-231 $Mki67^{-/-}$ cells (H). (F) Quantitative RT-PCR analysis of differentially expressed genes in 4T1 $Mki67^{-/-}$ cells; fold change in expression \pm SD is shown. Gene set enrichment analysis of ENCODE and ChEA Consensus transcription factors from ChIP-X gene sets in $Mki67^{-/-}$ 4T1 cells (G) and MDA-MB-231 $Mki67^{-/-}$ cells (I). FDR, false discovery rate adjusted p-values. (J) Left, heatmaps of ChIP-seq analysis of H3K27me3 in most down- and upregulated genes. Right, gene set enrichment analysis associated with these genes (K) The average values of the H3K27me3 (in green) and H3K4me3 (in apricot) ChIP-seq reads over the 10kb region surrounding the gene in 4T1 $Mki67^{-/-}$ cells were subtracted from the values of wild-type cells, and the plotted against the log of the fold-change for each gene in RNA-seq. (L) ChIP-seq profiles of histone marks at the Twist1 locus.

Fig. 4. Ki-67 promotes stem cell characteristics and controls the EMT in mammary carcinoma.

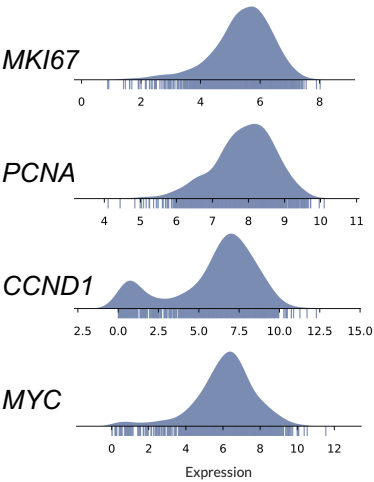
(A) Immunoblot analysis of *Hes1* expression in CTRL and two different clones (#1 and #2) of *Mki67*^{-/-} 4T1 cells. Immunoblot (B) and immunofluorescence (C; scale bar, 30µm) analysis of vimentin and E-cadherin expression in CTRL and *Mki67*^{-/-} 4T1 and MDA-MB-231 cells. (D) Aldehyde dehydrogenase activity measured using a flow-cytometry assay in 4T1 CTRL and *Mki67*^{-/-} cells. DAEB, inhibitor of ALDH was used as a negative control. Top, flow cytometry profiles; bottom, quantification of ALDH+ cells. Error bars, SEM (n=2 independent analyses). (E) Mammosphere formation assay of 4T1 CTRL or *Mki67*^{-/-} cells. Representative images (left; scale bar, 400 µm) and quantification (right; error bars, SEM, n=10) after 7 days.

Fig. 5. Ki-67 promotes tumour growth. (A) Dot plot analysis of differentially expressed genes (DEGs) in 4T1 *Mki67*^{-/-} cells versus tumours derived from grafting 4T1 *Mki67*^{-/-} cells into nude mice, showing a highly significant correlation. (B) Fluorescent IHC analysis of vimentin in 4T1 CTRL and *Mki67*^{-/-} tumours in NOD/SCID mice (scale bar, 30 µm). (C) Fluorescent IHC analysis of H3S10ph in 4T1 CTRL and *Mki67*^{-/-} tumours in NOD/SCID mice (red; pan cytokeratin (PCK) in green, DAPI in blue; scale bar 30 µm), top, and quantification, bottom. (D) IHC staining for Ki-67 and PCNA in 4T1 CTRL or *Mki67*^{-/-} tumours. Scale bar, 100 µm. 4T1 CTRL or *Mki67*^{-/-} orthotopic xenografts in NOD/SCID (E) and nude (F) mice. Tumour growth was monitored for 3 weeks. Error bars, SEM (n=6 mice). CD-31 staining of endothelial cells in CTRL and *Mki67*^{-/-} tumours in NOD-SCID mice: quantification of mean vessel density (MVD; G) and representative IHC images (H; scale bar 500 µm). (I) Sirius-red staining of collagen in CTRL and *Mki67*^{-/-} tumours in NOD-SCID mice; insets indicate tumours areas presented at higher magnification (scale bar, 250 µm). (J) Quantification of MVD in CTRL and *Mki67*^{-/-} tumours in nude mice.

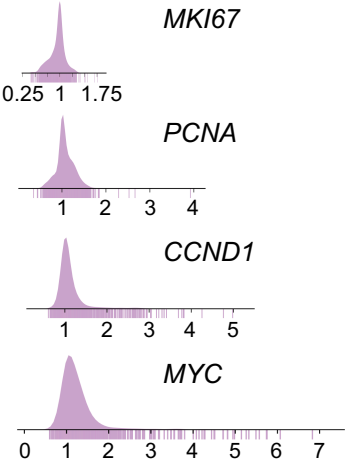
Fig. 6. Ki-67 promotes metastasis and anti-tumour immune responses. (A) Quantification of lung metastases in nude mice injected orthotopically with 4T1 CTRL or *Mki67*^{-/-} cells. Error bars, SEM. Top: representative images of lungs stained to visualise metastases (white nodules); scale bar, 10mm. (B) Lung tissue from nude mice injected via tail vein with 4T1 CTRL or *Mki67*^{-/-} cells (2 per condition) was dissociated after 2 weeks 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. (C) Tumour growth over 6 weeks of 4T1 CTRL or *Mki67*^{-/-} orthotopic xenografts in immunocompetent BALB/c mice. Error bars, SEM (n=8 mice), and quantification of lung metastases in each group (D). Error bars, SEM. (E) 4T1 CTRL or *Mki67*^{-/-} #2 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. (F) IHC analysis of 4T1 CTRL or *Mki67*^{-/-} tumours (week-4 post-transplantation) stained for Gr-1, a myeloid-derived suppressor cell (MDSC) marker. Scale bar, 100 µm. (G) 4T1 CTRL and *Mki67*^{-/-} cells were stained with anti-H2D^d-FITC and anti-H2K^d-PE, or control isotypes. Left, flow cytometry profiles. Right, quantification (n=1 of each clone).

Fig. 7. Ki-67 promotes cancer cell drug resistance. (A) Xenobiotic metabolism is a hallmark of genes downregulated in *Mki67*^{-/-} cells. (B) IC₅₀ of 4T1 CTRL or *Mki67*^{-/-} cells to indicated compounds, derived from dose-response experiments.

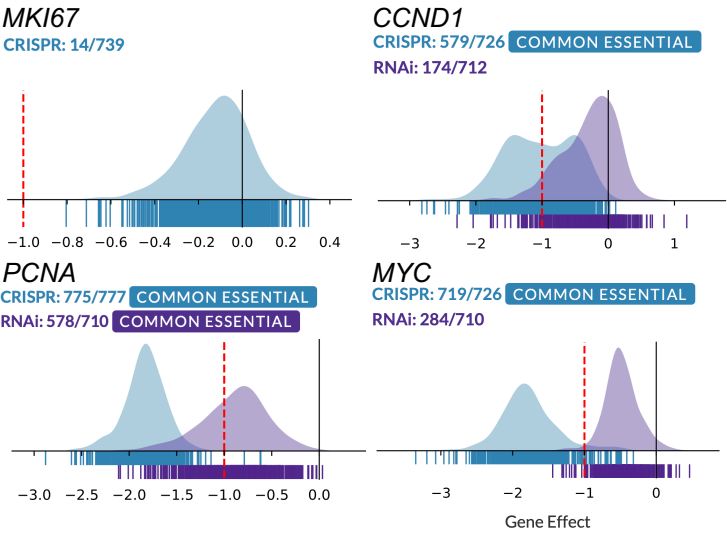
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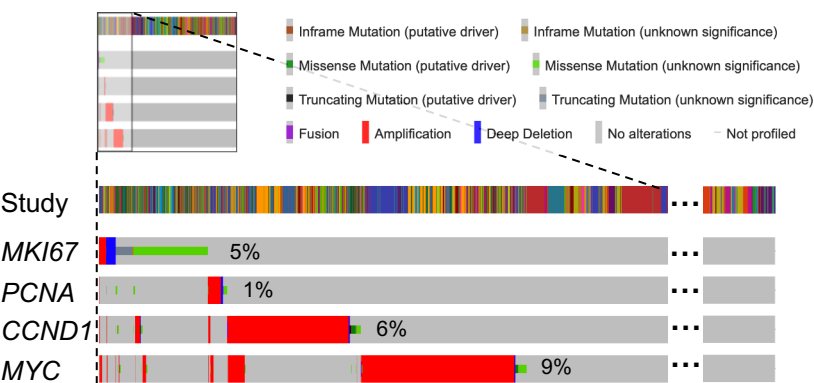
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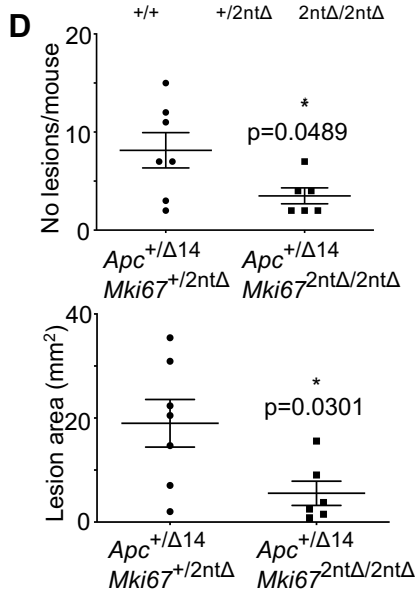
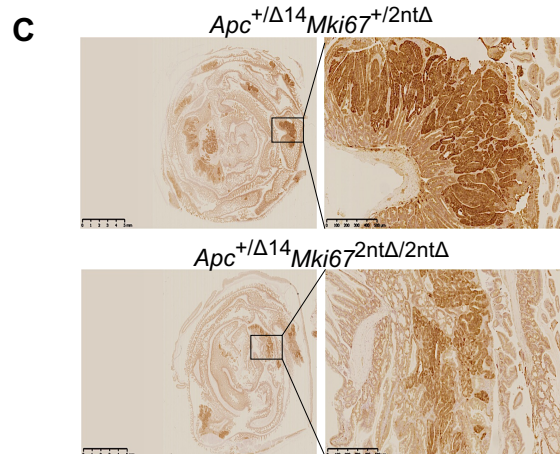
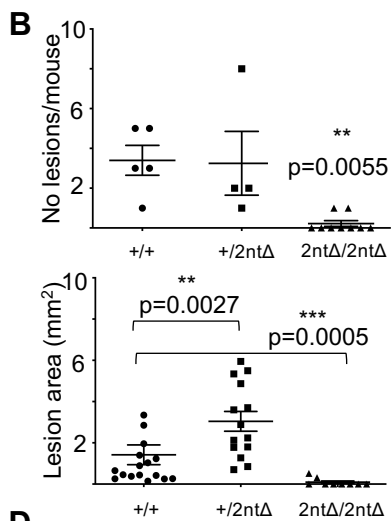
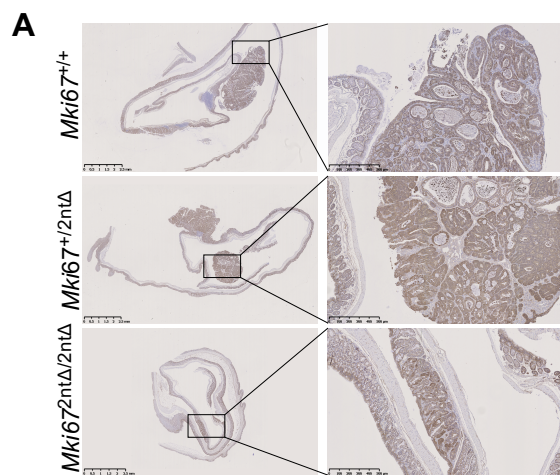


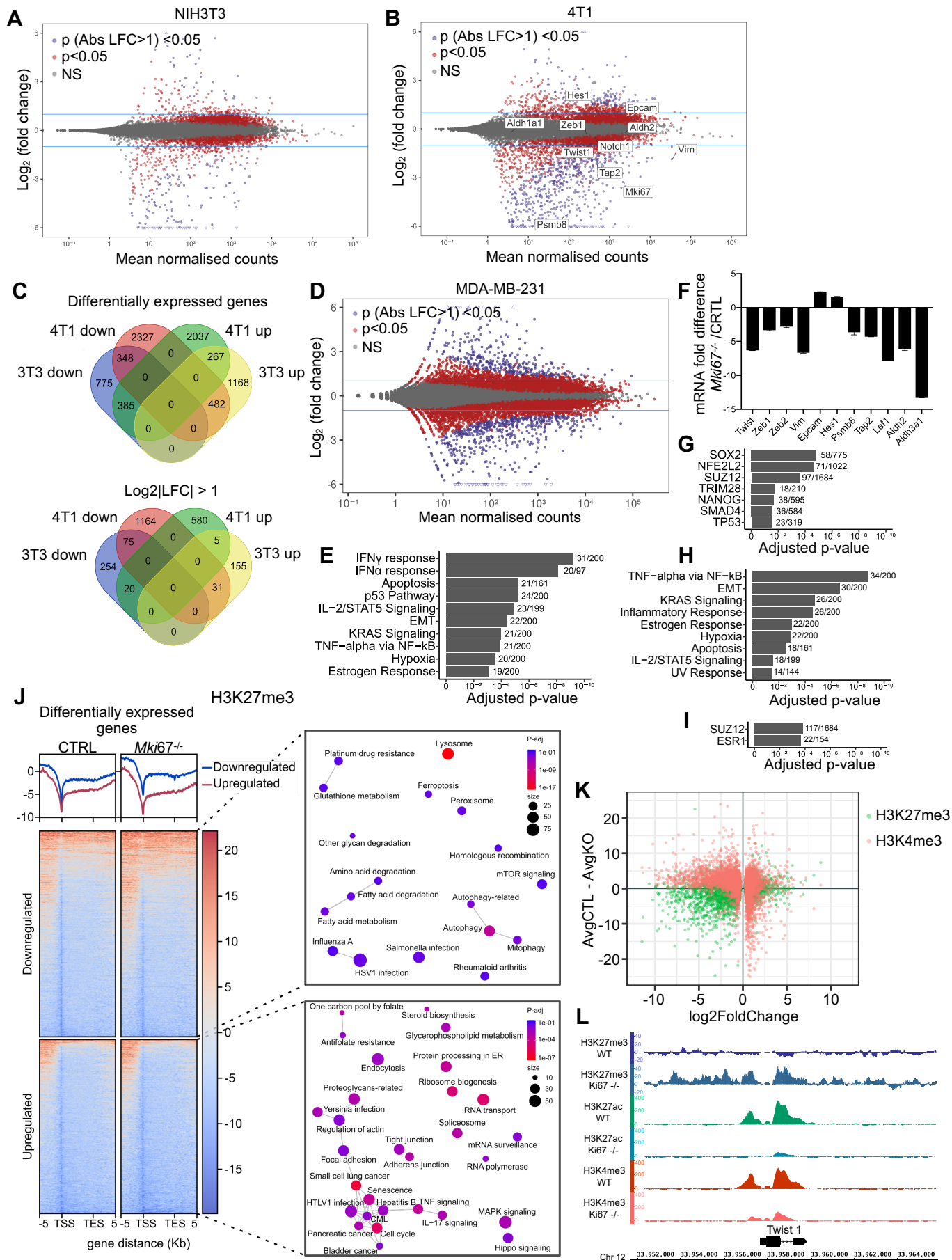
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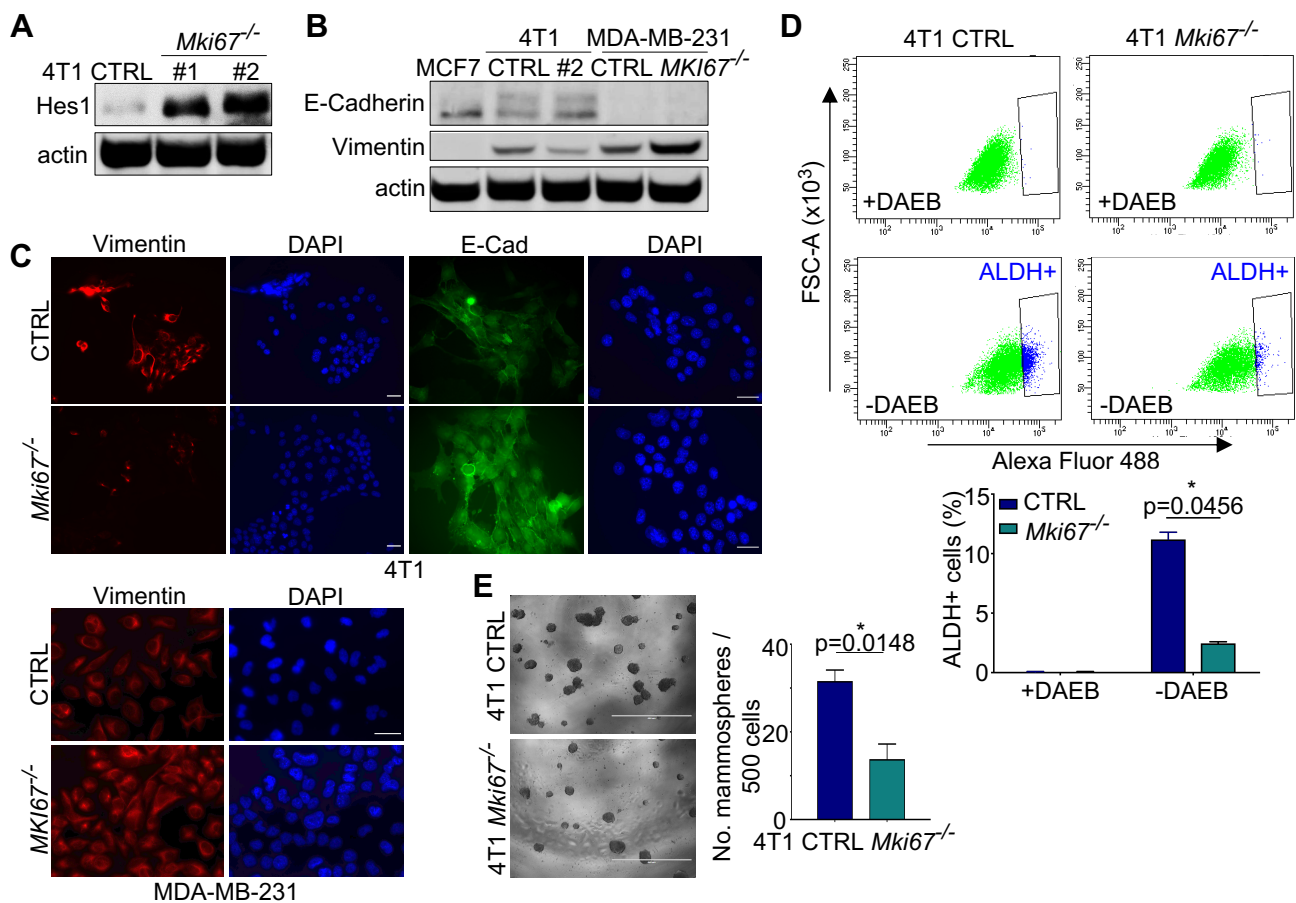


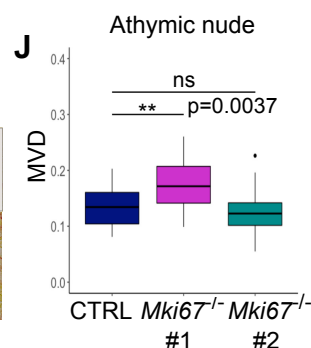
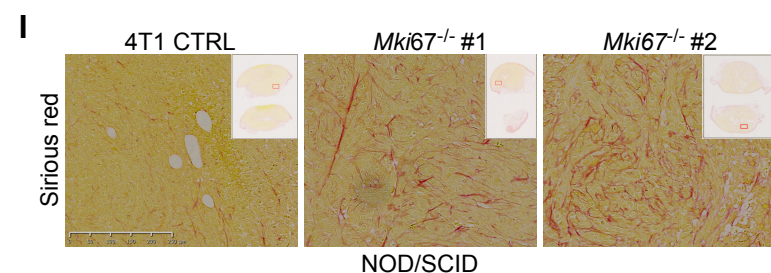
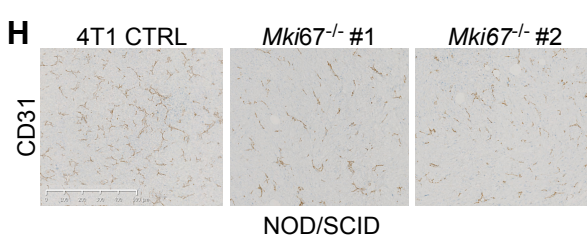
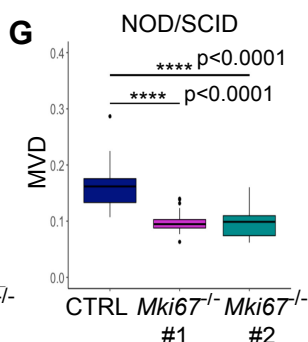
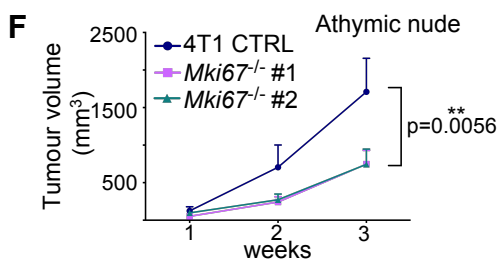
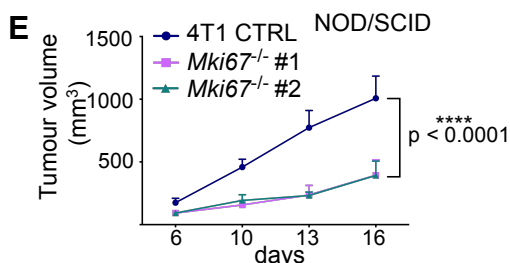
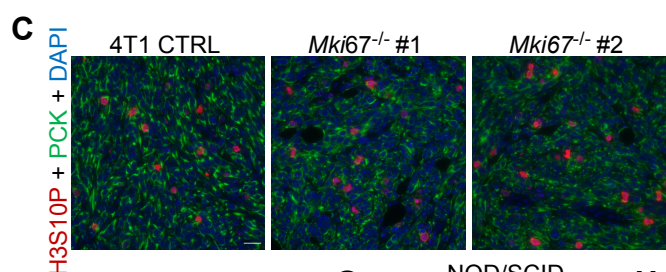
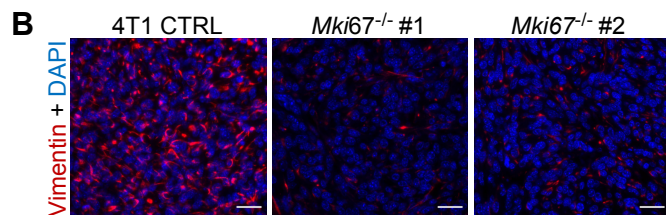
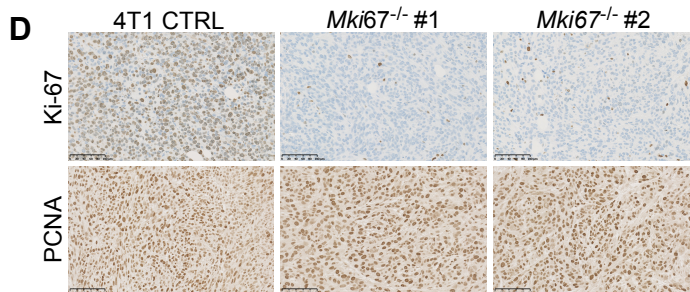
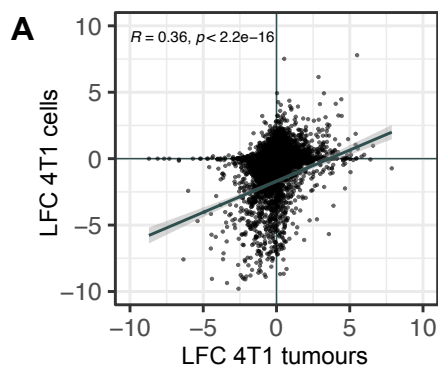
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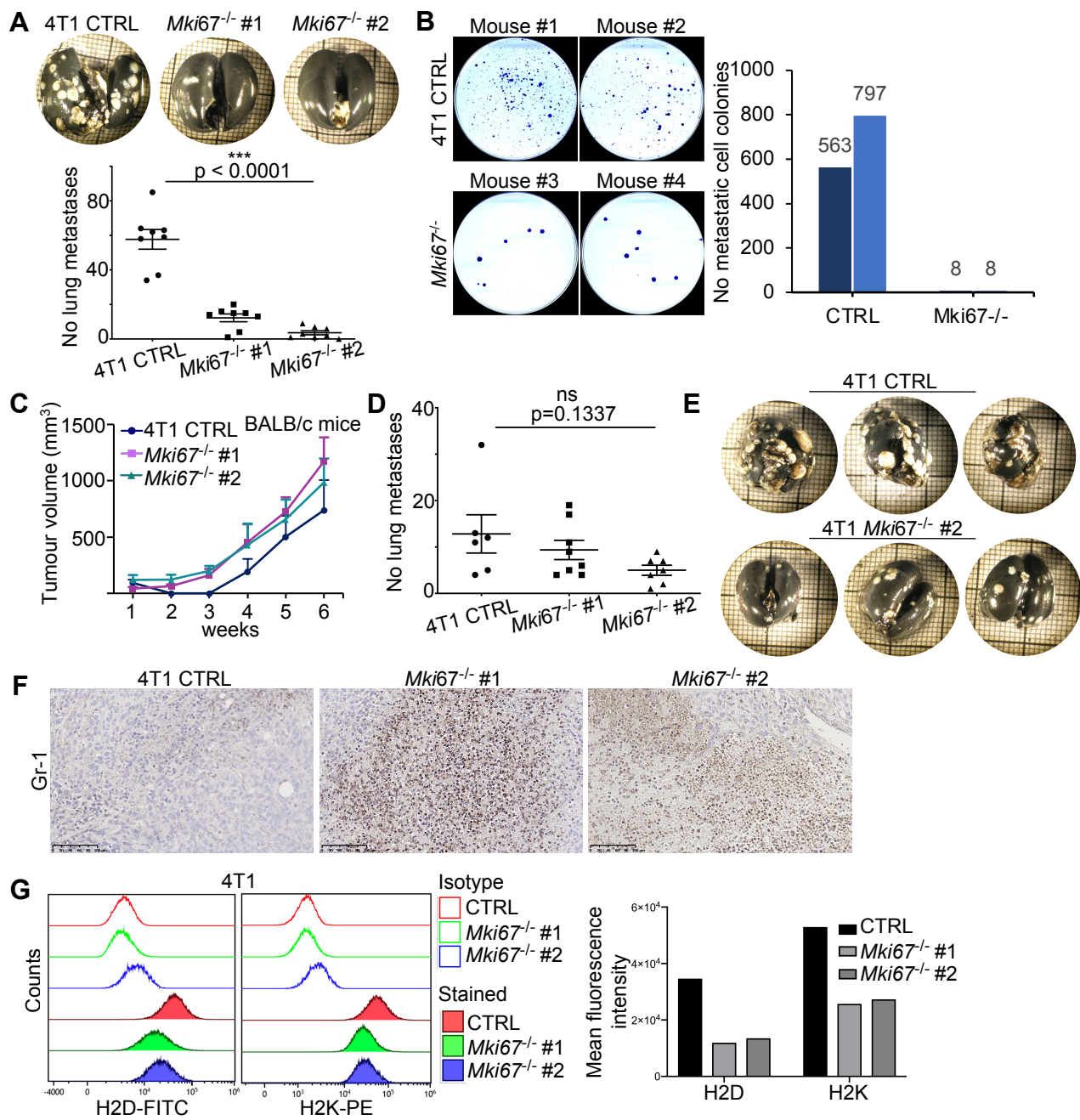




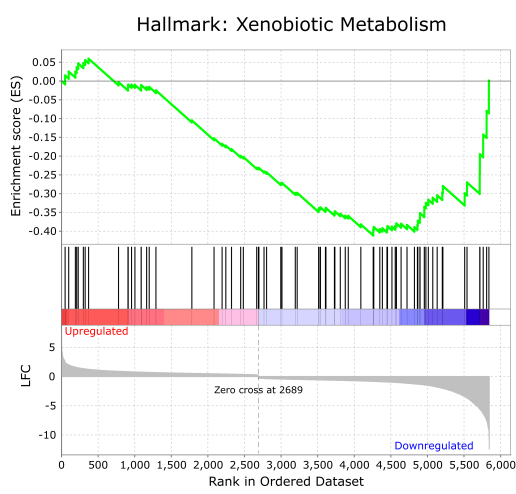








A



B

Compound	IC ₅₀ (μM)		
	4T1 CTRL	Mki67 ^{-/-} #1	Mki67 ^{-/-} #2
Camptothecin	4.1	1.5	1.0
Doxorubicin	1.2	0.5	0.6
Epirubicin	0.7	0.3	0.34
Daunorubicin	0.64	0.3	0.3
Mitoxantrone	1.3	0.4	0.49
Etoposide	21.4	7.8	9.1
Topotecan	15.8	2.6	1.9
Cytarabine	3.0	1.3	1.5
Floxuridine	0.1	0.1	0.0
Vorinostat	5.7	2.3	2.8
6-Thioguanine	N.A	N.A	N.A
Salinomycin	4.4	2.6	2.7