Figures and figure supplements

The cJUN NH₂-terminal kinase (JNK) signaling pathway promotes genome stability and prevents tumor initiation

Nomeda Girnius et al
Figure 1. JNK deficiency in mammary epithelial cells causes genomic instability and tumor formation. (A, B) Mammary gland tissue sections were prepared from parous Wap-Cre<sup>+</sup>/<sup>-</sup> Rosa26<sup>mTmG</sup>+/<sup>-</sup> female mice (n = 6) on day 21 post-weaning. These sections were stained with antibodies to cytokeratin 8 (CK8, red (A)) or cytokeratin 5 (CK5, red (B)), and GFP (green), and counterstained with DAPI (blue). Representative images are presented.

Figure 1 continued on next page
Figure 1 continued

(upper panel, scale bar = 48 μm). Boxed area was magnified (lower panel, scale bar = 24 μm). (C) Summary of the study cohort showing the total number of Mapk8<sup>loxP/loxP</sup> Mapk9<sup>loxP/loxP</sup> (ME<sup>WT</sup>) and Wap-Cre<sup>+/−</sup> Mapk8<sup>loxP/loxP</sup> Mapk9<sup>loxP/loxP</sup> (ME<sup>KO</sup>) mice examined, the number of mice exhibiting mammary intraepithelial neoplasia (MIN) or tumors (*p=0.037, **p=0.0084; Fisher’s Exact Test) (left panel). The type of carcinoma is presented (right panel). (D) Extracts prepared from ME<sup>KO</sup> and Wap-Cre<sup>+/−</sup> Trp53<sup>loxP/loxP</sup> (JNK<sup>WT</sup>) tumor cells were subjected to immunoblot analysis using antibodies to JNK and α-Tubulin. The ME<sup>KO</sup> tumors examined were representative of adenocarcinoma (#1), tumors with characteristics of both adenocarcinoma and adenosquamous carcinoma (#2), and adenosquamous carcinoma (#3). (E, F) Representative hematoxylin and eosin (H and E) -stained sections of adenosquamous carcinomas (E) and adenocarcinomas (F) from ME<sup>KO</sup> female mice are presented. Scale bar = 100 μm. (G) Exome sequencing was performed on ME<sup>KO</sup> tumor cell lines (n = 3). Mammary tissue from a virgin female of the same genotype (Wap-Cre<sup>+/−</sup> Mapk8<sup>loxP/loxP</sup> Mapk9<sup>loxP/loxP</sup>) was used as the reference genome. Circos plots showing copy number variations (CNVs) in ME<sup>KO</sup> tumor cells are presented. The outermost ring shows chromosome ideograms. The next track indicates high (red) and moderate (yellow) impact single nucleotide variants and indels marked by rectangles and triangles, respectively. The innermost track shows chromosome amplifications and deletions, with red and blue lines indicating chromosomal fragments present at log<sub>2</sub>(ratio tumor/normal)>0.2 or log<sub>2</sub>(ratio tumor/normal)<−0.2, respectively.

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Figure 1—figure supplement 1. Expression of estrogen and progesterone receptors in breast tumors caused by JNK deficiency in the mammary epithelium. (A, B) Representative adenocarcinoma (upper panel) and adenosquamous carcinoma (lower panel) sections stained for progesterone receptor (PR), estrogen receptor (ER) (Scale bar = 100 μm), and cytokeratin 8 (CK8, green) and cytokeratin 5 (CK5, red) (Scale bar = 50 μm) are presented. Immunofluorescent stains were counterstained with DAPI (left panels), and peroxidase-based staining was counterstained with hematoxylin (center and right panels) (A). Adenocarcinomas and adenosquamous carcinomas were scored for hormone receptor staining (B).

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**Table 1**

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**Figure 1—figure supplement 2.** Summary of exome sequence data. (A) Nomenclature Key: JNK^-^WT (tumors from parous Wap-Cre^-/^-Mapk8^-/- Mapk9^-/- Trp53^LoxP/LoxP^ mice); JNK^-^KO (tumors from parous Wap-Cre^-/^-Mapk8^LoxP/LoxP Mapk9^LoxP/LoxP Trp53^LoxP/LoxP^ mice); ME^-^KO (tumors from parous Wap-Cre^-/^-Mapk8^LoxP/LoxP Mapk9^LoxP/LoxP Trp53^LoxP/LoxP^ mice); and Control (mammary epithelium of non-parous mice of the same genotype). The NCBI SRA accession information (Project ID and Sample ID) for the exome sequence data is presented. The mean number of single nucleotide variants (SNVs) and insertions/deletions (Indels) is shown for each tumor genotype. An examination of frequent nucleotide changes revealed that ME^-^KO tumor cells favored...
Figure 1—figure supplement 2 continued

G to A (2/3 tumor cell lines) and JNK\textsuperscript{KO} favored C to T (4/6 tumor cell lines) replacements, but no favored replacements were identified in JNK\textsuperscript{WT} tumor cells. (B, C) High impact SNVs (B) and Indels (C) identified in JNK\textsuperscript{WT} (n = 2), ME\textsuperscript{KO} (n = 3) and JNK\textsuperscript{KO} (n = 6) cells are summarized. No well-established driver mutations were found among the high impact indels or SNVs identified and there were no recurring mutations within or across genotypes.

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Figure 2. JNK deficiency promotes tumor-associated gene expression. (A–D) RNA-seq analysis was performed using primary mammary epithelial cells (MEC, n = 3) and MEKO tumor cell lines (n = 3). K-means clustering was performed on differentially expressed genes and is presented as a heatmap (A). Pathway over-representation analysis using the KEGG database was performed on genes from each of the clusters. The pathways with the 10 lowest $p_{adj}$ values are presented (B). The mean expression Jun, Junb, Jund, Fos, and Fosb mRNA is presented as a heatmap (C). Ingenuity Pathway Analysis of the RNA-seq data was used to predict signaling pathway activity (D). (E) Wnt7b and Wnt10a expression in MEC (n = 3) and MEKO tumor cells (n = 3) is presented as the mean fragments per kilobase of exon model per million mapped fragments (FPKM) ± SEM. (F) WNT target gene expression (Axin2, Ccnd1, and Myc) in MEC (n = 3) and MEKO (n = 3) cells is presented as the mean FPKM ± SEM.

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Figure 2—figure supplement 1. Gene set enrichment analysis demonstrates that JNK deficiency causes decreased expression of a 'DNA Repair' gene signature. GSEA plot of a 'DNA Repair' gene signature by MEKO tumor cells compared with primary mammary epithelial cells (MEC) (p<0.01).
DOI: https://doi.org/10.7554/eLife.36389.009
Figure 3. JNK deficiency accelerates tumor formation in a mouse model of breast cancer. (A) Mammary tumor-free survival was monitored in cohorts of 26 Wap-Cre\textsuperscript{+/+} Trp53\textsuperscript{LoxP/LoxP} (JNK\textsuperscript{WT}) mice and 32 Wap-Cre\textsuperscript{+/+} Trp53\textsuperscript{LoxP/LoxP} Mapk8\textsuperscript{LoxP/LoxP} Mapk9\textsuperscript{LoxP/LoxP} (JNK\textsuperscript{KO}) mice. Animals euthanized before...
Figure 3 continued

A palpable mammary tumor had formed were censored in the log-rank analysis. (B) Tissue sections were prepared from JNK\textsuperscript{WT} mammary tumors (n = 11) and JNK\textsuperscript{KO} mammary tumors (n = 35). Representative images of H and E-stained sections from JNK\textsuperscript{WT} (upper panel) and JNK\textsuperscript{KO} (lower panel) mice are presented. Scale bar = 100 μm. (C) Adenocarcinoma tissue sections from JNK\textsuperscript{WT} mice (upper panel) and JNK\textsuperscript{KO} mice (lower panel) were stained with antibodies to (from left to right) estrogen receptor (ER), progesterone receptor (PR), cytokeratins 5 (red) and 8 (green) (CK5 and CK8 respectively), PCNA (Scale bars = 50 μm), and cleaved caspase 3 (Scale bar = 100 μm). Immunofluorescent stains were counterstained with DAPI, and peroxidase-based staining was counterstained with hematoxylin. Representative images are presented. (D, E) Exome sequencing was performed on JNK\textsuperscript{WT} (n = 2) and JNK\textsuperscript{KO} (n = 6) tumor cell lines. Mammary tissue from a virgin female of the same genotype (Wap-Cre\textsuperscript{+/−} Trp53\textsuperscript{LoxP/LoxP} for JNK\textsuperscript{WT} and Wap-Cre\textsuperscript{+/−} Trp53\textsuperscript{LoxP/LoxP} Mapk8\textsuperscript{LoxP/LoxP} Mapk9\textsuperscript{LoxP/LoxP} for JNK\textsuperscript{KO}) was used as the reference genome. Representative Circos plots showing CNVs are presented for JNK\textsuperscript{WT} (D) and JNK\textsuperscript{KO} (E) tumor cells. The outermost ring shows chromosome ideograms. The next track indicates high (red) and moderate (yellow) impact single nucleotide variants and indels marked by rectangles and triangles, respectively. The innermost track shows chromosome amplifications and deletions, with red and blue lines indicating chromosomal fragments present at log\(_2\)(ratio tumor/normal)>0.2 or log\(_2\)(ratio tumor/normal)<−0.2, respectively.

DOI: https://doi.org/10.7554/eLife.36389.011
Figure 3—figure supplement 1. Tumors in JNK<sup>KO</sup> mice are primarily adenocarcinomas and display a spectrum of hormone receptor expression patterns. (A) The JNK<sup>WT</sup> and JNK<sup>KO</sup> mouse cohorts are summarized. (B) Adenocarcinomas from JNK<sup>WT</sup> and JNK<sup>KO</sup> mice were stained for PR and ER and scored for expression. Numbers in parentheses indicate percentages.

DOI: https://doi.org/10.7554/eLife.36389.012
Figure 3—figure supplement 2. Exome sequencing of Control and JNK-deficient tumor cells. (A) Circos plots showing copy number variations (CNVs) in additional JNK\textsuperscript{KO} tumor cell lines are presented. Mammary tissue from a virgin female of the same genotype (Wap-Cre\textsuperscript{+/-} Trp53\textsuperscript{LoxP/LoxP} Mapk8\textsuperscript{LoxP/LoxP} Mapk9\textsuperscript{LoxP/LoxP}) was used as the reference genome. The outermost ring shows chromosome ideograms. The next track indicates high (red) and moderate (yellow) impact single nucleotide variants and indels marked by rectangles and triangles, respectively. The innermost track shows chromosome amplifications and deletions, with red and blue lines indicating chromosomal fragments present at log\textsubscript{2}(ratio tumor/normal)>0.2 or log\textsubscript{2}(ratio tumor/normal)<−0.2, respectively. (B) Kras expression in MEC (n = 3), JNK\textsuperscript{WT} (n = 2), and JNK\textsuperscript{KO} (n = 2) cells was measured by RNA sequencing and is presented as mean FPKM ± SEM. DOI: https://doi.org/10.7554/eLife.36389.013
Figure 3—figure supplement 3. Gene set enrichment analysis demonstrates increased expression of a ‘KRAS Signaling’ gene signature in breast tumor cells. GSEA plots of a ‘KRAS Signaling’ gene signature by JNK$^{WT}$ and JNK$^{KO}$ tumor cells compared with primary mammary epithelial cells (MEC) ($p<0.01$).

DOI: https://doi.org/10.7554/eLife.36389.014
Figure 4. RNA-seq analysis demonstrates that a sub-set of tumor-associated gene expression requires JNK. (A, B) RNA isolated from primary mammary epithelial cells (MEC, n = 3) and also JNK<sup>WT</sup> (n = 2), ME<sup>KO</sup> (n = 3), and JNK<sup>KO</sup> (n = 2) tumor cell lines was sequenced. The heatmap presents...

Figure 4 continued on next page
k-means clustering \((k = 4)\) of genes differentially expressed in any of the pairwise comparisons \((q < 0.05, |\log_2 \text{Fold Change}| > 0.75, \text{mean})\) (A). Pathway over-representation analysis was performed on each of the four clusters using the KEGG database (B). The pathways with the lowest \(p_{\text{adj}}\) values for each cluster are presented. (C) Comparative analysis was performed on genes differentially expressed between MEC and the tumor cell lines using Ingenuity Pathway Analysis (IPA). Heatmaps show the predicted activation (Activation z-score) of canonical pathways involved in immune (left panel) and integrin/cytoskeleton (right panel) signaling (cutoff score = 1.31; equates to \(p = 0.049\) using Fisher’s Exact Test).

DOI: https://doi.org/10.7554/eLife.36389.017
Figure 4—figure supplement 1. Gene expression analysis of control and JNK-deficient tumor cells. (A) Summary of the RNA sequencing data together with database accession information (GEO accession numbers and Sample ID). The number of samples per biological group is shown in Figure 4—figure supplement 1 continued on next page.
parentheses. (B) Venn diagram showing differentially expressed (DE) genes in JNK\textsuperscript{WT} (n = 2), ME\textsuperscript{KO} (n = 3), and JNK\textsuperscript{KO} (n = 2) tumors compared to MEC (n = 3). (C) Ingenuity Pathway Analysis was used to predict pathway activation and inhibition in JNK\textsuperscript{WT} (n = 2), ME\textsuperscript{KO} (n = 3), and JNK\textsuperscript{KO} (n = 2) tumor cells. The heatmap shows pathways ranked (top to bottom) by their score (total -log\textsubscript{10}p of Fisher’s Exact Test across the tumors). The top 100 pathways with lowest -log\textsubscript{10}p are shown. Coloring corresponds to the activation z-Score, with green representing inhibited pathways and red activated pathways. A cutoff of score = 1.31 was set (equates to p=0.049; Fisher’s Exact Test). DOI: https://doi.org/10.7554/eLife.36389.018
Figure 4—figure supplement 2. Comparison of signaling pathway activity in control and JNK-deficient tumor cells. (A) Two independently-derived JNK<sup>WT</sup> tumor cell lines (JNK<sup>WT</sup> 1 and JNK<sup>WT</sup> 2) were left untreated (-) or treated (+) with UV. The expression levels of tubulin, p-JNK, JNK, p-c-JUN (Ser 63), c-JUN, and tubulin were analyzed by western blotting. (B) Western blot analysis of JNK<sup>WT</sup> and JNK<sup>KO</sup> cells for JNK, p-ERK, ERK2, p-p38, p38, p-AKT (T308), p-AKT (S473), AKT, and tubulin.
Figure 4—figure supplement 2 continued

were exposed (+) to 60 J/m² ultraviolet light. Lysates from these cells were harvested at 30 min post-irradiation and were examined by immunoblot analysis by probing with antibodies to α-Tubulin, p-JNK, JNK, p-cJUN, and cJUN. (B) JNK<sup>WT</sup> (n = 2) and JNK<sup>KO</sup> (n = 6) tumor cell lines were cultured and protein lysates were prepared for immunoblot analysis. Lysates were probed for JNK, p-ERK, ERK2, p-p38, p-AKT (T308), p-AKT (S473), AKT, and α-Tubulin. Representative blots showing one JNK<sup>WT</sup> cell line and three JNK<sup>KO</sup> cell lines are presented.

DOI: https://doi.org/10.7554/eLife.36389.019
Figure 5. Stem cell populations are comparable in JNKWT and JNKKO tumor cells. (A) JNKWT and JNKKO tumor cells formed mammospheres when grown in suspension. Two independent cell lines were tested for each genotype. Representative phase contrast (upper panel) and H and E-stained agarose-embedded sphere sections (lower panel) are presented. Representative images are presented. Scale bar = 100 μm. (B) The number of mammospheres formed per 1000 plated cells each passage (P) was quantitated for JNKWT and JNKKO tumor cells. Two independent cell lines were tested for each genotype. The data presented are the mean ± SEM (n = 3 independent experiments). No significant differences were observed (p>0.05). (C) Representative agarose-embedded mammosphere sections stained with antibodies to cytokeratin 5 (CK5, red) and cytokeratin 8 (CK8, green), and counterstained with DAPI are presented. Scale bar = 50 μm. (D) RNA was isolated from JNKWT and JNKKO tumor cell mammospheres at different passages to quantify mRNA expression of Bmi1, Nanog, and Pou5f1. Two independent cell lines were tested for each genotype. The data presented are the mean ± SEM (n = 2 independent experiments). No significant differences were observed (p>0.05).

DOI: https://doi.org/10.7554/eLife.36389.024
Figure 5—figure supplement 1. JNK-deficient tumor cells do not exhibit enhanced tumor stem cell activity. (A) JNK\textsuperscript{WT} (n = 2) and ME\textsuperscript{KO} (n = 3) tumor cells grown in suspension formed mammospheres. Representative phase contrast (upper panel) and agarose-embedded H and E-stained sections (lower panel) are shown. Scale bar = 100 μm. (B) Sphere formation per 1000 tumor cells plated over two passages (P) was quantitated for two JNK\textsuperscript{WT} cell lines and three ME\textsuperscript{KO} cell lines (mean ± SEM; n = 3 independent experiments). DOI: https://doi.org/10.7554/eLife.36389.025
Figure 6. JNK\textsuperscript{WT} and JNK\textsuperscript{KO} tumor cells exhibit similar phenotypes. (A) JNK\textsuperscript{WT} (n = 2) and JNK\textsuperscript{KO} (n = 6) tumor cell lines were cultured (8 hr) in growth media and the change in confluence was measured using an IncuCyte ZOOM (mean ± SEM). No significant differences (p>0.05) were observed. Similar Figure 6 continued on next page...
data were obtained in four independent experiments. (B) Monolayers of JNK<sup>WT</sup> (n = 2) and JNK<sup>KO</sup> (n = 4) cells were wounded and cell migration rates were assessed by measuring the change in wound width 48 hr after wounding using an IncuCyte ZOOM (mean ± SEM). No significant differences (p>0.05) were observed. Similar data were obtained in two independent experiments. (C) JNK<sup>WT</sup> (n = 2) and JNK<sup>KO</sup> (n = 6) tumor cell chemotaxis in response to a serum gradient in the absence (Control) or presence of Matrigel was examined (mean ± SEM). No significant differences were observed (p>0.05). Similar data were obtained in two independent experiments. (D) Monolayers of JNK<sup>WT</sup> (n = 2) and JNK<sup>KO</sup> (n = 3) tumor cells were wounded, overlayed with 0.5 mg/ml collagen I in growth serum, and cultured for up to 48 hr in media containing 2% serum. Tumor cell migration into the collagen-filled wound was quantitated by measuring cell density in the initial wound area using an IncuCyte ZOOM (mean ± SEM). No significant differences were observed (p>0.05). Similar data were obtained in two independent experiments. (E-G) Orthotopic transplantation of JNK<sup>WT</sup> and JNK<sup>KO</sup> tumor cells (two independent cell lines per genotype) into the mammary fat pads of 26 (JNK<sup>WT</sup>) and 27 (JNK<sup>KO</sup>) syngeneic wild-type host mice was performed using 2 × 10<sup>6</sup> tumor cells (E). Orthotopic transplantation of 0.5 × 10<sup>6</sup> tumor cells was performed using 30 (JNK<sup>WT</sup>) and 26 (JNK<sup>KO</sup>) syngeneic wild-type host mice (F). No significant differences were observed (p>0.05). Representative H and E-stained tumor sections from JNK<sup>WT</sup> (upper panel) and JNK<sup>KO</sup> (lower panel) tumors are presented (G). Scale bar = 100 μm. (H, I) JNK<sup>WT</sup> (n = 2) and JNK<sup>KO</sup> (n = 4) tumor cell lines were cultured in soft agar and colony formation was quantitated (mean ± SEM) (H). Similar data were obtained from two independent experiments and representative images of crystal violet-stained colonies are presented (I). Scale bar = 100 μm. (J) Two JNK<sup>WT</sup> and five JNK<sup>KO</sup> tumor cell lines were cultured in suspension (24 hr) and apoptotic cells (7AAD<sup>+</sup> annexin V<sup>+</sup>) were quantitated by flow cytometry (mean ± SEM; n = 7 for JNK<sup>WT</sup> and n = 16 for JNK<sup>KO</sup>). DOI: https://doi.org/10.7554/eLife.36389.028
Figure 7. JNK deficiency promotes early disease lesions. (A) Eighteen weeks after gene deletion, tissue sections were prepared from mammary glands of JNK\textsuperscript{WT} (n = 5) and JNK\textsuperscript{KO} (n = 12) female mice. The mice were not matched for estrus cycle. Representative H and E-stained sections are presented. Scale bar = 100 \textmu m. (B, C) Proliferation was examined in mammary glands at 18 weeks after gene ablation by staining tissue sections with an antibody to PCNA (JNK\textsuperscript{WT} n = 4 mice, JNK\textsuperscript{KO} n = 5 mice). Representative PCNA-stained and DAPI counterstained glands are presented (B, Scale bar = 50 \textmu m). The percent of PCNA\textsuperscript{+} cells was quantified (mean ± SEM) (C). DOI: https://doi.org/10.7554/eLife.36389.030