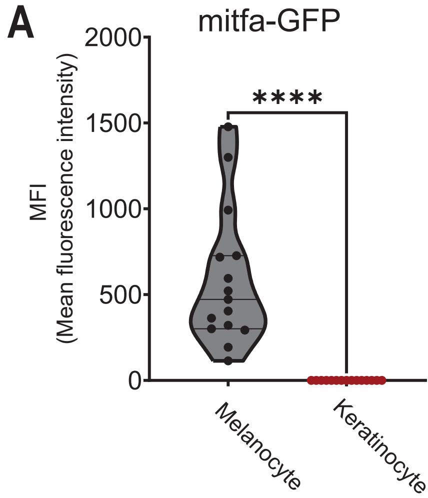
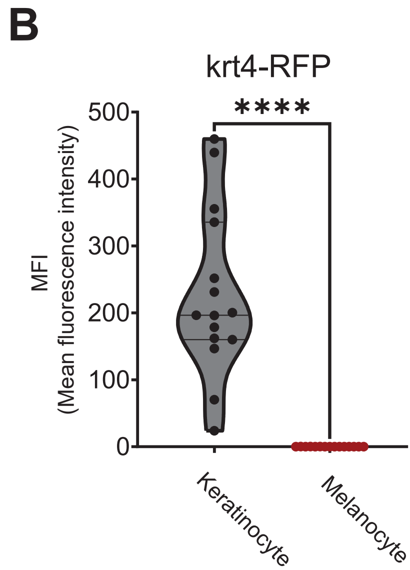
Supplementary Fig.1: Switching requires direct contact between nascent melanoma cells and keratinocytes in vivo





C D E F G

Melanocyte (mitfa-GFP) | Switched keratinocyte (krt4-RFP) | Merge

Melanocyte | Switched Keratinocyte | Merged

Supplementary Fig. 1: Switching requires direct contact between nascent melanoma cells and keratinocytes in vivo.

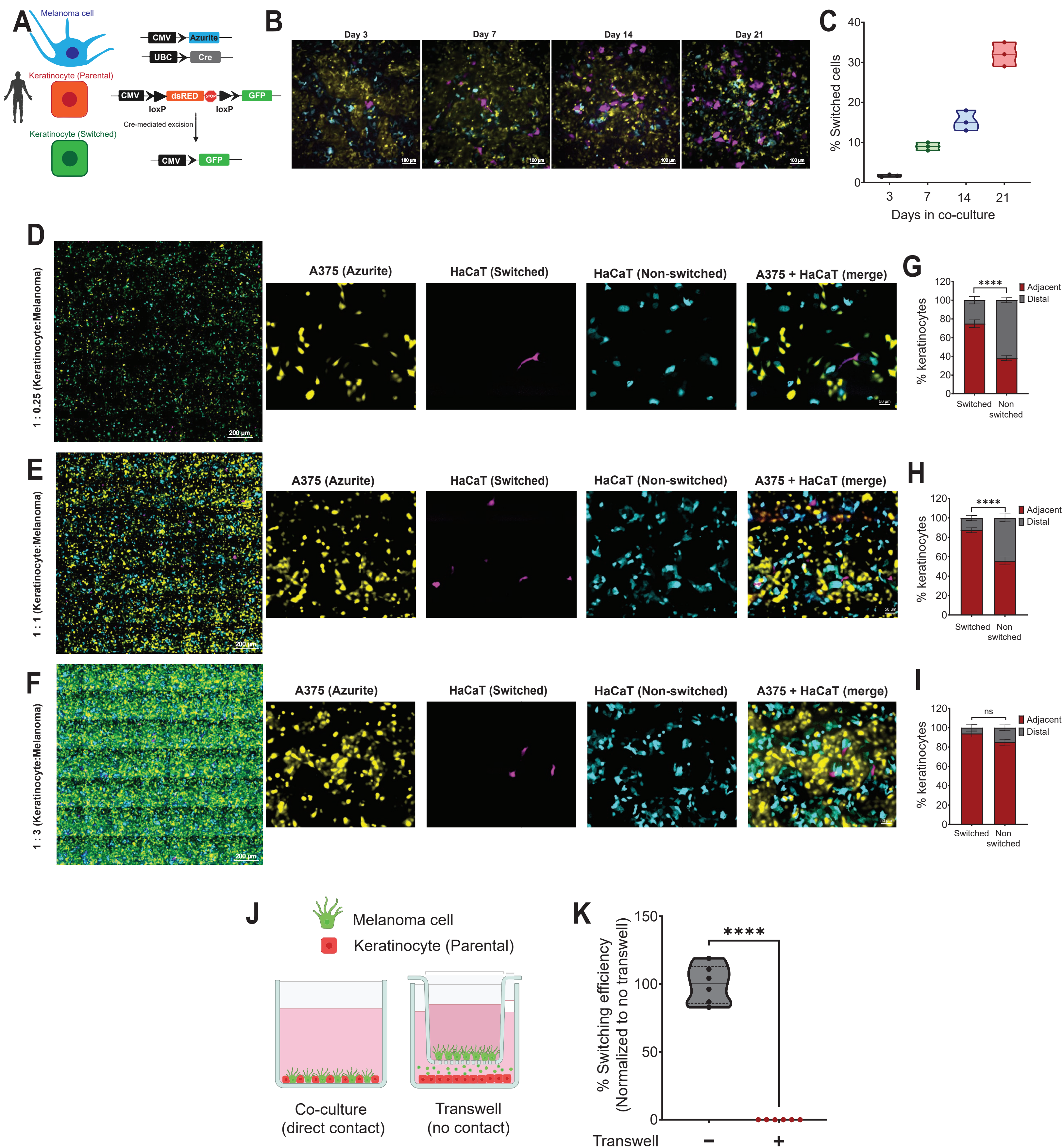
(A): MFI (Mean fluorescence intensity) of GFP in melanocytes and keratinocytes in F0 zebrafish embryos injected with mitfa-GFP/krt4-RFP and imaged at 3 days post fertilization (dpf) in 3 biological replicates. Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(B): MFI (Mean fluorescence intensity) of RFP in melanocytes and keratinocytes in F0 zebrafish embryos expressing krt4-RFP/mitfa-GFP and imaged at 3 days post fertilization (dpf) in 3 biological replicates. Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(C - F): Representative images of 3 dpf zebrafish embryos injected with the previously described constructs (Fig. 1A). Switched keratinocytes (magenta) are primarily located directly adjacent to a nascent melanoma cell (yellow). Individual cells are pseudocolored as indicated.

(G): Imaris based 3D reconstruction of melanoma/keratinocyte contacts in 3 dpf zebrafish embryos. Switched keratinocytes (magenta) are in direct contact with nascent melanoma cells (yellow), marked by dotted red boxes. Individual cells are pseudocolored as **(C – F)**.

Supplementary Fig.2: Switching requires direct contact between melanoma cells and keratinocytes in vitro



Supplementary Fig. 2: Switching requires direct contact between melanoma cells and keratinocytes in vitro.

(A): Schematic representation of the genetic reporter system to detect melanoma/keratinocyte communication in human co-cultured cells. (Created using BioRender.com)

(B): Representative images of melanoma/keratinocyte co-cultures incubated for 3, 7, 14 and 21 days in co-culture. Data is pooled from 3 biological replicates (n = 3).

(C): % Switched cells in melanoma/keratinocyte co-cultures incubated for 3, 7, 14 and 21 days in co-culture. Data is pooled from 3 biological replicates (n = 3).

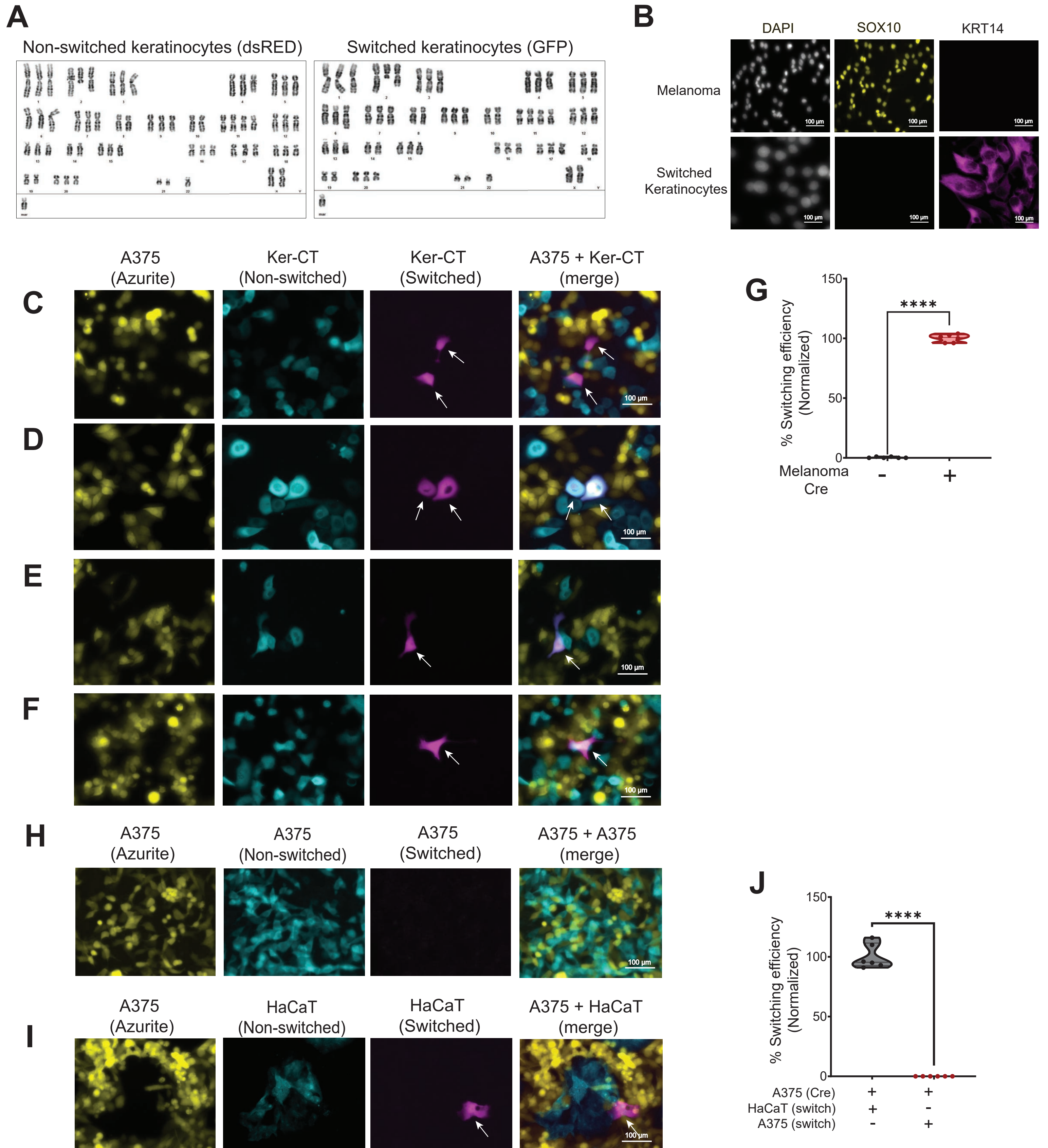
(D, E, F): Representative well-level images of melanoma/keratinocyte co-cultures with A375 melanoma cells (yellow) over-expressing Cre and Azurite and HaCaT switched keratinocytes over-expressing the floxed dsRED to GFP switch construct (turqoise/magenta), in three different melanoma/keratinocyte ratios (0.25:1, 1:1 and 3:1). Switched keratinocytes are primarily located directly adjacent to a melanoma cell. Individual cells are pseudocolored as indicated.

(G, H, I): Bar plot showing % switched/non-switched keratinocytes (GFP/dsRED positive) in co-culture in direct contact with a melanoma cell (Azurite positive). Data is pooled from 4 biological replicates (n = 12). P values generated by chi-squared test with **** = p < 0.0001.

(J): Schematic representation of Transwell assay for detecting melanoma/keratinocyte communication (using the Cre-loxP system) +/- direct cell-cell contact using a 400 nm transwell chamber. (Created using BioRender.com)

(K): % Switching efficiency calculated as number of switched cells per well in keratinocytes +/- direct contact with melanoma cells (A375), normalized to no transwell (positive control). Data is pooled from 3 biological replicates (n = 6). Error bars: SD, P values generated by unpaired t-test with **** = p < 0.0001.

Supplementary Fig.3: Switching does not involve cell fusion between keratinocytes and melanoma cells



Supplementary Fig. 3: Switching does not involve cell fusion between keratinocytes and melanoma cells.

(A): Karyotypic analysis of switched (GFP positive) vs non-switched (dsRED positive) keratinocytes post FACS highlighting the presence of HaCaT specific chromosomal markers in both cell types without additional chromosomal changes in switched keratinocytes.

(B): Immunostaining for SOX10 (melanoma marker) and KRT14 (keratinocyte marker) in monocultures of switched keratinocytes and melanoma cells to identify evidence of cell fusion between melanoma cells and keratinocytes in co-culture. Individual cells are pseudocolored as indicated.

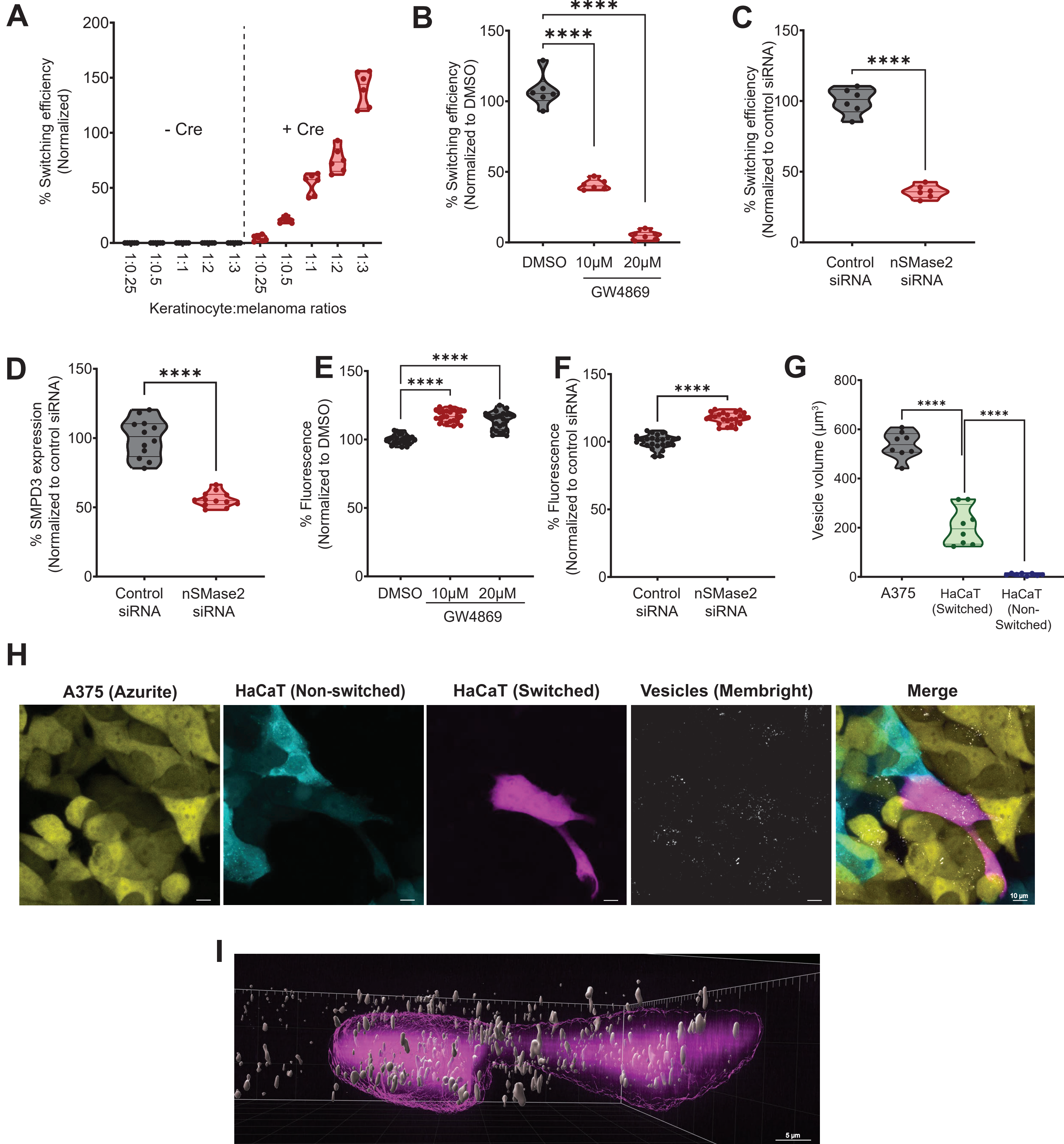
(C - F): Representative images of 48-hour melanoma/keratinocyte co-cultures with A375 melanoma cells over-expressing Cre and Azurite (yellow) and switched Ker-CT keratinocytes over-expressing the floxed dsRED to GFP switch construct (magenta, indicated by white arrows) (Ratio: 1:3, Keratinocyte:Melanoma). Individual cells are pseudocolored as indicated.

(G): Quantification of switching efficiency represented as % switching efficiency per well in melanoma/keratinocyte co-cultures with Ker-CT keratinocytes and A375 melanoma cells +/- Cre. Data is pooled from 3 biological replicates (n = 6) (Ratio: 1:3, Keratinocyte:Melanoma). Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

(H - I): Representative images of 48-hour melanoma/melanoma or melanoma/keratinocyte co-cultures with one population of A375 melanoma cells over-expressing Cre and Azurite (yellow) and a second population of A375 melanoma cells/HaCaT keratinocytes over-expressing the floxed dsRED to GFP switch construct (turquoise/magenta) (Ratio: 1:3, Keratinocyte:Melanoma or Melanoma:Melanoma). Individual cells are pseudocolored as indicated.

(J): Quantification of switching efficiency represented as % switching efficiency per well in melanoma/melanoma or melanoma/keratinocyte co-cultures (Ratio: 1:3, Keratinocyte:Melanoma or Melanoma:Melanoma). Data is pooled from 3 biological replicates (n = 6). Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

Supplementary Fig.4: Exosome-like vesicles are transferred from melanoma cells to keratinocytes



Supplementary Fig. 4: Exosome-like vesicles are transferred from melanoma cells to keratinocytes.

(A): % Switching efficiency calculated as number of switched cells per well with different melanoma/keratinocyte ratios. Data is pooled from 3 biological replicates (n = 6). Error bars: SD.

(B): % Switching efficiency calculated as number of switched cells per well normalized to control (DMSO) upon treatment with increasing concentrations of nSMase2 inhibitor, GW4869 in melanoma/keratinocyte co-cultures for 48 hours pooled from 3 biological replicates (Ratio: 1:3, Keratinocyte:Melanoma) (n = 6). Error bars: SD, P values generated by one-way ANOVA with multiple comparisons with **** = $p < 0.0001$.

(C): % Switching efficiency calculated as number of switched cells per well normalized to control siRNA when melanoma cells are treated with nSMase2 targeting siRNA pooled from 3 biological replicates (n = 12) (Ratio: 1:3, Keratinocyte:Melanoma). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(D): % SMPD3 Expression in melanoma cells calculated using qPCR using an nSMase2 primer pair normalized to control siRNA when co-cultures are treated with nSMase2 targeting siRNA pooled from 4 biological replicates (n = 12). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(E): Melanoma proliferation calculated as % Fluorescence in melanoma cells treated with GW4869 as indicated. Data is pooled from 4 biological replicates (n = 24) and is normalized to DMSO treatment condition. Error bars: SD, P values generated by one-way ANOVA with multiple comparisons **** = $p < 0.0001$.

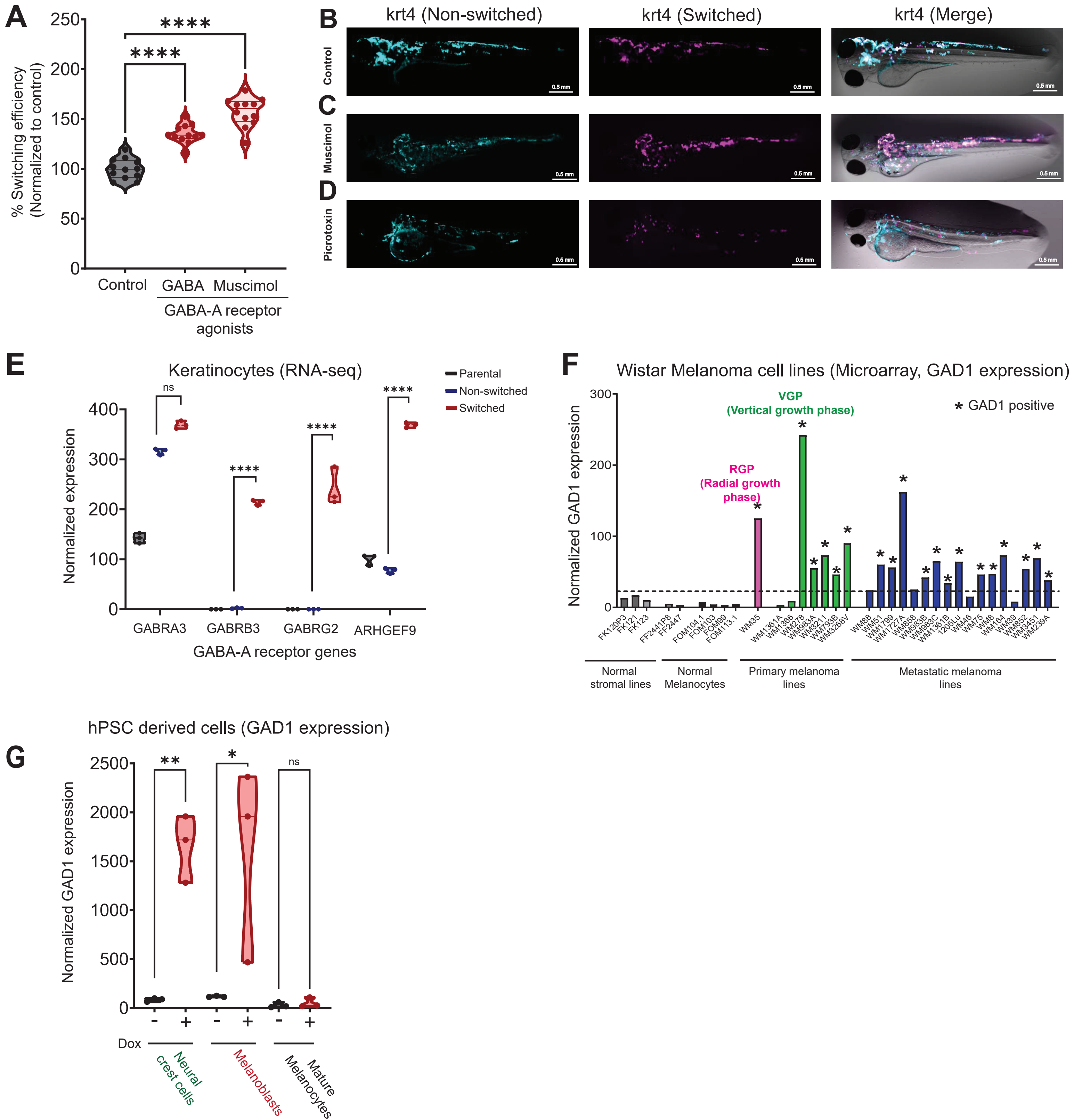
(F): Melanoma proliferation calculated as % Fluorescence in melanoma cells treated with control or nSMase2 siRNA as indicated. Data is pooled from 4 biological replicates (n = 24) and is normalized to control siRNA condition. Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

(G): Melanoma internalized vesicle volume (in μm^3) calculated as Membright dye positive area in melanoma cells, parental keratinocytes and switched keratinocytes. Data is pooled from 4 biological replicates (n = 12). Error bars: SD, P values generated by one-way ANOVA with multiple comparisons with **** = $p < 0.0001$.

(H): Representative image of a 48-hour melanoma/keratinocyte co-culture with A375 melanoma cells over-expressing Cre and Azurite (yellow) stained with the vesicle staining Membright dye (white) and non-switched keratinocytes expressing dsRED (turquoise), switched keratinocytes expressing GFP (magenta) (Ratio: 1:3, Keratinocyte:Melanoma). Individual cells are pseudocolored as indicated.

(I): Imaris based 3D reconstruction of **(H)**, melanoma/keratinocyte contacts and internalized vesicles stained with Membright in 48-hour melanoma/keratinocyte co-cultures. Switched keratinocytes (magenta) are in direct contact with melanoma cells (yellow) and have internalized melanoma vesicles (white). Individual cells are pseudocolored as indicated.

Supplementary Fig.5: GABAergic signaling is active in skin and melanoma cells



Supplementary Fig. 5: GABAergic signaling is active in skin and melanoma cells.

(A): % Switching efficiency calculated as number of switched cells per well normalized to control media upon treatment with GABA-A agonists, GABA (100 µM) and muscimol (10 µM) in melanoma/keratinocyte co-cultures pooled from 4 biological replicates (n = 12) (Ratio: 1:3, Keratinocyte:Melanoma). Error bars: SD, P values generated by one-way ANOVA with multiple comparisons with **** = $p < 0.0001$.

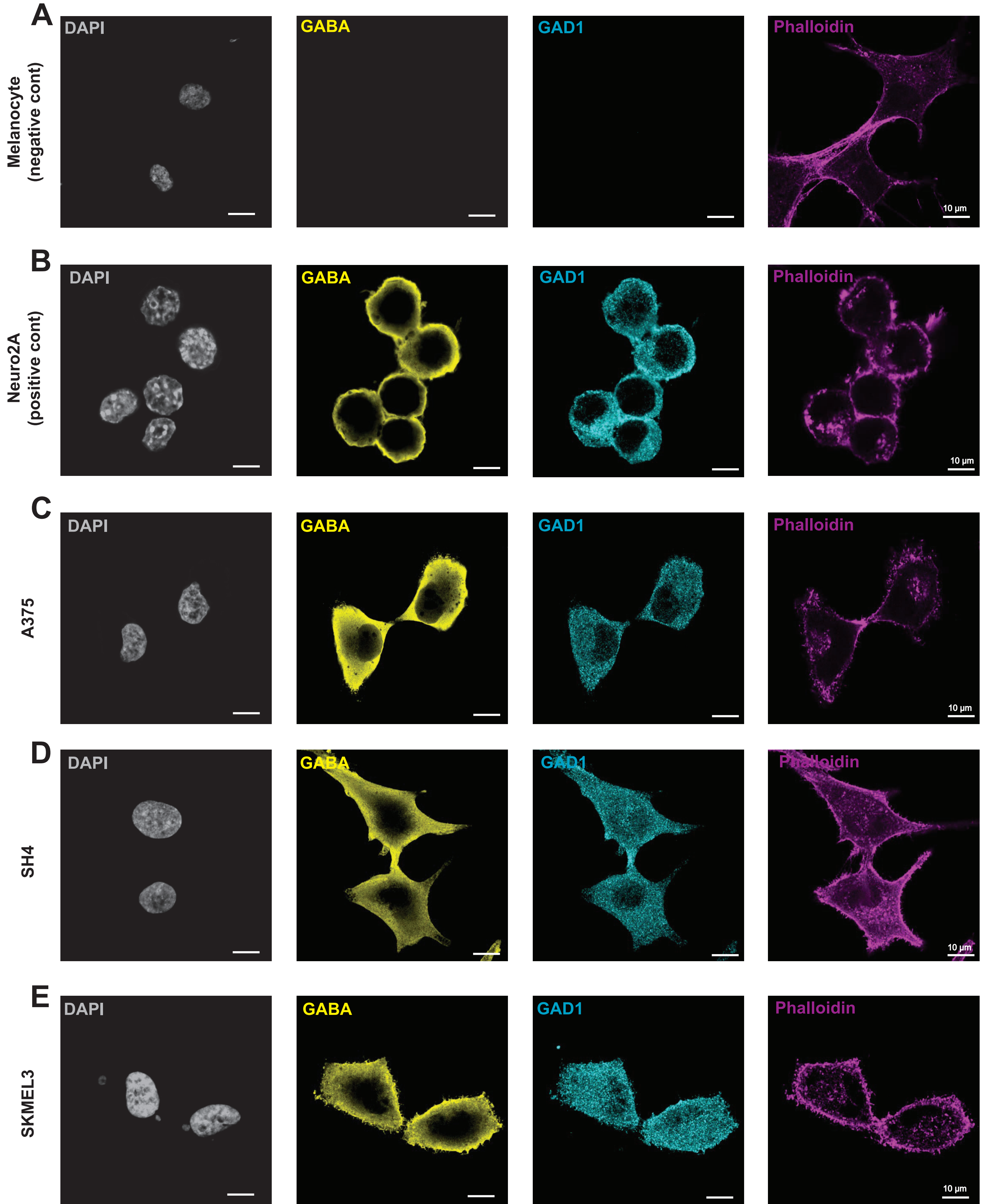
(B - D): Representative images of 3 dpf zebrafish embryos injected with the previously described constructs (Fig. 1A) and treated with control water **(B)**, water containing muscimol, 10 µM **(C)** or water containing picrotoxin, 100 µM **(D)**. The image shows parental keratinocytes (turquoise) and switched keratinocytes (magenta) in the whole zebrafish embryo. Individual cells are pseudocolored as indicated.

(E): Normalized expression of GABA receptor subunits (GABRA3, GABRB3 and GABRG2) and collybistin (ARHGEF9) in parental, non-switched and switched keratinocytes from RNA-seq analysis. Data represent three biological replicates. Error bars: SD, P values were calculated using DeSeq2 with **** = $p < 0.0001$.

(F): Normalized expression of GAD1 in Wistar melanoma cell lines representing primary melanoma (RGP and VGP), metastatic melanoma, primary melanocytes (FOM lines), keratinocytes (FK lines) and fibroblasts (FF lines). * represents cell lines that are GAD1 positive.

(G): Normalized expression of GAD1 in triple knockout (RB1, P53, P16) human pluripotent stem cell (hPSC) lines differentiated into neural crest cells, melanoblasts and melanocytes (data from *2*). Addition of doxycycline induces expression of $BRAF^{V600E}$ in this system. Only neural crest cells and melanoblasts induce GAD1 expression in response to doxycycline and can form tumors in vivo. Data represent three biological replicates. Error bars: SD, P values were calculated using DeSeq2 with ** = $p < 0.01$ and * = $p < 0.05$.

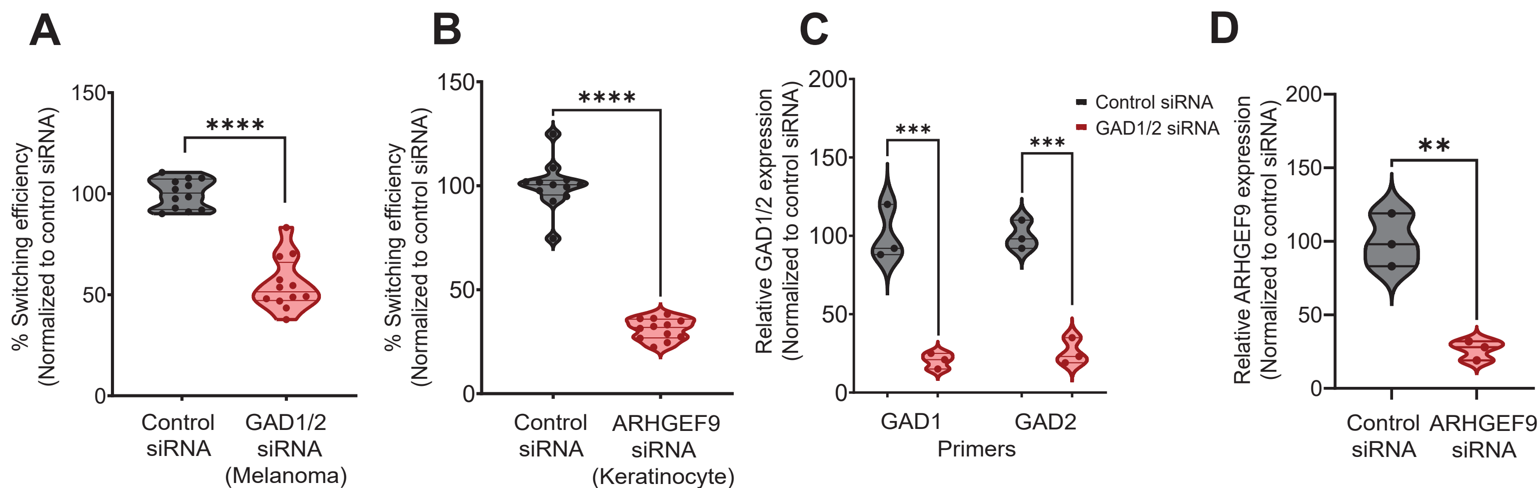
Supplementary Fig.6: Melanoma cells express *GAD1* and produce GABA



Supplementary Fig. 6: Melanoma cells express *GAD1* and produce GABA.

(A - E): Immunostaining for GABA (yellow), GAD1 (turquoise) and cytoskeleton with phalloidin (magenta) in melanocytes (negative control) **(A)**, Neuro2A cells (positive control) **(B)** and melanoma cell lines, A375 **(C)**, SH4 **(D)** and SKMEL3 **(E)**. Individual cells are pseudocolored as indicated.

Supplementary Fig.7: Disruption of GABA signaling blocks melanoma/keratinocyte communication



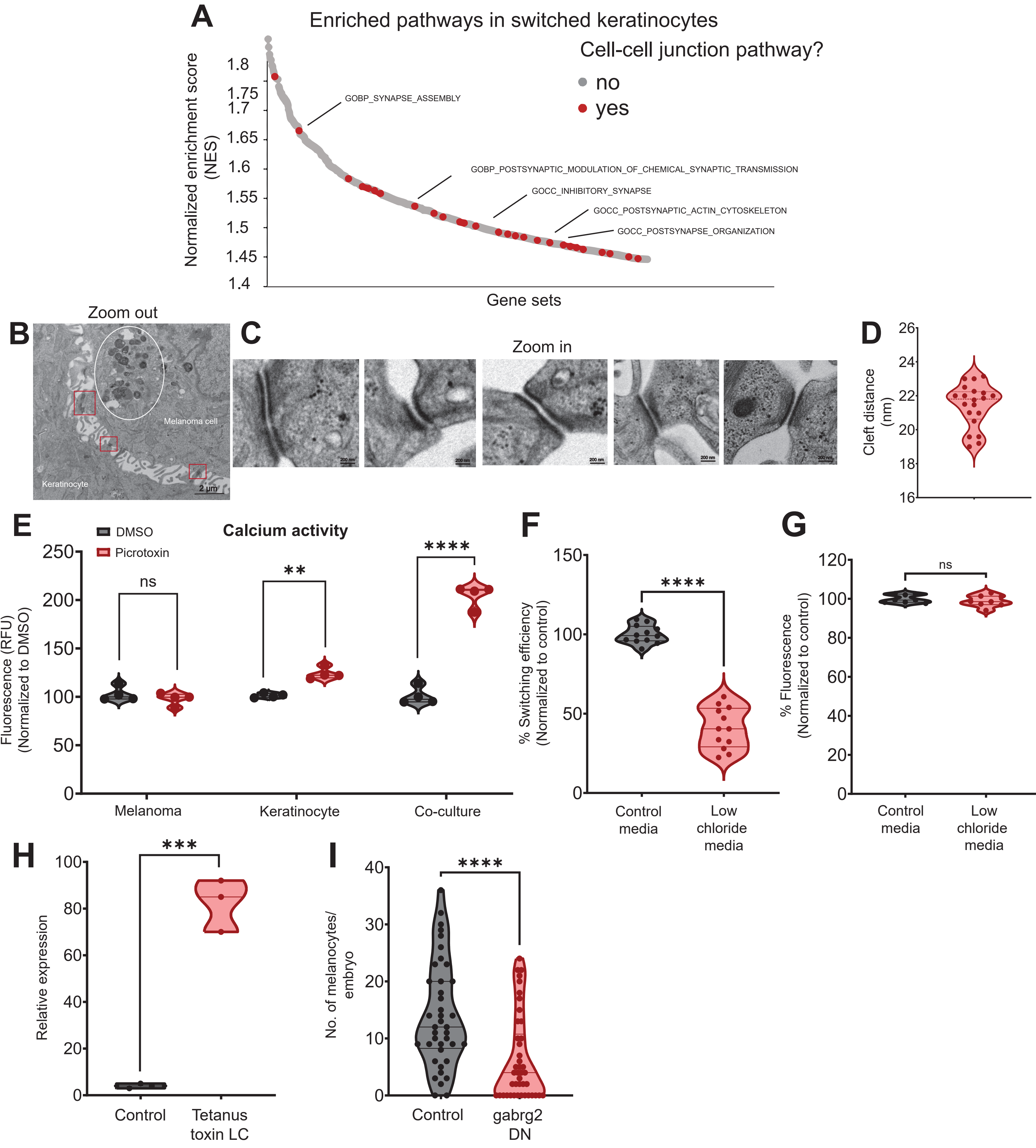
Supplementary Fig. 7: Disruption of GABA signaling blocks melanoma/keratinocyte communication.

(A and B): % Switching efficiency calculated as number of switched cells per well normalized to control siRNA pooled from 3 biological replicates upon GAD1/2 only **(A)** or ARHGEF9 only **(B)** knockdown (n = 12) (Ratio: 1:3, Keratinocyte:Melanoma). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(C): Normalized expression of GAD1 and GAD2 in control siRNA and GAD1/2 siRNA treated melanoma cells (A375). Data represent three biological replicates. Error bars: SD, P values were generated by unpaired t-test with **** = $p < 0.0001$.

(D): Normalized expression of ARHGEF9 in control siRNA and ARHGEF9 siRNA treated keratinocytes (HaCaT). Data represent three biological replicates. Error bars: SD, P values were generated by unpaired t-test with ** = $p < 0.01$.

Supplementary Fig.8: Specialized cell-cell junction signatures are enriched in melanoma cells and keratinocytes



Supplementary Fig. 8: Specialized cell-cell junction signatures are enriched in melanoma cells and keratinocytes.

(A): Waterfall plot of enriched pathways from GSEA analysis of switched vs parental keratinocytes. Synapse/specialized cell-cell junction related pathways are highlighted in red.

(B): Transmission electron microscopy (zoomed out) of melanoma/keratinocyte co-cultures with melanosome-like vesicles in melanoma cells (white circle) with specialized cell-cell junctions showing electron-dense structures (red boxes). Representative images are shown.

(C): Transmission electron microscopy (zoomed in) of melanoma/keratinocyte co-cultures with specialized cell-cell junctions showing electron-dense structures. Representative images are shown.

(D): Cleft distance quantified in nm in melanoma/keratinocyte co-cultures.

(E): Calcium spike activity in monocultures (melanoma cells and keratinocytes) and co-cultures upon picrotoxin (100 μM) addition (Ratio: 1:5, Melanoma:Keratinocyte). RFU is relative fluorescence units measured using the calcium dye, Cal-520. Data represent 3 biological replicates per condition, P values calculated using multiple two-tailed paired t-test, ** p value < 0.01, *** p < 0.001.

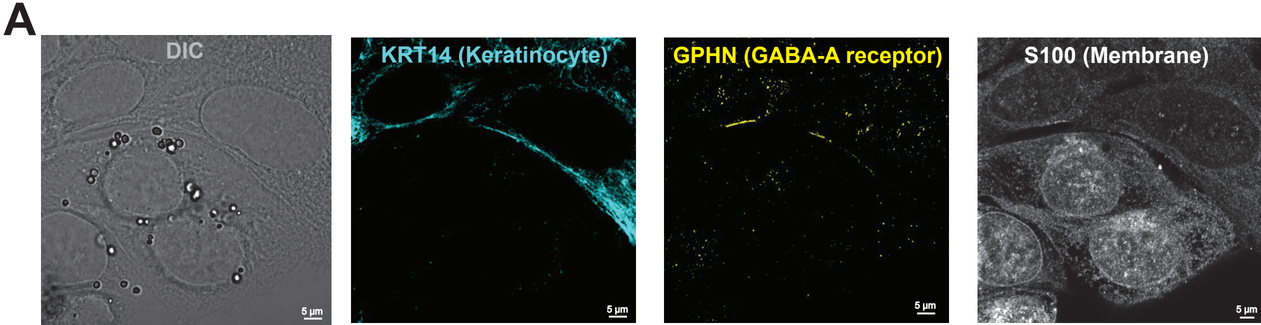
(F): % Switching efficiency calculated as number of switched cells per well normalized to control (regular media) upon growth in low chloride media of melanoma/keratinocyte co-cultures (to deplete intracellular chloride). Data is pooled from 4 biological replicates (n = 12). Error bars: SD, P values generated by unpaired t-test with **** = p < 0.0001.

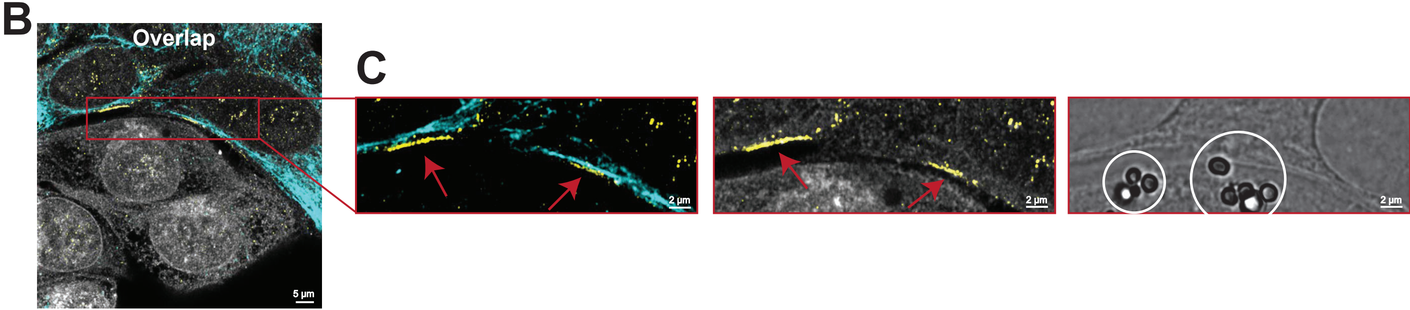
(G): Melanoma/keratinocyte co-culture proliferation calculated as % Fluorescence normalized to control (regular media) upon growth in low chloride media of melanoma/keratinocyte co-cultures (to deplete intracellular chloride). Data is pooled from 4 biological replicates (n = 12). Error bars: SD, P values generated by unpaired t-test.

(H): Relative expression of tetanus toxin light chain measured using qPCR in melanoma cells expressing control vector construct or Tetanus toxin light chain construct. Data represent 3 biological replicates per condition, P values calculated using unpaired t-test, *** p < 0.001.

(I): Number of pigmented melanocytes per embryo in +/- gabrg2 DN conditions. Data represent n = 40 control fish and n = 40 gabrg2 fish pooled from 3 biological replicates. Error bars: SD, P values generated by unpaired t-test, **** p < 0.0001.

Supplementary Fig.9: Melanoma vesicles are present near specialized GABAergic cell-cell junctions in co-cultures



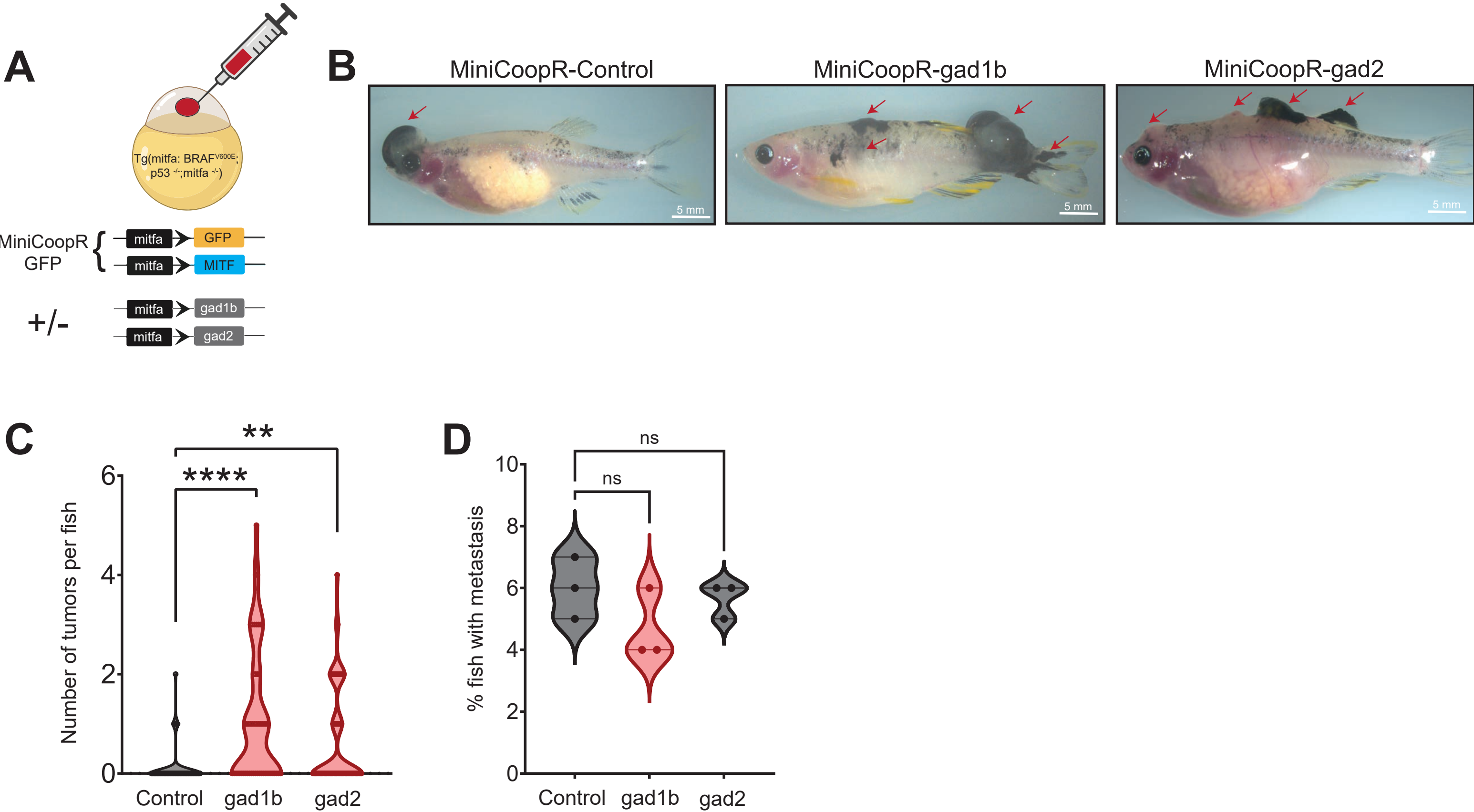


Supplementary Fig. 9: Melanoma vesicles are present near specialized GABAergic cell-cell junctions in co-cultures.

(A): Representative zoomed out image of immunostaining for KRT14 (keratinocyte marker), S100 (membrane marker) and gephyrin (GABA-A receptor marker) with DIC imaging in human melanoma/keratinocyte co-cultures (Ratio: 1:3, Keratinocyte:Melanoma). Individual cells are pseudocolored as indicated.

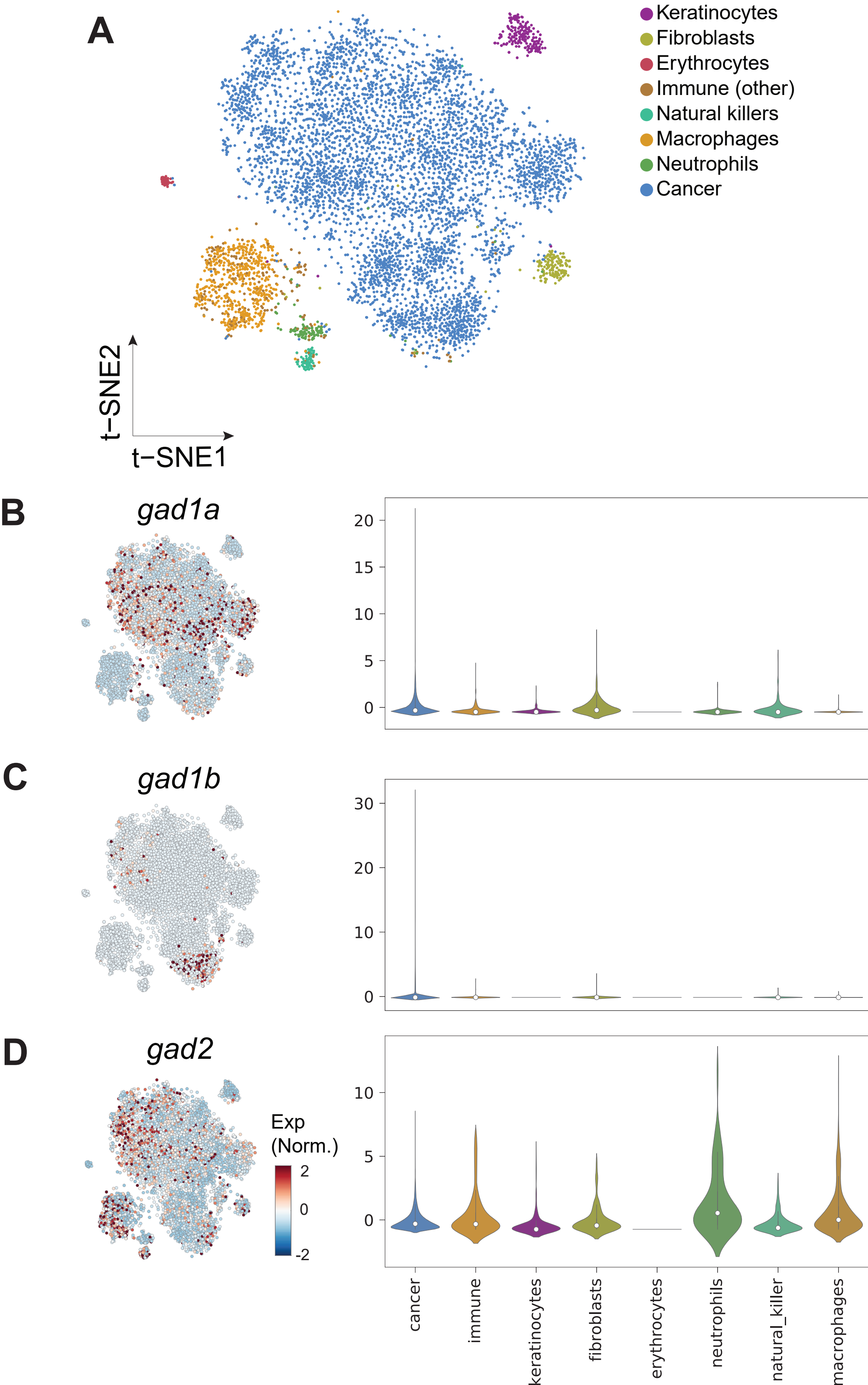
(B and C): Zoomed in images showing gephyrin positive clusters in keratinocytes (red arrows) and melanoma vesicles (white circles). Individual cells are pseudocolored as indicated above.

Supplementary Fig.10: *gad* activation correlates with higher tumor burden in melanoma



Supplementary Fig. 10: gad activation correlates with higher tumor burden in melanoma.
(A): Schematic representation of the MiniCoopR melanocyte rescue and melanoma development system in zebrafish with over-expression of *gad1b* or *gad2* gene in a melanocyte specific manner. (Created using BioRender.com)
(B): Representative images of 16-week-old zebrafish injected with MiniCoopR rescue plasmids showing control (GFP), gad1b and gad2 over-expressing tumors. gad over-expressing zebrafish have multiple tumors per fish (red arrow) when compared to control fish.
(C): Violin plots showing quantification of melanoma initiation frequency expressed as number of tumors per fish in 16-week-old zebrafish over-expressing GFP or gad1b or gad2 under a melanocyte specific (mitfa) promoter. Data represent n = 55 control (GFP) fish, n = 53 gad1b and n = 63 gad2 fish pooled from 3 biological replicates. P values generated by one-way ANOVA with multiple comparisons, ** $p < 0.01$, **** $p < 0.0001$.
(D): Violin plots showing % fish with kidney metastasis in 20-week-old zebrafish over-expressing GFP or gad1b or gad2 under a melanocyte specific (mitfa) promoter. Data represent n = 55 control (GFP) fish, n = 53 gad1b and n = 63 gad2 fish pooled from 3 biological replicates. P values generated by one-way ANOVA with multiple comparisons, ** $p < 0.01$, **** $p < 0.0001$.

Supplementary Fig.11: GABA producing enzymes are expressed in zebrafish melanoma cells

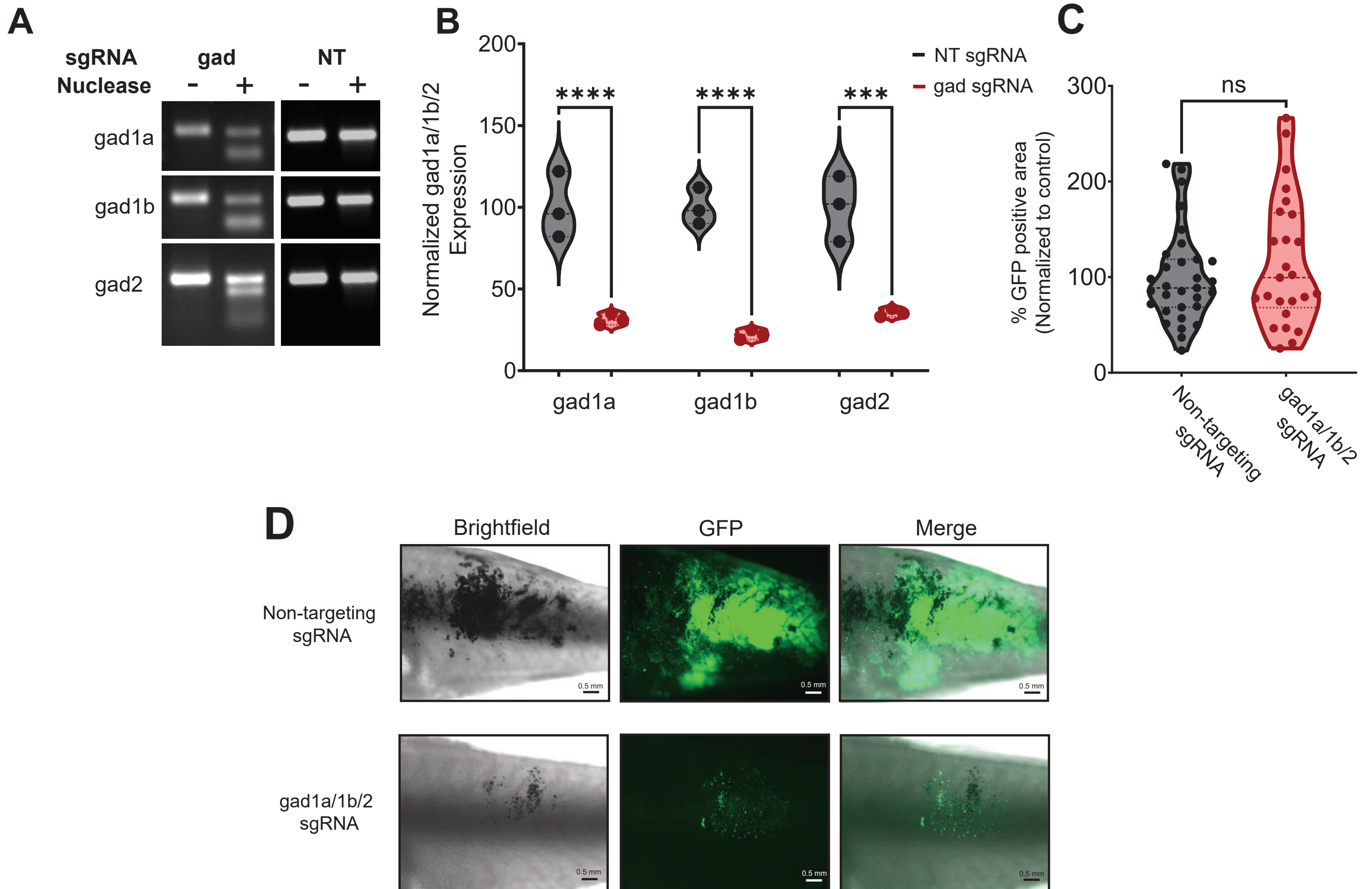


Supplementary Fig. 11: GABA producing enzymes are expressed in zebrafish melanoma cells.

(A): tSNE analysis of 7278 individual zebrafish melanoma cells with colors indicating individual tumor and microenvironmental cell types (data from *61*).

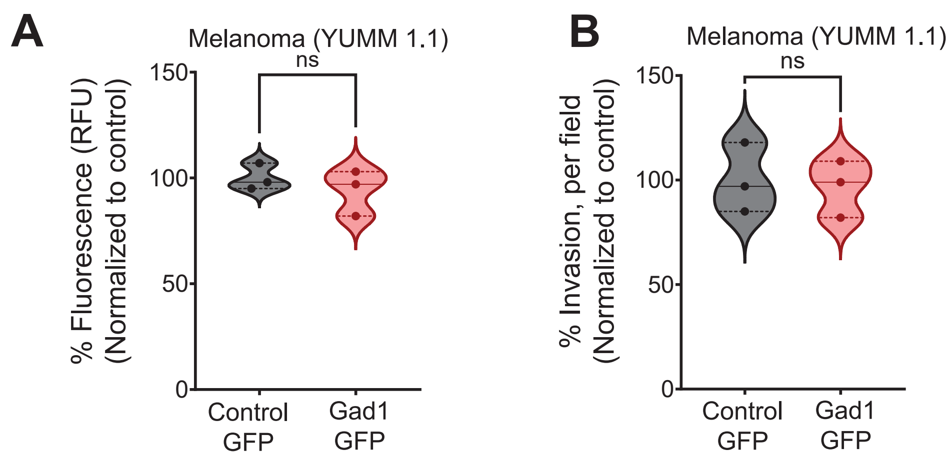
(B - D): Transcript levels for *gad1a* **(B)**, *gad1b* **(C)** and *gad2* **(D)** in single-cell RNA-seq analysis of zebrafish melanoma with individual cell clusters (left) and violin plots showing quantification of the transcripts in individual cell types (right). gad1a and gad1b are primarily expressed in tumor cells while gad2 is expressed in tumor and immune cell populations.

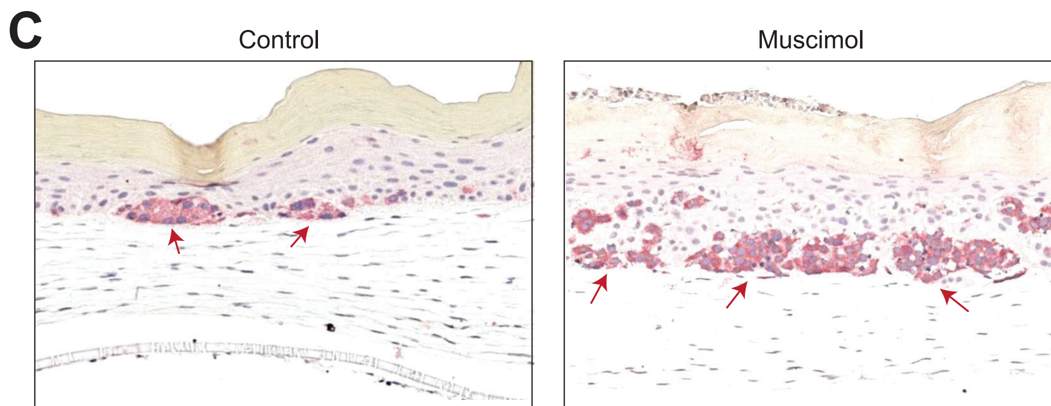
Supplementary Fig.12: Disruption of GABA synthesis blocks tumor initiation in melanoma

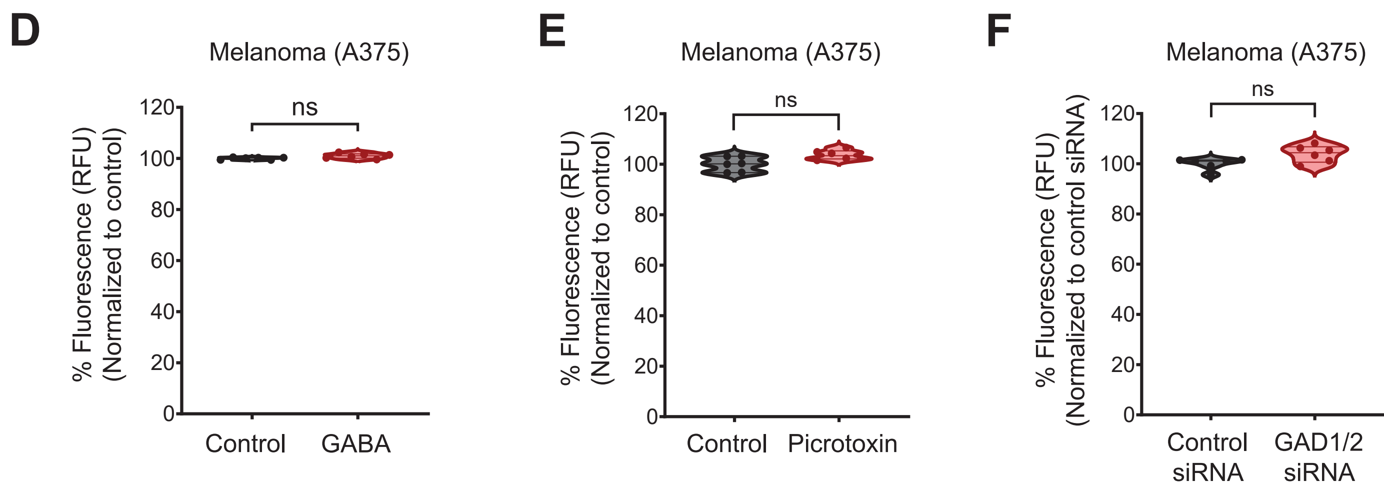


Supplementary Fig. 12: Disruption of GABA synthesis blocks tumor initiation in melanoma.
(A): Surveyor nuclease assay-based validation demonstrating targeted editing of *gad1a, gad1b* and *gad2* genes in zebrafish melanoma.
(B): Normalized expression of gad1a, gad1b and gad2 in non-targeting sgRNA and gad targeting sgRNAs expressing melanoma cells. Data represent three biological replicates. Error bars: SD, P values were generated by unpaired t-test with **** = $p < 0.0001$.
(C): Quantification of tumor area calculated as GFP positive area and normalized to the non-targeting control plasmid group. Data represent n = 53 non-targeting sgRNA fish and n = 46 gad1a/1b/2 sgRNA fish pooled from 3 biological replicates. Error bars: SD, P values generated by Mann Whitney test.
(D): Representative images of transgenic fish electroporated with melanocyte specific Cas9 and a non-targeting sgRNA or gad1a/1b/2 sgRNA, 10 weeks post electroporation.

Supplementary Fig.13: GAD expression/GABA treatment is pro-tumorigenic in a non-cell autonomous manner







Supplementary Fig. 13: GAD expression/GABA treatment is pro-tumorigenic in a non cell-autonomous manner.

(A): Melanoma cell proliferation calculated as % Fluorescence in YUMM 1.1 melanoma cells expressing control-GFP or Gad1-GFP as indicated. Data is pooled from 3 biological replicates ($n = 3$) and is normalized to control-GFP treatment condition. Error bars: SD, P values generated by unpaired t-test.

(B): Melanoma cell invasion calculated as % Invasion per field in YUMM 1.1 melanoma cells expressing control-GFP or Gad1-GFP as indicated. Data is pooled from 3 biological replicates ($n = 3$) and is normalized to control-GFP treatment condition. Error bars: SD, P values generated by unpaired t-test.

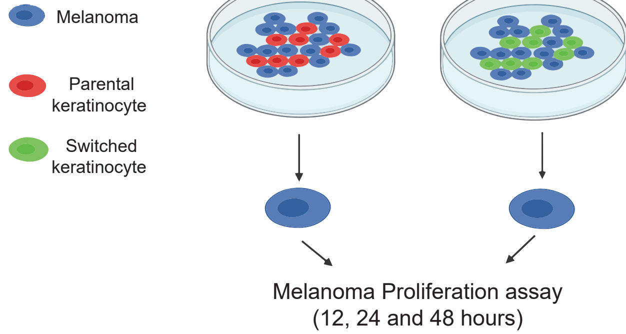
(C): Representative images of immunohistochemistry (IHC) performed on 3D melanoma skin reconstructs treated with control media or muscimol containing media, with melanoma cells stained with an anti-$BRAF^{V600E}$ antibody in Day 24 skin reconstructs, with keratinocytes and fibroblasts in 3D reconstruct media, melanoma positive areas are indicated with red arrows.

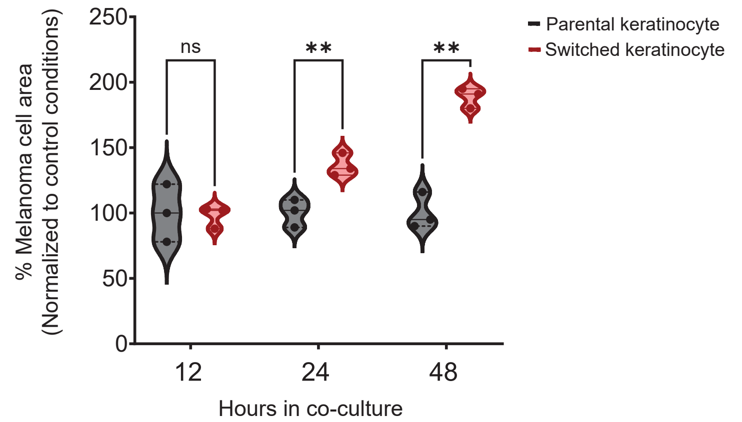
(D): Melanoma proliferation calculated as % Fluorescence in A375 melanoma cells treated with GABA (100 μM) as indicated. Data is pooled from 3 biological replicates ($n = 6$) and is normalized to control treatment condition. Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

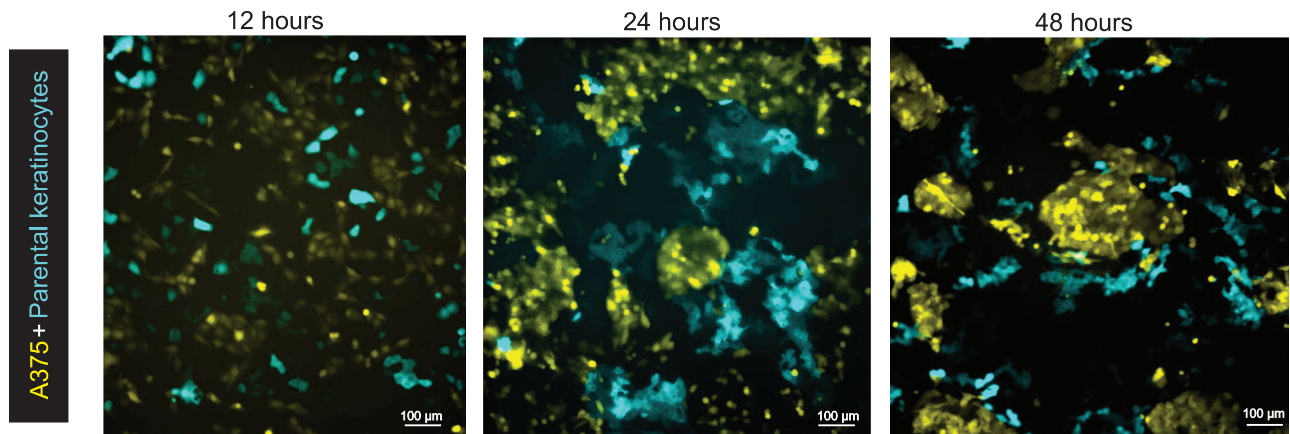
(E): Melanoma proliferation calculated as % Fluorescence in A375 melanoma cells treated with Picrotoxin (100 μM) as indicated. Data is pooled from 3 biological replicates ($n = 6$) and is normalized to control treatment (DMSO) condition. Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

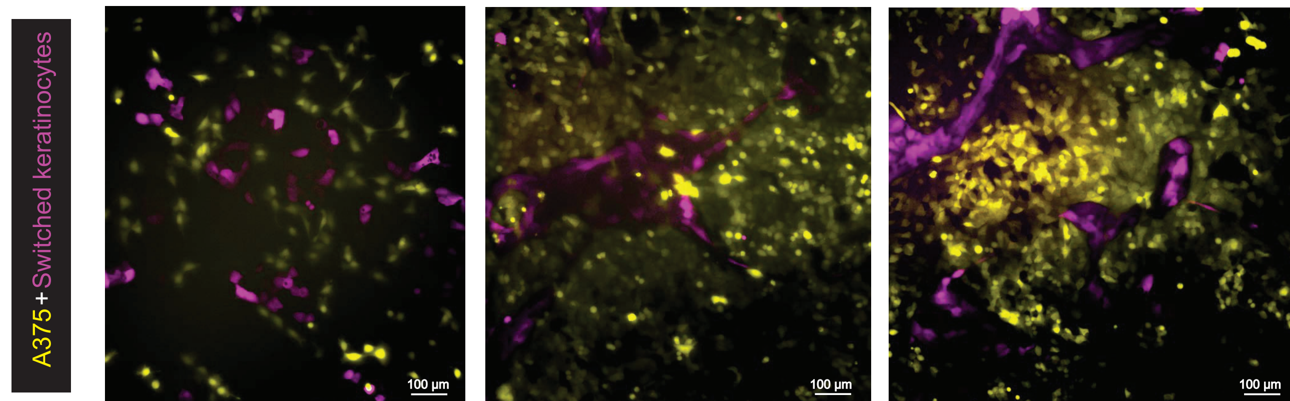
(F): Melanoma proliferation calculated as % Fluorescence in A375 melanoma cells treated with control siRNA or GAD1/2 siRNA as indicated. Data is pooled from 3 biological replicates ($n = 6$) and is normalized to control siRNA treatment condition. Error bars: SD, P values generated by unpaired t-test with **** = $p < 0.0001$.

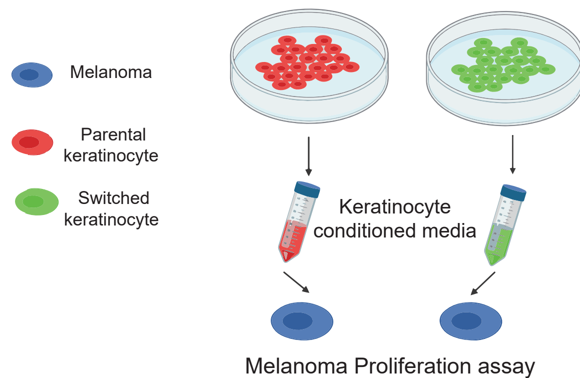
Supplementary Fig.14: Switched keratinocytes are pro-tumorigenic in melanoma

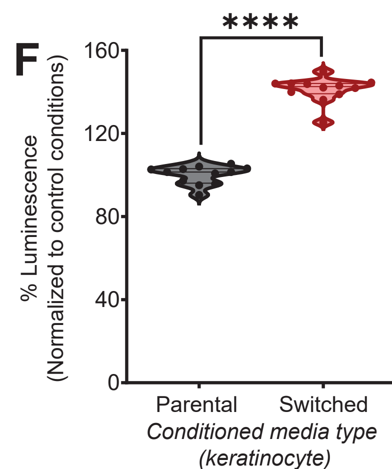












Supplementary Fig. 14: Switched keratinocytes are pro-tumorigenic in melanoma.

(A): Schematic representation of a melanoma proliferation assay in melanoma/keratinocyte co-cultures (Created using Biorender.com).

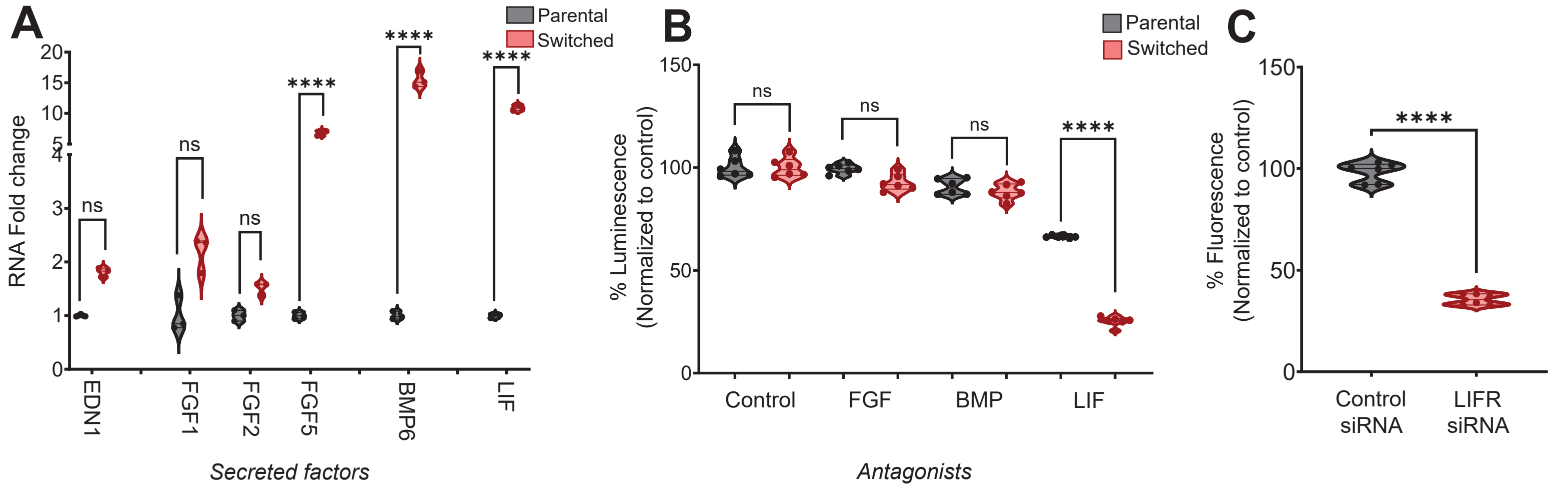
(B): Melanoma proliferation calculated as % azurite positive area when co-cultured with parental or switched keratinocytes for 12, 24 and 48 hours. Data is pooled from 3 biological replicates (n = 3) (Ratio: 1:5, Melanoma:Keratinocyte) and normalized to melanoma/parental keratinocyte condition. Error bars: SD, P values generated by unpaired t-test with **** = p < 0.0001.

(C - D): Representative images of melanoma/keratinocyte co-cultures with A375 melanoma cells (yellow) and HaCaT parental keratinocytes (turquoise) or HaCaT switched keratinocytes (magenta) at 12, 24 and 48 hours (Ratio: 1:5, Melanoma:Keratinocyte).

(E): Schematic representation of luminescence-based melanoma proliferation assay. Melanoma cells are treated with 24-hour conditioned media from parental or switched keratinocytes for 48 hours and proliferation is measured using the CellTiter-Glo assay (Created using BioRender.com).

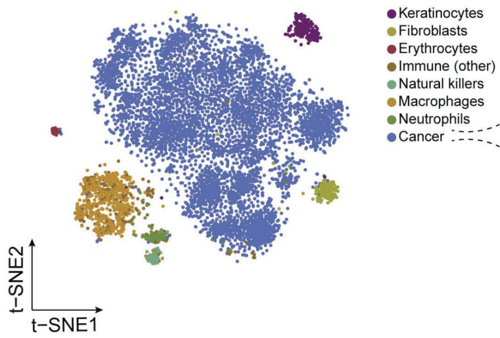
(F): Melanoma proliferation calculated as % Luminescence in melanoma cells treated with conditioned media as indicated. Data is pooled from 4 biological replicates (n = 12) and is normalized to control (parental keratinocyte) conditioned media. Error bars: SD, P values generated by unpaired t-test with **** = p < 0.0001.

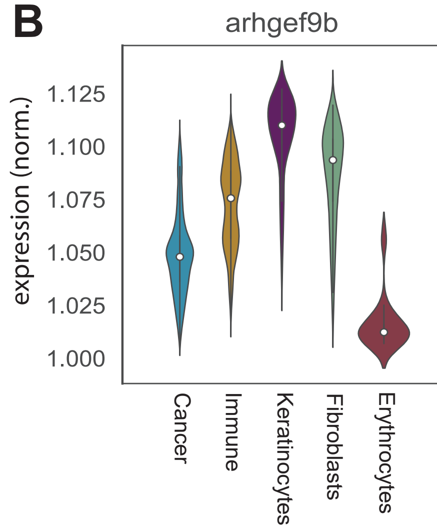
Supplementary Fig.15: *LIF* expression in keratinocytes drives melanoma cell proliferation

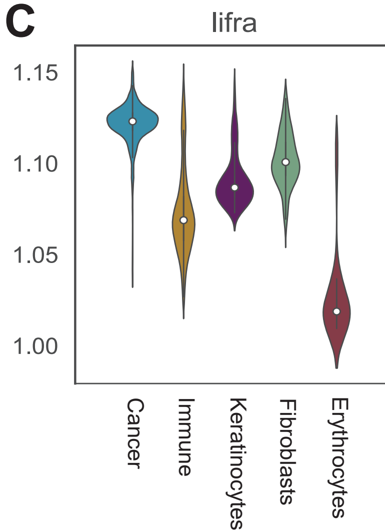


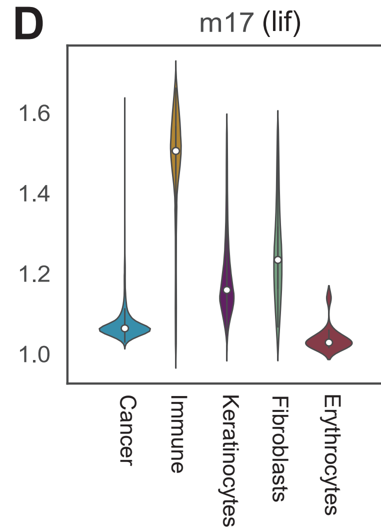
Supplementary Fig. 15: *LIF* expression in keratinocytes drives melanoma cell proliferation.
(A): Fold change differences in expression of secreted factors derived from RNA-seq analysis comparing switched vs parental keratinocytes. Data represent 3 biological replicates, Error bars: SD, P values calculated using DeSeq2.
(B): Melanoma proliferation calculated as % Luminescence in melanoma cells treated with parental or switched keratinocyte conditioned media for 48 hours +/- antagonists for the receptors of the indicated factors. Data represent 3 biological replicates (n = 12) and each condition (parental or switched) is normalized to its corresponding conditioned media control (no antagonist). Error bars: SD, P values generated by multiple two-tailed paired t-test with **** = $p < 0.0001$.
(C): Melanoma proliferation calculated as % fluorescence in melanoma cells treated with control or LIFR targeting siRNA pooled from 3 biological replicates (n = 6). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

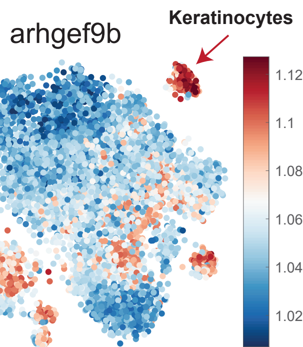
Supplementary Fig.16: Zebrafish melanoma scRNAseq identifies GABA and LIF associated components in the TME

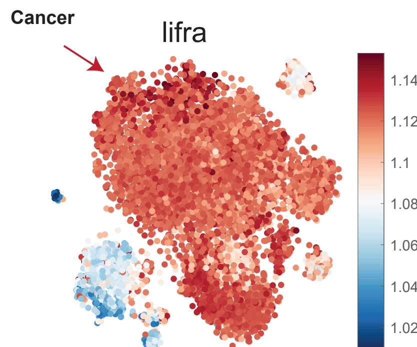


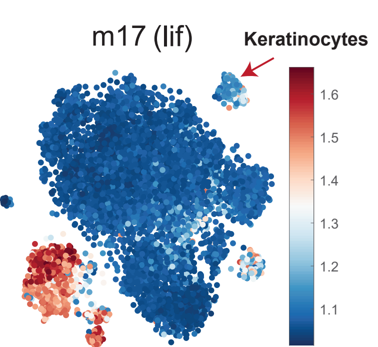










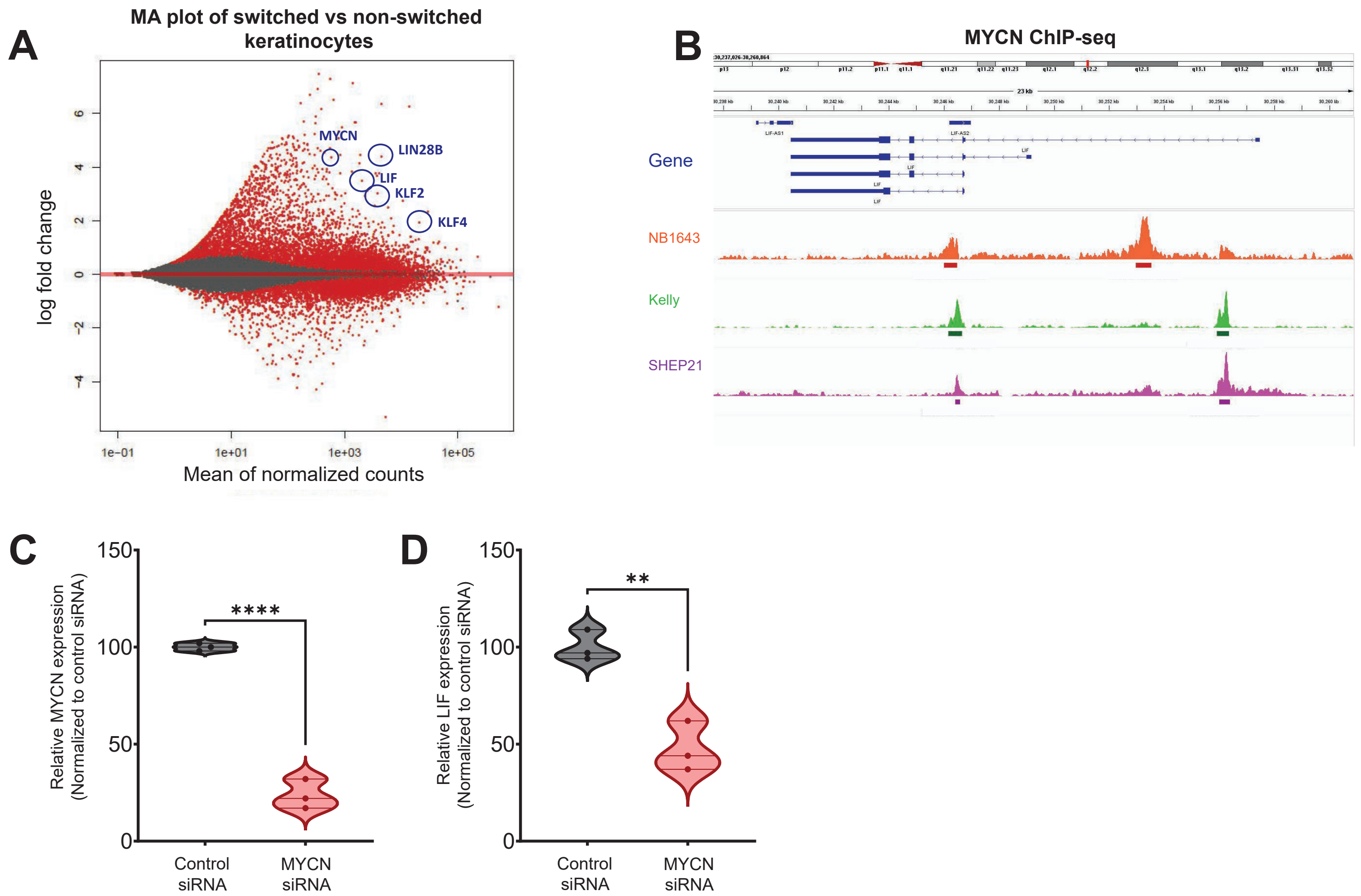


Supplementary Fig. 16: Zebrafish melanoma scRNAseq identifies GABA and LIF associated components in the TME.

(A): tSNE analysis of 7278 individual zebrafish melanoma cells with colors indicating individual tumor and microenvironmental cell types (data from *61*).

Transcript levels for arhgef9b **(B)**, lifra **(C)** and m17(lif) **(D)** in single-cell RNA-seq analysis of zebrafish melanoma with violin plots (top) showing quantification of the genes in individual cell types and individual cell clusters (bottom). Red arrows indicate the populations of interest for each gene.

Supplementary Fig.17: *MYCN* drives *LIF* expression in keratinocytes



Supplementary Fig. 17: *MYCN* drives *LIF* expression in keratinocytes.

(A): MA plot showing log fold change in transcript abundance in switched vs non-switched keratinocytes. Blue circles indicate genes of interest.

(B): IGV snapshot showing ChIP-seq tracks with MACS2 peaks showing MYCN binding at the *LIF* gene locus in neuroblastoma cell lines, NB1643, Kelly and SHEP21.

(C): Normalized MYCN expression in switched keratinocytes treated with control siRNA or MYCN targeting siRNA pooled from 3 biological replicates ($n = 3$). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.

(D): Normalized LIF expression in switched keratinocytes treated with control siRNA or MYCN targeting siRNA pooled from 3 biological replicates ($n = 3$). Error bars: SD, P values generated by two-tailed unpaired t-test with **** = $p < 0.0001$.