# Inhibition of DNA methylation promotes tumor sensitivity to netrin-1 interference.

Mélodie Grandin[1], Pauline Mathot[1], Guillaume Devailly[1], Yannick Bidet[2], Akram Ghantous[3], Clementine Favrot[1], Benjamin Gibert[1], Nicolas Gadot[4], Isabelle Puisieux[5], Zdenko Herceg[3], Jean-Guy Delcros[1], Agnès Bernet[1], Patrick Mehlen[1$], Robert Dante[1$]

**Appendix Figure S1 is related to Figure 3.**

**Appendix Figure S2 is related to Figure 3.**

**Appendix Figure S3 is related to Figures 3 and 4.**

**Appendix Figure S4 is related to Figures 3 and 4.**

**Appendix Figure S5 is related to Figure 5.**

**Appendix Figure S6 is related to Figure 5.**

**Appendix Table S1 is related to Experimental Procedures**

**Appendix Figure S1 - The netrin-1 blocking antibody net1-mAb triggered apoptotic cell death in breast cancer cell lines upon 5-azacytidine treatment.**

A, B Gene expression was measured by Q-RT-PCR after 72 hr in MDA-MB-231 (A) and HMLER (B) cells treated daily with 2 µM of 5-azacytidine (Aza). *PBGD* expression level was used as internal control. Error bars, s.e.m of at least 3 independent experiments, two-tailed unpaired Student's t test, ns = not significant.

C, F MDA-MB-231 and HMLER cell lines were treated with Aza, 2 µM for 72h, and/or net1-mAb (10 µg/mL, 48h) and/or recombinant netrin-1 (5 µg/mL, 48h). Caspase-3 activities (C and E) and cellular mortality (D and F) were determined in 3 independent experiments. **** P<0.0001. ANOVA1. Error bars = s.e.m

**Appendix Figure S2** – **Tumor growth inhibition induced by treatment combining DAC and therapeutic netrin-1 antibody, *in vivo*.**

A. When tumors reached 100 $mm^3$, mice were injected subcutaneously with decitabine (0.5mg/kg) or PBS and/or intraperitoneally with NET1-mAb-T (10 mg/kg) or a Control IgG (Iso-mAb (10 mg/kg). Tumor volumes were measured twice a week. Statistical significance of differences between PBS-groups and DAC + NET1-mAb-T-groups was determined by two-way ANOVA and post-hoc Tukey-test. (n = 6 mice per group). **** P<0.0001.

B. Activated caspase-3, median number of cells per $mm^2$ were measured using antibody against cleaved caspase-3 and the DNA fragmentation median number of cells per $mm^2$ were determined by TUNEL assay (B); mean values from 4 tumors per group. **** P<0.0001, ANOVA1

C. Representative tumor sections corresponding to the histograms (B), bars represent 50 µm.

**Appendix Figure S3 – Angiogenesis and cell proliferation in patient-derived xenografts treated net1-mAb and/ or decitabine (DAC).**

A, D Paraffin embedded tumor xenografts were stained using CD31 for angiogenesis and Ki-67 for cell proliferation in MDA-MB-231 (A, B) and PDX HBC 146 (C, D).

(A and C) Vessel numbers in tumors were measured by CD31 immunohistochemistry staining and expressed as median number of vessels per $mm^2$

(A and C) Mean proliferation rate assessed by Ki- 67 staining; proliferative cells were divided by non-proliferative cells. Error bars = s.e.m. ANOVA1; *** P<0.001, n = not significant, ANOVA1

(B and D) Representative sections of CD31 and KI67 staining for angiogenesis and proliferation count.

**Appendix Figure S4 - Combined treatment led to a reduced number of metastasis formation, and was not associated with changes in angiogenesis or proliferation.**

A, B. MDA-MB-231 cells previously treated with control IgG1, DAC and/or net1-mAb alone or in combination were grafted on the chorio-allantoic membrane (CAM) of 10 days old chicken embryos. Error bars = s.e.m, Kruskal–Wallis test.

**Appendix Figure S5 - Genes upregulated by decitabine treatment in MDA-MB231 and HMLER cell lines.**

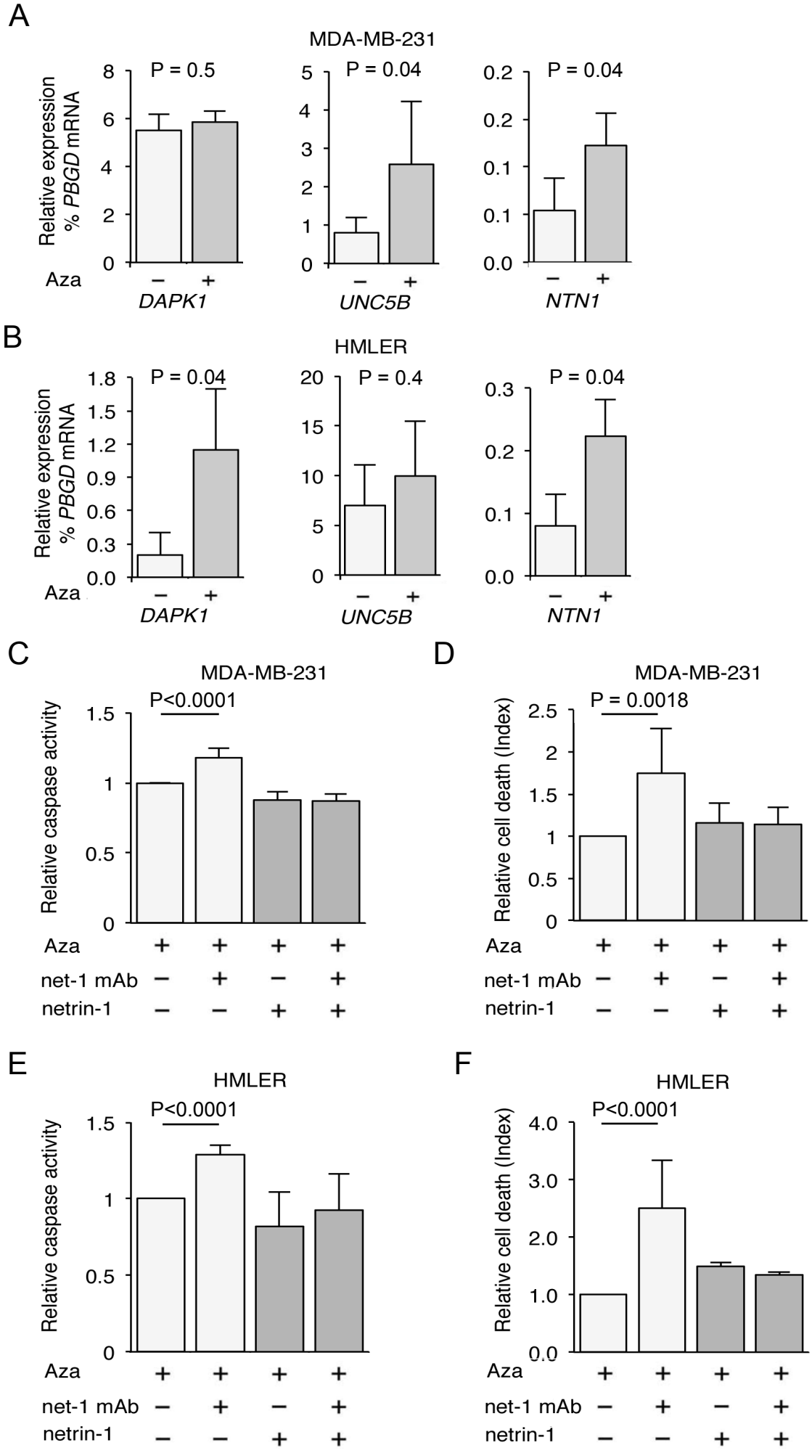
A. Venn diagram showing the overlap of genes upregulated by decitabine treatment in MDA-MB-231 and HMLER cell lines.

B. Gene ontology and KEGG pathways identified from of common DAC-upregulated genes between MDA-MB-231 and HMLER cell lines.

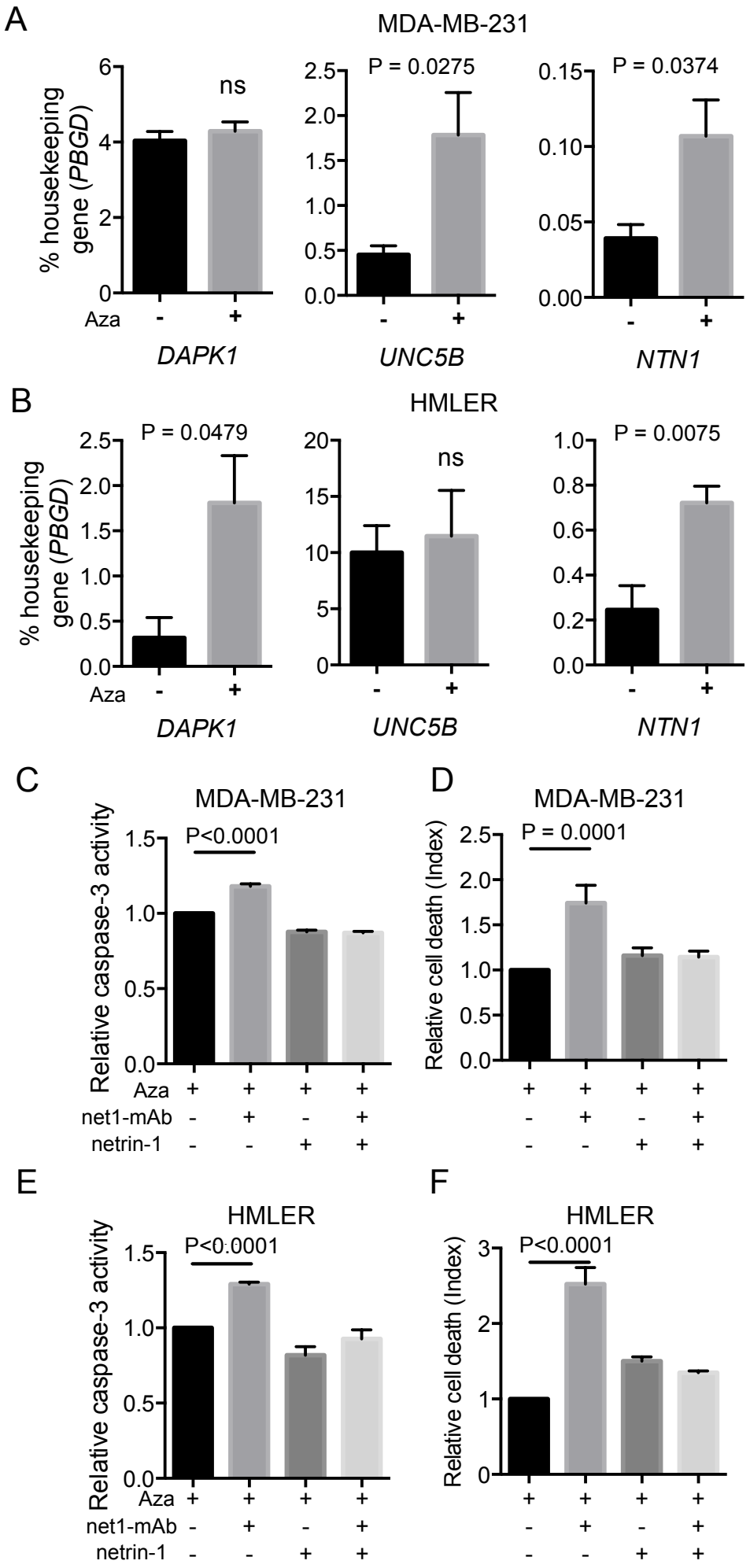
**Appendix Figure S6 – Expression of genes involved in the YAP signaling pathway and effect of *IRF7* depletion on *DAPK1*, *UNC5B*, and *NTN1* expression.**

A, B Q-RT-PCR assays of the expression of genes linked to the netrin-1 dependence receptor pathway, *DAPK1*, *UNC5B*, and *NTN1*, and genes linked to the YAP signaling pathway, *YAP*, *TAZ*, and *CTGF* in MDA-MB-231 (A) and HMLER (B) cells. Error bars = s.e.m. *** P<0.001, ns = not significant. ANOVA1

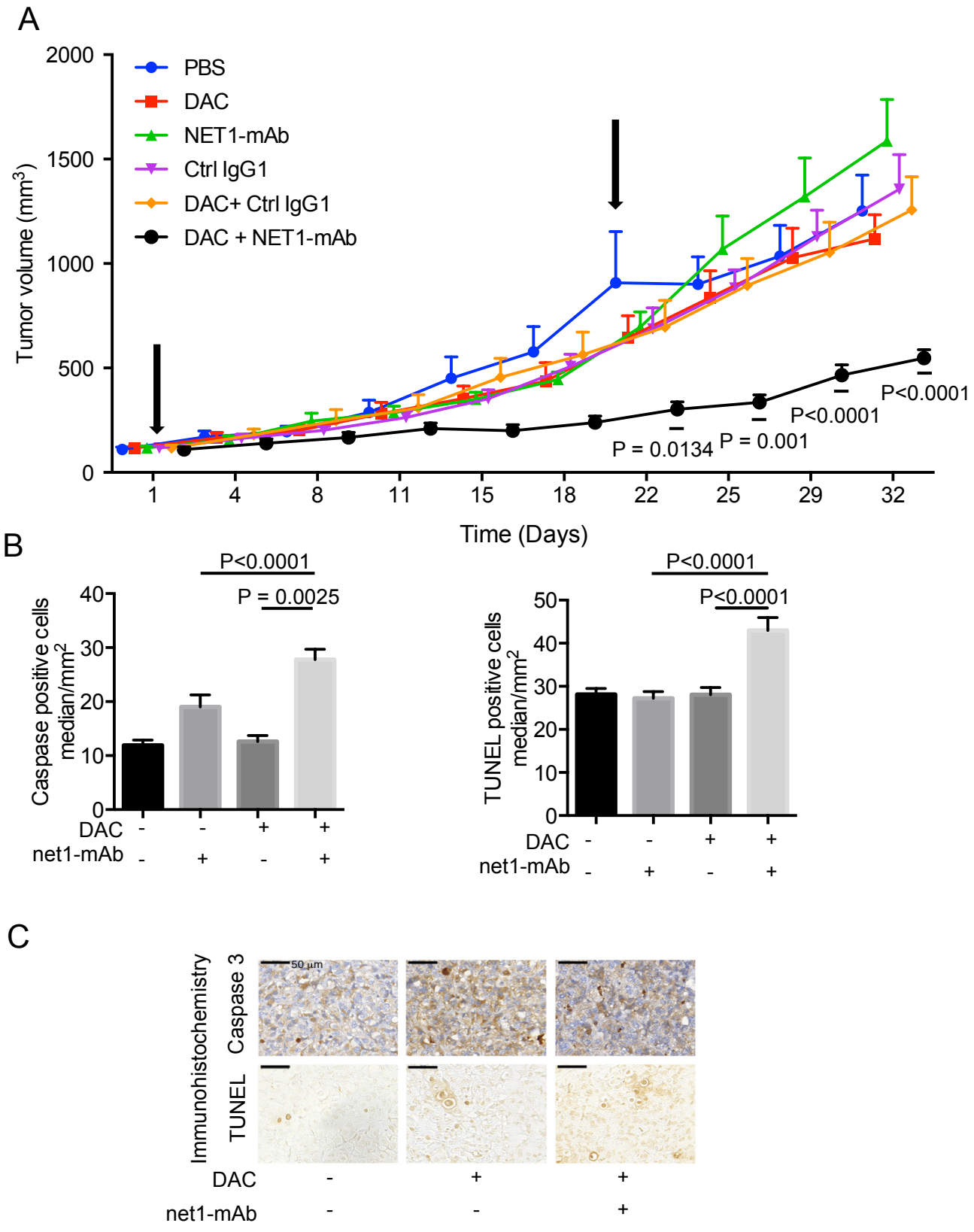
C, D Q-RT-PCR assays of the expression of *DAPK1*, *UNC5B*, *NTN1*, and *IRF7* upon treatment combining DAC treatment and transient transfection with a siRNA targeting *iRF7* in MDA-MB-231 (C) and HMLER (D) cells. **** P<0.0001, ns = not significant. ANOVA1.



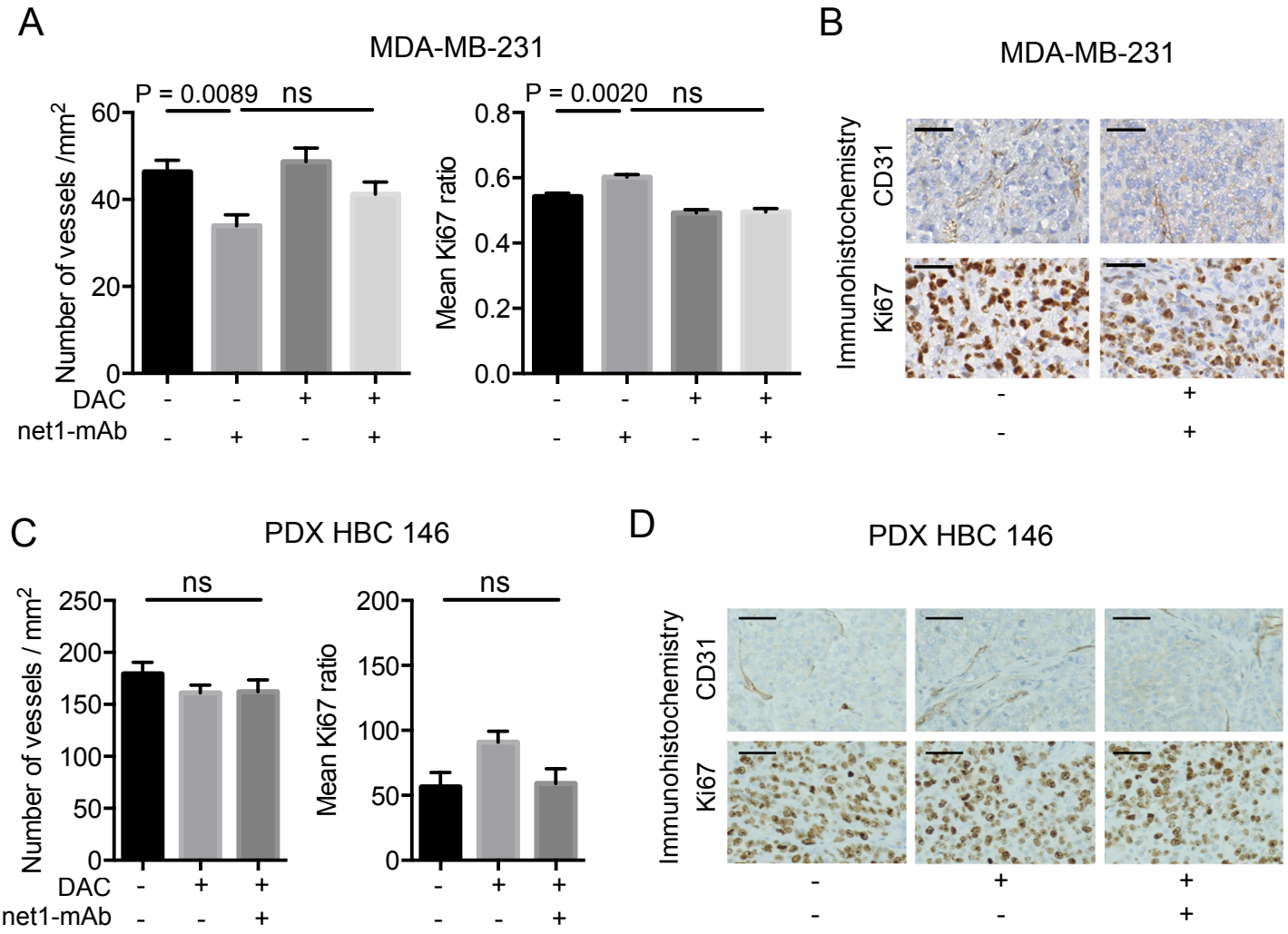

Appendix Figure S1. Grandin et al.




Appendix Figure S1. Grandin et al.

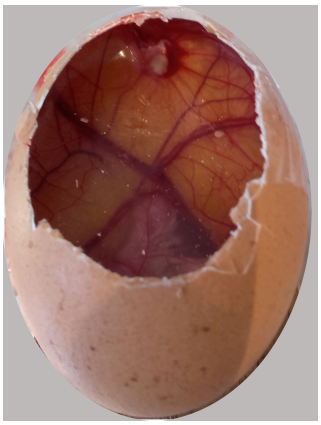



Appendix Figure S2. Grandin et al.

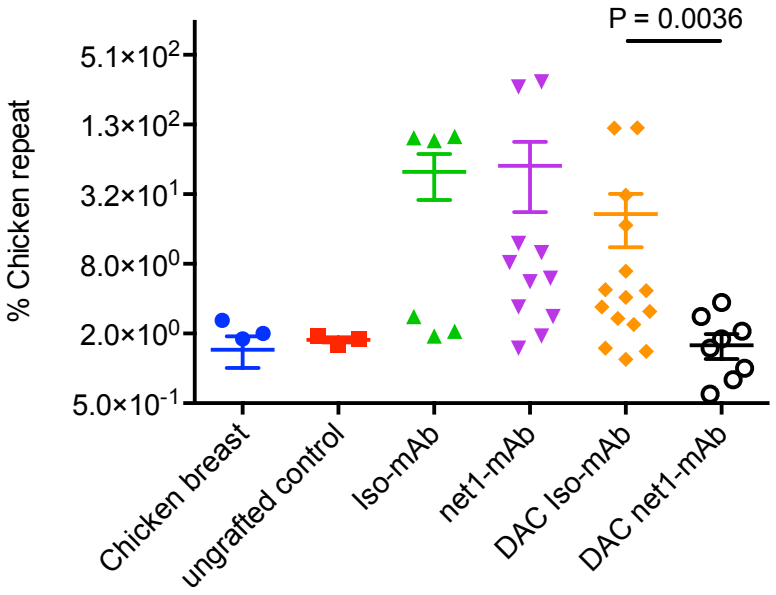



Appendix Figure S3. Grandin et al.

A

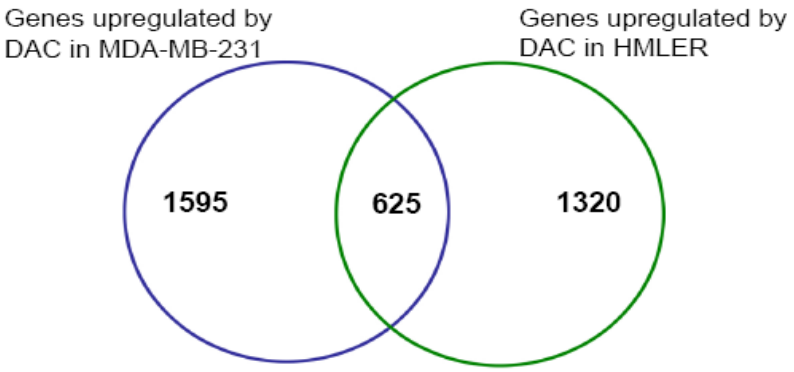


B




Appendix Figure S4. Grandin et al.

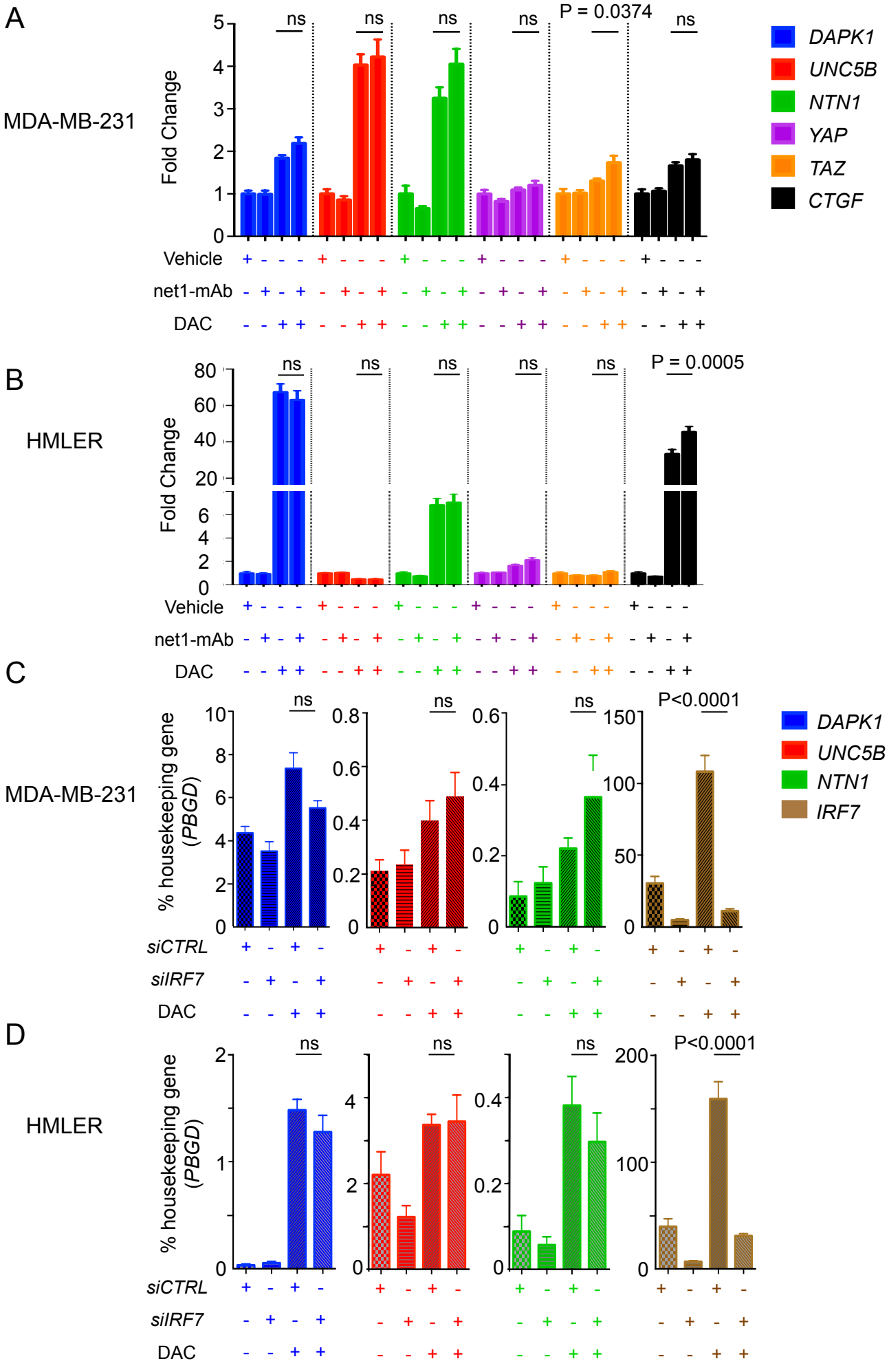
A




B

| Set | Rank | Name | p value | ajusted p value |
|---|---|---|---|---|
| KEGG pathways | 1 | Osteoclast differentiation | 2.40E-07 | 1.42E-05 |
| | 2 | MAPK signaling pathway | 7.50E-06 | 8.85E-05 |
| | 3 | Rheumatoid arthritis | 5.86E-06 | 8.85E-05 |
| | 4 | Cytokine-cytokine receptor interaction | 6.85E-06 | 8.85E-05 |
| | 5 | Pathways in cancer | 5.18E-06 | 8.85E-05 |
| | 6 | NOD-like receptor signaling pathway | 9.69E-06 | 9.53E-05 |
| | 7 | African trypanosomiasis | 2.54E-05 | 2.00E-04 |
| | 8 | Leishmaniasis | 2.80E-05 | 2.00E-04 |
| | 9 | Type I diabetes mellitus | 5.81E-05 | 4.00E-04 |
| | 10 | Malaria | 1.00E-04 | 6.00E-04 |
| Gene Ontology | 1 | Response to molecule of bacterial origin | 1.06E-06 | 0.0014 |
| | 2 | Response to lipid | 1.84E-05 | 0.0046 |
| | 3 | Response to bacterium | 2.76E-05 | 0.0046 |
| | 4 | Regulation of cell proliferation | 2.43E-05 | 0.0046 |
| | 5 | Immune response | 3.27E-05 | 0.0048 |
| | 6 | Cell proliferation | 4.70E-05 | 0.0053 |
| | 7 | Cytokine activity | 0.0003 | 0.0286 |

Appendix Figure S5. Grandin et al.




Appendix Figure S6. Grandin et al.

## Appendix Table S1 - L ist of primers

| **Q-RT-PCR Primers** | Forward (5' to 3') | Reverse (5' to 3') |
|---|---|---|
| *PBGD* | GAGTGATTCGCGTGGGTACC | GGCTCCGATGGTGAAGCC |
| *GAPDH* | CGGAGTCAACGGATTTGGTCGTAT | AGCCTTCTCCATGGTGGTGAAGAC |
| *DAPK1* | GATAGAAATGTCCCCAAACCTCG | TCTTCTTGGATCCTTGACCAGAA |
| *UNC5B* | CCCGCCACACAGATCTACTT | CAGTAATCCTCCAGCCCAAA |
| *NTN1* | TGCAAGAAGGACTATGCCGTC | GCTCGTGCCCTGCTTATACAC |
| *MBD2* | CCCACAACGAATGAATGAACAGC | TGAAGACCTTTGGGTAGTTCCA |
| *UNC5A* | ATCACCAAGGACACAAGGTTTGC | GGCTGGAAATTATCTTCTGCCGAA |
| *UNC5C* | GCAAATTGCTGGCTAAATATCAGGAA | GCTCCACTGTGTTCAGGCTAAATCTT |
| *DCC* | AGCCAATGGGAAAATTACTGCTTAC | AGGTTGAGATCCATGATTTGATGAG |
| *YAP* | CGCTCTTCAACGCCGTCA | AGTACTGGCCTGTCGGGAGT |
| *TAZ* | CCAGTGCCTCAGAGGTCCA | ATCTGCTGCTGGTGTTGGTG |
| *CTGF* | CCAATGACAACGCCTCCTG | TGGTGCAGCCAGAAAGCTC |
| *IRF7* | ACTGTGACACCCCCATCTTC | GCTGCTATCCAGGGAAGACACA |
| **Pyrosequencing** | | |
| Amplification primers | | |
| *NTN1* | BIO-TTATGGTTATTTATAAGTTTATGGA | TAACCCAATCCTACAACAC |
| *DAPK1* | BIO-TTTTTTGGATTGTGGAAATGTATAA | AACCCTAAACTACTACCTCTCCTCC |
| *GAPDH unmodified* | CTCTTGCTACTCTGCTCTGG | GCTAAGTTTAGCCTGCCTGG |
| *GAPDH* modified | GTATTTGTTGATGGGTTAAGG | ATAAAAACAAATCCCCTACCC |
| Sequencing primers | | |
| NTN1-a | TAACCCAATCCTACAACAC | |
| NTN1-b | CAATCCTACAACAC | |
| DAPK1-a | AACCCTTAAATCAA | |
| DAPK1-b | TTCTCTATAATTTA | |
| **DNA methylation analysis** | | |
| *NTN1* | ACACTGACGACATGGTTCTACATGAGTTGTGGTGAGTTTTTATTTT<br>TACGGTAGCAGAGACTTGGTCTAACAACATCCTTTAATCATCTTACAAC | |
| *DAPK1* | ACACTGACGACATGGTTCTACAGAGGTTTTTAGTGGATATGGGATT<br>TACGGTAGCAGAGACTTGGTCTTCCACCTCCAAAATTCAAATAATT | |
| **Human DNA analysis in Chicken lungs** | | |
| Alu | CACCTGTAATCCCAGCACTTT | CCCAGGCTGGAGTGCAGT |
| Avian Repeat | ATAGAATGGCCTGGGTTGAAAAG | AAGTTTTTCACACAGAGGGTGGT |
| **siRNA** | | |
| IRF7 | CAGCCUCUAUGACGACAUC | |