**Welz L. et al.: Epithelial XBP1 coordinates TP53-driven DNA damage responses and suppression of intestinal carcinogenesis**

**Supplementary Figure - Index**



**Supplementary Tables – Index**



**Figure S1: Validation of conditional knockout**

Validation of successful *Rnaseh2b* and *Xbp1* knockout in small intestinal *H2b/Xbp1*$^{fl/fl}$, *Xbp1*$^{\Delta IEC}$, *H2b*$^{\Delta IEC}$ and *H2b/Xbp1*$^{\Delta IEC}$ organoids, representative of a minimum of 3 individual experiments. (A) qPCR analysis, with n = 3 technical replicates. Relative mRNA expression is normalized to *Actb*. Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. ** p < .01; *** p < .0001; **** p < .00001. (B) Knockout of RNASEH2B was additionally validated on protein level. ß-Actin served as a loading control.

**Figure S2: *H2b/Xbp1*$^{\Delta IEC}$ tumor organoids exhibit autonomous Wnt pathway activation**

(A) qRT-PCR analysis of either non-tumor (NT) or tumor (T) *H2b/Xbp1*$^{\Delta IEC}$ organoids, unstimulated or treated with 2 µM of the Wnt inhibitor IWP-2 for 72 hours, representative of a minimum of 3 individual experiments with n = 3 technical replicates. Expression levels are displayed as relative mRNA levels. Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. **** p < .00001.

**Figure S3: Comparison of clinical phenotype of single- vs. co-housed aged *H2b/Xbp1*$^{\Delta IEC}$ mice**

(A) Basal phenotyping analysis of 52 weeks old single- (n = 7, 6 females, 1 male) vs. co-housed (n = 4, 3 females, 1 male) *H2b/Xbp1*$^{\Delta IEC}$ mice, consisting of body weight, SI length, colon length, liver weight in correlation to body weight, spleen weight in correlation to body weight, amount and size of SI tumors per animal. Each dot represents one individuum. In co-housing conditions *H2b/Xbp1*$^{\Delta IEC}$ and *H2b/Xbp1*$^{fl/fl}$ mice were kept in a 1:1 ratio. Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test.

**Figure S4: Clinical phenotype in aged *H2b/Xbp1*$^{\Delta IEC}$ mice**

(A) Basal phenotyping analysis of 52 weeks old WT (n = 10, 10 males), *Xbp1*$^{\Delta IEC}$ (n = 7, 1 female, 6 males), *H2b*$^{\Delta IEC}$ (n = 10, 4 females, 6 males), non-tumor (NT) and tumor (T)

*H2b/Xbp1*$^{\Delta IEC}$ (n = 21, 12 females, 9 males) mice; (B) Basal phenotyping analysis of 8 – 12 weeks old WT (n = 10, 10 males), *Xbp1*$^{\Delta IEC}$ (n = 7, 1 female, 6 males), *H2b*$^{\Delta IEC}$ (n = 10, 4 females, 6 males) and *H2b/Xbp1*$^{\Delta IEC}$ (n = 21, 12 females, 9 males) mice. Analysis parameters include body weight, SI length and colon length. Each dot represents one individuum. Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. * $p < .05$; ** $p < .01$; *** $p < .0001$.

**Figure S5: *Xbp1* deficiency limits intestinal epithelial regeneration in 8 - 12 weeks old mice**

Representative images of the SI of 8 - 12 weeks old *H2b/Xbp1*$^{fl/fl}$ (n = 3, 1 female, 2 males), *Xbp1*$^{\Delta IEC}$ (n = 2, 1 female, 1 male), *H2b*$^{\Delta IEC}$ (n = 3, 2 females, 1 male) and *H2b/Xbp1*$^{\Delta IEC}$ (n = 3, 1 female, 2 males) mice, stained for (A) γH2Ax, with white arrowheads indicating γH2Ax$^{+}$-nuclei, (C) TUNEL, (E) Ki67 and (G) Lysozyme. Each dot in the accompanying statistical analyses represents one individuum, for which the mean number of (B) γH2Ax$^{+}$, (D), TUNEL$^{+}$, (F) Ki67$^{+}$ and (H) Lysozyme$^{+}$ cells was assessed in a total of 50 crypts. Data are expressed as mean ± standard error of the mean and significance was determined using one-way ANOVA. * $p < .05$, *** $p < .001$.

**Figure S6: *Xbp1* deficiency limits intestinal epithelial regeneration in the context of DNA damage *in-vitro*.**

(A) Western Blot of γH2Ax and PARP of ModeK cells, treated with 2.5 µM AraC for 2, 4, 8 and 24 hours, representative of a minimum of 3 individual experiments with n = 3 technical replicates. GAPDH served as a loading control. (B) MTS assay of ModeK cells, representative of a minimum of 3 individual experiments with n = 5 technical replicates, treated with PBS or 2.5 µM AraC for 24 hours. After medium exchange, cells recovered for 48 hours. (C) Representative images of ModeK cells stimulated with PBS or 2.5 µM AraC for 24 hours. (D) FACS Annexin assay of ModeK cells, representative of a minimum of 3 individual experiments with n = 3 technical replicates, treated with 2.5 µM AraC for 0, 2, 4, 8, 24 and 48 hours, with (E) corresponding dot plots at timepoints 8 and 48 hours. (F) FACS Annexin assay of *Xbp1*$^{fl/fl}$ or *Xbp1*$^{\Delta IEC}$ intestinal organoids, representative of a minimum of 3 individual experiments with n = 3 technical replicates, stimulated with PBS or 2.5 µM AraC for 24 hours, with corresponding dot plots (G). Colony forming assay of untreated intestinal organoids of the indicated genotypes, demonstrating the amount (H) or diameter (I) of regrown organoids on day 10 after reseeding, with representative images provided in (J). Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. * $p < .05$, ** $p < .01$, *** $p < .0001$. Ann.: Annexin.

**Figure S7: Deletion of *Xbp1* drives mucosal inflammation upon DSS-induced colitis**

Representative H&E images of small intestines of mice with the respective genotypes being opposed to (A) acute and (C) chronic DSS-induced colitis, with (B, D) corresponding histopathological scoring of the SI; each dot represents the histopathological score of one individual mouse. For histopathological evaluation of chronic DSS colitis, two independent chronic DSS-induced colitis models were individually analyzed before data were pooled for final assessment. Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. * $p < .05$, ** $p < .01$, **** $p < .00001$.

**Figure S8: *H2b/Xbp1*$^{\Delta IEC}$ mice display a distinct transcriptional signature**

(A) Top DEG between *H2b*$^{\Delta IEC}$ vs. *H2b/Xbp1*$^{\Delta IEC}$ compared to *H2b/Xbp1*$^{fl/fl}$ organoids. (B) Top 10 GO BP in genes downregulated and (C) upregulated in *H2b*$^{\Delta IEC}$ vs. *H2b/Xbp1*$^{\Delta IEC}$ organoids but not in *H2b/Xbp1*$^{fl/fl}$ vs. *H2b/Xbp1*$^{\Delta IEC}$.

**Figure S9: DDIT4L regulates endogenous 4E-BP1 phosphorylation in intestinal epithelial cells**
(A) iCtrl ModeK cells were transfected with siRNA against Ddit4l and p53 for 24h and protein lysates were probed against (p)4E-BP1. ß-Actin was used as loading control. Representative image of a minimum of n = 3 individual experiments. (B) iCtrl ModeK cells were transfected with siRNA against Ddit4l and stimulated with 2.5 µM AraC for 24 or 48 hours. Protein lysates were probed against (p)4E-BP1. ß-Actin was used as loading control. Representative image of minimum of n = 3 individual experiments.

**Figure S10: Deficiency of *Xbp1* restores proliferation in *H2b*$^{\Delta IEC}$ organoids**
(A) CellTiter-Glo assay of untreated intestinal *H2b/Xbp1*$^{fl/fl}$, *Xbp1*$^{\Delta IEC}$, *H2b*$^{\Delta IEC}$ and *H2b/Xbp1*$^{\Delta IEC}$ organoids after a growing period of 72 hours. Representative data of n = 3 individual experiments with n = 3 technical replicates. *H2b/Xbp1*$^{fl/fl/\Delta IEC}$ organoids are abbreviated as "*H/X*$^{fl/fl/\Delta IEC}$". Data are expressed as mean ± standard error of the mean and significance was determined using an unpaired Student's t-test. * $p < .05$, ** $p < .01$, *** $p < .0001$.

**Supplemental Materials and Methods:**

**cDNA Synthesis and Gene Expression Analysis**
Using the RNeasy Kit (Qiagen), mRNA was isolated from ModeK cells washed in PBS, PBS-washed Matrigel containing organoids or from snap-frozen tissue. cDNA was synthesized using Revert Aid Premium cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. To examine gene expression, cDNA samples were subjected to quantitative reverse transcription polymerase chain reaction using either SYBR Green or TaqMan assays purchased from Applied Biosystems., Reactions were carried out on the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) and relative transcription levels were determined utilizing either *Actb* or *Gapdh* as housekeepers. Deploying PrimerBLAST-Software (NIH), primer sequences were designed, while TaqMan probes were derived from Thermo Fisher Scientific (for primer sequences and TaqMan probe IDs see Supplementary Table 1).

**Supplementary Table 1: Primers used for gene expression analysis**

| Gene name | Forward sequence (5' à 3') SYBR GREEN | Backward sequence (5' à 3') SYBR GREEN |
|---|---|---|
| ***Actb*** | GATCGGTGGCTCCATCCTGGC | CGCAGCTCAGTAACAGTCCGCC |
| ***Gapdh*** | CCGGGGCTGGCATTGCTCTCA | CTTGCTCAGTGTCCTTGCTGGGG |
| ***p53*** | CTAGCATTCAGGCCCTCATC | TCCGACTGTGACTCCTCCAT |
| ***Xbp1*** | TGGCCGGGTCTGCTGAGT | ACAGGGTCCAACTTGTCCAG |

| Gene name | Species | Taqman probe ID |
|---|---|---|
| ***Actb*** | murine | 00607939 |
| ***Ccnd1*** | murine | 00432359 |
| ***Ccng1*** | murine | 00438084 |
| ***Cd44*** | murine | 01277161 |
| ***Ddit4l*** | murine | 00513313 |
| ***Mdm2*** | murine | 01233138 |
| ***Rnaseh2b*** | murine | 01317628 |

| | | |
|---|---|---|
| ***Sesn2*** | murine | 00460679 |
| ***Sox9*** | murine | 00448840 |

**Transmission Electron Microscopy**

For electron microscopy, tissue was fixed at 4 °C with 3 % glutaraldehyde, washed with PBS, exposed to 2 % osmium tetroxide for 30 minutes, dehydrated in series with increasing ethanol concentrations and embedded in Araldite. Ultrathin (60 nm) sections were cut, mounted on carbon-coated copper grids (Science Service GmbH) and contrasted with saturated solution of uranyl acetate (Merck KGaA) in H2O. The grids were examined with a JEOL 1400 plus transmission electron microscope at 120 kV operating voltage.

**Immunohistochemistry and Immunofluorescence**

For immunohistochemical or immunofluorescence staining, 5 µm sections of paraffin-embedded ileum swiss rolls were deparaffinized with Xylol substitute (Roth), incubated in citrate buffer for 3 minutes and subsequently blocked with blocking serum (Vectastain for BrdU) for 20 minutes. For anti-BrdU staining, mice were pulsed with 10 mg / kg bodyweight BrdU 1.5 h before sacrifice. Primary antibodies used were mouse anti-BrdU (1 : 10 dilution; BD Biosciences), rabbit anti-p-4E-BP1 (1 : 300 dilution in BSA; Cell Signalling), goat anti-γH2Ax (1 : 500 dilution in BSA; Cell Signalling) and goat anti-lysozyme (1 : 500 dilution in BSA; Santa Cruz). Incubation of primary antibodies was performed at 4 °C overnight. Sections were washed and incubated with secondary antibodies (goat anti-mouse IgG for immunohistochemistry, 1 : 1000 in BSA; Jackson ImmunoResearch; donkey Alexa Fluor 488 for immunofluorescence, 1 : 500 in BSA; Invitrogen) and DAB substrate (Vectastain ABC Kit). Stainings were developed with DAB. Slides were counterstained with haematoxylin or DAPI and mounted. For TUNEL assay, slides were subjected to Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore) according to manufacturer' protocol. Slides were visualized by an AxioImager Z1 microscope (Zeiss). Images were captured by a digital camera system (AxioCam HrC/HrM, Zeiss). Measurements were made using a semi-automated image analysis software (AxioVision version 08 / 2013). For the analysis of immunohistochemistry, each dot in the accompanying statistical analysis represents one individual mouse, for which the mean number of positively stained cells with the respective antibody was assessed in a total of 30 - 50 crypts.

**Immunoblot Analysis**

Cells were lysed using sodium dodecyl sulfate (SDS)–based DLB buffer with 1 % Halt Protease inhibitor cocktail (Thermo Fisher Scientific), heated at 95 °C for 5 minutes and exposed to ultra-sonication for 5 seconds twice. Lysates were centrifuged at 16,000 g for 15 minutes at 4 °C to remove cell remnants. For protein extraction of organoids, Matrigel was removed by several centrifugation steps at 4 °C followed by lysis as described earlier. Afterwards, equal amounts of lysates containing Laemmli buffer were electrophoresed on 12 % polyacrylamide gels under standard SDS-PAGE conditions before being transferred onto polyvinylidene fluoride membranes (GE Healthcare). Protein-loaded membranes were blocked with 5 % milk in tris buffered saline and Tween 20 (TBST) before being incubated with the primary antibody at 4 °C overnight followed by incubation with a horseradish peroxidase–conjugated secondary antibody for 1 hour at indicated concentrations (Supplementary Table 2). Proteins were detected using the Amersham ECL Prime Western Blot Detection Reagent (GE Healthcare).

**Supplementary Table 2: Antibodies used for Western Blot analysis**

| Primary western | Origin | Dilution | Company | Article number |
|---|---|---|---|---|

| blot antibody | | | | |
|---|---|---|---|---|
| **ß-Actin** | mouse | 1 : 1000 in 5 % BSA in TTBS | Sigma Aldrich | A-5441 |
| **ß-Tubulin** | rabbit | 1 : 1000 in 5 % BSA in TTBS | Abcam | ab6046 |
| **GAPDH** | mouse | 1 : 1000 in 5 % BSA in TTBS | Santa Cruz | sc-365062 |
| **PARP** | rabbit | 1 : 1000 in 5 % BSA in TTBS | Cell Signaling Technology | 9542 |
| **p53** | mouse | 1 : 1000 in 5 % BSA in TTBS | Cell Signaling Technology | 2524T |
| **pS6** | rabbit | 1 : 1000 in 5 % BSA in TTBS | Cell Signaling Technology | 5364 |
| **p-4E-BP1** | rabbit | 1 : 2000 in 5 % BSA in TTBS | Cell Signaling Technology | 2855 |
| **RNASEH2B** | rabbit | 1 : 2000 in 5 % BSA in TTBS | received from collaborator[1] | - |
| **S6** | rabbit | 1 : 1000 in 5 % BSA in TTBS | Cell Signaling Technology | 2317 |
| **yH2Ax** | rabbit | 1 : 1000 in 5 % BSA in TTBS | Cell Signaling Technology | 2577 |
| **4E-BP1** | rabbit | 1 : 2000 in 5 % BSA in TTBS | Cell Signaling Technology | 9644 |

| Secondary western blot antibody | Origin | Dilution | Company | Article number |
|---|---|---|---|---|
| **rabbit IgG, HRP-linked** | sheep | 1 : 3000 in 5 % BSA in TTBS | GE Healthcare | NA934-1ML |
| **mouse IgG, HRP-linked** | sheep | 1 : 3000 in 5 % BSA in TTBS | GE Healthcare | NA931-1ML |

**Organoid Culture, Colony Formation Assay and CellTiter-Glo 3D Viability Assay**

Intestinal crypts were isolated from healthy tissue of young (8 - 12 weeks old) mice and cultured in Matrigel (Corning) as previously described[2]. As culture medium for the organoids, a conditioned medium containing Wnt3A, R-spondin1 and Noggin was prepared using L-WRN cells (CRL-3276, ATCC) according to the manufacturers' protocol. When crypts were

freshly seeded or when organoids were passaged, the 50 % L-WRN medium was supplemented with 10 µM Y-27632 (STEMCELL Technologies). For passaging, Matrigel was dissolved in Cell Recovery Solution (Corning) and organoids were enzymatically dissociated using TrypLE Express (Gibco, Thermo Fisher Scientific). To perform colony formation or CellTiter-Glo assays, organoids were dispersed into single cell solutions using TrypLE Express. For colony formation assays, 1,0000 cells per organoid genotype were reseeded onto 24-well plates in order to assess proliferative capacities by determining both organoid diameters and numbers. For CellTiter-Glo assays, 1,500 single cells were replated onto 96-well plates before after 24 hours, CellTiter-Glo 3D reagent (Promega) was added to each well in 1 : 1 ratio and mixtures were homogenized by vigorous pipetting. Plates were then incubated at RT for 30 minutes in the dark, with gentle shaking and luminescence recorded using an automatic plate reader (CLARIOstar, BMG Labtech). Images were acquired using the Leica M205FCA microscope equipped with a Leica DFC9000 GT camera and the Leica LAS X software (4x magnification). For rescue experiments, organoids in both assays were treated with either DMSO or 1 µM Rapamycin (LC Laboratories) dissolved in DMSO.

**Murine Intestinal Epithelial ModeK Cells, MTS Assay**

i*Xbp1* ModeK cells were generated by transfection of the respective cells with plasmids that intracellularly were transcribed into Xbp1-intering RNA sequences[2]. Knockdown was validated by qPCR analysis. For immortalization, we used the SV40 Virus (simian vacuolating virus). Cells were cultivated at 37 °C with DMEM GlutaMAX™ medium (Gibco,) with 10 % FCS (Merck Millipore). Medium was exchanged every other day. Viability of ModeK cells cultivated on 96-well plates was assessed by measuring MTS incorporation using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

**FACS Annexin Cell Death Assay**

ModeK cells and organoids of the respective genotypes were seeded onto 24-well plates. For FACS-based cell death assay using Annexin V-FITC (Immunotools) and 7-AAD (7-Aminoactinomycin; BD Biosciences), cells were dissociated using TrypLE Express (Thermo Fisher Scientific). Cells were then incubated in Annexin V and 7-AAD before FACS assay was performed using FACSCalibur (BD Biosciences, Germany). 10,000 cells were gated excluding doublets or non-dissociated cell groups. The fraction of cells positive for Annexin staining was considered as dead cells.

**Transfection of ModeK Cells with siRNA (small interfering RNA)**

For *in-vitro* transfection, Viromer© Blue (Lipocalyx) was used according to the manufacturers' protocol. SiRNA against *Ddit4l* (#GS73284) and *p53* (#GS22059) was derived from Qiagen and used at a concentration of 10 µM.

**Stimulants for ModeK Cells and Organoids**

As stimulants for ModeK cells and organoids, AraC (received as a gift from a collaborator at the Institute of Biochemistry, CAU Kiel), IWP-2 (Tenu-Bio) and Rapamycin (LC Laboratories) were used.

**In-silico RNA Sequencing Data Analysis**

RNA sequencing expression profiles and clinical data for colorectal cancer (COAD – Colon Adenocarcinoma and READ – Rectum Adenocarcinoma) were downloaded from The Cancer Genome Atlas (TCGA, http://gdac.broadinstitute.org/). Only samples from primary tumors, for which clinical survival data was available, were considered in the analysis (n = 376). RNA sequencing expression data was transformed to log2 (Transcripts-per-Millions +1).

**Survival Analysis**

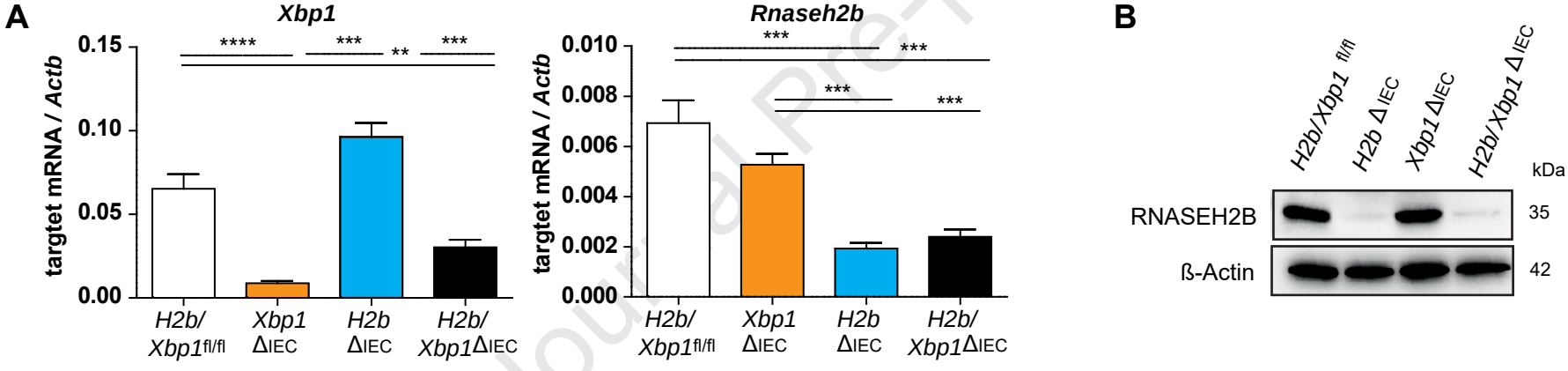
Overall survival analysis was performed using the R package survival. Samples were divided into two groups based on their *XBP1* gene expression ("*XBP1* high" and "*XBP1* low") by using the maximum Harrell's c-index. The Cox proportional-hazards model was used to evaluate the association between *XBP1* gene expression and the survival time of the patients. Kaplan-Meier survival curves with log rank tests were plotted to compare the overall survival between the two groups. Additionally, the hazard ratio (HR) with a 95 % confidence interval was provided for comparison of the groups. P-values were adjusted for multiple testing based on their false discovery rate (FDR) according to the Benjamini-Hochberg procedure.

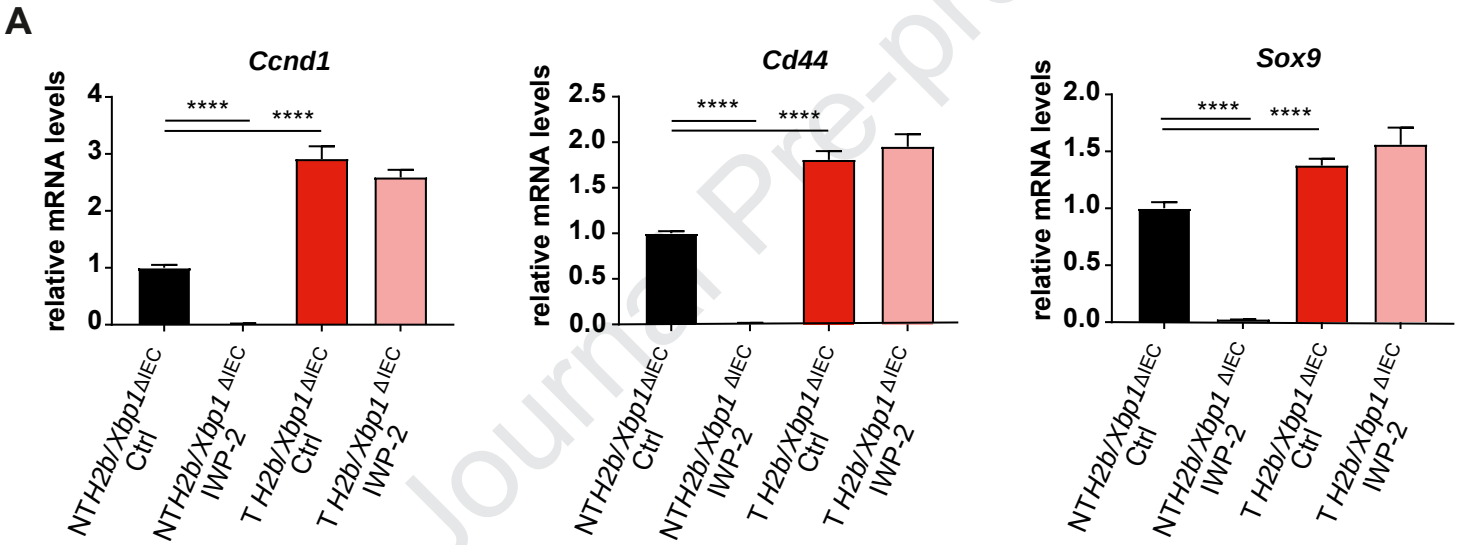
**Pathway Activity Analysis**

Pathway activity scores for 14 cancer related pathways (Androgen, Estrogen, WNT, EGFR, MAPK, PI3K, VEGF, JAK-STAT, TGF-b, TNF-a, NFkB, Hypoxia, Trail and p53-mediated DNA damage response) were estimated using PROGENy. This method embarks on a compendium of pathway-responsive gene signatures derived from perturbation experiments to infer pathway activity. To prepare the gene expression data for PROGENy[3], raw count data was imported to the R package DESeq2. Additionally, a linear model was applied to check for differences between the two groups ("*XBP1* high" and "*XBP1* low").

**Supplemental References**

1. Aden K, Bartsch K, Dahl J, et al. Epithelial RNase H2 Maintains Genome Integrity and Prevents Intestinal Tumorigenesis in Mice. Gastroenterology 2019;156.
2. Aden K, Breuer A, Rehman A, et al. Classic IL-6R signalling is dispensable for intestinal epithelial proliferation and repair. Oncogenesis 2016;5:e270–e270. Available at: https://doi.org/10.1038/oncsis.2016.71.
3. Schubert M, Klinger B, Klünemann M, et al. Perturbation-response genes reveal signaling footprints in cancer gene expression. Nat Commun 2018;9:20. Available at: https://doi.org/10.1038/s41467-017-02391-6.





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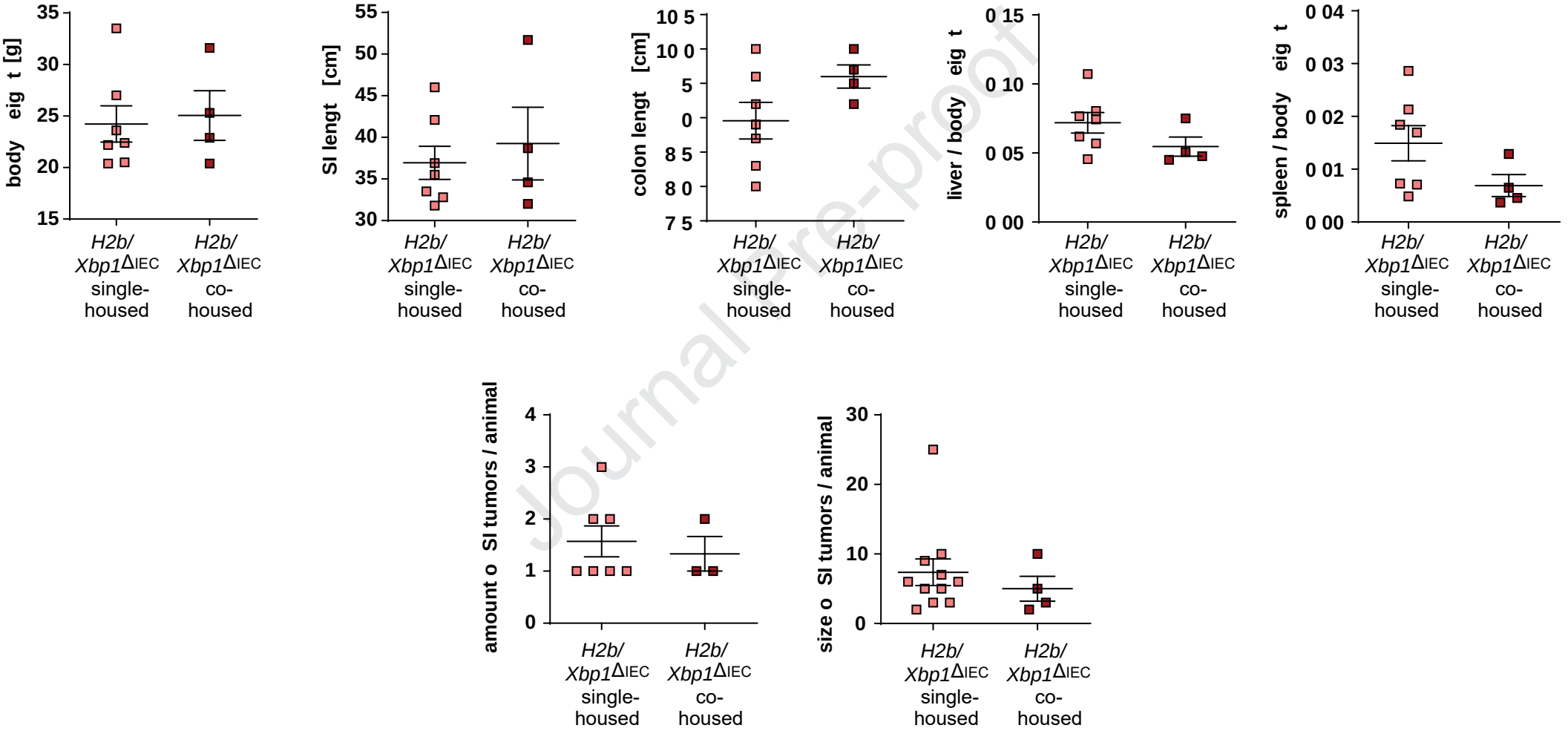

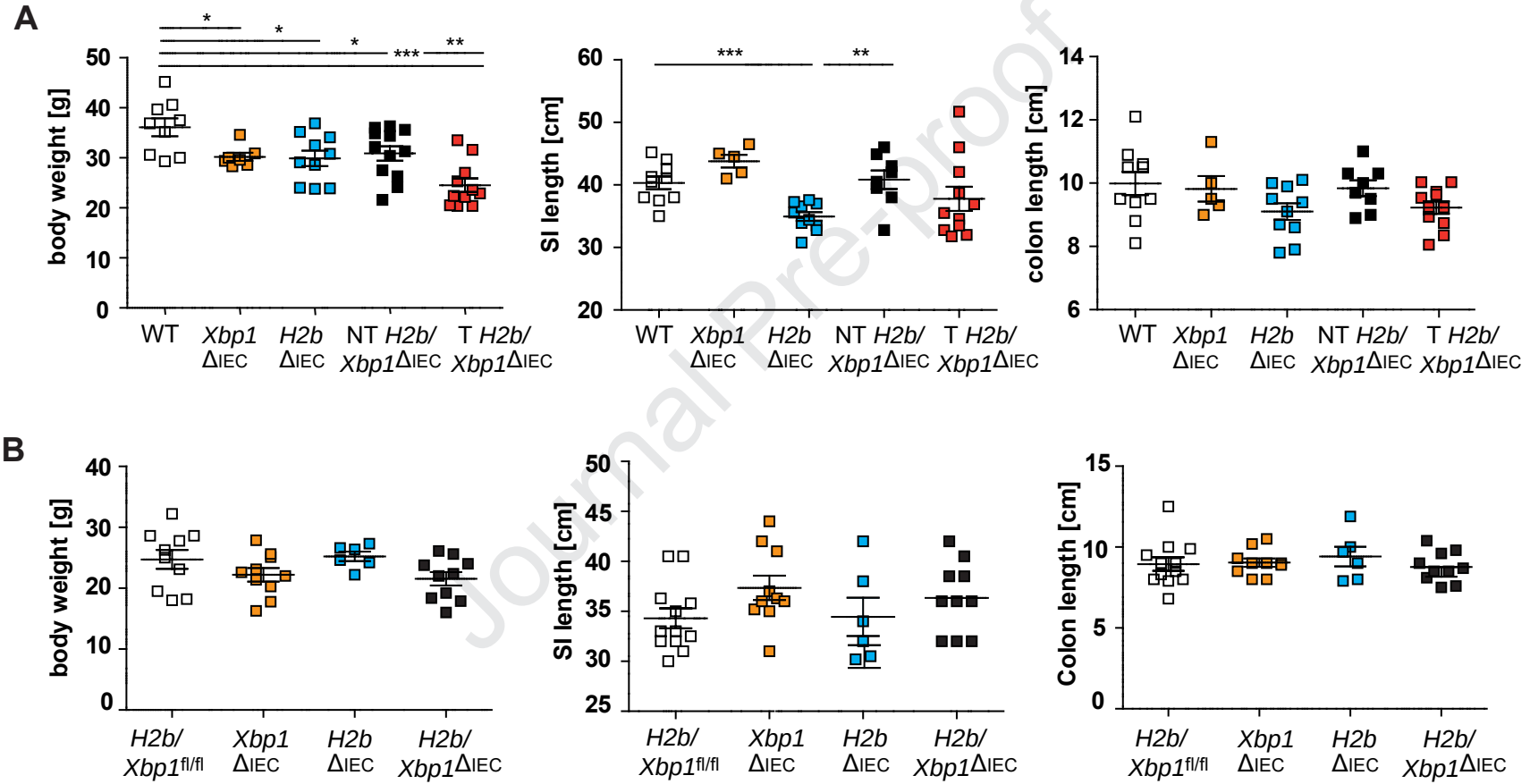

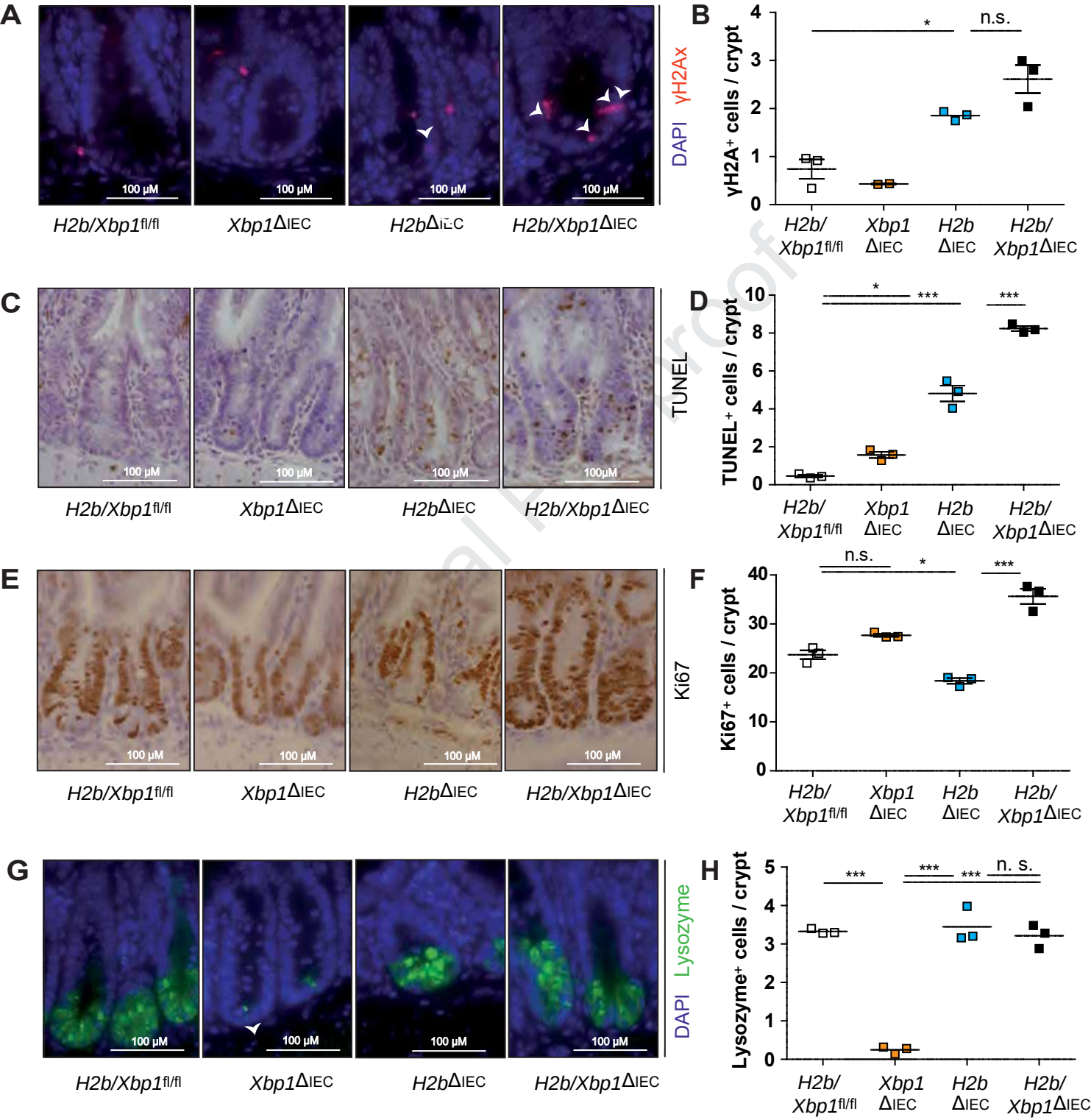
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C
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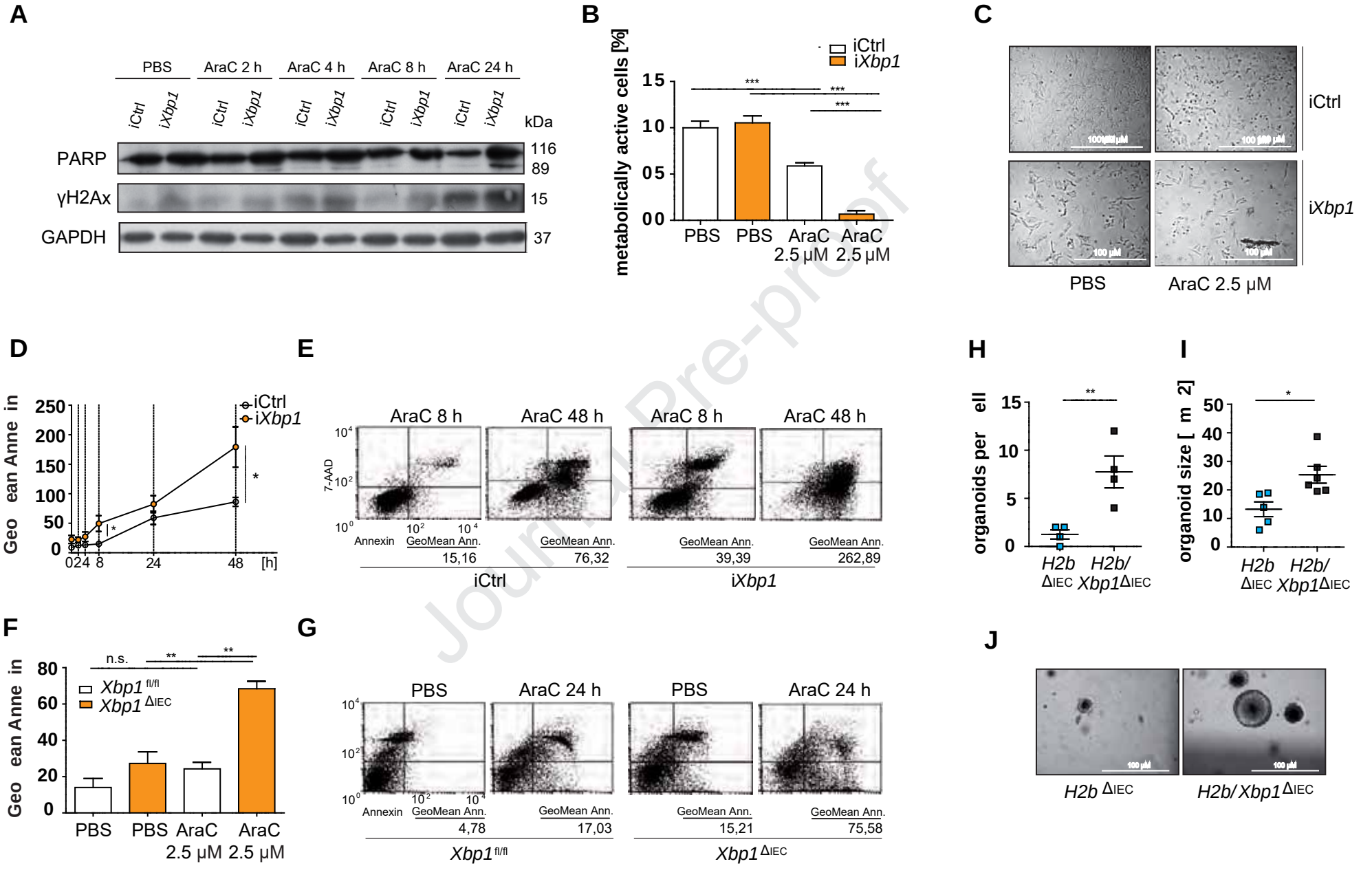

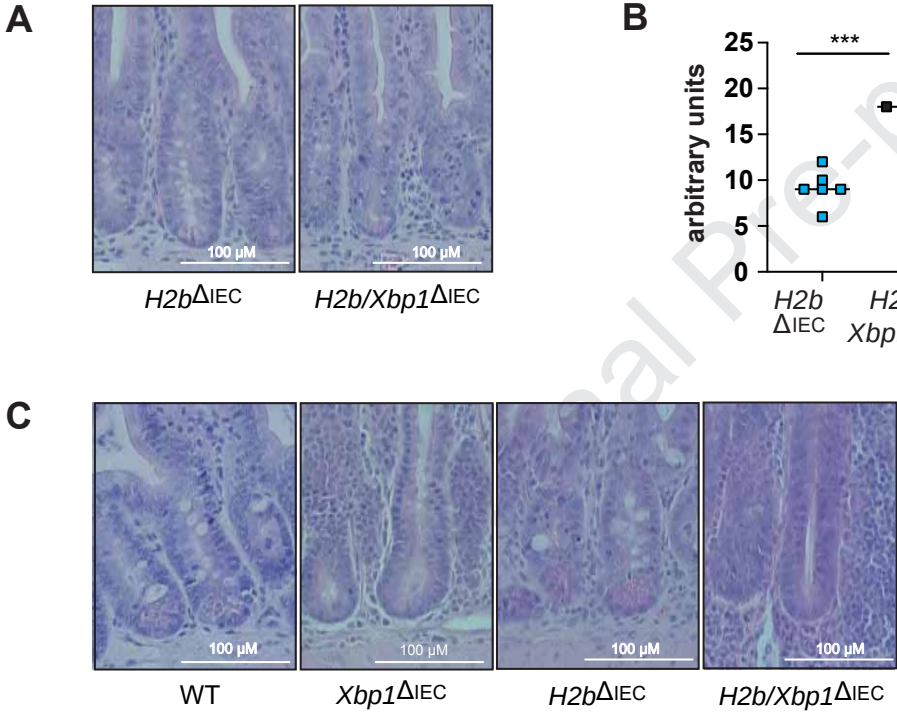

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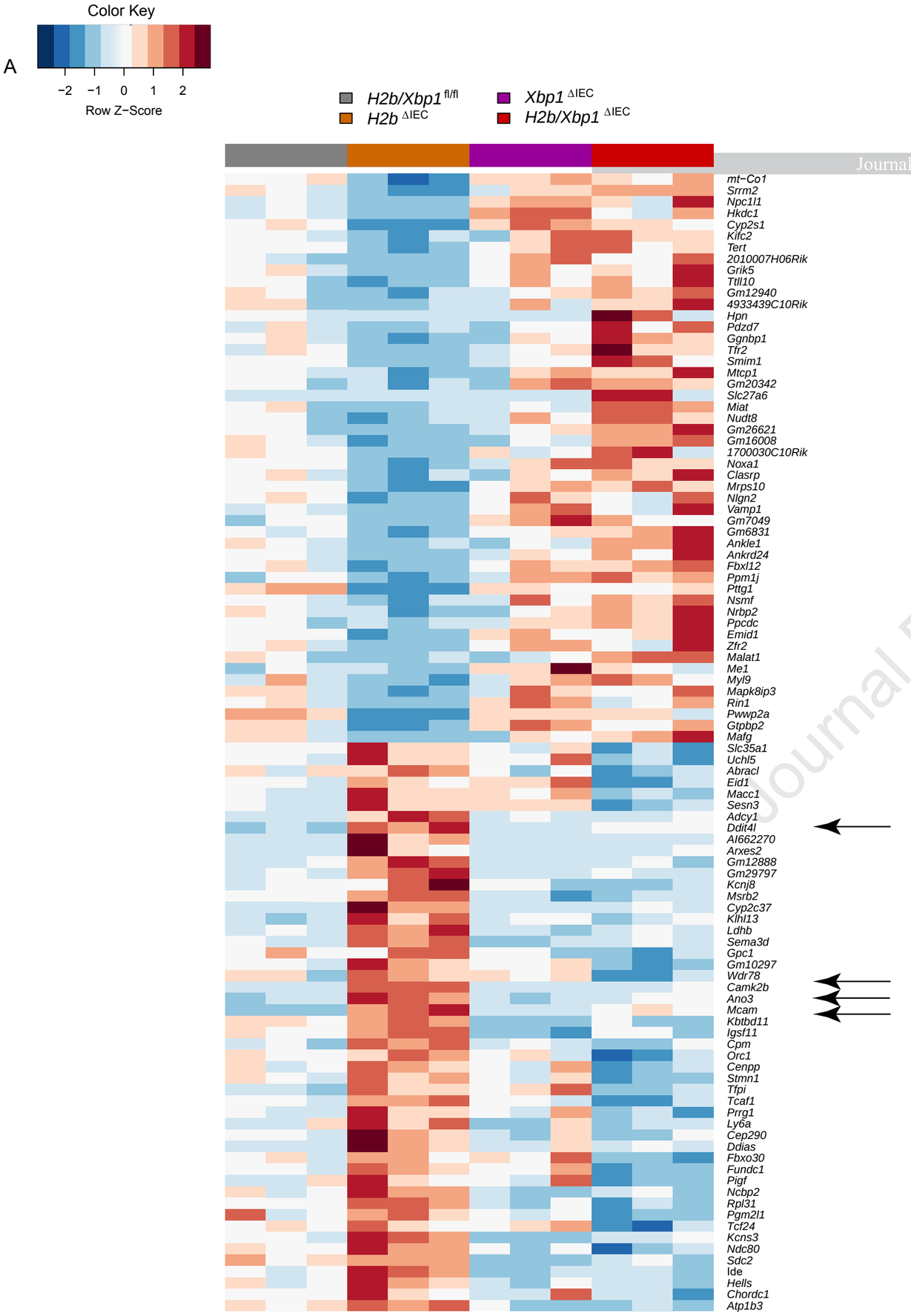


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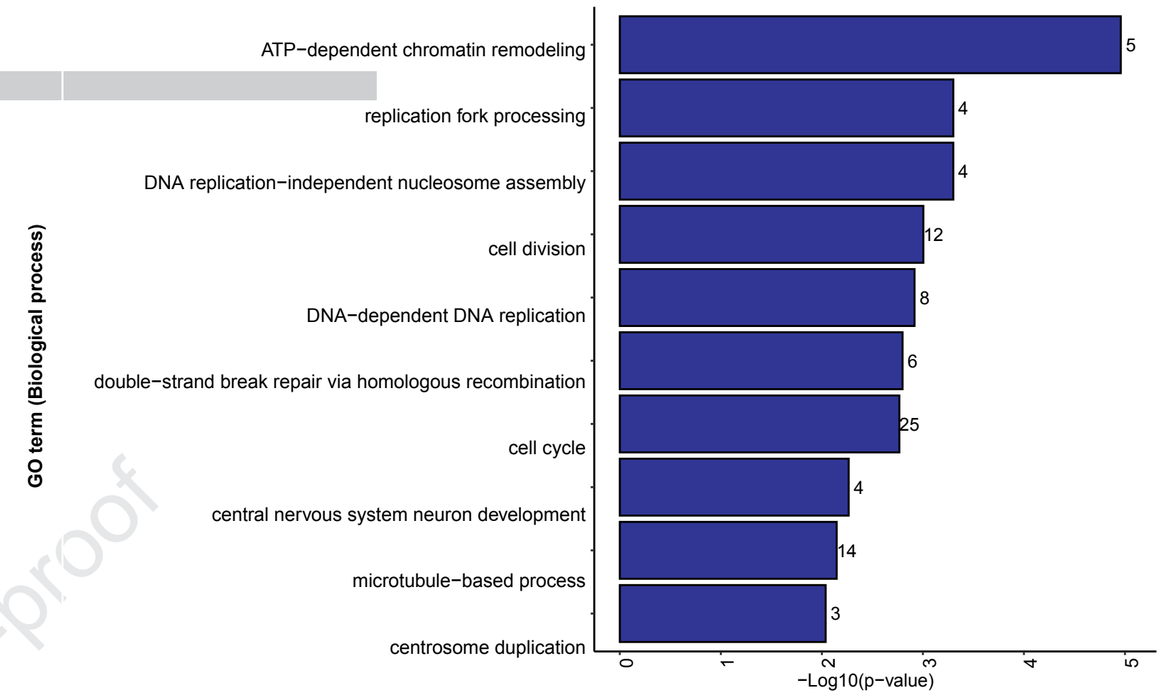


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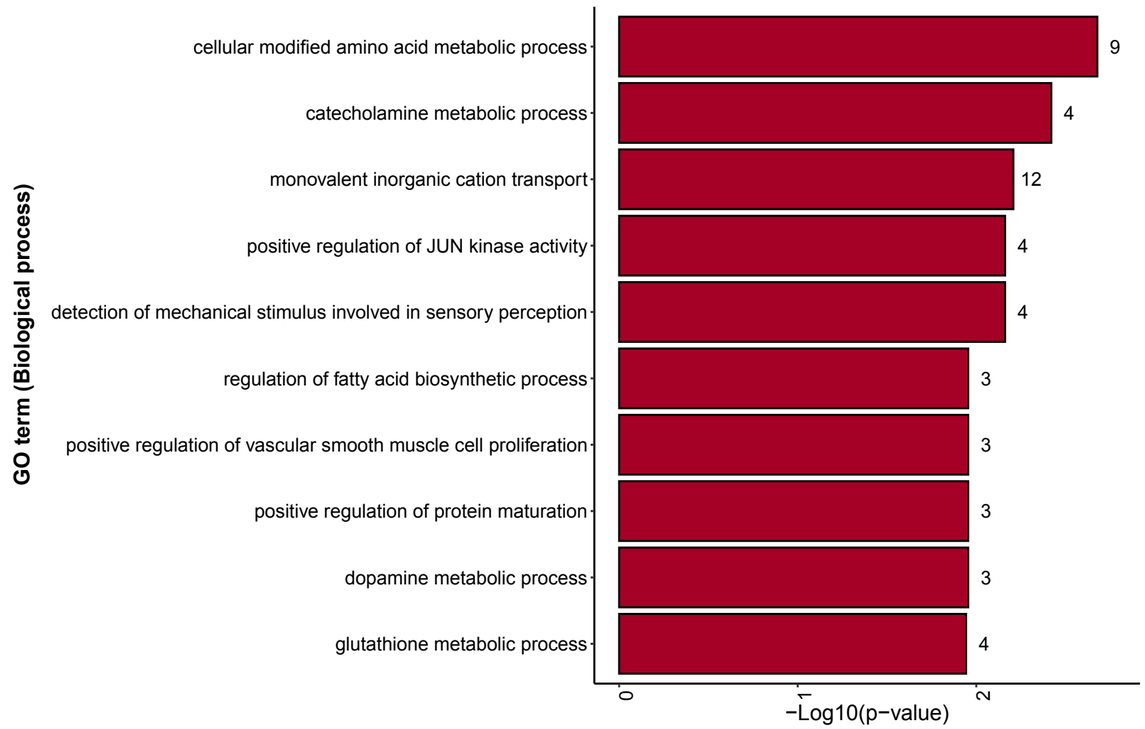

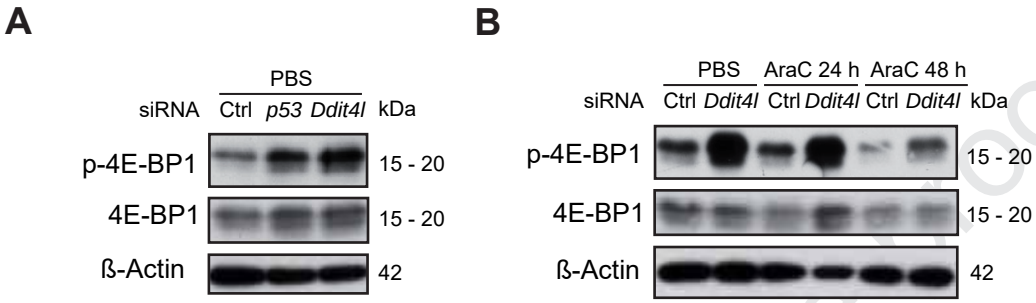

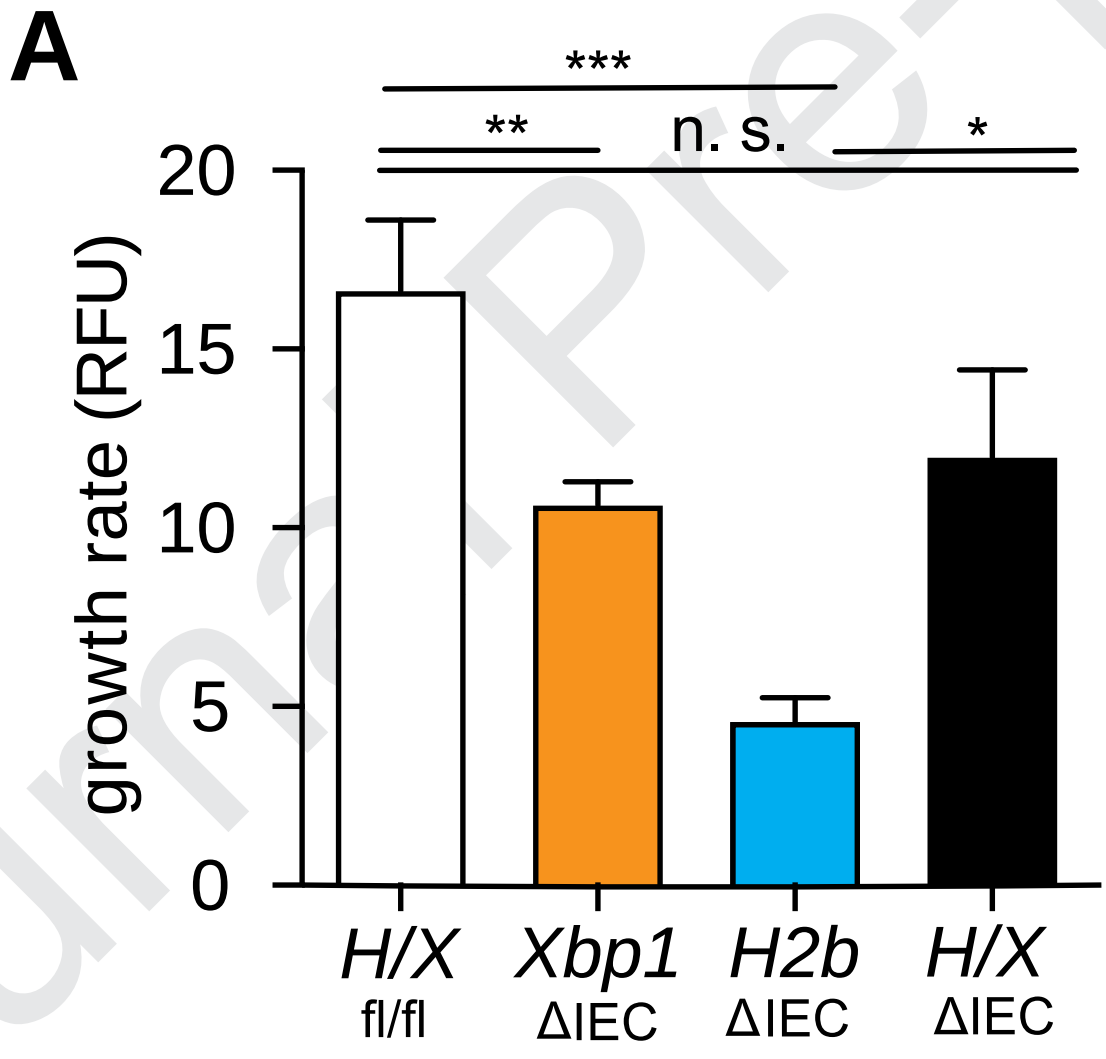
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