The WT1-BASP1 complex is required to maintain the differentiated state of taste receptor cells

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Abstract

The WT1-BASP1 complex acts as a transcriptional repressor. Here we analyse the role of BASP1 in the development of the peripheral taste system and find that it is expressed during the terminal developmental stages and throughout adulthood in the mature taste cells along with WT1. Using a conditional BASP1 mouse we demonstrate that BASP1 is critical for the function of adult peripheral taste cells and acts to maintain their differentiated state by inhibiting the WT1-dependent activation of Wnt and Shh pathway target genes. Our results uncover a central role for the WT1-BASP1 complex in the maintenance of the differentiated state.

Introduction

The WT1 transcription factor plays a critical role in the development and of multiple organs and tissues (Hastie 2017). WT1 null mice display complete agenesis of the kidneys, gonads, adrenal glands and spleen among other sites. WT1 is also needed for tissue maintenance in the adult, with these sites involving some overlap with development targets along with additional tissues also requiring WT1 to maintain their differentiated state (Chau et al. 2011). WT1 can either drive cell proliferation or promote differentiation but the mechanisms involved in this dichotomy are not clear (Toska and Roberts 2014; Hastie 2017).

WT1 acts in concert with a transcriptional cofactor, BASP1. BASP1 binding switches the function of WT1 from an activator to a repressor (Toska and Roberts 2014) and regulates the ability of WT1 to control differentiation in several model cell lines including kidney podocyte cells (Green et al. 2009), epicardial cells (Essafi et al. 2011) and blood cells (Goodfellow et al. 2011). Recent work has also shown that in the absence of BASP1, WT1 has an important role in maintaining multipotency (Blanchard et al. 2017). BASP1 blocks this function and is associated with driving iPSCs to differentiate.

WT1 null mice also display developmental defects in several sensory tissues including the retinal ganglion cells (Wagner et al. 2002), olfactory epithelia (Wagner et al. 2005) and, as shown by us, peripheral taste cells (Gao et al. 2014). Peripheral taste cells are continuously replaced throughout an organism's lifetime (Barlow and Klein 2015). Taste buds are comprised of at least three different mature taste cell types that have unique roles within the buds. They originate from Keratin 14 (Krt14+) progenitor

cells that become either non-taste epithelium or post-mitotic precursors that express sonic hedgehog (Shh+). These post-mitotic Shh+ cells further differentiate into fully differentiated Keratin 8 (Krt8)-expressing Type I, II or III taste cells in the taste bud (Liu et al. 2013; Barlow and Klein 2015). Both the Shh and Wnt/β-catenin signaling pathways regulate the specification of taste cell fate and are required for taste cell renewal (Castillo et al. 2014; Gaillard et al. 2015; Gaillard et al. 2017). However, the underlying mechanisms regulating Wnt and Shh signaling in adult taste cells and the renewal process are still unclear.

The goal of this study was to analyse the role of BASP1 in the taste cell renewal process. We find that deletion of BASP1 in differentiated taste cells leads to their reduced function, a loss of several differentiation markers, and the upregulation of WT1 target genes that are normally primarily expressed in the progenitor cells. Our findings reveal that the WT1-BASP1 complex plays a central role in the maintenance of the differentiated state in this system.

Results and Discussion

Our previous work demonstrated a key role for WT1 in the development of the peripheral taste system (Gao et al. 2014). Since BASP1 is a central transcriptional cofactor of WT1, we sought to determine if it acts with WT1 during the development of the circumvallate papillae (CV) which is present on the back of the tongue and houses hundreds of taste buds. We analyzed BASP1 expression at different developmental stages during CV formation using double label immunohistochemistry alongside either Krt8+ (a marker of the developing taste cells; Figure 1A), or GAP43 (a gustatory nerve marker that identifies the nerve that will innervate the emerging taste cells; Figure 1B). During development, Krt8 is expressed in the placode that will form the CV papillae but BASP1 expression is absent in the placode. At E12.5 and E15.5, BASP1 is present in the developing gustatory nerve (GAP43+) but is not co-expressed with Krt8. By P7, BASP1 is expressed in both the Krt8+ cells and GAP43+ expressing gustatory nerve. This expression pattern is maintained in the adult taste system. Thus, unlike WT1 (Gao et al. 2014), BASP1 is not expressed in the developing taste cells but is switched on around birth. BASP1 expression is maintained in the adult taste buds while its expression in the gustatory nerve is reduced after birth.

As reported in our previous work (Gao et al. 2014), adult mouse taste cells express WT1, and we find that it shows a large degree of colocalization with BASP1 (Figure 2A). The adult taste buds contain three major mature cell types (Finger 2000); the type I glial cells that support the function of type II and type III cells which are responsible for the transduction of taste. We used specific markers to determine the degree of WT1 and BASP1 co-localization with each of these cell types. NTPDase2 is a

marker specific to the type I glial cells and has strong co-localization with WT1 expression (Figure 2B) but does not co-localize with BASP1 (Figure 2C). In the taste responsive type II (PLCβ2 positive) and III (NCAM positive) cells both WT1 and BASP1 are expressed. These data demonstrate that WT1 is expressed in all three types of differentiated taste cells but BASP1 is only expressed in the type II and III cells with known roles of transducing taste stimuli. Our data also demonstrate that WT1, but not BASP1, is expressed in the progenitor (Krt14+) cells (Figure 2D). This suggests a likely role for WT1 in the formation of new taste cells which is consistent with WT1 function in the CV during embryonic development. WT1 is also expressed in the Shh+ cells, while nuclear BASP1 is expressed in a subset of the Shh+ cells (n=7 of 27 cells). This raises the possibility that BASP1 expression is initiated as the Shh+ cells undertake the final transition to terminal differentiation (Figure 2E). Taken together our results demonstrate that WT1 is expressed in both the progenitors and differentiated taste cells of the CV while BASP1 expression is generally limited to the differentiated cells.

Further experiments evaluated the effects of BASP1 expression in the mature taste cells as it relates to their function. We crossed a floxed BASP1 mouse (Supplementary Figure 1) with a Krt8-Cre-ER mouse to delete BASP1 in differentiated type I, II and III taste cells. Mice were treated with tamoxifen for 8 days, sacrificed a week later, and immunohistochemistry was then performed on tongue sections to confirm knockout of BASP1 in the taste buds (Figure 3A). RNA was also prepared from taste bud cells to confirm that BASP1 mRNA in taste cells was reduced (Figure 3A, at right). The specific deletion of the BASP1 gene in Krt8-expressing cells (Krt8-BASP1-KO; KO) did not lead to any obvious change in the taste bud morphology compared to

controls (CTL) and the number of taste buds in the CV papillae of BASP1-KO mice was not significantly different from control mice (Figure 3B). Furthermore, the expression levels of Krt8 in the taste buds was not altered in the KO mice (Figure 3C).

To evaluate if the loss of BASP1 in the taste cells affected their function, we isolated taste receptor cells from the CV and compared the taste-evoked calcium responses between BASP1-KO and CTL mice using live cell imaging. Two taste stimuli were used to activate Type II cells: sweet sucralose (20mM) and bitter denatonium benzoate (5mM, Den). Potassium chloride (50mM, HiK) was used to depolarize Type III taste cells and activate voltage gated calcium channels (VGCCs). There was a significant reduction in the number of responsive cells for all three stimuli in BASP1-KO mice compared to CTLs (Figure 3D). Moreover, the remaining taste-evoked calcium signals for denatonium and sucralose were significantly reduced in amplitude (percent increase over baseline) in KO mice compared to CTLs (Figure 3E). While the number of HiK responsive cells was reduced, the response amplitude to HiK in the remaining responsive cells did not change significantly in the KO mice. Representative traces are shown in Supplementary Figure 2. Taken together, these data reveal that the ability of the taste receptor cells to respond to stimuli was significantly impaired when BASP1 was absent.

Our results so far demonstrate that the expression of BASP1 in the differentiated taste cells is required for their function in taste detection and that the absence of BASP1 significantly impacted their ability to respond to stimuli. We next analyzed the effect of BASP1 deletion on functional markers for each of the taste cell types. Deletion of BASP1 (Figure 4A) caused a significant increase in the expression of the type I cell

marker, NTPDase2 (Figure 4B), but reduced both PLCβ2 (type II cell marker; Figure 4C) and NCAM (type III cell marker; Figure 4D) expression. Not all type II and type III functional proteins were affected by BASP1 deletion as gustducin (type II marker) and pgp9.5 (type III marker) expression levels were maintained in the taste buds of the BASP1 KO mice (Supplementary figure 3). Therefore, the reduction in PLCβ2 and NCAM expression was not due to a decrease in the overall number of type II and III cells (Figure 4E), but was instead due to the lower expression of these proteins in the cells (Figure 4C and D, graphs). This reduced expression of PLCβ2 and NCAM is consistent with the impaired responses of the taste cells that we observed (Figure 3).

Our previous work identified the LEF1 and PTCH1 genes as targets for regulation by WT1 during development of the peripheral taste system (Gao et al. 2014). These genes encode components of the Wnt and Shh signalling pathways, respectively, that are important during the initial development of the peripheral taste system. These signalling pathways also play a central role during taste cell renewal in the precursor cells but are generally switched off in the fully differentiated mature adult taste cells (Barlow and Klein 2015). We next tested the effect of knocking out BASP1 in the differentiated (Krt8-expressing) taste cells on the expression levels of LEF1 and PTCH1. RNA was prepared from the taste bud cells of either Krt8-BASP1-KO (KO) mice or control mice (CTL) and qPCR was used to determine the levels of LEF1 and PTCH1 mRNA relative to control GAPDH mRNA (Figure 5A). Deletion of BASP1 in the Krt8-positive taste cells led to a significant increase in both LEF1 and PTCH1 expression compared to CTL.

We then determined if WT1 and BASP1 localize to the promoter regions of the LEF1 and PTCH1 genes in the adult taste buds. Taste cells were isolated from the CV papillae of either CTL or K8-BASP1-KO mice and subjected to Chromatin Immunoprecipitation (ChIP) using WT1, BASP1, or control (IgG) antibodies. The results are expressed as fold enrichment at the LEF1 and PTCH1 promoters over a control genomic region (Figure 5B). While WT1 was localized at the LEF1 and PTCH1 promoters in both CTL and K8-BASP1-KO taste cells, BASP1 was only localized to the promoters of the LEF1 and PTCH1 genes in CTL taste cells and its binding was lost in the K8-BASP1-KO taste cells.

Immunohistochemical analysis evaluated the levels of LEF1 and PTCH1 protein expression in the CV of K8-BASP1-KO mice compared to CTLs (Figure 5C). LEF1 and PTCH1 are normally expressed at a low level in the mature taste cells and are generally confined to the progenitor cells. Loss of BASP1 in the differentiated taste cells (Krt8+) caused an upregulation in the expression of both LEF1 and PTCH1 throughout the mature cells of the taste bud when compared to CTLs (Figure 5C). Taken together these results demonstrate that WT1-BASP1 acts as a transcriptional repressor complex in the fully differentiated taste cells of the adult CV taste cells and that it normally inhibits the expression of Wnt and Shh signaling components in these differentiated cells.

Thus, the loss of BASP1 in Krt8 cells allows the activation of genes that are normally only expressed in the taste progenitor cells, causes the disruption of specific cell type markers and a severe reduction in taste cell function. These data suggest that the WT1/BASP1 complex regulates multiple pathways which are needed to maintain

taste cells in their differentiated state. Our previous work demonstrated that WT1 regulates the expression of LEF1 and PTCH1 genes during embryonic development of the peripheral taste system, from the early developmental stages through birth (Gao et al. 2014). We have now shown that BASP1 is not expressed while the initial taste cells are developing but is expressed in the differentiated cells shortly after birth. In parallel, WT1, but not BASP1, is expressed in the progenitor K14+ cells for the adult taste bud, which constitutively express LEF1 and PTCH1. In the K14+ cells, these signalling pathways direct the differentiation pathway to Shh+ cells and subsequently the mature taste cells of the bud (Kapsimali and Barlow 2013; Barlow and Klein 2015; Gaillard et al. 2015). The selective expression of BASP1 in a subset of Shh+ cells suggests that BASP1 binding is needed to drive the Shh+ cells to differentiate into either type II or type III cells while WT1 alone is sufficient to drive differentiation of type I cells since WT1 is expressed in all three cell types. We hypothesize that BASP1 binding to WT1 in Shh+ cells causes the silencing of the Wnt and Shh pathways along with the upregulation of gene expression pathways that are important for the specific functions of type II and III cells. Future experiments will test that hypothesis.

Based on these data, we propose that WT1 has important roles in the progenitor cells which generate taste cells while the sustained presence of the WT1-BASP1 complex is required to maintain taste cells in their differentiated state. Such a mechanism is consistent with the recently reported roles of WT1 and BASP1 in stem cells in which WT1 promotes multipotency while the WT1-BASP1 complex promotes the differentiated state (Blanchard et al. 2017).

Methods

Animals

Floxed BASP1 mice were generated in a C57BL/6 background by Cyagen BioSciences (Supplementary Figure 1). Animals were cared for in compliance with the University at Buffalo Animal Care and Use Committee. BASP1fl$^{+/-}$ mice were mated with Krt8-Cre/ERT2^{+} mice (017947, The Jackson Laboratory) to obtain BASP1fl$^{+/-}$Krt8-Cre/ERT2^{+} mice. These offspring were bred to obtain BASP1fl$^{+/+}$Krt8-Cre/ERT2^{+} mice which were used as BASP1-KO group. The BASP1fl$^{-/-}$;Krt8-Cre/ERT2^{+} or BASP1fl$^{+/+}$;Krt8-Cre/ERT2^{-} littermates were used as wild type CTLs. PCR primers used to genotype the conditional BASP1 knockout allele: 5'- TGCCCTGCCTGCAGGTCAAT-3' (Forward); 5'-CCGAGTCTGTACAAAAGCCACC-3' (Reverse). Tamoxifen was dissolved in corn oil mixed with ethanol (9:1 by volume) to a stock concentration of 20mg/ml as previously described (Miura et al. 2014). BASP1-KO and CTL mice were gavaged with tamoxifen (100mg/Kg body weight) every 24 hours for 8 consecutive days. Mice were sacrificed and tissues were collected 7 days after the last gavage. Primers used to genotype the Cre induced BASP1-KO allele: 5'-CGAGAGATTTGTGATGTATGATGAGCAG-3' (F); 5'-CCGAGTCTGTACAAAAGCCACC -3' (R). Taste cells were collected and calcium imaging performed as described in (Hacker et al. 2008; Maliphol et al. 2013; Dutta Banik et al. 2018).

Immunohistochemistry

Fluorescent immunohistochemistry was performed as described in (Gao et al. 2014) except for assays with LEF1 antibodies which were fixed for 10 seconds in 4%

paraformaldehyde/0.1 M phosphate buffer (PB, pH 7.2) at room temperature. Fluorescence intensity was quantitated using Image J as previously described (Dutta Banik et al. 2018). PTCH1 immunohistochemistry was as follows; Mice were anesthetized with sodium pentobarbital (40 mg/kg; Patterson Veterinary) and then transcardially perfused with a 0.025% heparin solution in 1% sodium nitrite, followed by a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.2). Following perfusion, the tongues were removed and placed into 4% paraformaldehyde for 2 h, followed by 4 °C overnight cryoprotection in 20% sucrose. On the following day, tongues were frozen in O.C.T. Compound (Sakura Finertek USA) and 12μm sections of the CV papillae were cut. Slides were incubated in 3% hydrogen peroxide blocking reagent for 5 minutes. After washing the sections in buffer, the section were incubated in serum blocking reagent, avidin blocking reagent and biotin blocking reagent for 15 minutes respectively. Sections were then incubated in PTCH1 or control antibodies for 2 hr at RT before an overnight incubation at 4°C. On the next day, sections were washed three times for 15 min each in buffer and incubated with biotinylated secondary antibody for 60 minutes, followed by three washes in buffer 15 minutes each time. The sections were then incubated in HSS-HRP for 30 minutes, followed by three x 2min washes in buffer. Binding of primary antibody to the sections was visualized using a diaminobenzidine tetrahydrochloride (DAB) chromagen (R&D Biosystems).

Anti-Shh (sc-365112) and anti-gustducin (sc-395) were from Santa Cruz, anti-Krt8 from Developmental Studies Hybridoma Bank, anti-Lef1 from Signal Transduction (C12A5), anti-GAP43 from Aves (GAP43), anti-NTPDase2 from Ectonucleotidases-ab.com (CD39L1), anti-PLCβ2 from GeneTex (GTX133765), anti-NCAM (AB5032), anti-

PGP9.5 (AB108986) and anti-Ptch1 (AB53715) were from Abcam, and anti-Krt14 from Biolegend (905301). WT1 and BASP1 antibodies were raised against a peptide (WT1) or recombinant protein (BASP1) by Pacific Immunology then purified by affinity chromatography. Antibody specificity was verified as described in (Toska et al. 2014).

RNA and ChIP analysis

Total RNA was extracted from taste cells using the Nucleospin RNA XS kit (Clontech). Unamplified total RNA was DNase treated and then reverse transcribed using the Bio-Rad cDNA synthesis kit. Primers for analysis of GAPDH, LEF1 and PTCH1 are described in (Gao et al. 2014). ChIP assays were performed as described before (Wang et al. 2010) using primers for LEF1, PTCH1 and control regions described in (Gao et al. 2014). Datasets are an average of three biological repeats with standard deviation and significance calculated by students t-test.

Author contributions

K.F.M. and S.G.E.R. conceived the study, designed the experiments, analyzed the data and prepared the manuscript. Y.G. performed most of the experiments and D.D.B. performed the PTCH1 immunohistochemistry. Y.G. and D.D.B. analyzed the data and edited the manuscript.

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Figures legends

Figure 1: BASP1 is expressed at the terminal stages of CV development.

(A) Immunohistochemistry of CV at different stages of development (indicated at right). BASP1 is red and Krt8 is green and merged image is shown. Higher magnification of the merged image (boxed section) is shown at right. **(B)** As in part A except that BASP1 (red) and GAP43 (green) were detected. Scale bars=10μM.

Figure 2: WT1 and BASP1 are expressed in overlapping cell types of the adult CV.

(A) WT1 and BASP1 are expressed in adult taste cells. Immunohistochemistry of CV taste buds to detect WT1 (green) and BASP1 (red) and merged image. Higher magnification of the merged image (boxed section) is shown at right. **(B)** Immunohistochemistry of CV taste buds to detect WT1 (red) and either NTPDase2 (red; type I cells), PLCβ2 (red; type II cells) or NCAM (red; type III cells). Merged images are shown at right. **(C)** As in part B except that BASP1 (green) was detected instead of WT1. **(D)** Left panels; Immunohistochemistry of CV taste buds to detect WT1 (red) and Krt14 (green). Merged image is shown at bottom. Right panels; Immunohistochemistry of CV taste buds to detect WT1 (red) and Shh (green). Merged image is shown at bottom right. **(E)** As in part D except that BASP1 (red) was detected instead of WT1. Scale bars=10μM.

Figure 3: Knockout of BASP1 in the adult CV affects the detection of tastants by the taste cells.

(A) Immunohistochemistry of CV taste field to detect BASP1 (red) in control (CTL) mice and Krt8-BASP1-CRE mice treated with Tamoxifen for 7 days. Scale bar=50μM. At right qPCR to determine BASP1 expression relative to GAPDH in RNA isolated from control (black bar, CTL) and Krt8-BASP1 knockout (blue bar, KO) mice. (*, $p<0.05$). **(B)** BASP1 knockout in Krt8 cells was performed as in part A. After staining with DAPI the number of cells per bud was counted. Mean with standard deviation are reported. Horizontal bars represent mean average intensity. **(C)** As in part A except that staining was with Krt8 antibodies. At right is quantitation of immunoreactivity per taste bud using Image J. **(D)** Chi-square analysis compared the overall number of responsive taste cells in control (CTL) and K8-BASP1-KO mice. The percentage of responsive taste cells is shown (***, $p < 0.001$ and **, $p<0.01$) for 5mM denatonium, 20mM sucralose and 50mM potassium chloride (HiK). **(E)** The response amplitudes (percent increase over baseline) for the responsive cells from part D were analysed and compared for the CTL and BASP1 KO mice (***, $p < 0.001$ and **, $p<0.01$).

Figure 4: Knockout of BASP1 in the adult CV leads to a disruption of type II and type III cells markers.

(A) Immunohistochemistry of CV taste buds using anti-BASP1 (red) in control (CTL) mice and Krt8-BASP1-CRE (KO) mice that had been treated with tamoxifen for 8 days. Scale bar=50μM. **(B)** As in part A except that NTPDase2 (type I marker) was tested. A plot of signal intensity per taste bud is shown at right. Horizontal bars represent mean intensity (***, $p<0.001$). **(C)** As in part A except that anti-PLCβ2 (type II marker) was used. A plot of signal intensity per taste bud is shown below. Horizontal bars represent

mean intensity (***, $p<0.001$). **(D)** As in part A except that NCAM labelling (type III marker) was measured. A plot of signal intensity per taste bud is shown below. Horizontal bars represent mean intensity (***, $p<0.001$). **(E)** The number of PLCβ2- (above) and NCAM- (below) positive cells per taste bud in wild type (CTL) and Krt8-BASP1-KO (KO) cells is shown. Error bars are standard deviation of the mean.

Figure 5: The WT1-BASP1 complex represses LEF1 and PTCH1 expression in the differentiated cells of the CV.

(A) Quantitative PCR (qPCR) was used to detect LEF1 (above) and PTCH1 (below) expression relative to GAPDH in mRNA isolated from taste cells of control (black bar, CTL) and Krt8-BASP1 knockout (blue bar, KO) mice (*, $p<0.05$). **(B)** ChIP analysis of isolated taste cells from adult mice. WT1, BASP1 or control (IgG) antibodies were used. After normalization to input DNA, fold enrichment at the LEF1 (top) or PTCH1 (bottom) promoter regions over a control genomic region is shown in CTL mice (black bars) and Krt8-BASP1-KO (blue bars) mice (* $p<0.05$). **(C)** Immunohistochemical analysis of LEF1 expression in the CV of CTL mice or Krt8-BASP1-KO mice (left panels). Expression levels of PTCH1 in the CV of control (CTL) mice or Krt8-BASP1-KO mice were also measured (right panels). Scale bars= 20μm.

Figure 1

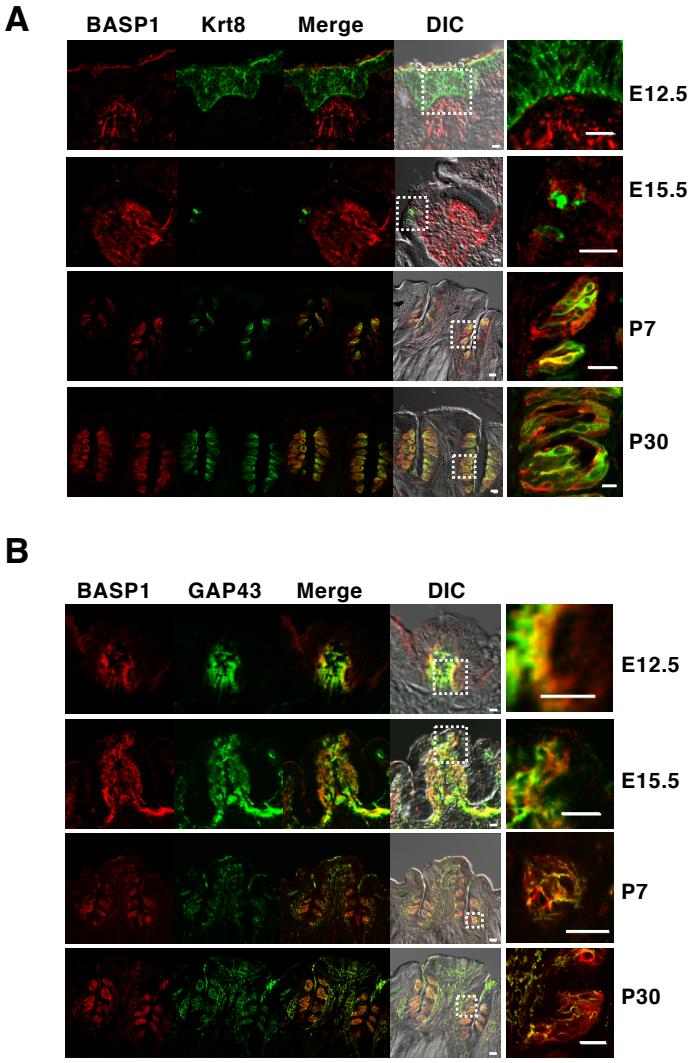
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Figure 2

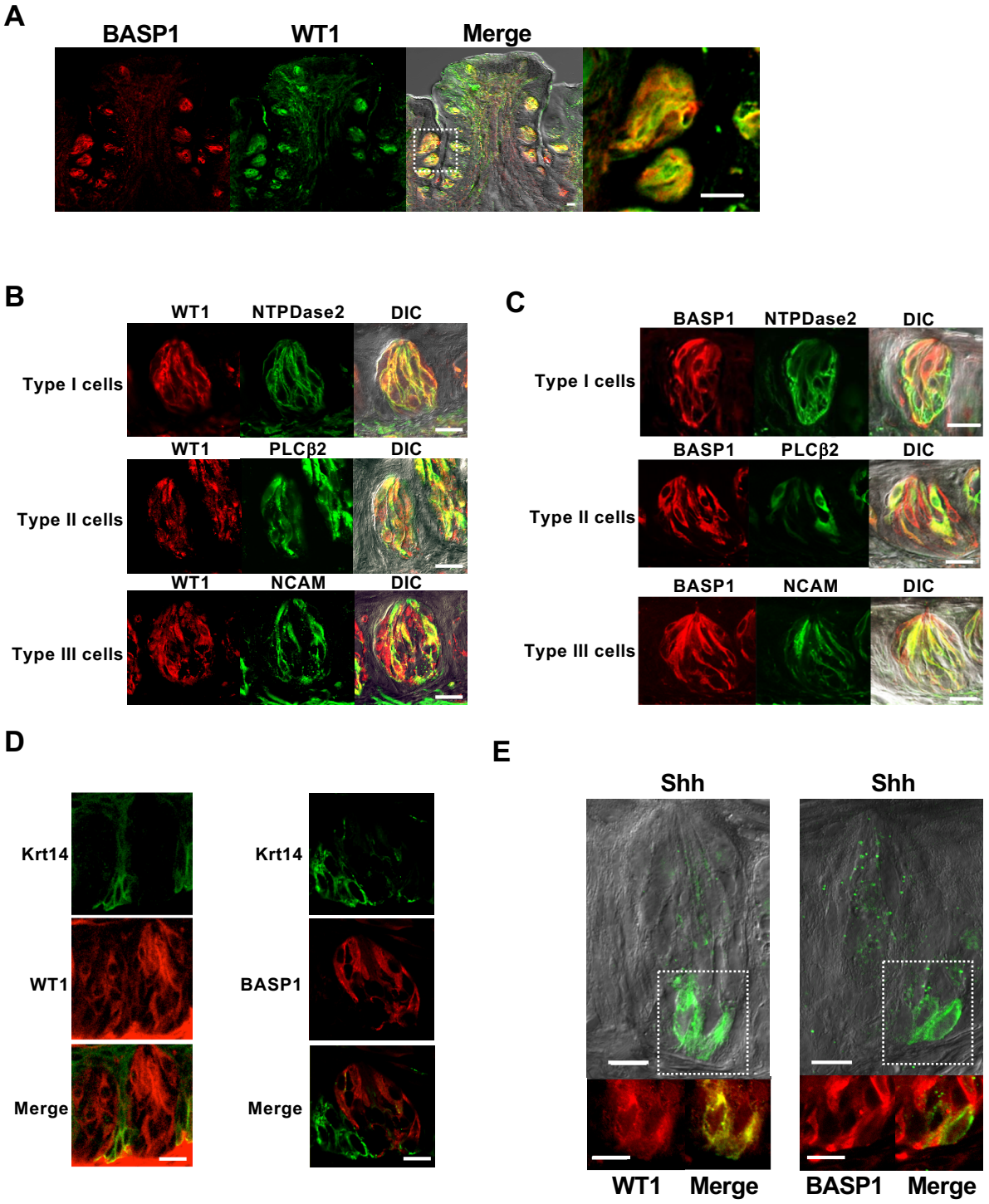
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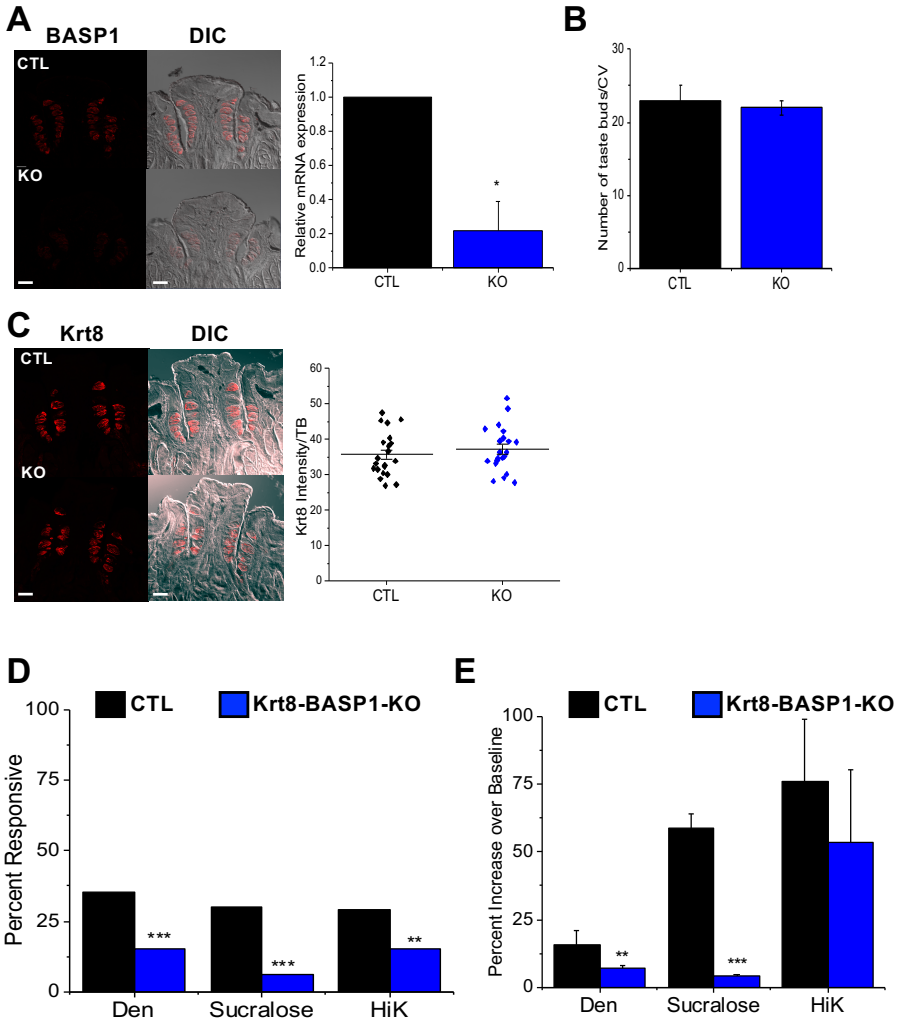
Figure 3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20
10
0
CTL
KO
C
Krt8
DIC
CTL
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Krt8 Intensity/TB
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E
CTL
Krt8-BASP1-KO
Percent Increase over Baseline
100
75
50
25
0
**
***
Den
Sucralose
HiK

Figure 4

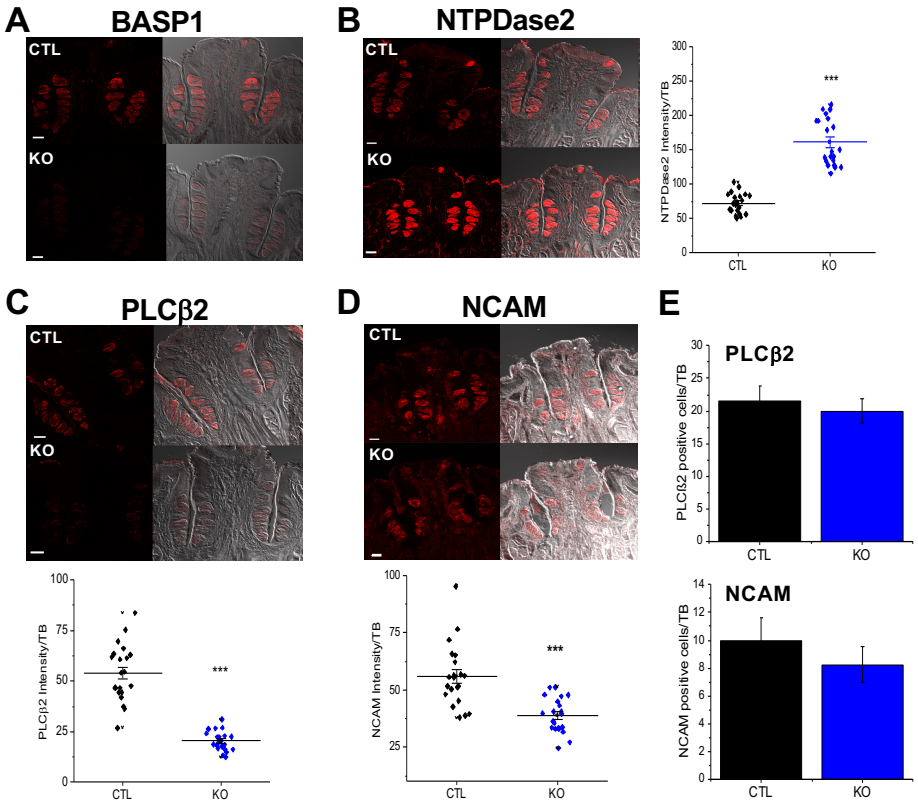
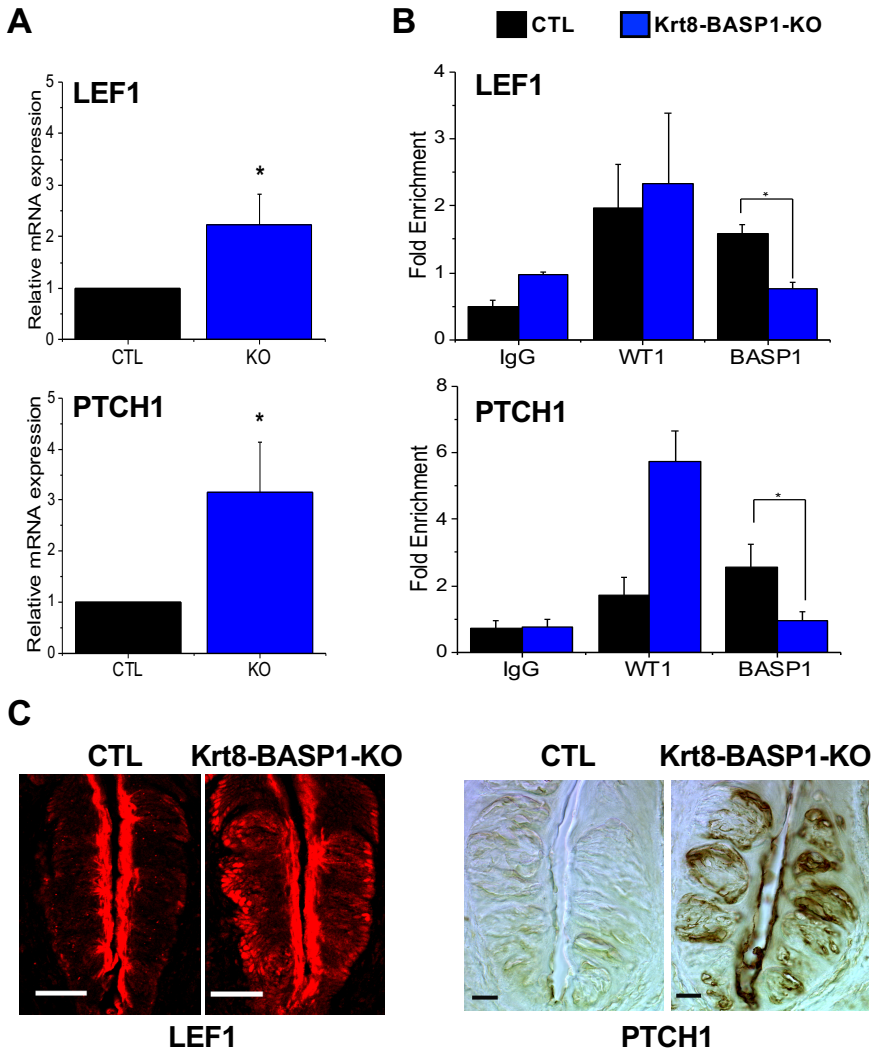

A
BASP1
CTL
KO
B
NTPDase2
CTL
KO
NTPDase2 Intensity/TB
300
250
200
150
100
50
0
***
CTL
KO
C
PLCβ2
CTL
KO
PLCβ2 Intensity/TB
100
75
50
25
0
***
CTL
KO
D
NCAM
CTL
KO
NCAM Intensity/TB
100
75
50
25
***
CTL
KO
E
PLCβ2
PLCß2 positive cells/TB
30
25
20
15
10
5
0
CTL
KO
NCAM
NCAM positive cells/TB
14
12
10
8
6
4
2
0
CTL
KO

Figure 5


A
LEF1
Relative mRNA expression
CTL
KO
PTCH1
B
CTL
Krt8-BASP1-KO
Fold Enrichment
IgG
WT1
BASP1
C
CTL
Krt8-BASP1-KO
LEF1
PTCH1