ENaC in Cholinergic Brush Cells

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Cholinergic polymodal chemosensory cells in the mammalian urethra (urethral brush cells = UBC) functionally express the canonical bitter and umami taste transduction signaling cascade. Here, we aimed to determine whether UBC are functionally equipped for the perception of salt through ENaC (epithelial sodium channel). Cholinergic UBC were isolated from ChAT-eGFP reporter mice (ChAT = choline acetyltransferase). RT-PCR showed mRNA expression of ENaC subunits Scnn1a, Scnn1b, and Scnn1g in urethral epithelium and isolated UBC. Scnn1a could also be detected by next generation sequencing in 4/6 (66%) single UBC, two of them also expressed the bitter receptor Tas2R108. Strong expression of Scnn1a was seen in some urothelial umbrella cells and in 65% of UBC (30/46 cells) in a Scnn1a reporter mouse strain. Intracellular $[\text{Ca}^{2+}]$ was recorded in isolated UBC stimulated with the bitter substance denatonium benzoate (25 mM), ATP (0.5 mM) and NaCl (50 mM, on top of 145 mM Na$^+$ and 153 mM Cl$^-$ baseline in buffer); mannitol (150 mM) served as osmolarity control. NaCl, but not mannitol, evoked an increase in intracellular $[\text{Ca}^{2+}]$ in 70% of the tested UBC. The NaCl-induced effect was blocked by the ENaC inhibitor amiloride ($IC_{50} = 0.47 \mu M$). When responses to both NaCl and denatonium were tested, all three possible positive response patterns occurred in a balanced distribution: 42% NaCl only, 33% denatonium only, 25% to both stimuli. A similar reaction pattern was observed with ATP and NaCl as test stimuli. About 22% of the UBC reacted to all three stimuli. Thus, NaCl evokes calcium responses in several UBC, likely involving an amiloride-sensitive channel containing $\alpha$-ENaC. This feature does not define a new subpopulation of UBC, but rather emphasizes their polymodal character. The actual function of $\alpha$-ENaC in cholinergic UBC—salt perception, homeostatic ion transport, mechanoreception—remains to be determined.

Keywords: chemosensory cells, cholinergic, ENaC, urethra, urethral brush cells, salt

INTRODUCTION

Bitter, sweet, umami, salty, sour, and fatty are the six recognized taste qualities detected by taste buds (Chaudhari and Roper, 2010). In type II sensory cells in the oropharyngeal taste buds, bitter, sweet, and umami perception is mediated by the canonical taste transduction signaling cascade, including G protein-coupled taste receptors, the taste-specific G protein $\alpha$-gustducin, phospholipase C$\beta$2 (PLC$\beta$2), and the transient potential receptor cation channel subfamily M member 5 (TRPM5) (Chaudhari and Roper, 2010). Other classes of G protein-coupled receptors respond to short- and long-chain fatty acids (Chaudhari and Roper, 2010).
In contrast, acid (protons) and salt (sodium chloride) are monitored by ion channels, directly leading to depolarization of the taste cell. Nonselective cation channels formed by polycystic kidney disease 2-like 1 protein (PKD2L1) and polycystic kidney disease 2-like 3 protein (PKD1L3) were proposed as candidates for sour taste receptors (Huang et al., 2006; Ishimaru et al., 2006; LopezJimenez et al., 2006; Chaudhari and Roper, 2010). An ion channel that is long been thought to mediate salt perception is the amiloride-sensitive epithelial sodium channel, ENaC (Heck et al., 1984; Avenet and Lindemann, 1988; Lindemann et al., 1998; Lin et al., 1999; Lindemann, 2001; Chandrashekar et al., 2010). It is predominately expressed in epithelial cells of the colon, lung, kidney, sweat and salivary glands, where it is a major regulator of sodium absorption and, thereby, essential for fluid homeostasis (Duc et al., 1994; McDonald et al., 1995; Garty and Palmer, 1997). ENaC is also expressed in the urothelium (Carattino et al., 2005; Du et al., 2007; Birder et al., 2010; Birder and Andersson, 2013). The canonical heteromeric ion channel consists of three subunits (α, β, γ) (Canessa et al., 1994b), encoded by the genes Scnn1a, Scnn1b, and Scnn1c. A fourth δ-subunit with distinct characteristics was identified and the presence of this subunit changes the biophysical characteristics as well as molecular regulation of this ion channel. Mice, however, lack a functional gene for this subunit and its physiological function remains unclear (Giraldez et al., 2012; Wichmann et al., 2018). ENaC is a constitutively active ion channel. Still, its expression, membrane abundance and open probability are tightly regulated by extrinsic and intrinsic factors. These include hormones, intracellular kinases and intramembrane lipids, as well as the extracellular sodium concentration, pH and mechanical stimuli (Chraibi and Horisberger, 2002; Althaus et al., 2007; Baines, 2013; Kleymann et al., 2018). The ion conductivity of αβγ-ENaC is limited to monovalent cations (Li⁺ > Na⁺ > K⁺) (Kellenberger and Schild, 2002).

Extraoral chemosensory cells, monitoring the composition of the mucosal lining fluid, have been described in the respiratory, gastrointestinal and urogenital tract. Like type II taste cells, they express the canonical taste transduction signaling cascade (taste receptors, α-gustducin, PLCβ2, TRPM5) (Höfer et al., 1996; Höfer and Drenckhahn, 1998; Finger et al., 2003; Krasteva et al., 2011, 2012; Deckmann et al., 2014; Schütz et al., 2015). They respond to bitter substances and bacterial products with a release of acetylcholine and initiate avoidance reflexes, thereby appearing as serving terminals situated at entrances into the body (Finger and Kinnamon, 2011; Lee and Cohen, 2015; Deckmann and Kummer, 2016). These cholinergic epithelial cells also express villin, a structural protein of microvilli. Such cells have originally been termed “brush cells” in the respiratory tract, and this term has also been adopted to the villin-positive, cholinergic chemosensory cells of the urethra (urethral brush cells = UBC) (Deckmann et al., 2014). In line with the sentinel concept, UBC respond to heat-inactivated uropathogenic Escherichia coli and are connected to sensory nerve fibers (Deckmann et al., 2014). Bitter application into the urethral lumen reflexively triggers enhanced detrusor activity, which has been interpreted as a protective reflex, as potential hazardous content is expelled from the urethra through micturition (Deckmann et al., 2014; Kummer and Deckmann, 2017).

Most cholinergic UBC are polymodal chemosensory cells, responding both to bitter substances and to glutamate with an increase in intracellular calcium concentration ([Ca²⁺]i) (Deckmann et al., 2014). This discriminates them from type II taste bud cells, which are generally responsive either to bitter, representing an aversive stimulus, or to umami, an attractive stimulus (Nelson et al., 2001; Chaudhari and Roper, 2010). At the urethral mucosa, both stimuli represent a potential danger signal, since many bacterial products have bitter quality and glutamate (umami) facilitates bacterial growth in urine. Here, we aimed to determine whether their polyomodal properties extend beyond taste receptor mediated qualities, focusing upon the perception of salt.

**MATERIALS AND METHODS**

**Animals**

Mice expressing enhanced green fluorescent protein (eGFP) under the control of the promoter of the acetylcholine synthesizing enzyme, choline acetyltransferase, (ChAT-eGFP; B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J; Stock No. 007902) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice expressing tdTomato, a bright red fluorescent protein, under the control of the promoter of Scnn1a, the coding gene sequence of α-ENaC (Scnn1atdTomato; Guy et al., 2015) were kindly provided by J. Guy and J. Staiger (Institute for Neuroanatomy, University Medical Center Goettingen, Georg-August-University Goettingen, Germany). This study was carried out in accordance with the recommendations of European Communities Council Directive of 24th November 1986 (86/609/EEC). The protocol was approved by the local authorities (Animal Welfare Officer at the University of Giessen and the Committee for Animal Welfare, Dept. V54, Regierungspräsidium Giessen, Germany; reference no. 572_M).

**Cell Isolation**

Cell isolation was performed as described previously (Deckmann et al., 2014). In brief: Urethrae were dissected, cut into small pieces, and enzymatically digested in dispase (2 mg/mL; Sigma-Aldrich/Merck, Darmstadt, Germany) and trypsin/PBS (1:1, Invitrogen, Carlsbad, CA, USA). After mechanical dissociation, cells were separated through a cell strainer (pore size 70 µm; BD Bioscience, Franklin Lakes, NJ, USA). The ChAT promoter is constitutively active in cholinergic chemosensory cells (Tallini et al., 2006). Hence, UBC constitutively express eGFP which served to sort them via FACS and to identify them with a fluorescence microscope.

**RT-PCR**

Total RNA from dissected urethra or pooled isolated cells (n = 4 samples, sorting based on ChAT-eGFP expression by FACS; BD
FASCARIA III cell sorter, settings and analysis were performed with a BD FACSDiva v6.1.3; BD Bioscience, Franklin Lakes, NJ, USA) was extracted using the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Extracted total RNA from kidney was used as positive control. RT-PCR was performed as described previously (primer sequences: Table S1; Deckmann et al., 2014).

**Next Generation Sequencing**

Next generation sequencing was performed as described elsewhere (Scholz et al., 2016). In brief: isolated single eGFP-positive cells were identified, picked and transferred to a PCR tube using a combined confocal laser-scanning-patch-clamp setup (Leica TCS SP5, Leica Microsystems/Luigs-Neumann, Wetzlar/Ratingen, Germany). Cell lysis, cDNA generation and amplification were performed using the Sigma SeqPlex RNA Amplification Kit (Sigma-Aldrich/Merck, Darmstadt, Germany). For library preparation, the Illumina Nextera XT DNA sample preparation protocol (Part # 15031942 Rev. C) was used. Samples run together with a 2 × 75 bp read length using the MiSeq Reagent Kit v3 (150 cycles) and the Illumina MiSeq Desktop Sequencer (Illumina, San Diego, CA, USA). The sequencing reads were aligned to the mm9 reference genome and transcriptome using TopHat2 (2.0.9). The TopHat setup (Leica TCS SP5, Leica Microsystems/Luigs-Neumann, Wetzlar/Ratingen, Germany). Cell lysis, cDNA generation and amplification were performed using the Sigma SeqPlex RNA Amplification Kit (Sigma-Aldrich/Merck, Darmstadt, Germany). For library preparation, the Illumina Nextera XT DNA sample preparation protocol (Part # 15031942 Rev. C) was used. Samples run together with a 2 × 75 bp read length using the MiSeq Reagent Kit v3 (150 cycles) and the Illumina MiSeq Desktop Sequencer (Illumina, San Diego, CA, USA). The sequencing reads were aligned to the mm9 reference genome and transcriptome using TopHat2 (2.0.9). The TopHat output files were saved in BAM format and evaluated by Cuffdiff2 (2.1.1). All samples were compared and evaluated in one calculation cycle, allowing the algorithm to estimate the Fragments Per Kilobase Million (FPKM) values at the transcript level resolution and to control for variability across the replicate libraries.

**Immunohistochemistry and Whole-Mount Immunostaining**

Specimen preparations and analyses were performed as described previously (Krasteva et al., 2011). In brief: urethrae used for immunohistochemistry (N = 3) and gall bladders used for whole-mount immunostaining (N = 2) were fixed using transcardiac perfusion with Zamboni solution (2% paraformaldehyde/15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4). Fixed organs were dissected, washed in 0.1 M phosphate buffer (0.1 M NaH2PO4, 0.1 M Na2HPO4), and either included overnight in 18% sucrose in 0.1 M phosphate buffer and frozen in liquid nitrogen or mounted on a block of silicon elastomer using insect pins. Primary antibody was applied to 4–18 tissue sections from every individual animal. Primary antibodies were chicken anti-RFP (NB101-97371; 1:200 dilution; Novus Biologicals, Littleton, CO, USA) and rabbit anti-TPRM5 (1:2,000) (Deckmann et al., 2014). Secondary antibodies were goat-anti rabbit Ig conjugated to Alexa 488 (1:500; Thermo Fisher Scientific Inc. Waltham, MA, USA) and donkey-anti chicken Ig conjugated to Cy3 (1:2,000; Dianova, Hamburg, Germany). Nuclei were labeled with 4′,6-diamidino-2-phenylindol (DAPI; 1 μg/ml; Sigma-Aldrich/Merck, Darmstadt, Germany). All sections were rinsed and coverslipped with carbonate-buffered glycerol (pH 8.6). Sections were evaluated by epifluorescence microscopy (Axioplan 2, Zeiss, Wetzlar, Germany) or with a confocal laser scanning microscope (LSM 710, Zeiss, Wetzlar, Germany). Specificity of secondary reagents was validated by omission of primary antibodies.

**Measurement of Intracellular Calcium Concentration**

Measurement of intracellular calcium concentration ([Ca2+]i) was performed as described previously (Deckmann et al., 2014). In brief: isolated cells were loaded with the fluorescent calcium indicator Calcium Orange® AM (0.01 μg/μl; Thermo Fisher Scientific Inc., Waltham, MA, USA) and plated on coverslips. [Ca2+]i was analyzed with a confocal laser scanning microscope (LSM 710 with ZEN 2010 B SP1, Zeiss, Wetzlar, Germany). Lasers and filters were: eGFP: excitation with Argon laser at 488 nm; recording of emission at 495–553 nm with optical filters MBS-488/561/633; Calcium Orange: excitation with DPSS561-10 laser at 561 nm; recording of emission at 566-683 nm with optical filters MBS-558/561. Regions of interest were selected manually and fluorescence intensities at the start of the recording period were set arbitrarily at 100%. Test stimuli and concentrations were denatonium benzoate (25 mM; Molekula, Munich, Germany), ATP (0.5 mM; Sigma-Aldrich/Merck, Darmstadt, Germany) and NaCl (1–150 mM; Carl Roth, Karlsruhe, Germany), and inhibitors and controls included the osmolarity control mannitol (1–150 mM; Sigma-Aldrich/Merck, Darmstadt, Germany) and the ENaC inhibitor amiloride (0.01–100 μM; Sigma-Aldrich/Merck, Darmstadt, Germany). All recordings were done during continuous superfusion with Tyrode III buffer (NaCl 130 mM; HEPES 10 mM; glucose 10 mM; KCl 5 mM; MgCl2 1 mM; CaCl2 8 mM; sodium pyruvate 10 mM; NaHCO3 5 mM; 2.5 mM/L/min; 37°C). Stimuli were added under continuous flow of Tyrode III into the chamber, so that indicated concentrations were reached initially and then washed out. Since baseline concentration of Na+ in the buffer was 145 mM, the total concentration after addition of 1–150 mM ranged from 146 to 295 mM.

**Statistical Analysis**

Data were analyzed for normal distribution by the Kolmogorov-Smirnov test. Multiple comparison analysis was performed by Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test. P values were regarded as statistically significant. Analyses were performed by GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

**RESULTS AND DISCUSSION**

RT-PCR revealed mRNA expression of the ENaC subunits Scnn1a, Scnn1b, and Scnn1g in the urethral epithelium (Figure 1A) and in pooled isolated UBC (Figure 1B). Next generation sequencing (NGS) of six isolated single eGFP-positive cells showed a heterogeneous expression pattern of Scnn1a, b, g (Figure 2). Scnn1a was detected in 4/6 cells (66.6%), Scnn1b and Scnn1g only in 1/6 cells. Canonical ENaC is composed of the α-, β-, and γ-subunit (Canessa et al., 1994a), but the ENaC α-subunit alone is able to
FIGURE 1 | RT-PCR of urethral epithelium and isolated UBC. (A) RT-PCR of urethral epithelium. (B) RT-PCR of UBC. Cells were isolated and sorted based on ChAT-eGFP expression by flow cytometry; agarose gel; α-ENaC (Scnn1a; 82 bp), β-ENaC (Scnn1b; 115 bp), γ-ENaC (Scnn1g; 150 bp), β-actin (300 bp), eGFP (180 bp); ± RT = aliquots processed with/without reverse transcription; kidney = positive control; H2O = water control.

FIGURE 2 | Expression of ENaC genes and markers of chemosensory cells in single UBC determined by NGS. Heatmap displaying the detection levels as normalized FPKM in GFP-positive UBC. FPKM, Fragments Per Kilobase Million.
form amiloride-sensitive homomers in vitro (Canessa et al., 1994b).

To further validate Scnn1a expression in cholinergic UBC, urethral tissue sections of a Scnn1a reporter mouse strain were labeled for cholinergic UBC. In view of often experienced methodological problems in detecting ChAT by immunohistochemistry in peripheral cells, we set out to establish a technically more reliable marker for immunohistochemical detection of cholinergic UBC. Villin-antibodies, an often used marker for brush cells in general, appeared not suitable for this purpose as there is a considerable number of villin-positive but ChAT- and TRPM5-negative slender epithelial cells in the murine urethra, in addition to the villin/ChAT/TRPM5-positive cells (Deckmann et al., 2014). These two phenotypes represent truly different cell populations, since genetic ablation of the transcription factor Skn-1a/Pou2f3 selectively prevents the development of TRPM5-positive (i.e., cholinergic UBC) but not of villin-positive but TRPM5-negative urethral cells (Yamashita et al., 2017).

We used TRPM5-immunolabeling as a marker for cholinergic UBC in Scnn1a-tdTomato reporter mice. In these mice, strong expression of Scnn1a was observed in several cells of the urethral epithelium (Figures 3A,B). Among them were umbrella cells, which build up the luminal lining in the proximal parts of the urethra being covered with an urothelium and which can be readily identified by virtue of their position and morphology. This is in line with the previously reported ENaC-immunoreactivity at the luminal membrane of umbrella cells in the rat urinary bladder (Smith et al., 1998) and functional investigation of this cell type (McCloskey et al., 2017). Notably, this cell layer did not consistently express tdTomato with positive and negative umbrella cells occurring in a mosaic pattern (Figure 3A). Although heterogeneity of umbrella cells with respect to other characteristics such as uroplakin expression has also been reported in select localizations such as the human ureter (Riedel et al., 2005), this labeling pattern might reflect incomplete expression of tdTomato in potentially Scnn1a-expressing cells. To test for this possibility, we looked for tdTomato expression in the gall bladder whose mucosal surface is known for homogeneous ENaC expression (Li et al., 2016). In two gall bladder whole-mount preparations, strong tdTomato expression was observed in epithelial cells covering only about 21% (case 1: 26.8%, case 2: 16.0%) of the mucosal surface whereas nearly 80% remained unlabeled (Figures 3C,D). Gall bladder whole-mounts were also incubated with TRPM5-antibody in order to label cholinergic chemosensory brush cells that are also present in this epithelium (Schütz et al., 2015).
FIGURE 4 | UBC response to NaCl is ENaC dependent and not an osmolarity effect. Urethral epithelial cells of ChAT-eGFP reporter mice were isolated and UBC were identified due to eGFP fluorescence. Experiments were performed during continuous superfusion with Tyrode III buffer containing 145 mM Na\(^{+}\). NaCl, mannitol and (Continued)
Two out of 69 TRPM5-positive cells expressed Scnn1a-tdTomato (Figures 3C,D).

Among non-umbrella cells with nuclei located in deeper layers of the urethral epithelium, we detected co-localization of TRPM5-immunoreactivity and Scnn1a-tdTomato signal (30 of 46 TRPM5-positive cells, 65%, N = 3 animals) as well as TRPM5-positive cells without Scnn1a-tdTomato signal (Figure 3B). These observations support our findings in RT-PCR experiments and single cell sequencing that a subpopulation of UBC expresses α-ENaC.

Functionally, application of NaCl evoked significant increases in [Ca\(^{2+}\)] as well as TRPM5-positive cells with nuclei located in deeper layers of the urethral epithelium, we detected co-localization of TRPM5-immunoreactivity and Scnn1a-tdTomato signal (30 of 46 TRPM5-positive cells, 65%, N = 3 animals) as well as TRPM5-positive cells without Scnn1a-tdTomato signal (Figure 3B). These observations support our findings in RT-PCR experiments and single cell sequencing that a subpopulation of UBC expresses α-ENaC.

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One of the NaCl-evoked [Ca\(^{2+}\)] increase during water deprivation or high-salt intake (Kummer, 2016; Kummer and Deckmann, 2017). Fourth, this response to the NaCl stimulus was sensitive to amiloride, whereas baseline [Ca\(^{2+}\)] in UBC (in the presence of 145 mM Na\(^+\) in Tyrode III) was not (Figure 4G). Fourth, this response to the NaCl stimulus was sensitive to amiloride, whereas baseline [Ca\(^{2+}\)] in UBC (in the presence of 145 mM Na\(^+\) in Tyrode III) was not (Figure 4G). Fourth, this response to the NaCl stimulus was sensitive to amiloride, whereas baseline [Ca\(^{2+}\)] in UBC (in the presence of 145 mM Na\(^+\) in Tyrode III) was not (Figure 4G).
and water homeostasis, e.g., concentration of primary urine in the kidney (Kellenberger and Schild, 2002). Given the low number of cholinergic UBC in the urethra and their minimal exposure to the luminal surface, this function appears rather unlikely for this particular cell type. However, ENaC is also a mechanosensitive ion channel, reacting to shear stress (Althaus et al., 2007; Guo et al., 2016). This opens the possibility that ENaC-subunit carrying UBC may be involved in sensing urine flow in the urethra. Notably, as mechanical strain affects the entire epithelium and is not restricted to the luminal membrane, it will reach UBC without a clear connection to the luminal surface (“closed type,” see Figure 3B and Deckmann and Kummer, 2016). Cholinergic UBC are connected to sensory nerve fibers and, reflexively, initiate micturition in response to a bitter stimulus in the urethral lumen (Deckmann et al., 2014). This has been interpreted as a protective reflex in that potentially hazardous content will be flushed out (Deckmann et al., 2014; Kummer and Deckmann, 2017). Voiding efficiency is augmented by sensory feedback from the urethra, where flow sensors are physiologically well characterized but not yet defined anatomically (Todd, 1964; Peng et al., 2008; Danziger and Grill, 2015). Thus, mechanosensitivity of cholinergic UBC may serve to augment the reflex response they have initiated.

To test for polymodal properties, cholinergic UBC were successively exposed to NaCl and ATP (N = 90; 70% responded to NaCl), to NaCl and denatonium (N = 36; 67% responded to NaCl), and to all three stimuli (N = 37; 65% responded to NaCl, Figure 5). When responses to both NaCl and denatonium were tested on 36 UBC, all three possible response patterns occurred in a balanced distribution (Figure 5): 42% NaCl only, 33% denatonium only, 25% both stimuli. These percentages are roughly reflected by the (immuno)histochemical (65% of UBC expressing Scnn1a-tdTomato signal) and by the NGS data with 4/6 cells (67%) expressing Scnn1a, and 2 of them (33%) expressing additionally a known receptor for denatonium, i.e., Tas2r108 (Figure 2). Of course, the small total number of cholinergic UBC subjected to NGS (N = 6) precludes a systematic quantitative analysis.

We have previously shown that a substantial number of denatonium-responsive UBC also reacts to monosodium glutamate (Deckmann et al., 2014). In terms of oropharyngeal gustation, these substances reflect an aversive (denatonium: bitter) and an attractive (monosodium glutamate: umami) stimulus, and, accordingly, are perceived by distinct cell populations, which still are considered as subtypes of type II taste cells (Chaudhari and Roper, 2010). The present data show...
an even broader diversity of UBC properties in that some of them share features also with type I cells of taste buds, expressing ENaC and being responsive to NaCl (Vandenbeuch et al., 2008). These findings further substantiate the polymodal character of cholinergic UBC. As far as further distinctive criteria are missing, we interpret the multiple combinations of responsiveness to various chemosensory stimuli and gene expression of related signaling components as phenotypic variation of a broadly tuned, polymodal chemosensory cell rather than defining multiple, clearly separated cell types.

CONCLUSION

In sum, we could show that a fraction of cholinergic UBC expresses α-ENaC and responds to the salty stimulus NaCl in an amiloride-sensitive manner. This feature does not define a new subpopulation of UBC, but rather emphasizes their polymodal character.

AUTHOR CONTRIBUTIONS

KD designed research and performed statistical analysis. KD, CK, PatS, PauS, MK, and SO performed research and analyzed data.

KD and WK obtained funding. KD, WK, AP, and MA drafted the manuscript. Work was supervised by WK and KD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00089/full#supplementary-material

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Frontiers in Cell and Developmental Biology | www.frontiersin.org 10 August 2018 | Volume 6 | Article 89

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