



Ectopically expressed olfactory receptors OR51E1 and OR51E2 suppress proliferation and promote cell death in a prostate cancer cell line

Received for publication, November 24, 2020, and in revised form, February 21, 2021 Published, Papers in Press, February 26, 2021,

<https://doi.org/10.1016/j.jbc.2021.100475>

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Olfactory receptors (ORs), the largest family of G protein-coupled receptors, are expressed in the nasal epithelium where they mediate the sense of smell. However, ORs are also found in other non-nasal tissues, but the role of these ectopic ORs in cell signaling, proliferation, and survival is not well understood. Here, using an inducible expression system in the lymph node carcinoma of the prostate (LNCaP) cell line, we investigated two ectopic ORs, OR51E1 and OR51E2, which have been shown to be upregulated in prostate cancer. We found that, consistent with previous studies, OR51E1 stimulated adenylyl cyclase in response to treatment by short-chain to medium-chain organic acids (C3–C9) but not by acetate. OR51E2 responded to acetate and propionate but not to the longer chain organic acids. Stimulation of LNCaP cells with butyrate inhibited their growth, and the knockdown of the endogenous OR51E1 negated this cytostatic effect. Most significantly, overexpression of OR51E1 or OR51E2 suppressed LNCaP cell proliferation. Overexpression of another ectopic OR OR2AT4, β 2-adrenergic receptor, or treatment of cells with forskolin did not suppress cell proliferation, indicating that a rise in cAMP is not sufficient to induce cytostasis. Overexpression of OR51E1 caused an upregulation of cytostatic and cell death markers including p27, p21, and p53, strongly increased annexin V staining, and stimulated extracellular signal-regulated protein kinases 1 and 2. Overexpression and/or activation of OR51E1 did not affect human embryonic kidney 293 cell proliferation, indicating that cytotoxicity of OR51E1/OR51E2 is specific for LNCaP cells. Together, our results further our understanding of prostate cancer etiology and suggest that ectopic ORs may be useful therapeutic targets.

G protein-coupled receptors (GPCRs) are responsible for the detection of a variety of extracellular stimuli that range from small molecules such as biogenic amines and metabolites to proteins. GPCR signaling regulates rapid cellular responses involving cAMP, Ca^{2+} , phosphorylation, and long-term processes such as gene expression and cell growth. GPCRs are the largest family of human genes (~800), and within GPCRs, the

largest subclass is olfactory receptors (ORs): there are more than 380 ORs in human genome (1).

Nomenclature describing ORs is independent between the species. Human receptors are called ORs (*olfactory receptors*) and classified with numbers and letters, *e.g.*, OR51E1, which is one of the receptors we are investigating in this article. The ~1000 mouse ORs are abbreviated as Olfr; Olfr558 is the ortholog of OR51E1 (2, 3). ORs were originally cloned from the nasal epithelium (4), but several years ago, expression of specific OR genes was detected in the airways, gut, blood vessels, brain, and other organs in rodents and human tissues and cell lines (2, 3, 5–10).

It is obvious that ectopic ORs do not take part in the perception of smell. Understanding of their function has lagged behind that of other GPCRs, and the majority of ORs remain to be orphan receptors (7, 11). One reason for this delay is that the expression of functionally active ORs and Olfrs has not been technically possible for many years. Most GPCRs can be produced in a functionally active state *in vitro* using standard methods such as transient transfection of model cell lines. ORs, however, require the presence of special chaperones and/or modification with signal peptides and other sequences for receptor stability and trafficking to the surface (12–14). These discoveries facilitated expression of ORs in specially engineered cell lines *via* transient transfection, thereby allowing investigation of their function. It was shown that ORs and Olfrs activate Gs and increase intracellular cAMP. However, it still has not been established if ORs can signal only through Gs or, like many nonolfactory GPCRs, can also activate other G proteins and/or other signal transduction pathways. Regardless, the ability of ORs to increase the cAMP level has been used for their deorphanization. These efforts have primarily been focused on finding ligands relevant to the sense of smell, and therefore, the currently established OR ligands are small volatile chemicals (11).

In this article, we investigate the function of two ORs, OR51E1 and OR51E2. They have been detected in several tissues and, as discussed later, are particularly interesting because of their association with prostate cancer. OR51E1 and OR51E2 are ~60% identical and have orthologs in mice. OR51E1 is ~95% identical to Olfr558, and OR51E2 is

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~93% identical to Olfr78. ORs are poorly conserved between species (15), and such high degree of conservancy is rare even for the GPCR family at large, which indicates that OR51E1 and Olfr558 play an essential physiological role. Deorphanization efforts showed that Olfr558 and Olfr78 can be activated by butyric and propionic acids, respectively (11, 16).

Studies in mice have shown that the *Olfr78* gene is expressed in blood vessels of the kidney, where this receptor can regulate blood pressure in response to propionic acid produced by gut microbiota (16). Olfr558 appears to be responsible for sensing nutrients such as butyrate and isovaleric acid in the gut (17). The human ortholog OR51E1 is also activated by butyrate and other aliphatic acids (18). Some investigators reported activation of OR51E2 by other chemicals, e.g., rose ketone β -ionone and lactate (19–22). However, other studies did not find agonism of β -ionone for OR51E2 (23). Such controversies in the OR field are not uncommon and are likely to be caused by the technical difficulties with detection and functional analysis of both endogenous and overexpressed ORs.

It has been established that the level of the OR51E1 and OR51E2 gene expression increases more than 10-fold in prostate cancer (24–26). Analysis of hundreds of human prostate specimens showed that this upregulation occurs in about two-thirds of malignant *versus* benign tumors or normal tissue (24, 27–30). Because of their association with prostate cancer, OR51E1 and OR51E2 have been referred to as prostate-specific GPCRs (PSGR2 and PSGR, respectively) (25, 27, 29). Changes in the expression level of OR51E2/PSGR in mouse models have been associated with activation of NF- κ B and protein kinases (28).

Here, we studied OR51E1 and OR51E2 in the human metastatic prostate cell line lymph node carcinoma of the prostate (LNCaP) using a novel expression system that allows robust detection and functional analysis of ORs. We show that activation of the endogenous OR51E1 and OR51E2 by their aliphatic acid agonists inhibits cell proliferation. Surprisingly, we discovered that overexpression of these ORs possesses not only the robust cytostatic effect but also causes cell death. Our data show that these effects are selective for OR51E1 and OR51E2 and provide initial insights into the mechanisms underlying these phenomena.

Results

OR51E1 and OR51E2 are endogenously expressed in the prostate cancer cell line LNCaP (Fig. 1A; (19, 23)). One of our goals was to study long-term effects of the OR51E1 upregulation, and for that purpose, we sought generation of a stable cell line. During our initial attempts, we found that none of the puromycin-resistant clones expressed the full-length receptor. We reasoned that high levels of OR51E1 were toxic to the cells, resulting in the selection of only the clones where its expression was suppressed. To circumvent this problem, we utilized an inducible system based on tetracycline promoter and augmented the OR51E1 complementary DNA (cDNA)

with sequences facilitating OR expression (13) and the FLAG tag for detection (Fig. 1, B–F); we similarly modified the cDNAs of other GPCRs that we used in our experiments.

Expression, stability, and activity of OR51E1

The selected puromycin-resistant cells had no detectable FLAG immunoreactivity prior to induction with doxycycline, showing the absence of significant leaking of the tetracycline promoter (Figs. 1, B and D and 2, A and B). Within several hours after induction, the cells produced the OR51E1 or OR51E2 proteins of the predicted ~40 kDa molecular weight, and as expected for glycosylated proteins such as GPCRs, the bands were fuzzy (Figs. 1 and 2). Indeed, a treatment with deglycosylation enzymes caused the main OR51E1 band to shift down to ~32 kDa, and the band also became sharper (Fig. 1C). About 20% of FLAG immunoreactivity was also detected in the area of ~70 kDa, which corresponds to the GPCR dimers, also a general characteristic of GPCR behavior on SDS-PAGE. Noteworthy, the commercial antibody against OR51E1, which we tested, did not detect any specific protein band in cells overexpressing the recombinant OR51E1 (Fig. 1F); this is not surprising given the notorious lack of activity of commercial anti-GPCR antibodies (31–33). According to immunofluorescence microscopy with the anti-FLAG antibody, overexpressed OR51E1 was present on the cell surface indicating effective trafficking to the plasma membrane (Fig. 1G).

To test the functional activity of recombinant ORs, we assayed cAMP using two independent methods: indirect, by detecting PKA-mediated phosphorylation (Fig. 2) and direct, using a novel fluorescence-based real-time cAMP biosensor (34–36) (Fig. 3).

The PKA activity was assessed using the antibody against the phosphorylated peptide corresponding to the phosphorylation consensus motif of PKA substrates RxxS*/T* (Fig. 2). This antibody revealed OR51E1-dependent phosphorylation of an 82 kDa protein. The identity of this protein is unknown, but it was also phosphorylated upon stimulation of LNCaP cells with forskolin (Fig. 2A), indicating that this phosphorylation occurs *via* the cAMP pathway. Phosphorylation of the p82 kDa protein depended on the OR identity and the nature of the stimulants. For example, OR51E1 was stimulated by valeric and nonanoic acids, whereas OR51E2 was not. Instead, OR51E2 was stimulated by acetate and propionate; OR51E1 was insensitive to acetate, and its response to propionate was weaker than to the longer aliphatic acids (Fig. 2, C and D). As a control in our studies, we also expressed a different OR, OR2AT4; it had the expected apparent molecular weight and an expression level similar to OR51E1 and OR51E2. OR2AT4 was not activated by either propionic or butyric acids; however, it did not respond to its purported agonist, Sandalore (37, 38) either (Fig. 2B). We also did not detect phosphorylation of p82 in OR51E1- and OR51E2-expressing cells upon stimulation with β -ionone (Fig. 2, C and D).

Direct measurement of cAMP using the fluorescent biosensor showed a rapid and reversible cAMP rise upon

application of butyrate in the cells expressing OR51E1. Such response did not occur in control LNCaP cells or the cells expressing OR51E2 (Fig. 3A). Under the same conditions, cAMP was not increased upon stimulation of OR51E1- or OR2AT4-expressing cells with Sandalore (Fig. 3B).

Since some investigations implicated ORs in stimulation of free Ca^{2+} fluxes (21, 23), we tested if in addition to cAMP, activation of ORs could influence Ca^{2+} in our system (Fig. 3, C and D). We used our LNCaP cell lines expressing OR51E1 and

OR2AT4, the OR that was previously reported to elicit Ca^{2+} responses (37). We also generated human embryonic kidney 293 (HEK293) cell lines expressing these two ORs and used the prototypical Gq-coupled GPCR M3 muscarinic cholinergic receptor (M3R) as a positive control. OR51E1 expressed in HEK293 cells responded to butyrate and some other related compounds, as evident from our PKA assay (Fig. 3D), indicating the receptor was functionally active. As expected, stimulation of M3R by its agonist oxotremorine M caused robust Ca^{2+} responses (Fig. 3E). Butyrate, valerate, or

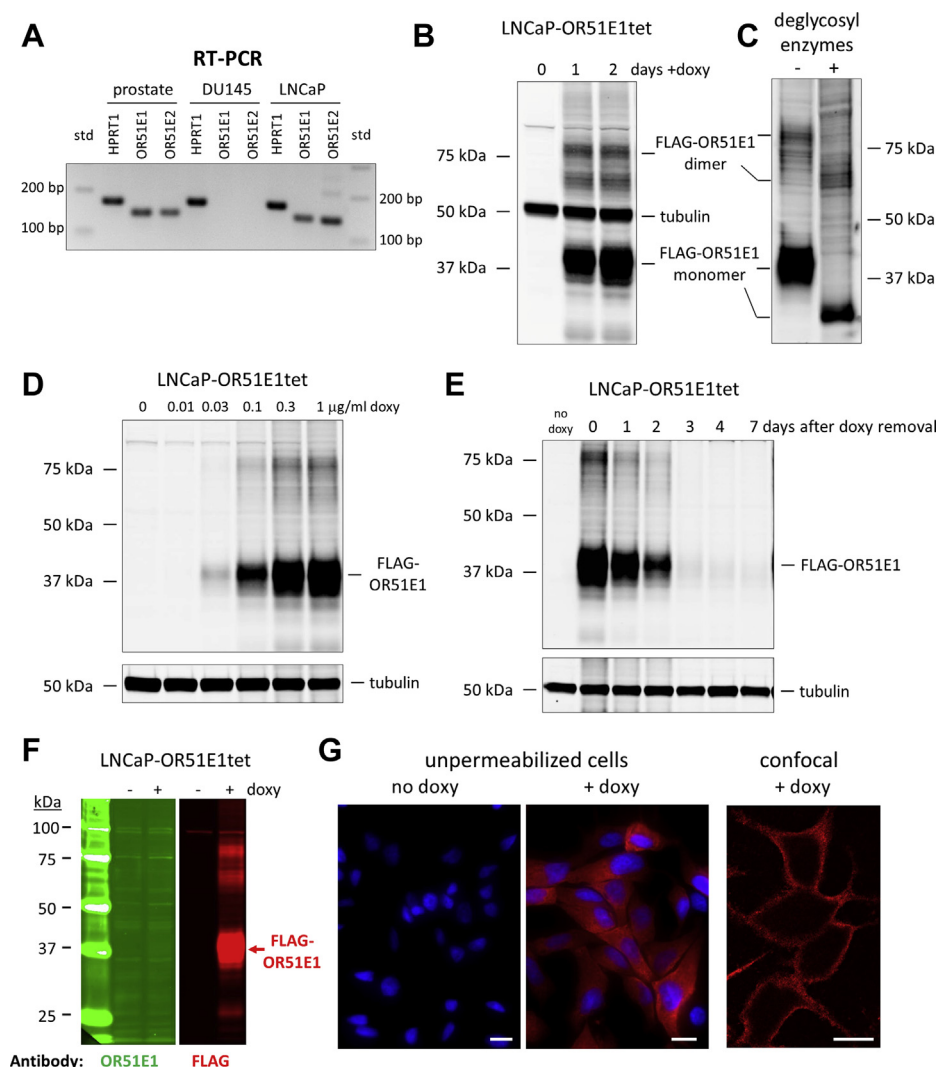


Figure 1. Expression of the endogenous and recombinant OR51E1 in LNCaP cells. A, *OR51E1* gene is endogenously expressed in LNCaP cells. RNA was isolated from the human prostate DU145 and LNCaP cells and subjected to RT-PCR with primers specific to *HPRT1* (a housekeeping gene), *OR51E1* and *OR51E2*. B, overexpression of OR51E1 in LNCaP cells. LNCaP-OR51E1tet cells were generated and analyzed as described in Experimental procedures section. The cells were grown and induced with 1 $\mu\text{g}/\text{ml}$ doxycycline or buffer (0), then harvested after 1 or 2 days. The total lysates were subjected to the Western blot analysis with anti-FLAG and antitubulin antibodies. Positions of protein markers are indicated on the left. C, overexpressed OR51E1 is glycosylated in LNCaP cells. The extract from cells expressing OR51E1 was left untreated (–) or treated with the deglycosylation enzyme mix (+), and the samples were analyzed using Western blot with anti-FLAG antibodies. D, dose dependence of OR51E1 expression on doxycycline concentration. LNCaP-OR51E1tet cells were grown in the presence of the indicated concentrations of doxycycline for 2 days and then subjected to the Western blot analysis as in B. E, inducible expression of OR51E1 is reversible. LNCaP-OR51E1tet cells were grown in the presence of 1 $\mu\text{g}/\text{ml}$ doxycycline for 3 days. Doxycycline was then removed for the indicated number of days (0–7), after which the cells were harvested and subjected to the Western blot analysis with anti-FLAG and antitubulin antibodies. F, a commercial antibody against OR51E1 does not work. LNCaP-OR51E1tet cells were grown without or with doxycycline and then subjected to the Western blot analysis. The same membrane was costained with rabbit anti-OR51E1 (green) and mouse anti-FLAG (red) antibodies. G, overexpressed OR51E1 is successfully trafficked to the cell surface. LNCaP-OR51E1tet cells were grown either without or with 1 $\mu\text{g}/\text{ml}$ doxycycline for 2 days, then fixed, stained with the anti-FLAG antibody (red) without cell membrane permeabilization and analyzed by open-field microscopy. Blue—staining of the nuclei with 4',6-diamidino-2-phenylindole. The image on the right—the cells were permeabilized before staining with the antibody, then stained, and analyzed using confocal microscopy. White scale bars represent 20 μm . LNCaP, lymph node carcinoma of the prostate.

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Sandalore did not influence Ca^{2+} in cells overexpressing either M3R, OR2AT4, or OR51E1 (Fig. 3F).

Our data from the real-time biosensor and PKA phosphorylation assays were consistent with our measurements of cAMP using the PerkinElmer TR-FRET kit (PerkinElmer), which we will report elsewhere. Here, to further characterize agonist specificity of OR51E1, we tested 15 structurally related compounds for their ability to elevate cAMP *via* OR51E1 and found that eight of them activated the receptor and seven did not (Fig. 4). Overall, our results on activation of OR51E1 by aliphatic acids but not structurally similar compounds (Fig. 4) were consistent with those earlier studies where the OR

expression at the protein level was convincingly demonstrated (11, 18). Having established the functional activity of the overexpressed OR51E1 and OR51E2, we focused on their cancer-related properties, *i.e.*, effects on cell growth and survival.

Cytostatic effect of OR51E1 and OR51E2 on LNCaP cells

Incubation of control LNCaP cells with 0.3 mM propionate or 0.1 mM butyrate caused a considerable reduction of their growth rate. These results were consistent with the earlier report by Hatt *et al.* (30) that treatment of LNCaP cells with

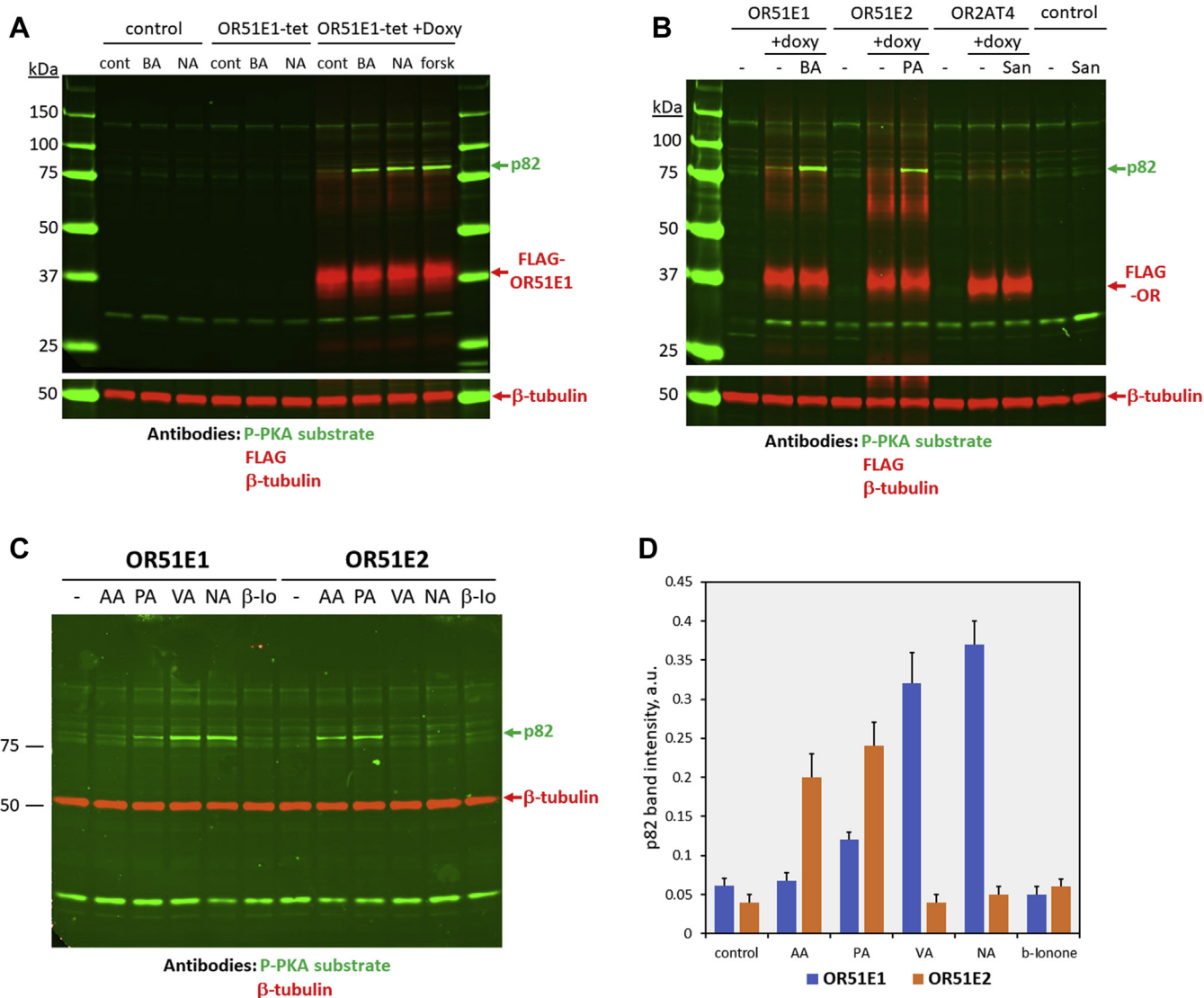


Figure 2. Stimulation of overexpressed ORs with their known agonists results in activation of PKA. A, control LNCaP cells and uninduced or doxycycline-induced LNCaP-OR51E1tet cells were incubated for 10 min with either 1 mM butyric acid (BA), 1 mM nonanoic acid (NA), or 10 μ M forskolin. Cells were then harvested and analyzed by Western blot with anti-FLAG (red) and anti-phospho-PKA substrate (green) antibodies (see text for details). Note the appearance of a green protein band with a molecular weight of \sim 82 kDa (p82). Panel below, the same membrane was later reprobbed with an antitubulin antibody (red). B, LNCaP-OR51E1tet, LNCaP-OR51E2tet, LNCaP-OR2AT4tet, and control LNCaP cells were grown, and then the expression of the ORs was induced with 1 μ g/ml doxycycline for 1 day. The cells were then stimulated with the putative agonists of the ORs for 10 min or incubated with vehicle. PA, 1 mM propionic acid; and San, 0.5 mM Sandalore. The expression of the receptors and PKA activity was analyzed by immunoblot as in A. C, doxycycline-induced LNCaP-OR51E1 and LNCaP-OR51E2 cells were treated for 10 min with 1 mM of either acetic acid (AA), PA, valeric acid (VA), or NA, or 0.25 mM β -ionone (β -lo). They were harvested and subjected to the Western blot analysis with anti-phospho-PKA substrate (green) and antitubulin (red) antibodies. D, quantification of the results in C. The intensity of p82 band was measured and normalized to the intensity of the tubulin band in the same lane. Bars show the mean values for OR51E1 and OR51E2 for each agonist; the experiment was performed 3 times. ORs, olfactory receptors.

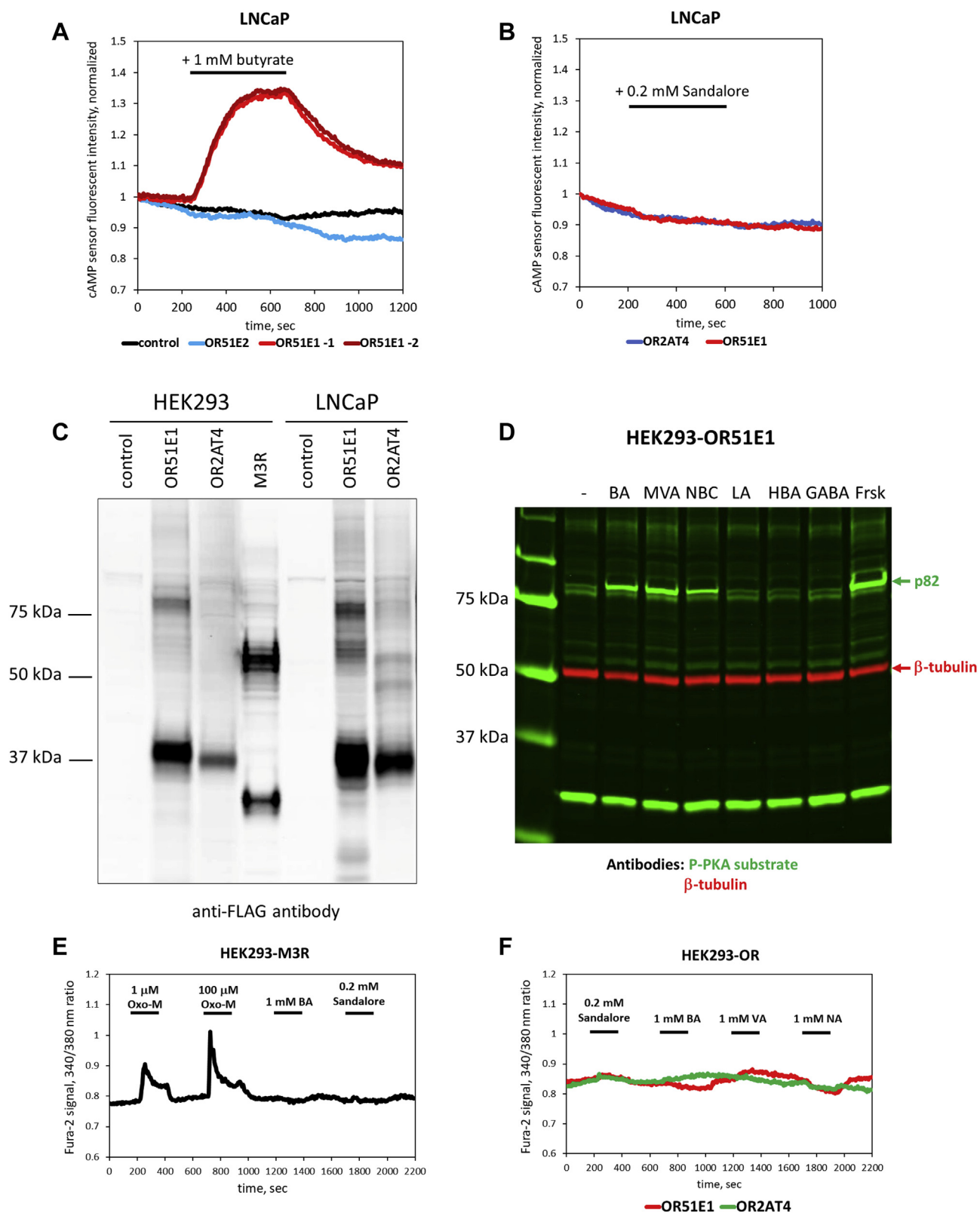


Figure 3. Stimulation of OR51E1 elevates intracellular cAMP but not free Ca^{2+} . A, control LNCaP cells and induced LNCaP-OR51E1tet or LNCaP-OR51E2tet lines were infected with BacMam virus encoding the upward cAMP sensor. Cells were then challenged with 1 mM butyrate for the indicated periods, and the sensor fluorescent intensity was measured in a flow cell using a fluorescence microscope, as described in [Experimental procedures](#) section. The experiment was performed 5 times; representative traces are shown. B, LNCaP-OR51E1tet or LNCaP-OR2AT4 lines were transduced with the BacMam cAMP sensor. Cells were challenged with 0.2 mM Sandalore for the indicated periods, and the sensor fluorescent intensity was measured. The experiment was performed 3 times; representative traces are shown. C, Western blot analysis (anti-FLAG antibody) of inducible cell lines overexpressing OR51E1, OR2AT4, and muscarinic M3 (M3R) receptors after a 2-day induction with doxycycline. Note that the receptor expression levels were similar between HEK293 and LNCaP lines. D, doxycycline-induced HEK293-OR51E1 cells were treated for 10 min with 1 mM of either butyric acid (BA), 3-methylvaleric acid

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OR51E1 agonist nonanoic acid induced cell senescence. We also found that when butyrate and propionate were added together, the cytostatic effect became stronger (Fig. 5A). It is not yet clear whether OR51E1 and OR51E2 activate the same cytostatic mechanism or engage distinct pathways. Importantly, we found almost no effect of these aliphatic acids on another prostate cancer cell line, DU145, which does not express *OR51E1* and *OR51E2* genes (Fig. 1A). The combination of butyrate and propionate had a statistically significant inhibitory effect on the growth of DU145 cells. This can be explained by the presence of other receptors or other mechanisms activated by the high concentrations of these aliphatic acids. In LNCaP cells, the involvement of OR51E1 in suppression of cell proliferation was shown by the shRNA-mediated knockdown of OR51E1 in LNCaP, which eliminated the cytostatic effect of butyrate (Fig. 5B). Interestingly, using our methods, we were unable to detect butyrate-mediated upregulation of cAMP stimulation of control LNCaP cells (Figs. 2A and 3A). Evidently, a detectable rise in cAMP requires a higher level of the receptor.

To facilitate the studies of overexpressed ORs on cell proliferation, we used the Incucyte (Sartorius) instrument (Fig. 6). This is a robotic microscope that can take images of cells during their long-term culture in an incubator and is capable of quantitative image analysis, e.g., calculation of cell confluence. As expected, under our conditions, the untreated LNCaP cells grew exponentially with a doubling time of about 28 h. After induction of OR expression with 1 $\mu\text{g}/\text{ml}$ doxycycline, their growth notably slowed down and virtually stopped after 24 h. The suppression was dependent on concentration of doxycycline ($\text{EC}_{50} = 0.1 \mu\text{g}/\text{ml}$), and the timing of the cytostasis onset corresponded to the appearance of the FLAG-tagged OR51E1 (Fig. 1B). The suppression of cell growth was also reversible: after the change to doxycycline-free medium, the level of OR51E1 dropped within 1 to 3 days (Fig. 1D), and the cells resumed proliferation (data not shown). These results supported our hypothesis that high level of the functionally active OR51E1 was toxic to the LNCaP cells.

Ligand, receptor, and cell-type specificity of OR-mediated cytostasis

To test if agonist stimulation could enhance the cytostatic effect of OR51E1 overexpression, we stimulated cells with butyrate after inducing the expression of OR51E1 by doxycycline (Fig. 6B). The addition of butyrate virtually stopped cell proliferation.

Next, we tested if the effect of OR51E1 was unique or if overexpression of other GPCRs could also suppress LNCaP cell growth. We analyzed cells that expressed OR51E2 and OR2AT4; we also generated an LNCaP cell line expressing the prototypical Gs-coupled GPCR, β_2 adrenergic receptor ($\beta_2\text{AR}$). Since all the

receptors were FLAG tagged, we were able to roughly compare their expression level by immunoblot with anti-FLAG antibody. The expression of $\beta_2\text{AR}$ was at least 10-fold higher than that of OR51E1, whereas the levels of all three ORs were similar. To compare the effects on cell growth, we decreased doxycycline for $\beta_2\text{AR}$ -expressing cells to titrate the $\beta_2\text{AR}$ level down approximately to the levels of OR51E1, OR51E2, and OR2AT4 (Fig. 6E). We found that under these conditions, $\beta_2\text{AR}$ had no effect. Interestingly, at the approximately 10-fold higher expression level, $\beta_2\text{AR}$ could also reduce LNCaP cell growth and OR2AT4 also had detectable cytostatic activity (Fig. 6D). However, OR51E1 and OR51E2 inhibited cell proliferation much more effectively than either $\beta_2\text{AR}$ or OR2AT4.

The observed cytostatic effect could potentially be explained by the increase in cAMP signaling because of stimulation of OR51E1 with butyrate in both control and OR51E1tet cells. However, our results showed that cytostasis occurs even when OR51E1 expression is induced in the absence of an agonist (Fig. 6, B, C, and F). This indicates that the overexpressed receptor possesses a basal cytostatic activity that may be independent of cAMP. To test this idea, we grew LNCaP cells in the presence of the direct adenylyl cyclase activator forskolin, which was applied at the concentration (1 μM) sufficient to saturate PKA-mediated phosphorylation (Figs. 2A and 3D). Consistent with previous studies, which did not detect forskolin-mediated cytostasis or even observed a slight mitogenic activity (e.g., (39)), our data showed no forskolin effect (Fig. 6F). It thus appears that elevation of cAMP is not sufficient to inhibit cell proliferation, and OR51E1 can engage additional mechanisms.

We then asked if OR51E1 could cause cytostasis in cells other than LNCaP. We found that OR51E1 overexpression had no effect on proliferation of HEK293 cells (Fig. 6G). Yet, activation of OR51E1 with butyrate and other agonists stimulated cAMP and PKA activity (Fig. 3D). These findings suggest that LNCaP cells not only endogenously express OR51E1 and OR51E2 but also possess a unique downstream pathway that is coupled to these ORs and can control proliferation of these cells.

OR51E1 overexpression promotes LNCaP cell death

To begin investigation of the molecular mechanism(s) underlying the OR51E1/OR51E2-mediated suppression of LNCaP cell proliferation, we tested the behavior of markers for the state of cell cycle, signal transduction, and cell death. We found that after doxycycline-induced expression of OR51E1, the cells became positive for staining with annexin V (Fig. 7, A and B), a marker of early/late stage apoptosis and necrosis. This did not occur in identically treated control LNCaP cells.

We also found that upregulation of OR51E1 increased phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Fig. 7C). Activation of ERK required

(MVA), 5-norbornene-2-carboxylic acid (NBC), lactic acid (LA), 3-hydroxybutyric acid (HBA), gamma-aminobutyric acid (GABA), or 1 μM forskolin (Frsk). Harvested cells were subjected to the Western blot analysis with anti-phospho-PKA substrate (green) and antitubulin (red) antibodies. E and F, doxycycline-induced HEK293-M3Rtet, HEK293-OR51E1tet, and HEK293-OR2AT4tet lines were used to test changes in intracellular free calcium concentration using Fura-2 dye. Cells were challenged with the indicated concentrations of muscarinic agonist oxotremorine-M (Oxo-M), BA, valeric acid (VA), nonanoic acid (NA), and Sandalore. The experiment was performed 3 times; representative traces are shown. HEK293, human embryonic kidney 293 cells; LNCaP, lymph node carcinoma of the prostate.

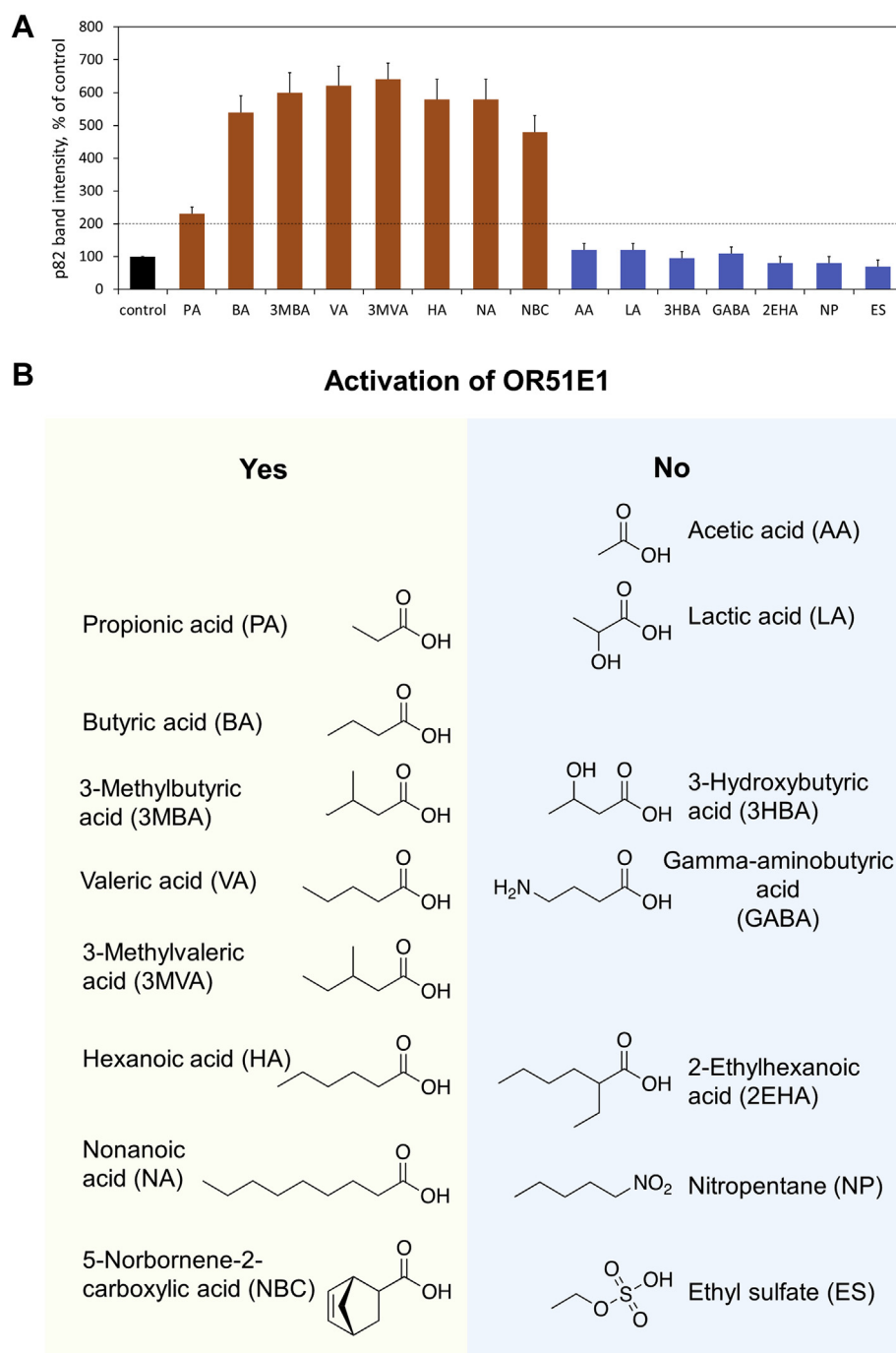


Figure 4. Compounds tested with OR51E1. A, a summary of the tested compounds activity at 1 mM in the PKA stimulation assay. The experiments were performed 3 to 5 times for each compound, and the bar graphs show mean \pm SD for these experiments. B, structures of the compounds tested for their ability to stimulate intracellular cAMP *via* OR51E1 at 1 mM. A compound was considered active if it produced a response at least 100% above the basal value (control) in the PKA activity assay.

at least 24 h, suggesting that the OR51E1 pathway involves a rather slow series of molecular events. In similar experiments with other ORs, activation of ERK1/2 occurred upon the induced expression of OR51E2 but not OR2AT4.

Probing the cell lysates with antibodies against other markers showed an increase in cleaved poly(ADP-ribose) polymerase

(Fig. 7D), which is often referred to as a marker of apoptosis. We also detected upregulation of a tumor suppressor protein p53. Activation of p53 can lead to upregulation of cell cycle inhibitors p27 Kip1 and p21 Waf1/Cip1, which we indeed observed. Thus, it appears that upregulation of OR51E1 suppresses cell growth *via* cell cycle arrest and by promoting cell death.

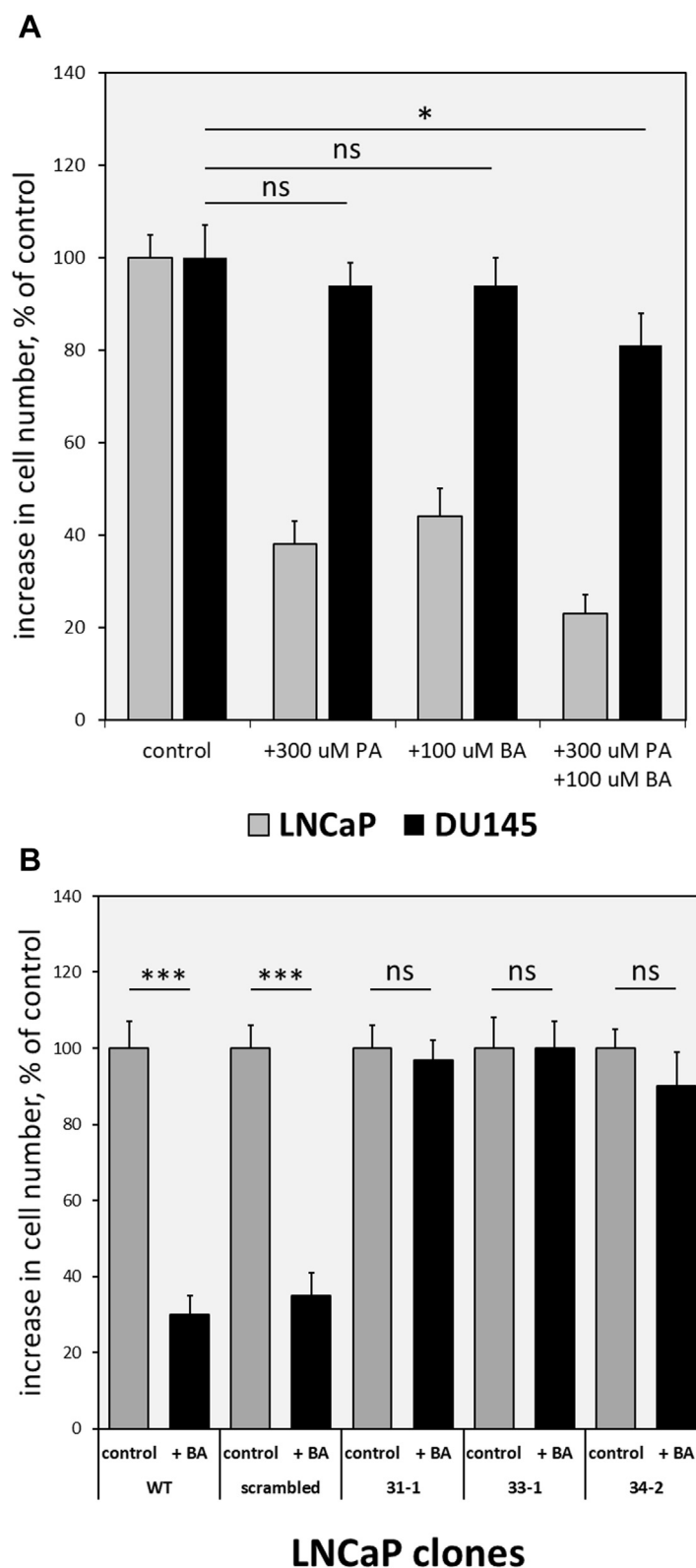


Figure 5. Butyrate inhibits proliferation of LNCaP cells via endogenous OR51E1. *A*, control LNCaP (gray) and DU145 (black) cells were grown either in the absence of any compounds (control) or in the presence of the indicated concentrations of butyrate acid (BA) and propionate acid (PA). The cells were counted after 7 days in culture as described in [Experimental procedures](#) section. The increase in the cell number in untreated cells was set as 100%, and the increase in treated cells was normalized to this value. The experiment was performed 4 times, and the bar graphs show mean \pm SD for these experiments. *B*, WT LNCaP cells, a stable clone expressing a scrambled shRNA, and three different stable clones expressing three different OR51E1 shRNA constructs (e.g., 31-1) were grown for 7 days either in the absence (control) or in the presence of 0.1 mM butyrate (BA) and then counted as in *A*. The experiment was performed 3 times. * $p < 0.05$ and *** $p < 0.001$. LNCaP, lymph node carcinoma of the prostate; ns, not significant.

Discussion

Despite the recently increased interest in ectopically expressed ORs, the role of this large and ubiquitous gene family remains understudied. Functional analyses are hindered

by the absence of ligands for most ORs, and there are technical challenges with the detection of these GPCRs at protein and mRNA levels. Although there are rare exceptions (40), antibodies against GPCRs are notoriously ineffective ((31–33); e.g.,

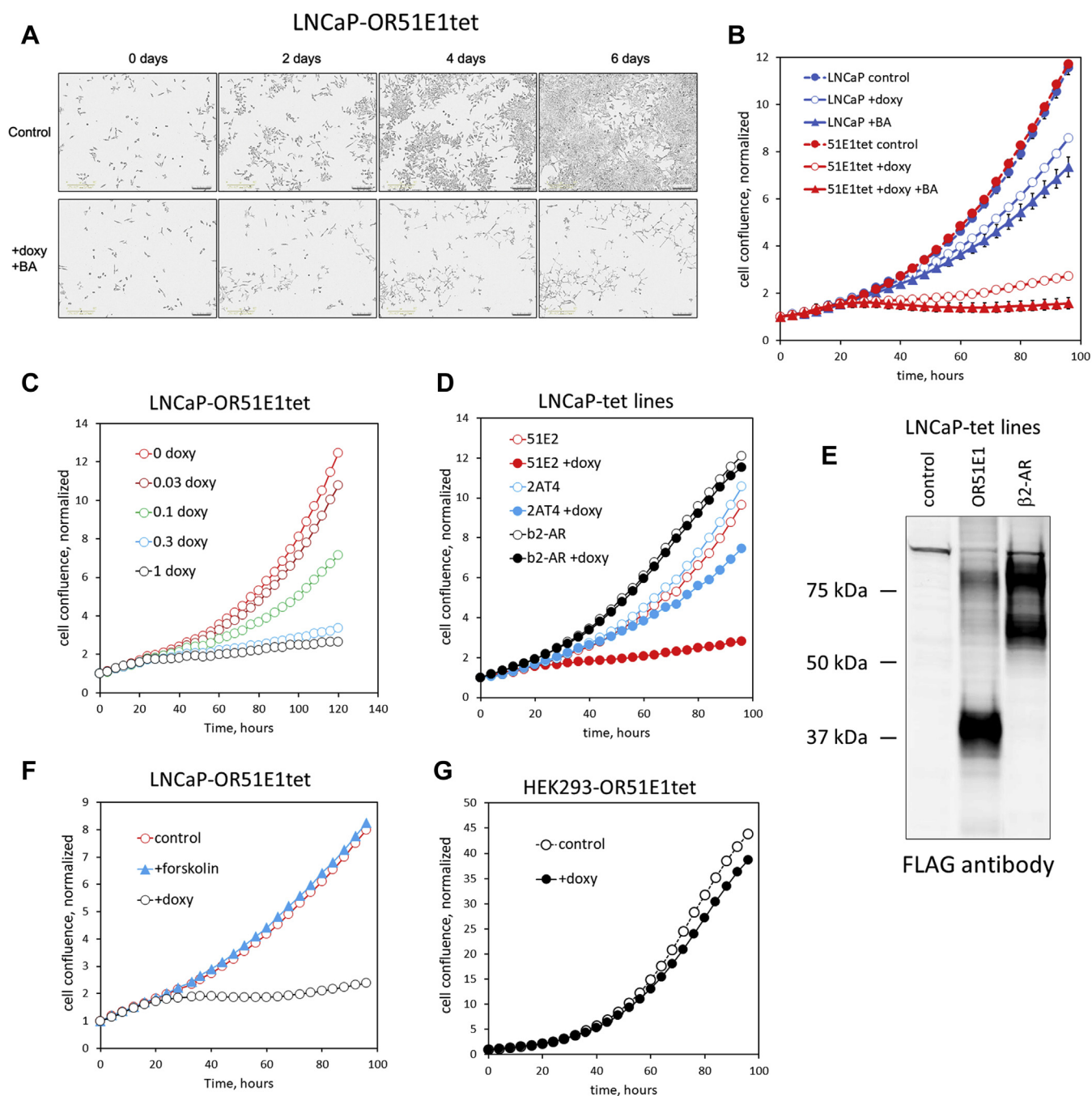


Figure 6. Overexpression of OR51E1 inhibits LNCaP cell proliferation. A, LNCaP-OR51E1tet cells were grown in Incucyte as described in [Experimental procedures](#) section. Cells were either induced in the presence of 1 $\mu\text{g/ml}$ doxycycline (doxy) and 0.2 mM butyrate (BA) or left untreated (control), and phase contrast images were taken at the indicated times. The experiment was performed more than 5 times; representative photographs are shown. B, control LNCaP cells and LNCaP-OR51E1tet cells were grown in Incucyte either in the absence or in the presence of 1 $\mu\text{g/ml}$ doxy and/or 0.2 mM BA, and cell confluence was monitored. The experiment was performed 4 times, and each data point represents the mean \pm SD. Some error bars are within the symbols. C, LNCaP-OR51E1tet cells were grown in the presence of the indicated concentrations of doxy (micrograms per milliliter). The experiment was performed 3 times. D, inducible LNCaP-tet lines overexpressing OR51E2, OR2AT4, or β 2-adrenergic receptor (β 2-AR) were grown either in the absence or in the presence of doxy (+doxy) experiment performed 3 times. E, Western blot analysis of induced LNCaP-tet lines overexpressing OR51E1 or β 2-AR. F, LNCaP-OR51E1tet cells were grown either in the absence (control) or in the presence of 1 μM forskolin or 1 $\mu\text{g/ml}$ doxy. G, HEK293-OR51E1tet cells were grown either in the absence (control) or in the presence of 1 $\mu\text{g/ml}$ doxy, and cell confluence was monitored. The experiment was performed 3 times. HEK293, human embryonic kidney 293 cells; LNCaP, lymph node carcinoma of the prostate; tet, tetracycline.

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Fig 1E), and we have not been able to find an example in the literature where antibodies detected an endogenous OR protein with all appropriate controls. Moreover, most OR and other GPCR genes are monoexonic, which may cause amplification of the genomic DNA contaminating the samples, resulting in false-positive detection of mRNA upon RT-PCR and/or transcriptomics. Without unambiguous demonstration that a given OR was actually present or absent in the studied cells or tissue, the functional effects of the putative ligand could be explained by its action on another target(s).

In this article, we report novel findings about ectopic OR51E1 and OR51E2, the two ORs that have been studied relatively well. The mouse homologs of these receptors, *Olf558* and *Olf78*, were deorphanized several years ago, and the agonism of short-to-medium carbonic acids was confirmed for the human orthologs OR51E1 and OR51E2 (18). The expression pattern of these receptors was investigated in mice, other animals (41), and human tissues and cell lines (21, 42–45). In mice, the expression of *Olf78* gene was shown in the kidney blood vessels where it mediates regulation of blood pressure by propionate produced by the gut microbiome (16). In the carotid body, this receptor is proposed to regulate blood flow through detection of lactate produced in hypoxia (22). In both studies, the function of *Olf78* was supported by analyses of the gene knockout. We discovered the expression of the *Olf558* gene in the ocular tissues, particularly a subset of arterioles, using *in situ* mRNA hybridization (46). In humans, the presence of OR51E1 was reported in several cell lines and tissue biopsies (e.g., (42, 43)), but many of these studies rely only on RT-PCR or unvalidated antibodies and should be interpreted with caution. The most well-established site of OR51E1 and OR51E2 expression is the prostate, where the pathologically high expression is linked to malignant cancer (24, 25, 47, 48). Both genes are highly expressed in LNCaP cells (30, 42), and so we chose this model to study their signaling and potential oncogenic activity.

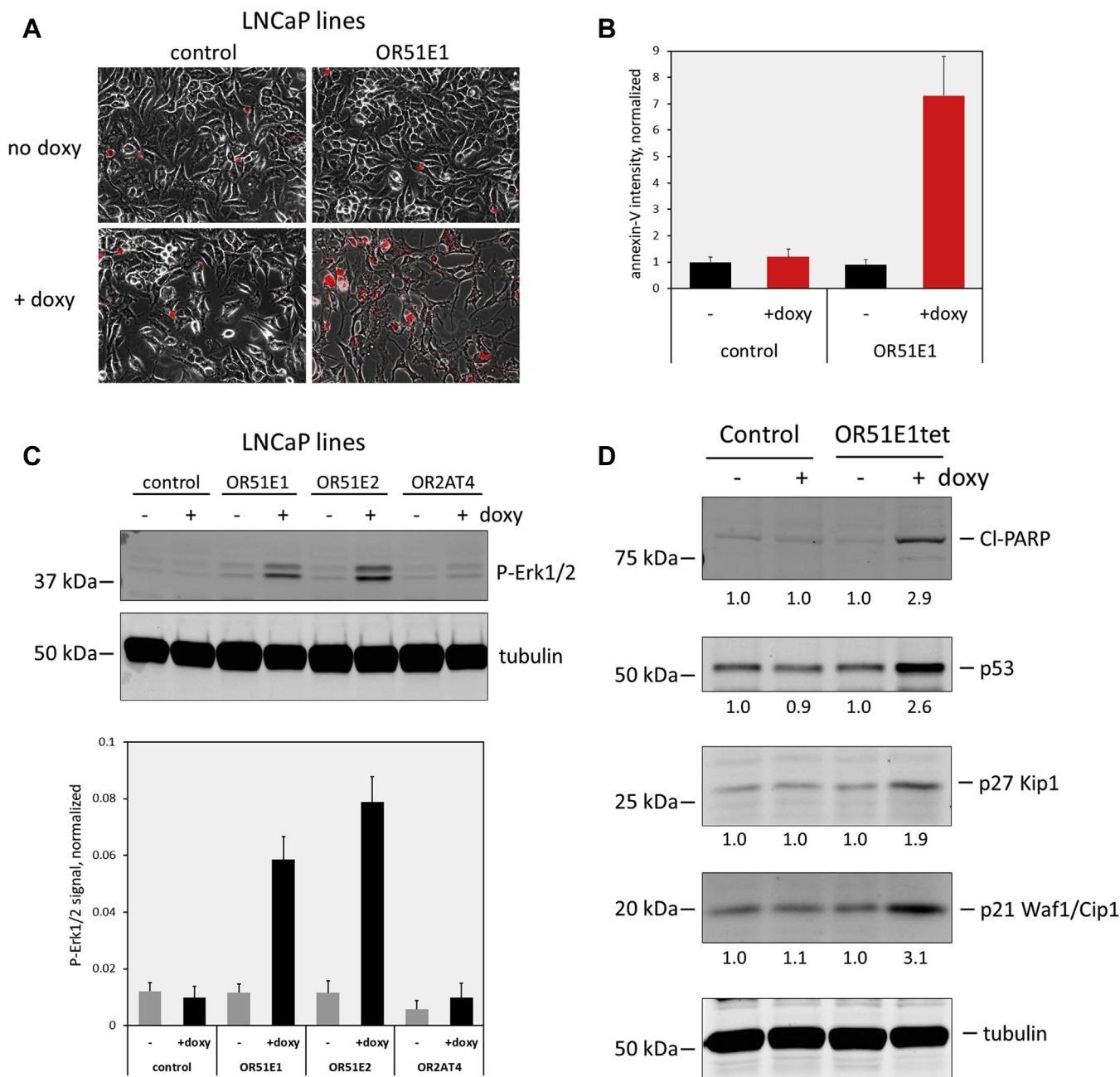
Prior to investigating the effects of OR51E1 and OR51E2 on LNCaP cell biology, we devoted a considerable effort to control experiments validating the functionality of the overexpressed receptors. Our immunoblot and microscopy results show robust OR protein expression beyond any doubt, which allowed us to correlate the functional changes with the presence and the level of the receptor. Only a few other articles demonstrated that the investigated ORs were actually expressed upon transfection in HEK293 cells and showed membrane localization (13, 14, 18, 49). With the endogenous OR51E1 and OR51E2, our experiments were limited to detection of mRNA because good antibodies for native proteins are not available. We demonstrated, however, that the diminished sensitivity to butyrate is associated with the knockdown of this receptor (Fig. 5). Furthermore, the OR51E1 is one of the few ORs that has two exons, and, as in the earlier study (46), we used primers that flank the sole intron. In our experiments, OR51E1 and OR51E2 mediated activation of adenylyl cyclase by aliphatic acids, and the cAMP responses to these ligands only occurred in the presence of OR. The agonist specificity was consistent with previous studies (18): OR51E1

responded to acids with longer aliphatic groups than OR51E2, which prefers propionate (Fig. 2). Together, these findings demonstrated robust expression and functional activity of these receptors.

Along with the results from other laboratories, our data began to shed light on structural requirements for OR51E1 agonists (Fig. 4). One key requirement is the presence of a carboxyl group at one end of the ligand. It seems that it cannot be replaced with a sulfate group, as ethyl sulfate was inactive. The hydrophobic tail should be at least two carbons long, since acetate was inactive, while propionate stimulated the receptor. The hydrophobic tail allows for some branching, as evident from the activity of, e.g., 3-methylbutyrate. In contrast, it appears that hydrophilic substitutions are not tolerated: neither 3-hydroxybutyrate nor gamma-aminobutyrate activated OR51E1. More work will be needed to better understand the structure–activity relationship for OR51E1 ligands, and our expression system provides an effective platform for such analyses.

Understanding the potential involvement of OR51E1/OR51E2 in cancer requires investigation of not only rapid signaling such as activation of adenylyl cyclase but also events on a longer time scale. While transient transfection was successfully used for deorphanization (11, 18), it does not allow keeping cells over several division cycles. Therefore, we generated stable LNCaP cell lines that can be maintained indefinitely and induced to express ORs in culture. Using these cells permitted us to perform well-controlled experiments comparing cell proliferation rate. Our central finding is that overexpression of OR51E1 and OR51E2 has a strong cytostatic effect, and that this effect is, at least in part, determined by the ability of these receptors to promote cell death. While this conclusion is supported by cell morphology, annexin V staining, and other markers (Fig. 7), elucidation of the specific cell death mechanism will require further experimentation.

It seems intuitively that a gene overexpressed in cancerous cells should drive uncontrolled proliferation, cell motility, or other properties associated with malignancy. Our data are unexpected because they show that OR51E1 and OR51E2 not only do not promote growth but also are toxic to cells. This conclusion is supported by both the overexpression and knockdown of the endogenous receptors (Fig. 5). The cytostatic activity appears to be specific for these two receptors and their ligands, which also suggests that the observed phenomena are physiologically relevant. At the moment, we can only speculate why these two ORs are upregulated in cancer, and if there is, indeed, a strong cause-and-effect relationship between this upregulation and prostate oncogenesis *in vivo*. Nevertheless, our findings allow us to propose two hypotheses that may explain the observed phenomena. One is that these ORs are responsible for the negative control of proliferation in the normal prostate epithelium. Cancerous transformation impairs this mechanism, e.g., by a mutation in the downstream signaling; the cells increase the expression of these OR genes to control the aberrant growth, but the mechanism is no longer effective. An alternative idea is that the increase in the OR51E1/OR51E2 level occurs to compensate for deficiency of



the endogenous agonist. The evidence that these receptors can be agonized by short aliphatic acids is overwhelming, but it is not known if they are the only physiological stimulators of these ORs. It is possible that in human prostate epithelial cells, OR51E1 and OR51E2 are involved in contact inhibition by sensing the presence of other cells. Such mechanism can

explain why stimulation of ORs or overexpression, which presumably mimics the basal activity, can stop proliferation of LNCaP cells *in vitro*.

In addition to the discovered role of OR51E1/OR51E2 in cytotoxicity and cytostasis, there are two other aspects of our work that warrant discussion. The first one concerns some

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differences between our findings and previous publications. We did not detect cAMP responses to β -ionone, a chemical often referred to as an agonist of OR51E2 (21, 23, 44) and to several other potential OR51E2 agonists identified in a computational screen (19). Previous studies showed that β -ionone can influence both cAMP and Ca^{2+} in human retinal pigment epithelium and melanoma cells (21, 23, 44). In our hands, while the observed cAMP responses to aliphatic acids were robust and comparable to those elicited by forskolin, we detected no Ca^{2+} responses to any ligands in our cells overexpressing functional ORs. We used OR2AT4 as a negative control in our cytostasis and other experiments and found that its putative agonist Sandalore (37) stimulated neither Ca^{2+} nor cAMP responses, even though the full-length OR2AT4 was present. One possible explanation of the controversies is that in some of the earlier investigations the functional OR protein was absent in the tested cells because the cDNA lacked the modifications necessary for receptor expression. As mentioned previously, experiments with anti-OR antibodies did not have reliable controls, whereas the putative ligands may have acted *via* OR-unrelated mechanisms, including those involved in the luciferase-based cAMP assays used in some of those studies. Indeed, shRNA experiments failed to prove the association with OR51E2 activity in retinal pigment epithelium cells (44). Furthermore, in some of the earlier studies, Ca^{2+} responses used to deorphanize ORs had very slow kinetics that are not typical for G protein signaling. On the other hand, some of the differences between our results and previous reports could be explained by the distinct behavior of ORs in various cellular systems. Indeed, our data show that in HEK293 cells OR51E1 does not cause cytotoxicity, and it is known that GPCRs can couple to distinct downstream pathways in different environments.

Another potentially important finding revealed in our study is the apparent lack of a direct connection between the ability of OR51E1/OR51E2 to upregulate cAMP and their effect on cell proliferation and survival. Consistent with earlier studies (39), we found that forskolin does not inhibit cell growth, indicating that rising cAMP and activation of PKA are not sufficient to induce cytostasis. In contrast, even in the absence of their agonists, overexpression of these ORs is sufficient to inhibit growth and promote cell death. The simplest explanation is that the basal activity of these ORs is biased toward the pathway that is distinct from the *G_s*-cAMP signaling. Thus far, we identified only some of the downstream effects of this pathway: upregulation of cell cycle arrest and cell death markers and activation of ERK1/2. Elucidating the pathway that connects OR51E1/OR51E2 to these events will show if targeting these mechanisms may be beneficial for understanding and treatment of prostate cancer.

Experimental procedures

Chemicals

Aliphatic acids, forskolin, and other reagents were purchased from MilliporeSigma. Stock solutions of all compounds

tested as potential agonists of ORs were adjusted such that their pH was ~ 7.2 . Sandalore was a generous gift from Dr R. Paus (University of Miami).

Antibodies

Anti-FLAG (catalog no. F1804) and anti-OR51E1 (catalog no. SAB2700210) antibodies were from MilliporeSigma. The following antibodies were from Cell Signaling Technologies: tubulin (catalog no. 86298), phospho-PKA substrate (catalog no. 9621), phospho-ERK1/2 (T202/Y204; catalog no. 9101), cleaved poly(ADP-ribose) polymerase (catalog no. 9541), p27 Kip1 (catalog no. 3698), and p21 Waf1/Cip1 (catalog no. 2947). Anti-p53 antibody (catalog no. sc-126) was from Santa Cruz Biotechnology, Inc. PE-annexin-V was from BD Biosciences (catalog no. 556421).

DNA constructs

The cDNA encoding human $\beta 2$ -adrenergic and M3R receptors in pcDNA3.1 were purchased from cDNA.org. Open reading frames of OR51E1, OR51E2, and OR2AT4 were cloned out of human genomic DNA using PCR. Human genomic DNA was purified from LNCaP cells. All receptor cDNAs were first subcloned into pcDNA3.1 vector containing the following sequence: 5'-GGATCCgtgtgccaccatgagaccatgatcctgtgctcctggccctgctgaccctaggcctggctGACTACAAAGACGATGACGACAAGatgaacgggaccgaggcccaacttctacgtgcctttctccaacaagacgggctgtgtGGCGCGCCgtgtgtgtgtgtGCGGC CGCgtgtgtgtTCTAGA-3'. It contains the following tags: Lucy (a leucine-rich 17-amino acid cleavable signal peptide) (13), FLAG, and 20 N-terminal amino acids of rhodopsin. It also contains AscI and NotI sites for cloning receptor cDNAs downstream from the tags. The receptor cDNAs together with N-terminal tags were then subcloned into pSBtet-GP (50) using PCR and SfiI sites to replace the luciferase insert. pSBtet-GP was a gift from Eric Kowarz (Addgene plasmid no. 60495; <http://n2t.net/addgene:60495>; Research Resource Identifiers portal: Addgene_60495). All constructs were verified by sequencing.

OR51E1 shRNA plasmid kit containing four unique 29-mer shRNA constructs in retroviral vector was purchased from Origene (catalog no. TR302775).

Cell culture and generation of stable cell lines

Dulbecco's modified Eagle's medium, RPMI 1604, PBS, and Hank's balanced salt solution (HBSS) were acquired from Thermo Fisher Scientific. Plasticware and disposables were from VWR. HEK293T cells were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1 \times penicillin/streptomycin. Human prostate cancer cell lines LNCaP and DU145 were purchased from American Type Culture Collection. They were cultured in RPMI medium 1640 with 10% fetal bovine serum and 1 \times penicillin/streptomycin. For transfection, cells were plated in 60 mm dishes. When they reached $\sim 60\%$ confluence, the cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) and a mixture of 5 μg of the

appropriate pSBtet-GP construct and 0.4 μg of plasmid pCMV(CAT)T7-SB100. pCMV(CAT)T7-SB100 is required to facilitate stable integration of pSBtet constructs into genomic DNA (51). pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid no. 34879; <http://n2t.net/addgene:34879>; Research Resource Identifiers portal: Addgene_34879). The transfecting medium was replaced the next day, and one more day later, the cells were replated into 100 mm dishes in the medium containing 1 $\mu\text{g}/\text{ml}$ puromycin.

Western blot analysis

Cells were grown in 12-well plates under conditions required for a particular experiment, *e.g.*, in the presence of the indicated concentrations of doxycycline for the indicated number of days. Typically, once cells reached 80 to 90% confluence, they were rinsed with PBS and harvested by the addition of 200 μl of 1 \times SDS-PAGE sample buffer. Cell lysates were briefly sonicated to destroy chromosomal DNA and resolved on SDS-PAGE, followed by immunoblotting. Incubation with primary antibodies was usually done overnight; the secondary antibodies labeled with infrared IRDye 800CW or 680RD were from LI-COR, Inc. The immune complexes were visualized using Odyssey (LI-COR) infrared fluorescence detection system; the scanning settings were set to avoid signal saturation. For quantitative analysis, the signal of the band of interest (*i.e.*, P-ERK1/2) was normalized to the signal for tubulin in the same lane on the immunoblot.

Protein deglycosylation

LNCaP-OR51E1tet cells were grown on a 100 mm culture dish till 50% confluence and then induced with 1 $\mu\text{g}/\text{ml}$ doxycycline. Two days later, they were rinsed with PBS and harvested by scraping into 0.5 ml of a low osmosis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM DTT, and protease inhibitors). Cells were broken by passing through a needle/syringe 10 times. The homogenate was centrifuged for 15 min at 14,000 rpm, and the supernatant was discarded. The membrane pellet was extracted with 0.5 ml of 0.5% Triton-X100/low osmosis buffer and centrifuged again for 15 min at 14,000 rpm. The supernatant (extract) was used for subsequent experiments. For deglycosylation, 40 μl of the extract was mixed with 5 μl of 10 \times deglycosylation buffer 2 (denaturing buffer) and either 5 μl of water (untreated) or 5 μl of deglycosylation enzyme mix II (New England Biolabs; catalog no. P6044). The samples were incubated first at room temperature for 30 min, followed with an overnight incubation at 37 $^{\circ}\text{C}$. The next day the samples were mixed with 50 μl of 2 \times SDS sample buffer and analyzed by Western blot.

PKA activity assay

LNCaP cells were grown in 12-well plates, induced by 1 $\mu\text{g}/\text{ml}$ doxycycline for 1 day if required. Prior to stimulation with various compounds, they were serum starved for 1 h. The cells were stimulated for 10 min by the addition of concentrated stocks of compounds in HBSS, after which the culture medium was quickly aspirated, and the cells were harvested in 200 μl of

1 \times SDS-PAGE sample buffer. Immunoblotting was done with antibodies against phospho-PKA substrate consensus sequence (RxxS*/T*) (rabbit polyclonal) and either FLAG or tubulin (mouse monoclonal).

cAMP sensor assay

We used a protein sensor that increases its fluorescence upon binding of cAMP (Montana Molecular, Inc) as described earlier (34–36, 52). Briefly, LNCaP cells were plated on poly-L-lysine-coated 12-mm glass coverslips in 24-well plates and then treated with 1 mg/ml doxycycline. The next day cells were infected with the BacMam virus encoding the red upward cAMP sensor using the following mix per well: 0.56 ml culture medium with 1 $\mu\text{g}/\text{ml}$ doxycycline and 3 μM suberoylanilide hydroxamic acid, and 40 μl of the virus. The day after, coverslips were secured in a flow chamber and mounted on the stage of a Nikon TE2000 inverted fluorescence microscope. The cells were continuously superfused by gravity flow with HBSS. To stimulate the cells, the flow was switched to agonist-containing HBSS for a specified time and then changed back to the agonist-free buffer. Images were collected in real time every 5 s using a 20 \times objective lens and recorded using MetaFluor software (Molecular Devices). The excitation wavelength was 550 nm, with the emission set to 570 nm. Groups of 10 to 30 cells were selected as regions of interest for signal quantification. Traces shown here are averages of \sim 10 regions of interest per a coverslip. All experiments were repeated 3 to 4 times with similar results.

Intracellular-free Ca^{2+} assay

HEK293T cells were grown on poly-L-lysine-coated 12-mm glass coverslips, washed with the culture medium, and then incubated at 37 $^{\circ}\text{C}$ in the culture medium containing 2 μM Fura-2-acetoxymethyl ester (Thermo Fisher Scientific) for 60 min. After loading Fura-2-acetoxymethyl ester, the cells were kept at ambient temperature for no longer than 0.5 h before imaging. Coverslips were secured in a flow chamber and mounted on the stage of a Nikon TE2000 inverted fluorescence microscope. The cells were continuously superfused with HBSS by gravity flow. To stimulate the cells, the flow was switched to agonist-containing HBSS for a time required by a specified experiment and then back to the agonist-free buffer. Images were collected in real time every 5 s using a 20 \times UV objective lens and recorded using MetaFluor software (Molecular Devices). The excitation wavelengths were 340 nm (Ca^{2+} bound) and 380 nm (Ca^{2+} free), with the emission set to 510 nm. The 340/380 ratio is representative of intracellular-free $[\text{Ca}^{2+}]$. Individual cells or clusters of 10 to 20 cells were selected as regions of interest for signal quantification. Traces shown in the figures are averages of 2 to 4 independent experiments with three replicate coverslips per each experiment.

Cell proliferation assays

Cell proliferation was assessed in two ways. In one assay, after a period of growth, cells were collected from the plates, and their number was determined using a traditional cell

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counter. In the other method, the number of cells was assessed by imaging of the growing adherent cell culture in the Incucyte instrument through calculating cell confluency. For the traditional cell count method, cells were plated in 60 mm dishes at 10^5 /dish. They were allowed to grow in the presence of the indicated compounds for 4 days, at which point some of the cultures would reach ~50% confluence. At this point, the cells were trypsinized and transferred to 100 mm dishes where they were allowed to grow for three more days. Then the cells were trypsinized and counted.

For the second assay, the cells were plated in 12-well plates at 2×10^4 /well. On the next day, the cell culture medium in each well was replaced with 4 ml of the medium containing indicated compounds, and the plate was placed in the Incucyte instrument residing inside a CO₂ incubator. The instrument has a robotic camera capable of photographing the same areas of the well; we programmed the instrument to take images every 4 h at 16 locations in every well. The software then analyzed the photographs and calculated cell confluence at each time point; these values were exported into Microsoft Excel and plotted against the time in culture. The cells were allowed to grow for 4 to 5 days until they start reaching ~50% confluence.

Immunocytochemistry

LNCaP-OR51E1tet cells were grown on poly-L-lysine-coated 12-mm glass coverslips for 2 days either in the absence or the presence of 1 µg/ml doxycycline. They were then rinsed with PBS and fixed in 4% paraformaldehyde/PBS for 10 min. The cells were rinsed with PBS, some coverslips were treated with 0.5% Triton X-100/PBS, and then blocked with 10% bovine serum albumin/PBS and incubated with mouse anti-FLAG antibody diluted 1:200 in PBS overnight at 4 °C. They were then washed 3 times with PBS and incubated with goat antimouse secondary antibody labeled with Alexa Fluor 555 dye for 1 h. The coverslips were washed 3 times with PBS, dried, and mounted on glass slides for either open-field or confocal microscopy.

Annexin V staining

LNCaP cells were grown on poly-L-lysine-coated 12-mm glass coverslips for 3 days either in the absence or the presence of 1 µg/ml doxycycline. The cells were then rinsed with HBSS and incubated with PE Annexin V diluted 1:20 in the 1× dilution buffer (BD Biosciences) for 30 min. They were then rinsed with HBSS and fixed in 4% paraformaldehyde/HBSS for 10 min. The coverslips were rinsed once more with HBSS, dried, and mounted on glass slides for microscopy. For quantification, the total intensity of the PE signal within the frame was measured and divided by the total intensity of nuclear (4',6-diamidino-2-phenylindole) staining in the same frame.

Statistics

Data are reported as means ± SD. GraphPad Prism software (version 6.07; GraphPad Software) was used for statistical

analysis. Groups of data were compared using ANOVA or two-tailed unpaired Student's *t* tests, with values of *p* < 0.05 considered statistically significant.

Data availability

All the relevant data described are contained within the article.

Acknowledgments—The authors thank Drs K. Burstein and P. Rai (University of Miami) for helpful discussions and sharing some reagents and protocols.

Author contributions—V. S. and A. P. conceived and designed the research. A. P. performed all experiments, analyzed, and prepared data for presentation. V. S. provided the resources and wrote the first draft; and both authors wrote the article.

Funding and additional information—Supported in part by National Institutes of Health grants R56DK119262 (to V. S.) and the subcontract IE92847 from R44GM125390 (to Anne-Marie Quinn). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: β2AR, β2 adrenergic receptor; cDNA, complementary DNA; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; GPCR, G protein-coupled receptor; HBSS, Hank's balanced salt solution; HEK293, human embryonic kidney 293 cells; LNCaP, lymph node carcinoma of the prostate; M3R, M3 muscarinic cholinergic receptor; OR, olfactory receptor; PSGR, prostate-specific G protein-coupled receptor.

References

1. Niimura, Y. (2009) Evolutionary dynamics of olfactory receptor genes in chordates: Interaction between environments and genomic contents. *Hum. Genomics*. **4**, 107–118
2. Glusman, G., Yanai, I., Rubin, I., and Lancet, D. (2001) The complete human olfactory subgenome. *Genome Res*. **11**, 685–702
3. Zhang, X., and Firestein, S. (2002) The olfactory receptor gene superfamily of the mouse. *Nat. Neurosci*. **5**, 124–133
4. Buck, L., and Axel, R. (1991) A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* **65**, 175–187
5. Fukuda, N., Yomogida, K., Okabe, M., and Touhara, K. (2004) Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. *J. Cell Sci*. **117**, 5835–5845
6. Kang, N., and Koo, J. (2012) Olfactory receptors in non-chemosensory tissues. *BMB Rep*. **45**, 612–622
7. Foster, S. R., Roura, E., and Thomas, W. G. (2014) Extrasensory perception: Odorant and taste receptors beyond the nose and mouth. *Pharmacol. Ther.* **142**, 41–61
8. Dalesio, N. M., Barreto Ortiz, S. F., Pluznick, J. L., and Berkowitz, D. E. (2018) Olfactory, taste, and photo sensory receptors in non-sensory organs: It just makes sense. *Front. Physiol*. **9**, 1673
9. Lee, S. J., Depoortere, I., and Hatt, H. (2019) Therapeutic potential of ectopic olfactory and taste receptors. *Nat. Rev. Drug Discov*. **18**, 116–138
10. Shepard, B. D., Koepsell, H., and Pluznick, J. L. (2019) Renal olfactory receptor 1393 contributes to the progression of type 2 diabetes in a diet-induced obesity model. *Am. J. Physiol. Renal Physiol*. **316**, F372–F381

11. Saito, H., Chi, Q., Zhuang, H., Matsunami, H., and Mainland, J. D. (2009) Odor coding by a mammalian receptor repertoire. *Sci. Signal.* **2**, ra9
12. Saito, H., Kubota, M., Roberts, R. W., Chi, Q., and Matsunami, H. (2004) RTP family members induce functional expression of mammalian odorant receptors. *Cell* **119**, 679–691
13. Shepard, B. D., Natarajan, N., Protzko, R. J., Acres, O. W., and Pluznick, J. L. (2013) A cleavable N-terminal signal peptide promotes widespread olfactory receptor surface expression in HEK293T cells. *PLoS One* **8**, e68758
14. Dey, S., Zhan, S., and Matsunami, H. (2011) Assaying surface expression of chemosensory receptors in heterologous cells. *J. Vis. Exp.* <https://doi.org/10.3791/2405>
15. Adipietro, K. A., Mainland, J. D., and Matsunami, H. (2012) Functional evolution of mammalian odorant receptors. *PLoS Genet.* **8**, e1002821
16. Pluznick, J. L., Protzko, R. J., Gevorgyan, H., Peterlin, Z., Sipos, A., Han, J., Brunet, I., Wan, L. X., Rey, F., Wang, T., Firestein, S. J., Yanagisawa, M., Gordon, J. I., Eichmann, A., Peti-Peterdi, J., et al. (2013) Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 4410–4415
17. Bellono, N. W., Bayrer, J. R., Leitch, D. B., Castro, J., Zhang, C., O'Donnell, T. A., Brierley, S. M., Ingraham, H. A., and Julius, D. (2017) Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. *Cell* **170**, 185–198.e116
18. Halperin Kuhns, V. L., Sanchez, J., Sarver, D. C., Khalil, Z., Rajkumar, P., Marr, K. A., and Pluznick, J. L. (2019) Characterizing novel olfactory receptors expressed in the murine renal cortex. *Am. J. Physiol. Renal Physiol.* **317**, F172–F186
19. Abaffy, T., Bain, J. R., Muehlbauer, M. J., Spasojevic, I., Lodha, S., Bruguera, E., O'Neal, S. K., Kim, S. Y., and Matsunami, H. (2018) A testosterone metabolite 19-hydroxyandrostenedione induces neuroendocrine trans-differentiation of prostate cancer cells via an ectopic olfactory receptor. *Front. Oncol.* **8**, 162
20. Xie, H., Liu, T., Chen, J., Yang, Z., Xu, S., Fan, Y., Zeng, J., Chen, Y., Ma, Z., Gao, Y., He, D., and Li, L. (2019) Activation of PSGR with beta-ionone suppresses prostate cancer progression by blocking androgen receptor nuclear translocation. *Cancer Lett.* **453**, 193–205
21. Gelis, L., Jovancevic, N., Veitinger, S., Mandal, B., Arndt, H. D., Neuhaus, E. M., and Hatt, H. (2016) Functional characterization of the odorant receptor 51E2 in human melanocytes. *J. Biol. Chem.* **291**, 17772–17786
22. Chang, A. J., Ortega, F. E., Riegler, J., Madison, D. V., and Krasnow, M. A. (2015) Oxygen regulation of breathing through an olfactory receptor activated by lactate. *Nature* **527**, 240–244
23. Neuhaus, E. M., Zhang, W., Gelis, L., Deng, Y., Noldus, J., and Hatt, H. (2009) Activation of an olfactory receptor inhibits proliferation of prostate cancer cells. *J. Biol. Chem.* **284**, 16218–16225
24. Wang, J., Weng, J., Cai, Y., Penland, R., Liu, M., and Ittmann, M. (2006) The prostate-specific G-protein coupled receptors PSGR and PSGR2 are prostate cancer biomarkers that are complementary to alpha-methylacyl-CoA racemase. *Prostate* **66**, 847–857
25. Xu, L. L., Stackhouse, B. G., Florence, K., Zhang, W., Shanmugam, N., Sesterhenn, I. A., Zou, Z., Srikantan, V., Augustus, M., Roschke, V., Carter, K., McLeod, D. G., Moul, J. W., Soppett, D., and Srivastava, S. (2000) PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. *Cancer Res.* **60**, 6568–6572
26. Xia, C., Ma, W., Wang, F., Hua, S., and Liu, M. (2001) Identification of a prostate-specific G-protein coupled receptor in prostate cancer. *Oncogene* **20**, 5903–5907
27. Xu, L. L., Sun, C., Petrovics, G., Makarem, M., Furusato, B., Zhang, W., Sesterhenn, I. A., McLeod, D. G., Sun, L., Moul, J. W., and Srivastava, S. (2006) Quantitative expression profile of PSGR in prostate cancer. *Prostate Cancer Prostatic Dis.* **9**, 56–61
28. Rodriguez, M., Luo, W., Weng, J., Zeng, L., Yi, Z., Siwko, S., and Liu, M. (2014) PSGR promotes prostatic intraepithelial neoplasia and prostate cancer xenograft growth through NF-kappaB. *Oncogenesis* **3**, e114
29. Weng, J., Ma, W., Mitchell, D., Zhang, J., and Liu, M. (2005) Regulation of human prostate-specific G-protein coupled receptor, PSGR, by two distinct promoters and growth factors. *J. Cell Biochem.* **96**, 1034–1048
30. Massberg, D., Jovancevic, N., Offermann, A., Simon, A., Baniahmad, A., Perner, S., Pungsrinont, T., Luko, K., Philippou, S., Ubrig, B., Heiland, M., Weber, L., Altmuller, J., Becker, C., Gisselmann, G., et al. (2016) The activation of OR51E1 causes growth suppression of human prostate cancer cells. *Oncotarget* **7**, 48231–48249
31. Marchalant, Y., Brownjohn, P. W., Bonnet, A., Kleffmann, T., and Ashton, J. C. (2014) Validating antibodies to the cannabinoid CB2 receptor: Antibody sensitivity is not evidence of antibody specificity. *J. Histochem. Cytochem.* **62**, 395–404
32. Jo, M., and Jung, S. T. (2016) Engineering therapeutic antibodies targeting G-protein-coupled receptors. *Exp. Mol. Med.* **48**, e207
33. Nooh, M. M., and Bahouth, S. W. (2017) Visualization and quantification of GPCR trafficking in mammalian cells by confocal microscopy. *Methods Cell Biol.* **142**, 67–78
34. Tewson, P. H., Martinka, S., Shaner, N. C., Hughes, T. E., and Quinn, A. M. (2016) New DAG and cAMP sensors optimized for live-cell assays in automated laboratories. *J. Biomol. Screen.* **21**, 298–305
35. Pronin, A. N., Wang, Q., and Slepak, V. Z. (2017) Teaching an old drug new tricks: Agonism, antagonism, and biased signaling of pilocarpine through M3 muscarinic acetylcholine receptor. *Mol. Pharmacol.* **92**, 601–612
36. Tewson, P., Martinka, S., Shaner, N., Berlot, C., Quinn, A. M., and Hughes, T. (2018) Assay for detecting Galphai-mediated decreases in cAMP in living cells. *SLAS Discov.* **23**, 898–906
37. Busse, D., Kudella, P., Gruning, N. M., Gisselmann, G., Stander, S., Luger, T., Jacobsen, F., Steinstrasser, L., Paus, R., Gkogkolou, P., Bohm, M., Hatt, H., and Benecke, H. (2014) A synthetic sandalwood odorant induces wound-healing processes in human keratinocytes via the olfactory receptor OR2AT4. *J. Invest. Dermatol.* **134**, 2823–2832
38. Cheret, J., Bertolini, M., Ponce, L., Lehmann, J., Tsai, T., Alam, M., Hatt, H., and Paus, R. (2018) Olfactory receptor OR2AT4 regulates human hair growth. *Nat. Commun.* **9**, 3624
39. Ueda, T., Bruchofsky, N., and Sadar, M. D. (2002) Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J. Biol. Chem.* **277**, 7076–7085
40. Holleman, J., and Marchese, A. (2014) The ubiquitin ligase deltex-3l regulates endosomal sorting of the G protein-coupled receptor CXCR4. *Mol. Biol. Cell* **25**, 1892–1904
41. Priori, D., Colombo, M., Clavanzani, P., Jansman, A. J., Lalles, J. P., Trevisi, P., and Bosi, P. (2015) The olfactory receptor OR51E1 is present along the gastrointestinal tract of pigs, co-localizes with enteroendocrine cells and is modulated by intestinal microbiota. *PLoS One* **10**, e0129501
42. Giandomenico, V., Cui, T., Grimelius, L., Oberg, K., Pelosi, G., and Tsolakis, A. V. (2013) Olfactory receptor 51E1 as a novel target for diagnosis in somatostatin receptor-negative lung carcinoids. *J. Mol. Endocrinol.* **51**, 277–286
43. Cui, T., Tsolakis, A. V., Li, S. C., Cunningham, J. L., Lind, T., Oberg, K., and Giandomenico, V. (2013) Olfactory receptor 51E1 protein as a potential novel tissue biomarker for small intestine neuroendocrine carcinomas. *Eur. J. Endocrinol.* **168**, 253–261
44. Jovancevic, N., Khalfaoui, S., Weinrich, M., Weidinger, D., Simon, A., Kalbe, B., Kernt, M., Kampik, A., Gisselmann, G., Gelis, L., and Hatt, H. (2017) Odorant receptor 51E2 agonist beta-ionone regulates RPE cell migration and proliferation. *Front. Physiol.* **8**, 888
45. Han, Y. E., Kang, C. W., Oh, J. H., Park, S. H., Ku, C. R., Cho, Y. H., Lee, M. K., and Lee, E. J. (2018) Olfactory receptor OR51E1 mediates GLP-1 secretion in human and rodent enteroendocrine L cells. *J. Endocr. Soc.* **2**, 1251–1258
46. Pronin, A. N., Levay, K., Velmsheshev, D., Faghihi, M. A., Shestopalov, V. I., and Slepak, V. Z. (2014) Expression of olfactory signaling genes in the eye. *PLoS One* **9**, e96435
47. Rodriguez, M., Siwko, S., Zeng, L., Li, J., Yi, Z., and Liu, M. (2016) Prostate-specific G-protein-coupled receptor collaborates with loss of PTEN to promote prostate cancer progression. *Oncogene* **35**, 1153–1162

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48. Stone, L. (2015) Prostate cancer: PTEN loss and PSGR overexpression promote cancer progression. *Nat. Rev. Urol.* **12**, 364
49. Wu, L., Pan, Y., Chen, G. Q., Matsunami, H., and Zhuang, H. (2012) Receptor-transporting protein 1 short (RTP1S) mediates translocation and activation of odorant receptors by acting through multiple steps. *J. Biol. Chem.* **287**, 22287–22294
50. Kowarz, E., Loscher, D., and Marschalek, R. (2015) Optimized sleeping beauty transposons rapidly generate stable transgenic cell lines. *Bio-technol. J.* **10**, 647–653
51. Mates, L., Chuah, M. K., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D. P., Schmitt, A., Becker, K., Matrai, J., Ma, L., Samara-Kuko, E., Gysemans, C., Pryputniewicz, D., Miskey, C., *et al.* (2009) Molecular evolution of a novel hyperactive sleeping beauty transposase enables robust stable gene transfer in vertebrates. *Nat. Genet.* **41**, 753–761
52. Wang, Q., Pronin, A. N., Levay, K., Almaca, J., Fornoni, A., Caicedo, A., and Slepak, V. Z. (2017) Regulator of G-protein signaling Gbeta5-R7 is a crucial activator of muscarinic M3 receptor-stimulated insulin secretion. *FASEB J.* **31**, 4734–4744