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Histone deacetylase inhibitor activity in royal jelly might facilitate caste switching in bees

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Worker and queen bees are genetically indistinguishable. However, queen bees are fertile, larger and have a longer lifespan than their female worker counterparts. Differential feeding of larvae with royal jelly controls this caste switching. There is emerging evidence that the queen-bee phenotype is driven by epigenetic mechanisms. In this study, we show that royal jelly—the secretion produced by the hypopharyngeal and mandibular glands of worker bees—has histone deacetylase inhibitor (HDACi) activity. A fatty acid, (*E*)-10-hydroxy-2-decenoic acid (10HDA), which accounts for up to 5% of royal jelly, harbours this HDACi activity. Furthermore, 10HDA can reactivate the expression of epigenetically silenced genes in mammalian cells. Thus, the epigenetic regulation of queen-bee development is probably driven, in part, by HDACi activity in royal jelly.

Keywords: *Apis mellifera*; epigenetic; HDAC inhibitor; phenotypic plasticity; royal jelly

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INTRODUCTION

The honey-bee (*Apis mellifera*) exemplifies a phenomenon in nature called environmentally driven phenotypic plasticity (Maleszka, 2008) which describes how diet dictates the ability of different phenotypes to arise from one genome. In social insects such as bees, it leads to the establishment of castes, with a differentiation between reproduction (queen) and labour (workers; Page Jr, 1991). This phenotypic difference is determined at an early larvae stage, and the imprint is established by distinct feeding regimens for queen and worker larvae (Page Jr & Peng, 2001). For the first 3 days of life, all larvae are fed royal jelly, a substance composed of a mixture of hypopharyngeal and mandibular-gland secretions that is made by nurse bees. Worker larvae are then switched to a diet that contains predominantly honey and pollen (worker jelly; Haydak, 1970). The queen larva, however, remains on a royal-jelly diet and is fed it in abundance. This diet results in a queen bee that is morphologically, behaviourally and physiologically distinct from her worker-bee counterparts, and also has a prolonged lifespan; at least 20 times longer than a regular worker-bee (Winston, 1987). This dietary input has pleiotropic effects: activating the insulin signalling cascade, affecting brain-releasing hormones and regulating global epigenetic changes (Maleszka, 2008). In other social insects such as ants, termites and some wasps, it is less clear how a selective diet or the environment leads to caste determination (Schwander *et al*, 2010).

In mammals, nutrition has a direct influence on gene expression and development, by affecting the epigenetic state of the genome (Dolinoy *et al*, 2007). The enzymes that maintain a specific epigenetic state include DNA methyltransferases, histone acetylases and deacetylases, and histone methyltransferases and demethylases. Any of these enzymes could be targeted by nutritional factors. What we can learn about the mechanisms regulating phenotypic plasticity in insects will probably be relevant to our understanding of the way in which diet and nutrition regulate fetal programming and disease in humans. Recent reports show that honey-bees have a fully operational DNA-methylation system (Wang *et al*, 2006), as opposed to flies, which do not have CpG methylation. The comparison between DNA-methylation patterns in the brains of queen and worker bees shows that there is no difference in the number of CpGs that are methylated (Lyko *et al*, 2010). However, there are distinct loci that are differentially methylated, especially in exonic regions, which affect alternative splicing. Importantly, small interfering RNA (siRNA)-induced knockdown of *Dnmt3* levels in newly emerged

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larvae results in the development of queen bees with fully developed ovaries (Kucharski *et al*, 2008). Thus, injection of honey-bee larvae with *Dnmt3* siRNA can replace royal jelly consumption and influence caste development. These data indicate that a component of royal jelly has the ability to control epigenetic pathways.

To investigate whether royal jelly harbours epigenetic regulatory activity, we used the *K-ras*-transformed NIH 3T3 cell reporter system. It has been shown previously that in these cells the activated Ras pathway epigenetically silences expression of the proapoptotic *Fas* gene (Fenton *et al*, 1998; Peli *et al*, 1999). Furthermore, this system has been subjected to a genome-wide RNA-interference screen to identify factors required for Ras-mediated epigenetic silencing (Gazin *et al*, 2007). This screen identified several transcriptional repressors including Dnmt1, Hdac9, Ezh2 and Sirt6. We therefore reasoned that this epigenetic reporter system is suited for the screening of potential royal jelly epigenetic regulatory activity. Indeed, when *K-ras*-transformed NIH 3T3 cells were treated with this hypopharyngeal gland secretion, the *Fas* gene was reactivated. We have identified the active ingredient as (*E*)-10-hydroxy-2-decenoic acid (10HDA) and demonstrate that this compound has histone deacetylase inhibitor (HDACi) activity.

RESULTS

Royal jelly reactivates an epigenetically silenced locus

As siRNA-induced knockdown of *Dnmt3* levels in bee larvae resulted in the development of queen bees (Kucharski *et al*, 2008), we theorized that royal jelly harbours activity that can regulate DNA methylation. We used the *K-ras*-transformed NIH 3T3 cell reporter system to measure the presence of epigenetic regulatory activity in royal jelly (supplementary Fig S1A online). These cells were treated with two concentrations of royal jelly (0.5% and 1%) for up to 3 days. By day 2, we observed re-expression of the *Fas* gene (Fig 1A). Furthermore, the regained expression was similar to that obtained with 5-aza-2′-deoxycytidine (5-Aza) treatment, a well-characterized inhibitor of DNA methylation (Stresemann & Lyko, 2008). Next, we fractionated royal jelly using an ultrafiltration membrane with a 3 kDa cut-off. The flow-through, which contains the low-molecular-weight fraction, harboured the epigenetic regulatory activity (Fig 1B). This result—together with the fact that proteinase K treatment of royal jelly did not reduce its activity in this assay (data not shown)—indicates that the active component of royal jelly is probably a small molecule of non-proteinaceous origin.

10HDA is the active component of royal jelly

Royal jelly is a complex mixture consisting of amino acids, sugars, proteins, fatty acids and mineral salts. A main component of royal jelly is 10HDA, which can compose 2–6% of royal jelly; this translates into a 100–300 mM concentration in royal jelly. We focussed on 10HDA because of its abundance, and published evidence that it is biologically active in mammalian cells, for which a mechanism has yet to be attributed. Again, by using the same *K-ras*-transformed NIH 3T3 cell reporter system, we show that a 5 mM solution of 10HDA restores Fas expression (Fig 1C). This demonstrates that a single component of royal jelly—10HDA—has the ability to activate gene expression, possibly through epigenetic reprogramming.

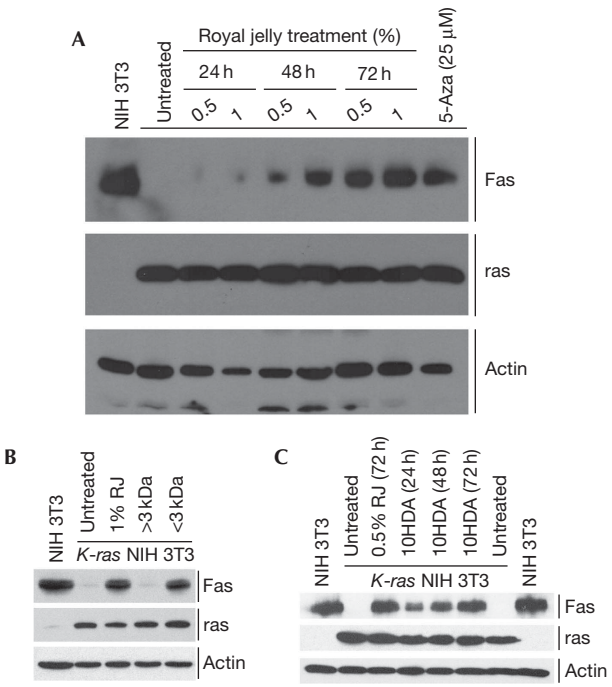

Fig 1 | Royal jelly and 10HDA can reactivate the expression of an epigenetically silenced *Fas* locus. (**A**) Western blot analysis showing that a 1% and 0.5% royal jelly (RJ) suspension can restore Fas expression in NIH 3T3 *K-ras* cells. Cells were cultured in RJ for 1–3 days. 5-Aza-2-deoxycytidine (5-Aza) treatment acts as a control for reactivated Fas expression. (**B**) The fraction of RJ that is smaller than 3 kDa in size harbours this epigenetic regulatory activity. (**C**) A 5 mM solution of 10HDA, a main component of RJ, is able to reactivate the expression of Fas in NIH 3T3 *K-ras* cells. Actin acts as a loading control. 10HDA, (*E*)-10-hydroxy-2-decenoic acid.

To establish that 10HDA functions broadly and that the reactivation of Fas expression is not idiosyncratic to the NIH 3T3 *K-ras* cell reporter system, we tested 10HDA in a second epigenetic reporter system, which is based on the reactivation of a silenced green fluorescent protein (GFP) locus (supplementary Fig S1B online). This ear fibroblast cell line (GF2–4) harbours a GFP gene that is heavily CpG methylated, as determined by bisulphite genomic sequencing (Kang *et al*, 2007). Treatment of GF2–4 cells with 5-Aza results in the reactivation of the GFP locus (Kang *et al*, 2007). Furthermore, a synergistic effect of a DNA demethylating agent and an HDACi on the expression of GFP was reported. We treated GF2–4 cells with either 10HDA alone or in combination with 5-Aza and observed that 10HDA functions synergistically with the 5-Aza (Fig 2A,B and supplementary Fig S2 online). 10HDA alone is unable to activate the silenced GFP locus. In this assay, 10HDA behaves in a similar manner to sodium butyrate, a widely documented HDACi. These data indicate that 10HDA inhibits a pathway involved in transcription repression that runs parallel to the DNA methylation pathway, and does not function as a DNA demethylating agent *per se*.

10HDA does not inhibit DNA methylation

We studied a third model system—SW48 cells (a human colon cancer cell line) containing a hypermethylated cytomegalovirus

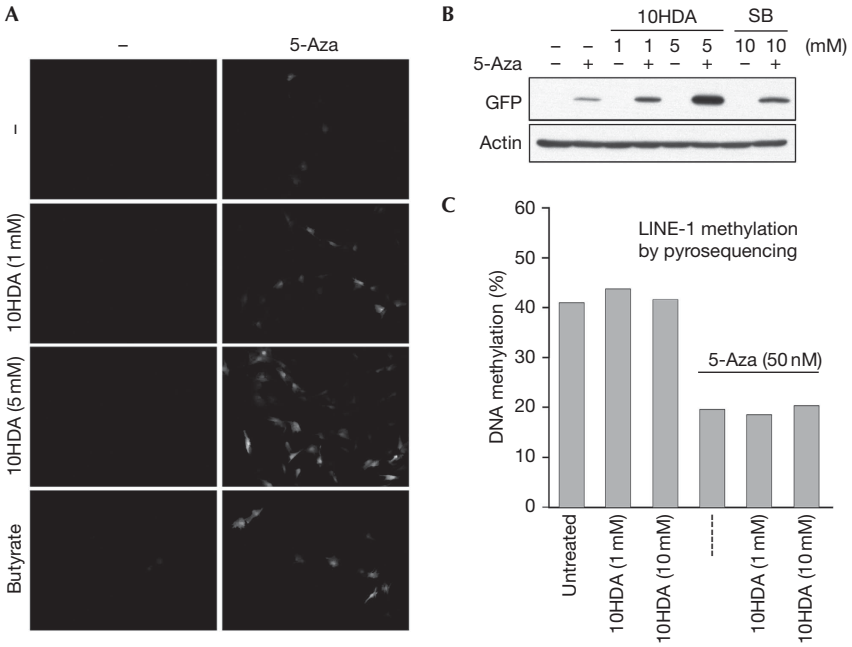

Fig 2 | 10HDA can reactivate epigenetically silenced green fluorescent protein loci. (**A**) 10HDA (5 or 1 mM) can restore GFP-expression in pig-ear fibroblasts when used in combination with 5-Aza (100 nM). (**B**) Quantification by western blot analysis shows a dose-dependent effect of 10HDA. 10HDA shows a stronger effect on GFP expression in pig-ear fibroblasts than sodium butyrate (SB), a known HDACi. Actin acts as a loading control. (**C**) 10HDA does not alter the degree of DNA methylation at LINE-1 repeat elements. SW48 colon-cancer cells were treated with 10HDA or a combination of 10HDA and 5-Aza (50 nM). 10HDA alone does not reduce the level of DNA methylation at LINE-1 repeat elements, as determined by pyrosequencing. The experiment was performed twice, with three replicates each. 5-Aza, 5-Aza-2-deoxycytidine; GFP, green fluorescent protein; HDACi, histone deacetylase inhibitor; LINE-1, long interspersed nucleotide elements; 10HDA, (*E*)-10-hydroxy-2-decenoic acid.

(CMV) immediate early promoter driving expression of GFP (supplementary Fig S1C online; Si *et al*, 2010). As observed in the ear fibroblast cell line, 10HDA alone had no effect, but markedly potentiated the ability of 5-Aza to activate GFP (supplementary Fig S3D online). In the same cell line, both royal jelly and 10HDA can reactivate epigenetically silenced p21 expression (supplementary Fig S3A,B online). To test directly whether 10HDA can block DNA methylation, we performed a pyrosequencing assay for LINE-1 (long interspersed nuclear element) repeats, as a measure of global hypomethylation in these cells. Although 5-Aza treatment reduces DNA methylation by 50% in this assay, 10HDA was not able to reduce the levels of LINE-1 DNA methylation alone, or to potentiate the effect of 5-Aza in sequential combination treatment (Fig 2C). Importantly, royal-jelly treatment (0.4% and 4%, v/v) did not have any effect on DNA methylation levels at LINE-1 elements either (supplementary Fig S3C online). We also evaluated the methylation level of the CMV promoter (by pyrosequencing) after 10HDA treatment and observed the same result—10HDA does not reduce the DNA methylation level at this specific promoter (data not shown). Thus, 10HDA treatment of SW48 cells does not reduce the DNA methylation levels at LINE-1 repeat elements or at a specific promoter that can be regulated by this compound. 10HDA is thus not a DNA methyltransferase inhibitor. It is possible that 10HDA is not a direct regulator of epigenetic factors, but instead interferes with the extracellular signal-regulated kinase (ERK) or phosphoinositide 3-kinase (PI3K) signalling pathways, which link *K-ras* to the epigenetic regulators (supplementary Fig S4A online). To test this possibility we treated cells with 10HDA, U0126 (an ERK inhibitor) and LY294002 (a PI3K inhibitor). 10HDA does not affect either of these signalling pathways (supplementary Fig S4B online), suggesting that it directly targets an epigenetic effector molecule.

10HDA is an HDACi

On the basis of the fact that 10HDA (i) functions synergistically with DNA demethylating agents (Fig 2A,B), but does not inhibit DNA methylation itself (Fig 2C) and (ii) is structurally similar to known HDACi compounds (Fig 3A), we investigated whether it was an HDACi. First, NIH 3T3 *K-ras* cells were treated with sodium butyrate, 10HDA and a 5% royal jelly suspension for 3 days. When core histones from these cells were analysed with pan-acetyl-lysine antibodies, acetylation levels were increased by all three treatments (Fig 3B). These data indicate that 10HDA blocks HDAC activity or promotes histone acetyltransferase activity. Next, we directly tested whether 10HDA and royal jelly inhibit HDAC activity using an *in vitro* assay. Both royal jelly and 10HDA harbour HDACi activity (Fig 3C). Importantly, the royal jelly used in this experiment was composed of 2% 10HDA, which at a 1% dilution translates into a 1 mM 10HDA solution. Thus, most of the HDACi activity in royal jelly can be attributed to 10HDA. (*E*)-9-oxodec-2-enoic acid (90DA) is an analogue of 10HDA that is produced by the queen bee (Plettner *et al*, 1996), and it also has similar HDACi activity (supplementary Fig S5 online). This activity of 90DA acid might be important for maintaining the royal phenotype, if it is produced and consumed by the queen herself.

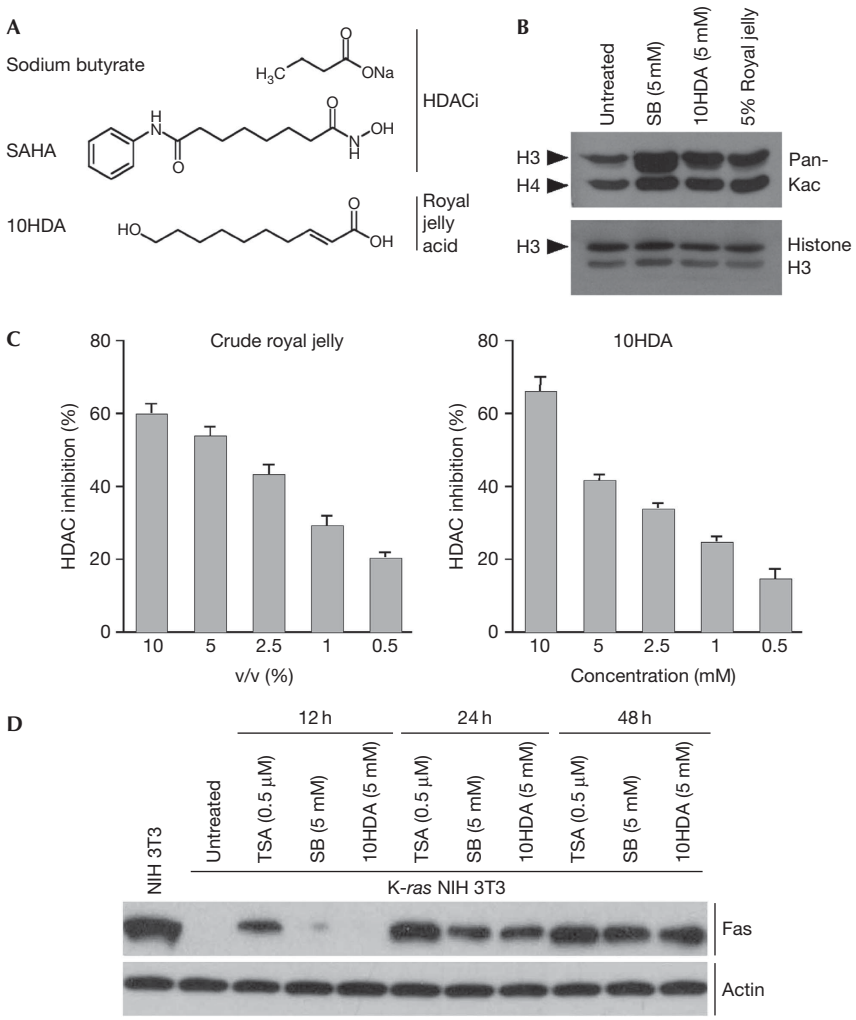

Fig 3 | 10HDA is an HDAC inhibitor. (**A**) 10HDA, also referred to as royal jelly (RJ) acid, has a few structural similarities to the known HDACis, sodium butyrate (SB) and SAHA (suberoylanilide hydroxamic acid). (**B**) NIH 3T3 *K-ras* cells were treated with SB (5 mM), 10HDA (5 mM) or a 5% RJ suspension for 72 h. Core histones were then isolated and western blot analysis was performed with a pan-acetyl-lysine antibody. Increased histone acetylation levels were observed in the treated samples. (**C**) 10HDA and diluted RJ inhibit HDAC activity *in vitro*. The data were generated using an HDAC colorimetric activity assay kit. Error bars represent s.d. calculated from three independent experiments. (**D**) Trichostatin A (TSA), SB and 10HDA are able to reactivate the expression of Fas in NIH 3T3 *K-ras* cells. Fas expression at three different time points was assayed. Actin acts as a loading control. HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; 10HDA, (*E*)-10-hydroxy-2-decenoic acid.

Next, we revisited the *K-ras*-transformed NIH 3T3 cell reporter system that we used initially (Fig 1) and compared the efficacy of trichostatin A (TSA), sodium butyrate and 10HDA in reactivating the *Fas* gene over a 2-day period (Fig 3D). Sodium butyrate and 10HDA behave similarly and show activity within 24 h, whereas TSA was able to reactivate the *Fas* gene within 12 h. Finally, we performed individual inhibitor assays using recombinant HDAC1, 3, 8, 10 and 11 (supplementary Fig S6 online). 10HDA inhibits all of these recombinant HDACs with and half-maximal inhibitory concentration (IC_{50}), in the range of 5–8 mM.

DISCUSSION

Royal jelly has been bestowed with mythical qualities because of its long-recognized ability to induce the differentiation of bee larvae into queens. As the queen bee is fertile, longer-lived and more substantial than her worker counterparts, royal jelly is consumed in large quantities all over the world as a health supplement. The consequences of human consumption of royal jelly are unknown, although several health benefits have been claimed, which lack scientific evidence.

Royal jelly is a complex mixture produced in the hypopharyngeal and mandibular glands of nurse bees and used to feed the queen throughout her life (Haydak, 1970). It contains approximately 65% water, 12% crude protein and 10% monosaccharides. The remainder of the royal jelly is composed of an ether-soluble fraction of fatty acids. This fatty acid component is comprised primarily of a single compound—10HDA (Barker *et al*, 1959). 10HDA contributes 2–6% of the wet weight of royal jelly, depending on the source, making it the dominant small molecule in this secretion. If we start with the lower 10HDA (molecular

weight = 186) concentration (2%), then this fatty acid is present at a level of 107 mM in royal jelly, which is thus the minimum concentration that the larvae are exposed too. In tissue culture, we see epigenetic regulatory effects of 10HDA at 0.5–1 mM; concentrations more than 100-fold lower than the developing larvae are soaked in. Thus, although 10HDA is not a potent HDACi (IC_{50} approximately 5 mM), it is present at concentrations that will elicit *in vivo* effects.

10HDA has several pharmacological activities, including antitumour activity (Townsend *et al*, 1959) and inhibitory effects on vascular endothelial growth factor-induced angiogenesis (Izuta *et al*, 2009). Interestingly, valproic acid (a known HDACi) is an inhibitor of angiogenesis (Hrebackova *et al*, 2010) and several HDACis are used in cancer treatment (Beumer & Tawbi, 2010). Furthermore, neural stem-cell differentiation into neurons is stimulated by 10HDA (Hattori *et al*, 2007), a phenomenon that is also observed using TSA (Balasubramaniyan *et al*, 2006), sodium butyrate (Yao *et al*, 2010) and valproic acid (Yu *et al*, 2009). Thus, many of the reported pharmacological activities of 10HDA might be due to its ability to inhibit HDACs.

There are four annotated histone deacetylases encoded in the honey-bee (*A. mellifera*) genome, with orthologues in both class I and class II. 10HDA inhibits class I and class II HDACs, as determined by using a HDAC colorimetric assay, specific *in vitro* assays using a panel of recombinant HDACs, and western blot analysis with pan-acetyllysine antibodies. Most HDACis is function by chelating a crucial zinc atom in the active site of the enzyme (Finnin *et al*, 1999; Bertrand, 2010). Carboxylic acids are a subset of these, and 10HDA can be classed within this group, which includes valproic acid and butyric acid (Bertrand, 2010). Thus, similarly to the other members of this group, 10HDA has proven to be a broad-spectrum class I and class II inhibitor, which causes an increase in lysine acetylation levels and generates a transcriptionally permissive state.

Recent experiments in the Maleszka laboratory have been pivotal in highlighting the importance of epigenetics in bee caste-switching (Kucharski *et al*, 2008). It is intriguing that no gross changes in CpG methylation levels were observed when comparing DNA in the brains of queen and worker bees (Lyko *et al*, 2010), which is consistent with the notion that royal jelly does not harbour activity that inhibits DNA methylation, but instead targets histone-modifying enzymes, such as HDACs. Indeed, on its own, 10HDA is sufficient to reactivate an epigenetically silenced locus, but does not inhibit DNA methylation. However, it is possible that royal jelly does harbour a compound that can selectively target DNA methyltransferases at specific loci, which would function synergistically with 10HDA to promote the generation of the queen bee. Alternatively, extended hyperacetylation might induce selective hypomethylation of DNA at specific loci—a phenomenon that has been observed in mammalian cells (Wu *et al*, 2008). It is important to note that for the first 3 days of development, all bee larvae are fed royal jelly, and workers only get worker jelly on the fourth day; thus, this induced hyperacetylated state might be a means by which to reprogramme the epigenetic code in early development.

METHODS

Antibodies, chemicals and royal jelly. The NIH 3T3 *K-ras* cells and the parental NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC; CRL-6361 and CRL-1658). The pig-ear fibroblast cell line (GF2–4) was a gift from Dr Jeung-Whan Han (Kang *et al*, 2007). Royal jelly was obtained from GloryBee Foods (Eugene, OR, USA; Cat. no. 12366). 5-aza-2′-deoxycytidine and sodium butyrate were obtained from Sigma-Aldrich (St Louis, MO, USA). The following antibodies were used: Fas from MBL (Woburn, MA, USA; Cat. no. JM-3070-100), K-ras from Abcam (Cambridge, MA, USA; Cat. no. ab16795), actin from Sigma-Aldrich (Cat. no. A3853), histone H3 from Millipore (Billerica, MA, USA; Cat. no. 06-755) and pan-acetyllysine from Cell Signalling (Danvers, MA, USA; Cat. no. 9441). 10HDA for preliminary experiments was obtained from Matreya LLC (Pleasant Gap, PA, USA) and larger amounts of it, as well as of 90DA, were prepared following a new, improved procedure (details for the synthesis and characterization are provided as supplementary information online). The fractionation of royal jelly was performed using Amicon-Ultra spin columns (Millipore).

Pyrosequencing. Pyrosequencing was performed as described previously (Figueiredo *et al*, 2009).

Histone extraction and HDACi assays. Histones are extracted using the TCA-precipitation method (Butler *et al*, 1986). HDAC activity was determined using an HDAC colorimetric kit from Enzo Lifesciences (Plymouth Meeting, PA, USA; Cat. no. ALX-850-294-KI01). This kit uses total HeLa cell nuclear extract as the enzyme source.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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