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Science **338**, 1462 (2012);
DOI: 10.1126/science.1225638

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

Pheromonal Induction of Spatial Learning in Mice

Sarah A. Roberts,[1] Amanda J. Davidson,[1] Lynn McLean,[2] Robert J. Beynon,[2] Jane L. Hurst[1]

Many mammals use scent marking for sexual and competitive advertisement, but little is known about the mechanism by which scents are used to locate mates and competitors. We show that darcin, an involatile protein sex pheromone in male mouse urine, can rapidly condition preference for its remembered location among females and competitor males so that animals prefer to spend time in the site even when scent is absent. Learned spatial preference is conditioned through contact with darcin in a single trial and remembered for approximately 14 days. This pheromone-induced learning allows animals to relocate sites of particular social relevance and provides proof that pheromones such as darcin can be highly potent stimuli for social learning.

Scent marks deposited in the environment are used widely by mammals and other vertebrates to advertise location, identity, and status to other conspecifics (*1*). Males in particular invest heavily in territorial scent marks and countermarks to advertise their competitive ability (*2*, *3*). These scent marks are important for female preference between males and for regulating interactions between competitors (*2*, *4*, *5*). However, surprisingly little is known about how scent marks attract conspecifics to particular sites and to the scent owner. It is assumed that this involves active detection and orientation toward odor molecules emanating from the scent source (*6*). At its simplest, animals may detect a plume of air- or water-borne odor molecules and orient along a concentration gradient toward the source (*6*) or follow trail pheromones left on the substrate, detected at a much closer distance (*7*–*9*). The role of learning and spatial memory in scent mark communication has received considerably less attention. We hypothesized that learning stimulated by specific pheromones is an essential component of the response to scent marks that are left in static locations to advertise use of the site by a particular scent owner.

Studies examining the rewarding properties of sexual experience in rodents demonstrate that multiple daily encounters with the opposite sex in one specific location (*10*, *11*), or just with attractive scents from the opposite sex (*11*–*13*), can induce remembered preference for the location itself through associative learning. However, the stimuli in scent marks that induce spatial learning and the rapidity of learning have not been examined. We used the attraction of female house mice to urine scent marks that male mice deposit throughout their defended territory (*14*, *15*) to determine whether specific pheromones may play a role. Outbred wild-stock house mice were used to ensure that both signal and response reflect natural behavior across different genotypes (*16*). Because female laboratory mice demonstrate a conditioned place preference after several daily encounters with cage bedding soiled by males (*13*), we first tested whether this is specifically conditioned by urine that males use for territorial marking and whether repeated encounter is required for learning. Females were given two small Petri dishes placed in opposite halves of a clean test arena, sited on different textured floor tiles as spatial cues. During 10-min daily learning sessions, one dish contained male urine (50 μL) and the other a water control. Conditioned place preference (CPP) was tested with no urine present 24 hours after the last learning session. Females spent more time in the urine dish than in the control dish over three daily learning sessions and developed a CPP for the remembered location when tested 24 hours later (Fig. 1A). This confirmed that the scent that conditions female place preference is in male mouse urine. Repeated exposure was not necessary to induce CPP, which was as strong after three, two, or only one brief daily exposure to the location of male urine (Kruskal-Wallis $\chi^2 = 0.71$, 2 df, $P = 0.70$) (Fig. 1, A to C). Even after only a single learning session, females spent approximately five times longer in the remembered location of male urine as compared with the control, showing as much bias as when urine was present. Learned preference occurred after just 13.6 ± 3.0 s of close sniffing at the male urine stimulus 24 hours earlier. In contrast, females spent relatively little time near equivalent urine from an unfamiliar female and showed no conditioned preference for its location (Fig. 1D), responses that differed from attraction to male urine location [learning day: matched-pair *t* test (t_{21}) = 2.37, $P = 0.028$; CPP: $t_{21} = 3.24$, $P = 0.004$].

Place preference for male scent location was remembered for a surprisingly long period. Pref-

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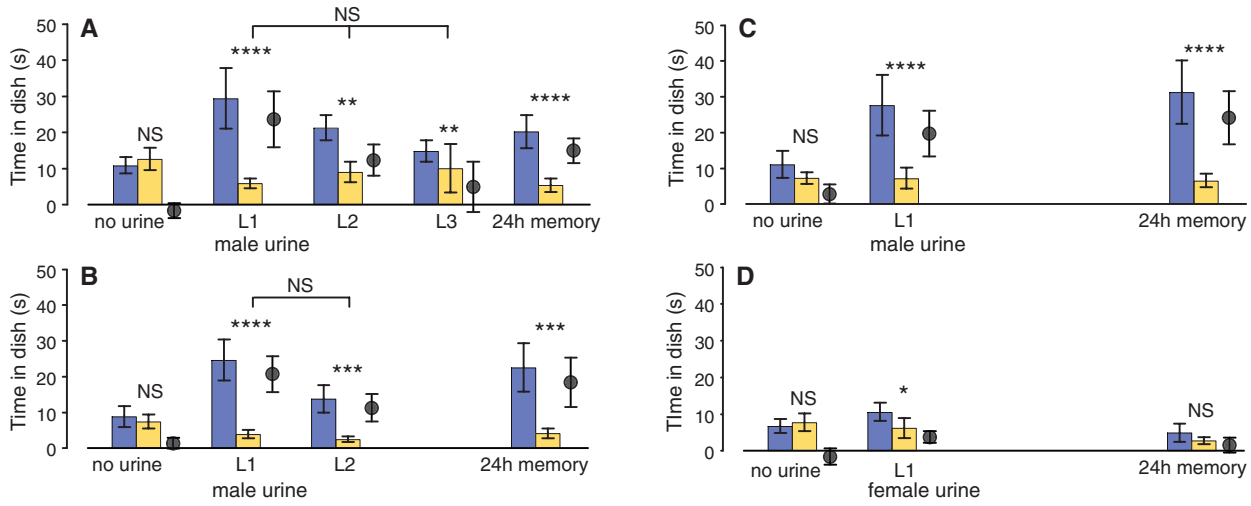


Fig. 1. Female sexual attraction to male urine scent marks and conditioned place preference. After confirming no side bias (no urine), female mice were given test urine versus water in two dishes in 10-min daily learning sessions (L1 to L3). CPP was tested 24 hours later with no urine present (24-hour memory). Females were given (**A**, **B**, and **C**) unfamiliar male or (**D**) female urine for three [(A) $n = 10$ subjects], two [(B) $n = 12$ subjects] or one [(C) and (D) $n = 12$ subjects] learning sessions. Greater time spent in the urine (blue bars) versus control dish (yellow bars) was assessed using one-tailed paired *t* tests (data log transformed to meet parametric assumptions): $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$. Circles show matched-pair difference in time spent in test minus control dish. Data are means ± SEM.

erence was evident when the interval between a single 10-min scent exposure and test of CPP was 14 days, although no CPP was retained after an interval of 28 days (Fig. 2A and fig. S1). However, extinction occurred rapidly once animals encountered no scent in a previously conditioned site: When tested on two successive days with no scent present, CPP was absent on the repeated test day (Fig. 2, B and C). Revisiting the remembered site between tests itself did not disrupt preference if male scent was still present (Fig. 1B). Thus, females form a memory of male scent mark location through associative learning on a single encounter that can be remembered for at least 14 days; however, they rapidly update remembered spatial associations if male scent is absent when revisiting a site. Mice are most likely to be using physical cues (floor tiles with different surface textures) and visual landmarks (overhead red light source) to remember scent location, although they could use acoustic cues (supplementary materials, materials and methods).

Associative learning of scent location could be a general response to male-conspecific odors. Many species-specific androgen-dependent volatile components in adult male mouse urine (*17*–*19*) could allow females to detect male scent marks. However, when nasal contact with urine was prevented by use of a mesh screen, airborne volatiles failed to condition any preference for scent location (fig. S2A). Airborne odor was sniffed during learning (t_{11} = 2.00, P = 0.039), but females spent no more time near this than near water, which is consistent with previous findings that male airborne volatiles do not stimulate instinctive sexual attraction in mice (*14*, *20*). Instinctive sexual attraction is elicited by one specific involatile sex pheromone expressed by adult males: a major urinary protein (MUP) named darcin (MGI:3651981, *Mup20*) that is detected on nasal contact (*15*). To test our hypothesis that darcin conditions females to show the same preference toward associated spatial cues, we expressed darcin as a recombinant protein (r-darcin) in *Escherichia coli* along with two other control MUPs: r-MUP7 (MGI:3709615) and r-MUP11 (MGI:3709617). Spatial conditioning was induced only by darcin, whether presented alone or added

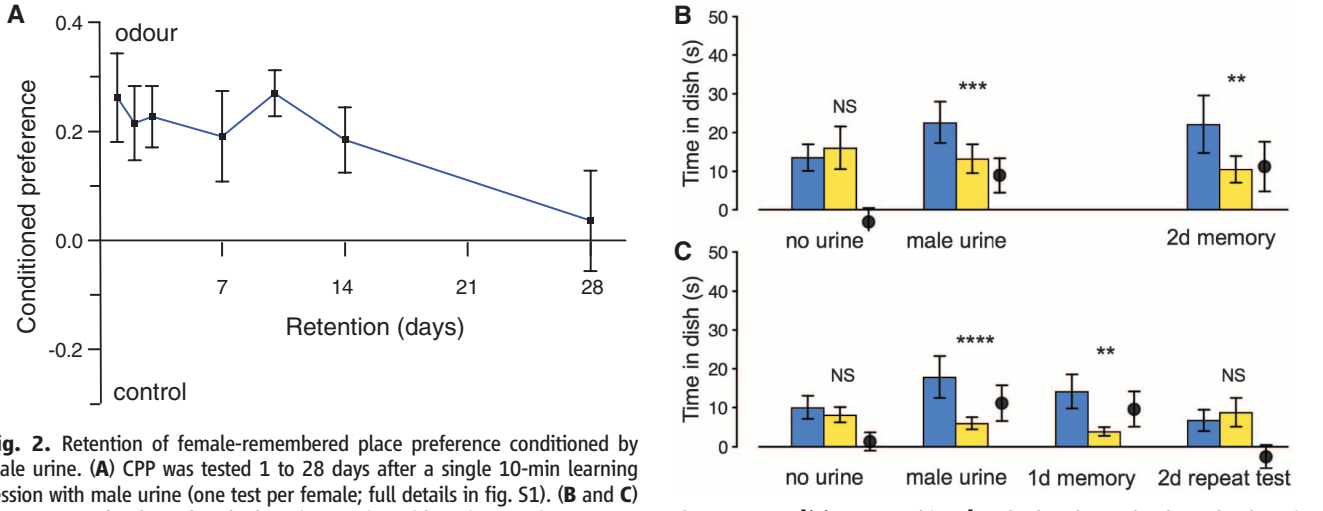
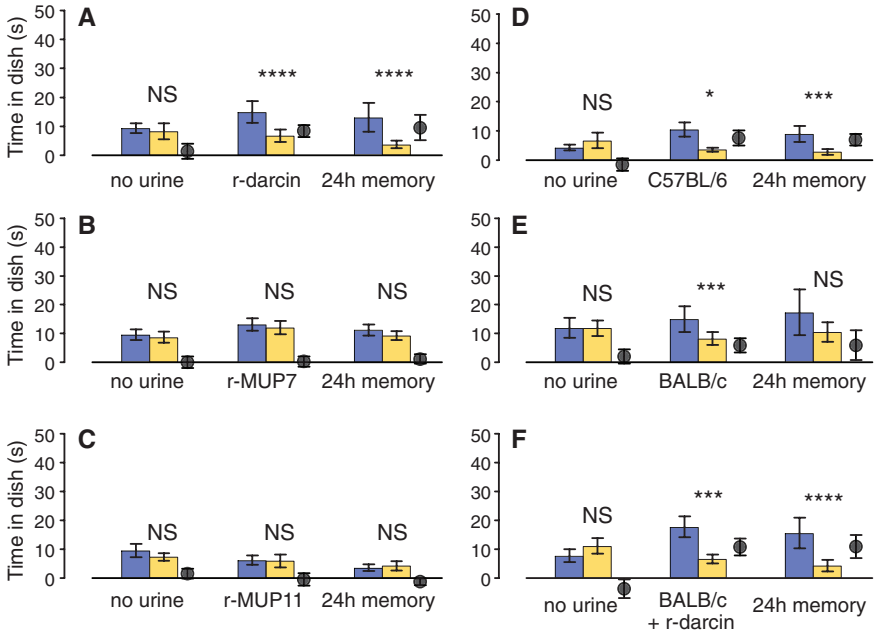


Fig. 2. Retention of female-remembered place preference conditioned by male urine. (**A**) CPP was tested 1 to 28 days after a single 10-min learning session with male urine (one test per female; full details in fig. S1). (**B** and **C**) CPP was tested 2 days after the learning session with no intervening exposure to the test arena [(B) n = 11 subjects], or both 1 day and 2 days after learning [(C) n = 20 subjects]. Key and statistical tests are as in Fig. 1.

Fig. 3. Darcin stimulates female conditioned preference for male urine location. CPP was assessed 24 hours after a single 10-min learning session with test stimulus versus (**A** to **C** and **F**) control buffer or (**D** and **E**) water. (A) r-darcin (1 μg/μl buffer, n = 18 subjects); (B) r-MUP7 (1 μg/μl buffer, n = 37 subjects) (supplementary materials, materials and methods); (C) r-MUP11 (1 μg/μl buffer, n = 18 subjects); (D) C57BL/6 male urine containing 8 μg/μl protein, including 1 μg/μl natural darcin (n = 10 subjects); (E) BALB/c male urine containing <0.1 μg/μl darcin (n = 11 subjects); (F) BALB/c male urine plus r-darcin (1 μg/μl, n = 12 subjects). Greater time in test dish was assessed by Wilcoxon [(A) to (C)] or t tests (data log transformed). Key is as in Fig. 1.



to male urine. Females spent more time with r-darcin than with a buffer control when present and showed a CPP that was just as strong 24 hours later (Fig. 3A). Female response to r-darcin alone was as strong as that toward intact male urine containing the same amount of darcin (inbred laboratory strain C57BL/6) (Fig. 3D). The lack of attraction to other r-MUPs (Fig. 3, B and C) differed from r-darcin both for learning (χ^2 = 9.03, 2 df, P = 0.011) and CPP (χ^2 = 9.78, 2 df, P = 0.008). Further, females showed no attraction or CPP for inbred BALB/c male urine (Fig. 3E), which has naturally high levels of MUP7 and MUP11 (*21*) but lacks normal adult male expression of darcin (*15*), unless r-darcin was added (Fig. 3F).

Darcin not only reliably conditions spatial preference, it also induces female learned attraction to the airborne urinary odor of the male scent owner (*15*). When given prior contact with male urine containing darcin, females learned an attraction to airborne urinary volatiles from this familiar urine but not toward unfamiliar urinary volatiles (fig. S2). However, there was no second-order conditioning, by which contact with darcin conditions attraction to airborne volatiles and the attractive airborne volatiles then condition remembered preference for airborne scent location, even after multiple learning sessions (fig. S2, B and C). Only direct contact with darcin itself conditions a remembered preference for male scent location.

Male scent marks advertise to females but also convey information and a competitive signal to other males. Males are strongly motivated to monitor and countermark signals from potential competitors, particularly those within a competitive male's own scent-marked territory (*2*, *5*). We tested whether male mice remember the location of another male's scent marks and whether this is induced by darcin. Singly housed adult males (representing competitive individual territory owners) spent time near unfamiliar male urine as expected and showed a strong conditioned preference for this location 24 hours later (Fig. 4A). This CPP was evident, if weaker, 14 days after urine encounter (Fig. 4B). Males spent time near airborne urinary volatiles from an unfamiliar male when nasal contact was prevented (unlike females), but there was no CPP for the airborne scent location (Fig. 4C). Exactly as in females, place preference was conditioned only through contact with darcin, which elicited a CPP even when presented alone (Fig. 4D). The lack of response to other r-MUPs (Fig. 4, E and F) differed significantly from the response to r-darcin on both learning day (χ^2 = 7.09, 2 df, P = 0.029) and CPP test (χ^2 = 9.59, 2 df, P = 0.008). BALB/c male urine without darcin failed to condition place preference (Fig. 4G) unless this male sex pheromone was added (Fig. 4H). Even when presented alone, preference for the location of r-darcin was remembered for 14 days by both sexes (z score = –2.57, P = 0.004; effect of sex, Mann-Whitney z score = –0.87, P = 0.41), which confirms that darcin is as potent as intact male urine in stimulating prolonged memory of male scent location.

Male mice express darcin themselves but spent very little time near their own urine during learning trials (Fig. 4, I and J)—substantially less than near unfamiliar male urine (Mann-Whitney z score = –3.78, P < 0.0005). Although own urine elicited a very weak CPP (Fig. 4I), this was much weaker than that conditioned by urine from another male (z score = –2.92, P = 0.004) (Fig. 4A). Thus, individual scent "signatures" in urine allow males to recognize own urine quickly, reducing contact with darcin (3.7 ± 0.6 s of close-contact sniffing versus 16.5 ± 1.5 s toward unfamiliar male urine) and minimizing CPP to own scent marks.

We have discovered a new mechanism of spatial learning induced by a specific pheromone that underlies the ability of animals to relocate and spend time at sites where they have previously encountered male scent. Darcin induces single-trial learning of place preference that is

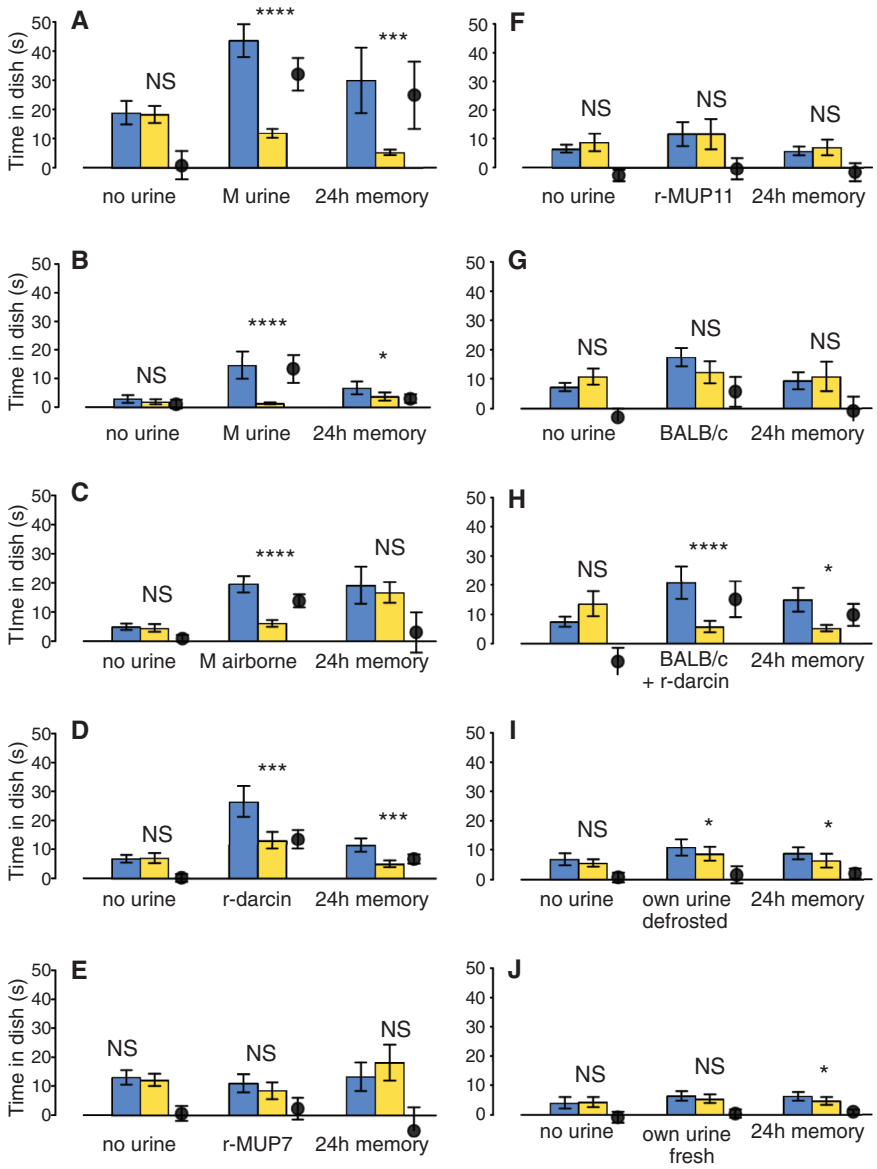


Fig. 4. Darcin stimulates conditioned preference for male urine location among competitor males. CPP was assessed 24 hours or 14 days after a single 10-min learning session with test stimulus versus (**A** to **C**, **G**, **I**, and **J**) control water or (**D** to **F** and **H**) buffer. Unfamiliar wild-stock male urine (A) 24 hours or (B) 14 days after contact, or (C) 24 hours after exposure to airborne urinary volatiles; (D) r-darcin (1 μg/μl buffer); (E) r-MUP7 (1 μg/μl buffer); (F) r-MUP11 (1 μg/μl buffer); (G) BALB/c male urine containing <0.1 μg/μl darcin; (H) BALB/c male urine plus r-darcin (1 μg/μl); n = 12 wild-stock males tested for each. Own urine (I) frozen prior to testing (n = 20 subjects) or (J) collected immediately before learning session (n = 11 subjects). Greater time in test dish was assessed by Wilcoxon [(D) to (H)] or t tests (data log transformed). Key is as in Fig. 1.

remembered for ~2 weeks, although extinction is rapid once animals learn that the involatile pheromone is no longer present. This suggests that darcin is a particularly salient social cue for attracting mice of both sexes. It appears to activate a specific mechanism of associative learning so that instinctive attraction to spend time near this pheromone is extended both to its learned location and to airborne odors associated with the pheromone (*15*). Single-trial learning of associated odors is induced by another pheromone from rabbit mammary glands to improve pup ability to localize nipples efficiently (*22*), but spatial learning is unlikely to be involved.

This establishes a new role for mammalian pheromones in stimulating learned as well as instinctive social responses. Pheromone-induced learning may be much more important than previously recognized, allowing animals to remember and rapidly relocate scent-marked sites of particular social relevance and driving the flexible individual-specific social responses that typify mammals. Even though all adult male mice produce the same sex pheromone, pheromone-induced learning strongly reinforces attraction to a particular individual male and his location. Learned attraction to the individual-specific airborne odor associated with darcin further targets attraction to other scent marks emitting the same individual's odor, resulting in contact with darcin and conditioned preference for other scent-marked sites as well as to the individual male himself. Thus, pheromone-induced learning reinforces attraction to a particular male much more effectively than does simple attraction to the pheromone alone. The reliable and rapid learning induced by darcin among both female and male mice provides a valuable and tractable new model to investigate the neural pathways and mechanisms involved in spatial learning and in the learning of complex individual-specific social odors in response to a specific pheromone stimulus. It may also help to establish how such social information about individual conspecifics is stored and integrated in the brain.

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Acknowledgments: We thank J. Waters, R. Spencer, S. Jopson, and R. Humphries for technical help and members of the Mammalian Behavior & Evolution Group for helpful discussions. The study was funded by research grants to J.L.H. and R.J.B. from the Biotechnology and Biological Science Research Council (BBC503897 and BB/J002631/1) and the Natural Environment Research Council (NE/G018650). The data reported in this study are provided as raw data in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6113/1462/DC1
Materials and Methods
Figs. S1 and S2
References (*23*–*27*)
Data File S1

4 June 2012; accepted 29 October 2012
10.1126/science.1225638

EZH2 Oncogenic Activity in Castration-Resistant Prostate Cancer Cells Is Polycomb-Independent

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Epigenetic regulators represent a promising new class of therapeutic targets for cancer. Enhancer of zeste homolog 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2), silences gene expression via its histone methyltransferase activity. We found that the oncogenic function of EZH2 in cells of castration-resistant prostate cancer is independent of its role as a transcriptional repressor. Instead, it involves the ability of EZH2 to act as a coactivator for critical transcription factors including the androgen receptor. This functional switch is dependent on phosphorylation of EZH2 and requires an intact methyltransferase domain. Hence, targeting the non-PRC2 function of EZH2 may have therapeutic efficacy for treating metastatic, hormone-refractory prostate cancer.

Factors involved in maintaining the epigenetic state of the cell are frequently altered in cancer and are promising therapeutic targets. The expression of EZH2 (enhancer of zeste homolog 2) is correlated with prostate cancer progression, especially to its lethal castration-resistant state (CRPC) (*1*). EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which silences transcription through trimethylation of Lys^{27} on histone H3 (H3K27me3) (*2*). Most studies have focused on PRC2-mediated repression as the oncogenic mechanism of EZH2. In addition, tumor suppressors such as *DAB2IP* have been reported as EZH2 or PRC2 targets (*3*). However, substantial studies have indicated that both *Drosophila* E(z) (enhancer of zeste) and EZH2 have potential functions other than that of a transcriptional repressor (*4*–*6*), although the mechanisms are unclear.

We used the LNCaP cell line as a model of androgen-dependent prostate cancer and LNCaP-abl (abl), its androgen-independent derivative (*7*), to study EZH2 function in the progression of prostate cancer to CRPC. As is the case for clinical tumors (*1*), EZH2 levels in abl cells were much higher than in LNCaP cells (Fig. 1A). EZH2 silencing had a more profound effect on the androgen-independent growth of abl cells than on the androgen-dependent growth of LNCaP cells (Fig. 1B and fig. S1). The requirement of EZH2 for androgen-independent growth was confirmed in an in vivo mouse xenograft CRPC model using CWR22Rv1 cells (Fig. 1C).

Next, we explored EZH2-dependent genes in LNCaP and abl cells. Although similar numbers of genes were up- or down-regulated after EZH2 silencing in LNCaP cells, many more genes were significantly down-regulated upon EZH2 depletion in abl cells, and these EZH2-stimulated genes were highly expressed in abl cells (Fig. 1D). EZH2 silencing by means of two independent small interfering RNAs (siRNAs) confirmed the derepression of the EZH2-repressed gene *DAB2IP* in LNCaP cells and the down-regulation of several EZH2-stimulated genes in abl cells (fig. S2A). We found similar results in two other hormone-refractory cell lines, C4-2B and CWR22Rv1 (fig. S2B). We then examined

www.sciencemag.org/cgi/content/full/338/6113/1462/DC1

Supplementary Material for

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Published 14 December 2012, *Science* **338**, 1462 (2012)
DOI: 10.1126/science.1225638

This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/338/6113/1462/DC1)

Data file S1

Materials and Methods

Subjects and urine donors

Subjects were 192 captive-bred and 9 wild caught adult female *Mus musculus domesticus* and 99 captive-bred and 2 wild caught adult males (F0-F5) aged 8-15 months, from a colony derived from wild ancestors captured from four different populations in the northwest of England, UK. Females were housed in 45 x 28 x 13cm cages (MB1, North Kent Plastics, UK) in single-sex small family groups (2-4 sisters per cage during the test period) in a different room from the urine donors. Subject males were housed singly in 43 x 11.5 x 12cm cages (M3, North Kent Plastics, UK). While naïve female mice isolated from any prior contact with adult male scent show conditioned place preference for male soiled bedding (*13*), such scent deprivation is completely unnatural as wild animals are normally surrounded by scents from conspecifics of both sexes. To ensure that animals had more normal exposure to conspecific scent cues prior to testing, soiled bedding from captive-bred conspecifics of the same and opposite sex was added regularly to subject and donor cages during the testing period. Some subjects were used in more than one test, always with different unfamiliar test stimuli and with a minimum of 5 weeks between successive tests (female subjects: 71/201 (35%) used twice, 3/201 (1.5%) used three times; male subjects: 16/101 (16%) used twice, 11/101 (11%) used three times).

Urine donors were 86 captive-bred adult male and 8 captive-bred female wild-stock house mice aged 9-13 months, derived from the same colony as the subjects but were unrelated and unfamiliar. To ensure that responses generalised across individual donors, urine from at least 8 different donors was used to test each group of 12 subjects. In addition, urine from males of two inbred laboratory strains with highly consistent and well established urinary MUP and volatile components (*15,21,23-25*) was used for specific tests (obtained from Harlan, UK at 3-4 weeks old). Urine from BALB/c strain males (n = 10, aged 6-9 months) was used to test response to urine without normal adult male levels of the male sex pheromone darcin. Urine from C57BL/6 strain males (n=6, aged 6-12 months) was used to establish response to intact urine containing 1µg/µl natural darcin. All donor males were housed singly in 43 x 11.5 x 12cm cages (M3, North Kent Plastics, UK) as wild-derived males can be highly aggressive towards other males and the urine scent of subordinate males is unattractive to females. Females were housed in small single-sex groups of 2-4 in 45 x 28 x 13cm cages. Urine was collected by holding a donor mouse by the scruff of the neck over a clean 1.5ml Eppendorf tube. Urine from wild-stock donors was not pooled as each individual excretes a different genetic identity signature. For tests using inbred laboratory strain donors where all individuals are genetically identical, urine from 3-6 donors of the same strain was pooled for testing, using different combinations of donors to create each stimulus pool. Urine was collected up to 1-2 weeks prior to testing and stored at -20^{o}C until use except for own fresh urine, which was collected 1-2 hours prior to testing.

Throughout, all animals were housed on a reversed 12:12h light cycle with lights off at 0800. Tests were conducted during the active dark period under dim red lighting. Mice were maintained on Corn Cob Absorb 10/14 substrate with paper wool nest material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, St Louis, MO, USA). Cardboard tubes and red plastic mouse houses (Tecniplast UK Ltd)

were provided for home cage enrichment. All animal care protocols were in accordance with the University of Liverpool Animal Welfare Committee requirements and UK Home Office guidelines for animal care.

Conditioned place preference tests

Tests were conducted in clean 45 x 28 x 13cm arenas (MB1 cage base fitted with a perforated Perspex lid) containing two different 14.5cm x 14.5cm tiles to provide internal spatial cues: one pottery tile on the right hand side, one clear plastic tile on the left hand side approximately 4cm apart and 2.5cm from each side, stuck down with a reusable adhesive (Blu-Tack, Bostik Limited, UK). Plastic Petri dishes (55mm diameter) in the centre of each tile (dishes approx 12-14cm apart) contained 55mm diameter glass microfibre filters (Whatman, grade GF/C, stuck down with double sided adhesive tape). Additional external spatial cues were provided by the location of red lighting in the room and a radio that provided an even background noise always in the same location.

Each test consisted of three stages: (a) an initial 10min habituation to the test arena (day 0), (b) one or more daily 10min learning sessions when a urine stimulus (the unconditioned stimulus) was presented in one of the two Petri dishes, and (c) a 10min conditioned place preference test with no scent present, conducted 24h after the last learning day, or after a longer interval to assess memory duration. On the habituation day, both Petri dishes contained 50µL of ddH_20 streaked on the filter paper to familiarise subjects with the test arena and to confirm no significant side bias in the absence of a scent stimulus. On learning days, 50µL of stimulus urine was streaked as 5 x 10 µL on the filter paper of one Petri dish to mimic scent marks, and 50µL of control ddH_20 similarly streaked in the other dish. The position of the stimulus was randomised between subjects but balanced to ensure that an equal number of each stimulus type was presented on each side. To test conditioned place preference, both Petri dishes contained 50µL of ddH_20. After each trial, arenas, tiles and Petri dishes were cleaned thoroughly with 70% ethanol.

Most tests were conducted over three successive days, with 24h between each stage. However, to assess the number of repeated exposures to urine required to establish a significant conditioned place preference for the associated location, urine was presented in the same location for 1-3 daily learning sessions. To establish extinction of the conditioned place preference response in the absence of scent, conditioned place preference was assessed on two successive days. To assess the duration of memory for the location of male urine, the conditioned place preference test was conducted at increasing intervals after a single learning session (1, 2, 3, 7, 10, 14, 28d intervals for females; 1, 14d intervals for males), using different individuals for each time interval.

To assess response to recombinant MUP stimuli, 50µL of the recombinant MUP in 50mM phosphate, 20mM NaCl pH7.4 buffer was used as the test stimulus and 50µL of the same buffer replaced ddH_20 on all days of the test. In most tests, subjects were given full contact with the test stimulus on the learning day(s). However, to assess response to airborne volatiles alone from the scent stimulus, both Petri dishes were covered with 55mm mesh tea strainers that prevented contact with the scent source (mesh holes 1mm x 1mm, subjects on average 25mm away from the stimulus). To assess the possibility of second-order conditioning by airborne volatiles after direct contact with urine, mice were

pre-exposed to contact with 50μL of male urine for 30min in an empty clean MB1 cage prior to transfer to the test arena where urine and control stimuli were covered with mesh to prevent contact during the learning session.

To reduce variability in the oestrus stage of test females and in their recent experience of scents from other conspecifics, soiled nest material and substrate from opposite sex individuals was introduced into the subject's home cage 60-64h prior to the first learning day. Thus females were likely to be in proestrus on the first learning day and in oestrus on the following test day (*26*). Similarly, all male subjects received soiled nest material and substrate from females and from other males to equalise recent experience of conspecific scents. Subject behaviour towards the two Petri dishes was recorded remotely on DVD for all stages. Floor tiles were labelled as A and B, and transcription of DVD recordings was carried out blind to the position of the test stimulus during each trial using an event recording program. We recorded time on each tile (all four paws on the tile), total time in each Petri dish (whole body or head inside dish) and time sniffing the stimulus in each Petri dish (head in the dish and nose making sniffing movements). Initial trials of response to male urine indicated that total time in the urine dish rather than time on the surrounding tile was the best measure of attraction to male urine on learning days, and thus time in the dishes was used to assess conditioned place preference. When Petri dishes were covered with mesh, the total time in the dish was recorded as time on the mesh. For each set of conditioned place preference tests, subject animals remained in the test room throughout to avoid any disruption caused by room change.

Expression and purification of recombinant MUPs

The primary sequences of darcin (accession numbers NP_001012323/XP_355497), 18645Da MUP (accession GB/AAH91744.1) and 18694Da MUP (accession NP_001157998.1) were used to direct *de novo* gene synthesis for maximal expression in *E.coli*. These MUPs are encoded by *Mup* genes *Mup20* (darcin, 18893Da MUP20), *Mup7* (18645Da MUP7) and *Mup9, 11, 16, 18* and 19 (18694Da, referred to here as MUP11) in the C57BL/6 strain genome using the MGI numbering scheme. In laboratory strains, darcin and MUP7 show male-specific expression while MUP11 is expressed by mice of each sex (*21*). Among wild mice, darcin is expressed consistently by male but not by female mice while expression of MUPs with the same mass as MUP7 is more variable between the sexes (*15*). The genes were codon optimised for expression in *E.coli* and cloned into pET28b via *Nco*I and *Xho*I restriction sites (Entelechon GmBH, Regensburg, Germany). The plasmids were used to transform BL21(λ)DE3 cells and each recombinant MUP (r-MUP) expressed in Luria Broth containing kanamycin (30μg/ml). At OD_{600nm} of between 0.6-0.8, the expression of each r-MUP was induced by the addition of isopropyl β-D-1 thiogalactopyranoside to a final concentration of 1mM. Five hours post-induction, cells were harvested by centrifugation at 2,000 x g and the cell pellets stored at -20°C prior to further purification. Harvested cells were lysed using Bugbuster protein extraction reagent (Novagen, Nottingham, UK) containing Complete™ EDTA-free protease inhibitor cocktail (Roche, Burgess Hill, UK). The r-MUP, present in the soluble fraction of the bacterial cell lysate, was purified by virtue of the hexahistidine tag on nickel affinity columns according to manufacturers protocols (Novagen). Column

fractions containing r-MUP were pooled and dialysed against 50mM phosphate, 20mM NaCl pH7.4. This preparation was used without further processing. Both r-darcin and r-MUP11 fold into the eight-stranded beta barrel structure typical of MUPs (*27*). The purity of each r-MUP was assessed by SDS-PAGE analysis and protein concentration was determined by protein assay. Recombinant MUPs were tested at a concentration of 45-55µg in 50µl buffer, to mimic the natural concentration of darcin observed in C57BL/6 males (approximately 10-14% of total MUP) and in wild male mice (*15*). To test response to r-darcin in the normal context of male urine, r-darcin (55µg) was also added to 50µl BALB/c male urine as males of this strain lack expression of darcin but show normal expression of other urinary MUPs (*15,21*) and typical levels of adult male urinary volatiles (*23-25*).

Data analysis

We expected mice to show significantly greater attraction to a scent stimulus compared to a water or buffer control on learning days, and to spend more time in the remembered location of a scent stimulus if this stimulated conditioned place preference. To assess the strength of attraction, we analysed the real difference in time spent in the test versus control dish. Where log transformations of total time in each dish (s+1) approximated normality (Kolmogorov-Smirnov and Shapiro-Wilks tests, $P > 0.05$), we assessed whether animals spent greater time in the stimulus dish compared to the control dish with matched-pair t-tests (one-tailed to assess greater time in the test stimulus dish); ANOVAs or independent Student's t-tests compared the strength of bias (time in stimulus minus control dish) between specific tests. When data could not be transformed to approximate normality, non-parametric Wilcoxon matched-pair tests assessed greater time in the stimulus compared to the control dish; Friedman (for repeated measures), Kruskal-Wallis or Mann-Whitney tests compared the strength of bias. As there was natural variation in time spent in the dishes between sets of test animals (even during habituation with no scent present), figures include the matched-pair difference in time between test and control dish to allow comparison of the strength of attraction between days and between tests. Note that the directional (one-tailed) test of greater time in the test dish is conservative for concluding that there was no response to specific urinary components and to the extinction of response, when responses might be weak. In accordance with ethical requirements to minimise the number of test animals required, all tests were initially run with n=12 subjects, which was sufficient to show the strong response expected to an intact male urine stimulus (occasional tests were eliminated when mice failed to visit the stimulus, showed stereotypical behaviour during the test or a problem occurred with the DVD recording). When a conditioned place preference response was not statistically significant but showed a tendency towards significance ($P < 0.1$), statistical power was increased to confirm that there was no response by increasing sample sizes to n = 18 or 20 subjects for all test stimuli being run simultaneously and compared directly. In one case, (response to r-MUP7, Fig. 3B), an unusual response was observed where there was a weakly significant bias in the conditioned place preference test ($z = -1.81$, $P = 0.037$) despite no attraction on the learning day ($z = -0.62$, $P = 0.28$). To confirm that this was a chance effect, the test was repeated with an independent set of n=20 females, which confirmed no response on the learning ($z = -0.52$, $P = 0.31$) or test

day ($z = 0.67$, $P = 0.76$). The full combined dataset (n=37 females) are presented in Fig. 3B.

A tendency for attraction to male scent to reduce over repeated learning trials was not significant over either a three day (Fig. 1A; repeated measures ANOVA on log transformed data, $F_{2,18} = 0.68$, $P = 0.52$) or two day learning period (Fig. 1B; matched pair t-test, $t_{11} = 1.16$, $P = 0.27$). Although attraction to spend time near male urine did not differ over successive learning sessions, time spent sniffing the urine decreased significantly as the urine stimulus became familiar (3 day learning: Friedman repeated measures test, $\chi^2=7.20$, 2d.f., $P=0.03$; 2 day learning: Wilcoxon matched pair test, $z = -2.35$, $P = 0.01$).

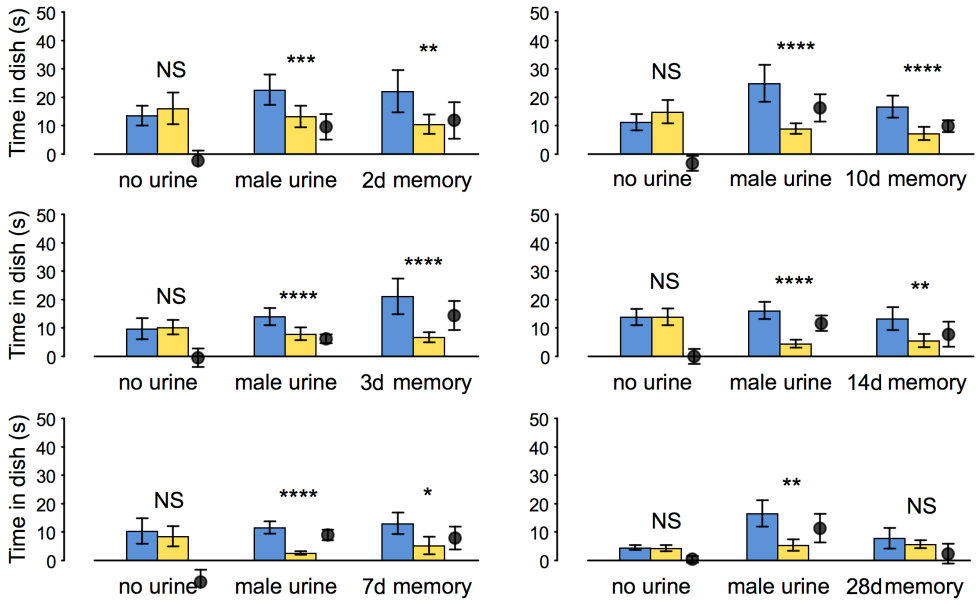

Fig. S1. Retention of remembered female place preference conditioned by male urine scent marks.

Females were presented with two Petri dishes on opposite sides of a test arena for 10min to confirm no side bias (no urine). The following day they were given 50µl of male urine versus water streaked out on filter paper in the Petri dishes for 10min. Conditioned place preference was tested with no urine present after 2d (n=11), 3d (n=12), 7d (n=11), 10d (n=12), 14d (n=20), or 28d (n=12), using different females for each test. Greater time spent in the urine (blue bars) versus control dish (yellow bars) was assessed using 1-tailed paired t-tests (data log transformed to meet parametric assumptions): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. Circles show matched-pair difference in time spent in test minus control dish. Data are means ± s.e.m.

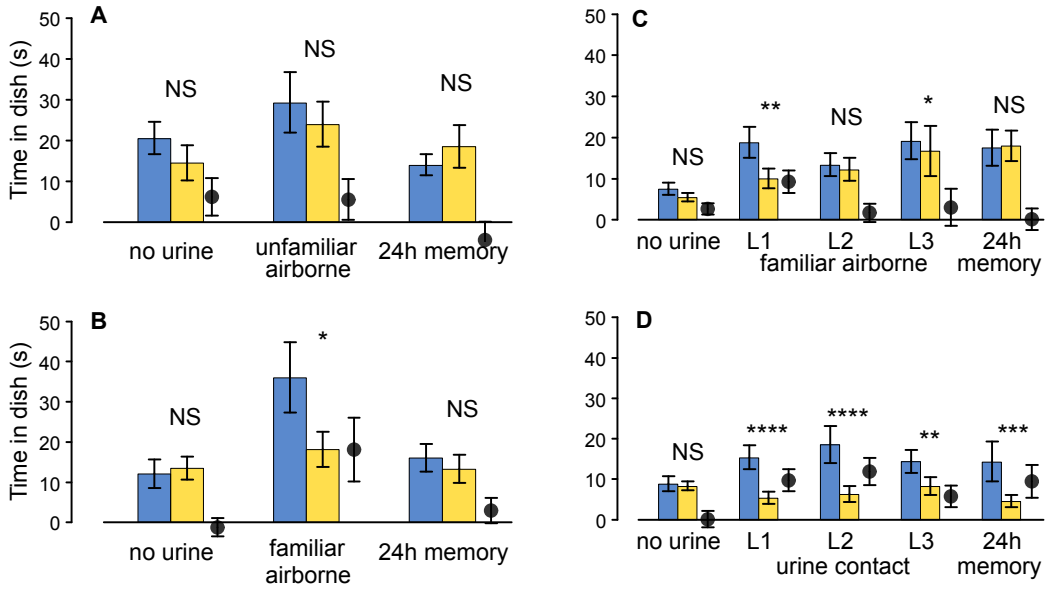

Fig. S2. Male airborne urinary volatiles do not stimulate conditioned place preference.

On learning days, females were given contact with 50µL of male urine each day prior to introduction to the test arena and then presented with airborne volatiles only from unfamiliar male urine (A) or from the same familiar male urine (B,C) versus water on opposite sides of a test arena for 10min for one (A,B) or three (C) learning days. Conditioned place preference was tested 24h later with no odour present (24h memory). As a control for (C), another set of females were given direct contact with male urine in the test arena during the learning day (D). Greater time spent in the urine (blue bars) versus control dish (yellow bars) was assessed by 1-tailed paired Wilcoxon (A-C) or t-tests (data log transformed to meet parametric assumptions): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. Circles show matched-pair difference in time spent in test minus control dish. Data are means ± s.e.m., n=12(A,B), 14(C), 15(D).

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