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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.
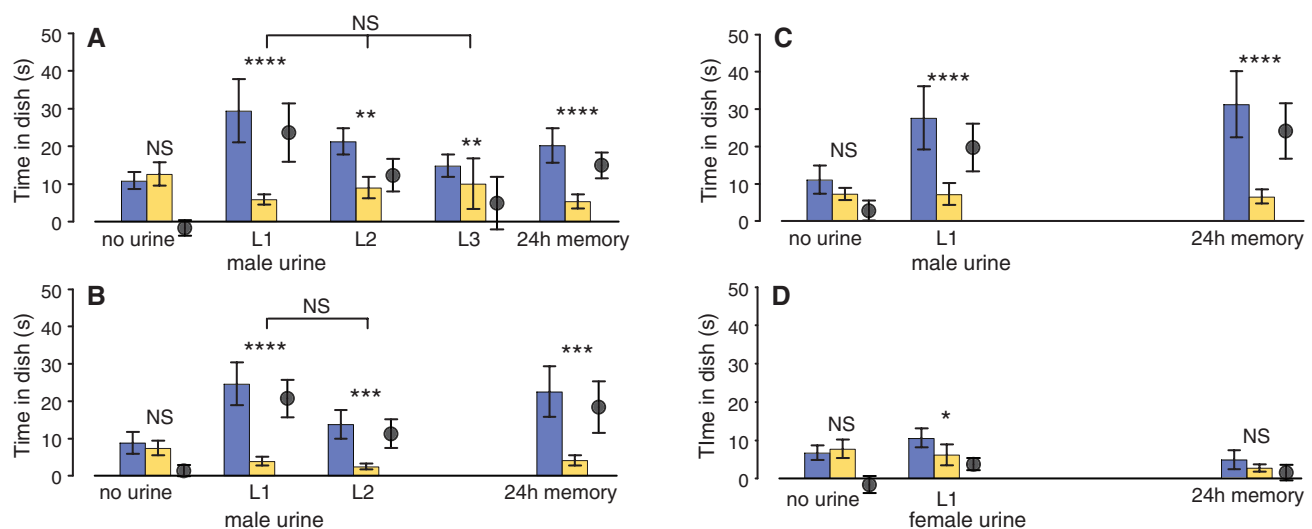
Scent marks deposited in the environment are used widely by mammals and other vertebrates to advertise location, identity, and status to other conspecifics (*1*). Males in particular invest heavily in territorial scent marks and countermarks to advertise their competitive ability (*2*, *3*). These scent 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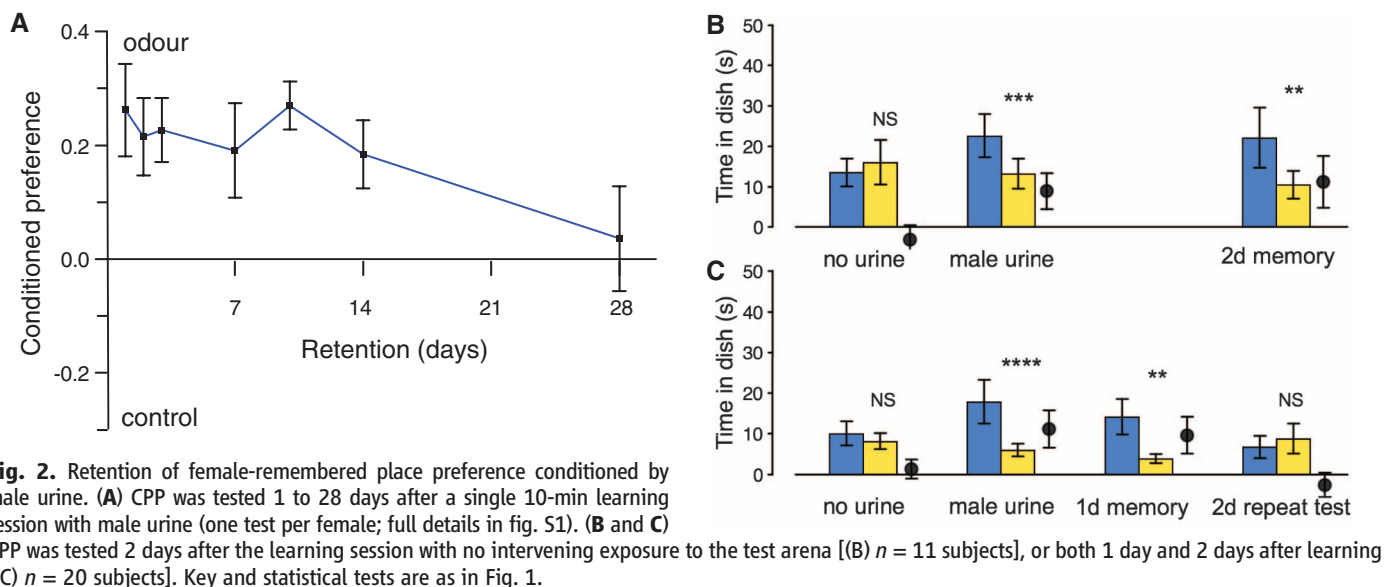] or one [(**C**) and (**D**) n = 12 subjects] learning sessions. Greater time spent in the urine (blue bars) versus control dish (yellow bars) was assessed using one-tailed paired *t* tests (data log transformed to meet parametric assumptions): *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001. Circles 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.
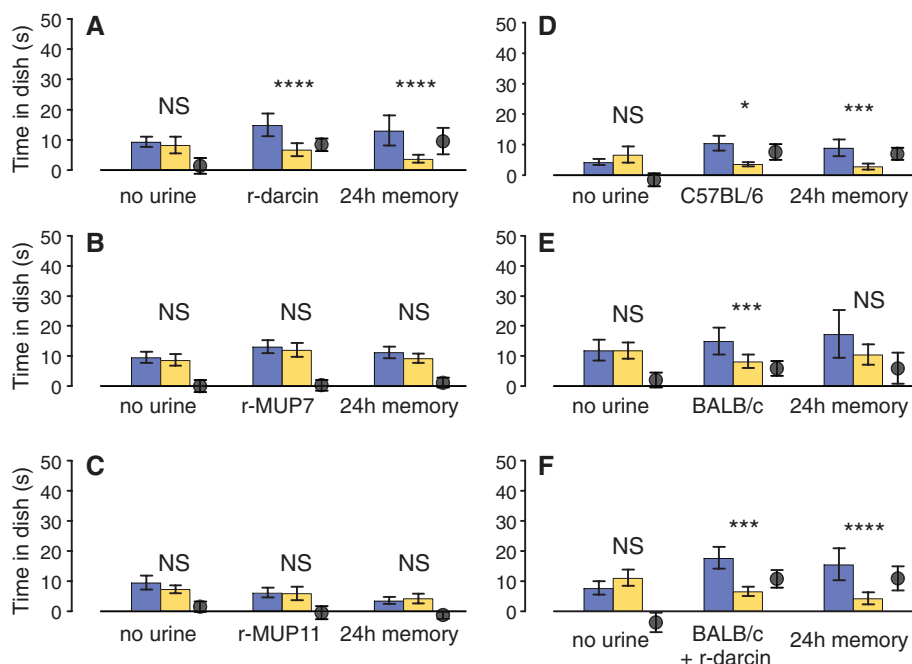
to male urine. Females spent more time with r-darcin than with a buffer control when present and showed a CPP that was just as strong 24 hours later (Fig. 3A). Female response to r-darcin alone was as strong as that toward intact male urine containing the same amount of darcin (inbred laboratory strain 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
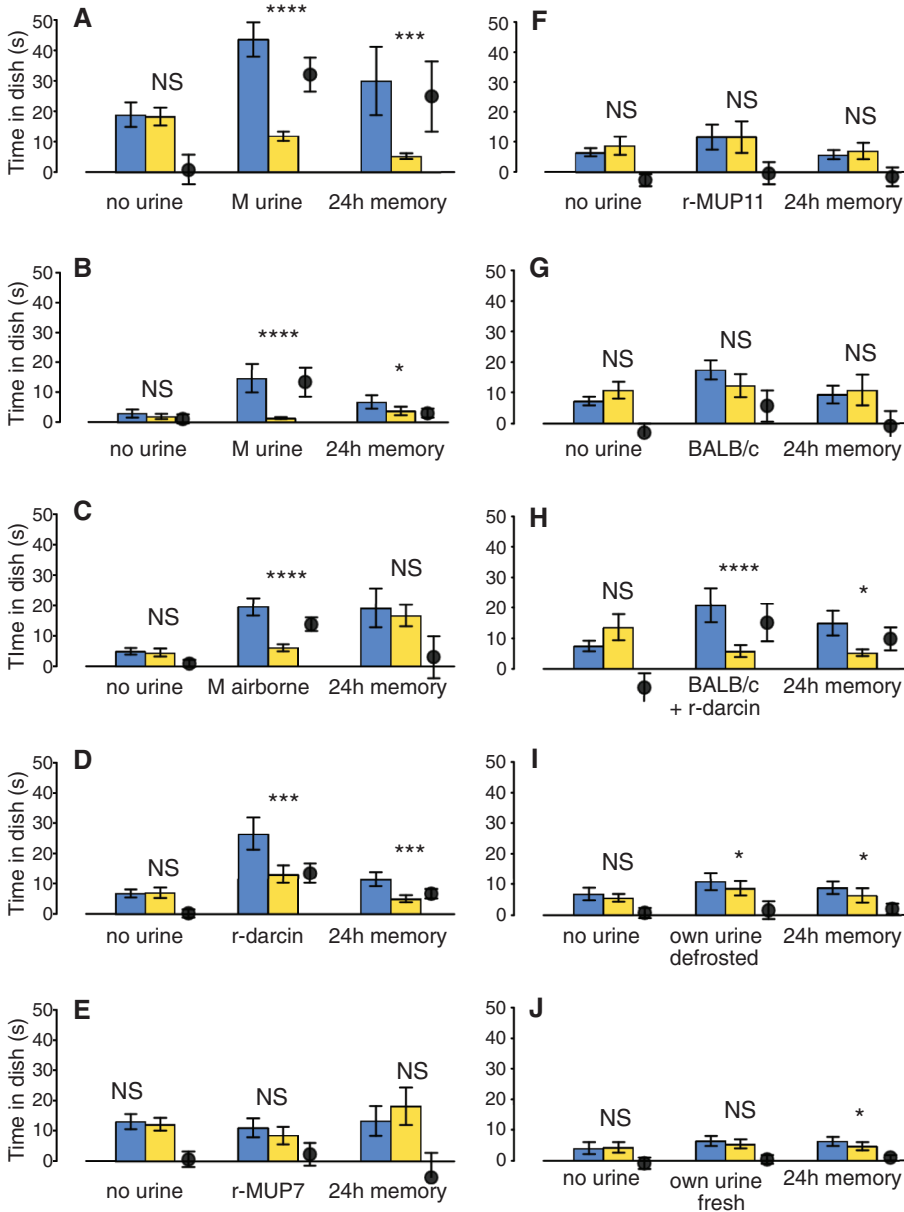
Fig. 4. Darcin stimulates conditioned preference for male urine location among competitor males. CPP was assessed 24 hours or 14 days after a single 10-min learning session with test stimulus versus (**A** to **C**, **G**, **I**, and **J**) control water or (**D** to **F** and **H**) buffer. Unfamiliar 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 buffer); 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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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