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

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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

the profiles of EZH2-dependent genes in two clinical prostate cancer cohorts (*8*, *9*). Although the set of EZH2-repressed genes in LNCaP cells exhibited lower expression in CRPC and marginal negative correlation with EZH2 level, the set of EZH2-stimulated genes identified in abl cells had significantly higher expression levels and positive correlation with EZH2 in these metastatic, hormone-refractory prostate tumors (Fig. 1, E and F, and fig. S3). These results suggest a potentially important functional switch of EZH2 from transcriptional repression to gene activation in CRPC.

To determine whether the gene activation function of EZH2 is the effect of direct binding, we conducted chromatin immunoprecipitation sequencing (ChIP-seq) of EZH2 and H3K27me3. Although EZH2 and H3K27me3 colocalized at the majority of sites in both LNCaP and abl cells, we identified a subset of EZH2 sites that lack nearby H3K27me3 in abl cells (Fig. 2A). These EZH2 sites lacking H3K27me3 were validated with the use of four different EZH2 antibodies (fig. S4A) and by EZH2 silencing (fig. S4B). We defined EZH2 "ensemble" peaks as those with both EZH2 and H3K27me3 enrichment, and "solo" peaks as those with only EZH2 binding. A majority of both ensemble and solo binding sites were located at the promoter regions or gene bodies (fig. S5A). Although ensemble peaks in LNCaP and abl cells overlapped considerably, very few solo peaks overlapped between the two cell lines (fig. S5B). The genes near EZH2 binding sites displayed even more striking differences (fig. S5C). This finding suggests that EZH2 gains a unique set of chromatin binding sites that lack H3K27me3 in abl cells. In addition, the solo peaks were enriched for the active histone marks H3K4me2 and H3K4me3 (i.e., dimethylation and trimethylation of Lys[4] on histone H3) and RNA polymerase II (Pol II) (Fig. 2B), suggesting the potential function of

these peaks in gene activation. Indeed, although ensemble binding was enriched near the transcription start sites of EZH2-repressed genes, solo binding was enriched near EZH2-stimulated genes (Fig. 2C and fig. S6). Strikingly, EZH2 depletion decreased the levels of the active marks at these solo sites (fig. S7), indicating a mechanism of EZH2 in gene activation via modulation of active chromatin states. Genes directly activated by EZH2 in abl cells were significantly overexpressed in gene signatures derived from independent metastatic, hormone-refractory prostate tumors (fig. S8A), and survival analysis supported the prognostic power only of EZH2-activated genes with solo peak binding in abl

cells [Fig. 2D (*8*) and fig. S8, B to D (*10*)]. Taken together, these data support the importance of EZH2 gene activation function in CRPC.

We then tested the involvement of another PRC2 subunit, SUZ12, in the EZH2 solo peaks in CRPC. SUZ12 ChIP-seq signals displayed strong correlation with both H3K27me3 (0.57) and EZH2 ensemble peaks (0.66), but little correlation with EZH2 solo peaks (0.27) (fig. S9A). Silencing of SUZ12 drastically reduced EZH2 binding at ensemble peaks but had no effect at the solo peaks (fig. S9B). Silencing of either SUZ12 or another PRC2 core component, EED, increased the expression of the EZH2-repressed gene *DAB2IP* but had no effect on EZH2-
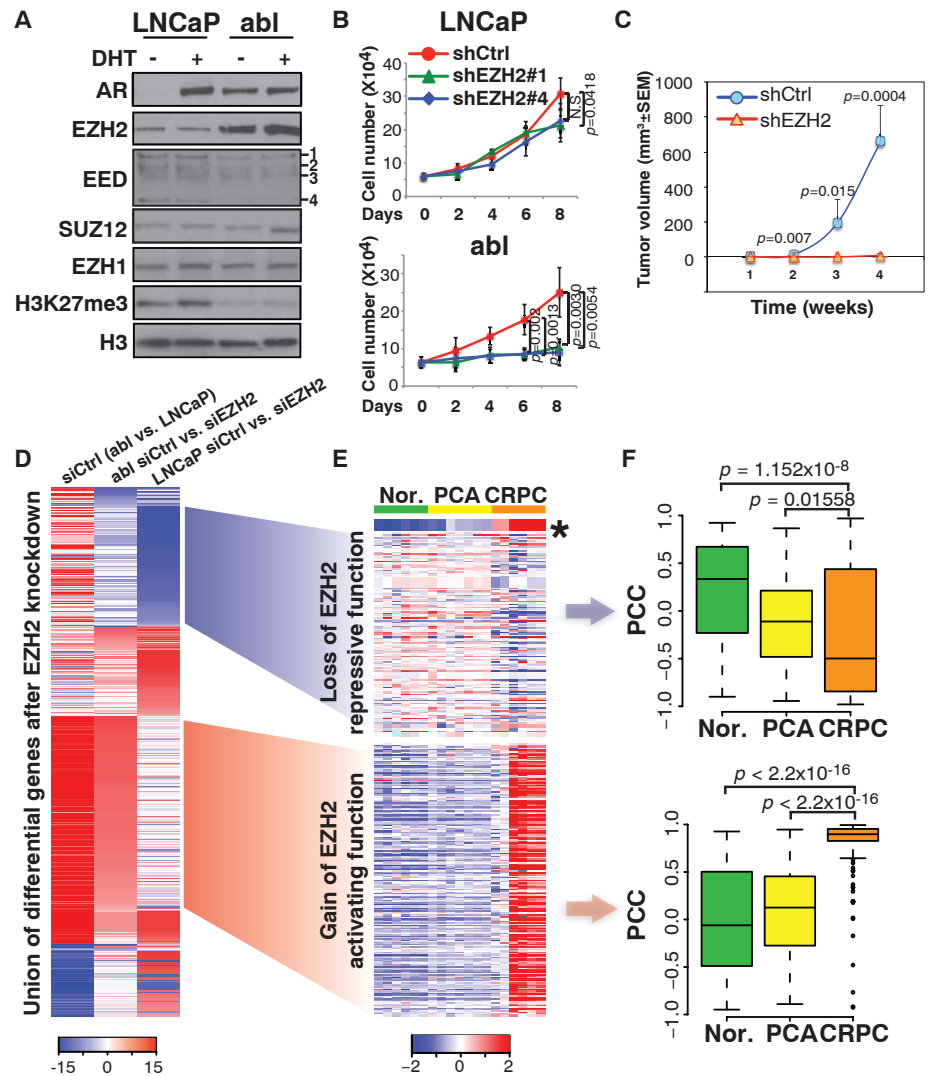
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Fig. 1. Overexpression of EZH2-stimulated genes in clinical CRPC samples. (**A**) Immunoblot of nuclear extracts from LNCaP and abl cells treated without (−) or with (+) 5α-dihydrotestosterone (DHT) to control for effects of cell growth on protein expression. EED isoforms are numbered (*22*). EZH1, enhancer of zeste homolog 1. (**B**) Growth of cells transduced with lentiviral short hairpin RNAs (shRNAs) targeting scrambled control (shCtrl) or EZH2 (shEZH2#1 and #4). (**C**) Tumor growth curve of castrated male *scid* mice injected with CWR22Rv1 cells with or without EZH2 silencing. (**D**) Clustering of the union of differentially expressed genes in LNCaP and abl cells after transfection with siRNAs against control (siCtrl) or EZH2 (siEZH2). (**E** and **F**) Heat map of expression levels (E) and box plots of Pearson correlation coefficients (PCC) (F) of EZH2-repressed (top) and EZH2-stimulated (bottom) genes with EZH2 level in the Varambally *et al.* (*9*) cohort. Nor., normal tissues; PCA, primary tumors. Asterisk in (E) denotes EZH2 level.

that Akt is the kinase for EZH2 phosphorylation at Ser[21] (16), we found higher levels of active Akt in abl cells (Fig. 4A). Although both LNCaP and abl cells were PTEN-null, the Akt phosphatase PHLPP-1 (18) was decreased in abl cells (fig. S18B), which may contribute to the activation of the phosphatidy-linositol 3-kinase (PI3K)–Akt signaling in abl cells. To gain further insight into the involvement of EZH2 phosphorylation in the EZH2 interaction with

Fig. 3. Requirement of methyltransferase activity and interaction with AR for the EZH2 transactivation function. (**A**) Immunoblot of nuclear extracts from LNCaP and abl cells after gel filtration fractionation. Molecular mass standards are indicated. (**B**) Box plots of minimum to maximum reverse transcription polymerase chain reaction (RT-PCR) values for EZH2-activated genes in abl cells after replacement with wild-type or mutant EZH2 as indicated. (**C** and **D**) Growth of abl cells (C) and LNCaP cells (D) in hormone-depleted medium after replacement with wild-type or mutant EZH2 as indicated. (**E**) Coimmunoprecipitation of EZH2 and AR in LNCaP and abl cells without (–) or with (+) DHT.
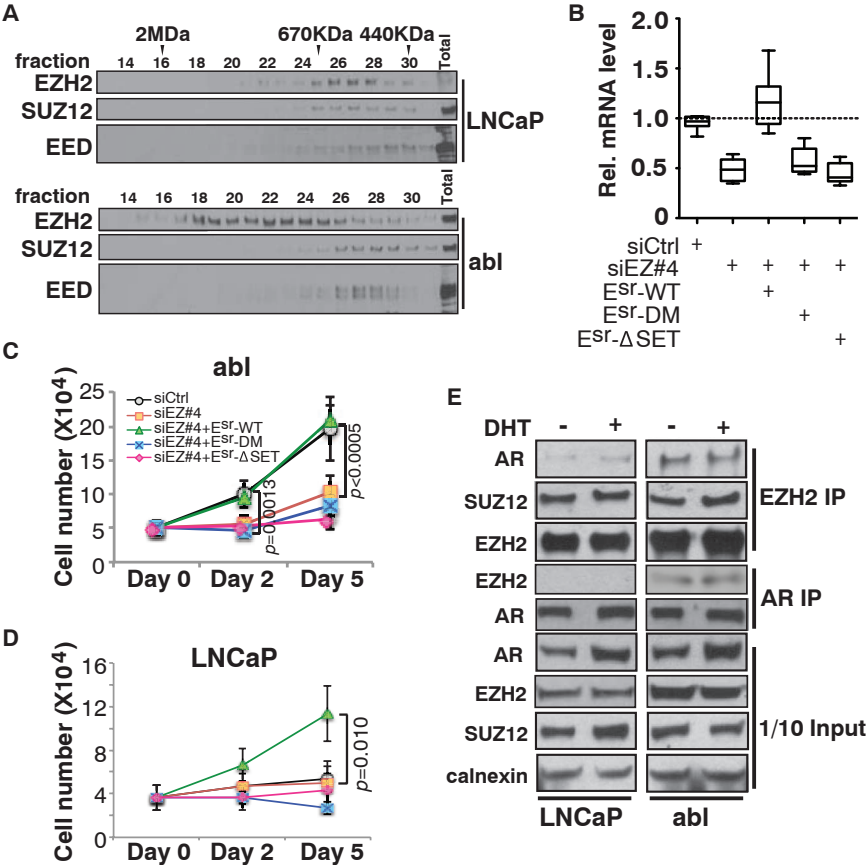
Fig. 4. Critical role of EZH2 phosphorylation at Ser[21] for its functional switch. (**A**) Immunoprecipitation (IP) in LNCaP and abl cells, using control immunoglobulin (IgG) or antibodies specific to EZH2 followed by immunoblotting with the indicated antibodies. (**B**) Box plots of ChIP–quantitative PCR values for EZH2 recruitment to selected ensemble or solo sites, using phosphorylation-specific antibodies. (**C**) Growth of abl cells in androgen-depleted medium after replacement with wild-type or mutant EZH2 as indicated. (**D**) Immunoblot of nuclear extracts from abl cells after gel filtration fractionation. Molecular mass standards are indicated. (**E**) Analysis of EZH2, Ser[21]-phosphorylated EZH2 [pEZH2(S21)], and H3K27me3 protein levels by quantitative immunohistochemistry in neoadjuvant prostate tumors (PCA) and CRPC.
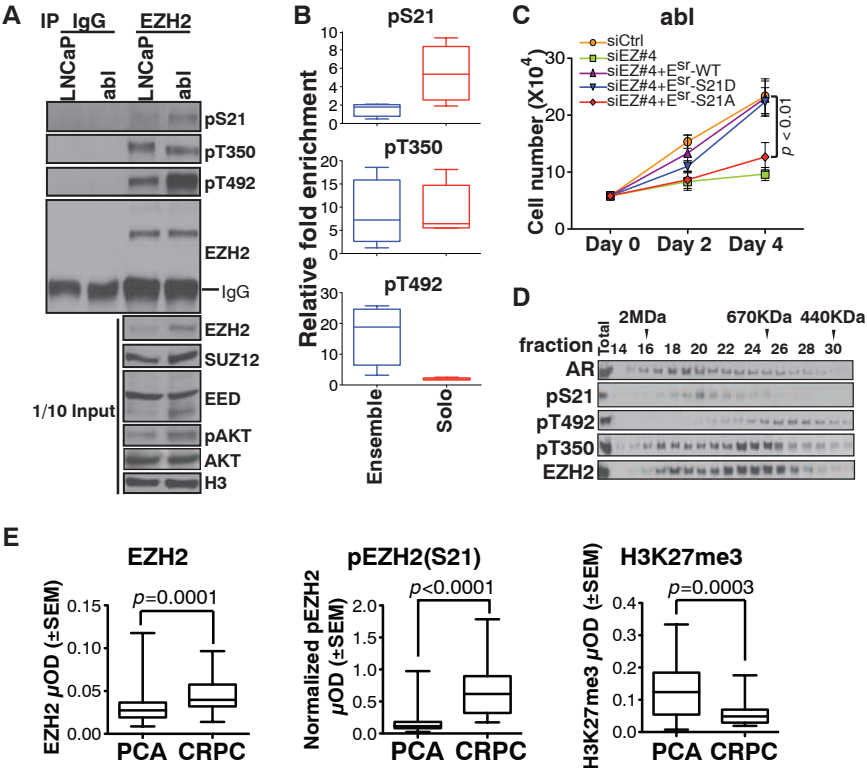
AR, we investigated the copurification of phosphorylated forms of EZH2 and AR by gel filtration and found that pS21 EZH2 predominantly coeluted with AR in a high–molecular weight complex (Fig. 4D). These results suggest a potential role for EZH2 phosphorylation at Ser[21] to promote its association with an AR-containing complex.

The importance of EZH2 phosphorylation at Ser[21] in prostate cancer progression was further analyzed by immunohistochemistry in tissue microarrays containing early-stage prostate tumors from a neoadjuvant androgen deprivation therapy trial and metastatic, hormone-refractory tumors (Fig. 4E and fig. S19). As previously reported (1), the level of EZH2 in CRPC was higher than during early-stage disease, and pS21 EZH2 was even more significantly increased in CRPC. Intriguingly, H3K27me3 levels significantly decreased with prostate cancer progression, consistent with our observation that the global level of H3K27me3 in abl cells was considerably lower than in LNCaP cells (Fig. 1A). This result further supports our conclusion that the oncogenic activity of EZH2 in CRPC is independent of its Polycomb-repressive function.

This study demonstrates that phosphorylation of EZH2 at Ser[21], mediated directly or indirectly by the PI3K-Akt pathway, can switch its function from a Polycomb repressor to a transcriptional coactivator of AR (and potentially other factors). Rescue experiments and the lack of correlation with H3K27me3 levels support a role for EZH2-directed methylation of sub-strates other than H3K27, including potential nonhistone proteins. The current rationale for EZH2 inhibitor design is based primarily on targeting its Polycomb-repressive activity and uses H3K27me3 as the pharmacodynamic readout (19). However, the observed loss-of-function mutations of EZH2 in myelodysplastic syndrome and acute leukemia raise concerns that such inhibitors might exhibit important hematologic side effects (20, 21). Our finding of an altered function for EZH2 in CRPC cells raises the potential to develop inhibitors that specifically target the EZH2 activation function while sparing its PRC2-repressive function. In addition, our finding that EZH2 cooperates with AR-associated complexes and requires phosphorylation to support CRPC growth suggests novel combination therapies for the treatment of metastatic, hormone-refractory prostate cancer (fig. S20).

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Airn Transcriptional Overlap, But Not Its lncRNA Products, Induces Imprinted Igf2r Silencing

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Mammalian imprinted genes often cluster with long noncoding (lnc) RNAs. Three lncRNAs that induce parental-specific silencing show hallmarks indicating that their transcription is more important than their product. To test whether Airn transcription or product silences the Igf2r gene, we shortened the endogenous lncRNA to different lengths. The results excluded a role for spliced and unspliced Airn lncRNA products and for Airn nuclear size and location in silencing Igf2r. Instead, silencing only required Airn transcriptional overlap of the Igf2r promoter, which interferes with RNA polymerase II recruitment in the absence of repressive chromatin. Such a repressor function for lncRNA transcriptional overlap reveals a gene silencing mechanism that may be widespread in the mammalian genome, given the abundance of lncRNA transcripts.

Macro long noncoding (lnc) RNAs such as Airn (1), Kcnq1ot1 (2), or Nespas (3) that silence imprinted gene clusters offer important epigenetic models for the numerous lncRNAs mapped in the mammalian genome (4–6). In the Igf2r imprinted cluster, the paternally expressed Airn (antisense Igf2r RNA noncoding) macro lncRNA silences in cis the paternal alleles of Igf2r, Slc22a3, and Slc22a2 (1). Airn may use different silencing mechanisms, because Igf2r is silenced in all embryonic, extraembryonic, and adult tissues that express Airn, whereas Slc22a2 and Slc22a3 are only silenced in some extraembryonic lineages (7, 8). In support of this, Slc22a3 silencing in the placenta depends on the Airn lncRNA product recruiting EHMT2 histone methyl-transferase, whereas Igf2r silencing does not (9). Igf2r silencing is also not dependent on Polycomb-group proteins or DNA methylation (10, 11). Thus, the mechanism by which Airn silences Igf2r, the only gene in this cluster with an essential embryonic function (12), remains unknown. Airn transcription overlaps the Igf2r promoter but not the Slc22a3 or Slc22a2 promoters (fig. S1A), indicating that silencing could depend on Airn transcriptional overlap independent of the Airn lncRNA product.

To test the role of Airn transcription versus product in Igf2r silencing, we used homologous recombination in embryonic stem (ES) cells to insert polyadenylation (polyA) cassettes on the paternal chromosome that truncate Airn to different

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