Hepcidin regulation in prostate and its disruption in prostate cancer

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Abstract

Hepcidin is a circulating peptide hormone made by the liver that is a central regulator of systemic iron uptake and recycling. Here we report that prostate epithelial cells also synthesize hepcidin, and that synthesis and secretion of hepcidin are markedly increased in prostate cancer cells and tissue. Prostatic hepcidin functions as an autocrine hormone, decreasing cell surface ferroportin, an iron exporter, increasing intracellular iron retention, and promoting prostate cancer cell survival. Synthesis of hepcidin in prostate cancer is controlled by a unique intersection of pathways that involves BMP4/7, IL6, Wnt, and the dual BMP and Wnt antagonist, SOSTDC1. Epigenetic silencing of SOSTDC1 through methylation is increased in prostate cancer, and is associated with accelerated disease progression in prostate cancer patients. These results establish a new connection between iron metabolism and prostate cancer, and suggest that prostatic dysregulation of hepcidin contributes to prostate cancer growth and progression.

Introduction

Hepcidin is a peptide hormone secreted by the liver that is a master regulator of systemic iron homeostasis (1). When iron is sufficient, hepcidin is synthesized. Hepcidin binds to ferroportin, an iron efflux pump expressed in enterocytes and macrophages of the reticuloendothelial system. Binding of hepcidin to ferroportin triggers ferroportin degradation, thereby blocking iron delivery from enterocytes to the systemic circulation, as well as blocking delivery of iron catabolized by macrophages to the circulation (2). Thus hepcidin reduces levels of systemic iron when iron is abundant. Conversely, when iron is insufficient, hepcidin synthesis is reduced, enabling increased iron uptake and recycling, and restoring iron homeostasis. Inappropriate hepcidin synthesis is triggered under inflammatory conditions and can contribute to the anemia of chronic disease by reducing iron uptake in patients with chronic inflammation, infection, and cancer (3).

Three major drivers of hepatic hepcidin transcription have been described. These are bone morphogenetic proteins (BMPs), particularly BMP6 (4), inflammatory cytokines, particularly IL6, and transferrin-bound iron (Tf-Fe). These effectors cross-talk in a regulatory pathway that is incompletely understood (5, 6).

Hepcidin is elevated in the serum of patients with cancer. This is generally considered an indirect consequence of the increased levels of cytokines present in cancer patients and the stimulatory effect of these cytokines on hepatic hepcidin synthesis (5). However, hepcidin synthesis is not restricted to the liver (7, 8) and tumor tissue itself may also play a role in the increased hepcidin observed in cancer (7). Further, due to its ability to potentiate iron retention in cells, dysregulated hepcidin may contribute not only to altered systemic iron regulation, but exert local effects on tumor growth and malignant potential.

Prostate cancer is the most common cancer in men, and the second leading cause of cancer-related mortality among men (9). Locally advanced prostate cancer is treated effectively by androgen deprivation therapies; however, treatments for recurrent metastatic prostate cancer, which is generally androgen-insensitive, are substantially less effective (10). In this manuscript, we report that hepcidin is synthesized in prostate cancer cells and contributes to prostate cancer cell survival. Further, we demonstrate that hepcidin synthesis in prostate cancer is controlled by a unique regulatory cascade that is distinct from the pathway controlling hepcidin synthesis in the liver, and that alterations in this pathway are associated with biochemical recurrence in prostate cancer patients. These data establish a new link between iron metabolism and prostate cancer, identify an unanticipated role for hepcidin in prostate cancer, and suggest new targets for regulating prostate cancer growth and metastases.

Materials and Methods

Cell culture

Non-malignant prostate epithelial cells (PEC) (11) were cultured in keratinocyte – SFM medium (GIBCO) supplemented with 50 mg/liter bovine pituitary extract and 5 µg/liter

human recombinant EGF (GIBCO). Cells were used at passages 4 – 8. Non-malignant PrEC cells (Lonza) were cultured in Lonza PrEGM media with supplements (Lonza PrEGM Bullet-kit). DU145 cells were cultured in EMEM medium (ATCC) containing 10% FBS (BenchMark). LNCaP cells were cultured in RPMI-1640 (GIBCO) containing 10% FBS. PC3 cells were cultured in F-12K medium (GIBCO) containing 10% FBS. All cells were maintained at 37° C in a humidified incubator at 5% CO_2. DU145 and PC3 cell lines used in these studies were obtained from the ATCC and re-authenticated using STR profiling (ATCC: DU145 July 15, 2014 and PC3 November 18, 2014). LNCaP cells were purchased from the ATCC for this study and were passaged 3 – 6 times.

Western Blots

For ferroportin analysis, non-reduced samples were used; other samples were reduced. Western blots were probed with antibodies to Phospho-Smad-1-5-8 (Cell Signaling), total Smad-1 (Cell signaling), Smad-5, Phospho-STAT3 (Cell Signaling), STAT3 (Cell Signaling), hepcidin (Fitzgerald), ferroportin (Novus Biologicals), GAPDH (Fitzgerald), or β-actin (Abcam).

Real-time qPCR

RT-qPCR was performed essentially as described (12). Additional details, including primer sequences, are provided in **Supplemental Methods**.

Neutralizing antibody and recombinant protein treatments

Cells were treated with 1 or 3 μg/ml anti-BMP4, anti-BMP6, anti-BMP7, or anti-IL6 neutralizing antibodies (R&D Systems). 3 μg/ml Isotope matched anti-IgG was used as a control (R&D Systems). Human recombinant BMP6 (R&D systems), BMP4 and BMP7 (GIBCO) were used at 50 – 200 ng/ml for 24 hrs. Cells were treated with human recombinant IL6 (Peprotech) for 24 hours.

ELISA analysis for secreted Hepcidin and IL6

Hepcidin was measured in conditioned RPMI-1640 media using an ELISA from Bachem or USCN Life Science Inc.; both assays gave similar results in direct comparisons. IL6 was measured using an ELISA from R&D systems.

Labile iron pool Assay

Cells were cultured in 96-well plates overnight. The labile iron pool was measured after treatment with 800 nM hepcidin (Peptide International) or 200 μM ferric ammonium citrate (Sigma) for 4 hours (see **Supplemental Methods**).

Immunofluorescence

Cells were plated on an 8 chamber slide (BD Falcon) for 24 hrs prior to treatment with 800 nM hepcidin (Peptide International) for 24 hours. Cells were fixed with 4% paraformaldehyde for 15 minutes and blocked with 5% BSA at 4°C overnight. Anti-human ferroportin 38C8 (Amgen) was applied for one hour followed by rhodamine-red conjugated secondary antibody (Jackson ImmunoResearch).

Cell viability

Viability was measured using an MTS (Promega) or clonogenic assay. See **Supplemental Methods** for details.

Statistical analysis

Statistics are reported as the mean ± standard deviation. Unless otherwise noted, significant differences between control and treatment groups were determined using two-tailed unpaired Student's t tests.

De-methylation studies of SOSTDC1

DU145 cells were cultured in 2 μM 5-Aza-2'-deoxycytidine (Sigma) or vehicle (DMSO) control for 96h with media changes every 24h. RNA was isolated using Trizol reagent (Invitrogen), and a Micro-to-Midi total RNA purification system (Invitrogen). Genomic DNA was isolated using Trizol and Gentra Puregene reagent (Qiagen).

DNA Methylation Assay

DNA samples were modified in preparation for sequencing as described previously (13). Amplified PCR products were subcloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA and approximately 20 individual colonies for each sample picked. Plasmid DNA was directly amplified (Templiphi amplification kit; GE healthcare, Piscataway, NJ) and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems). Data were compared with the UCSC genome reference sequence to assess the methylation status of each CpG site (BiQ Analyzer software). Clones with a minimum of 95% bisulfite conversion rate were included in subsequent analyses.

Published Methylation Data and analysis

DNA methylation data from 92 primary PCa samples and 86 adjacent normal prostate tissues was downloaded from Gene Expression Omnibus (GSE26126)(14) and analyzed using the Bioconductor lumi package as described (15). The β (methylation) value, a continuous variable between 0 and 1 (with β values approaching 1 indicating complete methylation and 0 indicating no methylation), was calculated as described (16). Association between phenotype and DNA methylation was assessed using a generalized linear model and PROC GENMOD or PROC LIFETEST in SAS software (SAS Institute Inc, Cary, NC) (15).

Luciferase reporter Assays

DU145 cells were transfected with 0.1μg TCF/LEF firefly luciferase (Qiagen) or hepcidin luciferase construct (17) and constitutively active Renilla luciferase. 24 hrs post-transfection, cells were treated with 20 mM lithium chloride (Calbiochem), 50 μM endo-IWR (R&D Systems), 50 ng/ml Wnt3a (R&D Systems), or 100 ng/ml rSOSTDC1/USAG1 (R&D Systems). Luciferase activity was measured using the Dual-Luciferase Assay System (Promega).

Immunohistochemistry (IHC)

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded sections using a mouse monoclonal anti-human hepcidin antibody (19D12 Amgen) or anti-SOSTDC1 rabbit antiserum (18). Additional details are provided in **Supplemental Methods**.

Results

Hepcidin is expressed and regulates ferroportin in prostate cells

We first tested whether hepcidin was expressed in a representative selection of cultured prostate cancer cells as well as non-malignant prostate epithelial cells. We examined cancer cells whose proliferation is insensitive (DU145, PC3) and sensitive (LNCaP) to androgen. Hepcidin was secreted by prostate cells and was markedly elevated in all prostate cancer cells relative to benign prostate cells (Figure 1A). Hepcidin transcripts were similarly elevated in prostate cancer cells (**Supplemental Figure 1A**). Amounts of hepcidin secreted by DU145 cells were comparable to amounts secreted by HepG2 cells (a cell line frequently used to model hepatocyte secretion of hepcidin): 66 ± 2 pg/ml/10^6 cells/24hrs cells in HepG2 cells vs. 59 ± 8pg/ml/10^6cells/24hrs in DU145 cells.

Hepcidin increases intracellular iron by binding to and triggering the degradation of the iron efflux pump, ferroportin (2). We tested whether this pathway was conserved in prostate cells. As shown in **Figure 1B,** the increase in hepcidin in prostate cancer cells was associated with a decrease in ferroportin in these cells when compared to non-malignant prostate cells. Further, the addition of recombinant hepcidin to non-malignant prostate epithelial cells expressing high ferroportin substantially reduced ferroportin (**Figure 1C,D**), and increased intracellular iron (**Figure 1E**). To directly assess whether hepcidin secreted by prostate cells functions in an autocrine fashion to regulate ferroportin, we treated prostate cancer cells with anti-hepcidin antibody. As shown in **Figure 1F**, antibody-mediated blockade of hepcidin increased ferroportin. Thus, prostate cells synthesize functional hepcidin and its target ferroportin, and the ferroportin/hepcidin regulatory axis is a functional autocrine pathway in prostate cells.

Hepcidin expression is increased in human prostate cancer samples

To assess whether hepcidin was similarly increased in prostate tissue from cancer patients, we queried a database of gene expression in tissue isolated from human subjects (19). Hepcidin expression was observed in normal prostate, and was significantly upregulated in prostate cancer (**Figure 2A**). Hepcidin was further upregulated in metastatic disease (**Figure 2A**). Ferroportin, the target of hepcidin, was downregulated in prostate cancer, and further downregulated in metastatic prostate cancer (**Figure 2A**). Next, we used immunohistochemistry to examine hepcidin levels in prostate tissue containing malignant as well as benign glands. As shown in **Figure 2B** and **Supplemental Figure 2**, hepcidin immunoreactivity was observed in malignant glands, localized along the cell membranes with accentuation towards the glandular lumina. Faint cytoplasmic staining was also noted. Stromal cells were negative for hepcidin. Thus, prostate epithelial cells and not adjacent stroma are the major source of hepcidin in prostate cancer.

Hepcidin secretion controls labile iron and affects survival of prostate cancer cells

We next assessed the consequences of endogenous hepcidin synthesis in prostate cancer cells. Since hepcidin reduces iron export by triggering the degradation of ferroportin, the increase in hepcidin observed in prostate cancer cells should be associated with an increase in metabolically available iron (the labile iron pool, LIP). As shown in **Figure 2C**, DU145 prostate cancer cells do indeed exhibit a larger labile iron pool than benign prostate cells. We tested whether the hepcidin-mediated increase in labile iron promotes cell survival by incubating cells with anti-hepcidin antibody. To avoid confounding effects of exogenous iron, these experiments were performed in serum-starved cells. As seen in **Figure 2D**, blockade of hepcidin reduced viability of prostate cancer cells. We confirmed this result by performing a clonogenic assay on cells treated with two different anti-hepcidin antibodies compared to cells treated with control IgG. As seen in Figure 2E, treatment with anti-hepcidin antibodies resulted in a statistically significant decrease in cell number. Consistent with these results, treatment with deferoxamine (DFO), an iron chelator, also reduced prostate cancer cell viability (Supplemental Figure 3). Thus, elevated hepcidin contributes to prostate cancer cell survival.

IL6 and BMP4/7 regulate hepcidin synthesis in prostate cells through an autocrine loop

We next focused on mechanisms that regulate hepcidin synthesis in prostate cells. We first examined pathways known to regulate hepcidin in hepatocytes: IL6 and BMPs. We treated DU145 prostate cancer cells with IL6, an inflammatory cytokine known to be upregulated in prostate cancer (20). IL6 increased hepcidin transcripts (**Supplemental Figure 1B**), phosphorylation of STAT3 (**Figure 3A**), and secretion of hepcidin (**Figure 3B**), suggesting that IL6 triggers hepcidin synthesis through STAT3. To test whether IL6 induces hepcidin through an autocrine loop, we incubated DU145 cells with anti-IL6 antibody. As shown in **Figure 3C**, anti-IL6 antibody reduced hepcidin secretion, indicating that endogenous synthesis of IL6 contributes to the increase in hepcidin observed in prostate cancer cells.

We next assessed whether synthesis of hepcidin in prostate cells is also dependent on BMPs. We focused on BMP6 and the related BMPS 2, 4 and 7, since BMP6 plays a major role in iron-mediated transcriptional induction of hepcidin in the liver (4). BMPs 4, 6 and 7 were all functional in triggering SMAD signaling in prostate cells **(Supplemental Figure 4)**. However, only BMP4 and to a greater extent BMP7 induced hepcidin secretion in DU145 cells (**Figure 3D**). BMP6, the major driver of hepcidin synthesis in the liver, had relatively little effect on hepcidin transcripts (**Supplemental Figure 1C**) or secretion (**Figure 3D)** in prostate cells. To test the role of autocrine BMP signaling in hepcidin induction, we treated DU145 cells with antibodies to BMP4, BMP6 or BMP7. As shown in **Figure 3E**, depletion of BMP7 (and to a lesser degree BMP4) by neutralizing antibodies inhibited hepcidin secretion in these cells. Consistent with the inability of BMP6 to induce hepcidin in DU145 cells, antibody to BMP6 was unable to block a decrease in hepcidin transcripts (**Supplemental Figure 1D**) or hepcidin secretion (**Figure 3E**). A preferential ability of BMP7 to induce hepcidin was also seen in PC3 and LNCaP prostate cancer cells (**Supplemental Figure 5**).

Hepcidin synthesis in prostate cells is controlled by the BMP antagonist SOSTDC1

BMP antagonists are a major mechanism through which the activity of BMPs is regulated (21). In particular, the BMP antagonist Sclerostin domain containing 1 protein (SOSTDC1) directly binds to and inhibits the activity of BMPs 2, 4 and 7 (22, 23). Since SOSTDC1 is down-regulated in cancer (18, 24-26), we tested whether downregulation of this antagonist contributed to the increased hepcidin expression seen in prostate cancer cells.

We first verified that SOSTDC1 attenuates BMP4/7 signaling in prostate cancer cells by treating cells with either BMP4 or BMP7 in the presence or absence of recombinant SOSTDC1 and assessing SMAD signaling. As shown in **Figure 4A**, SOSTDC1 attenuates BMP4/7 mediated SMAD phosphorylation in these cells. We then tested whether SOSTDC1 would diminish hepcidin synthesis. As shown in **Figure 4B**, treatment of cells with recombinant SOSTDC1 reduced hepcidin secretion 3-5 fold, as well as reducing hepcidin transcripts (**Supplemental Figure 1E**).

Hepcidin is regulated by Wnt signaling in prostate cells

An unusual feature of SOSTDC1 is its ability to interfere with canonical Wnt signaling as well as block BMP signaling (27). The potent effect of SOSTDC1 on hepcidin synthesis led us to query whether SOSTDC1 exerted its inhibitory effect on hepcidin synthesis by simultaneously targeting both BMP and Wnt pathways. We first tested the involvement of the Wnt pathway in hepcidin synthesis. In the canonical pathway, Wnt ligands prevent the degradation of β-catenin by inhibiting a destruction complex composed of APC, Axin, GSK3β and casein kinase; stabilized β-catenin is then able to interact with TCF/LEF transcription factors to trigger the transcription of downstream genes (28). We treated DU145 prostate cancer cells with LiCl, an inhibitor of GSK3β and activator of the Wnt pathway (28) (**Supplemental Figure 6**), and measured effects on hepcidin transcripts. As shown in **Figure 4C**, LiCl treatment increased hepcidin transcripts, an effect that was attenuated by SOSTDC1. Hepcidin transcripts were also increased in cells treated with Wnt3a (**Figure 4C**). Conversely, endo-IWR, a small molecule stabilizer of Axin that acts as a Wnt pathway antagonist (29), markedly inhibited hepcidin synthesis **(Figure 4C)**.

Sequence inspection revealed a potential TCF/LEF transcription factor binding site at –55/-48 nt of the human hepcidin promoter (**Figure 4D**), suggesting that activation of TCF/LEF transcription factors by Wnt signaling could directly drive hepcidin transcription. To test this possibility, we performed transient transfections with an hepcidin-luciferase reporter construct in DU145 cells. As shown in **Figure 4D**, treatment of transfectants with either LiCl or Wnt3a increased hepcidin promoter-driven luciferase activity, an effect that was antagonized by SOSTDC1 (**Figure 4D**). Conversely, IWR-mediated inhibition of luciferase activity was blocked in cells co-treated with IWR and LiCl or Wnt3a (**Figure 4D**). Interestingly, a similar effect was not observed in HepG2 hepatocellular carcinoma cells **(data not shown)**, suggesting that Wnt-mediated hepcidin induction may be tissue restricted. Further, combining SOSTDC1 and anti-IL6 antibody to simultaneously inhibit Wnt, BMP4/7 and IL6 signaling almost completely prevented hepcidin synthesis (**Figure 4E)**, suggesting that in conjunction these pathways drive most or all of hepcidin synthesis in prostate cancer cells.

SOSTDC1 expression is suppressed by promoter methylation in prostate cancer cells

We asked whether the increase in hepcidin in prostate cancer could be related to decreased levels of SOSTDC1. We first used qRT-PCR to measure SOSTDC1 expression in prostate cells. As shown in **Figure 5A and B**, SOSTDC1 mRNA and secretion was dramatically suppressed in DU145 prostate cancer cells when compared to normal prostate epithelial cells. We tested whether this was due to DNA methylation, an important mechanism of gene silencing in cancer (30). DNA methylation often occurs at CpG sites in the proximal promoter, and inhibits the access of transcription factors to the promoter. We identified a candidate CpG region approximately 100 nt from the transcriptional start site of the SOSTDC1 gene. As shown in **Figure 5C**, this region was heavily methylated in prostate cancer cells. Treatment with 5-aza-dC demethylated this region, and dramatically increased expression of SOSTDC1 (**Figure 5D**).

Methylation of SOSTDC1 in prostate tumors is associated with increased probability of cancer recurrence

To test whether the increased methylation of SOSTDC1 that we observed in prostate cancer cells was also observed in prostate tumors, we queried a publicly available database of DNA methylation profiles from prostate tumors and adjacent prostate tissue (14). This database also contained information on time to prostate cancer recurrence, where recurrence was scored by measuring prostate specific antigen (PSA), a biochemical marker of prostate cancer. As shown in **Figure 6A**, the SOSTDC1 promoter was more extensively methylated in prostate tumors than in normal prostate. As predicted from this result, immunohistochemistry revealed that SOSTDC1 expression was reduced in prostate cancer in comparison to benign prostatic glands in the same patient sample. The reduction was particularly noticeable in areas of high grade (**Figure 6B and Supplemental Figure 7**).

To determine the prognostic impact of SOSTDC1 methylation, we assessed the association of SOSTDC1 methylation with biochemical recurrence of prostate cancer. As shown in **Figure 6C**, methylation of SOSTDC1 was associated with increased risk of prostate cancer recurrence. A model of hepcidin regulation in prostate cancer is shown in **Figure 7**.

Discussion

Our data demonstrate that normal prostate cells synthesize hepcidin, and that hepcidin synthesis is markedly upregulated in prostate cancer cells (**Figure 1**) and tissue (**Figure 2**). Hepcidin acts in an autocrine fashion and exerts important effects on both normal and malignant prostate cells. It reduces levels of the iron efflux pump, ferroportin (**Figure 1**), increases metabolically available iron (**Figure 1,2**), and promotes survival (**Figure 2**). Since prostate cells represent not only a source but also a local target of hepcidin activity (**Figure 1**), this autocrine regulatory axis may contribute to normal prostate biology. In addition, although hepcidin has primarily been studied for its role in regulation of systemic iron uptake and recycling, these results demonstrate that local synthesis of hepcidin by peripheral tissues also have important effects.

We observed that hepcidin was elevated in metastatic as compared to localized prostate cancer (Figure 2A). Metastatic prostate cancer eventually evolves into disease that no longer responds to androgen deprivation therapy. Although we did not study the regulation of hepcidin by androgen, we found that hepcidin synthesis was elevated in both androgen receptor positive and androgen receptor negative cell lines (Figure 1), suggesting that hepcidin may not be a direct target of androgen signaling. Thus, elevated levels of prostatic hepcidin may persist despite androgen deprivation therapy, possibly contributing to prostate cancer progression by enhancing prostate cancer cell survival (Figure 2E, 2D). Future studies will be required to directly test this hypothesis.

The regulation of hepcidin synthesis in prostate cancer exhibits several distinctive features. First, it utilizes different BMPs than are used to control systemic hepcidin synthesis in the liver, with BMP4/7 rather than BMP6 exhibiting a prominent role (**Figure 3**). BMPs and their receptors are expressed in normal prostate tissue as well as in prostate cancer (31, 32), and both BMP6 (33) and BMP7 (34) are elevated in prostate cancer. Since BMP6 and BMP7 activated SMAD signaling in prostate cells (**Supplemental Figure 5**), defects in signaling are unlikely to account for the difference in hepcidin response to these BMPs.

A second unanticipated feature of hepcidin regulation in the prostate is the involvement of Wnt signaling (**Figure 4**), which has not previously been implicated in hepcidin regulation. Transfection of prostate cancer cells with a luciferase reporter gene driven by 2.7 kb of the hepcidin promoter suggests that hepcidin is a direct downstream target of Wnt (**Figure 4**). Constitutive Wnt signaling has been implicated in many cancers, including prostate cancer (35). Thus, aberrant activation of this pathway may increase hepcidin expression in other cancers as well.

The third component of hepcidin regulation in prostate cancer is IL6, which also regulates expression of hepatic hepcidin in response to inflammation. In the prostate, autocrine secretion of IL6 makes a significant contribution to hepcidin secretion (**Figure 3,4**). IL6 is synthesized by prostate cancer cells (36) and affects their differentiation, therapy resistance, and proliferation (37). Higher levels of IL6 have been associated with tumor burden, bone metastasis and poor survival of prostate cancer patients (38). Intriguingly, high levels of circulating hepcidin are also significantly correlated with the presence of metastases in patients with prostate cancer (39). Thus upregulation of hepcidin may be one mechanism through which IL6 contributes to prostate cancer progression.

A fourth and new member of the regulatory network controlling hepcidin synthesis in prostate cancer is SOSTDC1, a BMP antagonist notable for its ability to antagonize Wnt as well as BMP signaling (40). BMP antagonists are secreted proteins that control BMP activity by binding directly to BMPs and preventing them from productively engaging their receptors (21). SOSTDC1 antagonizes BMPs-2, –4, and –7; downregulation of SOSTDC1 has been observed in kidney (18), breast (24) and gastric (25, 26) cancers, implicating SOSTDC1 as a potential tumor suppressor.

We observed SOSTDC1 plays a central role in regulation of hepcidin in prostate cells through its dual inhibition of BMP and Wnt signaling (**Figure 4**). Our results demonstrate

that SOSTDC1 is downregulated in prostate cancer cells (**Figure 5**) and prostate cancer tissue (**Figure 6**). Corroborating this result, we observed that SOSTDC1 is silenced in prostate cancer cell lines by promoter methylation (**Figure 6**). Silencing of SOSTDC1 has substantial clinical impact, since methylation of the SOSTDC1 promoter in patient samples was associated with more rapid disease progression (**Figure 6**).

These four key observations allow us to develop a model for the regulation of prostate cancer cell growth through hepcidin-mediated control of iron efflux (**Figure 7**).

Data presented here provide novel mechanistic insights into pathways governing hepcidin synthesis in cancer cells, and for the first time demonstrate a critical role for hepcidin in prostate cancer. We previously observed that expression of ferroportin was associated with breast cancer progression and affected growth of tumor xenografts in mice (7). Here we demonstrate that prostate cancer cells synthesize hepcidin, and that through its effects on ferroportin, hepcidin is a principal regulator of intracellular iron and survival in these cells. We show that hepcidin synthesis in prostate cancer cells is governed by a unique pathway involving Wnt and SOSTDC1, signaling molecules not previously implicated in hepcidin regulation in any tissue. Further, we demonstrate for the first time that epigenetic mechanisms, in particular methylation of SOSTC1, play an important role in this process. Future studies will assess the role of Wnt and SOSTDC1 in regulating hepcidin synthesis in other cancer types.

In addition to hepcidin and ferroportin, other proteins contribute to maintenance of intracellular iron, including ferritin, which stores intracellular iron, TfR1, which is involved in iron import, STEAP family proteins, which are involved in intracellular iron trafficking, and the iron regulatory proteins IRP1 and IRP2, among others (41). Ultimately, the contribution of these will need to be evaluated to create a comprehensive picture of iron metabolism in prostate cancer.

Our work suggests that hepcidin itself, and/or the pathways that regulate its synthesis, may be a potential therapeutic target or biomarker of prostate cancer progression (39). The discovery of the ferroportin/hepcidin regulatory axis has not only increased fundamental understanding of mechanisms involved in maintenance of iron balance, but has engendered a vigorous search for pharmacological agents that can target this axis (5). Hepcidin has been a particularly attractive target, and drugs that can increase or mimic hepcidin as well as drugs that can down-regulate hepcidin are both under active investigation as potential therapies (5, 42, 43). Notably, inhibition of hepcidin significantly inhibited prostate cancer cell survival (**Figure 2**), suggesting that agents that inhibit hepcidin might find additional application as modulators of tumor growth. Further, since prostate cancer cells exhibit a mechanism of hepcidin regulation that is distinct from that regulating systemic hepcidin, it may be possible to differentially inhibit these two pathways. In particular, it may be fruitful to explore whether perturbation of SOSTDC1, a critical link between iron metabolism and prostate cancer, may be used in attaining such selective intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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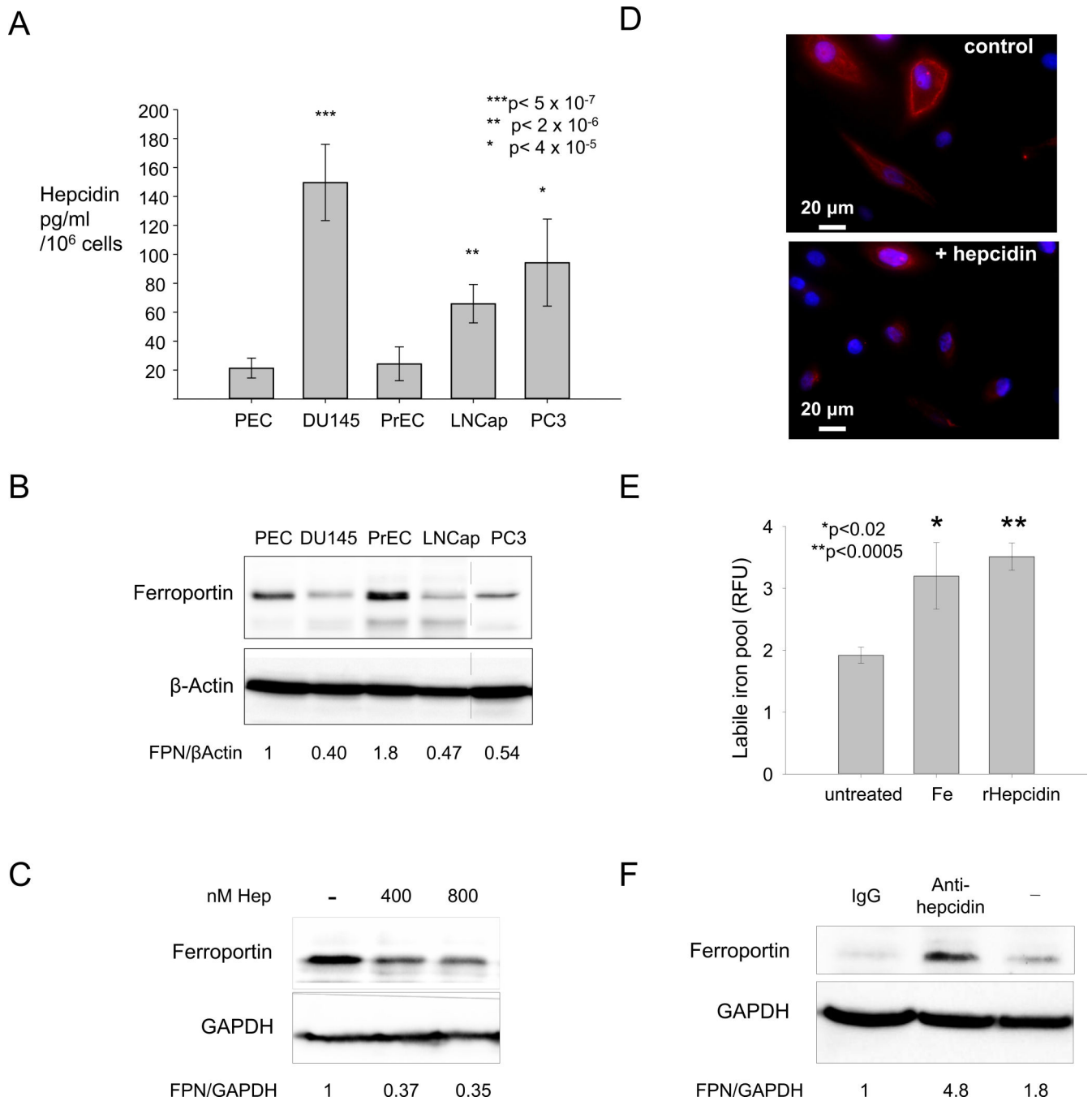
Figure 1. Hepcidin is upregulated in prostate cancer cells and regulates ferroportin and intracellular iron in prostate cells

(A) Hepcidin in conditioned medium from normal prostate epithelial cells (PEC) and prostate cancer cell lines. (B) Western blot of pro-hepcidin and ferroportin in normal prostate cells and prostate cancer cell lines. Shown is a single exposure of one blot; dashed line indicates where an irrelevant lane was cropped out; (C) PEC cells were treated with hepcidin in growth medium for 4hr and levels of ferroportin assessed by western blot (D) Immunofluorescence of ferroportin in untreated PEC and PEC treated for 24 hours with 800 nM hepcidin; ferroportin (red) and nuclei (blue). (E) labile iron pool in PEC treated with

iron (ferric ammonium citrate) or hepcidin. (F) Western blot of ferroportin in PC3 cells treated with anti-hepcidin antibody or IgG control for 24 hours. Means and standard deviations of triplicate determinations; data are representative of one of three independent experiments.
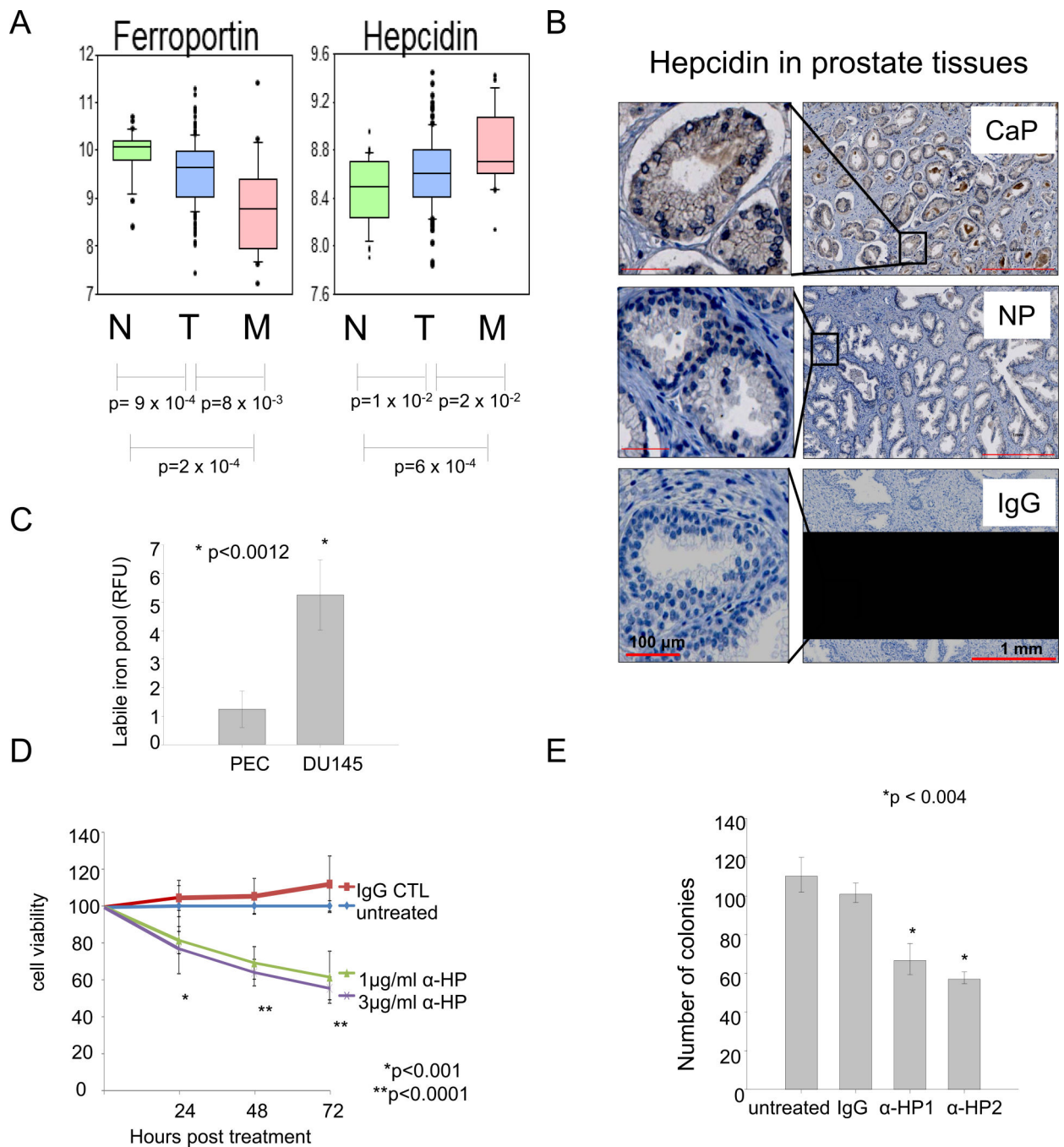
Figure 2. Hepcidin is upregulated and ferroportin is down-regulated in prostate cancer patient samples

(A) Transcript levels of ferroportin and hepcidin in normal prostate (N; green, n=29), primary tumor (P; blue, n=131) and metastatic tumor (M; red, n=19). Data from (19). (B) Immunohistochemistry of hepcidin in prostate tissue. Top: Prostate cancer [Gleason score (3+5) = 8] .Middle: Benign prostatic glands and stroma. Bottom: Control IgG staining. CaP, prostate cancer; NP, normal prostate glands. (C) Labile iron pool in normal PEC and DU145 prostate cancer cells. (D) MTS assay of DU145 cells treated with either IgG control or anti-hepcidin antibody. (E) Clonogenic assay of DU145 using 3 μg/ml anti-hepcidin antibody

from Amgen (#1) or Abcam (#2). Means and standard deviations of triplicate determinations; data are representative of one of three independent experiments. P values reported in Fig. 2D represent differences between anti-hepcidin-treated (1 μg/ml and 3 μg/ml) and IgG control; difference between untreated and IgG control was not significant (p>0.05).
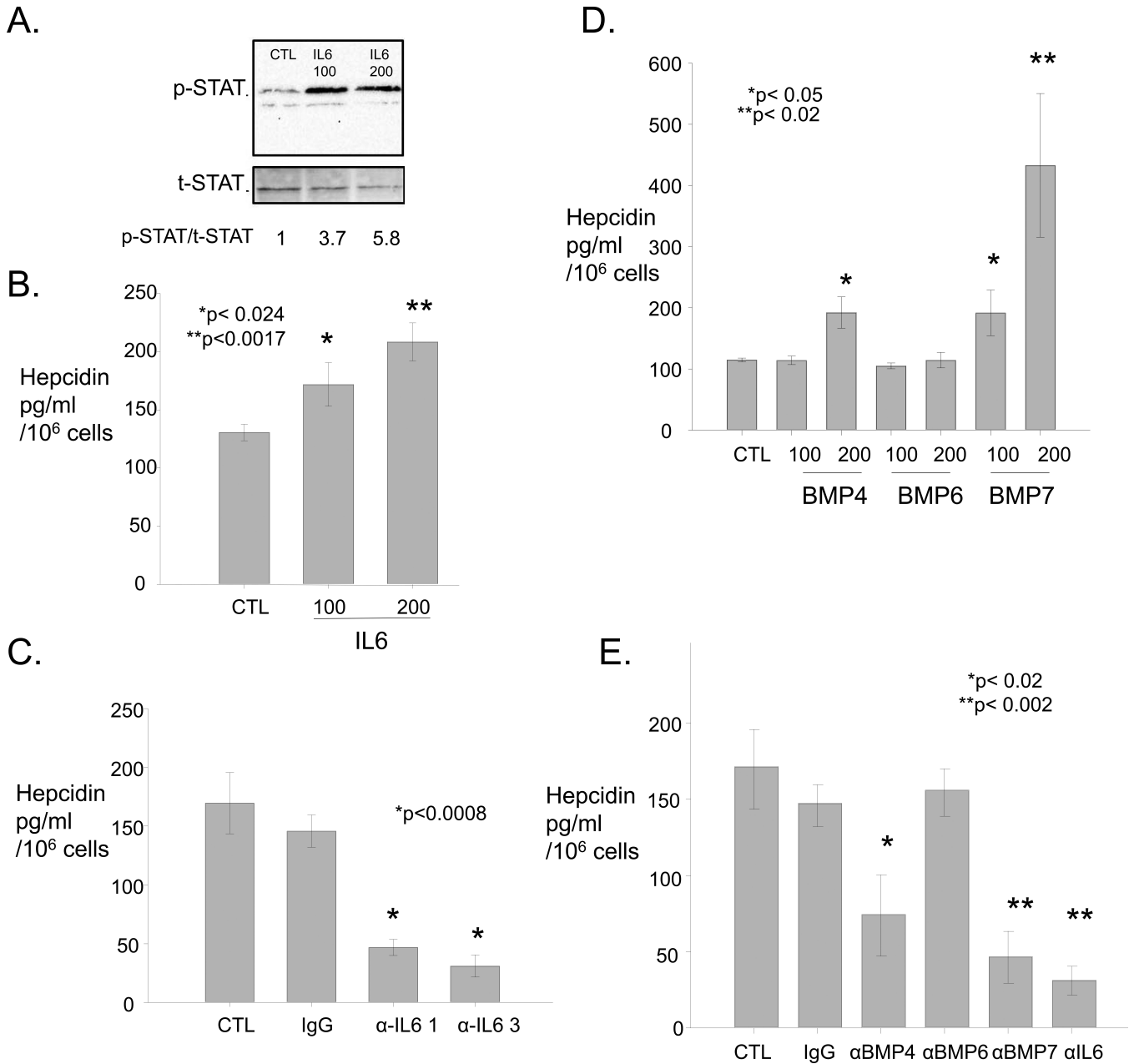
Figure 3. IL6 and BMP4/7 mediate induction of hepcidin in prostate cancer cells
(A) Phosphorylated and total STAT3 in DU145 cells treated with IL6. (B) Extracellular hepcidin following treatment of DU145 cells with 100 or 200 ng/ml IL6. (C) Extracellular hepcidin in DU145 cells treated with anti-IL6 antibody or IgG control antibody. (D) Extracellular hepcidin in DU145 cells treated with 100 ng/ml or 200 ng/ml BMP4, BMP6, or BMP7. (E) Extracellular hepcidin in DU145 cells treated with 3μg/ml anti-BMP4, anti-BMP6, anti-BMP7, anti-IL6 antibody, or IgG control antibody. Means and standard deviations of triplicate determinations; data are representative of one of three independent experiments.
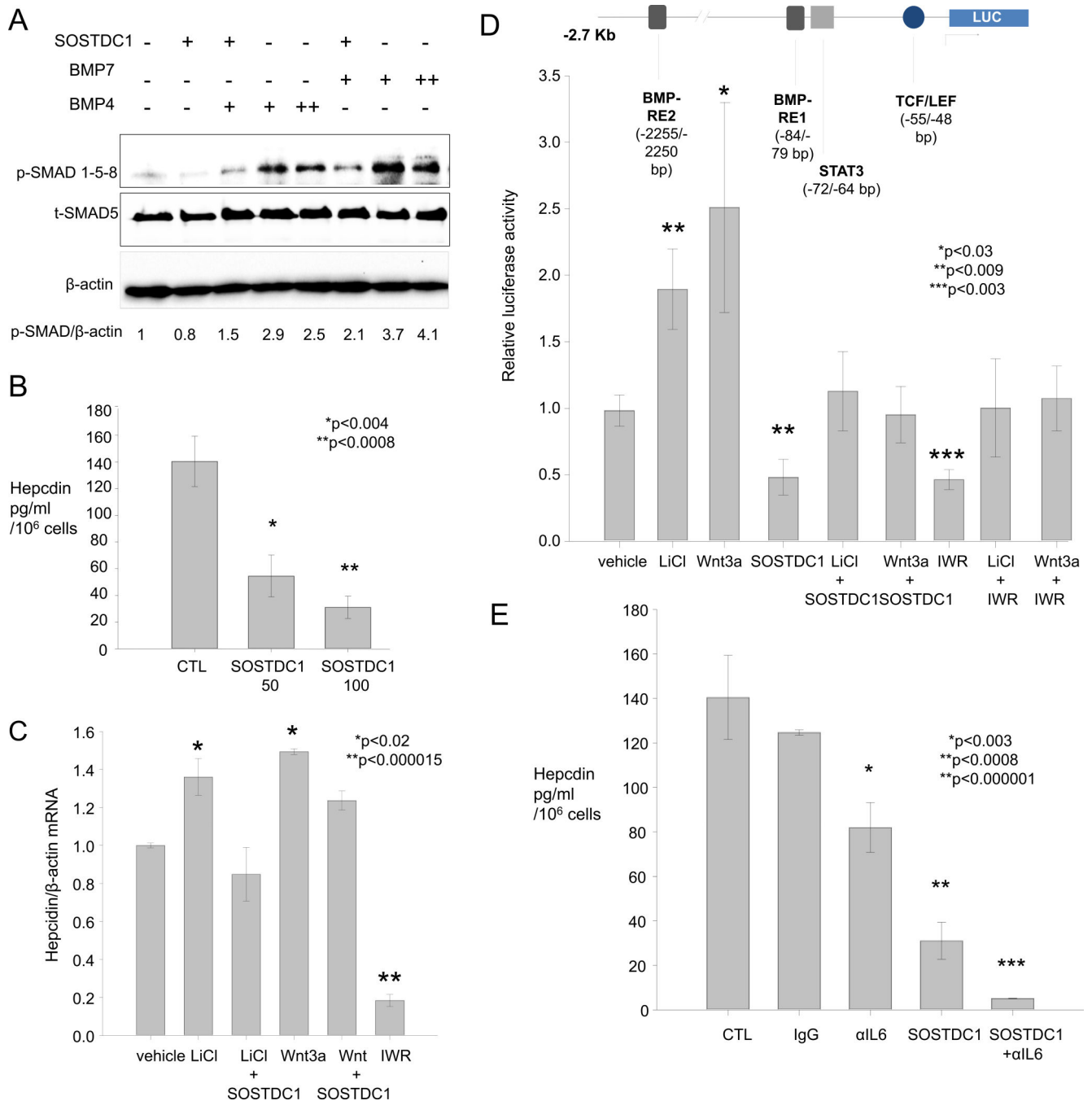
Figure 4. SOSTDC1 antagonizes BMP and Wnt-mediated induction of Hepcidin
(A) Phosphorylation of Smad-1-5-8 in DU145 cells treated with BMP4 or BMP7 in the
presence or absence of SOSTDC1 for 24 hours. (B) Extracellular hepcidin in DU145 cells
treated with 50 or 100 ng/ml rSOSTDC1 for 24 hours. (C) Hepcidin transcript levels in
DU145 cells treated with 20 mM LiCl, 20 mM LiCl plus 100 ng/ml SOSTDC1, 50 ng/ml
Wnt3a, 50 ng/ml Wnt3a plus 100 ng/ml SOSTDC1 or 50 uM endo-IWR for 24 hrs. (D)
Hepcidin promoter-driven luciferase activity in DU145 cells treated as in C. Inset is a
cartoon of the location of BMP and STAT3 (44) sites and a candidate TCF/LEF site in the

human hepcidin promoter. (E) Hepcidin secretion in DU145 cells treated with 1 μg/ml anti-IL6 antibody, 1 ug/ml control IgG, 100 ng/ml recombinant SOSTDC1, or the combination of anti-IL6 and SOSTDC1 for 24 hours. Means and standard deviations of triplicate determinations; data are representative of one of three independent experiments.
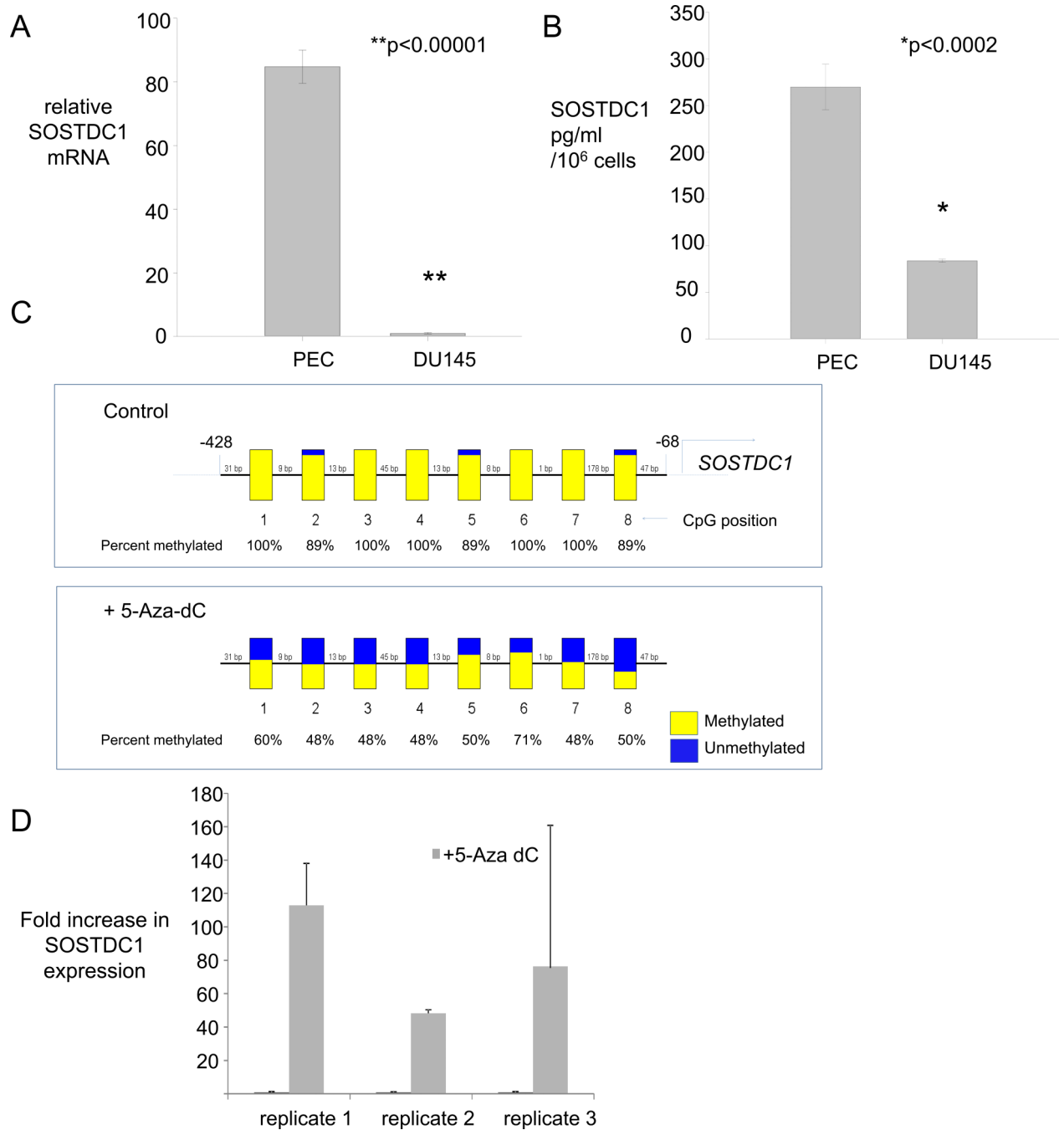
Figure 5. Expression of SOSTDC1 is controlled by promoter methylation in prostate cancer cells
(A) Transcript levels of SOSTDC1 in DU145 and normal PEC cells. (B) Extracellular SOSTDC1 in DU145 and PEC cells. (C) Methylation of the SOSTDC1 promoter at the designated positions in DU145 cells treated with DMSO or 5-Aza-2'-deoxycytidine. (D) SOSTDC1 transcripts in DU145 cells treated with 2 μM 5-Aza-2'-deoxycytidine or DMSO in 3 independent experiments. Means and standard deviations of triplicate determinations; data in A and B are representative of one of three independent experiments.
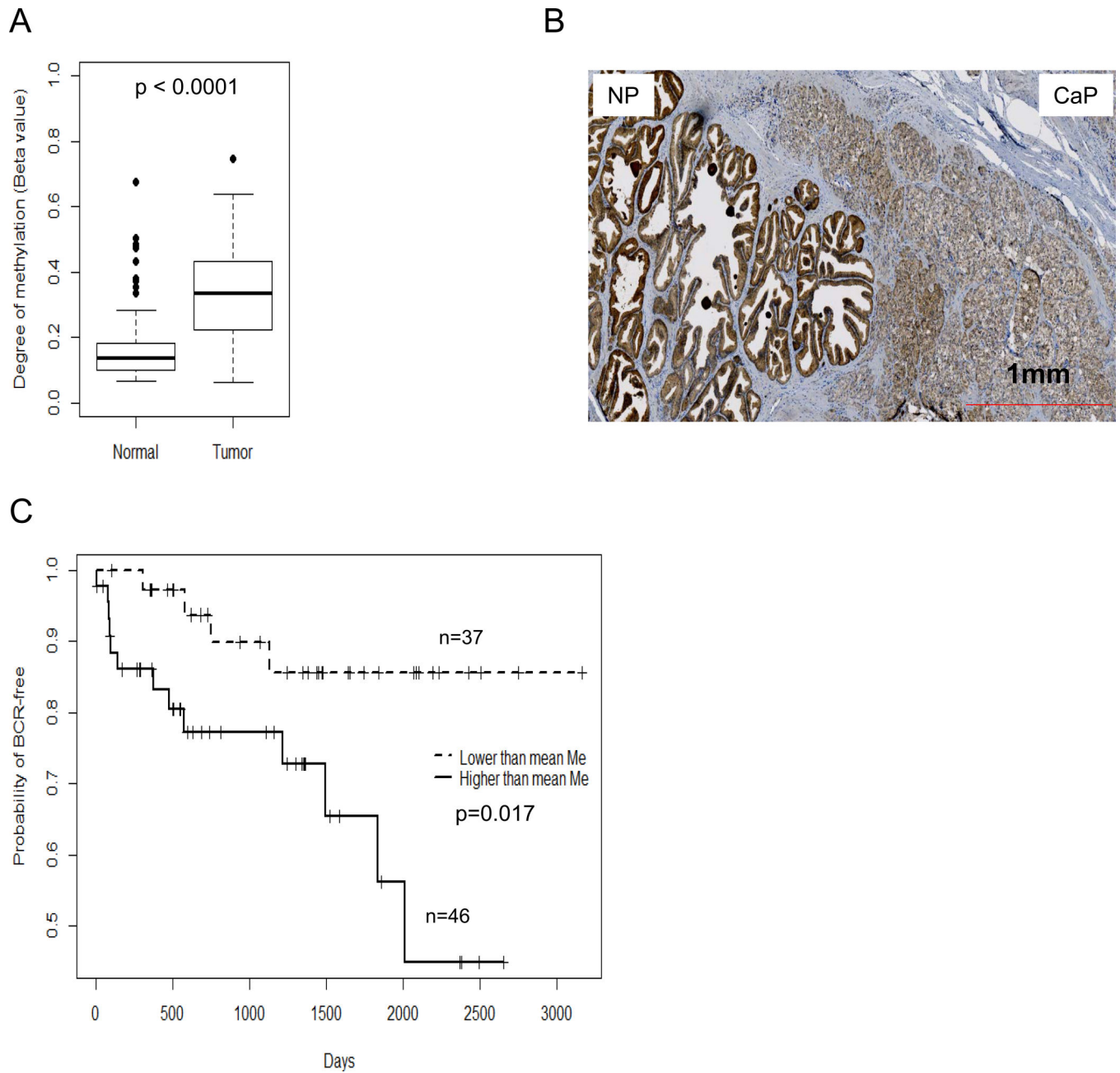
A

B

C

Figure 6. SOSTDC1 promoter is hypermethylated in prostate tumors
(A) Methylation of SOSTDC1 in benign and malignant human prostate tissue (data from (14)). (B) SOSTDC1 expression in prostate tissue, analyzed by immunohistochemistry (IHC). Benign prostatic glands at left are more intensely staining than the high grade [Gleason score (5+4) = 9] prostatic carcinoma to the right. (C) Probability of biochemical recurrence in prostate cancer patients with SOSTDC1 methylation in tumor tissue that is above or below the mean.
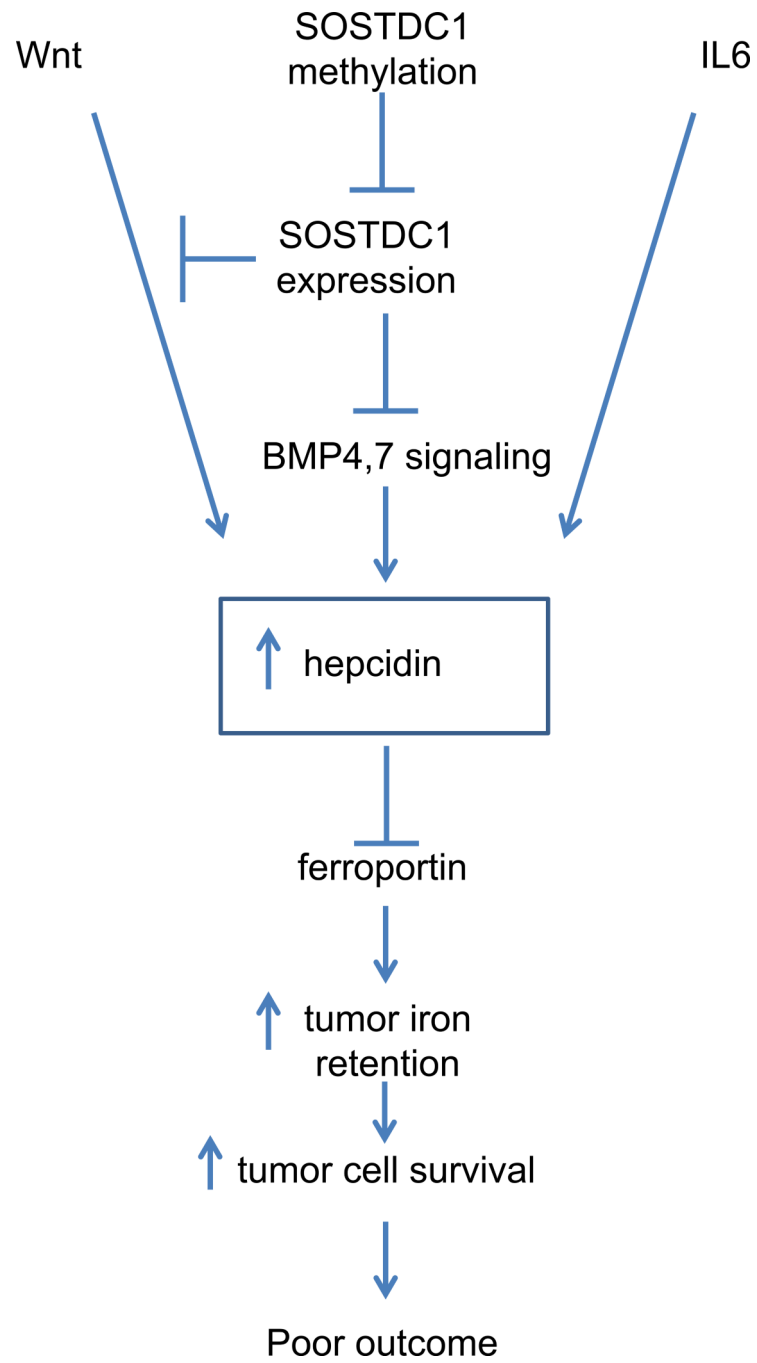
Figure 7.
Working model of transcriptional control of hepcidin in the prostate.