Coordinate Loss of MAP3K7 and CHD1 Promotes Aggressive Prostate Cancer

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Abstract

Prostate cancer subtypes are poorly defined and functional validation of drivers of ETS rearrangement–negative prostate cancer has not been conducted. Here, we identified an ETS subtype of aggressive prostate cancer (ERG: MAP3K7del/CHD1del) and used a novel developmental model and a cell line xenograft model to show that cosuppression of MAP3K7 and CHD1 expression promotes aggressive disease. Analyses of publicly available prostate cancer datasets revealed that MAP3K7 and CHD1 were significantly codetiled in 10% to 20% of localized tumors and combined loss correlated with poor disease-free survival. To evaluate the functional impact of dual MAP3K7–CHD1 loss, we suppressed Map3k7 and/or Chd1 expression in mouse prostate epithelial progenitor/stem cells (PrP/SC) and performed tissue recombination experiments in vivo. Dual shMap3k7–shChd1 PrP/SC recombinants displayed massive glandular atypia with regions of prostatic intraepithelial neoplasia and carcinoma apparent. Combined Map3k7–Chd1 suppression greatly disrupted normal prostatic lineage differentiation; dual recombinants displayed significant androgen receptor loss, increased neuroendocrine differentiation, and increased neural differentiation. Clinical samples with dual MAP3K7–CHD1 loss also displayed neuroendocrine and neural characteristics. In addition, dual Map3k7–Chd1 suppression promoted E-cadherin loss and mucin production in recombinants. MAP3K7 and CHD1 protein loss also correlated with Gleason grade and E-cadherin loss in clinical samples. To further validate the phenotype observed in the PrP/SC model, we suppressed MAP3K7 and/or CHD1 expression in LNCaP prostate cancer cells. Dual shMAP3K7–shCHD1 LNCaP xenografts displayed increased tumor growth and decreased survival compared with shControl, shMAP3K7, and shCHD1 xenografts. Collectively, these data identify coordinate loss of MAP3K7 and CHD1 as a unique driver of aggressive prostate cancer development. Cancer Res; 75(6): 1021–34. ©2015 AACR.

Introduction

Lethal prostate cancer affects approximately 10% of patients diagnosed (1). Currently, we have a poor understanding of how to identify these approximately 10% of patients; therefore, characterization of the molecular signatures that define lethal prostate cancer is of high priority. Comprehensive genomic profiling has revealed that the prostate cancer genome is extremely complex as evidenced by the high frequency of genetic alterations, including mutations, copy-number alterations (CNA), translocations, and fusions as well as mRNA up-/downregulation and DNA methylation events (2–7).

ETS rearrangements are the most common genomic alterations in prostate cancer and occur in approximately 50% of primary tumors. Therefore, prostate cancers are typically classified as being ETS+ or ETS−. ETS rearrangements frequently cooccur with PTEN deletions (ETS+PTEN−−) subtype; ref. 3). Multiple reports have demonstrated that aberrant expression of ETS transcription factors (e.g., ERG, ETV1) can synergize with PTEN loss to promote tumor progression in mouse models (8–12). Genomic alterations associated with ETS+ tumors include SPO1 mutations (6%–15%; refs. 5, 13), SPINK1 overexpression (10%; refs. 14, 15), MAP3K7 deletions (18%–38%; refs. 3, 7, 16, 17), and CHD1 deletions and mutations (15%–27%; refs. 3, 4, 6, 7, 18–20). However, collaborative subtypes of ETS+ tumors have not been described.

We and others previously validated MAP3K7 and CHD1 as prostate tumor suppressors (17–21). Both genes have important functions in normal cellular processes and deletion of either gene has adverse clinical implications (3, 16, 17, 19, 20). MAP3K7 encodes for the protein mitogen-activated kinase kinase kinase 7 (also called TGFβ-activated kinase-1 or TAK1), a downstream
target of multiple signaling molecules, including TGFβ (22), TNFα (23), IL-1 (24), TLR (25), Wnt (26), and TRAIL (27). Loss of MAP3K7 is associated with high-grade prostate cancer in multiple independent datasets (16, 17, 21). CHD1 loss is associated with increased biochemical recurrence in several datasets (3, 20).

Recently, we and others reported that MAP3K7 (6q15) and CHD1 (5q21.1) are significantly codelated in prostate tumors (18, 20). However, the functional significance of dual MAP3K7 and CHD1 loss has not been evaluated. In this study, we used a mouse progenitor/stem cell model and the LNCaP xenograft model to demonstrate that MAP3K7 loss and CHD1 loss synergize to promote prostate tumor progression through alteration of normal prostatic differentiation and loss of E-cadherin. These data functionally define an ERG+ MAP3K7del/CHD1del subtype of aggressive prostate cancer.

Materials and Methods

Dataset analyses

Array comparative genomic hybridization data, mRNA expression data, and clinical data for the MSKCC (3), TCGA (http://cancer.genome.nih.gov/), Broad/Cornell-1 (5), Broad/Cornell-2 (7), and Michigan (6) prostate cancer datasets were downloaded from the cBioPortal for Cancer Genomics (31, 32). Data were processed according to the methods described in the source publications. Further statistical analyses of these already processed data were done in the R statistical computing program (see Supplementary Materials and Methods).

Cell culture

Mouse prostate epithelial progenitor/stem cells (PrP/SC) were isolated and maintained as described previously (33, 34). LNCaP cells (ATCC) were cultured in RPMI (Gibco) supplemented with 10% FBS (Gemini) and 1% penicillin–streptomycin (Gibco). Cell line identity was verified by spectral karyotyping.

Lentiviral plasmid construction and infection

shControl and shMap3K7 plL7-EGFP lentiviral vector design and PrP/SC transduction were described previously (21). Other plasmids used were: pLKO.1-puro-shChd1 (Sigma), pLKO.1-puro-shMAP3K7 (Sigma), pLKO.1-neo-shCHD1 (shCHD1 targeting sequence cloned into Addgene plasmid 13425), and pLenti-PGK-Blast-V5-luc (Addgene plasmid 19166; ref. 35). Targeting sequences were: GCCAGGAGACATACAGTATT (mChd1-1), CAGTGTGTGTATGAGAAT (hMAP3K7), CCCGTTTATCAAGAGCTTAA (hCHD1), and CAACAGATGAAAGGCCACAAA (shControl; see Supplementary Materials and Methods).

Monolayer growth assays

Monolayer growth assays were performed as described previously (see Supplementary Materials and Methods; ref. 21).

Clonogenic assays

Clonogenic growth assays were performed as described previously (21) and quantified using Incucyte ZOOM (Essen Bioscience; see Supplementary Materials and Methods).

Western blot

Standard techniques were performed for protein isolation, quantification, and immunoblotting. Antibodies used were: MAP3K7/TAK1 (Cell Signaling Technology), CHD1 (Bethyl Laboratories), α-tubulin (Cell Signaling Technology), V5-HRP (Invitrogen), and goat anti–rabbit-HRP (Santa Cruz Biotechnology). Horseradish peroxidase signal was detected with Pierce ECL 2 Substrate (Thermo Scientific). Quantification was performed with ImageJ. The densities of MAP3K7 and CHD1 bands were first normalized to α-tubulin then to the normalized values of shControl cells.

PrP/SC xenografts

All animal procedures were conducted with approval from University of Colorado’s Institutional Animal Care and Use Committee in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Tissue recombination of PrP/SCs with fetal rat urogenital mesenchyme (UGM) and subsequent renal grafting were performed as described previously (see Supplementary Materials and Methods; refs. 21, 34).

Graft immunohistochemistry

Graft processing, histology, and immunohistochemistry was performed as described previously (see Supplementary Materials and Methods; ref. 21).

LNCaP xenografts

V5-luciferase–labeled LNCaP cells (4 × 10^6) in 50% Matrigel (BD Biosciences) were s.c. injected into the right flank of NOD/SCID mice (Charles River Laboratories). Bioluminescence was measured weekly using the Xenogen IVIS200 imager and Living Image software (Caliper Life Sciences). Mice were imaged 10 minutes after i.p. injection with α-luciferin (Gold Biotechnology). Tumor growth was measured weekly using calipers; tumor volume (TV) was calculated with the formula TV = length × (width)^2/2. Animals were removed from the study if their tumor reached 20 mm in any direction, TV was greater than 3,000 mm^3, or if their tumor became ulcerated.

Clinical sample immunohistochemistry

Study subjects were a subset of treatment-naïve men who underwent radical prostatectomy at Stanford University Medical Center between 1983 and 1998. Institutional Review Board approval for human subjects research was obtained at Stanford University. Fifty patient samples, selected on the basis of the presence of high-grade tumors, were evaluated for MAP3K7, CHD1, and E-cadherin expression. Immunohistochemistry and scoring of expression were performed as described previously (21). Antibodies used were: MAP3K7/TAK1 (Cell Signaling Technology), CHD1 (Bethyl Laboratories), and E-cadherin (Cell Signaling Technology; see Supplementary Materials and Methods).
Statistical analysis

All statistical analyses were performed with the R statistical computing program. Specific tests used are indicated in the figure legends of Figs. 1–7 and in Supplementary Materials and Methods. Post-hoc analyses for all ANOVAs were performed using the Tukey multiple comparisons test. A two-tailed P value of <0.05 was considered statistically significant.

Results

MAP3K7del/CHD1del tumors define a subset of aggressive prostate cancer

To determine the clinical significance of combined loss of MAP3K7 and CHD1 in prostate tumors, five publicly available microarray-based prostate cancer CNA datasets were analyzed (Supplementary Table S1; refs. 6, 7, 17, 18, 20). MAP3K7 and CHD1 were significantly co-deleted in 10% to 20% of localized prostate tumors (Fig. 1A, black bars and Supplementary Table S2) and 20% to 25% of metastases (Supplementary Table S3). Notably, 65% to 85% of CHD1 deletions were accompanied by a MAP3K7 deletion (Fig. 1A, black bars); the MAP3K7/CHD1 del subtype was extremely rare (Fig. 1A, red bars). Conversely, MAP3K7 deletions occurred with and without CHD1 deletions at roughly equal frequencies (Fig. 1A, black and green bars). Consistent with previous reports, MAP3K7 deletions and/or CHD1 deletions were significantly inversely correlated with TMPRSS2:ERG fusion (Fig. 1B, Supplementary Table S2; refs. 6, 7 and in Supplementary Materials and Methods). All PrP/SC lines illustrated in Fig. 1B were recombined with UGM and grafted under the renal capsules of recipient nude mice (n = 4 grafts/cell line; see Supplementary Materials and Methods). After 12 weeks, kidneys were removed and grafts evaluated (Supplementary Table S4). The 109 MSKCC tumors were then stratified according to mRNA expression of both MAP3K7 and CHD1. Patients with both MAP3K7 and CHD1 expression in the lower quartile (bottom 25%) had a significantly higher rate of biochemical recurrence (P = 0.02) compared with patients with intermediate expression (middle 50%) of these genes (Fig. 1D, middle). Patients with high expression (top 25%) of both MAP3K7 and CHD1 had the lowest recurrence rate (Fig. 1D, right). Kaplan–Meier analyses of MAP3K7 and CHD1 mRNA and CNA data were repeated in the ERG fusion–negative subset of tumors illustrated in Fig. 1B. Although the same trends in data seen in Fig. 1C and D were observed, these analyses were not significant due to decreased sample size when ERG fusion–positive tumors were excluded (Supplementary Fig. S2). Finally, both MAP3K7 expression and CHD1 expression correlated with CNA in the MSKCC dataset (Fig. 1E). Taken together, these data identify an ERG+ subset of aggressive prostate cancer characterized by concurrent loss of MAP3K7 and CHD1.

Loss of CHD1 is deleterious to growth of wild-type PrP/SCs, but not shMap3k7 PrP/SCs

To determine how combined loss of MAP3K7 and CHD1 contributes to prostate tumorigenesis, we used a developmental model in which mouse PrP/SCs maintain stem cell characteristics over long-term culture and recapitulate normal prostate development in vitro using tissue recombination and renal grafting in a recipient host (34). We previously manipulated expression of Map3k7 in our PrP/SC model using a lentiviral shMap3k7–GFP construct and demonstrated that suppression of Map3k7 expression increased PrP/SC growth in vitro and promoted prostate tumorigenesis in vivo (21). In this study, multiple shControl–GFP or shMap3k7–GFP PrP/SC lines were infected with a nontargeting control (shControl–puro) or two different shChd1–puro lentiviruses and stable cell lines were established (Fig. 2A and B). Chd1 suppression significantly decreased proliferation of shControl PrP/SCs (P < 0.0001; Fig. 2C). Strikingly, Chd1 knockdown had attenuated effects on growth of PrP/SCs that had already lost Map3k7 expression (Fig. 2D). In clonogenic growth assays, Map3k7 suppression robustly increased PrP/SC clonogenic proliferation and colony size consistent with our previous findings (Fig. 2E and Supplementary Fig. S3; ref. 21). Chd1 suppression did not significantly alter clonogenic growth of shControl or shMap3k7 PrP/SCs (Fig. 2E–G and Supplementary Fig. S3). Collectively, these data show that the genetic background in which CHD1 is lost is important.

Cosuppression of Map3k7 and Chd1 in PrP/SCs promotes tumor progression

We next evaluated how combined loss of Map3k7 and Chd1 affects prostate development in vivo using a tissue recombination system described previously (21, 34). All PrP/SC lines illustrated in Fig. 2A were recombined with UGM and grafted under the renal capsules of recipient nude mice (n = 4 grafts/cell line; see Supplementary Materials and Methods). After 12 weeks, kidneys were removed and grafts evaluated (Supplementary Fig. S4). For detailed experimental findings and experimental controls see Supplementary Table S5 and Supplementary Fig. S5. As expected, shControl grafts differentiated into benign ductal glands characterized by a p63+/CK5+/CK14+ basal layer surrounding an AR+/CK18+ secretory luminal layer (Figs. 3A and 4A). Normal secretions of mouse dorsolateral secretory protein (mDLP) into the lumens were observed (Supplementary Fig. S6). Nuclei of shControl PrP/SCs were unremarkable (Fig. 3D). Three shControl grafts contained foci displaying mild hyperplasia as observed previously (Supplementary Fig. S7; ref. 21). shMap3k7 grafts displayed a mixture of benign, high-grade prostatic intraepithelial neoplasia (PIN), and carcinoma phenotypes consistent with our previous findings (Fig. 3A; ref. 21). mDLP secretions were abnormal and variable (Supplementary Fig. S6). shMap3k7 nuclei were pleomorphic and had prominent nucleoli (Fig. 3D). shChd1 grafts were mostly benign, although a few foci of low-grade PIN were observed. mDLP expression was mostly normal (Supplementary Fig. S6) and the p63+ basal layer was intact (Fig. 3A). Interestingly, androgen receptor (AR) expression was variable; although most foci were strongly AR positive, a few areas had weak or completely negative AR expression, suggesting that Chd1 may be involved in AR regulation and/or luminal cell differentiation (Fig. 3A). Nuclear atypia in shChd1 grafts was characterized by multinucleated cells and prominent nucleoli (Fig. 3D). These data indicate that Chd1 loss alone is not sufficient to induce prostate tumorigenesis.

Strikingly, all grafts from shMap3k7–shChd1 PrP/SCs (hereinafter referred to as shDouble) displayed massive glandular atypia with little or no normal glandular development observed. Both high-grade PIN and invasive carcinoma phenotypes were
MAP3K7 and CHD1 deletion and mRNA downregulation correlate with increased biochemical recurrence. A, frequency of single and cooccurring MAP3K7 and CHD1 deletions in four prostate cancer datasets. B, oncoprint of MAP3K7 and CHD1 CNAs in ERG fusion-positive and -negative tumors (MSKCC dataset, n = 157 primary tumors). C, Kaplan–Meier analysis of recurrence-free survival in patients with MAP3K7 and/or CHD1 deletions (MSKCC, n = 157; log-rank test). D, left, correlation of MAP3K7 and CHD1 mRNA expression (MSKCC, n = 109; P < 0.0001). Patients were divided into top (purple), middle (blue), and bottom (black) quartiles for MAP3K7 and CHD1 mRNA expression. Samples in which the gene expression of CHD1 and MAP3K7 did not fall into the same quartiles are shown as open circles. Middle and right, Kaplan–Meier analysis of recurrence-free survival in patients with lower, middle, or upper quartile expression of MAP3K7 and CHD1. E, oncoprint of MAP3K7 and CHD1 CNAs and mRNA expression (MSKCC, n = 109). Samples are ranked from lowest (blue) to highest (red) mRNA Z-score. CHD1 and MAP3K7 samples were ranked independently.

Figure 1.
detected (Fig. 3B). AR expression was significantly decreased in shDoubles compared with all other recombinants \(P < 0.01\) with numerous glands completely filled with AR\(^{-}\)/p63\(^{-}\) cells (Fig. 3B and C). Often, these areas were adjacent to foci that were strongly AR positive. Other glands contained clusters of cells growing within the lumen, suggestive of intraductal carcinoma of the prostate (IDC-P). Interestingly, IDC-P is an aggressive subtype of Gleason grade 4/5 (36). Still other glands were characterized by an outer layer of AR\(^{-}\)/p63\(^{-}\) columnar epithelial cells surrounding vacuole-like structures within the lumens. Cells that resembled goblet cells and signet ring cells were also present (Fig. 3B, arrows). Numerous shDouble cells were multinucleated, had...
Figure 3.
Dual shMap3k7–shChd1 recombinants display high-grade PIN and intraductal carcinoma in vivo. A, left, hematoxylin and eosin (H&E) staining of representative shControl, shMap3k7, and shChd1 tissue recombinants after 12 weeks in vivo (×100, ×400; scale, 50 μm). Right, IHC with AR and p63 antibodies. Arrowheads, p63++ cells.

B, representative images of shDouble recombinants stained with hematoxylin and eosin, AR, and p63. Recombinants from three different shDouble cell lines are shown. Arrowheads, p63++ cells. Arrows, goblet or signet ring-like cells. C, AR quantification. Data, means ± SEM (one-way ANOVA; P < 0.01; bars with different letters above are statistically significantly different). D, representative high-magnification images from shControl, shMap3k7, shChd1, and shDouble recombinants (×1,000). Arrowheads, multinucleated cells. Arrows, cells with prominent nucleoli. Arrows with tails, large cells.
and target genes assessed, only activity occurred with MAP3K7. KLK3 and KLK2 were inversely correlated with MAP3K7. 1D) of the MSKCC and TCGA datasets. No correlation with MAP3K7 decreased or absent in numerous regions. shDouble recombinants, CK18 expression was epithelial marker CK18 and basal epithelial markers CK5 and SC differentiation, we examined the expression of the luminal differentiation rather than specifically causes AR loss. To determine whether MAP3K7 and CHD1 are associated with changes in AR expression in human tumors, we evaluated AR and target gene (TMPRSS2, KLK3, KLK2, FKB5, NKK3-1, and ACPP) expression in the MAP3K7–CHD1 quartiles (generated in Fig. 1D) of the MSKCC and TCGA datasets. No correlation with MAP3K7 and CHD1 was observed for AR expression. Of the AR target genes assessed, only FKB5 was positively correlated with MAP3K7 and CHD1 expression, the expected result if loss of AR activity occurred with MAP3K7 and CHD1 loss. In contrast, KLK3 and KLK2 were inversely correlated with MAP3K7 and CHD1 expression, suggestive of increased AR activity (Supplementary Fig. S8 and Supplementary Table S6). The other targets were variable and inconsistent across the datasets. These data suggest either that dual MAP3K7–CHD1 loss has no overall effect on AR/AR target gene expression in clinical samples or that the effects are variable and locus dependent.

Cosuppression of Map3k7 and Chd1 inhibits normal differentiation of PrP/SCs

To assess the impact of Map3k7 and Chd1 suppression on PrP/SC differentiation, we examined the expression of the luminal epithelial marker CK18 and basal epithelial markers CK5 and CK14. shChd1 recombinants strongly expressed CK18 in most regions. In shMap3k7 recombinants, CK18 expression was decreased or absent in numerous regions. shDouble recombinants, comprised of mostly abnormal acini, displayed almost no CK18 expression (Fig. 4A and C). Normal expression of CK5 and CK14 was observed in shChd1 grafts whereas shMap3k7 grafts contained numerous CK5–/CK14+ cells particularly in regions of carcinoma (consistent with p63 loss). A few shMap3k7 acini displayed increased CK5 and CK14 expression indicative of basal cell hyperplasia, a phenotype observed in our previous study (Supplementary Fig. S9 and Fig. 4C; ref. 21). shDouble recombinants displayed a CK5/CK14 expression pattern similar to that observed in shMap3k7 grafts, including a few foci with basal cell hyperplasia (Fig. 4A–C). The differentiation of shDouble recombinants was further altered by the loss of AR expression (which occurred in shChd1 but not shMap3k7 grafts). shDouble recombinants predominantly displayed an “undifferentiated” phenotype characterized by cells completely negative for AR, p63, CK18, CK14, and CK5 expression (Fig. 4B). Collectively, these data demonstrate that both Map3k7 and Chd1 are necessary for normal differentiation of the PrP/SC.

Map3k7–Chd1-deficient PrP/SCs undergo lineage switching

Because the AR+/p63+/CK18+/CK14+/CK5+ cells present in shDouble recombinants are not characteristic of luminal or basal epithelial cells, we hypothesized that these cells may have undergone altered lineage differentiation. In addition to luminal and basal cells, the epithelial compartment of the normal prostate contains rare neuroendocrine cells thought to regulate growth and differentiation through crosstalk with other prostatic cells. Increased neuroendocrine differentiation is observed in a small subset of highly aggressive prostate cancers and is associated with poor prognosis (37, 38). Synaptophysin (SYP), a neuroendocrine cell marker, was expressed sporadically by single cells in shControl, shChd1, and shMap3k7 recombinants whereas shDouble recombinants displayed occasional clusters of SYP+ cells suggestive of increased neuroendocrine differentiation (Fig. 4D and E). Consistent with these observations, both SYP and CHGA (Chromogranin A, another neuroendocrine marker) mRNA expression were significantly inversely correlated with expression of MAP3K7 and CHD1 in human prostate tumors (Fig. 4H and I; Supplementary Fig. S10 and Supplementary Table S6). Because Chd1 suppression has been reported to promote neural differentiation of ES cells (30), we also evaluated our tissue recombinants for expression of Nestin, a neural stem cell marker. Unexpectedly, both shControl and shChd1 recombinants were almost completely negative for Nestin staining whereas shMap3k7+ and shDouble recombinants displayed increased Nestin expression (Fig. 4F and G). In human prostate tumors, NES (Nestin) gene expression was significantly inversely correlated with MAP3K7 and CHD1 expression (Fig. 4I; Supplementary Fig. S10 and Supplementary Table S6). Taken together, these data suggest that MAP3K7del/CHD1del tumors may represent a distinct prostate cancer subtype with neuroendocrine and neural features.

Another distinguishing characteristic of shDouble recombinants is the presence of numerous glands that produced abnormal secretory products and that were lined with a mixture of columnar, goblet-like, and signet ring-like cells (Fig. 3B and D). Columnar epithelial cells and goblet cells are frequently located adjacent to mucous membranes in benign and malignant tissues whereas signet ring cells are predominantly seen in cancers; both goblet cells and signet ring cells can secrete mucus. Rare subtypes of prostate cancer arising from these cells include mucinous adenocarcinoma, signet ring cell carcinoma, and mucinous carcinoma with signet ring cells; the presence of signet ring cells is associated with extremely poor prognosis (39). We hypothesized that cosuppression of Map3k7 and Chd1 could promote differentiation into mucin-secreting cells; Alcian Blue staining confirmed the presence of acidic mucin within the glands of shDouble recombinants. Only one shDouble graft was negative for mucin whereas all shControl and shMap3k7 grafts were mucin negative. Notably, one gland out of all shChd1 grafts was mucin positive (Fig. 5A and B). Overall, these data show that combined loss of Map3k7 and Chd1 drastically alters PrP/SC fate by altering cellular differentiation.

Cosuppression of Map3k7 and Chd1 promotes loss of E-cadherin

E-cadherin loss is an important step in tumor progression in a number of cancers, including prostate cancer (40). Aberrant mucin expression is associated with E-cadherin loss in several types of cancer (41–43) and mucins have been shown to promote invasion by inhibiting E-cadherin–mediated cell–cell interactions (44, 45). We evaluated E-cadherin expression in the recombinants; most glands of shControl and shChd1 grafts were strongly E-cadherin positive. In contrast, E-cadherin expression was decreased or absent in many foci of shMap3k7 and shDouble grafts, suggesting that Map3k7 loss promotes...
E-cadherin suppression (Fig. 5C and D). Interestingly, the mucin-secreting cells of shDouble recombinants did not express E-cadherin (Fig. 5E). shMap3k7 and shDouble recombinants also displayed significantly increased proliferation (Ki67) compared with shControl or shChd1 grafts (P < 0.01; Fig. 5F and G). These data demonstrate that dual Map3k7–Chd1 suppression promotes invasive disease associated with loss of E-cadherin.

Cosuppression of MAP3K7 and CHD1 promotes growth of LNCaP xenografts

To determine whether the aggressive phenotype observed in PrP/SC recombinants is reproducible in a human-derived model, we examined the effects of combined MAP3K7 and CHD1 suppression on growth of LNCaP prostate cancer cells in vitro and in vivo. Although the LNCaP model was derived from a lymph node metastasis, LNCaP cells remain androgen sensitive, are modestly aggressive, and are TMPRSS2:ERG fusion negative (but do overexpress ETV1). We used a strategy similar to the one described in Fig. 2A to perform dual knockdown of MAP3K7 and CHD1 in LNCaP cells (Fig. 6A and B). MAP3K7 suppression alone did not significantly affect growth of LNCaP cells. However, as observed in the PrP/SC model, CHD1 knockdown was deleterious to shControl cells, but not LNCaP cells deficient in MAP3K7 expression (Fig. 6C). Interestingly, CHD1 knockdown also altered the morphology of both shControl and shMAP3K7 LNCaP cells; these cells had more filopodial extensions suggestive of a more invasive phenotype (Supplementary Fig. S11). To assess the effects of cosuppression of MAP3K7 and CHD1 on LNCaP growth...
in vivo, all four LNCaP cell lines were labeled with V5-luciferase (Supplementary Fig. S12A and S12B) and s.c. injected into NOD/SCID mice (n = 12 mice/cell type). shDouble xenografts displayed higher bioluminescence signals compared with shControl, shMap3k7, and shChd1 xenografts from days 14 to 56 (Fig. 6D and E). By 56 days, some mice with shDouble xenografts had to be removed from the study due to tumor burden. In addition, shDouble tumors were palpable by 28 days after implantation, and the average tumor volume of shDouble xenografts was higher than all other groups over time (Fig. 6F). The bioluminescence...
intensity and tumor volume of shMAP3K7 xenografts were not significantly different than shControl xenografts. Notably, loss of CHD1 had a protective effect on xenograft tumor formation. On average, mice implanted with shCHD1 xenografts did not develop palpable tumors until after 84 days (Supplementary Fig. S12C). Kaplan–Meier analysis revealed that mice with shDouble xenografts had a significantly decreased survival rate relative to shControls \( (P = 0.02; \text{Fig. 6G}) \). Survival of mice with shMAP3K7 xenografts was similar to shControl mice whereas shCHD1 mice had a significantly longer survival time than controls \( (P = 0.008; \text{Fig. 6G}) \). These data suggest that neither MAP3K7 loss nor CHD1 loss alone can increase xenograft proliferation, but their dual loss can synergize to promote aggressive tumor growth and decreased survival.

MAP3K7, CHD1, and E-cadherin protein expressions are coordinately decreased in high-grade tumors

Because combined suppression of MAP3K7 and CHD1 promotes tumor progression in the PrP/SC and LNCaP models, we wondered whether co-loss of MAP3K7 and CHD1 protein expression occurs in aggressive human prostate tumors. We also evaluated E-cadherin expression in tumors because co-suppression of Map3k7 and Chd1 promoted E-cadherin loss in the PrP/SC model. Fifty high-grade primary tumors from a cohort at Stanford University were analyzed for MAP3K7, CHD1, and E-cadherin protein expression; these samples were heterogeneous, multifocal, and contained regions of benign glands as well as Gleason grade 3, 4, and/or 5 prostate cancer. Benign glands expressed high levels of cytoplasmic MAP3K7 in both basal and luminal cells. Strong nuclear expression of CHD1 was observed predominantly in the basal cells of normal glands. E-cadherin was strongly expressed on the cell membranes of benign glands. In contrast, MAP3K7, CHD1, and E-cadherin expressions were largely decreased or absent in high-grade tumors. Regions of combined MAP3K7, CHD1, and E-cadherin loss were observed in numerous cancer samples (Fig. 7A). We previously described a scoring system for quantification of MAP3K7 expression and demonstrated that MAP3K7 expression inversely correlated with Gleason grade within and across patient samples (Supplementary Fig. S13A and Fig. 7B; ref. 21). Using the same scoring system as MAP3K7 (Supplementary Fig. S14), we observed that E-cadherin expression progressively decreased with higher Gleason grade (Supplementary Fig. S13C; Fig. 7D). Because CHD1 is localized in the nucleus, a different system was developed to evaluate CHD1 expression based on a scoring index that combines the individual staining intensities of multiple tumor nuclei into one "H-Score" (see Supplementary Materials and Methods). Benign glands strongly and ubiquitously expressed CHD1, and therefore were not scored. Using this scoring system, loss of CHD1 expression was significantly associated with higher tumor grade (Supplementary Fig. S13B, Fig. 7C). In addition, MAP3K7 and E-cadherin protein expression was significantly correlated with each other, but CHD1 expression was not associated with either MAP3K7 or E-cadherin expression likely due to the different scoring systems used (Supplementary Table S7). We extended these observations to tumors in the MSKCC and TCGA cohorts and found that tumors with low expression of both MAP3K7 and CHD1 mRNA had significantly lower CHD1 (E-cadherin) gene expression (Fig. 7E and F, Supplementary Table S6). In summary, these data demonstrate that MAP3K7 loss, CHD1 loss, and E-cadherin loss are important, concurrent events in prostate cancer progression.

Discussion

Although numerous oncogenes and tumor suppressors have been implicated in prostate tumorigenesis, prostate cancer subtypes remain poorly defined. ETS+ tumors, in particular, have not been well characterized. Recently, we and others have identified a subset of ETS+ prostate cancer in which MAP3K7 and CHD1 are significantly codeleted \( (18, 20) \). In this study, we demonstrated that dual suppression of MAP3K7 and CHD1 promotes aggressive tumorigenesis in both PrP/SC and LNCaP models. Our data indicate that MAP3K7 and CHD1 contribute to tumor progression in different ways (Fig. 7G). CHD1 knockdown significantly decreased growth of both PrP/SCs and LNCaP cells, a phenotype observed previously in ES cells and prostate cell lines BPH-1, PC3, and DU145 \( (20, 30) \). Consistent with these in vitro findings, CHD1 suppression did not promote prostate tumorigenesis in either of our in vivo models. shCHD1 LNCaP xenografts actually displayed a significantly increased time to tumor development compared with controls. Interestingly, both PrP/SCs and LNCaP cells deficient in MAP3K7 were largely resistant to the deleterious effects of CHD1 suppression on proliferation. MAP3K7 mediates MAPK signaling in inflammatory cells in response to cellular stress \( (22) \). Previously, we showed that loss of Map3k7 in PrP/SCs inhibited activation of two downstream targets, p38 and JNK, in response to TNF\( \alpha \) in vitro and in shMap3k7 recombinants in vivo \( (21) \). If CHD1 loss activates a stress response that decreases cell growth, then MAP3K7-deficient cells may be incapable of responding to this stress signal and can proliferate normally upon suppression of CHD1. Both our in vitro and in vivo findings support a model in which CHD1 loss provides a protumorigenic advantage independent of increased growth. In support of this, CHD1-deficient LNCaP cells displayed increased filopodia formation, a phenotype associated with invasive ability, and others have observed that CHD1 knockdown in benign prostatic cell lines PANC2 and RWPE-1 increases invasion \( (19) \).

We also demonstrated that MAP3K7 loss and CHD1 loss had different, but synergistic effects on PrP/SC differentiation (Fig. 7G). Dual Map3k7-Chd1-deficient recombinants displayed decreased prostatic luminal cell differentiation, but increased neuroendocrine and neural differentiation. These findings are significant given the inverse correlation of SYP, CHGA, and NES expression with MAP37 and CHD1 expression in human prostate tumors. MAP3K7\(^{\text{del}}\)/CHD1\(^{\text{del}}\) tumors may define a unique subtype of prostate cancer with neuroendocrine/nerve characteristics. Dual Map3k7-Chd1-deficient recombinants also contained features of other rare prostate cancer subtypes, including mucin-producing goblet and signet ring cells. Consistently, the many differentiation patterns observed in shDouble recombinants recapitulate the heterogeneity present in human cancers. Recently, a stomach cancer with both neuroendocrine and mucinous signet ring cell phenotypes was described \( (46) \). Likewise, prostate cancer is a heterogeneous and multifocal disease \( (47) \) and neuroendocrine, mucinous and signet-ring prostate carcinomas do occur. Further investigation of MAP3K7 and CHD1 expressions in specific histologic subtypes of prostate cancer is warranted.

Loss of E-cadherin, a critical mediator of cell–cell adhesions, is a defining feature of tumor progression in epithelial cancers. In the PrP/SC model, E-cadherin expression was greatly decreased in shMAP3K7 and shDouble recombinants, but not shChd1 recombinants, indicating that E-cadherin loss may be mediated by MAP3K7 (Fig. 7G). Consistent with this finding, MAP3K7 inhibi-
Figure 7.
MAP3K7, CHD1, and E-cadherin are coordinately lost in high-grade tumors. A, fifty whole-mount prostatectomy samples were stained with MAP3K7, CHD1, and E-cadherin antibodies. Representative images from four samples are shown. For each sample, the same region was imaged for all three stained sections. Arrowheads, benign glands. B–D, quantification of MAP3K7, CHD1, and E-cadherin protein expressions. MAP3K7 and E-cadherin expressions were scored on a scale of 0 (no expression) to 3 (strong expression; one-way ANOVA; *, $P < 0.0005$); CHD1 expression was scored on a scale of 0 to 300 (Welch t-test; *, $P < 0.0005$). E and F, correlation of CDH1 (E-cadherin) mRNA expression with MAP3K7 and CHD1 expression (MSKCC and TCGA). Data, means ± SEM (one-way ANOVA; *, $P < 0.0005$; **, $P < 0.001$; ***, $P < 0.01$; ****, $P < 0.05$; NS, not significant). G, model of single or dual Map3k7 and Chd1 loss during PrP/SC differentiation.
bition has been associated with E-cadherin loss and epithelial-to-
mesenchymal transition in a squamous cell carcinoma model (48) and in obliterative bronchiolitis, a lung disease (49).

Codeligation of MAP3K7 and CHD1 is observed in both metastatic and pre-metastatic tumors confirming that loss of these genes is important for tumor progression. However, given the small increase in codeligations observed in metastatic tumors (from 10%–20% in localized tumors to 20%–25% in metastases), MAP3K7 and CHD1 may not be metastasis drivers, but rather are critical mediators of progression to invasive carcinoma. Consistent with this hypothesis, aggressive, invasive phenotypes are displayed by MAP3K7–CHD1-deficient PtPr/Kc and LNCaP cells in vivo, but no metastases were observed in either model. Likely another genetic "hit" is required to promote metastasis. CHD1 deletions have been associated with other genomic alterations besides MAP3K7 deletions, including SPOP mutations, PDE4D deletions, LRPIB deletions, and SPINK1 overexpression (5, 13, 15, 18). Incorporation of other alterations into our shMAP3K7–shCHD1 models will provide further insight into the progression of ETS prostate cancer. In summary, we have identified a novel molecular subtype of aggressive prostate cancer, ERG/MAP3K7/CHD1(49), and validated that combined suppression of MAP3K7 and CHD1 promotes tumor progression using two in vivo models. These findings have important prognostic and therapeutic implications for prostate cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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