p27 transcriationally coregulates cJun to drive programs of tumor progression

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The CDK inhibitor, p27, was discovered as a mediator of growth arrest by transforming growth factor type β (TGF-β) that impedes cell cycle progression by inhibiting cyclin-dependent kinases (CDKs) (1–3). p27 is invariably deregulated in human cancers, but unlike typical tumor suppressors, mutations or deletions of the CDKN1B gene encoding p27 are rare. p27 can functionally disrupt in cancers by excess proteolysis, by decreased translation, or by C-terminal phosphorylation (4, 5).

The phosphatidylinositol 3′-kinase (PI3K) pathway is activated in most human cancers (6) by genetic changes activating receptor tyrosine kinases, p53, or its effectors cJun (7) or by loss of its negative regulator, phosphatase and tensin homolog (8). PI3K activated kinases phosphorylate p27 at two sites, T157 and T198. Phosphorylation at T157 in the p27 nuclear localization signal delays nuclear import (9), and T198 phosphorylation stabilizes the protein (10, 11), leading to accumulation of p27 in the cytoplasm. Notably, up to 60% of newly diagnosed breast cancers express activated pAKT19, and this correlates with detection of both nuclear and cytoplasmic p27 (9) and with detection of p27pT198 (12) by immunohistochemical analysis. Despite strong cytoplasmic p27 expression, nuclear p27 remains present in all AKT-activated cancers, and cancers with both nuclear and cytoplasmic p27 have a worse prognosis than those with exclusively nuclear p27 (9, 13). Proteomic analysis showed that levels of activated AKTps473, p70S6kps389, and p90RSKps359 are all strongly correlated with phosphorylated p27pT198 in over 700 primary human breast cancers from The Cancer Genome Atlas (TCGA) and The Cancer Proteome Atlas (TCPA) (14), supporting that PI3K-activated kinases phosphorylate p27 in human cancer. It is increasingly clear that p27pT157pT198 drives tumor metastasis via multiple mechanisms. Phosphorylation of p27 at T157 and T198 (9, 15–19) impairs its CDK inhibitory action (20, 21) and promotes binding to RhoA/ROCK1 to disrupt the actin skeleton and enhance cell motility and invasion (18, 22). Increased p27pT157pT198 also facilitates metastases in PI3K-activated cancer models (13, 14) and contributes to epithelial–mesenchymal transformation (EMT) by activating STAT3-dependent TWIST1 induction (14).

p27 is regulated by both the PI3K and TGF-β pathways. Members of the TGF-β family of cytokines bind heterotrimeric TGF-β receptors to activate SMADs, which homo- and heterodimerize and translocate to the nucleus to activate gene expression programs (23, 24). The TGF-β pathway regulates tissue differentiation and morphogenesis in development and activates cytostatic and apoptotic processes to maintain tissue homeostasis (24). Although TGF-β mediates cell cycle arrest via p27 in normal epithelial cells (1, 2), these cytostatic effects are disrupted in cancers, and aberrant TGF-β signaling stimulates EMT, invasion, and metastasis (23, 24). The PI3K and TGF-β pathways have been shown to cooperate to mediate EMT (7), but mechanisms underlying this are not fully known. The present work reveals a previously unknown mechanism whereby oncogenic activation of the PI3K and TGF-β pathways cooperates to drive EMT and metastasis. We identify a role for
p27 in which it functionally interacts with cJun as a transcriptional coregulator. p27 and cJun interaction, nuclear localization, and the distribution and extent of p27 and cJun recruitment to chromatin are increased by C-terminal p27 phosphorylation. At a large subset of target genes, cJun binding is p27 dependent, suggesting that p27 may be an obligate cJun coactivator at these sites. TGFβ2 is identified as a p27/cJun target gene required for p27-driven metastasis in vivo. p27/cJun complexes activate oncogenic target gene programs associated with EMT and cancer metastasis, and these target genes are preferentially expressed in primary human breast cancers with high levels of activated p27T117.

**Results**

**p27 Drives an EMT Gene Expression Program.** Prior work showed that C-terminal p27 phosphorylation mediates activation of TWIST1 to drive a morphologic EMT (14). To investigate effects of p27 on metastatic gene programs more broadly, global gene expression assayed by RNA sequencing (RNA-seq) was compared in MDA-MB-231 (hereafter 231), a breast cancer line with low metastatic ability; in MDA-MB-231-1833, a bone-tropic highly metastatic derivative line (25) (hereafter 1833); and in MDA-MB-231-1833shp27, in which p27 was stably depleted (13). To test the effects of phosphorylated p27T157pT198, we used 231 transduced with p27 bearing phosphomimetic threonine-to-aspartic acid mutations at T157 and T198 (14). Since as little as two- to threefold p27 overexpression arrests the cell cycle and would not permit study of phosphomimetic p27, p27 was also mutated to p27CK to abolish cyclin-CDK binding (26), yielding p27CK-DD (14). Notably, comparison of 231 transduced with either p27CK− or p27CK−DD showed only the phosphomimetic p27CK−DD induces a morphologic EMT and confers excess metastasis, indicating that these phosphorylations are critical to p27-driven EMT (14).

Gene ontology (GO) analysis revealed activation of programs associated with cell motility, migration, and extracellular matrix (ECM) organization in 231p27CK− compared with 231, and in 1833 vs. 1833shp27 (Fig. 1). Differentially expressed genes in these lines were compared in an established “EMT core signature” derived by overexpression of master EMT regulators in human mammary epithelial cells (27). Gene set enrichment analysis (GSEA) showed that genes up-regulated in this signature were increased in 231p27CK−DD compared with 231 and in highly metastatic 1833 vs. 1833shp27, while genes down-regulated in the EMT signature were also decreased in the highly metastatic lines (Fig. 1B and SI Appendix, Fig. S1A). EMT core signature genes differentially expressed between 231p27CK−DD vs. 231 and 1833 vs. 1833shp27 are shown in Fig. 1C. Genes down-regulated during EMT were decreased in 231p27CK−DD and in 1833 compared with both 231 and 1833shp27, while genes activated in the EMT signature were increased in both highly metastatic lines (Fig. 1C; see gene list in SI Appendix, Table S1). Thus, p27 knockdown reverses the expression of an EMT gene profile, supporting the notion that p27 drives metastasis, in part, by activating an EMT transcription program.

Comparison of EMT markers and drivers showed that 1833 expressed negligible E-Cadherin and Vimentin compared to 231 (13, 14), p27 depletion decreased expression of the master EMT transcription factors (EMT-TFs) SNAI1, SNAI2, and ZEB2 in highly metastatic 1833 and LuL2 (Fig. 1E), confirming findings on RNA-seq (SI Appendix, Fig. S1B), while p27CK−DD transduction up-regulated EMT-TFs in both breast (231) and bladder (UMUC3) lines (Fig. 1F).

Fig. 1. p27 activates an EMT gene expression program. (A) GO analysis of genes governing cell migration, motility, and ECM in 231 vs. 231p27CK−DD and in 1833 vs. 1833shp27. FDR, fold discovery rate. (B) GSEA shows differential expression of EMT core signature genes (27) up-regulated (EMT UP) and down-regulated (EMT Down) in the indicated lines. Normalized enrichment score (NES), FDR, and P values are shown. (C) Heatmap of mean fold changes in differentially expressed EMT core signature genes in parental 231, 231p27CK−DD, 1833, and 1833shp27. Genes altered by p27 knockdown revert away from the Taube et al. (27) EMT core signature. Genes are listed in SI Appendix, Table S1. (D) Effects of stable p27 depletion in 1833 vs. 207 and the EMT markers E-Cadherin (E-cad) and Vimentin (Vim) by Western blot (β-actin as loading control; data representative of more than three repeat assays). (E) Effects of stable p27 depletion (shp27) on qPCR expression of EMT-TFs (SNAI1, SNAI2, and ZEB2) in PI3K-activated lines (1833 and LuL2) vs. scramble shRNA controls (Scr). (F) Effects of stable p27CK−DD expression on expression of EMT-TFs in 231 and UMUC3 vs. vector-only control lines. In E and F, means ± SEM graphed from three or more replicates of three or more biologic assays (***P < 0.001, ****P < 0.0001). 231DD, 231p27CK−DD. See also SI Appendix, Fig. S1 A and B.

**p27T157pT198 Activates TGF-β Signaling by Inducing TGB2 Expression.** To further identify p27-regulated gene programs, we focused on genes both stringently up-regulated by p27CK−DD transduction in 231 and down-regulated in 1833 by p27 depletion. A total of 489 genes were increased by over twofold (P < 0.005, q < 0.1) in 231p27CK−DD vs. 231, while 229 genes were down-regulated by at least one half (fold change > 0.5, P < 0.005, q < 0.1) by p27 depletion in 1833. Of these, 82 genes were both up-regulated in 231p27CK−DD vs. 231 and also down-regulated in 1833shp27 vs. 1833 (Fig. 2 and SI Appendix, Table S2). GO analysis showed that the 82 genes that are both up-regulated by p27CK−DD and in 1833shp27 were associated with important oncogenic pathways, including TGF-β, focal adhesion, ECM receptor interaction, and PI3K-AKT signaling pathways (Fig. 2B and SI Appendix, Fig. S1C). GSEA also showed TGF-β signaling...
activation in 231p27CK−DD compared with 231, while p27 knockdown impaired TGF-β pathway activation in 1833 (Fig. 2 C and D and SI Appendix, Fig. S1D and Table S3). Similarly, treatment with the PI3K/mammalian target of rapamycin (mTOR) inhibitor, PF04691502 (hereafter PF1502), at a dose known to inhibit PI3K in these lines and to decrease both p27pT157 and p27pT198 (13), down-regulated genes associated with TGF-β pathway activation (SI Appendix, Fig. S1 D and E). Thus, TGF-β signaling is activated by p27CK−DD transduction in 231 and inhibited by p27 knockdown and by PI3K inhibition in 1833.

Up-regulation of TFGFB2 by p27 was confirmed by qPCR in three highly metastatic lines: 1833, 4175, and LuL2. In all three lines, p27 depletion decreased TFGFB2 expression (Fig. 2E). Furthermore, p27CK−DD transduction increased TFGFB2 expression and TGF-β2 secretion in the immortal, nontransformed mammary epithelial line MCF12A and in the low-metastatic 231 and UMUC3 lines (Fig. 2 F and G). In MCF12A, TGF-β2 induced a morphological change from an epithelial to a more mesenchymal phenotype (SI Appendix, Fig. S1F). TGF-β2 up-regulated SNAI1 and SNAI2 expression in MCF12A, 231, and UMUC3 (SI Appendix, Fig. S1G) and increased Matrigel invasion by 231 and UMUC3 (Fig. 2G and SI Appendix, Fig. S1H), confirming its importance to EMT in these models. In 231, EMT activation by p27CK−DD was shown by increased Matrigel invasion, decreased E-cadherin, and higher EMT-TF expression. All of these were reversed by TFGFB2 depletion (Fig. 2 H–J), indicating that TGF-β2 is a key driver of EMT activation by p27. Thus, C-terminal phosphorylated p27 appears to activate an EMT program, in part, by inducing TFGFB2 expression.

p27/cJun Complex Formation and Nuclear Localization Are Regulated by p27 Phosphorylation. To investigate how p27 up-regulates TFGFB2 expression, a phosphoprotein array was compared in MCF12A and MCF12Ap27CK−DD. Notably, p27CK−DD expression significantly increased serine 63-phosphorlated, activated cJun (hereafter cJunpS63) in MCF12A (Fig. 3A). p27CK−DD transduced 231 and UMUC3 also had more cJunpS63 than parental lines, while p27 depletion in metastatic 1833 and LuL2 lines decreased cJunpS63 (Fig. 3B). cJun forms heterodimeric AP-1 transcription factor complexes that contribute to transformation (30). An in silico search revealed AP-1 consensus binding sites upstream of the TFGFB2 coding sequence.

To test whether p27 might interact with cJun to regulate TFGFB2 expression, we next assayed if cellular p27 binds cJun. Treatment of LuL2 with PF1502 over 48 h decreased AKTpS473 and reduced p27 phosphorylation at T198 (Fig. 3C, Left) without affecting p27 or cJun levels. cJun was detected in cellular p27 immunoprecipitates in LuL2. PI3K inhibition decreased both p27pT198 and p27-bound cJun (Fig. 3C). Furthermore, more p27-associated
Fig. 3. Association of cJun with p27 is increased by p27 phosphorylation. (A) Activated cJun (cJunp563) in MCF12A controls (Vector) and MCF12Ap27CK–DD (p27CK–DD) by dot blot (Left) and densitometry values graphed as mean ± SEM (Right). (B) Western blots of cJun, cJunp563, and β-actin in the indicated lines. (C) LuL2 treated for 48 h with the PI3K inhibitor PF1502 (PI3Ki). Western blots show input (Left) for p27 immunoprecipitation (IP) blotting to detect p27-associated cJun (Middle). Densitometric quantitation of p27-bound cJun (Right). See also SI Appendix, Fig. S2A and B. (D) Nucleoprotein (N) and cytoplasmic (C) fractions of LuL2 (D) and 1833 (E) were immunoblotted (Left), and p27-associated cJun and JNK were detected by IP blot (Middle) with densitometry (Right). (F) In situ PLA shows p27/cJun complexes in the indicated lines and in 1833 treated with PF1502 (PI3Ki), indicated by red fluorescent dots. (G) Dots (mean ± SEM) graphed from triplicate PLAs; ANOVA with post hoc comparisons (**P < 0.01, ***P < 0.001). (Scale bar: 10 μm.) See also EMSA data in SI Appendix, Fig. S2C.

cJun was detected in p27CK–DD–transduced 231 and UMUC3 than in vector control cells, as assayed by densitometry (SI Appendix, Fig. S2A and B). These observations suggest that the interaction of p27 with cJun is increased by p27 phosphorylation.

Levels of p27-associated cJun and Jun N-terminal kinase (JNK) were assessed in nuclear and cytoplasmic fractions. In both LuL2 and 1833, JNK was largely cytoplasmic, while cJun was predominantly nuclear (Fig. 3 D and E, Left). p27-associated cJun was two- to threefold higher in nuclear vs. cytoplasmic fractions, while p27-associated JNK was detected only in the cytoplasm (Fig. 3 D and E, Right). Proximity ligation assays (PLAs) confirmed the interaction between nuclear p27 and cJun and that this interaction is greater in 231p27CK–DD than in control 231 cells (Fig. 3 F and G). Electrophoretic mobility shift assay (EMSA) revealed that p27 can bind an AP1 consensus motif (SI Appendix, Fig. S2C).

**cJun and p27 Are Corecruited to a Site Upstream of TGFB2.** Since p27 could associate with cJun and bind to AP1 motifs, we next assayed if nuclear p27/cJun complexes might mediate the TGFB2 gene induction observed in p27–activated cells. Publicly available cJun ChIP sequencing (ChIP-seq) ENCODE data revealed that cJun binds a 5′-TGAG/CTCA-3 AP1 consensus site upstream of the TGFB2 transcriptional start site (TSS) in human cells (Fig. 4A). ChIP-qPCR showed that cJun and p27 are corecruited to this AP1 site. More p27 and cJun were associated with this AP1 motif in 231p27CK–DD and 1833 than in 231 vector controls. Notably, chromatin association of both p27 and cJun decreased with p27 depletion and with loss of p27 phosphorylation following PI3K/mTOR inhibition with PF1502 (Fig. 4 B and C). Neither p27 nor cJun bound to irrelevant sites (SI Appendix, Fig. S3A). Sequential ChIP assays with cJun followed by re-ChIP with p27 antibodies indicated that cJun and p27 co-occupy this TGFB2 site, with greater recruitment in p27CK–DD–expressing and PI3K-activated cells, and with loss of both p27 and cJun from this site upon p27 knockdown and PI3K inhibition (Fig. 4D). Thus, C-terminal p27 phosphorylation appears to increase cJun/p27 corecruitment to this AP1 motif upstream of TGFB2. The bladder cancer models showed similar patterns of p27 and cJun recruitment to this TGFB2–AP1 site (SI Appendix, Fig. S3B). H3K27Ac, coactivator CBP/p300, and RNA polymerase II (Pol II) were more abundant at this TGFB2 site in p27TP57pT98 or p27CK–DD–expressing cells than in 231 controls and decreased significantly with p27 depletion and PI3K inhibition in 1833 (Fig. 4 E–G). Because p27 knockdown decreases TGFB2 expression and because p27CK–DD transduction induces TGFB2...
to increase TGF-β2 secretion, these data suggest a model in which C-terminal p27 phosphorylation promotes p27 and cJun recruitment as well as interaction with CBP/p300 and Pol II at this enhancer to induce TGFβ2 expression (SI Appendix, Fig. S3C).

cJun and TGF-β2 Mediate p27-Driven Metastasis from Primary Tumors in Vivo. Since p27 appears to drive a TGF-β2–dependent EMT, we assayed the functional contribution of C-terminally phosphorylated p27, cJun, and TGF-β2 to metastasis in vivo. NOD-SCID mice were injected orthotopically with 231, 1833, 1833shp27, and 231p27CK-DD, and with 231p27CK-DD depleted of either JUN or TGFβ2 (depletion shown in SI Appendix, Fig. S4B). After removal of primary tumors at 300 mm3, mice were monitored for metastasis from the primary site. Orthotopic injection of each of 1833 and 231p27CK-DD yielded significantly more metastases in liver, lymph nodes, and lung than vector control 231 cells (Fig. 5A–C). p27 depletion in 1833 and loss of cJun or TGF-β2 expression in 231p27CK-DD significantly decreased p27-driven metastasis in vivo (Fig. 5A–C). While 1833 and 231p27CK-DD formed more metastasis than 231, primary tumor growth and Ki67 staining did not differ significantly between groups (SI Appendix, Fig. S4 B and C). TGF-β2 levels, assayed by immunohistochemistry, were elevated in primary tumors from 1833 and 231p27CK-DD compared with 231 and were decreased in 1833shp27 tumors and in both JUN- and TGFβ2-depleted 231p27CK-DD–derived tumors (Fig. 5D and SI Appendix, Fig. S4D). Thus, cJun activation and TGFβ2 induction appear to be required for p27-driven metastasis in this breast cancer model.

Identification of cJun/p27-Regulated Target Genes. To test if p27 binds more broadly to chromatin and to identify phosphorylation-dependent patterns of chromatin occupancy by p27 and cJun, ChIP-seq was performed in 231, 231p27CK-DD, and 1833. As a control, we also performed p27 ChIP-seq in 1833shp27. This revealed that p27 is broadly recruited to chromatin and that a significant proportion of cJun ChIP-seq peaks also contain p27 (Fig. 6A), suggesting that cJun and p27 may function together at common sites. Notably, 231p27CK-DD showed greater recruitment not only of p27 but also of cJun to chromatin, even at sites not shared by p27. The latter may result from p27-mediated cJun activation (Fig. 3B). Genomic distribution analysis showed that a little over half of the sites bound by cJun, p27, and both cJun/p27 are located at common sites. Importantly, ChIP-seq heatmaps showed that the p27 signal was abrogated in p27-depleted 1833shp27 cells (Fig. 6C). Interestingly, the chromatin occupancy of cJun was also modulated by p27 depletion: among a
Fig. 6. Genome-wide DNA binding of p27 and cJun. (A) Venn diagrams show mean p27- and cJun-DNA binding peaks in 231, 231p27CK−DD, 1833, and 1833shp27 from two independent ChIP-seq assays, each with two biologic replicates. (B) Genomic distribution of DNA binding peaks for p27, cJun, and for peaks occupied by both p27 and cJun in the indicated lines. (C and D) DNA binding heatmaps of p27 (C) and cJun Chip (D) in 1833 and 1833shp27. See also SI Appendix, Fig. S5A and see SI Appendix, Fig. S5B for cJun-regulated pathways affected by loss of p27. (E) Venn diagrams show mean numbers of target genes (+/− 5 kb from the TSS) bound by cJun only, p27 only, and both cJun and p27 in 231, 231p27CK−DD, and 1833. (F) GO analysis (WikiPathway 2016) showing major signaling pathways related to 919 p27 target genes and 590 cJun/p27 cotarget genes bound in all three lines from E. See also SI Appendix, Fig. S5C. (G) Binding motif search shows top transcription motif bound by cJun only, p27 only, and cJun/p27 for binding motifs +/− 5 kb from the TSS for each line. See also SI Appendix, Fig. S5D for binding at all sites. (H) Heatmaps show expression profile from RNA-seq of genes bound by both cJun and p27 in ChIP-seq in all three lines. (I) Signal intensities of p27 and cJun were quantitated at target genes bound by both cJun and p27 in all three cell lines. Significant differences calculated with paired t test, and P values adjusted by Bonferroni–Hochberg. See also SI Appendix, Fig. S5E and F and Table S4 for signal intensities of cJun and p27 binding to group 1 and group 2 genes, respectively. 231DD, 231p27CK−DD.
little over 10,000 cJun-bound peaks detected in 1833, 5,294 sites were shared by p27 and were lost or decreased upon p27 knockdown, while 2,853 cJun-bound peaks were unaffected. In addition, 3,365 new, exclusively cJun-bound peaks were acquired (Fig. 6D and SI Appendix, Fig. S5A). GO analysis of 1,364 genes annotated by the 5,294 cJun-bound peaks that are lost with p27 depletion in 1833 showed that these p27-regulated cJun targets are associated with Notch, apoptosis, and cytokine signaling (SI Appendix, Fig. S5B). Thus, p27 may be required for cJun recruitment to an important fraction of cJun-regulated genes.

Evaluation of binding at target genes (+5 kb from the TSS) revealed that p27 is recruited to over 2,000 target genes in each of the three lines (Fig. 6E, Top Right Venn diagram). Notably, GO analysis of the 919 p27 targets common to all three lines reveals that p27 binds genes involved in focal adhesion, actin cytoskeleton, integrin signaling, and Wnt pathways (Fig. 6F, Top). GO analysis of cJun targets common to all three lines identifies genes governing cell surface adhesion, integrin and growth factor pathways (SI Appendix, Fig. S5C). There were 590 cJun/p27 target genes commonly bound in all three lines (Fig. 6E, Bottom and SI Appendix, Table S4). These are associated with pathways similar to those for each factor alone and also include hepatocyte growth factor and Rac1/Pak1 signaling (Fig. 6F, Bottom). p27 and cJun were also corecrueted to 252 novel target genes in both 231p27CK−/− and 1833 compared with the control 231 (Fig. 6E, Bottom).

DNA binding motif analysis revealed that AP-1 binding consensus motifs, including JunB, Fra1, and BATF, were the top-enriched DNA motifs for p27 and cJun individually and account for nearly one-third of the sites bound by both cJun and p27 (binding motifs ±5 kb from the TSS, Fig. 6G; genome-wide binding motifs in SI Appendix, Fig. S5D). Thus, p27 and cJun are commonly recruited to genes bearing cJun consensus motifs.

**Differential Expression of cJun/p27 Target Genes in Lines with Different Metastatic Potential.** To evaluate the potential for p27 and cJun to coregulate gene expression, patterns of chromatin annotation were compared with RNA-seq gene expression data. Of the 919 annotated by p27 in all three lines, over half were differentially expressed in 231p27CK−/− and 1833 compared with 231 (546/919) and in 1833 (456/919) compared with 231 (any fold change vs. 231, q > 0.1). cJun/p27 target genes bound in all three lines showed two differential expression patterns. Group 1 genes were up-regulated in 231p27CK−/− and in 1833 compared with 231 controls, while group 2 genes were down-regulated. cJun/p27 target genes activated in both highly metastatic lines identify putative oncogenes (group 1, Fig. 6H), while those down-regulated in p27-null 231 (Fig. 6E, Bottom). cJun/p27 target genes that were not bound by either p27 (Fig. 6E, Bottom) and whose binding is only acquired when highly phosphorylated cJun and p27 to an AP-1 site +2 kb from MYO10 and MYO10 expression were greater in 231p27CK−/− and 1833 than in 231 and were down-regulated in 1833shp27 (SI Appendix, Fig. S6 A–D).

Differential cJun/p27 binding to, and induction of, two other target genes, PAU/SERPINE1 [a known prognostic factor for breast cancer (32)] and KLF8 [an EMT mediator (33)], were also verified (SI Appendix, Fig. S6 E–L). Together, p27 appears to bind cJun and promote recruitment to, and transactivation of, gene programs that contribute to tumor progression and metastasis.

**p27/cJun Target Genes Are Differentially Expressed in Breast Cancers with High p27pT157.** We next assayed if primary human breast cancers with C-terminally phosphorylated p27 would show differential expression of cJun/p27-regulated genes identified herein. Among primary breast cancers in the TCGA database, 846 had gene expression, p27pT157 levels on reverse phase protein array, and outcome data available. Of these, cancers in the top decile of p27pT157 expression showed significantly worse overall survival (OS) (Fig. 7G, P = 0.028). A subset of 392 genes, differentially expressed in the “p27pT157 high” breast cancers vs. all others, was also coordinately differentially expressed in p27-activated 231p27CK−/− and 1833 compared with 231 and 1833shp27. Of these 392 differentially expressed genes, 25% (97/392) were Jun-bound and 16% (63/392) showed both p27 and cJun binding in the Chip-seq and included the validated target, PAI1. Univariate and multivariate analysis of each of these genes in a training dataset of 702 breast cancers identified the 30 p27-regulated genes differentially expressed both in p27-activated cell lines and in the cancers with high p27pT157 that contribute most importantly to patient outcome. Principle component analysis showed that these 30 genes cluster patients into two groups (Fig. 7H) that have significantly different OS on Kaplan–Meier analysis (Fig. 7I, P = 0.036). The prognostic value of this p27-regulated gene signature for OS was validated using receiver operating characteristic (ROC) curve analysis in an independent breast cancer validation cohort and yielded an area under the ROC curve (AUC) of 0.63 at 5 y and of 0.73 at 6 y of follow-up (Fig. 7J). The coordinate expression of p27-regulated gene drivers of poor patient outcome both in p27-activated cancer lines and in primary breast cancers with high C-terminal p27 phosphorylation supports the biologic relevance of p27-driven gene regulation to metastatic tumor progression.

**Discussion.** The cyclin-CDK inhibitor p27 is a ubiquitously expressed, critical negative regulator of the G1 to S phase cell cycle transition (4), inhibits cyclin-CDK complexes in the nucleus of quiescent cells but accumulates in the cytoplasm in early G1 (34). Transient C-terminal p27 phosphorylation by pAKT in early G1 delays nuclear p27 import (9) and promotes cyclin D-CDK4/6 assembly and activation (21, 35, 36) as Src and cyclin E-Cdk2 phosphorylate p27 to mediate its proteolysis and promote G1 transit (37, 38). The coordinate phosphorylation of p27 at T157 and T198 also promotes its binding to RhoA/ROCK1 (18) to alter the actin cytoskeleton in normal cells, mediating changes in cell shape required for execution of later cell cycle phases (39, 40).

In the last two decades, p27 has been found to act as both tumor suppressor and oncogene and as a critical regulator of development. The present work opens the possibility that these roles might be modulated by a transcriptional regulatory action of p27. p27 deregulation is a hallmark of human cancers. The tumor-suppressor, CDK-inhibitory function of p27 is impaired through miRNA-mediated decreases in p27 translation and by accelerated p27 proteolysis in Src-activated cells (4). p27 also acquires prooncogenic functions through its C-terminal phosphorylation by P3K-activated kinases (41). C-terminal p27 phosphorylation at T157 and T198 attenuates CDK inhibitory action and increases cyclin D-CDK activation (21, 35) and disrupts the actin cytoskeleton through RhoA/ROCK1 inactivation (18, 22) to promote cancer metastasis (42). Enforced expression of p27 in the cytoplasm of malignant
Fig. 7. cJun/p27-regulated target genes govern cancer programs of tumor progression. (A) Heatmap of expression of 61 p27/cJun target genes common to all three lines whose expression is up-regulated in 231p27CK−/− (231DD) and 1833 lines vs. 231. See also SI Appendix, Table S4. (B) Mean amplitudes of target gene binding by cJun (Top) and p27 (Bottom) are shown for the differentially expressed genes in A in the indicated cell lines. (C) GO analysis shows the top signaling pathways related to the differentially expressed genes identified in A. (D) Heatmap of expression of 43 newly acquired cJun/p27 target genes bound in 231DD and 1833, but not in 231, that are differentially expressed in the indicated cell lines. See also SI Appendix, Table S5. (E) Mean amplitude of target gene binding by cJun (Top) and p27 (Bottom) are shown for the differentially expressed genes in D in the indicated cell lines. (F) GO analysis shows the main signaling pathways related to the genes identified in D. See SI Appendix, Fig. S6 for validation of cJun/p27 cotarget genes. (G) Kaplan–Meier (KM) graph showing differential OS among women whose breast cancers have high vs. low p27pT157 on reverse phase protein array (RPRA) from n = 846 cases in the TCGA/TCGA dataset. (H) Principle component analysis of 30 p27-regulated genes expressed among n = 703 breast cancers. (I) KM graph shows overall breast cancer survival according to differential expression of the p27-regulated 30 gene profile among the training set (n = 703 cases). (J) The prognostic value of this p27-regulated 30 gene signature for OS was validated using ROC analysis in an independent breast cancer validation cohort. AGE-RAGE, advanced glycation end products-receptor for advanced glycation end products; AMPK, 5′ AMP-activated protein kinase; FP, false positive; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR, peroxisome proliferator-activated receptor; TP, true positive.
cells with high endogenous PI3K activation is sufficient to increase cell invasion and metastasis (43, 44). Although aberrantly detected in cyttoplasm, p27 is never exclusively cyttoplasmic, nor is nuclear p27 limited in PI3K-activated cancers (9, 15, 16). Delayed nuclear import and increased stability of C-terminally phosphorylated p27 permit novel protein interactions in both cytoplasm and nucleus that drive onconeogenesis. Our prior work showed that p27CK−DD, but not p27CK−, interacts with and activates STAT3 to induce TWIST1 expression and a morphologic EMT and showed that C-terminal p27 phosphorylation critically mediates metastasis (14). Here, we identify a mechanism of onconeogene cooperation between the PI3K pathway and cJun and reveal a previously unknown p27/cJun partnership that provides new insight into the profound effects of p27 on tumor metastasis. It is not clear that any of the p27 phenotypes resulting from these phosphorylations has primacy over any other. Rather, we posit that they would act together to promote transformation in the context of constitutive onconeogene PI3K activation. We show that p27 is broadly recruited to chromatiner and cooperates with cJun to activate gene programs that govern focal adhesion, cytoskeleton, and signaling regulators of cell motility and metastasis.

Several lines of evidence have suggested a role for p27 in transcriptional regulation during embryogenesis. As CDK inhibitor, p27 acts to coordinate cell cycle exit with terminal differentiation (40, 41). p27 also plays CDK-independent developmental roles in collaboration with tissue-specific transcription factors, including MYOD and neurogenin 2. In mice, p27 interacts functionally with the proneural factor neurogenin 2 in neuronal differentiation (45), and in Xenopus laevis, the p27 homolog Xic1 cooperates with the myogenic factor MYOD to mediate myogenesis (46, 47). These effects are cell cycle independent, since a cdknx mutant encoding a xic1 devoid of cyclin-CDK binding restored differentiation defects in cdknx-null frogs (46, 47). Tissue differentiation processes affected in p27-null mice (46) also rescued by p27 knockdown (51). These developmental actions may reflect transcriptional roles of p27, whose potential regulation by periodic AKT activation and p27 phosphorylation have yet to be explored. Further evidence for an interaction in transcription came from a ChIP-on-chip survey in NIH 3T3 cells that showed that p27 binds gene promoters in complex with p130, E2F4, HDAC1, and SIN3A (52). This complex appears to mediate SOX2 repression during ES cell differentiation and in quiescent mouse embryonic fibroblasts (MEFs) (53). A recent genomic survey confirmed broad chromatin association of p27 in quiescent MEFs (54), but biologic targets and function were not characterized.

The present work provides a comprehensive comparison of p27−chromatin binding in human cancer cells with different metastatic potential and reveals the unexpected finding that p27 functionally cooperates with cJun to regulate transcriptional programs associated with cell adhesion and metastasis. cJun participates in homo- and heterodimeric AP-1 transcription factor complexes to activate drivers of transformation, proliferation, apoptosis, and metastasis in human cancers (30). JNKs phosphorylate and activate cJun (55). cJun regulates EMT during differentiation (55) and drives cancer cell motility and invasion (56), and its levels correlate with poor breast cancer patient outcome (57). Here, we show that cJun interacts with p27 and that its transcriptional activity is importantly regulated by p27. p27 phosphorylation increases its coprecipitation with cJun and cJun activation and increases the magnitude of, and changes the distribution of, cJun/p27 coregulation to chromatin. A significant proportion of cJun-annotated sites are shared by p27, and cJun recruitment to these sites decreased dramatically with p27 depletion. Thus, p27 phosphorylation may not only promote its interaction with cJun complexes, but also facilitate their stable chromatiner association.

Approximately half of p27 binding sites are promoter proximal, and cJun/AP-1 consensus motifs were the most common DNA binding motif. p27 targets in our PI3K-activated lines were nearly twice as abundant as reported in quiescent MEFs (54). The inactivation of PI3K/AKT and the loss of p27pT157pT198 in quiescence (21) may account for these differences. AKT activation is required for G1 to S phase progression, and, in normal mammary epithelial cells, AKT activation and p27 phosphorylation at T157 and T198 peak in mid-G1 (21). Cyclic changes in p27pT157pT198 abundance may modulate p27-regulated transcription across the cell cycle. In cancers with onogenic PI3K activation, the increased C-terminal p27 phosphorylation may abrogate the coresspfunctiomic of p27 observed in quiescent cells and alter both coregulator and transcriptional target gene selection, directing constitutive p27/cJun association to drive proonogenic changes in gene expression. p27 is a central node, integrating PI3K and TGF-β signaling pathways to maintain expression of a profile of EMT genes. Both TGF-β2 and TGF-β2-induced gene profiles are up-regulated in p27-activated models. TGF-β2 is a known EMT driver (58) that promotes metastasis in a variety of malignancies, including gliomas (59) and pancreatic (60, 61) and breast cancers (62). cJun/p27 complexes are corecruited to TGFβ2 to drive its expression as well as associate with chromatiner more broadly to modulate transcription of genes critical for cell adhesion, cytoskeletal regulation, and signaling. Metastasis from primary orthoptic tumors was reduced by p27 depletion in 1833 and by JUN or TGFβ2 depletion in 231p27CK−DD, supporting the functional importance of these pathways in metastasis.

A large number of cJun/p27 target genes were differentially expressed in p27-activated models with control 231. These cJun/p27 target genes associate with proonogenic signaling, including p53, cancer-related miRNAs, HIF-1, focal adhesion, and ECM pathways. In addition, in the highly metastatic lines, a new set of target genes were acquired that govern metabolic and HIF-1/hypoxia-regulated pathways. cJun/p27 target genes identified in 231p27CK−DD and 1833, but not activated in 231, may require a threshold of p27 phosphorylation for binding and gene induction. The binding and expression of the validated cJun/p27 targets—PAI1/SERPINE1, MYO10, p27-KLF8—were p27 dependent and increased in metastatic lines. All play roles in EMT or in cancer cell motility and metastasis and are associated with early breast cancer metastasis (31–33).

While cyclin-CDKs have long been known to govern transcription via pRb-family phosphorylation and activation of E2Fs, the present work identifies a novel proonogenic function for p27 as a transcriptional coregulator of cJun. In over 60% of human cancers, PI3K/AKT constitutively activates effectors AKT, SGK1, and p90Rsk, all of which phosphorylate p27 (4, 42). AKT activation is associated with both cytoplasmic p27 (9, 15, 16) and detection of C-terminally phosphorylated p27 in primary human breast cancers (12, 14). That genes differentially regulated by p27 in our cell line models were also differentially expressed in primary breast cancers with high p27pT157 supports the biologic relevance of this mechanism of p27 action in vivo. Moreover, this p27-regulated gene profile is associated with poor cancer survival, indicating its relevance to disease progression. Together, our findings support a model in which C-terminal phosphorylation promotes p27 interaction with cJun, leading to p27/cJun coregulation to, and activation of, oncogenic genes that drive programs of EMT and cancer metastasis.

Materials and Methods
All materials and methods, including the source of p27 phosphomimetic mutant-expressing cells, lentivirus production and infection, siRNA-mediated knockdown of p27, real-time qPCR, Western blotting, immunoprecipitation, nuclear and cytoplasmic fractionation, transwell invasion assay, ChIP assay, orthotopic xenograft assay, immunohistochemistry, PLA, EMSA, RNA-seq, RNA-seq bioinformatic analysis, ChIP-seq and bioinformatic analysis thereof, analysis of p27-regulated gene expression in primary human breast cancers from TCGA/TCGA, and statistical analysis and references pertaining to these methods are detailed in SI Appendix, Supplemental Materials and Methods. Reagents and resources are listed in SI Appendix, Table S6. Animal work was compliant with University of Miami Institutional Animal Care and Use Committee.
Data Availability
Data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession no. GSE112446.

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