

## Original Research

## CDK5RAP3 is a co-factor for the oncogenic transcription factor STAT3

Susana P. Egusquiaguirre; Suhu Liu; Isidora Tošić<sup>1</sup>; Kevin Jiang; Sarah R. Walker; Maria Nicolais; Tzuen Yih Saw; Michael Xiang; Katarina Bartel; Erik A. Nelson; David A. Frank

Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215; Departments of Medicine, Brigham and Womens Hospital and Harvard Medical School, Boston, MA 02115, United States

## Abstract

The transcription factor STAT3 regulates genes governing critical cellular processes such as proliferation, survival, and selfrenewal. While STAT3 transcriptional function is activated rapidly and transiently in response to physiologic signals, through a variety of mechanisms it can become constitutively activated in the pathogenesis of cancer. This leads to chronic expression of genes that underlie malignant cellular behavior. However, STAT3 is known to interact with other proteins, which may modulate its function. Understanding these interactions can provide insights into novel aspects of STAT3 function and may also suggest strategies to therapeutically target the large number of cancers driven by constitutively activated STAT3. To identify critical modulators of STAT3 transcriptional function, we performed an RNAinterference based screen in a cellbased system that allows quantitative measurement of STAT3 activity. From this approach, we identified CDK5 kinase regulatorysubunit associated protein 3 (CDK5RAP3) as an enhancer of STAT3dependent gene expression. We found that STAT3 transcriptional function is modulated by CDK5RAP3 in cancer cells, and silencing CDK5RAP3 reduces STAT3mediated tumorigenic phenotypes including clonogenesis and migration. Mechanistically, CDK5RAP3 binds to STAT3regulated genomic loci, in a STAT3dependent manner. In primary human breast cancers, the expression of CDK5RAP3 expression was associated with STAT3 gene expression signatures as well as the expression of individual STAT3 target genes. These findings reveal a novel aspect of STAT3 transcriptional function and potentially provide both a biomarker of enhanced STAT3dependent gene expression as well as a unique mechanism to therapeutically target STAT3.

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## Introduction

The transcription factor STAT3 is activated constitutively in a large fraction of human cancers, including approximately 70% of breast cancers [1,2]. When activated by IL6 and other cytokines, STAT3 becomes phosphorylated on a unique carboxyterminal tyrosine residue, which leads to the formation of an active dimer that translocates from the cytoplasm to the nucleus, and binds to nine base pair sequences in the regulatory regions of target genes (TTCN<sub>3</sub>GAA). Under normal, physiologic conditions, STAT3 is activated for a period of 30 to 90 minutes, after which it is rapidly dephosphorylated and shuttles out of the nucleus. This tight reg-

ulation of STAT3 function reflects the fact that target genes of STAT3 control critical cellular processes such as proliferation, survival, and selfrenewal. When activated inappropriately in a cancer cell, either through activation of kinases, loss of negative regulatory factors, or a combination, it leads to continuous expression of genes that drive the malignant behavior of a cell [3,4].

Abundant evidence suggests that the activation of STAT3 is not a bystander event in oncogenesis, but it directly drives cancer pathogenesis [2,5,6]. Since loss of STAT3 function in normal cells can be tolerated [7], inhibiting STAT3 function is a promising strategy for targeted cancer

Corresponding author at: Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, United States.  
e-mail address: david\_frank@dfci.harvard.edu (D.A. Frank).

<sup>1</sup> I.T. also holds an appointment in the Department of Biochemistry, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia.

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therapy. However, as with transcription factors as a class, inhibiting the function of STAT3 with small molecules is challenging. One approach to overcome this obstacle is to identify critical cofactors of STAT3 function that may be amenable to inhibition.

To identify novel modulators of STAT3 function in an unbiased fashion, we utilized an RNA interference based approach to identify gene products that enhanced STAT3 transcriptional function. The goal was to identify novel aspects of STAT3 dependent gene expression, to gain insight into modulators of STAT3 function in cancer, and to potentially identify new strategies to target this oncogenic transcription factor.

## Materials and methods

### Cell lines

SKBR3 cells (received from Lyndsay Harris, DanaFarber Cancer Institute) were maintained in RPMI containing 10% fetal bovine serum. U3Aluciferase cells, parental 2fTGHluciferase cells (both containing stably integrated STAT3 dependent luciferase reporter genes, and originally provided by George Stark, Lerner Research Institute, Cleveland Clinic, Cleveland, OH) [8], and MCF7 cells (kindly provided by Francis Kern, Southern Research Institute, Birmingham, AL) were maintained in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum. MCF10A cells (received from ATCC, crl10317) expressing STAT3C (with a FLAG epitope) under an inducible promoter [9] were maintained in DMEM/F12 containing 5% horse serum, 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 0.5 g/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma) and 10 g/ml insulin (Sigma). To induce STAT3C expression, cells were stimulated with the indicated concentration of doxycycline (Sigma) for 24 hours for mRNA analyses and luciferase reporter assays. All cells were passaged for less than three months after thawing. All cells were maintained in a humidified incubator at 37 C with 5% CO<sub>2</sub>, and were authenticated by short tandem repeat DNA profiling.

### RNA interference screen

The screen was performed in U3A cells, which lack STAT1 [10], to avoid any confounding effects from this highly related transcription factor. Three Dharmacon SMARTpools (Lafayette, CO), comprising a total of 7292 target genes most likely to contain unique STAT3 modifiers (with four duplexes per target), were screened in 384 well plates in triplicate: Human 1 Pool (Kinases/Phosphatases), Human 2 Pool (G protein coupled receptors), and Human 3 Pool (the Remaining Druggable Genome). In parallel, a screen for viable cell number was performed (using ATP dependent bioluminescence) to be able to normalize changes in STAT3 dependent luciferase with any change in viability. Each small interfering RNA (siRNA) pool (containing four distinct siRNAs) was added at 25 nM/well in OptiMEM and Lipofectamine RNAiMAX (Invitrogen), after which U3Aluciferase cells were seeded (2000 cells/well). Cells were incubated for 72 hours, after which IL6 was added (5 ng/ml). After five hours, cells were equilibrated for 15 minutes at room temperature, after which luciferase activity was detected with a BrightGlo assay (Promega) and viable cell number was determined using Cell TiterGlo.

### Cytokine treatments

Cells were treated with interleukin (IL)6 (10 ng/mL; Peprotech) for 15 minutes for immunoblots, 30 minutes for cellular fractionations and for chromatin immunoprecipitation analysis, 90 minutes for mRNA analyses, and 6 hours for luciferase reporter assays. IL6 was added to the media every 48 hours for clonogenic and migration analysis.

### RNA interference

Cells (5 × 10<sup>5</sup> cells per well in a 6well plate) were transfected using Lipofectamine RNAiMAX with 10 nM of siRNA targeting CDK5RAP3#1 or CDK5RAP3#17 (D012957010002 or D012957170002, respectively; Dharmacon), STAT3 (D003544020010, Dharmacon), or nontargeting siRNA Control (D0012100305, Dharmacon). Cells were transfected with siRNA for 72 hours prior to biological experiments.

### Immunoblot analyses and nuclear fractionation

Cells (5 × 10<sup>5</sup> cells per well in a 6well plate) were lysed on ice for 15 minutes in RIPA lysis buffer (Boston BioProducts, Boston, MA) with phosphatase and complete protease inhibitors (Roche). Immunoblots were probed with antibodies to CDK5RAP3 (ab70776, Abcam, Cambridge, MA), phosphoSTAT3 (Tyr705) (9131, Cell Signaling Technology, Inc. Danvers, MA), STAT3 (sc482, Santa Cruz), FLAG (F1804, Sigma), PARP (9542S, Santa Cruz Biotechnology), tubulin (T5168, SigmaAldrich), and actin (A2228, SigmaAldrich). Cellular fractionation was performed using the Active Motif Nuclear Extract Kit (Cat. No. 40010, Carlsbad, CA). Band intensity was quantitated using ImageJ software (National Institutes of Health).

### Luciferase reporter assays

Cells (5 × 10<sup>4</sup> cells per well in a 24well plate) were reverse transfected using Lipofectamine RNAiMAX with 10 nM of siRNA targeting CDK5RAP3 (constructs #1 and #17), or siRNA targeting STAT3, or a nontargeting siRNA control. 48 hours after transfection, 1 g of a STAT3 dependent luciferase reporter (m67luc luciferase reporter plasmid, J. Bromberg, Memorial SloanKettering Cancer Center, New York, NY) was transfected into the cells in combination with 0.1 g *Renilla* luciferase transfection control (Promega) using Lipofectamine 2000 (Invitrogen). 24 hours after transfection, the cells were stimulated for 6 hours with IL6, then lysed and quantitated by a dual luciferase assay (Promega), and read on a Luminoskan Ascent Luminometer (ThermoLab Systems, Helsinki, Finland). STAT3 and NFκB dependent luciferase production was normalized to *Renilla* luciferase values.

### mRNA expression analyses (RT-qPCR)

Total cellular RNA was isolated using RNeasy Mini kits (Qiagen). RNA quality was evaluated on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific), and reverse transcribed with TaqMan (Applied Biosystems, Foster City, CA) to generate cDNA. Quantitative polymerase chain reaction (qPCR) was performed in quadruplicate using Power SYBR master mix (Applied Biosystems) on a QuantStudio 6 Flex RealTime PCR system (Applied Biosystems). Specificity of amplification was confirmed by melt curve analysis. Cycle threshold (C) values for target genes were normalized to the endogenous reference gene GAPDH, and the fold change was determined by dividing the expression in each sample by that of the unstimulated control sample. Primer sequences (Supplementary Table 1) were designed from the UCSC genome browser reference mRNA sequences using Primer3.

### Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [11]. Briefly, cells (5 × 10<sup>6</sup>) were fixed in 1% formaldehyde for 10 minutes, sonicated in 15 second pulses using a Qsonica sonicator, and lysates immunoprecipitated overnight at 4 C with an antibody for STAT3 (sc482, Santa Cruz Biotechnology), Polymerase II (sc9001, Santa Cruz Biotechnology) or CDK5RAP3

(ab70776, Abcam, Cambridge, MA). Quantitative PCR was performed using the indicated primers (Supplementary Table 1), and signal detected was normalized to input.

#### Quantitation of viable cell number

Cells (3000 cells per well in a 96well plate) were transfected with siRNA targeting CDK5RAP3#1 or CDK5RAP3#17, STAT3, or a nontargeting siRNA control in triplicate. 72 hours later, viable cell number was quantitated by measuring intracellular ATP using Cell TiterGlo (Promega) on a Luminoskan Ascent luminometer.

#### Clonogenic assays

Cells ( $3 \times 10^3$  cells per well in a 96well plate) were transfected with siRNA targeting CDK5RAP3#1 or CDK5RAP3#17, STAT3, or a nontargeting siRNA control for 24 hours, then trypsinized and seeded in a 6well plate. After 7 days, colonies were washed in PBS, then fixed and stained in 0.5% crystal violet and 6% glutaraldehyde. Normalized Number of Colonies and Normalized Colony Intensity were then quantitated with CellProfiler™ software (Broad Institute, Boston, USA) (<http://cellprofiler.org/>).

#### Migration assays

Cells ( $1 \times 10^6$  cells per well in a 6well plate) were transfected for 48 hours with siRNA targeting CDK5RAP3#1 or CDK5RAP3#17, STAT3, or a nontargeting siRNA control, then the confluent monolayer was disrupted with a linear scratch made with a sterile P200 pipette tip. Cells were washed in PBS, incubated in serumfree media to prevent proliferation, and imaged at 24 hours intervals.

#### Gene expression analysis from primary breast cancers

The GSE5460 [1] breast cancer microarray dataset was downloaded from the Gene Expression Omnibus. Phosphorylation of STAT3 on tyrosine 705 (STAT3\_PY705) from dataset GSE5460 was published previously [1]. Gene expression was downloaded from the Cancer Genome Atlas (TCGA) breast invasive carcinoma dataset from cBioportal on December 12, 2016.

Geneset enrichment analysis was performed using xapps.gsea.Main from the Broad Institute of MIT and Harvard (<http://software.broadinstitute.org>) [11,12], based on STAT3 gene expression signatures [13,14]. Correlation analysis between the mRNA levels of CDK5RAP3 and STAT3 target genes were conducted with GraphPad Prism 7 software (La Jolla, CA).

#### Statistical analyses

Results were presented as SD. Twotailed Student t tests for paired samples were performed with GraphPad Prism 7 software (La Jolla, CA). Values of  $p < 0.05$  were considered significant (\*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ ; \*\*\*\*,  $P < .0001$ ).

## Results

#### RNA interference screen

Inappropriate activation of the oncogenic transcription factor STAT3 is a common event in cancer. However, it is likely that the expression of other proteins, which can positively or negatively affect STAT3 function, may be important modulators of the oncogenic activity of STAT3.

To identify novel proteins that modulate STAT3 transcriptional function, we performed an RNA interference screen using a cellbased readout of STAT3 transcriptional activity [8]. We performed a cell viability based counterscreen to exclude siRNAs that lead to nonspecific toxicity or downregulate proteins that are necessary for transcription more broadly, as the U3A cells in which the screen was performed are not dependent on STAT3 for their survival. We chose U3A cells, as these cells lack STAT1, and all IL6induced luciferase activity is STAT3dependent. We targeted 7292 genes using pools of four siRNAs for each gene, and scored each target based on the ratio of STAT3dependent luciferase activity to viable cell number (as quantitated by ATPdependent bioluminescence). As an internal positive control, siRNAs targeting STAT3 itself led to a value of 0.2. We then chose the 1% of targets with the lowest STAT3toviability ratio for further analysis, in which each of the four siRNAs were tested individually.

The gene that showed the most consistent effect in decreasing STAT3dependent gene expression, while leaving viability unaffected, was CDK5 kinase regulatorysubunit associated protein 3 (CDK5RAP3, also called C53/LZAP; Figure 1A). We identified ten other genes whose targeting led to STAT3inhibitory effects almost as great as CDK5RAP3 (Figure 1B). None of these 11 genes had known STAT3 modulatory effects, obvious intersections with STAT3 activation, or common known functional characteristics. CDK5RAP3 is a regulator of cell cycle progression pathways, which has been described as having both tumor suppressor and cancer promoting activity [15,16]. However, its definitive cellular role has been controversial.

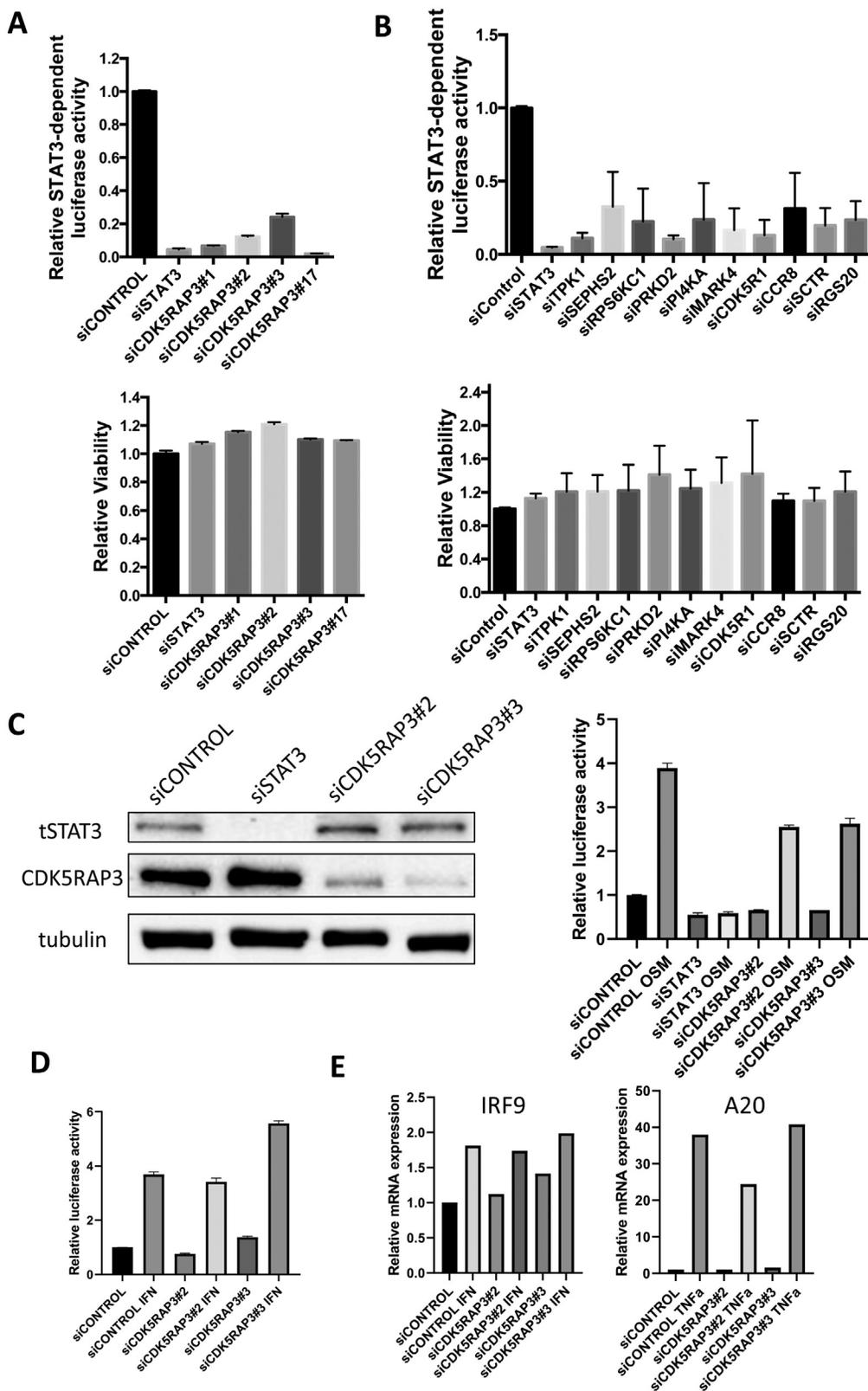
Given the identification of CDK5RAP3 as a potential enhancer of STAT3 transcriptional function, we first wished to validate its effect on STAT3 activity. Although the probability of offtarget effects of all of these multiple siRNAs to CDK5RAP3 seemed remote, we first confirmed in independent experiments that siRNAs that target CDK5RAP3 do, in fact, decrease expression of this protein in U3A cells (Figure 1C). To determine whether CDK5RAP3 might similarly modulate the highly homologous (although biologically distinct) family member STAT1, we used the same siRNA to CDK5RAP3 in parental 2fTGH cells also stably expressing a luciferase reporter gene under the control of a STAT promoter. Knockdown of CDK5RAP3 had no consistent effect on luciferase activity induced by the STAT1activating cytokine interferon (Figure 1D). To further exclude nonspecific transcriptional inhibition resulting from depletion of CDK5RAP3, we treated 2fTGH cells with either interferon, to induce the activation of STAT1, or TNF, to induce the activation of NFB. We then quantitated expression of the highly specific STAT1 target gene *IRF9* and the highly specific NFB target gene *A20* (*TNFAIP3*) by quantitative RTPCR. Depletion of CDK5RAP3 by siRNA had no consistent effect on the expression of these genes, further indicating that CDK5RAP3 is not a general regulator of transcription. This is also consistent with the lack of effect of CDK5RAP3 knockdown on cellular viability (Figure 1A).

#### CDK5RAP3 modulates STAT3 transcriptional activity in breast cancer cells

STAT3 plays an important role in mammary gland physiology, and is frequently activated inappropriately in breast cancer. To determine the effect of CDK5RAP3 on STAT3 function in models that are more relevant in terms of physiology and pathogenesis, we next utilized two breast cancer cell lines that do not depend on STAT3 for viability, but in which STAT3 phosphorylation can be induced by cytokine stimulation: MCF7, which is a hormone receptor expressing line, and SKBR3, which contains Her2 amplification. We transfected these cells with a luciferase reporter gene under the control of a STAT3regulated promoter, to allow a broad assessment of STAT3 transcriptional function. When treated with the

cytokine IL6, which is frequently found in the tumor microenvironment [13], prominent luciferase activity was induced (Figure 2A). When the cells were treated with siRNA targeting STAT3, luciferase activity returned essentially to baseline in MCF7 cells and was reduced by approximately 70% in SKBR3 cells. This finding suggests that the IL6-induced transcriptional activity is mediated by STAT3 in the MCF7 cells. It is pos-

sible that some residual transcriptional activity regulating the STAT-dependent promoter is mediated by IL6 induced STAT1 activity in the SKBR3 cells. When the cells were treated with either of two distinct siRNAs targeting CDK5RAP3 (chosen based on optimal efficacy), the induction of STAT3-dependent luciferase was significantly decreased in both MCF7 cells (by approximately 70%) and SKBR3 cells (by approxi-



mately 50%; Figure 2A). Again, the smaller effect in the SKBR3 cells could reflect some STAT1dependent transcription, which, as shown previously, is not dependent on CDK5RAP3 (Figure 1D). Since the STAT3dependent luciferase reporter provides a global measure of STAT3 activity, we next examined specific endogenous genes known to be regulated by STAT3. We found that silencing CDK5RAP3 suppressed the induction of these genes in both MCF7 and SKBR3 cells to extents similar to that seen with knockdown of STAT3 itself (Figure 2B).

In cancer cells driven by constitutive STAT3 activation, such as triple negative breast cancer [13], STAT3 is chronically phosphorylated on tyrosine 705 and found within the nucleus. To determine whether CDK5RAP3 could also enhance the activity of constitutively active STAT3, we employed a mutant, activated form of STAT3 (STAT3C). We used nontransformed mammary epithelial MCF10A cells, in which the expression of STAT3C (with a FLAG epitope) was under the control of a doxycyclineresponsive promoter. In this system, there is minimal expression of FLAGSTAT3C in the absence of doxycycline, and the expression of STAT3C can be titrated to physiological levels of STAT3 transcriptional activity (Figure 2C). These cells also stably express an m67luciferase STAT reporter gene, to allow an integrated measure of STAT transcriptional activity. When these cells were treated with doxycycline, there was a prominent induction in STAT3dependent transcriptional activity that was completely abrogated by siRNA to STAT3 (Figure 2D). Similarly, when the cells were transfected with either of two distinct siRNAs targeting CDK5RAP3, the induction of STAT3dependent luciferase was completely lost (Figure 2D). To determine whether this role for CDK5RAP3 with chronic STAT3 activation extended to endogenous STAT3regulated genes, we assessed the mRNA expression of a panel of wellannotated STAT3 target genes. All of these genes showed decreases in expression in the presence of siRNA targeting CDK5RAP3 (Figure 2E).

#### *CDK5RAP3 is necessary for colony formation and migration in breast cancer cells*

Given the identification of CDK5RAP3 as a modulator of STAT3 transcriptional activity, and the critical role of STAT3 in breast tumorigenesis, these studies raise the possibility that CDK5RAP3 may contribute to malignant cellular behavior. To test this hypothesis, we determined the effect of depleting CDK5RAP3 by RNA interference on the phenotype of these cells. As would be expected in cells lacking constitutive STAT3 activation, the silencing of STAT3 or CDK5RAP3 had no effect on the viability of MCF7 and SKBR3 cells (Figure 3A). To determine if silencing CDK5RAP3 affects other STAT3mediated tumorigenic phenotypes, we first performed clonogenesis assays. Silencing CDK5RAP3 significantly decreased both the number and the size of IL6induced colonies in MCF7 and SKBR3 cells (Figure 3B). We next assessed the effect of CDK5RAP3 on the motility of these cells, using standard wound

closure assays. We found that silencing CDK5RAP3 significantly reduced the motility of both MCF7 and SKBR3 cells in the presence of IL6, both 24 and 48 hours after knockdown (Figure 3C). This suggests that CDK5RAP3 enhances the tumorigenic phenotype in MCF7 and SKBR3 cells.

#### *CDK5RAP3 does not alter tyrosine phosphorylation or nuclear localization of STAT3*

Having shown that CDK5RAP3 enhances STAT3 transcriptional activity and modulates the biology of breast cancer cells, we next wished to determine the mechanism by which it exerted this effect. We first considered the possibility that CDK5RAP3 modulates the activating tyrosine phosphorylation of STAT3. To test this hypothesis, we transfected MCF7 or SKBR3 cells with siRNA targeting CDK5RAP3, and then stimulated the cells with IL6. Silencing CDK5RAP3 had no significant effect on the tyrosine phosphorylation of STAT3 (Figure 4A). We then considered the possibility that CDK5RAP3 altered the nuclear localization of STAT3. We isolated cytoplasmic and nuclear fractions from MCF7 and SKBR3 cells transfected with siRNA targeting CDK5RAP3 in untreated cells or cells stimulated with IL6. Silencing CDK5RAP3 had no effect on the nuclear localization of tyrosine phosphorylated STAT3 (Figure 4B and C). Taken together, these findings suggest that CDK5RAP3 may be modulating the transcriptional function of nuclear phosphorylated STAT3.

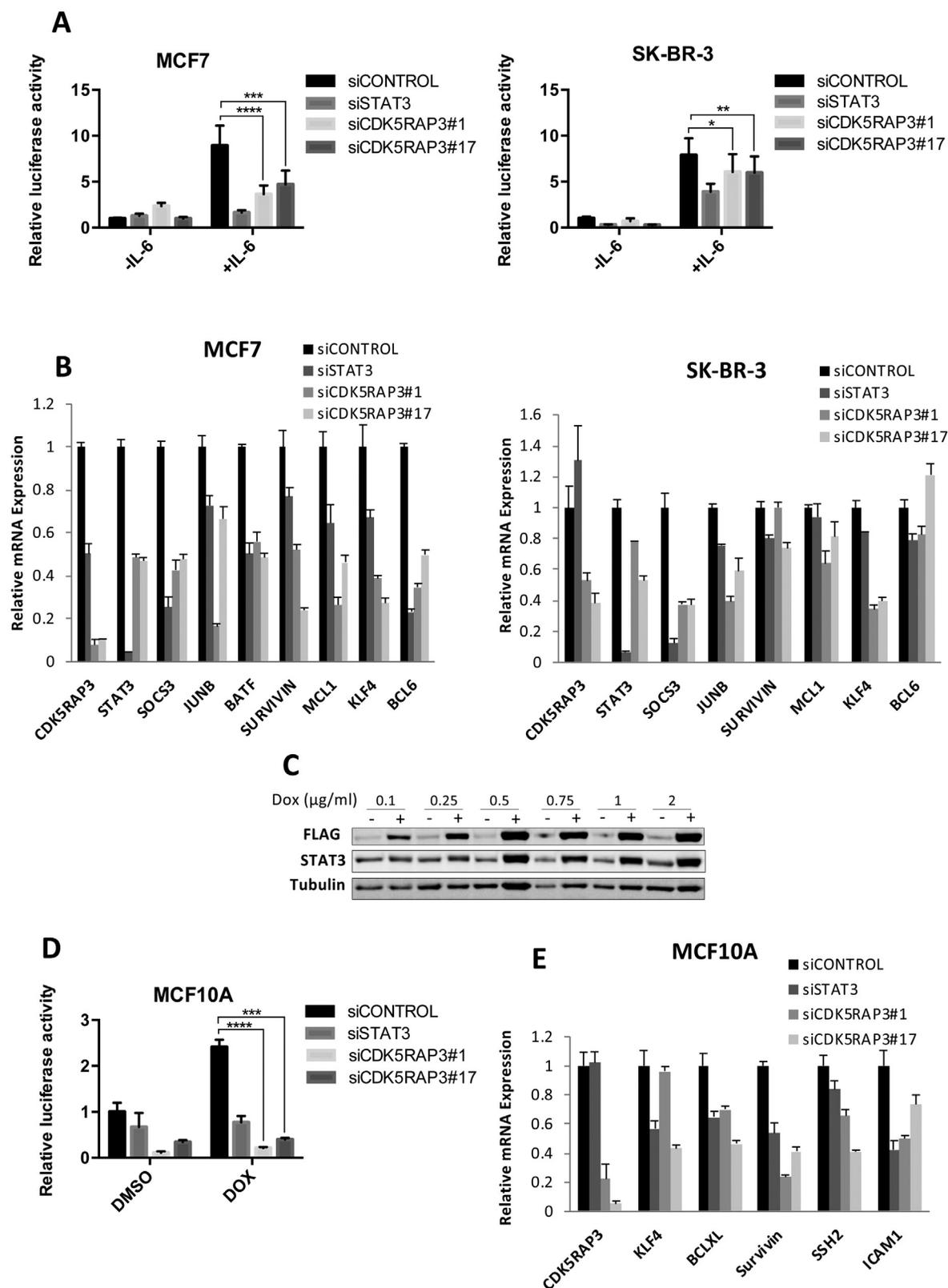
#### *CDK5RAP3 associates with the promoters of STAT3 target genes and enhances transcription*

Since CDK5RAP3 did not alter STAT3 tyrosine phosphorylation or nuclear localization, we considered the hypothesis that CDK5RAP3 affected STAT3 function at gene promoters. To determine whether CDK5RAP3 affects IL6mediated STAT3 recruitment to cognate binding sites, we performed chromatin immunoprecipitation (ChIP) in MCF7 cells at five genes we had shown to be regulated by STAT3 (Figure 2B). We first performed ChIP with an antibody to RNA polymerase II. At all five of these genes, recruitment of RNA polymerase II was decreased by CDK5RAP3 knockdown (or, as a positive control, STAT3 knockdown), confirming that CDK5RAP3 is directly regulating transcription at these genes (Figure 5A). We next performed ChIP to STAT3, to determine whether CDK5RAP3 was necessary for the binding of STAT3 to its cognate genomic binding sites. Of the five genes, at two (*MCL1* and *JUNB*), we could not show increased STAT3 binding following IL6 treatment. This could reflect the fact that the loci interrogated in this directed ChIP were not the STAT3regulated sites in these cells. Alternatively, these loci could have been bound by dephosphorylated STAT3 prior to IL6 treatment, which was replaced by transcriptionally active phosphorylated STAT3 following IL6 treatment. The decrease in binding seen at *JUNB* with STAT3 depletion would be consistent with this model, although it

**Figure 1.** An RNA interference screen identifies CDK5RAP3 as an enhancer of STAT3 transcriptional activity. (A) In a large scale RNA interference screen, four distinct siRNAs targeting CDK5RAP3 decreased IL-6-driven STAT3-dependent transcriptional activity in U3A cells (top) without affecting cell viability (bottom). Data are expressed normalized to the non-targeting control siRNA as the mean SD (n = 3). (B) Ten other genes were identified from this screen that decreased STAT3-dependent transcription to lesser extents. Data represent the mean of the four siRNA constructs for each gene, normalized to the non-targeting control siRNA as the mean SD (n = 3). (C) siRNAs to STAT3 and CDK5RAP3 deplete their respective target proteins in U3A cells after 48 hour incubation (left; representative of n = 3) and decrease STAT3-dependent luciferase activity (right; n = 3). 48 hours after introduction of the siRNAs, cells were treated with oncostatin M (OSM; 10 ng/ml) or media control for 6 hours, after which a luciferase assay was performed (n = 4). (D) 2fTGH-luciferase cells were incubated with the indicated siRNAs for 48 hours. They were then treated with interferon- (IFN; 25 ng/ml) or media control for six hours, after which a luciferase assay was performed. (E) 2fTGH cells were treated with media control or interferon- (IFN; 25 ng/ml) or TNF (25 ng/ml) for six hours. RT-PCR was then performed for the highly selective STAT1 target gene IRF9 (left) and the highly selective NF- $\kappa$ B target gene A20 (right), respectively (representative experiments shown; n = 4).

is impossible to conclude with certainty. Interestingly, the binding of STAT3 also decreased with knockdown of CDK5RAP3. However, the mechanistic implications of this finding are uncertain at this time. At the other three genes (*BATF*, *STAT3* and *BCL6*), there was no reduction in STAT3 binding following CDK5RAP3 silencing (Figure 5B). This suggests that CDK5RAP3 is not necessary for STAT3 binding to cognate reg-

ulatory sites. We then considered the possibility that CDK5RAP3 was directly associated with these promoters to which STAT3 was bound. To test this hypothesis, we performed ChIP at these loci with an antibody to CDK5RAP3. At all five genes, CDK5RAP3 binding was enhanced following IL6 stimulation, suggesting that CDK5RAP3 was recruited to these sites in conjunction with activated STAT3 (Figure 5C). It should



be noted that binding above background could not be detected for CDK5RAP3, in the presence or absence of IL6, at the nonexpressed gene *rhodopsin* or gene desert regions in the genome, suggesting that CDK5RAP3 binding to DNA was not a widespread event. When STAT3 was depleted by RNA interference, CDK5RAP3 no longer localized to these sites, suggesting that STAT3, either directly or indirectly, was necessary for this CDK5RAP3-DNA interaction. We also considered the possibility that CDK5RAP3 and STAT3 physically associate, though we were unable to coprecipitate these proteins under endogenous conditions. Taken together, these findings suggest that CDK5RAP3 can modulate STAT3-dependent gene expression by affecting STAT3 binding, at least at some sites, and may also modulate transcription by directly associating with STAT3-dependent promoters.

#### *CDK5RAP3 is associated with STAT3-dependent gene expression in primary breast cancers*

Having identified CDK5RAP3 as a STAT3 transcriptional activity modulator in breast cancer cells, we next wished to determine if this relationship occurs in primary human breast tumors. Using data from The Cancer Genome Atlas (TCGA) and from the breast cancer microarray dataset GSE5460 [1], we first segregated breast cancers based on the activating tyrosine phosphorylation of STAT3. Since we did not observe any effect of CDK5RAP3 on the phosphorylation of STAT3 (Figure 3A), nor did we detect any consistent effect of STAT3 on CDK5RAP3 expression (Figure 2B), we predicted that there would be no difference in CDK5RAP3 expression based on STAT3 phosphorylation. Indeed, this is what was found in primary breast cancers (Figure 6A). However, as detected in the initial RNA interference screen, and validated in cell-based systems, CDK5RAP3 clearly enhances STAT3-dependent gene expression in model systems. Thus, it would be predicted that CDK5RAP3 expression would positively correlate with expression of STAT3 target genes in these breast cancer samples. To test this hypothesis, we first used gene set enrichment analysis (GSEA) to determine the relationship between CDK5RAP3 expression and a validated STAT3 gene expression signature [13,17]. We found a strong correlation between CDK5RAP3 mRNA expression and the presence of a STAT3 gene expression signature (Figure 6B), suggesting that the association between CDK5RAP3 and STAT3 transcriptional function is present in primary breast cancers. We then looked at the correlation between expression of CDK5RAP3 and four genes we found to be highly responsive to STAT3 (Figure 2B), *MCL1*, *JUNB*, *SOC3* and *BCL6*. The expression of all four of these genes positively correlated with CDK5RAP3 levels (Figure 6C). Taken together, these data support the hypothesis that CDK5RAP3 enhances STAT3 activity in both breast cancer model systems and primary breast cancers.

Since STAT3 is inappropriately activated across a spectrum of human cancers, we looked on a more global scale for alterations in *CDK5RAP3* in

human cancers. Using the COSMIC database (<https://cancer.sanger.ac.uk/cosmic/browse/tissue>), we found that point mutations in *CDK5RAP3* were infrequent, and distributed across the entire gene (Figure 7A). Similarly, deletions were rare in this gene, and insertions were not found, suggesting that genetic alterations that altered the function of the protein were uncommon. By contrast, the most common gene abnormality was gain in copy number, and overexpression of the gene was fairly common across a wide range of human cancers. Coupled with the effects on gene expression in breast cancer (Figure 6B and C), this raises the possibility that enhanced CDK5RAP3 expression may be a common mechanism to amplify STAT3-dependent gene expression, although this hypothesis remains to be tested more rigorously.

## Discussion

Using an unbiased large scale strategy, we have identified CDK5RAP3 as an enhancer of STAT3 transcriptional activity. Although CDK5RAP3 has been suggested to have a number of roles in gene regulation and oncogenesis, it had not been previously associated with the oncogenic transcription factor STAT3. CDK5RAP3 was first identified as a binding partner of cyclin-dependent kinase 5 (CDK5) activator, p35<sup>ncK5a</sup>, in yeast 2hybrid screening [15]. CDK5RAP3 has two putative LXXLL motifs mediating binding to nuclear receptors, and a leucine zipper region responsible for protein dimerization [18,19]. CDK5RAP3 can associate with a nuclear coactivator, cAMP response element-binding protein (CREB)-binding protein (CBP), suggesting that CDK5RAP3 may also function as a transcriptional coactivator or corepressor [20]. Thus, although CDK5RAP3 has a number of cellular roles, it is plausible that it may function as a transcriptional modulator.

The finding that CDK5RAP3 can bind directly to genomic loci known to be bound by STAT3, in a STAT3-dependent manner, suggests a novel function for this protein. CDK5RAP3 may modulate STAT3 function by two nonmutually exclusive mechanisms. At some genes, such as *MCL1* and *JUNB*, CDK5RAP3 enhances STAT3 binding (Figure 5). It may do so by modulating chromatin structure, or acting as a scaffold for other transcriptional regulators. At other sites, it does not seem to affect the binding of STAT3. However, at all STAT3 binding sites examined, the presence of CDK5RAP3 enhances the recruitment of RNA polymerase II, and resultant gene expression. Thus, the binding of CDK5RAP3 to DNA, either directly or through a partnering DNA binding protein, may denote transcriptionally active sites. We considered the possibility that STAT3 and CDK5RAP3 physically associate. However, using a variety of buffer conditions, we were unable to coprecipitate these two proteins under endogenous expression conditions. This may reflect the fact that they only interact indirectly, perhaps by binding to adjacent chromatin loci, or that their interactions are too weak or transient to be detected by coprecipitation.

**Figure 2.** CDK5RAP3 expression modulates STAT3 activity in breast cancer cells. MCF7 and SK-BR-3 cells were transfected with two different siRNAs targeting CDK5RAP3, STAT3, or a non-targeting control. (A) 48 hours after siRNA transfection, a STAT3-dependent luciferase reporter (m67-luciferase) was transfected into cells, and then, 24 hours after transfection of the luciferase reporter, cells were stimulated for 6 hours with IL-6 (or media control), and a luciferase assay was performed (n = 3). (B) 72 hours after siRNA transfection, cells were stimulated with IL-6 for 90 minutes and were analyzed by qRT-PCR for expression of endogenous STAT3 target genes (normalized to GAPDH and to the non-targeting control siRNA; n = 3). (C) MCF10A cells, in which the expression of STAT3C (with a FLAG epitope) was under the control of a doxycycline-responsive promoter, were stimulated with doxycycline (or vehicle control) for 24 hours at the indicated concentrations and were analyzed by immunoblot for the expression of FLAG and STAT3 in whole cell lysates (with tubulin serving as a loading control). These MCF10A cells were transfected with two different siRNAs targeting CDK5RAP3, STAT3, or a non-targeting control for 72 hours, were then stimulated with doxycycline (2 g/ml) for 24 hours and were analyzed by (D) a luciferase reporter assay for STAT3-dependent transcriptional activity (n = 3), and (E) qRT-PCR for expression of endogenous STAT3 target genes (normalized to GAPDH and to the non-targeting control siRNA; n = 3).

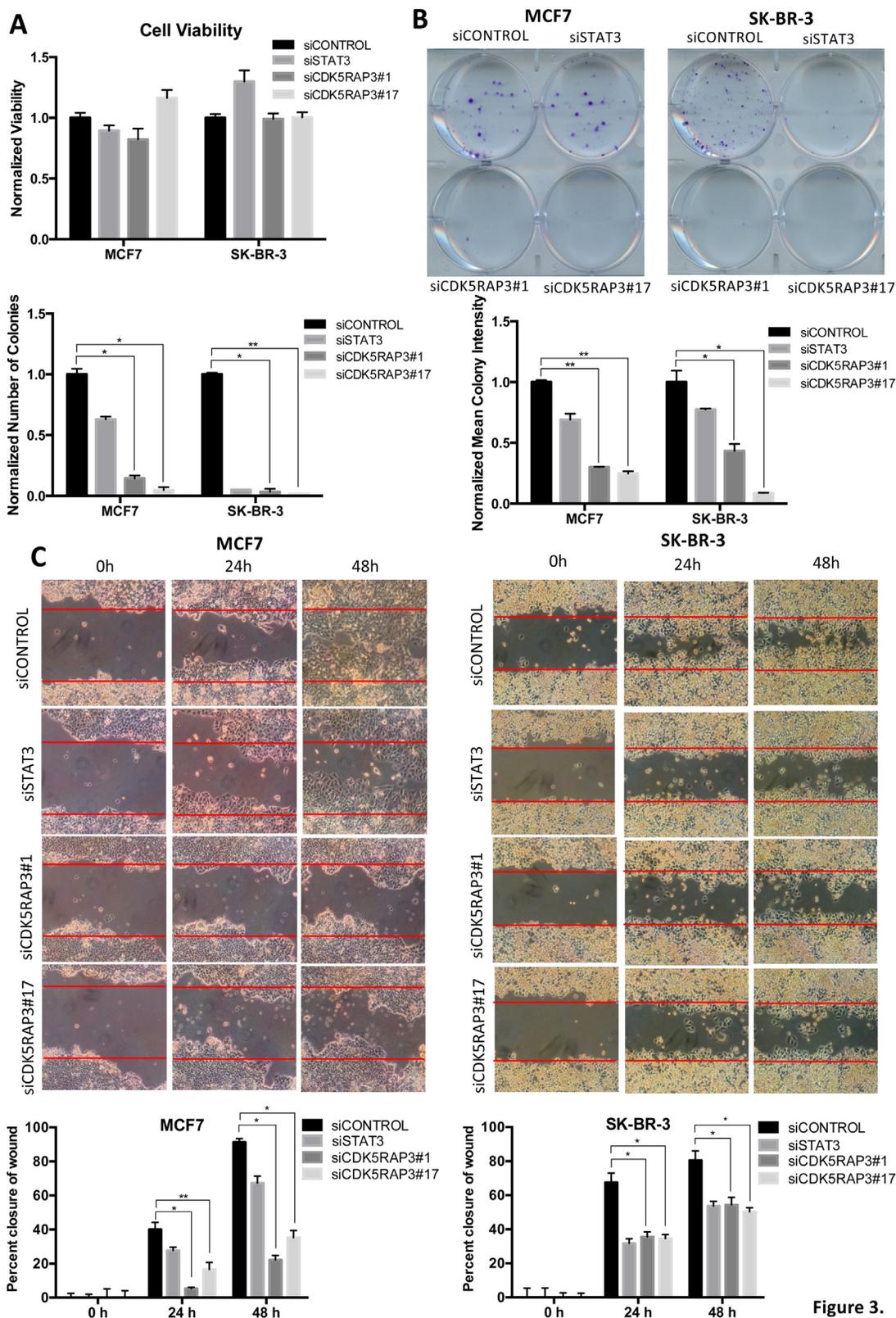
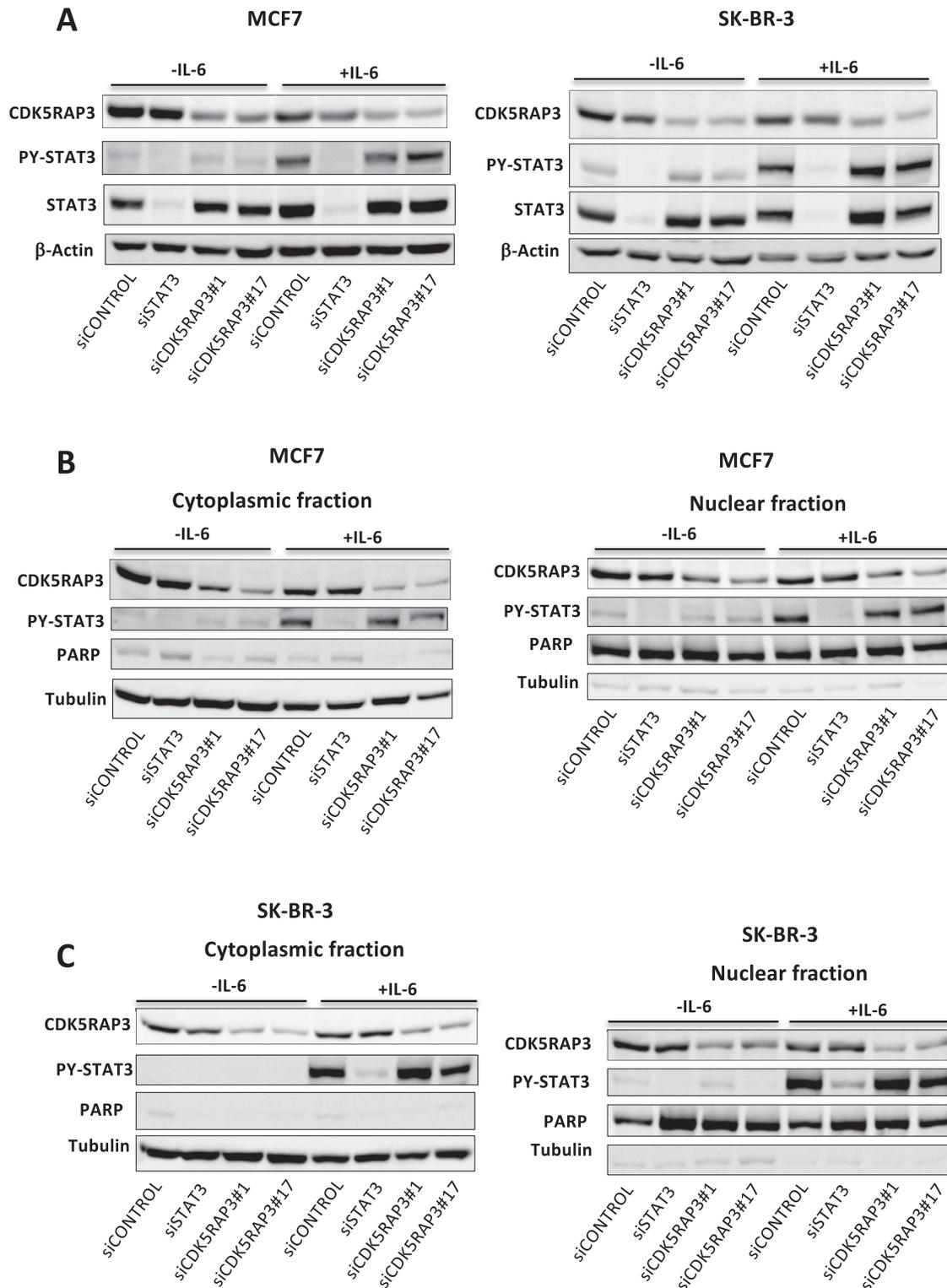


Figure 3.

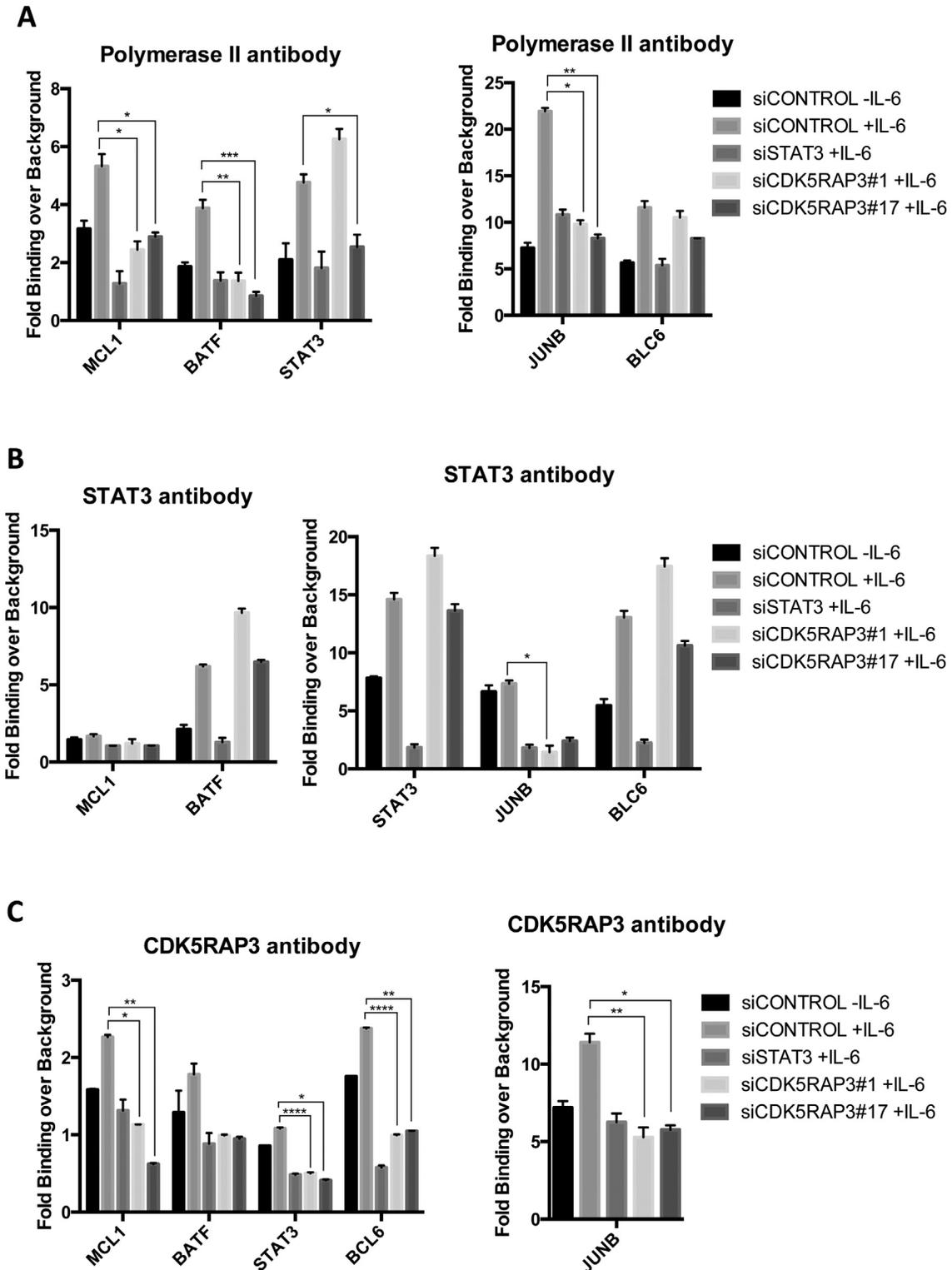
**Figure 3.** CDK5RAP3 enhances breast cancer tumorigenic phenotypes *in vitro*. (A) MCF7 and SK-BR-3 cells were transfected with two different siRNAs targeting CDK5RAP3, STAT3, or a non-targeting control, after which relative viable cell number was quantitated by ATP-dependent bioluminescence. Cells treated as in (A) were then incubated with IL-6 (and were replenished with fresh IL-6 every 48 hours) and were analyzed for (B) clonogenicity after 7 days (top). Quantitation (bottom) of the number of colonies (left) and mean colony intensity, as an indicator of cell number (right), both normalized to the control siRNA is shown (bottom)  $n = 3$ ; and (C) migration (as assessed by wound healing assays) after 24 hours and 48 hours ( $n = 3$ ).



**Figure 4.** CDK5RAP3 does not alter tyrosine phosphorylation or nuclear localization of STAT3. MCF7 and SK-BR-3 cells were transfected with two different siRNAs targeting CDK5RAP3, STAT3, or a non-targeting control. Cells were left untreated or stimulated with IL-6, and were then analyzed by immunoblot for CDK5RAP3 and phosphorylated and total STAT3 in whole cell lysates (A), and cytoplasmic and nuclear fractions. (B, C). Loading controls included -actin (whole cell lysates), tubulin (cytoplasmic fraction), and PARP (nuclear fraction).

CDK5RAP3 may also promote tumorigenesis through other mechanisms. For example, CDK5RAP3 expression is frequently upregulated in lung adenocarcinoma [21] and hepatocellular carcinoma (HCC) [22]. The mechanism by which CDK5RAP3 is overexpressed is still unclear, though it has been reported that the chromosomal region 17q, which con-

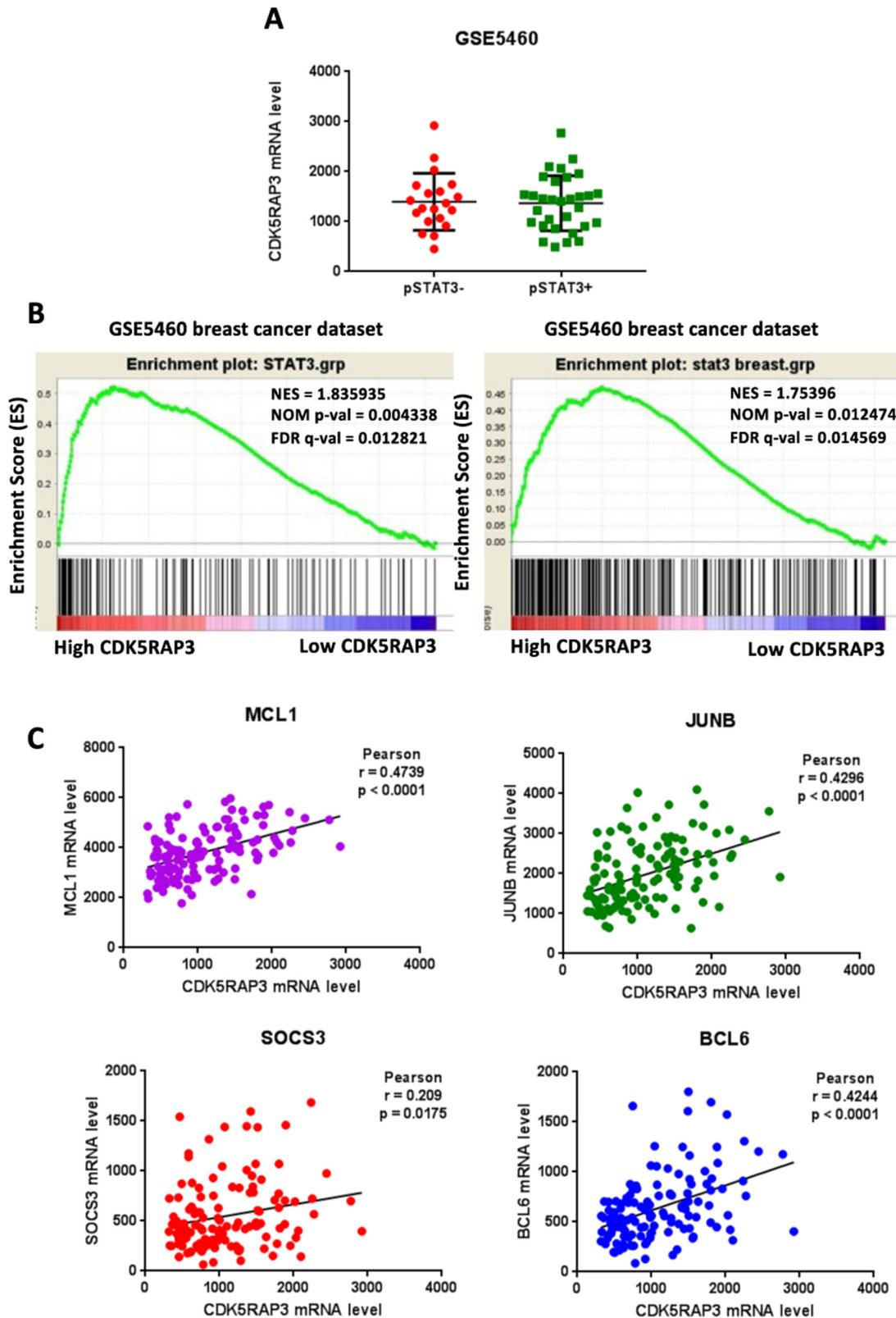
tains *CDK5RAP3*, is frequently amplified in HCCs [23]. Interestingly, this region is close to the loci of STAT3 and STAT5 (A and B), as well as BRCA1 (Figure 7B). CDK5RAP3 may also contribute to HCC metastasis by activating p21activated protein kinase 4 (PAK4), thereby promoting cell invasion [22]. In addition, overexpression of CDK5RAP3 can



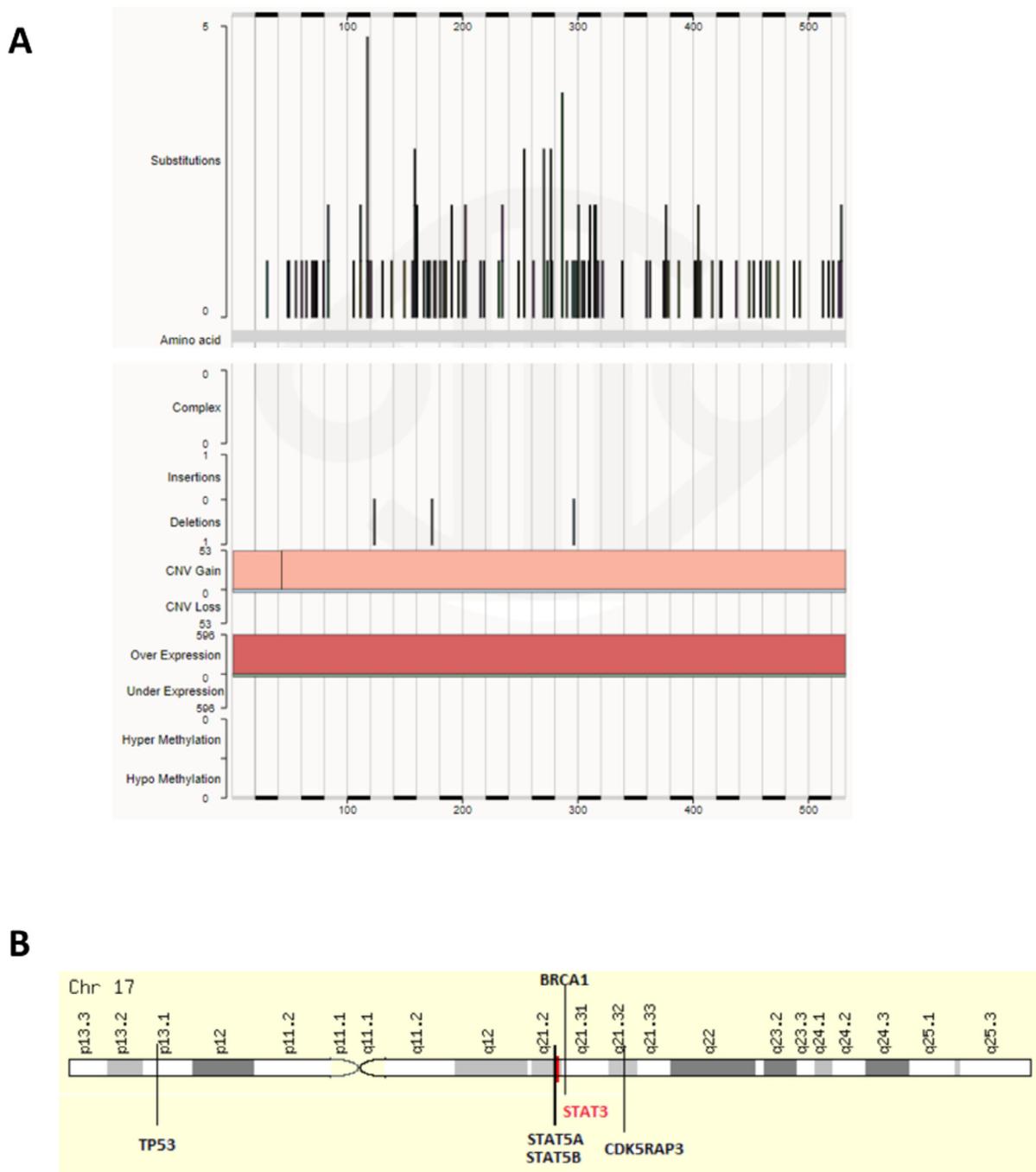
**Figure 5.** CDK5RAP3 associates with the promoters of STAT3 target genes and enhances transcription. MCF7 cells were transfected with two different siRNAs targeting CDK5RAP3, STAT3, or a non-targeting control. Cells were then stimulated with IL-6 and were analyzed by chromatin immunoprecipitation with (A) an antibody to RNA polymerase II, (B) an antibody to STAT3, or (C) an antibody to CDK5RAP3, followed by qPCR at the indicated STAT3 target genes (representative of  $n = 2$ ).

down regulate the tumor suppressor  $p14^{ARF}$  [15]. Thus, CDK5RAP3 may exert protumorigenic effects both through its modulation of STAT3 as well as via other mechanisms.

The finding that CDK5RAP3 enhances STAT3 transcriptional activity and biological effects raises several other considerations. First, it has been found that approximately 70% of breast cancers have constitutive activa-



**Figure 6.** CDK5RAP3 expression correlates with STAT3 transcriptional activity in primary breast cancers. (A) Primary breast cancers from dataset GSE5460 (129 patient samples) were segregated based on STAT3 tyrosine phosphorylation, and CDK5RAP3 mRNA levels were compared. (B) GSEA was performed on GSE5460 breast cancer microarray dataset (129 breast cancer samples sorted according to CDK5RAP3 mRNA level). The top 50 samples with highest and lowest CDK5RAP3 mRNA levels were compared for expression of a STAT3 gene expression signature. Statistical significance was defined as FDR q value  $< 0.25$  and normalized p value  $< 0.05$ . (C) Correlation of expression of STAT3 target genes (*MCL1*, *JUNB*, *SOCS3*, and *BCL6*) with CDK5RAP3 mRNA levels was performed for samples in The Cancer Genome Atlas (TCGA) Breast Cancer provisional data set (N = 526 patients).



**Figure 7.** Alterations in the *CDK5RAP3* gene in cancer. (A) Distribution of genetic abnormalities in *CDK5RAP3* across its 532 amino acids in 35,277 unique samples (COSMIC Database v90, Sanger Institute, October 2019). (B) Location of *CDK5RAP3*, and other relevant genes, on chromosome 17.

tion of STAT3 [1], and considerable evidence suggest that STAT3 may be an important therapeutic target in this disease. Since cancers with higher levels of CDK5RAP3 expression have greater STAT3 target gene expression (Figure 6B and C), it is possible that CDK5RAP3 may serve as a biomarker for cancers more susceptible to the therapeutic effect of STAT3 inhibitors. As we understand more about the transcriptional roles of CDK5RAP3, it is also possible that targeting this protein therapeutically will also show efficacy in STAT3-dependent cancers.

In conclusion, starting from a large scale RNA interference screen, we have found that CDK5RAP3 enhances the transcriptional activity of STAT3 in model systems of breast cancer, and that a correlation between CDK5RAP3 expression and STAT3-dependent gene expression also occurs

in primary breast cancers. These findings reflect a novel aspect of transcriptional regulation mediated by STAT3. Furthermore, given the contributions of STAT3 to malignant phenotypes of cancer cells, understanding the interaction between STAT3 and CDK5RAP3 may provide insights into novel strategies to target the oncogenic effects of STAT3.

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neo.2019.10.002>.

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