PAK4 suppresses RELB to prevent senescence-like growth arrest in breast cancer

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Overcoming cellular growth restriction, including the evasion of cellular senescence, is a hallmark of cancer. We report that PAK4 is overexpressed in all human breast cancer subtypes and associated with poor patient outcome. In mice, MMTV-PAK4 overexpression promotes spontaneous mammary cancer, while PAK4 gene depletion delays MMTV-PyMT driven tumors. Importantly, PAK4 prevents senescence-like growth arrest in breast cancer cells in vitro, in vivo and ex vivo, but is not needed in non-immortalized cells, while PAK4 overexpression in untransformed human mammary epithelial cells abrogates H-RAS-V12-induced senescence. Mechanistically, a PAK4 – RELB - C/EBPβ axis controls the senescence-like growth arrest and a PAK4 phosphorylation residue (RELB-Ser151) is critical for RELB-DNA interaction, transcriptional activity and expression of the senescence regulator C/EBPβ. These findings establish PAK4 as a promoter of breast cancer that can overcome oncogene-induced senescence and reveal a selective vulnerability of cancer to PAK4 inhibition.

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Breast cancer is the most common malignancy in women and causes significant lethality. It is therefore imperative to find molecular actors that may be exploited as therapeutic targets.

Cellular senescence is a stress-inducible fail-safe mechanism that complements apoptosis in preventing the proliferation of damaged cells, while also contributing to ageing. The senescent phenotype is highly heterogeneous and can be induced by different stimuli, including by oncogenes in untransformed cells (oncogene-induced senescence, OIS). Importantly, the identification of senescent cells in several pre-malignant or benign conditions suggests that senescence potently prevents cancer by acting as a major barrier to cancer development. While no universal senescence-specific markers exist, senescent cells are characterized by a durable proliferative arrest often accompanied by increased senescence-associated β-galactosidase (SA-β-gal) activity and a senescence-associated secretory phenotype (SASP) consisting of a plethora of secreted molecules that influence senescence through complex and poorly understood autocrine and paracrine mechanisms.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) acts as a master regulator of the SASP. NF-κB signaling is categorized into canonical (or classical) and non-canonical (or alternative) pathways, based on which components engage in the signaling cascade. Canonical NF-κB signaling entails the processing of the precursor form NF-κB1 into the active form p50 and nuclear accumulation of RELA-p50 dimers. Distinctly, noncanonical NF-κB signaling involves the processing of NF-κB2 into p52 and assembly of RELB-p52 complexes. While canonical NF-κB signaling (explicitly RELA) as well as CCAAT/enhancer-binding protein beta (C/EBPβ) are key transcriptional SASP regulators and thereby firmly linked to cellular senescence, the potential involvement of noncanonical NF-κB signaling in senescence has remained largely unknown.

The serine/threonine p21-activated kinases (PAKs) are recognized for their privileged position at the intersection of major signaling pathways required for oncogenesis. Among PAKs, PAK4 plays a pivotal role in various cancer-associated cellular events, which includes regulating different aspects of proliferation. In agreement, PAK4 is frequently overexpressed in cancer and correlates to poor patient prognosis in several cancer forms. Yet, the potential functional role of PAK4 during cancer development in vivo has remained unknown and our understanding of the PAK4 signaling pathways that affect tumorigenesis is so far sparse.

Here we show that PAK4 is overexpressed in breast cancer associated with poor prognosis. PAK4 overexpression abrogates OIS in untransformed human mammary epithelial cells (HMECs), leads to mammary tumors in mice and suppresses senescence-like growth arrest in various breast cancer models, controlled by a PAK4–RELB–C/EBPβ regulatory axis. Our results reveal a critical function of PAK4 in breast cancer pathogenesis and identify a druggable vulnerability that may be exploited for breast cancer therapy.

Results
PAK4 expression is linked to breast cancer patient outcome. The PAK4 gene is located at a chromosomal region (19q13.2) frequently amplified in breast cancer with basal-like features and consistently, PAK4 was found overexpressed in a small set of human breast cancer specimens. In addition, we reported that high PAK4 levels correlate with poor survival of endocrine-treated breast cancer patients. However, expression levels and copy number variation of PAK4 in relation to breast cancer patient outcome has not yet been examined in more general and larger sets of breast cancer patients. To this end, we analyzed the METABRIC dataset and found that PAK4 transcript expression was approximately twofold higher in breast tumors compared with their normal counterparts. PAK4 mRNA levels were high across all breast cancer subtypes both when using the PAM50 signature (Fig. 1b) and the IC10 classification (Fig. 1c).

PAK4 overexpression in breast cancer relative to normal breast tissues was confirmed in two independent breast cancer datasets (Supplementary Fig. 1a). PAK4 protein levels displayed a similar trend within a panel of six human breast cancer cell lines (Supplementary Table 1), most exhibiting PAK4 overexpression as compared with two independent batches of primary, non-immortalized HMECs (Supplementary Fig. 1d).

To analyze the clinical outcome of breast cancer patients in the METABRIC cohort, patients were stratified according to quartiles of PAK4 expression. Higher PAK4 expression was associated with worse disease-specific survival (DSS) in the entire cohort (Fig. 1d) as well as in patients that did not receive systemic adjuvant treatment (Fig. 1e). High expression levels of PAK4 also correlated with poor overall survival (OS) (Supplementary Fig. 1e). These conclusions withstand multivariate analyses, including lymph node status, breast cancer subtype, tumor size, and grade (Supplementary Tables 2 and 3).

PAK4 overexpression in cancer varies widely and may be due to both mRNA upregulation and/or gene amplification. PAK4 is the most amplified PAK in breast cancer (~8%), while PAK4 amplification is only detected in ~2% of breast tumors in The Cancer Genome Atlas (TCGA) cohort. Using cBioPortal, we replicated this finding and also expanded the analysis to the METABRIC dataset, where we found a comparable fraction of tumors with PAK4 amplification (Supplementary Table 4). Interestingly, patients carrying tumors with PAK4 amplification tended to exhibit worse prognosis (Supplementary Fig. 1f, g). We also analyzed PAK4 copy number and mutational status in the breast cancer cell lines used throughout the study, but no relevant alterations were found (Supplementary Table 1). Together, this indicates that PAK4 overexpression in breast cancer correlates with unfavorable disease outcome.

PAK4 overexpression promotes mammary tumors. While grafted immortalized mouse mammary epithelial cells overexpressing PAK4 and breast cancer cells with PAK4 depletion shed some light on the potential relevance of PAK4 in breast cancer growth in vivo, the role of PAK4 during cancer development has not yet been examined.

To this end, transgenic MMTV–PAK4-overexpressing mice were generated in an inbred FVB/N strain (Supplementary Fig. 2a, b). Young MMTV–PAK4 mice were healthy, fertile, and had no overt phenotypic differences from wild-type (wt) mice. Importantly, from 6 months of age, PAK4-overexpressing virgin and nulliparous females exhibited lesions that eventually developed into mammary tumors in 25% of the cases (Fig. 2a, b). MMTV–PAK4 tumors morphologically resembled invasive lobular breast cancer and exhibited cribriform and fibrotic morphologies (Supplementary Fig. 2c). We also extracted genomic DNA from three mammary tumors that arose in virgin MMTV–PAK4 female mice (20–24 months old) plus liver tissue to conduct whole-exome sequencing (WES). The PAK4 construct was visible in the sequencing coverage profiles as evident by very distinct exon/intron boundaries (Supplementary Data 1). Coverage for PAK4 was about ten times higher than for genes surrounding the PAK4 locus suggesting multiple integration sites.

Interestingly, activating Ras mutations, including G12C and G12D, were found in two out of three MMTV–PAK4 tumors (Supplementary Data 1).
PAK4 depletion impairs PyMT-driven mammary tumorigenesis. To further assess the function of PAK4 during mammary cancer development, we generated an additional genetically engineered mouse based on the widely used and relevant Polyoma Middle T (PyMT) transgenic breast cancer mouse model that is in part driven by PyMT-induced RAS signaling\textsuperscript{21,22}. In this model, PyMT oncoprotein expression in the mammary epithelium is driven by the mouse mammary tumor virus (MMTV) promoter\textsuperscript{23}. After a short latency, PyMT mice stochastically develop focal mammary tumors expressing biological markers consistent with those expressed in human breast cancers and that recreate stages similar to human breast cancer progression\textsuperscript{22}. Importantly, PAK4 protein levels were elevated in PyMT-driven mammary tumors as compared with paired adjacent mammary tissues (Supplementary Fig. 2d–f). However, MMTV–PyMT tumors do not exhibit PAK4 amplification\textsuperscript{23}.

We crossed PAK4\textsuperscript{fl/fl} mice\textsuperscript{24} with MMTV-Cre mice/line D\textsuperscript{25} to generate mice carrying conditional PAK4 depletion in the mammary epithelium (MMTV-Cre:PAK4\textsuperscript{fl/fl}, hereafter referred to as PAK4\textsuperscript{MEp–/–} for simplicity). No significant defects in mammary gland morphogenesis were apparent in PAK4\textsuperscript{MEp–/–} mice\textsuperscript{26}. PAK4\textsuperscript{MEp–/–} mice were then interbred with MMTV-Cre and MMTV–PyMT mice to generate MMTV–PyMT:MMTV-Cre, PAK4\textsuperscript{MEp–/–} (PyMT;PAK4\textsuperscript{MEp–/–}) and MMTV–PyMT:MMTV-Cre (PyMT;PAK4\textsuperscript{MEp+/+}) control mice (Fig. 2c). PyMT-driven mammary tumorigenesis was examined in virgin females in the presence or absence of endogenous PAK4.

By 12 weeks of age, the PyMT oncogene induced extensive hyperplasia and mammary intraepithelial neoplasia in PyMT;PAK4\textsuperscript{MEp+/+} control animals. In contrast, PyMT;PAK4\textsuperscript{MEp–/–} glands were only mildly hyperplastic and exhibited fewer and smaller foci intermingled with normal ductal structures (Fig. 2d–e)
and Supplementary Fig. 2g). Consistently, PyMT;PAK4MEp−/− female mice developed palpable tumors significantly later than PyMT;PAK4MEp+/+ mice, with the median tumor onset delayed on average by 22 days (Fig. 2f). This delay in tumor onset was also reflected in the median animal survival (Fig. 2g).

MMTV–PyMT also drives mammary tumor development in male mice, providing a different mammary tumor model with a considerably longer latency than in females. Strikingly, the effects of PAK4 gene depletion on tumor formation were even more prominent in the male PyMT (Fig. 2h).

Given the inherent caveats of the mouse strains herein employed, we evaluated the extent of PAK4 depletion in late stage mammary tumors that arose in females of both genotypes by measuring PAK4 protein levels by different techniques. While there was an overall consistent decrease in the levels of PAK4 in PyMT;PAK4MEp−/− tumors compared with controls, there was still substantial PAK4 expression in some PyMT;PAK4MEp−/− tumors, with significant variation between animals detected by immunoblot (Supplementary Fig. 2h, i). To analyze the spatial distribution of remaining PAK4 expression in the tumors, we...
detected PAK4 mRNA levels using in situ hybridization (ISH/ RNAScope). We found an overall lower PAK4 expression in early lesions compared with late-stage PyMT-driven tumors; that PAK4 expression is consistently less in the tissues harvested from PyMT;PAK4MEp<−/− mice as compared with control; that residual PAK4 expression occurred specifically in tumor areas of PyMT; PAK4MEp<−/− tissues (Supplementary Fig. 2j, k). This indicates that PAK4 was not completely depleted from the mammary tissues of PyMT;PAK4MEp<−/− mice and PAK4 may therefore still contribute to tumorigenesis. ISH analysis of control tissues also points to the existence of small sub-populations of PyMT cells that express low or no PAK4. The behavior of such cells is likely not affected by Cre-mediated PAK4 depletion and hence, these cells may contribute as well to the tumor masses. In addition, we performed immunohistochemistry (IHC) with an anti-PAK4 antibody that we generated for this purpose (pab #73). The IHC results were in agreement with the other techniques (IB and ISH) used to evaluate PAK4 expression in these murine tissues (Supplementary Fig. 2l, m). The PAK4 labeling score correlated negatively with the mouse survival time (Supplementary Fig. 2n).

While several aspects might contribute to the variation of PAK4 expression observed, the most likely explanation stems from the previously recognized stochastic nature of transgene expression, including MMTV-Cre 25. In our model, the expression of Cre recombinase and PyMT is not coupled within the exact same mammary epithelial cells, meaning that, stochastically, a mosaic pattern of transgene expression is expected, where some cells will co-express Cre and PyMT and other cells will only express Cre, PyMT, or none of the transgenes 25. Due to the strong nature of the PyMT oncogene, it is likely that cells where only PyMT is expressed may eventually contribute to subpopulations of tumor cells, where PAK4 is not subjected to Cre-mediated knockout. To address this complexity, we have included IHC for Cre in tumors that arose in mice of both genotypes and scored the Cre expression (Supplementary Fig. 2o, p). A mosaic pattern of Cre expression was indeed present, particularly in PyMT; PAK4MEp<−/− mice. A mosaic pattern of PyMT expression is less relevant here because only PyMT-transformed cells would develop into tumors. Interestingly, we observed a selectivity against Cre-positive cells in tumors that arose in PyMT;PAK4MEp<−/− mice (Supplementary Fig. 2o, p), which likely underscores the here observed role of PAK4 in tumor development. This heterogeneity makes it challenging to relate PAK4 expression to other molecular events in this model. Nevertheless, our mouse model provides compelling evidence that mammary epithelial disruption of PAK4 results in impaired PyMT-induced tumorigenesis.

PAK4 knockdown in HMECs, ex vivo bulk tumor cells derived from breast cancer patients (patient-derived cells, PDCs) arrested proliferation upon PAK4-siRNA treatment (Supplementary Fig. 3j). Expression of a siRNA-resistant PAK4 mutant restored the proliferation of breast cancer cells (Supplementary Fig. 3k), while the proliferation of breast cancer cells highly expressing kinase-dead EGFP-PAK4-M350 was impaired to an extent similar to that of siPAK4-transfected cells (Supplementary Fig. 3l). This suggests that PAK4-M350 acts as a dominant negative with respect to the studied phenotype. Moreover, breast cancer cells were generated using CRISPR/Cas9 genome editing with single guide RNAs (sgRNAs) targeting PAK4. The growth of sgPAK4 cells was dramatically inhibited as compared with parental cells, accompanied by increased SA-β-gal activity (Fig. 3g–i), consistent with the effect of siRNA-mediated PAK4 knockdown. Different PAK4-depleted breast cancer cell lines exhibited arrest in the G0/G1 or G2/M phases of the cell cycle (Supplementary Fig. 3m–o), as previously reported for cancer cells undergoing senescence 26.

Finally, we examined malignant cells of more diverse histological origins. PAK4 is overexpressed in pancreatic adenocarcinoma 27. Consistently, PAK4 siRNA induced SA-β-gal in three out of four tested human pancreatic adenocarcinoma cell lines as well as in cells derived from the KPC transgenic mouse model of pancreatic adenocarcinoma 27 (Supplementary Fig. 3p, q). Given PAK4 overexpression in ovarian tumors 28, we also analyzed ovarian cancer cells and found that PAK4 knockdown induced SA-β-gal in OVCAR-3 cells, but not in TOV-21G or Caov-3 cells (Supplementary Fig. 3r). Thus, eleven out of fourteen cancer cell lines here examined exhibited increased SA-β-gal activity upon PAK4 knockdown.
Taken together, this shows that inhibition of PAK4 triggers a senescence-like response in cancer cells of different histological origins.

PAK4 overexpression overcomes H-RAS-V12-induced senescence. Considering also that MMTV–PAK4 overexpression may have facilitated KRAS-driven mammary tumors (Fig. 2b and Supplementary Data 1), and that oncogenic RAS-signaling in untransformed cells activates OIS3, we tested the hypothesis that PAK4 may overcome oncogenic RAS-induced senescence. We utilized an established model where inducible H-RAS-V12 causes senescence-linked growth arrest in untransformed HMECs33. Indeed, while induction of H-RAS-V12 in this model induced an almost complete proliferation block, consistent with previous results from this model33, overexpression of EGFP–PAK4
partially rescued proliferation (Fig. 3k). This suggests that PAK4 overexpression may overcome the OIS barrier to cancer.

**PAK4 inhibition induces senescence-like programs in vivo.** We then expanded our analyses to three distinct in vivo models. First, we analyzed X-Gal-stained mammary tissues from our model of PyMT-driven tumorigenesis with conditional PAK4 knockout (Fig. 2 and Supplementary Fig. 2) harvested at 8–11 weeks of age. This time point was selected because senescent cells are abundant in the preneoplastic stage of certain cancers4. While most lesions displayed X-Gal positivity, we found an increase, albeit modest, in X-Gal positivity in PyMT;PAK4MEp−/− lesions as compared with control PyMT mice (Fig. 4a–c).
Next, we treated MMTV–PyMT mice with the PAK4 inhibitor PF-03758309. Because PF-03758309 has been previously shown to block the growth of multiple human tumor xenografts, we used acute treatment where PF-03758309 was injected intramuscularly for 5 days (Fig. 4d) followed by X-Gal staining. PF-03758309-treatment resulted in a higher proportion of X-Gal-positive cells compared with vehicle-treated samples (Fig. 4e, f).

By performing analyses of gene expression data across the METABRIC cohort, we also found that higher expression of PAK4 was strongly correlated with known modules that reflect biological processes in breast cancer (Supplementary Fig. 4a). Of relevance, we observed a positive correlation between PAK4 expression and the breast cancer proliferation score (Fig. 4k). These results indicate that PAK4 expression is associated with better prognosis in the HER2-positive breast cancer subtype (Supplementary Fig. 5d, e), in which PAK4 expression was the highest (Fig. 1b).

We then examined if the induction of growth arrest upon PAK4 knockdown in cancer cells functionally involved RELB. Indeed, RELB co-depletion with PAK4 restored the proliferative capacity of Hs 578T cells, defining RELB as essential for growth arrest upon PAK4 depletion (Fig. 5e and Supplementary Fig. 5c).

Thus, PAK4 is a negative regulator of NF-κB signaling in breast cancer cells, regulating senescence-like growth arrest via the noncanonical NF-κB subunit RELB.

**RELB-Ser151 phosphorylation promotes cell proliferation.** FLAG-tagged as well as endogenous PAK4 immunoprecipitated endogenous RELB, while FLAG-tagged RELB immunoprecipitated endogenous PAK4 (Fig. 6a and Supplementary Fig. 6a–c). Interestingly, PAK4 strongly phosphorylated the RELB homology domain of RELB (RELB–RHD) (Fig. 6b, c and Supplementary Fig. 6d, e), consistent with NF-κB activity being controlled by phosphorylation. Mapping Mass Spectrometry (MS) analysis identified serine 151 (Ser151) as a putative PAK4 site within RELB–RHD (Supplementary Fig. 6f). Phosphorylation of RELB–Ser151 is also indicated in the Phosida database (Supplementary Fig. 6g). Importantly, phospho-null RHD-S151A (Ser→Ala substitution) abolished the PAK4 phosphorylation of the RHD, validating the MS results (Fig. 6c). Sequence alignment showed conservation of RELB–Ser151 across species (Fig. 6d).

In line with the notion that RELB signaling promotes growth arrest, overexpression of FLAG-tagged RELB wt led to reduced Edu incorporation in Hs 578T breast cancer cells, while expression of a FLAG-tagged phospho-mimicking RELB–S151E (Ser→Glu substitution) did not affect proliferation (Fig. 6e, f).

These results indicate that PAK4 is a RELB-kinase and that RELB phosphorylation on Ser151 is a novel mechanism controlling cell proliferation.
Fig. 5 PAK4 inhibits senescence-like growth arrest via the NF-κB subunit RELB. a Column clustered heatmaps of NF-κB (RELA) target genes that are differentially expressed in Hs 578T cells upon siPAK4. Genes are by column and samples by row. The color intensity represents column Z-score, where red indicates highly and blue lowly expressed. Selected NF-κB target genes are indicated. b RT-qPCR validation of differentially expressed NF-κB target genes in Hs 578T cells. mRNA was prepared 72 h after transfection with the indicated siRNAs (n = 3 per group). c Correlation between PAK4 expression and an NF-κB signature in breast tumors (T) and normal tissue (N) in the METABRIC dataset. d Correlation between the expression of PAK4 and the alternative NF-κB subunit RELB in breast tumors (T) and normal tissue (N) in the METABRIC dataset. e Quantification of EdU+ cells in Hs 578T cells 4 days after transfection with the indicated siRNAs (n = 3 per group). Data are represented as mean ± SEM in b and e. p-values by two-tailed unpaired t test are indicated in b and by one-way ANOVA followed by Tukey’s post hoc test in e. The Spearman correlations and p-values by Spearman’s test are indicated in c and d for tumors (T, blue, n = 1992) and normal tissue (N, gray, n = 144).
Fig. 6 Phosphorylation of RELB-Ser151 promotes cell proliferation. a Immunoblot (IB) analysis for RELB and FLAG of FLAG-immunoprecipitates (IP) derived from COS-7 cells transfected with the indicated vectors. b Radioactive kinase assay with human recombinant His-tagged PAK4 and GST-tagged RELB full length. c Radioactive kinase assay using recombinant His-tagged PAK4 with GST, GST-tagged REL-homology domain of RELB (GST-RHD) or non-phosphorylatable GST-RHD-S151A. d Sequence alignment of Ser151 present in human RELB across different species. e Quantification of EdU+ cells in HaCaT cells 36 h after transient transfection with the indicated constructs (n = 4 per group). f Representative immunoblot of protein overexpression for experiments quantified in e. Data are represented as mean ± SEM in e and the p-value by one-way ANOVA followed by Tukey’s post hoc test is indicated. Results in a–c are representative of three independent repeats. Coomassie-stained gels are shown to the left as loading control in b and c.

Consistently, PAK4 overexpression in breast cancer cells inhibited RELB binding to DNA, while PAK4 depletion increased RELB DNA-binding (Fig. 7d, e and Supplementary Fig. 7a, b). To examine Ser151-phosphorylation effects on RELB transcriptional activity, we overexpressed FLAG-tagged RELB wt and RELB–S151E and compared, by qPCR array, the expression of candidate genes from our lists of DE genes upon PAK4 knockdown derived by RNA-Seq (Supplementary Fig. 7c and Supplementary Data 3 and 4). Interestingly, C/EBPβ, an important senescence regulator, emerged as the top altered candidate (Fig. 7d and Supplementary Fig. 7c). In agreement, we found a positive correlation between RELB and C/EBPβ expression in the METABRIC patient dataset (Fig. 7c). Further, PAK4 silencing silenced C/EBPβ expression (Fig. 7d) and co-depletion of C/EBPβ with PAK4 partially rescued the inhibited proliferation induced by PAK4 knockdown (Fig. 7d and Supplementary Fig. 7d). These data demonstrate that phosphorylation of Ser151 abrogates RELB-DNA-binding and consequently negatively influences RELB-mediated transcription (i.e., C/EBPβ expression) impacting cell proliferation (Fig. 7d).

Discussion
Here we identify PAK4 as an attractive breast cancer drug target candidate given its overexpression in breast cancer patients negatively affecting prognosis and its key function in evading senescence-like growth arrest in breast cancer cells via the non-canonical NF-κB component RELB.

The lack of investigations on the potential role of PAK4 in tumorigenesis in vivo prompted us to establish two transgenic models, with PAK4 overexpression and depletion, respectively, specifically in the mouse mammary gland. PAK4 wt overexpression caused hyperplasia and, at a later stage, mammary tumors in a fraction of the mice comparable to the overexpression of catalytically active PAK140. This incomplete penetrance as well as the late tumor onset may suggest that similarly to PAK1, PAK4 itself acts as a relatively weak oncogene and/or to facilitate tumor formation driven by other oncogene(s), the latter supported by the activating KRAS mutations that we observed in these tumors and the finding that PAK4 overexpression overcame RAS-induced senescence in mammary epithelial cells.

The PAK4 conditional depletion was made in the context of PyMT-driven mammary tumorigenesis. PyMT mice lacking PAK4 developed mammary tumors significantly later than control mice. This was likely a consequence of the observed reduced burden of hyperplasia and pre-malignant lesions detected in early stage glands of PAK4-depleted animals. Also this impaired PyMT-driven tumor initiation is consistent with a role for PAK4 in senescence evasion, an early event in tumorigenesis. This notion is corroborated by our observations of a restored senescence-like response in PAK4-depleted breast cancer xenografts and in PyMT tumors with conditional PAK4 knockout and PyMT tumors treated in vivo with a PAK4 inhibitor that has
proved successful in blocking the growth of multiple human tumor xenografts.\textsuperscript{34}

The restored senescence-like response in vivo upon PAK4 inhibition is supported by extensive in vitro phenotypic characterization and transcriptome analyses employing a wide variety of tools (siRNAs, shRNA, CRISPR/Cas9, and protein overexpression). It is also in agreement with earlier reports showing that PAK4 promotes cell cycle progression when overexpressed, and hinders such progression when removed or inhibited.\textsuperscript{19,20} Beyond that, our data show that PAK4 inhibition almost
invariably arrests cancer cell proliferation, while inducing additional senescence-like features (including morphological changes, increased SA-β-gal activity, and gene expression changes indicative of a senescent-like phenotype). Our data thus suggest that, apart from glioblastomas, epithelial cancer cells of various origins require PAK4 to avoid senescence, adding to the idea of generalized PAK4 addiction in cancer.

NF-κB signaling has diverse and complex roles in cancer with reports that NF-κB seems to both contribute to promotion and suppression of tumorigenesis depending on the cellular context. However, while canonical NF-κB signaling (essentially via RELA) has been extensively associated with the senescent phenotype, the potential role in senescence for the noncanonical NF-κB pathway has remained elusive. We identify PAK4 as an inhibitor of NF-κB signaling in breast cancer and show that the senescence-like growth arrest upon PAK4 knockdown in cancer cells functionally requires the noncanonical NF-κB subunit RELB. RELB is a known transcriptional target of both types of NF-κB signaling, meaning that the increased mRNA levels of NFκB1, NFκB2, and RELB, as well as the activation of RELB could act in synergy with increased RELB levels upon PAK4 depletion. Furthermore, in line with recent studies that indicate that post-translational modifications of NF-κB subunits play a critical role in fine-tuning transcriptional activity, we identified PAK4 as a RELB kinase. Adding to the four previously validated RELB phosphosites, we identified Ser151 as a PAK4-site within the RHD of RELB, whose phosphorylation status regulated the RELB binding to DNA and consequently, RELB transcriptional activity. RELB–Ser151 phosphorylation prevented RELB interaction with DNA possibly due to electrostatic repulsion with the DNA phosphate backbone. Such a modification may not only affect the binding of different homo- or heterodimers of NF-κB to cognate κB sites, but also regulate DNA-binding and release, thereby fine-tuning transcription. It may also affect the recruitment of various transcriptional coactivators and/or repressors to the promoters of NF-κB target genes. The RELB–Ser151 PAK4 phosphorylation site also affected the expression of C/EBPβ, whose expression is critical for senescence. Growth arrested PAK4-depleted cells show increased binding of RELB to DNA consistent with a previous report of RELB–DNA-binding activities during therapy-induced senescence. Interestingly, NF-κB (explicitly RELA) and C/EBPβ can cooperatively regulate the SASP. Here we present a direct link between RELB and C/EBPβ, raising the interesting question that these previously considered distinct pathways may be regulated in an integrated manner to control cell fate.

Further raising the potential clinical relevance, we found that PAK4 is overexpressed across all breast cancer subtypes, specifically upregulated during the disease development and correlated with poor prognosis. In addition, we found that the small fraction of patients carrying tumors that harbor PAK4 amplification tend to exhibit worse prognosis, suggesting that although not a very frequent alteration, PAK4 amplification may still be a clinically relevant feature. Importantly, we found that a metagene signature of NF-κB signaling, as well as RELB expression itself, negatively correlated with PAK4 expression in the METABRIC breast cancer patient dataset. These correlations were only apparent in breast cancer but not in normal breast tissue, adding to the notion that it may be possible to selectively target the addiction to PAK4 of cancer cells. In addition, we show that the low PAK4/high RELB pattern of expression correlates with better prognosis in HER2-positive breast cancer patients. This is consistent with the observation that HER2-positive patients exhibited the highest PAK4 expression among the PAM50 and IC10 breast cancer subtypes and also consistent with the strong correlation observed between PAK4 expression and HER2 signaling in the METABRIC dataset. PDCs were also sensitive to PAK4 abrogation ex vivo while primary non-immortalized HMECs were not, reemphasizing the notion of PAK4 as a potential breast cancer treatment target.

In summary, our findings establish PAK4 as a promoter of breast cancer development, possibly through overcoming the barrier of OIS. By showing also a competitive advantage conferred by PAK4 to established breast cancer cells, acting through the noncanonical NF-κB signaling subunit RELB to prevent senescence-like growth arrest, we reveal a selective vulnerability of cancer cells to PAK4 inhibition that may be explored as a therapeutic strategy.

Methods
Mice. All experimental animal procedures in this study were performed in accordance with Swedish and European Union guidelines and approved by the institutional ethical commissions (Stockholms Södra och Länkäpings djurförsöks- setiska nämnden S19-09; S169-09; S86-11; S23-12; S169-12; S66-14 and 42-16). Animals were housed in group in standardized cages with a 12:12 h light:dark cycle with unrestricted access to food and water. At the experimental endpoint, animals were sacrificed by cervical dislocation after isoflurane anesthesia. Both males and females were used as specified in each section.

To generate the MMTV-myc-PAK4 sequence, we amplified by PCR the coding sequence of mouse PAK4 from a commercial cDNA clone IRAY:p686C0391D (Sourcebiosciences) and cloned the amplified cDNA in pCNA vector containing myc tag sequence at the 5’ end of the multiple cloning site. Then, we isolated the myc-PAK4 fragment by digestion with Kpn1 and NotI, and inserted it into the psp72 MMTV–LTR plasmid (a kind gift from M. Glukova, Paris). The MMTV-myc-PAK4 sequence was isolated from the vector by digestion with SalI, gel purified, and microinjected at 3.4 ng/μL in the pronucleus of fertilized oocytes from FVB/N mice (Charles River) according to standard protocols. MMTV–PAK4 is available upon request for noncommercial use.
with 2 mM glutamine (25030, Life Technologies) and 10% FBS. OVCAR-3 cells were used to evaluate PAK4 expression in mammary tumors and adjacent mammary tissue and to study the intratumoral effects of PF-03758309.

Female 6–8-week-old BALB/c mice (CanCg:Foxn1nu/fl, Charles River) were used in the xenograft model. BALB/c mice acclimatized for 1 week before being randomly assigned per experimental condition.

Intratumoral injection of PF-03758309. PF-03758309 has been previously characterized as a PAK4 inhibitor34. PF-03758309 (Anthem Biosciences, Banga- toon, Sweden) was dissolved in dimethyl sulfoxide (DMSO, D8797, Sigma).

The compound was administered intratumorally at 6 mg/kg body weight in 100 μl of PBS to 10-week-old MMTV-PyMT females (FVB/N) with palpable tumors (n = 10). The largest palpable tumor at the experimental starting point was injected daily for 5 consecutive days. Vehicle animals (n = 5) received the same number of injections of the solvent. At the experimental endpoint, tumors were harvested and processed according to subsequent analyses.

Small-interfering RNAs (siRNAs). The siRNAs used in this study were synthesized by Qiagen, Life Technologies and GenePharma. siRNA targeting sequences were as follows: PKAP4: #1: AAGGGCGTGTATACGAGG, PKAP4: #2: CGAAAGTGTTGGGAGATGTT, PKAP4: #3: GGAGAAATGCCATACGAGG.

Patient-derived primary breast cancer cells. Fresh scrambles were collected from primary breast cancer after surgical procedures in compliance with approved ethical permission. Permits were obtained from the regional ethics board at Karolinska Institutet in Stockholm and from the Stockholm medical biobank (ethical approval #02–061 with amendments 2013–1065–32 and 2015–2259; and ethical approval 2016/957–31 with amendment 2017/742–32). All patient material was anonymized. Single cells were generated immediately as previously described. Cells were then differentiated in DMEM/F-12 Glutamax medium (11960, Life Technologies) supplemented with 5% FBS, 10 ng/mL epidermal growth factor (EGF, PHG0311, Life Technologies), 5 μg/mL insulin (I5500, Sigma), 1 μg/mL hydrocortisone (H0888, Sigma), and 10 μg/mL cholera toxin (C8052, Sigma). All the experiments were performed within five generations of these cultures that were then discarded.

Mammary Epithelial Basal Medium (CC-3153, Lonza) supplemented with MEGM BulletKit (CC-3051, Lonza) supplemented with 10% FBS and 1 mM sodium pyruvate (11360, Life Technologies). HMECs harboring 4-Hydroxytamoxifen (4-HOT)-inducible RASV12 were kindly provided by David Beach, Barts and The London School of Medicine and Dentistry, UK, were grown in phenol red-free Medium 199 (M0650, Sigma) supplemented with 15% FBS. SUM-159 cells were differentiated in DMEM/F-12 (11960, Life Technologies) supplemented with 10% FBS and 1 mM sodium pyruvate (11360, Life Technologies). HMECs were grown in MMEM supplemented with 10% FBS, 10 ng/mL EGF (PHG0311, Life Technologies), 5 μg/mL insulin (I5500, Sigma), 5 μg/mL cholera toxin (C8052, Sigma). All the experiments were done in a blinded fashion with mice being randomly selected for experiments. The person performing the measurements was blinded to the treatment groups. At the experimental endpoint, xenografts were resected, photographed, weighted, and processed according to subsequent analyses.

In vitro quantification of the Compound was administered intratumorally at 6 mg/kg body weight in 100 μl of PBS to 10-week-old MMTV-PyMT females (FVB/N) with palpable tumors (n = 10). The largest palpable tumor at the experimental starting point was injected daily for 5 consecutive days. Vehicle animals (n = 5) received the same number of injections of the solvent. At the experimental endpoint, tumors were harvested and processed according to subsequent analyses.

siRNAs. For CRISPR/Cas9-mediated PKAP4 gene ablation, two single guide (sg) RNA sequences against PKAP4 (sgPKAP4: #1: 5′-TGTCGGAGCTGTCTGCCGCTG-3′ and sgPKAP4: #2: 5′-GTGCACCGGGCTGTCAGGTCGAGAG-3′) were designed with CRISPR DESIGN38 (https://crispr.mit.edu). Plasmids for the lentivirus vector-mediated CRISPR-Cas9 (jentiCRISPR2v, cat#52961) and packaging (CMV-VSV- G, cat#4854 and psPAX2, cat#12260, generously provided by Eyal Gottlieb, The Beaton Institute, Glasgow, UK). Oligonucleotides for siRNA were annealed and cloned into the lentivirus transfer vector jentiCRISPR2v at the BsmBI restriction site39 and the specific target sequence was verified by DNA sequencing.

Plasmids and mutagenesis. Flag-PAK4, Flag-BAP, EGFP, EGFP-PKAP4 wt, and EGFP-PKAP4-M305 constructs have been previously described31. An siRNA-resistant form of PKAP4 was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (200518, Agilent) and the EGFP–PKAP4 construct. The silent mutations were introduced into the siRNA.
target region using the following primers: 5′-GCTTCTCAAGCGTTGTATCATGAGGGAC-3′ and 5′-GATGCCTCTCATGAAACACCTGGTGAAG-3′. FL-TAG (FLAG-FL-RELB) was transfected by cloning into the full-length human RELB cDNA52 (gift from Sam Okret, Karolinska Institutet, Huddinge, Sweden) into the vector p3 × FLAG-CMV-10 (E4401, Sigma) within the pGEX-4T-1 vector (289949, GE Healthcare) (named GST-RHD) within the vectors used in this study. The primers were used to fuse them in N-terminal with FLAG. The GST-tagged full-length human RELB cDNA was amplified using primers to fuse it in N-terminal with Flag expression vector, full-length human PAK4 including a C-terminus for each 100 mm cell culture dish. DNA was transfected for each 100 mm cell culture dish. Cancer cells were reverse transfected with siRNAs (10 μM) using Lipofectamine 2000 (11668, Invitrogen) according to the manufacturer’s suggestion. Routinely 8 μg of DNA was transfected for each 100 mm cell culture dish. Cells were harvested for the various experimental purposes at the timepoints specified in the figures. The efficacy of gene suppression/expression was monitored after transfection by immunoblot, RT-qPCR, or immunostaining.

**CRISPR/Cas9 gene editing.** For PAK4 gene ablation, we established BT-549 cells with stable expression of Cas9 and two single guide RNA sequences against PAK4 (sgPAK4). sgPAK4 was specific to the PAK4 gene and was obtained from HEK293T cells by co-transfecting lentiviral vector, psPAX2, and pCMV-VSV-G at a ratio of 4:3:2 using Lipofectamine 2000. After 24 h, the media was replaced with fresh media. The supernatant containing the viral particles was collected every 24 h for 2 days, filtered through a 0.45 μm pore membrane (83.1826, Seradex), and centrifuged for 15 min at 1500 × g at 4 °C with the Lenti-X concentrator (631231, Clontech). The pellet, containing viral particles, was dissolved in medium and used for cell infection. Cells were transfected in 2 mL of media with 100 μM of viral supernatant in six-well plates. At 48 h post-transduction, cells were selected with 1 μg/mL puromycin (P8833, Sigma) for 48 h, and then cells were expanded in the regular culture medium and used for assays at the timepoints specified in the figures. The effectiveness of CRISPR gene knockout was confirmed by immunoblotting with the indicated antibody.

**SA-β-gal activity.** For cultured cells, SA-β-gal staining was performed at the indicated time points using the Senesence β-Galactosidase Staining Kit (9860, Cell Signaling) or as described previously25. Images were acquired with a Nikon widefield, 10 × 0.45 NA objective and MII cell scan software. Regions of interest were selected according to a standardized pattern, in which X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the total number of cells were manually counted in ImageJ/Fiji using the Cell Counter plugin (National Institutes of Health, NIH) to determine the percentage of X-Gal cells. An additional quantitative assay of SA-β-gal activity using cell extracts was employed as previously described26. This method uses 4-methylumbelliferyl-β-D-galactopyranoside (MUG, M1633, Sigma) as substrate instead of X-Gal. The fluorescence product was measured using an automated plate reader (SpectraMax Gemini, Molecular Devices) with excitation at 360 nm and emission at 465 nm. SA-β-gal staining of cryosections was performed on freshly harvested tissues at the experimental endpoint. Briefly, after resection, tumors were immediately snap frozen in liquid nitrogen, embedded in OCT cryomount (45830, Histolab), cryosectioned (4-μm thickness), and mounted onto SuperFrost Plus slides.
ethyl-20-deoxouridine (EdU, Life Technologies) or 30 μM S-Bromo-2'-deoxouridine (BrUd, B5002, Sigma). Cells were fixed and assayed for EdU incorporation using an EdU assay kit (C10635, Life Technologies) according to the manufacturer’s instructions or cells were fixed and stained for BrdU using a BrdU mAb G3G4 (1:100, G3G4, Developmental Studies Hybridoma Bank, Department of Biomedical Sciences, University of Iowa, Iowa City, IA) as described previously.

EdU/BrDU imaging was performed on a Nikon A1 confocal microscope using a Plan Apo 60×/1.4 objective, producing a pixel resolution of 0.8 μm. In total, 3 × 3 or 4 × 4 image montages were acquired and stitched. Nuclear outlines were identified using Hoechst 33342 (14533, Sigma) staining. The percentage of EdU/BrDU+ cells in total cells was analyzed with the NIS-Elements software (Nikon).

For EdU/BrDU analysis by flow cytometry a minimum of 10,000 events was analyzed on a BD FACSCalibur or a BD FACSCantoII.

HMECs harboring 4-Hydroxytamoxifen (OHT)-inducible RASG12V were EdU-stained and analyzed by FACS 48 h after transient transfection with the indicated plasmids and simultaneously treated with 12.5 nM OHT (H7904, Sigma) or vehicle. Colony formation assay

For colony formation assays on plastic six-well plates, cells were replated 4 days after lentiviral infection at single cell density (15,000 cells per well) in regular cell culture medium. At the indicated timepoints post-seeding, cells were rinsed twice with PBS, fixed with 4% formaldehyde (F1635, Sigma) for 20 min at room temperature and stained with 0.01% Crystal Violet in ddH2O (0.01% [w/v], C3886, Sigma) for 30 min at room temperature. Plates were then washed extensively and allowed to dry. Plates were scanned and analyzed with ImageJ/Fiji software (National Institutes of Health, NIH) using the Colony Area plugin.

Mammary gland wholemounts and sections. The fourth inguinal mammary glands were dissected from 12 weeks old female mice (n = 6 per genotype), wholemounted onto SuperFrost Plus slides (631-0108, VWR), fixed in Carnoy’s fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 2 h at room temperature, stained with a solution of carmine (C1022, Sigma) and aluminum potassium sulfate (A7167, Sigma) overnight at room temperature, de-stained in 70% ethanol, further washed in 100% ethanol, cleared of fat in xylene (28975, VWR) overnight and mounted using DPX (44581, Sigma) or stored in xylene until scanning. ImageJ/Fiji (National Institutes of Health, NIH) was used for morphometric analysis. Raw images were cropped to include only the fourth mammary gland, converted to 8-bit and sharpened. The area occupied by carmine staining (mammary epithelium) was thresholded and presented as a percentage of the total mammary fat pad area.

Paraffin-embedded tissues (sectioned at 4-μm thickness) were routinely stained with hematoxylin and eosin for morphological evaluation according to standard procedures.

Cell lysis. Adherent or pelleted cells harvested by trypsinization (15400, ThermoFisher) were washed with PBS and lysed on ice in cold PAK-lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, and 1% NP-40 or PBSTSD lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.14 M NaCl, 2.8 mM KCl, 10 mM Na2HPO4, and 1.5 mM KH2PO4) containing freshly added protease and phosphatase inhibitors cocktails.

Total protein from snap frozen tissue was extracted with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails as above.

To prepare nuclear extracts, cells were lysed with cytoplasmic buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl2, and freshly added protease and phosphatase inhibitors as above), incubated on ice for 15 min, homogenized in a glass Dounce homogenizer with 30 strokes of a tight-fitting glass pestle Kontes B, and checked under the microscope to verify that 95% of the cells displayed trypan blue (T8154, Sigma) staining. Nuclei were pelleted by centrifugation at 350 g for 5 min at 4 °C, washed with cytoplasmic buffer three times, dissolved in nuclear buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and freshly added protease and phosphatase inhibitors) and kept on ice for 30 min before vigorous vortexing. Nuclear extracts were then transferred to 16,000 g for 20 min at 4 °C.

Protein concentration was determined with the Pierce bichromic acid assay (22225, ThermoFisher) kit according to the manufacturer’s instructions.

Immunoblotting. Equal amounts of denatured protein were subjected to 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) using molecular weight markers (26619, Fermentas) to confirm the expected size of the target proteins and transfection efficiency. Transfer of proteins to PVDF membrane was performed under semi-dry conditions at 200 mA for 1 h. Membranes were blocked by immersing the membranes in blocking buffer containing 4% nonfat milk (70166, Sigma) in TBST buffer for 1 h on a shaker at room temperature or overnight at 4 °C. Membranes were probed with the following primary antibodies: α-PAK4 polyclonal antibody (1:1000, C1E4 #4922, Cell Signaling; 1:1000, #06–1105, Millipore; 1:1000, EPI613Y #GTX12691, Genetex and 1:1000, clone 17.3 LS-C554950–100, LSBio), α-p53 (1:1000, DO-1 #sc-126, Santa Cruz); α-p21 (1:1000, C19 #sc-397, Santa Cruz); α-ACTIN (1:1000, JLA20, Developmental Studies Hybridoma Bank); α-VINCULIN (diluted 1:1000, Sigma); α-GAPDH (1:10000, #MAB574, Millipore) and α-pRB (1:1000, #554136, BD–Phar- mingen). Antibodies against ACTIN, VINCULIN, GAPDH, and pRB served as control for protein loading. Next, appropriate peroxidase-conjugated anti-mouse (715–035–150, Jackson Immunoresearch) or anti-rabbit (111–035–144, Jackson Immunoresearch) secondary antibodies were diluted 1:3000.

Bound antibodies were visualized with the Pierce enhanced chemiluminescence Plus Western Blotting Substrate detection system (32132, ThermoFisher) according to the manufacturer’s instructions.

Quantity One analysis software (Bio-Rad) and ImageJ/Fiji (National Institutes of Health, NIH) were used for densitometric analysis of western blots. Quantification results were background subtracted and normalized to a loading control.

Uncropped and unprocessed scans of all blots are supplied in the Source Data file.

Co-immunoprecipitations. Immunoprecipitations were performed by incubating the same amount of the relevant whole-cell lysates with the appropriate antibodies (500–1000 μg of whole cell lysate, 2 μg of antibody) overnight at 4 °C with gentle agitation to allow immunocomplexes to form. Prior to inclusion of the specific antibodies, samples were precleared by incubation with protein G-Sepharose beads (sc-2002, Santa Cruz) and 2 μg of α-mouse IgG (I5381, Sigma), for 1 h at 4 °C. Following the overnight incubation and when applicable, the immunocomplexes were collected by the addition of protein G-Sepharose beads for 3 h at 4 °C and washed three times with lysis buffer. Protein complexes were subjected to SDS loading buffer and eluates were resolved on a 10% SDS-PAGE for immunoblotting with relevant antibodies. Unless otherwise indicated, FLAG-tagged proteins were immunoprecipitated with EZView Red ANTI-FLAG M2 Affinity Gel (F2426, Sigma). Otherwise, the following antibodies were used: monoclonal mouse α-PAK4 (clone OTI1C7 #CF807297, OriGene), monoclonal mouse α-RELB (clone 17.3 FLS-C354950–100, LSBio), and α-mouse IgG (I5381, Sigma) as control.

NF-xB DNA-binding activity. DNA-binding activity of the mammalian NF-xB subunit RELB was measured in 20 μg of nuclear extracts using the TransAM NF-xB Family transcription factor assay kit (11467468, Active Motif) in accordance with the manufacturer’s protocol.

Exome sequencing. Genomic DNA was extracted from flash-frozen liver tissue and from three mammary tumors that arose in virgin MMTV–PAK4 female mice (20–24 months old) using the DNeasy Blood & Tissue Kit (69504, Qiagen) as specified by the manufacturer. DNA concentrations were determined using an Implantable Bioanalyzer (Agilent) and a Pro-QED fragment standard library. Multiplexed exome samples were then sequenced via the Illumina HiSeq2000 platform at the SciLifeLab in Stockholm.

RNA sequencing. Total RNA was isolated from Hs 578T and BT-549 cells 72 h after transient transfection with PAK4-targeting (siPAK4#1) or control siRNA using the NEasy Mini Kit (74104, Qiagen) as specified by the manufacturer. RNA was sequenced with an Illumina HiSeq2000 Bioanalyzer (Agilent) and a Pro-QED fragment standard library. Multiplexed exome samples were then sequenced via the Illumina HiSeq2000 platform. Library construction and sequencing was performed at the SciLifeLab in Stockholm.

Confirmed end reads were aligned to the human mm9 genome using BWA (bwa-bwa.sourceforge.net) with additional processing using Picard (broadinstitute.github.io/picard/) and Samtools. Somatic variants were called using MuTect and annotated using AnnoVAR.

NF-xB target genes were derived from a third party dataset (in fetal lung fibroblasts—https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) to our knowledge.

Figure 2A was generated using the ggplot2 package in R. Explicit read abundance at each reference transcript (Ensembl build 73) was corrected via reference transcriptome (Ensembl build 73). Explicit read abundance at each reference transcriptome (Ensembl build 73) was corrected via reference transcriptome (Ensembl build 73). Explicit read abundance at each reference transcriptome (Ensembl build 73).
Reverse transcription quantitative PCR array (RT-qPCR). For RNA Seq validation, a fast 48× TaqMan-based qPCR array (H413257, Life Technologies) was customized and used to profile Hs 57T7 cells upon transient transfection of FLAG-RELB-RHD. The bacteria were grown in Luria Broth (LB) media for 48 h. qPCR reactions were carried out in biological triplicates.

The array consisted of a selection of 44 genes that were found to be DE upon PKA4 knockdown by RNA-Seq and that have been shown to play a role in senescence-associated phenotypes plus four housekeeping genes. The following TaqMan gene expression assays were used: 18s RNA (Assay ID: Hs99999901_s1, housekeeping gene), BID (Assay ID: Hs00609632_m1), BIRC3 (Assay ID: Hs00985031_g1), BRAT1 (Assay ID: Hs00378008_m1), CCNG1 (Assay ID: Hs017111_12_m1), CD82 (Assay ID: Hs01017982_m1), CDX1 (Assay ID: Hs00367777_m1), CEBPB (Assay ID: Hs00167958_c1), CEBPD (Assay ID: Hs00174681_m1), CEBPG (Assay ID: Hs01535411_m1), E2F2 (Assay ID: Hs00231667_m1), E2F3 (Assay ID: Hs00245201_m1), E4F1 (Assay ID: Hs00174118_m1), E6 (Assay ID: Hs00174981_m1), GADD45A (Assay ID: Hs00167958_m1), GAPDH (Assay ID: Hs99999905_s1, housekeeping gene), GUSB (Assay ID: Hs00999908_m1, housekeeping gene), HES1 (Assay ID: Hs00820788_m1), HEY1 (Assay ID: Hs01141510_m1), HEY2 (Assay ID: Hs00025622_m1), HIPRT1 (Assay ID: Hs99999909_m1, housekeeping gene), ICAM1 (Assay ID: Hs00146932_m1), IL8 (Assay ID: Hs00174118_m1), IGF1 (Assay ID: Hs00174103_m1), ILG1 (Assay ID: Hs00153537_m1), MAPK6 (Assay ID: Hs00092389_m1), MCM10 (Assay ID: Hs00860315_m1), NFKB1 (Assay ID: Hs00765730_m1), NFKB2 (Assay ID: Hs01008611_m1), PCNA (Assay ID: Hs00427241_g1), POLA1 (Assay ID: Hs00413835_m1), PDK1 (Assay ID: Hs00174103_m1), RELB (Assay ID: Hs00242302_m1), REL (Assay ID: Hs00095034_m1), SPP1 (Assay ID: Hs00095010_m1), TIMP2 (Assay ID: Hs00234278_m1), TIMP3 (Assay ID: Hs00227744_m1), TNF (Assay ID: Hs00244199_m1), TRAF2 (Assay ID: Hs000184112_m1), and TWIST1 (Assay ID: Hs00820703_s1, housekeeping gene). The total RNA was isolated using the RNeasy Mini Kit (74104, Qiagen) as specified in the manufacturer's instructions at the BEA core facility in Stockholm. RT-qPCR reactions were carried out in biological triplicates.

Generation of anti-PAK4 antibodies. For anti-PAK4 antibody production, a PAK4 NH2-terminal sequence (aa 1–16) was amplified by PCR and cloned into the PET11b vector (EMD Biosciences, Burlington, MA, USA). His-tagged fusion protein was expressed in B. subtilis (Bacillus subtilis) and purified using Glutathione Sepharose 4B (GE Healthcare). The expression construct was validated against the SwissProt database and with variable modifications, Deamidated (NQ), Oxidized (M), Phospho (ST), Phospho (Y) and with Coomassie Blue dye (443283M, VWR). The radioactivity incorporated into the substrate was visualized and quantified by autoradiography and Phosphor Imager analysis (Molecular Imager FX, Bio-Rad).

Mass spectrometric analysis. GST-RHD (GST-tagged hRELB aa 102–400) was phosphorylated by His-PAK4 in vitro and analyzed by MS. Gel lanes with proteins were excised manually from Coomassie-stained gels. The lanes, cut in several pieces, were processed and digested by trypsin using a robotic protein handling system (MasilPREF, Waters). Proteins were reduced with 10 mM dithiothreitol (DTT, Sigma) in 100 mM ammonium bicarbonate and incubated at 40 °C for 30 min with 5 mM iodoacetamide (Sigma) in 100 mM ammonium bicarbonate for 40 min at 20 °C. The gels were fixed in 7% acetic acid and 20% methanol and stained with Coomassie Blue dye (443283M, VWR). The radioactivity incorporated into the substrate was visualized and quantified by autoradiography and Phosphor Imager analysis (Molecular Imager FX, Bio-Rad).

Detection of phosphorylation sites within RELB. The RELB gene was searched for in the Phosphosida and PhosphositePlus databases in accordance with the suppliers’ guide. Literature was curated for additional residues. Residues previously identified in murine RELB were converted to the corresponding human residue for display.

Clinical data and gene expression profiling. The role of PAK4 gene expression in clinical samples was explored on the previously published METABRIC data14. Briefly, 1980 patients were included in this study that performed transcriptional profiling (Illumina HT-12 v3 platform) of 1992 primary tumor specimens and tumor adjacent normal tissue (144 samples) from biobanks in the UK and Canada. All clinical-pathological characteristics, PAM50 subtypes, Integrative subtypes, and survival outcome information were obtained from the original publication. The normalized data were downloaded from the European genome-phenome archive platform (https://www.ebi.ac.uk/ega/studies/EGAS000000000083, accessed on Mar 2015). The 3 PAK4 annotated probes had Spearman correlation coefficients 0.53, 0.03, and 0.03. The most varying probe that targets all PAK4 transcript variants (ILMN_1728887) was selected for further analysis. A gene expression based proliferation module score was computed as previously described15. PAK4 gene expression was explored in the Oncomine cancer microarray database and web-based data-mining platform (accessed in December 2016)16. PAK4 mRNA levels were compared in breast carcinomas versus normal breast tissues in the TCGA16 (n = 93 carcinomas versus n = 61 normal) and Zhao17 (n = 38 carcinomas versus n = 3 normal) datasets.

The somatic mutation and copy number segmentation data of the breast cancer cell lines were downloaded from the CCLE data portal (www.portals.broadinstitute.org/ccle, accessed on Dec 2016)18. Prior knowledge about and recurrence of mutations were analyzed using OncokCB19 (www.oncokb.org), Cancer Hotspots20,21, (www.cancerhotspots.org), and 3D Hotspots22 (www.3dhotspots.org). Copy number data were processed by using the GISTIC2.0 module on GenePattern23 (www.genepattern. broadinstitute.org) running default parameters. Copy number alteration and clonal cancer spectrum analysis (n = 987) were downloaded from Broad GDAC Firehose (https://ezid.ccb Molc.org?uri=10.3980/C11G0K49) (www.gdac.broadinstitute.org). Patients were stratified according to their PAK4
copy-number status (all other versus amplification) and further analyzed using the Kaplan-Meier method and compared by the logrank (Mantel-Cox) test carried out with SPSS Statistics software version 23 (IBM Corporation, Armonk, NY, USA).

**Statistics.** Group size was based on previous experience. No statistical method was used to predetermine sample size. Unless otherwise noted, each experiment was repeated three or more times. Data shown in column graphs represent mean ± standard error of the mean (SEM) or mean ± standard deviation (SD), as indicated in the figure legends, and individual data points are plotted. Statistical analysis was performed with GraphPad Prism 6.0. Details of statistical testing can be found in the figure legends and in the source data file. All datasets were tested for Gaussian distribution using the Kolmogorov–Smirnov test.

Statistical analysis of the METABRIC clinical data is described in detail below. The association between PAK4 and clinical-pathological characteristics was assessed by Mann–Whitney tests (two groups) or Spearman correlation test (continuous variable). OS and DSS endpoints were used to explore the prognostic ability of PAK4. Deaths of an unknown cause were excluded in analyses of the DSS endpoint. Some sensitivity analyses including deaths of an unknown cause as an event of the DSS endpoint demonstrated consistency in the results. In all patients as well as in the subgroup of untreated patients (not receiving any adjuvant treatment), PAK4 association with both endpoints was investigated in univariable and multivariable Cox models stratified by site (as suggested in the original publication). The multivariable Cox models were adjusted by tumor size, lymph node status, tumor grade, age at diagnosis, and PAM50 subtype. The univariable prognostic role of PAK4 categorized according to quartiles was visualized by the Kaplan–Meier method.

Association between PAK4 expression and modules that reflect biological processes in breast cancer29,30, NF-κB signaling29, and NF-κB subunits in tumor clinical samples was quantified by Spearman’s rank correlation. The analysis is exploratory. All statistical analyses were done with R statistical software v3.1.0.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The RNA-Seq data reported here has been deposited in the NCBI GEO under ID code: GSE122817. Exome-Seq data are available in SRA under the accession code PRJNA545882. Remaining primary data of interest is provided in the source data file of this paper.

**Code availability**

R code used in this study has been deposited on GitHub (https://github.com/CostaNatComm19/analysisPublicData).

Received: 11 July 2018 Accepted: 17 July 2019

Published online: 09 August 2019

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Acknowledgements

We thank Audrey Minden, Lars Holmgren, Rainer Heuchel, Matthias Lühe, David Beach, Sam Okret, and Ralf Mariedfeld for providing various mice, cells, and reagents as specified in Methods. We thank Zhihun Li for assistance with kinase assays, Agnete Andersson for support with the xenografts and for performing ISH, Isha Raj for help with the structural modeling, Sara Göransson and Xavier Serra-Picamal for assistance with imaging and image analysis of X-Gal experiments in cells, and Jens Henrik Norum and Erik Fredlund for assistance with exome sequencing analysis. We thank all Stromblad laboratory members for valuable comments and discussion. This study was supported by grants to S.S. from the Swedish Research Council, Radiumhemmets Forskningsfonder and the Swedish Cancer Society and the Breast Cancer Theme Center at KI. T.C. was supported by the Portuguese Foundation for Science and Technology (RFUI/BD/473368). The mAbs anti-ACTIN JLA20 and anti-BrdU G3G4 were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242. This study makes use of data generated by the Molecular Taxonomy of Breast Cancer International Consortium supported by Cancer Research UK and the British Columbia Cancer Agency Branch. Microscopy was performed at the Live Cell Imaging facility/Nikon Center of Excellence, Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden, supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Research Council, the Centre for Innovative Medicine, the board of research at KI and the Jonasson donation to the School of Technology and Health, Royal Institute of Technology, Sweden. We also thank the core facility Bioinformatics and Expression Analysis, which is supported by the board of research at the KI and the research committee at the Karolinska Hospital. Proteomic analysis was carried out at the Proteomics Karolinska. The authors also acknowledge support from Science for Life Laboratory, the National Genomics Infrastructure, NGI, and Uppmax for providing assistance in massive parallel sequencing and computational infrastructure.

Author contributions

T.C. conceived, designed, and performed most experiments and analyses. T.Z., H.O., M.M.B., M.Z., P.R., R.K., M.S., P.H.V., U.R. and X.G. contributed to experiments and analyses. J.L. and J.S. analyzed clinical data. E.T. and P.D. contributed the MMTV–PAK4 transgenic mouse model. N.R. performed RNA-Seq analyses. R.M. and J.H. provided PDGs. P.R. and K.P. provided PyMT-derived samples and cells. P.D.A. advised and assisted in data interpretation. S.S. conceived, designed and supervised the study, interpreted data, and provided financing. T.C. and S.S. wrote the manuscript and all authors approved the final version.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11510-4.

Competing interests: The authors declare no competing interests.

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Peer review information: Nature Communications thanks Jonathan Chernoff and other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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