Potassium Channel KCNA1 Modulates Oncogene-Induced Senescence and Transformation

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Abstract

Oncogene-induced senescence (OIS) constitutes a failsafe program that restricts tumor development. However, the mechanisms that link oncogenesis to senescence are not completely understood. We carried out a loss-of-function genetic screen that identified the potassium channel KCNA1 as a determinant of OIS escape that can license tumor growth. Oncogenic stress triggers an increase in KCNA1 expression and its relocation from the cytoplasm to the membrane. Mechanistically, this relocation is due to a loss of protein kinase A (PKA)-induced phosphorylation at residue S446 of KCNA1. Accordingly, sustaining PKA activity or expressing a KCNA1 phosphomimetic mutant maintained KCNA1 in the cytoplasm and caused escape from OIS. KCNA1 relocation to the membrane induced a change in membrane potential that invariably resulted in cellular senescence. Restoring KCNA1 expression in transformation-competent cells triggered variation in membrane potential and blocked RAS-induced transformation, and PKA activation suppressed both effects. Furthermore, KCNA1 expression was reduced in human cancers, and this decrease correlated with an increase in breast cancer aggressiveness. Taken together, our results identify a novel pathway that restricts oncogenesis through a potassium channel-dependent senescence pathway. Cancer Res; 73(16); 5253–65. ©2013 AACR.

Introduction

Cellular senescence is induced by various cellular stresses such as telomere shortening or oncogenic stress and it results in a stable form of cell-cycle arrest and the appearance of new cellular features such as in morphology or senescence-associated β-galactosidase activity (SA-β-Gal). Senescence by its antiproliferative power is considered as a barrier against tumor development (1–4).

The concept of oncogene-induced senescence (OIS) as a tumor-suppressive mechanism emerged in the late 90s, with the observation that activated oncogenes cause senescence of normal cells in culture (5). After years of debate about the relevance of this observation, a set of in vivo studies revealed the presence of senescence in premalignant tumors and its absence in malignant tumors (4). In addition to confirmation of the relevance of oncogene-induced senescence, molecular insights into its regulation have emerged. Key cell-cycle regulators such as those directly regulating the p16-pRb and p53 pathways are reported to affect oncogene-induced senescence (6). Recently, some unexpected mechanisms impacting these pathways have been shown to regulate senescence, for example, regulation by DNA damage, by secreted factors such as cytokines, and by autophagy (7–10).

Most of these pathways involved a central role of the p53 as well as of the p16–Rb pathways in human cells (11), but the experimental evidence were mainly obtained using only human fibroblasts. Emerging evidences support the existence of other mechanisms regulating senescence escape. To date, little is known about these mechanisms (12–18). Similar data generated in human epithelial cells or in other lineages are rather rare and seem to display a more complex picture of the genetic events involved in senescence escape (18). As an example, poststasis human mammary epithelial cells (HMEC), which are not expressing the p16-INK4A (19), enter in senescence in response to an oncogenic stress in a DNA damage-p53-independent pathway (17, 18).

In this study, we have carried out a functional loss-of-function short hairpin RNA (shRNA) genetic screen to identify senescence regulators displaying tumor-suppressive activity involved in OIS-escape in human epithelial cells. By this means, we have identified the importance of a potassium channel KCNA1 as a regulator of OIS. In particular, we observed a fine regulation of the localization of this channel to the cell membrane controlled by the protein kinase A (PKA). In addition, we...
provide evidence that change in KCNA1 localization impacts on membrane potential, cellular senescence, and transformation processes.

Materials and Methods

Cell culture

HMECs (Lonza) were cultured in mammary epithelial cell growth medium (Promocell) and penicillin/streptomycin 100 U/mL (Invitrogen). Virus-packaging GP293 cells (Clontech), PlatE (20) cells, and NIH3T3 cells (American Type Culture Collection) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS (Hyclone Perbio) and gentamycin (Invitrogen) at 80 μg/mL.

Genetic screening

One hundred thousand HMEC-TM cells were seeded into 10 cm dishes. The following day, each dish was infected with a pool of the short hairpin RNA (shRNA) library (10,000 shRNAs per pool and in 7 pools; Decode RNAi viral screening pools, Thermo Scientific) and treated every 2 days with 400 nmol/L 4-OHT, a concentration that did not impact HMEC-T proliferation (data not shown). Thirty days later, gDNA was prepared from 2 pools out of 7 with clones that escaped senescence. PCR was used to amplify shRNA-coding DNA according to the manufacturer’s recommendations for the two conditions. They were next cloned into the pGEMTeasy vector (Promega). One hundred clones were sequenced and shRNA in each clone were identified.

Vectors

KCNA1–shRNA-encoding retroviral vectors were used to knock down KCNA1 (SourceBioScience LifeSciences, NK1_p3205F1410Q, NK1_p3205J1710Q). KCNA1 cDNA [Open Biosystems (MHS1010-98052646)] was excised by EcoRI and cloned into the pLPX (Clontech) retroviral vector. The plasmids pNLCA1M1 (AN3, S218E, S222D):ER, pBabe-puro-BRAF:ER (21) and pBabe-puro-H-RASV12 (5) were used when indicated. PRKACB and PRKACG obtained from Addgene (Plasmid 20596 and 20597) have been described previously (22). The retroviral vectors pMSCV-IRES-GFP expressing PRKAR2A or R1A were described previously (23).

Immunoblotting and immunofluorescence

Immunoblot analyses were conducted as described previously (24). For immunofluorescence, cells were fixed either with 4% paraformaldehyde (PFA) to detect protein at the membrane or with methanol at −20°C for 10 minutes to detect protein at all locations. Immunofluorescence staining was conducted as described previously (24). Stainings were superimposed using ImageJ software. Primary antibodies used were: anti-KCNA1 (75-105, external; NeuroMab) used throughout the study, anti-KCNA1 (75-007, internal; NeuroMab) used when mentioned, anti-phosphoERK (9101; Cell Signaling Technology), anti-FLAG (200472; Stratagene), anti-phospho-histone3Ser10 (ab19555; Abcam), anti-cyclinA (sc-751; Santa Cruz Biotechnology), anti-RAS (610001, BD), and anti-tubulin (T6199, Sigma).

SA-β-Gal analysis, colony-formation assays, and soft agar experiments

Cells were seeded into 6-well plates (at 5 × 10^4 cells/well). One day later, the various treatments began. Six to 10 days after the initial treatment, the cells were fixed with 4% PFA and processed as described previously (25).

Statistical analysis

Graphs are presented with SD as error bars, and Student t test was used to determine the P value. *, P < 0.05; **, P < 0.01; and ***, P < 0.001, unless specified otherwise in the figure legends. See the Supplementary Materials and Methods for details.

Results

KCNA1 downregulation induces an OIS escape

To study OIS in human epithelial cells, we used poststasis (which does not express p16INK4a; ref. 19) HMECs. Early passage cells (<3 passages) were immortalized by retroviral transduction of hTert and of 4-hydroxy tamoxifen (4-OHT) inducible oncogene MEKER (HMEC-TM cells) and subsequently used for the different experiments for no more than 10 passages. To identify new senescence regulators, we carried out a loss-of-function genetic screen. The library was composed of seven pools of shRNA, each pool containing 10,000 shRNA. Our strategy was to infect HMEC-TM cells with an shRNA library; treat them with 4-OHT every 2 days for 30 days to activate MEK to completely block cell growth, as seen with control GFP-expressing cells. shRNA in cells that escaped senescence were identified (Fig. 1A; Supplementary Fig. S1B), and its expression was shown to be decreased in breast cancer (26) and nothing much was reported about its potential function in cancer.

To validate a role of KCNA1, HMEC-TM were treated with 4-OHT for 3 days, and this resulted in phosphorylation of ERK (the MEK substrate), downregulation of the late mitosis marker P-S10-H3 (Supplementary Fig. S1C), decreased cell growth (Supplementary Fig. S1D and 1E), the appearance of SA-β-Gal (Supplementary Fig. S1F), and increased levels of a known set of senescence markers including interleukin (IL)-8 (27), Sprouty2 (28), Dec1, and Dr (Supplementary Fig. S1G; ref. 29). Nevertheless 10 days after oncogenic stress induction by the 4-OHT treatment, the cells were able to regrow (data not shown), suggesting that, in these experimental settings, the senescent phenotype is not stable; this may be due to p16 inactivation and/or immortalization by the hTert. Therefore, we used these cells as a model presenting the OIS hallmarks and also used nonimmortalized HMEC that are known to display an irreversible senescence upon oncogenic stress induction to confirm a role of KCNA1 in OIS escape (17). Two independent KCNA1-targeting shRNAs were created and confirmed by quantitative reverse transcription (qRT)-PCR and immunofluorescence their ability to downregulate KCNA1 (Fig. 1B and C). KCNA1 knockdown with these new shRNAs enabled cells to escape MEK-induced growth arrest according...
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Figure 1. KCNA1 downregulation promotes escape from OIS hallmarks. A, design of the loss-of-function shRNA genetic screen in HMEC-TM cells. B, HMEC-TM cells were infected with a control or with two independent KCNA1-targeting shRNA vectors. After selection, RNAs were prepared and KCNA1 expression was measured by qRT-PCR. Relative KCNA1 mRNA expression was normalized to actin mRNA expression. The experiments shown are representative of three repeats. C, cells were treated with 4-OHT for 3 days, fixed by methanol, and knockdown of KCNA1 protein checked by immunofluorescence. The experiments shown are representative of two repeats. D, infected and selected HMEC-TM cells were seeded and treated 3 times with 4-OHT (400 nmol/L). Six to 10 days after the first 4-OHT treatment, the following assays were conducted. Cell extracts were prepared and analyzed by immunoblotting with antibodies detecting 2 proliferation markers, cyclin A and P-S10-H3. Tubulin was monitored to check the protein loading. The experiments shown are representative of two repeats. E, cells were PFA-fixed and cell growth was monitored with crystal violet staining. The experiments shown are representative of four repeats. F, 8 days after 4-OHT treatment, the cells were counted and number of population doublings calculated. The experiments shown are representative of two repeats. G, after fixation, SA-β-Gal activity assays were conducted. The experiments shown are representative of two repeats. H, RNA were prepared and IL-8 expression analyzed by qRT-PCR. Expression of IL-8 mRNA was normalized against actin mRNA. *, P < 0.05; **, P < 0.01 and ***, P < 0.001.

To the maintenance of cyclin A (accumulated in S, G2, and early M phases), of P-S10-H3 (accumulated in late M phase; Fig. 1D), the ability to proliferate and form colonies (Fig. 1E and F), and to escape MEK-induced expression of the SA-β-Gal senescence marker (Fig. 1G) or MEK-induced IL-8 expression (Fig. 1H). It also favored an escape from RAF-induced OIS hallmarks.
(Supplementary Fig. S2A and B). The ability of KCNA1 knockdown to escape OIS in nonimmortalized HMEC-MEK:ER (HMEC-M) was confirmed by checking cell proliferation (Supplementary Fig. S3A), SA-β-Gal staining (Supplementary Fig. S3B), and IL-8 expression (Supplementary Fig. S3C).

**KCNA1 is relocated to the membrane by the oncogenic stress**

To verify KCNA1 expression is regulated by oncogenic stress, KCNA1 expression levels were monitored. Both KCNA1 transcripts (Fig. 2A) and KCNA1 protein (Fig. 2B and C and Supplementary Fig. S3D) were found to increase in response to oncogenic stress in immortalized and nonimmortalized HMEC. KCNA1 being a potassium channel involved in potassium efflux from the cell, we next investigated its subcellular localization by confocal microscopy during OIS. Interestingly, KCNA1 was found to localize at the cell membrane (Fig. 2C). To confirm its localization at the membrane during OIS, further immunofluorescence studies were conducted using antibodies recognizing the extracellular and intracellular part of KCNA1 on nonpermeabilized cells. Only the antibody that recognizes the extracellular KCNA1 labeled the cells, confirming the membrane localization of KCNA1 (Fig. 2D and Supplementary Fig. S3D). Both antibodies labeled the cells in a similar fashion in permeabilized cells confirming the specificity of the antibodies for KCNA1. Importantly, we also observed higher apical membrane KCNA1 levels in human prostatic–intraepithelial neoplasias, known to display OIS (7, 30), than in the adjacent normal tissue (Fig. 2E). This is consistent with our in vitro observations. Taken together, these results show that KCNA1 expression and localization are regulated by oncogenic stress and are involved in OIS. Interestingly, HMEC in replicative senescence also displayed an increased and membrane-localized KCNA1 (Supplementary Fig. S3E and 3F), suggesting it might have a role in senescence beyond oncogenic–stress-induced senescence.

Having shown that KCNA1 downregulation favors escape from oncogene-induced senescence, we next examined the effect of its constitutive expression. Surprisingly, constitutive expression of KCNA1 (Supplementary Fig. S4A and 4B) did not significantly reduce cell growth (Supplementary Fig. S4C) or induce the appearance of the SA-β-Gal senescence marker (Supplementary Fig. S4D). In addition, ectopically expressed KCNA1 failed to locate at the membrane according to the
absence of labeling by immunofluorescence using the antibody recognizing the extracellular part of KCNA1 (Supplementary Fig. S4E, green panel). However, inducing the oncogenic stress allowed the overexpressed KCNA1 to relocate to the membrane (Supplementary Fig. S4E, red panel). To further confirm that a mechanism specifically related to oncogenic stress must be involved in triggering KCNA1 relocation to the membrane, we biotinylated the extracellular part of membrane-bound proteins of live cells with or without the induction of the oncogenic stress. Biotinylated KCNA1 was only observed in cells where oncogenic stress was induced. As expected, KCNA1 was biotinylated and, thus, membrane located upon oncogenic stress (Supplementary Fig. S4F). Accordingly, a low level of oncogenic stress, induced with a low dose of 4-OHT, was sufficient to trigger senescence hallmarks in cells ectopically expressing KCNA1 (Supplementary Fig. S4G–S4J). The same treatment when applied to control cells caused a growth slowdown but no senescence (Supplementary Fig. S4G–S4J). In addition, we observed the translocation of KCNA1 to occur as early as 8 hours after oncogenic stress induction (Supplementary Fig. S4K). Our data show that oncogenic stress induces KCNA1 relocation to the membrane, participating in oncogene-induced senescence. This translocation of KCNA1 to the membrane, probably, is an early event.

**Phosphorylation of KCNA1 at Ser446 by PKA is involved on its cytoplasmic retention**

We, next, wanted to discover the mechanism controlling the KCNA1 membrane relocation upon oncogenic stress. Electrophysiologic studies have shown that the function of KCNA1 is regulated by its phosphorylation at Ser46 by the protein kinase PKA (31). As phosphorylation is well known to affect subcellular localization, we examined whether phosphorylation by PKA might influence KCNA1 relocation. To this end, we used a pharmacologic inhibitor (H-89) and a PKA activator cocktail (forskolin + IBMx, F/I). Inhibition of PKA by H-89 resulted in endogenous KCNA1 relocation to the membrane even in the absence of oncogenic stress (Fig. 3A), whereas activation of PKA by F/I inhibited KCNA1 relocation in response to oncogenic stress (Fig. 3A). Ectopically expressed active PKA subunits (Supplementary Fig. S5A and 5B) also blocked endogenous KCNA1 membrane localization upon oncogenic stress (Supplementary Fig. S5C), whereas PKA inhibition by constitutively expressing PKA regulatory subunits resulted in KCNA1 increased expression and membrane localization (Supplementary Fig. S5D). Inhibition of PKA by H-89 also resulted in membrane localization of ectopically expressed KCNA1 (Fig. 3B), whereas PKA activation by F/I blocked ectopically expressed KCNA1 relocation upon oncogenic stress, according to the result of membrane proteins biotinylation experiments (Fig. 3C).

In vitro, we found the purified KCNA1 protein to be phosphorylated by recombinant PKA, and this phosphorylation was abolished by the Ser46Ala mutation (Fig. 3D). In addition, by using a phosphoS/T PKA substrate-specific antibody on immunoprecipitated KCAN1, we observed a loss of KCNA1 phosphorylation after oncogenic stress induction (Fig. 3E). Accordingly, the KCNA1 S446A-mutant expressed in cells could localize to the membrane in the absence of oncogenic stress, in contrast to wild-type (WT) KCNA1 (Fig. 3F and G) present at a similar level (Supplementary Fig. S5E). Conversely, a phosphorylation-mimetic KCNA1-mutant (S446E) failed to relocate to the membrane in response to oncogenic stress, unlike WT KCNA1 (Fig. 3H and I), both proteins being expressed at similar levels (Supplementary Fig. S5F). In summary, KCNA1 relocation to the membrane is repressed by PKA phosphorylation at Ser-446, and this repression might be lost upon oncogenic stress.

**Sustaining PKA activity and retaining KCNA1 in the cytoplasm allow OIS bypass**

The effect of PKA-mediated phosphorylation on KCNA1 relocation in response to oncogenic stress suggests that PKA activity must be regulated by oncogenic stress. Accordingly, we found oncogenic stress to cause a strong decrease in PKA activity, as estimated by measuring total phosphorylation of PKA substrates. This decrease was countered by F/I PKA activator treatment or by expressing PKA catalytic subunits (Fig. 4A and Supplementary Fig. S6A) and mimicked by H-89 PKA inhibitor treatment (Fig. 4A). Functionally, maintaining PKA activity by ectopically expressing constitutively active PKA subunits or by treating cells with F/I resulted in escape from senescence in immortalized (Fig. 4B–E and Supplementary Fig. S6B) or nonimmortalized HMEC (Supplementary Fig. S7). H-89 PKA inhibitor treatment, which induced KCNA1 plasma membrane localization (Fig. 3A and B), induced a premature senescence (Fig. S6C and D).

We next took advantage of the KCNA1 S446E phospho-mimetic mutant, which failed membrane localization upon oncogenic stress, and hypothesized that this mutant may act as a dominant negative form as this channel forms an homotetramer to allow potassium exit. If so, we expected this mutant to induce an OIS bypass as KCNA1 knockdown did. Indeed, ectopic expression of KCNA1 S446E allowed the cells to escape the growth arrest (Fig. 5A and B, for immortalized HMECs, and Supplementary Fig. S3A, for nonimmortalized HMECs), the appearance of the SA-β-Gal senescence marker (Fig. 5C, for immortalized HMECs, and Supplementary Fig. S3B, for nonimmortalized HMECs), and the increase of IL-8 mRNA expression (Fig. 5D, for immortalized HMECs, and Supplementary Fig. S3C, for nonimmortalized HMECs) induced by the oncogenic stress. Further confirming a dominant negative role of KCNA1 S446E mutant, we observed its expression inhibited endogenous (Supplementary Fig. S3D) or constitutively expressed WT KCNA1 (Fig. 5E) to relocate at the membrane upon oncogenic stress induction.

These results support the view that, by inhibiting PKA-induced KCNA1 S446 phosphorylation, oncogenic stress triggers relocation of KCNA1 to the membrane, a mechanism participating in senescence induction.

**PKA–KCNA1–membrane potential pathway impacts OIS**

As KCNA1 is a channel regulating potassium efflux through the plasma membrane (32) and, as it has relocated to the membrane in response to oncogenic stress, we next investigated whether directly adding potassium to the media might
PKA phosphorylates S446 of KCNA1 and has an impact on KCNA1 localization. A, HMEC-TM cells were seeded and left untreated or treated with H-89 PKA inhibitor, with or without 4-OHT, or with the F/I PKA activator, with or without 4-OHT. After 3 daily treatments, the cells were fixed with PFA and subjected to KCNA1 immunofluorescence staining. The experiments shown are representative of two repeats. B and C, HMEC-TM cells expressing KCNA1 were treated with the indicated compounds, and membrane-bound proteins were biotinylated and purified by avidin beads. An immunoblot against total (input) and bound (beads) KCNA1 was next conducted. The experiments shown are representative of two repeats. D, HEK 293T cells were transfected with His-tagged WT- or S446A-mutant KCNA1. The WT and mutant KCNA1 proteins were affinity purified. Eluted KCNA1 protein was phosphorylated alone or with PKA and separated by SDS-PAGE. WT and S446A-mutant KCNA1 phosphorylation by PKA (phosphoimage) and the protein levels (immunoblot) are shown. The experiments shown are representative of two repeats. E, HMEC-TM constitutively expressing KCNA1 were left untreated or were treated three times by 4-OHT at 400 nmol/L. KCNA1 was then immunoprecipitated and an immunoblot was conducted against KCNA1 and against the phosphoS/T PKA substrates. F, HMEC-TM cells were infected with a vector encoding WT or S446A KCNA1, puromycin selected, and seeded. Immunofluorescence analysis against total or membrane KCNA1 were conducted. The experiments shown are representative of three repeats. G, HMEC-TM cells expressing the indicated KCNA1 proteins were biotinylated and the membrane-bound proteins purified. Total (input) and membrane bound (beads) KCNA1 were analyzed by immunoblot. The experiments shown are representative of two repeats. H, HMEC-TM cells were infected with a vector encoding WT or S446E KCNA1, puromycin selected, seeded, and treated with 4-OHT. Membrane-located KCNA1 was visualized by immunofluorescence. The experiments shown are representative of three repeats. I, HMEC-TM cells expressing WT or S446E mutant were treated with or without 4-OHT. Membrane-bound proteins were purified by avidin beads after biotinylation of proteins located at the membrane. Total and membrane-located KCNA1 were then analyzed by immunoblot. The experiments shown are representative of two repeats.
Impact senescence. Interestingly, this treatment massively induced cellular senescence hallmarks as it resulted in a growth arrest (Fig. 6A and B) and the appearance of SA-β-Gal–positive cells (Fig. 6C) or increased IL-8 mRNA expression (Fig. 6D). Further suggesting a role of the extracellular potassium, an inhibitor of the sodium/potassium ATPase pump known to block potassium entry blocked cell growth (Fig. 6E and F), increased the proportion of SA-β-Gal–positive cells (Fig. 6G), and increased IL-8 mRNA expression (Fig. 6H).

Interestingly, the oncogenic stress was found to cause a variation in membrane potential (ΔV(m); Fig. 6I). This oncogenic–stress-induced variation was largely suppressed in KCNA1-knockdown cells, indicating that KCNA1 mediates it (Fig. 6I). Accordingly, the F/I PKA activator cocktail, which inhibits KCNA1 relocation to the membrane, suppressed the oncogenic–stress-induced membrane potential change, whereas the PKA inhibitor H-89, which induces KCNA1 membrane relocation in the absence of oncogenic stress, induced a membrane potential change (Fig. 6J). The phosphomimetic KCNA1 S446E mutant, in which membrane location by the oncogenic stress was prevented, did not display oncogenic–stress-induced membrane potential change (Fig. 6K). Accordingly, directly adding potassium (Fig. 6L) or blocking the sodium/potassium ATPase pump (Fig. 6M) provoked membrane potential changes. Together, these results support the view that KCNA1 membrane localization upon oncogenic stress results in membrane potential changes. These changes can be prevented by KCNA1 knockdown, by its retention to the
cytoplasm through PKA activation, and by adding the phosphorymimetic KCNA1 mutant. Conversely, these changes are mimicked by adding potassium or blocking potassium entry by the sodium/potassium ATPase pump inhibitor.

**PKA–KCNA1 pathway impacts oncogene-induced transformation**

Our results, thus far, show that the PKA–KCNA1 pathway regulates oncogenic-stress-induced senescence through a membrane potential change. We, next, investigated whether this pathway might regulate the transformation process. For this we used NIH3T3 cells, spontaneously immortalized cells derived from mouse embryonic fibroblasts. These cells can be transformed by oncogenic RAS (33). When expressed alone in NIH3T3 cells, KCNA1 failed to localize to the membrane, but when coexpressed with oncogenic RAS, it was found at the membrane (Fig. 7A and B). The ability of oncogenic RAS to promote KCNA1 relocation to the membrane was inhibited by

![Figure 5](image_url)

**Figure 5.** KCNA1 phosphomimetic mutant allows to bypass OIS hallmarks. A–D, HMEC-TM cells were infected with an empty or S446E KCNA1 vector, puromycin selected, seeded, and treated with or without 4-OHT for 3 days. A, 4 days later, cells were PFA-fixed and crystal violet-stained. The experiment is representative of three repeats. B, 4 days later, cells were counted and the number of population doublings calculated. The experiment is representative of two independent repeats. C, 4 days later, cells were analyzed for an SA-β-Gal activity test. The experiments shown are representative of two repeats. D, RNA were prepared and IL-8 expression analyzed by qRT-PCR. Expression of IL-8 mRNA was normalized against actin mRNA. E, HMEC-TM were infected with KCNA1 WT or with KCNA1 WT + S446E mutant. After 4-OHT treatment, HMEC-TM cells were fixed with methanol or PFA and total- or membrane-bound KCNA1 were respectively detected by immunofluorescence. The experiments shown are representative of two repeats. *, P < 0.05 and †††, P < 0.001.
constitutive PKA subunit expression (Fig. 7A and B) or PKA induction by F/I (Supplementary Fig. S8A) as on human epithelial cells. As expected, RAS-induced relocation of KCNA1 to the membrane resulted in membrane potential alteration (Fig. 7C), while its sequestration in the cytoplasm caused by F/I-triggered PKA induction maintained the membrane potential (Fig. 7C). Importantly, RAS-induced transformation was blocked when KCNA1 and oncogenic RAS were coexpressed.
Figure 7. The KCNA1 pathway regulates RAS-induced transformation and predicts the risk of developing breast metastases. A, NIH3T3 cells were infected with indicated vectors and selected. Total or membrane-associated KCNA1 was detected by immunofluorescence. The experiments shown are representative of two repeats. B, cell extracts were prepared and analyzed by immunoblot using an antibody directed against RAS. C, NIH3T3 cells expressing the indicated proteins were untreated or treated with F/I for 3 days. The membrane potential was measured with DiBAC4. The experiments shown are representative of two repeats. D, NIH3T3 cells expressing indicated proteins were seeded at the same density. Five days later, representative images were taken and presented. The experiments shown are representative of two repeats. E, 25,000 NIH3T3 cells were seeded in agar. Eleven days later, representative images were taken and presented. The experiments shown are representative of three repeats. F, summary of the KCNA1 mRNA expression found in various cancers as compared with normal counterparts using the oncomine database. G–I, RNA from human primary breast cancer samples were analyzed for KCNA1 mRNA expression by qRT-PCR and normalized against actin mRNA. PCRs were conducted in triplicates. G, relative KCNA1 mRNA expression in samples coming from patient displaying or not a metastasis in 3 years was represented. Means ± SEM are represented. P values were determined using the Mann–Whitney U test. H–I, cancer samples were next, split in 2 groups (high and low KCNA1 levels) and Kaplan–Meier analyses of the probability to live without metastases (H) and to survive (I) were conducted. P values were calculated using a log-rank test.
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and restored by expression of constitutively active PKA catalytic subunits (Fig. 7D and E) and by F/J treatment (Supplementary Fig. S8B). Interestingly, the phospho-metric S446E KCNA1 mutant was unable to localize at the membrane (Supplementary Fig. S8D), to induce a membrane potential change (Supplementary Fig. S8C), and to block transformation (Supplementary Fig. S8D) upon RAS expression. This confirms the inhibitory function of PKA-mediated S446 phosphorylation.

A search in the Oncomine database, a cancer microarray database allowing to conduct differential expression analyses comparing most major types of cancer with respective normal tissues, showed that some cancers were found to display an unusually low KCNA1 mRNA level (Fig. 7F). For example, six analyses showed a lower KCNA1 transcript level in breast cancer samples than in normal counterparts (Fig. 7F), and this finding was confirmed at protein level in an independent study (26). We, next, analyzed KCNA1 mRNA expression levels of nontreated breast primary tumors of 41 patients. Twenty displayed metastasis and died before 36 months and 21 showed no metastasis and survived beyond 36 months. Interestingly, KCNA1 mRNA expression was found to be significantly higher in the group displaying no metastasis (Fig. 7G). In order to examine whether low levels of KCNA1 might correlate with an increased risk of developing metastasis and of dying, we conducted a Kaplan–Meier analysis. Tumors displaying higher KCNA1 expression than the median value (>0.00137; including this tumor) was included in the group "high", whereas tumors expressing lower levels than the median value (<0.00137) were included in the group "low". Interestingly, low levels of KCNA1 were associated with an increased risk of developing metastasis and of dying (Fig. 7H and I). Among these 41 primary tumors, 34 were fully characterized for HER-2, ER, and PR expression. We cannot observe any correlation between KCNA1 mRNA levels and luminal A or triple-negative breast cancer subgroups (Supplementary Fig. S9A). The number of samples for luminal B or HER-2− subtypes was too low to draw any conclusion; nevertheless, our results might suggest that KCNA1 mRNA levels are lower in HER-2− breast tumors (Supplementary Fig. S9A). As expected, triple-negative subgroups displayed higher risk of metastasis and of dying than the luminal A group (Supplementary Fig. S9B and 9C), showing that KCNA1 was an independent variable for metastasis and survival. Taken together, these data support a functional role of KCNA1 in cancer.

Discussion

Our results show that a downregulation of KCNA1, a voltage-gated delayed potassium channel that is phylogenetically related to the Drosophila Shaker channel, leads to an OIS escape. Interestingly, KCNA1 localization is tightly regulated during OIS by PKA activity. Indeed, we show that an inhibition of PKA activity results in a loss of S446 KCNA1 phosphorylation and a subsequent localization of KCNA1 to the membrane. The KCNA1–PKA pathway regulates the membrane potential and directly impacting the membrane potential induces senescence in nontransformed cells. Interestingly, restoring KCNA1 at the membrane inhibits oncogene-induced cellular transfor-

mation in NIH3T3 cells. In those cells, KCNA1 blocks oncogene-induced transformation without inducing cellular senescence, suggesting a more general tumor-suppressive function of KCNA1.

These data strongly support that the function of KCNA1 as a regulator of the extracellular concentration of potassium is required for its antioncogenic effects. However, to definitively prove it, it would be interesting to generate a KCNA1 point mutant able to translocate to the plasma membrane in response to the oncogenic stress, but unable to open and to allow potassium exit.

Evidence for the tumor-suppressive activity for KCNA1 is also provided by the decreased expression of KCNA1 observed in breast cancers (26) and also our observation that low level of KCNA1 correlates with increase aggressiveness of primary breast tumors. Furthermore, the KCNA1 gene is known to be repressed by the polycomb repressive complexes and by DNA methyltransferases (34, 35), two systems that are involved in the repression of tumor-suppressive genes (36, 37), and this supports a possible tumor-suppressive role for KCNA1.

We observed that oncogenic stress as well as a direct PKA inhibition results in an increased KCNA1 expression. Interestingly, cAMP and PKA activity has been associated with KCNA1 mRNA destabilization (38). However, we show that the membrane localization of KCNA1 is due to its increase in RNA or protein expression, because overexpression of KCNA1 by ectopic expression does not result in increased membrane localization. The membrane localization occurs only after the induction of oncogenic stress, by PKA inhibition or by expressing a mutated the S446A, a nonphosphorylatable amino acid. We then end up with a model where PKA controls the level and the localization of KCNA1. Regulation of the tumor-suppressive function of KCNA1 in this study by the PKA, which is considered to be a possible target for cancer therapy due to its involvement in tumor initiation and progression in numerous cancers (39), suggests that targeting PKA in cancer may activate the KCNA1 tumor-suppressive pathway.

Our data also indicate that in nontransformed cells, the oncogenic stress results in an inhibition of the PKA activity. It has been reported that OIS induces feedback loops aiming to switch off the oncogenic signal (28). As PKA activity positively regulates the Raf–MEK pathway (40, 41), we speculate that the decrease in the PKA activity during OIS that we observed can be due to activation of such loops.

The use of KCNA1 S446E phosphomimetic mutant seems to behave as a dominant negative form as it favors OIS escape and inhibits membrane potential changes during the oncogenic stress. KCNA1 exerts its potassium channel function under an homotetramer form, and our results support the view that, in the tetramer, the phosphorylated form is dominant over the nonphosphorylated one.

Interestingly, the OIS in the HMECs, the cellular model we used, does not involve p16 as it is not expressed because of its hypermethylation (19), and does not required p53 as previously shown (17) and confirmed by us (data not shown). A major future challenge will, thus, be to find out how the exit of potassium and membrane potential changes regulate senescence outcome.
Thus, overall, our data support the view that the PKA-controlled KCNA1 membrane localization switch regulates the response to oncogenic stress. When functional, this pathway should contribute to sensing the aberrant oncogenic signal by responding to senescence induction and blocking of transformation. When the pathway is nonfunctional, this could favor disabling of the senescence program and increase the oncogene-induced transformation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Lallet—Daher, C. Wiel, D. Gitenay, B. Le Calve, S. Aubert, D. Vindrieux, D. Bernard

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