

RESEARCH ARTICLE

TRPC1 is a differential regulator of hypoxia-mediated events and Akt signalling in PTEN-deficient breast cancer cells

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ABSTRACT

Hypoxia is a feature of the tumour microenvironment that promotes invasiveness, resistance to chemotherapeutics and cell survival. Our studies identify the transient receptor potential canonical-1 (TRPC1) ion channel as a key component of responses to hypoxia in breast cancer cells. This regulation includes control of specific epithelial to mesenchymal transition (EMT) events and hypoxia-mediated activation of signalling pathways such as activation of the EGFR, STAT3 and the autophagy marker LC3B, through hypoxia-inducible factor-1 α (HIF1 α)-dependent and -independent mechanisms. TRPC1 regulated HIF1 α levels in PTEN-deficient MDA-MB-468 and HCC1569 breast cancer cell lines. This regulation arises from effects on the constitutive translation of HIF1 α under normoxic conditions via an Akt-dependent pathway. In further support of the role of TRPC1 in EMT, its expression is closely associated with EMT- and metastasis-related genes in breast tumours, and is enhanced in basal B breast cancer cell lines. TRPC1 expression is also significantly prognostic for basal breast cancers, particularly those classified as lymph node positive. The defined roles of TRPC1 identified here could be therapeutically exploited for the control of oncogenic pathways in breast cancer cells.

KEY WORDS: Breast cancer, Ca²⁺, Hypoxia, Akt, PTEN, TRPC1, Signal transduction

INTRODUCTION

The microenvironment of breast tumours is dynamic and can be a key factor in contributing to breast cancer progression (Bissell and Hines, 2011). One essential feature of the breast tumour microenvironment is hypoxia (Semenza, 2016). Hypoxia in breast tumours is a trigger for angiogenesis and other processes important in metastasis, and in the preparation of the metastatic niche (Semenza, 2013). Hypoxia is also crucial for the promotion of breast cancer cells to a more invasive phenotype via epithelial to mesenchymal transition (EMT) (Cooke et al., 2012; Lester et al., 2007).

EMT in breast cancer cells is associated with a remodelling of cellular adhesion through the loss of epithelial markers such as E-cadherin, increased levels of specific transcription factors such as Snail, Zeb and Twist family proteins, and the expression of mesenchymal markers such as the intermediate filament protein vimentin (Tomaskovic-Crook et al., 2009; Ye and Weinberg, 2015). EMT is also associated with the acquisition of resistance to many antineoplastic agents and increased invasiveness (Ye and Weinberg, 2015). We recently identified the Ca²⁺ signal as a regulator of the induction of the EMT marker vimentin by epidermal growth factor (EGF) (Davis et al., 2013). Hence, components of Ca²⁺ signalling in breast cancer cells may represent opportunities to selectively exploit and control specific metastatic and resistance pathways associated with EMT.

Ca²⁺-permeable ion channels represent important and selective regulators of intracellular Ca²⁺ homeostasis and responses to external stimuli, and are encoded by over 40 genes. Transient receptor potential (TRP) ion channels (Wu et al., 2010) have clear physiological and pathological roles, and often act as sensors of changing environmental cues. TRPV1 is a heat-responsive ion channel, TRPM8 senses cold, and TRPM2 is sensitive to oxidative stress (Wu et al., 2010) and is fundamental in signalling cascades responsible for acetaminophen (paracetamol)-induced liver damage (Kheradpezhough et al., 2014). TRPC5 is important in semaphorin 3A-induced neuronal growth cone collapse (Kaczmarek et al., 2012) and fear-related behaviour (Riccio et al., 2009), and is also associated with the acquisition of multidrug resistance in breast cancer cells (Ma et al., 2012, 2014). TRPC1 was the first TRP ion channel identified in mammals (Wu et al., 2010) and it is expressed in a variety of cell types (Cheng et al., 2013; Nilius and Szallasi, 2014); however, a detailed understanding of its role(s) in many contexts remains unclear and controversial. TRPC1 has also been described as contributing to store-operated Ca²⁺ entry, often in association with the canonical store-operated Ca²⁺ channel component Orai1 and the Ca²⁺ store sensor STIM1 (Cheng et al., 2013).

TRPC1 is associated with pathophysiological processes in hypoxia in smooth muscle cells of the pulmonary system, being important in pulmonary vascular remodelling associated with chronic hypoxia (Malczyk et al., 2013) and hypoxia-induced enhancement of vascular tone (Xia et al., 2014). Chronic hypoxia is also an inducer of TRPC1 expression in pulmonary arterial smooth muscle cells via hypoxia-inducible factor 1 α (HIF1 α) (Wang et al., 2006). Despite the link between hypoxia and TRPC1 in the vascular system, no studies have explored the role of TRPC1 in the response to hypoxia in breast cancer cells. We have assessed the potential role of TRPCs in hypoxia-induced EMT in breast cancer cells and show that a specific remodelling of TRPC1 expression is a feature of hypoxia-associated EMT in breast cancer cells via HIF1 α . We also define TRPC1 as a regulator of specific hypoxia-induced changes in

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EMT marker induction. Unexpectedly, this regulation is through both HIF1 α -dependent and -independent mechanisms and involves the differential regulation of specific cellular signalling pathways.

RESULTS

TRPC1 expression is increased during hypoxia and is required for hypoxic induction of Snail and Claudin-4

We assessed the mRNA expression pattern of TRPC family genes in MDA-MB-468 breast cancer cells, with the exception of *TRPC2* (a pseudogene in humans; Yildirim and Birnbaumer, 2007), in both hypoxic and normoxic conditions. *TRPC4*, -5, -6 and -7 mRNA was not detectable in either hypoxic or normoxic samples (Fig. 1A). While *TRPC3* mRNA levels were not significantly changed upon hypoxia, *TRPC1* mRNA levels were significantly increased (Fig. 1A,Bi). Hypoxia-induced *TRPC1* upregulation was also observed in MDA-MB-231 and HCC1569 breast cancer cells (Fig. S1). *TRPC1* mRNA upregulation in MDA-MB-468 cells was specific to hypoxia, as *TRPC1* mRNA levels were modestly downregulated upon addition of EGF, another EMT inducer (Fig. 1Bii). Hypoxia-induced upregulation of *TRPC1* was dependent on HIF1 α , as siRNA-mediated silencing of *HIF1A* (Fig. 1Ci) attenuated hypoxia-induced *TRPC1* mRNA upregulation (Fig. 1Cii). Assessment of potential HIF binding sites in the promoter region of *TRPC1* identified binding motifs for HIF1 α and HIF1 β (Fig. S2). However, only the silencing of *HIF1A* reduced hypoxia-mediated induction of *TRPC1* mRNA levels (Fig. 1Cii); siRNA against *HIF1B* or *HIF2A* had no effect on the increase in *TRPC1* expression (Fig. S3). Analysis of the gene expression profile of *TRPC1* in human breast cancer cell lines, revealed a significant upregulation of *TRPC1* in Basal B cell lines (which exhibit enhanced mesenchymal-like features; Blick et al., 2010; Neve et al., 2006) compared with other subgroups (Fig. 1D).

To determine whether TRPC1 is involved in the induction of hypoxia-induced EMT, we assessed the effect of its siRNA-mediated silencing on EMT markers. Hypoxia (24 h) resulted in significant upregulation of the mesenchymal markers vimentin, Snail (also known as SNAI1), Twist (TWIST1) and N-cadherin (CDH2), and downregulation of the epithelial marker claudin-4 (CLDN4) (Fig. 1F). Silencing of TRPC1 (Fig. 1E) under hypoxic conditions significantly attenuated the induction of the EMT transcription factor Snail and partially prevented the reduction of the epithelial marker claudin-4 (24 h); however, induction of the other EMT markers by hypoxia was independent of TRPC1 (Fig. 1F). TRPC1 is therefore the first identified ion channel regulator of the EMT transcription factor Snail and epithelial marker claudin-4. This regulation appears to be hypoxia specific as silencing of TRPC1 did not have any significant effect on the basal levels of Snail and claudin-4 in normoxia (Fig. S4). The differential regulation of hypoxia-mediated events was further explored through assessment of cellular signalling pathways activated by hypoxia in breast cancer cells.

TRPC1 is a regulator of hypoxia-induced EGFR and STAT3 activation

EGFR and STAT3 signalling are two key hypoxia-associated signalling pathways (Selvendiran et al., 2009; Pawlus et al., 2014; Peng et al., 2006) that also have important roles in EMT induced by hypoxia (Cui et al., 2016; Misra et al., 2012) and EGF (Lo et al., 2007), although their potential regulation by TRPC1 is unknown. We assessed EGFR at Y1173 (an important site of EGFR autophosphorylation; Kondratov et al., 2010) and STAT3 phosphorylation at Y705 in MDA-MB-468 cells under hypoxic

conditions for 6, 24 and 48 h. Phosphorylation of EGFR was significantly enhanced after 24 and 48 h of hypoxia, with no significant concomitant change in total EGFR protein (Fig. 2A). Likewise, total STAT3 protein levels were not altered by hypoxia, but phosphorylation of STAT3 was significantly increased at 24 h (Fig. 2A). Silencing of *TRPC1* with siRNA significantly reduced hypoxia-induced EGFR and STAT3 phosphorylation, with no change in total EGFR or STAT3 levels, respectively (Fig. 2B,C). Given the upregulation of TRPC1 with hypoxia but not EGF (Fig. 1B), the selective role of TRPC1 in hypoxia was explored by silencing *TRPC1* and assessing EGFR and STAT3 phosphorylation induced by EGF. *TRPC1* silencing did not alter EGF-induced phosphorylation of EGFR (Fig. 2D) or inhibit STAT3 phosphorylation (Fig. 2E); instead, augmentation of STAT3 phosphorylation was evident (Fig. 2E). These findings provide further evidence for the selectivity of TRPC1 in hypoxia and identify a role for TRPC1 in hypoxia-induced transactivation of EGFR.

Regulation of non-stimulated and store-operated Ca²⁺-entry pathways by TRPC1 after hypoxia

TRPC1 has been implicated in store-operated Ca²⁺ entry in some cell types (Cheng et al., 2013). Under normoxic conditions in MDA-MB-468 breast cancer cells, TRPC1 is a regulator of non-stimulated (constitutive) Ca²⁺ influx but not of store-operated Ca²⁺ entry (Davis et al., 2012). To assess whether hypoxia alters the nature of the contribution of TRPC1 to Ca²⁺ influx, we assessed the effects of *TRPC1* silencing on non-stimulated (constitutive) Ca²⁺ influx and store-operated Ca²⁺ entry after hypoxia (Fig. 3). Silencing of *TRPC1* had no effect on the cytosolic free Ca²⁺ ([Ca²⁺]_{CYT}) increases associated with store depletion induced by the sarco/endoplasmic reticulum Ca²⁺ ATPase inhibitor cyclopiazonic acid (CPA) (Fig. 3A,B, peak 1). However, *TRPC1* silencing significantly augmented store-operated Ca²⁺ influx (Fig. 3A,B, peak 2) and reduced non-stimulated Ca²⁺ influx compared with the non-targeting control (Fig. 3A,B). Hence, hypoxia (24 h) was not associated with a change in the nature of the contribution of TRPC1 to basal Ca²⁺ influx or store-operated Ca²⁺ entry, as the effects were identical to those seen with normoxia (Davis et al., 2012).

TRPC1 regulates HIF1 α levels and Akt activation in MDA-MB-468 breast cancer cells

As TRPC1 modified specific EMT markers and cellular signalling cascades induced by hypoxia, we sought to determine whether TRPC1 was involved in the early induction of the hypoxic transcriptional regulator HIF1 α . Pronounced accumulation of HIF1 α protein was evident at 6 h, and this early induction was significantly inhibited by silencing of *TRPC1* (Fig. 4A and Fig. S6A). We assessed whether this regulation was solely through the reduced ability of cells to sense reduced O₂ levels by silencing *TRPC1* and examining basal protein and mRNA levels of HIF1 α under normoxic conditions. Consistent with its status as a PTEN-deficient breast cancer cell line (Li et al., 1997; Zundel et al., 2000), MDA-MB-468 breast cancer cells exhibited some basal expression of HIF1 α protein, which was almost completely abolished upon *TRPC1* silencing (Fig. 4B), and this effect was not mediated through a decrease in *HIF1A* mRNA levels (Fig. 4C). Hence, the effect of TRPC1 on HIF1 α protein did not occur via transcriptional modulation but rather via effects on HIF1 α translational and/or degradation.

Hypoxia-induced HIF1 α protein stabilisation is predominantly via inhibition of proteasome-mediated degradation as a result of low levels of O₂ (Salceda and Caro, 1997). To assess if TRPC1 regulation of HIF1 α protein was through this O₂-dependent

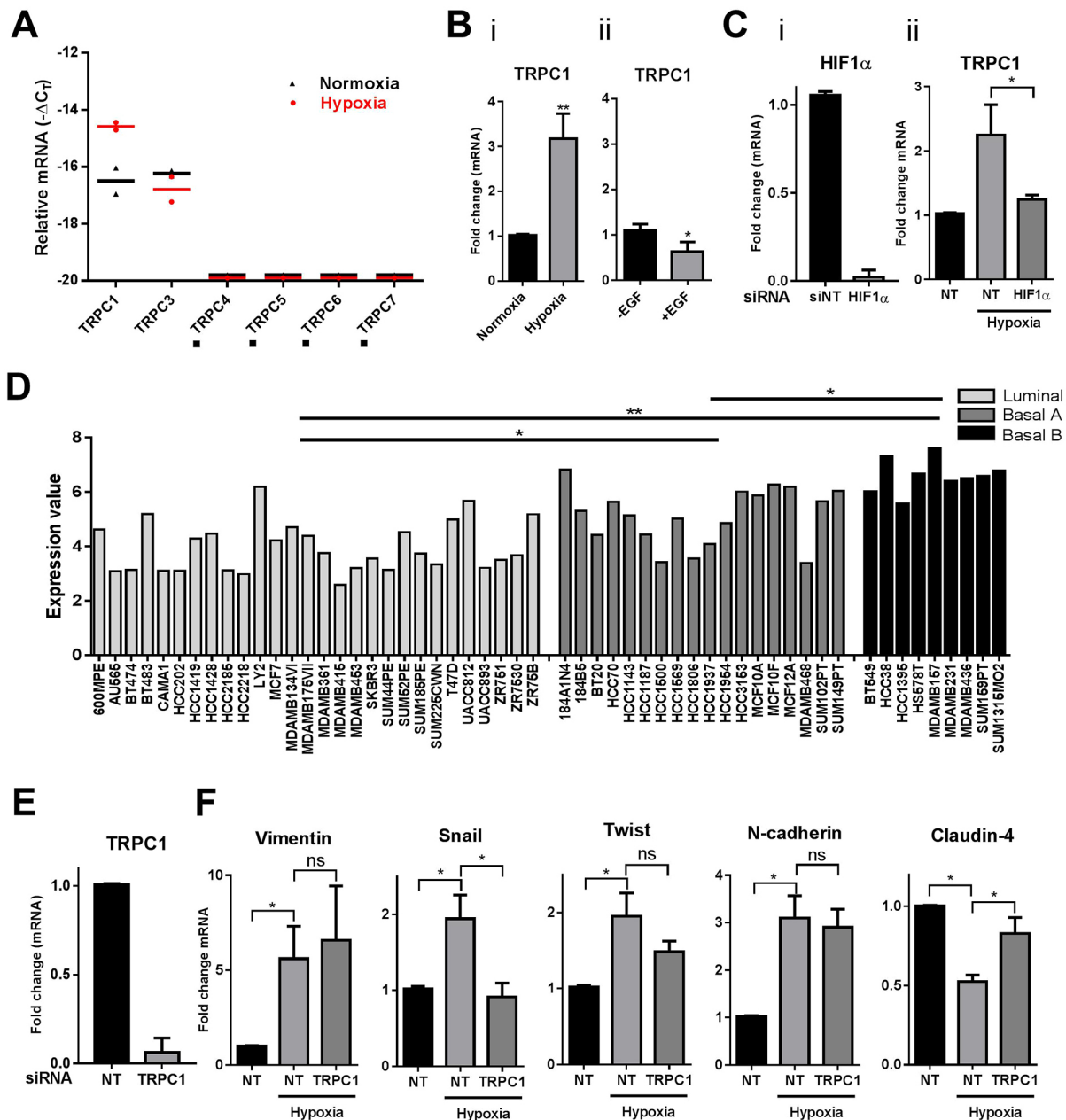


Fig. 1. *TRPC1* expression is upregulated by hypoxia via *HIF1α* and its silencing attenuates hypoxia-induced changes in *Snail* and *claudin-4*.

(A) Transcriptional profile of TRPC isoforms [relative mRNA ($-\Delta\Delta C_T$)] showed selective upregulation of *TRPC1* with hypoxia (24 h) [■ denotes a target below the limit of detection ($C_T > 35$)]. (B) *TRPC1* induction in MDA-MB-468 cells 24 h after hypoxia (i) or 50 ng/ml EGF (ii). * $P < 0.05$, ** $P < 0.01$ compared with control (unpaired *t*-test), $n = 3$, mean \pm s.d. (C) siRNA-mediated silencing of *HIF1A* relative to non-targeting control (NT) (i) and its effect on hypoxia-induced *TRPC1* mRNA upregulation (24 h hypoxia, ii). * $P < 0.01$ (one-way ANOVA, with Bonferroni's multiple comparisons), $n = 3$, mean \pm s.d. (D) *TRPC1* assessment in gene expression data of 50 breast cancer cell lines and 5 non-malignant breast cell lines, including three subtypes of luminal, basal A and basal B/mesenchymal. Data are from Array Express (accession no. E-MTAB-181) (Heiser et al., 2012) and are normalised log₂-transformed values; * $P < 0.001$, ** $P < 0.0001$ (one-way ANOVA, with Tukey's multiple comparisons). (E) Confirmation of *TRPC1* siRNA-mediated silencing. (F) Effect of *TRPC1* silencing on hypoxia-mediated induction of EMT markers in MD-MB-468 cells. * $P < 0.01$ (one-way ANOVA, with Bonferroni's multiple comparisons), ns, not significant; $n = 3$, mean \pm s.d.

proteolysis mechanism, we treated *TRPC1*-silenced and control cells with the proteasome inhibitor MG132 (Salceda and Caro, 1997). As expected, MG132 treatment increased *HIF1α* protein levels (Fig. 4D) and siRNA-mediated silencing of *TRPC1* reduced the MG132-induced increases in *HIF1α* (Fig. 4D). *TRPC1* does not, therefore, regulate *HIF1α* via proteasome-mediated degradation.

Another possible intersection between *HIF1α* and *TRPC1* could be via Akt (AKT1). *HIF1α* translation is upregulated by Akt (Pore et al., 2006), and Akt activity is enhanced in PTEN-deficient cells

(Cantley and Neel, 1999; deGraffenried et al., 2004; Soria et al., 2002), which include MDA-MB-468 cells (Li et al., 1997). We therefore assessed the effects of *TRPC1* silencing on the constitutive activation of Akt and the role of the Akt pathway on *HIF1α* levels in MDA-MB-468 cells. These studies were performed under normoxic conditions since we showed that *TRPC1* could regulate *HIF1α* levels in the absence of hypoxia (see Fig. 4B). *TRPC1* silencing significantly reduced basal Akt phosphorylation but not total levels of Akt protein (Fig. 4E), whereas basal phosphorylation of both

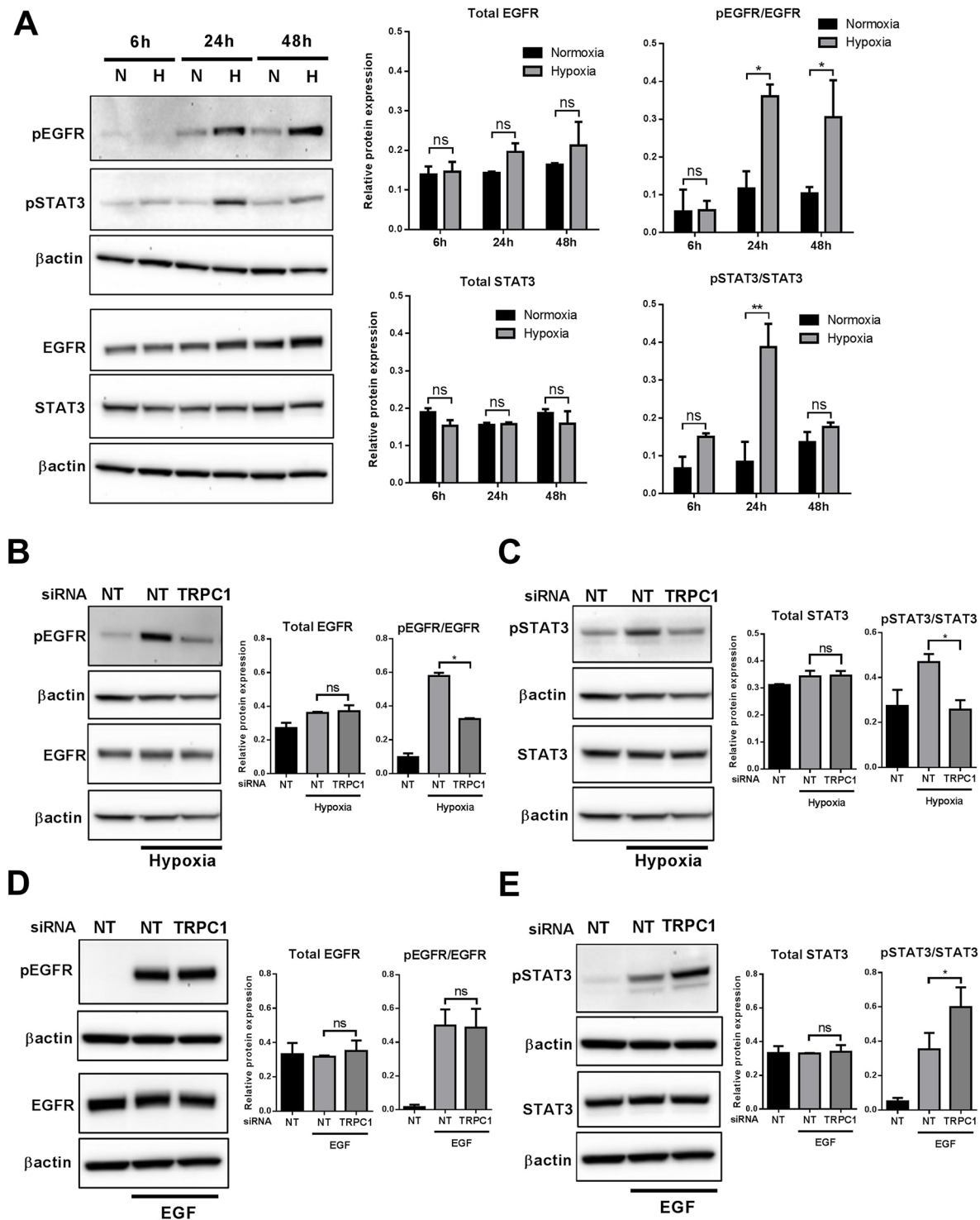


Fig. 2. TRPC1 promotes hypoxia but not EGF-induced EGFR(Y1173) and STAT3(Y705) phosphorylation. (A) MDA-MB-468 breast cancer cells were serum reduced (24 h) and exposed to hypoxia for 6, 24 and 48 h; representative immunoblot and densitometry analysis of total EGFR and STAT3 (normalised to β-actin) and phosphorylated EGFR (Y1173) and STAT3 (Y705) (normalised to β-actin and total EGFR and STAT3). * $P < 0.01$, ** $P < 0.001$ (two-way ANOVA, with Bonferroni's multiple comparisons); ns, not significant. (B) Representative immunoblot and densitometry analysis of the effect of *TRPC1* silencing on total EGFR protein and phosphorylation with hypoxia (24 h). * $P < 0.001$ (one-way ANOVA, with Bonferroni's multiple comparisons). (C) Representative immunoblot and densitometry analysis of the effect of *TRPC1* silencing on total STAT3 protein and phosphorylation with hypoxia (24 h). * $P < 0.01$ (Bonferroni one-way ANOVA). (D) Representative immunoblot and densitometry analysis of the effect of *TRPC1* silencing on total EGFR protein and phosphorylated levels with EGF (20 min). (E) Representative immunoblot and densitometry analysis of the effect of *TRPC1* silencing on total STAT3 protein and phosphorylation with EGF (20 min). * $P < 0.05$ (Bonferroni one-way ANOVA). The same membrane was blotted for both STAT3 and EGFR (both total and phosphorylated), and therefore the same β-actin loading controls are used for comparisons in B and C, and D and E, respectively. All densitometry data presented are the mean \pm s.d. of three independent experiments and normalised to β-actin. Relative protein expression is defined as quantification of fraction of total protein band intensity after normalisation to β-actin loading control.

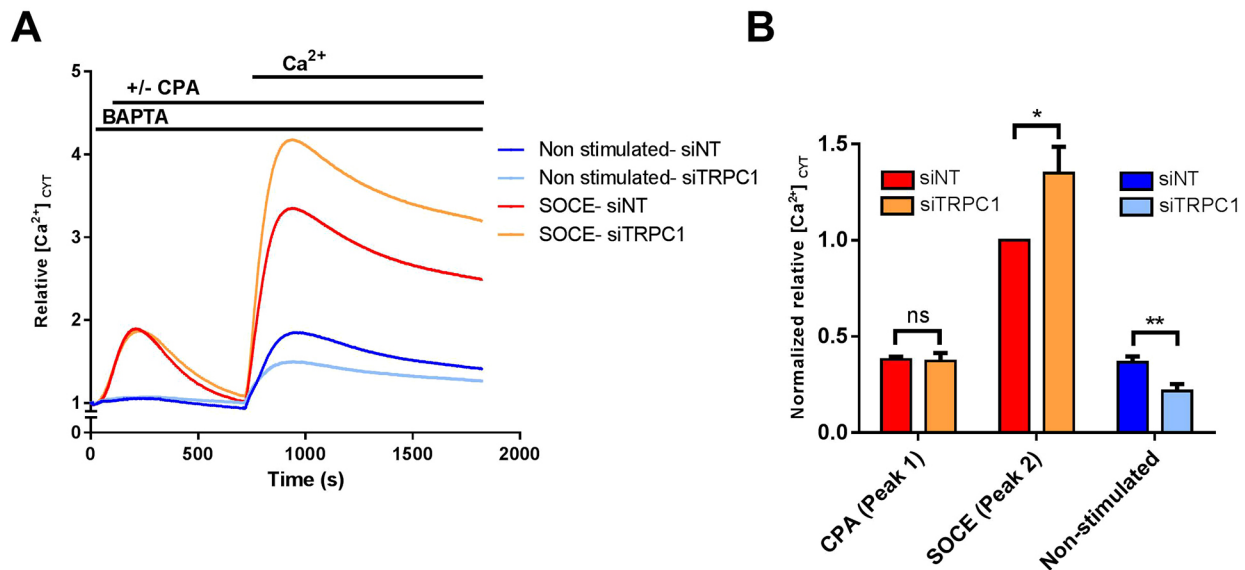


Fig. 3. *TRPC1* silencing attenuates non-stimulated Ca^{2+} influx and augments store-operated Ca^{2+} entry after hypoxia in MDA-MB-468 breast cancer cells. (A) Mean $[\text{Ca}^{2+}]_{\text{cyt}}$ levels during assessment of non-stimulated Ca^{2+} influx (DMSO control) and store-operated Ca^{2+} entry (SOCE; 10 μM CPA) with or without *TRPC1* siRNA silencing after exposure to hypoxia (24 h). (B) Quantification of relative $[\text{Ca}^{2+}]_{\text{cyt}}$ (mean \pm s.d., $n=3$) normalised to the maximum peak height of the store-operated Ca^{2+} entry of the non-targeting siRNA control. For each group, the siTRPC1 is compared with its non-targeting (NT) control. * $P<0.05$, ** $P<0.01$ (unpaired *t*-test).

EGFR and STAT3 were unaffected in normoxia (Fig. 4E), unlike the decrease seen with *TRPC1* silencing and hypoxia-induced EGFR and STAT3 phosphorylation (Fig. 2B,C). MK-2206 2HCl, an Akt-selective inhibitor, concentration-dependently phenocopied the suppression of basal HIF1 α levels by *TRPC1* silencing (Fig. 4F), implicating Akt as the conduit between *TRPC1* and HIF1 α translation. Similarly, in *PTEN*-null HCC1569 breast cancer cells (Chou et al., 2009), *TRPC1* silencing (Fig. 4Gi) significantly reduced both basal Akt phosphorylation (Fig. 4Gii) and basal HIF1 α expression (Fig. 4Giii). To confirm this observation, we overexpressed *TRPC1* using a commercially available *TRPC1* plasmid (Fig. 4Hi) and examined the levels of HIF1 α and Akt phosphorylation (Fig. 4Hii). The data show that *TRPC1* overexpression is sufficient to induce HIF1 α and Akt phosphorylation, and this induction of HIF α is inhibited by Akt inhibition (Fig. 4Hii). These results highlight the important role of *TRPC1* in the Akt–HIF1 α pathway in *PTEN*-deficient breast cancer cells, even under normoxic conditions.

HIF1A silencing attenuates hypoxia-mediated changes in *SNAIL* gene expression and non-stimulated Ca^{2+} influx

Since we showed that HIF1 α is a *TRPC1*-sensitive protein in this model, we assessed whether HIF1 α is an intermediate between *TRPC1* and the induction of EMT markers and cellular signalling cascades induced by hypoxia. To achieve this aim, we silenced *HIF1A* and compared these effects with those observed upon *TRPC1* silencing. Some of the effects of *TRPC1* silencing were independent of HIF1 α . Silencing of *HIF1A* phenocopied the effects of *TRPC1* silencing on hypoxia-induced changes in the expression of Snail but not claudin-4 (Fig. 5A). Despite the ability of HIF1 α to regulate *TRPC1* expression (as shown in Fig. 1C), *HIF1A* silencing had the opposite effect of *TRPC1* silencing on hypoxia-mediated changes in EGFR and STAT3 phosphorylation (Fig. 5B). Although this result may be unexpected, it is not totally surprising given that *TRPC1* expression is not reduced to the same degree by silencing of *HIF1A* compared with *TRPC1*. Furthermore, HIF1 α as a hypoxic transcriptional regulator, may influence other pathways that may

have opposing effects to those of *TRPC1* on EGFR and STAT3 activity. However, these results do clearly define effects of *TRPC1* that are independent of HIF1 α , namely hypoxia-induced changes in claudin-4 expression and EGFR and STAT3 phosphorylation. The differential contribution of HIF1 α to the effects of *TRPC1* silencing was also evident when assessing Ca^{2+} homeostasis. *HIF1A* silencing reduced constitutive Ca^{2+} influx in a similar manner to that seen with silencing of *TRPC1*; however, *HIF1A* siRNA did not promote store-operated Ca^{2+} entry, as was seen upon *TRPC1* siRNA treatment (Fig. 5C). Hence, some of the responses of breast cancer cells to hypoxia are clearly influenced by *TRPC1*, but the contribution of *TRPC1* appears to involve both HIF1 α -dependent and -independent mechanisms.

***TRPC1* modulates hypoxia-induced changes in the autophagy marker LC3B via regulation of EGFR**

In addition to EMT, one other event associated with hypoxia is autophagy. Autophagy is a key hypoxia-induced survival mechanism seen in some cancer cells (Hu et al., 2012), and is Ca^{2+} signalling dependent (Cárdenas et al., 2010; Decuypere et al., 2013; Kondratskyi et al., 2013; Parys et al., 2012). *TRPC1* silencing significantly reduced the protein levels of the hypoxia-induced autophagy marker LC3BII (also known as MAP1LC3BII, where II indicates the lipidated form) (Fig. 6A and Fig. S6B). Silencing of *EGFR* also significantly attenuated hypoxia-induced elevation of LC3BII levels, whereas silencing of either *STAT3* or *HIF1A* had no significant effect (Fig. 6B). These results suggest that hypoxia increases LC3BII levels via a *TRPC1* pathway that is dependent on EGFR phosphorylation but is independent of *TRPC1*-mediated regulation of hypoxia-induced changes in HIF1 α levels or STAT3 activity.

***TRPC1* is associated with breast cancer molecular subtypes with mesenchymal features and poor prognosis in basal breast cancers**

Collectively, the results above implicate *TRPC1* in some of the pathways associated with hypoxia-induced EMT. We assessed

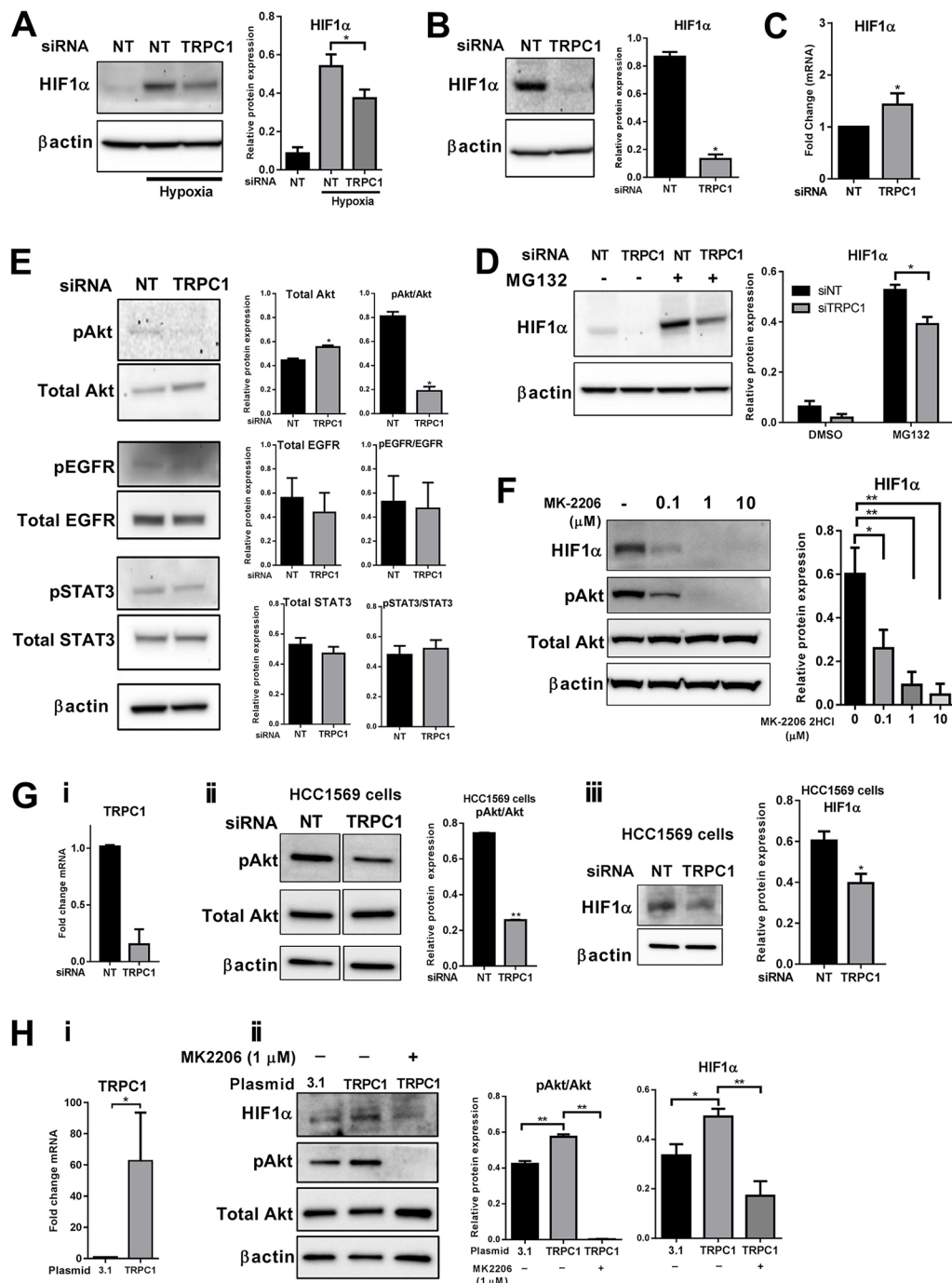


Fig. 4. TRPC1 regulation of HIF1 α translation and basal activation of Akt in MDA-MB-468 breast cancer cells in normoxia. (A) Representative immunoblot (left) and quantitative analyses (right) of the effect of *TRPC1* siRNA-mediated silencing on hypoxia-induced (6 h) increases in HIF1 α protein compared with NT siRNA in normoxia and hypoxia ($n=3$, mean \pm s.d., $*P<0.05$, one-way ANOVA, with Bonferroni's multiple comparisons). (B) Immunoblot (left) and quantitative analysis (right) of HIF1 α protein levels in MDA-MB-468 cells with *TRPC1* and NT siRNA both under normoxic conditions ($n=3$, mean \pm s.d., $*P<0.001$, unpaired *t*-test). (C) Assessment of the effect of *TRPC1* silencing on *HIF1A* mRNA levels in normoxia ($n=3$, mean \pm s.d., $*P<0.05$, unpaired *t*-test). (D) Effect of *TRPC1* silencing on MG132 proteasome inhibitor-induced increases in HIF1 α protein ($n=3$, mean \pm s.d., $*P<0.001$, two-way ANOVA, with Bonferroni's multiple comparisons). (E) Levels of total and phosphorylated levels of Akt, EGFR and STAT3 in the absence and presence of *TRPC1* siRNA in normoxia ($n=3$, mean \pm s.d., $*P<0.001$, unpaired *t*-test). (F) Representative immunoblot (left) and quantitative analysis (right) of HIF1 α protein levels in MDA-MB-468 cells treated with the Akt inhibitor MK-2206 2HCl for 24 h ($n=3$, mean \pm s.d., $*P<0.01$, $**P<0.001$, one-way ANOVA, with Bonferroni's multiple comparisons). (G) Confirmation of *TRPC1* silencing (i), and representative immunoblot and quantitative analysis of the basal level of Akt phosphorylation (ii) and HIF1 α protein (iii) in HCC1569 cells with *TRPC1* silencing ($n=3$, mean \pm s.d., $*P<0.01$, $**P<0.0001$ unpaired *t*-test). All densitometry data are presented as the mean of three independent experiments. The uncropped full blots of Gii are shown in Fig. S5. Relative protein expression is defined as quantification of fraction of total protein band intensity after normalisation to β -actin loading control. (H) *TRPC1* plasmid increases *TRPC1* levels compared with PCDNA3.1 control plasmid (3.1) (i; $n=3$, mean \pm s.d., $*P<0.05$, unpaired *t*-test). *TRPC1* overexpression increases levels of Akt phosphorylation and HIF1 α protein in MDA-MB-468 cells and these increases are reduced by the Akt inhibitor MK2206 (1 μ M) (ii; $n=3$, mean \pm s.d., $*P<0.05$, $**P<0.001$, one-way ANOVA, with Bonferroni's multiple comparisons).

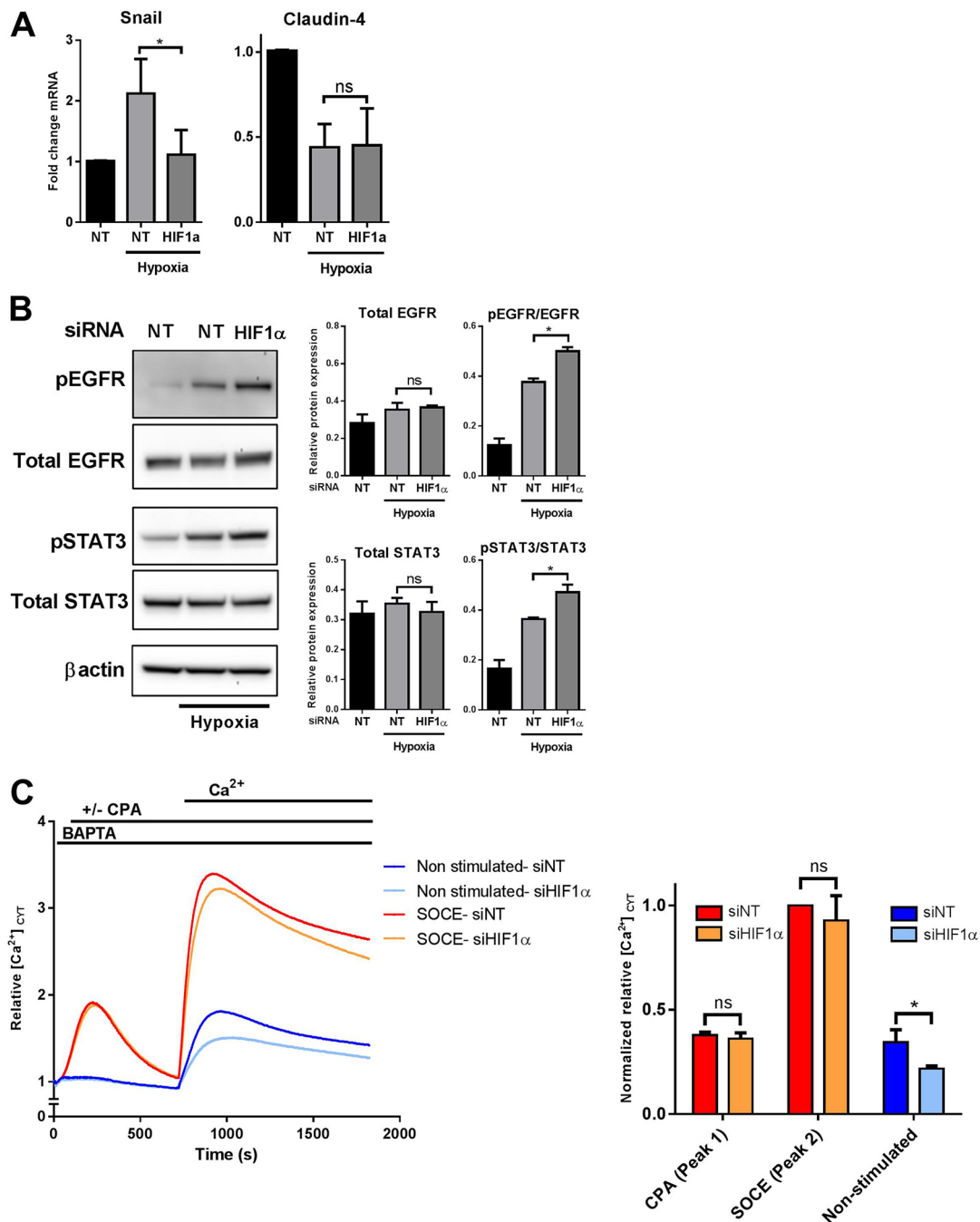


Fig. 5. Assessment of the involvement of HIF1α in hypoxic events regulated by TRPC1. (A) Effect of *HIF1A* siRNA-mediated silencing on the levels of hypoxia (24 h)-mediated upregulation of the mesenchymal marker *Snail1*, and hypoxia-mediated downregulation of the epithelial marker *Claudin-4* (24 h hypoxia) ($n=3$, mean±s.d., * $P<0.05$, one-way ANOVA, with Bonferroni's multiple comparisons). (B) Representative immunoblot (left) and quantitative analyses (right) of the effect of *HIF1A* silencing on the levels of total and phosphorylated EGFR(Y1173) and STAT3(Y705) with 24 h hypoxia. All densitometry data are presented as the mean±s.d. of three independent experiments (* $P<0.01$, one-way ANOVA, with Bonferroni's multiple comparisons). (C) Mean [Ca²⁺]_{CYT} levels during assessment of non-stimulated Ca²⁺ influx (DMSO control) and store-operated Ca²⁺ entry (SOCE; 10 μM CPA) with or without *HIF1A* silencing after exposure to hypoxia (24 h) (left), quantification of relative [Ca²⁺]_{CYT} normalised to the maximum peak height of the store-operated Ca²⁺ entry of the non-targeting siRNA control (right). For each group, the siHIF1α was compared with its non-targeting control. Values are mean±s.d., $n=3$; * $P<0.05$, unpaired *t*-test. Relative protein expression is defined as quantification of the fraction of total protein band intensity after normalisation to β-actin loading control.

whether such an association was also present in clinical breast cancers given that some breast cancer subtypes are more enriched in EMT markers than others. Our assessment of PAM50 breast cancer molecular subtypes (Harrell et al., 2012), showed that the claudin-low (C-Low) subtype (predominantly associated with EMT and stem cell characteristics; Hennessy et al., 2009), had the highest

levels of *TRPC1* expression, with significantly higher levels than basal, HER2, luminal A and luminal B breast cancers [Fig. 7A, expression data from The University of North Carolina (UNC) (Harrell et al., 2012); see Materials and methods]. Consistent with this result, assessment of the recently defined triple-negative breast cancer (TNBC) subtypes identified the highest levels of *TRPC1* in

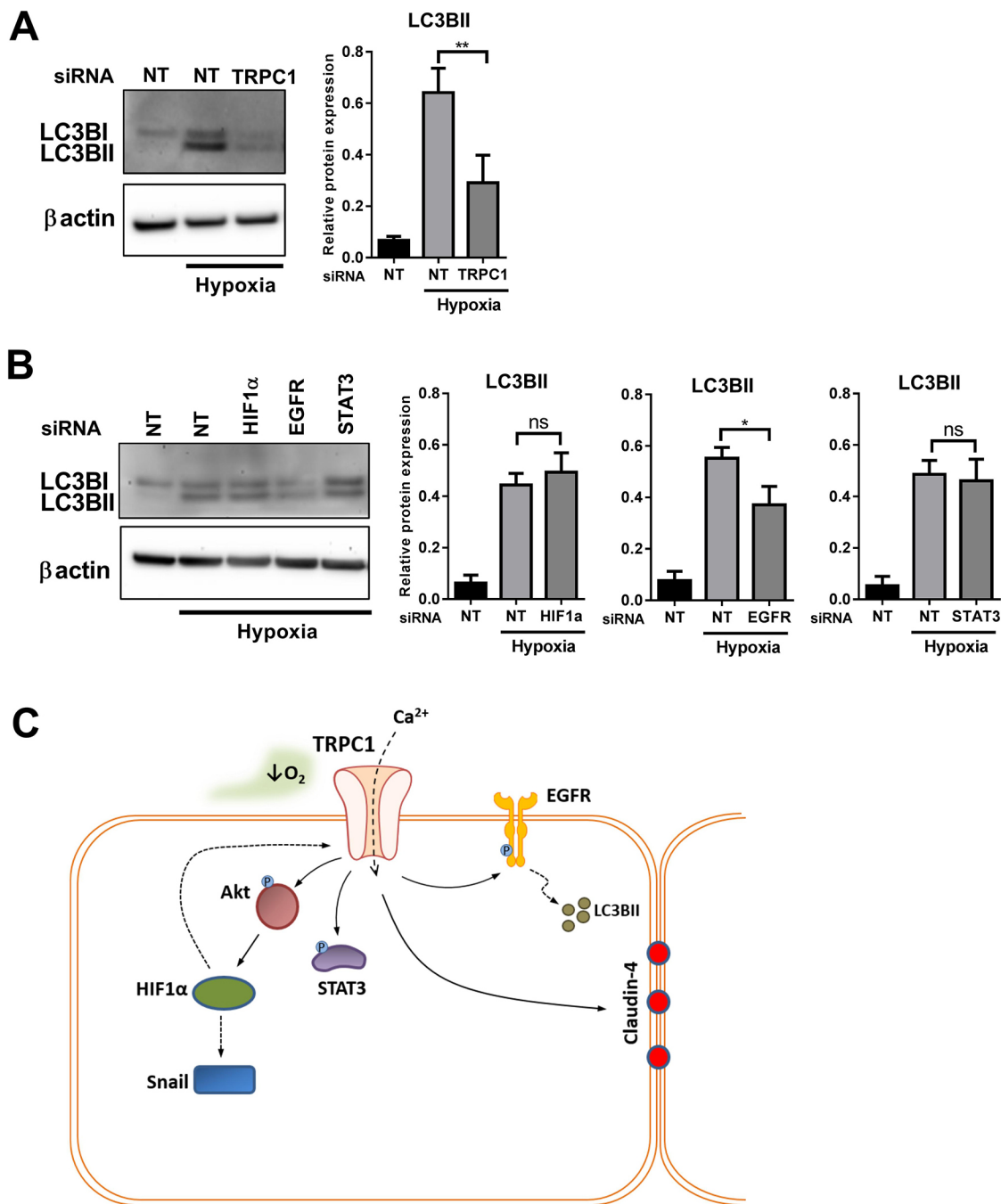


Fig. 6. Regulation of hypoxia-mediated induction of the autophagy marker LC3BII by TRPC1 and EGFR. (A) Assessment of the effect of *TRPC1* silencing on hypoxia (48 h)-mediated upregulation of LC3BII protein. Representative immunoblot (left) and quantitative analyses (right) of the LC3BII band as the active autophagosome membrane-bound form. (B) Representative immunoblot (left) and quantitative analyses (right) of LC3BII levels with siRNA-mediated silencing of *HIF1 α* , *EGFR* and *STAT3*. All densitometry data are presented as the mean of three independent experiments ($n=3$, mean \pm s.d., * $P<0.05$, ** $P<0.01$, one-way ANOVA, with Bonferroni's multiple comparisons). Relative protein expression is defined as quantification of fraction of total protein band intensity after normalisation to β -actin loading control. (C) Schematic representation of proposed hypoxia-associated events regulated by TRPC1. TRPC1 regulates HIF-1 α protein levels via an Akt-dependent pathway. TRPC1 promotes EMT through upregulation of hypoxia-induced *SNAI1* mRNA expression via a HIF-1 α -dependent mechanism and downregulation of *CLDN4*. TRPC1 regulates activation of hypoxia-induced STAT3 and EGFR phosphorylation and hypoxia-induced LC3BII levels via effects on EGFR.

the mesenchymal (MES) subtype, with significantly greater levels than basal-like immune-activated (BLIA) and basal-like immune-suppressed (BLIS) subtypes (Fig. 7B). High levels of TRPC1 are associated with a significantly poorer prognosis in basal breast cancers (Fig. 7C), particularly in basal breast cancers with positive lymph node involvement [Fig. 7D; data sourced from Kaplan–

Meier (KM) plotter; Györfi et al., 2010]. To provide greater insight into those breast cancers with high levels of *TRPC1* expression, the top 100 genes most positively (Table S1) and negatively (Table S2) associated with *TRPC1* in breast cancers were assessed by gene ontology. Genes positively associated with *TRPC1* were significantly enriched for pathways and processes associated with

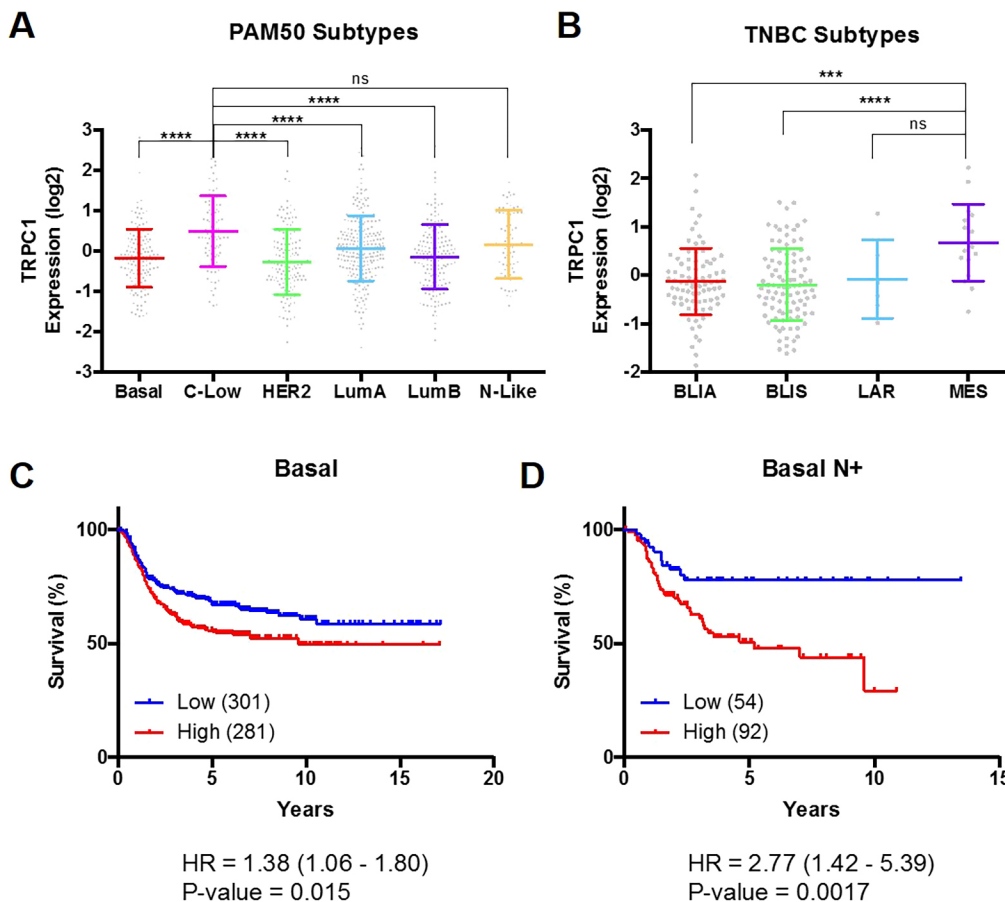


Fig. 7. *TRPC1* expression is enriched in mesenchymal subtypes of breast cancer and stratifies the survival of basal patients with metastatic relapse. (A) Expression of *TRPC1*, log₂ transformed, across the five intrinsic subtypes of breast cancer, including the basal and normal-like derived claudin-low (C-Low). (B) Expression of *TRPC1*, log₂ transformed, across the four subtypes of triple-negative breast cancer. Expression data sourced from UNC (Harrell et al., 2012). Statistical significance for A and B was obtained via a one-way ANOVA with multiple comparisons corrected via Tukey test. *** $P < 0.001$, **** $P < 0.0001$; ns, not significant. (C,D), Kaplan–Meier curves for the stratification of patient relapse-free survival by *TRPC1* expression level in all basal tumours and those that are lymph node-positive (N+). Patient numbers in the high or low expression groups are indicated in brackets, log rank P -values and hazard ratios (HRs) are displayed below each graph; 95% confidence intervals in brackets. Patient survival outcome data sourced from KM-Plotter (see Materials and methods for details).

the mesenchymal phenotype such as those related to wound healing, migration, adhesion and development (Table 1 and Fig. S7). Indeed, these processes are shown to be a consequence of EMT (Tomaskovic-Crook et al., 2009), and the relationship between the positively associated genes with *TRPC1* and these processes further supports the role of *TRPC1* in EMT. In contrast, negatively associated genes were associated with features often reduced as a consequence of EMT, such as chromatin assembly, nucleosome organization and M phase (Table 1 and Fig. S8). This analysis of breast cancer subtypes provides further evidence for an association between *TRPC1* and some aspects of EMT.

DISCUSSION

As a primary tumour develops, the associated hypoxic environment initiates events such as the induction of a more invasive phenotype through EMT (Azimi and Monteith, 2016; Lester et al., 2007) and cell survival via autophagy (Hu et al., 2012). The studies presented here provide evidence for *TRPC1* as a critical regulator of processes induced by hypoxia via HIF1 α -dependent and -independent mechanisms. Our studies have also defined a role for *TRPC1* in the control of HIF1 α expression under normoxic conditions in PTEN-deficient breast cancer cells through an Akt-dependent mechanism.

Initial evidence for a selective role of *TRPC1* in hypoxia was the upregulation of *TRPC1* with hypoxia-induced EMT but not EGF-induced EMT. Increases in *TRPC1* channel expression were sensitive to the silencing of a master regulator of hypoxia responses, *HIF1A* (Weidemann and Johnson, 2008), consistent with the prediction of *TRPC1* as a HIF1 α target gene (Ortiz-Barahona et al., 2010). Cellular signalling can selectively control

different pathways to EMT induction (Kondratov et al., 2010) and the subsequent array of events that include increased migration, resistance to therapeutic agents and the remodelling of cellular adhesion (Tomaskovic-Crook et al., 2009; Ye and Weinberg, 2015). Our studies provide evidence for *TRPC1* as a regulator of hypoxia-mediated EMT pathways associated with changes in claudin-4 and Snail. The observation that *TRPC1* is highest in the claudin-low (PAM50) and MES (TNBC) molecular subtypes, provides compelling evidence for an association between *TRPC1* and aspects on the EMT pathway, and is consistent with its increased expression in the basal B cell line subgroups, which show enhanced mesenchymal-like features (Neve et al., 2006). The relationship between genes positively correlated with *TRPC1* and pathways remodelled in EMT (e.g. wound healing, migration, development, adhesion; Tomaskovic-Crook et al., 2009), further support the placement of *TRPC1* as an ion channel regulator of aspects of EMT and therefore potentially metastasis (Semenza, 2013) and/or therapeutic resistance (Fischer et al., 2015). Indeed, we found that elevated *TRPC1* levels were associated with poor survival in basal breast cancers, particularly those with lymph node involvement.

TRPC1 is not a master regulator of all changes associated with EMT induced by hypoxia since some EMT markers were insensitive to *TRPC1* silencing. As *TRPC1* regulates hypoxia-mediated changes in EMT markers via HIF1 α -dependent and -independent mechanisms (Fig. 6C), our work also reflects the specificity of different signals in inducing EMT. The role of *TRPC1* in hypoxia-mediated EMT is analogous to the role of the ion channel TRPM7 in the control of EGF-induced protein expression of the EMT marker vimentin, but not Twist and N-cadherin (Davis

Table 1. TRPC1 expression associates positively with mesenchymal ontologies and negatively with proliferation ontologies

Gene ontology term	Reference	Observed	Expected	Fold Enrichment	P-value (raw)	P-value (adjusted)
Top 100 positively associated genes by expression						
Regulation of wound healing	77	5	0.42	11.87	6.26E–05	2.00E–3
Extracellular matrix organisation	211	9	1.15	7.8	2.18E–06	3.00E–04
Cellular response to growth factor stimulus	277	10	1.51	6.6	2.58E–06	3.00E–04
Ossification	282	9	1.54	5.84	2.27E–05	1.00E–03
Muscle contraction	254	8	1.39	5.76	7.29E–05	2.10E–03
Blood vessel morphogenesis	433	10	2.37	4.22	1.00E–04	2.30E–03
Coagulation	503	11	2.75	4	8.72E–05	2.30E–03
Cell migration	841	17	4.6	3.7	2.30E–06	3.00E–04
Cell adhesion	954	19	5.22	3.64	6.41E–07	2.00E–04
Organ morphogenesis	802	15	4.39	3.42	2.50E–05	1.00E–03
Regulation of cell proliferation	1177	18	6.44	2.8	5.10E–05	1.70E–03
Response to external stimulus	1323	20	7.23	2.76	2.08E–05	9.00E–04
Regulation of cellular component organisation	1214	18	6.64	2.71	7.64E–05	2.20E–03
Anatomical structure formation involved in morphogenesis	1594	22	8.72	2.52	2.99E–05	1.10E–03
Cellular developmental process	2829	30	15.47	1.94	1.00E–04	2.30E–03
Top 100 negatively associated genes by expression						
Chromatin assembly	117	5	0.59	8.45	4.42E–02	4.42E–02
Nucleosome organisation	128	5	0.65	7.72	5.00E–04	4.92E–02
M phase	539	10	2.73	3.67	4.00E–04	4.72E–02

Gene ontology (GO) analysis produced a hierarchical tree of GO terms, the end point term was taken and is summarised in the table. For the full GO tree, please see Figs S2 and S3.

et al., 2013). Hence, TRPC1 and other ion channels such as TRPM7, are involved in very specific aspects of EMT remodelling and may therefore function as fine-tuners of EMT induction. They therefore represent opportunities to therapeutically target specific aspects of the mesenchymal phenotype in breast cancer.

TRPC1 silencing inhibited hypoxia- but not EGF-mediated stimulation of EGFR(Y1172) and STAT3(Y705) phosphorylation. Specific regulation by TRPC1 of hypoxia but not EGF responses, indicated that this channel is not just a simple regulator of Ca²⁺-dependent housekeeping functions required for activation of the EGFR and STAT3 signalling pathways. Instead, TRPC1 is a specific regulator of processes associated with the transactivation of EGFR during hypoxia, which occurs through a HIF1 α -independent mechanism. We also demonstrated that TRPC1-mediated regulation of EGFR activity during hypoxia is manifest as effects on the autophagy marker LC3B (Fig. 6). In A549 lung carcinoma cells, TRPC1 silencing inhibits EGF-induced phosphorylation of EGFR at a different phosphorylation site (Y1068) (Tajeddine and Gailly, 2012), suggesting that TRPC1 selectively regulates signalling cascades through differential control of autophosphorylation sites (Nyati et al., 2006) and/or cell-type-specific roles for TRPC1 in EGFR signalling. Cell-type-specific roles for TRPC1 are also reflected in the regulation of store-operated Ca²⁺ entry (Cheng et al., 2013; Davis et al., 2012). Indeed, in A549 but not MDA-MB-468 cells, TRPC1 is a regulator of store-operated Ca²⁺ entry (Davis et al., 2012; Tajeddine and Gailly, 2012). This highly context-dependent role for TRPC1 in store-operated Ca²⁺ entry may explain the major differences in phenotypes between *Trpc1*- and *Orail* (the classical store-operated Ca²⁺ entry component)-knockout mice: *Orail*-null mice are associated with much more severe effects than *Trpc1*-knockout mice (Liu et al., 2007), including compromised immunity (Kim et al., 2011) and defects in osteoblast function (Robinson et al., 2012). TRPC1 is not the only Ca²⁺-influx pathway where its contribution is dependent upon the cell identity because silencing of *ORAI3* inhibits store-operated Ca²⁺ entry in oestrogen-receptor-positive but not oestrogen-negative breast cancer cell lines (Motiani et al., 2010).

TRPC1 is not responsible for store-operated Ca²⁺ entry in either normoxia (Davis et al., 2012) or after hypoxia in MDA-MB-468 breast cancer cells; indeed, TRPC1 silencing produces a modest increase in store-operated Ca²⁺ entry. However, our studies do show that TRPC1 is a major regulator of non-stimulated Ca²⁺ influx after hypoxia. The contribution of TRPC1 on basal [Ca²⁺]_{CYT} is associated with the pathogenesis of chronic hypoxia-induced pulmonary hypertension (Wang et al., 2015) and in cardiac myocytes, where the contribution of TRPC1 with TRPC4 to basal Ca²⁺ influx plays an important role in the development of cardiac hypertrophy (Camacho Londoño et al., 2015). TRPC1-mediated basal influx of Ca²⁺ in MDA-MB-468 breast cancer cells appears to be a trigger for the normoxic expression of HIF1 α expression via a PTEN–Akt-dependent pathway.

Mutations in the PTEN tumour suppressor gene are a characteristic of a variety of tumour types (Li et al., 1997), and in breast cancer they are associated with metastasis and poor prognosis (Saal et al., 2005, 2007). Loss of PTEN is a well characterised inducer of HIF1 α expression in the absence of hypoxia, via enhancement of Akt activity (deGraffenried et al., 2004; Pore et al., 2006); however, the potential role of Ca²⁺ signalling in this process has not been fully explored. Our studies in PTEN-deficient MDA-MB-468 breast cancer cells showed that TRPC1 silencing almost completely abolished the expression of HIF1 α under normoxic conditions, as well as basal Akt but not basal EGFR or STAT3 phosphorylation. The association between TRPC1 and the Akt–HIF1 α axis (Pore et al., 2006) was further supported by the inhibition of basal HIF1 α expression in MDA-MB-468 breast cancer cells with pharmacological inhibition of Akt, the reduction in Akt phosphorylation and HIF1 α levels in HCC1569 cells with TRPC1 silencing, and the role of TRPC1 downregulation and Akt inhibition in neurotoxin-induced ER stress in dopaminergic neurons reported by Selvaraj et al. (2012). Given that *Trpc1*-null mouse phenotypes have been described as not dramatic (Nilus and Szallasi, 2014) and include modest body weight increases, defective saliva secretion (Liu et al., 2007; Nilus and Szallasi, 2014) and altered recovery from anaphylaxis (Medic et al., 2013), targeting TRPC1

may offer a unique mechanism to regulate some of the cellular events associated with PTEN loss in some cancers without global systemic toxicity.

In conclusion, this study provides new insights into changes in TRPC ion channels that are associated with hypoxia in breast cancer cells and the role of TRPC1 in these processes. The contribution of TRPC1 is dependent on specific oncogenic mutations such as the tumour suppressor PTEN and the recruitment of HIF1 α -dependent and -independent pathways. This new understanding of the role of TRPC1 in the response of breast cancer cells to hypoxia may enable more effective targeting of cancer metastasis and/or survival mechanisms.

MATERIALS AND METHODS

Cell culture and induction of hypoxia

MDA-MB-468 and MDA-MB-231 human breast cancer cell lines were obtained from The Brisbane Breast Bank, UQCCR, Australia and American Type Culture Collection (ATCC) respectively, and cultured and maintained as previously described (Azimi et al., 2015, 2017). HCC1569 cells were obtained from the ATCC and maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS at 37°C and 5% CO₂. For hypoxia studies, 24 h post plating MDA-MB-468 breast cancer cells were serum reduced (0.5% FBS, 24 h) and then maintained in 1% O₂, 5% CO₂ and 94% N₂ for periods stated in results. For studies of EGF-induced EMT, cells were serum reduced (0.5% FBS, 24 h) 24 h post plating and then treated with 50 ng ml⁻¹ EGF (E9644; Sigma-Aldrich) for stated periods. Cell lines were routinely tested to confirm the absence of mycoplasma (MycoAlert). STR profiling was performed using the StemElite ID Profiling Kit (Promega).

Quantitative real-time PCR

Total RNA was isolated and purified from cultured cells using an RNeasy Plus Mini Kit (74134; Qiagen). cDNA was synthesised using an Omniscript RT Kit (205111, Qiagen) and was amplified using TaqMan Fast Universal PCR Master Mix (4352042; Applied Biosystems) and TaqMan gene expression assays in a StepOnePlus instrument (Applied Biosystems) under universal cycling conditions. The relative target quantity was determined using the comparative CT ($\Delta\Delta CT$) method by normalising to 18S rRNA as previously described (Aung et al., 2009). The TaqMan gene expression assays used in this study included: vimentin (Hs00185584_m1), Twist (Hs00361186_m1), Snail (Hs00195591_m1), N-cadherin (Hs00983062_m1), claudin-4 (Hs00976831_s1), HIF-1 α (Hs00153153_m1), TRPC1 (Hs00608195_m1), TRPC3 (Hs00162985_m1), TRPC4 (Hs01077392_m1), TRPC5 (Hs00202960_m1), TRPC6 (Hs00989190_m1), TRPC7 (Hs00220638_m1), STAT3 (Hs00374280_m1) and EGFR (Hs01076092_m1).

Immunoblotting

Cells were lysed in protein lysis buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science). Samples were resolved on NuPAGE Novex 4–12% BisTris gels (Invitrogen) or Mini-Protean TGX Stain-Free Precast Gels (Bio-Rad) and transferred to a PVDF membrane. The following primary antibodies were purchased from Cell Signaling and were diluted 1:1000: phospho-Akt (Ser473) (4051), Akt (9272), EGFR (2232), phospho-EGFR (Tyr1173) (4407), STAT3 (9139) and phospho-STAT3 (Tyr705). Anti-HIF-1 α antibody was purchased from BD Bioscience (610959, 1:500 dilution) and β -actin antibody was purchased from Sigma-Aldrich (A5441, 1:10,000 dilution). HRP-conjugated secondary antibodies goat anti-mouse (170-6516) and goat anti-rabbit (170-6516) were purchased from Bio-Rad and used at a 1:10,000 dilution. Primary antibodies were incubated with membrane overnight at 4°C and secondary antibodies for 1 h at room temperature. Chemiluminescence blots were imaged using a Bio-Rad VersaDoc Imaging System or a Bio-Rad ChemiDoc Imaging System. Bands were quantified by Quantity One (v.4.6.7, Bio-Rad) and normalised to β -actin. Phosphorylated proteins were then quantified relative to their total protein. Results are expressed as fraction of total protein band intensity.

siRNA transfection

Dharmacon ON-TARGETplus SMARTpool siRNA (100 nmol per well) and DharmaFECT4 Transfection Reagent (0.1 μ l per well) were used for siRNA silencing (GE Dharmacon). The following ON-TARGETplus SMARTpool siRNAs were used in this study: *TRPC1* (L-004191-00), *HIF1A* (L-004018-00), *EGFR* (L-003114-00) and *STAT3* (L-003544-00). MDA-MB-468 cells were seeded at 6×10^4 (48 h hypoxia) or 1×10^5 (6 h and 24 h hypoxia) cells per well in a 96-well plate. Post plating (24 h), cells were transfected with siRNA for 48 h. Cells were then serum starved (0.5% FBS) for 24 h and exposed to hypoxic conditions (1% O₂) for the indicated time periods. For experiments involving *TRPC1* silencing in HCC1569 cells, 5000 cells per well were seeded in a 96-well plate and left to adhere for 24 h. Cells were then treated with *TRPC1* siRNA for 48 h and were subsequently serum deprived (0.5% FBS). After 24 h cells in serum-deprived medium, cells were treated with 0.1% DMSO in serum-deprived medium for 24 h, 0.5 μ M acetic acid was added for 20 min, before addition of cell lysis buffer for protein isolation.

Additional experiments with different siRNAs (Dharmacon siGENOME) are presented in Fig. S6A,B. The following Dharmacon siGENOME siRNAs were used in this study: non-targeting (D-001206-14-05) and *TRPC1* (M-004191-02). Effectiveness of each siRNA was confirmed through greater than 80% silencing of the relevant target gene as quantified using real-time RT-PCR.

Akt pharmacological inhibition

For pharmacological inhibition studies, cells were seeded at 2×10^5 cells per well (96-well plate) and left to adhere for 24 h before serum deprivation (0.5% FBS) for a further 24 h. Cells were then incubated with Akt inhibitor MK-2206 2HCl or vehicle control (0.05% DMSO) for 24 h.

Measurement of intracellular free Ca²⁺

Cytosolic free Ca²⁺ ([Ca²⁺]_{CYT}) was assessed using a Fluorometric Imaging Plate Reader (FLIPR^{TETRA}, Molecular Devices) and the BD PBX no-wash Ca²⁺ Assay Kit (BD Biosciences) as described previously (Grice et al., 2010). Cells were seeded at 1×10^4 cells per well in 96-well black plates (Corning Costar). *TRPC1* and *HIF1A* were silenced using siRNA as described above. Post hypoxia (24 h), cells were loaded for 60 min at 37°C with 2 μ M PBX Ca²⁺ indicator dye in a solution comprising 5% (v/v) PBX Signal Enhancer and 500 μ M probenecid in physiological salt solution (PSS; 5.9 mM KCl, 1.4 mM MgCl₂, 10 mM HEPES, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 140 mM NaCl, 11.5 mM glucose, 1.8 mM CaCl₂). For assessment of store-operated Ca²⁺ entry, BAPTA (500 μ M, Invitrogen) was added (for chelation of extracellular Ca²⁺) followed by the addition of cyclopiazonic acid (10 μ M, Sigma) [for inhibition of sarco/endoplasmic reticulum Ca²⁺-ATPases (SERCA) pump and subsequent depletion of ER Ca²⁺]. Store-operated Ca²⁺ influx was assessed by the addition of CaCl₂ (0.6 mM) 700 s after the addition of cyclopiazonic acid. For the assessment of non-stimulated Ca²⁺ influx, following addition of BAPTA, 0.6 mM CaCl₂ was added in the absence of cyclopiazonic acid. Fluorescence was assessed at 470–495 nm excitation and 515–575 nm emission wavelengths. Data analyses were performed using ScreenWorks Software (v.2.0.0.27, Molecular Devices). Fluorescence response over baseline was assessed as a relative measure of [Ca²⁺]_{CYT}.

TRPC1 plasmid DNA transfection

TRPC1 plasmid was distributed by Addgene and was originally donated by Craig Montell (Addgene plasmid # 24408) (Xu et al., 1997). The pcDNA3.1 vector was used for a control. MDA-MB-468 cells (4×10^5 cells) were transfected with 1.2 μ g plasmids in a 20 μ l solution of SE Cell Line Kit (Lonza) using DS-120 programme of a 4D-Nucleofector unit (Lonza) and subsequently plated in 96-well plates.

Analysis of TRPC1 expression in breast cancer cell lines

For analyses of the *TRPC1* gene expression profile across a panel of breast cancer cell lines, we used a publically available microarray dataset from Array Express (accession no. E-MTAB-181) (Heiser et al., 2012) representing three subgroups of Luminal (predominantly luminal;

corresponding to luminal and HER2 breast cancer subtypes), basal A (mixed basal/luminal; corresponding to basal breast cancer subtype) and basal B (corresponding to mesenchymal subgroup with increased invasive properties; corresponding to claudin-low breast cancer subtype) (Neve et al., 2006; Prat et al., 2010).

Analysis of *TRPC1* expression in breast tumours

Breast tumour gene expression data were sourced from the University of North Carolina (UNC) cohort (Harrell et al., 2012). The UNC dataset classified its 855 tumours into the five intrinsic PAM50 molecular subtypes and further sub-divided the basal-like and normal-like subtypes into claudin-low tumours, basal-like (basal, 140), claudin-low (C-Low, 90), HER2-enriched (HER2, 144), luminal A (LumA, 243), luminal B (LumB, 162) and normal-like (N-Like, 76). Triple-negative breast cancers (TNBCs) from the UNC cohort [based on immunohistochemistry (IHC) of the oestrogen (ER) and progesterone receptors (PR) and HER2] were assigned a TNBC subtype based on the expression of subtype-specific gene signatures (Burstein et al., 2015). This analysis classified the 200 TNBCs into 83 basal-like immune-activated (BLIA), 95 basal-like immune-suppressed (BLIS), 6 luminal androgen receptor (LAR) and 16 mesenchymal (MES).

Survival analysis of basal tumours and association with *TRPC1* levels

The online tool, Kaplan–Meier plotter (Györfy et al., 2010) was utilised to produce survival curves for breast cancer patients. Two cohorts were used in this study, all patients with basal tumours and patients with basal tumours and classified as lymph-node positive (N+). Patient relapse-free survival (RFS) was stratified based on *TRPC1* (Affymetrix probe ID: 205802_at) expression using the ‘Auto select best cutoff,’ feature. Patient numbers in the low and high expression groups are displayed on the graphs, as are their hazard ratios (HRs) and *P*-values.

Identification of genes most correlated to *TRPC1* expression in breast cancers

The top 100 genes positively and negatively correlated to *TRPC1* expression were sourced from TCGA microarray data (available at www.cbioportal.org) (Cerami et al., 2012; Gao et al., 2013; Koboldt et al., 2012). This dataset contains 463 tumours with microarray expression data. Correlations to *TRPC1* expression were calculated through both Pearson and Spearman methodologies, Pearson was used as a cut-off for the top 100. For the complete list of positive and negative correlated genes, please see Tables S1 and S2, respectively.

Gene ontology analysis

The top 100 genes positively and negatively correlated to *TRPC1* expression were input into the online tool WEB-based GENE SeT AnaLysis Toolkit (WebGestalt, <http://bioinfo.vanderbilt.edu/webgestalt/>) (Wang et al., 2013). Gene ontology (GO) results from this analysis were determined as significant based on an adjusted *P*-value for multiple testing (Benjamini and Hochberg method) of a hypergeometric test (raw *P*-value) with a significance cut-off level of 0.05 and a minimum of 2 genes allocated to the GO term. GO hierarchical trees produced from this analysis are available in Figs S7 and S8, and summarised in Table 1.

TRPC1 promoter analysis

Genomic data for human mammary epithelial cell (HMEC) histone methylation was sourced from ENCODE through the IGV browser (Dunham et al., 2012; Robinson et al., 2011). Both Phastcons and CpG islands were sourced from the Broad Institute data registry ([http://data.broadinstitute.org/igvdata/\\$\\$_dataServerRegistry.txt](http://data.broadinstitute.org/igvdata/$$_dataServerRegistry.txt), accessed through IGV <http://software.broadinstitute.org/software/igv> Version 2.3.81). Mapped HIF1 ChIP-Seq data was sourced from GSE59937 (Zhang et al., 2015). The HIF1 motif (RCGTGM) was sourced from Schödel et al. (2011). Relevant data was then loaded into the Integrative genomics viewer (IGV, Mac desktop application v.2.3.81; Robinson et al., 2011) and a snapshot taken of human chromosome 3:142,442,229–142,445,500, build hg19.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.6.05 for Windows (GraphPad software). Details of each test employed are described in each figure legend. Data are presented as mean±s.d.

Competing interests

G.R.M. and S.J.R.-T. are associated with QUE Oncology Inc.

Author contributions

Conceptualization: I.A., M.A.B., E.T., S.J.R.-T., G.R.M.; Methodology: I.A., M.J.G.M., E.K., D.T., K.I.D.S.Y.; Software: I.A., M.J.G.M.; Validation: I.A.; Formal analysis: I.A., M.J.G.M., E.K., D.T., K.I.D.S.Y.; Investigation: I.A., M.J.G.M., E.K., D.T., K.I.D.S.Y.; Data curation: I.A.; Writing – original draft: I.A., M.J.G.M., E.K., S.J.R.-T., G.R.M.; Writing – review and editing: I.A., M.J.G.M., E.K., D.T., K.I.D.S.Y., M.A.B., E.T., S.J.R.-T., G.R.M.; Visualization: I.A.; Supervision: M.A.B., S.J.R.-T., G.R.M.; Project administration: S.J.R.-T., G.R.M.; Funding acquisition: S.J.R.-T., G.R.M.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.196659.supplemental>

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