The breast cancer oncogene IKKε coordinates mitochondrial function and serine metabolism

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Abstract

IkB kinase ε (IKKε) is a key molecule at the crossroads of inflammation and cancer. Known to regulate cytokine secretion via NFkB and IRF3, the kinase is also a breast cancer oncogene, overexpressed in a variety of tumours. However, to what extent IKKε remodels cellular metabolism is currently unknown. Here, we used metabolic tracer analysis to show that IKKε orchestrates a complex metabolic reprogramming that affects mitochondrial metabolism and consequently serine biosynthesis independently of its canonical signalling role. We found that IKKε upregulates the serine biosynthesis pathway (SBP) indirectly, by limiting glucose-derived pyruvate utilisation in the TCA cycle, inhibiting oxidative phosphorylation. Inhibition of mitochondrial function induces activating transcription factor 4 (ATF4), which in turn drives upregulation of the expression of SBP genes. Importantly, pharmacological reversal of the IKKε-induced metabolic phenotype reduces proliferation of breast cancer cells. Finally, we show that in a highly proliferative set of ER negative, basal breast tumours, IKKε and PSAT1 are both overexpressed, corroborating the link between IKKε and the SBP in the clinical context.

Keywords ATF4; breast cancer; IKKε; mitochondrial metabolism; serine biosynthesis

Subject Categories Cancer; Metabolism; Signal Transduction

DOI 10.15252/embr.201948260 | Received 10 April 2019 | Revised 29 June 2020 | Accepted 9 July 2020

EMBO Reports (2020) e48260

Introduction

Chronic inflammation, triggered by the tumour stroma or driven by oncogenes, plays a central role in tumour pathogenesis (Netea et al., 2017). A key step leading to inflammation in both compartments is activation of the transcription factor nuclear factor kB (NFkB), mediated via canonical or alternative, non-canonical pathways. Key players in both pathways are the members of the IkB kinase (IKK) family, which, by phosphorylating IkB, induce its proteasome-mediated degradation, a step required for the release of NFkB from its IkB-imposed cytosolic localisation, thus leading to its nuclear translocation (Clément et al., 2008).

Evidence in support of the crucial role played by the IKK family in inflammation-induced malignant transformation was provided by the reduction of tumour incidence following the deletion of the canonical IKK family member IKKβ in intestinal epithelial and myeloid cells in a mouse model of colitis-associated cancer development (Greten et al., 2004). Soon after, the non-canonical member of the IKK family, IKKε, was shown to induce breast cancer (Boehm et al., 2007) and to be overexpressed in ovarian (Guo et al., 2009), prostate (Péant et al., 2011) and non-small cell lung cancers (Guo et al., 2013), pancreatic ductal carcinoma (Cheng et al., 2011) and glioma (Guan et al., 2011). In particular, IKKε was shown to induce breast cancer via mechanisms involving CYLD (Hutti et al., 2009) and TRAF2 (Zhou et al., 2013), ultimately mediating NFkB activation (Boehm et al., 2007).

Beyond cancer, IKKε is a key regulator of both innate and adaptive immunity, activating NFkB and interferon regulatory factor 3 (IRF3), inducing type I interferon signalling (Clément et al., 2008; Zhang et al., 2016), although activation of the interferon response is not essential for IKKε-mediated cellular transformation (Boehm et al., 2007). On the other hand, IKKε has been shown to regulate central carbon metabolism both in immune and cancer cells. In dendritic cells (DCs), IKKε, together with its closest homologue TANK binding kinase 1 (TBK1), is required for the switch to aerobic glycolysis induced by activation of the Toll-like receptors (TLRs) and activation of DCs. Glycolysis is the main glucose catabolic pathway whereby, through a series of reactions, cells metabolise glucose...
to pyruvate, which, in the presence of oxygen, is in turn oxidised to 
CO₂ in the mitochondrial matrix via the TCA cycle to produce ATP 
using the mitochondrial respiratory chain. Lack of oxygen prevents 
the mitochondrial utilisation of pyruvate, meaning glucose is instead 
converted into lactate (anaerobic glycolysis). In contrast, aerobic 
glycolysis refers to a metabolic condition whereby glucose is not 
fully oxidised in the mitochondria, even in the presence of oxygen, 
and is utilised for the production of amino acids, lipids and nucleo-
tides via pathways branching out from glycolysis and the TCA cycle. 
Accordingly, aerobic glycolysis in DCs allows fatty acid synthesis, 
which is required for the expansion of the endoplasmic reticulum 
and Golgi, supporting DC activation (Everts et al, 2014). Allowing 
the production of key cellular constituents, aerobic glycolysis is 
most frequently observed in highly proliferative cells, such as acti-
vated immune cells and cancer cells (Andrejeva & Rathmell, 2017). 
In agreement, IKKε also regulates glucose uptake in pancreatic 
ductal adenocarcinoma and mitochondrial function in mouse 
embryonic fibroblasts (MEFs) (Reilly et al, 2013; Zubair et al, 
2016). In addition, we have recently demonstrated that IKKε and the 
serine biosynthesis pathway (SBP) are important for the acquisition 
of malignant traits in breast epithelium exposed to macrophage 
conditioned medium and accordingly, expression of SBP enzymes 
correlates with inflammation in breast cancer (Wilcz-Villega et al, 
2020). Our findings are in line with the known function of the SBP 
in cancer. Indeed, phosphoglycerate dehydrogenase (PHGDH), the 
first enzyme of the pathway, is amplified in breast cancer and mela-
noma, where it functions as an oncogene (Locasale et al, 2011; 
Possemato et al, 2011) and the SBP is also the target of a series of 
oncogenes (Amelio et al, 2014; Yang & Vousden, 2016). However, a 
comprehensive investigation of the role of IKKε as regulator of cellular 
metabolism in cancer has not yet been carried out. Taking an 
unbiased approach, we followed the fate of C13 glucose upon modu-
lation of IKKε expression. We found that IKKε inhibits the mitochon-
dria and indirectly controls the SBP via activation of ATF4, 
ultimately driving the upregulation of the SBP enzymes, in particu-
lar phosphoserine aminotransferase 1 (PSAT1). Importantly, we also 
demonstrate that IKKε-mediated regulation of cellular metabolism is 
independent of the canonical signalling pathway via NFκB/IRF3. 
Moreover, we have identified a subset of basal, estrogen receptor 
negative (ER−) highly proliferative breast tumours where IKKε and 
PSAT1 are both overexpressed, confirming the pathophysiological 
role of our findings. These results identify an additional role for 
IKKε in breast cancer, adding regulation of cellular metabolism to 
the canonical oncogenic mechanisms. Thus, our data suggest a 
synergistic mechanism of action by which alterations of cellular 
metabolism and inflammation driven by the IKKε oncogene support 
tumour growth and proliferation.

Results

IKKε rewires cellular metabolism

To investigate the effect of IKKε activation on metabolism, we used 
two cellular model systems: (i) doxycycline-inducible Flp-In 293 HA-
IKKε-expressing cells and their respective GFP-expressing controls 
(Flp-In 293 HA-GFP cells) and (ii) two breast cancer cell lines, T47D 
and MDA-MB-468, where the kinase was silenced via siRNA. HEK-
293 cells do not express endogenous IKKε, and thus, we could set its 
expression to a level that matched those observed in IKKε expressing 
breast cancer cell lines (Boehm et al, 2007) (Fig 1A). Liquid chro-
matography–mass spectrometry (LC–MS) analysis of steady-state 
metabolite levels revealed that induction of IKKε expression affected 
a large fraction of the measured metabolites (26 out of 32, Fig 1B 
and Dataset EV1). To account for any possible effect of doxycycline 
on cell metabolism, we compared cells with doxycycline-induced 
expression of IKKε versus GFP (Ahler et al, 2013). Of particular 
interest, IKKε increased cellular glucose and glutamine levels, along 
with a group of amino acids, including serine and glycine. The 
increased intracellular level of serine was likely a consequence of 
increased biosynthesis as we observed a significant increase in the 
level of 13C₆-glucose-derived serine (m⁺3 isotopologue), suggesting 
that IKKε positively regulates the SBP (Fig 1C). A key enzyme in the 
SBP is phosphoserine aminotransferase 1 (PSAT1), which transfers 
nitrogen from glutamine-derived glutamate to phosphohydroxypropyr-
vate, generating phosphoserine for the final dephosphorylation step 
of serine biosynthesis (Fig 1D). Using 15N₂-glutamine labelling, we 
confirmed increased levels of labelled serine (m + 1) in IKKε 
expressing cells (see Fig 1C and Dataset EV1), consistent with an 
increase in PSAT1 transamination activity, supporting our hypothe-
sis that serine biosynthesis was activated by IKKε. In contrast, we 
observed a significant reduction in the accumulation of the TCA 
cycle intermediates citrate m + 2 and malate m + 2 from 13C₆-glucose, 
indicating that IKKε reduces pyruvate dehydrogenase (PDH) activity 
(Fig 1E). Fractional enrichment analysis of the above metabolites 
showed that in addition to increased serine biosynthesis, IKKε also 
augmented serine uptake from the media, shown by an increase in 
serine m + 0 isotopologue (Fig 1F). Moreover, the fraction of 13C₆-
glucose-derived citrate and malate (m + 2, pyruvate dehydrogenase 
generated) was reduced in IKKε expressing cells, causing reduction in 
their total levels, indicating that no other carbon sources (e.g. gluta-
mine) compensate for the lack of pyruvate entering the TCA cycle 
(Fig 1F). Finally, the fraction of 15N labelled serine derived from gluta-
mine was also increased, indicating higher glutamine usage in serine 
biosynthesis as nitrogen source (Fig 1G).

We then investigated whether IKKε has a similar metabolic func-
tion in breast cancer cell lines, where it is constitutively expressed. 
Since IKKε has been shown to be an oncogene in different breast 
cancer subtypes (Boehm et al, 2007), we used T47D and MDA-MB-
468 cell lines to model estrogen receptor positive (ER⁺) and triple-
negative breast cancer, respectively (Subik et al, 2010). After 
silencing the kinase (Fig 2A and B), 13C₆-glucose and 15N₂-glutamine 
labelling analysis confirmed the overall effect of IKKε on cellular 
metabolism. In the serine and glycine biosynthesis pathways, IKKε 
silencing exerted the opposite effect as compared to IKKε induction 
in the Flp-In 293 model (Fig 2C–F and Dataset EV1). Similarly, IKKε 
knockdown resulted in increased levels of the TCA cycle metabolites 
citrate and malate m + 2 isotopologues, derived from 13C₆-glucose via 
PDH (Fig 2E and F). Taken together, these data indicated that in 
cancer cells IKKε redirects a significant fraction of glucose-derived 
carbons to the SBP and reduces pyruvate oxidation in the TCA cycle. 

IKKε inhibits mitochondrial function via PDH

Considering the effect on the TCA cycle observed via tracer 
compounds (see Figs 1 and 2), we assumed that IKKε alters
Figure 1.
mitochondrial oxidative function. Indeed, mitochondrial oxygen consumption rate (OCR) was suppressed by IKKε induction in the Flp-In 293 HA-IKKε cell line, accompanied by reduced mitochondrial membrane potential (ΔΨm), as assessed by respirometry and steady-state tetramethyl-rhodamine methylester (TMRM) intensity imaging. Of note, when using Flp-In 293 cells that express variant mutants of HA-IKKε, which feature mutations disrupting the function of IKKε’s kinase domain (KD-m) and Ubiquitin-like domain (UbLD-m), we confirmed that both functional domains of the kinase (Ikeda et al., 2007) were required to exert inhibition of mitochondria (Fig 3A and B). Furthermore, IKKε silencing resulted in significantly higher OCR in a set of breast cancer cells (Figs 3C and EV1A). IKKε primarily affected ATP-coupled respiration, without significantly inhibiting uncoupled or reserve OCR, as shown by measuring respiration in the presence of oligomycin (inhibitor of the ATP synthase), and the uncoupler FCCP, respectively (Fig EV1B–F). Moreover, the effect was integral to the mitochondria, since mitochondria isolated from IKKε expressing cells showed reduced respiration compared to those isolated from GFP-expressing controls (Fig 3D).

In order to elucidate the mechanism by which IKKε regulates mitochondrial metabolism, we compared the phosphoproteomes of three independent control (GFP) and IKKε expressing Flp-In 293 clones. Multivariate analysis showed that the two clones highly expressing IKKε grouped together in principal component analysis (PCA) and were separated from controls and cells expressing IKKε at low levels (Fig 3E and F). These results suggested that IKKε induces a dose-dependent effect in the phosphoproteome of these cells. We identified more than 3,000 phosphopeptides quantified in four technical replicates, which interestingly included the E1 subunit of the pyruvate dehydrogenase complex (PDHA1 - pS232) (Dataset EV2).

Phosphorylation of PDHA1 on S232 is known to inhibit PDH activity and is also reported to be necessary for tumour growth (Gollas et al., 2016), and thus, we hypothesised that IKKε regulates pyruvate entry in the TCA cycle and consequently electron provision for the respiratory chain. Of note, other phosphosites of PDHA1 were either unchanged or less phosphorylated, indicating that the increase in pS232 is not due to higher level of expression of the protein (Dataset EV2). In agreement with our hypothesis, PDH activity was reduced in IKKε expressing cells (Fig 3G), and the effect was reverted by inhibiting pyruvate dehydrogenase kinase using dichloroacetic acid (DCA) (Stacpoole, 1989). DCA restored both IKKε-mediated reduction of ΔΨm and inhibition of respiration in Flp-In 293 mitochondria, but had no effect in control cells (Fig 3H and I), indicating that diminished pyruvate oxidation by the PDH complex is the limiting factor of respiratory activity in IKKε overexpressing

**Figure 1.** IKKε induces remodelling of cellular carbon metabolism by activating the serine biosynthesis pathway (SBP) and suppressing pyruvate oxidation.

A. Top panel: Scheme illustrating the tetracycline-inducible Flp-In 293 system that controls the expression of HA-IKKε or HA-GFP. Bottom panel: Representative Western blot showing induced expression of HA-IKKε in Flp-In 293 cells treated with doxycycline (Dox, 50 ng/ml) for 16 h compared to endogenous IKKε in T47D, MDA-MB-231 and MDA-MB-468 breast cancer cell lines.

B. Heatmap and hierarchical clustering of metabolite concentrations in Flp-In 293 HA-GFP and Flp-In 293 HA-IKKε cells treated with doxycycline (Dox, 50 ng/ml, 16 h; n = 5 technical replicates).

C. Serine production from glucose (serine m + 3,13C2-glucose labelling, left panel) and glutamine (serine m + 1,15N2-glutamine labelling, right panel) in Flp-In 293 HA-GFP or Flp-In 293 HA-IKKε cells treated with doxycycline (50 ng/ml, 16 h; n = 5 technical replicates).

D. Schematic representation of the 13C2-glucose and 15N2-glutamine labelling strategy to assess the effect of HA-IKKε induction on glycolysis, the TCA cycle and serine metabolism.

E. Contribution of pyruvate and glucose-derived carbon to TCA cycle metabolites in Flp-In 293 HA-GFP or Flp-In 293 HA-IKKε cells treated with doxycycline (50 ng/ml, 16 h; n = 5 technical replicates).

F. Fractional enrichment of serine, malate and citrate 13C-isotopologues in Flp-In 293 HA-GFP and Flp-In 293 HA-IKKε cells treated with doxycycline (50 ng/ml, 16 h; n = 5 technical replicates).

G. Fractional enrichment of the serine 15N-isotopologue in Flp-In 293 HA-GFP and Flp-In 293 HA-IKKε cells treated with doxycycline (50 ng/ml, 16 h). m + 1 shows the naturally occurring 13C isotopologue (n = 5 technical replicates).

Data Information: In (C, E–G), metabolite levels were normalised to the internal standard HEPES. In (D) and (E–G), data are presented as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (two-tailed Student’s t-test or Mann–Whitney test).

Source data are available online for this figure.

**Figure 2.** The effect of IKKε silencing on metabolism of breast cancer cell lines.

A, B Representative Western blot showing the level of IKKε in IKBKE (IKKε)-silenced (A) T47D and (B) MDA-MB-468 breast cancer cell lines.

C, D Heatmap and hierarchical clustering of metabolite concentrations in (C) IKBKE (IKKε)-silenced T47D cells and (D) IKBKE (IKKε)-silenced MDA-MB-468 cells (n = 5 technical replicates).

E. Glycine production, representative of serine production, from glutamine (glutamine m + 2,15N2-glutamine labelling) and glucose (glucose m + 2,13C2-glucose labelling); and contribution of pyruvate and glucose-derived carbon to TCA cycle metabolites (citrate m + 2, malate m + 2,13C2-glucose labelling) in IKBKE (IKKε)-silenced and MDA-MB-468 breast cancer cell lines (n = 5 technical replicates).

F. Serine production from glutamine (glutamine m + 1,15N2-glutamine labelling), and serine and glycine production from glucose (glucose m + 2, serine m + 3,13C2-glucose labelling) as well as contribution of pyruvate and glucose-derived carbon to TCA cycle metabolites (malate m + 2,13C2-glucose labelling) in IKBKE (IKKε)-silenced MDA-MB-468 cells (n = 5 technical replicates).

Data Information: In (C, D), metabolite levels were normalised to the internal standard HEPES. In (D, F), metabolite levels were normalised to total ion count. In (C, D), metabolite levels were scaled to maximum and minimum levels of each metabolite. In (E, F), data are presented as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (two-tailed Student’s t-test).

Source data are available online for this figure.
Figure 2.
cells. In line with this conclusion, we also showed that IKKε overexpressing cells rely less on pyruvate for their respiration in comparison with control cells, expressing GFP (Fig 3I).

**IKKε: activates the SBP transcriptional response via ATF4**

Our tracer experiments indicated that in addition to inhibiting mitochondria, IKKε also stimulated serine biosynthesis (Figs 1 and 2). Since mitochondrial dysfunction has previously been shown to induce activation of activating transcription factor 4 (ATF4) and to regulate SBP gene transcription (Bao et al, 2016; Khan et al, 2017), we hypothesised that IKKε-induced mitochondrial inhibition elicits a similar ATF4-mediated response, in turn inducing serine biosynthesis. Confirming this hypothesis, ATF4 was induced in IKKε expressing cells (Fig 4A), while c-Myc, another known regulator of serine metabolism (Nikiforov et al, 2002; Sun et al, 2015; Anderton et al, 2017), remained unchanged (Fig EV2A). We therefore assessed the overall level of the three enzymes of the pathway: PHGDH, PSAT1 and phosphoserine phosphatase (PSPH). We observed that IKKε induction in Flp-In 293 HA-IKKε cells led to a marked (2–6 fold) increase in the transcription of all three SBP enzyme mRNAs (Fig 4B), which was also reflected at the protein level (with the exception of PHGDH). Of note, consistently with the role of IKKε as key mediator of the innate immune response, IRF3 was also phosphorylated upon induction of the kinase, confirming the activation of canonical kinase signalling in addition to the upregulation of ATF4 and SBP enzymes (Fig 4C). Importantly, silencing of ATF4 abolished the transcriptional upregulation of SBP enzymes mediated by IKKε and reduced their protein levels (Fig 4D and E), demonstrating the requirement of the transcription factor for the upregulation of SBP enzymes by the kinase. Moreover, while the enzymes of the SBP were upregulated in Flp-In 293 HA-IKKε cells, we did not observe differences in the level of expression of serine hydroxymethyltransferase 2 (SHMT2), the main mitochondrial enzyme involved in serine catabolism (Stower & Schirch, 1990), supporting the hypothesis that IKKε primarily acts on serine biosynthesis. The lack of changes in SHMT2 expression is also in agreement with the lack of c-Myc involvement in the IKKε-induced pathway (Fig EV2B, and see (Nikiforov et al, 2002)).

Confirming the role of IKKε/ATF4 observed in our HEK model cell line, silencing of IKKε in a panel of breast cancer cell lines had the opposite effect. Upon siRNA-mediated knockdown of IKKε, downregulation of PSAT1 at the transcriptional level was observed in 5 out of 9 breast cancer cell lines tested (ZR-75-1, T47D, MDA-MB-468, Cal120 and HCC1143) (Fig 5A). Downregulation at the protein level was also observed in all cell lines where the protein was detected (with the exception of MDA-MB-231). At the protein level, we also observed downregulation of PHGDH in ZR-75-1, MDA-MB-468 and MCF7 cells and of PSPH in ZR-75-1, MDA-MB-453 and MDA-MB-468 cells (Fig 5B–E), while no increase in SHMT2 was observed (Fig EV2C and D). Moreover, reduction of the SBP enzymes was also observed in breast cancer cell lines upon the silencing of ATF4 (Fig 5F and G), in agreement with previous data (DeNicola et al, 2015).

Altogether, these data indicated that IKKε orchestrates a complex metabolic reprogramming that encompasses the inactivation of mitochondrial metabolism and the consequent transcriptional activation of the SBP, mediated by ATF4.

Importantly, siRNA of PSAT1 in Flp-In 293 HA-IKKε or in the breast cancer cell lines panel had no effect on oxygen consumption, further supporting that IKKε-mediated regulation of the SBP is a secondary event to regulation of the mitochondria (Fig EV3A and B).

**IKKε: induces SBP gene transcription via a non-canonical mechanism**

Next, we tested the involvement of the canonical downstream signalling pathways known to be activated by the kinase. Silencing of the transcription factors IRF3 and p65 (Clément et al, 2008) did not abolish the induction of SBP enzyme gene transcription by
Figure 4.
IKKε (Fig EV3C–E). Of note, these experiments indicated that IRF3 is required to maintain the transcription of PHGDH in basal conditions, but has no role in IKKε-mediated induction. Finally, since IKKε induces the secretion of a range of cytokines (Barbie et al., 2014), we tested the possibility that the induction of SBP enzyme gene transcription is mediated by an autocrine loop. Extracellular media conditioned by HA-GFP or HA-IKKε expressing Flp-In 293 cells was collected and applied on three different receiving cell lines: Flp-In 293 HA-GFP, not expressing IKKε (Fig EV4A) and the T47D and ZR-75-1 breast cancer cell lines, constitutively expressing IKKε (Fig EV4B and C). Media conditioned by IKKε-expressing cells had no effect on the SBP enzymes, even though cytokine-mediated JAK-STAT signalling was observed in all three receiving cell lines, as demonstrated by induction of STAT1, phospho-STAT1 and OAS1 (an interferon-inducible gene, only in ZR-75-1).

Altogether, these results demonstrated that IKKε induces SBP enzyme gene transcription by a cell-autonomous mechanism which, however, does not include its canonical downstream targets.

**Pharmacological inhibition of IKKε-induced metabolic changes reduces cell proliferation**

To test the functional consequences of the IKKε-mediated metabolic rewiring on tumour proliferation, we assessed the outcome of inhibiting the two key metabolic reactions of serine biosynthesis on cell proliferation in a panel of breast cancer cell lines. Significantly reduced breast cancer cell proliferation was observed upon treatment with NCT502, a recently described inhibitor of the IKKε (Fig EV4C). IKKε (pS396) and SBP enzymes in Flp-In 293 HA-GFP or Flp-In 293 HA-IKKε cells treated with doxycycline (Dox) for 16 h. Data are expressed as fold changes, relative to levels in non-treated Flp-In 293 HA-GFP cells and normalised to β-Actin (n = 4 biological replicates).

**IKKε and PSAT1 are overexpressed in a common, highly proliferative subset of breast cancer**

In order to explore IKKε and SBP enzyme gene expression status in breast cancer patient samples, we analysed the METABRIC dataset (Curtis et al., 2012), which includes data from 1981 breast cancer patients with pathological and clinical details. IKBKE and PSAT1 mRNA were significantly upregulated (above the 95% confidence interval) in 200 (10.1%) and 664 (33.5%) samples, respectively, and 107 (5.4%) samples showed overexpression of both mRNAs. This indicated a highly significant association between the two genes, as confirmed chi-square independence test (Fig 6C). Given that breast cancer is a heterogenous disease, commonly classified into 5 to 10 intrinsic subtypes (Perou et al., 2000; Curtis et al., 2012), the association of IKBKE and PSAT1 might be driven by subtype-specific expression. Thus, in order to identify subtypes with significant overexpression compared to the total population, we compared the expression values of IKBKE and the SBP genes in all Pam50 subtypes (Parker et al., 2009; Jiang et al., 2016) and in the estrogen receptor (ER) positive and negative populations. This analysis indicated that both IKBKE and PSAT1, similarly to PHGDH and PSPH, are significantly upregulated in an ER-negative Pam50:basal subpopulation of tumours, with the highest proliferation index (Nielsen et al., 2010) (Fig 6D–H). ATF4-driven overexpression of PSAT1 in ER-negative tumours has been previously shown in a different dataset (Gao et al., 2017) and here we also have found strong association of these two genes (Fig 6I and J). Importantly, while PSAT1 is overexpressed in almost all ER-negative samples (378 out of 435), only 79 samples overexpress IKBKE, indicating that PSAT1 is regulated by multiple inputs. However, above 90% of samples overexpressing IKBKE (72 out of 79) also overexpress PSAT1. Due to the large fraction of PSAT1 overexpressing samples in the ER-negative subset and the overall low expression and detected variability of IKBKE and ATF4 mRNAs in the dataset, only low but still statistically significant levels of correlation could be found between these genes (Fig 6K and L). However, these gene expression patterns clearly show that IKKε-mediated expression of PSAT1 and the SBP enzymes, demonstrated in our in vitro experiments, is potentially also functional in a subset of clinical samples, suggesting the pathophysiological importance of the pathway in breast cancer. Importantly, we also confirmed the correlation between IKKε and PSAT1 expression in a set of breast cancer cases (Wilcz-Villega et al., 2020).
Figure 5.
Here, we described a novel fundamental mechanism by which IKKε, a key player in the innate immune response, regulates cellular metabolism. We show that the kinase orchestrates a complex metabolic reprogramming culminating in the regulation of the serine biosynthesis pathway. The mechanism involved in IKKε-mediated regulation of the SBP is inhibition of carbon supply to the mitochondria, leading to the transcriptional upregulation of SBP genes via a mitochondrial-nuclear retrograde signalling pathway targeting ATF4, ultimately activating serine biosynthesis. The overall metabolic switch induced by IKKε supports cancer cell proliferation and is present in a subset of breast tumours, providing potentially important pharmacological targets. The pathway described here is reminiscent of recent data showing that the uptake of pyruvate in mitochondria regulates the SBP (Baksh et al., 2020).

While such mechanistic details of the function of IKKε as a newly identified modulator of the SBP and mitochondria have not been reported before, previous studies implicated IKKε in the regulation of cellular metabolism. Consistent with our data, IKKε was shown to inhibit OCR in MEFs (Reilly et al., 2013) and regulate glycolysis in DCs, although in this system the kinase did not affect mitochondrial metabolism (Everts et al., 2014). Similarly, in pancreatic ductal adenocarcinoma, IKKε was shown to stimulate glucose uptake, but did not inhibit mitochondrial respiration (Zubair et al., 2016). Thus, IKKε appears to modulate cellular metabolism in a tissue- and context-specific manner, and our study pinpoints and extends the breadth of the specific cellular targets utilised by the kinase to exert these heterogeneous responses. Importantly, in addition to the previously known canonical NFκB and IRF3 signalling pathways (Clément et al., 2008), IKKε can engage the mitochondrial-nuclear ATF4-mediated signalling. Whether NRF2, previously demonstrated to regulate the SBP upstream of ATF4 (DeNicola et al., 2015), is also involved in the signalling induced by IKKε, remains to be tested. While here we have shown that in breast cancer cells IKKε-mediated changes in metabolism support proliferation, these metabolic alterations might also facilitate other cellular functions (Jones & Bianchi, 2015), for example, cytokine secretion in immune cells (Chang et al., 2013; Tannahill et al., 2013; Rodriguez et al., 2019; Yu et al., 2019). Apart from providing novel mechanistic details of IKKε-mediated cellular metabolic changes, this work also indicates the necessity of further research to better understand the physiological and pathological role of IKKε in order to efficiently and selectively target tumour cells relying on this oncogene. Our observations suggest that drugs targeting IKKε-regulated metabolic pathways can specifically target breast cancer cells without affecting other cell types, considering that it is only in these cells that IKKε has been reported to regulate the SBP. Indeed, our gene expression analysis indicated that the IKKε-mediated pathway defines a subset of ER⁺, basal breast tumours, and thus, evaluation of the IKKε-mediated metabolic and gene expression phenotype can help to further stratify breast cancer for treatment. Our stratification is also in agreement with previous

**Figure 5.** The SBP is primarily regulated by an IKKε-mediated transcriptional response.

A qRT-PCR analysis of PHGDH, PSAT1 and PSPH mRNA levels in a panel of IKKε-silenced breast cancer cell lines. Data are expressed as fold changes, relative to levels in a non-silenced control of each cell line and normalised to β-Actin (n ≥ 3 biological replicates).

B Representative Western blot showing the levels of IKKε and the SBP enzymes in IKKε-silenced ZR-75-1, T47D, MDA-MB-468 and MCF7 breast cancer cell lines.

C–E Levels of the SBP enzymes in a panel of IKKε (IKKε-silenced breast cancer cell lines. (C) PHGDH, (D) PSAT1 and (E) PSPH levels in indicated cell lines normalised to Vinculin. Densitometry analysis quantified single sample density as a percentage of total blot density per cell line prior to vinculin normalisation (n ≥ 3 biological replicates).

F qRT-PCR analysis of PHGDH, PSAT1 and PSPH mRNA levels in ATF4-silenced ZR-75-1, T47D and MDA-MB-468 breast cancer cell lines. Data are expressed as fold changes, relative to levels in non-silenced control cells and normalised to β-Actin (n = 3 biological replicates).

G Representative Western blot showing the levels of PHGDH, PSAT1 and PSPH in ATF4-silenced ZR-75-1, MDA-MB-468, MDA-MB-231, T47D and HCC1143 breast cancer cell lines.

Data Information: In (A, C–F), data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. In (A, F), one-sample t-tests were performed using log-transformed fold change values for all samples, except Sum44 PHGDH in (A), in which case a Wilcoxon signed rank was performed using log-transformed fold change values. In (C–E), two-tailed paired t-tests were performed.

Source data are available online for this figure.

**Discussion**

Here, we described a novel fundamental mechanism by which IKKε, a key player in the innate immune response, regulates cellular metabolism. We show that the kinase orchestrates a complex metabolic reprogramming culminating in the regulation of the serine biosynthesis pathway. The mechanism involved in IKKε-mediated regulation of the SBP is inhibition of carbon supply to the mitochondria, leading to the transcriptional upregulation of SBP genes via a mitochondrial-nuclear retrograde signalling pathway targeting ATF4, ultimately activating serine biosynthesis. The overall metabolic switch induced by IKKε supports cancer cell proliferation and is present in a subset of breast tumours, providing potentially important pharmacological targets. The pathway described here is reminiscent of recent data showing that the uptake of pyruvate in mitochondria regulates the SBP (Baksh et al., 2020).

While such mechanistic details of the function of IKKε as a newly identified modulator of the SBP and mitochondria have not been reported before, previous studies implicated IKKε in the regulation of cellular metabolism. Consistent with our data, IKKε was shown to inhibit OCR in MEFs (Reilly et al., 2013) and regulate glycolysis in DCs, although in this system the kinase did not affect mitochondrial metabolism (Everts et al., 2014). Similarly, in pancreatic ductal adenocarcinoma, IKKε was shown to stimulate glucose uptake, but did not inhibit mitochondrial respiration (Zubair et al., 2016). Thus, IKKε appears to modulate cellular metabolism in a tissue- and context-specific manner, and our study pinpoints and extends the breadth of the specific cellular targets utilised by the kinase to exert these heterogeneous responses. Importantly, in addition to the previously known canonical NFκB and IRF3 signalling pathways (Clément et al., 2008), IKKε can engage the mitochondrial-nuclear ATF4-mediated signalling. Whether NRF2, previously demonstrated to regulate the SBP upstream of ATF4 (DeNicola et al., 2015), is also involved in the signalling induced by IKKε, remains to be tested. While here we have shown that in breast cancer cells IKKε-mediated changes in metabolism support proliferation, these metabolic alterations might also facilitate other cellular functions (Jones & Bianchi, 2015), for example, cytokine secretion in immune cells (Chang et al., 2013; Tannahill et al., 2013; Rodriguez et al., 2019; Yu et al., 2019). Apart from providing novel mechanistic details of IKKε-mediated cellular metabolic changes, this work also indicates the necessity of further research to better understand the physiological and pathological role of IKKε in order to efficiently and selectively target tumour cells relying on this oncogene. Our observations suggest that drugs targeting IKKε-regulated metabolic pathways can specifically target breast cancer cells without affecting other cell types, considering that it is only in these cells that IKKε has been reported to regulate the SBP. Indeed, our gene expression analysis indicated that the IKKε-mediated pathway defines a subset of ER⁺, basal breast tumours, and thus, evaluation of the IKKε-mediated metabolic and gene expression phenotype can help to further stratify breast cancer for treatment. Our stratification is also in agreement with previous

**Figure 6.** The pathophysiological role of IKKε-induced metabolic and gene expression alterations in breast cancer.

A Correlation of change in OCR (ΔOCR) in a panel of IKKε (IKKε-silenced breast cancer cell lines (from Fig 3C) and the change in cell confluence (Δconfluency) upon treatment of the panel of cell lines with NCT502 (from Fig EV5A).

B Correlation of ΔOCR in a panel of IKKε (IKKε-silenced breast cancer cell lines (from Fig 3C) and the Δconfluency upon treatment of the panel of cell lines with 6-Diazo-5-oxo-L-norleucine (DON) (from Fig EV5C).

C Association between IKKε (IKKε) and PSAT1 mRNA overexpression evaluated by a chi-squared independence test. The + sign indicates samples with significant (P < 0.05) overexpression of IKKε or PSAT1. Number and percentage of samples, as well as the chi-square values are shown.

D–I Expression of IKKε (IKKε) and the SBP enzymes PSAT1, PHGDH and PSPH in the METABRIC dataset. The expression of a proliferation-related gene set and ATF4 is also shown. Samples were stratified by PAM50 intrinsic subtypes and ER status. Brown–Forsythe and Welch ANOVA test with unpaired t with Welch correction were applied. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

J Association between ATF4 and PSAT1 mRNA overexpression evaluated by chi-squared independence test. The + sign indicates samples with significant (P < 0.05) overexpression of ATF4 or PSAT1. Number and percentage of samples, as well as the chi-square values are shown.

K L Correlation of IKBKE (IKKε) and Psat1 (K) or ATF4 and Psat1 (L) expression in the ER-negative sample subset.

Data Information: In (A, B), cell confluency was measured using the IncuCyte Zoom, OCR was measured using Seahorse XF96e or XF24 analysis. Data are n ≥ 3 biological replicates. In (A, B, K, L) linear regression, correlation coefficients (Pearson’s correlation, Spearman’s rho) significance of difference from slope = 0 are shown. Source data are available online for this figure.
Figure 6.
studies, where strong correlation between PSAT1 expression and tumour proliferation has been found in ER+ tumours (Coloff et al., 2016; Gao et al., 2017). Of note, IKKε, along with the JAK/STAT pathway, has been reported to regulate a cytokine network promoting cellular proliferation in a subset of triple-negative breast tumours (Barbie et al., 2014), and PSAT1 overexpression is also a feature of a small fraction of ER+ tumours along with the JAK-STAT pathway (De Marchi et al., 2017). While we have shown that IKKε activates the JAK-STAT pathway (Fig EV4) along with PSAT1 overexpression, JAK-STAT activation per se was not sufficient for the induction of the enzyme. This indicates that both IKKε and PSAT1 are defining features in order to identify tumours where the pathway is actively promoting proliferation.

Finally, further work is required to investigate whether the IKKε-mediated metabolic phenotype described here, especially regarding the regulation of the SBP, occurs in different cellular systems where IKKε is known to be activated. This could help to develop new therapeutic strategies applicable in a broad range of inflammation-related diseases, beyond cancer.

Materials and Methods

Plasmids

DNA fragments encoding wild-type human IKKε (Uniprot: Q14164) were amplified separately by PCR using primers containing Kpn1 (5’) and EcoR1 (3’) restriction sites. For 5’-tttgtaccacaagccagcagagtagacagacacagcaggta-3’ Rev 5’-gatggtatctgcaatctagggcttgggaa-3’

The PCR products were double digested by these two enzymes and ligated to vector pcDNA5.5 (a kind gift from Dr Tencho Tenev), which provides a 2xHA tag at the c-terminal, to generate the pcDNA5.5-unt-IKKε plasmid. For mutant variants of IKKε with disrupted functional domains, the kinase domain mutant (KD-m) was created by site-directed mutagenesis, using primers to introduce a K38A mutation to the wild-type IKKε sequence. For 5’-gatggtatctgcaatctagggcttgggaa-3’ Rev 5’-gatggtatctgcaatctagggcttgggaa-3’

UbLD-M-IKKε plasmid, encoding the ubiquitin-like domain mutant (UbLD-m) variant of IKKε (containing L353A and F354A mutations) was a kind gift from Prof Ivan Dikic. Both KD-m and UbLD-m IKKε variants were amplified and ligated into the pcDNA5.5 vector using the same Kpn1 and EcoR1 double digestion and ligation method as the wild-type kinase, generating the pcDNA5.5-KD-M-IKKε and pcDNA5.5-UbLD-M-IKKε plasmids.

Cells

To generate Flp-In 293 cells expressing either wild type, kinase domain mutant (KD-m) or ubiquitin-like domain mutant (UbLD-m) IKKε, Flp-In 293 cells (Invitrogen) were transfected with either pcDNA5.5-unt-IKKε, pcDNA5.5-KD-M-IKKε (K38A), pcDNA5.5-UbLD-M-IKKε (L353A,F354A) or pcDNA5.5-GFP, together with a pOG44 plasmid at a molar ratio of 1:9. cDNA plasmids were mixed with Lipofectamine LTX (15338100, Thermo Fisher Scientific) or Fugene HD (E2311, Promega) according to the manufacturer’s instruction and transfected into the different cell lines for 48 h.

Stable cell lines and single cell clones expressing wild-type IKKε (wt) or mutant IKKεs, with disruption of either kinase domain or ubiquitin-like domain function (KD-m or UbLD-m), in a doxycycline-dependent manner were selected with 300 µg/ml hygromycin (Calbiochem). All Flp-In 293 cells were cultured in DMEM (Sigma-Aldrich). The panel of breast cancer cell lines were kindly provided by Dr. Alice Shia and Prof. Peter Schmid. MDA-MB-231, MDA-MB-468, MDA-MB-175, ZR75.1, T47D, HCC1143, MCF7 were cultured in RPMI-1640 (Sigma-Aldrich), Cal120 and MDA-MB-453 were cultured in DMEM (Sigma-Aldrich) and SUM44 in DMEM (Sigma-Aldrich) and 1 nM estrogen (Sigma-Aldrich). For all cell lines, medium was supplemented with 10% FBS, penicillin-streptomycin and Normocin (InvivoGen). Serum-free medium was custom made DMEM without serine, with 10% dialysed FBS and penicillin-streptomycin. All cells were cultured with environmental conditions of 37°C, 5% CO2.

Drugs

The following drugs were used: 6-Diazo-5-oxo-l-norleucine (Don, D2141, Sigma-Aldrich); Sodium dichloroacetate (DCA, 347795, Sigma-Aldrich); NCT-502 and PHGDH inactive (19716 and 19717, Cayman); Doxycycline (Dox, D9891, Sigma-Aldrich), Oligomycin (sc-203342, Santa Cruz Biotechnology); FCCP (sc-203578, Santa Cruz Biotechnology); Antimycin (sc-202467, Santa Cruz Biotechnology); Rotenone (sc-20342, Santa Cruz Biotechnology); Cyt.C (C2037, Sigma-Aldrich) CB-839 (10-4556, Focus Biomolecules); Adenosine diphosphate (ADP, A2754, Sigma-Aldrich).

siRNA transfection

The following oligos were transfected for siRNA-mediated knockdown: AllStars Negative Control siRNA (1027281, Qiagen); Hs_ATF4_9 FlexiTube siRNA (SI04236337, Qiagen); Hs_IKBKE_6 FlexiTube siRNA, (SI02622339, Qiagen); Hs_IKBKE_7 FlexiTube siRNA (SI02622326, Qiagen); Hs_IKBKE_8 FlexiTube siRNA (SI02655317, Qiagen); Hs_IKBKE_9 FlexiTube siRNA (SI02655324, Qiagen); Hs_IKBKE_10 FlexiTube siRNA (SI02626526, Qiagen); Hs_PAT1_10 FlexiTube siRNA (SI03019709, Qiagen, UK); Hs_PAT1_12 FlexiTube siRNA (SI03222412, Qiagen, UK); Hs_PAT1_14 FlexiTube siRNA (SI04265625, Qiagen, UK); Hs_PAT1_15 FlexiTube siRNA (SI04272212, Qiagen, UK); Hs_REL_A_5 FlexiTube siRNA (SI00301672, Qiagen).

For transfection, siRNA was mixed with DharmaFect 4 (T200402, Dharmacon), and cells were transfected according to the transfection reagent manufacturer’s instruction for 48 h or 72 h prior to measurements. Cells were transfected with a final concentration of 50 nM siRNA, and a pool of all 4 IKBKE-targeting oligos was used for suppression of IKBε, a pool of all 4 PSAT1-targeting oligos was used for suppression of PSAT1, and single targeting oligos were used for the suppression of ATF4, p65 and IRF3.

Oxygen consumption and extracellular acidification rate measurements

An XF24 Extracellular or XF96 Extracellular Flux analyser (Sea-horse Biosciences, Agilent Technologies) was used to determine the bioenergetic profiles in breast cancer cell lines. Cells were plated in six-well corning dishes first and then transfected with siRNA 24 h
after plating. Twenty-four hours after transfection, cells were trypsinised, counted and plated into a 24 or 96-well Seahorse plate. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were assessed in Seahorse medium according to the manufacturer protocols. Respiratory parameters were assessed as described in Fig EV1B. Oxygen consumption rate (OCR) of Flp-In 293 cells was measured using an Oroboros high-resolution respirometer (Oroboros) at 37°C, in Seahorse XF assay medium containing 4.5 g/l glucose, 1 mM pyruvate and 25 mM Hepes, and the assay was performed as in Fig EV1B.

For measurements in isolated mitochondria, Flp-In 293 cells were first washed with PBS and collected in homogenisation buffer (250 mM sucrose, 5 mM Hepes, pH 7.4, 0.5 mM EGTA), and Protease inhibitor cocktail (1187358001, Roche) and then homogenised in a glass/glass, tight potter by 100 strokes on ice, followed by centrifugation for 5 min at 800 g at 4°C. The supernatant, containing mitochondria, was centrifuged again at 9,000 g. The pellet was resuspended and adjusted to a protein concentration of 0.8 mg/ml in OCB buffer (125 mM KCl, 20 mM MOPS, 10 mM Tris pH7.2–7.3, 0.2 mM EGTA, 2.5 mM KH2PO4, 2.5 mM MgCl2). 10 mM glutamate and 5 mM malate were added to the mitochondrial suspension before the experiment, and OCR was measured in OCB buffer using the Oroboros high-resolution respirometer. ADP (final concentration 0.25 mM), Cyt.C (10 μM), oligomycin (5 μM) were injected step by step, and 50 μM FCCP was added in 1 μl steps until maximum respiratory capacity was detected. At the end of the run, antimycin (5 μM final concentration) was injected. Data were then analysed by the Datalab 5.5 (Oroboros) software.

**Cell proliferation assay**

Cells were plated in Corning 96-well plates at a density between 2,000 and 10,000 cells per well for different cell lines. Cell proliferation rate was then measured using the Incucyte ZOOM instrument (Essen Biosciences) for 3–7 days, and proliferation rate was analysed with the Incucyte Zoom 2015A software (Essen Biosciences).

**Metabolic labelling and metabolome analysis**

Flp-In 293 cells and breast cancer cell lines (T47D and MDA-MB-468) were first plated separately in six-well plates in five technical replicates per each condition. IKKε expression in Flp-In 293 cells was then induced by 50 ng/ml doxycycline, and breast cancer cells were transfected with siRNA to suppress IKKε. Two hours after induction for the Flp-In 293 cells, and 48 h after siRNA transfection for the breast cancer cell lines, cells were induced with either 13C6-glucose (CLM-1396-5, Cambridge Isotope Laboratories) medium or 15N2-glutamine (NLM-1328-0.25, Cambridge Isotope Laboratories) medium for 14 h. Cells were then washed three times with PBS, and metabolites were extracted using cold extraction buffer (50% methanol, 30% acetonitrile, 20% ultrapure water, 100 mg/ml HEPES) at a ratio of 1 ml extraction buffer/106 cells. After 15-min incubation time on methanol and dry ice, cells were placed on a shaker for 15 min using a thermal mixer at 4°C and incubated for 1 h at –20°C. Cell lysates were centrifuged, and the supernatant was collected and transferred into autosampler glass vials, which were stored at –80°C until further analysis.

Samples were randomised in order to avoid bias due to machine drift and processed blindly. LC–MS analysis was performed using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer coupled to a Dionex U3000 UHPLC system (Thermo Fisher Scientific). The liquid chromatography system was fitted with a Sequent ZIC-pHILIC column (150 mm × 2.1 mm) and guard column (20 mm × 2.1 mm) from Merck Millipore and temperature maintained at 45°C. The mobile phase was composed of 20 mM ammonium carbonate and 0.1% ammonium hydroxide in water (solvent A) and acetonitrile (solvent B). The flow rate was set at 200 μl/min with the gradient described previously (Mackay et al, 2015). The mass spectrometer was operated in full MS and polarity switching mode. The acquired spectra were analysed using Xcalibur Qual Browser and Xcalibur Quan Browser software (Thermo Fisher Scientific).

**Phosphoproteomics**

**Sample preparation**

Flp-In 293 single cell clones for IKKε (Clones 1, 2 and 3) and GFP (Clones 1, 2 and 3) were seeded in 6-well plate in three replica for each condition. After 24 h of seeding, cells were induced with doxycycline for 16 h. Cells were first washed with ice-cold PBS containing 1 mM Na2VO4 and 1 mM NaF and then lysed in a lysis buffer containing 8M Urea, 20 mM HEPES, 1 mM Na2VO4, 1 mM NaF, 1 mM B-Glycerol phosphate and 0.25 mM Na2HPO4. After incubation on ice for 5 min, the cells were then scraped and collected in Eppendorf tubes and stored at –80°C. For sample analysis, cell lysates were thawed, protein digested with trypsin, and phosphopeptides were enriched using TiO2 as described in (Wilkes & Cuilliau, 2017).

**Nanoflow-liquid chromatography tandem mass spectrometry (LC–MS/MS)**

Dried samples were dissolved in 0.1% TFA (0.5 μg/μl) and run in a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) connected to a nanoflow ultra-high pressure liquid chromatography (UPLC, NanoAcquity, Waters). Peptides were separated using a 75 μm × 150 mm column (BEH130 C18, 1.7 μm Waters) using solvent A (0.1% FA in LC–MS grade water) and solvent B (0.1% FA in LC–MS grade ACN) as mobile phases. The UPLC settings consisted of a sample loading flow rate of 2 μl/min for 8 min followed by a gradient elution with starting with 5% of solvent B and ramping up to 35% over 100 min followed by a 10-min wash at 85% B and a 15-min equilibration step at 1% B. The flow rate for the sample run was 300 nL/min with an operating back pressure of about 3,800 psi. Full scan survey spectra (m/z 375–1,800) were acquired in the Orbitrap with a resolution of 30,000 at m/z 400. A data-dependent analysis (DDA) was employed in which the five most abundant multiply charged ions present in the survey spectrum were automatically mass-selected, fragmented by collision-induced dissociation (normalised collision energy 35%) and analysed in the LTQ. Dynamic exclusion was enabled with the exclusion list restricted to 500 entries, exclusion duration of 30 s and mass window of 10 ppm.

**Database search for peptide/protein identification and MS data analysis**

Peptide identification was by searchers against the Swiss-Prot database (version 2013-2014) restricted to human entries using the
Mascot search engine (v 2.5.0, Matrix Science). The parameters included trypsin as digestion enzyme with up to two missed cleavages permitted, carbamidomethyl (C) as a fixed modification and Pyro-glu (N-term), Oxidation (M) and Phospho (STY) as variable modifications. Datasets were searched with a mass tolerance of ± 5 ppm and a fragment mass tolerance of ± 0.8 Da.

The automated programme Pescal (Cutillas & Vanhaesebroeck, 2007) was used to calculate the peak areas of the peptides identified by the mascot search engine. Proteins were identified with at least two peptides matched to the protein and a mascot score cut-off of 20 was used to filter false-positive detection. The resulting quantitative data were parsed into Excel files for normalisation and statistical analysis. Significance was assessed by t-test of log2 transformed data. When required, P-values were adjusted using the Benjamini–Hochberg method. Results are shown as log2 fold IKK± over control.

**Western blot**

Protein levels were assessed using Western blotting. Cells were lysed in a lysis buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 1.5 mM MgCl2, 1% Triton, 10% glycerol) containing complete protease inhibitor cocktail (Roche) and, where necessary, HALT bromophenol blue). SDS-PAGE was performed using either 10 or 4–20% gels (Biorad) and, where necessary, HaloPrep bromphenol blue. SDS-PAGE was performed using either 10 or 4–12% Bis-Tris Protein gels (Invitrogen) and resolved using SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue). SDS-PAGE was performed using either 10 or 4–12% NuPAGE™ Bis-Tris Protein gels (Invitrogen) and resolved by SDS-PAGE in loading buffer (40% Glycerol, 30% β-Mercaptoethanol, 6% SDS, bromophenol blue).

**High-content imaging and measurement of mitochondrial membrane potential (ΔΨm)**

Cells were seeded in thin, clear bottom black 96-well plates (BD Falcon) at medium density (4,000 cells/well) 24 h before the experiments. Prior to imaging cells were loaded with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) and 30 nM tetramethyl-rhodamine-methylster (TMRM) for 30 min. TMRM was present during imaging in the solution (DMEM w/o phenol red). Images were acquired with the ImageXpress Micro XL (Molecular Devices) high-content wide field digital imaging system using a Lumencor SOLA light engine illumination, ex377/50 nm em447/60 nm (Hoechst) or ex562/40 nm and ex624/40 nm (TMRM) filters, and a 60X, S PlanFluor ELWD 0.70 NA air objective, using laser-based autofocus. Sixteen fields/well were acquired. Images were analysed with the granularity analysis module in the MetaXpress 6.2 software (Molecular Devices) to find mitochondrial (TMRM) and nuclear (Hoechst) objects with local thresholding. Average TMRM intensities per cell were measured and averaged for each well. The mean of wells was then used as individual data for statistical analysis to compare each condition.

**PDH activity measurement**

PDH activity was measured on whole cell lysates using the pyruvate dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit (ab109902, Abcam).

**qRT–PCR**

mRNA levels were assessed using quantitative real-time PCR (qRT–PCR). Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) as per the manufacturer’s protocol. RNA yield was quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific), and 1 μg of RNA was reverse transcribed to cDNA using the Omniscript RT Kit (Qiagen). qPCR was performed using the TaqMan™ assay system.

The following TaqMan™ gene expression probes were used: PHGDH (Hs00198333_m1, Thermo Fisher Scientific); PSAT1 (Hs00795278_mH, Thermo Fisher Scientific); PSPH (Hs004826 | EMBO reports e48260 | 2020 15 of 18
190154_m1, Thermo Fisher Scientific); ACTB (β-Actin, 4310881E, Applied Biosystems).

Assay mixtures were prepared consisting of 10 μl TaqMan™ Master Mix (Applied Biosystems), 1 μl TaqMan™ gene probe & 1 μl cDNA, topped up to 20 μl with 8 μl RNase free H₂O. The qPCR reaction was carried out using either the 7500 Real Time or the QuantiStudio 5 Real-Time PCR systems (Applied Biosystems), and the process was 2 min at 50°C, followed by 10 min holding at 95°C, then 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Relative mRNA quantifications were obtained using the comparative Ct method, and data were analysed using either the 7500 software v2.3 or QuantiStudio Design & Analysis Software (Applied Biosystems).

Generation of conditioned medium

Flp-In 293 HA-GFP or HA-IKKε cells were treated for 16 h with 50 ng/ml doxycycline in 1 ml of medium per well of a 6-well plate, allowing secretion of potential signalling factors into the medium. Following induction, medium was collected and filtered using a 0.22 μm pore size filter and stored at 4°C till use.

Gene expression analysis of clinical samples

The METABRIC dataset (Curtis et al., 2012) was obtained from Synapse: https://www.synapse.org/#!Synapse:syn1688369 (METABRIC Data for Use in Independent Research). All analysis was carried out using Bioconductor R packages. Overexpression of all genes was determined by fitting a Gaussian distribution to the central subpopulation shifted to zero and then determining samples which had expressions greater than 1.96 times the standard deviation from zero.

Data availability

Datasets generated as part of this study through labelled metabolite analysis and phosphoproteomic analysis are both provided in full as part of this manuscript as Datasets EV1 and EV2, respectively.

Statistical analysis

Data are presented as mean ± either standard deviation (SD) or standard error of the mean (SEM) as indicated in the figure legends. Statistical analysis tests were performed using GraphPad Prism (version 8), and specific tests were performed as indicated in the figure legends. Statistical significance was assumed at P < 0.05 and is noted on figures using *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 where appropriate.

Expanded View for this article is available online.

Acknowledgements

We are grateful to Dr. Alice Shia and Prof. Peter Schmid (BCI – QMUL) for providing the breast cancer cell line panel and to Dr. Tencho Tenev and Prof. Pascal Meier (CJR, London) for useful advice and discussion, to Dr Gunnel Hallden (BCI – QMUL) for providing reagents, to Prof. Ivan Dickic (Institute of Biochemistry II – Goethe University, Germany) for providing plasmids containing IKKε mutants and to the Barts Cancer Institute FACS facility for their support. KaB is supported by the Barts London Charity (Grant Reference Number: 467/2053), and WJ is supported by the Medical Research Council (MRC) PhD programme. PC is funded by the BBSRC (BB/M006174/1) and Barts and The London Charity (297/2249). GS is funded by University College London COMPLEX/British Heart Foundation Fund (SP/08/004), the BBSRC (BB/L020874/1) and the Wellcome Trust (207815/Z/211/Z) in the UK, and the Italian Association for Cancer Research (AIRC, IG22221). CF and ASHc are funded by the Medical Research Council, core fund to the MRC Cancer Unit (MRC_MC_UU_12022/6).

Author contributions

RX performed experiments to characterise the effect of IKKε on mitochondrial oxygen consumption rate and to test the sensitivity of breast cancer cell lines to different drugs. WJ performed the experiments to characterise the mechanism through which IKKε regulates cellular metabolism (qRT-PCR and WB). RX did the cloning of IKKε mutants and generated the cell lines. EW-V helped with the phosphoproteomic experiment together with VR and PC that also performed the MS for the in vitro kinase assay. ASHC and CF performed the MS experiment with metabolic tracers and analysed the data. AN and CC helped with phosphoproteomic data analysis. By contributed to the OCR measurements, SOB to the characterisation of the role of ATF4 and YW to the cloning. GS performed the experiments to measure mitochondrial membrane potential and analysed the data. RBB, GS and KeB analysed the gene expression datasets. KaB designed the study and wrote the manuscript with the help of RX, WJ, PC, GS and CF.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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