



Induction of store-operated calcium entry (SOCE) suppresses glioblastoma growth by inhibiting the Hippo pathway transcriptional coactivators YAP/TAZ

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Abstract

Glioblastomas (GBM) are the most aggressive brain cancers without effective therapeutics. The Hippo pathway transcriptional coactivators YAP/TAZ were implicated as drivers in GBM progression and could be therapeutic targets. Here we found in an unbiased screen of 1650 compounds that amlodipine is able to inhibit survival of GBM cells by suppressing YAP/TAZ activities. Instead of its known function as an L-type calcium channel blocker, we found that amlodipine is able to activate Ca²⁺ entry by enhancing store-operated Ca²⁺ entry (SOCE). Amlodipine as well as approaches that cause store depletion and activate SOCE trigger phosphorylation and activation of Lats1/2, which in turn phosphorylate YAP/TAZ and prevent their accumulation in the cell nucleus. Furthermore, we identified that protein kinase C (PKC) beta II is a major mediator of Ca²⁺-induced Lats1/2 activation. Ca²⁺ induces accumulation of PKC beta II in an actin cytoskeletal compartment. Such translocation depends on inverted formin-2 (INF2). Depletion of INF2 disrupts both PKC beta II translocation and Lats1/2 activation. Functionally, we found that elevation of cytosolic Ca²⁺ or PKC beta II expression inhibits YAP/TAZ-mediated gene transcription. In vivo PKC beta II expression inhibits GBM tumor growth and prolongs mouse survival through inhibition of YAP/TAZ in an orthotopic mouse xenograft model. Our studies indicate that Ca²⁺ is a crucial intracellular cue that regulates the Hippo pathway and that triggering SOCE could be a strategy to target YAP/TAZ in GBM.

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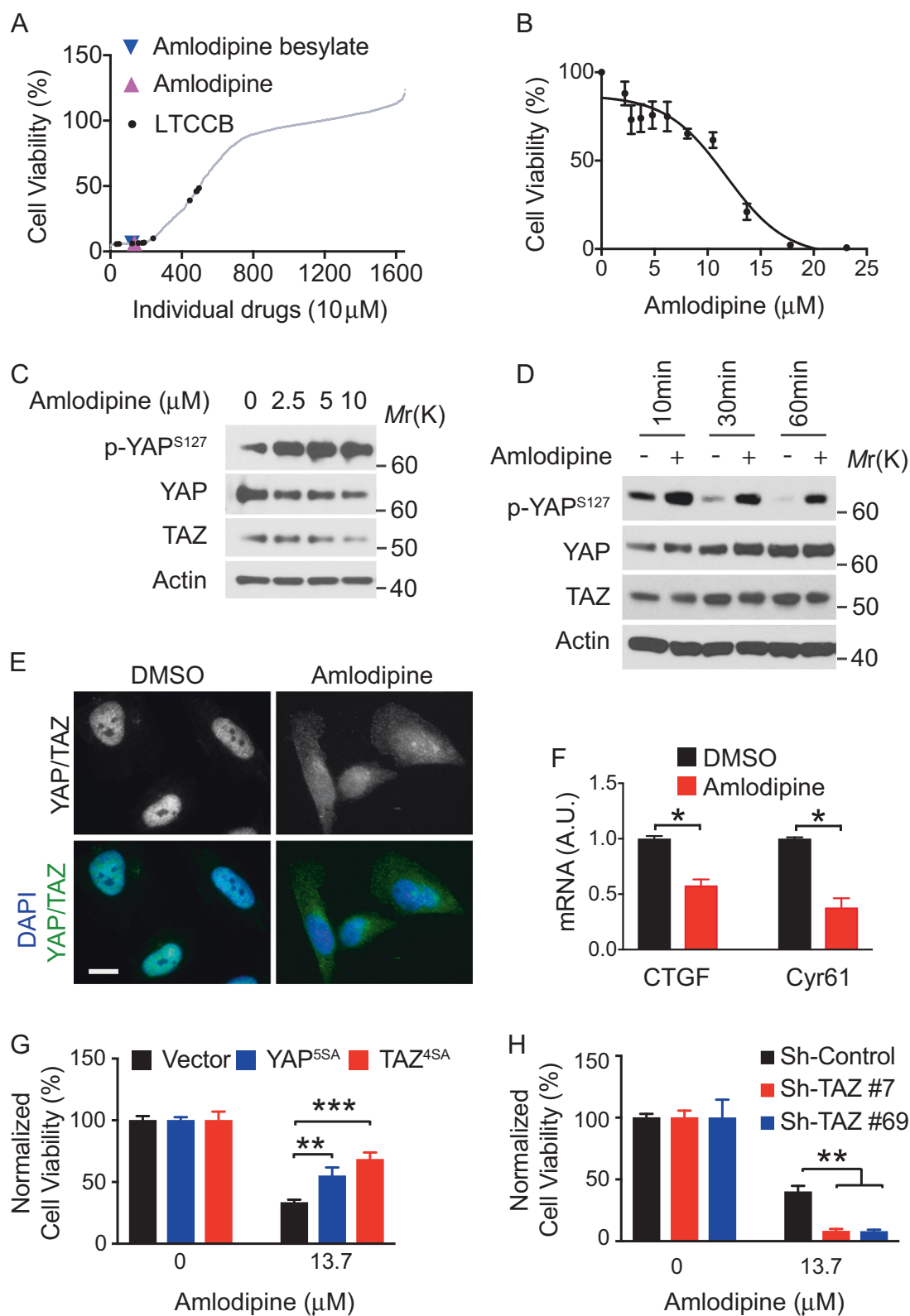
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Introduction

Glioblastomas (GBM) are the most aggressive brain cancers. Median survival of patients with GBM is only 12–17 months [1]. Currently, surgery followed by radiotherapy and chemotherapy is still the major treatment, although the outcome is usually poor. Development of targeted therapies for these cancers based on oncogenic mutations and signaling pathways could alter the prognosis. Integrated genomic and gene expression signature studies classified GBM into several subtypes differing in treatment responses and survival rates [2, 3]. Among these subtypes, the mesenchymal group associates with worst prognosis [2]. Gene regulatory network analysis and comprehensive analysis of brain tumor samples by immunohistochemistry found transcriptional coactivator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP) as drivers in GBM mesenchymal transformation [4, 5].

YAP and TAZ (YAP/TAZ) are two paralogous nuclear effectors of the Hippo signaling pathway, which is a



conserved signaling network governing cellular growth and survival [6]. This pathway contains a core serine/threonine kinase cascade, including MST1/2 kinases and their

substrates Lats1/2 kinases. The upstream growth control signals from cell–cell contact, cell–matrix contact, and extracellular soluble factors, as well as intracellular

◀ **Fig. 1** Amlodipine inhibits cell survival and YAP/TAZ in LN229 cells. **a** An in vitro screen for small molecules, which inhibit LN229 cells, revealed the inhibitory effects of LCCB. **b** Cells were treated by amlodipine at the indicated concentrations for 3 days and subjected to MTT assay. $n = 5$. **c** Cells were treated by amlodipine at the indicated concentrations for 24 h and subjected to western blotting. Representative blots from three independent experiments. **d** Cells were treated with or without amlodipine (10 μ M) for the indicated times and subjected to western blotting. Representative blots from three independent experiments. **e** Cells were treated by DMSO or amlodipine for 30 min and subjected to IF staining. Representative images from three independent experiments. Scale bar = 20 μ m. **f** Cells were seeded in DMSO- or amlodipine-containing media for 3 h and subjected to qRT-PCR analysis. $n = 2$. **g, h** Cells stably transduced by TAZ^{4SA}, YAP^{5SA}, or vector alone in (**g**) or by TAZ shRNAs or a scramble shRNA control in (**h**) were treated by DMSO or amlodipine for 3 days and subjected to MTT assay. In **g, h**, the MTT reading of amlodipine-treated cells in the indicated genetically modified groups were normalized to DMSO-treated cells in the correspondent groups. In **g**, $n_{\text{vector}} = 6$, $n_{\text{YAP5SA}} = 3$, $n_{\text{TAZ4SA}} = 4$. In **h**, $n = 4$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

metabolic levels can lead to activation of Lats1/2, which in turn phosphorylate and inhibit YAP/TAZ by preventing their accumulation in the nucleus. The Hippo pathway thus suppresses the downstream oncogenic transcription and promotes quiescence. Loss of this growth control machinery could lead to enlarged organs and even tumorigenesis due to cell hyperproliferation and dysfunctional cell removal via apoptosis. Consistently, YAP/TAZ activation is widely found in multiple human cancers [7, 8]. Recent studies have also found that hyperactivation of YAP/TAZ is associated with resistance to canonical chemotherapies, radiotherapies, and targeted therapies [9–12]. Therefore, drugs targeting YAP/TAZ have been of recent interest in cancer treatment [13].

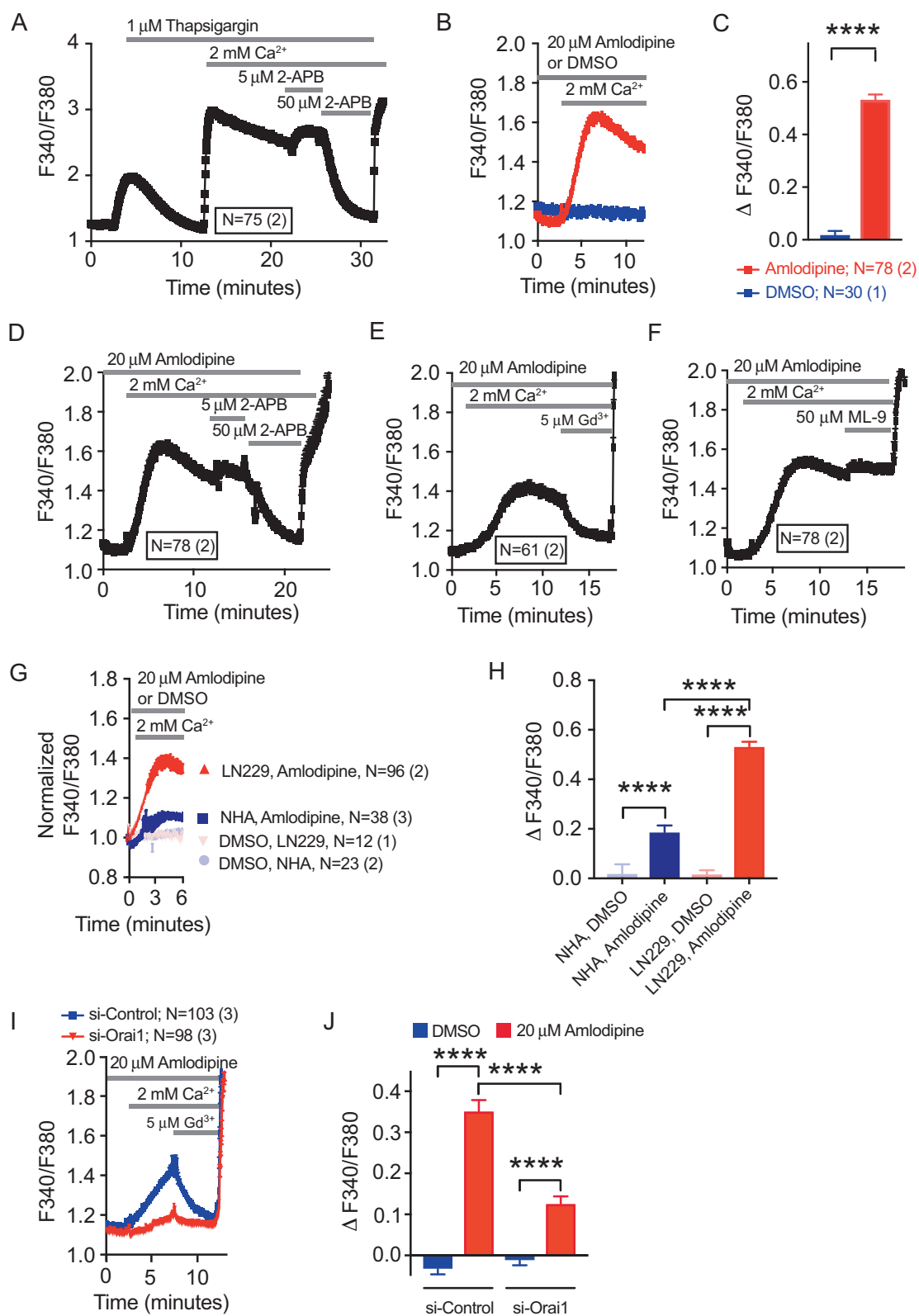
Calcium (Ca^{2+}) is a fundamental intracellular signal that regulates a variety of cellular functions. Elevation of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) could paradoxically promote both cell proliferation and cell death. It has long been realized that cancer cells hijack the Ca^{2+} -signaling toolkit to benefit their proliferation and migration; therefore targeting Ca^{2+} transport has been proposed for cancer treatment [14]. On the other hand, cancer cells also develop strategies to avoid Ca^{2+} -induced cell death; and these strategies may also be explored for cancer therapies [15]. Store-operated Ca^{2+} entry (SOCE) is the most ubiquitous Ca^{2+} signaling pathway in non-excitable cells. It is activated upon depletion of the internal Ca^{2+} reserves of the endoplasmic reticulum (ER) [16]. The activation process involves sensing of Ca^{2+} store depletion by the ER protein STIM1, which aggregates in ER–plasma membrane junctional areas to trap and activate the SOCE channel, formed by Orai proteins (Orai1–3) [17]. The STIM/Orai signaling nexus has been implicated in tumorigenesis and has been proposed to be a viable target for therapeutic interventions [18].

Here we conducted an unbiased screen using a library containing 1650 compounds, most of which are Food and Drug Administration (FDA)-approved drugs. From the screen, we found that amlodipine inhibits GBM cells' survival by suppressing YAP/TAZ activities. Unexpectedly, we found that, in addition to its canonical function as a L-type calcium channel blocker (LTCCB), amlodipine is able to activate Ca^{2+} entry through SOCE via Orai channels. Thus elevation of intracellular Ca^{2+} inhibits YAP/TAZ by activating the core serine/threonine kinase cascade of the Hippo pathway. This process depends on inverted formin-2 (INF2)-mediated Ca^{2+} -induced actin remodeling and protein kinase C (PKC) beta II. Correspondingly, elevation of PKC beta II expression inhibits GBM cell growth and tumorigenesis by inhibiting YAP/TAZ. We propose that the SOCE–PKC beta II axis could be used to inhibit YAP/TAZ-active GBM.

Results

Amlodipine inhibits survival of GBM cells by suppressing YAP/TAZ activities

YAP/TAZ are activated during the development of GBM. To identify methods of inhibiting GBM growth, we carried out a small-molecule screen using a library containing 1650 compounds, most of which are FDA-approved drugs. Our screen used the LN229 human GBM cell line, which shows increased expression of TAZ [19], presumably due to gene amplification (The Cancer Genome Atlas database). We confirmed that TAZ is essential for cell growth and tumorigenesis of LN229 cells by short hairpin RNA (shRNA)-mediated knockdown of TAZ in these cells (Figure S1A–S1F). From the screen, we found a set of LTCCBs that can effectively inhibit the survival of these cells (Fig. 1a). Among these LTCCBs, both amlodipine and amlodipine besylate show around 95% inhibition. We confirmed that amlodipine can inhibit LN229 cell survival at $\text{IC}_{50} = 11 \mu\text{M}$ (Fig. 1b). We noted a discrepancy of amlodipine efficacy between the screen and this confirmation assay. This may be due to different amlodipine sources or dilution methods used in these different assays. We then examined the effects of amlodipine on YAP/TAZ and found that amlodipine induces YAP phosphorylation and reduces their expression levels in a dose-dependent manner after 24 h of treatment (Fig. 1c). To determine whether reduced expression levels of YAP/TAZ were a consequence of amlodipine-induced cell death, we conducted time course studies. We found that as little as 10 min of amlodipine treatment can induce mild increase of YAP phosphorylation (Fig. 1d). We also saw YAP/TAZ cytoplasmic translocation after as little as 30 min of treatment (Fig. 1e). This



cytoplasmic translocation was followed by inhibition of the expression of YAP/TAZ target genes CTGF and Cyr61 (Fig. 1f). These results suggested that amlodipine induces

YAP/TAZ phosphorylation, which may promote their degradation. To test this notion, we stably expressed TAZ^{4SA} or YAP^{5SA}, which cannot be regulated by

◀ **Fig. 2** Amlodipine induces Ca^{2+} entry in LN229 cells. **a** Ca^{2+} measurements using the standard Ca^{2+} off/ Ca^{2+} on protocol and the dye Fura-2-AM showing that 1 μM thapsigargin activated Ca^{2+} release in nominally Ca^{2+} free bath solutions and SOCE when extracellular Ca^{2+} (2 mM) was replenished. Subsequent addition of 5 μM 2-APB potentiated SOCE while 50 μM 2-APB inhibited SOCE in LN229 cells. **b, c** In the absence of extracellular Ca^{2+} , 20 μM amlodipine did not activate a Ca^{2+} signal. However, in the presence of extracellular Ca^{2+} , amlodipine activated significant and sustained Ca^{2+} entry. **d, e** 50 μM 2-APB and 5 μM Gd^{3+} inhibited amlodipine-activated calcium entry while 5 μM 2-APB potentiated Ca^{2+} entry. **f** The SOCE inhibitor ML-9 (50 μM), which acts through disruption of STIM1 aggregation, did not inhibit amlodipine-activated Ca^{2+} entry. **g, h** Amlodipine activated a significant Ca^{2+} entry in both LN229 and NHA cells compared to vehicle control, DMSO. Compared to NHA cells, LN229 cells had significantly more amlodipine-activated Ca^{2+} entry. **i** Knockdown of Orai1 substantially inhibited amlodipine-activated Ca^{2+} entry when compared to si-Control. Later addition of 5 μM Gd^{3+} inhibited amlodipine-activated Ca^{2+} entry in both si-Control- and si-Orai1-transfected cells. **j** Compared to vehicle, amlodipine activated significant Ca^{2+} entry, and knockdown of Orai1 statistically inhibited amlodipine-activated Ca^{2+} entry when compared to si-Control. All data were derived from the indicated number of total cells and independent experiments detailed in outlined boxes. 10 μM ionomycin was included at the end of all tracings as a Fura-2 loading control. **** $P < 0.0001$

phosphorylation [20, 21]. Compared to the native forms, expression levels of both mutants appear to be less reduced by amlodipine (Figure S1G). However, amlodipine still reduces their expression, especially TAZ^{4SA}, at higher concentrations. This is probably due to the secondary effect of cell death or a phosphorylation-independent mechanism.

To examine whether inactivation of YAP/TAZ accounted for the inhibition of cell survival by amlodipine, we assessed LN229 cells stably expressing the constitutively active mutant, TAZ^{4SA} or YAP^{5SA}. Cells expressing TAZ^{4SA} or YAP^{5SA} were less inhibited by amlodipine (Fig. 1g). Conversely, silencing TAZ expression by shRNAs made these cells more sensitive to amlodipine (Fig. 1h). These results indicated that amlodipine inhibits the survival of LN229 cells by suppressing YAP/TAZ activities. We also examined the effects of amlodipine in normal human astrocytes (NHA) and found that it can also inhibit their survival similarly as LN229 cells (Figure S1H). Notably, amlodipine can also induce YAP/TAZ phosphorylation in NHA (see below).

Amlodipine activates Ca^{2+} entry through SOCE in LN229 cells

While contemplating how amlodipine, an LTCCB, could induce YAP/TAZ phosphorylation, we anticipated that amlodipine inhibits Ca^{2+} entry thereby regulating YAP/TAZ. Surprisingly, we found that amlodipine increased cytosolic Ca^{2+} in LN229 cells in a dose-dependent manner (Figure S2A). To further understand these unexpected

effects on Ca^{2+} signaling and since GBM cells are non-excitable cells, we considered that amlodipine could be activating SOCE. SOCE is a ubiquitous Ca^{2+} entry pathway in non-excitable cells that is activated by luminal ER Ca^{2+} store depletion. Following store depletion, the ER transmembrane proteins, STIM, aggregates and migrates toward the ER-plasma membrane junctions to trap and activate the plasma membrane pore-forming protein Orai to stimulate SOCE. There are three homologs of Orai (Orai1–3), and in most cells only Orai1 is necessary for SOCE [17]. There are few pharmacological inhibitors of SOCE that can be useful in identifying SOCE. These include the compound 2-aminoethoxydiphenyl borate (2-APB) that at high concentrations (50 μM) inhibits Orai1 and Orai2 but potentiates Orai3. The lanthanide ion Gd^{3+} when used at relatively low concentrations (<5 μM) is a relatively specific inhibitor of all Orai channels. The myosin-light-chain kinase inhibitor ML-9 (at 50 μM) inhibits STIM1 aggregation [22–26]. To characterize the SOCE pathway in LN229 cells, the ER internal stores were passively depleted using either thapsigargin (1 μM), a pharmacological inhibitor of the sarcoplasmic/ER Ca^{2+} ATPase (SERCA) pump, or using relatively low microsomal concentrations of the Ca^{2+} ionophore ionomycin (100 nM) [27, 28]. Intracellular Ca^{2+} measurements were achieved using the ratiometric dye Fura-2. Fura-2 is excited alternately at 340 and 380 nm and fluorescence emission is collected at 510 nm. The ratio of Fura-2 emission (F340/F380) upon excitation at 340 nm (which increase on Ca^{2+} binding) to its emission upon excitation at 380 nm (which decrease on Ca^{2+} binding) is directly proportional to the amount of cytosolic Ca^{2+} . Addition of either thapsigargin (Fig. 2a) or ionomycin (Figure S2B) to LN229 cells in a nominally Ca^{2+} free external bath solution caused a typical passive release of Ca^{2+} from ER stores, reminiscent of widely documented Ca^{2+} release signals in many non-excitable cell types. Subsequent restoration of Ca^{2+} (2 mM) to the bath solution revealed the activation of the Ca^{2+} entry phase across the plasma membrane, which is mediated through the SOCE pathway. As expected from the established pharmacological features of SOCE [22, 23], the compound 2-APB potentiated SOCE at 5 μM and inhibited at 50 μM (Fig. 2a and S2B), demonstrating that LN229 cells bear the typical pharmacological signature of SOCE mediated through Orai1 channels. Leak control recordings in LN229 cells were performed in the absence of store depletion and showed insignificant Ca^{2+} entry upon restoration of 2 mM Ca^{2+} to the bath solution (Figure S2C). These results indicated that SOCE is functional in LN229 cells.

To investigate whether amlodipine either causes Ca^{2+} release from ER stores or activates Ca^{2+} entry across the plasma membrane in LN229 cells, we added 20 μM amlodipine in the absence of external Ca^{2+} for several minutes

followed by restoration of 2 mM Ca^{2+} to the external milieu. In the absence of extracellular Ca^{2+} , amlodipine did not activate a Ca^{2+} signal, suggesting that amlodipine does not cause Ca^{2+} release from intracellular stores (Fig. 2b–f). However, when extracellular Ca^{2+} was replenished, amlodipine consistently activated a significant and sustained Ca^{2+} signal in all cells (Fig. 2b–f), suggesting that amlodipine activates a Ca^{2+} entry route across the plasma membrane. To pharmacologically characterize the amlodipine-activated Ca^{2+} entry pathway, we tested the effects of the drug 2-APB on this pathway. Similar to SOCE activated by thapsigargin and ionomycin, amlodipine-activated Ca^{2+} entry was potentiated by 5 μM 2-APB and inhibited by 50 μM 2-APB, suggesting that amlodipine activated Ca^{2+} entry through the SOCE channel Orai1 (Fig. 2d). When used at relatively low concentrations (1–5 μM), the lanthanide ion Gd^{3+} is a specific inhibitor of SOCE through Orai channel isoforms [22, 24, 25]. Consistently, Gd^{3+} at 5 μM inhibited amlodipine-activated Ca^{2+} entry (Fig. 2e). ML-9 (50 μM) was reported to inhibit SOCE through a store-independent mechanism by causing rapid reversal of STIM1 aggregation [26], a process that is required for activation of SOCE through Orai1 channels. Interestingly, 50 μM ML-9 was unable to inhibit amlodipine-activated Ca^{2+} entry (Fig. 2f), further supporting that the effect of amlodipine on Orai1 channels is independent of store depletion and of STIM1 aggregation. We also examined the effects of amlodipine in NHA. Although amlodipine can activate Ca^{2+} entry in NHA, the magnitude of this Ca^{2+} entry is significantly smaller than in LN229 cells (Fig. 2g, h, S2D and S2E). This is consistent with the findings that SOCE in response to passive store depletion by thapsigargin is much smaller in NHA (Figure S2F) compared to LN229 cells (Figure S2H; blue trace), as reported previously [29]. To further examine the role of Orai1 channels in mediating the effect of amlodipine, we silenced the expression of Orai1, the pore-forming subunit of SOCE channels, by using small interfering RNA (siRNA) in LN229 cells (Figure S2G). As expected, knockdown of Orai1 significantly reduced SOCE in response to thapsigargin (Figure S2H; Figure S2I shows leak controls) as well as Ca^{2+} entry in response to amlodipine treatment (Fig. 2i, j). Collectively, these results showed that SOCE is more robust in LN229 cells compared to NHA and that amlodipine activates Ca^{2+} entry through Orai1 channel-mediated SOCE in LN229 cells.

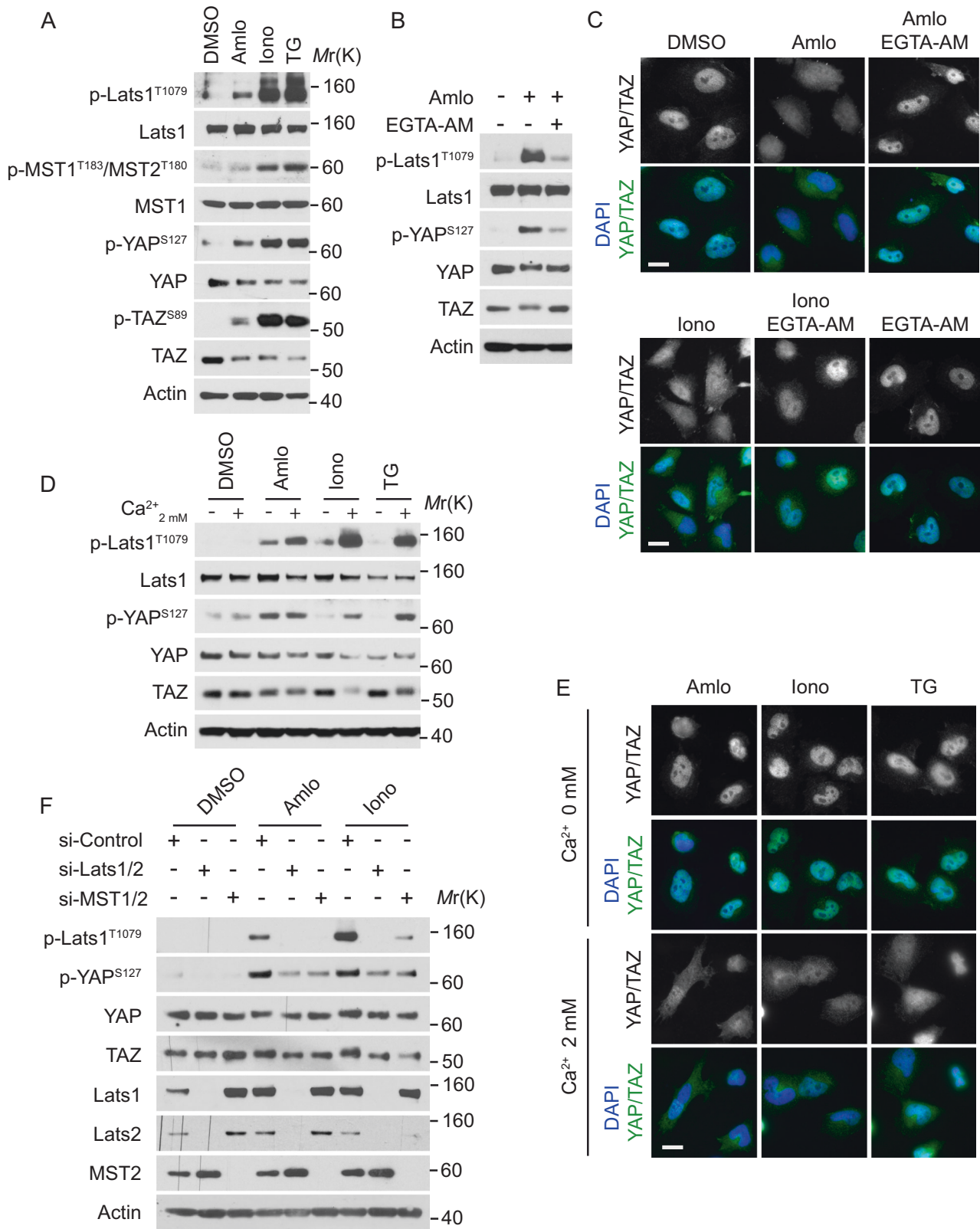
In the absence of extracellular Ca^{2+} , 20 μM amlodipine caused an increase in emitted Fura-2 fluorescence after excitation at both 340 and 380 nm (Figure S2J). Unlike the opposite effect on F340 (increase) and F380 (decrease) observed upon Ca^{2+} binding to Fura-2, this simultaneous increase in both F340 and F380 that translates into a decrease of F340/F380 ratio suggested that amlodipine emits

green fluorescence upon excitation in the ultraviolet (UV) range, thus blunting the Fura-2 signal. Indeed, addition of 20 μM amlodipine to HEK293 cells prior to activation of SOCE with 1 μM thapsigargin caused a significant decrease in Fura-2 fluorescence signal throughout the recording (Fig. S2K, red trace) as previously described [30]. Significantly, washout of amlodipine restored the Fura-2 signal in response to 1 μM thapsigargin to control levels without amlodipine (Figure S2K), strongly suggesting that amlodipine blunts the Fura-2 signal and thus leads to underestimation of the overall intracellular Ca^{2+} concentrations. Indeed, we show a robust Ca^{2+} signal activated by amlodipine when using the single excitation–single emission Ca^{2+} dye Fluo-4, which is excited in the blue range (Figure S2A), further ruling out potential non-specific effects of amlodipine. Taken together, these data suggested that amlodipine activates Ca^{2+} entry through SOCE mediated by Orai1 channels. Amlodipine activates Orai1-mediated Ca^{2+} entry independently of depletion of ER Ca^{2+} stores.

Activation of SOCE inhibits YAP/TAZ activities through the Hippo pathway

The above results suggested that SOCE-mediated cytosolic Ca^{2+} elevation triggers YAP/TAZ phosphorylation. To test this notion, we used two specific SOCE activators, thapsigargin (1 μM) and ionomycin (1 μM), to trigger SOCE and increase intracellular Ca^{2+} . We found that ionomycin and thapsigargin can each robustly induce YAP/TAZ phosphorylation and the cytoplasmic translocation of YAP/TAZ in LN229 cells (Fig. 3a and S3A). Correspondingly, we saw the up shift of TAZ protein bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 3a), a typical phenomenon indicating protein phosphorylation. Therefore, we used this phenomenon as an indication of TAZ phosphorylation in the following studies.

To further investigate whether these effects are dependent on cytosolic Ca^{2+} , we used the cytosolic Ca^{2+} chelator ethylene glycol tetraacetic acid–acetoxymethyl ester (EGTA–AM). We found that adding EGTA–AM suppresses the effects of amlodipine on YAP/TAZ phosphorylation as well as cytoplasmic translocation (Fig. 3b, c upper panel). Similarly, the effect of ionomycin on YAP/TAZ cytoplasmic translocation was also compromised by EGTA–AM (Fig. 3c lower panel). We then used a Ca^{2+} -free medium and found that under this condition, neither ionomycin nor thapsigargin induced YAP/TAZ phosphorylation and their cytoplasmic translocation (Fig. 3d, e). On the other hand, reintroduction of Ca^{2+} into the medium restored the effects for ionomycin and thapsigargin, suggesting a crucial role for Ca^{2+} entry from the extracellular milieu. When similar Ca^{2+} -free medium was used to examine the effect of amlodipine, we found that amlodipine can still induce YAP



phosphorylation to some extent (Fig. 3d). We reasoned that a residual amount of Ca²⁺ in the nominally Ca²⁺ free medium or on the cell membranes might contribute this

observed phenomenon. To test this, we added a very low concentration of EGTA (25 μM) into the Ca²⁺-free medium and found that in this condition the induction of YAP

◀ **Fig. 3** Elevation of cytosolic Ca^{2+} inhibits YAP/TAZ through the Hippo pathway in LN229 cells. **a** Cells were treated by amlodipine (Amlo, 10 μM), ionomycin (Iono, 1 μM), or thapsigargin (TG, 1 μM) for 30 min and subjected to western blotting. Representative blots from three independent experiments. **b** Cells were treated by Amlo (5 μM) with or without EGTA-AM (12.5 μM) for 30 min and subjected to western blotting. Representative blots from two independent experiments. **c** Cells were treated by Amlo (5 μM) and Iono (0.5 μM) with or without EGTA-AM (12.5 μM) for 30 min and subjected to IF staining. Representative images from two independent experiments. **d, e** Cells were treated in media with or without Ca^{2+} containing Amlo (10 μM), Iono (1 μM), or TG (1 μM) for 30 min and subjected to western blotting (**d**) or IF staining (**e**). Representative blots and images from two independent experiments. **f** LN229 cells were transfected by siRNAs against Lats1 and Lats2 (Lats1/2), MST1 and MST2 (MST1/2), or scramble siRNA (control), treated by Amlo or Iono, and subjected to western blotting. Representative blots from three independent experiments. Scale bars = 20 μm

phosphorylation by amlodipine was largely blocked (Figure S3D). Based on these results, we concluded that elevation of cytosolic Ca^{2+} through SOCE induces YAP/TAZ phosphorylation and their translocation into the cytosol.

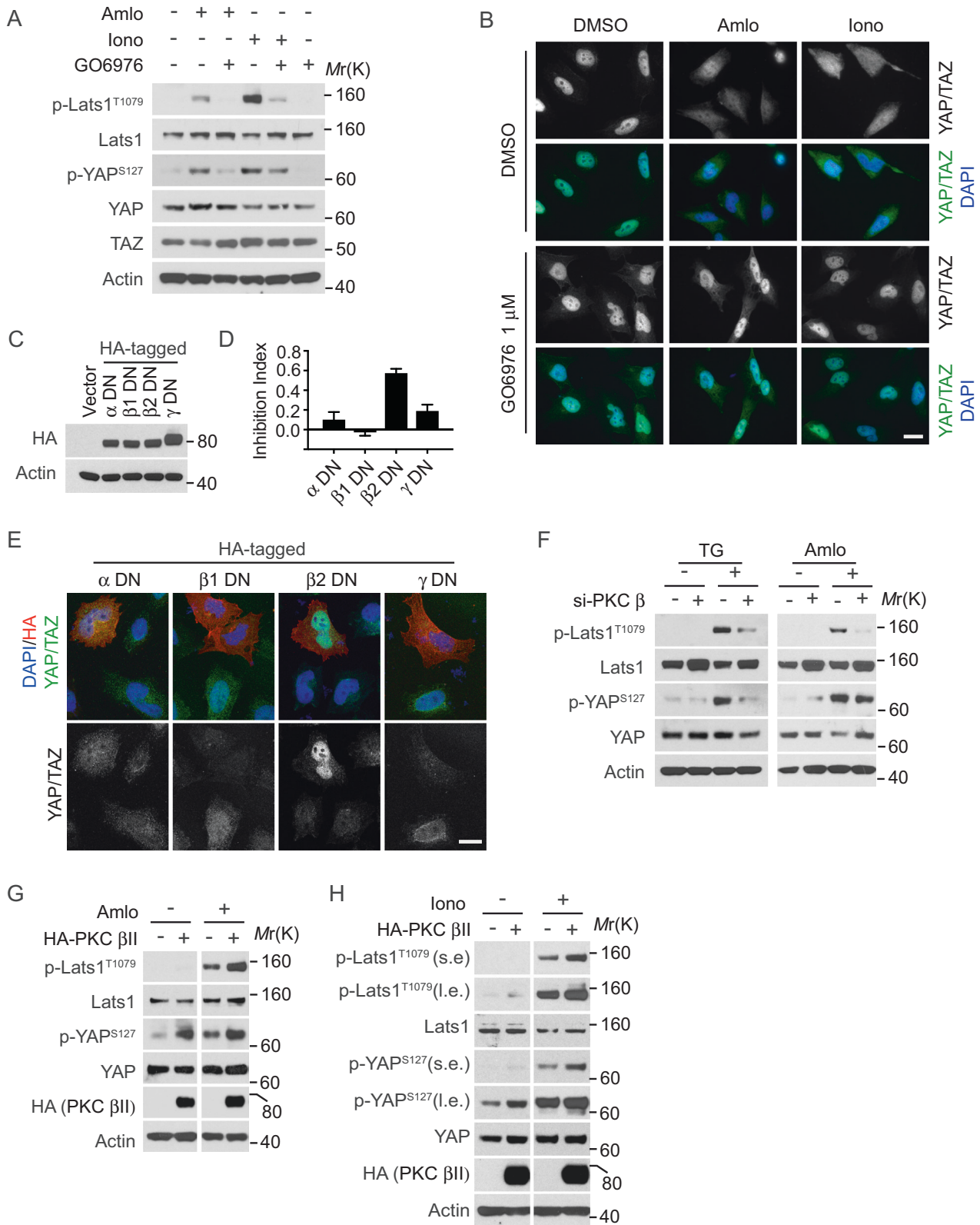
YAP/TAZ phosphorylation indicates activation of their kinases, Lats1/2. Indeed, we found that Lats1 phosphorylation on threonine 1079 (T1079), an essential step for Lats1 activation [31], was robustly induced by amlodipine, ionomycin, or thapsigargin in LN229 cells (Fig. 3a). We also found that the comparable threonine (T1041) in Lats2, which is the homology of Lats1, was also phosphorylated under the treatments of ionomycin or thapsigargin (Figure S3B). In the Hippo pathway, MST1/2 are major kinases in charge of the phosphorylation of Lats1/2, although several other kinases were recently identified to have similar functions [32, 33]. We found that both ionomycin and thapsigargin induce MST1/2 phosphorylation (Fig. 3a), suggesting their activation. To test whether the effects of SOCE on the Hippo pathway are limited to LN229 cells, we examined several other GBM cell lines, including LN18 and DBTRG-05MG cells. In these cells, treatment by amlodipine, ionomycin, or thapsigargin can also robustly induce Lats1 and YAP phosphorylation, as well as YAP/TAZ cytoplasmic translocation (Figure S3C and data not shown). Interestingly, when examining NHA, we found that thapsigargin can only slightly induce TAZ phosphorylation but not phosphorylation of Lats1 and YAP (Figure S3E) compared to LN229 cells (Fig. 3a). This is consistent with the observation that SOCE induced by thapsigargin in NHA is much weaker than in LN229 cells (Figure S2F vs. S2H, blue trace). However, we found that amlodipine can still induce Lats1 and YAP/TAZ phosphorylation (Figure S3E). Given SOCE induced by amlodipine in NHA is also weaker than in LN229 cells (Fig. 2g, h), the effects on the Hippo pathway might be SOCE independent.

In our subsequent studies, we focused on using Lats1 and YAP phosphorylation as readout in LN229 cells to examine

the mechanisms involved. Our experiments indicated that the effect of amlodipine, ionomycin, or thapsigargin on Lats1 phosphorylation also depends on increased intracellular Ca^{2+} through Ca^{2+} entry from the extracellular space (Fig. 3b–d and S3D). These results suggested that elevation of cytosolic Ca^{2+} inhibits YAP/TAZ by activating Lats1. To further establish this connection, we knocked down Lats1 with a pool of siRNAs and found that ionomycin-induced YAP/TAZ phosphorylation was not affected (Figure S3F). However, when both Lats1 and Lats2 were depleted by siRNAs, amlodipine- or ionomycin-induced YAP/TAZ phosphorylation was inhibited (Fig. 3f). These results suggested that Lats1 and Lats2 are both involved in mediating YAP/TAZ phosphorylation. In agreement with this, depletion of Lats2 alone had no effect on ionomycin-induced YAP/TAZ phosphorylation either (Figure S3F). Moreover, YAP/TAZ cytoplasmic translocation induced by amlodipine, ionomycin, or thapsigargin also requires Lats1 and Lats2 (Figure S3G). We further found that depletion of both MST1 and MST2 inhibits amlodipine- and ionomycin-induced phosphorylation of Lats1, YAP, and TAZ, as well as amlodipine-, ionomycin-, or thapsigargin-induced YAP/TAZ cytoplasmic translocation (Fig. 3f and S3G). Notably, TAZ protein levels appear to be still reduced by amlodipine or ionomycin in Lats1/2- or MST1/2-depleted cells. This might be due to other mechanisms independent on the core kinase cascade. Nevertheless, these results indicated that activating the MST1/2–Lats1/2 kinase cascade is a major mechanism to inhibit YAP/TAZ by elevation of cytosolic Ca^{2+} .

Ca^{2+} -induced Lats1 activation and YAP/TAZ inhibition depends on conventional PKCs (cPKCs)

Calcium is an intracellular messenger, which binds to multiple effectors and regulates downstream signaling. Among these effectors, cPKCs were found to be involved in the Hippo pathway regulation by 12-O-tetradecanoylphorbol-13-acetate (TPA, also known as PMA) and G-protein-coupled receptors (GPCRs) [34]. We examined the role of cPKCs by using a cPKC-specific inhibitor GO6976 [35]. We found that GO6976 blocked amlodipine- or ionomycin-induced Lats1 and YAP phosphorylation (Fig. 4a), as well as YAP/TAZ cytoplasmic translocation in LN229 cells (Fig. 4b). This inhibition was dose-dependent, with a partial effect at as low as 0.25 μM and full inhibition at 1 μM (Figure S4A–C). These results supported that the inhibition by GO6976 is unlikely an off-target effect. We also observed similar inhibitory effects of GO6976 (1 μM) in LN18 and DBTRG-05MG cells (data not shown). These results suggested that cPKCs act downstream of Ca^{2+} to activate Lats1/2.



There are four cPKC isoenzymes (alpha, beta I, beta II, and gamma). To investigate whether one particular isoenzyme could be the major mediator in Ca²⁺ regulation of

YAP/TAZ, we transiently expressed a dominant-negative (DN) mutant of each isoenzyme [36] in LN229 cells (Fig. 4c). Interestingly, we found that the beta II DN mutant

◀ **Fig. 4** cPKC mediates Ca^{2+} -induced Lats1 activation and YAP/TAZ inhibition in LN229 cells. **a, b** Cells were treated by Amlor or Iono with or without GO6976 (1 μM) and subjected to western blotting (**a**) or IF staining (**b**). Representative blots and images from three independent experiments. **c–e** Cells were transfected by the indicated HA-tagged dominant-negative (DN) mutants of individual cPKC isoenzymes, treated by amlodipine, and subjected to western blotting (**c**) and IF staining (**e**) as indicated. Quantification of the effect of each PKC DN mutant on YAP/TAZ localization was shown in (**d**) (see Methods). Representative images from three (for beta II DN) or two (for the others) independent experiments. **f** Cells were transfected by siRNAs against PKC beta or scramble siRNA, treated by TG or Amlor, and subjected to western blotting. Representative blots from three independent experiments. **g, h** Cells transfected by HA-tagged PKC beta II were treated by Amlor (**g**), Iono (**h**), or DMSO as a control and subjected to western blotting. s.e. short exposure, l.e. long exposure. Representative blots from three independent experiments. Scale bar = 20 μm

is the most effective one in suppressing amlodipine-induced YAP/TAZ cytoplasmic translocation (Fig. 4d, e), while these DN mutants have no effects on YAP/TAZ localization in dimethyl sulfoxide (DMSO)-treated cells (Figure S4D). We obtained similar results when examining ionomycin-induced YAP/TAZ cytoplasmic translocation (data not shown). These results suggested that PKC beta II is a major mediator of Ca^{2+} -induced YAP/TAZ inhibition in LN229 cells. To examine this notion, we employed a pool of siRNAs to knockdown PKC beta II (Figure S4E) and found that PKC beta II depletion suppressed thapsigargin- and amlodipine-induced phosphorylation of Lats1 and YAP (Fig. 4f). To further investigate whether PKC beta II mediates Ca^{2+} -induced Hippo pathway activation, we transiently expressed PKC beta II in LN229 cells and found that it enhanced amlodipine- and ionomycin-induced Lats1 and YAP phosphorylation (Fig. 4g, h). These results indicated that PKC beta II is a major mediator of Ca^{2+} -induced Lats1/2 activation.

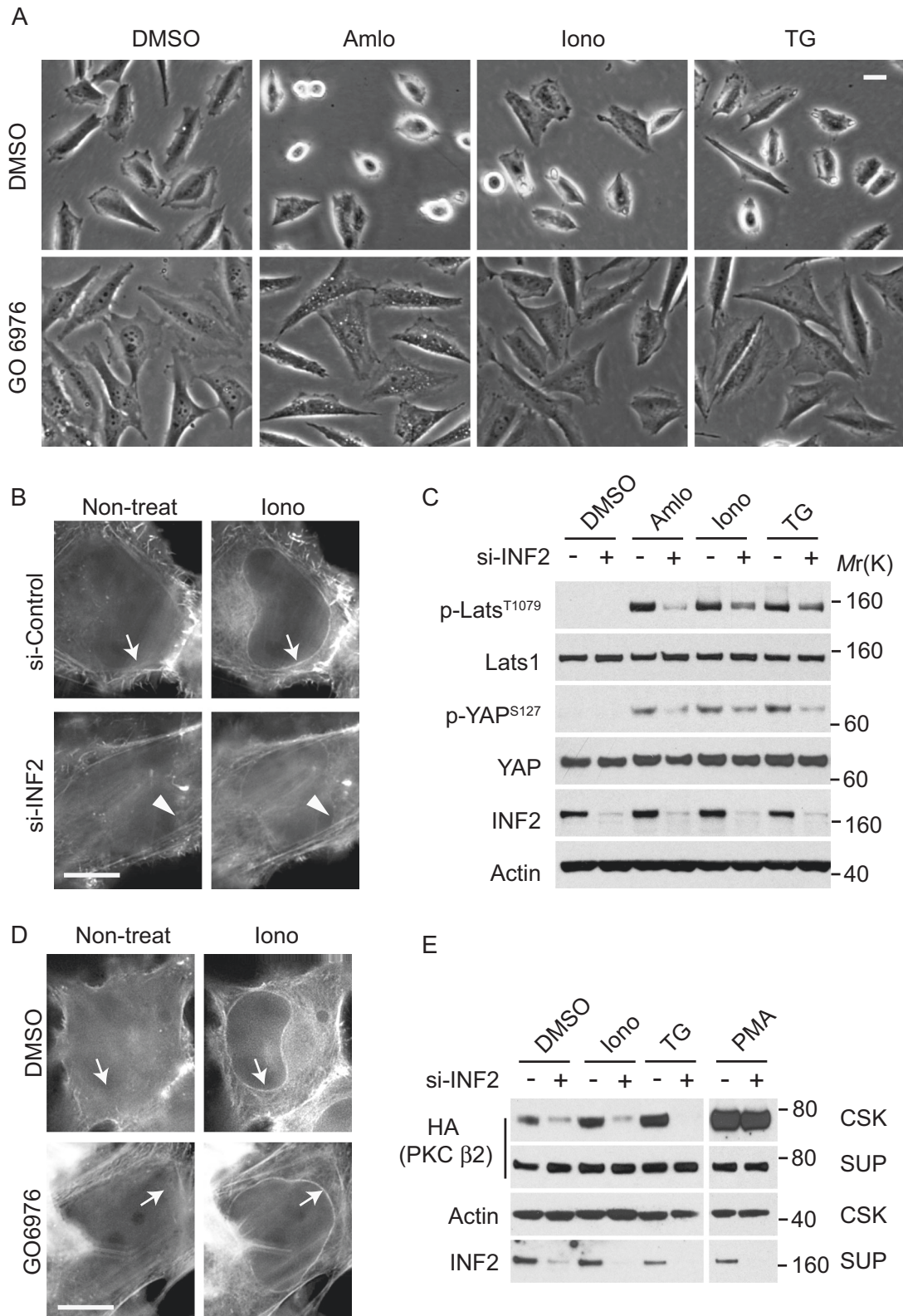
Interestingly, we observed a mild induction of YAP phosphorylation by PKC beta II without the treatment of amlodipine or ionomycin (Fig. 4g, h). In these experiments, we also examined the localization of YAP/TAZ and did not observe marked difference between vector- and PKC beta II-transfected cells (data not shown). It was probably due to the technical limitation of imaging YAP/TAZ localization, which cannot detect the small difference as shown by western blotting. Nevertheless, these results suggested that PKC beta II needs to be activated to trigger the robust effects on the Hippo pathway. Since PMA is a potent activator of cPKCs as well as novel PKCs (nPKCs), we examined whether activation of cPKCs by PMA can also activate Lats1/2 and inhibit YAP/TAZ. Interestingly, in contrast to ionomycin and thapsigargin, PMA (100 nM) treatment does not affect phosphorylation of Lats1, YAP, or TAZ (Figure S4F) nor does it affect cytoplasmic-nuclear distribution of YAP/TAZ in LN229 cells (Figure S4G). To

examine whether PMA in our experiments is an active compound, we used PKC beta II localization as readout. It was reported previously that PMA can induce PKC beta II to associate with the actin cytoskeleton [37]. We observed a similar effect in LN229 cells treated by PMA (100 nM) (Fig. 5e). This result suggested that PMA is active. Overall, the above results suggested that PKC beta II is required but not sufficient to fully induce the Hippo pathway and YAP/TAZ phosphorylation. Additional effectors associating with Ca^{2+} signalling are likely required for this process.

INF2-mediated CaAR is required for Ca^{2+} -induced Hippo pathway activation

To explore additional effectors in Ca^{2+} activation of the Hippo pathway, we compared cell morphologies under different treatments. When treated by amlodipine, ionomycin, or thapsigargin, the periphery of LN229 cells retracts and these cells appeared to detach from the culture dishes (Fig. 5a). The retraction and detachment can be blocked by co-treatment with GO6976 (Fig. 5a), suggesting that cPKCs are responsible for these morphological changes. In contrast, PMA treatment induces these cells to flatten without detachment within 2 hours (Figure S5A and data not shown). Given it has been shown that cell detachment activates the Hippo pathway [38], we focused on those cells in which amlodipine or ionomycin treatment produced no apparent morphological changes, hoping to answer whether cell detachment associated with amlodipine or ionomycin treatment is necessary for inducing YAP/TAZ cytoplasmic translocation. Interestingly, we found that these cells nevertheless showed significant amount of YAP/TAZ cytoplasmic translocation (Figure S5B). These results suggested that YAP/TAZ cytoplasmic translocation occurs before cell detachment. Notably, this assay does not rule out the possibility that early stages of cell periphery retraction are required for the YAP/TAZ regulation.

F-actin remodeling has been found to regulate the Hippo pathway although the mechanism remains unclear [39]. Our observations of changes in cell morphology induced by amlodipine, ionomycin, or thapsigargin implied actin cytoskeletal reorganization. It has been reported recently that Ca^{2+} can induce actin filaments to assemble at the perinuclear rim and ER [40, 41]. The actin filament assembly is accompanied by cortical actin filament disassembly. Such actin remodeling was called calcium-mediated actin reset (CaAR). We performed time-lapse imaging of F-actin labeled by EGFP-Lifeact [42] and found that ionomycin and thapsigargin can induce the assembly of actin filaments at the perinuclear rim (Fig. 5b and S5C, arrows). These observations suggested that CaAR also occurs in LN229 cells. It has been previously shown that CaAR in 3T3 fibroblasts and HeLa cells depends on a formin



family protein, INF2 [40, 41]. To examine whether CaAR was responsible for the Hippo pathway regulation by Ca²⁺, we used siRNAs to silence the expression of INF2 in

LN229 cells. We found that knockdown of INF2 effectively blocks the perinuclear assembly of actin filaments induced by ionomycin or thapsigargin (Fig. 5b and S5C,

◀ **Fig. 5** INF2-mediated CaAR is required for Ca^{2+} -induced Hippo pathway activation and PKC beta II association with the actin cytoskeleton. **a** LN229 cells were treated by amlodipine, ionomycin, or thapsigargin with or without GO6976 for 30 min and subjected to phase-contrast imaging. Representative images from three independent experiments. Scale bar = 20 μm . **b** LN229 cells stably transduced by EGFP-Lifeact were transfected by siRNAs against INF2 or scramble siRNA, treated by ionomycin and subjected to live cell time-lapse imaging for EGFP. Representative images from 40 examined cells. Scale bar = 10 μm . **c** LN229 cells were transfected by siRNAs against INF2 or scramble siRNA; treated by amlodipine, ionomycin, or thapsigargin; and subjected to western blotting. Representative blots from two independent experiments. **d** LN229 cells stably transduced by EGFP-Lifeact were treated by ionomycin with or without GO6976 (1 μM) and subjected to live cell time-lapse imaging for EGFP. Representative images from 40 examined cells. Scale bar = 10 μm . **e** LN229 cells stably transduced by HA-tagged PKC beta II were transfected by siRNAs against INF2 or scramble siRNA; treated by ionomycin, thapsigargin; or PMA for 20 min; and subjected to cytoskeletal fractionation into a Triton-X100 soluble fraction (SUP) and insoluble cytoskeletal fraction (CSK), followed by western blotting. Representative blots from two independent experiments

arrowheads), suggesting that INF2 is also essential for CaAR in these cells. Under this circumstance, we examined the Hippo pathway and found that amlodipine, ionomycin, or thapsigargin treatment was unable to induce the phosphorylation of Lats1 and YAP as effectively as in control cells (Fig. 5c). These results indicated that INF2-mediated CaAR is essential for the Hippo pathway activation by Ca^{2+} .

INF2-mediated CaAR is required for PKC beta II association with the actin cytoskeleton

Our results above have revealed that two factors, cPKCs and CaAR, are involved in the Hippo pathway activation by Ca^{2+} . Next, we sought to dissect their possible causal relations. Since cPKCs are required for the aforementioned cell morphological changes and the Hippo pathway activation, we investigated whether cPKCs were also involved in CaAR. We found that the perinuclear assembly of actin filaments induced by ionomycin or thapsigargin was not affected by the cPKCs inhibitor GO6976 (Fig. 5d and S5D, arrows). These results suggested that cPKCs were not required for the actin filament assembly. On the other hand, given that PKC beta II is a major cPKC involved in the Hippo pathway activation by Ca^{2+} , we investigated whether CaAR affects the PKC beta II activity. It has been shown that PKC beta II is activated by binding to F-actin [37], therefore we reasoned that CaAR may induce the interaction between PKC beta II and F-actin. Indeed, we found an enrichment of hemagglutinin (HA)-tagged PKC beta II in the Triton-X100 insoluble actin cytoskeletal compartment after the treatment of ionomycin or thapsigargin (Fig. 5e). Interestingly,

knockdown of INF2 eliminates PKC beta II from this actin compartment (Fig. 5e). Because the role of INF2 in CaAR is to mediate the actin remodeling [40, 41], this result suggested that INF2-mediated actin remodeling is required for the PKC beta II translocation to the cytoskeleton. Notably, PMA can also robustly induce PKC beta II translocation to an actin cytoskeletal compartment in this assay [37]. However, this translocation does not appear to depend on INF2 (Fig. 5e). In addition, PMA treatment cannot induce the perinuclear assembly of actin filaments (Figure S5E). These differential dependencies on INF2 by PKC beta II translocations in response to Ca^{2+} and PMA may explain why Ca^{2+} , but not PMA, activates the Hippo pathway. Collectively, these results suggested that INF2-mediated CaAR induces translocation of PKC beta II to certain actin compartments and activate the Hippo pathway.

PKC Beta II suppresses GBM growth by inhibiting YAP/TAZ

To study whether cPKCs mediate Ca^{2+} inhibition of YAP/TAZ activities, we examined the transcription of CTGF and Cyr61. Ionomycin treatment downregulates the transcription of these genes in LN229 cells. However, such inhibition was abolished when the cells were co-treated by the cPKC inhibitor, GO6976 (Fig. 6a). Additionally, we found that cells stably expressing recombinant PKC beta II showed reduced expression of CTGF and Cyr61. In contrast, if the cells also express the TAZ^{4SA} constitutively active mutant, PKC beta II cannot inhibit the expression of CTGF and Cyr61 (Fig. 6b). We also examined YAP^{5SA} constitutively active mutant and found that its expression is able to antagonize the effect of PKC beta II on Cyr61 expression (Figure S6A). Interestingly, YAP^{5SA} by itself reduces CTGF expression in this condition. This is probably due to a perturbation of other machineries required for CTGF expression. Nevertheless, under this circumstance, co-expression of PKC beta II showed no further inhibition of CTGF expression (Figure S6A). This result is also consistent with the notion that PKC beta II cannot inhibit CTGF expression in the presence of YAP^{5SA}. These results supported the notion that PKC beta II inhibits YAP/TAZ transcriptional activities by activating the Hippo pathway.

Since TAZ is required for LN229 cell growth and tumorigenesis (Figure S1A-F), we examined whether inhibiting TAZ with PKC beta II suppressed oncogenic properties of LN229 cells. We first performed a two-dimensional colony-formation assay and found that LN229 cells stably expressing PKC beta II exhibited reduced clonogenicity (Fig. 6c). However, PKC beta II expression in TAZ^{4SA}- or YAP^{5SA}-transduced cells does not inhibit their colony-forming abilities (Fig. 6c and S6B). To further examine the

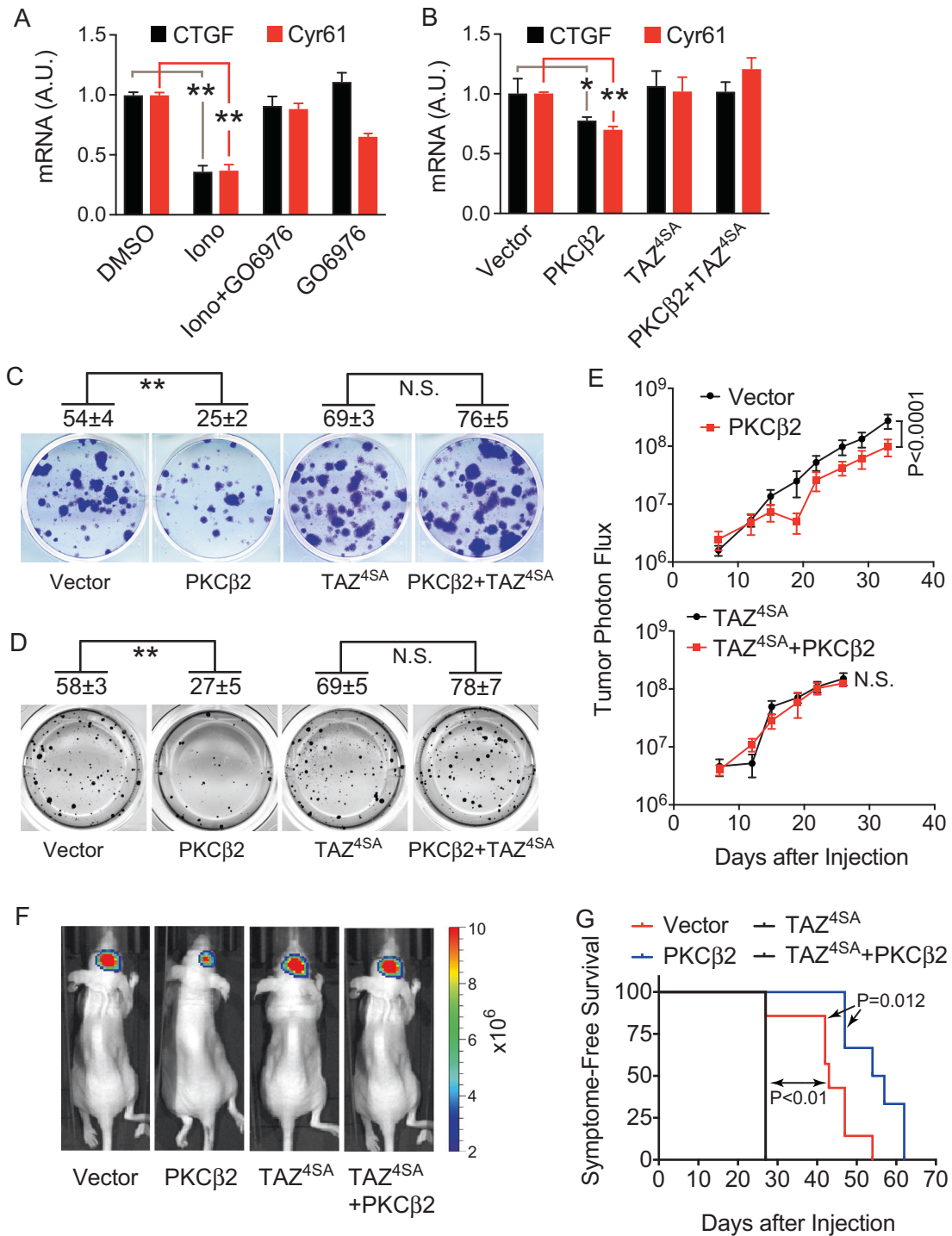


Fig. 6 PKC beta II inhibits YAP/TAZ-mediated transcription and GBM growth. **a** Cells were allowed to attach on petri dishes for 3 h in media containing ionomycin with or without GO6976 (1 μM) followed by qRT-PCR analysis for the indicated gene expression. $n = 2$, $**P < 0.01$. **b** Cells stably transduced by the indicated genes were allowed to attach on petri dishes for 3 h followed by qRT-PCR analysis for the indicated gene expression. $n = 2$, $*P < 0.05$, $**P < 0.01$. **c**, **d** Cells stably transduced by the indicated genes were subjected to the colony-formation assay (**c**) or the tumorsphere assay (**d**). Colonies (**c**) or spheres (**d**) numbers in each well were counted and indicated. $n = 3$,

$**P < 0.01$. N.S., $P > 0.05$. **e** Cells expressing luciferase were transduced by the indicated genes, followed by intracranial injection into nude mice. Tumors were monitored by bioluminescence imaging. $n = 6$ for vector, $n = 7$ for PKC beta II, $n = 5$ for TAZ^{4SA}, and $n = 5$ for TAZ^{4SA}+PKC beta II. The P values at day 33 are shown. Two-way ANOVA was used in this case. N.S., $P > 0.05$. **f** Representative mice bioluminescence imaging at day 26 from **e** are shown. **g** Survival curves are shown from the mice in **e** and Log-rank tests were performed

effects of PKC beta II on oncogenic properties, we conducted a three-dimensional tumorsphere assay, which reflects the stemness of tumor cells. We found that expression of PKC beta II inhibited tumorsphere formation by these cells, and co-expression of TAZ^{4SA} or YAP^{5SA} active mutant abolishes this inhibition (Fig. 6d and S6C). These results suggested that PKC beta II is able to suppress the oncogenic properties of GBM cells by inhibiting YAP/TAZ activities and that PKC beta II may have a tumor-suppressor function in GBM. To test this notion, we employed the orthotopic mouse GBM model and found that PKC beta II expression in LN229 cells significantly reduced tumor growth in the brain (Fig. 6e, upper panel and Fig. 6f). However, when co-expressing with TAZ^{4SA} or YAP^{5SA} in LN229 cells, PKC beta II cannot inhibit the tumor growth (Fig. 6e, lower panel, Fig. 6f, and S6D). In addition, mice with those tumors derived from PKC beta II-transduced LN229 cells survived longer than those from vector-transduced cells (Fig. 6g). However, this difference was eliminated when cells also expressed TAZ^{4SA} or YAP^{5SA} (Fig. 6g and S6E). Therefore, our results above indicated that PKC beta II is able to inhibit GBM growth and prolong mouse survival by inhibiting YAP/TAZ.

Discussion

In this study, we uncovered a way to inhibit YAP/TAZ in GBM by activating Lats1/2 kinases (Fig. 7). Elevation of cytosolic Ca²⁺ by triggering SOCE induces phosphorylation and activation of Lats1/2. We then identified that PKC beta II is a major mediator for Ca²⁺-induced Lats1/2 phosphorylation. Ca²⁺ induces PKC beta II to accumulate in an actin cytoskeletal compartment. Such PKC beta II translocation depends on INF2. Depletion of INF2 by siRNAs disrupts both Ca²⁺-induced PKC beta II translocation to the actin cytoskeleton and Ca²⁺-induced Lats1/2 activation. Furthermore, we showed that elevation of cytosolic Ca²⁺ or PKC beta II expression inhibits YAP/TAZ-mediated gene transcription and that PKC beta II expression inhibits GBM cell growth, stemness, and tumorigenesis by inhibiting YAP/TAZ. Collectively, these findings suggested that Ca²⁺-induced F-actin remodeling provides a scaffold for PKC beta II to activate the Hippo pathway and inhibit YAP/TAZ.

In human primary GBM cells, knockdown of STIM1 or Orai1 inhibited cell invasion [29]. Blocking Orai1 channels pharmacologically or by RNAi in the rat GBM cell line C6 attenuated cell proliferation and induced apoptosis [43]. These early studies suggested that SOCE contributes to GBM cell migration, proliferation, and survival. Our results here showed that pharmacological activation of SOCE channels can inhibit cell survival. This suggests that, while

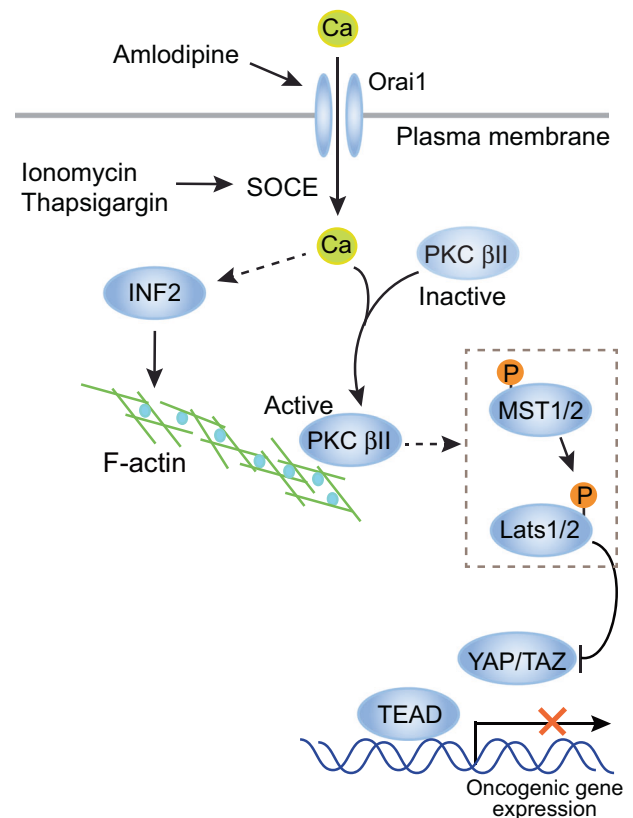


Fig. 7 Model illustrating the mechanism by which Ca²⁺ inhibits YAP/TAZ-mediated transcriptional program. Elevation of cytosolic Ca²⁺ by the indicated approaches induces INF2-mediated actin cytoskeleton remodeling, which leads to new actin-filament assembly. At the same time, Ca²⁺ induces PKC beta II translocation to the newly formed F-actin compartment, where PKC beta II is activated. Active PKC beta II induces activation of the core Hippo kinase cascade, including MST1/2 and Lats1/2 phosphorylation. Lats1/2 then phosphorylate YAP/TAZ and shut down the downstream transcriptional program of oncogenic genes

SOCE provides a growth advantage to GBM cells under resting conditions, robust pharmacological activation of SOCE can inhibit survival through Ca²⁺ overload-induced YAP/TAZ inhibition. Given a higher level of SOCE exists in GBM cells than in normal astrocytes [29], Ca²⁺ overload might be easier to achieve in tumor cells. This may provide a potential intervention window for therapeutic purposes. Notably, our results indicated that the concentrations of amlodipine to activate Orai1 channels and inhibit cell survival are around 10 μM, which is much higher than the maximum steady-state plasma concentration (~0.04 μM) in clinical studies for its original usage as a hypertension drug [44]. Therefore, finding a way to effectively activate SOCE in tumor cells is needed in the future.

The role of PKC in regulating the Hippo pathway has been previously studied [34]. It was found that PMA induces YAP/TAZ dephosphorylation or phosphorylation depending on differential cellular contexts. In HEK293A

cells, PMA induces YAP/TAZ dephosphorylation, and cPKCs promote this effect. Our studies also identified a role of cPKCs in Hippo pathway regulation. However, the Ca^{2+} -induced Hippo pathway regulation appears to be different from the previously reported PMA-induced regulation. First, our results indicated that cPKCs promote YAP/TAZ phosphorylation. Second, we found that PMA treatment cannot induce YAP/TAZ phosphorylation as effectively as ionomycin and thapsigargin do in the same cells. Third, our studies suggested that MST1/2 are essential for Ca^{2+} -induced regulations of Lats1/2 and YAP/TAZ. Furthermore, we found that, unlike Ca^{2+} , PMA cannot induce CaAR-like actin remodeling. This difference in CaAR induction may determine the differential effects of Ca^{2+} and PMA on the Hippo pathway.

Our studies revealed that the DN PKC beta II mutant effectively suppresses the cytoplasmic translocation of YAP/TAZ, whereas the DN mutants of other cPKC isoforms have little or no effects. The differential effects among these mutants may reflect the different extents of involvement by individual cPKC in Ca^{2+} -induced Hippo pathway activation. One possibility is due to differences in expression levels of these cPKCs. Our quantitative PCR (qPCR) analyses indicated that the expression of the PKC beta I isoform is about 30-fold lower than of PKC beta II isoform in LN229 cells (data not shown). However, the expression of PKC alpha is readily detected in these cells. Therefore, the involvement of individual cPKC isoenzymes in Ca^{2+} -induced Hippo pathway activation cannot be explained solely by the differences in their expression. Our studies found a Ca^{2+} -induced accumulation of PKC beta II in an actin cytoskeletal compartment. Notably, PKC beta II is the only PKC that contains a F-actin-binding motif at its V5 carboxyl terminus and can be activated by F-actin [37, 45]. Therefore, it is likely that Ca^{2+} -induced actin cytoskeleton remodeling provides a scaffold for PKC beta II to regulate the Hippo pathway.

The actin cytoskeleton is an important component in transducing upstream signals to YAP/TAZ in Lats1/2-dependent and -independent manners [39, 46]. It has been proposed that stress fibers or other unknown cell spreading-associated F-actin networks serve as platforms to regulate certain Hippo pathway regulators [39]. Consistently, Angiotensin, which is a direct inhibitor of YAP/TAZ, was found to be sequestered by F-actin [47]. However, how Lats1/2 are regulated by F-actin was unknown. Here our studies suggested a different perspective by which actin cytoskeleton regulates the Hippo pathway. Instead of simple disruption of F-actin, assembly of new actin filaments may actively regulate the Hippo pathway. Therefore, in addition to sequestering a YAP/TAZ inhibitory factor, such as Angiotensin [47], by the old F-actin [39], the newly formed

actin filaments may provide a scaffold to launch Lats1/2 activators, such as PKC beta II. Although MST1/2 are still important for Lat1/2 phosphorylation, it remains to be determined whether PKC beta II activates Lats1/2 by direct phosphorylation or indirect mechanisms. CaAR occurs in response to a variety of physiological cues, such as GPCR activation and mechanical stimulation [40, 41], and it would be interesting to examine whether similar regulation of the Hippo pathway by CaAR also exists in other Hippo pathway regulatory circumstances.

Taken together, our results indicate that Ca^{2+} is an intracellular cue in activating the Hippo pathway and inhibiting YAP/TAZ. The regulation occurs through Ca^{2+} -mediated actin reset and depends on INF2 and PKC beta II. In this process, Ca^{2+} -induced F-actin re-assembly may provide a scaffold for PKC beta II to induce Lats1/2 phosphorylation by MST1/2. In addition, we found that amlodipine activates Ca^{2+} entry through an Orai channel isoform in a store-independent manner. This provides a way to elevate cytosolic Ca^{2+} thereby inhibiting YAP/TAZ in tumor cells. Our findings provide a novel perspective on how the Hippo pathway is regulated by the actin cytoskeleton and suggest robust SOCE activation as a strategy to suppress YAP/TAZ signaling in GBM as well as other cancers.

Materials and methods

Mice

Six-to-eight-week-old female nude mice (Nu(NCr)-Foxn1nu from Charles River, Strain Code: 490) were used in the studies. For tumorigenesis experiments, LN229 cells were first transduced in vitro with a retrovirus vector expressing firefly luciferase. These cells were then transduced in vitro with retrovirus or lentivirus vectors expressing the indicated shRNAs or cDNAs. For each mouse, 3×10^5 cells were injected intracranially. Bioluminescence imaging was used to monitor tumor growth in the brains. Briefly, mice were anesthetized with isoflurane. 1.875 mg of luciferin dissolved in 125 μl of phosphate-buffered saline (PBS; 15 mg/ml) was intraperitoneally injected into mice. Ten minutes later, the mice were placed in the IVIS imaging chamber (Xenogen, Alameda, CA) and imaged for 1 min with the camera set at the highest sensitivity. Photons emitted from the brain region were quantified using the LivingImage software (Xenogen). Luciferase activity was presented as the photons emitted/s. All experiments were performed in accordance with the Penn State University Institutional Animal Care and Use Committee guidelines.

Cells

LN229 (CRL-2611), LN18 (CRL-2610), and DBTRG-05MG (CRL-2020) human GBM cell lines were from ATCC. NHA (CC-2565) were from LONZA. LN229, LN18, and DBTRG-05MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, 10-013-CV) supplemented with 10% fetal bovine serum (FBS; Gibco, 10437028) and 1% Antibiotic–Antimycotic Solution (Corning, 30-004-CI) at 37 °C with 5% CO₂. NHA were cultured in NHA growth medium according to the standard protocol as described in the instructions for the Human Astrocyte Cell System (LONZA). None of these cell lines were listed in the database of misidentified cell lines maintained by ICLAC and NCBI Biosample. These cell lines were not authenticated in this study. All cell lines were examined to be mycoplasma negative before experiments.

Unless otherwise indicated, experiments were performed with cells grown to 50% confluency. For compound treatment experiments, cells were seeded in complete medium (10% FBS) overnight and treated with DMEM medium containing 2% FBS and the indicated compounds for the indicated times before harvest. For NHA, the NHA growth medium was used in the treatment. For calcium-dependent experiments, after overnight culture in complete medium, cells were pre-incubated in calcium-free medium (Invitrogen, #21068028) supplemented with 2 mM Glutamine and 2% FBS for 1 h. Calcium-free or calcium-containing DMEM (containing 2 mM CaCl₂) with the indicated compounds was then added to cells. For compound combination experiments, cells were preincubated with GO6976 or EGTA-AM for 1 h before the indicated compound mixture was added to the cells.

Antibodies and compounds

Anti-Phospho-LATS1 (Thr1079) (8654), anti-Phospho-MST1 (Thr183)/MST2 (Thr180) (3681), anti-Phospho-YAP (Ser127) (13008), anti-Phospho-TAZ (Ser89) (59971), anti-YAP (12395), anti-TAZ (4883), anti-Lats1 (3477), anti-Lats2 (5888), anti-MST1 (14946), anti-MST2 (3952), anti-YAP/TAZ (8418) (an antibody recognizing both YAP and TAZ), and anti-alpha-tubulin (3873) antibodies were from Cell Signaling Technology. Anti-INF2 (ABT-61) antibody was from Millipore. Anti-HA (MMS-101P) antibody was from BioLegend. Anti-β-Actin (A5316) and anti-Orail1(O8264) antibodies were from Sigma-Aldrich. Goat anti-mouse immunoglobulin G–horseradish peroxidase (IgG-HRP) (sc-2005), goat anti-rabbit IgG-HRP (sc-2004) antibodies were from Santa Cruz Biotechnology. Anti-alpha-tubulin (12G10) was from Developmental Studies Hybridoma Bank. Amlodipine (S1905), amlodipine besylate (S1813), and GO6976 (S7119) were from Selleck

Chemicals. Ionomycin (I9657), thapsigargin (586005), and PMA (P1585) were from Sigma-Aldrich. EGTA-AM (324628) was from Calbiochem.

Vectors

pBabe-neo-TAZ (4SA) and pBabe-neo-YAP (5SA) were described previously [20, 21] and generously provided by Kun-Liang Guan. pHACE-PKC alpha DN (21235), pHACE-PKC beta I DN (16381), pHACE-PKC beta II DN (16385), and pHACE-PKC gamma DN (21239) were described previously [36] and gifts from Bernard Weinstein via Addgene. mEGFP-Lifeact-7 (54610) was a gift from Michael Davidson via Addgene. To generate pHACE-PKC beta II wild type, pHACE-PKC beta II DN (16385) was subjected to site-directed mutagenesis using the Quick-change Mutagenesis Kit (Stratagene). To generate pBabe-puro-PKC beta II wild type, HA-tagged PKC beta II wild type from pHACE-PKC beta II wild type was subcloned into the pBabe-puro vector.

Small-molecule screen

LN229 cells were seeded in 20 µl of DMEM containing 2% FBS per well into 384-well plates (Corning) using an automated plate filler (BioTek). After 24 h, 10 µl of compound solution were transferred into the 384-well plates resulting in ~10 µM final drug concentration. Each plate also included DMSO only negative control and staurosporine (2 µM) positive control. After 72 h incubation, cell viability was determined in each well using the PrestoBlue Cell Viability Reagent (Invitrogen) and read in a multi-mode plate reader (BMG Labtech). Luminescence data were normalized to DMSO as 100% viable and staurosporine as 0% viable.

Immunoblotting

Immunoblotting procedure was described previously [48]. Briefly, cells were lysed in SDS-lysis buffer (10 mM Tris pH 7.5, 1% SDS, 50 mM NaF, 1 mM NaVO₄) and subjected to SDS-PAGE on 4–12% Bis-Tris SDS-PAGE gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore). Membranes were incubated in blocking buffer: 5% skim milk/TBST (0.1% Tween, 10 mM Tris at pH 7.6, 100 mM NaCl) for 1 h at room temperature and then with primary antibodies diluted in 5% bovine serum albumin (BSA)/TBST overnight at 4 °C. After three washes, the membranes were incubated with goat anti-rabbit HRP-conjugated antibody (1:5000; Santa Cruz sc-2054) or goat anti-mouse HRP-conjugated antibody (1:10,000; Santa Cruz sc-2005) at room temperature for 2 h and subjected to chemiluminescence using ECL (Pierce #1856136).

Immunofluorescent staining

Immunofluorescent staining was described previously [48]. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 20 min and incubated in permeabilization buffer (PDT: 0.3% sodium deoxycholate, 0.3% Triton X-100 in PBS) for 30 min on ice. They were then blocked with 5% BSA/PBS at 4 °C for 1 h and followed by incubating overnight at 4 °C with primary antibodies diluted in 2.5% BSA/0.05% Triton X-100/PBS. After washing with 0.1% Triton X-100/PBS, cells were incubated with secondary antibodies diluted in 2.5% BSA/0.05% Triton X-100/PBS for 2 h at 4 °C. Cells were washed with 0.1% Triton X-100/PBS, rinsed with PBS, and mounted in ProLong Gold Mountant (Invitrogen #P10144). When indicated, nuclei were stained with 4,6-diamidino-2-phenylindole.

Quantification of YAP/TAZ localization

The percentages of cells showing cytoplasmic YAP/TAZ signal more than or equal to nuclear YAP/TAZ signal were calculated. For inhibition index shown in Fig. 4d, the above percentages were calculated for HA-positive cells (HA⁺%) and HA-negative cells (HA⁻%), respectively. Inhibition index = $1 - \text{HA}^+ \% / \text{HA}^- \%$.

Subcellular fractionation

Cytoskeleton fractionation was described previously [37]. Briefly, cells were rinsed twice with ice-cold PBS followed by adding 1% Triton X-100 solubilization buffer (15 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 1% (v/v) Triton X-100, and 1X proteinase inhibitors). After incubation on dish at 4 °C for 10 min, cells were harvested into a 1.5 ml tube. Cell lysates were centrifuged at 21,000 × g at 4 °C for 40 min. The supernatant was saved as the soluble fraction (SUP). After washing with 1% Triton X-100 solubilization buffer, the pellet was suspended with SDS-lysis buffer (10 mM Tris pH 7.5, 1% SDS, 50 mM NaF, 1 mM NaVO₄) and assayed as the Triton insoluble fraction (CSK).

Gene expression and silencing

Transient transfections were performed using Fugene 6 (Promega), Lipofectamine 2000 (Invitrogen), or DharmaFECT 1 (Dharmacon) by following the manufacturer recommended protocols. Retroviral generation and infection were described previously [48]. Lentiviral vectors encoding shRNAs targeting TAZ (#7: TRCN0000370007; #69: TRCN0000019469) were from Sigma-Aldrich. ON-TARGETplus SMARTpool siRNAs against Lats1, Lats2, MST1, MST2, PKC beta, INF2, or ON-TARGETplus siCONTROL were from Dharmacon. siRNA for Orai1

(sequence: CGUGCACAAUCUCAACUCGUU) was from Integrated DNA Technologies. For si-Orai1 control, All-Stars Negative Control siRNA (SI03650318) was from Qiagen.

Quantitative reverse transcriptase PCR (qRT-PCR)

qRT-PCR was carried out according to standard protocols. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). cDNAs were synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, 1708891), and qPCR was carried out on a CFX96 Touch Real-Time PCR Detection System with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725271). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference to normalize the input cDNA. Primer sequences used: PKC beta II forward, GACCAAACACCCAGGCAAAC, reverse, GATGGCGG GTGAAAAATCGG; CTGF forward, GCAGGCTAGAGA AGCAGAGC, reverse, ATGTCTTCATGCTGGTGCAG; Cyr61 forward, ACTTCATGGTCCCAGTGCTC, reverse, TGGTCTTGCTGCATTTCTTG; and GAPDH forward, GGAGCGAGATCCCTCCAAAAT, reverse, GGCTGTTGT CATACTTCTCATGG.

The primers were designed using PrimerBank.

Live cell imaging

Live cell microscopy was performed on *DeltaVision Elite* Inverted Microscope (Applied Precision Inc., WA) at 37 °C and 5% CO₂ using 60× oil immersion objective. LN229 cells stably transduced by EGFP-Lifeact were plated on 35 mm glass bottom microwell petri dishes (MatTek corporation, MA) at a density of 5×10^5 cells. Two hours prior to image acquisition, culture media was replaced with DMEM supplemented with 2% FBS. Throughout imaging acquisition, dishes were maintained in a sealed heated chamber so that temperature and CO₂ levels were both kept constant. Image fields were pre-selected with *z* sections spaced at 0.2 μm and total thickness of 4 μm (20 slices). Baseline conditions were recorded for 5 min, followed by treatment of either thapsigargin or ionomycin, both final concentration at 1 μM diluted in DMEM with 2% FBS. Time-lapse images were acquired every minute with total recording time of 40 min. Final images were processed via deconvolution using the same working software.

Ca²⁺ measurements

For intracellular Ca²⁺ measurements using Fura-2, LN229 cells were cultured on 30-mm glass coverslips for 24 h in complete media. Coverslips were then mounted in a Teflon chamber and incubated at 37 °C for 30 min in complete culture medium with 2 μM Fura-2 AM. Cells

were then washed a total of 4 times with HEPES-buffered saline solution (140 mM NaCl, 1.13 mM MgCl₂, 4.7 mM KCl, 2 mM CaCl₂, 10 mM D-glucose, and 10 mM HEPES; pH 7.4) and kept in this solution at room temperature for 10 min prior to Ca²⁺ imaging. To record and analyze Fura-2 fluorescence from LN229 cells, a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH, USA) was used. Fura-2 was alternately excited at 340 and 380 nm and emitted fluorescence was collected at 510 nm. For each pixel, the emission ratio of 340/380 nm was represented. The addition of amlodipine causes an equal increase of emitted fluorescence upon excitation at both the 340 and 380 nm, suggesting that amlodipine emits fluorescence upon UV excitation. This amlodipine fluorescence was subtracted from the overall recordings. Amlodipine fluorescence masks the Fura-2 signal in a dose-dependent manner and leads to underestimation of the Ca²⁺ signal activated by amlodipine. Solutions with thapsigargin, ionomycin, and amlodipine were suspended in HEPES-buffered saline solution. Ca²⁺ traces were averages from many cells per coverslip and multiple independent experiments as indicated on respective figures. For intracellular Ca²⁺ measurements using Fluo-4 NW, assay was carried out according to the manufacturer's protocols (Invitrogen, F36206). Briefly, cells were loaded with 2 μM Fluo-4 NW in the HEPES-buffered saline solution at 37 °C for 30 min. After 30 min of incubation at 37 °C, calcium flux assays were performed with on-line compound addition in a FlexStation 3 plate reader (Molecular Devices), with simultaneous data collection. Dose–response curves were generated by SoftMaxPro (Molecular Devices).

Colony-formation assay

LN229 cells were trypsinized, resuspended, and counted. Four hundred cells were seeded in complete culture medium per well of a 6-well plate and cultured for 10–12 days. The plates were then washed with PBS and stained with crystal violet. Colonies' images were scanned and scored using Image J.

Tumorsphere assay

LN229 cells were trypsinized, resuspended, and counted. Five hundred cells were seeded in neural sphere medium (DMEM/F12, Corning #15-090-CV, L-glutamine, 2 mM, Invitrogen #25030-081, N-2 supplement, 1×, Invitrogen #17502048, B-27 Supplement, 1×, Invitrogen #17504044, BSA, 50 μg/ml, Sigma, epidermal growth factor and basic fibroblast growth factor, 20 ng/ml each, R&D systems, Antibiotic–Antimycotic Solution, 1% Corning #30-004-

CI) containing 0.34% low melting temperature agarose (Invitrogen #18300012) per well in 24-well Ultra Low Cluster Plates (Costar). After solidification at 4 °C for 5 min, the plates were incubated at 37 °C for 2–3 weeks. Tumorspheres were stained by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Invitrogen), scanned, and scored using Image J.

MTT cell viability assay

Cells were seeded in DMEM medium containing 2% FBS at a density of 3000 cells per well in 96-well plates. After overnight culture, cells were treated with the indicated agents for 3 days. Viable cell numbers were determined using the MTT (Invitrogen #M6494) reagent according to the manufacturer's protocols (Invitrogen). Each assay consisted of two or three replicate wells. Data were presented as normalized to control cells (treated by DMSO).

Statistical methods

For statistical analyses (including animal studies), samples sizes were chosen based on whether the differences between groups are biologically meaningful and are statistically significant. No data were excluded from the analyses. For cell experiments, all cells in one experiment were from the same pooled parental cells. All mice were from the same cohort. The mice were randomly picked to implant different types of cells. For data collection relying on objective instruments, such as plate reader, qPCR, microscopy software, animal IVIS system, and western blotting, the investigators were not blinded to group allocation during data collection. For animal studies, the investigators were not blinded to group allocation during data collection. The variance is similar between the groups that are being statistically compared. Statistical significance was determined by unpaired two-tailed Student's *t* test unless indicated otherwise. All center values shown are mean, and all error bars shown are standard error of the mean (s.e.m). All statistical calculations and plotting were performed using Graphpad Prism 7.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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