DOI: 10.1002/cbic.200800255

Chemical Genetic Analysis of FOXO Nuclear–Cytoplasmic Shuttling by Using Image-Based Cell Screening

Fabian Zanella,[a] Aranzazú Rosado,[a, b] Beatriz García,[a] Amancio Carnero,[a] and Wolfgang Link*[a]

FOXO proteins are direct targets of PI3K/Akt signaling and they integrate the signals of several other transduction pathways at the transcriptional level. FOXO transcription factors are involved in normal cell homeostasis and neoplasia, and they are regulated by multiple post-transcriptional modifications. In cancer research, the regulation of the FOXO factors is receiving increasing attention as their activation has been linked to cell-cycle arrest and apoptosis. Hence, FOXO proteins have been proposed to act as tumor suppressors. Here, we applied a chemical biology approach to study the mechanisms that influence the intracellular localization of the FOXO family member FOXO3a. We established a high-throughput cellular-imaging assay that monitors the nuclear–cytoplasmic translocation of a GFP–FOXO3a fusion protein in tumor cells. Nuclear accumulation of fluorescent signals upon treatment with the known PI3K inhibitors LY294002, wortmannin, PIK-75, and PI-103 was dose dependent and agreed well with the IC_{50} values reported for PI3Kα inhibition in vitro. Additionally, we identified 17 compounds from a panel of 73 low-molecular-weight compounds capable of inducing the nuclear accumulation of GFP–FOXO. These compounds include chemicals known to interfere with components of the PI3K/Akt signaling pathway, as well as with nuclear export and Ca^{2+}/calmodulin (CaM)-dependent signaling events. Interestingly, the therapeutic agent vinblastine induced efficient nuclear translocation of the FOXO reporter protein. Our data illustrate the potential of chemical genetics when combined with robust and sensitive high-content-screening technology.

Introduction

Mammalian FOXO proteins are the orthologues of *Candida elegans* DAF16, and they pertain to the O class of forkhead transcription factors that have a characteristic forkhead box DNA binding domain. FOXO proteins function as transcriptional regulators in the cell nucleus and they bind as monomers to their consensus DNA binding sites. They are components of highly conserved signal-transduction pathways that link growth and stress signals to the control of gene expression. The ever-growing list of target genes for these factors contains many elements that function in metabolism, apoptosis, resistance to oxidative stress, and cell-cycle inhibition, and includes glucose-6-phosphatase, phosphoenolpyruvate carboxykinase,[1] FasL, Bim,[2,3] MnSOD, catalase, $p27^{KIP1}$, p130, and cyclin G_2.[4–6] FOXO factors are inactivated in a variety of cancers and indeed, FOXO3a was found mainly in the cytoplasm in human primary breast cancer where nuclear exclusion was closely correlated with poor survival.[7] Moreover, the expression of a constitutively active form of FOXO1 diminished tumorigenesis in cells with aberrant PI3K/Akt activity in nude mice.[8] In fact, the simultaneous disruption of the three principal FOXO genes leads to thymic lymphoma and haemangiomas, indicative that FOXO factors are bona fide tumor suppressors.[9]

Despite these consequences of their inactivation and somewhat surprisingly, no inactivating mutations in the FOXO genes have yet been reported. Hence, inactivation of FOXO proteins in human cancer seems to be mainly due to aberrant upstream signaling. Subcellular localization of FOXO proteins plays a major role in the regulation of their activity. Nuclear–cytoplasmic shuttling of FOXO factors is controlled by a sophisticated signaling network that integrates information from PI3K/Akt and stress-induced signaling pathways via the Jun N-terminal kinase (JNK), the mammalian sterile 20-like kinase MSTI, and the NAD-dependent deacetylase SIRT1.[10] In the absence of growth factor signaling, FOXO factors are localized in the nucleus and are transcriptionally active. By contrast, FOXO transcription factors are phosphorylated by several kinases in response to growth and survival factors, including AKT, SGK, and CK1,[11] and phosphorylation by DYRK1A and IKKβ has been shown to drive FOXO factors out of the nucleus.[7,11] Akt negatively regulates FOXO proteins through the phosphorylation of residues at three highly conserved RXRXXS/T consensus sites; this leads to conformational changes that facilitate 14-3-3 binding and that activate CRM-1-mediated nuclear export. Stress signals have been shown to antagonize Akt signaling by preventing the binding of FOXO to 14-3-3 by phosphorylation of FOXO at S207 by MST1 and the phosphorylation of 14-3-3

[a] *F. Zanella, A. Rosado, B. García, Dr. A. Carnero, Dr. W. Link*
Experimental Therapeutics Program
Centro Nacional de Investigaciones Oncologicas (CNIO)
Melchor Fernandez Almagro 3, 28029 Madrid (Spain)
E-mail: wlink@cnio.es

[b] *A. Rosado*
Current address: NKI-AVL
Plesmanlaan 121, 1066 CX, Amsterdam (The Netherlands)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

by JNK.[12,13] Furthermore, oxidative stress promotes Ral-mediated, JNK-dependent phosphorylation of FOXO4.[14] Additionally, monoubiqutination and SIRT-dependent deacetylation of FOXO proteins in response to increased cellular oxidative stress is thought to induce their nuclear translocation.[15,16] Despite considerable efforts, our understanding of how non-Akt-mediated regulation of FOXO factors affects their subcellular localization is very limited. To define the circuit that regulates the subcellular transport of FOXO proteins, we performed chemical genetic studies to interrogate the FOXO shuttling system.

Results

U2foxRELOC cells respond to inhibition of the PI3K/Akt pathway

To generate a system suitable to monitor nuclear–cytoplasmic shuttling of FOXO protein in a high-throughput-screening (HTS) format, we stably transfected a GFP–FOXO3a reporter plasmid into U2OS cells and prepared cell clones. As a proof of principle, we treated U2foxRELOC cells with LY294002, a broad spectrum PI3K inhibitor widely used to suppress the activation of PI3K/Akt signaling. Whereas the GFP fusion protein was present in both the cytoplasm and nucleus in U2foxRELOC cells, with significantly more GFP in the cytoplasm than in the nucleus, when exposed to LY294002 for 1 h almost all the GFP–FOXO3a translocated to the nucleus of these cells (Figure 1). To analyze whether treatment with LY294002 compromised the integrity of the cell cytoplasm, we used cell-tracker orange as a vital cytoplasmic counterstain. The overall morphology of U2foxRELOC cells remained unaffected by exposure to LY294002 (Figure 1F), indicating that the kinetics of the assay allowed us to reduce the incubation time necessary to attain unambiguous responses when cells were exposed to different compounds. This is an important factor to minimize the possible toxic effects that might interfere with the analysis, as well as other indirect effects.

Dose-response analysis of PI3K inhibitors with different IC_{50} values by using automated U2foxRELOC

We next explored the feasibility of extracting quantitative data from the U2foxRELOC system by analyzing the effects of PI3K

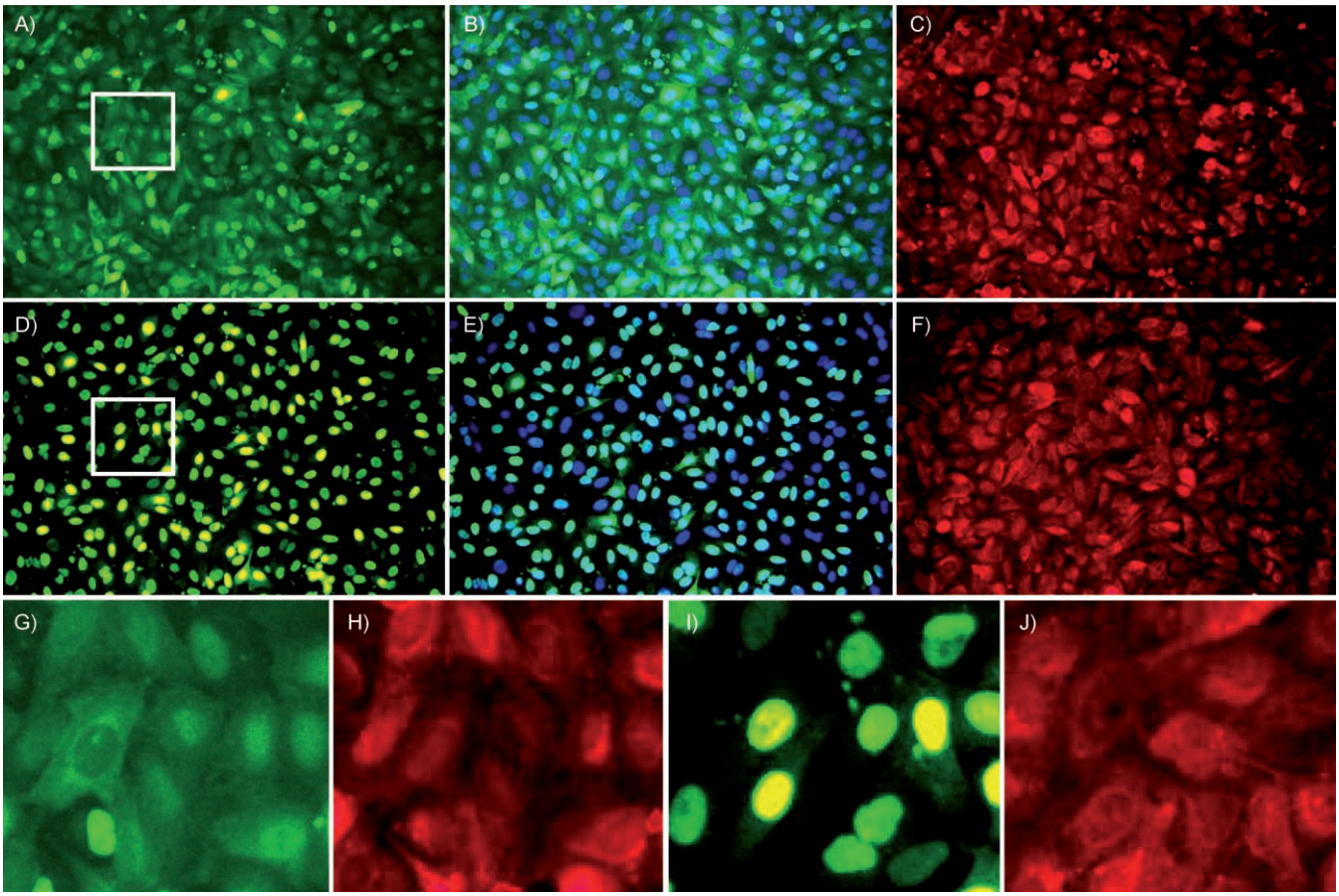


Figure 1. Translocation of GFP–FOXO following PI3K inhibition. U2foxRELOC cells stably expressing GFP–FOXO fusion protein were seeded in 96-well plates, incubated for 12 h, preincubated with cell-tracker orange dye, and treated with DMSO (A–C) or 20 μM LY294002 (D–F). After 1 h at 37 °C, the cells were washed, fixed, and stained with DAPI in a fully automated manner by using a robotic workstation. The cells were photographed by using a BD Pathway HT cell-imaging platform. A) and D) GFP; B) and E) GFP and DAPI merged images; C) and F) cell-tracker orange. G) a close-up view of the region in (A) outlined by the square. I) Close-up view of the region in (D) outlined by the square. H) and J) Corresponding cell-tracker orange images.

inhibitors that act on the recombinant p110α protein with different IC_{50} values. The IC_{50} in vitro value of the competitive, pan-PI3K inhibitor LY294002 is 500 nM and hence, it is about 60-fold less potent than the imidazopyridine inhibitor PIK-75 (7.8 nM), about 135-fold less potent than the synthetic selective class I PI3K inhibitor PI-103 (IC_{50} = 3.7 nM) and about 900-fold less potent than the fungal metabolite wortmannin (IC_{50} = 0.57 nM).[17] U2foxRELOC cells were exposed to eleven different concentrations of the PI3K inhibitors for 1 h in culture, the final concentrations of the inhibitors ranging from 50 μM to 1.6 nM. The morphological integrity of the assayed cells was confirmed by cell-tracker orange fluorescence of cytoplasm of viable cells (data not shown). The data obtained from these experiments agree well with the IC_{50} values for the corresponding compounds in biochemical assays (Figure 2). Thus, LY294002 induced nuclear translocation of the FOXO reporter protein at 50 μM, and 33.3 μM, and was slightly less efficient at 11.1 μM. However, exposure of U2foxRELOC cells to 3.7 μM LY294002 failed to affect the intracellular localization of the fluorescent signal. By contrast, wortmannin triggered nuclear shuttling of GFP–FOXO even at low nanomolar concentrations. PI-103 produced fluorescent precipitates at 50 μM and 33.3 μM, visible in both the DAPI and the GFP channel, although nuclear accumulation of GFP–FOXO was also evident under these conditions. Importantly, exposure of U2foxRELOC cells to concentrations as low as 46 nM of PI-103 or 15 nM of wortmannin was sufficient to induce the nuclear translocation of the FOXO reporter protein. The slightly greater potency of PI-103 in comparison with PIK-75 on the recombinant p110α protein was reflected by the small difference in the minimal effective concentration at which accumulation of the fluorescent signal could be detected in the U2foxRELOC system. In contrast to PI-103, PIK-75 was unable to induce significant translocation of the reporter protein at 46 nM. Hence, the U2foxRELOC appeared to be a very sensitive system to detect inhibitors of the PI3K/Akt pathway with different potencies.

Chemical interrogation of nuclear–cytoplasmic shuttling of FOXO by using the U2foxRELOC-based assay

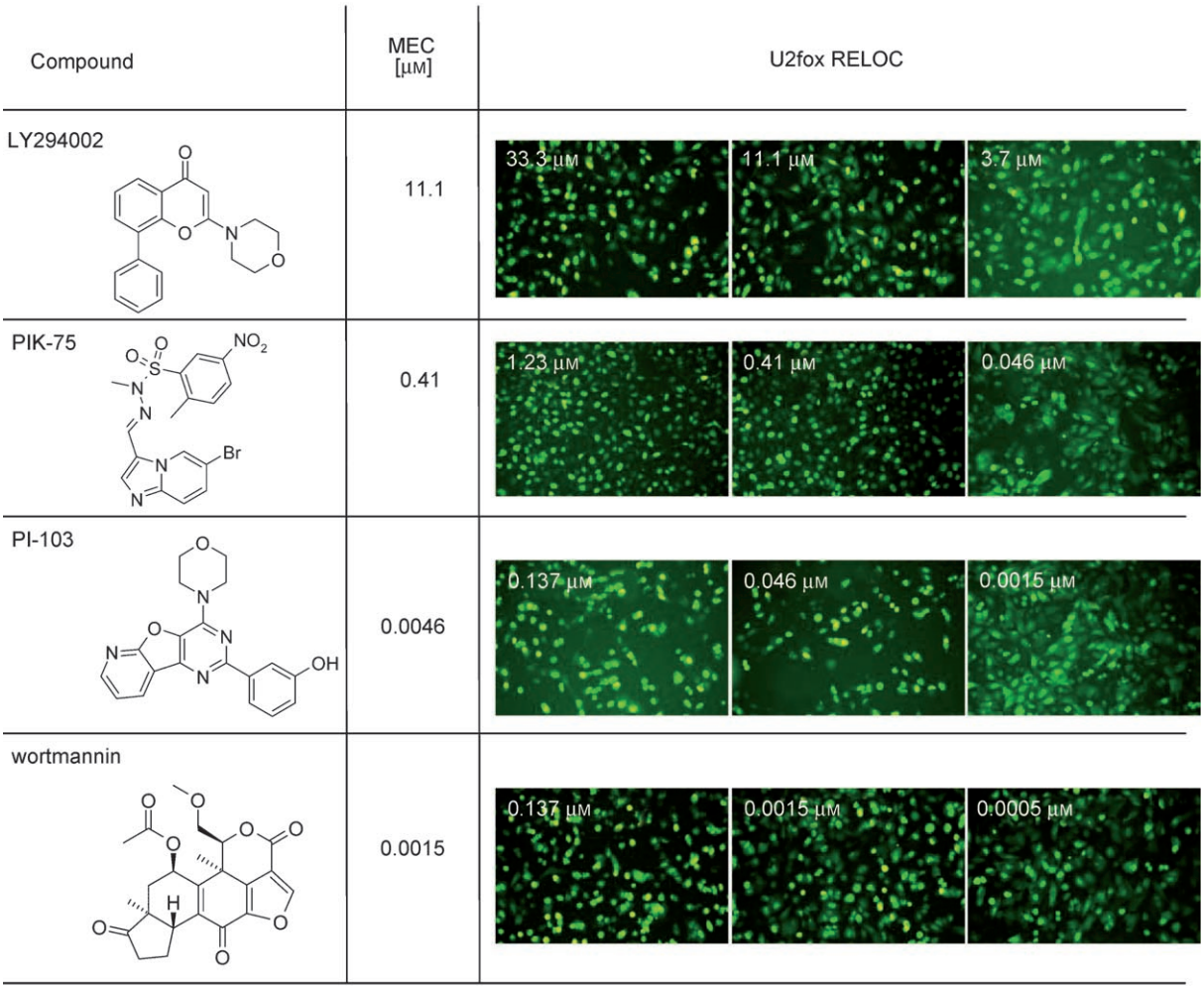
We applied a chemical biology approach to study the signaling network that regulates the intracellular localization of FOXO transcription factors. Accordingly, we screened a panel of compounds with known biological activity in the U2foxRELOC cell system (see Table S1 in the Supporting Information for the complete list of compounds studied). The initial test panel consisted of 73 compounds known to interfere with the major signal transduction pathways. A major concern when using small molecules for pathway analysis is their specificity for their corresponding target. To draw useful conclusions, it is recommended to use two structurally unrelated compounds for each target[18] and therefore, we included several independent small-molecule inhibitors for the same molecular target wherever possible. Likewise, to distinguish on target and off target effects, different concentrations of the compounds were assessed in the primary screen. We exposed U2foxRELOC cells to equal volumes of test compounds at final concentrations in a range of two orders of magnitude around their IC_{50} value. As reference compounds we used LY294002 and the nuclear export inhibitor leptomycin B. To determine the cutoff threshold for primary hits, the nuclear accumulation of fluorescence triggered by LY294002 in the U2foxRELOC assay was defined as 100% activity. Primary hits were defined as those samples that have an activity above 60% and several test compounds fulfilled these criteria (Figure 3 A).

Ras farnesyltransferase inhibitor, manumycin A was shown to be capable of inducing FOXO translocation into the nucleus. Several small molecule compounds known to interfere with the PI3K/Akt pathway, one of the major signaling branches downstream of Ras, also induced nuclear FOXO translocation. Namely, the Akt inhibitors, Akt inhibitor VIII, Akt inhibitor X, PI-103, wortmannin, D000, and UCN01, were active in this assay at concentrations previously reported to affect targets related to PI3K/Akt signaling, demonstrating the capacity of the U2foxRELOC system to identify inhibitors of the PI3K/Akt pathway. In contrast, known activators of the PI3K/Akt pathway including EGFP, IGF, PDGF, and insulin produced little nuclear localization of GFP–FOXO. However, insulin was the only factor that decreased the number of cells with nuclear fluorescent signal below the level of vehicle-treated cells. Rapamycin, is a widely used inhibitor of mTOR that has no effect on GFP–FOXO localization; this indicates that the rapamycin-sensitive mTOR complex acts downstream of the PI3K/Akt-associated signaling events relevant for the regulation of FOXO activity.

To analyze the involvement of alternative downstream Ras signaling pathways in FOXO translocation, we tested several compounds previously shown to interfere with different elements in the MAPK cascade. Inhibition of Raf1, Mek1/2, JNK, or p38α MAP kinase upon treatment with GW5074, arctigenin, U0126, PD98059, SP600125, JNK inhibitor VIII, or SB202190, did not induce nuclear translocation of GFP–FOXO. Conversely, the p38 MAP kinase inhibitor SB203580 triggered the accumulation of nuclear fluorescence, although at a concentration that has been shown to decrease Akt activity in several cell lines.[19,20] As anticipated, staurosporine, a relatively nonselective protein kinase inhibitor that blocks many kinases to a differing extent was capable of inducing the nuclear accumulation of the FOXO reporter protein. We analyzed the effect of six different tyrosine kinase inhibitors on translocation in the U2foxRELOC assay, including tyrphostatin SU1498, tyrphostin AG 82, tyrphostin AG 1478, tyrphostin AG 1433, tyrphostatin SU 1498, genistein, and PP1 analogue. Only the broad tyrosine kinase specific inhibitor genistein induced GFP–FOXO nuclear translocation.

From a subgroup of drugs currently used in anticancer chemotherapy and known to act through different molecular mechanisms, including etoposide, camptothecin, cisplatin, oxaliplatin, flavopiridol, gemcitabine, paclitaxel, and vinblastine, only the latter drug displayed activity in the U2foxRELOC assay. Vinblastine has been shown to activate the JNK pathway.[21] Whether FOXO translocation mediated by these drugs is due to the activation of JNK and in turn, the phosphorylation of 14-3-3 protein, or the direct inactivating phosphorylation of FOXO3a remains to be determined. Roscovitine is a

A)



B)

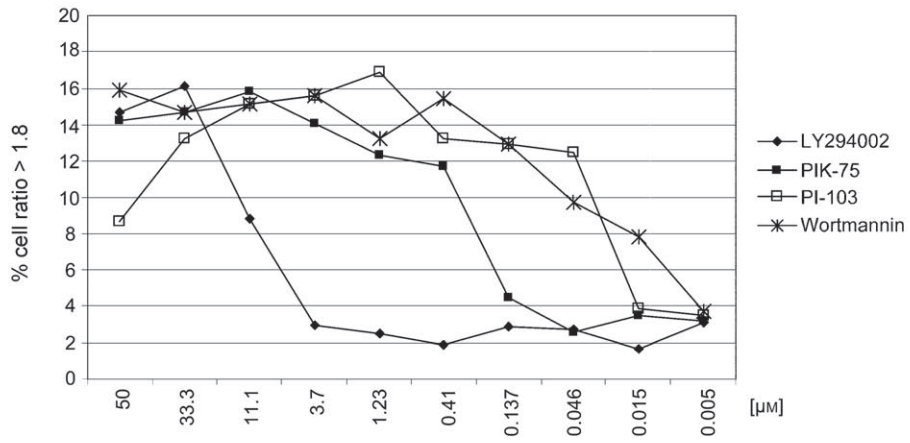
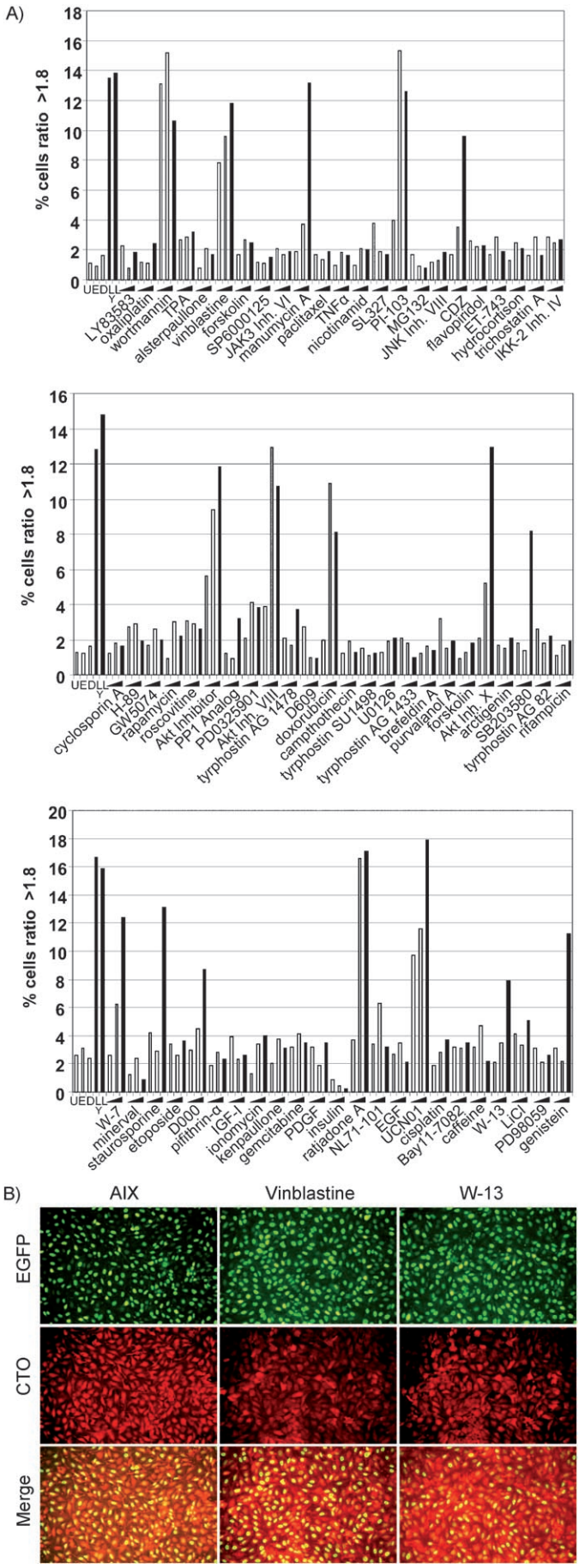


Figure 2. Dose-response relationship of the nuclear–cytoplasmic shuttling of FOXO following PI3K inhibition. A) We used LY294002, wortmannin, PIK-75, and PI-103, four structurally unrelated PI3K inhibitors that inhibit the recombinant p110α protein with IC_{50} values of 500, 7.8, 3.7, and 0.57 nM, respectively.[17] U2foxRELOC cells were seeded in 96-well plates, incubated for 12 h, and treated with eleven different concentrations of LY294002, PIK-75, PI-103, or wortmannin ranging from 50 μM to 1.6 nM. After 1 h at 37 °C in medium containing one of each of the compounds, the cells were washed, fixed, and stained with DAPI and photographed by using automated microscopy. The minimal effective concentration (MEC) is the lowest dose of each corresponding compound that induced GFP–FOXO translocation, as determined by assessing 11 different concentrations. B) The graph shows the proportion of cells exhibiting nuclear/cytoplasmic (Nuc/Cyt) ratios of fluorescence intensity greater than 1.8 for each treatment.



potent pan-CDK inhibitor currently undergoing phase II clinical testing that failed to induce FOXO protein translocation into the nucleus. Hence, despite promoting a cytoplasmic localization of FOXO1,[22] CDK-mediated phosphorylation is not involved in the nuclear–cytoplasmic shuttling of the FOXO3a-reporter protein. Likewise, other CDK inhibitors, such as kenpaullone, alsterpaullone, and purvalanol A, had no effect in this assay.

As expected, exposure of U2foxRELOC cells to the nuclear export inhibitor ratjadone A resulted in the nuclear accumulation of fluorescent signal. In agreement with previous data,[23] the calmodulin antagonists W-7, W-13, and calmidazolium chloride (CDZ) also produced a positive result in the U2foxRELOC assay. The nuclear accumulation of fluorescent signal was not attributable to shrinkage of the cell cytoplasm, as witnessed by vital staining when using cell-tracker orange fluorescent dye (Figure 3B and data not shown).

Further analysis of the compounds that produce FOXO relocation

After screening the initial panel of compounds, we validated the compounds of interest to identify those whose activity was not specific for FOXO3a nuclear translocation using independent image-based translocation assays. To rule out general perturbations affecting the fluorophore, we tested the hit compounds using a U2gfpRELOC assay based on U2OS cells stably expressing GFP alone. GFP localization was unaffected upon exposure to the compounds identified in the U2foxRELOC assay (data not shown). Likewise, when analyzed in the U2nesRELOC assay, a cell-based system to detect inhibition of the general export machinery, only the two known nuclear export inhibitors, ratjadone A and leptomycin B, were shown to induce nuclear trapping of the reporter protein (ref. [24] and data not shown).

As specificity is closely correlated with the concentrations used, we determined the minimal dose necessary to induce the nuclear accumulation of GFP–FOXO for each hit compound (Table 1). We performed dose-response experiments at eleven different concentrations and ratjadone A, leptomycin B, PI-103, wortmannin, and vinblastine were identified as the most potent activators of GFP–FOXO nuclear translocation, acting in the low nanomolar range. Submicromolar concentrations of D000 were sufficient to induce nuclear shuttling of the report-

Figure 3. Nuclear accumulation of the GFP–FOXO reporter protein induced by the test compounds. A) We exposed U2foxRELOC cells to three different concentrations of the 73 compounds for 1 h. Bar graphs show the percentage of the cells in each well exhibiting nuclear/cytoplasmic (Nuc/Cyt) ratios of fluorescence intensity greater than 1.8. Low, medium, and high concentrations are indicated by light gray, gray, and dark gray bars, respectively. Untreated wells are indicated by U, control wells containing dimethyl sulfoxide, ethanol, LY294002, or leptomycin B are indicated by D, E, Ly, and L, respectively. The data shown represent three independent experiments. B) The morphology of U2foxRELOC cells remained unaffected by exposure to Akt inhibitor X (AIX), vinblastine, or W13. U2foxRELOC cells were preincubated with cell-tracker orange dye and exposed to the Akt inhibitor X (AIX, 5 μM), vinblastine (100 nM), or W13 (20 μM). After 1 h at 37 °C, the cells were processed as described in Figure 1.

Table 1. The compounds capable of inducing the nuclear accumulation of GFP–FOXO and their main molecular targets.

COMPOUND	TARGET	MEC [μM][a]
manumycin A	Ras-farnesyltransferase	11.1
LY294002	PI3K	11.1
wortmannin	PI3K	0.015
PI-103	PI3K	0.046
PIK-75	PI3K	0.41
D000	PI3Kδ	11.1
Akt inhibitor	Akt	11.1
Akt inhibitor VIII	Akt	3.7
Akt inhibitor X	Akt	3.7
UCN01	PDK1, other protein kinases	0.045
staurosporine	protein kinases	11.1
leptomycin B	CRM1	0.045
ratjadone A	CRM1	0.045
genistein	tyrosine protein kinases	11.1
vinblastine	tubulin	0.045
W7	CaM	11.1
W13	CaM	11.1
calmidazolium chloride	CaM	11.1

[a] The minimal effective concentration (MEC) is the lowest dose of each corresponding compound that induced GFP–FOXO translocation, as determined by assessing 11 different concentrations.

er protein whereas micromolar concentrations of manumycin A, LY294002, Akt inhibitor, Akt inhibitor VIII, Akt inhibitor X, staurosporine, genistein, CDZ, W-7, and W-13 were required to obtain the same result.

Analysis of calcium signaling

We investigated whether the nuclear accumulation of the FOXO-reporter protein upon exposure to the calmodulin (CaM) inhibitors W-7, W-13, and CDZ was mediated by specific inhibition of CaM. We took advantage of a closely related naphthalene-sulfonamide analogue that displays a very different inhibitory profile on CaM. W-7 and W-13 inhibit the CaM regulated activity of Ca^{2+}-modulin-dependent phosphodiesterase at IC_{50} values of 28 μM and 68 μM, respectively.[25] The related W-12, a compound that lacks chlorine, is a much less effective CaM inhibitor[25] than W-7 or W-13, and it was used to distinguish CaM-specific inhibitory effects from nonspecific drug effects. Indeed, W-12 failed to produce nuclear translocation of the FOXO reporter protein even at 50 μM (Figure 4B). Conversely, the minimal effective concentrations of W-7 or W-13 necessary to induce nuclear localization of GFP–FOXO were in good agreement with the IC_{50} values reported previously for CaM inhibition by these two naphthalene-sulfonamide analogues. Taken together these data indicate that CaM is involved in regulating FOXO translocation.

To further study the implication of Ca^{2+} signaling in FOXO shuttling, we used different chemical probes capable of altering the cellular Ca^{2+} homeostasis. When the reporter cells were loaded with the intracellular Ca^{2+}-chelator, BAPTA-AM, we confirmed the nuclear accumulation of the fluorescent signal, in agreement with a role for Ca^{2+} signaling in mediating the nuclear translocation of FOXO3a. In addition, the extracellular Ca^{2+}-chelator EGTA also produced the nuclear accumulation of the FOXO reporter protein, providing further evidence that intracellular and extracellular Ca^{2+} regulates the subcellular localization of FOXO proteins. In contrast, the increase in cytosolic Ca^{2+} induced by caffeine, thapsigargin, or ionomycin did not promote the nuclear retention of GFP–FOXO, nor did they restore its cytoplasmic localization when applied together with W-7, W-13, and CDZ (Figure 4 and data not shown).

The molecular mechanisms by which the CaM antagonists exert their effect on FOXO translocation were further explored by examining the impact of inhibiting of multifunctional calcium/CaM-dependent protein kinases. KN-62 and K-93 inhibit CaMKI, CaMKII, and CaMKIV, yet they had no effect on the subcellular localization of FOXO. Likewise, when using ML-7 to inhibit the calcium/CaM-dependent protein kinase, myosin light-chain kinase (MLCK), failed to induce nuclear accumulation of the fluorescent reporter. Furthermore, inhibiting the CaM-kinase-kinases (CaMKK), upstream activators of the calcium/CaM-dependent protein kinases, CaMKI and CaMKIV, did not reproduce the effect of CaM inhibition on FOXO translocation. To explore the effect on Akt/PI3K signaling of the chemical probes that interfere with Ca^{2+} signaling and that induce nuclear FOXO localization, we monitored the phosphorylation of Akt. In Western blots probed with a specific antibody against Akt Ser473, a dramatic decrease in Akt phosphoylation on Ser473 was evident upon treatment with W-7, W-13, Bapta AM, or EGTA. These data indicate a direct relationship between blocking CaM activity and decreased Akt phosphorylation.

Discussion

Chemical genetics has emerged as an exciting new research field that explores the interface between chemistry and biology. In this study, we demonstrate the potential of chemical genetics combined with high-content screening to dissect out the complex regulation of the subcellular localization of FOXO transcription factors. We established U2foxRELOC, a quantitative cell-based high-content screening assay that monitors the nuclear–cytoplasmic translocation of a GFP–FOXO3a fusion protein. Herein, U2foxRELOC was used to obtain quantitative information about the impact of different PI3K inhibitors on downstream signaling, to perform a large-scale chemical genetic study with a panel of test compounds. We also used this system to analyze the effect of chemical probes that modulate Ca^{2+} signaling on the nuclear–cytoplasmic shuttling of a FOXO reporter protein. High-throughput cellular imaging has enabled us to measure the minimal effective concentration of different PI3K inhibitors necessary to induce nuclear shuttling of a FOXO reporter protein. Using this approach we screened a panel of 73 compounds with a known mechanism of action and we identified 17 compounds that induced the nuclear accumulation of the fluorescent reporter. The majority of these small molecules are known inhibitors of the PI3K/Akt pathway confirming the essential role of signaling through PI3K, PDK1, and Akt in regulating the subcellular localization of FOXO proteins. Interestingly, D000 a compound claimed to specifically

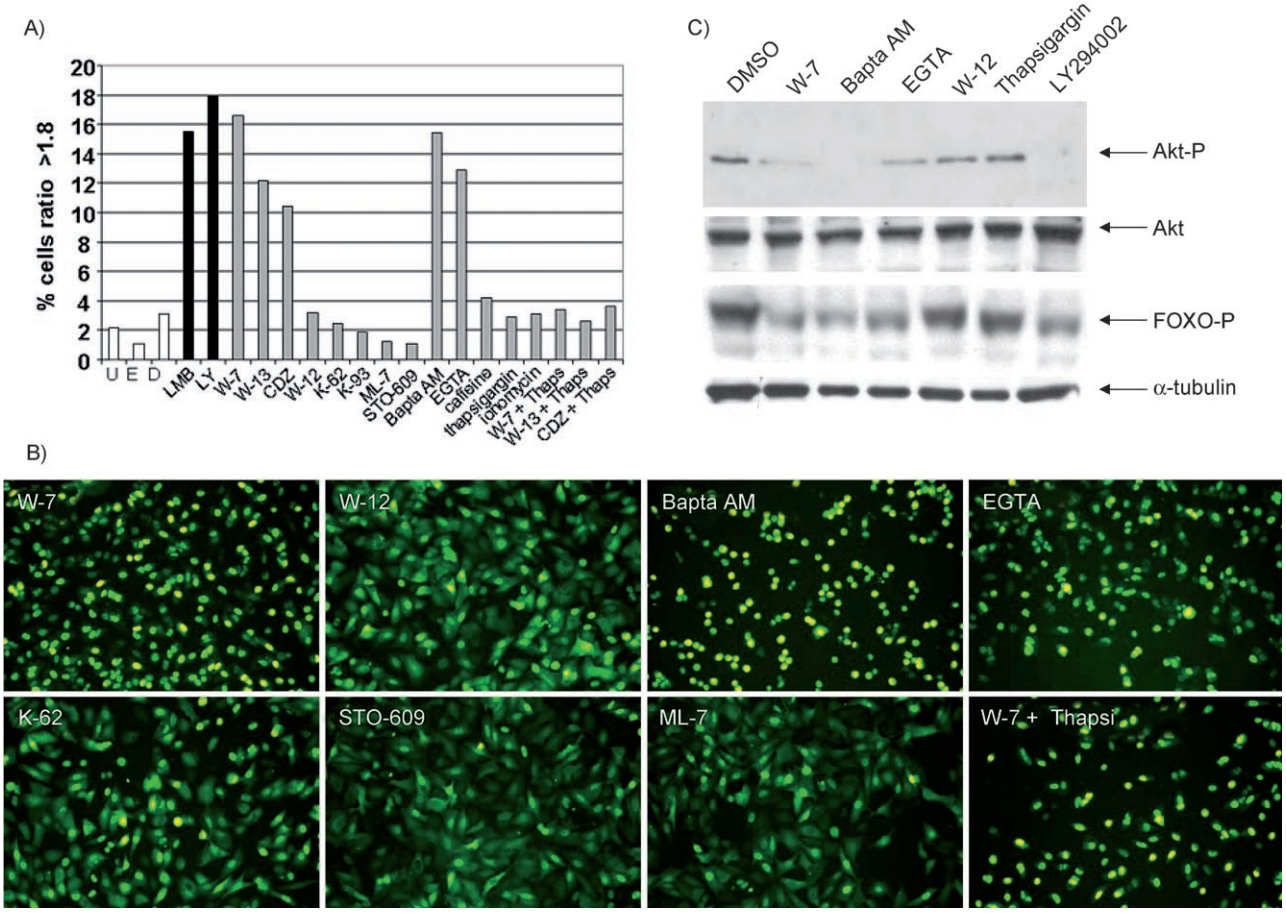


Figure 4. A) Nuclear accumulation of the GFP–FOXO reporter protein following treatment with chemical probes that interfere with Ca^{2+} signaling. Bar graphs show the percentage of cells in each well exhibiting nuclear/cytoplasmic (Nuc/Cyt) ratios of fluorescence intensity greater than 1.8. Untreated wells are indicated by U, control wells containing dimethyl sulfoxide, ethanol, LY294002, or leptomycin B are indicated by D, E, LY, and LMB, respectively. We exposed U2-foxRELOC cells to W7 (20 μM), W13 (20 μM), calmidazolium chloride (CDZ, 20 μM), W12 (50 μM), KN62 (30 μM), KN93 (30 μM), ML-7 (30 μM), STO-609 (1 $\mu g\,mL^{-1}$), Bapta AM (100 μM), EGTA (5 mM), caffeine (4 mM), thapsigargin (200 nM), ionomycin (200 nM), and to thapsigargin (200 nM) in the presence of W7 (20 μM), W13 (20 μM), or CDZ (20 μM). The data shown represent three independent experiments. B) Representative images of treated cells by using the high-throughput format of the U2foxRELOC system. Images of fixed and DAPI-stained cells were taken by automated microscopy 1 h after drug exposure. Images corresponding to U2foxRELOC cells exposed to W7 (20 μM), W12 (200 μM), Bapta AM (100 μM), EGTA (5 mM), KN62 (30 μM), STO-609 (1 $\mu g\,mL^{-1}$), ML-7 (30 μM), or W7 (20 μM) in the presence of thapsigargin (200 nM) are shown. C) Immunoblot analysis of total lysates from U2foxRELOC cells exposed to DMSO (1%), W7 (20 μM), Bapta AM (100 μM), EGTA (5 mM), W12 (50 μM), thapsigargin (200 nM), or LY294002 (20 μM) for 1 h. A representative experiment is shown and the relevant proteins are indicated by arrows.

inhibit p110δ,[26] was capable of inducing the translocation of the GFP–FOXO reporter. These data are in agreement with our previous observation that a constitutively active form of p110δ efficiently induced the activation of Akt in Rat1 fibroblasts.[27] However, whether the nuclear shuttling of the FOXO reporter protein on exposure to D000 is due to the specific inhibition of the delta isoform of PI3K remains to be determined.

We tested an extensive panel of chemotherapeutic agents reported to target a variety of cellular macromolecules including topoisomerase I, topoisomerase II, thymidylate synthetase, DNA, tubulin, and cyclin-dependent kinases. However, the majority of these drugs failed to produce nuclear trapping of GFP–FOXO. Although the tubulin-targeting agent paclitaxel has been shown to induce nuclear translocation in MCF7 cells,[28] it had no impact on the subcellular translocation of GFP–FOXO in the U2foxRELOC assay. These differences might be due to the experimental setting or the different cell lines used in these experiments. Indeed, FOXO translocation in MCF7 cells was identified after 16 h in the presence of paclitaxel, whereas U2foxRELOC were exposed to paclitaxel for only 1 h.[28] Conversely, we identified the vinca alkaloid vinblastine as a very potent FOXO translocating agent, active in the low-nanomolar range. Vinblastine is a microtubule-depolymerizing drug whose mode of action has been characterized.[29] Our data suggest that the molecular mechanisms employed for the nuclear translocation of FOXO factors following exposure to the small tubulin-binding molecules paclitaxel and vinblastine differ. Paclitaxel and vinblastine bind to different sites in tubulin and they promote microtubule bundling or microtubule disassembly in vitro, respectively. Whether these contrasting effects on microtubule dynamics account for the different behavior observed in the U2foxRELOC assay remains to be determined.

The inhibition of the Ca^{2+}-binding protein CaM produces the nuclear accumulation of FOXO proteins in cell assays based on the immunodetection of transiently expressed reporter protein.[23] We confirmed this observation using U2 foxRELOC, a technology based on the stable expression of a genetically tagged FOXO reporter protein. Importantly, we extended previous data regarding the implication of Ca^{2+}-signaling in the regulation of FOXO transcription factors by analyzing the effect of several chemical probes in the U2 foxRELOC assay and on Akt phosphorylation.

Chemical genetic analysis of the Ca^{2+}-dependent regulation of FOXO localization revealed the important role of intra- and extracellular calcium concentrations. Calcium/calmodulin-regulated FOXO-translocation is not directly mediated either by multifunctional or dedicated calcium/CaM-dependent protein kinases, or by upstream CaM-kinase-kinases. This is consistent with a model in which low calcium concentrations decrease the activity of CaM, in turn inhibiting Akt and the translocation of FOXO proteins into the cell nucleus. Akt associates with CaM in mouse mammary carcinoma cells and has been proposed as a CaM-binding protein.[30] However, whether a decrease in CaM-binding induces the nuclear translocation of FOXO proteins by directly affecting Akt activity remains to be explored. CaM expression is altered in several cancers and its inhibition might be a strategy to restore the tumor suppressor activity of the FOXO factors.

In summary, our data demonstrate that U2 foxRELOC is a sensitive and robust assay system suitable for identifying small-molecule inhibitors of signaling events that regulate the subcellular localization of FOXO proteins. The signaling events identified here include PI3K/Akt signaling, nuclear export, and calcium/CaM-dependent signaling. This work illustrates the power of chemical genetics combined with image-based cellular screening to analyze signaling pathways. Moreover, our data raise expectations that a more extensive chemical interrogation of the nuclear–cytoplasmic shuttling of FOXO could lead to the identification of new molecular targets and small molecules that might aid the development of more potent therapeutic agents to treat tumors.

Experimental Section

Compound supply and recombinant proteins: A complete list of compounds used in the present study is given in Table 1 of the Supporting Information. The PI3K inhibitors PIK-75 and PI-103 were synthesized according to published patent specifications. UCN01 and flavopiridol were kindly provided by the NCI, National Institutes of Health, cisplatin was provided by C. Navarro, minerval was generously provided by P. Escriba, and gemcitabine was a gift from Eli Lilly Pharmaceuticals (Indianapolis, IN). All other chemicals were purchased from commercial sources. Akt inhibitor, Akt inhibitor VIII, Akt inhibitor X, alsterpaullone, Bapta-AM, Bay11–7082, ionomycin, JAK3 inhibitor VI, JNK inhibitor VIII, kenpaullone, KN62, KN93, LY294002, MG132, ML-7, NL71–101, PD98059, PP1, purvalanol A, ratjadone A, SB202190, SB203580, W-13 HCl, W-7 HCL, W-12 HCL, and wortmannin were purchased from Calbiochem (San Diego, CA, USA). Brefeldin, cyclosporin A, forskolin, genistein, H89, leptomycin B, rapamycin, roscovitine, thapsigargin, tyrphostatin AG 1478, tyrphostatin SU1498, and U0126 were purchased from LC Laboratories. (Woburn, MA, USA); D-609, LY83583, manumycin A, pifithrin-α cyclic, rifampicin, tyrphostin AG 82, and tyrphostin AG 1433 were purchased from Alexis Biochemicals (San Diego, CA, USA); Caffeine, calmidazolium chloride, EGTA, etoposide, GW5074, hydrocortisone, nicotinamid, oxaliplatin, paclitaxel, staurosporine, STO-609, 12-O-tetradecanoylphorbol-13-acetate (TPA), trichostatin A, and vinblastine were purchased from Sigma–Aldrich.

Arctigenin, SL327 and SP6000125 were purchased from Biaffin (Kassel, Germany); lithium chloride (LiCl) was purchased from Merck; D000 was purchased from Labotest (Niederschoena, Germany); epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) were purchased from RELIATech A.S. (Braunschweig, Germany) and human insulin-like growth factor-I (IGF-I) and human insulin were purchased from Roche Diagnostics. Stock solutions of the test compounds were deposited in three different concentrations onto 96-well mother plates, transferred to multiple replica plates, and frozen at $-80\,^{\circ}C$.

Generation and maintenance of U2 foxRELOC and U2gfpRELOC cells: U2-OS cells obtained from the ATCC were cultivated as indicated. These cells were transfected at confluence with the plasmid containing the GFP–FOXO3a fusion protein (a gift from T. Finkel) as described by Zanella et al,[24] or that containing EGFP alone, by using the effectene transfection reagent (Qiagen). Selection was performed with G418 (1 mg mL^{-1}, Calbiochem) for one week and the resistant colonies were then cultured selecting those that best expressed the reporter by FACS, as well as the most homogeneous population. The selected clones, designated as U2 foxRELOC or U2gfpRELOC, were then cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS (Sigma), antibiotics (penicillin, streptomycin), antimycoplasm (plasmocin), and G418 at 100 $\mu g\,mL^{-1}$.

Compound administration and relocalization assay: The U2 foxRELOC-based assay was formatted in 96-well plates and workflow has been automated. All liquid handling for compound treatment, washing, fixing, and staining steps was performed by a robotic workstation.[31] Clonal U2 foxRELOC cells were seeded at a density of 1.0×10^5 cells mL^{-1}, in black-walled clear-bottomed 96-well microplates (BD Biosciences), in a final volume of 200 μL per well distributed by using a multidrop automatic dispenser. Cells were allowed to attach for 12 h at $37\,^{\circ}C$ in an atmosphere of 5% CO_2, and each test compound was then automatically administered to the assay plates in 2 μL by using a robotic workstation (Biomek 1000, Beckman). Treated cells were then incubated for 1 h before the culture medium was aspired, the cells were washed with PBS twice, and they were fixed in paraformaldehyde (100 μL, 6%) for 30 min at RT. The fixed cells were then washed twice with PBS and stained with DAPI (Invitrogen) for 20 min at RT to define the nucleus. The DAPI solution was removed by aspiration and finally, the plates were washed with PBS twice and stored in the dark at $4\,^{\circ}C$ before analysis. Vital staining with the cell-tracker orange (CMTMR) fluorescent dye (Invitrogen) was performed following the manufacturer's guidelines. As such, cell-tracker orange working solution (5 μM) was added to the assay plates and incubated at $37\,^{\circ}C$. After 30 min the dye solution was replaced with fresh medium, and the cells were incubated for another 30 min. Cells were then fixed and processed as described above.

Assay readout: Assay plates were read on the BD Pathway™ 415 Bioimager equipped with a 488/10 nm EGFP excitation filter, a 380/10 nm DAPI excitation filter, a 515 LP nm EGFP emission filter, and a 435 LP nm DAPI emission filter. Images were acquired in the

DAPI and GFP channels of each well by using 20× dry objective. The plates were exposed for 0.066 ms (Gain 31) to acquire DAPI images and 0.55 ms (Gain 30) for GFP images.

Data analysis: Data was exported from the BD Pathway Bioimager as text files and imported into the data analysis software BD Image Data Explorer for processing. The nuclear/cytoplasmic (Nuc/Cyt) ratios of fluorescence intensity were determined by dividing the intensity of the GFP fluorescence from the nucleus by that in the cytoplasm. We applied a threshold ratio of greater than 1.8 to define nuclear accumulation of fluorescent signal for each cell. Based on this procedure we calculated the percentage of cells per well exhibiting nuclear translocation. Compounds that induced nuclear accumulation of the fluorescent reporter above 60% of the signal obtained from wells treated with 20 μM LY294002 were considered as hits.

Western blot analysis: Subconfluent cells were incubated under different conditions and washed twice with TBS prior to lysis. Lysis buffer was added containing 50 mM Tris HCl, 150 mM NaCl, 1% NP40, 2 mM Na_3VO_4, 100 mM NaF, 20 mM $Na_4P_2O_7$, and protease inhibitor cocktail (Roche Molecular Biochemicals). The proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were incubated overnight at 4 °C with antibodies specific for Akt, phospho-Ser-473-Akt (Cell Signaling Technology), phospho-Thr32-FOXO3a (Upstate), and α-tubulin (Sigma), they were washed and then incubated with IRDye800 conjugated anti-mouse and Alexa Fluor 680 goat anti-rabbit IgG secondary antibodies. The bands were visualized by using an Odyssey infrared imaging system (Li-Cor Biosciences).

Acknowledgements

This work was supported by a grant from the Spanish MCyT BIO2002-00197 and the Spanish MEC (project BIO2006-02432). F.Z. is recipient of a Marie Curie Fellowship. The authors acknowledge the supply of PIK-75 and PI-103 synthesized by D. Soilán and R. Álvarez at the Medicinal Chemistry Department, CNIO, the expert technical assistance of E. Gonzalez and F. Blanco, the assistance of the staff at BD Biosciences for their contribution in establishing the technology, and C. Blanco, J. F. Martinez, and O. Renner for helpful discussions and critical reading of this manuscript.

Keywords: antitumor agents · chemical genetics · FOXO · high-throughput screening

[1] A. Barthel, D. Schmoll, K. D. Kruger, G. Bahrenberg, R. Walther, R. A. Roth, H. G. Joost, *Biochem. Biophys. Res. Commun.* **2001**, *285*, 897.
[2] A. Brunet, A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, M. E. Greenberg, *Cell* **1999**, *96*, 857.
[3] M. Stahl, P. F. Dijkers, G. J. P. L. Kops, S. M. A. Lens, P. J. Coffer, B. M. T. Burgering, R. H. Medema, *J. Immunol.* **2002**, *168*, 5024.
[4] G. J. Kops, R. H. Medema, J. Glassford, M. A. Essers, P. F. Dijkers, P. J. Coffer, E. W. Lam, B. M. Burgering, *Mol. Cell. Biol.* **2002**, *22*, 2025.
[5] N. Nakamura, S. Ramaswamy, F. Vazquez, S. Signoretti, M. Loda, W. R. Sellers, *Mol. Cell. Biol.* **2000**, *20*, 8969.
[6] L. Martinez-Gac, M. Marques, Z. Garcia, M. R. Campanero, A. C. Carrera, *Mol. Cell. Biol.* **2004**, *24*, 2181.
[7] M. C. Hu, D. F. Lee, W. Xia, L. S. Golfman, F. Ou-Yang, J. Y. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, M. C. Hung, *Cell* **2004**, *117*, 225.
[8] S. Ramaswamy, N. Nakamura, I. Sansal, L. Bergeron, W. R. Sellers, *Cancer Cell* **2002**, *2*, 81.
[9] J. H. Paik, R. Kollipara, G. Chu, H. Ji, Y. Xiao, Z. Ding, L. Miao, Z. Tothova, J. W. Horner, D. R. Carrasco, S. Jiang, D. G. Gilliland, L. Chin, W. H. Wong, D. H. Castrillon, R. A. DePinho, *Cell* **2007**, *128*, 309.
[10] A. van der Horst, B. M. Burgering, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 440.
[11] L. P. van der Heide, M. F. Hoekman, M. P. Smidt, *Biochem. J.* **2004**, *380*, 297.
[12] M. K. Lehtinen, Z. Yuan, P. R. Boag, Y. Yang, J. Villen, E. B. Becker, S. DiBacco, N. de La Iglesia, S. Gygi, T. K. Blackwell, A. Bonni, *Cell* **2006**, *125*, 987.
[13] J. Sunayama, F. Tsuruta, N. Masuyama, Y. Gotoh, *J. Cell Biol.* **2005**, *170*, 295.
[14] M. A. Essers, S. Weijzen, A. M. de Vries-Smits, I. Saarloos, N. D. de Ruiter, J. L. Bos, B. M. Burgering, *Embo J.* **2004**, *23*, 4802.
[15] A. van der Horst, A. M. de Vries-Smits, A. B. Brenkman, M. H. van Triest, N. van den Broek, F. Colland, M. M. Maurice, B. M. Burgering, *Nat. Cell Biol.* **2006**, *8*, 1064.
[16] D. Frescas, L. Valenti, D. Accili, *J. Biol. Chem.* **2005**, *280*, 20589.
[17] C. Chaussade, G. W. Rewcastle, J. D. Kendall, W. A. Denny, K. Cho, L. M. Gronning, M. L. Chong, S. H. Anagnostou, S. P. Jackson, N. Daniele, P. R. Shepherd, *Biochem. J.* **2007**, *404*, 449.
[18] J. Bain, L. Plater, M. Elliott, N. Shpiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alessi, P. Cohen, *Biochem. J.* **2007**, *408*, 297.
[19] J. W. Mockridge, M. S. Marber, R. J. Heads, *Biochem. Biophys. Res. Commun.* **2000**, *270*, 947.
[20] Y. Taniyama, M. Ushio-Fukai, H. Hitomi, P. Rocic, M. J. Kingsley, C. Pfahnl, D. S. Weber, R. W. Alexander, K. K. Griendling, *Am. J. Physiol. Cell Physiol.* **2004**, *287*, C494.
[21] C. Brantley-Finley, C. S. Lyle, L. Du, M. E. Goodwin, T. Hall, D. Szwedo, G. P. Kaushal, T. C. Chambers, *Biochem. Pharmacol.* **2003**, *66*, 459.
[22] H. Huang, K. M. Regan, Z. Lou, J. Chen, D. J. Tindall, *Science* **2006**, *314*, 294.
[23] T. R. Kau, F. Schroeder, S. Ramaswamy, C. L. Wojciechowski, J. J. Zhao, T. M. Roberts, J. Clardy, W. R. Sellers, P. A. Silver, *Cancer Cell* **2003**, *4*, 463.
[24] F. Zanella, A. Rosado, F. Blanco, B. R. Henderson, A. Carnero, W. Link, *Assay Drug Dev. Technol.* **2007**, *5*, 333.
[25] T. Tanaka, T. Ohmura, H. Hidaka, *Pharmacology* **1983**, *26*, 249.
[26] C. Sawyer, J. Sturge, D. C. Bennett, M. J. O'Hare, W. E. Allen, J. Bain, G. E. Jones, B. Vanhaesebroeck, *Cancer Res.* **2003**, *63*, 1667.
[27] W. Link, A. Rosado, J. Fominaya, J. E. Thomas, A. Carnero, *J. Cell. Biochem.* **2005**, *95*, 979.
[28] A. Sunters, P. A. Madureira, K. M. Pomeranz, M. Aubert, J. J. Brosens, S. J. Cook, B. M. Burgering, R. C. Coombes, E. W. Lam, *Cancer Res.* **2006**, *66*, 212.
[29] B. Gigant, C. Wang, R. B. Ravelli, F. Roussi, M. O. Steinmetz, P. A. Curmi, A. Sobel, M. Knossow, *Nature* **2005**, *435*, 519.
[30] T. B. Deb, C. M. Coticchia, R. B. Dickson, *J. Biol. Chem.* **2004**, *279*, 38903.
[31] A. Rosado, F. Zanella, B. Garcia, A. Carnero, W. Link, *PLoS ONE* **2008**, *3*, e1823.

Received: April 15, 2008
Published online on August 29, 2008

