Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity

Nileeka Balasuriya1,4, Maya T. Kunkel3,4, Xuguang Liu1, Kyle K. Biggar1, Shawn S-C. Li1, Alexandra C. Newton3*, and Patrick O’Donoghue1,2*

1Department of Biochemistry, The University of Western Ontario, London, ON N6A 5C1, Canada.
2Department of Chemistry, The University of Western Ontario, London, ON N6A 5C1, Canada.
3Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA.
4These authors contributed equally.
*Corresponding authors: anewton@ucsd.edu (A.C.N.), patrick.odonoghue@uwo.ca (P.O.)

Running title: Phosphorylation of Thr308 is irreplaceable for Akt activity

Keywords: activation loop, Akt/protein kinase B, genetic code expansion, hydrophobic motif, phosphomimetics, phosphorylation, phosphoseryl-tRNA synthetase (SepRS), tRNA<sub>sep</sub>

Abstract
The proto-oncogene Akt/protein kinase B (PKB) is a pivotal signal transducer for growth and survival. Growth factor stimulation leads to Akt phosphorylation at two regulatory sites (Thr308, Ser473), acutely activating Akt signaling. Delineating the exact role of each regulatory site is, however, technically challenging and has remained elusive. Here, we used genetic code expansion to produce site-specifically phosphorylated Akt1 in order to dissect the contribution of each regulatory site to Akt1 activity. We achieved recombinant production of full length Akt1 containing site-specific pThr and pSer residues for the first time. Our analysis of Akt1 site-specifically phosphorylated at either or both sites revealed that phosphorylation at both sites increases the apparent catalytic rate 1500-fold relative to un-phosphorylated Akt1, an increase attributable primarily to phosphorylation at Thr308. Live imaging of COS7 cells confirmed that phosphorylation of Thr308, but not Ser473, is required for cellular activation of Akt. We found in vitro and in the cell that pThr308 function cannot be mimicked with acidic residues nor could un-phosphorylated Thr308 be mimicked by an Ala mutation. An Akt1 variant with pSer308 achieved only partial enzymatic and cellular signaling activity, revealing a critical interaction between the γ-methyl group of pThr308 and Cys310 in the Akt1 active site. Thus, pThr308 is necessary and sufficient to stimulate Akt signaling in cells and the common use of phosphomimetics is not appropriate for studying the biology of Akt signaling. Our data also indicate that pThr308 should be regarded as the primary diagnostic marker of Akt activity.

Introduction
The proto-oncogene Akt/protein kinase B (PKB) is a central transducer of growth and survival signaling (1). There are three isozymes of Akt in mammals. Akt1, Akt2, and Akt3 include kinase domains with extensive homology to those of protein kinases A, G and C, defining them as members of the AGC family of Ser/Thr protein kinases (2). Akt
transduces signals in the phosphoinositide 3-kinase (PI3K) signaling cascade, which is one of the most commonly deregulated pathways in human cancer (3,4). Thus, enormous efforts are directed at understanding the mechanisms of activation of Akt and how to target these enzymes therapeutically (5).

Akt activity in the cell is acutely controlled by growth factor-dependent phosphorylation mechanisms (6). Following activation by agonist-bound receptor tyrosine kinases at the plasma membrane, PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) to generate the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) (7). This second messenger engages Akt at the plasma membrane by binding its autoinhibitory pleckstrin homology (PH) domain, resulting in a conformational change that exposes the kinase domain for phosphorylation (8). The phosphoinositide dependent kinase 1 (PDK-1) phosphorylates a conserved Thr on the activation loop (Thr308 in Akt1), leading to partial activation of Akt (9). Full activation of Akt1 results from a second phosphorylation event in the C-terminal tail, a regulatory region referred to as the ‘hydrophobic motif’ (Ser473 in Akt1) that was originally identified in protein kinase C and S6 kinase (10-12). Phosphorylation at Ser473 in Akt1 depends on the mammalian target of rapamycin complex 2 (mTORC2) (13). In addition, Akt is co-translationally and constitutively phosphorylated at another C-terminal site termed the ‘turn motif’ (Thr450 in Akt1) by mTORC2, a modification that regulates the stability of the enzyme (14). Given that Akt is activated by phosphorylation of Thr308 and Ser473, phospho-specific antibodies to these sites are widely used as diagnostic markers (15-17). To a striking degree, many clinical (18-21) and biochemical (22) studies rely solely on Ser473 phosphorylation as a proxy for Akt activity.

The activation of Akt by phosphorylation is well established (8), yet the contribution of each specific phosphorylation site towards the maximal Akt activity and pathogenesis is less well defined. This knowledge gap resulted from the inability previously to prepare Akt variants in site-specifically phosphorylated forms. Earlier work established production of partially active and truncated Akt1 in E. coli. The attempt was unsuccessful in producing a sufficient amount of full length Akt1 to determine activity (23). Instead this study relied on a construct lacking the PH domain with Ser473 substituted by glutamate, and the authors were unable to show how the phosphomimetic compared to pSer at position 473 (23). A protocol for active Akt1 production in insect (Sf9) cells has been established (24). The ability to generate ppAkt1 required a complex and low yield in vitro procedure to phosphorylate Akt with 2 additional purified upstream kinases in the presence of lipid vesicles (24). Protein production in Sf9 cells fails to produce Akt1 with site-specific or programmed phosphorylation. The resulting protein is a mixture of singly and doubly phosphorylated species (25) and includes phosphorylation at Thr450 in addition to potentially other modifications, so the ability to isolate the activity of each regulatory site individually and in precise combinations has remained elusive. As demonstrated below, we have developed a facile and efficient approach that is a novel combination of in vivo enzymatic phosphorylation with genetic code expansion to produce pAkt1 and ppAkt1 variants with specifically programmed phosphorylation (Figure 1A).

Because of the previous technological barriers to producing specifically phosphorylated kinase variants, a significant
literature continues to accumulate for Akt (23,26-32) and other kinases (33), in which ‘phosphomimetic’ substitutions are used as a genetic tool to interrogate the biological consequences of phosphate on a site of interest (33). The rationale behind these experiments is that the acidic residues, Asp and Glu, are negatively charged like phosphate. Yet the carboxylate of an acidic amino acid has considerably less electronegativity than a phosphate and a significantly smaller hydration sphere and volume (34,35). A complementary approach involves nullifying the effect of a particular phosphorylation site by introducing a non-phosphorylatable Ala mutation (26); however, an Ala is considerably smaller and less polar than a Ser or Thr, and Ala lacks the capacity to form hydrogen bonds. In light of these observations, the widespread use of Ala and phosphomimetic mutants in signaling studies begs the question of how appropriate these mutations are to interrogate the cellular function of Akt or other phosphorylated proteins.

Here we provide, for the first time, a quantitative analysis of the contribution of phosphate at positions 308 and 473 in the catalytic activity of Akt1 and, additionally, examine the effects of substitutions of acidic residues or Ala at these positions. To do this, we developed an optimal strategy to produce recombinant full-length human Akt1 in Escherichia coli with genetically encoded phosphoserine (pSer) introduced at specific phosphorylation sites. We measured the activity of singly and doubly phosphorylated Akt1 variants with phosphate at positions 308 (pAkt1S308, pAkt1T308) and/or 473 (pAkt1S473) in comparison to inactive and un-phosphorylated Akt1 as well as Akt1 variants with phosphomimetic Glu or Asp mutations. With a Förster resonance energy transfer (FRET)-based Akt activity sensor (BKAR) (36), we conducted complementary experiments of specific Akt1 variants in live cells. Our data reveal that, in comparison to un-phosphorylated enzyme, phosphorylation of Thr308 alone increases the apparent catalytic rate by nearly 400-fold, which is sufficient to observe maximal signaling in cells. Phosphorylation of only Ser473 boosts Akt1 activity by ~80-fold over un-phosphorylated enzyme, however, our data suggest that phosphorylation at Ser473 alone may not be sufficient to elicit Akt1 signaling in cells. Thus, phosphorylation of Thr308 is necessary and sufficient for the activity of Akt. Importantly, our cell based observations and biochemical data confirm that Ala does not mimic a non-phosphorylated Thr308 and further that acidic residues fail to activate Akt1. Phosphomimetic substitutions, therefore, do not mimic phosphorylation in Akt1 at either of its two key regulatory phosphorylation sites.

**Results**

**Activation of full-length pAkt1 variants by genetic code expansion.** We used *in vitro* radioactive kinase assays with γ-[32P]-ATP to test the activity of full length Akt1 variants that were made with site-specifically incorporated pSer residues (Figure S1, S2, S3). The assays quantify the ability of each Akt1 variant to phosphorylate an Akt1 substrate peptide (CKRPRAASFAE) that is derived from the natural Akt1 target glycogen synthase kinase 3β (GSK-3β). Using genetic code expansion (37,38), we reassigned the UAG stop codon to genetically encode pSer in recombinant Akt1 proteins produced in *E. coli*. For example, we expressed pAkt1S473 with a genetically-encoded pSer residue inserted in response to a UAG codon at position 473 in the Akt1 construct. We used Multiple Reaction Monitoring Tandem Mass Spectrometry (MRM-MS/MS) to unambiguously identify
pSer at position 473 (Figure S3). Genetically-encoded pAkt1$^{S473}$ showed a clear signal for phosphorylation at position 473 with no evidence of de-phosphorylation (Figure S3A,B). In wild type or un-phosphorylated Akt1, Ser473 is readily detected with no evidence of pSer473 (Figure S3C,D).

For pThr incorporation at position 308, we relied on enzymatic phosphorylation by the upstream kinase PDK1 (23). We produced phosphorylated pAkt1$^{T308}$ as well as the doubly-phosphorylated ppAkt1$^{T308,S473}$ by co-expressing PDK1 in E. coli with either the wild-type Akt1 or pAkt1$^{S473}$ constructs, respectively (Figure 1A). We confirmed phosphorylation at Thr308 (Figure S4) by Parallel Reaction Monitoring Tandem Mass Spectrometry (PRM-MS/MS). The MS/MS data identified an insignificant level of Thr308 in the pAkt1$^{T308}$ sample, indicating essentially quantitative phosphorylation of Thr308 by PDK1 in E. coli. Finally, we confirmed site-specific phosphorylation of the ppAkt1$^{T308,S473}$ variant using PRM-MS/MS (Figure S2). In the MS/MS analysis, we achieved up to 90% coverage of the full length Akt1 (Figure S2D), and we were unable to identify significant peptides with modifications other than the anticipated and programmed phosphorylations. The data indicate that both genetic code expansion and PDK1 phosphorylation are highly site-specific, and that endogenous modification or de-phosphorylation by E. coli enzymes is absent or minimal.

We first measured the activity of Akt phosphorylated at both positions (ppAkt1$^{T308,S473}$). Doubly phosphorylated Akt1 (Figure S4) had an apparent reaction rate of 44 ± 6 fmol/min/pmol of enzyme (Figure 1, Table 1, Figure S5, S6). We performed these kinase assays in conditions of sub-saturating ATP. We chose these conditions so that Akt1 protein variants with a wide range of activities could be assayed using the same experimental conditions. In order to compare the relative activities of Akt1 and pAkt1 variants, we determined an apparent catalytic rate ($k_{\text{app}} = \frac{v}{[\text{Akt1}]}$) based on the initial velocity ($v_0$) observed in the kinase assays. Under sub-saturating ATP, our reaction velocities are far below $V_{\text{max}}$ for Akt and related kinases (24,39); $k_{\text{app}}$ is not intended to estimate $k_{\text{cat}}$. Interestingly, the bacterially expressed ppAkt1 construct consistently migrated slightly faster than the other pAkt1 variants and Akt1 mutants on sodium dodecyl sulphate (SDS)-PAGE (Figure S1, Figure S2A). We determined that this was not a result of premature termination of the translation product because PRM-MS/MS unambiguously identified intact full length and doubly phosphorylated ppAkt1$^{T308, S473}$ (Figure S2B-D). We found no evidence of truncation or stopping at the UAG473 codon, or de-phosphorylation according to mass spectrometry. Phosphorylation at Thr450 is known to specifically decrease the electrophoretic mobility of Akt1 expressed in mammalian cells (40). Our bacterially expressed pAkt1 variants show no evidence of phosphorylation at Thr450. These observations together suggest that phosphorylation status impacts the mobility of Akt1 on SDS-PAGE.

Akt1 variants that contain pThr308 were so highly active under the conditions of our assays that we reduced the Akt1 concentration 10-fold in order to establish a linear range of activity to determine $v_0$ (Figure S5). Un-phosphorylated Akt1 was essentially inactive, showing a low basal activity of 0.03 ± 0.01 fmol/min/pmol of enzyme (Table 1). Thus, in comparison to un-phosphorylated enzyme, phosphorylation at both activating sites increased the catalytic rate of Akt1 by 1500-fold. We note this is the first time that the activity of Akt1 has been definitively
measured and reported for homogenously pure variants with site-specifically programmed phosphorylation at both key regulatory sites and without phosphate occupancy at other sites (e.g., pThr450).

**Phosphorylation of Thr308 is necessary and sufficient for Akt1 activation.** We next examined the contribution of phosphate at each site individually to the maximal activity of Akt1. Based on measurements of the initial velocity, the activity of mono-phosphorylated pAkt1<sup>T308</sup> corresponded to ~27% that of the doubly-phosphorylated species (Figure 1, Table 1). Thus, phosphorylation at the PDK-1 site alone results in an over 400-fold increase in activity compared to the un-phosphorylated enzyme. These data establish that phosphorylation at Thr308 alone is sufficient for robust activation of Akt. Purified, monophosphorylated pAkt1<sup>S473</sup> was considerably less active than protein with phosphate at Thr308 (Figure 1). pAkt1<sup>S473</sup> had activity that corresponded to about 5% of the activity of the doubly-phosphorylated species (Table 1). Although significantly reduced relative to that of the doubly-phosphorylated species, activity from pAkt1<sup>S473</sup> still represents an 80-fold increase over the un-phosphorylated Akt1 enzyme. Thus, genetic code expansion with pSer enables production of active Akt1 without the need to purify and activate upstream Akt1 kinases (24). In the absence of substrate peptide, we were able to detect low, but above background, phosphorylation that may be attributed to auto-phosphorylation by pAkt1<sup>S473</sup> (Figure 2).

Genetic code expansion was also used to produce pAkt1<sup>S308</sup>. The Akt enzyme containing pSer308 had above background but low activity (Figure 3A). Western blot analysis revealed a band co-migrating with full-length Akt but also a species with an apparent MW of ~35 kDa (Figure S7). This could result either from degradation or premature truncation from stopping at UAG308, which would produce a protein of 37.6 kDa. We adjusted our estimated rate for this enzyme by accounting for the fraction of full-length pAkt1<sup>S308</sup> in our preparation. Although it is possible we underestimated the activity Akt1 with pS308 (Figure 3A), the data show that pSer cannot substitute for pThr308 to produce optimally active Akt1.

**Enzymatic activity of phosphomimetic Akt1 variants.** We next tested the activity of purified phosphomimetic Akt1 mutants (Figure S6). In these experiments, we programmed either Ser473 phosphorylation (Figure 2A) or unphosphorylated Ser473 (Figure 3A) and examined the effect of residue substitutions at position 308. Replacement of Thr308 with Asp increased the activity of pAkt1<sup>S473</sup> by a modest 2-fold (from 2.5 to 4.6 fmol/min/pmol Akt), resulting in enzyme that displayed only 10% of the activity of doubly-phosphorylated enzyme (Figure 2A, S6, Table 1). Surprisingly, replacing the Thr308 with an Ala eliminated the activity of the mono-phosphorylated pAkt1<sup>S473</sup> (Figure 2A), demonstrating that Ala308 is not an appropriate substitute for unphosphorylated Thr.

In the context of Akt1 that was not phosphorylated at Ser473 (Figure 3B), Akt1<sup>D308</sup> demonstrated 5-fold higher enzyme activity in comparison to un-phosphorylated Akt1, but this was still ~100-times lower than that of pAkt1<sup>T308</sup> (Figure 3A, S6, Table 1). Akt1<sup>E308</sup> was >200-fold less active than pAkt1<sup>T308</sup> (Figure 3A, S6). Interestingly, production of pAkt1<sup>S308</sup> by our genetic code expansion approach resulted in an enzyme with only 3-fold more activity than Akt1<sup>D308</sup> (Figure 3A), suggesting that Asp308 may, to some extent, mimic pSer308, but not the natural pThr308 residue in Akt1.
Phosphomimetic substitution at Ser473 also failed to activate Akt1. We tested Ser473 mutations in the context of un-phosphorylated Akt1 (Figure 3B, S6) and in Akt1 variants phosphorylated at Thr308 (Figure 2B). For un-phosphorylated Akt1, we examined the effect of Asp and Glu substitutions at position 473 (Figure S6). Substitution with Asp at position 473 lead to essentially inactive enzyme (Figure 3B, S6, Table 1). Replacement of Ser473 with Glu resulted in an insignificant increase above the activity of un-phosphorylated Akt1 enzyme (Figure 2C, S6, Table 1). In the context of pAkt1T308 (Figure 2B), phosphomimetic mutations (D and E) failed to stimulate activity above pAkt1T308 with S473. Indeed, all S473 variants reduced pAkt1T308 activity with S473A showing the most significant reduction (Tables 1, 2).

Akt1 mutant activity in live cells. Having assessed the activity of Akt mutants in vitro, we next examined the activity of Akt1 phosphorylation site mutants in live cells. To this end, we utilized our quantitative FRET-based kinase activity reporter, BKAR, and examined Akt signaling (36). Co-expression of kinase constructs with FRET-based activity sensors affords a sensitive assay for examining agonist-evoked signalling in a cellular context (41). As the response to endogenous Akt activity is so low, it provides an ideal system to evaluate signaling by introduced Akt variants as a significant response can be observed from the overexpressed kinase.

Serum-starved COS-7 cells expressing BKAR and mCherry-tagged Akt variants were stimulated with epidermal growth factor (EGF) followed by Akt inhibition with GDC 0068. In real time, we monitored resulting changes in the BKAR FRET ratio (CFP/FRET) that are reflective of cellular Akt activity (Figure 4A). For these experiments, cells selected for analysis had comparable levels of Akt1 expression as assessed by quantifying mCherry levels; thus, in this system, one can clearly compare the relative signaling competence between wild type and mutant Akt. We have documented previously that the mCherry tag does not significantly impact Akt1 activity (41).

Overexpression of wild type (WT) Akt1 resulted in a significant BKAR response to EGF treatment compared to the response observed from endogenous Akt (Figure 4A, blue vs. grey trace). Interestingly, despite the level of overexpressed Akt in these experiments being in vast excess compared to endogenous Akt, substitution of either the non-phosphorylatable Ala or the ‘phosphomimetic’ Asp or Glu at the Thr308 site yielded an Akt1 enzyme that displayed no signaling in the cell (Figure 4A, orange and purple traces, Figure S8). These data establish the requirement of pThr308 for detectable Akt1 activation in cells and show that T308D is not a constitutively active Akt1 variant.

In contrast to the strict requirement for phosphorylation at Thr308, either Ala, Asp or Glu substitution at the Ser473 site were tolerated without detrimental effects on cellular Akt activity as assessed using BKAR (Figure 4A, green and red trace, Figure S8). Importantly, a double mutation of both 308 and 473 to Asp resulted in an inactive enzyme. Thus, the activity of Akt monophosphorylated at Thr308 is necessary and sufficient for maximal signaling in the context of cells.

Mutant Akt1 kinases are phosphorylated following EGF stimulation. We next examined the phosphorylation state of Thr308 and Ser473 of the mutant Akt kinases in our cell-based assay (Figure 4B). In experiments in Figure 5, we reduced the expression of the
tagged Akt variants to be equivalent to the endogenous Akt expression level as determined by Western blot. EGF stimulation (all even numbered lanes) induced phosphorylation of both endogenous and wild-type Akt1 (lane 4) as assessed by Western blot (Figure 5). Importantly, phosphorylation of Thr308 in the Ser473 mutants (lanes 10 and 12) was at the same level as the WT Akt1 (lane 4) demonstrating that PDK1 phosphorylation downstream of PI3K signaling was intact. Analysis of Ser473 phosphorylation revealed that, while this site was phosphorylated downstream of PI3K signaling, the level of phosphorylation at Ser473 was reduced in the T308A and T308D mutants (lanes 6 and 8) compared to WT Akt1 (lane 4). This suggests that lack of phosphate on Thr308 impairs phosphorylation of Ser473. Even though the level of Ser473 phosphorylation was reduced in these mutants, the level of total overexpressed Akt protein was saturating in Figure 4A. Given the excessive amount of Akt overexpressed in the experiments in Figure 4A, this lack of activity observed using BKAR indicates that T308A, T308D and T308E (Figure S8) are clearly not able to signal, and any catalytic activity observed in these variants is below the threshold to result in detectable cellular activity.

**Impact of Ser substitution at Thr308 in cells.**
As the recombinant Akt1^{T308} had similar activity as the pAkt1^{S308} protein *in vitro*, we were interested to examine the impact of Ser at 308 in the overexpressed mCherry-Akt system using BKAR. Overexpression of mCherry-Akt-T308S resulted in a similar BKAR response to that of WT Akt1 (data not shown). As this imaging system is monitoring an excess of the overexpressed mutant Akt, we attempted to express reduced levels of the kinase in order to discern whether there was a difference in cellular activity between WT Akt1 and Akt1 with Ser at position 308. Indeed, titrating down the expression levels and selecting for the lowest expressing cells based on mCherry intensity, we observed a reduction in Akt1 signaling output from the Ser308 enzyme compared to the WT enzyme with Thr308 (Figure 5A, blue and red traces). In the impaired kinase (Akt1^{S308}), we then substituted the 473 site with Ala (Ser308, Ala473), however, even in the context of an impaired Akt kinase, mutation of the 473 site did not influence Akt signaling (Figure 5A, green trace). Under the same conditions noted here to analyze minimal levels of overexpressed kinase, we were unable to detect a reduced level in signaling from the S473A mutant compared to WT Akt1 (Figure S9). This result is in agreement with our observations that mutation of Ser473 to Ala in the context of Akt1 phosphorylated at residue 308 either on Thr (Figure 4, green trace) or Ser (Figure 5, green trace) does not impact signaling in cells. Furthermore, the data indicate that the ability of pAkt1^{T308}S473A to signal is greater than that of Akt1 T308S.

Western blot analysis confirmed that phosphorylation of the 308 site (even with a Ser substitution) was intact following EGF stimulation (Figure 5B). Our data in live cells indicate that phosphate, specifically, not an acidic residue, at position 308 is the most critical component to induce Akt activity. In the cell, as we found *in vitro*, phosphomimetic substitutions of Asp or Glu at the 308 site are unable to propagate Akt signaling, and, in agreement with our enzymatic data, substitution of Thr308 to Ser leads to reduced Akt activity and signaling.

**Discussion**
Using genetic code expansion and enzymatic phosphorylation, we produced fully active
Akt1 directly from *E. coli* for the first time. We validated our biochemical findings in cells using a genetically-encoded reporter for Akt signaling. This allowed us to 1) measure the specific activity of Akt1 site-specifically and exclusively phosphorylated at either or both regulatory sites, Thr308 and Ser473, 2) systematically examine the role of phosphate at each site in modulating the intrinsic catalytic activity of the pure enzyme, and 3) examine the effectiveness of amino acid substitutions to mimic phosphorylated or un-phosphorylated residues in cells.

**Generation of active Akt1.** We demonstrated the compatibility of enzymatic phosphorylation with genetically-encoded phosphoserine incorporation by producing site-specific and doubly-phosphorylated Akt1 (Figure 1A). This was achieved by co-expressing the upstream kinase PDK1, to induce Thr308 phosphorylation, and simultaneously genetically encoded pSer473 in Akt1. To our knowledge, this is the first demonstration of protein production in *E. coli* with programmed pSer and pThr residues in the same protein. Consistent with numerous studies (24), our in vitro kinase assays revealed robust activity of ppAkt1 activity in the absence of PIP3, suggesting PIP3 is not required for Akt1 activity *per se*. This contradicts a recent study proposing that PIP3 binding may be essential for both Akt1 activation and activity in cells (42). As noted above, producing site-specifically and doubly phosphorylated, and thus fully-activated Akt1 has previously not been achieved via recombinant expression *in vivo* in *E. coli* given the complicated nature of Akt activation by multiple kinases, chaperones, and lipid second messengers (24). Our experiments demonstrate a novel approach, taking advantage of genetic code expansion and enzymatic phosphorylation, that overcomes the existing limitations in both methods to produce fully-active human kinases.

**Role of phosphorylation in activating Akt1.** The kinetic mechanisms of Akt activation by phosphorylation are less well characterized relative to other kinases such as PKA (43) and PKC (44), stemming in part from the difficulty in obtaining Akt in specifically phosphorylated forms. Most studies relied on immunoprecipitating Akt from unstimulated and growth factor-stimulated cells. Akt1 activity is then determined using a kinase activity assay in the immunoprecipitates. Such studies typically report a 10–50-fold stimulation of the activity of Akt immunoprecipitated from growth factor-treated compared to unstimulated cells, e.g., (45-49). Alessi and co-workers showed that Akt1 had 45-fold higher activity when immunoprecipitated from cells treated with IGF-1 compared to untreated cells (6). In the context of an Akt1 mutant with Ala present at position 473, the increase was only 5-fold, and if Ala was present at position 308, there was essentially no stimulated activity. Similar results were reported by Hemmings and coworkers for Akt3 (50). These data are qualitatively similar to the results from our study, yet our quantitative analysis defines Thr308 as the critical regulator of Akt activity and its phosphorylation alone increases activity to approximately one-third that of the doubly-phosphorylated enzyme. Our unique ability to produce recombinant fully-phosphorylated Akt1 from bacteria has revealed that specific phosphorylation at both regulatory sites increases the intrinsic catalytic activity by over three orders of magnitude, defining the stringency with which Akt signaling is ‘silent’ in the absence of agonist.
stimulation and the resulting phosphorylation of Akt.

Several lines of evidence support our finding that phosphorylation of Thr308, but not Ser473, is necessary and sufficient for the activation of the intrinsic catalytic activity of Akt. First, Vogt and colleagues concluded that phosphorylation of this site, but not Ser473, was sufficient for the oncogenic potential of Akt (26). Specifically, they showed that mutating Thr308 prevented the phosphorylation of Akt substrates and transformation of chicken embryonic fibroblasts. Secondly, Jacinto and coworkers showed that in cells lacking mTORC2, Akt was not phosphorylated on Ser473, yet retained the ability to phosphorylate a subset of Akt substrates (14). Based on this and other reports from the literature, phosphorylation of Ser473 was suggested to determine substrate specificity of Akt. Supporting a role of Ser473 in the cellular function of Akt, loss of phosphorylation of this site by overexpressing the PH domain Leucine-rich repeat Protein Phosphatase (PHLPP) (which dephosphorylates Ser473) results in increased apoptosis (51) and, conversely, enhancing phosphorylation at Ser473 by depletion of PHLPP results in suppression of tumors in a xenograft model. Although these effects on apoptosis may arise from non-Akt functions of PHLPP (52), they support a role of Ser473 phosphorylation in Akt biology. Phosphorylation at Ser473 may function to tune rather than activate Akt signaling. However, our results establish that the phosphorylation state of Thr308 is a superior read-out for the activation state of Akt. Whereas phosphorylation at Thr308 alone is able to activate Akt1, phosphorylation of Ser473 alone leads to relatively weak activity in vitro, and no detectable activity in the cell. Neither the Asp308 nor Glu308 Akt1 mutants had EGF-stimulated activity in cells. Although the pAkt\textsuperscript{5473} Thr308Asp mutant had 10% of the activity of the doubly-phosphorylated Akt1 in vitro (Table 2), we observed no cellular Akt1 activity and impaired Ser473 phosphorylation in Akt1 variants with Asp, Glu or Ala mutations at position 308. Even if these variants are active in the cell, the low intrinsic activity of the Asp308 mutant, for example, may be too low to overcome the opposing de-phosphorylation reaction catalyzed by cellular phosphatases acting on Akt substrates, including the BKAR reporter. Akt1 Thr308Asp mutants can no longer be regarded as constitutively active Akt1 variants (30-32).

The phosphorylation status of Akt1 is indeed clinically relevant. Whereas pSer473 is the most commonly used biomarker (53,54), pThr308 was identified as a better prognostic biomarker in human non-small cell lung cancer (55) and acute myeloid leukemia (56). Our study underscores the relevance of examining the phosphorylation state of Thr308 as a general marker for the activation state of Akt in basic research and clinical settings.

**Acidic residues do not function as phosphomimetics in Akt1.** Koshland and colleagues first introduced the use of acidic amino acids to mimic phosphate, showing that an Asp mimicked the functional effect of phosphate to inactivate isocitrate dehydrogenase (57). There are other examples where the negative charge of the amino acid effectively mimics that of phosphate and, indeed, nature has used the trick in reverse: it is estimated that 5% of pSer sites may have evolved from an ancestor with Glu or Asp at the homologous position (34). However, acidic residues are not necessarily ‘phosphomimetics’: their negative charge, hydration sphere, and size are considerably
different from a phosphorylated Ser or Thr residue. Acidic amino acids have a net negative charge of 1 compared to 2 for phosphate at neutral pH, the hydration sphere is 4 waters compared to 14 for phosphate, and their volume is considerably smaller than the phospho-amino acids they replace.

Because kinases are often activated by phosphorylation, Glu and Asp mutations are routinely used to mimic the function of phosphorylated serine or threonine residues in many kinases (33), including Akt1 (26,29). Our current study reveals that acidic residues do not mimic phosphorylation in Akt1. This is an important finding given the widespread and continued use of phosphomimetics in interrogating Akt signaling, and kinase signaling generally, in cells. The phosphomimetic variants that were active in our assays are 30 to 210-fold less active than their monophosphorylated counterparts. Based on our data in live cells, Thr308 phosphomimetic variants fail to activate Akt signaling (Figure 4A). Although, Akt1 Thr308Asp was the most active of the phosphomimetic mutants in vitro, this level of activity is insufficient to produce a cellular response. This result is consistent with a study from Vogt and colleagues who reported that Thr308Asp was an ‘unsuitable’ substitution for pThr308 (26,29). Their data showed that Akt Thr308Asp did not cause the phosphorylation of cellular Akt substrates such as GSK-3β nor did it induce oncogenic transformation, functions that were robustly mediated by wild-type Akt. Nonetheless, phosphomimetics constructs of Akt pervade the literature on Akt signaling mechanisms. Because phosphomimetics do not result in physiologically relevant activation of the kinase activity of Akt, it is possible that any biological effects of such mutations may arise from scaffolding functions or non-catalytic mechanisms, as increasingly described for protein kinase family members (58).

An interesting finding from our study is that pSer is not well tolerated at position 308. According to the in vitro kinase assays, pAkt1S308 is more active than phosphomimetic 308 mutants, yet pAkt1T308 is ~25-fold more active than pAkt1S308. Furthermore, when phosphorylated post-translationally in the cell, pAkt1S308 also showed reduced activity compared to that of pAkt1T308. To the best of our understanding, there is no literature-based evidence on the effect of pS308 on the activation of Akt1 in vivo or in vitro. Our results indicate that the γ-methyl group of Thr308 optimally positions the phosphate to create the ordered hydrogen-bonding network between the phosphate of pThr308, His194 and Arg273, which facilitates the binding to ATP and substrate at the Akt1 active site (26). Indeed, this observation is supported by crystallographic structures of Akt in partially active form and in complex with substrate peptide and an ATP analog (59,60). The structure shows pThr308 is involved in salt bridge interactions with Arg273 and Lys297, and a water-mediated hydrogen bond with His194 is also evident (Figure 6). In the structure, steric complementarity can be seen between the γ-methyl of pThr308 and Cys310 (Figure 6B). In agreement with our cell-based and biochemical data, the structure suggests the interaction between the γ-methyl of pThr308 and Cys310 optimally orients the phosphate for interaction with is positively charged counterparts in the salt bridges. We hypothesize that pS308, which lacks this methyl group, will rotate more freely in the active site, perhaps shifting the enzyme in and out of its active confirmation.

Our study also revealed that Ala is not a mimic of un-phosphorylated Thr308. Ala at position 308 resulted in a 10-fold reduction in
the basal activity of Akt1 mono-phosphorylated at Ser473. Taylor and colleagues also found that Ala at the activation loop of protein kinase A (PKA) results in a more severe kinetic defect compared to having an un-phosphorylated Thr at the activation loop \((61,62)\). The detrimental effect of Ala may arise from a loss of hydrogen bonding ability. The Ala mutation favors a helical conformation of the activation loop that may also drive structural ensembles away from the active conformation or it may favor an inhibited conformation due to interactions with other domains or proteins (A. Kornev and S. Taylor, personal communication).

**Akt1 activation threshold in cells.** Live cell imaging experiments revealed that all forms of Akt1 that had phosphorylation of Thr at position 308 had maximal signaling output following EGF stimulation of cells, regardless of the amino acid at position 473 (Table 2). In contrast, no activity was observed if the residue at position 308 was not phosphorylated, regardless of the amino acid at position 473 (Table 2). Based on *in vitro* data, we can estimate a threshold to observe Akt activity in cells: our *in vitro* studies reveal that phosphorylation of Ser473 alone stimulated Akt1 activity 80-fold over the un-phosphorylated enzyme, yet this level of Akt1 activity is apparently below the threshold required to elicit Akt1 signaling in the cell. *In vitro*, the activity of mono-phosphorylated Akt1 at Thr308 was reduced by less than 3-fold compared to the maximally-active and doubly-phosphorylated enzyme, and this level of activity is sufficient for maximal signaling output in cells. In addition, pAkt1\(^{T308}\) S473A was 10-fold less active than the doubly phosphorylated enzyme, yet this enzyme was sufficient for robust Akt1 signaling in the cell. Taken together, our data suggest that Akt1 variants whose intrinsic catalytic activity is greater than \(-10%\) that of doubly phosphorylated enzyme are robustly active in cells. This novel finding further suggests that in cells only a fraction of Akt needs to be activated by phosphorylation for full Akt-dependent signaling.

Although previous reports have identified a 10-fold activity increase associated with Ser473 phosphorylation in immunoprecipitates, the stoichiometry of phosphorylation was unknown \((6)\). Furthermore, the phosphorylation state of Akt1 may differentially impact substrates based on the local concentration or abundance of the substrate. For example, if Akt1 is poised on a scaffold and co-localized with substrate, a lack of Ser473 phosphorylation may be irrelevant as the local concentration of substrate is high. For phosphorylation of an untethered or low abundant substrate, that 3-fold difference in activity may reduce or eliminate Akt1 signaling in a substrate-specific manner. Indeed, previous work suggested that phosphorylation at Ser473 may increase the activity of Akt1 for particular substrates, e.g., FoxO1/3a \((14)\), yet significant future efforts are required to define the impact of Akt1 phosphorylation status on substrate selectivity.

Mutation and phosphorylation state can impact Akt1 activity directly, but also indirectly by altering the accessibility of phosphatases. For example, previous studies showed that Ala substitution in PKC at the hydrophobic motif increased phosphatase sensitivity of PKC at additional sites \((63)\). Our finding that Akt1 mutants with very low activity did not signal in cells suggests that localized activation needs to exceed a threshold to outcompete phosphatases in the cellular environment. Thus, the dynamic range of Akt signaling in a cell is such that sufficient Akt needs to be activated to overcome...
phosphatase suppression, but additional changes in phosphorylation site occupancy may or may not be needed for maximal signaling, particularly if localized to protein scaffolds and microdomains (64).

**Conclusion**

We established a robust protocol to generate and characterize fully active and differentially active Akt1 variants. These Akt1 variants will next be employed as a unique set of tools to investigate Akt1 substrate specificity and to screen for potential drug candidates against the most potent and active forms of the oncogenic kinase. The ability to produce optimally active Akt1 will have broad implications for drug discovery efforts, as we hypothesize that drug screening against a fully active Akt1 enzyme will more likely produce a potent and selective inhibitor.

We presented a systematic study of the impact of phosphorylation and phosphomimetic substitutions on the activity of Akt1 in the test tube and in living cells. Our data suggest that phosphomimetic substitutions should be tested for their ability to mimic phosphorylation, and Ala substitutions should also be assessed for their ability to act as non-phosphorylatable counterparts. We found these common assumptions simply do not hold for Akt1 and complementary experiments in vitro and in the cell were invaluable in reaching this conclusion. Viewing the cell-based assays in isolation of the kinase activity data may have led to the erroneous conclusion that phosphorylated Thr308 is necessary, sufficient, and irreplaceable for activation of Akt in vitro and in cells. We showed that no other residue is tolerated at that site, including acidic amino acids, which are frequently used as ‘phosphomimetics’. Our findings also question the generally assumed validity of using Ala mutation to mimic a non-phosphorylated form of residues like Ser and Thr. In total, our results beg caution in using amino acid substitutions to examine the role of phosphorylation in protein function and in the biology of cell signaling.

**Experimental procedures**

**Bacterial strains and plasmids.** The full length human AKT1 gene was cloned into a pUC18-derived vector (pDS1(38)) and pCDFDuet1 vector (see SI methods). Ser473 and Thr308 sites were mutated by site-directed mutagenesis in *E. coli* DH5α to amber (TAG), Asp (GAC), or Glu (GAG) codons according to previously described methods (65). All clones were verified by DNA sequencing.
Genomics Research Center, Robarts Research Institute, London; Genewiz Inc. NJ, USA). Phosphoproteins were produced by genetically encoding pSer at UAG codons with the 2nd generation pSer incorporation system (pDS-pSer2, kanamycin resistant) (38,66). Recombinant Akt1 and pAkt1 variants were over expressed in E. coli BL21(DE3) (Invitrogen, California, USA); exogenous pSer (5 mM) was added to the expression media for production of pAkt1 variants (see SI methods for a detailed protocol). For the imaging studies, the mouse AKT1 gene (a gift from A. Toker; 98.3% amino acid identity to human Akt1) was subcloned into pcDNA3 (Invitrogen) containing mCherry at its amino-terminus. Mutations were generated by QuikChange mutagenesis (Agilent Genomics) and confirmed by Sanger sequencing (Eton Bioscience). Generation of BKAR was previously described (36).

**Protein production and purification.** For phosphoprotein production, pDS1-Akt1 expression plasmid variants with TAG codons at the indicated phospho-site were co-transformed with pDS-pSer2 into E. coli BL21(DE3). For unphosphorylated and phosphomimetic Akt1 variants, the appropriate pDS1-Akt1 plasmid was transformed alone into BL21 (DE3). Cells were grown and pelleted at 5000 × g as described in SI methods. The cell pellets were re-suspended in lysis buffer (20 mM Hepes, 150 mM NaCl, 3 mM β-mercaptoethanol, 3 mM Dithiothreitol (DTT), 10 mM Imidazole) at 10 ml per gram of cells. Lysis buffer for phosphoproteins contained phosphatase inhibitors (1 mM Na3VO4 and 5 mM NaF). One tablet of ethylenediaminetetraacetic acid (EDTA)-free mini protease inhibitor cocktail (Roche, Mississauga, ON, Canada) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to the cell suspension (typically 200 ml). Cells were treated with lysozyme (1 mg/ml) for 20 min, shaking at 4°C and lysed using French Pressure Cell Press (American Instrument Co. Inc. Maryland, USA) at 1000 psi. Cell lysates were centrifuged at 38,000 × g for 1 h at 4°C. The supernatant was filtered through a 1.2 µm filter, mixed with Ni-NTA affinity beads (Thermo Scientific) and pre-equilibrated with lysis buffer for 1 h. Finally, proteins bound to beads were purified under gravity flow. Elution fractions were further purified using Superdex200 gel filtration column attached to an AKTA Pure L1 fast protein liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, UK) (see SI Methods). Protein yields ranged from 20-100 µg/l E. coli culture.

**In vitro Akt1 kinase assay.** For the kinase activity assay, we established a set of common conditions to assay a series of Akt1 variants of widely varying activity. To achieve this, we worked in a regime of sub-saturating ATP, thus, our apparent reaction constants (kapp) serve well to compare the Akt1 variants to one another, but these rates are significantly lower than kcat. Our data represent single turnover kinetics. Akt1 activity was determined using 200 µM substrate peptide CKRPRAASFAE (SignalChem, Vancouver, BC, Canada) derived from the natural Akt1 substrate, glycogen synthase kinase (GSK-3β). The reported KM of Akt1 for a similar substrate peptide is 18 µM (67), so the peptide concentration is in excess. Assays were performed in 3-(N-morpholino)propanesulfonic acid (MOPS, 25 mM, pH 7.0), β-glycerolphosphate (12.5 mM), MgCl2 (25 mM), ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA, 5 mM, pH 8.0), EDTA (2 mM), ATP (20 µM) and 0.4 µCi (33 nM) γ-[32P]-ATP in a 30
µl reaction volume, at 37°C in 30 min time courses. Unless otherwise indicated, reactions were initiated by the addition of 18 pmol of the indicated Akt1 variant (to yield a concentration of 600 nM) and quenched by spotting 5 µl from each reaction on P81 paper at specified time points (68). Following washes with 1% phosphoric acid (3 × 10 min) and 95% ethanol (1 × 5 min), P81 paper was air-dried and exposed to a phosphor screen. The 32P-peptide products were imaged and quantitated using a Storm 860 Molecular Imager and ImageQuant TL software (Molecular Dynamics, Caesarea, Israel).

**Cell culture media and conditions.** COS-7 cells were maintained in DMEM (Cellgro) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO2. For cell imaging experiments, cells were plated onto sterilized glass coverslips in 35-mm dishes prior to transfection with 1 µg pcDNA3-BKAR with or without 1 µg pcDNA3-mCherry-Akt using Lipofectamine 3000 (Invitrogen). For experiments in which low expression levels of Cherry-Akt were desired, cells were transfected with 1 µg pcDNA3-BKAR and 0.05 µg pcDNA3-mCherry-Akt. Cells were imaged within 24 hours following transfection. For Western blot experiments, in which average overexpressed Akt levels were desired to be at a similar level to endogenous Akt, COS-7 cells were transfected with 0.05 µg pcDNA3-mCherry-Akt using Lipofectamine 3000 (Invitrogen) in 6-well dishes.

**Cell imaging.** Transfected COS-7 cells were serum-starved at least 4 hours (as long as overnight) prior to imaging. Cells were washed one time in Hank’s Balanced Salt Solution (HBSS, Cellgro) supplemented with 1 mM CaCl2 and imaged in this HBSS in the dark at room temperature. Data were collected on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software (Universal Imaging, Corp.). Optical filters were obtained from Chroma Technologies and Semrock. Data were collected through a 10% neutral density filter. Cyan fluorescent protein (CFP) and FRET images were obtained every 15 seconds through a 420/20 nm excitation filter, a 450 nm dichroic mirror and a 475/40 nm or 535/25 nm emission filter for CFP and FRET, respectively. Cherry images were acquired through a 560/25 nm excitation filter, a 593 nm dichroic mirror and a 629/53 nm emission filter. Excitation and emission filters were switched in filter wheels (Lambda 10-2, Sutter). Integration times were 200 ms for CFP and FRET and 150 ms for mCherry. Cells were stimulated with 50 ng/ml EGF (PeproTech, Inc.) and then treated with 20 µM GDC 0068 (Selleckchem.com) at the indicated times. Data were normalized to the first 3 minutes of the experiment for each cell. Error bars represent 1 standard error of the mean.

**Western Blotting.** Within 24 hours after COS-7 cell transfection, cells were serum-starved for at least 4 hours. Cells were rinsed once in HBSS/1 mM Ca2+ and then left untreated or stimulated with 50 ng/ml EGF for 10 minutes at room temperature. Cells were lysed in 50 mM Na2HPO4, 1 mM Na2P2O7, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 (supplemented with 1 mM DTT, 200 µM benzamidine, 40 µg/ml leupeptin, 300 µM phenylmethylsulfonyl fluoride, and 1 µM microcystin) and cleared by a high-speed centrifugation for 2.5 min. The cleared lysates were analyzed by Western blotting to determine the relative amounts of Akt-Thr308 (Phospho-Akt (Thr308), Cell Signaling #9278) or Akt-Ser473 (Phospho-Akt (Ser473)(D9E) XP,
Cell Signaling #4060) phosphorylation. Total Akt levels were assessed with an antibody against all Akt isozymes (Anti-AKT1/2/3, Abcam #126811). Western blots were developed using chemiluminescence.

Acknowledgements
We are grateful to Ilka Heinemann and David Litchfield for critical discussions and suggestions on the manuscript and to Alexandr Kornev and Susan Taylor for helpful discussions. This work was supported from the Natural Sciences and Engineering Research Council of Canada [RGPIN 04282-2014 to P.O.]; Canada Foundation for Innovation [229917 to P.O.]; the Ontario Research Fund [229917 to P.O.]; Canada Research Chairs [950-229917 to P.O.]; the Canadian Cancer Society Research Institute innovation grant [704324 to P.O. and S.L.]; the Canadian Breast Cancer Foundation [to S.L.]; and National Institutes of Health [R03 CA178524 to M.T.K. and R35 GM122523 and R01 GM 43154 to A.C.N.]. N.B. holds an Ontario Graduate Scholarship. K.K.B. held a Banting Postdoctoral Fellowship.

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
References

1. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274

2. Hanada, M., Feng, J., and Hemmings, B. A. (2004) Structure, regulation and function of PKB/AKT—a major therapeutic target. Biochim Biophys Acta 1697, 3-16

3. Altomare, D. A., and Testa, J. R. (2005) Perturbations of the AKT signaling pathway in human cancer. Oncogene 24, 7455-7464

4. Dai, D. L., Martinka, M., and Li, G. (2005) Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. J Clin Oncol 23, 1473-1482

5. Wong, K. K., Engelman, J. A., and Cantley, L. C. (2010) Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev 20, 87-90

6. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balph. Curr. Biol. 7, 261-269

7. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. Curr Biol 5, 1394-1403

8. Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., and Hirsch, E. (2014) PI3K/AKT signaling pathway and cancer: an updated review. Ann Med 46, 372-383

9. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balph. Curr. Biol. 7, 261-269

10. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. Curr Biol 5, 1394-1403

11. Tsutakawa, S. E., Medzhiradszky, K. F., Flint, A. J., Burlingame, A. L., and Koshland, D. E., Jr. (1995) Determination of in vivo phosphorylation sites in protein kinase C. J Biol Chem 270, 26807-26812

12. Pearson, R. B., Dennis, P. B., Han, J.-W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H., and Thomas, G. (1995) The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J. 14, 5279-5287

13. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307, 1098-1101

14. Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J., and Su, B. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127, 125-137
15. Iacovides, D. C., Johnson, A. B., Wang, N., Boddapati, S., Korkola, J., and Gray, J. W. (2013) Identification and quantification of AKT isoforms and phosphoforms in breast cancer using a novel nanofluidic immunoassay. *Mol Cell Proteomics* **12**, 3210-3220

16. LoRusso, P. M. (2016) Inhibition of the PI3K/AKT/mTOR Pathway in Solid Tumors. *J Clin Oncol*

17. Massihnia, D., Avan, A., Funel, N., Maftouh, M., van Krieken, A., Granchi, C., Raktoe, R., Boggi, U., Aicher, B., Minutolo, F., Russo, A., Leon, L. G., Peters, G. J., and Giovannetti, E. (2017) Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of Akt inhibitors with gemcitabine in pancreatic cancer. *J Hematol Oncol* **10**, 9

18. Liao, Y., Yuan, S., Chen, X., Zhu, P., Li, J., Qin, L., and Liao, W. (2017) Upregulation of BRCA1-associated RING Domain 1 Promotes Hepatocellular Carcinoma Progression by Targeting Akt Signaling. *Sci Rep* **7**, 7649

19. Xiao, J., Yu, W., Hu, K., Li, M., Chen, J., and Li, Z. (2017) miR-92a promotes tumor growth of osteosarcoma by targeting PTEN/AKT signaling pathway. *Oncol Rep* **37**, 2513-2521

20. Parker, L., Levinger, I., Mousa, A., Howlett, K., and de Courten, B. (2016) Plasma 25-Hydroxyvitamin D Is Related to Protein Signaling Involved in Glucose Homeostasis in a Tissue-Specific Manner. *Nutrients* **8**

21. Tang, H., Wu, Y., Liu, M., Qin, Y., Wang, H., Wang, L., Li, S., Zhu, H., He, Z., Luo, J., Wang, H., Wang, Q., and Luo, S. (2016) SEMA3B improves the survival of patients with esophageal squamous cell carcinoma by upregulating p53 and p21. *Oncol Rep* **36**, 900-908

22. Yang, Y., Huang, Y., Wang, Z., Wang, H. T., Duan, B., Ye, D., Wang, C., Jing, R., Leng, Y., Xi, J., Chen, W., Wang, G., Jia, W., Zhu, S., and Kang, J. (2016) HDAC10 promotes lung cancer proliferation via AKT phosphorylation. *Oncotarget* **7**, 59388-59401

23. Klein, S., Geiger, T., Linchevski, I., Lebendiker, M., Itkin, A., Assayag, K., and Levitzki, A. (2005) Expression and purification of active PKB kinase from Escherichia coli. *Protein Expr Purif* **41**, 162-169

24. Zhang, X., Zhang, S., Yamane, H., Wahl, R., Ali, A., Lofgren, J. A., and Kendall, R. L. (2006) Kinetic mechanism of AKT/PKB enzyme family. *J Biol Chem* **281**, 13949-13956

25. Fabbro, D., Batt, D., Rose, P., Schacher, B., Roberts, T. M., and Ferrari, S. (1999) Homogeneous purification of human recombinant GST-Akt/PKB from Sf9 cells. *Protein Expr Purif* **17**, 83-88

26. Hart, J. R., and Vogt, P. K. (2011) Phosphorylation of AKT: a mutational analysis. *Oncotarget* **2**, 467-476

27. Tobisawa, T., Yano, T., Tanno, M., Miki, T., Kuno, A., Kimura, Y., Ishikawa, S., Kouzu, H., Nishizawa, K., Yoshida, H., and Miura, T. (2017) Insufficient activation of Akt upon reperfusion
because of its novel modification by reduced PP2A-B55alpha contributes to enlargement of infarct size by chronic kidney disease. Basic Res Cardiol 112, 31

28. Warsi, J., Fezai, M., Fores, M., Elvira, B., and Lang, F. (2015) Up-Regulation of Voltage Gated K+ Channels Kv1.3 and Kv1.5 by Protein Kinase PKB/Akt. Cell Physiol Biochem 37, 2454-2463

29. Liu, P., Begley, M., Michowski, W., Inuzuka, H., Ginzberg, M., Gao, D., Tsou, P., Gan, W., Papa, A., Kim, B. M., Wan, L., Singh, A., Zhai, B., Yuan, M., Wang, Z., Gygi, S. P., Lee, T. H., Lu, K. P., Toker, A., Pandolfi, P. P., Asara, J. M., Kirschner, M. W., Sicinski, P., Cantley, L., and Wei, W. (2014) Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. Nature 508, 541-545

30. Zhuo, D. X., Zhang, X. W., Jin, B., Zhang, Z., Xie, B. S., Wu, C. L., Gong, K., and Mao, Z. B. (2013) CSTP1, a novel protein phosphatase, blocks cell cycle, promotes cell apoptosis, and suppresses tumor growth of bladder cancer by directly dephosphorylating Akt at Ser473 site. PLoS One 8, e65679

31. Munoz, C., Almilaji, A., Setiawan, I., Foller, M., and Lang, F. (2013) Up-regulation of the inwardly rectifying K(+) channel Kir2.1 (KCNJ2) by protein kinase B (PKB/Akt) and PIKfyve. J Membr Biol 246, 189-197

32. Berndt, N., Yang, H., Trinczek, B., Betzi, S., Zhang, Z., Wu, B., Lawrence, N. J., Pellecchia, M., Schonbrunn, E., Cheng, J. Q., and Sehti, S. M. (2010) The Akt activation inhibitor TCN-P inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane. Cell Death Differ 17, 1795-1804

33. Dissmeyer, N., and Schnittger, A. (2011) Use of phospho-site substitutions to analyze the biological relevance of phosphorylation events in regulatory networks. Methods Mol Biol 779, 93-138

34. Pearlman, S. M., Serber, Z., and Ferrell, J. E., Jr. (2011) A mechanism for the evolution of phosphorylation sites. Cell 147, 934-946

35. Hunter, T. (2012) Why nature chose phosphate to modify proteins. Philos Trans R Soc Lond B Biol Sci 367, 2513-2516

36. Kunkel, M. T., Ni, Q., Tsien, R. Y., Zhang, J., and Newton, A. C. (2005) Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. J Biol Chem 280, 5581-5587

37. Park, H. S., Hohn, M. J., Umehara, T., Guo, L. T., Osborne, E. M., Benner, J., Noren, C. J., Rinehart, J., and Söll, D. (2011) Expanding the genetic code of Escherichia coli with phosphoserine. Science 333, 1151-1154

38. George, S., Aguirre, J. D., Spratt, D. E., Bi, Y., Jeffery, M., Shaw, G. S., and O’Donoghue, P. (2016) Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. FEBS Lett 590, 1530-1542

39. Adams, J. A., and Taylor, S. S. (1992) Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent
protein kinase as measured by viscosity experiments. *Biochemistry* **31**, 8516-8522

40. Toker, A., and Newton, A. C. (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* **275**, 8271-8274

41. Kunkel, M. T., and Newton, A. C. (2009) Spatiotemporal Dynamics of Kinase Signaling Visualized by Targeted Reporters. *Curr. Protoc. Chem Biol.* **1**, 17-28

42. Ebner, M., Lucic, I., Leonard, T. A., and Yudushkin, I. (2017) P(3,4,5)P3 Engagement Restricts Akt Activity to Cellular Membranes. *Mol Cell* **65**, 416-431

43. Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968) An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *J Biol Chem* **243**, 3763-3765

44. Antal, C. E., and Newton, A. C. (2014) Tuning the signalling output of protein kinase C. *Biochemical Society transactions* **42**, 1477-1483

45. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-31524

46. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* **271**, 21920-21926

47. Andjelkovic, M., Maira, S.-M., Cron, P., Parker, P. J., and Hemmings, B. A. (1999) Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. *Mol. Cell Biol.* **19**, 5061-5072

48. Scheid, M. P., Marignani, P. A., and Woodgett, J. R. (2002) Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol Cell Biol* **22**, 6247-6260

49. Hauge, C., Antal, T. L., Hirschberg, D., Doehn, U., Thorup, K., Idrissova, L., Hansen, K., Jensen, O. N., Jorgensen, T. J., Biondi, R. M., and Frodin, M. (2007) Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation. *Embo J* **26**, 2251-2261

50. Brodbeck, D., Cron, P., and Hemmings, B. A. (1999) A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. *J Biol Chem* **274**, 9133-9136

51. Gao, T., Furnari, F., and Newton, A. C. (2005) PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell** **18**, 13-24

52. Newton, A. C., and Trotman, L. C. (2014) Turning off AKT: PHLPP as a drug target. *Annual review of pharmacology and toxicology* **54**, 537-558

53. Slipicevic, A., Holm, R., Nguyen, M. T., Bohler, P. J., Davidson, B., and Florenes, V. A. (2005) Expression of activated Akt
and PTEN in malignant melanomas: relationship with clinical outcome. *Am J Clin Pathol* **124**, 528-536

54. Tsao, A. S., McDonnell, T., Lam, S., Putnam, J. B., Bekele, N., Hong, W. K., and Kurie, J. M. (2003) Increased phospho-AKT (Ser(473)) expression in bronchial dysplasia: implications for lung cancer prevention studies. *Cancer Epidemiol Biomarkers Prev* **12**, 660-664

55. Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R., and Tavare, J. M. (2011) Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *Br J Cancer* **104**, 1755-1761

56. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038

57. Thorsness, P. E., and Koshland, D. E. (1987) Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J. Biol. Chem.* **262**, 10422-10425

58. Hu, J., Ahuja, L. G., Meharena, H. S., Kannan, N., Kornev, A. P., Taylor, S. S., and Shaw, A. S. (2015) Kinase regulation by hydrophobic spine assembly in cancer. *Mol Cell Biol* **35**, 264-276

59. Lippa, B., Pan, G., Corbett, M., Li, C., Kauffman, G. S., Pandit, J., Robinson, S., Wei, L., Kozina, E., Marr, E. S., Borzillo, G., Knauth, E., Barbacci-Tobin, E. G., Vincent, P., Troutman, M., Baker, D., Rajamohan, F., Kakar, S., Clark, T., and Morris, J. (2008) Synthesis and structure based optimization of novel Akt inhibitors. *Bioorg Med Chem Lett* **18**, 3359-3363

60. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* **9**, 940-944

61. Steichen, J. M., Kuchinskas, M., Keshwani, M. M., Yang, J., Adams, J. A., and Taylor, S. S. (2012) Structural basis for the regulation of protein kinase A by activation loop phosphorylation. *J Biol Chem* **287**, 14672-14680

62. Adams, J. A., McGlone, M. L., Gibson, R., and Taylor, S. S. (1995) Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. *Biochemistry* **34**, 2447-2454

63. Bornancin, F., and Parker, P. J. (1997) Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state [published erratum appears in *J Biol Chem* 1997 May 16;272(20):13458]. *J Biol Chem* **272**, 3544-3549

64. Antal, C. E., and Newton, A. C. (2013) Spatiotemporal dynamics of phosphorylation in lipid second
messenger signaling. *Mol Cell Proteomics* **12**, 3498-3508

65. Edelheit, O., Hanukoglu, A., and Hanukoglu, I. (2009) Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnol* **9**, 61

66. Lee, S., Oh, S., Yang, A., Kim, J., Soll, D., Lee, D., and Park, H. S. (2013) A facile strategy for selective incorporation of phosphoserine into histones. *Angew Chem Int Ed Engl* **52**, 5771-5775

67. Reuveni, H., Livnah, N., Geiger, T., Klein, S., Ohne, O., Cohen, I., Benhar, M., Gellerman, G., and Levitzki, A. (2002) Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry* **41**, 10304-10314

68. Turowec, J. P., Duncan, J. S., French, A. C., Gyenis, L., St Denis, N. A., Vilk, G., and Litchfield, D. W. (2010) Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods Enzymol* **484**, 471-493
Table 1. Specific activity of Akt1 variants.

| Akt1 variant                        | Akt1 amount (pmol) | Initial velocity ($v_0$) (fmol/min) | Apparent catalytic rate, $k_{app}$ (fmol/min/pmol Akt1) | Activation (fold increase) |
|------------------------------------|--------------------|-------------------------------------|-----------------------------------------------------|----------------------------|
| Akt1 (unphosphorylated)            | 18                 | 0.6 ± 0.2                           | 0.03 ± 0.01                                         | 1                          |
| pAkt1                              |                    |                                     |                                                     |                            |
| pS473                              | 18                 | 46 ± 5                              | 2.5 ± 0.3                                           | 83                         |
| pS308                              | 6                  | 2.9 ± 0.2                           | 0.48 ± 0.03                                         | 16                         |
| pT308*                             | 1.8                | 22 ± 4                              | 12 ± 2                                              | 400                        |
| ppAkt1 (pT308, pS473)*             | 1.8                | 79 ± 11                             | 44 ± 6                                              | 1467                       |
| Phosphomimetics                    |                    |                                     |                                                     |                            |
| E473                               | 18                 | 1.5 ± 0.1                           | 0.083 ± 0.006                                       | 2.7                        |
| E308                               | 18                 | 1.0 ± 0.1                           | 0.056 ± 0.006                                       | 1.9                        |
| D473                               | 18                 | 0.40 ± 0.03                         | 0.022 ± 0.002                                       | 0.7                        |
| D308                               | 18                 | 2.6 ± 0.1                           | 0.14 ± 0.01                                         | 5                          |
| pAkt1S473 mutants                  |                    |                                     |                                                     |                            |
| D308                               | 18                 | 83 ± 1                              | 4.6 ± 0.1                                           | 153                        |
| A308                               | 18                 | 5.4 ± 0.3                           | 0.30 ± 0.02                                         | 10                         |
| pAkt1T308 mutants                  |                    |                                     |                                                     |                            |
| D473                               | 18                 | 188 ± 11                            | 10.4 ± 0.6                                          | 348                        |
| E473                               | 18                 | 120 ± 16                            | 6.6 ± 0.8                                           | 222                        |
| A473                               | 18                 | 74 ± 3                              | 4.1 ± 0.2                                           | 137                        |

Initial velocities ($v_0$) and apparent catalytic rates ($v_0$/[Akt1]) of each enzyme variant were calculated using linear regression analysis of the activity plots.

*In order to measure the initial velocity accurately, the most active variants were assayed at a 10-fold reduced enzyme concentration (Figure S5).
Table 2. Akt enzyme activity comparison in vitro and in COS7 cells.

| Akt1 sites | Akt1 activity |
|------------|---------------|
|            | in vitro (%)  | in cell |
| 308 pThr   | 100           | +       |
| pSer       |               |         |
| Thr pThr   | <1            | N.D.    |
| Ser        |               |         |
| pThr       | 27            | N.D.    |
| Ser        |               |         |
| Thr pThr   | 24            | +       |
| Asp        |               |         |
| Thr pThr   | 15            | +       |
| Glu        |               |         |
| pThr       | 9             | +       |
| Ala        |               |         |
| Thr pThr   | 5             | N.D.    |
| pSer       |               |         |
| Asp pSer   | 10            | 0       |
| Glu pSer   | N.D.          | 0       |
| Ala pSer   | <0.001        | 0       |
| pSer pSer  | N.D.          | +*      |
| pSer       | 1             | N.D.    |
| Ser        |               |         |
| pSer       | N.D.          | +*      |
| Ala        |               |         |
| Thr Asp    | <1            | N.D.    |
| pSer       |               |         |
| Thr Glu    | <1            | N.D.    |
| Asp Ser    | <1            | N.D.    |
| Glu Ser    | <1            | N.D.    |

Enzyme activity determined biochemically is given as a percentage of the maximally active ppAkt1. Agonist-evoked enzyme activity in cells is denoted by a plus (+) sign. No detectible activity is denoted by zero (0). N.D. Not determined.

*Observed a reduced response compared to WT when the Akt1 mutant indicated was expressed at a reduced level, similar to the level of endogenous Akt (Figure 3B).
Figure 1. A novel route to doubly phosphorylated and active Akt1. (A) Schematic representation of recombinant Akt1 biosynthesis with pSer473 genetically encoded in response to the UAG codon and pThr308 enzymatically phosphorylated in vivo in E. coli. Genetically encoded pSer incorporation requires phosphoseryl-tRNA synthetase (SepRS), a UAG-decoding tRNA\textsuperscript{Sep}, and the elongation factor mutant (EFSep). (B) Enzyme activity of differentially phosphorylated Akt1 variants with a GSK-3β substrate peptide. Akt1 quantitatively phosphorylated at both 308 and 473 (ppAkt\textsuperscript{S473, T308}, blue diamonds) showed maximal activity compared to the un-phosphorylated Akt1 (gray circles), and singly phosphorylated Akt1 variants: pAkt\textsuperscript{T308} (black cross) and pAkt\textsuperscript{S473} (brown diamonds). The reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation. Lower activity variants show above background kinase activity (inset).
Figure 2. Activity of singly phosphorylated Akt1 variants with regulatory site mutations. (A) Akt1 enzyme activity is shown for variants with S473 phosphorylated and T308 un-phosphorylated (brown diamond), or mutated: T308D (purple triangles), T308A (orange squares). Controls include unphosphorylated Akt1 (gray circles), and pAkt1\textsuperscript{S473} in the absence of substrate peptide (-peptide, green crosses). (B) In comparison to pAkt\textsuperscript{T308} activity (black crosses) and in the context of Akt1 phosphorylated at T308, mutations S473D (blue triangles), S473E (magenta crosses) or S473A (cyan squares) resulted in marginally reduced activity. All reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation.
Figure 3. Activity of T308S and phosphomimetic Akt1 variants. (A) In comparison to pAkt1\textsuperscript{T308} activity (black crosses), mutations of T308 to either D (pink squares), E (green triangles), or pS (red diamonds) resulted in low but above background kinase activity (inset). (B) Kinase activity was also measured in the context of an unphosphorylated Akt1 (gray circles) with phosphomimetic mutations S473D (green squares) and S473E (peach squares). All reported values represent the mean of triplicate experiments with error bars indicating 1 standard deviation.
Figure 4. Cellular activity of Akt1 variants. (A) Serum-starved COS-7 cells expressing BKAR alone (gray) or BKAR with Cherry-tagged Akt WT (blue), T308A (orange), T308D (purple), S473A (green), S473D (red), or T308D, S473D (DD, yellow) were imaged, stimulated with EGF, and then treated with the Akt inhibitor GDC 0068. Multiple cells were included from at least two independent experiments for analysis. FRET ratios from each cell were normalized and their average plotted over time. Error bars represent SEM. (B) Following a 10-minute treatment with EGF, lysates from serum-starved COS-7 cells expressing the indicated Cherry-tagged Akt were analyzed by Western blotting for Akt activation using phospho-specific antibodies toward the activation loop (α-p308) and hydrophobic motif (α-p473). The asterisk (*) marks migration of Cherry-Akt and the hyphen (-) marks endogenous Akt.
Figure 5. Reduced, but active, signaling from Akt T308S. (A) Serum-starved COS-7 cells expressing BKAR with or without minimally detectable levels of Cherry-tagged Akt were imaged during stimulation with EGF followed by treatment with the Akt inhibitor GDC 0068. Normalized average FRET ratios for WT (blue), T308S (red) and T308S, S473A (green) in comparison to BKAR alone (gray) are shown. Data were analyzed from cells expressing equal low levels of Cherry-Akt. Multiple cells were included from at least two independent experiments. FRET ratios from each cell were normalized and their average plotted over time. Error bars represent SEM. (B) Following a 10-minute treatment with EGF, lysates from serum-starved COS-7 cells expressing the indicated Cherry-tagged Akt were analyzed by Western blotting for Akt activation using phospho-specific antibodies toward the activation loop (α-p308) and hydrophobic motif (α-p473). The asterisk (*) marks migration of Cherry-Akt and the hyphen (-) marks endogenous Akt.
Figure 6. Structure of human Akt in complex with GSK-3β substrate peptide. (A) Structure of the active human pAkt\textsuperscript{T308} (S473D) kinase domain (gray, cartoon) show in complex with GSK-3β peptide (purple) and a non-hydrolyzable ATP analog (ATP' = ANP-PNP). The target of Akt1 phosphorylation on the GSK-3β substrate peptide is indicated (*). Key resides are labeled and in addition to the regulatory phosphorylation sites (pThr308, and phosphomimetic mutation S473D), other Ser/Thr phosphorylation sites are highlighted (yellow). (B) A 90° rotated and close up view of the Akt1 active site, focused on the position of pThr308. The phosphate at position 308 makes extensive salt bridge interactions with Arg273 and Lys297; pThr308 also participates in a water mediated hydrogen bond network with His194. The C\textgamma methyl group on pThr308 forms a hydrophobic interaction with Cys310 as indicated in the van der Waals surface representation (transparent). The figure includes structural data from PDB codes 3CQU (59) and 1O6K (60).
Genetic code expansion and live cell imaging reveal that Thr308 phosphorylation is irreplaceable and sufficient for Akt1 activity
Nileeka Balasuriya, Maya T. Kunkel, Xuguang Liu, Kyle K. Biggar, Shawn S-C. Li, Alexandra C. Newton and Patrick O'Donoghue

J. Biol. Chem. published online May 17, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.002357

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts