Title: The activity of the anti-apoptotic fragment generated by the caspase-3/p120 RasGAP stress-sensing module displays strict Akt isoform specificity.

Authors: Vanli G, Peltzer N, Dubuis G, Widmann C

Journal: Cellular signalling

Year: 2014 Dec

Volume: 26

Issue: 12

Pages: 2992-7

DOI: 10.1016/j.cellsig.2014.09.009

The activity of the anti-apoptotic fragment generated by the caspase-3/p120 RasGAP stress-sensing module displays strict Akt isoform specificity

Güliz Vanli*, Nieves Peltzer*[#], Gilles Dubuis, Christian Widmann

Department of Physiology, University of Lausanne, Switzerland

* Equal contribution

Correspondence to Christian Widmann, Department of Physiology, Bugnon 7, 1005 Lausanne, Switzerland, Phone: +41 21 692 5123, Fax: +41 21 692 5505, E-mail: Christian.Widmann@unil.ch.

[#] Current address: Centre for Cell Death, Cancer and Inflammation, UCL Cancer Institute, London, UK

Keywords: RasGAP; fragment N; Akt phosphorylation; Isoform specificity

Abbreviations. PH, pleckstrin homology; PI3K, phosphatidylinositide 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; mTORC2, mammalian target of rapamycin 2 complex; PDK1, phosphoinositide-dependent kinase 1; FasL, Fas ligand; GFP, green fluorescent protein; KO, knock-out; PFA, paraformaldehyde; BSA, bovine serum albumin; TCL-1, T-cell lymphoma-1; MTCP-1, mature T-cell proliferation; JIP1, c -Jun N-terminal kinase-interacting protein; DTC, disseminated tumor cells.

Abstract

The caspase3/p120RasGAP module acts as a stress sensor that promotes pro-survival or pro-death signaling depending on the intensity and the duration of the stressful stimuli. Partial cleavage of p120 RasGAP generates a fragment, called fragment N, which protects stressed cells by activating Akt signaling. Akt family members regulate many cellular processes including proliferation, inhibition of apoptosis and metabolism. These cellular processes are regulated by three distinct Akt isoforms: Akt1, Akt2 and Akt3. However, which of these isoform(s) is/are required for fragment N mediated protection has not been defined. In this study, we investigated the individual contribution of each isoform in fragment N-mediated cell protection against Fas ligand induced cell death. To this end, DLD1 and HCT116 isogenic cell lines lacking specific Akt isoforms were used. It was found that fragment N could activate Akt1 and Akt2 but that only the former could mediate the protective activity of the RasGAP-derived fragment. Even over-expression of Akt2 or Akt3 could not rescue the inability of fragment N to protect cells lacking Akt1. These results demonstrate a strict Akt isoform requirement for the anti-apoptotic activity of fragment N.

1. Introduction

Maintaining cellular homeostasis requires that many biological parameters, such as cell volume, electrolyte concentration, osmolarity, acidity, membrane potential, concentrations of intracellular ions, nutrients, oxygen and reactive oxygen species, are kept within ranges that allow the maintenance of cellular activities required to sustain the functions and the life of cells [1]. Too great of a perturbation of these parameters can trigger the activation of stress sensors that will mount appropriate cellular responses to maintain homeostasis or to induce cell death if cellular stress or damage cannot be resolved [2, 3].

The caspase3/p120RasGAP module is one of such stress sensors. It can switch signaling from pro-survival to pro-death depending on the intensity and duration of the stressful stimuli [2]. Under mild stress conditions, the proteolytic activity of caspase-3 slightly increases [4, 5]. In these conditions p120RasGAP is cleaved at position 455 into an amino-terminal fragment, called fragment N, which protects cells by activating the anti-apoptotic Akt kinase [4, 6, 7]. If the extent of the stress increases above a certain threshold however, fragment N is further cleaved at position 157 and it loses its capacity to stimulate Akt [4, 6]. Knock-in mice bearing an uncleavable version of RasGAP, and hence that are unable to produce fragment N, are impaired in their ability to stimulate Akt and are more sensitive to pathophysiological insults [8].

Akt exists in highly homologous isoforms in mammals that are encoded by different genes: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) [9-11]. These three isoforms show about 80% amino acid identity and they share a common N-terminal pleckstrin homology (PH) domain, a catalytic domain, and a C-terminal

regulatory domain [12]. Targeted deletion of Akt1, Akt2 and Akt3 or combined deletion of the isoforms by homologous recombination revealed that Akt isoforms have redundant as well as non-redundant functions. $Akt1^{-/-}$ mice have reduced body weight and display increased risk of neonatal mortality [13, 14]. $Akt2^{-/-}$ mice develop glucose metabolism dysfunctions, including increased fasting plasma glucose levels and peripheral insulin resistance, but this generally does not evolve to full-blown diabetes [15, 16]. $Akt3^{-/-}$ mice display reduced brain size [17]. The data obtained with these single knock-out mice highlight some of the non-redundant functions of Akt isoforms. The redundant functions of the Akt kinases can be revealed in mice lacking more than one Akt isoform. For example, mice lacking both Akt1 and Akt2 display drastic growth deficiencies and they die shortly after birth [18]. Moreover, the $Akt1^{-/-}/Akt3^{-/-}$ genotype is embryonically lethal [19]. This indicates that Akt1 and Akt2 (or Akt3) share some redundant functions in allowing successful embryonic development and survival. Another example of redundancy comes from the observation that disruption of one Akt1 allele in the $Akt2^{-/-}$ background worsened glucose metabolism dysfunction, culminating in the development of severe diabetes [20]. However, Akt1 involvement in glucose metabolism is only revealed in the absence of Akt2, indicating that Akt2 is the main isoform controlling glucose handling in mice and that Akt1 only has accessory or modulatory functions in this response.

The mechanism by which Akt is activated has been extensively investigated, in particular following growth factor receptor activation or stimulation of the insulin receptor [21, 22]. Stimulation of Akt activity requires translocation to the plasma membrane and subsequent phosphorylation on key residues. Membrane translocation is achieved when phosphatidylinositide 3-kinase (PI3K) is activated

following receptor tyrosine kinase activation. This activation increases locally the production of phosphatidylinositol 3,4,5-triphosphate (PIP3). This lipid is a ligand for the PH domain of Akt allowing its recruitment to membranes. There, Akt is phosphorylated on at least two residues. The first one is found within the activation loop in the catalytic domain (Thr308 in Akt1, Thr309 in Akt2 and Thr305 in Akt3) and is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) [23, 24]. The second one corresponds to a serine residue (Ser473 in Akt1, Ser474 in Akt2, Ser472 in Akt3) found in the hydrophobic region of the kinase carboxy-terminal tail and it is phosphorylated by mammalian target of rapamycin 2 complex (mTORC2) [25]. It has been suggested that phosphorylation at Ser473 is preceding and facilitating the phosphorylation at the Thr308 site [25, 26] Nevertheless, phosphorylation on Ser473 is usually considered as the signature for kinase activity, since it stabilizes the active conformation state of Akt [25, 27, 28].

In certain conditions, Akt can also be phosphorylated on these residues by other kinases, such as DNA-PK and ILK [23]. Recently, additional residues in the C-terminus of Akt (Ser477 and Thr479) have been found to be phosphorylated by the cyclin-dependent kinase Cdk2. These phosphorylation events appear to facilitate, or to functionally compensate for Ser473 phosphorylation [29].

Activated Akt phosphorylates a wide range of downstream effectors that bear the consensus motif RXRXXS/T-bulky hydrophobic, where X denotes any amino acid and S/T denote serine and threonine residues [30]. The anti-apoptotic functions of Akt are mediated following the phosphorylation of a series of substrates including Bad, IKKβ, and FOXO transcription factors [31-37].

As indicated above, the anti-apoptotic fragment N produced by the caspase-3/p120RasGAP stress sensor activates Akt and generates a potent cell survival signal. But which specific Akt isoform is involved in this protective response is unknown. Here therefore, we investigated which of the Akt isoform(s) was/were involved in fragment N mediated cell survival using DLD1 and HCT116 cell lines lacking specific Akt isoforms.

2. Material and Methods

2.1 Reagents

The anti-Akt1, anti-Akt2, anti-phospho Akt1 (Ser 473),anti- phospho Akt2 (Ser 474), pan Akt and anti-actin monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA; cat. no. 2967, 2964, 9271, 8599, 4685, 4970 respectively). Secondary antibodies used for Western blotting were Alexa Fluor 680–conjugated anti-rabbit (Molecular Probes, Eugene, OR; cat. no. A21109) or IRDye 800–conjugated anti-mouse antibodies (Rockland, Gilbertsville, PA; cat. no 610-132-121). The Fas ligand (FasL) used here corresponds to a hexameric form of a fusion protein between FasL and the Fc portion of IgG1 [38] and was a generous gift from Dr. Pascal Schneider (University of Lausanne, Switzerland). Antibodies were diluted in bovine serum albumin (BSA) (Sigma-Aldrich; cat. no. A7906).

2.2 Plasmids

The pEGFP-C1 (#6) plasmid encodes a green fluorescent protein (GFP) (Clontech). The pcDNA3 (#1) plasmid is a eukaryotic cytomegalovirus promoter-driven expression vector. HA-hRasGAP[1-455](D157A).dn3 (#352) encodes a tagged and caspase-resistant version of the 1–455 amino acid fragment (fragment N) of human RasGAP (previously called N-D157A.dn3) [6]. HA-hRasGAP[1–455](D157A).lti (#353) encodes the same fragment but in a vector allowing for the production of lentiviruses (previously called N-D157A.lti) [4]. Full length human Akt1 gene was excised from Myr-HA-hAkt1.dn3 (#845) (Addgene plasmid no.

9008) using BamHI and EcoRI. This digestion released the intact Akt1-coding sequences lacking the sequences coding for the Myr and HA tags. Full length human Akt2 gene was excised from Myr-HA-hAkt2.dn3 (#846) (Addgene plasmid no. 9016) [39] using the same enzymes (this also removed the Myr and HA- tags). Then, the Akt1- and Akt2-containing excised fragments were sub-cloned into the LeGO.iG2 (Addgene plasmid no. 27341) plasmid (#809) opened with the same restriction enzymes. Akt3 gene was excised from Myr-HA-hAkt3 (#861) (addgene plasmid no. 9017) using EcoRI and BssHI to release the full-length tag-free human Akt3 cDNA. After this digestion, the hanging ends were filled using the T4 polymerase. The Akt3 cDNA fragment was then sub-cloned into the StuI-linearized LeGO.iG2 plasmid. The orientation of the Akt3 construct within this vector was checked by restriction analysis. The hAkt1.LeGO-iG2 (#843), hAkt2.LeGO-iG2 (#844), and hAkt3.LeGO.iG2 (#862) plasmids were used to produce lentiviruses expressing GFP and full length human Akt1, Akt2 and Akt3, respectively.

2.3 Cell Lines

HCT116 human colorectal cancer cell line and the DLD1 human colorectal cancer cell line harbor the H1047R mutation in exon 20 (kinase domain) and the E545K mutation in exon 9 (helical domain) of the PIK3CA gene, respectively. Both cell lines also harbor heterozygous G13D KRAS mutations [40]. These cells lines have been genetically engineered to inactivate the Akt1, Akt2, and PDPK1 genes through targeted homologous recombination. Akt3 is not expressed in these cell lines [41]. The parental DLD1 cell line and its derivatives were maintained in RPMI 1640 (Invitrogen catalogue no. 61870) containing 10% fetal bovine serum (PAA

Laboratories GmbH, catalogue no. A15-151). The parental HCT116 cell line and its derivatives were maintained in DMEM (Invitrogen, catalogue no. 61965) containing 10% fetal bovine serum. All the cell lines were grown in a 37°C and 5% CO_2 atmosphere.

2.4 Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in MonoQ-c buffer [6]. Equal amounts of proteins were migrated in a polyacrylamide gel and transferred onto a Trans-Blot nitrocellulose membrane (Bio-Rad catalogue no. 10484060). Membranes were blocked with 5% bovine serum albumin and incubated over-night at 4°C with specific primary antibodies. Blots were washed with TBS/Tween 0.1%, incubated with specific secondary antibodies and visualized with the Odyssey infrared imaging system (LICOR Biosciences, Bad Homburg, Germany). The integrated densities of the Akt1 and Akt2 bands were quantitated using the Image J software (National Institutes of Health, Bethesda, MD, USA) and were then normalized to the corresponding actin levels.

2.5 Cell transfection

HCT116 cell lines were co-transfected with a fragment N-expressing plasmid (#352) or with an empty pcDNA3 vector (#1), together with a GFP-expressing plasmid (#6). Transfection was performed using the calcium phosphate precipitation method, as previously described [42].

2.5 Lentivirus preparation

Recombinant lentiviruses were produced according to a previously described method [43]. The minimal amounts of viral supernatant that induce the expression of the protein of interest in more than 95% of the cells (as assessed by immunofluorescence) were used.

2.5 Apoptosis assay

Cells were infected with empty viruses or fragment N-expressing viruses and 48 hours later were treated with 5 ng/ml FasL for 18 additional hours. For rescue experiments, cells were first infected with empty viruses or fragment N-expressing viruses, and 24 hours later were infected again with Akt1, Akt2, or GFP-expressing viruses. After a 24 hour additional period, cells were treated with 5 ng/ml of FasL for 18 more hours. The cells were then fixed in 2% paraformaldehyde (PFA) and apoptosis was scored by counting the pycnotic nuclei (visualized with Hoechst 33342) [6]. When apoptosis was scored in transfected cells, only the GFP-positive cells were considered.

2.6. Statistics

Statistical analyses were done with Microsoft Office Excel 2003 SP1 using the two-tailed unpaired Student t test. Significance is indicated by an asterisk when $p <$

0.05/n, where p is the probability derived from the t test analysis and n is the number of comparisons done (Bonferroni correction).

3. Results

3.1. Fragment N stimulates both Akt1 and Akt2 phosphorylation

Akt isoform-specific antibodies recognizing the phosphorylated serine residues at position 473/474 were used to determine which Akt isoform are activated by fragment N. The specificity of these antibodies was verified by using DLD1 and HCT116 cells lacking Akt1, Akt2, or both. Additionally, cells lacking PDK1, the kinase phosphorylating Akt on threonine 308/309, were also used to determine the impact of this phosphorylation event on the phosphorylation of residues 473/474. Figure 1 shows that fragment N leads to increased Ser473/474 phosphorylation on Akt1 and Akt2 in the parental cell lines (compare lanes 1 and 2). The ability of fragment N to induce the phosphorylation of both Akt1 (Ser473) and Akt2 (Ser474) suggests that fragment N is acting on the phosphorylation process itself that is common to both isoforms. Yet, even though Akt1 and Akt2 are activated by similar mechanisms [44, 45], the increase in fragment N-mediated Ser473/474 phosphorylation was apparently more pronounced for Akt2 than for Akt1. Fragment N-induced phosphorylation of residues 473/474 did not appear to be affected by the absence of PDK1. In the present case therefore, there was no clear modulation of serine 473/474 phosphorylation, which is mediated by mTORC2, by the phosphorylation event on threonine 308/309, which is mediated by PDK1, as shown in other situations [46, 47]. As expected, no signal was detected using the anti-Akt1 and the anti-Akt2 phospho-specific antibody in cells lacking Akt1 and Akt2, respectively.

3.2 Akt1 is required for fragment N mediated protection

The relative contribution of each Akt isoform to fragment N-mediated protection was assessed by measuring Fas ligand (FasL)-mediated apoptosis in the cells described in Figure 1. Figure 2 shows that fragment N did not protect DLD1 and HCT116 cells lacking Akt1. In contrast, fragment N successfully reduced the apoptotic response in the parental and in the Akt2 knock-out (KO) cell lines. These observations suggest that Akt2 is dispensable for fragment N-mediated protection. The protective ability of fragment N was also abolished in cells lacking PDK1. This is expected as Akt1 needs to be phosphorylated both on Ser473 by mTORC2 and on Thr308 by PDK1 for optimal activation [22].

3.3 Reconstitution of Akt1, but not of Akt2 or Akt3, expression in Akt1 KO cells restores fragment N-induced protection

To verify that the inability of fragment N to protect Akt1 KO cells was indeed due to the absence of Akt1 and not a consequence of off-target mutations or alterations, Akt1 was reintroduced by lentiviral infection in DLD1 and HCT116 Akt1 KO cells. Figure 3 shows that reconstitution of Akt1 expression in Akt1 KO cells allowed them to be protected by fragment N against FasL-induced apoptosis.

The results described above indicate that Akt1 is the sole Akt isoform that mediates the protective response activated by fragment N. However, one could argue that Akt1 and Akt2 are equally capable of mediating this protective response but that the cells used in this study express much less Akt2 than Akt1 so that removing the latter globally reduces more total Akt levels than removing the former. The intensity of the Akt1 and Akt2 isoform bands on the Western blot revealed with a pan-specific antibody (lower blots in Figure 1A-B) are indeed

compatible with the notion that Akt1 is expressed more than Akt2 in DLD1 and HCT116 cells. Consequently, even if fragment N uses both Akt isoforms, removing Akt1 may have a stronger impact on the protective response than removing Akt2. To assess this point, Akt1 and Akt2 were expressed to similar levels in DLD1 and HCT116 Akt1 KO cells following infection with a lentivirus encoding cytomegalovirus promoter-driven Akt1 and Akt2 constructs (Figure 3A). Overexpression of Akt1 and Akt2 yielded a 2.7$\pm$0.8 and 3.1$\pm$0.7 fold increase over endogenous levels in DLD1 cell line, respectively and a 3.7$\pm$0.8 and 2.1$\pm$0.1 fold increase over endogenous levels in HCT116 cell lines, respectively. Figure 3B shows that re-expression of Akt1, but not of Akt2, in Akt1 KO cells allowed them to be protected by fragment N against FasL-induced apoptosis. Akt3 is not supposed to be expressed in DLD1 and HCT116 cell lines [41] and hence is not uniquely required for fragment N-mediated cell protection. Akt3 may however be able to substitute for Akt1 to allow this activity of fragment N. To assess this point, Akt3 was ectopically expressed in Akt1 KO DLD1 cells and the ability of fragment N to protect these cells against FasL-induced apoptosis was investigated. The ectopic expression of Akt3 in these cells induced an unexpected increase in the basal apoptosis (compare the 5^{th} and the 6^{th} bars with the 9^{th} and 10^{th} bars in Supplemental Figure 2). FasL nevertheless more or less doubled this death response but this was not antagonized by fragment N expression (compare the 11^{th} and the 12^{th} bars in Supplemental Figure 2). Hence, Akt3 cannot substitute for Akt1 in mediating the protective ability of fragment N. Overall, it can be concluded from these experiments that Akt1 is the sole Akt isoform that mediate the anti-apoptotic activity of fragment N.

4. Discussion

Akt isoform-specific signaling is achieved in several ways, including tissue specific expression, differential subcellular localization, distinct catalytic activities, different signaling targets, and differential activation of the isoforms [48]. In a study that was conducted in HEK293T cells, Lee *et al*. [49] concluded that isoform specific Akt signaling is achieved most prominently by differential expression, rather than differences in intrinsic enzyme activity. In contrast, we provide evidence here that Akt1 is the sole Akt isoform mediating fragment N-mediated protection. In this case, therefore, there is a strict intrinsic Akt isoform specificity that cannot be explained by differential expression levels. Akt1, but not Akt2, has been shown to be able to inhibit starvation-induced apoptosis in the single disseminated tumor cells (DTC)-derived LC-M1 cell line[50]. Akt1 appears therefore to carry an intrinsic protective activity that it does not share with Akt2. This may however by cell type-specific as there are examples, in tumor cell lines in particular, that removal of Akt2 affects survival [51]. In these cases, however, the pro-survival triggering signals have not been characterized and consequently, how Akt2 participates in cell survival remains ill-defined. Even though, the distribution of Akt3 expression is restricted to specific tissues such as brain and testis; Akt3 is overexpressed in some cancer types; such as breast and prostate cancers as well as malignant gliomas and melanomas [52-54]. In a recent study, knockdown of Akt3 in a glioblastoma cell line was shown to reduce cell viability and cell cycle progression and to increase apoptosis [55]. Yet, in the context of fragment N mediated cell protection, Akt3 is dispensable.

Isoform specific signaling can also be achieved by a differential capacity of Akt isoforms to interact with regulatory partners. Proteins such as T-cell lymphoma-1 (TCL-1), mature T-cell proliferation 1 (MTCP-1) and c-Jun N-terminal kinase (JNK)-interacting protein 1 (JIP1) were shown to bind Akt in a marked isoform-specific manner. This is believed to play a role in differential activation of Akt1 and Akt2 isoforms [56-61]. In the case of fragment N-induced Akt activation, it appears that such type of regulation is not taking place, as the RasGAP-derived fragment induces the phosphorylation of Akt1 and Akt2 to similar extent (if anything Akt2 is more strongly activated by fragment N than Akt1). Hence the upstream signaling molecules participating in fragment N-mediated Akt1 and Akt2 phosphorylation are likely not different.

In conclusion, the present work provides evidence that fragment N activates both Akt1 and Akt2 but only Akt1 is required for the protective functions of the RasGAP-derived fragment N. In this case therefore, the isoform specific-survival response is not due to a differential activation of the Akt isoforms but is rather a consequence of their distinct intrinsic signaling properties.

Acknowledgements

This study was supported by Swiss National Science Foundation (Grant No. 31003A_141242).

References

1. Chovatiya, R. and R. Medzhitov, *Stress, inflammation, and defense of homeostasis.* Molecular Cell. **54**(2): p. 281-288.
2. Khalil, H., et al., *Caspase-3 and RasGAP: a stress-sensing survival/demise switch.* Trends in Cell Biology, 2014. **24**(2): p. 83-89.
3. Fulda, S., et al., *Cellular stress responses: cell survival and cell death.* International Journal of Cell Biology, 2010. **2010**.
4. Yang, J.Y., et al., *Partial cleavage of RasGAP by caspases is required for cell survival in mild stress conditions.* Mol Cell Biol, 2004. **24**(23): p. 10425-36.
5. Zhang, J., et al., *Visualization of caspase-3-like activity in cells using a genetically encoded fluorescent biosensor activated by protein cleavage.* Nat Commun, 2013. **4**.
6. Yang, J.Y. and C. Widmann, *Antiapoptotic signaling generated by caspase-induced cleavage of RasGAP.* Mol Cell Biol, 2001. **21**(16): p. 5346-58.
7. Yang, J.-Y. and C. Widmann, *The RasGAP N-terminal Fragment generated by caspase cleavage protects cells in a Ras/PI3K/Akt-dependent manner that does not rely on NFκB activation.* Journal of Biological Chemistry, 2002. **277**(17): p. 14641-14646.
8. Khalil, H., et al., *Caspase-3 protects stressed organs against cell death.* Molecular and Cellular Biology, 2012. **32**(22): p. 4523-4533.
9. Jones, P.F., T. Jakubowicz, and B.A. Hemmings, *Molecular cloning of a second form of rac protein kinase.* Cell Regul, 1991. **2**(12): p. 1001-9.
10. Cheng, J.Q., et al., *AKT2, A Putative Oncogene Encoding a Member of a Subfamily of Protein-Serine/Threonine Kinases, is Amplified in Human Ovarian Carcinomas.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(19): p. 9267-9271.
11. Konishi, H., et al., *Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins.* Biochem Biophys Res Commun, 1995. **216**(2): p. 526-34.
12. Scheid, M.P. and J.R. Woodgett, *PKB/AKT: functional insights from genetic models.* Nat Rev Mol Cell Biol, 2001. **2**(10): p. 760-768.
13. Chen, W.S., et al., *Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene.* Genes & Development, 2001. **15**(17): p. 2203-2208.
14. Yang, Z.Z., et al., *Protein kinase B alpha/Akt1 regulates placental development and fetal growth.* Journal of Biological Chemistry, 2003. **278**(34): p. 32124-32131.
15. Cho, H., et al., *Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).* Science, 2001. **292**(5522): p. 1728-1731.
16. Garofalo, R.S., et al., *Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta.* Journal of Clinical Investigation, 2003. **112**(2): p. 197-208.
17. Tschopp, O., et al., *Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis.* Development, 2005. **132**(13): p. 2943-54.
18. Peng, X.-d., et al., *Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2.* Genes & Development, 2003. **17**(11): p. 1352-1365.

19. Yang, Z.-Z., et al., *dosage-dependent effects of Akt1/Protein kinase Bα (PKBα) and Akt3/PKBγ on thymus, skin, and cardiovascular and nervous system development in mice.* Molecular and Cellular Biology, 2005. **25**(23): p. 10407-10418.
20. Hay, N., *Akt isoforms and glucose homeostasis – the leptin connection.* Trends in Endocrinology & Metabolism, 2011. **22**(2): p. 66-73.
21. Song, G., G. Ouyang, and S. Bao, *The activation of Akt/PKB signaling pathway and cell survival.* Journal of Cellular and Molecular Medicine, 2005. **9**(1): p. 59-71.
22. Toker, A. and S. Marmiroli, *Signaling specificity in the Akt pathway in biology and disease.* Advances in Biological Regulation, 2014(0).
23. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.
24. Yang, J., et al., *Molecular mechanism for the regulation of protein kinase b/akt by hydrophobic motif phosphorylation.* Molecular Cell, 2002. **9**(6): p. 1227-1240.
25. Sarbassov, D.D., et al., *Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex.* Science, 2005. **307**(5712): p. 1098-101.
26. Scheid, M.P., P.A. Marignani, and J.R. Woodgett, *multiple phosphoinositide 3-Kinase-Dependent steps in activation of protein kinase B.* Molecular and Cellular Biology, 2002. **22**(17): p. 6247-6260.
27. Yang, J., et al., *Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP.* Nat Struct Biol, 2002. **9**(12): p. 940-4.
28. Bellacosa, A., et al., *A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities.* Cancer Biol Ther, 2004. **3**(3): p. 268-75.
29. Liu, P., et al., *Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus.* Nature, 2014. **508**(7497): p. 541-545.
30. Alessi, D.R., et al., *Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase.* FEBS Letters, 1996. **399**(3): p. 333-338.
31. Tzivion, G., M. Dobson, and G. Ramakrishnan, *FoxO transcription factors; regulation by AKT and 14-3-3 proteins.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2011. **1813**(11): p. 1938-1945.
32. Kloet, D.E.A. and B.M.T. Burgering, *The PKB/FOXO switch in aging and cancer.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2011. **1813**(11): p. 1926-1937.
33. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-241.
34. Datta, S.R., et al., *survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis.* Developmental Cell, 2002. **3**(5): p. 631-643.
35. Nidai Ozes, O., et al., *NF-[kappa]B activation by tumour necrosis factor requires the Akt serine-threonine kinase.* Nature, 1999. **401**(6748): p. 82-85.
36. Romashkova, J.A. and S.S. Makarov, *NF-[kappa]B is a target of AKT in anti-apoptotic PDGF signalling.* Nature, 1999. **401**(6748): p. 86-90.
37. Kane, L.P., et al., *Akt-Dependent Phosphorylation Specifically Regulates Cot Induction of NF-κB-Dependent Transcription.* Molecular and Cellular Biology, 2002. **22**(16): p. 5962-5974.
38. Holler, N., et al., *two adjacent trimeric Fas ligands are required for fas signaling and formation of a death-inducing signaling complex.* Molecular and Cellular Biology, 2003. **23**(4): p. 1428-1440.
39. Ramaswamy, S., et al., *Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway.* Proceedings of the National Academy of Sciences, 1999. **96**(5): p. 2110-2115.
40. Samuels, Y., et al., *Mutant PIK3CA promotes cell growth and invasion of human cancer cells.* Cancer Cell, 2005. **7**(6): p. 561-573.

41. Ericson, K., et al., *Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation.* Proceedings of the National Academy of Sciences, 2010. **107**(6): p. 2598-2603.
42. Jordan, M., A. Schallhorn, and F.M. Wurm, *Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation.* Nucleic Acids Research, 1996. **24**(4): p. 596-601.
43. Annibaldi, A., et al., *Revisiting G3BP1 as a RasGAP binding protein: sensitization of tumor cells to chemotherapy by the RasGAP 317-326 sequence does not involve G3BP1.* PLoS One, 2011. **6**(12).
44. Chin, Y.R., et al., *Targeting Akt3 Signaling in Triple-Negative Breast Cancer.* Cancer Research, 2014. **74**(3): p. 964-973.
45. Woodgett, J.R., *Recent advances in the protein kinase B signaling pathway.* Current Opinion in Cell Biology, 2005. **17**(2): p. 150-157.
46. Williams, M.R., et al., *The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells.* Current Biology, 2000. **10**(8): p. 439-448.
47. Ellwood-Yen, K., et al., *PDK1 attenuation fails to prevent tumor formation in PTEN-deficient transgenic mouse models.* Cancer Research, 2011. **71**(8): p. 3052-65.
48. Gonzalez, E. and T.E. McGraw, *The Akt kinases Isoform specificity in metabolism and cancer.* Cell Cycle, 2009. **8**(16): p. 2502-2508.
49. Lee, R.S., et al., *Relative Expression Levels Rather Than Specific Activity Plays the Major Role in Determining In Vivo AKT Isoform Substrate Specificity.* Enzyme Res, 2011. **720985**(10): p. 22.
50. Grabinski, N., et al., *Distinct functional roles of Akt isoforms for proliferation, survival, migration and EGF-mediated signalling in lung cancer derived disseminated tumor cells.* Cellular Signalling, 2011. **23**(12): p. 1952-1960.
51. Koseoglu, S., et al., *AKT1, AKT2 and AKT3-dependent cell survival is cell line-specific and knockdown of all three isoforms selectively induces apoptosis in 20 human tumor cell lines.* Cancer Biology & Therapy, 2007. **6**(5): p. 755-762.
52. Stahl, J.M., et al., *Deregulated Akt3 activity promotes development of malignant melanoma.* Cancer Research, 2004. **64**(19): p. 7002-7010.
53. Knobbe, C.B. and G. Reifenberger, *Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas.* Brain Pathol, 2003. **13**(4): p. 507-18.
54. Mure, H., et al., *Akt2 and Akt3 play a pivotal role in malignant gliomas.* Neuro-Oncology, 2010. **12**(3): p. 221-232.
55. Paul-Samojedny, M., et al., *Knockdown of AKT3 (PKB gamma) and PI3KCA suppresses cell viability and proliferation and induces the apoptosis of glioblastoma multiforme T98G cells.* Biomed Research International, 2014.
56. Laine, J., et al., *The protooncogene TCL1 Is an Akt kinase coactivator.* Molecular Cell, 2000. **6**(2): p. 395-407.
57. Pekarsky, Y., et al., *Tcl1 enhances Akt kinase activity and mediates its nuclear translocation.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(7): p. 3028-3033.
58. Künstle, G., et al., *Identification of Akt association and oligomerization domains of the Akt kinase coactivator TCL1.* Molecular and Cellular Biology, 2002. **22**(5): p. 1513-1525.
59. Kim, A.H., et al., *Akt1 Regulates a JNK scaffold during excitotoxic apoptosis.* Neuron, 2002. **35**(4): p. 697-709.
60. Kim, A.H., T. Sasaki, and M.V. Chao, *JNK-interacting Protein 1 promotes Akt1 activation.* Journal of Biological Chemistry, 2003. **278**(32): p. 29830-29836.
61. Franke, T.F., et al., *PI3K/Akt and apoptosis: size matters.* Oncogene, 2003. **22**(56): p. 8983-8998.

FIGURE CAPTIONS

Figure 1. **Overexpression of fragment N results in increased Akt1 and Akt2 phosphorylation.** DLD1 and HCT116 cells were infected with an empty lentivirus or with a fragment N-encoding lentivirus. Forty-eight hours later, the cells were lysed and the extent of isoform-specific Akt phosphorylation was assessed by Western blot. These experiments were repeated 2-3 times with similar results (Supplemental Figure 1).

Figure 2. **Fragment N-mediated cell survival is abolished in cells lacking Akt1.** DLD1 isogenic cell lines were infected with an empty lentivirus or lentivirus encoding for fragment N (panel A). Alternatively, HCT116 isogenic cell lines were transfected with GFP-expressing plasmid together with empty pcDNA3 or with pcDNA3 encoding fragment N (panel B). Forty-eight hours following the infection or transfection step, cells were treated with 5 ng/ml FasL for 18 hours and apoptosis was scored.

Figure 3. **Akt2 cannot rescue fragment N-mediated protection in Akt1 KO cells.** A. The indicated cells were infected with viruses expressing either Akt1 or Akt2. Forty-eight hours after, the cells were lysed and the expression levels of each isoform were assessed by using isoform specific antibodies by Western blot. B. The indicated cells were infected with an empty lentivirus or a lentivirus encoding for fragment N. Twenty-four hours later, the cells were infected with Akt1- or Akt2-expressing viruses. Forty-eight hours after this second infection, the cells were treated with FasL (5 ng/ml) and 18 hours later, apoptosis was scored.

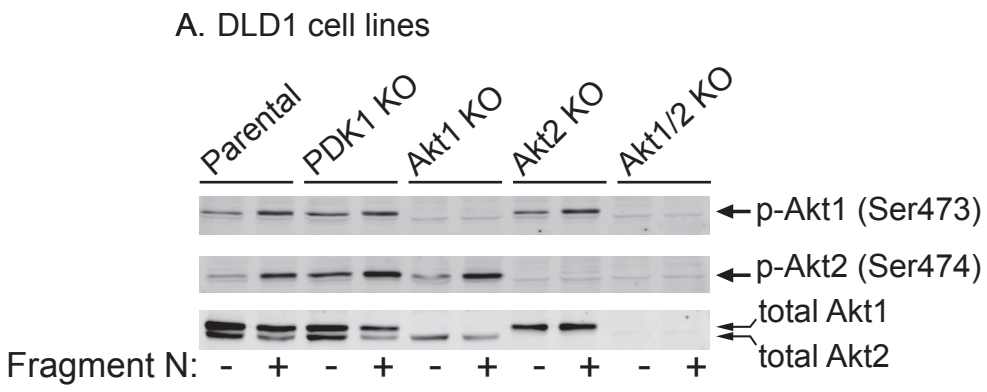
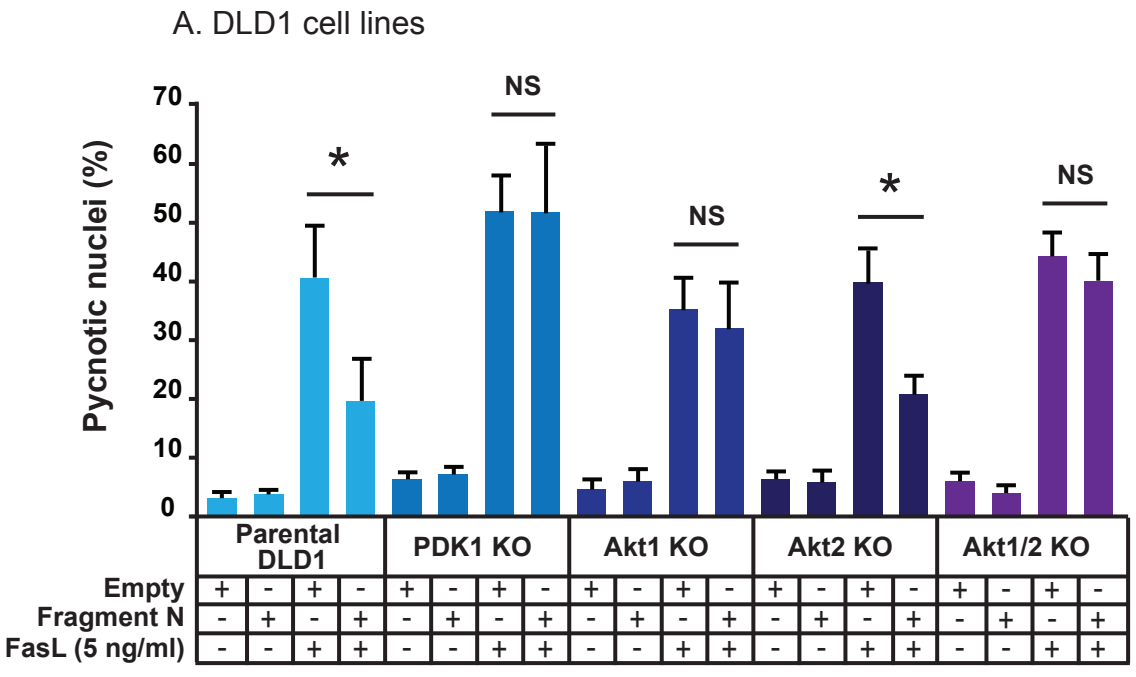

A. DLD1 cell lines
Parental
PDK1 KO
Akt1 KO
Akt2 KO
Akt1/2 KO
p-Akt1 (Ser473)
p-Akt2 (Ser474)
total Akt1
total Akt2
Fragment N: - + - + - + - + - +
B. HCT116 cell lines
Parental
PDK1 KO
Akt1 KO
Akt2 KO
p-Akt1 (Ser473)
p-Akt2 (Ser474)
total Akt1
total Akt2
Fragment N: - + - + - + - +

Figure 1


A. DLD1 cell lines
Pycnotic nuclei (%)
70
60
50
40
30
20
10
0
*
NS
NS
*
NS
Parental DLD1
PDK1 KO
Akt1 KO
Akt2 KO
Akt1/2 KO
Empty
Fragment N
FasL (5 ng/ml)

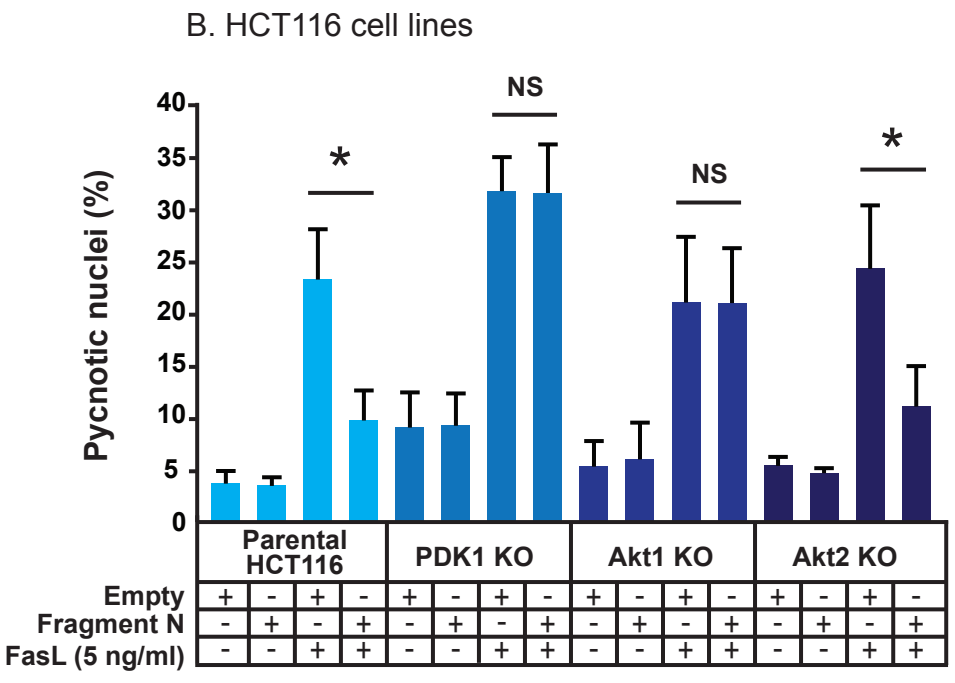
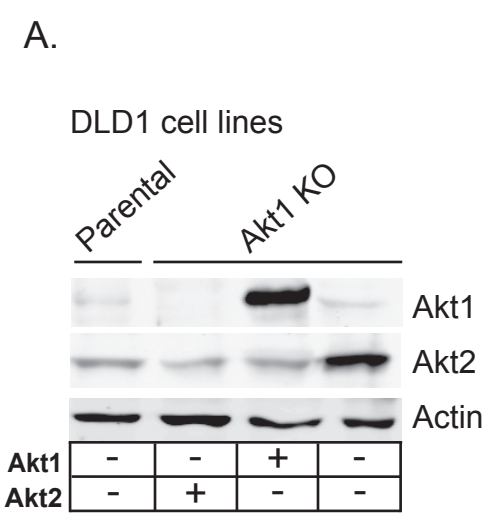
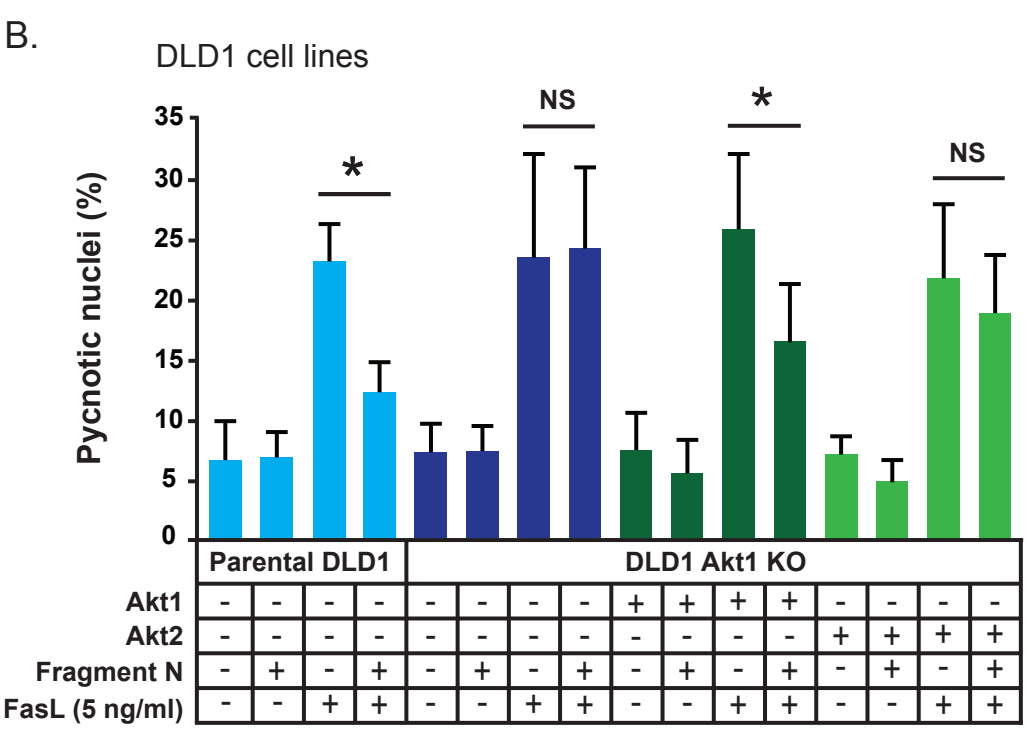
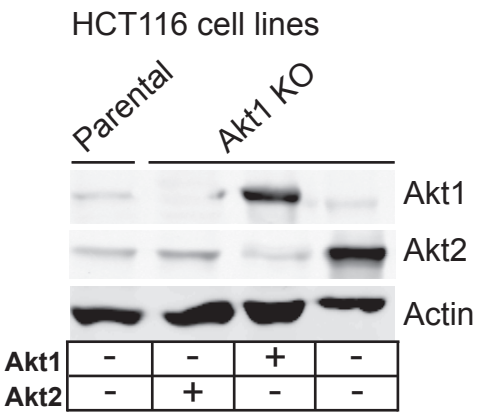

B. HCT116 cell lines
Pycnotic nuclei (%)
40
35
30
25
20
15
10
5
0
*
NS
NS
*
Parental HCT116
PDK1 KO
Akt1 KO
Akt2 KO
Empty
Fragment N
FasL (5 ng/ml)

Figure 2


A.
DLD1 cell lines
Parental
Akt1 KO
Akt1
Akt2
Actin
Akt1 - - + -
Akt2 - + - -


B.
DLD1 cell lines
Pycnotic nuclei (%)
35
30
25
20
15
10
5
0
*
NS
*
NS
Parental DLD1
DLD1 Akt1 KO
Akt1 - - - - - - - - + + + + - - - -
Akt2 - - - - - - - - - - - - + + + +
Fragment N - + - + - + - + - + - + - + - +
FasL (5 ng/ml) - - + + - - + + - - + + - - + +


HCT116 cell lines
Parental
Akt1 KO
Akt1
Akt2
Actin
Akt1 - - + -
Akt2 - + - -

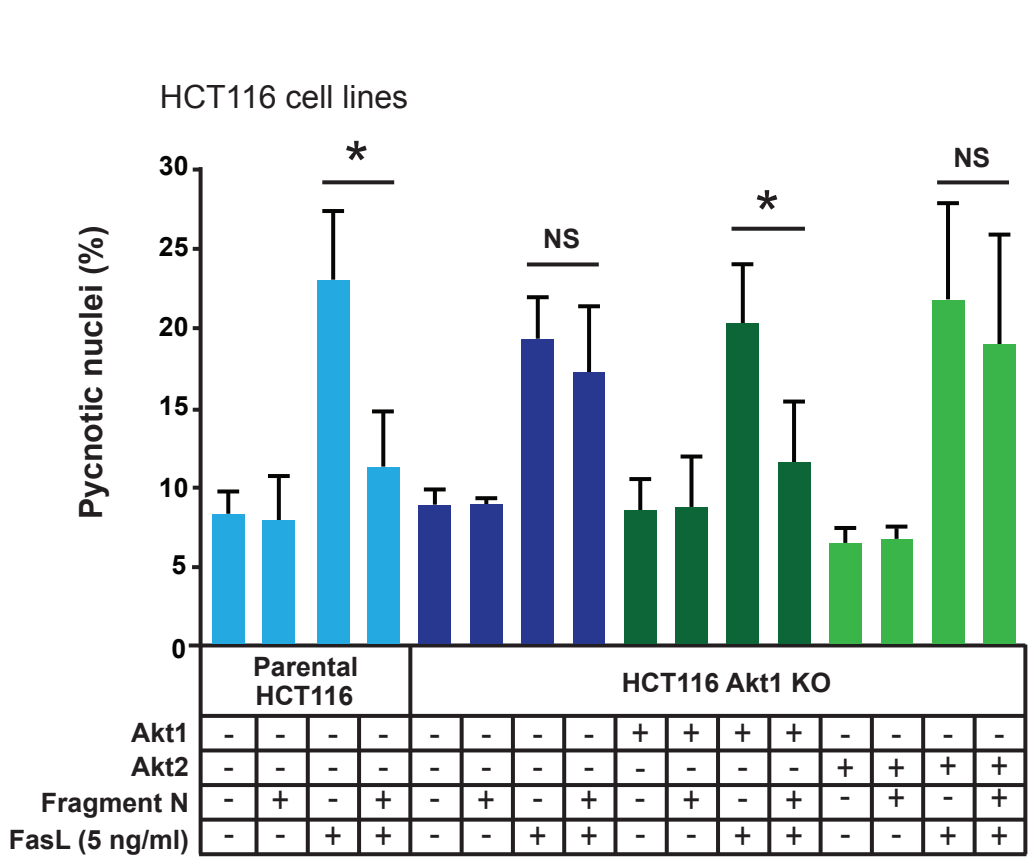
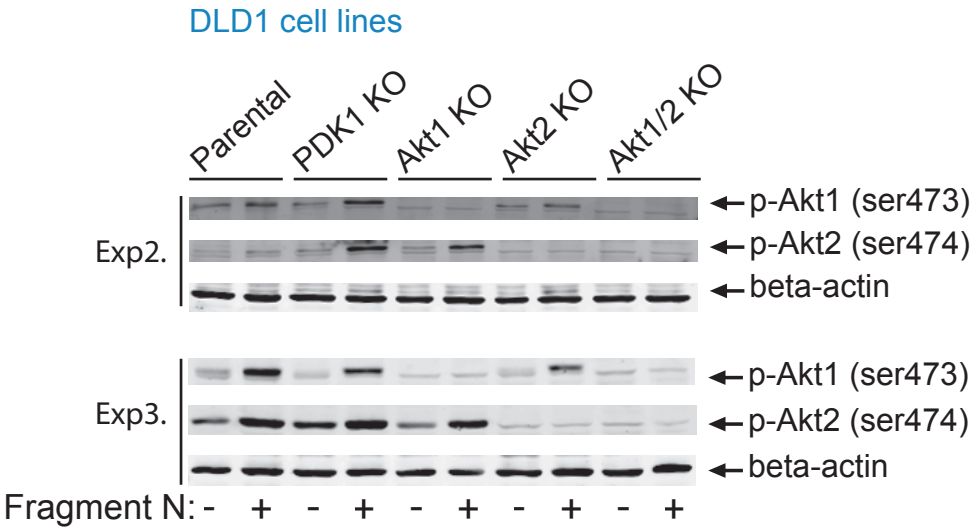
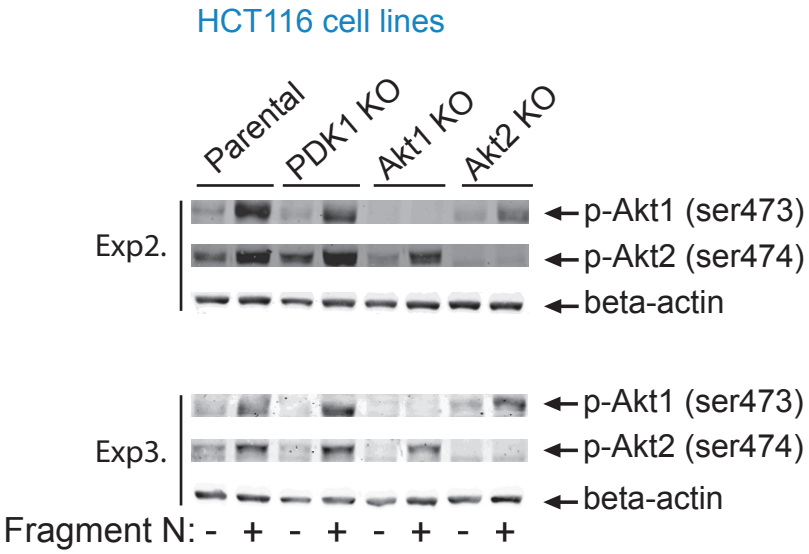

HCT116 cell lines
Pycnotic nuclei (%)
30
25
20
15
10
5
0
*
NS
*
NS
Parental HCT116
HCT116 Akt1 KO
Akt1 - - - - - - - - + + + + - - - -
Akt2 - - - - - - - - - - - - + + + +
Fragment N - + - + - + - + - + - + - + - +
FasL (5 ng/ml) - - + + - - + + - - + + - - + +

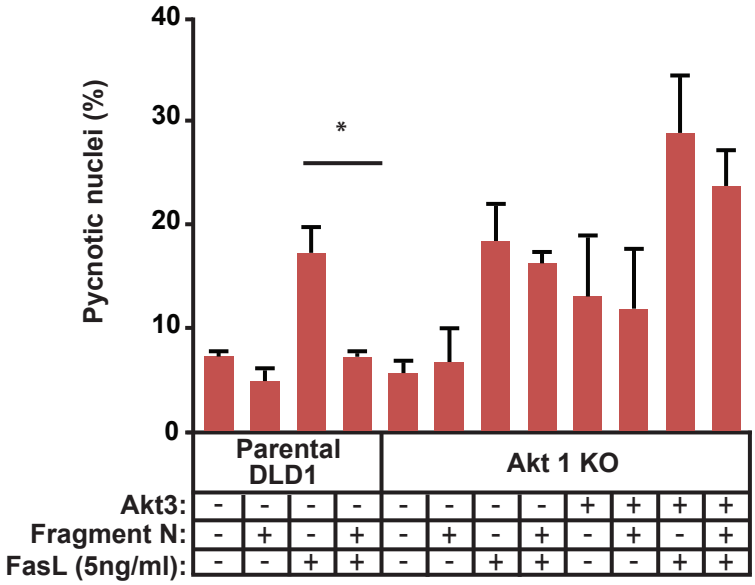
Figure 3





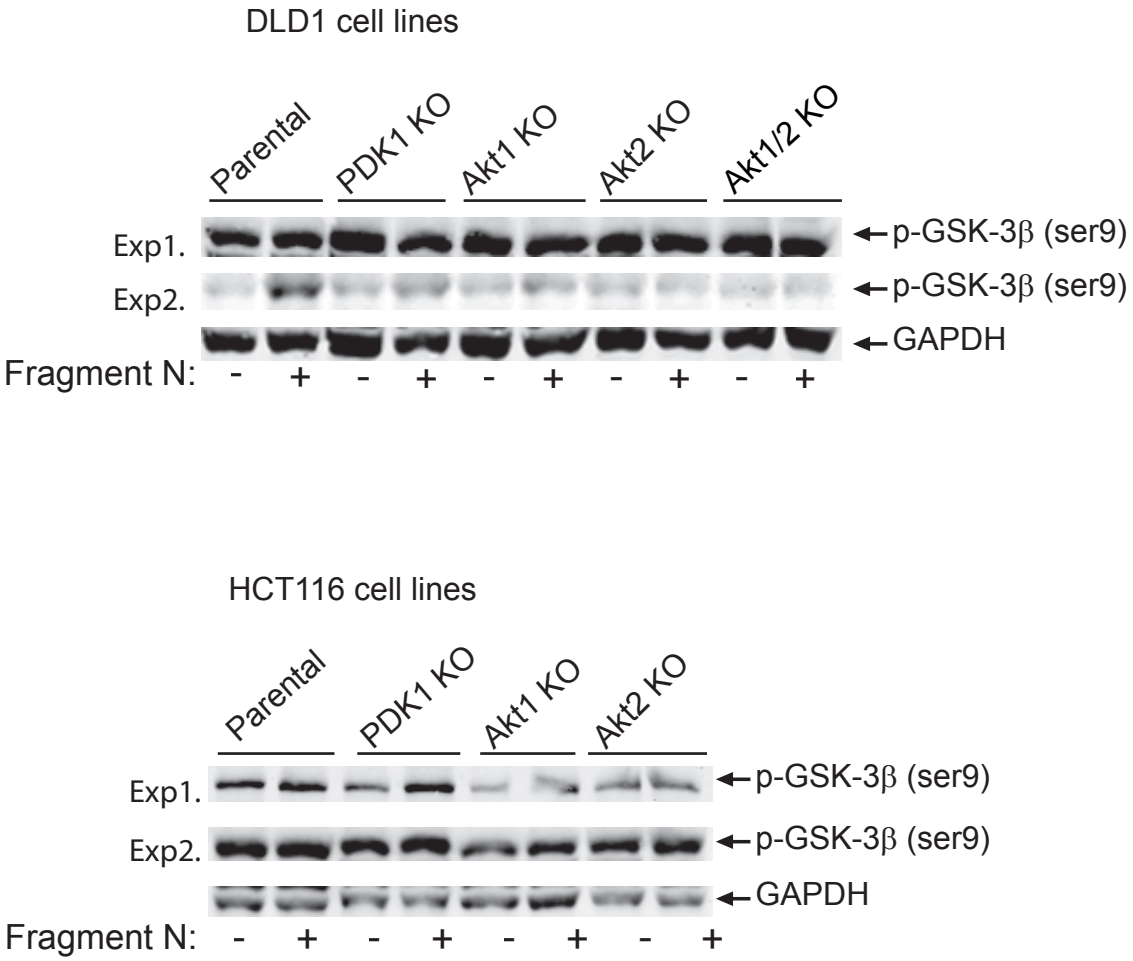
Supplemental Figure 1

Replicate data from the experiments shown in Figure 1.



Supplemental Figure 2

The indicated cell lines were infected with an empty lentivirus or a lentivirus that express fragment N. Twenty-four hours after, the cell lines were either infected with Akt3 expressing or control virusus. Forty-eight hour after this second infection cells were treated either with Fas ligand (5ng/ml) or with the vehicle and the apoptosis was scored (*p=0.001).



Supplemental Figure 3

DLD1 and HCT116 cells were infected with an empty lentivirus or with a fragment N-encoding lentivirus. Forty-eight hours later, the cells were lysed and the extent GSK-3β (Ser9) phosphorylation was assessed by Western blot.