RESEARCH COMMUNICATION

Cell type specificity of PI3K signaling in Pdk1- and Pten-deficient brains

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Loss of PTEN causes unregulated activation of downstream components of phosphatidylinositol 3-kinase (PI3K) signaling, including PDK1, and disrupts normal nervous system development and homeostasis. We tested the contribution of Pdk1 to the abnormalities induced by *Pten* deletion in the brain. Conditional deletion of *Pdk1* caused microcephaly. Combined deletion of *Pdk1* and *Pten* rescued hypertrophy, but not migration defects of Pten-deficient neurons. Pdk1 inactivation induced strikingly different effects on the regulation of phosphorylated Akt in glia versus neurons. Our results show Pdk1-dependent and Pdk1-independent abnormalities in Pten-deficient brains, and demonstrate cell type specific differences in feedback regulation of the ubiquitous PI3K pathway.

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The phosphatidylinositol 3-kinase (PI3K) signaling pathway is activated by extracellular signals and regulates crucial biological processes including metabolism, survival, proliferation, growth, and migration. Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5 trisphosphate (PIP3), and the lipid phosphatase PTEN reverses this reaction, thus negatively regulating the pathway (Engelman et al. 2006).

PTEN is an important tumor suppressor in sporadic brain, endometrium, and prostate cancers. In addition, inherited mutations in PTEN cause cancer predisposition, macrocephaly, and hamartomas in multiple tissues (Chalhoub and Baker 2009). In mice, germline deletion of *Pten* causes embryonic lethality, while heterozygous mice are tumor-prone (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999).

PDK1 is required to phosphorylate and activate AKT, a key downstream effector of PI3K signaling, in response to elevated PIP3. PDK1 also phosphorylates additional downstream substrates including S6K and SGK in a PIP3-

independent manner (Rintelen et al. 2001; Mora et al. 2004). *Pdk1* is indispensable in development, as germline deletion causes embryonic lethality (Lawlor et al. 2002). The PI3K pathway is evolutionarily conserved. PTEN and PDK1 are epistatic in *Drosophila*, in which *dPDK1* deficiency rescued the lethality of *dPTEN*-deficient flies (Rintelen et al. 2001). Similarly, Pdk1 is an important downstream mediator of Pten-deficient cancer in mice, where *Pdk1* haploinsufficiency delayed the onset of multiple tumor types in *Pten*^{+/-} mice (Bayascas et al. 2005). In addition to tumorigenic phenotypes, loss of Pten or Pdk1, which activates or abrogates PI3K signaling, respectively, can influence cell size and/or cell number depending on the context of tissue type and developmental stage analyzed (Williams et al. 2000; Lawlor et al. 2002; Mora et al. 2003, 2005; Hashimoto et al. 2006; Haga et al. 2008; Chalhoub and Baker 2009).

Conditional deletion of *Pten* in the mouse CNS has elucidated multiple roles in brain development and maintenance, causing increased cell proliferation and size, and neuronal migration defects (Groszer et al. 2001; Li et al. 2002; Marino et al. 2002; Fraser et al. 2004; Yue et al. 2005). *Pten* deletion in neural progenitors resulted in enhanced proliferation and self-renewal in vitro (Groszer et al. 2001). However, *Pten* deletion in post-mitotic neurons caused hypertrophy without effects on proliferation. In these models, pronounced macrocephaly was attributable to a cell-autonomous effect of Pten loss in neurons (Backman et al. 2001; Kwon et al. 2001), or a wider effect of Pten loss in multiple cell types including neurons and glia (Fraser et al. 2004, 2008), depending on the expression pattern of Cre recombinase used. In addition, a migration defect was observed in Pten-deficient cerebellar neurons. At the molecular level, hyperphosphorylation of Akt and S6, a substrate of S6K, was detected predominantly in Pten-deficient neurons (Kwon et al. 2001, 2003; Fraser et al. 2004), indicating activation of downstream effectors regulated by Pdk1.

To determine the role of Pdk1 in mature brains, and the contribution of Pdk1 to abnormalities in Pten-deficient brains, we generated mice with brain-specific inactivation of *Pten*, *Pdk1*, or combined deletion of both. Here, we report that Pdk1 deficiency caused microcephaly, and combined deletion of *Pdk1* and *Pten* rescued the neuronal hypertrophy, but not the migration defect of cerebellar neurons in Pten-deficient brains. Pdk1 loss caused strong feedback up-regulation of Akt phosphorylation at Ser 473 in glia, but not in neurons. Our in vivo results show that Pdk1 is not required for all abnormalities associated with Pten deficiency in the brain, and demonstrate cell type specific differential regulation of the ubiquitously expressed PI3K pathway.

Results and Discussion

Pdk1 deficiency caused microcephaly and rescued macrocephaly in *Pten*-deficient brains

To assess the contribution of Pdk1 to Pten-deficient brain hypertrophy in vivo, we used a GFAP-cre transgenic line directing Cre-mediated deletion throughout the brain in both neurons and glia, with nearly complete activity in the hippocampus (hereafter referred to as *hp-cre*) (Bajenaru et al. 2002; Fraser et al. 2004). *hp-cre* mice were bred to

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Pten; *Pdk1*-floxed mice to generate the four genotypes analyzed in this study: *Pten*^{loxP/loxP}; *Pdk1*^{loxP/+}; *cre* (*Pten* cKO), *Pten*^{loxP/+}; *Pdk1*^{loxP/loxP}; *cre* (*Pdk1* cKO), *Pten*^{loxP/loxP}; *Pdk1*^{loxP/loxP}; *cre* (*Pten*; *Pdk1* dKO), and *No-cre* mice (control). *Pdk1* cKO mice had a significantly decreased brain size relative to controls (Fig. 1). Conditional deletion of *Pten* caused pronounced macrocephaly, as reported previously (Kwon et al. 2001; Fraser et al. 2004). CT scans to measure brain volume showed that combined deletion of *Pten* and *Pdk1* fully rescued the *Pten*-deficient macrocephaly, with *Pten*; *Pdk1* dKO brains showing a similar reduction in size as *Pdk1* cKO brains compared with controls (Fig. 1; Supplemental Fig. S1). Additionally, severe ataxia and seizures observed in *Pten* cKO mice were never observed in age-matched *Pten*; *Pdk1* dKO littermates. Furthermore, astrogliosis and microgliosis associated with the disorganization and enlargement of the hippocampus in *Pten* cKO mice (Fraser et al. 2004) were rescued in *Pten*; *Pdk1* dKO brains (Supplemental Fig. S2A; data not shown).

Pdk1 deficiency rescued cell-autonomous hypertrophy of *Pten*-deficient neuronal nuclei and somata

We verified deletion of *Pten* in hippocampus by *Pten* immunohistochemistry (IHC) (Supplemental Fig. S2A,B). *Pten*-deficient neurons also displayed elevated phosphorylation of S6 (p-S6), consistent with enhanced mTORC1 signaling leading to downstream activation of S6K (Fig. 2A). Full activation of S6K also requires phosphorylation by *Pdk1* (Collins et al. 2003). IHC showed that p-S6 was absent in *Pten*; *Pdk1* dKO or *Pdk1* cKO hippocampi, consistent with efficient deletion of *Pdk1* (Fig. 2A). We used this indirect assessment because *Pdk1* antibodies were not reliable for IHC.

To determine the contribution of *Pdk1* to the cell-autonomous hypertrophy of *Pten*-deficient neurons, we measured the size of *Pten*-positive and *Pten*-negative granule neurons from the dentate gyrus within the hippocampal region from mice with single or combined deletion of *Pten* and *Pdk1*, and controls (Fig. 2B; Supplemental Fig. S2B). We evaluated nuclear diameter because

Figure 1. *Pdk1* is indispensable for brain size regulation. Sagittal H&E-stained sections from mice with the indicated genotypes, in which *hp-cre* drives conditional deletion. The dramatic hypertrophy of *Pten* cKO was rescued in *Pten*; *Pdk1* dKO brains, which show a similar size reduction to *Pdk1*-deficient brains when compared with control. Bar, 2 mm. Mice were 10 wk old.

Figure 2. *Pdk1* is required for cell-autonomous hypertrophy of *Pten*-deficient neurons. (A) IHC for p-S6 (Ser 235/236) in the dentate gyrus from the indicated genotypes combined with *hp-cre*. Elevated levels of p-S6 in *Pten* cKO neurons were abrogated in *Pten*; *Pdk1* dKO, consistent with deletion of *Pdk1*. Bar, 100 μm. (B) The nuclear diameter of granule neurons was measured using *Pten* IHC to distinguish between *Pten*-positive and *Pten*-negative neurons. The mean ± SEM of 50 measured nuclei per brain section from six to eight mice is shown. *P* values ([***] *P* < 0.0001; [ns] not significant, *P* > 0.05) were determined by a Student's unpaired *t*-test. Mice were 10 wk old.

the neuronal soma boundary is not consistently visible in all cells and we showed previously that nuclei and somata were coordinately enlarged in *Pten*-deficient neurons (Kwon et al. 2001, 2003; Fraser et al. 2004, 2008). The enlarged nuclear diameter of *Pten*-deficient neurons was fully rescued to normal size by the concomitant deletion of *Pdk1*, demonstrating a requirement for *Pdk1* in the cell-autonomous hypertrophy of *Pten*-deficient neurons. *Pdk1* deletion did not cause a significant decrease in the nuclear size of dentate gyrus granule neurons, perhaps due to the contribution of some remaining *Pdk1*-positive neurons included in the measurements, as we did not have an IHC assay to allow exclusion of *Pdk1*-expressing neurons.

Pdk1 deficiency did not rescue the cerebellar neuron migration defect in *Pten*; *Pdk1* dKO mice

We also evaluated the cerebellum to determine the contribution of *Pdk1* to hypertrophy and migration defects in cerebellar granule neurons that we identified previously in *Pten* cKO mice. However, a significant reduction in the

number of *Pten*-deficient neurons present in *Pten;Pdk1 dKO* cerebella compared with *Pten cKO* was reproducibly observed (Supplemental Fig. S3A). This suggests that cells with combined deletion of *Pten* and *Pdk1* had severely compromised survival, consistent with the requirement for PI3K activation in granule cell progenitor survival demonstrated previously (Dudek et al. 1997; Kenney et al. 2004; Zhao et al. 2006). Therefore, we employed an alternative *Gfap-cre* transgenic line with later onset of Cre activity in the postnatal cerebellar granule cells (Kwon et al. 2001). For simplicity of discussion, this line is referred to as *cb-cre* hereafter. Similar numbers of *Pten*-deficient cerebellar granule neurons were observed in the *Pten cKO* and *Pten;Pdk1 dKO* mice with *cb-cre*, likely because the later onset of Cre activity, around postnatal day 14, bypassed the developmental stage at which *Pdk1* was required for survival (Supplemental Fig. S3B; Kwon et al. 2001). This allowed a controlled comparison of *Pten*-deficient cerebellar granule neurons in the absence and presence of *Pdk1*. Consistent with the size rescue observed in the hippocampus with *hp-cre;Pten;Pdk1 dKO* mice, the overall size of the *cb-cre;Pten cKO* cerebellum and the hypertrophy of individual *Pten*-deficient cerebellar granule neurons were completely rescued in *cb-cre;Pten;Pdk1 dKO* mice (Supplemental Fig. S4).

Strikingly, *Pdk1* deletion failed to rescue migration defects of *Pten*-deficient cerebellar granule neurons. During cerebellar development, granule cell progenitors proliferate in the external germinal layer (EGL) at the surface of the cerebellum, then exit the cell cycle and migrate to their final position in the internal granule layer (IGL), where they fully differentiate (Behesti and Marino 2008). Ectopically positioned granule neurons are easily visualized by expression of GABA_A Rα6, which is normally restricted to mature granule neurons of the IGL. As expected, cerebella from *cb-cre;Pten cKO* mice contained ectopic GABA_A Rα6-positive granule neurons at the pial surface and in the molecular layer (Fig. 3; Supplemental Fig. S5). These neurons were hypertrophic and expressed elevated levels of p-S6, as observed in other *Pten*-deficient neurons (Fig. 3). Surprisingly, concomitant deletion of *Pdk1* did not rescue this migration defect. Ectopic GABA_A Rα6-expressing granule neurons were consistently observed in *Pten;Pdk1 dKO* cerebella, but not in control cerebella (Fig. 3; Supplemental Fig. S5). *Pdk1* was clearly deleted in ectopic granule neurons of *Pten;Pdk1 dKO* cerebella, as these cells did not show the hypertrophy or elevated p-S6 associated with *Pten*-null neurons (Fig. 3).

While there are a number of examples of AKT activation influencing migration (Kolsch et al. 2008), the failure of *Pdk1* loss to rescue the migration defect of *Pten*-deficient cerebellar granule neurons indicates that at least a component of this phenotype is AKT-independent. PTEN may also influence cell migration through alternative mechanisms. For example, elevated PIP3 can regulate cytoskeletal rearrangement and cell migration and motility through an AKT-independent pathway involving small GTPases, such as RAC1/CDC42 (Kolsch et al. 2008). Alternatively, *in vitro* studies have implicated the protein phosphatase activity of PTEN in adhesion and migration, independent of its effects on the PI3K pathway (Tamura et al. 1998; Raftopoulou et al. 2004). Mutant mouse models that selectively target the lipid or protein phosphatase activity of *Pten* will help to further

Figure 3. *Pdk1* deficiency did not rescue the migration defect in *Pten cKO* cerebellar granule neurons. IHC for p-S6 (Ser 235/236) on cerebella from the indicated genotypes combined with *cb-cre*. *Pten cKO* cerebella contained ectopic neurons that were hypertrophic and contained elevated p-S6. Ectopic granule neurons persist in *Pten;Pdk1 dKO* cerebella, but they do not show hypertrophy or elevated p-S6, consistent with deletion of *Pdk1*. Insets show IHC for GABA_A Rα6 demonstrating that ectopic cells are cerebellar granule neurons. Bar, 50 μm. Mice were 8 wk old.

resolve the mechanistic basis of migration defects in *Pten*-deficient neurons.

Pdk1 deletion blocked phosphorylation of downstream effectors

To verify deletion of *Pdk1* and the concomitant loss of phosphorylation of downstream components in the PI3K signaling cascade, we performed Western blot analysis on cerebellar and hippocampal protein extracts in *Pdk1 cKO* and *Pten;Pdk1 dKO* brains. Cerebellar protein extracts from the *cb-cre* line showed substantial loss of *Pten*, *Pdk1*, or both as predicted by their corresponding genotype (Supplemental Fig. S6A). Akt is fully activated when phosphorylated by *Pdk1* on Thr 308 (T308) and by a second kinase, most commonly the mTorC2 complex, on Ser 473 (S473) (Mora et al. 2004; Sarbassov et al. 2005). High p-Akt (S473 and T308) levels were present in *Pten cKO* extracts, as expected for activated PI3K signaling. In *Pten;Pdk1 dKO* cerebella, although p-Akt S473 was still elevated, phosphorylation at T308 was abolished by the deletion of *Pdk1*, which would prevent full activation of Akt. As expected, the enhanced levels of phosphorylation of the Akt substrate Gsk3β observed in *Pten cKO* cerebellum were restored to levels similar to controls in *Pten;Pdk1 dKO* cerebella. In addition, conditional deletion of *Pdk1*, alone or in combination with *Pten*, substantially decreased levels of p-S6. Similar loss of p-Akt T308 and decreased phosphorylation of S6, as well as decreased phosphorylation of GSK3β and an additional Akt substrate, Fkhr, were associated with *Pdk1* deficiency in hippocampal protein extracts from the *hp-cre* line (Fig. 4). Taken together, these data demonstrate that *Pdk1* was effectively codeleted along with *Pten* in the cerebellum and hippocampus using *cb-cre* and *hp-cre*, respectively.

Figure 4. *Pdk1* deficiency blocked phosphorylation of downstream effectors of the PI3K pathway and induced up-regulation of p-Akt S473. Western blot analysis of protein lysates from hippocampi of *hp-cre* mice. (Lane 1) *Pten cKO*. (Lane 2) *Pten;Pdk1 dKO*. (Lane 3) *Pdk1 cKO*. (Lane 4) Control. *Pten cKO* mice showed increased phosphorylation of Akt at S473 and T308, and increased phosphorylation of downstream substrates GSK3 β , S6, and Fkhr. (Lane 2) This enhanced phosphorylation of Akt T308 and substrates was blocked by *Pdk1* deletion. (Lane 3) Note the high levels of p-Akt S473 in *Pdk1 cKO*. β -Actin is shown as a loading control. The arrow denotes a specific band. Mice were 10 wk old.

Cell type specific feedback regulation of p-Akt S473 in *Pdk1*-deficient brains

Western blot analysis of *Pdk1*-deficient brain regions showed complete loss of Akt phosphorylation in *cb-cre;Pdk1*-deficient cerebella (Supplemental Fig. S6A), in which Cre activity was restricted to neurons (Kwon et al. 2001). In contrast, in hippocampal extracts in which Cre activity was present in neurons and glia (Fraser et al. 2004), *Pdk1* deletion alone abrogated phosphorylation of its substrate, Akt T308, but unexpectedly induced phosphorylation of Akt S473 to levels comparable with those induced by deregulated PI3K signaling in *Pten*-deficient hippocampi (Fig. 4). The up-regulation of p-Akt S473 was blocked by the dual PI3K/mTOR inhibitor BEZ235 (Supplemental Fig. S6B; Maira et al. 2008).

To investigate the selective up-regulation of p-Akt S473 in *hp-cre* mice, we used IHC. As expected in the *cb-cre* line, *Pten cKO* granule neurons displayed elevated levels of p-Akt S473 in the IGL of the cerebellum. *Pten*-deficient regions of *Pten;Pdk1 dKO* cerebella had similar elevated p-Akt S473 levels to *Pten cKO*, although the density of staining was higher due to the size rescue of cerebellar granule neurons. In contrast, *Pdk1 cKO* cerebella were similar to controls with no detectable p-Akt S473 signal in cerebellar granule neurons (Supplemental Fig. S6C), indicating that *Pdk1* deletion does not alter the phosphorylation status of Akt S473 in mature cerebellar granule neurons. Interestingly, using the *hp-cre* line, which targets both neurons and glia, *Pdk1* deficiency led to a surprising p-Akt pattern (Fig. 5). As reported previously, *Pten cKO* brains had elevated levels of p-Akt S473 predominantly in neurons, including the hippocampus and cortex, and occasionally in a few reactive hippocampal astrocytes (Fraser et al. 2004). Unexpectedly, in addition

to the neuronal populations with elevated p-Akt S473 observed in *Pten cKO* mice, *Pten;Pdk1 dKO* brains also showed high levels of p-Akt S473 in cells with stellate morphology characteristic of glia (Fig. 5). The single deletion of *Pdk1*, without loss of *Pten*, caused elevated levels of p-Akt S473 only in cells with glial morphology in both the hippocampus and cortex, indicating a compensatory or feedback up-regulation of pAkt S473. To confirm the identity of these cells, we took advantage of the *lacZ* marker included in the *GFAP-cre* transgene. In adult brains, this transgene was shown to be expressed selectively in astrocytes (Bajenaru et al. 2002), while the broader Cre activity induced by the transgene is a reflection of Cre-mediated deletion earlier in development (Fraser et al. 2004). We double-stained brain cryosections using X-Gal histochemistry and IHC for p-Akt S473, and found a complete overlap between p-Akt and X-Gal-positive cells in *Pten;Pdk1 dKO* and *Pdk1 cKO* cerebral cortices. Further double-labeling showed elevated p-Akt S473 was restricted to astrocytes and Olig2-expressing cells of the oligodendroglial lineage, and was not found in neurons or microglia (Supplemental Fig. S7; data not shown). No overlap was seen between X-Gal and p-Akt-positive cells in *Pten cKO* brains (Fig. 5, right panels), highlighting the distinct cell type differences in response to disruption of different effectors in the PI3K pathway.

Feedback inhibition in PI3K signaling has been demonstrated, but differential regulation of this mechanism in normal cells has not been clearly shown. S6K can phosphorylate and inhibit IRS1, creating a negative feedback loop that limits insulin and IGF receptor signaling through the PI3K pathway (Um et al. 2004). Release of this feedback inhibitory pathway occurs in some, but not all, cancer cells in response to mTOR inhibition. This results in higher levels of AKT activation, and likely contributes to the failure of mTOR inhibitors in cancer

Figure 5. *Pdk1* deficiency caused elevation of p-Akt S473 selectively in glia. IHC with p-Akt S473 antibody on the hippocampus (left) and cortex (center). (Right) X-Gal histochemistry followed by p-Akt IHC revealed β -galactosidase-positive glia. The p-Akt-positive neurons in *Pten cKO* do not overlap with X-gal, while high levels of p-Akt are present in X-gal-positive glia within *Pten;Pdk1 dKO* and *Pdk1 cKO* cerebral cortices compared with control. Bars: left and center panels, 400 μ m; right panel, 100 μ m. Mice were 10 wk (left and center panels), and 2 wk old (right panel).

therapy. It remains unclear why cancer cells are heterogeneous in this response to mTOR inhibition (O'Reilly et al. 2006; Cloughesy et al. 2008). Deletion of *Pdk1* prevents full activation of S6k, and would therefore similarly relieve feedback inhibition and cause enhanced phosphorylation of p-Akt S473. Indeed, elevated phosphorylation of p-Akt S473 following *Pdk1* deletion has been reported in mouse embryonic stem cells, heart, and liver (Williams et al. 2000; Mora et al. 2003, 2005). However, p-Akt status was assessed by Western blot in these experiments, precluding interpretation of the differences in pathway regulation between cell types within a tissue under physiological conditions.

Our data show a striking cell type difference in pathway regulation. Deletion of the negative regulator Pten induced increased p-Akt S473 in neurons, but not glia. In contrast, deletion of the positive regulator Pdk1 caused increased p-Akt S473 in glia, but not neurons. This is consistent with a strong feedback in glia, which acts to limit PI3K signaling, even in the absence of Pten. The reason for this difference in regulation may reflect the biological properties and roles of these cell types. Except in response to specific mutations, mature neurons do not proliferate or regenerate, suggesting no need for a tight control of PI3K signaling through feedback regulation in these cells. In contrast, glia can re-enter the cell cycle following different cues. This response should be limited to minimize pathological conditions such as glial scars and the possibility of oncogenic transformation to generate gliomas (Yang and Herrup 2007). Glia, therefore, may require tighter control mechanisms on PI3K signaling, including feedback regulation.

In summary, our study shows that Pdk1 is a critical player in the Pten signaling pathway. Our results demonstrate clearly that Pdk1 loss rescues Pten-deficient brain hypertrophy in agreement with genetic studies showing epistasis between Pten and Pdk1 in flies and mice (Rintelen et al. 2001; Bayascas et al. 2005). However, Pdk1 loss did not rescue Pten-null neuronal migration defects. This may reflect AKT-independent regulation of migration through PI3K signaling, or may be due to PIP3-independent functions of PTEN in neuronal migration. Surprisingly, analysis of Akt phosphorylation revealed striking cell type differences in feedback up-regulation of p-Akt S473 in Pdk1-deficient glia compared with neurons in the mature brain. In vitro studies have elegantly shown the mechanistic connections among key effectors in the PI3K signaling pathway. Our in vivo analysis demonstrates that under physiological conditions, this ubiquitously expressed pathway may show functionally important differences in regulation between cell types that are important in normal development and disease.

Materials and methods

All experimental procedures were as described previously (Chalhoub et al. 2006) with the following modifications or additions: Pten antibody (Cell Signaling, #9559) was used at a 1/100 dilution for IHC, and 1/1000 dilution for immunoblot; Pdk1 antibody (Cell Signaling, #3062) was used at a 1/1000 dilution for immunoblot; p-Akt (T308) antibody (Cell Signaling, #9275) was used at a 1/1000 dilution for immunoblot; GABA_A R α 6 antibody (Santa Cruz Biotechnologies, #sc-7359) was used at 1/500 dilution for IHC following antigen retrieval. IHC was counterstained with hematoxylin.

For the X-Gal and p-Akt (S473) double stain, we perfused mice with 2% PFA to fix, removed brains, and post-fixed overnight in 2% PFA. After 24 h of equilibration in 25% sucrose/PBS, OCT-embedded brains were cryo-

sectioned. We performed X-Gal histochemistry as described (Chow et al. 2008), followed by 5 min of fixation in 4% PFA, then proceeded with p-Akt IHC as described previously (Chalhoub et al. 2006) without counterstain.

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