



RESEARCH ARTICLE

Open Access

# Differential PKA activation and AKAP association determines cell fate in cancer cells

Erik D Hedrick, Ekta Agarwal, Premila D Leiphrahpam, Katie L Haferbier, Michael G Brattain\* and Sanjib Chowdhury\*

## Abstract

**Background:** The dependence of malignant properties of colorectal cancer (CRC) cells on IGF1R signaling has been demonstrated and several IGF1R antagonists are currently in clinical trials. Recently, we identified a novel pathway in which cAMP independent PKA activation by TGF $\beta$  signaling resulted in the destabilization of survivin/XIAP complex leading to increased cell death. In this study, we evaluated the effect of IGF1R inhibition or activation on PKA activation and its downstream cell survival signaling mechanisms.

**Methods:** Small molecule IGF1R kinase inhibitor OSI-906 was used to test the effect of IGF1R inhibition on PKA activation, AKAP association and its downstream cell survival signaling. In a complementary approach, ligand mediated activation of IGF1R was performed and AKAP/PKA signaling was analyzed for their downstream survival effects.

**Results:** We demonstrate that the inhibition of IGF1R in the IGF1R-dependent CRC subset generates cell death through a novel mechanism involving TGF $\beta$  stimulated cAMP independent PKA activity that leads to disruption of cell survival by survivin/XIAP mediated inhibition of caspase activity. Importantly, ligand mediated activation of the IGF1R in CRC cells results in the generation of cAMP dependent PKA activity that functions in cell survival by inhibiting caspase activity. Therefore, this subset of CRC demonstrates 2 opposing pathways organized by 2 different AKAPs in the cytoplasm that both utilize activation of PKA in a manner that leads to different outcomes with respect to life and death. The cAMP independent PKA activation pathway is dependent upon mitochondrial AKAP149 for its apoptotic functions. In contrast, Praja2 (Pja2), an AKAP-like E3 ligase protein was identified as a key element in controlling cAMP dependent PKA activity and pro-survival signaling. Genetic manipulation of AKAP149 and Praja2 using siRNA KD had opposing effects on PKA activity and survivin/XIAP regulation.

**Conclusions:** We had identified 2 cytoplasmic pathways dependent upon the same enzymatic activity with opposite effects on cell fate in terms of life and death. Understanding the specific mechanistic functions of IGF1R with respect to determining the PKA survival functions would have potential for impact upon the development of new therapeutic strategies by exploiting the IGF1R/cAMP-PKA survival signaling in cancer.

**Keywords:** Colorectal cancer, IGF1R, AKAP149, Praja2, PKA, XIAP

## Background

The IGF1R signaling pathway plays a crucial role in cell growth, proliferation, survival, and differentiation [1-5]. IGF1R is often overexpressed and upregulated in many cancer types, including colorectal cancer (CRC) [6]. Thus, IGF1R has been shown to be a promising therapeutic target and both pharmacological and biological agents have been developed to inhibit IGF1R

for therapeutic applications in cancer. These agents include monoclonal antibodies, which specifically bind to IGF1R homodimers [3,7,8] and small molecular kinase inhibitors [3,7]. OSI-906 is a small molecule IGF1R kinase inhibitor that is currently in clinical trials [7]. OSI-906 targets both IR and IGF1R heterodimers [7]. This drug has been shown in previous studies to be an effective inhibitor of IGF1R signaling leading to a decrease in cellular proliferation and increased apoptosis [7]. OSI-906 has been shown to reduce tumor growth in athymic nude mice [7].

\* Correspondence: mbrattain@unmc.edu; schowdhury@unmc.edu  
Eppley Cancer Center, University of Nebraska Medical Center, Nebraska  
Medical Center, Omaha, NE 68198-5950, USA

Recently, we showed that TGF $\beta$  mediates its tumor suppressor and pro-apoptotic effects in part, through the activation of protein kinase A (PKA) in a cyclic AMP (cAMP) independent manner in colorectal cancer [9]. The TGF $\beta$  mediated cAMP independent PKA activation was Smad3-dependent and inhibited the expression of the X-linked inhibitor of apoptosis protein (XIAP) that has been shown to mediate aberrant cell survival and metastasis [9,10]. Cell fate in response to cellular stress is determined by multiple signals that determine whether pro-apoptotic or anti-apoptotic signals that normally function in equilibrium will ultimately predominate in response to the stress. For example, stress causes the mitochondria to release survivin and XIAP to the cytoplasm forming a survivin/XIAP complex to promote cell survival [11]. The survivin/XIAP complex that mediates caspase inhibition has been shown to be a key cell survival mechanism enabling the metastatic process [11,12]. The complex is critical for stabilization of XIAP to inhibit caspases. We recently demonstrated that TGF $\beta$ /PKA signaling leads to the disruption and subsequent destabilization of the survivin/XIAP complex to enable cell death by PP2A mediated inhibition of Akt phosphorylation of a stabilizing XIAP site (S87) and by the direct phosphorylation of survivin at S20 which disrupts complex formation by the 2 inhibitor of apoptosis (IAP) family members and leads to their destabilization thereby enabling cell death [9,13,14].

A-kinase anchoring proteins (AKAPs) are specialized anchoring proteins that recruit and compartmentalize PKA and other enzymes in the cytoplasm to specific subcellular locations and organelles for their enzymatic functions [15,16]. This is accomplished by the binding of PKA regulatory subunits (PKARI and/or PKARII) to specific AKAPs. In our previous work, we demonstrated the critical role of AKAP149 (also termed as D-AKAP1 and AKAP121) in regulating cAMP independent TGF $\beta$ /PKA signaling for its metastasis suppressor activity [9]. Recently, Lignitto et al. (2011) described the role of Praja2 (also termed as Pja2), an AKAP-like E3 ubiquitin ligase that plays a critical role in the cAMP-dependent activation of PKA. Praja2 acts as a pro-survival AKAP and promotes cell proliferation and growth [17].

In the current work, we investigated the molecular mechanisms that determine AKAP-PKA-driven cell fate in the IGF1R-dependent subsets of CRC cells using the IGF1R kinase inhibitor OSI-906. We report a newly identified form of crosstalk between the opposing IGF1R and TGF $\beta$  signaling pathways in which PKA activation is utilized by both pathways, but different AKAPs are utilized to organize opposing functions with regard to XIAP protein stability in controlling cell survival in CRC cells. This is the first report demonstrating the novel linkage of IGF1R/cAMP-PKA/Praja2/XIAP cell survival

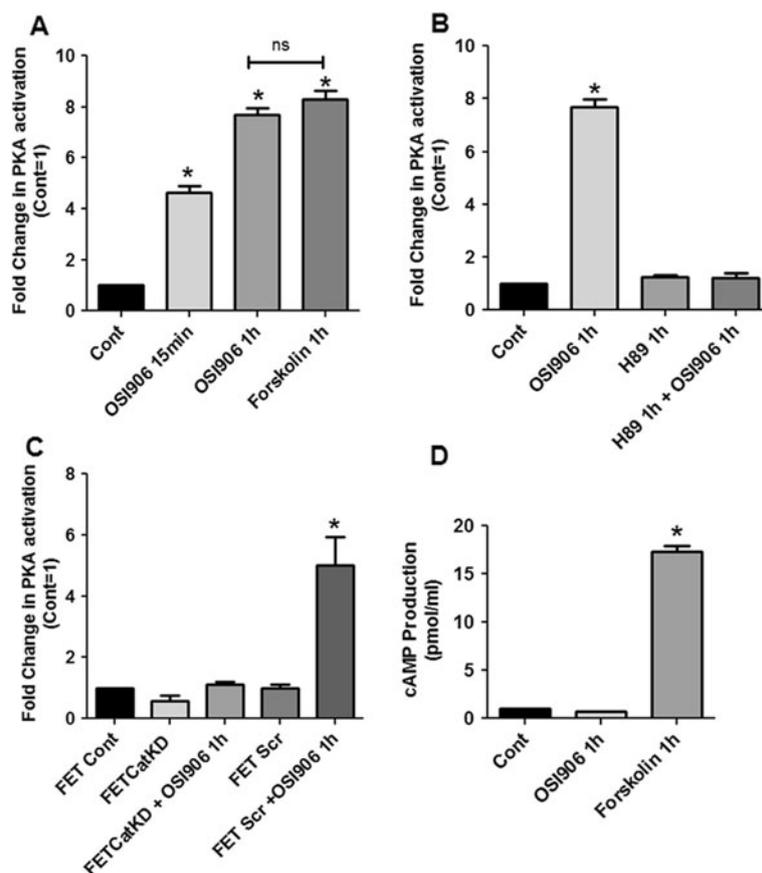
signaling in CRC. Identification of specific mechanistic functions of IGF1R/PKA signaling could have potential for impact upon the development of therapeutic strategies for IGF1R-dependent cancer by shifting the balance of these 2 pathways away from cell survival towards cell death.

## Results

### Inhibition of IGF1R by OSI-906 activates PKA

Zhang et al. (2004) first demonstrated that TGF $\beta$  could activate PKA in a Smad3-dependent manner independent of cAMP [18]. We demonstrated that TGF $\beta$  signaling can activate PKA by a Smad3-dependent, cAMP independent mechanism in IGF1R-dependent subsets of CRC cells leading to cell death through the disruption of the formation survivin/XIAP complexes which are necessary for caspase 3,7,9 inhibition to block apoptosis [9,13]. This raised the possibility that the inhibition of IGF1R in this subset might generate cell death through mechanisms involving TGF $\beta$ /PKA signaling mediated control of aberrant cell survival. Therefore, we tested the hypothesis that IGF1R inhibition by OSI-906 activates PKA in CRC cells in a cAMP independent, Smad3-dependent mechanism. To this end, IGF1R-dependent FET colon carcinoma cells [19] were treated with OSI-906 for specific times (15 mins and 1 h), and a non-radioactive protein kinase assay (Promega) was performed for measuring PKA activity. Following drug treatment, PKA activity increased by approximately 5-fold at 15 min and 7-fold by 1 h (Figure 1A). It was observed that OSI-906 mediated PKA activation was completely abolished following pretreatment of the cells with H89, a pharmacological PKA inhibitor (Figure 1B) thus indicating that PKA signaling was initiated by an endogenous cellular mechanism in response to blockade of IGF1R signaling. Similar results were confirmed in 2 additional IGF1R-dependent colon carcinoma cell lines GEO and CBS (Additional file 1: Figure S1). The OSI-906 mediated PKA activation was further confirmed by siRNA knockdown of PKA catalytic subunit. As shown in Figure 1C, knockdown of PKA catalytic subunit in FET cells (termed as FETCatKD) resulted in abrogation of OSI-906 mediated PKA activation. However, FET cells transfected with scrambled siRNA (termed FET Scr) showed PKA activation upon treatment with OSI-906.

To further confirm the activation of PKA by IGF1R inhibition, we used MK-0646, a recombinant humanized monoclonal antibody against IGF1R. Previous studies have reported that MK-0646 binds specifically to IGF1R and triggers internalization of its receptors and degradation which subsequently blocks IGF-I and IGF-II-mediated cell proliferation and survival [20]. As shown in Additional file 1: Figure S2, treatment with MK-0646 activated PKA in FET cells. We next determined whether

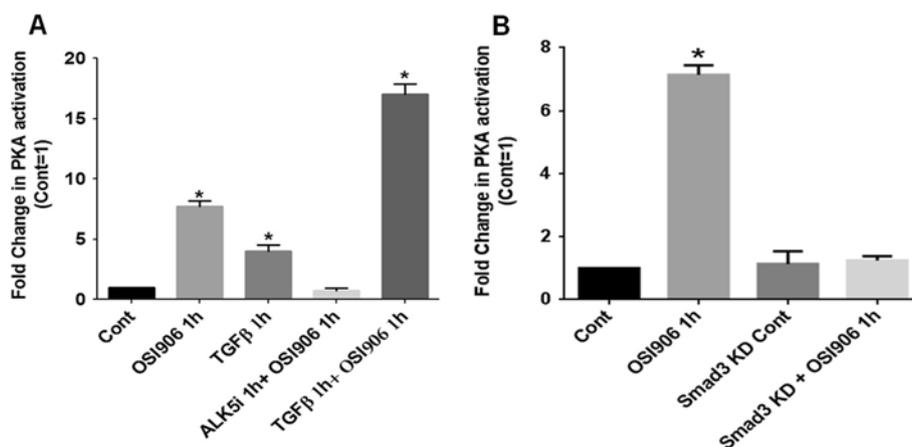


**Figure 1 OSI-906 mediates cAMP independent and AKAP dependent PKA activation in colon cancer cells.** FET cells treated with OSI-906 (1  $\mu$ M) activated PKA in a time dependent fashion (A). Forskolin (10  $\mu$ M) was used as a positive control. PKA inhibitor H89 (15  $\mu$ M) was used to inhibit PKA activation. Pretreatment with H89 followed by OSI-906 treatment abrogated the PKA activation (B). Knockdown of PKA Catalytic subunit (FETCatKD) abrogated OSI-906 mediated PKA activation. However, FET cells transfected with Scrambled siRNA (FET Scr) cells showed PKA activation following OSI-906 treatment. (C). PKA activation by OSI-906 is cAMP independent as determined via cAMP assay (D). All experiments were repeated three times independently with 3 replicas per repeat for the ANOVA analysis. (\* P < 0.001).

activation of PKA by OSI-906 is dependent upon cAMP activation by treating FET cells with OSI-906 and measuring cAMP levels using a non-radioactive cAMP enzyme immunoassay (Figure 1D). It was observed that OSI-906 was unable to increase cAMP production in contrast to Forskolin treatment which provided a significant increase in cAMP levels as expected. It should be noted that the levels of OSI-906 driven PKA activity in the absence of cAMP were similar to those induced by Forskolin in its cAMP-dependent PKA activation, thus indicating that the mechanism of cAMP independent PKA activation by OSI-906 was approximately as potent as that of Forskolin induction. 1way ANOVA with Bonferroni's multiple comparison test showed that while both treatment with OSI-906 1 h and Forskolin 1 h showed statistically significant increase in PKA activity (as indicated in Figure 1A), no statistically significant difference was observed comparing OSI-906 1 h vs. Forskolin 1 h.

#### OSI-906 mediated activation of PKA and cellular apoptosis requires TGF $\beta$ signaling

Based on cAMP independent PKA activation by OSI-906 treatment and our prior report showing TGF $\beta$ /PKA regulated aberrant cell survival, we hypothesized that OSI-906 mediated cAMP independent PKA activation requires TGF $\beta$  signaling in order to mediate its proapoptotic effects. To this end, FET cells were treated with either ALK5i (400 nM), an inhibitor of TGF $\beta$ R1 kinase activity, or exogenous TGF $\beta$  (5 ng/ml) [19]. We previously showed that pretreatment with ALK5i prior to TGF $\beta$  treatment inhibits the TGF $\beta$ /PKA-mediated cell death through the abrogation of survivin and XIAP downregulation [9,13]. This led us to the hypothesis that IGF1R inhibition leads to increased TGF $\beta$  mediated cAMP independent PKA activation which then mediates cell death. If this hypothesis were correct; blockade of TGF $\beta$  signaling would abrogate OSI-906 mediated PKA activation and downstream signaling. Figure 2A shows



**Figure 2** OSI-906 activates PKA in a TGF $\beta$  signaling dependent manner. FET cells pretreated treated with TGF $\beta$ RI inhibitor ALK5i (400 nM) abrogated OSI-906 (1  $\mu$ M) mediated PKA activation. FET cells treated with TGF $\beta$  (5 ng/ml) and OSI-906 together showed an increase in PKA activation (A). OSI-906 mediated PKA activation is Smad3-dependent as determined by knockdown of Smad3 in FET cells (B). All experiments were repeated three times independently with 3 replicas per repeat for the ANOVA analysis (\* P < 0.001).

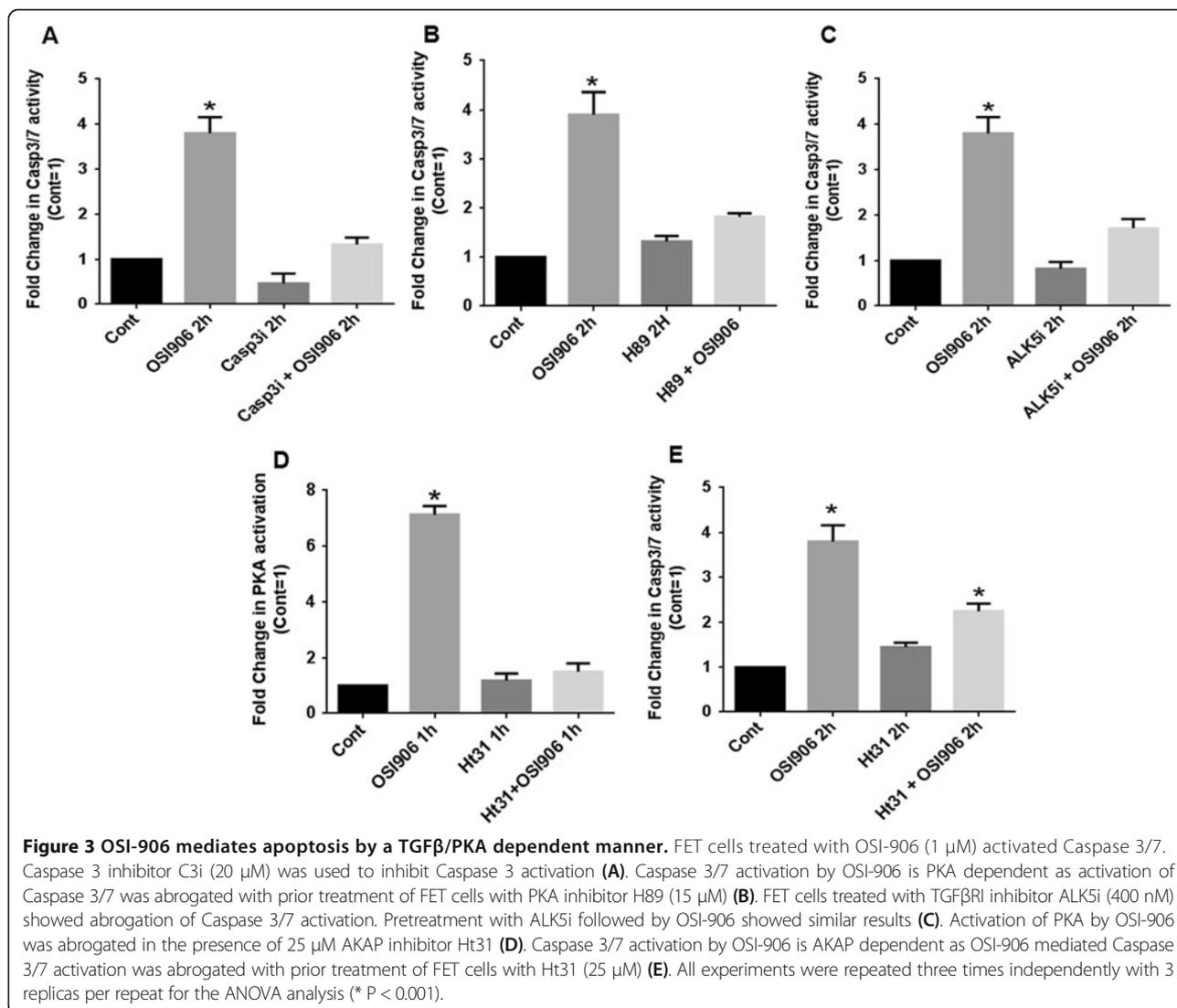
that pre-treatment of FET cells with ALK5i for 1 h suppressed OSI-906 mediated PKA activation thus indicating the dependence of functional TGF $\beta$  signaling for the OSI-906 effects on PKA. Treatment with TGF $\beta$  led to approximately 4-fold increase in PKA activation. However, a 16-fold increase in PKA activation was observed in cells treated with both OSI-906 and exogenous TGF $\beta$  for 1 h as shown in Figure 2A further confirming the role of TGF $\beta$  signaling in the OSI-906 effects on PKA activation. 2way ANOVA with Bonferroni's post-tests on TGF $\beta$ - and OSI-906-mediated PKA activity demonstrated a synergistic effect on PKA activation upon combination of TGF $\beta$  and OSI-906 treatment on FET cells ( $p < 0.0001$ ). Therefore, we had established that cross-talk between the IGF1R and TGF $\beta$  signaling pathways converge at PKA activation. Earlier work had emphasized the role of Smad3 in TGF $\beta$  mediated PKA activation and downstream pro-apoptotic signaling [9]. We also generated stable shRNA Smad3 knockdown in FET cells (designated FET S3KD) to genetically confirm that OSI-906 treatment was unable to activate PKA (Figure 2B).

Based on our previous report that TGF $\beta$ /PKA signaling mediated induction of cell death in CRC cells and the current finding that OSI-906 mediated PKA activation that was dependent on TGF $\beta$  signaling, we hypothesized that OSI-906 treatment would lead to TGF $\beta$ /PKA mediated cell death. We observed that OSI-906 treatment was able to increase caspase 3/7 activation by approximately 4-fold (Figure 3A). The caspase 3/7 activity was abrogated in the presence of a caspase 3 inhibitor (C3i) in both control and OSI-906 treated FET cells as expected (Figure 3A). Moreover, this caspase 3/7 activation was greatly abrogated in the presence of H89 to

block PKA activity (Figure 3B), indicating that OSI-906-mediated activation of the caspase 3/7 requires the activation of PKA. The requirement for TGF $\beta$  signaling for OSI-906/PKA mediated caspase 3/7 induction was observed in FET cells in the presence of ALK5i for 1 h followed by OSI-906 treatment for 4 h. Caspase 3/7 activation was greatly abrogated as compared to FET cells treated with OSI-906 (Figure 3C). We also observed PARP cleavage associated with OSI-906 treatment. However, pre-treatment with Alk5i followed by OSI-906 treatment abrogated PARP cleavage (Additional file 1: Figure S3).

#### OSI-906 mediated PKA activation and promotion of apoptosis requires AKAP

AKAPs mediate cytoskeleton dynamics, apoptosis, proliferation, growth, and other cellular processes [16]. Along with serving as anchors for PKA, AKAPs also serve as molecular scaffolds for substrates of PKA as well as other molecules and enzymes involved in specific PKA mediated biological processes [16]. Previously we showed that AKAPs are a critical component in TGF $\beta$ /PKA mediated cell survival regulation [9]. AKAP inhibitor Ht31, a synthetic thyroid anchoring peptide has been shown to be a very potent competitive inhibitor of PKARII/AKAP interaction, thereby preventing PKA anchoring. We performed PKA activity assays on FET cells to determine the extent of PKA activation following pre-treatment with Ht31 (25  $\mu$ M) prior to OSI-906 exposure (Figure 3D). In agreement with our earlier studies, Ht31 was able to block the OSI-906 mediated PKA activation. Since AKAP/PKA interaction appears to be a requirement for OSI-906 mediated PKA activation, we reasoned that these interactions should also be required for

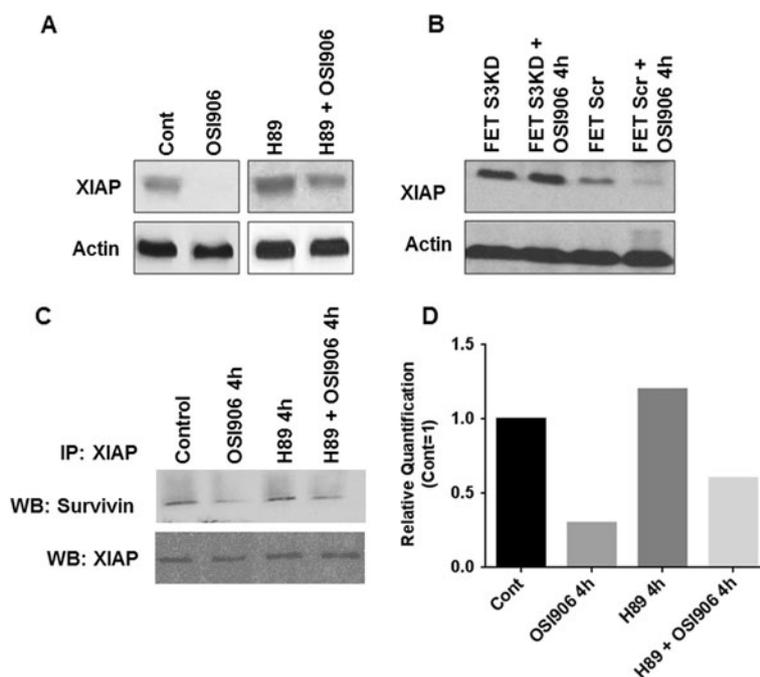


downstream signaling events leading to caspase 3/7 activation (Figure 3E). Addition of Ht31 inhibitor for 1 h prior to OSI-906 treatment for specified times partially abrogated the caspase 3/7 activity.

#### OSI-906 inhibits the expression of XIAP in a TGFβ/PKA dependent manner

XIAP plays a critical role in inhibiting apoptosis by binding to caspase 3, 7 and 9 and thereby inhibiting their pro-apoptotic activity. XIAP and survivin form a stabilizing complex that plays an important role in cell survival and metastasis [21,22]. We have reported that TGFβ/PKA signaling converges on XIAP downregulation in mediating its pro-apoptotic effects in FET cells [9]. Furthermore, it has been demonstrated that PKA activation leads to phosphorylation of survivin on Ser20 in the cytosol. This phosphorylation of survivin has been shown to disrupt survivin/XIAP binding leading to XIAP degradation [11].

We hypothesized that OSI-906 mediated PKA activation would lead to a TGFβ dependent XIAP degradation. It was observed that OSI-906 treatment led to the inhibition of XIAP expression (Figure 4A). Moreover, the OSI-906 mediated downregulation was dependent upon PKA activation as well as TGFβ signaling. When FET cells were pre-treated with H89 for 1 h prior to OSI-906 treatment, the downregulation of XIAP was abrogated and XIAP levels were stabilized (Figure 4A). To illustrate the necessity of TGFβ signaling in the OSI-906 mediated XIAP loss, stable shRNA Smad3 knockdown cell lines (FETS3KD) were utilized as described above. It was observed that OSI-906 treatment of FETS3KD cells had no effect on XIAP protein expression (Figure 4B). Furthermore, inhibition of IGF1R led to abrogation of the survivin/XIAP complex as determined by immunoprecipitation (IP) assay (Figure 4C). However, pre-treatment with H89 prevented survivin/XIAP abrogation by OSI-906. The western blots



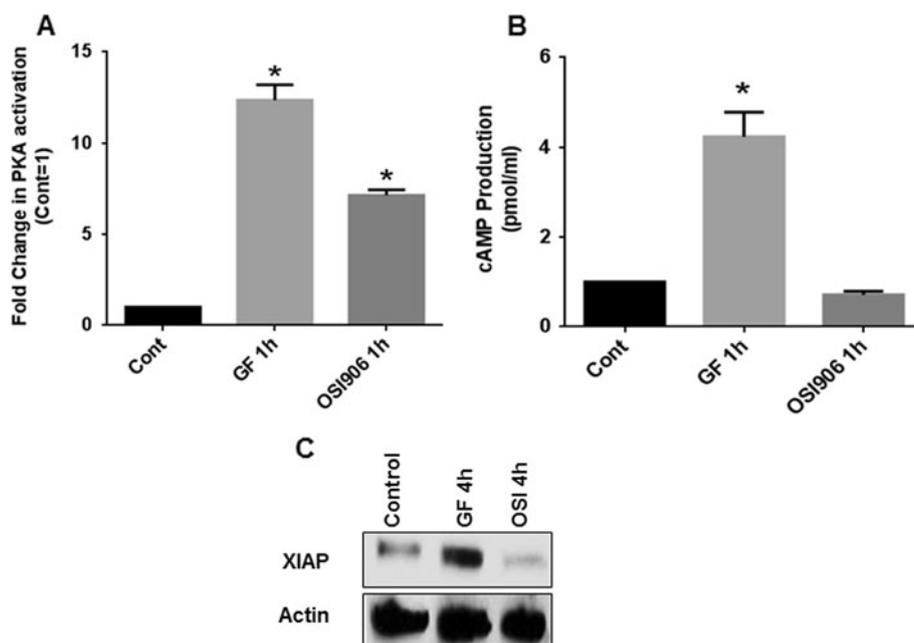
**Figure 4 OSI-906 regulates XIAP in a TGF $\beta$ /PKA dependent manner.** FET cells treated with OSI-906 (1  $\mu$ M) showed effective downregulation of XIAP. This observed downregulation is PKA dependent as downregulation of XIAP was abrogated in FET cells treated with PKA inhibitor H89 (15  $\mu$ M) (A). OSI-906 mediated XIAP downregulation is dependent on Smad3 as determined by shRNA knockdown of Smad3 in FET cells (termed as FETS3KD). FETS3KD cells treated with OSI-906 showed no change in XIAP expression. However, FET Scr cells showed XIAP downregulation following treatment with OSI-906 (B). FET cells treated with OSI-906 effectively dissociated XIAP from Survivin as determined by immunoprecipitation (IP) assay (C). OSI-906 mediated XIAP/Survivin dissociation is PKA dependent as dissociation was abrogated when FET cells were treated with H89 and/or pretreated with H89 prior to OSI-906 treatment. Relative quantification of the IP has been performed showing reduced interaction between survivin/XIAP following OSI-906 treatments (D).

were quantified using the NIH Image J software to demonstrate the quantitative difference in survivin protein that was immunoprecipitated with XIAP antibody following OSI-906 treatment (Figure 4D). Most studies involving survivin/XIAP complex formation have been demonstrated using overexpression methods as only a small fraction of the total cellular survivin and XIAP form a complex. In our study, we used the endogenous protein as opposed to overexpression approaches to eliminate any artifacts in the interaction that may arise due to overexpression of survivin or XIAP in the parental cells. Earlier work from our laboratory has demonstrated that TGF $\beta$  treatment causes the dissociation of survivin/XIAP complex through the TGF $\beta$ /PKA signaling pathway in poorly metastatic CRC cells with functional TGF $\beta$  tumor suppressor signaling (designated CBSRII cells) as opposed to their highly metastatic counterparts deficient in functional TGF $\beta$  tumor suppressor signaling (designated CBS cells) [9].

#### Differential activation of PKA by modulation of IGF1R signaling determines cell fate

As demonstrated above, OSI-906 mediates PKA activation in a cAMP independent, TGF $\beta$ /Smad3/AKAP149 dependent manner, leading to increased apoptosis. It is

well known that classical cAMP-dependent PKA activation leads to increased cell survival signaling by transcriptional upregulation of several oncogenes. We hypothesized that ligand mediated activation of IGF1R signaling activates PKA in a cAMP-dependent manner and would lead to increased cell survival. Previous work from our laboratory have extensively used Transferrin and Insulin (jointly termed here as GF or growth factors) in combination to activate IGF-1R-mediated cell survival signaling in colon cancer cells [23-27]. Wang et al has shown that the mitogenicity of insulin (at the concentration used) in cultures of these cells is not through insulin receptor but instead through the IGF1R [27]. Intriguingly, GF mediated activation of IGF1R lead to a robust 10-fold activation of PKA (Figure 5A). This PKA activation by IGF1R was found to be cAMP-dependent as determined by cAMP assay (Figure 5B). We further observed an increased expression of XIAP following IGF1R activation (Figure 5C). This is in direct contrast to the OSI-906 mediated cAMP independent PKA activation and XIAP downregulation. Thus, the dichotomy of PKA activation decides the downstream survival or apoptotic cellular signaling fate in these subsets of CRC cells.



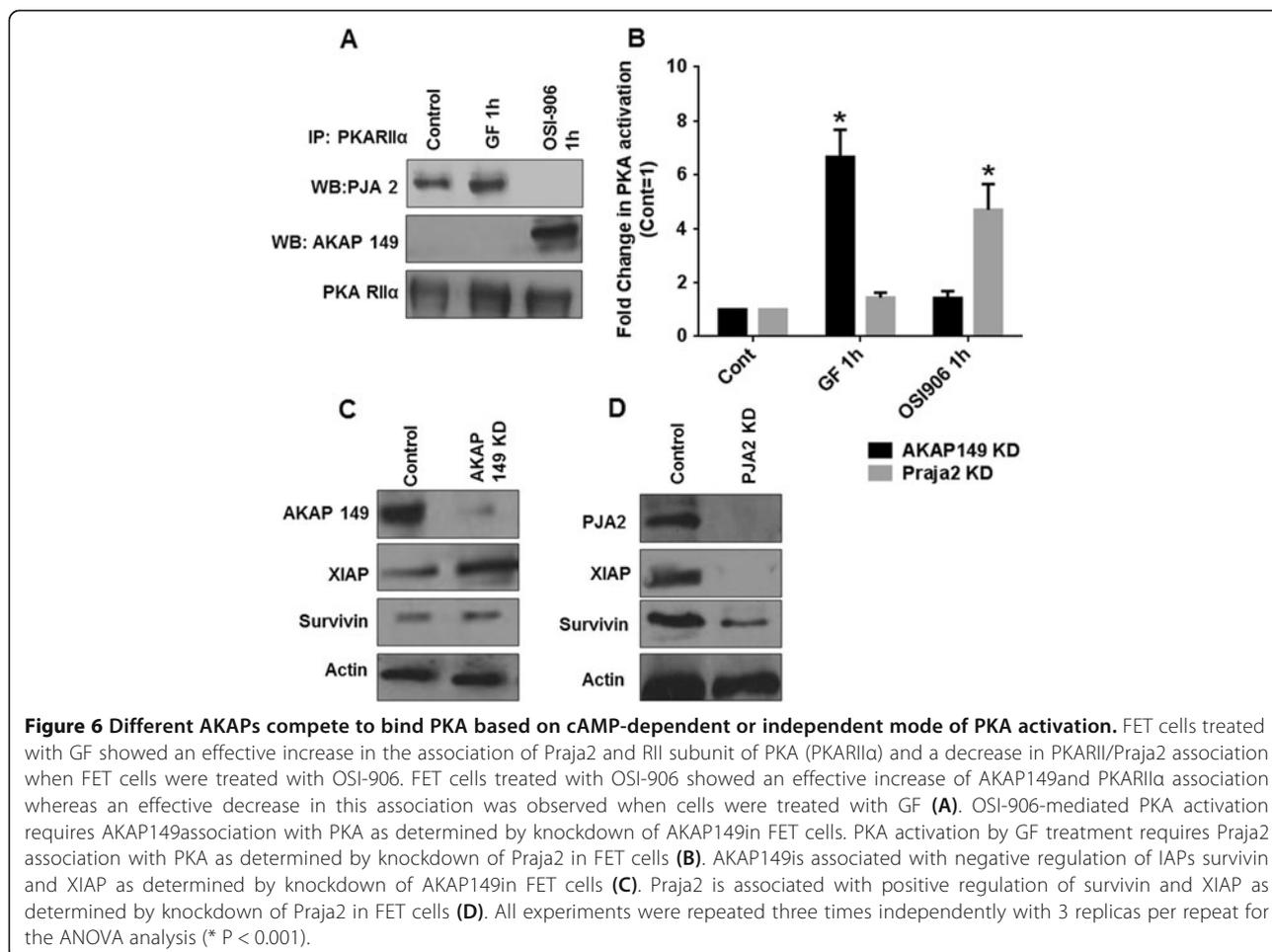
**Figure 5 Differential PKA activation decides cell survival or apoptotic fate.** FET cells treated with Growth Factors (or GF that includes a combination of Transferrin and Insulin) or OSI-906 (1  $\mu$ M) activated PKA (A). FET cells treated with GF showed cAMP-dependent activation of PKA whereas FET cells treated with OSI-906 showed cAMP-independent activation of PKA as determined by cAMP assay (B). FET cells treated with GF showed an upregulation of XIAP, whereas cells treated with OSI-906 showed a downregulation of XIAP (C). All experiments were repeated three times independently with 3 replicas per repeat for the ANOVA analysis (\*  $P < 0.001$ ).

### Different AKAPs compete to bind PKA based on PKA activation

Earlier work from our laboratory demonstrated the role of mitochondrial AKAP149 as playing a key role in regulating the TGF $\beta$ /PKA effects on cell survival by regulating XIAP mediated cell survival and inducing cell death in CRC cells [9]. This is critical since it is mitochondrial XIAP and survivin that move to the cytoplasm following stress response [11]. AKAP149 siRNA knockdown prevented TGF $\beta$  mediated PKA activation and XIAP downregulation [9]. Recently, Lignitto et al. [17] identified Praja2 as an AKAP-like E3 ligase scaffold which associates with the PKARII subunit in the presence of elevated concentrations of cAMP. Praja2 increases PKA activation by liberating the catalytic subunits from the regulatory subunits in a cAMP-dependent fashion. It was further demonstrated that Praja2 binds to AKAP149 [17]. We hypothesized that Praja2 recruits PKA and facilitates pro-survival functions following ligand mediated cAMP-dependent IGF1R/PKA activation. We observed that Praja2 required ligand mediated activation of IGF1R/PKA signaling to associate with the PKA RII subunit by IP analysis. However, in the presence of OSI-906, the association between Praja2 and PKA RII is completely abrogated in FET cells (Figure 6A). As demonstrated above, both activation and inhibition of IGF1R lead to a robust activation of PKA either

by cAMP-dependent or independent mechanisms, respectively. Importantly, the 2 opposing mechanisms of PKA activation by OSI-906 and ligand-mediated IGF1R activation selectively bind to different AKAPs (AKAP149 or Praja2) (Figure 6A). Treatment of FET cells with OSI-906 for 1 h lead to a robust binding of PKA RII with AKAP149. However, unlike Praja2 association with PKA RII under cAMP-dependent conditions, no association of PKA RII and AKAP149 was observed under GF treatment (Figure 6A). Interestingly, robust increase in AKAP149/PKA RII association was observed under treatment with TGF $\beta$  (Additional file 1: Figure S4). These results implicate AKAP149 as a pro-apoptotic AKAP, whereas Praja2 as a pro-survival AKAP that promotes aberrant cell survival in CRC cells.

We further hypothesized that the elimination of Praja2 using siRNA knockdown would lead to increased pro-apoptotic signaling. To test this hypothesis, we transiently knocked down Praja2 and AKAP149 proteins, respectively, by siRNA in FET cells. As shown in Figure 6B, siRNA knockdown of AKAP149 completely abrogated OSI-906 mediated PKA activation. However, ligand-mediated activation of IGF1R led to robust PKA activation in the AKAP149 knockdown cells. Interestingly, Praja2 siRNA knockdown showed the opposite results with an increase in OSI-906 mediated PKA activation (Figure 6B). Previously we showed that AKAP149 siRNA knockdown



led to an increase in XIAP mediated cell survival and inhibition of apoptosis [9]. As shown in Figures 6C and 6D, siRNA knockdown of AKAP149 leads to increased survivin and XIAP expression whereas transient knockdown of Praja2 leads to a decrease in survivin and XIAP expression. These data further confirms the role of AKAP149 and Praja2 in pro-apoptotic and pro-survival signaling, respectively.

## Discussion

We have shown that IGF1R and TGF $\beta$  engage in a previously unidentified form of crosstalk that controls cell survival by opposing modes of PKA activation with respect to dependence upon cAMP. The IGF1R kinase inhibitor OSI-906 was associated with TGF $\beta$  mediated PKA activation that was independent of cAMP, but was dependent on AKAP as shown by the use of an AKAP inhibitor Ht31. Previous work from our laboratory has established the role of AKAP149 in TGF $\beta$ /PKA tumor suppressor signaling. In the present work, we observed that OSI-906 also mediates its pro-apoptotic effects through AKAP149, as determined by immunoprecipitation and knockdown studies (Figures 6A-6D). The OSI-

906 mediated PKA activation is dependent on TGF $\beta$  signaling, as ALK5i (TGF $\beta$ R1 kinase inhibitor) or Smad3 KD resulted in abrogation of the OSI-906 mediated PKA activation. In addition, TGF $\beta$  treatment increased PKA activation. OSI-906 also induced caspase 3/7 mediated apoptosis and downregulation of XIAP which was dependent on TGF $\beta$  signaling and cAMP-independent PKA activation. The OSI-906 mediated downregulation of these IAPs was also abrogated in Smad3 KD cells. Survivin promotes survival by binding to the BIR3 domain of XIAP in the cytosol and prevents XIAP auto-ubiquitination, thus allowing XIAP to associate with caspases and prevent apoptosis [10,28-30]. Treatment with OSI-906 dissociated XIAP from survivin as demonstrated by co-immunoprecipitation analysis. However, this dissociation was inhibited when PKA activity was blocked by pre-treatment with H89 to abrogate PKA activity, thus illustrating the critical role of cAMP-independent PKA signaling in the OSI-906 mediated effects on apoptosis. Treatment with IGF1R-specific monoclonal activity MK-0646 also activated PKA although with a lower potency than OSI-906. This can be attributed to OSI-906 being a dual kinase IGF1R +

Insulin Receptor (IR) inhibitor which might account for a more potent effect on PKA activation compared to a blocking antibody against IGF1R.

Interestingly, IGF1R activation also led to robust activation of PKA in a cAMP-dependent manner. It was determined that the IGF1R mediated PKA activation was cAMP-dependent as reflected by elevated levels of cAMP following IGF1R activation leading to a pro-survival cellular environment. An important observation from this study was the demonstration of the dependence of IGF1R mediated cAMP/PKA aberrant cell survival signaling on Praja2. Praja2 is an AKAP which is associated with the RII regulatory subunit of PKA and is dependent upon elevated cAMP levels for binding of PKA [17]. Praja2 binds to PKARII through its regulatory subunit binding domain (RBD) [17]. This AKAP-like scaffolding protein contains a RING finger domain which confers E3 ubiquitin ligase activity enabling Praja2 mediated cell survival. Lignitto et al. [17] demonstrated that in the presence of elevated cAMP levels Praja2 binds to PKARII. The binding of Praja2 with PKARII causes ubiquitination of PKARII followed by its proteasomal degradation [17]. In line with the pro-survival function of Praja2, we showed that the knock-down of Praja2 in CRC cells blocked IGF1R induced PKA activation and downregulated survivin and XIAP expression.

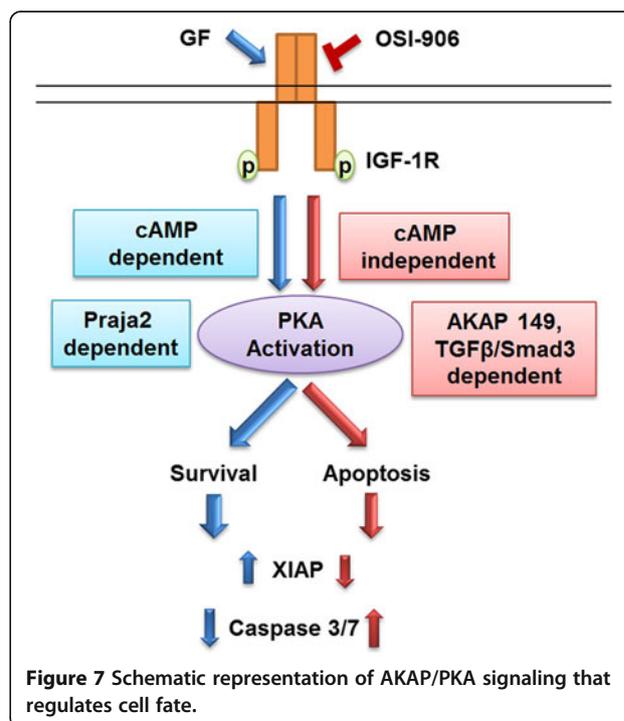
This study shows AKAP dependent PKA activation in the cytoplasm based on cAMP dependence or independence leads to activation of 2 opposing pathways to orchestrate cell fate in terms of life or death thus adding further levels of complexity to the many roles performed by PKA. Deregulation of AKAPs has been shown in numerous studies to lead to cancer [31]. For example, Gravin/PKA association induces cell cycle arrest and apoptosis via cyclin D1 downregulation and activation of caspase 3 respectively [31]. PKA also plays a key role in DNA replication via its association with AKAP95 [31].

In this study, we have elucidated dual mechanisms of PKA activation. The pro-apoptotic mechanism does not require cAMP, and directs PKA to induce apoptosis in CRC cells [9]. We also show that activation of PKA in a cAMP-dependent manner via IGF1R activation resulted in PKA induced cell survival. These findings have elucidated a dichotomous role for PKA in cell survival that is ultimately determined by directing cell fate at the level of cAMP dependence and selection of binding to AKAP149 or Praja2. In essence, cAMP dependency reflects a form of novel crosstalk between TGF $\beta$  and IGF1R signaling which influences the equilibrium between cell death and cell survival. Activation of IGF1R diverts this equilibrium in favor of cell survival by activating PKA in a manner that is cAMP/ Praja2 dependent. This activation leads to the upregulation of

XIAP and downregulation of caspase 3/7 activation. On the other hand, inhibition of IGF1R also leads to activation of PKA. This activation requires endogenous TGF $\beta$  signaling as well as AKAP149 association. The data indicate that the dual nature of PKA activation is accomplished by differential AKAP association. As observed, AKAP149 and Praja 2 associate with PKA in a cAMP independent or cAMP-dependent fashion respectively. This finding has been schematically represented in Figure 7. While Praja2 association leads to proteasomal degradation of PKA RII via its ubiquitin ligase activity, AKAP149 association may be enabling other molecules of the TGF $\beta$ /PKA pathway to be in close proximity with PKA to achieve specific pro-apoptotic processes. Specific molecular signatures associated with AKAP complex signaling will enhance our understanding of the functions of these AKAPs and how they might be exploited as novel targets for therapy.

## Conclusion

We have shown that IGF1R activation in cell lines of IGF1R-dependent CRC subset results in the induction of cAMP-dependent PKA activity that stimulates survivin/XIAP complex formation and mediates cell survival. Moreover, a different AKAP called Praja2 from that which generates cAMP independent activity (AKAP149) mediates this function. Thus, cellular fate with respect to life and death in response to stress in these cancer cells appears to be a cytoplasmic function that depends upon the dynamics of the interplay between these 2



**Figure 7** Schematic representation of AKAP/PKA signaling that regulates cell fate.

AKAPs and their anchoring functions of the components of the 2 opposing pathways that they harbor. Understanding the dynamics of the molecular controls by which the cells select life or death will be a significant addition to our conceptual understanding of how AKAPs function to organize overall cytoplasmic signaling in response to changes in cellular environment. Characterization of AKAP processing of life and death signals for determining cell fate may well identify additional factors that are potential novel targets for enhancing treatment that can counter aberrant cell survival properties of cancer cells by promoting the biological consequences of cAMP independent PKA activation.

## Methods

### Cell culture and reagents

The FET, CBS and GEO colon carcinoma cells were originally isolated from CRC primary tumors and have been extensively characterized [32,33]. These cells were maintained at 37°C in humidified atmosphere in a chemically defined serum free (SF) medium containing McCoy's 5A medium (Sigma-Aldrich) supplemented with amino acids, pyruvate, vitamins, antibiotics, and growth factors transferrin (4 µg/ml; Sigma), insulin (20 µg/ml; Sigma), and Epidermal Growth Factor (EGF) (10 ng/ml; R&D Systems). Cells were routinely subcultured with a 0.25% trypsin (Invitrogen) in Joklik's medium (Invitrogen) containing 0.1% EDTA. Cells were harvested on day 5 after the addition of specific pharmacological agents at different time points. OSI-906 was purchased from Chemitec. TGFβ1 was purchased from R&D. MK-0646 was obtained from Merck & Co.

### Antibodies, western blotting and immunoprecipitation

Survivin antibody (#2808) and PKA inhibitor H89 (# 9844) was obtained from Cell Signaling Technology. The PKA RII (# ab124400), XIAP (#s ab21278; ab28151), Praja2 (#s ab131118; ab56876), PARP (# ab4830) and Smad3 (# ab28379) antibodies were purchased from Abcam. Anti-Actin antibody was purchased from Sigma (# A2066). The AKAP inhibitor Ht31 was obtained from Promega (# V8211). Cells were lysed in TNESV lysis buffer (50 mmol/liter Tris (pH 7.5), 150 mmol/liter NaCl, 1% NP40, 50 mmol/liter NaF, 1 mmol/liter Na<sub>3</sub>VO<sub>4</sub>, 25 µg/ml β-glycerophosphate, 1 mmol/liter phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail (Roche, Indianapolis, IN) as previously described [9,13,34,35]. Western blotting was performed using previous established protocol from our laboratory [9,13,34-36]. Co-Immunoprecipitation (IP) was performed with 500 µg protein aliquots using magnetic beads (Millipore) according to the instructions provided by the manufacturer (# LSKMAGAG10) and previously established method [9]. Binding of

survivin to XIAP was tested by IP with an antibody to XIAP (Clone 2 F1) at a concentration of 2 µg/mL [37] which was selected as the optimal concentration of IP based on antibody titration and immunoblotted from survivin.

### RNA interference studies

PKAα Cat (sc-36240), Praja2 (sc-91836) and AKAP149 (sc-136355) siRNAs and Smad3 shRNA (sc-77326-SH) were obtained from Santa Cruz Biotechnology. Transfection was performed according to manufacturer's protocol and previously established methods in our laboratory [9].

### PKA activity assay

PKA activity was measured using the PepTag non-radioactive protein kinase assay (Promega, catalogue no. V5340) using kemptide (LRRASLG) adhering to the protocol provided by the manufacturer [9]. This assay utilizes brightly colored, fluorescent peptide substrate as mentioned above, that is highly specific to PKA.

### cAMP assay

For quantitative determination of cAMP, a non-radioactive Direct Cyclic AMP Enzyme Immunoassay kit (Enzo Life Sciences, catalogue no. ADI-901-066) was employed. The protocol provided by the manufacturer was used [9].

### Caspase activity assay

Caspase 3/7 activity was measured using the Apo-ONE Homogenous Caspase 3/7 assay (Promega, catalogue no. G7790) using caspase inhibitor Ac-DEVD-CHO (Promega, catalogue no. G5961). The Apo-ONE Homogeneous Caspase 3/7 kit contains the caspase 3/7 substrate rhodamine; Z-DEVD-R110 as a profluorescent substrate. Following mixture of buffer and substrate along with cell lysates under different treatment conditions cleavage of DEVD peptides occur by caspase 3/7 activity which is measured by fluorometric methods.

### Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were tested for statistical significance by using one-way Anova using GraphPad Prism 5 software. Only P values of <0.001 were considered significant. All experiments have been repeated three times independently with 3 replicas per repeat for the ANOVA analysis.

### Additional file

**Additional file 1: Figure S1.** PKA activity assay in IGF1R-dependent GEO and CBS cells. Treatment with OSI-906 (1 µM) leads to increase in PKA activation. Forskolin was used as a positive control. **Figure S2:** MK-0646 activates PKA in FET Cells. Treatment with MK-0646, a humanized recombinant monoclonal antibody against IGF-1R activates PKA FET cells.

Forskolin was used as a positive control. **Figure S3:** PARP activation by OSI-906 treatment is dependent on TGFβ/PKA signaling. Treatment with OSI906 (1 μM) for 4 h lead to PARP cleavage. Pretreatment with ALK5i (400nM) or H89 (10 μM) followed by OSI-906 treatment abrogate the PARP cleavage.

**Figure S4:** TGFβ Signaling regulates AKAP 149/ PKARIIa interaction in FET cells. Treatment with TGFβ (5 ng/mL) for 4 h lead to robust increase in AKAP149/PKA RII interaction. Pretreatment with H31 (25 μM) followed by TGFβ treatment partially abrogated the AKAP149/PKA RII interaction.

#### Abbreviations

CRC: Colorectal cancer; IGF1R: Insulin-like growth factor receptor 1; AKAP: A-kinase anchoring protein; PKA: Protein kinase A; XIAP: X-linked inhibitor of apoptosis.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

EDH carried out the different PKA, cAMP and Caspase assays in the study, analyzed the data and drafted the manuscript. EA, PDL, KHL helped with performing western blotting, drug treatments and transient transfection using siRNA. MGB and SC participated in the conception and design of the study and helped to draft the final manuscript. All authors read and approved the final manuscript.

#### Acknowledgements

This research study was funded by the National Cancer Institute R01 grants to MGB (CA054807, CA034432, CA038173 and CA072001).

Received: 28 May 2013 Accepted: 24 September 2013

Published: 1 October 2013

#### References

- Rodon J, DeSantos V, Ferry RJ Jr, Kurzrock R: **Early drug development of inhibitors of the insulin-like growth factor-I receptor pathway: lessons from the first clinical trials.** *Mol Cancer Ther* 2008, **7**(9):2575–2588.
- Ryan PD, Goss PE: **The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer.** *Oncologist* 2008, **13**(1):16–24.
- Valentinis B, Baserga R: **IGF-I receptor signalling in transformation and differentiation.** *Mol Pathol* 2001, **54**(3):133–137.
- Buck E, Gokhale PC, Koujak S, Brown E, Eyzaguirre A, Tao N, Rosenfeld-Franklin M, Lerner L, Chiu MI, Wild R, et al: **Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): rationale for cotargeting IGF1R and IR in cancer.** *Mol Cancer Ther* 2010, **9**(10):2652–2664.
- Godsland IF: **Insulin resistance and hyperinsulinaemia in the development and progression of cancer.** *Clin Sci (Lond)* 2009, **118**(5):315–332.
- Weber MM, Fottner C, Liu SB, Jung MC, Engelhardt D, Baretton GB: **Overexpression of the insulin-like growth factor I receptor in human colon carcinomas.** *Cancer* 2002, **95**(10):2086–2095.
- Mulvihill MJ, Cooke A, Rosenfeld-Franklin M, Buck E, Foreman K, Landfair D, O'Connor M, Pirritt C, Sun Y, Yao Y, et al: **Discovery of OSI-906: a selective and orally efficacious dual inhibitor of the IGF-1 receptor and insulin receptor.** *Future Med Chem* 2009, **1**(6):1153–1171.
- Pitts TM, Tan AC, Kulikowski GN, Tentler JJ, Brown AM, Flanigan SA, Leong S, Coldren CD, Hirsch FR, Varella-Garcia M, et al: **Development of an integrated genomic classifier for a novel agent in colorectal cancer: approach to individualized therapy in early development.** *Clin Cancer Res* 2010, **16**(12):3193–3204.
- Chowdhury S, Howell GM, Rajput A, Teggart CA, Brattain LE, Weber HR, Chowdhury A, Brattain MG: **Identification of a novel TGFβ/PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer.** *PLoS One* 2011, **6**(5):e19335.
- Dubreix-Daloz L, Dupoux A, Cartier J: **IAPs: more than just inhibitors of apoptosis proteins.** *Cell Cycle* 2008, **7**(8):1036–1046.
- Dohi T, Xia F, Altieri DC: **Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection.** *Mol Cell* 2007, **27**(1):17–28.
- Mehrotra S, Languino LR, Raskett CM, Mercurio AM, Dohi T, Altieri DC: **IAP regulation of metastasis.** *Cancer Cell* 2010, **17**(1):53–64.
- Chowdhury S, Howell GM, Teggart CA, Chowdhury A, Person JJ, Bowers DM, Brattain MG: **Histone deacetylase inhibitor belinostat represses survivin expression through reactivation of transforming growth factor beta (TGFβ) receptor II leading to cancer cell death.** *J Biol Chem* 2011, **286**(35):30937–30948.
- Agarwal E, Brattain MG, Chowdhury S: **Cell survival and metastasis regulation by Akt signaling in colorectal cancer.** *Cell Signal* 2013, **25**(8):1711–1719.
- Pidoux G, Tasken K: **Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins.** *J Mol Endocrinol* 2010, **44**(5):271–284.
- Welch EJ, Jones BW, Scott JD: **Networking with AKAPs: context-dependent regulation of anchored enzymes.** *Mol Interv* 2010, **10**(2):86–97.
- Lignitto L, Carlucci A, Sepe M, Stefan E, Cuomo O, Nistico R, Scorziello A, Savoia C, Garbi C, Annunziato L, et al: **Control of PKA stability and signalling by the RING ligase praja2.** *Nat Cell Biol* 2011, **13**(4):412–422.
- Zhang L, Duan CJ, Binkley C, Li G, Uhler MD, Logsdon CD, Simeone DM: **A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A.** *Mol Cell Biol* 2004, **24**(5):2169–2180.
- Wang J, Yang L, Yang J, Kuropatwinski K, Wang W, Liu XQ, Hauser J, Brattain MG: **Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells.** *Cancer Res* 2008, **68**(9):3152–3160.
- Donovan EA, Kummar S: **Role of insulin-like growth factor-1R system in colorectal carcinogenesis.** *Crit Rev Oncol Hematol* 2008, **66**(2):91–98.
- Schimmer AD, Dalili S: **Targeting the IAP family of caspase inhibitors as an emerging therapeutic strategy.** *Hematology Am Soc Hematol Educ Program* 2005:215–219.
- Schimmer AD, Dalili S, Batey RA, Riedl SJ: **Targeting XIAP for the treatment of malignancy.** *Cell Death Differ* 2006, **13**(2):179–188.
- Awwad RA, Sergina N, Yang H, Ziober B, Willson JK, Zborowska E, Humphrey LE, Fan R, Ko TC, Brattain MG, et al: **The role of transforming growth factor alpha in determining growth factor independence.** *Cancer Res* 2003, **63**(15):4731–4738.
- Boyd DD, Levine AE, Brattain DE, McKnight MK, Brattain MG: **Comparison of growth requirements of two human intratumoral colon carcinoma cell lines in monolayer and soft agarose.** *Cancer Res* 1988, **48**(9):2469–2474.
- Hu YP, Patil SB, Panasiwicz M, Li W, Hauser J, Humphrey LE, Brattain MG: **Heterogeneity of receptor function in colon carcinoma cells determined by cross-talk between type I insulin-like growth factor receptor and epidermal growth factor receptor.** *Cancer Res* 2008, **68**(19):8004–8013.
- Jiang D, Yang H, Willson JK, Liang J, Humphrey LE, Zborowska E, Wang D, Foster J, Fan R, Brattain MG: **Autocrine transforming growth factor alpha provides a growth advantage to malignant cells by facilitating re-entry into the cell cycle from suboptimal growth states.** *J Biol Chem* 1998, **273**(47):31471–31479.
- Wang D, Li W, Jiang W, Humphrey LE, Howell GM, Brattain MG: **Autocrine TGFα expression in the regulation of initiation of human colon carcinoma growth.** *J Cell Physiol* 1998, **177**(3):387–395.
- Andersen MH, Svane IM, Becker JC, Straten PT: **The universal character of the tumor-associated antigen survivin.** *Clin Cancer Res* 2007, **13**(20):5991–5994.
- Eckelman BP, Salvesen GS, Scott FL: **Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family.** *EMBO Rep* 2006, **7**(10):988–994.
- Fabregat I: **Dysregulation of apoptosis in hepatocellular carcinoma cells.** *World J Gastroenterol* 2009, **15**(5):513–520.
- Almeida MQ, Stratakis CA: **How does cAMP/protein kinase A signaling lead to tumors in the adrenal cortex and other tissues?** *Mol Cell Endocrinol* 2011, **336**(1–2):162–168.
- Brattain MG, Levine AE, Chakraborty S, Yeoman LC, Willson JK, Long B: **Heterogeneity of human colon carcinoma.** *Cancer Metastasis Rev* 1984, **3**(3):177–191.
- Brattain MG, Marks ME, McCombs J, Finely W, Brattain DE: **Characterization of human colon carcinoma cell lines isolated from a single primary tumour.** *Br J Cancer* 1983, **47**(3):373–381.
- Chowdhury S, Ongchin M, Sharratt E, Dominguez I, Wang J, Brattain MG, Rajput A: **Intra-Tumoral Heterogeneity in Metastatic Potential and Survival Signaling between Iso-Clonal HCT116 and HCT116b Human Colon Carcinoma Cell Lines.** *PLoS One* 2013, **8**(4):e60299.

35. Chowdhury S, Ongchin M, Wan G, Sharratt E, Brattain MG, Rajput A: **Restoration of PTEN activity decreases metastases in an orthotopic model of colon cancer.** *J Surg Res* 2013, **184**(2):755–760.
36. Chowdhury S, Sharratt E, Spornyak J, Brattain MG, Rajput A: **Anti-tumor activity of IGF1R kinase inhibitor PQIP in colon cancer.** *Clin Exp Pharmacol* 2013, **54**:005.
37. Jost PJ, Grabow S, Gray D, McKenzie MD, Nachbur U, Huang DC, Bouillet P, Thomas HE, Borner C, Silke J, *et al*: **XIAP discriminates between type I and type II FAS-induced apoptosis.** *Nature* 2009, **460**(7258):1035–1039.

doi:10.1186/1750-2187-8-10

**Cite this article as:** Hedrick *et al.*: Differential PKA activation and AKAP association determines cell fate in cancer cells. *Journal of Molecular Signaling* 2013 **8**:10.

**Submit your next manuscript to BioMed Central  
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)

