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Apoptin Interacts with and Regulates the Activity of Protein Kinase C Beta in Cancer Cells

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Running Title: Apoptin activates and is regulated by PKC β

Abbreviations: CAV = chicken anaemia virus; PKC = protein kinase C; HGyV = human Gyrovirus; Thr-108 = threonine-108; PLA = proximity ligation assay; CKAR = C kinase activity reporter; FRET = fluorescence resonance energy transfer; TCSPC = time-correlated single-photon counting; FLIM = fluorescence lifetime imaging microscopy; CFP/YFP/GFP/RFP = cyan / yellow / green / red fluorescent protein; Cdk2 = cyclin-dependent kinase 2; NF- κ B = nuclear factor kappa B; ANOVA = analysis of variance

Keywords: Chicken Anaemia Virus; Apoptin; Protein Kinase C; FRET; Novel Therapeutic Agents

Abstract

1
2 Apoptin, the VP3 protein from chicken anaemia virus (CAV), induces tumour cell-
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4 specific cell death and represents a potential future anti-cancer therapeutic. In tumour
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6 but not in normal cells, Apoptin is phosphorylated and translocates to the nucleus,
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8 enabling its cytotoxic activity. Recently, the β isozyme of protein kinase C (PKC β)
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10 was shown to phosphorylate Apoptin in multiple myeloma cell lines. However, the
11
12 exact mechanism and nature of interaction between PKC β and Apoptin remain
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14 unclear. Here we investigated the physical and functional link between PKC β and
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16 CAV-Apoptin as well as with the recently identified Apoptin homologue derived
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18 from human Gyrovirus (HGyV).
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26 In contrast to HCT116 colorectal cancer cells the normal colon mucosa cell lines
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28 expressed low levels of PKC β I and showed reduced Apoptin activation, as evident by
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30 cytoplasmic localisation, decreased phosphorylation and lack of cytotoxic activity.
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32 Co-immunoprecipitation and proximity ligation assay (PLA) studies identified
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34 binding of both CAV- and HGyV-Apoptin to PKC β I in HCT116 cells. Using Apoptin
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36 deletion constructs the N-terminal domain of Apoptin was found to be required for
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38 interacting with PKC β I. FRET-based PKC activity reporter (CKAR) assays by
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40 fluorescence lifetime imaging microscopy (FLIM) showed that expression of Apoptin
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42 in cancer cells but not in normal cells triggers a significant increase in PKC activity.
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50 Collectively, the results demonstrate a novel cancer specific interplay between
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52 Apoptin and PKC β I. Direct interaction between the two proteins leads to Apoptin-
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54 induced activation of PKC and consequently activated PKC β I mediates
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56 phosphorylation of Apoptin to promote its tumour-specific nuclear translocation and
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58 cytotoxic function.
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Introduction

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3 Apoptin was originally identified as the apoptosis-inducing VP3 protein from chicken
4 anaemia virus (CAV), the first member of the Gyrovirus genus [1]. In recent years
5 several other viruses resembling the overall organisation of CAV have been
6 identified, including the first human Gyrovirus HGyV [2] which contains a VP3
7 protein with similar properties as CAV-Apoptin [3]. Importantly, Apoptin has the
8 ability to selectively kill various human tumour or transformed cells with little
9 cytotoxic effect in normal cells [4-7]. However, the precise cellular mechanisms of
10 Apoptin-induced cell death and its mode of tumour selectivity remain unclear [8]. In
11 general, Apoptin triggers caspase-dependent cell death via the intrinsic apoptotic
12 pathway [9, 10], which occurs independently of p53 but seems to require the activity
13 of pro-apoptotic TAp73 isoforms from the p53 family [11, 12].

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31 CAV-Apoptin is a small protein of 14 kDa, rich in proline, serine, threonine and basic
32 amino acids. It contains a bipartite nuclear localisation signal and a nuclear export
33 signal, which facilitate shuttling of the protein between the nucleus and cytoplasm
34 [13-15], as well as several potential phosphorylation sites, including threonine-108
35 (Thr-108). In tumour cells but not in normal cells Apoptin is phosphorylated on
36 Thr-108 [16], which promotes its accumulation in the nucleus of tumour cells whereas
37 in normal cells it predominantly localises to the cytoplasm. Both phosphorylation and
38 nuclear localisation are important for its pro-apoptotic function in tumour cells [15].
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A number of kinases including cyclin-dependent kinase 2 (Cdk2) have been

1 implicated in Apoptin phosphorylation [17]. Recently, the beta isoform of protein
2 kinase C (PKC β) was shown to interact with Apoptin and regulate its phosphorylation
3 and cytotoxic activity in multiple myeloma cells [18].
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8 PKC is a family of serine-threonine kinases, comprising at least 13 known isoforms
9 that differ in their tissue distribution, subcellular localisation and function [19]. They
10 play key roles in the regulation of essential biological processes including
11 proliferation, apoptosis and differentiation. Consequently, elevated levels or
12 differential activation of PKC isoforms have been linked to various types of cancer
13 including colon cancer and leukaemias [20-22]. All isoforms share a similar structure,
14 comprising a highly conserved catalytic domain, a regulatory domain and an
15 autoinhibitory pseudosubstrate, and can be further subdivided into classical, novel or
16 atypical isoforms depending on the composition of regulatory elements [23].
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31 In unstimulated cells the majority of PKC isoforms are located in the cytosol and
32 translocate to the plasma membrane upon stimulation for full activation by lipid co-
33 activators. Although this event has served as the hallmark for PKC activation, it is
34 unclear to what extent translocation to the plasma membrane actually corresponds to
35 catalytic activity and substrate phosphorylation. Genetically encodable biosensors
36 based on fluorescence resonance energy transfer (FRET) between fluorescent proteins
37 provide a novel tool to study signalling processes in cells, such as activation of
38 kinases [24] or Rho GTPases [24-26]. FRET refers to a non-radiative type of energy
39 transfer between an excited donor fluorophore and an acceptor fluorophore in close
40 proximity. This process depletes the donor population in the excited state, reducing
41 the fluorescence intensity as well as the fluorescence lifetime of the donor and
42 sensitising emission from the acceptor fluorophore. For the detection of PKC activity
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1 Violin *et al.* developed CKAR (C kinase activity reporter) which comprises a PKC
2 substrate sequence linked to CFP and YFP as well as the FHA2 phosphothreonine-
3 binding domain [27]. Activation of PKC triggers a conformational change that
4 reduces FRET between the two fluorophores, which can be measured using
5 ratiometric imaging of CFP to YFP fluorescence intensity. In contrast to intensity-
6 based methods for the detection of altered FRET, fluorescence lifetime imaging
7 microscopy (FLIM) is independent of fluorophore concentrations and stoichiometry,
8 requires only measurement of donor fluorescence and represents a state-of-the-art
9 technology to study cellular signalling events [28, 29].
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23 Here we show that the activity of Apoptin in human cancer and normal cells derived
24 from colon mucosa correlates with respective expression levels of PKC β I. Both
25 CAV- and HGyV-Apoptin were found to interact and co-localise with PKC β I in the
26 nucleus of cancer cells. Using a modified CKAR reporter plasmid in a FLIM setup we
27 detected Apoptin-induced activation of PKC in cancer cells but not in normal cells.
28 Taken together, the data highlight an important role of PKC β I for the regulation of
29 Apoptin in human cancer cells, which indicates a novel anti-proliferative function for
30 PKC β I in response to apoptotic stimuli. In addition to elucidating the tumour-specific
31 regulation of Apoptin the information obtained could be important for the design of
32 future PKC β -targeted strategies.
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Results

PKC β I expression levels correlate with Apoptin activity

Apoptin phosphorylation and its apoptotic activity are regulated by the PKC isoforms β I and β II in multiple myeloma cell lines [18]. We therefore investigated the link between PKC β and Apoptin in the human colorectal cancer cell line HCT116, which we found to be highly sensitive to Apoptin [11]. HCT116 cells demonstrated significantly higher levels of PKC β I expression than NCM460 cells derived from normal colon mucosa (Fig. 1a) while PKC β II levels did not show any marked difference (Fig. S1a).

Western blot analysis of HCT116 and NCM460 cells expressing FLAG-tagged Apoptin showed a significantly lower Apoptin phosphorylation in normal cells compared to cancer cells by approximately 40% (Fig. 1b). This decreased phosphorylation status of Apoptin was associated with a cytoplasmic expression pattern of Apoptin in NCM460 cells as shown by fluorescence and confocal microscopy, whereas in HCT116 cells Apoptin was exclusively present in the nucleus (Fig. 1c, d). In contrast to HCT116 cells, NCM460 cells remained completely resistant to Apoptin-induced apoptosis following infection with an adenoviral vector to express Apoptin (Fig. 1e). Transient knockdown of PKC β I in HCT116 cells to nearly undetectable levels was associated with a significant decrease in Apoptin phosphorylation by approximately 50% measured as the ratio of phosphorylated Apoptin (P-Apoptin) to total GFP-Apoptin (Fig. 1f).

The differential activity and expression of Apoptin was confirmed using another normal colon mucosa cell line, NCM356, with significantly lower expression levels of

1 PKC β I (Fig. S1a-d). Overall the results suggest a correlation between PKC β I
2 expression levels and the tumour-specific activity of Apoptin, indicated by its
3 phosphorylation, nuclear localisation and cytotoxicity.
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7 **Apoptin interacts with PKC β I in the nucleus of colon cancer cells**

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11 A number of cellular proteins have been identified as Apoptin binding partners and
12 regulators of its function and tumour cell-specific cytotoxicity. To test whether
13 CAV-Apoptin and its newly discovered homologue HGyV-Apoptin can directly bind
14 to PKC β I in colon cancer cells, HCT116 cells were co-transfected with HA-tagged
15 PKC β I and plasmids expressing FLAG-tagged Apoptin. Both CAV- and
16 HGyV-FLAG-Apoptin were present in immunocomplexes pulled down from total cell
17 extracts using anti-FLAG antibodies (Fig. 2a). As expected from their respective
18 amino acid sequences, HGyV-Apoptin was detected at a slightly higher molecular
19 weight than CAV-Apoptin [3]. Similarly, the reciprocal immunoprecipitation using
20 anti-HA antibodies confirmed binding of Apoptin to PKC β I in HCT116 cells (data
21 not shown).
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40 The interaction between Apoptin and endogenous PKC β I was further analysed using
41 the proximity ligation assay (PLA) which allows the direct detection and localisation
42 of protein interactions *in situ* [30]. Staining of HCT116 cells transfected with
43 FLAG-Apoptin using antibodies against Apoptin and FLAG served as a positive
44 control for all PLA experiments (Fig. 2b). To detect binding of Apoptin to PKC β I,
45 HCT116 cells were transfected with CAV- or HGyV-FLAG-Apoptin or the pcDNA3
46 control plasmid and stained with antibodies directed against FLAG and PKC β I,
47 followed by PLA analysis. Red fluorescent PLA signals, indicating a site of protein
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1 interaction, were detected in cells expressing both types of Apoptin but not cells
2 transfected with the control vector (Fig. 2c). Corresponding to the expression pattern
3 of Apoptin these were predominantly localised in the nucleus, indicating nuclear
4 translocation of PKC β I in response to Apoptin expression. Quantification of positive
5 PLA foci showed a significantly increased number of signals in Apoptin-expressing
6 cells compared to control cells (Fig. 2c). The clear interaction between Apoptin and
7 PKC β I observed by PLA is unlikely to be affected by cell death as no significant
8 induction of apoptosis was observed at the same time point following transfection of
9 Apoptin plasmids (data not shown).

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23 We next investigated the importance of the N-terminal part of Apoptin for binding to
24 PKC β . This region contains a leucine-rich stretch and has been implicated in
25 interaction of Apoptin with other cellular proteins. In contrast to cells expressing full-
26 length GFP-Apoptin, HCT116 cells transfected with an Apoptin mutant lacking
27 amino acids 1 to 73 showed no significant increase in the total number of PLA signals
28 compared to the GFP control (Fig. 2d). This data suggests that the C-terminal half of
29 Apoptin alone is not sufficient to mediate binding to PKC β I and that interacting
30 regions are located within the N-terminus. Additional Apoptin mutants need to be
31 tested in order to precisely map the region/s required for interacting with PKC β I.

42 43 44 45 46 **FRET-based measurements of PKC activity**

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50 PKC β -mediated Apoptin phosphorylation and Apoptin-induced translocation of
51 PKC β to the nucleus suggest a novel regulatory loop between Apoptin and PKC β
52 activities. To monitor PKC activity we employed a modified version of the PKC
53 activity reporter CKAR [27] which consists of the two fluorophores GFP and RFP
54 linked by a PKC substrate sequence and the FHA2 phosphothreonine-binding domain
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1 (Fig. 3a). As illustrated, PKC-induced phosphorylation of the substrate triggers a
2 conformational change, resulting in a decrease of FRET from GFP to RFP and
3
4 consequently an increase in the fluorescence intensity and lifetime of the donor
5 fluorophore GFP. We detected alterations in FRET by measuring the two-photon
6 excited fluorescence lifetime τ of GFP using a time-correlated single-photon counting
7 system (TCSPC) established for FLIM [31]. Mapping of GFP lifetimes across single
8 cells expressing CKAR allowed for the visualisation of PKC activity and calculation
9 of average GFP lifetimes (Fig. 3b). Comparison of a number of cells transfected with
10 the reporter plasmid to cells expressing only the donor fluorophore GFP showed a
11 clear decrease of average GFP lifetimes from 2.14 to 1.86 ns, indicating FRET
12 between GFP and RFP with a FRET efficiency of 13.3% (Fig. 3b).
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28 The ability of CKAR used in this TCSPC/FLIM setup to measure alterations in PKC
29 activity was tested in HCT116 cells transfected with CKAR followed by treatment
30 with the PKC agonist TPA or Gö6976, an inhibitor of classical PKC isoforms. As
31 shown on representative FLIM images TPA treatment, as expected, triggered a shift
32 to higher GFP lifetimes, indicating activation of PKC, whereas GFP lifetimes were
33 reduced following treatment with Gö6976 (Fig. 3c). This was reflected in a
34 corresponding increase or decrease of average GFP lifetimes compared to untreated
35 cells, suggesting that CKAR is suitable for the detection of changes in PKC activity
36 following pharmacological modulation.
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51 **Apoptin expression induces cancer specific PKC activation**

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54 To investigate the effect of Apoptin on PKC activity, HCT116 cells were transfected
55 with CKAR followed by infection with an adenoviral vector to express Apoptin
56 (Ad-Apoptin). Apoptin expression was detected by Western blot analysis after
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5 hours and further increased until 16 hours post-infection with Ad-Apoptin (Fig. 4a).

Phosphorylated Apoptin was detectable after 8 and 16 hours, suggesting activity of the Apoptin kinase before or at that time point.

As shown on representative FLIM images Apoptin expression was associated with a time-dependent increase in PKC activity as indicated by a shift to higher GFP lifetimes in Apoptin-treated cells (Fig. 4b). Measurement of average GFP lifetimes confirmed a significant increase in PKC activity at 8 and 16 hours post-infection similar to control treatment with TPA. A later drop of GFP lifetimes to basal levels might be due to apoptosis as a similar reduction of PKC activity was observed in cisplatin-treated cells (data not shown). These results suggest Apoptin-induced activation of PKC, correlating with the expression and phosphorylation levels of Apoptin at the respective stage in human cancer cells.

Apoptin phosphorylation and therefore activity of the Apoptin kinase represents an important aspect of the tumour cell-specific cytotoxic function of Apoptin. We therefore investigated whether Apoptin expression in normal colon mucosa cells with lower expression levels of PKC β I (Fig. 1a, Fig. S1a) would trigger activation of PKC as observed in cancer cells. In contrast to HCT116 cells, which showed a significant increase in PKC activity following infection with Ad-Apoptin, PKC activation remained unaltered in normal NCM356 cells at any time point (Fig. 4c). Similarly, compared to HCT116 cells Apoptin only triggered a minor increase in PKC activity in normal NCM460 cells (Fig. 4d), suggesting that this differential effect of Apoptin on the activation status of PKC is not cell type-specific. Apoptin-induced PKC activation therefore seems to occur predominantly in tumour cells but not in normal cells and might depend on the presence of PKC β I.

Discussion

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2
3 Specific killing of tumour cells while minimising damage to normal tissues remains a
4 major challenge in the development of effective anti-cancer agents. In recent years,
5 several cellular or viral proteins that selectively induce cell death in cancer cells have
6 been identified, including Apoptin which was originally discovered as the
7 apoptosis-inducing protein from CAV [8]. Gaining further understanding of their
8 regulation and cellular targets is essential for their development into future therapeutic
9 agents as well as the identification of novel tumour-specific targets and processes.

10
11 In this study we investigated the physical and functional interaction between Apoptin
12 and PKC. The PKC signalling pathway has predominantly been associated with
13 survival, proliferation and inhibition of apoptosis and is therefore an important target
14 for the development of anti-cancer agents.

15
16 Tumour cell-specific phosphorylation of Apoptin on Thr-108 by a cellular kinase
17 represents an important step for its accumulation in the nucleus and cytotoxic activity
18 [16] and PKC β was identified as a potential Apoptin kinase, but so far only in
19 multiple myeloma cells [18]. Several cancer types, including multiple myeloma, show
20 increased expression or activity of PKC β , suggesting that PKC β might represent a
21 tumour-specific target responsible for sensitising cells to Apoptin [20-22].

22
23 We therefore investigated the dynamics of the interplay between Apoptin and PKC β I
24 in HCT116 cells, a colorectal cancer cell line expressing high levels of PKC β I and
25 displaying high sensitivity to Apoptin. Co-immunoprecipitation and PLA studies
26 convincingly showed direct binding and co-localisation of Apoptin and PKC β I. This
27 interaction seemed to require the N-terminal region of Apoptin which has been shown

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1 to be involved in both binding of Apoptin to other cellular proteins [32-34] as well as
2 Apoptin multimerisation [35]. Importantly, interaction with Apoptin triggered nuclear
3 translocation of PKC β I; to our knowledge this is a novel phenomenon in respect to
4 PKC β I activity. The importance of this nuclear localisation on the activity of PKC β I
5 is currently unclear, however it may cause changes to PKC β I accessibility for its
6 substrates and/or its function [18]. Notably, Apoptin has been shown to trigger
7 nuclear accumulation of the related kinase Akt, switching its pro-survival to pro-
8 apoptotic activity in prostate cancer cells [36].
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10 In contrast to HCT116 cells, two cell lines derived from normal colon mucosa were
11 found to express almost undetectable levels of PKC β I. Increased expression levels of
12 PKC β I in HCT116 cells correlated with enhanced activation of Apoptin,
13 demonstrated by increased expression and phosphorylation levels of Apoptin, its
14 translocation to the nucleus as well as induction of apoptosis in cancer cells while
15 normal colon mucosa cells remained resistant to Apoptin expression. Knockdown of
16 PKC β I in HCT116 cells reduced Apoptin phosphorylation, albeit not completely
17 which might be due to residual Apoptin phosphorylation by other regulatory kinases
18 such as PKC β II or Cdk2.
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21 Interestingly, HGyV-Apoptin, a recently identified VP3 homologue derived from the
22 first human Gyrovirus HGyV [2, 3], was also shown to interact with PKC β I. An
23 online kinase prediction assay of the HGyV-Apoptin amino acid sequence
24 (<http://www.cbs.dtu.dk/services/NetPhos>) revealed several potential phosphorylation
25 sites matching the PKC consensus motif (S/TXK/R or S/TXXK/R) including Thr-111
26 which, like Thr-108 of CAV-Apoptin, is adjacent to a putative NES within the
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respective protein. This suggests a similar mode of tumour-specific regulation of HGyV-Apoptin by PKC β I-mediated phosphorylation as identified for CAV-Apoptin.

Using CKAR, a FRET-based biosensor for PKC activation, we showed a transient increase in PKC activity following Apoptin expression in HCT116 cells similar to treatment with known PKC activators. The demonstrated increase in PKC activity correlated with the presence of phosphorylated Apoptin, suggesting a link between Apoptin expression, PKC activation and Apoptin phosphorylation. In contrast to HCT116 cancer cells, normal colon mucosa cell lines displayed significantly reduced and delayed activation of PKC following Apoptin expression. These data support an important role for PKC in the tumour cell-specific regulation of Apoptin.

Despite the biosensor's lack of discrimination between various classical and novel PKC isoforms [37], the reduced PKC activation in normal colon mucosa cells lacking PKC β I indicates specific activation of PKC β I by Apoptin. However, a potential involvement of other PKC isoforms, including PKC β II or PKC δ , cannot be excluded and should be further investigated. Importantly, PKC δ is generally considered to be a pro-apoptotic PKC isoform that is cleaved and activated in response to many pro-apoptotic stimuli [38] including Apoptin expression and might represent a link between Apoptin-induced PKC activation and induction of caspase-dependent apoptosis [18].

It remains to be determined how the presence of Apoptin triggers activation of PKC. Using *in vitro* kinase assays we have previously shown PKC-mediated Apoptin phosphorylation after incubation with PKC β alone without any additional PKC activators in the reaction [18], suggesting that the presence of Apoptin alone is sufficient to activate PKC.

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Allosteric activation of PKC β II has been suggested to involve exposure of arginine-19 to proteolysis, which is shielded by a cluster of acidic residues under non-stimulated conditions [39]. Apoptin is rich in basic amino acids, which could potentially bind to these acidic residues on PKC β to expose Arg-19 and thereby trigger PKC activation.

In conclusion, this study has established an important tumour-specific link between Apoptin and PKC β I in colon cancer cells. Remarkably, this interaction induces not only PKC-dependent phosphorylation of Apoptin but also nuclear translocation and activation of PKC. In addition to phosphorylating Apoptin, modulation of PKC activity by Apoptin could trigger other yet unknown cellular pathways shifting the balance from proliferation to apoptosis.

Materials and Methods

Cell Lines and Reagents

HCT116 cells were obtained from ATCC and maintained in McCoy's 5A medium supplemented with 10% foetal bovine serum, 50 µg/ml streptomycin and 100 µg/ml penicillin (PAA Laboratories). NCM356 and NCM460 cells were purchased from INCELL Corporation. Cisplatin was obtained from the Guy's Hospital Pharmacy. Adenoviral vectors for the expression of Apoptin (Ad-Apoptin) or GFP (Ad-GFP) were generated as described previously [40]. For viral infections, virus stocks were diluted in culture medium to achieve the desired MOI (multiplicity of infection) and added to the cells.

Transient Transfection

pCAV-FLAG-AP for the expression of CAV-FLAG-Apoptin was obtained from Dr. Jose Teodoro (Goodman Cancer Research Centre, Montreal, Canada), plasmids for full-length or truncated GFP-Apoptin (pEPI-GFP-Apoptin 1-121 or 74-121) were provided by Dr. Gualtiero Alvisi (Monash University, Clayton, Australia). The expression vector for HGyV-FLAG-Apoptin (pHGyV-FLAG-AP) was generated as described previously [3]. pHA-PKCβI was obtained from Professor Alexandra Newton (University of California, San Diego, CA). pGFP/RFP CKAR was modified from the original reporter construct pCFP/YFP CKAR [27] by Dr. Melanie Keppler (King's College, London). Control vectors used were pcDNA3.1 and pEGFP-C1 (Life Technologies).

HCT116 cells were transiently transfected using X-tremeGENE HP transfection reagent according to the manufacturer's protocol (Roche Applied Science). In short, a

1 mix of plasmid DNA and transfection reagent was prepared in OptiMEM reduced-
2 serum medium (Life Technologies) and added dropwise to the cells after incubation
3
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5 for 20 min at room temperature.
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7 8 **Transient siRNA Knockdown** 9

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11 siGENOME Human PRKCB siRNA SMARTpool targeting PKC β I (targeting
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13 sequences GAACCAAGGUCCCGGAAGA, GGAUGAAACUGACCGAUUU,
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15 CCAAGUCUAUGUCCAAGGA, AGAGUAAGGGCAUCAUUUA) and GAPDH
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17 control siRNA were obtained from Dharmacon (GE Healthcare). Cells were
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19 transfected with siRNAs using the X-tremeGENE siRNA transfection reagent as
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21 recommended by the manufacturer (Roche Applied Science). In short, siRNA and
22
23 transfection reagent were prepared separately in OptiMEM reduced- serum medium
24
25 (Life Technologies), mixed carefully and added dropwise to the cells after incubation
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27 for 20 min at room temperature.
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34 35 **Lentivirus production** 36

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38 Lentiviral expression constructs for GFP (Lenti-GFP) or CAV-GFP-Apoptin
39
40 (Lenti-GFP-AP) were constructed as previously described [18]. Lenti-FLAG-AP for
41
42 the expression of CAV-FLAG-Apoptin was generated by insertion of the
43
44 FLAG-Apoptin gene from pCAV-FLAG-AP into the lentiviral backbone used for
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46 Lenti-GFP.
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51 Lentiviruses were produced in HEK293T cells transfected with the second-
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53 generation packaging plasmid pCMV Δ 8.91 and plasmid pMDG encoding
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55 VSV-G-pseudotyped envelope as well as the construct of interest by calcium
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57 phosphate precipitation. The virus-containing supernatant was harvested at 24 and 48
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1 hours post-transfection, put through a 0.45 µm filter, concentrated by centrifugation
2 for 6 hours at 13,000 x g and stored at -70°C. For infection lentiviral stocks were
3 diluted in a minimal amount of medium supplemented with 5 µg/ml polybrene (Santa
4 Cruz Biotechnology) and added to target cells.
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10 **Flow cytometry**

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12 Cells were cultured on 12-well plates at a density of 1×10^5 cells per well and
13 infected the next day. Cells were collected after 40 hours after by trypsinisation and
14 washing in PBS. Apoptosis was quantified by staining with Annexin V-APC
15 (BD Biosciences) and propidium iodide (PI, Sigma-Aldrich). Data was acquired on a
16 BD FACSCantoII flow cytometer (BD Biosciences) and analysed using FlowJo
17 software (Tree Star, Inc.)
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29 **Immunoprecipitation**

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31 Cells were seeded on 6-well plates at a density of 2×10^5 cells per well and
32 transfected the next day as indicated. At 2 days post-transfection cells were collected
33 by scraping and washing with cold PBS and centrifuged for 15 min at 16,000 x g. The
34 obtained cell pellet was resuspended in 250 µl to 500 µl radioimmunoprecipitation
35 assay buffer (Cell Signalling), sonicated and incubated for 1 hour on ice. After
36 centrifugation at 4°C and 16,000 x g for 10 min the supernatant was transferred to a
37 new tube and incubated with 2 µg of anti-HA or anti-FLAG antibody for 30 min
38 under agitation at 4°C. 20 µl of washed Bio-Adembeads PAG (Ademtech) was added
39 and incubated with the sample for 3 hours at 4°C. Using a magnetic rack, beads were
40 washed and isolated from the solution and proteins were removed from the beads by
41 resuspending in Laemmli sample buffer and boiling for 5 min at 95°C.
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Western blot analysis

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3 Cells were cultured on 6-well plates at a density of 2×10^5 cells per well and treated
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5 the next day. Cells were collected by trypsinisation, washed in PBS and re-suspended
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7 in lysis buffer (2 mM $MgCl_2$, 25 mM HEPES, 2 mM EGTA, 0.1% Triton X-100)
8
9 supplemented with protease inhibitors. Following 30 min incubation on ice, protein
10
11 lysates were obtained by centrifugation for 15 min at 16,000 x g. 40 to 60 μ g of
12
13 protein was used for further SDS-PAGE and transfer of proteins to nitrocellulose
14
15 membranes (Sigma-Aldrich). Membranes were incubated with specific primary and
16
17 secondary antibodies as indicated and developed using an enhanced
18
19 chemiluminescent system.
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26 Antibodies used for immunoblotting were: β -actin, Tubulin, FLAG (Sigma-Aldrich),
27
28 PKC β I, PKC β II (Santa Cruz Biotechnology), GFP and HA (Cell Signalling).
29
30 Antibodies directed against total or Thr-108-phosphorylated Apoptin (P-Apoptin)
31
32 were obtained as described previously [18]. Secondary HRP-coupled anti-rabbit and
33
34 anti-mouse antibodies were obtained from GE Healthcare and Sigma-Aldrich,
35
36 respectively. The relative expression or phosphorylation levels of proteins were
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38 determined using ImageJ software.
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Immunofluorescence

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48 Cells were grown on Falcon 8-chamber culture slides (BD Biosciences) at a density of
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50 2×10^4 to 3×10^4 cells per well and transfected the next day. After 2 days cells were
51
52 washed in PBS, fixed in 4% paraformaldehyde for 10 min and permeabilised with
53
54 0.2% Triton X-100 for 10 min. After permeabilisation cells were washed in PBS,
55
56 blocked using 3% BSA in PBS-T for 30 min and incubated with a primary anti-FLAG
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1 antibody for 90 min and a secondary FITC-conjugated anti-mouse antibody (Sigma-
2 Aldrich) for 1 hour. Cells were washed between incubations in PBS and all steps were
3
4 carried out at 37°C. Cells were mounted in Vectashield mounting medium containing
5
6 DAPI (Vector Laboratories) to stain nuclei. Images were acquired on an Olympus
7
8 BX61 automated fluorescence microscope or an A1R Si Confocal System and Eclipse
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10 Ti-E Inverted Microscope (Nikon) for confocal microscopy.
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15 **Proximity Ligation Assay (PLA)**

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19 Cells were seeded in Falcon 8-chamber culture slides (BD Biosciences) at a density of
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21 3×10^4 cells per well and transfected the next day. After 2 days cells were washed in
22
23 PBS, fixed in 4% paraformaldehyde for 10 min, washed again and permeabilised with
24
25 0.2% Triton X-100 for 10 min. After permeabilisation cells were blocked in blocking
26
27 solution for 30 min and then incubated with the indicated primary antibodies for 2
28
29 hours at 37°C in a humidity chamber. All further steps were carried out according to
30
31 the manufacturer's instructions (Duolink In Situ kit, Olink) in a reaction volume of
32
33 80 µl per well with three washing steps in Buffer A between incubations. Briefly,
34
35 cells were incubated with PLA probes for 1 hour followed by a ligation reaction for
36
37 30 min and incubation with an amplification mix for 100 min. After mounting in
38
39 DAPI-containing mounting medium images were acquired on the Olympus BX61
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41 automated fluorescence microscope using Cell^F software (Olympus) and analysed
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43 using Blobfinder software (Olink) to automatically detect and quantify positive PLA
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45 signals.
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54 **Fluorescence Lifetime Imaging Microscopy (FLIM)**

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57 Cells were seeded on sterile coverslips in 24-well plates at a low density and 24 hours
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59 later transfected with pGFP/RFP CKAR or peGFP-C1. The next day cells were
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1 infected with Ad-Apoptin at an MOI of 10 to express Apoptin for 2 up to 24 hours
2 before fixation in 4% paraformaldehyde for 10 min. Control cells were treated with
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4 200 nM TPA (12-O-Tetradecanoylphorbol-13-Acetate, Cell Signalling) for 30 min or
5
6 50 nM Gö6976 (Calbiochem) for 120 min. To reduce autofluorescence fixed cells
7
8 were treated with 1 mg/ml sodium borohydride for 5 min and mounted on slides in
9
10 8 µl FluorSave mounting medium (Calbiochem).
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15 Time-domain FLIM was performed using a home-built two-photon laser-scanning
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17 microscope based on the modified Nikon Eclipse Ti-E inverted microscope. A mode-
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19 locked Ti:Sapphire laser (Coherent, Chameleon Vision) with repetition rate of
20
21 80 MHz and pulse width of ~140 fs was tuned to 890 nm providing a photoexcitation
22
23 source for GFP. GFP fluorescence (500/40 nm bandpass) was detected with a
24
25 40x/1.3NA objective lens and a hybrid photo multiplier tube (PMT, HPM-100) in
26
27 conjunction with a time-correlated single-photon counting (TCSPC, SCP-150) card
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29 (both from Becker & Hickl). Images were acquired at 256 x 256 pixels with peak
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31 photon counting rates kept below 10^7 photons per second to avoid pulse pile-up. The
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33 GFP fluorescence lifetime τ was calculated by fitting bi-exponential fluorescence
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35 decay models to the images using TRI2 software (Paul Barber, University of Oxford).
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37 Output files were analysed to produce a distribution of the GFP lifetime across the
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39 cells and calculation of average donor lifetimes in single cells.
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48 **Statistical Analysis**

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51 Statistical analysis of results obtained from at least three independent experiments
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53 was performed using GraphPad Prism 6 software and one- or two-way analysis of
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55 variance (ANOVA) or two-tailed Student's *t* test as appropriate and indicated.
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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Material

Fig. S1 Correlation of PKC β I Levels and Apoptin Activity in HCT116 and NCM356 Cells

Legends to Figures

Fig. 1 Correlation of PKC β I Levels and Apoptin Activity in Colon Cancer and Normal Cells

(a) PKC β I expression in HCT116 and NCM460 cells was investigated by Western blot analysis of total cell lysates, showing results from 2 separate cultures. Bars represent PKC β I expression normalised to β -Actin; error bars indicate SEM (n = 7). Statistical significance was determined by unpaired two-tailed Student's *t* test (*** P < 0.001). (b) HCT116 and NCM460 cells were infected with Lenti-GFP (GFP) or Lenti-CAV-FLAG-AP (Apo) at an MOI of 5 for 2 days. Total cell lysates were analysed by immunoblotting with the indicated antibodies; blots were cut and combined at the indicated line. Bars represent ratios of phosphorylated Apoptin (P-Apoptin) to FLAG-Apoptin; error bars indicate SEM (n = 4). Statistical significance was determined by unpaired two-tailed Student's *t* test (** P < 0.01). (c/d) HCT116 and NCM460 cells were transfected with CAV-FLAG-AP, fixed after 24 hours and stained with a primary mouse anti-FLAG and secondary FITC-conjugated anti-mouse antibody and DAPI for detection of nuclei. Representative widefield (c, original magnification 60x, bar = 10 μ m) and confocal (d, original magnification 100x, bar = 20 μ m) fluorescence images are shown. Bars represent the percentage of Apoptin-expressing cells with nuclear localisation of Apoptin; error bars indicate SEM (n = 3). Statistical significance was determined by unpaired two-tailed Student's *t* test (*** P < 0.001). (e) HCT116 and NCM460 cells were infected

1 with Ad-GFP or Ad-Apoptin at an MOI of 10 for 40 hours before cell death detection
2 by Annexin V/PI staining and FACS analysis. Bars represent the mean percentage of
3 Annexin V positive cells; error bars indicate SEM (n = 4). Statistical significance was
4 determined by two-way ANOVA (*** P < 0.001). (f) HCT116 cells were transfected
5 with PKC β I or GAPDH control siRNA and infected 24 hours later with Lenti-CAV-
6 GFP-AP at an MOI of 5. Total cell lysates were collected after 2 days for Western
7 blot analysis with the indicated antibodies. Bars represent ratios of phosphorylated
8 Apoptin (P-Apoptin) to total GFP-Apoptin; error bars indicate SEM (n = 3).
9 Statistical significance was determined by one-way ANOVA (* P < 0.05, ns P >
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29 **Fig. 2 Interaction and Co-localisation of CAV-Apoptin and HGyV-Apoptin with**
30 **PKC β I in the Nucleus of Cancer Cells**
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35 (a) HCT116 cells were co-transfected with CAV-FLAG-AP (CAV) or HGyV-FLAG-
36 AP (HGyV) and HA-PKC β I. Immunocomplexes pulled down with anti-FLAG
37 antibodies and total cell lysates prepared in RIPA buffer at 2 days post-transfection
38 were subjected to Western blot analysis with the indicated antibodies. (b) HCT116
39 cells were transfected with CAV-FLAG-AP or pcDNA3 followed by PLA staining
40 using anti-FLAG and anti-P-Apoptin antibodies and counterstaining of nuclei with
41 DAPI. Representative PLA images are shown (original magnification 60x,
42 bar = 10 μ m). (c) HCT116 cells were transfected with CAV-FLAG-AP, HGyV-
43 FLAG-AP or pcDNA3 and analysed by PLA at 2 days post-transfection using anti-
44 FLAG and anti-PKC β I antibodies. Representative images indicating nuclei
45 counterstained with DAPI and PLA signals are shown (original magnification 60x,
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1 bar = 10 μ m). Bars represent average number of PLA foci per cell calculated from at
2 least 100 cells (excluding cells with 0 signals); error bars indicate SEM (n = 3).
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4 Statistical significance was determined by one-way ANOVA (* P < 0.05, ** P <
5 0.01). (d) HCT116 cells were transfected with peGFP-C1 or the indicated pEPI-GFP-
6 Apoptin plasmids and analysed by PLA using anti-GFP and anti-PKC β I antibodies at
7 2 days post-transfection. Representative images indicating nuclei counterstained with
8 DAPI, PLA signals or GFP expression are shown (original magnification 60x,
9 bar = 10 μ m). Bars represent average number of PLA foci per cell, localised to either
10 the nucleus or the cytoplasm, calculated from at least 100 GFP-expressing cells; error
11 bars indicate SEM (n = 3). Statistical significance was determined by one-way
12 ANOVA (* P < 0.05, ns P > 0.05).
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32 **Fig. 3 Measurement of PKC activity by FRET/FLIM using CKAR**

34 (a) Structure of the PKC activity reporter CKAR, comprising GFP and RFP linked by
35 a PKC substrate sequence and the FHA2 phosphothreonine-binding domain of
36 Rad53p. PKC-induced phosphorylation of the substrate sequence triggers a
37 conformational change, resulting in reduced FRET measured as an increase of the
38 fluorescence lifetime τ of the donor GFP (adapted from [27]). (b) HCT116 cells were
39 transfected with pGFP/RFP CKAR or peGFP-C1 and fixed for FLIM analysis the
40 following day. A representative FLIM image of a single cell, indicating the
41 distribution of GFP lifetime (τ) is shown (bar = 10 μ m). Each symbol represents the
42 average τ of a single cell; lines indicate the mean value of τ for the entire sample. (c)
43 HCT116 cells were transfected with pGFP/RFP CKAR and treated the following day
44 with 200 nM TPA for 30 min or 50 nM Gö6976 for 120 min before fixing for FLIM
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1 analysis. Representative FLIM images indicating τ are shown (bar = 10 μm). Bars
2 represent changes in average τ compared to untreated cells, corresponding to PKC
3 activity, from at least 15 cells per sample; error bars indicate SEM (n = 5). Statistical
4 significance was determined by one-way ANOVA (* P < 0.05, *** P < 0.001).
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14 **Fig. 4 Apoptin-induced Activation of PKC Isoforms in Colon Cancer Cells but**
15 **not Normal Cells**
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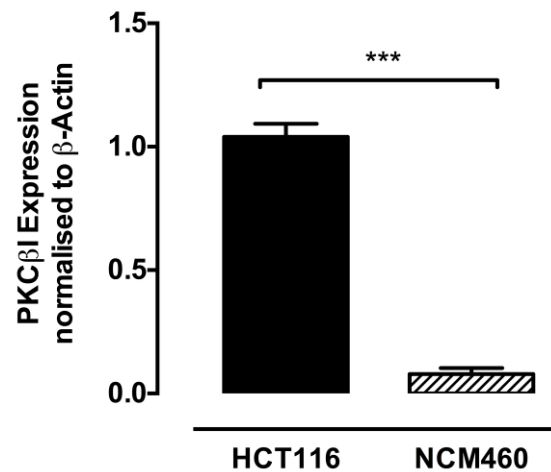
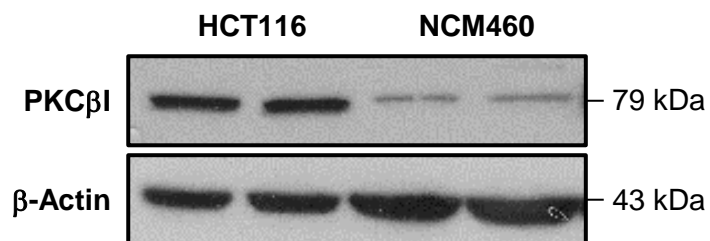
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20 (a) HCT116 cells were infected with Ad-Apoptin at an MOI of 10 and cell lysates
21 were collected at the indicated time points for Western blot analysis. Blots were cut
22 and combined at the indicated line. (b) CKAR-expressing HCT116 cells were
23 infected with Ad-Apoptin at an MOI of 10 before fixing for FLIM analysis at the
24 indicated time points. Representative FLIM images indicating τ are shown
25 (bar = 10 μm). Bars represent changes in average τ compared to untreated cells
26 calculated from at least 15 cells per sample; error bars indicate SEM (n = 3 for 2h/5h,
27 n = 5 for 8h/16h/24h). Statistical significance was determined by one-way ANOVA
28 (* P < 0.05, *** P < 0.001). (c/d) HCT116 and NCM356 (c) or NCM460 (d) cells
29 were transfected with pGFP/RFP CKAR and infected the following day with Ad-
30 Apoptin at an MOI of 10 for 8 or 16 hours before fixing for FLIM analysis. Bars
31 represent changes in average τ compared to untreated cells calculated from at least 10
32 cells per sample; error bars indicate SEM (n = 3). Statistical significance was
33 determined by two-way ANOVA (** P < 0.01).
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Fig. S1 Correlation of PKC β I Levels and Apoptin Activity in HCT116 and NCM356 Cells

(a) PKC β I and PKC β II expression levels in HCT116, NCM356 and NCM460 cells were determined by Western blot analysis. (b) HCT116 and NCM356 cells were infected with Lenti-GFP (GFP) or Lenti-CAV-FLAG-AP (Apo) at an MOI of 5. Total cell lysates were collected after 2 days for Western blot analysis with the indicated antibodies; blots were cut and combined at the indicated line. (c) HCT116 and NCM356 cells were transfected with CAV-FLAG-AP, fixed after 24 hours and stained with a primary mouse anti-FLAG and secondary FITC-conjugated anti-mouse antibody. Nuclei were detected by counterstaining with DAPI. Representative fluorescence images are shown (magnification 60x, bar = 10 μ m). (d) HCT116 and NCM356 cells were infected with Ad-GFP or Ad-Apoptin at an MOI of 10 and collected at 40 hours post-infection for cell death detection by Annexin V/PI staining and FACS analysis. Bars represent the percentage of Annexin V positive cells; error bars indicate SEM (n = 4). Statistical significance was determined by two-way ANOVA (***) P < 0.001).

a



b

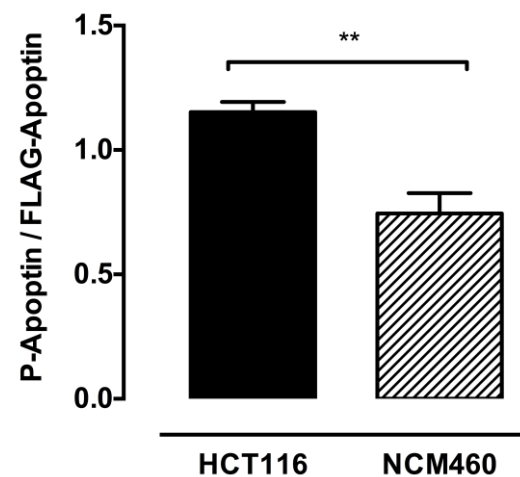
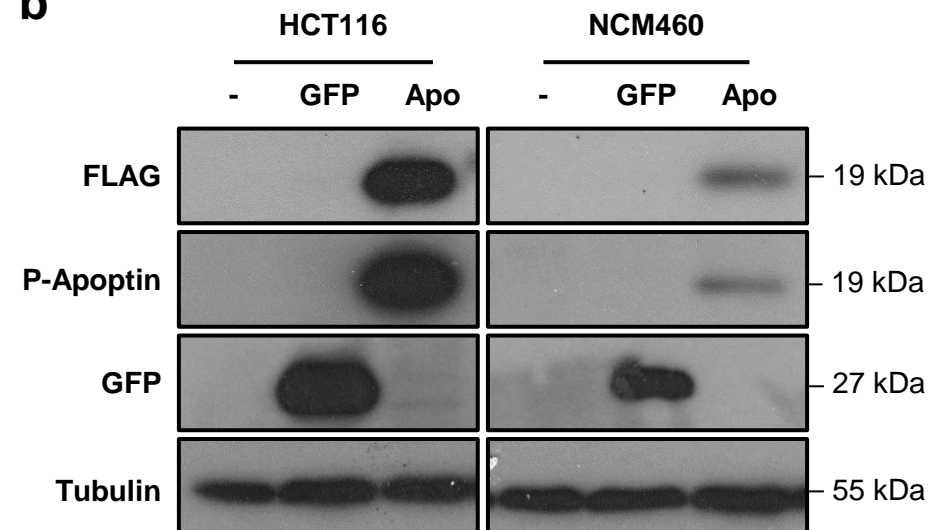
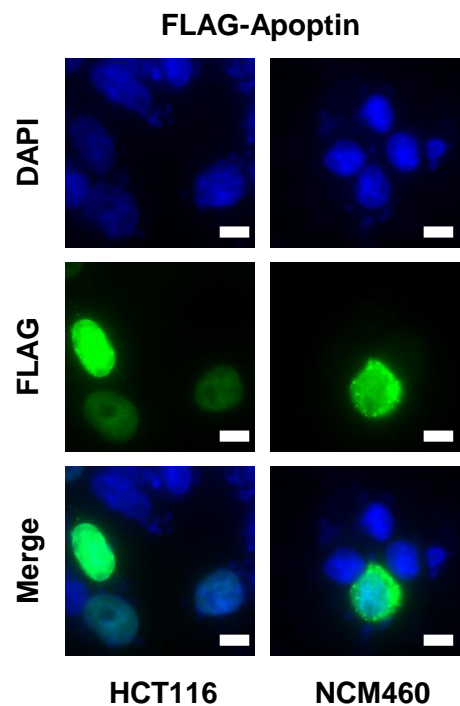
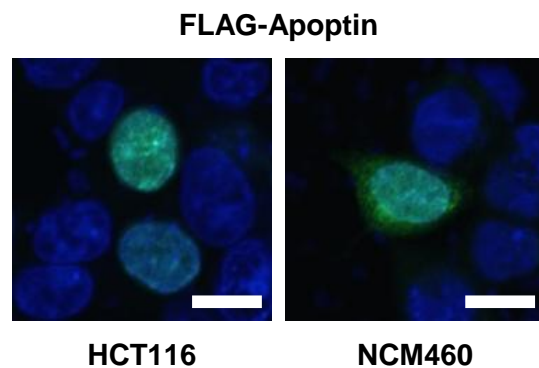


Fig. 1 continued

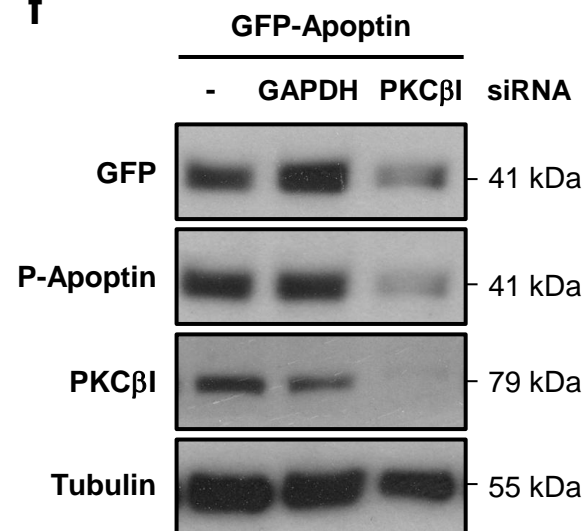
c



d



f



e

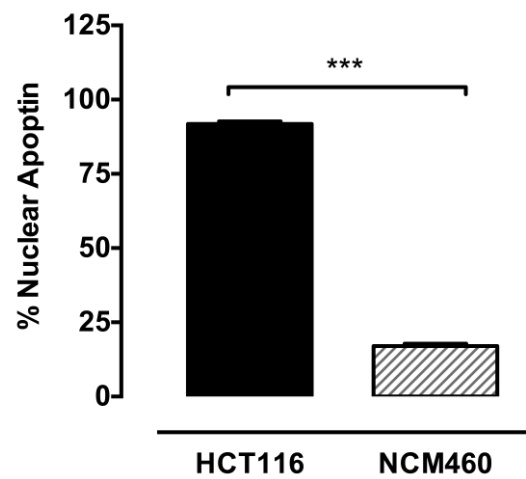
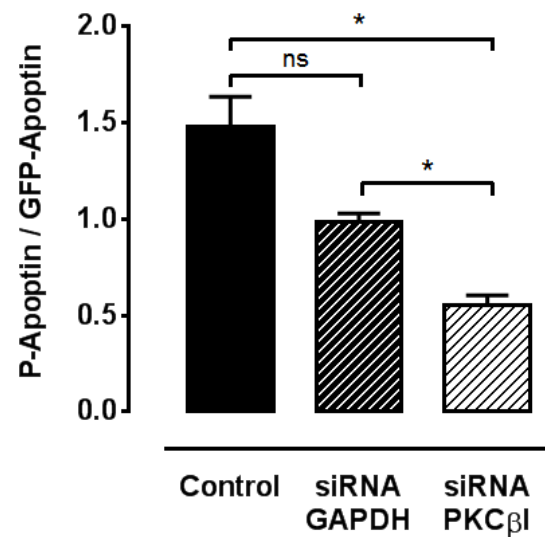
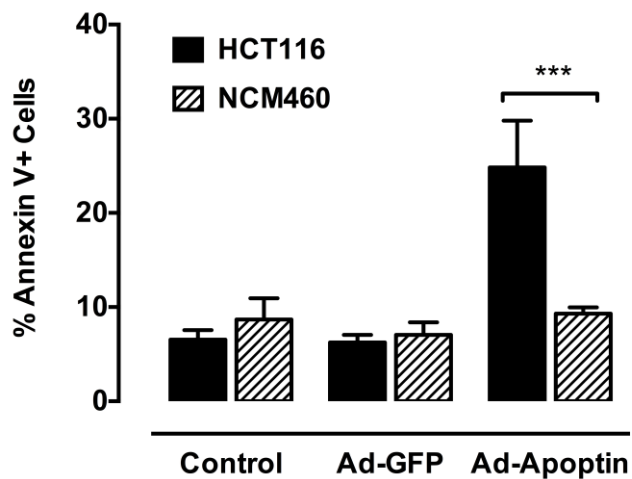


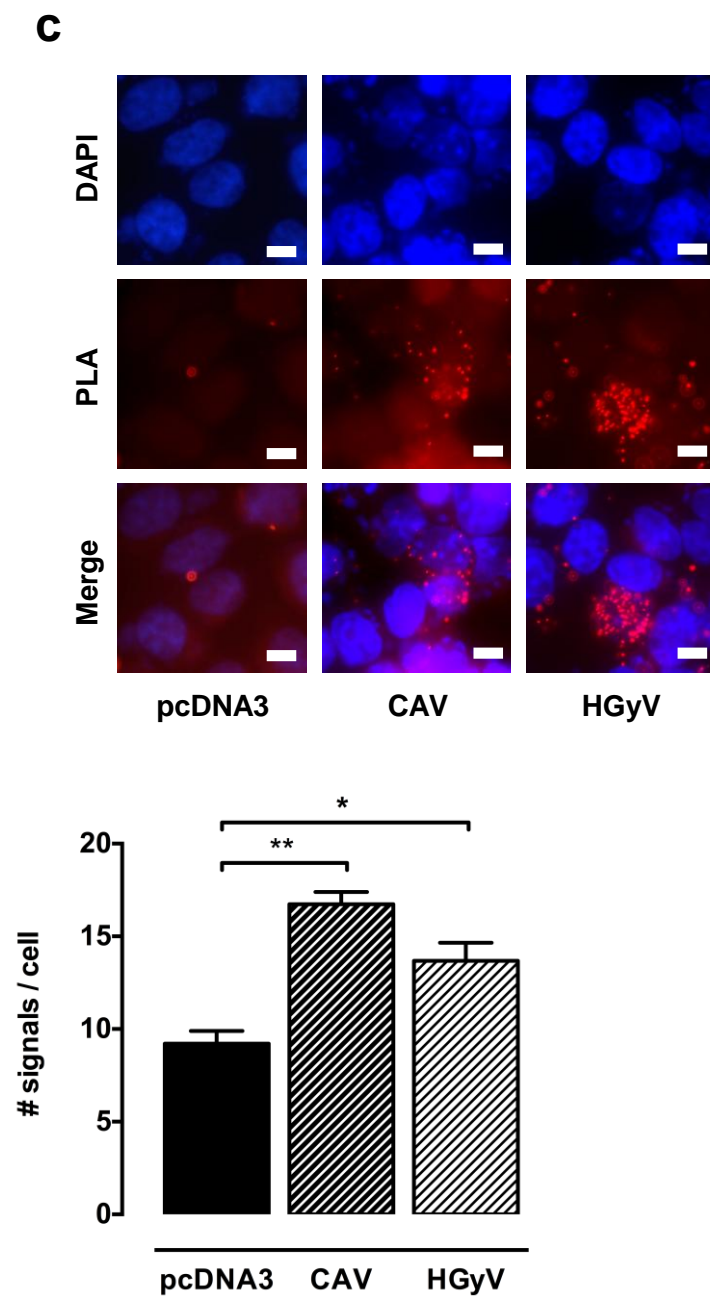
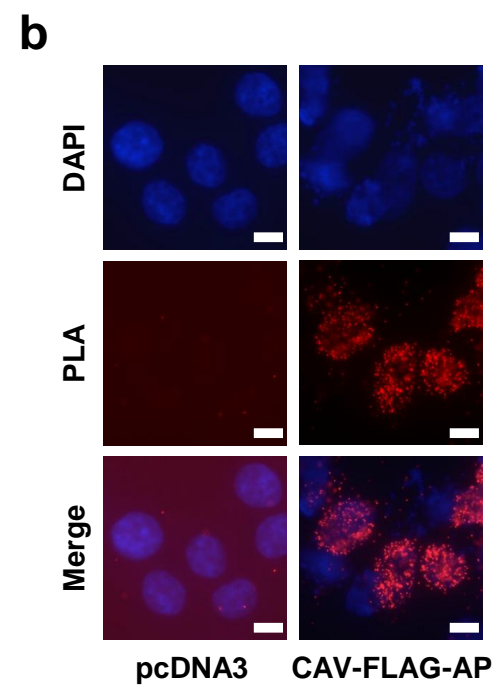
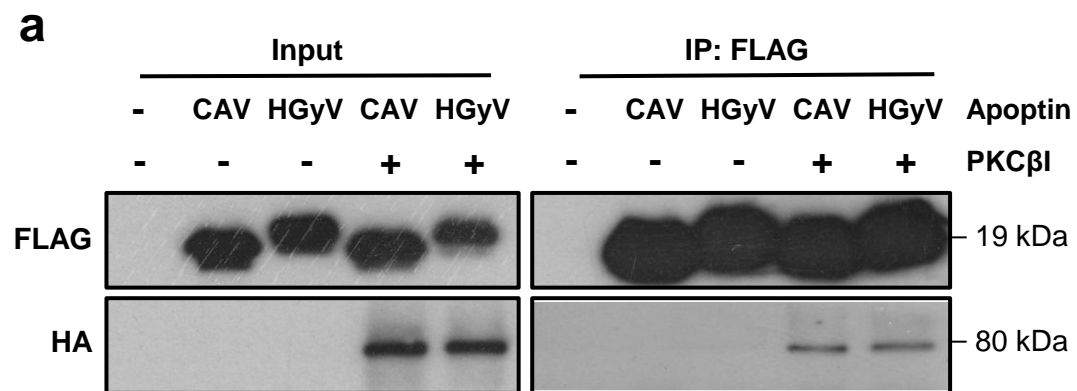
Fig. 2

Fig. 2 continued

d

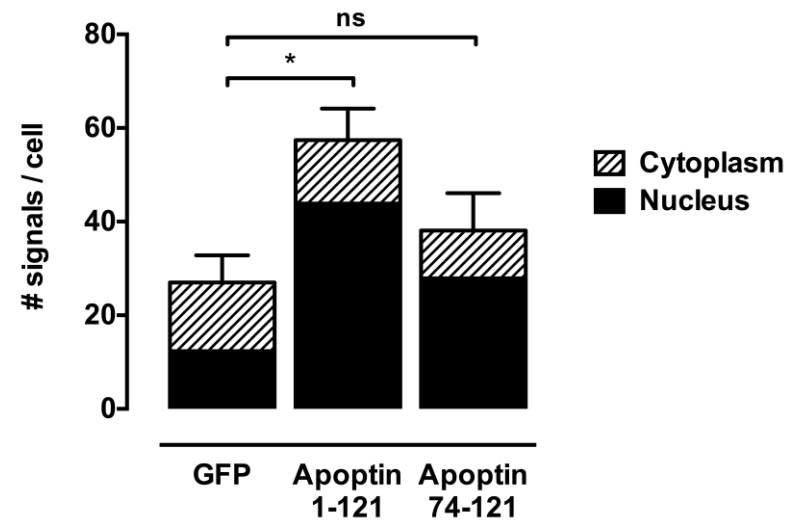
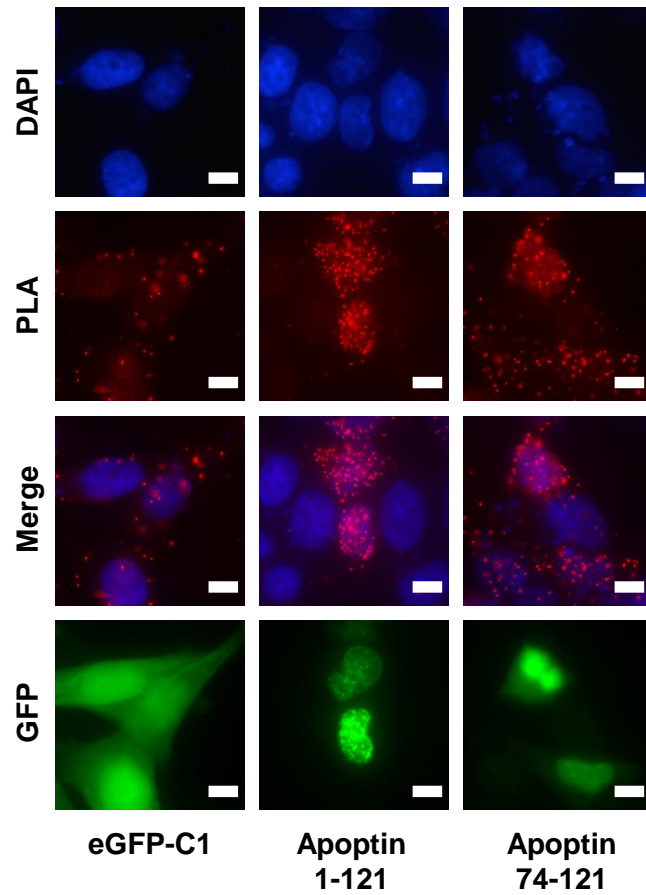


Fig. 3

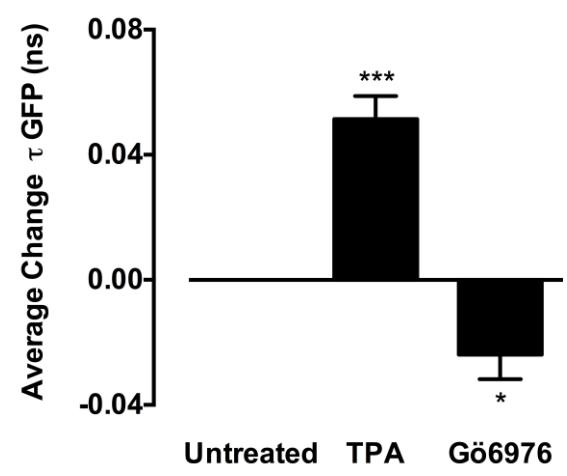
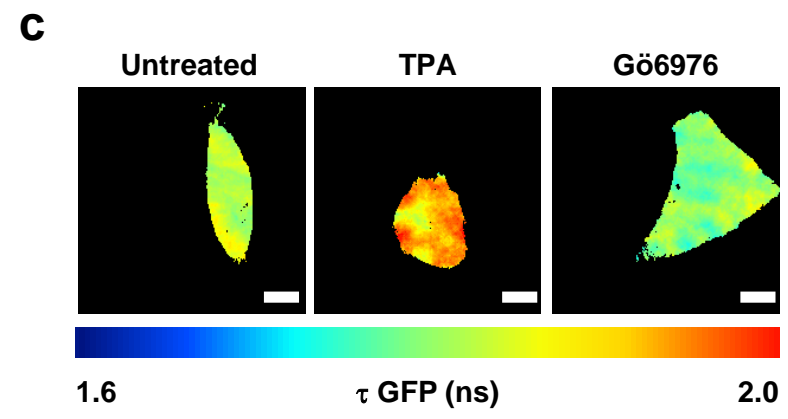
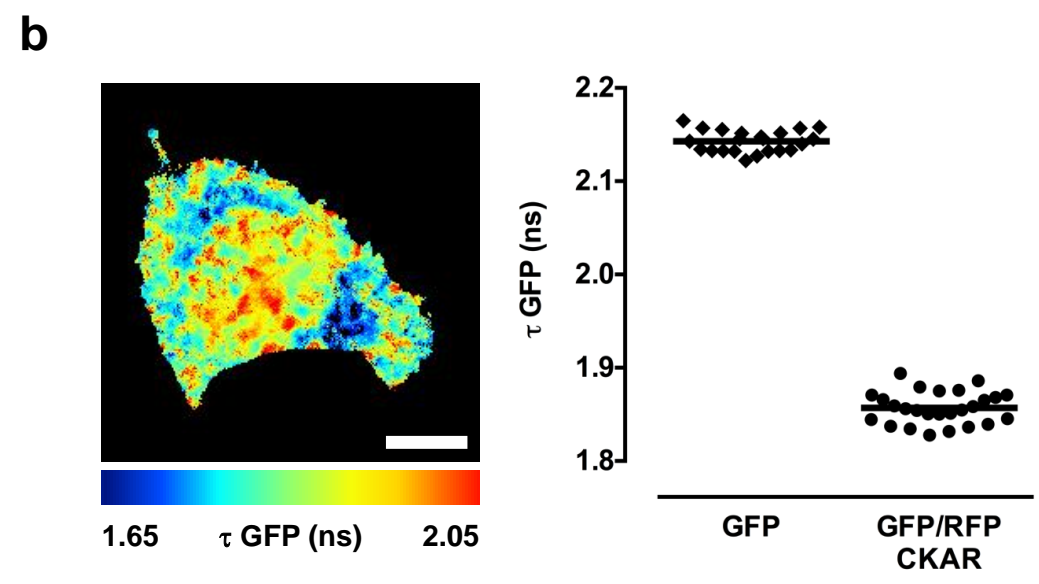
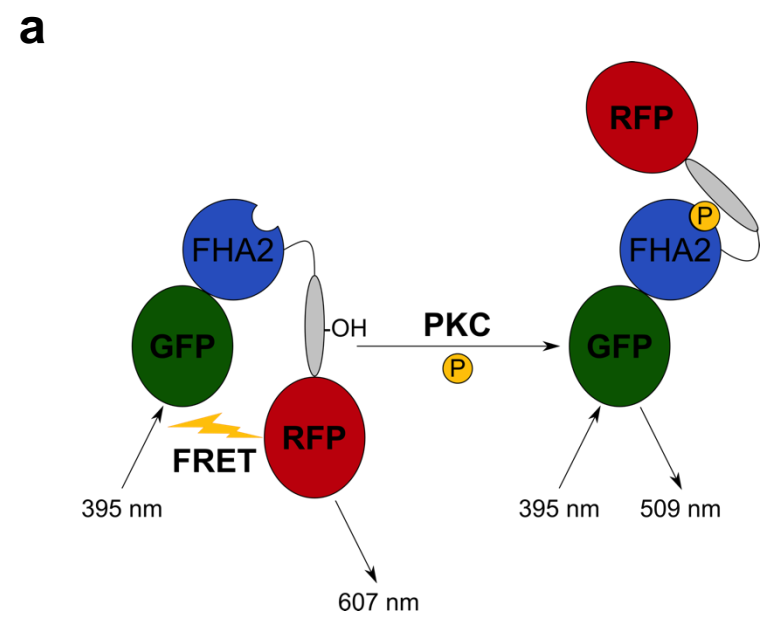


Fig. 4

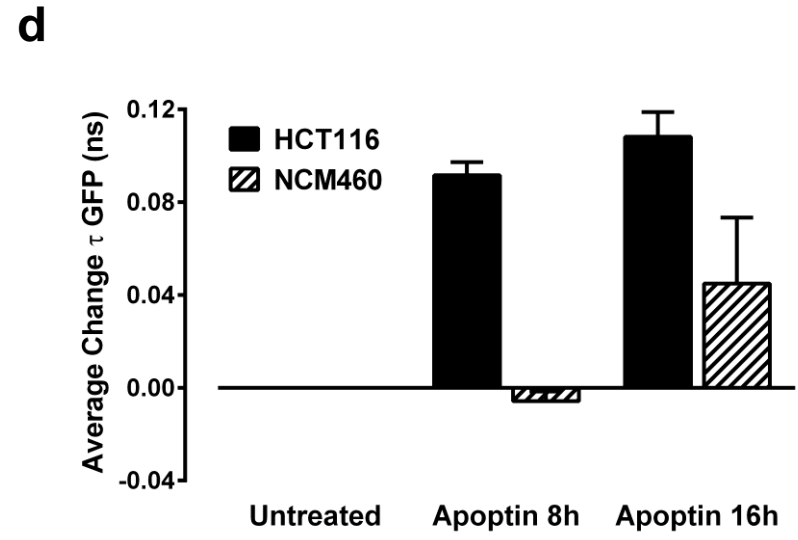
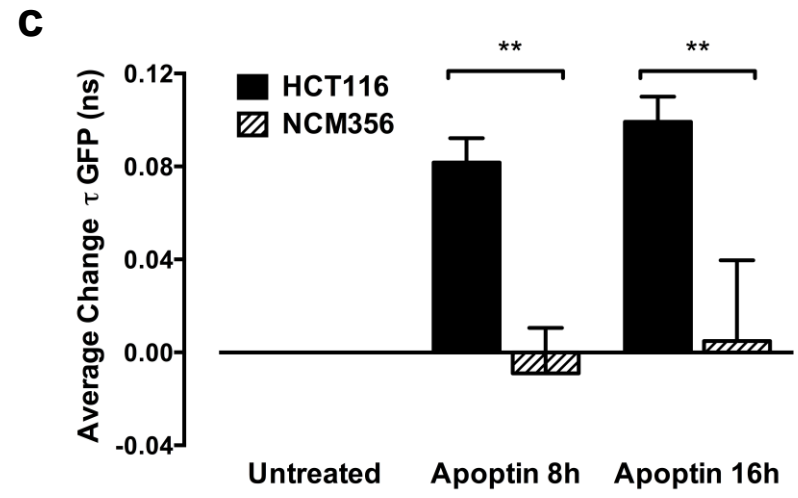
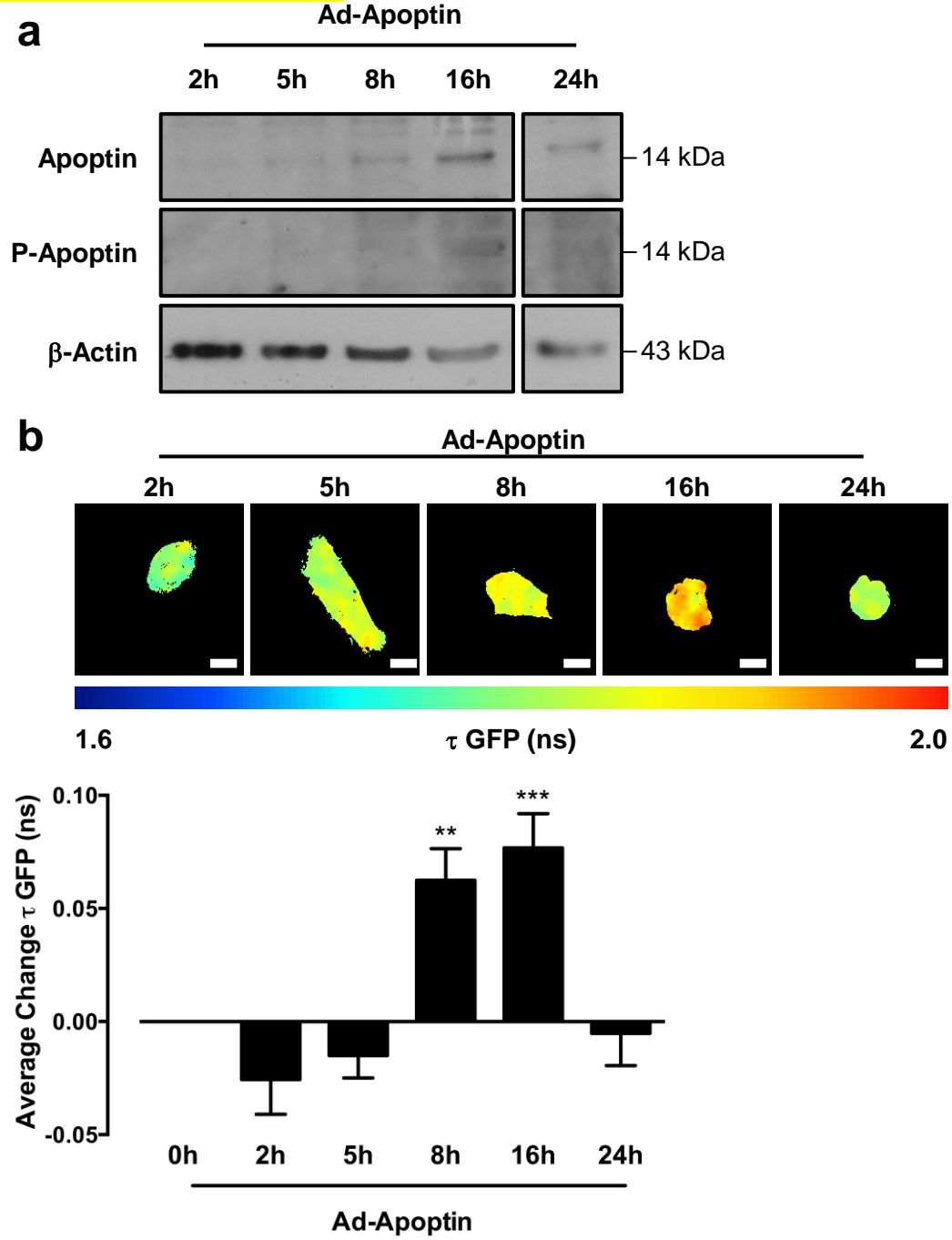
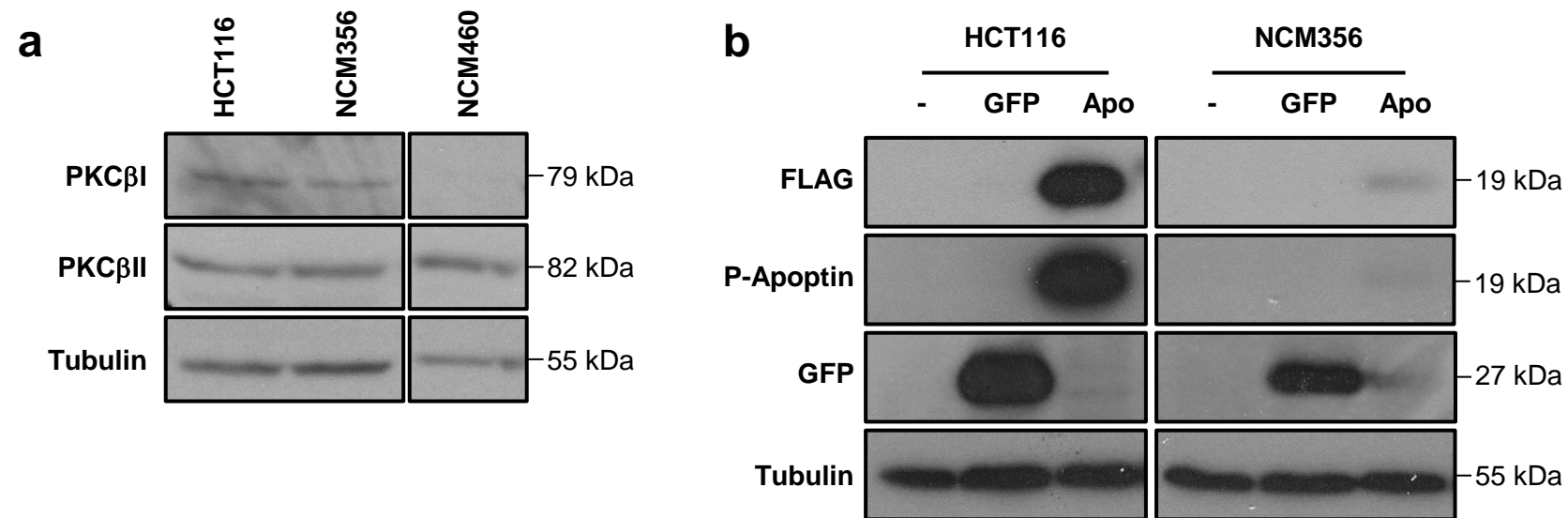
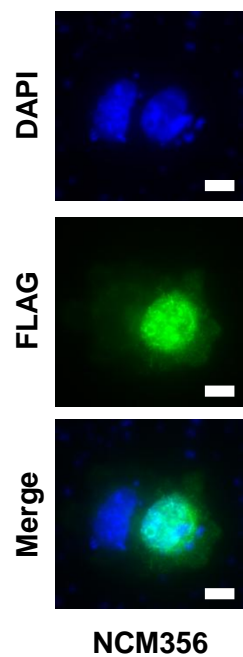


Fig. S1

c FLAG-Apoptin



d

