Tumor suppressor protein kinase Chk2 is a mediator of anoikis of intestinal epithelial cells

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Abstract

Resistance of carcinoma cells to anoikis, apoptosis that is normally induced by detachment of non-malignant epithelial cells from the extracellular matrix, is thought to be critical for carcinoma progression. Molecular mechanisms that control anoikis of non-malignant and cancer cells are understood poorly. In an effort to understand them we found that detachment of non-malignant intestinal epithelial cells triggers upregulation of Chk2, a pro-apoptotic protein kinase that has never been implicated in anoikis and has been thought to kill cells mainly under the conditions compromising genome integrity. We found that enforced downregulation of Chk2 protects intestinal epithelial cells from anoikis. Chk2 can kill cells by stabilizing p53 tumor suppressor protein or via p53-independent mechanisms, and we established that Chk2-mediated anoikis of intestinal epithelial cells is p53-independent. We further found that, unlike non-malignant intestinal epithelial cells whose anoikis is triggered by detachment-induced Chk2 upregulation, intestinal epithelial cells carrying oncogenic *ras*, a known inhibitor of anoikis, remain anoikis-resistant in response to enforced Chk2 upregulation. By contrast, drugs, such as topoisomerase I inhibitors, that can kill cells via Chk2-indpendent mechanisms, efficiently triggered anoikis of *ras*-transformed cells. Thus, oncogenic *ras* can prevent Chk2 from triggering anoikis even when levels of this protein kinase are elevated in cancer cells, and the use of therapeutic agents that kill cells in a Chk-2-independent, rather than Chk-2-dependent, manner could represent an efficient strategy for overcoming *ras*-induced anoikis resistance of these cells. We conclude that Chk-2 is an important novel component of anoikis-promoting machinery of intestinal epithelial cells.

Introduction

Resistance of carcinoma cells to anoikis, apoptosis induced by detachment of non-malignant epithelial cells from the extracellular matrix (ECM) [1,2], is thought to be critical for tumor progression [3]. It is known in this regard that many normal epithelia are organized *in vivo* as cellular monolayers that grow in contact with a form of the ECM called the basement membrane (BM). Detachment from the ECM causes apoptosis of the respective epithelial cells [4,5]. By contrast, carcinoma cells are well known to be able to promote the degradation of the ECM in the process of invasion of adjacent tissues [6] and form metastasis at distant sites [7,8], where these cells are no longer attached to the BM. Despite the fact that tumor cells often tend to be detached from the BM during cancer progression, these cells typically remain viable, and this viability is thought to be critical for the malignant growth of the respective tumors.

The notion that anoikis resistance of carcinoma cells is essential for their ability to form malignant tumors is supported by several lines of evidence. First, the ability of cancer cells to grow without adhesion to the ECM as colonies in soft agar represents a "gold standard" for malignant transformation [9,10]. Second, activation of proto-oncogenes, such as *ras* [11] and β-catenin [12] renders cancer cells anoikis-resistant. Third, inhibition of anoikis resistance of cancer cells blocks their ability to form primary tumors [11,13] and metastasis [8,14]. Finally, acquisition of anoikis resistance by non-malignant cells is sufficient for attainment of an overt tumorigenic phenotype by these cells [15]. Thus, anoikis resistance of cancer cells represents a potential therapeutic target. However, the mechanisms that control anoikis in normal and cancer cells are not well understood.

It is now known that anoikis susceptibility and resistance is controlled in normal and cancer cells, respectively, by a network of signals [4,5,11,13,16]. We found in this regard that anoikis of non-malignant intestinal epithelial cells is driven by detachment-induced downregulation of the anti-apoptotic protein Bcl-X_L [11] as well as by detachment-dependent upregulation of the pro-apoptotic protein Fas ligand [4]. We also established that *ras* oncogene suppresses anoikis of malignant intestinal epithelial cells by upregulating Bcl-X_L [11], as well as anti-apoptotic proteins cIAP2 and XIAP [16] and by downregulating a pro-apoptotic protein Bak [13]. We also established that β-catenin, another major oncoprotein, suppresses anoikis of cancer cells by downregulating a pro-apoptotic protein kinase DAPk-2 [12].

Whether or not all critical elements of the networks that control anoikis of non-malignant and cancer cells have been identified, is not known. In an effort to identify such elements we have discovered a novel mechanism that controls anoikis of intestinal epithelial cells. This mechanism involves detachment-induced upregulation of Chk2, a pro-apoptotic protein kinase that, to our knowledge, has not so far been implicated in anoikis. We further established that the ability of Chk2 to promote anoikis is blocked in *ras*-transformed intestinal epithelial cells.

Materials and methods

Cell Culture

IEC-18 and mouse colonocytes were cultured as described [4]. For suspension cultures cells were plated above a layer of 1% sea plaque-agarose polymerized in α-MEM.

Expression vectors

The expression vector carrying HA-tagged Chk2 was kindly provided by Dr. J. Chen, Mayo Clinic and Foundation, Rochester, Minnesota, USA. The expression vector carrying Flag-tagged Chk2 was kindly provided by Dr. K. Khanna, Queensland Institute of Medical Research, Brisbane, Australia. The T-REX system (Invitrogen) was used to generate cells expressing tetracycline-inducible Chk2. FLAG-Chk2 cDNA was placed into the pcDNA4-TO vector (a component of the T-REX system).

Western blot

This assay was performed as described [4]. The following antibodies were used. Anti-Chk2 (Millipore, catalogue #05-649), anti-p53 (Cell Signaling Technology, catalogue #2524), anti-phospho-Chk2 (Thr387) (AbCam, catalogue #ab55319), anti-CDK-4 (SantaCruz, catalogue #SC-601), anti-Fas ligand (SantaCruz, catalogue #SC-6237), anti-β-actin (Sigma, catalogue #A5441). Image quantification was performed as we previously described [16].

Chk2 mRNA detection

The expression of the Chk-2 mRNA was assayed by Rat Apoptosis Oligo GEArray microarray (SA Biosciences) according to manufacturer's instruction. Signals on the array were detected by ECL. The intensity of each signal was quantified by densitometry as described [16].

RNA interference

RNA interference was performed as we described [16]. siCONTROL non-targeting siRNA #1 (Dharmacon) was used as a control RNA. The sequences of the sense strands of the RNAs used in this study were as follows: control RNA (siCONTROL non-targeting siRNA #1 (Dharmacon)), UGUUGUUUGAGGGGAACGGTT; Chk2-specific siRNA-1, GUACCGGACU UACAGCAAGUU; Chk2-specific siRNA-2, GAGGAAGCCUUAAGUCAUCUU; Chk2-specific siRNA-3, CCUAAGAAUUGUUACAUUGUU; p53-specific siRNA-1 GUACUCAA UUUCCCUCAAUUU; p53-specific siRNA-2 CCACUAUCCACUACAAGUAUU; p53-specific siRNA-3 GCGACAGGGUCACCUAAUUUU. All RNAs were purchased from Dharmacon.

Analysis of apoptosis by flow cytometry

Apoptosis Detection kit from Chemicon was used for the assay. Cells were harvested, washed with phosphate buffered saline, and re-suspended in binding buffer provided by the manufacturer at a concentration of 10^6 cells/ml. 200μl of the resulting cell suspension was mixed with 3μl of Annexin V conjugated to FITC and 2μl of propidium iodide. This mixture was incubated for 15 minutes at room temperature. FACSCalibur system (BD Biosciences) was used for the analysis. Annexin V-positive, propidium iodide-negative cells were considered apoptotic.

Cell survival assays

The following assays were performed as we previously described: measurement of the ability of cells to form colonies in monolayer after being cultured in suspension[5], soft agar growth assay[13], Annexin V-binding assay[12], detection of apoptotic cellular nuclei[4] and Cell Death Elisa assay[13]. To measure the effect of ectopic Chk2 on apoptosis of IEC-18 cells they were co-transfected with 1 μg of either pcDNA3 (control vector) or pEFBos Chk2 expression vector and 0.2 μg pEGFP-C1 vector as previously described[4].

Statistical analysis

Statistical significance of data in Fig. 1-4, 6a and 6b, right was assessed by 2-tailed Student's t-test. 2-tailed chi-square test for goodness-of-fit and 2-tailed exact test for goodness-of-fit were used independently of each other in Fig. 6b, left and 6d for assessing statistical significance of data (respective results were statistically significant according to the results of both tests).

Results

Detachment of intestinal epithelial cells from the ECM triggers Chk2 upregulation.

In an effort to understand the mechanisms of anoikis of non-malignant intestinal epithelial cells, we found that detachment of highly anoikis-susceptible non-malignant intestinal epithelial cells IEC-18 (a cell line that we often use to study anoikis [4,5,11,13,16]) from the ECM triggers upregulation of a pro-apoptotic Ser/Thr protein kinase Chk2 (Fig. 1*a*). We also observed that detachment promotes Chk2 upregulation in non-malignant anoikis-susceptible mouse colonocytes [17] (Fig. 1*b,c*), another intestinal epithelium-derived cell line that we frequently utilize in our studies on anoikis [4,18]. Thus, detachment-induced upregulation of Chk2 is not unique to IEC-18 cells. We further found that detachment triggers a significant increase in Chk2 phosphorylation at Thr 387, one of the well established indicators of the increased activity of this protein kinase [19,20](Fig. 1*d*). The degree of increase in the levels of phospho-Chk2 following detachment was similar to that observed for the total Chk2 protein at all time points investigated (Fig. 1*e*).

Detachment-induced Chk2 upregulation observed by us occurred at the protein (Fig. 1) but not the mRNA (not shown) level, and we found that cycloheximide, a protein biosynthesis inhibitor [21], blocks Chk2 expression in attached IEC-18 cells (Fig. 2*a, c*) to a significantly more noticeable degree than that in the detached cells (Fig. 2*b, c*). These data indicate that detachment increases the stability of Chk2 protein.

Interestingly, Chk2 levels are reduced in a fraction of colorectal and other types of cancer [22], and loss of Chk2 expression or activity promotes tumor growth in mice [23]. So far this kinase has never been implicated in anoikis and has been thought to block cell growth mainly under the circumstances compromising genome integrity [22].

Chk2 contributes to anoikis of non-malignant intestinal epithelial cells.

To examine whether Chk2 plays a causal role in anoikis we tested whether this phenomenon can be suppressed by the ablation of Chk2 by RNA interference (RNAi). To achieve this, we blocked Chk2 expression in IEC-18 cells by three separate Chk2-specific small interfering RNAs (siRNAs) targeting different regions of Chk2 mRNA (Fig. 3*a*). We subsequently measured the susceptibility of siRNA-transfected cells to detachment-induced death by a clonogenicity assay that we often utilize in our studies of anoikis [5,18]. We found that enforced downregulation of Chk2 significantly enhanced the survival of IEC-18 cells after their detachment from the ECM (Fig. 3*b*). This increase in survival was paralleled by a noticeable decrease in the ability of detached cells to bind Annexin V (Fig. 3*c*) (Annexin V binding to dying cells represents a well established hallmark of apoptosis). Thus, Chk2 represents a component of anoikis-inducing machinery of non-malignant intestinal epithelial cells.

To test whether enforced increase in Chk2 expression can enhance apoptosis of attached and/or detached IEC-18 cells we co-transfected these cells with either a control expression vector or an expression vector coding for Chk2 together with that coding for the green fluorescent protein (GFP) (Fig. 3*d, left*) and assayed the nuclei GFP-positive cells for apoptotic morphology before and after detachment (Fig. 3*d, right*) as we previously published[4]. We found that exogenous Chk2 reproducibly caused approximately 1.5-fold increase of apoptosis of the attached IEC-18 cells (Fig. 3*d, right*) whereas detachment resulted in approximately 3.5-fold increase in death of these cells. Thus, Chk2 is capable of killing attached intestinal epithelial cells, when expression of this protein is increased in these cells. The fact that ectopic Chk2 did not kill the indicated attached cells to the same degree as detachment is not surprising as, in

addition to the upregulation of Chk2, detachment of the indicated cells triggers several other anoikis-inducing mechanisms, such as those driven by the downregulation of Bcl-X_L [11] and the upregulation of Fas ligand [4]. Thus, it is likely that simultaneous induction of all of the indicated pro-anoikis events in the attached cells is required to mimic the effect of detachment on their survival. We also found that exogenous Chk2 did not enhance apoptosis of detached cells at either 24h (not shown) or 48h (Fig. 3*d*) post-detachment. Thus, perhaps not unexpectedly, additional Chk2 upregulation in detached cells (that have already acquired increased Chk2 amounts following detachment and succumbed to apoptosis, see Fig. 1 and 3*b*, *c*) does not further enhance their death. Collectively, our data indicate that detachment-induced upregulation of Chk2 contributes to the execution of anoikis of intestinal epithelial cells.

Antagonists of Chk2 and p38 MAP kinases cooperate in protecting intestinal epithelial cells from anoikis.

We found previously that anoikis of intestinal epithelial cells (including IEC-18 cells) is mediated by detachment-induced activation of p38 MAP kinase and subsequent p38-dependent upregulation of a pro-apoptotic protein Fas ligand[4]. In an effort to investigate whether Chk2 and Fas ligand promote anoikis within the same signaling pathway or via mechanisms that act independently of each other we observed that ablation of Chk2 by RNAi does not alter Fas ligand expression in IEC-18 cells that were detached for 4h (Fig. 4*a*) (we found before that Fas ligand is upregulated in IEC-18 cells as early as 30min following detachment[4]). Thus, detachment-induced upregulation of Fas ligand does not appear to represent the consequence of detachment-triggered Chk2 upregulation. Furthermore, treatment of detached IEC-18 cells for 4h with SB 203580, a small molecule p38 MAP kinase inhibitor, did not block Chk2 expression in

these cells (Fig. 4*b*) (we found previously that exposure to SB 203580 downregulates Fas ligand in IEC-18 cells and blocks their anoikis[4]). Thus, detachment-induced Chk2 upregulation seems to occur independently of p38 MAP kinase. Collectively, these data indicate that detachment of intestinal epithelial cells triggers two anoikis-promoting mechanisms that are independent of each other, one mediated by Fas ligand and another one controlled by Chk2.

We further observed that simultaneous ablation of Chk2 by RNAi and treatment with SB 203580 protected IEC-18 cells from anoikis significantly more efficiently than either treatment alone (Fig. 4*c*). These data are consistent with a notion that Chk2 and p38MAP kinase, when activated by detachment, cooperate between each other in promoting anoikis of intestinal epithelial cells.

p53 is not involved in anoikis of non-malignant intestinal epithelial cells.

Chk2 was proposed to kill cells by phosphorylating and thus stabilizing a pro-apoptotic protein p53 [24]. In addition, Chk2 can trigger apoptosis via p53-independent mechanisms [25]. We thus tested whether p53 is involved in anoikis of non-malignant intestinal epithelial cells. We found in this regard that p53 levels do not increase in IEC-18 cells following their detachment. To the contrary, we observed a decrease in p53 levels further to detachment of these cells (Fig. 5*a*). These results suggested that Chk2 triggers anoikis of intestinal epithelial cells in a p53-independent manner.

To address the role of p53 in anoikis of these cells in a more definitive way, we blocked p53 expression in IEC-18 cells by three different siRNAs (Fig. 5*b*). Unlike the case with Chk2 (see Fig. 3), enforced inhibition of p53 expression did not rescue IEC-18 cells from anoikis (Fig.

5*c*). Thus, p53 does not contribute to anoikis of intestinal epithelial cells, which indicates that Chk2 triggers this process in a p53-independent manner.

Oncogenic *ras* prevents Chk2 from triggering anoikis of intestinal epithelial cells.

Given that detachment-induced upregulation of Chk2 can trigger anoikis of intestinal epithelial cells and that *ras* oncogene is an established major inhibitor of anoikis [11,18], we decided to test whether treatments resulting in Chk2 upregulation can facilitate anoikis of malignant intestinal epithelial cells carrying *ras*. Interestingly, we found that detachment-induced Chk2 upregulation does not occur in two independently derived previously published[11] highly malignant anoikis-resistant clones of IEC-18 cells ras-3 and ras-7 constitutively expressing oncogenic *H-ras* (Fig. 6*a*). We reasoned that *ras* oncogene could block anoikis of these cells by preventing detachment-induced Chk2 upregulation. Alternatively, given that oncogenic *ras* is already known to trigger multiple anti-apoptotic mechanisms[11,13,16,18], it is possible that *ras* has the ability to prevent Chk2 from triggering apoptosis regardless of the levels of this protein kinase in the *ras*-transformed cells. To test whether enforced upregulation of Chk2 in malignant intestinal epithelial cells carrying activated *ras* can overcome their anoikis resistance we decided to overexpress Chk2 in ras-3 cells. We first confirmed that similar to what we published before[11], ras-3 cells are significantly more anoikis-resistant than IEC-18 cells in that they display much lower degree of nuclear fragmentation (a hallmark of apoptosis) than the parental IEC-18 cells following detachment (Fig. 6, *b, left*) and a much more pronounced ability to form colonies in the absence of adhesion to the ECM in soft agar (Fig. 6*b, right*), a property that cancer cells typically display once they acquire the ability to survive without being attached to the ECM (we have demonstrated in this regard that the reversal of the anti-anoikis mechanisms mediated in the

ras-transformed cells by components of the cellular apoptotic machinery, such as Bak[13], Bcl-X_L[11], cIAP2 and XIAP[16] blocks the ability of these cells to form colonies in soft agar). We then generated a variant of ras-3 cells ras-3 tet-Chk2, in which Chk2 expression is controlled by a tetracycline-dependent promoter that remains blocked by a tetracycline repressor, unless cells are treated tetracycline (Fig. 6*c, left*). We found that treatment of ras-3 tet-Chk2 cells with tetracycline resulted in the expression of Chk2 in these cells at levels that strongly exceeded those of the endogenous Chk2 in these cells as well as in the control ras-3 tet cells expressing tetracycline repressor alone (Fig. 6*c, left*). However, the induction of abnormally high Chk2 expression in these cells did not increase their susceptibility to apoptosis following detachment from the ECM (Fig. 6*c, middle*). Likewise, enforced expression of Chk2 did not reduce the ability of ras-3 cells to survive and grow as colonies in soft agar (Fig. 6*c, right*). Collectively, these data indicate that oncogenic Ras prevents Chk2 from triggering anoikis even when increased expression of this kinase is enforced in malignant intestinal epithelial cells.

An important implication of our findings for the identification of therapeutic agents that are capable of promoting anoikis of cancer cells carrying oncogenic *ras* is that such agents will likely be able to kill detached cancer cells if they do not require Chk-2 for their pro-apoptotic effect. One class of compounds that are well known to induce programmed cell death in a Chk-2-independent fashion are topoisomearse I inhibitors [26,27], several of which are presently used for cancer treatment[28]. We found that treatment with some of these drugs, such as camptothecin (Fig. 6*d, left*) or SN38, the active metabolite of irinotecan (one of the therapeutically active camptothecin derivatives[29]) (Fig. 6*d, right*), at concentrations that were found to be achievable in the plasma of cancer patients treated with these drugs in clinical studies[29,30], significantly sensitizes ras-3 cells to anoikis.

In summary, we have demonstrated that detachment-induced increase in Chk2 expression represents a novel mechanism of detachment-induced apoptosis of intestinal epithelial cells and that cell death-promoting molecular events induced by such upregulation are suppressed in malignant intestinal epithelial cell carrying *ras* oncogene.

Discussion

Our studies have not only identified Chk2 as a novel element of the anoikis-promoting machinery of intestinal epithelial cells but also attributed a new role to Chk2 (a protein that has so far been known to promote apoptosis mainly in response to DNA damage [31]) as a mediator of anoikis. Even though the involvement of some DNA damage-associated events in control of anoikis can not, in principle, be excluded, it seems that it is the increase in the cellular levels of Chk2 that contributes to the execution of detachment-induced death of non-malignant intestinal epithelial cells. Presumably, this increase results in the enhanced phosphorylation of one or more of the known mediators of Chk2-driven apoptosis, such as E2F-1, PML and others [31]. The notion that Chk2 upregulation can by itself promote cell death is consistent with data obtained by others indicating that Chk2 is capable of autoactivation when overproduced in various types of cells[32,33].

The fact that Chk2 can act as a mediator of anoikis agrees with numerous observations indicating that cells composing colorectal and other types of tumors (and such cells tend to be anoikis-resistant) often display reduced levels and/or activity of Chk2 [22]. Furthermore, patients carrying loss-of-function mutations of Chk2 are known to be at increased risk of various types of cancer [22] which suggests that reduction of Chk2 activity plays a causal role in cancer progression. In support of this possibility, loss of Chk2 gene or blockade of Chk2 kinase activity was found to promote tumor growth in mice [23]. Our data suggest that one mechanism by which loss of Chk2 expression or activity contributes to cancer progression is by rendering respective tumor cells anoikis-resistant.

Interestingly, we found that Ras, an oncogene that frequently occurs in cancer, prevents detachment-induced Chk2 upregulation in intestinal epithelial cells. However, we found that Chk2 when expressed in *ras*-transformed cells at abnormally high levels does not promote their

anoikis. Thus, *ras* oncogene has the ability to prevent Chk2 from triggering anoikis. One possible explanation of the fact that *ras*-induced inhibition of Chk2 upregulation in detached cells does not by itself contribute to the anti-anoikis effect of Ras is that the indicated oncoprotein blocks this form of apoptosis in intestinal epithelial cells via multiple mechanisms, at least some of which could be redundant. The anti-anoikis mechanisms that we have identified so far are mediated by *ras*-induced upregulation of anti-apoptotic proteins Bcl-X_L [11], cIAP2 and XIAP [16] and *ras*-dependent downregulation of a pro-apoptotic protein Bak [13]. It can not be excluded that some of the anti-anoikis signaling events triggered by Ras play redundant roles in protecting malignant cells from anoikis. Thus, simultaneous inhibition of several of these pathways, rather than one, could be required for the demonstration of the involvement of some of the individual mechanisms, such as that mediated by Chk2, in the anti-anoikis effect of Ras.

Given that oncogenic Ras has the ability to prevent Chk2 from triggering anoikis, treatment aimed at promoting anoikis of malignant intestinal epithelial cells carrying oncogenic Ras will most likely be successful if based on agents that do not rely on Chk2 in their ability to cause apoptosis. According to our data, topoisomerase I inhibitors, such as camptothecin and SN38, the active metabolite of irinotecan (one of the therapeutically active camptothecin derivatives[29]) that are known not to require Chk2 for promoting apoptosis[26], are capable of triggering anoikis of *ras*-transformed intestinal epithelial cells. Perhaps not by coincidence, some of the topoisomerase I inhibitors are presently used in clinic as anti-cancer agents[28].

In summary, we have shown in this study that detachment-induced upregulation of Chk2 represents a novel mechanism of anoikis of intestinal epithelial cells and that the pro-anoikis mechanisms induced by such upregulation are blocked in *ras*-transformed intestinal epithelial cells.

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Figure legends

Figure 1. Detachment of non-malignant intestinal epithelial cells from the ECM triggers upregulation of Chk2. IEC-18 cells (*a*) and mouse colonocytes (*b*) were cultured attached to (att) or detached from (det) the ECM for indicated times and assayed for Chk2 expression by Western blot. CDK4 was used as loading control. (c) Levels of Chk2 in attached and detached colonocytes cultured as in (*b*) were quantified by densitometry. Values derived for Chk2 were normalized by those observed for CDK4 (loading control). Protein levels in the attached cells were arbitrarily designated as 1.0. The results represent the average of three independent experiments plus the SD. (*d*) IEC-18 cells were cultured attached to (att) or detached from (det) the ECM for indicated times and assayed for Chk2 phosphorylation at threonine 387 by Western blot. The same gel was re-probed with the anti-Chk2 antibody and then with the anti-CDK4 antibody as a loading control. (*e*) Levels of Chk2 (black bars) and phospho-Chk2 (grey bars) in attached and detached cells cultured as in (*d*) were quantified as in (*c*). The results represent the average of three (in case of Chk2) and four (in case of phospho-Chk2) independent experiments plus the SE. Values marked with an asterisk were significantly ($p<0.05$) different from those derived from the respective control experiments.

Figure 2. Cycloheximide blocks Chk2 expression in attached cells faster than in detached cells. IEC-18 cells were cultured attached to (a) or detached from (b) the ECM for the indicated times in the presence of DMSO (-) or 10μg/ml cycloheximide (CHX) (+) and assayed for Chk2 expression by Western blot. β-actin was used as a loading control. (c) Levels of Chk2 in attached (solid line) and detached (dotted line) cells cultured as in (*a and b*) were quantified by densitometry. Values derived for the indicated proteins were normalized by those observed for β-

actin (loading control). For each time point protein levels in the DMSO-treated cells were arbitrarily designated as 1.0. The results represent the average of three independent experiments plus the SD. Values marked with an asterisk were significantly ($p<0.05$) different from those derived from the respective control experiments.

Figure 3. Detachment-induced upregulation of Chk2 contributes to anoikis of non-malignant intestinal epithelial cells. (*a*) IEC-18 cells were transfected with a control RNA (cRNA) or Chk2-specific siRNA-1 (Chk2 siRNA-1), Chk2-specific siRNA-2 (Chk2 siRNA-2) or Chk2-specific siRNA-3 (Chk2 siRNA-3) and assayed for Chk2 expression by Western blot. CDK4 was used as a loading control. (*b*) Cells transfected as in (*a*) were placed in monolayer immediately or after being detached for 24h. Colonies formed by the viable cells were counted 7 days later. Results are expressed as a percentage of the number of colonies formed by cells plated in monolayer immediately after transfection and represent the average of the triplicates plus the S.D. This experiment was repeated twice with similar results. (c) Cells transfected as in (a) were placed in suspension for 24h and assayed for Annexin V binding by flow cytometry. The data represent the average of five experiments plus the S.E. (d, left) IEC-18 cells were co-transfected with a control vector (cont vector) or a vector coding for HA-tagged Chk2 (Chk2) and a GFP expression vector and assayed for Chk2 expression with an HA-specific antibody by Western blot (WB). The membrane was then re-probed with a Chk2-specific antibody (Chk2). CDK4 was used as a loading control. (*d, right*) Cells processed as in (*d, left*) were placed in monolayer or suspension for 48h and GFP-positive cells were assayed for nuclear morphology. Cells with fragmented or condensed nuclei were scored as apoptotic. An increase in the percentage of apoptotic cells (this percentage is indicated above each bar) was then calculated for each sample relative to that

observed for the adherent cells transfetced with a control vector (relative apoptosis). Percentage of apoptotic adherent cells transfected with a control vector in a total population of GFP-positive cells was arbitrarily designated as 1.0. Results represent the average of three independent experiments plus the SD. Values marked with an asterisk were significantly ($p<0.05$) different from those derived from the respective control experiments.

Figure 4. Chk2 and p38 MAP kinase antagonists cooperate between each other in protecting intestinal epithelial cell from anoikis. (*a*) IEC-18 cells transfected with a Chk2-specific siRNA2 were cultured in suspension for 4h and assayed for Fas ligand expression by Western blot. (*b*) IEC-18 cells were placed in suspension, treated with either DMSO (-) or 20 μM SB 203580 (+) for 4h and assayed for Chk2 expression by Western blot. CDK4 was used as a loading control in (*a*) and (*b*). (*c*) Cells treated/transfected (+) or not (-) with the indicated reagents as in (*a*) and (*b*) were cultured detached from the ECM for 20h and assayed for apoptosis as in Fig. 3c. Results represent the average of three independent experiments plus the S.E. Values marked with an asterisk were significantly ($p<0.05$) different from those derived from the respective control experiments.

Figure 5. p53 does not contribute to anoikis of non-malignant intestinal epithelial cells. (*a*) IEC-18 cells were cultured attached to (att) or detached from (det) the ECM for indicated times and assayed for p53 expression by Western blot. (*b*) IEC-18 cells were transfected with a control RNA (cRNA) or p53-specific siRNA-1 (p53 siRNA-1) or p53-specific siRNA-2 (p53 siRNA-2) or p53-specific siRNA-3 (p53 siRNA-3) and assayed for p53 expression by Western blot. (*c*). Cells transfected as in (*b*) were assayed for survival as in Fig. 3b. Results represent the average

of the triplicates plus the S.D. This experiment was repeated twice with similar results. CDK4 was used as a loading control in (*a*) and (*b*).

Figure 6. *ras*-transformed intestinal epithelial cells do not undergo anoikis in response to the upregulation of Chk2. (*a, left*) Indicated cell lines were cultured attached to or detached form the ECM for 24h and assayed for Chk2 expression by Western blot. CDK4 was used as a loading control. (*a, right*) Levels of Chk2 in attached and detached indicated cell lines cultured as in (*a, left*) were quantified by densitometry. Values derived for Chk2 were normalized by those observed for CDK4 (loading control). Protein levels in the attached IEC-18 cells were arbitrarily designated as 1.0. The results represent the average of three independent experiments plus the SD. (*b, left*) IEC-18 and ras-3 cells were cultured attached to or detached from the ECM for 48h and assayed for nuclear morphology characteristic of apoptosis as in Fig. 3*d*. The data represent the average of the duplicates plus the SD. 0% apoptosis was observed for attached ras-3 cells (*b, right*) IEC-18 and ras-3 cells were placed in monolayer or soft agar, and colonies formed by these cells were counted 7 days later. Results (the fraction of cells forming colonies in soft agar referred to as "% colonies") are expressed as a percentage of the number of colonies formed by cells plated in monolayer and represent the average of the triplicates plus the SD. (*c, left*) ras-3-tet cells, a derivative of ras-3 cells constitutively expressing tetracycline repressor and ras-3-tet-Chk2 cells constitutively expressing tetracycline repressor and Flag-tagged Chk2 under the control of tetracycline-inducible promoter were cultured in the absence (-) or in the presence (+) 100μg/ml tetracycline for 24h and assayed for Chk2 expression by Western blot (WB) with a Flag-specific antibody (Flag). The membrane was then re-probed with a Chk2-specific antibody (Chk2). The positions of endogenous (endog.) and exogenous (exog.) Flag-tagged Chk2 on the

gel are indicated. The membrane was further re-probed with an anti-CDK4 antibody as a loading control. (*c, middle*) Indicated cell lines were cultured as in (*c, left*), placed in suspension for 24 hours in the absence (-) or in the presence (+) of 100μg/ml tetracycline and assayed for chromosomal DNA fragmentation, a characteristic feature of apoptosis, by the Cell Death ELISA. Results (% apoptotic signal observed in detached IEC-18 cells) were calculated as a percent of a signal observed in detached IEC-18 cells. The data represent the average of the duplicates plus the SD. (*c, right*) Indicated cell lines were processed as in (*c, left*), and assayed for soft agar growth as in (*b, right*) in the absence (-) or in the presence (+) 100μg/ml tetracycline. Results represent the average of the duplicates plus the SD. (*d*) ras-3 cells were cultured attached to or detached from the ECM cultured for 24h in the absence (-) or in the presence (+) of 200nM (*left*) camptothecin or 200nM SN38 (*right*) and assayed for apoptosis by the Cell Death ELISA as in (*c, middle*). Results (% apoptotic signal observed in detached IEC-18 cells) were calculated as a percent of a signal observed in IEC-18 cells untreated with the drugs that were cultured in parallel with ras-3 cells. Results represent the average of the duplicates plus the S.D. All experiments were repeated twice with similar results. Values marked with an asterisk were significantly ($p<0.05$) different from those derived from the respective control experiments.

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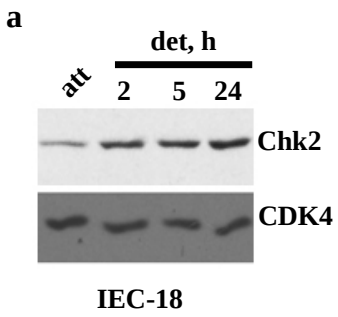
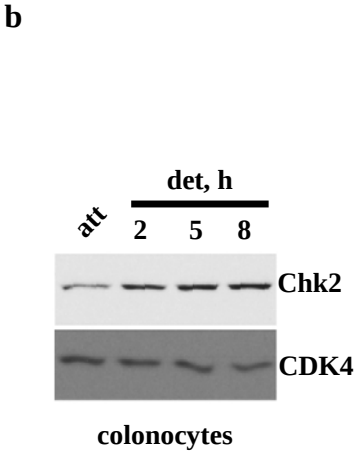
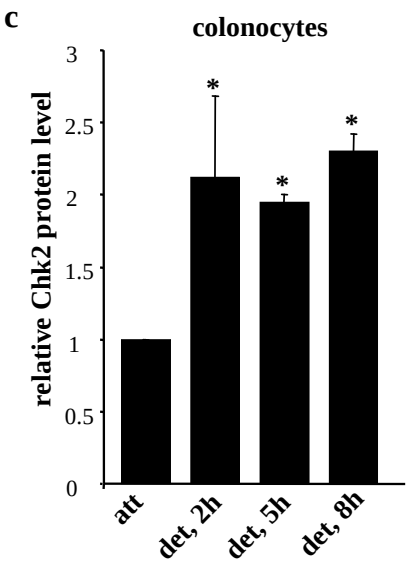
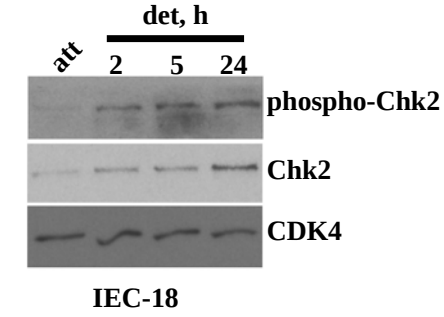
Figure 1


a
det, h
att
2
5
24
Chk2
CDK4
IEC-18


b
det, h
att
2
5
8
Chk2
CDK4
colonocytes


c
colonocytes
relative Chk2 protein level
3
2.5
2
1.5
1
0.5
0
att
det, 2h
det, 5h
det, 8h


d
det, h
att
2
5
24
phospho-Chk2
Chk2
CDK4
IEC-18

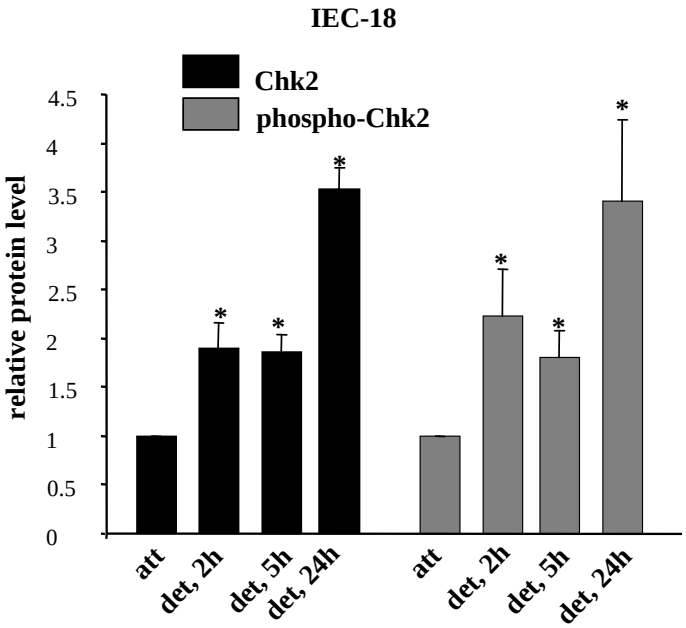

e
IEC-18
Chk2
phospho-Chk2
relative protein level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
att
det, 2h
det, 5h
det, 24h
att
det, 2h
det, 5h
det, 24h

Figure 2

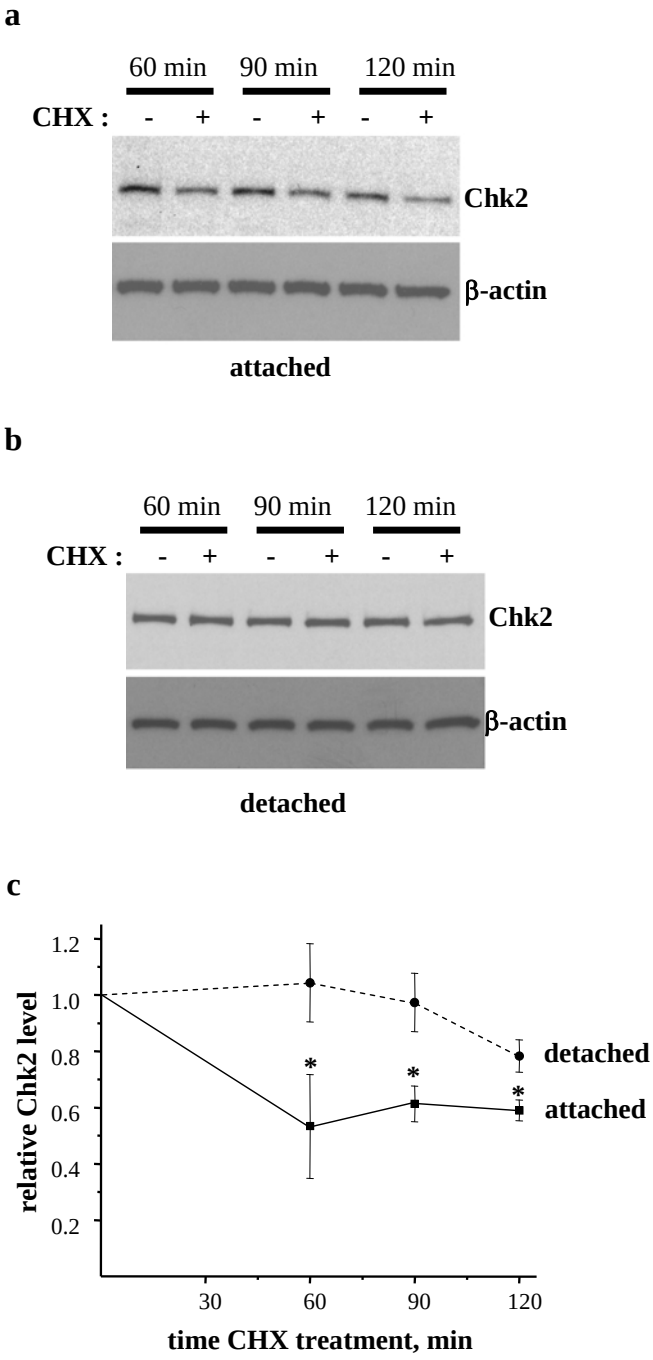

a
60 min
90 min
120 min
CHX :
- + - + - +
Chk2
β-actin
attached
b
60 min
90 min
120 min
CHX :
- + - + - +
Chk2
β-actin
detached
c
relative Chk2 level
1.2
1.0
0.8
0.6
0.4
0.2
30
60
90
120
time CHX treatment, min
detached
attached
*
*
*

Figure 3

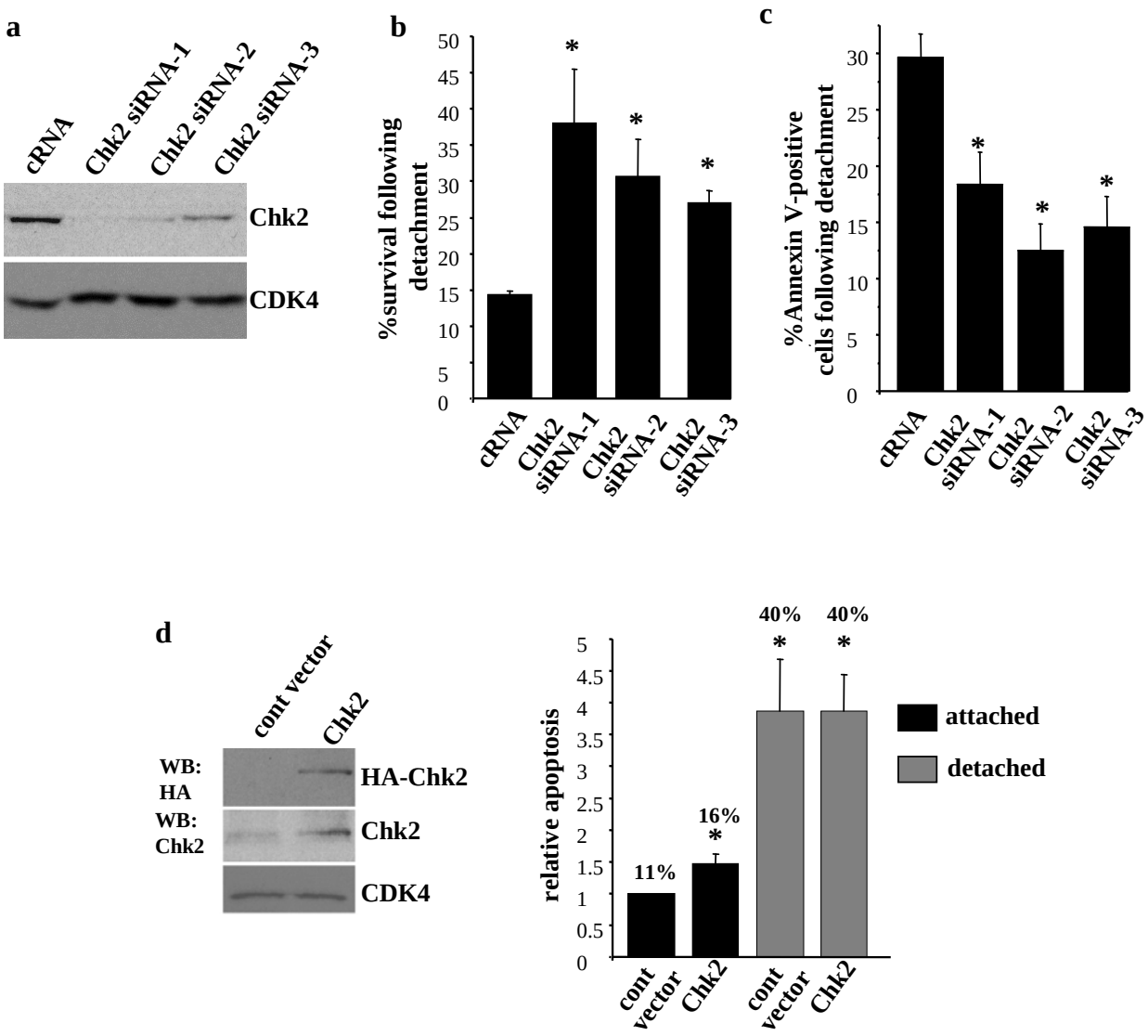

a
cRNA
Chk2 siRNA-1
Chk2 siRNA-2
Chk2 siRNA-3
Chk2
CDK4
b
%survival following detachment
50
45
40
35
30
25
20
15
10
5
0
*
*
*
cRNA
Chk2 siRNA-1
Chk2 siRNA-2
Chk2 siRNA-3
c
%Annexin V-positive cells following detachment
30
25
20
15
10
5
0
*
*
*
cRNA
Chk2 siRNA-1
Chk2 siRNA-2
Chk2 siRNA-3
d
cont vector
Chk2
WB: HA
HA-Chk2
WB: Chk2
Chk2
CDK4
relative apoptosis
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
11%
16%
*
40%
*
40%
*
attached
detached
cont vector
Chk2
cont vector
Chk2

Figure 4

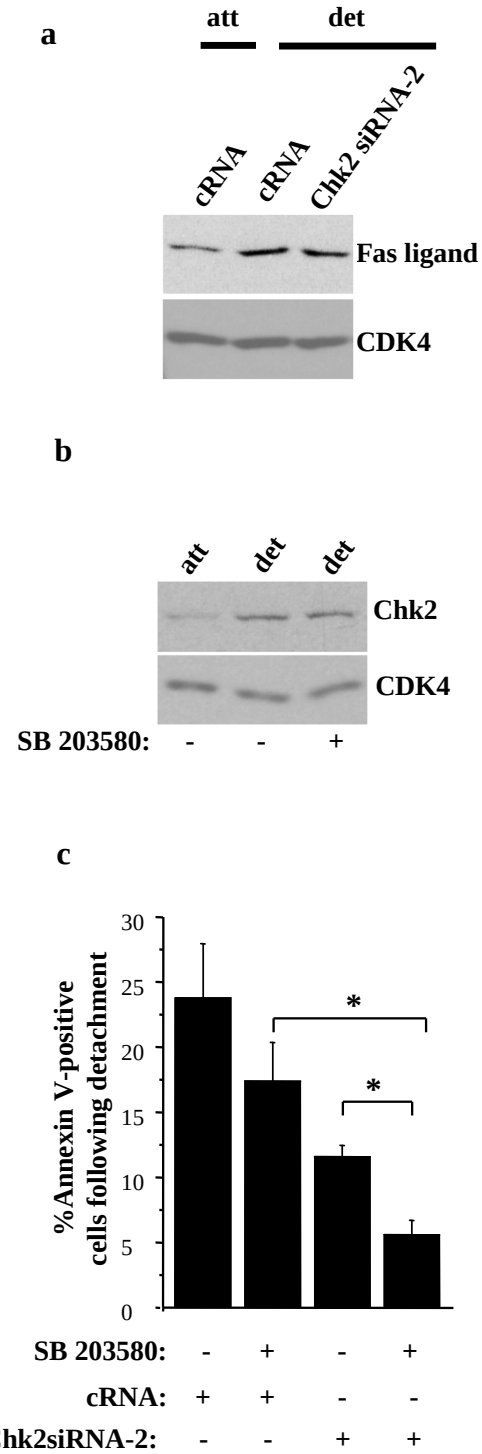

a
att
det
cRNA
cRNA
Chk2 siRNA-2
Fas ligand
CDK4
b
att
det
det
Chk2
CDK4
SB 203580: - - +
c
%Annexin V-positive cells following detachment
30
25
20
15
10
5
0
*
*
SB 203580: - + - +
cRNA: + + - -
Chk2siRNA-2: - - + +

Figure 5

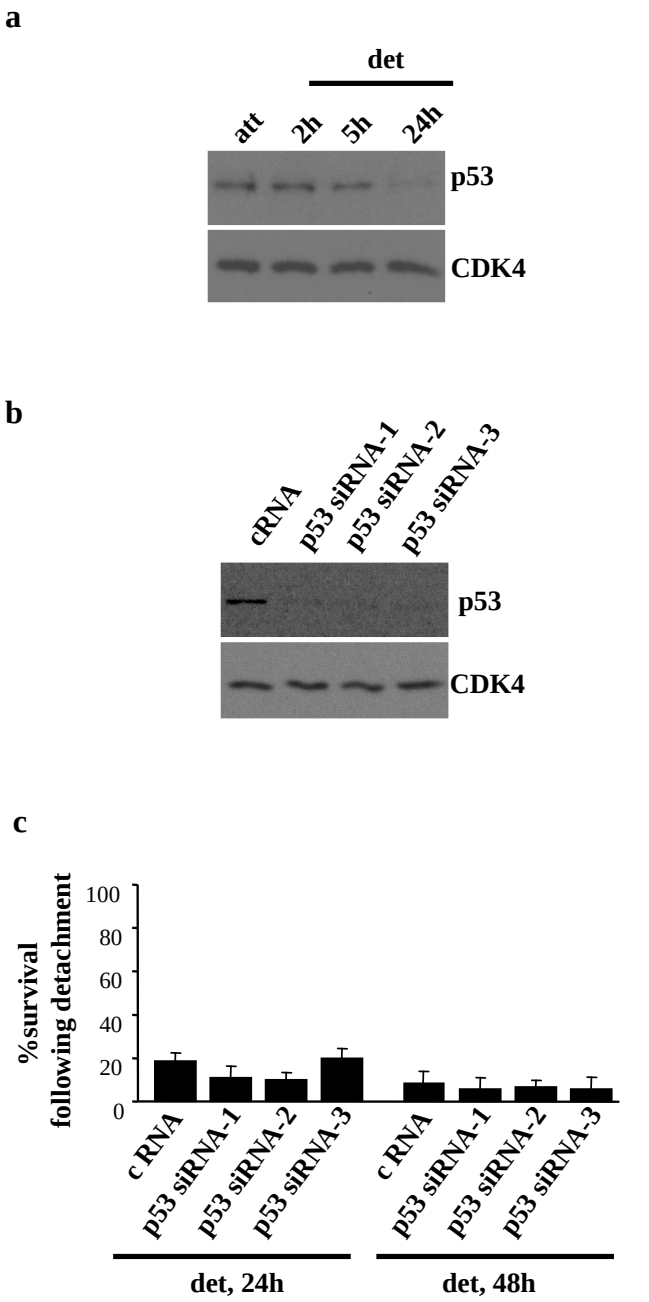
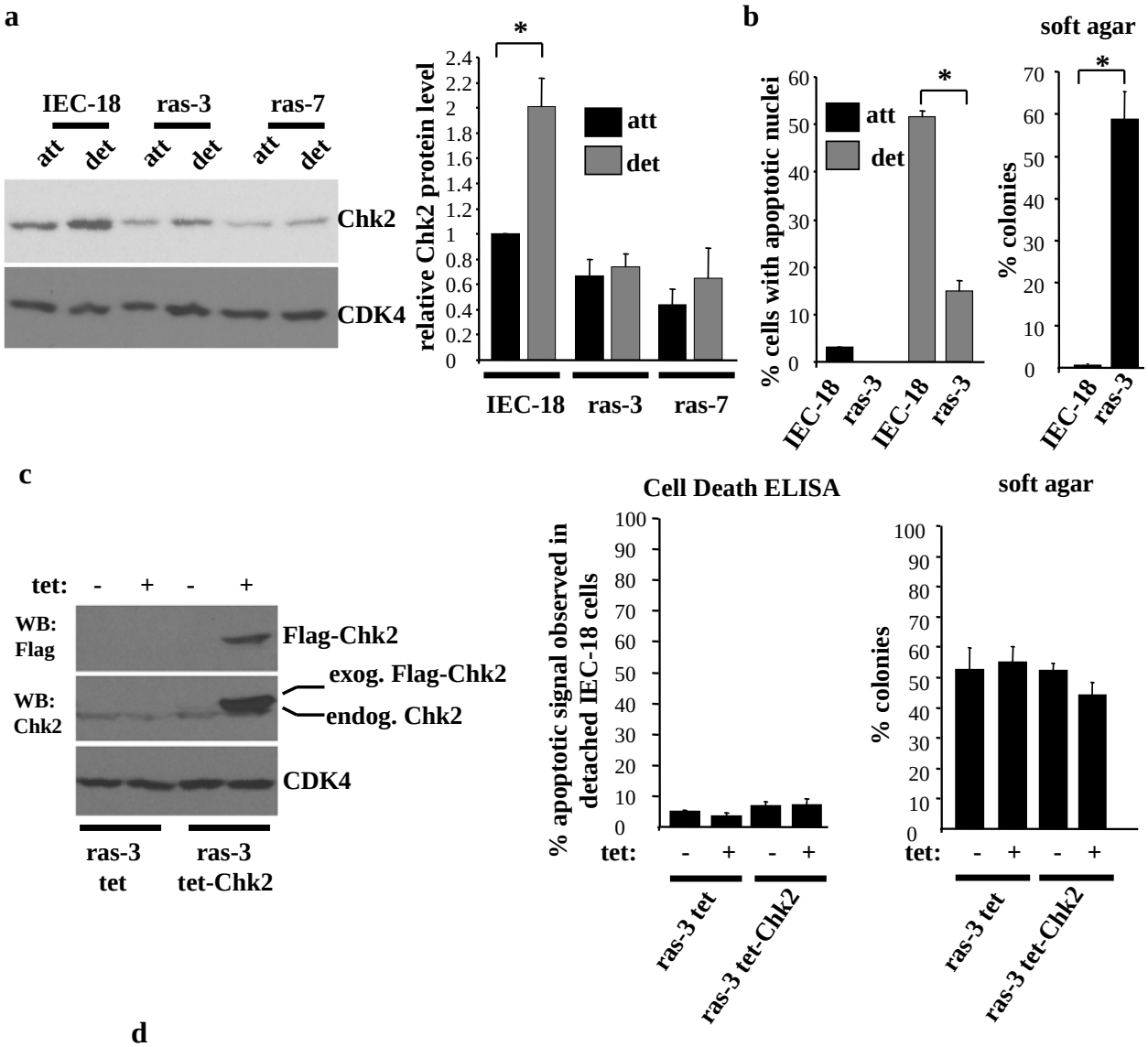

a
det
att
2h
5h
24h
p53
CDK4
b
cRNA
p53 siRNA-1
p53 siRNA-2
p53 siRNA-3
p53
CDK4
c
%survival following detachment
100
80
60
40
20
0
c RNA
p53 siRNA-1
p53 siRNA-2
p53 siRNA-3
c RNA
p53 siRNA-1
p53 siRNA-2
p53 siRNA-3
det, 24h
det, 48h

Figure 6


a
IEC-18
ras-3
ras-7
att
det
Chk2
CDK4
relative Chk2 protein level
att
det
IEC-18
ras-3
ras-7
b
soft agar
% cells with apoptotic nuclei
% colonies
IEC-18
ras-3
c
tet:
WB: Flag
WB: Chk2
Flag-Chk2
exog. Flag-Chk2
endog. Chk2
CDK4
ras-3 tet
ras-3 tet-Chk2
Cell Death ELISA
% apoptotic signal observed in detached IEC-18 cells
soft agar
% colonies
d
Cell Death ELISA
ras-3
% apoptotic signal observed in detached IEC-18 cells
camptothecin:
SN38: