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ORIGINAL ARTICLE

5-Fluorouracil signaling through a calcium–calmodulin-dependent pathway is required for p53 activation and apoptosis in colon carcinoma cells

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5-Fluorouracil (5-FU) is an anti-metabolite that is in clinical use for treatment of several cancers. In cells, it is converted into three distinct fluoro-based nucleotide analogs, which interfere with DNA synthesis and repair, leading to genome impairment and, eventually, apoptotic cell death. Current knowledge states that in certain cell types, 5-FU-induced stress is signaling through a p53-dependent induction of tumor necrosis factor-receptor oligomerization required for death-inducing signaling complex formation and caspase-8 activation. Here we establish a role of calcium (Ca^{2+}) as a messenger for p53 activation in response to 5-FU. Using a combination of pharmacological and genetic approaches, we show that treatment of colon carcinoma cells stimulates entry of extracellular Ca^{2+} through long lasting-type plasma membrane channels, which further directs posttranslational phosphorylation of at least three p53 serine residues (S15, S33 and S37) by means of calmodulin (CaM) activity. Obstructing this pathway by the Ca^{2+}-chelator BAPTA (1,2-bis(o-aminophenoxy)ethane- N,N,N′,N′-tetraacetic acid) or by inhibitors of CaM efficiently reduces 5-FU-induced caspase activities and subsequent cell death. Moreover, ectopic expression of p53 S15A in HCT116 $p53^{-/-}$ cells confirmed the importance of a Ca^{2+}–CaM–p53 axis in 5-FU-induced extrinsic apoptosis. The fact that a widely used therapeutic drug, such as 5-FU, is operating via this pathway could provide new therapeutic intervention points, or specify new combinatorial treatment regimes.

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INTRODUCTION

5-Fluorouracil (5-FU, Adrucil) is an anti-metabolite, which has been used in clinical practice for over 40 years. Some of its primary targets are colorectal and gastrointestinal cancers, but it is also used in treatment of breast, head and neck, and ovarian carcinomas. In cells, it is converted into three main fluoronucleotide analogs: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate, each able to induce cellular stress by distinct mechanisms. Fluorodeoxyuridine monophosphate acts as a covalent thymidylate synthase inhibitor, resulting in a thymineless cell-state due to reduced deoxyuridine monophosphate methylation. The lack of 2′-deoxythymidine triphosphate follows by an accumulation of fluorodeoxyuridine triphosphate and deoxyuridine triphosphate pools, which may overwhelm steady-state repair systems that normally exclude uracil from DNA. As a consequence of a depleted thymine pool, forced incorporation of uracil and subsequent impairment of DNA replication and repair may then trigger activation of specific cell death pathways.[1] It has also been suggested that loss of carcinogenic properties in colon and breast cancer cell lines may be caused by RNA stress through misincorporation of fluorouridine triphosphate,[2–4] thus inhibiting processes such as rRNA maturation, splicing of pre-mRNA and post-transcriptional modification of tRNAs.[1] In comparison with the generally accepted ATM (kinase ataxia telangiectasia mutated)/ATR (Rad3-related protein)–Chk1/Chk2 (checkpoint kinases 1 and 2, respectively)–p53 signaling pathway, leading to apoptotic cell death in response to severe DNA damage, significantly less has been reported regarding death signaling cascades originating from RNA damage, even though it has been proposed that transcriptional stress also can lead to p53 activation.[5] However, despite the extensive use of 5-FU in the clinic, the relative importance for each triggering point is not clearly established. A plausible explanation for this ambiguity can be that, as 5-FU and its metabolites have the potential to strain several discrete molecular mechanisms, treatment outcome is governed by the tumor cell context, which certainly differs between cell types. In support of this principle are data demonstrating a marked difference between 5-FU and its metabolite fluorodeoxyuridine monophosphate in their effect on the cell cycle progression in colon cancer, and the cell's ability to generate DNA double-strand breaks, monitored by conversion of H2AX into its active form γH2AX.[6] In addition, it seems that a cancer cell's efficacy to remove and repair DNA sites where uracil have been incorporated highly dictates its sensitivity to 5-FU.[7]

Once initiated, apoptosis is executed by caspases and several upstream regulatory factors, which direct their proteolytic activity, have been defined as either tumor suppressors or oncogenes.[8] Two main apoptotic routes exist, the extrinsic receptor-mediated and the intrinsic mitochondrial pathways. Although in the former, extracellular ligands stimulate members from the tumor necrosis

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factor (TNF) receptor family (CD95, TNF, DR4 or DR5) to form death-inducing signaling complexes (DISCs) required for caspase-8 activation, disruption of mitochondrial membrane integrity and release of pro-apoptotic factors control the latter pathway. Although the initial phases are characterized by distinct features, these apoptotic pathways converge in the activation of effector caspases (caspases-3, -6 and -7), which in turn target a broad spectrum of cellular proteins, thereby predestining cells to irreversible cell death. As a consequence of double-strand breaks in genomic DNA, many chemotherapeutic agents in current use have been reported to trigger apoptosis by the intrinsic pathway. Engagement of this conduit usually requires p53 function and mutations within the gene, or abnormalities in signaling events leading to its activation can produce multiple drug-resistant phenotypes.[9] However, 5-FU does not conform to this rule but has instead been described to induce a p53-dependent extrinsic, DISC-mediated apoptotic pathway. Accordingly, *in situ* immunohistochemical analyses of clinical colorectal cancer sections correlated with the tumor stage (stage I–IV), and treatment response demonstrated that p53 and DR5 expression decreased progressively for each stage, suggesting that these proteins are important markers of advanced tumors, and that p53 appears as a prognostic factor to predict recurrence-free survival.[10] It was also shown that inducible silencing of DR5 in mice led to accelerated growth of bioluminescent tumor xenografts and conferred 5-FU-resistance.[11] Similar data have been provided by using tumor cell lines, although conflicting evidences exist postulating DR4, DR5 or CD95 as the regulatory receptor for caspase-8 activation and apoptosis.[12–14] Interestingly, death receptor ligand does not seem to be required for DISC formation in this particular apoptosis pathway.[12] Most DR5 expression studies in clinical tumor sections do not experimentally verify whether the death receptor is induced as a consequence of chemotherapy, or if it is a response to tumorogenesis. On the other hand, an examination of the response of three human colon tumors to TNF-related apoptosis-inducing ligand (TRAIL) alone and in combination with chemotherapy drugs, using SCID mice engrafted with intact patient surgical colorectal cancer specimens, revealed that tumors treated with carnitine palmitoyltransferase-11 (CPT-II) showed increased membrane expression of DR5, also suggesting that CPT-II may increase sensitivity to TRAIL by upregulation of DR5.[15] In the present study, we extend the knowledge concerning 5-FU-induced cell death and introduce a calcium–calmodulin (Ca^{2+}–CaM)-dependent signaling event that is required for specific phosphorylation-mediated activation of p53 and subsequent DISC activity, events that are essential for 5-FU-induced apoptosis.

RESULTS

DR5 but not CD95 facilitates apoptosis induced by 5-FU in HCT116 cells

Previously, we described a pronounced p53-dependent upregulation of CD95 in 5-FU-treated HCT116 cells.[16] In addition, using the same experimental settings, CD95–DISC formation was revealed by immunoprecipitation. However, as shown by immunostaining, in response to 5-FU, both TNF-family receptors DR5 and CD95 are accumulated in the plasma membrane (Figure 1a), indicating that either one of them or both could have a vital role for efficient apoptosis. As conflicting evidences exist in this matter,[12,13,17] we decided to assess the contribution of each individual receptor to initiator caspase-8 and effector caspase-3 activation by means of small interfering RNA (siRNA) technology (Figures 1b and c). This experimental approach clearly demonstrated that DR5 but not CD95 is the sole receptor required for caspase-8 activation and further processing of caspase-2 and effector caspase-3 upon treatment with 5-FU. In addition, isolation of membrane protein fractions from untreated and induced cells revealed that in response to 5-FU accumulation of DR5 and DISC components, Fas-associated protein with death domain (FADD) and caspase-8 occur in plasma membranes (Figure 2a). DISC non-associated caspase-7, on the other hand, remained in cytosol irrespectively of treatment. As we used a protocol in which the total membrane protein pool was isolated, TOM 40 (translocase of the outer mitochondrial membrane) and tubulin served as a marker for fractionation efficiency. To rule out the existence of DISC components in cellular membranes other than the plasma membrane, obtained results were confirmed using immunostaining with specific antibodies targeting DR5 and FADD (Figure 2b). Moreover, as a correspondent localization pattern was not observed in HCT116 $p53^{-/-}$ cells using the same incubation time, we concluded that an efficient accumulation of DISC components DR5, FADD and caspase-8 in plasma membranes of 5-FU-treated cells is a p53-dependent or -facilitated event. Thus, in agreement with some previous reports,[11,12] these results indicate the DR5-receptor as a p53-dependent mediator of 5-FU-induced apoptosis, also suggesting that the CD95–DISC, formed for still unidentified reason(s), remains inactive with respect to caspase-8 activation, or that contribution of this complex is insignificant.[16]

Analysis of potential regulatory factors upstream of DR5–DISC formation

In contrast to classical extrinsic death pathways triggered by ligands of the TNF family, 5-FU-induced apoptosis most certainly emerges from either DNA or RNA damage. The question relating to how initial drug-triggering points are transduced to DISC formation and caspase-8 activity is indeed intriguing. p53 is obviously an important factor for the process but a detailed description of signaling events originating from 5-FU-induced cellular stress leading to p53 activity and subsequent DR5 oligomerization is to a large extent still lacking in current literature.[9,16] Therefore, a panel of inhibitors, including Ca^{2+}-chelator BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid), RIP1-kinase inhibitor NEC1, the antioxidant Trolox, pepstatin A, an inhibitor of acid proteases and the cathepsin B inhibitor CA-074, was added in combination with 5-FU to HCT116 cells, to target potential upstream controlling conduits (Figure 3a). Among the inhibitors used, three effectively abrogated effector caspase-3 processing. Two of these, pepstatin A and CA-074, are silencing lysosomal protease activity. However, neither of them had any effect on the most apical caspase-8 activation. Hence, we concluded that lysosomal proteases indeed have a role in 5-FU-induced apoptosis but appear to function as an enhancer of effector caspase activity, downstream of DISC formation. This is well in agreement with a recent report showing that lysosomal membrane permeability and the cytosolic release of cathepsins B, L and D indirectly depend on Bax/Bak and components of the apoptosome.[18] In comparison, BAPTA had a profound effect also on caspase-8 processing, indicating Ca^{2+} as a messenger acting upstream of the caspase cascade. Moreover, although the 5-FU-induced p53 level remained unaffected in the presence of BAPTA, phosphorylation of S15 was reduced considerably, thus positioning the effect of Ca^{2+} in advance of p53 posttranslational modifications (Figure 3a and Supplementary Figure S1).

By using lentiviral-mediated transfer of wild-type (wt) and mutated p53 cDNAs in p53 knock-out HCT116 cells, the p53 S15 phospho site was previously implicated in the apoptotic response to 5-FU, as determined by measurement of subG1 cells after propidium iodide staining.[19] Here, by taking advantage of the same methodology, we were able to further specify that in comparison with reintroduced wt p53, the S15A mutation abrogates DISC-facilitated caspase-8 processing and downstream cleavage of poly (ADP-ribose) polymerase (PARP; Figure 3b).

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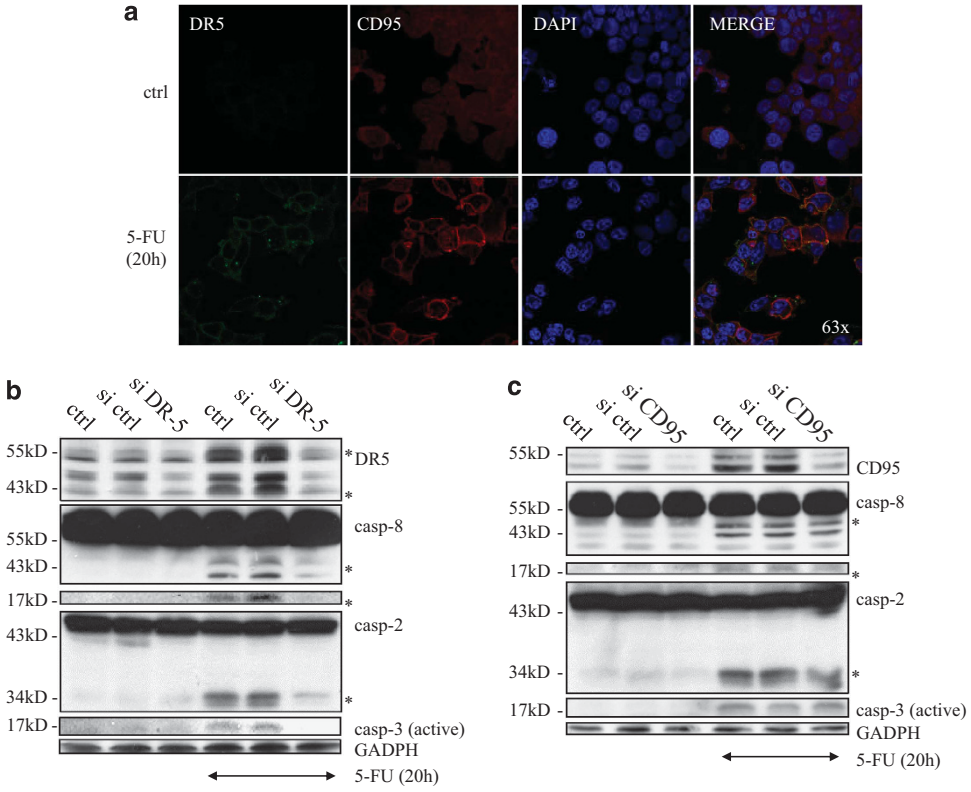

Figure 1. Both death receptors DR5 and CD95 translocate to plasma membranes in response to 5-FU treatment, but only silencing of the former protein reduces the processing of apical and effector caspases. HCT116 control cells and cells induced with 5-FU for 20 h were fixed in 4% formaldehyde for 20 min and exposed to specific antibodies targeting either CD95 or DR5 (**a**). Cell nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole). The effect of 5-FU with respect to processing of caspases -8, -2 and -3 was analyzed by SDS–PAGE, using total lysates from cells in which either DR5 (**b**) or CD95 (**c**) had been silenced by means of siRNA transfections. Non-targeting control siRNA was used to exclude the possibility that transfections *per se* interfered with analysis outcome and glyceraldehyde 3-phosphate dehydrogenase was used as a control for equal loading of samples. Processed caspase fragments and isoforms of DR5 are indicated with asterisks.

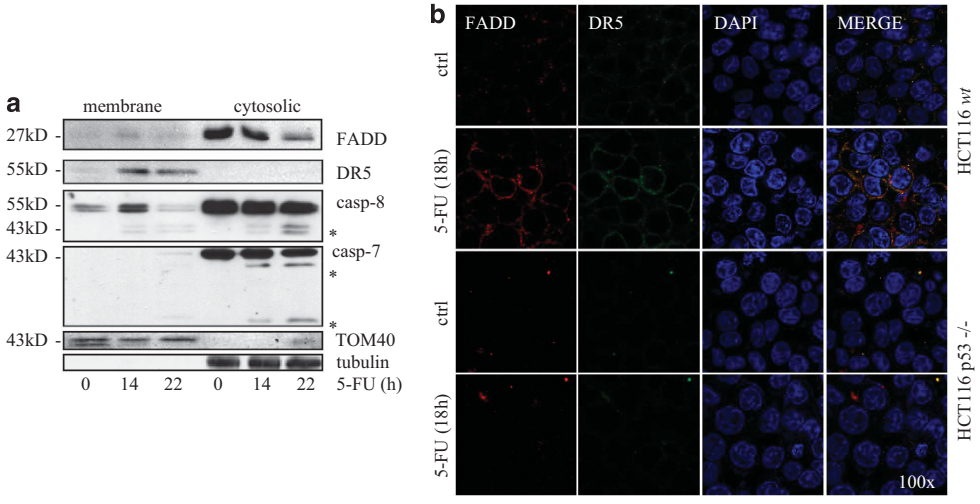

Figure 2. DISC-related factors accumulate in plasma membranes in a p53-dependent manner as a response to 5-FU treatment. HCT116 cells were treated as indicated in figures and subsequently fractionated, generating membrane and cytosolic protein lysates, which were analyzed by SDS–PAGE. Western blot membranes were next probed with antibodies directed to DISC-associated proteins DR5, FADD and caspase-8 (**a**). Detection of TOM 40 and tubulin served to indicate the purity of membrane and cytoplasmic fractions, respectively. Exposure of the DISC non-related caspase-7 was used as a negative control. Equal amount of protein material was loaded into each lane. Processed caspase fragments are indicated with asterisks. To confirm the presence of the DISC in plasma membranes and to examine the significance of p53 for translocation of DISC proteins to plasma membranes, HCT116 wt and HCT116 $p53^{-/-}$ cells were treated as indicated, fixed in paraformaldehyde and analyzed by indirect immunofluorescence using antibodies targeting DR5 and FADD (**b**). Cell nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole).

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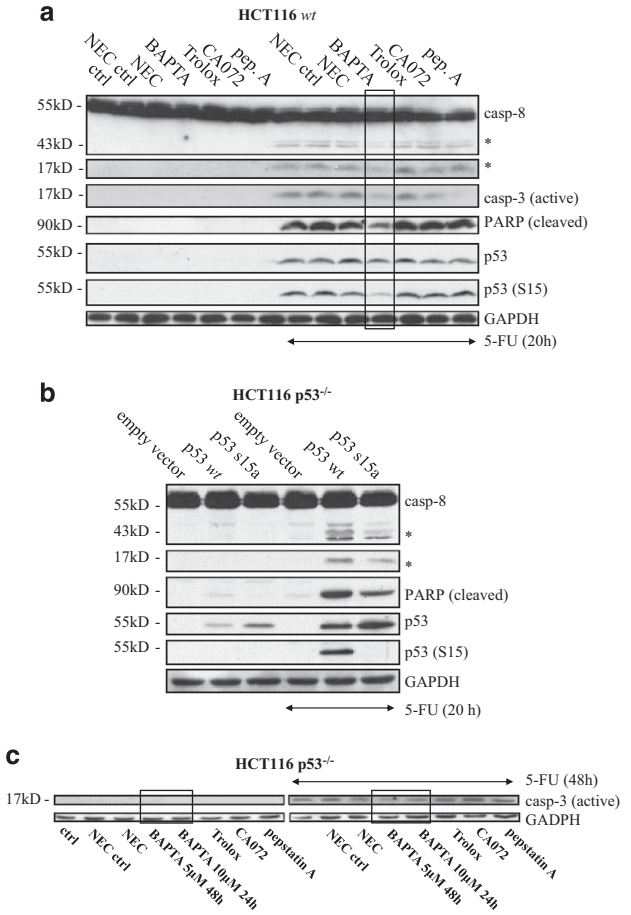

Figure 3. Chelation of Ca^{2+} interferes with p53 S15 phospho activation and initiation of the apoptotic cascade in 5-FU-induced HCT116 cells but not in HCT116 $p53^{-/-}$ cells. HCT116 wt (**a**) and $p53^{-/-}$ cells (**c**) were left untreated or induced with 5-FU as indicated, either alone or in combination with potential chemical inhibitors of DISC-mediated apoptosis. Cells were collected and total cell lysates were analyzed by SDS–PAGE. Following the blotting procedure, membranes with material originating from HCT116 wt cells were examined by using antibodies directed to caspase-8, processed caspase-3, cleaved PARP, p53 and its phospho activation of S15, whereas membranes with separated HCT116 $p53^{-/-}$ cell lysates were analyzed with respect to processed caspase-3 as a control for the p53-regulating properties of Ca^{2+}. Reintroduction of wt p53 and S15A-mutated p53 to p53-deficient HCT116 cells by means of lentiviral transduction served to confirm that the S15 phospho site is important for proper DISC-mediated caspase-8 processing and cleavage of PARP (**b**). In addition to PARP and caspase-8, the membrane in **b** was probed with antibodies targeting p53 and phospho-modified S15 to control correct expression of cDNAs. Probing of glyceraldehyde 3-phosphate dehydrogenase served as a control for equal loading of samples. Two conditions of BAPTA treatment and 5-FU co-treatment, 5 μM for 48 h or 10 μM added 24 h post 5-FU induction, were performed in HCT116 $p53^{-/-}$ cells to avoid toxicity. Results for BAPTA are decorated (**a** and **c**). Processed caspase fragments are indicated with asterisks.

With prolonged 5-FU treatment, it has not escaped our notice that also HCT116 $p53^{-/-}$ cells undergo a DR5- and caspase-8-dependent cell death. In fact, DR5 is correspondingly upregulated in these cells, but to a lesser extent compared with the parental cell line (manuscript in preparation). As neither BAPTA nor any of the other inhibitors tested obstructed the weak caspase-3 activity detected in HCT116 $p53^{-/-}$ cells 48 h post 5-FU treatment, we concluded that Ca^{2+} primarily exerts its effect on p53 activity as a response to stress induced by 5-FU (Figure 3c).

The Ca^{2+}-level in HCT116 cells is enhanced in response to 5-FU treatment

Ca^{2+} is a ubiquitous second messenger involved in many cellular processes, including cell death.[20,21] Ca^{2+} fluxes from the extracellular space are determined by the activity of voltage-operated, receptor-operated and store-operated membrane channels that normally are under tight control. Elevated Ca^{2+} level in the cytoplasmic compartment can also occur as a result of release from the endoplasmic reticulum, which is the major organelle involved in Ca^{2+} homeostasis. Changes in the intracellular Ca^{2+} levels in response to 5-FU treatment in HCT116 cells were monitored by using the Fluo-4AM fluorescent indicator. By fluorescence-activated cell sorting, we detected an increase in intracellular Ca^{2+} at 4 h, and a further enhancement at 5 h post treatment (Figure 4a). After 5 h, increased level of Ca^{2+} remained up to 15 h, which is the time point when initiation of caspase processing can be detected by SDS–polyacrylamide gel electrophoresis (PAGE; Figure 4b and ref 16, respectively). Examination of cellular Ca^{2+} by time-lapse confocal microscopy was then performed, and influx commencement noticed as early as 1.5 h after addition of 5-FU (Figure 4c). This is well in advance of the p53 S15 phosphorylation, which could be detected by western blotting 5 h post induction (Figure 4d). Thus, these data support our findings, indicating Ca^{2+} as a regulatory factor acting upstream of p53 activity in response to 5-FU. There are, however, some parameters that have to be considered in this respect. First, western blotting can be a sensitive or insensitive technique, depending on the specific antibody used. Thus, activation of p53 as a consequence of S15 phosphorylation may occur earlier than what SDS–PAGE results predict. Second, most likely, a critical threshold concentration of Ca^{2+} must be reached to trigger subsequent p53 activity. By our measurements, it is impossible to specify this threshold limit but a qualified guess would be that it is reached between 1.5 and 4 h post induction. Still, irrespectively of these uncertainties, elevation of Ca^{2+} and p53 activity, as determined by S15 phosphorylation, remains co-ordinated sequence of events.

Extracellular Ca^{2+} influx is required for 5-FU-induced p53 phosphorylation and caspase activation

To determine the source of Ca^{2+} required for apoptotic signaling in 5-FU-treated HCT116 cells, the following experiments were performed. To begin with, cells were cultured and treated in Ca^{2+}-free media and then analyzed with respect to p53 S15 phosphorylation and appearance of apoptotic markers, including caspase processing and PARP cleavage (Figures 5a and b). As the lack of environmental Ca^{2+} had a clear effect on all parameters tested, reducing phospho-p53 activation even more efficiently than BAPTA and decreasing caspases-3 and -8 processing, as well as PARP cleavage to control cell levels, we concluded that extracellular Ca^{2+} is the primary source required for development of specific apoptotic processes in 5-FU-treated HCT116 cells. Besides, verapamil, an inhibitor of long lasting (L)-type Ca^{2+} plasma membrane channels abrogated 5-FU-induced p53 S15 phosphorylation, as well as caspase-3 and -8 activities, in a concentration-dependent manner (Figure 5c). Although these data clearly state that a 5-FU-dependent cellular influx of Ca^{2+} is an early event obligatory for at least one p53 posttranslational modification, they do not entirely exclude the possibility that endoplasmic reticulum-derived Ca^{2+} has a role. Ca^{2+}-induced Ca^{2+} release is a process that amplifies cytosolic Ca^{2+} through either inositol 1,4,5-trisphosphate or different isoforms of sarco/endoplasmic reticulum ryanodine receptors. Although primarily associated with the physical control of muscle cells and neurons, Ca^{2+}-induced Ca^{2+} release has also been implicated in apoptosis regulation.[22] To investigate whether the external Ca^{2+} signaling relays on the internal Ca^{2+}-induced Ca^{2+} release excitable system for optimal phospho-p53

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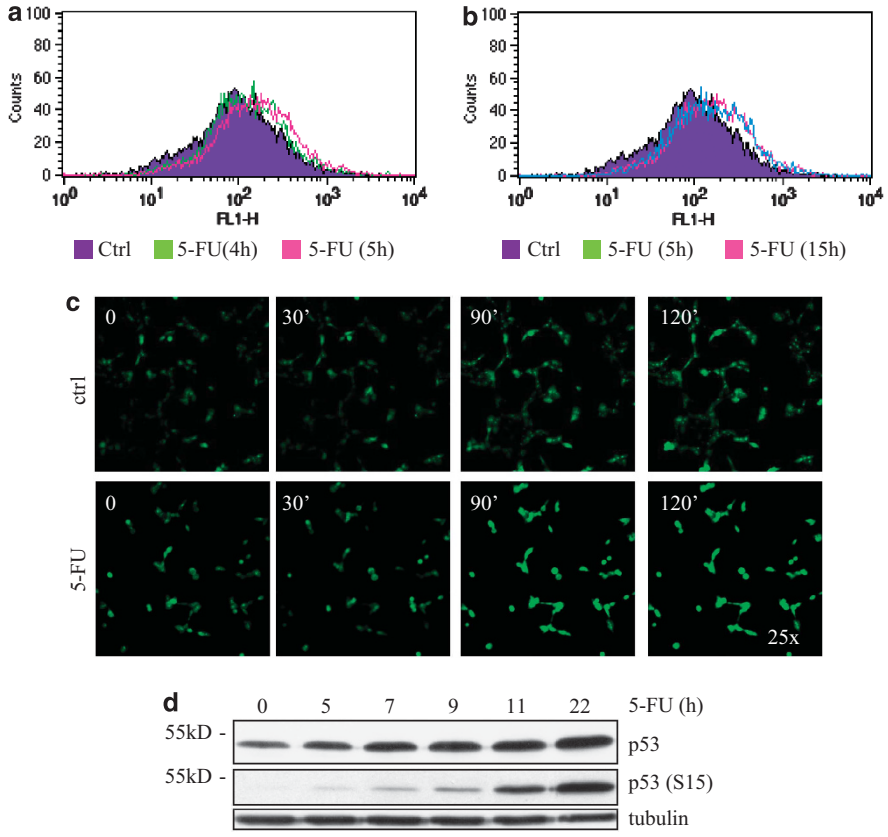

Figure 4. Increase in intracellular Ca^{2+} occurs as an immediate and persistent response to 5-FU, which is timely co-ordinated with the S15 phospho activation and stabilization of the tumor suppressor p53 in HCT116 cells. The Ca^{2+} probe Fluo-4AM was added to cells 30 min in advance of 5-FU and a comparison of Ca^{2+} levels was performed in between non-treated and treated samples. Elevated fluorescent intensity indicating elevated Ca^{2+} levels was observed by fluorescence-activated cell sorting using the FL-1H channel in cells treated for 4 and 5 h (**a**), as well as in cells induced for 15 h (**b**). Examination of the Fluo-4AM fluorescent signal by confocal microscopy revealed a rise in intracellular Ca^{2+} as early as 90 min post treatment (**c**). Analysis of S15 phosphorylation and stabilization of p53 using SDS–PAGE indicated activation of the tumor suppressor at 5 h post 5-FU treatment (**c**). Tubulin served as a marker for equal loading.

activation, intracellular Ca^{2+} stores were depleted by incubation of cells in the presence of thapsigargin for 1 h in advance of 5-FU treatment (Figure 5d). As the phospho activation of p53 S15 remained unaltered in 5-FU and thapsigargin co-treated cells compared with 5-FU control samples, we concluded that Ca^{2+} released from the endoplasmic reticulum does not significantly contribute to 5-FU-induced cell death signaling. Together, these data indicate that the Ca^{2+} required for early apoptotic signaling occurring in response to 5-FU originates from extracellular sources, and that translocation of ions through the plasma membranes at least partly is mediated by L-type Ca^{2+} channels.

CaM is a transitional factor for conducting Ca^{2+} signaling to p53 activity in response to 5-FU

To maintain normal cellular control and tissue integrity, p53 is regulated at the posttranslational level by protein–protein interactions and covalent modifications, including phosphorylation at over 20 phospho-acceptor sites.[23] The reports examining the role of kinases able to modulate p53 activity have led to much controversy within the field, but the general view seems to be that one or several kinases may act on the same residue in a cell- or stimuli-specific manner. Indeed, several acceptor sites of p53 are phosphorylated in response to 5-FU (Figure 6b) and, most likely, the majority of them contribute to treatment outcome in one or another way. However, our focus was to decipher the Ca^{2+}-dependent pathway described, and to analyze its importance for DR5-mediated cell death. Among kinase-dependent activity pathways acting on p53, only two are controlled by Ca^{2+} signaling. One of them involves serine/threonine kinase members included in a subgroup of the protein kinase C (PKC) family termed the classical group encompassing PKCs-α, -βI, -βII and -γ.[24–26] The other one is facilitated by the ubiquitous Ca^{2+}-sensing protein CaM and occurs through activation of members contained in the superfamily of CaM-dependent kinases.[27–29] As a selective inhibitor of PKC (PKC412) did not attenuate p53 S15 phosphorylation in any of the concentrations tested, we concluded that this kinase did not contribute to the 5-FU-induced and Ca^{2+}-dependent events leading to p53 activity. Interestingly, in presence of PKC412 processing of caspases-3 and -8 was inhibited, but obviously in a manner independent of the p53 S15 residue (Figure 6a). In sharp contrast, we observed abrogation of p53 S15 and S33 phosphorylations in parallel with decreased processing of caspases occurring in a concentration-dependent manner when two different CaM inhibitors, calmidazolium chloride or fluphenazine-N-2-chloroethane, were added to HCT116 cells in combination with 5-FU (Figure 6b). A decrease in p53 S46 phosphorylation was also noted, but only in cells pretreated with calmidazolium chloride and not fluphenazine-N-2-chloroethane. S37 phosphorylation was indeed blocked using both inhibitors but in a concentration-independent pattern dissimilar to reduction of caspase processing and phospho activation of S15 and S33, thus indicating this particular phospho site as being less important for the apoptotic process examined. Phosphorylation of the S6, 9 and 382 sites, on the other hand, remained unaffected in the presence of CaM inhibitors.

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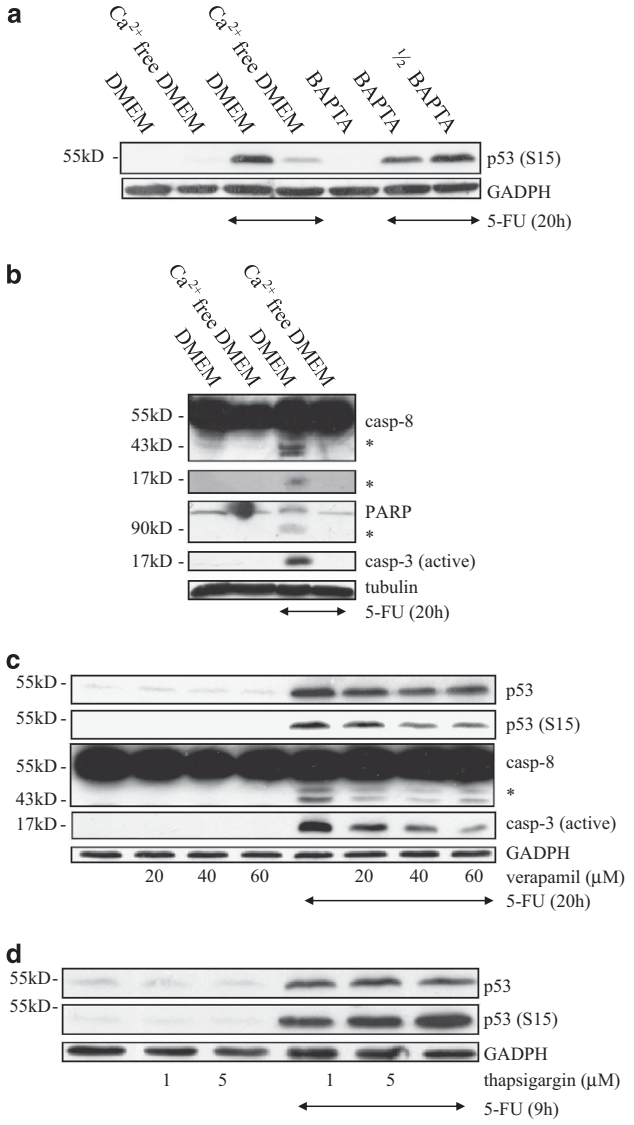

Figure 5. Influx of Ca^{2+} from extracellular sources, but not Ca^{2+} released from the ER, is required for efficient phospho activation of p53 and further induction of DISC-mediated apoptosis. HCT116 cells were exposed to 5-FU, either in combination with BAPTA using standard culture medium (Dulbecco's modified Eagle's medium) or alone using Ca^{2+}-free Dulbecco's modified Eagle's medium. Inactive (1/2) BAPTA was used as a negative control. Cells were then harvested at the time points indicated, and cell lysates were subjected to immunoblot analysis. Representative results for phospho activation of p53 S15 (**a**), caspase-8, active caspase-3 and cleaved PARP (**b**) are shown. The importance of extracellular Ca^{2+} for efficient p53 activation (S15) and caspase-3 and -8 activities were confirmed in immunoblotting by co-treatment of 5-FU and verapamil, an inhibitor of L-type Ca^{2+} plasma membrane channels (**c**). Immunoblot analysis of lysates from cells treated with thapsigargin 1 h in advance of 5-FU induction served to exclude the possibility that Ca^{2+} originating from the ER amplified p53 phosphorylation (S15; **d**). Tubulin and glyceraldehyde 3-phosphate dehydrogenase served as markers for equal loading. Processed caspase fragments are indicated with asterisks.

Interestingly, DR5 protein expression levels remained in the presence of CaM inhibitors, indicating mechanisms separated from death receptor transactivation to be involved in p53-mediated triggering of caspase-8 (data not shown). Other factors, which have been defined as regulators of p53 posttranslational modifications and activation, include Chk2 and ATM.[24,30,31] By using HCT116 Chk2$^{-/-}$ cells and a specific inhibitor of ATM (KU55933), we could conclude that these factors did not contribute significantly to any of the 5-FU-induced p53 phosphorylation events analyzed. Still, in Chk2$^{-/-}$ cells, processing of caspases-3 and -8 occurred at a slower rate compared with the parental cell line, implicating this factor in 5-FU-induced apoptosis by a yet unknown mechanism (Figure 6c). Using KU55933 in combination with 5-FU, on the other hand, drastically increased apoptotic rate as determined by caspase-3 processing and PARP cleavage (Figures 6d and e). Interestingly, the synergistic effect with respect to effector caspase processing induced by the ATM inhibitor occurred in the absence of a further increased apical caspases-8 activity, as compared with samples where 5-FU was used alone (Figure 6e), indicating that combinatorial treatment using 5-FU and KU55933 triggers an additional cell death mechanism, which is separated from the DR5–DISC. At later time points of 5-FU-induced cell death, KU55933 indeed obstructs several tested phospho-p53 modifications, except S33 and only partly S15. Importantly, at these time points, KU55933 control samples also contained active p53 and were positive for several apoptotic markers implying difficulties of data interpretation (data not shown). Validation of data obtained using KU55933 was generated by siRNA methodology (Figures 6f–g). In summary, it is likely that 5-FU stimulates apoptosis through a Ca^{2+}–CaM-dependent pathway, which in turn directs the DR5–DISC activity through p53. However, the specific kinase directed by Ca^{2+}–CaM with importance for discrete p53 phosphorylations remains unidentified. Interestingly, transactivation and dimerization of the DR5, analyzed by standard and non-denaturing SDS–PAGE, respectively, occurring in response to 5-FU, was neither affected by BAPTA nor calmidazolium chloride, indicating that p53 supports caspase-8 processing by mechanisms separated from these events (Figure 6h). It should also be noted that BAPTA and calmidazolium chloride, but not fluphenazine-N-2-chloroethane, diminished 5-FU-mediated autophagy, as determined by a reduction of the major selective autophagic substrate p62. As all three inhibitors interfere with activation of the caspase cascade, these results exclude autophagy to be functionally connected to the signaling route investigated (data not shown).

5-FU-induced Ca^{2+} deregulation is signaling through a p38MAPK-independent pathway

In a recent report, it was suggested that p38MAP kinase (p38MAPK) activation is a key determinant in the cellular response to 5-FU. As inhibition of p38MAPKα by the SB203580 compound or by short hairpin RNA interference obstructed 5-FU-associated apoptosis while autophagy was promoted, p38MAPK was suggested to act as a potential regulator of p53 and the balance between apoptotic and autophagic processes.[32] In turn, the mammalian mitogen-activated protein (MAP3K) apoptosis signal-regulating kinase 1 (ASK1) is a pivotal component in cytokine- and stress-induced apoptosis, which may act as a critical intermediate of Ca^{2+} signaling between Ca^{2+}–CaM-dependent protein kinase type II and p38MAPK[33,34] It is, therefore, possible that the Ca^{2+}-dependent apoptotic pathway disclosed by our data merges with the previously defined p38MAPK pathway.[32] To verify this hypothesis, 5-FU-treated HCT116 cells were pre-incubated with BAPTA and the p38MAPKα inhibitor SB203580, either separately or in combination (Figure 7). Transactivation of the cyclic AMP-dependent transcription factor-3 (ATF3), a target protein of both the p38MAPK and the CaM/calcineurin pathway acting via the myocyte-specific enhancer factor 2a functioned as a control for drug efficiency. Indeed, each compound efficiently obstructed effector caspase-3 processing, and a synergistic effect was revealed in combinatorial treatment. Caspase-8 processing, on the other hand, was only blocked by the addition of BAPTA but not of SB203580. Moreover, although only a minor destabilization of p53 could be observed in samples pretreated with BAPTA and no effect was observed with SB203580, they provided a synergistic

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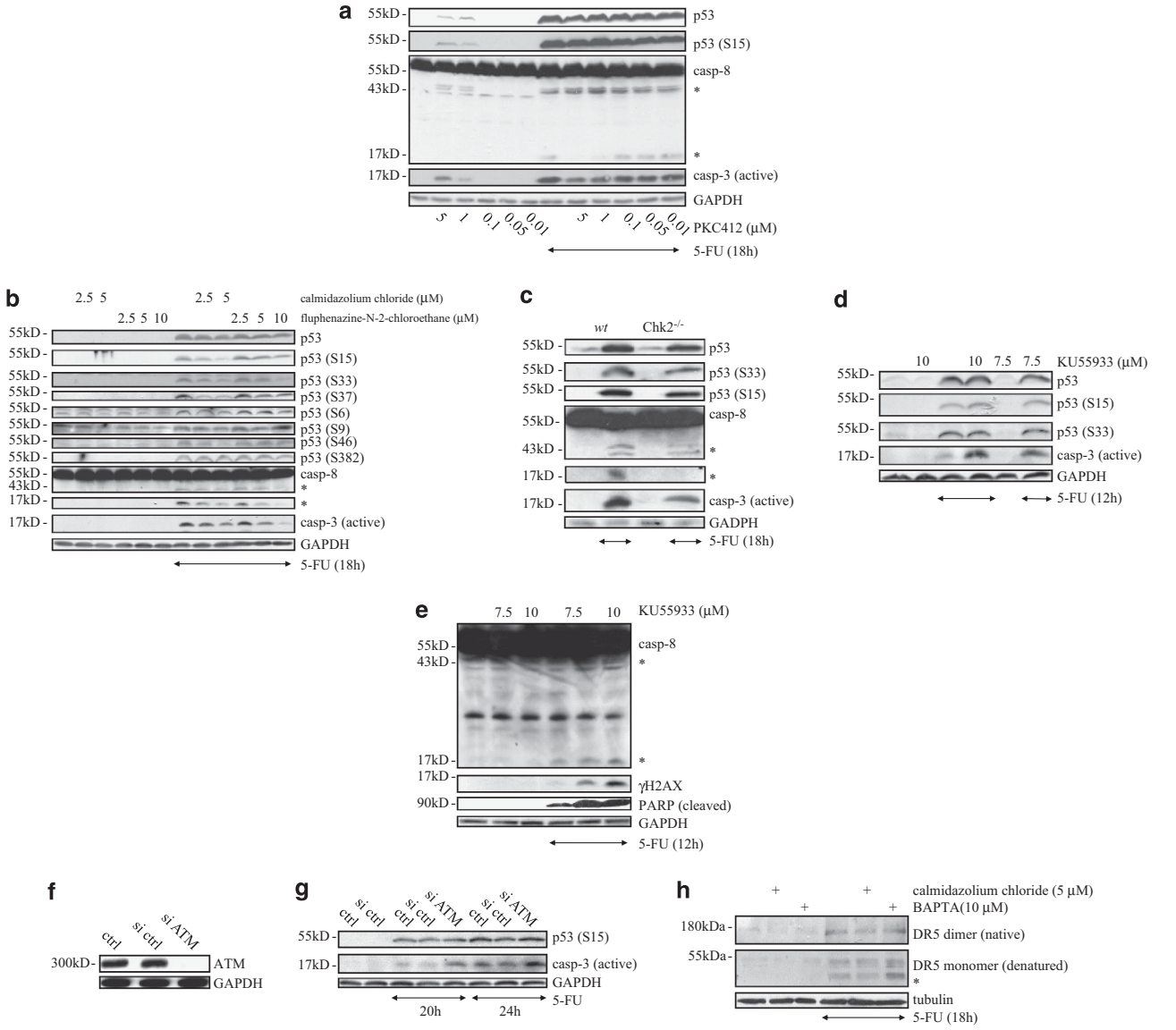

Figure 6. Two different chemical inhibitors targeting CaM, but neither inhibitors of protein kinase C nor ATM, nor Chk2 deficiency interferes with S15 and S33 p53 phosphorylation. HCT116 cells were treated with 5-FU, either alone or in combination with inhibitors to protein kinase C (PKC412; **a**), CaM (calmidazolium chloride and fluphenazine-N-2-chloroethane; **b**) or ATM (KU55933; **d** and **e**) at concentrations and the time points indicated. In HCT116 Chk2-deficient cells, no additional inhibitors were used in combination with 5-FU (**c**). Cells were then collected and protein lysates were analyzed by SDS–PAGE using antibodies targeting p53 and its phospho-modifications, as well as markers for DISC-mediated apoptosis. Silencing of ATM in HCT116 cells by transfection of specific siRNAs before 5-FU treatment was performed to validate data generated by KU55933 (**f** and **g**). In **h**, transactivation and dimerization of the DR5, occurring in response to 5-FU alone or in combination with either BAPTA or calmidazolium chloride, were analyzed by standard and non-denaturing SDS–PAGE, respectively. In all figures, glyceraldehyde 3-phosphate dehydrogenase served as markers for equal loading. Processed caspase fragments and the short isoform of DR5 are indicated with asterisks.

effect with respect to this event resulting in levels of the tumor suppressor, which were similar to controls. In conclusion, although these data do not exclude the possibility that pathways resulting from p38MAPK activity and cellular Ca^{2+} deregulation share some common features, they certainly expose features of specificity, especially with respect to the DR5-mediated caspase-8 activation, which seems to involve Ca^{2+} but not p38MAPK. Moreover, effective stabilization of p53 seems to require posttranslational modifications originating from both signaling circuits.

DISCUSSION

The association between alterations in intracellular Ca^{2+} homeostasis and various stages of the apoptotic signaling cascade is indisputable.[35] In the present report, we uncover a new Ca^{2+}-dependent apoptotic mechanism, which occurs in response to 5-FU and is mediated through CaM and p53 activities, regulating the caspase cascade via the DR5–DISC and initiation of caspase-8 processing (Figure 8). Importantly, the signaling route described could also be initiated by low doses of 5-FU, and the response of human A549 lung adenocarcinoma cells was similar to the one revealed in HCT116 cells (Supplementary Figures S3 and S2). The generality of the pathway was further validated by including the human breast adenocarcinoma MCF-7 and the human ovarian carcinoma CAOV-4 cell lines into the study. Both responded to 5-FU treatment in terms of p53 S15 phospho activation and in the case of CAOV-4, also with an accumulation of DR5 and processed caspase-3. As MCF-7 cells are caspase-3-deficient, an

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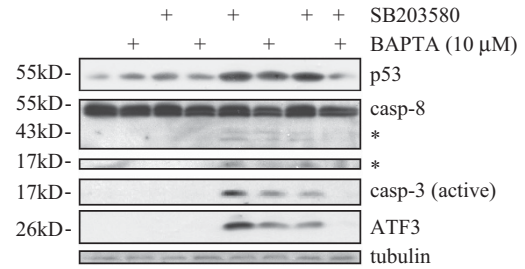

Figure 7. p38 regulates 5-FU-induced apoptosis in HCT116 cells by a caspase-8-independent mechanism. 5-FU co-treatment of HCT116 wt cells using either BAPTA (10 μM) or the specific p38 inhibitor SB203580 (10 μM) was used to compare apoptotic signaling facilitated by Ca^{2+} and p38, respectively. Protein lysates of treated and control cells were subjected to SDS–PAGE and probed with specific antibodies targeting p53, caspase-8 and caspase-3. AMP-dependent transcription factor-3 served as control for inhibitor efficiency and tubulin as markers for equal loading. Processed caspase-8 fragments are indicated with asterisks.

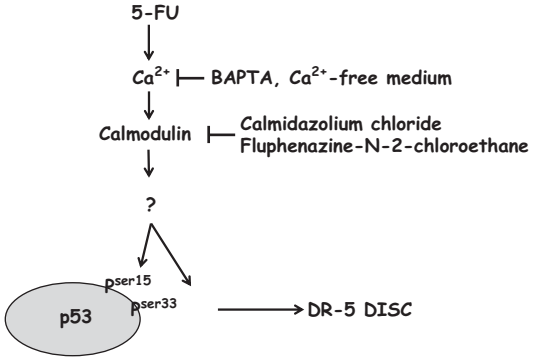

Figure 8. Schematic illustration of Ca^{2+}-regulated phospho activation of p53 and its implication in 5-FU-induced apoptosis.

investigation of the common apoptotic markers would be misleading and was, therefore, not performed. Nevertheless, both BAPTA and the CaM-inhibitor calmidazolium chloride clearly abrogated the p53 S15 phosphorylation in these cells. In contrast, although caspase-3 processing and DR5 transactivation were obstructed by the same set of inhibitors in 5-FU-induced CAOV-4 cells, no effect was detected with respect to phospho activation of p53 S15. Thus, the Ca^{2+}–CaM-mediated pathway might direct other p53 phospho sites, or a separate mechanism required for activation of the caspase cascade in this particular cell line (data not shown).

Evidences obtained from a wide variety of preclinical experimental studies, epidemiological findings and a few human clinical trials support the view that dietary Ca^{2+} can modulate and inhibit colon carcinogenesis.[36] More interesting for the present study, however, is the fact that adjuvant chemotherapy combining 5-FU with Ca^{2+} folinate can alter the natural history of resected colon cancer and offer an overall survival advantage in comparison with other 5-FU-based regimens or with 5-FU alone.[37]

According to the literature, 5-FU and its metabolites have several direct or indirect target points within cells, including DNA, RNA and ribosomal biogenesis.[1,4,38] Although DNA lesion represent the most well-documented incident causing activation of p53, recent reports also involve transcriptional stress and ribosome damage in signaling events, leading to specific posttranslational modifications of the tumor suppressor.[5,34] An accumulation of double-strand breaks in response to 5-FU in HCT116 cells is evident as the number of γH2AX foci observed by immunostaining increased over time (data not shown), but our data involving ATM inhibition by KU55933 and Chk2-deficient cells excluded the DNA damage response as a starting point for Ca^{2+}-induced p53 phospho activation. Thereby, we are not arguing that apoptosis proceed irrespectively of the DNA damage response. It is important to remember that all p53 sites analyzed were phosphorylated in response to 5-FU, but only three were silenced in the presence of inhibitors to CaM. The origin of the stress response that is able to deregulate cellular Ca^{2+} in our settings is, however, still unclear and further investigations are required to solve this issue. The combined effect of KU55933 and 5-FU augmented effector caspase-3 activity compared with samples treated with 5-FU alone, whereas no difference was detected for the more upstream caspase-8 activity. These results are in good agreement with a recent report, suggesting that KU55933 may target cancer cells resistant to traditional chemotherapy due to aberrant activation of Akt.[39] Experiments using verapamil were indicating high-voltage-gated Ca^{2+} channels of the L-type as the entry point for extracellular Ca^{2+} influx in response to 5-FU. This is well in line with the fact that the elevated Ca^{2+} level appeared as an immediate reaction to treatment and then remained until onset of the caspase cascade. The α1-subunit, which contains the voltage-sensing machinery and the drug/toxin-binding sites, form the Ca^{2+}-selective pore and is the primary factor operating in high-voltage-gated Ca^{2+} channels. Out of ten α1-subunits described in humans, four are specific for L-type channels, and current work aims to identify whether one or several α1-subunits are required for the process described, and to further specify the link between 5-FU and L-type high-voltage-gated Ca^{2+} channels. Here, two possibilities exist. Either 5-FU-specific cell stress induces a still unidentified signaling cascade activating one or several L-type pores, alternatively, 5-FU or its metabolites act directly on these pores. In line with our study and supporting the fact that at least the CaM-directed p53 S15 phospho-modification is important for 5-FU-induced apoptosis are findings coming from expression at physiological levels of p53 mutants in p53 knock-out HCT116 cells. Compared with cells expressing exogenous wt p53, the apoptotic response to 5-FU was more than 50% reduced in cells expressing S15A mutant p53, whereas a serine to alanine replacement on the 33 amino acid residue had no profound effect on cell death.[19]

BAPTA or CaM inhibitors reduced the capacity of 5-FU to induce apical caspase-8 processing through DR5–DISCs. Still, oligomerization of DR5 occurred also in the presence of these agents with an efficiency similar to cells treated with 5-FU alone. Evidently, a candidate target molecule for the pathway described is the cellular FLICE inhibitory protein (cFLIP), a catalytically inactive homolog of caspases-8 and -10 able to prevent their activation by obstructing binding sites on the DISC.[40] Thus, deregulation of cFLIP is a prerequisite for efficient initiation of DISC-mediated caspase-8 processing. Suppression of the cFLIP short and long isoforms in response to 5-FU occurred, however, not only in a p53- but also in a Ca^{2+}-independent fashion as determined by analyzing its expression level in the presence of BAPTA and in HCT116 $p53^{-/-}$ cells (Supplementary Figure S4 and S5). In fact, this is in agreement with recent reports showing that chemotherapy-induced downregulation of cFLIP splice forms occurs in cell lines irrespectively of their p53 status and, more specifically, that ATM kinase activity is required to trigger 5-FU- and neocarzinostatin-induced and p53-independent cFLIP downregulation, which in turn sensitized hepatocellular carcinoma cell lines to TRAIL.[41,42] Identification of the factor regulated by the Ca^{2+}–CaM–p53 axis described in this report conducting DR5–DISC activity thereby remains.

Previously, we suggested CD95 as an upstream regulatory element required for initiation of the caspase cascade in response to 5-FU.[16] This assumption was based on a prominent p53-dependent upregulation of this death receptor and

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co-immunoprecipitation studies. However, by means of RNA interference methodology, the present report concludes that DR5 is more important with respect to caspase-8 activity and apoptotic proceedings. On the basis of these results, a possible explanation for this inconsistency may be that the FAS–DISCs formed in the experimental system analyzed contributes insignificantly to the cell death process examined.

As TRAIL can induce apoptotic cell death in a variety of tumor cells by engaging specific death receptors, DR4 and DR5, whereas having low toxicity towards normal cells, it has been postulated as a future therapeutic option.[43] Our present report, as well as data obtained by others, is demonstrating that 5-FU-induced cell death also involves DR5. Disclosing 5-FU-induced death pathways might, therefore, conform to the highly interesting research field of TRAIL in tumor treatment and to processes relating to development of TRAIL resistance. In addition, although Ca^{2+} previously has been implicated in various cell death pathways, the novelty of our data indicating that the Ca^{2+}–CaM signaling is required for apoptosis triggered by 5-FU in certain cancer cell types must be emphasized. Response rates for 5-FU in advanced colorectal cancer are modest, and although combinatorial treatment using chemotherapeutic agents, such as oxaliplatin and irinotecan, has improved survival rates there is a need for new curative strategies. The fact that a widely used drug, such as 5-FU, is signaling by this mean may provide new therapeutic intervention points for combinatorial treatment regimens, as well as explanations for cell resistance.

MATERIALS AND METHODS

Cell culture

The HCT116 parental cell line and its variants, deficient in p53 or Chk2 (a gift from Professor Bert Vogelstein) were cultured in Dulbecco's modified Eagle's medium and the A549 cell line in RPMI1640 medium, both supplemented with 10% heat-inactivated fetal bovine serum and PenStrep (100 U/ml penicillin, 100 mg/ml streptomycin). Cell culture reagents were purchased from GIBCO (Invitrogen, San Diego, CA, USA). Treatment of cells with the anti-metabolite 5-FU (Teva Pharmaceutical Industries, Petach Tikva, Israel) was performed at a concentration of 768 μM, unless otherwise stated. Usage of thapsigargin, BAPTA (Invitrogen), Verapamil, pepstatin A (Sigma-Aldrich, St Louis, MO, USA), calmidazolium chloride, fluphenazine-N-2-chloroethane (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PKC412 (Novartis International, Basel, Switzerland), ATM inhibitor KU55933, p38 MAPK inhibitor SB203580 (Selleck Chemicals LLC, Houston, TX, USA), CA-074 Me, Trolox, Necrostatin-1 and its inactive control Necrostatin-1i (Merck, Darmstadt, Germany) were performed as indicated in figures.

Gel electrophoresis and immunoblotting

Cells were treated as indicated, washed in phosphate-buffered saline and lysed for 5 min in Complete Lysis-M (Roche Diagnostics, Mannheim, Germany) containing 1 × Complete Protease Inhibitor Cocktail (Roche Diagnostics) and 1 × Phosphatase inhibitor Cocktail 2 (Sigma-Aldrich). The BCA Protein assay (Thermo Fisher Scientific, Lafayette, IN, USA) was used to determine protein concentration. Equal quantities from each sample were subjected to SDS–PAGE and electroblotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then blocked and probed with the primary antibody of interest at 4 °C overnight. Finally, membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature, revealed by enhanced chemiluminescence (GE Healthcare Biosciences, Uppsala, Sweden) and exposed to SuperRX X-ray films (Fujifilm Corporation, Tokyo, Japan). To analyze DR5 clustering, cells were lysed for 20 min on ice using a non-reducing Triton buffer (20 mM Tris HCl (pH 7.5), 150 mM NaCl, 10% Glycerol, 0.2% Triton X-100 and 1 mM EDTA) supplemented with protease and phosphatase inhibitors. Samples were then treated with Benzonase nuclease (Sigma-Aldrich) for 15 min at room temperature, mixed in non-reducing sample buffer and subjected to SDS–PAGE.

Immunofluorescence

HCT116 and HCT116 $p53^{-/-}$ cells were seeded on coverslips, treated as indicated in figures and then fixed for 30min, using 4% formaldehyde in phosphate-buffered saline at 4 °C. Permeabilization and blocking was done in phosphate-buffered saline containing 0.3% Triton X-100 and 1% bovine serum albumin for 1 h. Incubations with primary antibodies (1:400) and secondary antibodies (1:200) were performed at 4 °C overnight and at room temperature for 60 min, respectively. Nuclei were counterstained with Hoechst 33,342 (10 μg/ml in phosphate-buffered saline). Finally, the coverslips were mounted using Vectashield H-1000 (Vector Laboratories Inc., Peterborough, UK) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss MicroImaging, Göttingen, Germany).

Antibodies

The following primary antibodies were used in western blotting: p53 mAb, clone DO1; glyceraldehyde 3-phosphate dehydrogenase polyclonal antibody (pAb), Tom 40 pAb, CD95 pAb, AMP-dependent transcription factor-3 pAb (Santa Cruz Biotechnology), phospho-p53 pAbs (Ser6, 9, 15, 33, 37, 46 and 382), cleaved-caspase-3 pAb, ATM monoclonal antibody (mAb), clone D2E2; phospho-H2A.X (Ser139) pAb (Cell Signaling, Danvers, MA, USA), α-Tubulin mAb, clone B-5-1-2; DR5 pAb (Sigma-Aldrich), PARP mAb, clone 4C10-5; Bax mAb, clone 6A7; caspase-7 mAb, clone B94-1; caspase-2 mAb, clone 35 (BD Biosciences, Franklin Lakes, NJ, USA), cFlip mAb, clone Dave-2 (Alexis, San Diego, CA, USA), FADD pAb (Upstate Biotechnologies, Lake Placid, NY, USA) and caspase-8 mAb, clone C15 (kindly provided by Professor PH Krammer and Dr I Lavrik, German Cancer Research Center, Heidelberg, Germany). Analysis of DR5 in immunofluorescence was performed using the mAb clone 11/B4 (kindly provided by Professor L Anděra, Academy of Sciences of the Czech Republic, Prague, Czech Republic). For immunofluorescence detection of FADD, γH2A.X and CD95, the antibodies described above were used. Fluorescent secondary antibodies directed to mouse and rabbit (Alexa488 and Alexa594) were purchased from Molecular Probes (Invitrogen).

RNA interference methodology

Silencing of protein expression in HCT116 cells was accomplished by transfection of 21-nucleotide RNA duplexes. Transfection of CD95 (L-0,03,776-00), DR5 (L-0,04,448-00) and control (D-0,01,810-10) ON-TARGET-*plus* SMARTpool siRNAs (Thermo Fisher Scientific) was performed using the INTERFERin transfection reagent (Polyplus transfection) according to the instructions of the manufacturer. Similarly, ATM was targeted by a pool of siRNAs (Flexitube siATM-8, -9 and -12), each individually purchased from Qiagen (Hilden, Germany) 1919. Briefly, 4×10^5 cells were transfected in normal cell medium using 10 nM final concentration of siRNA and 3.65 μl/ml INTERFERin.

Isolation of membrane proteins

Cells were treated as indicated in figures, and proteins found in cytosolic and membrane fractions were isolated using the Qproteome Cell Compartment Kit (Qiagen) according to the instructions of the manufacturer. Enrichment of DISC-containing proteins in cellular membranes was then assayed by SDS–PAGE.

Vectors and cloning

The wt p53 and p53 (S15A) cDNAs were kindly provided by Professor Moshe Oren (Weizmann Institute of Science, Rehovot, Israel). Subsequent to deletion of the stop codons in order to allow for green fluorescent protein expression, wt and mutated p53 sequences were subcloned into the *Xba*1–*Bam*H1 site of the pCDH–CMV–MCS–EF1–copGFP expression vector (System Biosciences, Mountain View, CA, USA). Lentiviral packaging was accomplished by using the LentiSuite according to the instructions of the manufacturer (System Biosciences). Then, transductions of HCT116 $p53^{-/-}$ cells were performed by incubation at normal culturing conditions in the presence of virus for 24 h.

Ca^{2+} measurements

Intracellular Ca^{2+} levels were monitored by using the Fluo-4AM fluorescent indicator (Invitrogen). In brief, 4 μM of the Ca^{2+} probe was added to cells 30 min in advance of 5-FU treatment. Time-lapse analysis of living cells was then performed using the Zeiss LSM 510 META confocal laser scanner microscope or the FACS Calibur system in combination with the CellQuest v.3.3 software (Becton-Dickinson, San Jose, CA, USA).

ABBREVIATIONS

Ca^{2+}, calcium; DISC, death-inducing signaling complex; 5-FU, 5-fluorouracil.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

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