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Reduced CaM/FLIP binding by a single point mutation in c-FLIP$_L$ modulates Fas-mediated apoptosis and decreases tumorigenesis

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We have previously demonstrated that calmodulin (CaM) binds directly to c-FLIP$_L$ in a Ca^{2+}-dependent manner. Deletion of the CaM-binding region (amino acid 197–213) results in reduced CaM binding, and increased Fas-mediated apoptosis and decreased tumorigenesis of cholangiocarcinoma cells. The present studies were designed to identify the precise amino acids between 197 and 213 that are responsible for CaM/FLIP binding, and their roles in mediating the anti-apoptotic function of c-FLIP$_L$. Sequence analysis of the CaM-binding region at 197–213 predicted three unique positively charged residues at 204, 207 and 209, which might be responsible for the CaM/FLIP binding. A point mutation at H204 of c-FLIP$_L$ was found to markedly reduce CaM binding, whereas point mutation at R207 or K209 did not affect c-FLIP$_L$ binding to CaM. Decreased CaM/FLIP binding was confirmed in cholangiocarcinoma cells overexpressing the H204 c-FLIP$_L$ mutant. Reduced CaM binding by the H204 mutant resulted in increased sensitivity to Fas-mediated apoptosis and inhibited tumor growth in mice compared with wild-type c-FLIP$_L$. Death-inducing signaling complex (DISC) analysis showed that the reduced CaM binding to H204 mutant resulted in less c-FLIP$_L$ recruited into the DISC. Concurrently, increased caspase 8 was recruited to the DISC, which resulted in increased cleavage and activation of caspase 8, activation of downstream caspase 3 and increased apoptosis. Therefore, these results demonstrate that the H204 residue is responsible for c-FLIP$_L$ binding to CaM, which mediates the anti-apoptotic function of c-FLIP$_L$, most likely through affecting recruitment of caspase 8 into the DISC and thus caspase 8 activation. These studies further characterized CaM/FLIP interaction and its function in regulating Fas-mediated apoptosis and tumorigenesis, which may provide new therapeutic targets for cancer therapy.

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Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, development of the immune system, embryonic development, metamorphosis, hormone-dependent atrophy and also in chemical-induced cell death.[1] Stimulation of apoptosis has been promoted as a potential therapy for many cancers, including cholangiocarcinoma. Results from our group and others have suggested that regulation of Fas-mediated apoptosis is a promising therapeutic avenue for cholangiocarcinoma.[2–9]

The Fas death receptor is a member of the tumor necrosis factor (TNF) receptor superfamily. In response to the binding of its ligand (FasL) or agonistic antibody, Fas undergoes oligomerization and its death domain (DD) binds to the adaptor molecule Fas-associated DD (FADD) through a homotypic interaction.[10] Then, FADD recruits and aggregates caspase 8 and/or cellular FLICE-like inhibitory protein (c-FLIP) to form the death-inducing signaling complex (DISC). Death or survival signals transmitted through the DISC are determined by the relative expression of caspase 8 or its homolog with no caspase activity, c-FLIP, recruited into the DISC.[11–14] Two cellular isoforms of FLIP have been identified, c-FLIP$_L$ (55 kDa) and c-FLIPs (26 kDa). c-FLIPs contain only the tandem death effector domains (DEDs). c-FLIP$_L$ contains not only the tandem DEDs, but also a protease-like domain, homologous to caspase 8, in which several amino acids important for protease activity are mutated, including the

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cysteine at the active site.[15] c-FLIP$_L$ has generally been thought to be an anti-apoptotic protein, however, some evidence suggests that it can also improve Fas-mediated apoptosis by forming a heterodimer with caspase 8, which helps achieve the initial cleavage step of procaspase 8.[16–18] With stable expression of caspase 8, c-FLIP$_L$ is believed to inhibit caspase 8 activation by interfering with the recruitment and/or activation of caspase 8 in the DISC.[16,17,19] However, the precise mechanism for the anti-apoptotic function of c-FLIP$_L$ in Fas-mediated apoptosis is not clearly understood.

Our previous studies have demonstrated that calmodulin (CaM) antagonists induce apoptosis through a Fas-related mechanism in a cholangiocarcinoma tumor model.[2,3,5,9] CaM is a small (17 kDa), acidic, dumbbell-shaped protein that binds Ca^{2+} through EF-hand motifs.[20] Its important role in apoptosis has been reported in several studies.[21–23] We have shown that CaM binds directly to Fas.[24] Further studies on the interaction of CaM with other proteins in the Fas-induced DISC showed that CaM also directly binds to c-FLIP$_L$ in a Ca^{2+}-dependent manner, but not FADD or caspase 8.[25] The CaM-binding region in c-FLIP$_L$ was identified between amino acids 197 and 213. Deletion of this region causes reduced binding between CaM and c-FLIP$_L$. Overexpression of c-FLIP$_L$ lacking this region results in increased Fas-mediated apoptosis in cholangiocarcinoma cells and decreased tumorigenesis compared with wild-type (WT) c-FLIP$_L$-overexpressing cells.[2,25] These findings indicate that CaM-c-FLIP$_L$ binding is important for the anti-apoptotic function of c-FLIP$_L$ in the Fas pathway.

Sequence comparison of the CaM-binding region at 197–213 on c-FLIP$_L$ with those of c-FLIP$_S$ and caspase 8 predicted three unique positively charged residues at 204, 207 and 209, which might be responsible for the CaM/FLIP binding. We found that point mutation at H204 significantly reduced the binding to CaM, whereas point mutation at R207 or K209 showed no effect. Reduced CaM binding by the c-FLIP$_L$ H204 mutant in cholangiocarcinoma cells increased their sensitivity to Fas-mediated apoptosis and decreased their tumorigenic potential in a nude mouse model compared with WT c-FLIP$_L$. DISC analysis, further demonstrated that reduced CaM binding to c-FLIP$_L$ resulted in less c-FLIP$_L$ and increased the caspase 8 recruited into the DISC, which resulted in increased activation of caspase 8, and thus activation of downstream signals and increased apoptosis. Our findings provide new evidence and mechanistic molecular insight to support the concept that CaM-c-FLIP$_L$ binding modulates the anti-apoptotic function of c-FLIP$_L$ in Fas-mediated apoptosis and consequently the tumorigenic potential of cancer cells.

MATERIALS AND METHODS

Cell Culture, Antibodies and Reagents

The cholangiocarcinoma cell line, SK-ChA-1, is one of three human cell lines from adenocarcinomas of the extrahepatic biliary tract established by Dr A Knuth (Ludwig Institute for Cancer Research, London, UK).[26] Cells were grown in RPMI 1640 (Invitrogen) supplemented with penicillin (5 units/ml), streptomycin (5 μg/ml) and 10% heat-inactivated fetal bovine serum. Cholangiocarcinoma cell line, Mz-ChA-1, was kindly provided by Dr Gregory J Gores (Mayo Clinic, Rochester, MN, USA) and maintained as previously described.[2,27] The FLIP antibody, NF-6, was obtained from Alexis Biochemicals. The caspase 3 antibody was from StressGen and antibodies to caspase 8 and FADD were purchased from BD Bioscience. Fas-activating antibody, clone CH-11, was from Upstate Biotechnology. The monoclonal antibody to CaM was developed as previously described.[28] Antibodies to Fas and GAPDH were purchased from Santa Cruz Biotechnology.

Expression and Purification of His-SUMO Fusion Proteins

The human c-FLIP$_L$ cDNA was subcloned into the pET28a vector with His-SUMO provided by Dr J Ma (Department of Biochemistry and Molecular Genetics, UAB, Birmingham, AL) under Bgl II and Xho I sites. Point mutations H204, R207 and K209 in c-FLIP$_L$ were generated with a QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The recombinant c-FLIP$_L$ proteins were expressed in *E. coli* BL21-Gold (DE3) (Stratagene). After induction with 0.1 mM IPTG, the cells were allowed to grow at 30°C overnight. Collected cells were lysed by adding lysosome and sonication. Proteins were purified by Ni-NTA Superflow Columns (Qiagen).

Protein Pulldown

Protein pulldown with CaM–Sepharose 4B (Amersham Biosciences) was performed as described previously.[24,25] Briefly, 20 μg of purified fusion protein or 300 μg of extracted protein from cholangiocarcinoma cells overexpressing WT c-FLIP$_L$ or the H204 mutant was incubated with CaM–Sepharose 4B in the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and proteinase inhibitor mix, pH 7.5) with 2 mM $CaCl_2$ or 2 mM EGTA overnight at 4°C. The beads were washed four times with lysis buffer and proteins were identified by western blot analysis.

Western Blot Analysis

Protein extracts from cells were prepared as described previously.[2,3] Concentrations of protein were determined with a BCA protein assay kit (Thermo Scientific). Proteins were separated by SDS–PAGE and transferred to Immobilon P membranes (Millipore) as described previously.[2,3] Membranes were blocked in 5% non-fat milk and incubated with primary antibodies overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies in the blocking buffer were incubated for 1 h at room temperature. Signals were detected using Immobilon Western chemiluminescent horseradish peroxidase substrate detection kit (Millipore). Bands were quantified by ImageQuant version 5.1 (GE Healthcare Lifesciences).

Generation of Cholangiocarcinoma Cells Stably Overexpressing WT c-FLIP$_L$ and H204 Mutant

Stable overexpression of c-FLIP$_L$ and the H204 c-FLIP$_L$ mutant in cholangiocarcinoma cells, SK-ChA-1, was accomplished using methods described previously.[29,30] The human WT c-FLIP$_L$ and H204 mutant cDNA were subcloned into the lentiviral vector under Bgl II and Xho I sites. Each construct was packed into lentivirus-like particles pseudotyped with the vesicular stomatitis virus glycoprotein as we previously described.[31] Transduction was performed by incubating cholangiocarcinoma cells with recombinant lentivirus, and stably transduced cells were selected with puromycin (2 μg/ml).

Transient Transfection Assays

The human WT c-FLIP$_L$ and H204 mutant cDNA were subcloned into the pcDNA3.1. vector under Bgl II and Xho I sites and confirmed by sequencing. Cholangiocarcinoma cells, SK-ChA-1 or Mz-ChA-1, were transfected with these constructs at a 1:3 ratio using Fugene 6 (Roche Applied Science). Twenty-four hours after transfection, cells were harvested or treated with Fas-activating antibody, CH-11, for apoptosis assay.

Assessment of Apoptosis

Apoptosis was induced with Fas-activating antibody, CH-11, as described previously.[4] Briefly, cholangiocarcinoma cells were exposed to 500 ng/ml of CH-11 antibody for 24 h. Apoptosis was determined by Annexin V-FITC and propidium iodide staining (BD biosciences) and analyzed by flow cytometry (BD biosciences).

Analysis of Fas-Mediated DISC

Immunoprecipitation for DISC analysis was performed as previously described.[32] Cholangiocarcinoma cells (5×10^7), SK-ChA-1, were incubated with or without 1 µg/ml Fas-activating antibody (CH-11) for 30 or 60 min at 37°C and then washed with PBS and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and proteinase inhibitor mix, pH 7.5) for 30 min on ice. In control cells, Fas-activating antibody (CH-11) was added to cell lysates at a final concentration of 1 µg/ml to immunoprecipitate non-stimulated Fas receptors. After centrifugation at 13 000 *g* for 15 min at 4°C, the supernatant was incubated with 20 µl of goat anti-mouse IgM agarose (Sigma) overnight at 4°C and analyzed by western blot analysis.

Mouse Xenograft Model

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham, Birmingham, AL, USA. Male athymic nu/nu mice (4 weeks, NCI-Frederick) were used for tumor inoculation. Briefly, cholangiocarcinoma cells, SK-ChA-1, stably overexpressing WT c-FLIP$_L$ or the H204 mutant (2×10^6 cells in 200 µl PBS/site) were inoculated subcutaneously into the flank area of mice. Tumor size and body weight were measured every 3 days and volumes were determined using the formula volume = length $\times$ width^2/2.

TUNEL Staining

At the end of the animal experiment, tumors were removed, fixed in 4% paraformaldehyde and embedded in paraffin. Consecutive tumor sections (8 µm) from each group were used for histological and immunohistochemial staining. To assess apoptotic cells in tumor tissues, TUNEL staining (DeadEnd Fluorometric TUNEL System; Promega) was performed following the manufacturer's protocol. For quantitative analysis, cell numbers were counted under a microscope ($\times$ 400). Four fields in each slide were counted and the percentage of apoptotic cells was determined.

Statistical Analysis

Results are expressed as means ± s.e. Differences between two groups were identified with Student's *t*-test. Significance was defined as $P < 0.05$.

RESULTS

c-FLIP$_L$ H204 Mutant has Reduced Binding to CaM *In Vitro*

Previous studies from our group demonstrated the CaM-binding region in c-FLIP$_L$ is amino acids 197–213.[25] To identify the precise amino acids between 197 and 213 that are responsible for CaM/FLIP binding, we further compared the 197–213 regions of c-FLIP$_L$, c-FLIP$_S$ and caspase 8. As shown in Figure 1a, c-FLIP$_L$ has more positive charges, which was largely due to three amino acids (H204, R207 and K209). Since c-FLIP$_S$ and caspase 8 do not bind to CaM, these three amino acids might be essential for the interaction between c-FLIP$_L$ and CaM. To test this hypothesis, H204, R207 and K209 were mutated to alanine separately with a QuikChange site-directed mutagenesis kit as mentioned in the Materials and Methods. These c-FLIP$_L$ mutations and WT were cloned into a His-SUMO vector and proteins were expressed in *E. coli* using IPTG induction and purified with Ni-NTA Superflow Columns. Binding to CaM was analyzed with a pulldown assay using CaM-Sepharose 4B. As shown in Figures 1b and d, only the H204 mutant showed reduced binding to CaM, whereas R207 and K209 had no effect. Furthermore Ca^{2+} did not significantly affect the H204 mutant binding to CaM in contrast to WT c-FLIP$_L$, in which CaM/FLIP binding was largely Ca^{2+}-dependent, being decreased substantially with the presence of EGTA (Figures 1c and e).

c-FLIP$_L$ H204 Mutant has Reduced Binding to CaM in Cholangiocarcinoma Cells and Sensitizes the Cells to Fas-Induced Apoptosis

To determine whether the reduced c-FLIP$_L$ binding to CaM by the H204 mutant also exists in cells, we stably overexpressed WT c-FLIP$_L$ and H204 mutant in cholangiocarci-

noma cells, SK-ChA-1, using the lentiviral expression vectors (Figure 2a). Whole cell lysates from these stable overexpression cells were used for protein pulldown assays with CaM-Sepharose 4B. Consistent with the *in vitro* data, the H204 mutant reduces c-FLIP$_L$ binding to CaM compared with WT c-FLIP$_L$ (Figures 2b and c). The CaM-c-FLIP$_L$

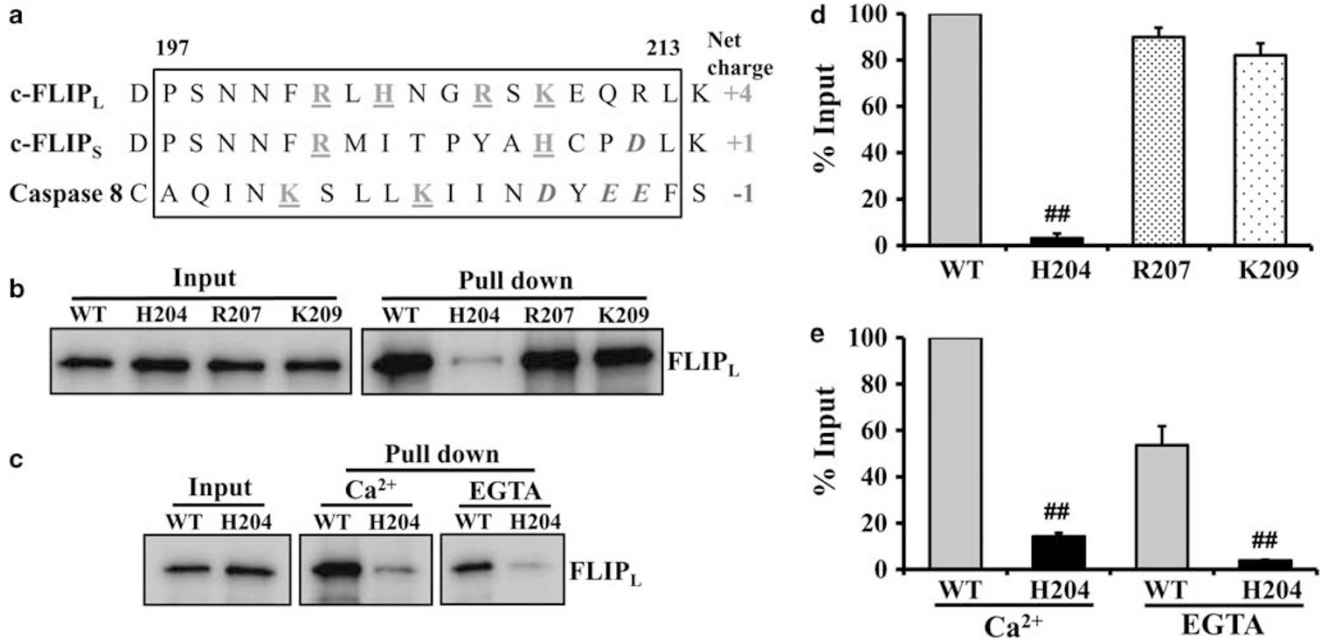

Figure 1 c-FLIP$_L$ H204 mutant has reduced binding to CaM *in vitro*. (**a**) Sequence comparison of the 197–213 regions from c-FLIP$_L$, c-FLIP$_S$ and caspase 8. Amino acids with positive (red, underline) or negative (blue, italic) charge are indicated. Protein pulldown assays with CaM-Sepharose 4B were performed using (**b**, **d**) various His-SUMO-FLIP$_L$ mutations or (**c**, **e**) His-SUMO-FLIP$_L$ WT and H204 mutant in the presence of Ca^{2+} or EGTA. Proteins binding to CaM-Sepharose 4B were determined by western blot analysis. Results shown are means ± s.e. ($n = 3$). $^{\#\#}P < 0.01$ for comparison with WT c-FLIP$_L$.

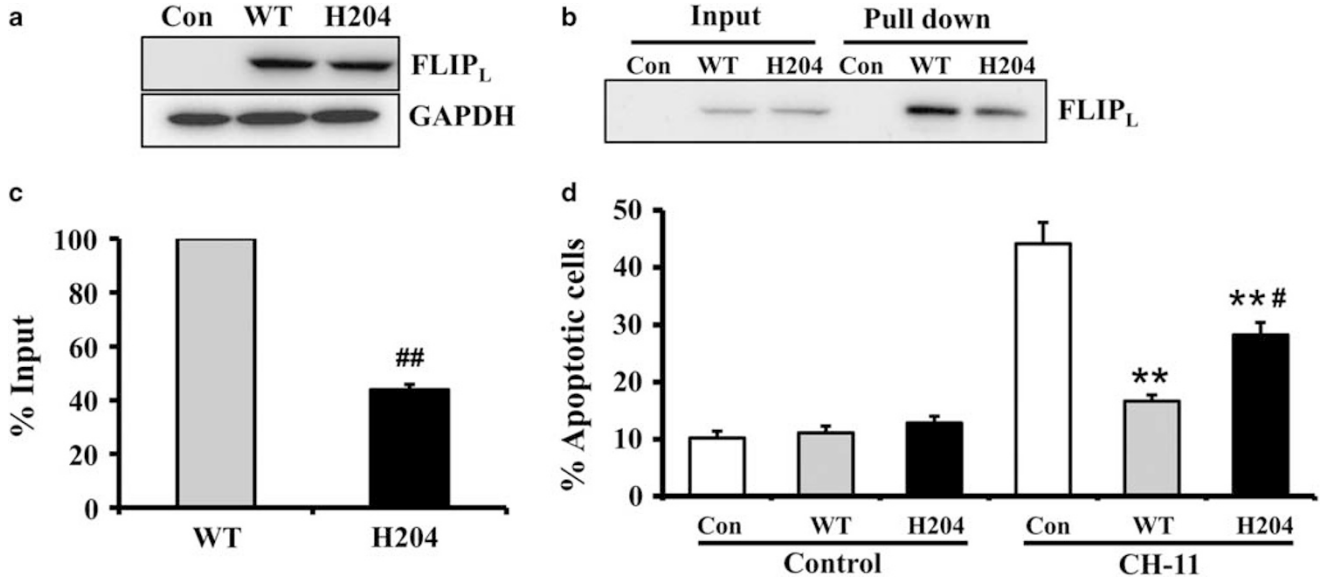

Figure 2 c-FLIP$_L$ H204 mutant has reduced binding to CaM in cholangiocarcinoma cells and sensitizes the cells to Fas-induced apoptosis. (**a**) Western blot analysis for c-FLIP$_L$ expression using cell lysates from cholangiocarcinoma cells, SK-ChA-1, stably overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant. (**b**, **c**) Protein pulldown assays with CaM-Sepharose 4B were performed using whole cell lysates from SK-ChA-1 cells stably overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant. The proteins pulled down were immunoblotted for c-FLIP$_L$. (**d**) Apoptosis was determined by Annexin V/propidium iodide staining in SK-ChA-1 cells stably overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant with or without CH-11, the Fas-activating antibody, treatment. Results shown are means ± s.e. (n = 3). $^{**}P < 0.01$ for comparison with controls. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ for comparison of the H204 mutant with WT c-FLIP$_L$.

interaction has been described as an important modulator of Fas-mediated apoptosis, which is an important pathway for apoptotic cell death in cholangiocarcinoma cells.[16,25] To determine the effect of the reduced CaM-c-FLIP$_L$ interaction by H204 on Fas-mediated apoptosis, cholangiocarcinoma cells overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant were treated with CH-11, the Fas-activating antibody, for 24 h. Apoptosis was determined by Annexin V/propidium iodide staining. As shown in Figure 2d, overexpression of WT c-FLIP$_L$ inhibited Fas-mediated apoptosis compared with control. The H204 mutant-overexpressing cells were partially resistant to Fas-mediated apoptosis as compared with controls, however, their sensitivity to Fas-mediated apoptosis was significantly higher than WT c-FLIP$_L$-overexpressing cells (Figure 2d). Thus the c-FLIP$_L$ H204 mutant partially rescues the inhibition of WT c-FLIP$_L$ on Fas-mediated apoptosis most likely because of the reduced binding with CaM.[25] Neither WT c-FLIP$_L$ nor the H204 mutant affected the levels of basal apoptosis (Figure 2d).

c-FLIP$_L$ H204 Mutant Reduces the Inhibitory effect of WT c-FLIP$_L$ on Fas-Induced Caspase Activation

To elucidate the mechanisms underlying the effects of WT c-FLIP$_L$ and the H204 mutant on Fas-mediated apoptosis, we determined the expression/activation of caspases in CH-11-treated cholangiocarcinoma cells (SK-ChA-1) overexpressing LacZ, WT c-FLIP$_L$ or the H204 mutant. As shown in Figures 3a and c, activation of caspase 3, a downstream caspase, was inhibited by WT c-FLIP$_L$ compared with control. Although compared with control the H204 mutant also inhibited activation of caspase 3, activated caspase 3 was higher than that in WT c-FLIP$_L$ cells (Figures 3a and c). These results are consistent with the apoptosis data (Figure 2d). c-FLIP$_L$ has been thought to act primarily as an anti-apoptotic protein that interferes with activation of caspase 8 as well as recruitment of caspase 8 into the DISC.[17,25] Therefore expression/activation of caspase 8 was determined by western blot analysis. As expected, both overexpression of WT c-FLIP$_L$ and the H204 mutant significantly blocked caspase 8 activation by CH-11 compared with controls (Figures 3a and b). However, the H204 mutant partially rescued the inhibition by WT c-FLIP$_L$ on caspase 8 activation, suggesting that the CaM binding to c-FLIP$_L$ modulates Fas-mediated apoptosis through activating caspase 8.

Effects of c-FLIP$_L$ H204 Mutant on Fas-Mediated DISC Formation

To determine the mechanism that enables the CaM-c-FLIP$_L$ interaction to modulate the sensitivity of cells to Fas-mediated apoptosis, Fas-mediated DISC analysis was performed. Cholangiocarcinoma cells, SK-ChA-1, overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant were exposed to CH-11 for 0,

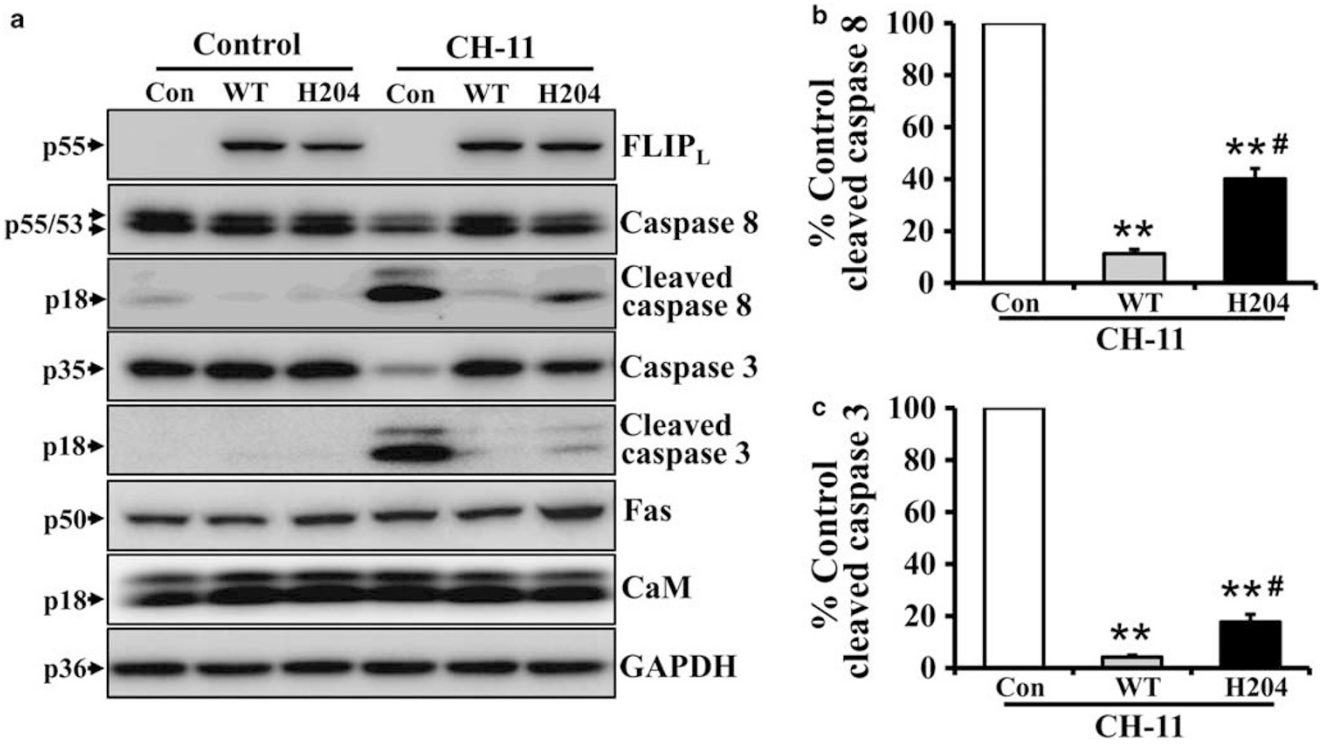

Figure 3 c-FLIP$_L$ H204 mutant reduces the inhibitory effect of WT c-FLIP$_L$ on Fas-induced caspase activation. Cholangiocarcinoma cells, SK-ChA-1, stably overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant were treated with CH-11 for 24 h. (**a**) Different apoptosis-related proteins as indicated were detected by western blot analysis. Cleavage of caspase 8 (**b**) and caspase 3 (**c**) are quantitated. Results shown are means ± s.e. (n = 3). $^{**}P < 0.01$ for comparison with controls and $^{\#}P < 0.05$ for comparison of the H204 mutant with WT c-FLIP$_L$.

30 and 60 min. The recruitment of c-FLIP$_L$, caspase 8, Fas, CaM and FADD into the DISC in response to CH-11 was analyzed. More WT c-FLIP$_L$ and H204 mutant were recruited into the DISC than in control cells because they were overexpressed (Figure 4). Compared with WT c-FLIP$_L$, less c-FLIP$_L$ H204 mutant was in the DISC, which supports our previous hypothesis that disrupted CaM-c-FLIP$_L$ binding causes a conformational change in c-FLIP$_L$ that affects its recruitment into the DISC.[25] Recruitment of caspase 8 into DISC was inhibited by overexpression of both WT c-FLIP$_L$ and the H204 mutant apparently because of its competition with caspase 8 for FADD binding.[10,33,34] Activated caspase 8 (p43/41) appeared at 30 min in c-FLIP$_L$ H204 mutant cells, which was earlier than 60 min in WT c-FLIP$_L$ cells (Figure 4). The amount of CaM in the DISC was decreased in c-FLIP$_L$ H204 mutant cells, which confirmed that reduced binding with CaM does exist both *in vitro* and *in vivo* (Figures 1 and 2).

Effects of c-FLIP$_L$ H204 Mutant on Cholangiocarcinoma Tumorigenesis in Mice

Increased sensitivity to Fas-mediated apoptosis by reduced binding between the H204 mutant and CaM (Figure 2d) suggests that the effects of WT c-FLIP$_L$ and the H204 mutant on tumorigenesis may be different. To test this hypothesis, tumorigenesis of WT- and H204 mutant-overexpressing cholangiocarcinoma cells (SK-ChA-1) was characterized in a nude mice xenograft model. Cholangiocarcinoma cells stably overexpressing WT c-FLIP$_L$ or the H204 mutant were inoculated subcutaneously into the flank area of 4-week-old male athymic nude mice. Tumor volumes were measured every 3 days. As shown in Figure 5a, H204 mutant overexpressing cells formed significantly smaller tumors than WT c-FLIP$_L$ cells from day 9 through day 15. Furthermore, TUNEL staining showed the increased apoptotic cells in H204 mutant tumors compared with WT c-FLIP$_L$ tumors (Figures 5b and c). These findings support the concept that the interaction between CaM and FLIP is important in cholangiocarcinoma pathogenesis.[2]

DISCUSSION

CaM interacts with a variety of proteins such as calcineurin, CaM kinases, myosin light-chain kinase, nitric oxide synthase and neuromodulin[35] and it affects numerous signaling pathways involved in such diverse processes such as inflammation, memory, muscle contraction, the immune response and ion channel functioning.[36] Previously, we have shown that CaM binds to two key proteins (Fas and c-FLIP$_L$, but not FADD or caspase 8) in the cell death pathway suggesting a novel role of CaM in Fas-mediated signaling.[24,25] CaM binds to c-FLIP$_L$ directly in a Ca^{2+}-dependent manner.[25] A CaM-binding region was identified in amino acids 197–213 of c-FLIP$_L$ and deletion of this region causes reduced binding between CaM and c-FLIP$_L$.[25] Furthermore, deletion of the CaM-binding region results in increased Fas-mediated apoptosis suggesting that CaM-c-FLIP$_L$ binding is important for the anti-apoptotic function of c-FLIP$_L$ in the Fas pathway.[25] To identify the precise amino acids between 197 and 213 that are responsible for CaM/FLIP binding, and their roles in mediating the anti-apoptotic function of c-FLIP$_L$, sequence comparison of this binding region among c-FLIP$_L$, c-FLIP$_S$ and caspase 8 predicted three positively charged residues at 204, 207 and 209, which might be responsible for the CaM/FLIP binding (Figure 1a). Considering these findings, it was reasonable to determine whether one of these three amino acid mutants affects c-FLIP$_L$ binding to

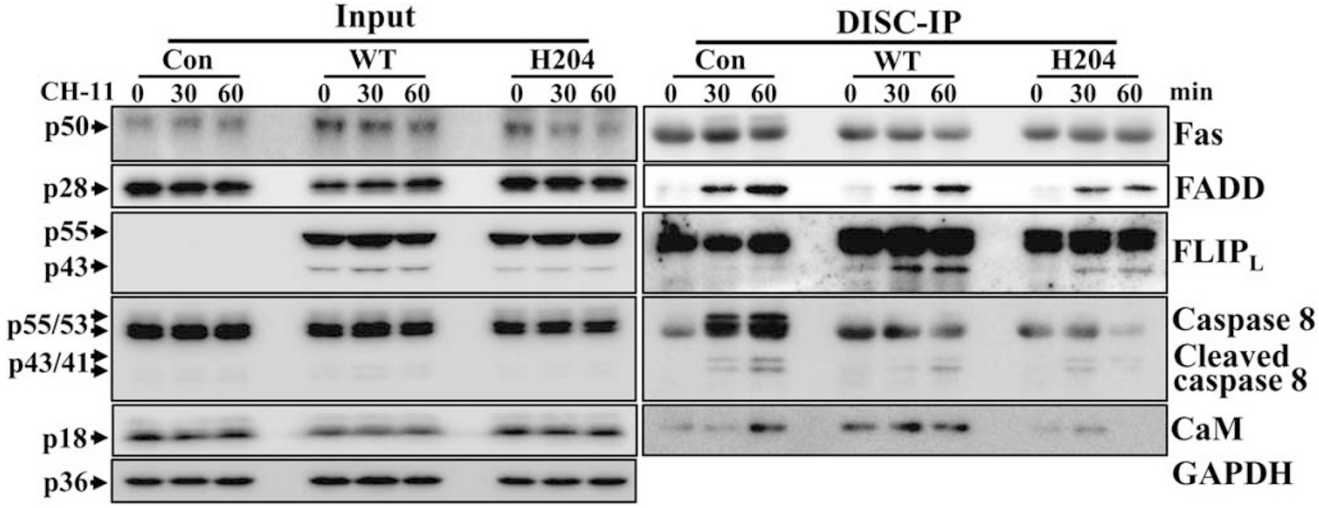

Figure 4 Effects of c-FLIP$_L$ H204 mutant on Fas-mediated DISC formation. Cholangiocarcinoma cells, SK-ChA-1, stably overexpressing LacZ, WT c-FLIP$_L$ or H204 mutant were exposed to CH-11 for 0, 30 and 60 min. Western blot analyses were performed with extracted proteins to determine the expression of DISC proteins including c-FLIP$_L$, caspase 8, Fas, CaM and FADD in response to CH-11 (left panel inputs). Immunoprecipitation for DISC analysis was performed to determine the recruitment of c-FLIP$_L$, caspase 8, Fas, CaM and FADD into DISC in response to CH-11 (right panel-DISC-IP).

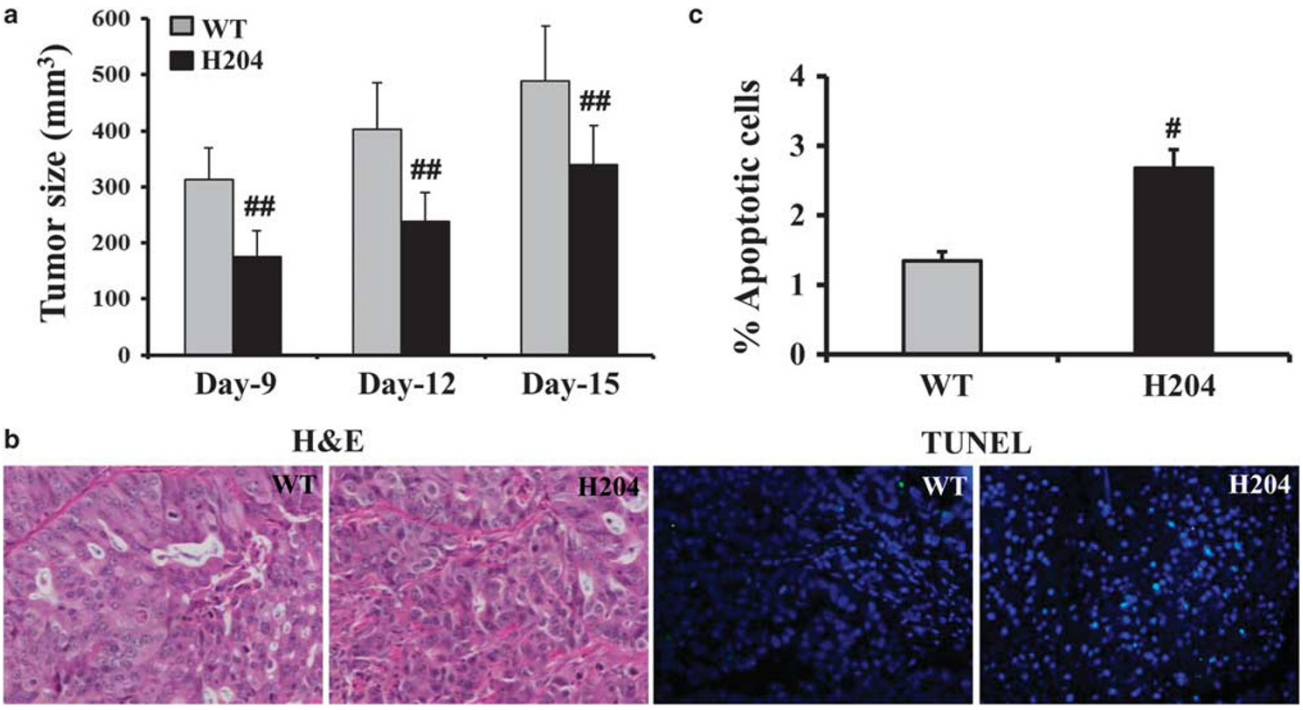

Figure 5 Effects of c-FLIP$_L$ H204 mutant on cholangiocarcinoma tumorigenesis in mice. Cholangiocarcinoma cells, SK-ChA-1, stably overexpressing WT c-FLIP$_L$ or H204 mutant were inoculated subcutaneously into the flank area of 4-week-old male athymic nude mice (five mice per group). (**a**) Tumor volumes were measured every 3 days for 15 days and determined using the formula volume = length × width^2/2. (**b**) At the end of the tumorigenesis experiment, tumors were processed as described in Materials and methods. Consecutive sections of tumors from each mouse were analyzed by H&E staining and TUNEL staining. Representative images of each group are shown. For quantitative analysis, cell numbers were counted and the percentage of positive cells was determined. (**c**) Percentage of apoptotic cells determined by TUNEL staining. Results shown are means ± s.e. ($n = 10$ for tumors and $n = 12$ for TUNEL staining). $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ for comparison of the H204 mutant with WT c-FLIP$_L$.

CaM. We found that only the H204 mutant showed reduced binding to CaM, which was confirmed by both using purified proteins and intact cells (Figures 1 and 2). Furthermore, the H204 mutant increased the sensitivity of cells to Fas-mediated apoptosis (Figure 2d) and showed increased apoptosis and decreased tumorigenesis in mice compared with WT c-FLIP$_L$ (Figure 5), which provided new evidence that the anti-apoptotic effect of c-FLIP$_L$ requires CaM binding. The same mechanism was confirmed by using transiently transfected cells and in an additional cholangiocarcinoma cell line, Mz-ChA-1 (Supplementary Figures 1 and 2).

The function of c-FLIP$_L$ in apoptotic signaling is considered to be primarily an anti-apoptotic protein although some evidence indicates that it can enhance Fas-mediated apoptosis when expressed at much lower and higher physiological levels.[16–18] During the early stages of death receptor signaling, the key regulatory protein, c-FLIP$_L$ is recruited to the DISC, where it competes with procaspase 8 and 10 binding to FADD and modulates activation of procaspase 8 and 10.[17,37,38] To elucidate how CaM-c-FLIP$_L$ binding modulates the anti-apoptotic function of c-FLIP$_L$, we determined the effects on expression/activation of apoptosis-related proteins. Reduced binding between c-FLIP$_L$ and CaM increased caspase 8 activation, which in turn leads to activated caspase 3 and increased apoptosis (Figure 3). Thus CaM-c-FLIP$_L$ binding modulates Fas-mediated apoptosis at least in part by modulating activation of caspase 8.

Inhibition of caspase 8 activation by c-FLIP$_L$ appeared to be largely due to the interfering with recruitment of caspase 8 into the DISC and reducing local concentrations of procaspase 8 for its autoproteolytic cleavage and activation.[17,19,39,40] To determine how reduced CaM binding affects c-FLIP$_L$ and caspase 8 recruitment to the DISC, we performed IP of the DISC after Fas activation. Less c-FLIP$_L$ H204 mutant was in the DISC compared with WT c-FLIP$_L$ (Figure 4), which supports our previous hypothesis that disrupted CaM-c-FLIP$_L$ binding may cause a conformational change in c-FLIP$_L$ and decrease its recruitment into the DISC.[25] The amount of caspase 8 recruited into the DISC decreased in c-FLIP$_L$-overexpressing cells (Figure 4), suggesting that inhibition of caspase 8 activation by c-FLIP$_L$ was mainly through reducing caspase 8 in the DISC. Activated caspase 8 (p43/41) in the DISC appeared at 30 min in control and c-FLIP$_L$ H204 mutant cells, which was earlier than the 60 min seen in WT c-FLIP$_L$ cells (Figure 4). Our previous publication has demonstrated the highest CaM-c-FLIP$_L$ binding in response to CH-11 stimulation occurred at 30 min and returned to the basal level at 60 min in cholangiocarcinoma

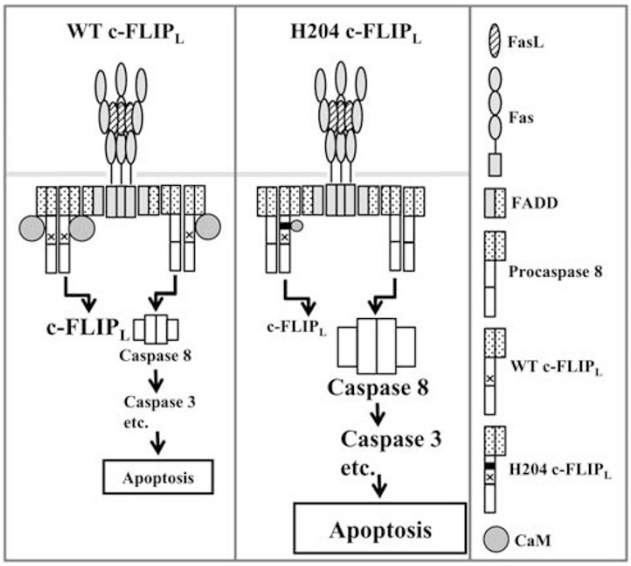

Figure 6 Proposed model of the effects of WT c-FLIP$_L$ and H204 mutant on Fas-mediated DISC formation. The model depicts the Fas-mediated DISC formation and apoptotic signaling affected by WT c-FLIP$_L$ and H204 mutant in cholangiocarcinoma cells. WT c-FLIP$_L$ competes with procaspase 8 binding to FADD, thus decreasing caspase 8 recruitment to the DISC and its activation, which inhibits Fas-mediated apoptosis. The H204 mutant in c-FLIP$_L$ has reduced binding to CaM, which results in less c-FLIP$_L$ recruited to the DISC. Consequently, more procaspase 8 is recruited to the DISC, forming high local concentrations, which leads to its autoproteolytic cleavage and activation, which in turn activates downstream signals and increases apoptosis.

cells.[25] That could explain why the largest difference between the effects of WT c-FLIP$_L$ and H204 mutant on caspase 8 activation was observed at 30 min. A proposed mechanism whereby CaM/FLIP binding modulates Fas-mediated apoptosis is shown in Figure 6. WT c-FLIP$_L$ competes with procaspase 8 binding to FADD and thus decreases caspase 8 recruitment to the DISC and its activation, thus inhibiting Fas-mediated apoptosis. The H204 mutant in c-FLIP$_L$ reduces its binding to CaM, which results in less c-FLIP$_L$ recruited to the DISC. Consequently, more procaspase 8 is recruited to the DISC, forming high local concentrations, which leads to its autoproteolytic cleavage and activation, which in turn activates downstream signals and increases apoptosis.[32,39,40]

Fas is well characterized as a death receptor in the apoptotic machinery, but activation of Fas has also been shown to induce cell proliferation and tissue regeneration.[41,42] Recently, c-FLIP has been implicated in signaling alternative pathways, linking the Fas receptor to the NF-κB, JNK, MAPK and ERK pathways.[25,43–46] These studies link the Fas-signaling pathway to survival pathways in addition to its well-known role in apoptosis. Our studies provide new evidence supporting the concept that the Fas-CaM-FLIP arm of the DISC might be important in modulating both apoptotic and survival signaling in response to Fas activation. Further characterizing the nature of CaM-FLIP interaction with respect to other death receptors such as DR4, DR5 and TNF-R1, may provide new therapeutic targets for cancer therapy.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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