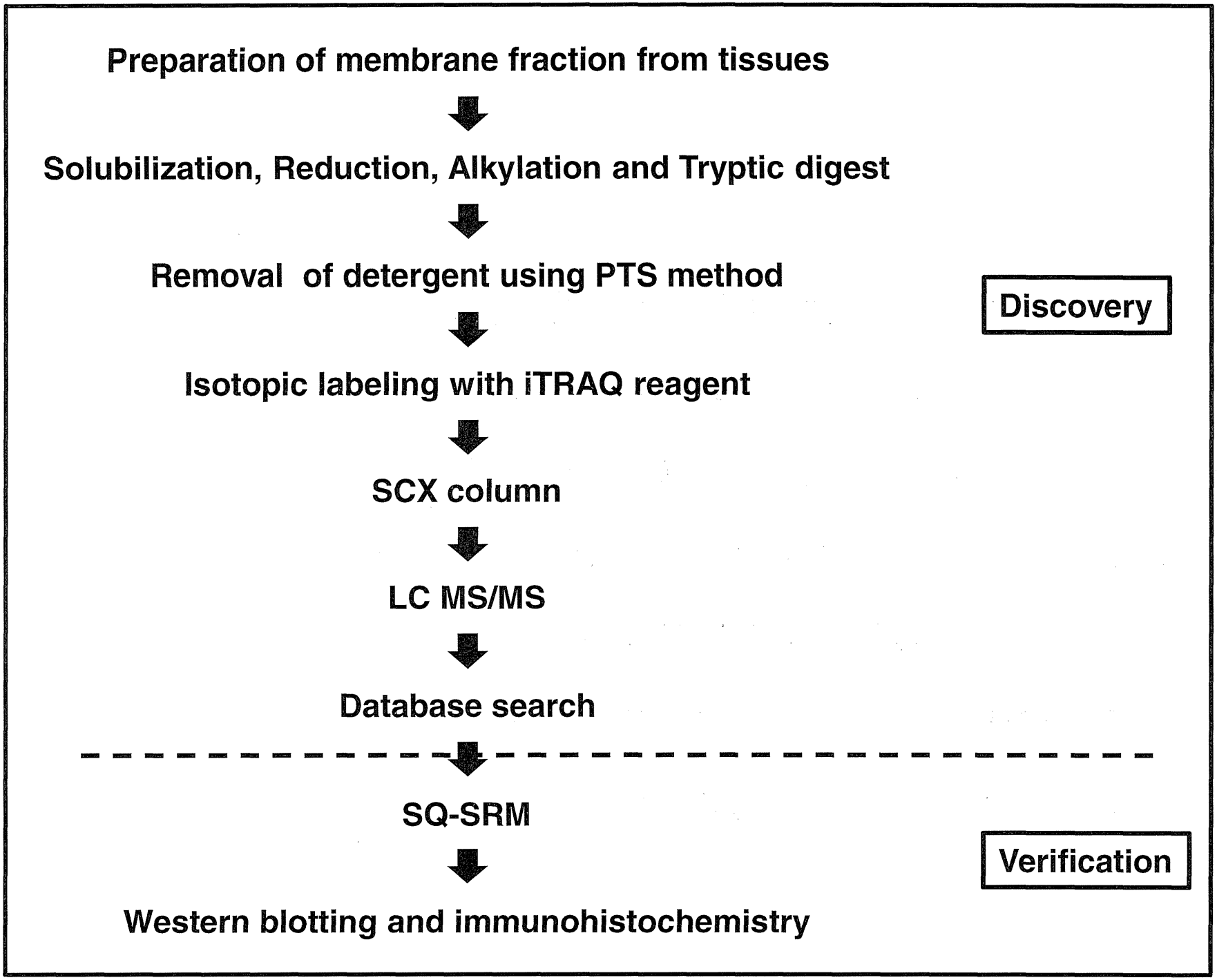
Preparation of membrane fraction from tissues
Solubilization, Reduction, Alkylation and Tryptic digest
Removal of detergent using PTS method
Isotopic labeling with iTRAQ reagent
SCX column
LC MS/MS
Database search
SQ-SRM
Western blotting and immunohistochemistry
Discovery
Verification

Figure 1

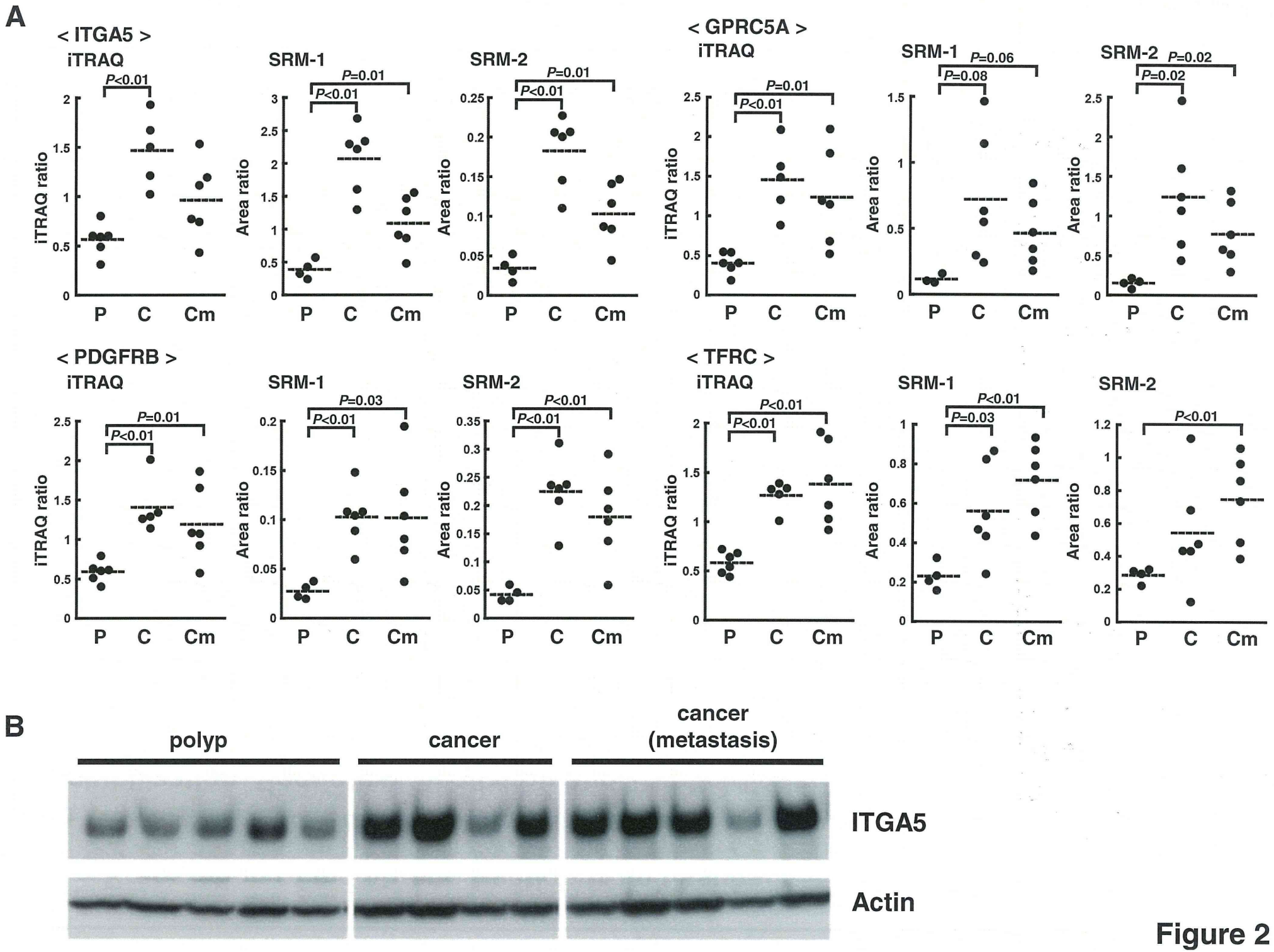
A
< ITGA5 >
iTRAQ
SRM-1
SRM-2
< GPRC5A >
< PDGFRB >
< TFRC >
iTRAQ ratio
Area ratio
P<0.01
P=0.01
P=0.03
P=0.06
P=0.08
P=0.02
P
C
Cm
B
polyp
cancer
cancer
(metastasis)
ITGA5
Actin

Figure 2

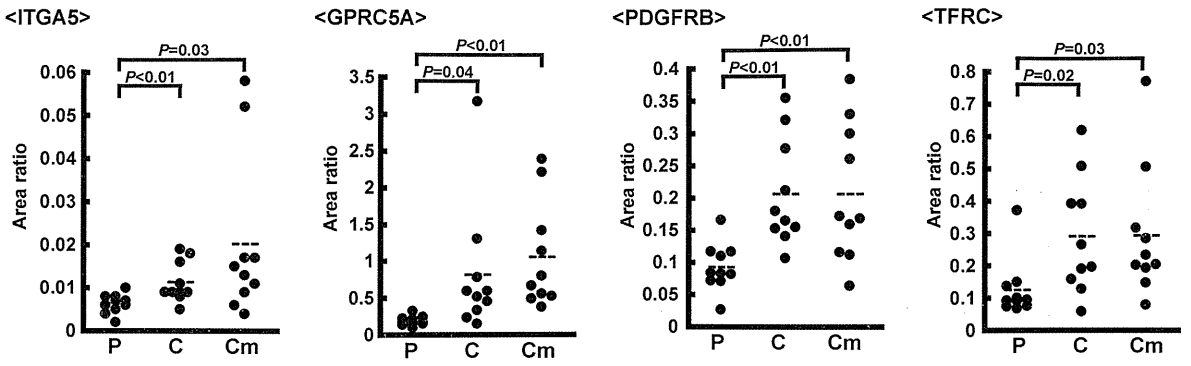
<ITGA5>
P=0.03
P<0.01
Area ratio
0.06
0.05
0.04
0.03
0.02
0.01
0
P
C
Cm
<GPRC5A>
P<0.01
P=0.04
Area ratio
3.5
3
2.5
2
1.5
1
0.5
0
P
C
Cm
<PDGFRB>
P<0.01
P<0.01
Area ratio
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
P
C
Cm
<TFRC>
P=0.03
P=0.02
Area ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
P
C
Cm

Figure 3

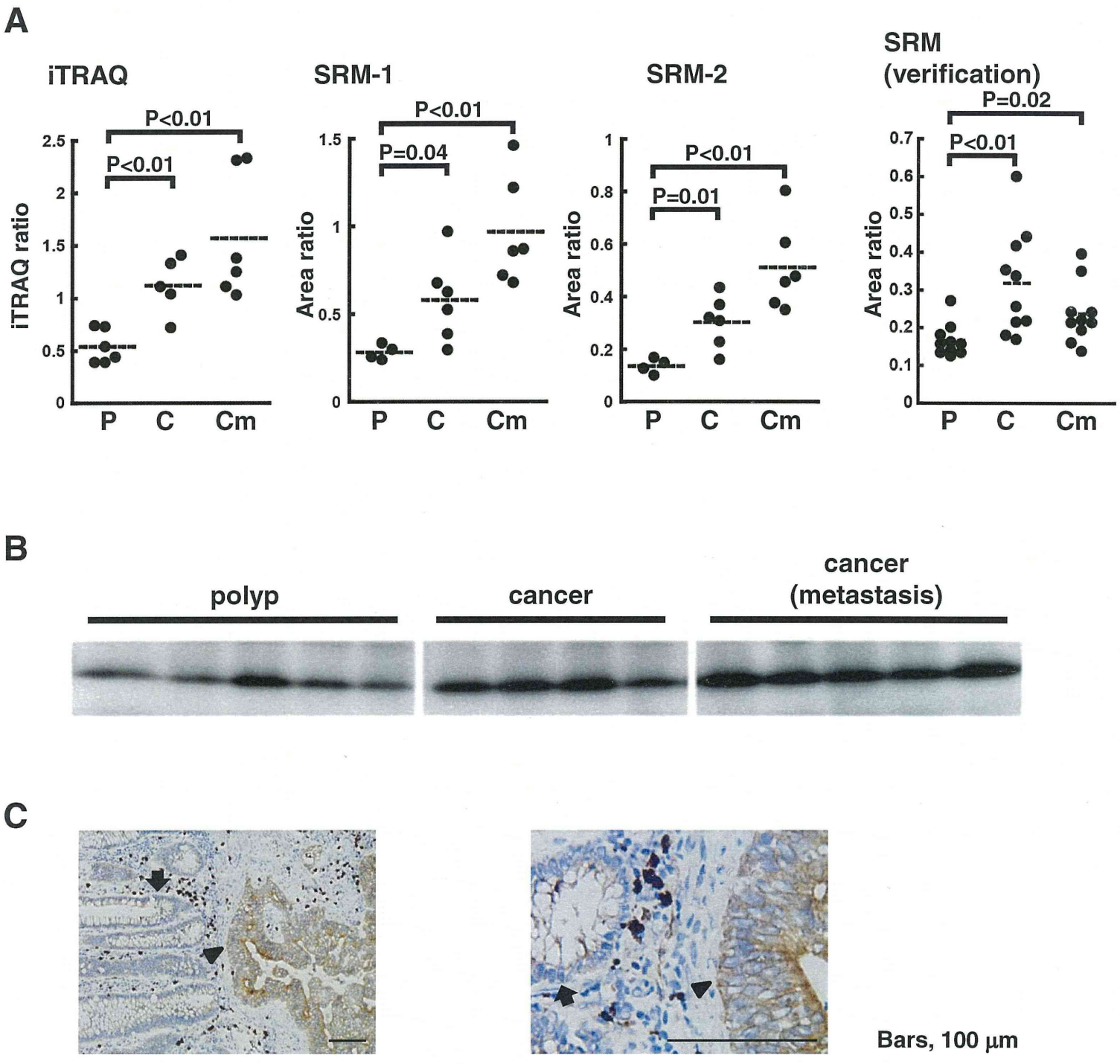
A
iTRAQ
SRM-1
SRM-2
SRM (verification)
P<0.01
P<0.01
P<0.01
P=0.04
P<0.01
P=0.01
P=0.02
P<0.01
iTRAQ ratio
Area ratio
Area ratio
Area ratio
P
C
Cm
B
polyp
cancer
cancer (metastasis)
C
Bars, 100 μm

Figure 4

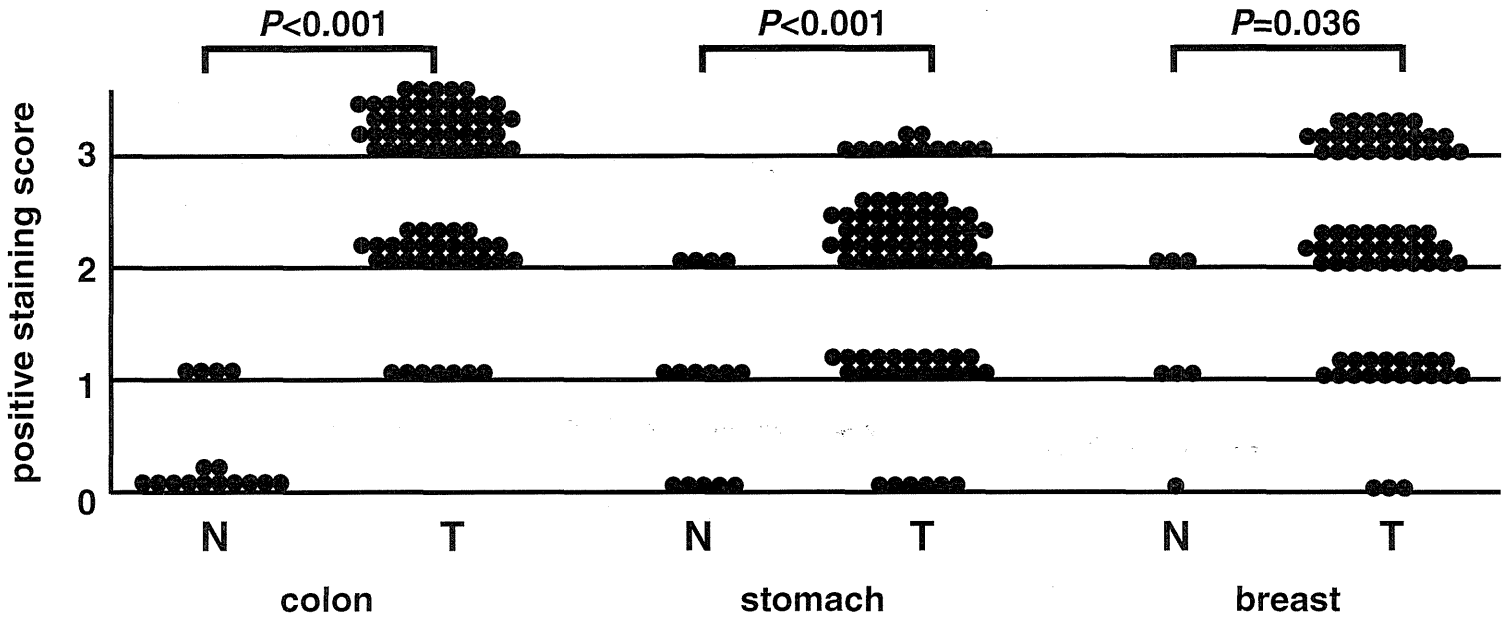
P<0.001
P<0.001
P=0.036
positive staining score
3
2
1
0
N
T
N
T
N
T
colon
stomach
breast

Figure 5

A novel mechanism of keratin cytoskeleton organization through casein kinase Iα and FAM83H in colorectal cancer

Takahisa Kuga[1,*], Hideaki Kume[1], Naoko Kawasaki[1], Misako Sato[1], Jun Adachi[1], Takashi Shiromizu[1], Isamu Hoshino[2], Takanori Nishimori[2], Hisahiro Matsubara[2] and Takeshi Tomonaga[1,*]

[1]Laboratory of Proteome Research, National Institute of Biomedical Innovation, Ibaraki, Osakai 567-0085, Japan
[2]Department of Frontier Surgery, Graduate School of Medicine, Chiba University, Chuo-ku, Chiba 260-8670, Japan
*Authors for correspondence (t-kuga@nibio.go.jp; tomonaga@nibio.go.jp)

Accepted 9 July 2013
Journal of Cell Science 126, 4721–4731

doi: 10.1242/jcs.129684

Summary

Keratin filaments form cytoskeletal networks in epithelial cells. Dynamic rearrangement of keratin filament networks is required for epithelial cells to perform cellular processes such as cell migration and polarization; however, the mechanism governing keratin filament rearrangement remains unclear. Here, we describe a novel mechanism of keratin cytoskeleton organization mediated by casein kinase Iα (CK-1α) and a newly identified keratin-associated protein, FAM83H. Knockdown of FAM83H induces keratin filament bundling, whereas overexpression of FAM83H disassembles keratin filaments, suggesting that FAM83H regulates the filamentous state of keratins. Intriguingly, keratin filament bundling is concomitant with the dissociation of CK-1α from keratin filaments, whereas aberrant speckle-like localization of CK-1α is observed concomitantly with keratin filament disassembly. Furthermore, CK-1α inhibition, similar to FAM83H knockdown, causes keratin filament bundling and reverses keratin filament disassembly induced by FAM83H overexpression, suggesting that CK-1α mediates FAM83H-dependent reorganization of keratin filaments. Because the N-terminal region of FAM83H interacts with CK-1α and the C-terminal region interacts with keratins, FAM83H might tether CK-1α to keratins. Colorectal cancer tissue also shows keratin filament disassembly accompanied with FAM83H overexpression and aberrant CK-1α localization, and FAM83H-overexpressing cancer cells exhibit loss or alteration of epithelial cell polarity. Importantly, knockdown of FAM83H inhibits cell migration accompanied by keratin cytoskeleton rearrangement in colorectal cancer cells. These results suggest that keratin cytoskeleton organization is regulated by FAM83H-mediated recruitment of CK-1α to keratins, and that keratin filament disassembly caused by overexpression of FAM83H and aberrant localization of CK-1α could contribute to the progression of colorectal cancer.

Key words: Keratin cytoskeleton, Casein kinase Iα, FAM83H, Colorectal cancer, Invasion

Introduction

Keratins are intermediate filament proteins that form cytoskeletal filament networks in epithelial cells (Moll et al., 2008; Oriolo et al., 2007; Windoffer et al., 2011). To form a filamentous structure, keratin proteins are nucleated, assembled into particles and then elongated (Miller et al., 1991; Miller et al., 1993; Windoffer et al., 2011). Keratin filaments are further bundled to form a mechanically stable cytoskeleton (Moll et al., 2008; Windoffer et al., 2011). Keratin filament assembly and bundling are dynamic and reversible processes (Flitney et al., 2009; Windoffer and Leube, 1999; Windoffer et al., 2004). The keratin cytoskeleton is an important structural stabilizer of epithelial cells (Moll et al., 2008). However, to achieve various dynamic cellular processes, including epithelial cell polarization and migration, keratin filament networks need to be dynamically reorganized (Ameen et al., 2001; Beil et al., 2003; Kölsch et al., 2010; Oriolo et al., 2007; Salas et al., 1997; Windoffer et al., 2011).

Although the molecular mechanism governing the rearrangement of keratin filaments is largely unclear, phosphorylation and protein–protein interactions of keratins have been shown to be involved (Izawa and Inagaki, 2006; Omary et al., 2006; Windoffer et al., 2011). Phosphorylation of keratins induces the disassembly of keratin filaments. Keratin filaments reconstituted using rat keratin 8 and keratin 18 were disassembled by *in vitro* phosphorylation of the keratins, but reassembled by their dephosphorylation (Yano et al., 1991). Upon treatment of epithelial cells with the protein phosphatase inhibitors okadaic acid and orthovanadate, phosphorylation levels of keratins were enhanced and keratin filaments were concomitantly disassembled (Strnad et al., 2001; Strnad et al., 2002); however, only a few kinases such as p38 MAPK and PKCζ were reported to be involved in keratin filament disassembly in different situations (Sivaramakrishnan et al., 2009; Wöll et al., 2007).

The bundling of keratin filaments seems to be regulated by the association of keratins with specific proteins (Windoffer et al., 2011). The plakin family proteins, which are linker proteins between keratin filaments and various cytoskeletal structures (Wiche, 1998), are major modulators of keratin filament bundling (Boczonadi et al., 2007; Liu et al., 2011; Long et al., 2006; Osmanagic-Myers et al., 2006); however, our knowledge about

Journal of Cell Science

crucial modulators of keratin filament bundling remains very limited. To further understand the mechanism governing the rearrangement of keratin filaments, identification of novel keratin-associated proteins and kinases responsible for the mechanism is required.

Similar to normal epithelial cells, epithelial cancer cells also express keratin proteins (Moll et al., 2008; Omary et al., 2009). Several studies have reported the involvement of keratins in cancer progression, invasion and metastasis (Omary et al., 2009). In several types of cancer, the expression levels of specific subtypes of keratin correlate with patient survival (Moll et al., 2008). In colorectal cancer, reduced expression of keratin 8 and keratin 20 was associated with shorter patient survival (Knösel et al., 2006). Furthermore, several reports have suggested a direct beneficial impact of keratins in cancer treatment. Forced expression of keratin 18 in MDA-MB-231 breast cancer cells strengthened cell–cell adhesions and markedly regressed tumor growth and metastasis in a xenograft model (Bühler and Schaller, 2005). Pancreatic carcinoma cells also exhibited decreased motility and tumorigenicity upon forced expression of keratin 18 (Pankov et al., 1997); however, additional studies are needed to elucidate the role of keratins in cancer.

FAM83H is an essential protein for amelogenesis because its truncated mutations cause autosomal-dominant hypocalcified amelogenesis imperfecta (Kim et al., 2008; Urzúa et al., 2011). However, very recent reports suggest the involvement of FAM83H in cancer. Microarray analysis of cancer tissues indicate increased expression of FAM83H in various types of cancer, including colorectal cancer (Sasaroli et al., 2011). Furthermore, Hatanaka and colleagues performed a focus formation assay using the cDNA library prepared from biliary tract cancer tissue and showed that fragmented FAM83H cDNA possesses transforming activity (Hatanaka et al., 2010). In a phospho-proteomic study of lung cancer cell lines that are sensitive or resistant to the anticancer drug dasatinib, phosphorylation levels of FAM83H decreased approximately tenfold in dasatinib-resistant cell lines (Klammer et al., 2012), implying the involvement of FAM83H in the resistance to dasatinib; however, there have been no reports on the precise mechanisms.

In this study, we determined that FAM83H and casein kinase Iα (CK-1α) are novel keratin-associated proteins and demonstrate that FAM83H-mediated recruitment of CK-1α to keratin filaments controls the filamentous state of keratins in colorectal cancer cells. Furthermore, we show that overexpression of FAM83H and CK-1α-mediated disassembly of keratin filaments occur in colorectal cancer tissue. FAM83H-overexpressing cancer cells exhibit loss or alteration of epithelial cell polarity and cell–cell adhesions – a hallmark of highly motile cancer cells. Given that FAM83H is involved in reorganization of the keratin cytoskeleton during the migration of colorectal cancer cells, keratin filament disassembly induced by FAM83H overexpression and aberrant localization of CK-1α might be important for the migration and invasion of colorectal cancer.

Results

Associations of FAM83H and CK-1α with keratins

We first searched for proteins associated with FAM83H using a proteomic approach. HCT116 colorectal cancer cells were transfected with a plasmid vector encoding FLAG-tagged FAM83H or the empty vector, and immunoprecipitation with anti-FLAG antibody was performed using extracts of these cells. Proteins present in the immunoprecipitates were identified using in-gel digestion and LC-MS/MS. By comparing the proteins identified from FAM83H-FLAG immunoprecipitates with those from the control, keratin proteins (subtypes 18 and 19) and CK-1α were identified as FAM83H-associated proteins (supplementary material Table S1).

Western blot analysis of these immunoprecipitates substantiated the association of FAM83H with CK-1α and keratin 8 and 18 (Fig. 1A). Furthermore, endogenous FAM83H and CK-1α were detected in immunoprecipitates using anti-keratin-18 antibody

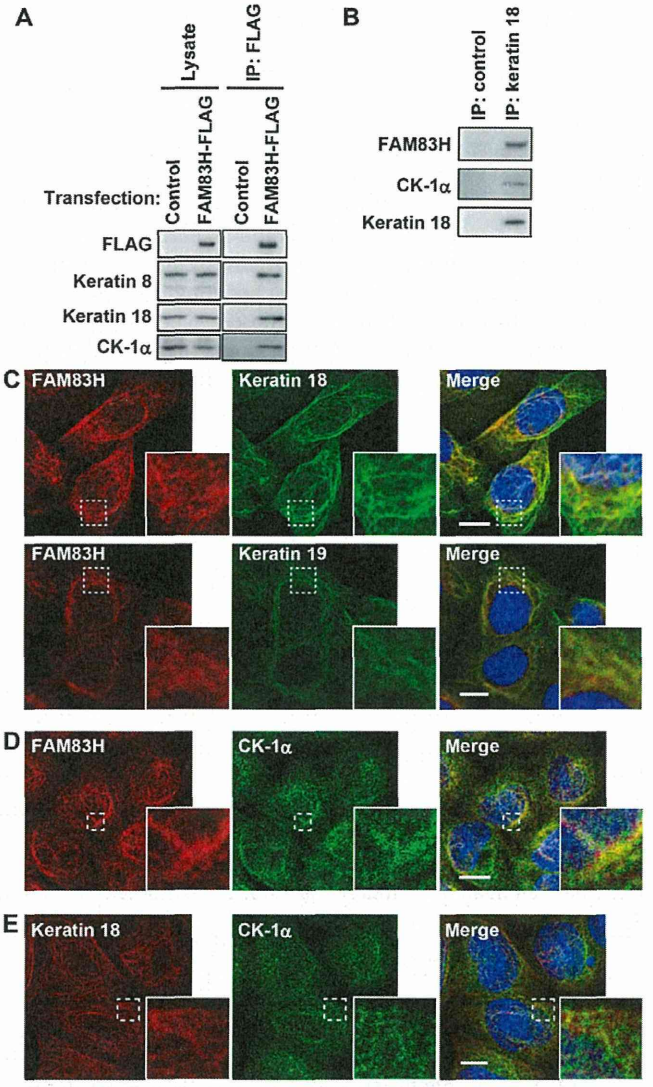

Fig. 1. Association of FAM83H and CK-1α with keratins. (A) Immunoprecipitation with anti-FLAG antibody was performed using cell lysates from HCT116 cells treated with FAM83H–FLAG vector or the empty vector (control) for 24 hours. The cell lysates and immunoprecipitates were analyzed by western blotting. (B) Immunoprecipitates with anti-keratin-18 or the control antibody (MPOC-21) from DLD-1 cell lysates were analyzed by western blotting. (C–E) DLD1 cells were stained with the indicated antibodies. Small panels show magnified images of the areas enclosed by dotted lines. DNA is stained with DAPI (blue). Scale bars: 10 μm.

(Fig. 1B). Fig. 1C shows subcellular colocalization of FAM83H with keratin 18 and keratin 19, and Fig. 1D,E show partial colocalization of CK-1α with FAM83H and keratin 18 [Pearson's correlation coefficient: FAM83H vs keratin 18, $r=0.58\pm0.20$ ($n=6$ cell images); FAM83H vs keratin 19, $r=0.49\pm0.06$ ($n=6$ cell images); FAM83H vs CK-1α, $r=0.35\pm0.06$ ($n=11$ cell images); keratin 18 vs CK-1α, $r=0.31\pm0.07$ ($n=11$ cell images)]. Given that organization of the keratin cytoskeleton is regulated by protein phosphorylation (Omary et al., 2006), these results suggest that FAM83H and CK-1α is involved in keratin cytoskeleton organization.

FAM83H regulates the filamentous state of keratins

To assess whether FAM83H regulates the organization of keratin filaments, HCT116 cells were treated with siRNA to knock down FAM83H and keratin filaments were visualized. Control cells formed an intricate network of thin keratin filaments in the cytoplasm (Fig. 2A). In sharp contrast, FAM83H-knockdown cells constructed a markedly rough network of thick keratin filaments (Fig. 2A). Next, we tested the effect of overexpression of FAM83H–FLAG on keratin filaments in HCT116 cells. Fig. 2B shows that FAM83H–FLAG overexpression completely disassembled keratin filaments (region 1), although adjacent cells without FAM83H–FLAG expression exhibited normal networks of keratin filaments (region 2). FAM83H–FLAG mainly colocalized with the disassembled keratin proteins (Pearson's correlation coefficient, $r=0.78\pm0.20$ ($n=3$ cell images); Fig. 2B, region 1). In DLD1 colorectal cancer cells, similar reorganization of keratin filaments by knockdown and overexpression of FAM83H was observed (supplementary material Fig. S1).

In addition, western blot analysis showed that these alterations of the keratin filament networks were not due to changes in the expression levels of keratin proteins because no changes in the expression of keratin 8, keratin 18 or keratin 19 were observed upon knockdown or overexpression of FAM83H (Fig. 2A,B). Given that keratin filaments are dynamic cytoskeletal elements capable of rapid and reversible restructuring (Windoffer et al., 2011), these results indicate that FAM83H controls the equilibrium between the assembly (bundling) and disassembly of keratin filaments.

CK-1α mediates FAM83H-dependent rearrangement of keratin filaments

Next, we examined whether CK-1α is involved in the organization of keratin filaments. HCT116 cells were treated with a CK-1 inhibitor, D4476, or with siRNA to knock down CK-1α (CK-1α siRNA) and analyzed by immunofluorescence for keratin filaments. Inhibition of CK-1α, similar to FAM83H knockdown, promoted bundling of keratin filaments without changes in the protein level of keratins (Fig. 3A,B), implying that FAM83H and CK-1α cooperate to regulate the filamentous state of keratins. On the basis of the amino acid sequence, FAM83H does not have any enzymatic domains; thus, CK-1α kinase activity could mediate the mechanism by which FAM83H rearranges keratin filaments.

To explore the requirement of CK-1α for FAM83H rearrangement of keratin filaments, HCT116 cells were transfected with FAM83H–FLAG in the presence of CK-1α siRNA or D4476. Immunofluorescence for keratin filaments showed that both CK-1α siRNA and D4476 obviously reversed

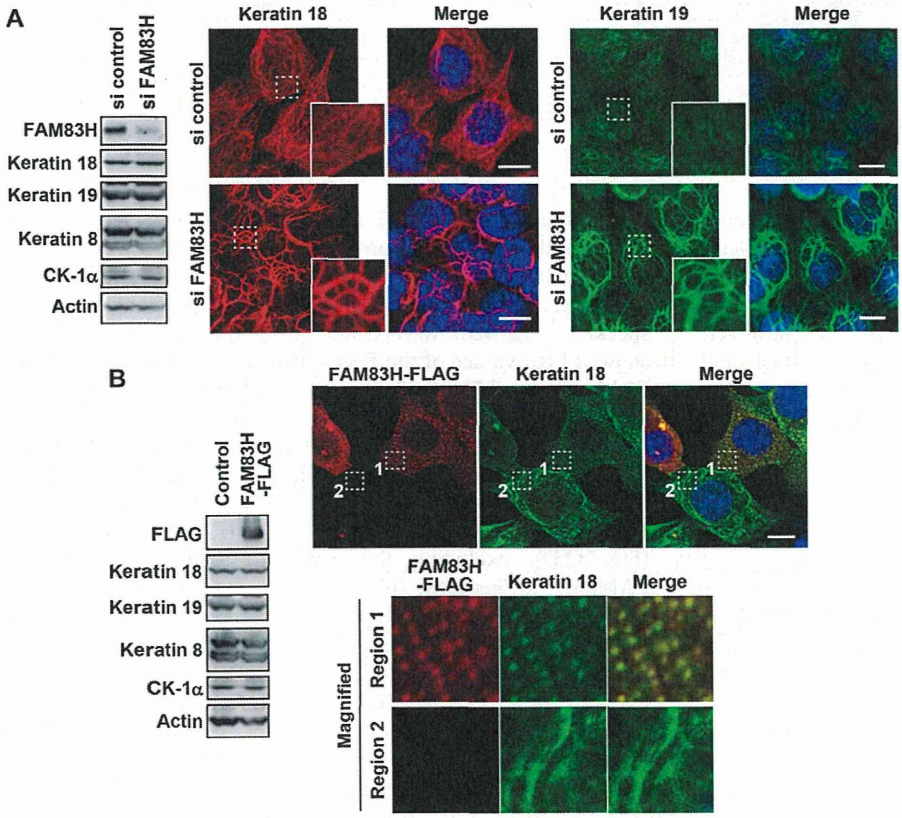

Fig. 2. Effects of knockdown and overexpression of FAM83H on the filamentous state of keratins. (A) HCT116 cells were treated with FAM83H siRNA or control siRNA for 2 days and analyzed by immunofluorescence and western blotting. Small panels show magnified images of the areas enclosed by dotted lines. (B) HCT116 cells were transfected with FAM83H–FLAG vector or the empty vector (control) and cultured for 24 hours. Western blotting and immunofluorescence were performed. Magnified images of the areas enclosed by dotted lines in cells with (region 1) and without (region 2) FAM83H–FLAG expression are shown. DNA is stained with DAPI (blue). Scale bars: 10 μm.

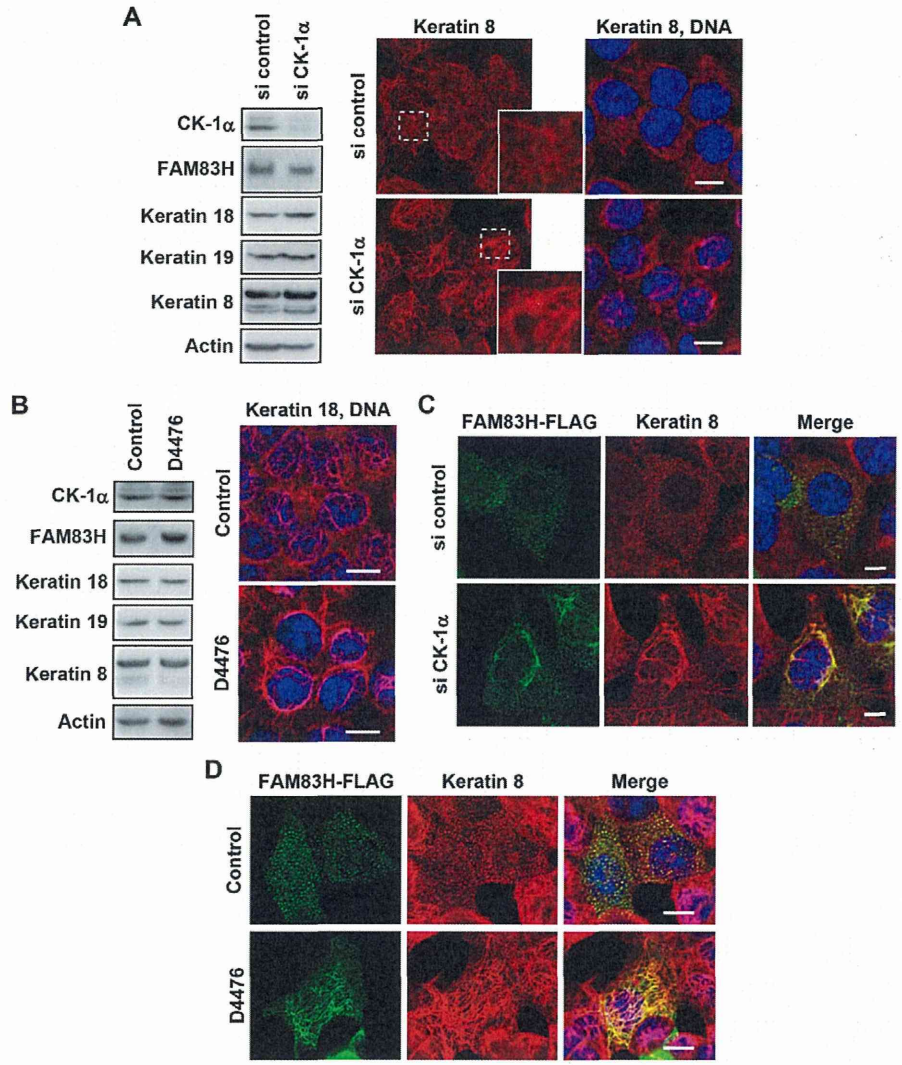

Fig. 3. CK-1α mediates FAM83H-dependent rearrangement of keratin filaments. (A) HCT116 cells were treated with CK-1α siRNA or control siRNA for 2 days and analyzed by immunofluorescence and western blotting. Insets show magnified images in the regions enclosed by dotted lines. (B) HCT116 cells were treated with D4476, a CK-1 inhibitor or DMSO (control) for 3 hours and analyzed by immunofluorescence and western blotting. Keratin 18 staining is shown in red. (C) HCT116 cells were transfected with CK-1α siRNA or control siRNA, followed by transfection of FAM83H–FLAG after 24 hours, and then cultured for 24 hours. Cells were analyzed by immunofluorescence. (D) HCT116 cells were transfected with FAM83H-FLAG and cultured for 24 hours. D4476 or DMSO (control) was added for the last 18 hours of the 24 hours culture. Cells were analyzed by immunofluorescence. DNA is stained with DAPI (blue). Scale bars: 10 μm.

the effect of FAM83H–FLAG on the disassembly of keratin filaments (Fig. 3C,D). These results suggest that CK-1α mediates FAM83H–dependent rearrangement of keratin filaments.

Keratin proteins have been subjected to phosphorylation to regulate their own structural state (Omary et al., 2006); however, we could not simply explain the mechanism of FAM83H- and CK-1α-mediated rearrangement of keratin filaments by phosphorylation, at least at Ser73 and Ser431 of keratin 8 and Ser33 and Ser52 of keratin 18 (see supplementary material Fig. S2).

FAM83H recruits CK-1α to keratin filaments

What is the precise mechanism by which FAM83H modulates the CK-1α-mediated rearrangement of keratin filaments? It is known that CK-1α is basically constitutive active and its subcellular localization is an important factor in its functional regulation (Knippschild et al., 2005); thus, FAM83H might regulate the subcellular localization of CK-1α. To test this hypothesis, we examined the effect of knockdown or overexpression of FAM83H on the subcellular localization of CK-1α. Immunofluorescence for CK-1α showed that FAM83H knockdown diminished the colocalization of CK-1α with keratin filaments (Fig. 4A). FAM83H knockdown decreased the value of the Pearson's correlation coefficient between CK-1α and keratin filaments (Fig. 4A). Conversely, FAM83H overexpression recruited CK-1α to disassembled keratin speckles (Fig. 4B). In FAM83H-overexpressing cells, CK-1α showed high values of the Pearson's correlation coefficient with FAM83H [$r=0.78\pm0.20$ ($n=3$ cell images)] and keratin 18 [$r=0.62\pm0.10$ ($n=6$ cell images)]. Coimmunoprecipitation assays using anti-keratin 18 antibody further showed that FAM83H knockdown abolished the physical interaction of CK-1α with keratin 18 (Fig. 4C). Neither knockdown nor inhibition of CK-1α affected the localization of FAM83H to keratins (Fig. 3C,D), excluding the possibility that CK-1α recruits FAM83H to keratins. These results indicate that FAM83H recruits CK-1α to keratin filaments to enable CK-1α to modulate the filamentous state of keratins.

FAM83H is a linker protein between CK-1α and keratins

To further elucidate the mechanism governing FAM83H-dependent rearrangement of keratin filaments, we performed proteomic analysis using dominant-negative truncated proteins of FAM83H (Lee et al., 2008; Urzúa et al., 2011; Wright et al.,

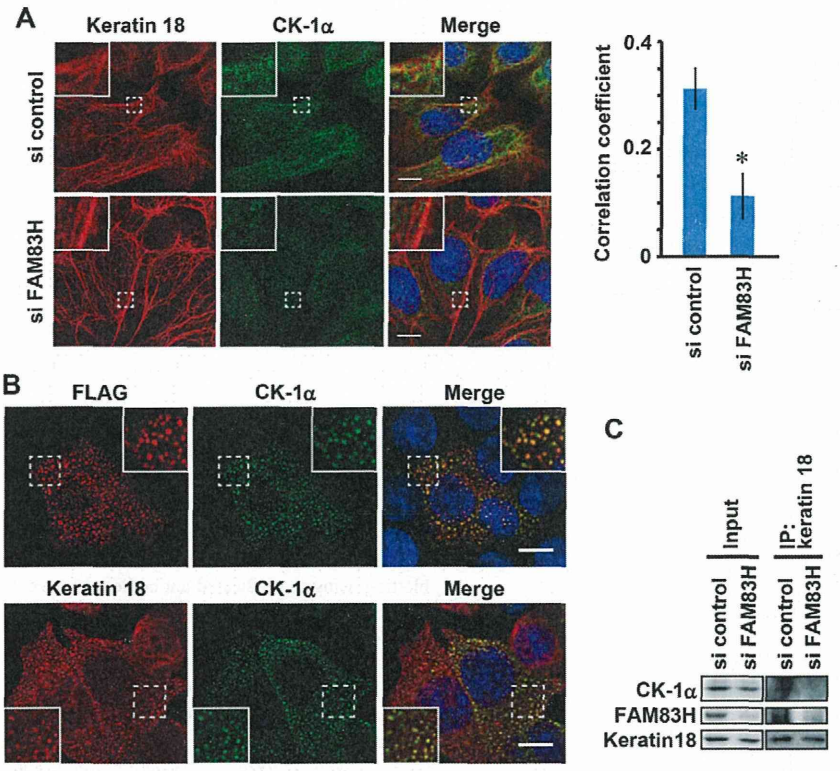

Fig. 4. FAM83H recruits CK-1α to keratin filaments. (A) DLD1 cells were transfected with FAM83H siRNA or control siRNA for 2 days and analyzed by immunofluorescence. Colocalization of CK-1α and keratin 18 was evaluated by the Pearson's correlation coefficient. Quantification data are presented as the mean ± s.d. calculated from at least ten cell images. Asterisk indicates a significant difference from the control (two-tailed Student's *t*-test, $*P<0.0001$). (B) HCT116 cells were transfected with FAM83H–FLAG for 24 hours and analyzed by immunofluorescence. DNA is stained with DAPI (blue). Insets show magnified images in the regions enclosed by dotted lines (A,B). Scale bars: 10 μm (A,B). (C) DLD1 cells transfected with FAM83H siRNA or control siRNA for 2 days were analyzed by coimmunoprecipitation assay using anti-keratin-18 antibody. Immunoprecipitates were examined by western blotting.

2009). Similar to FAM83H knockdown, transfection with a plasmid encoding FAM83H-truncated protein with a FLAG-tag [amino acids 1–286, FAM83H-286N–FLAG; 1–296, FAM83H-296N–FLAG] induced the aggregation of severely bundled keratin filaments in HCT116 cells (Fig. 5A and data not shown). LC-MS/MS analysis of coimmunoprecipitated proteins with FAM83H-286N–FLAG and FAM83H–FLAG (full-length) showed that FAM83H-286N–FLAG immunoprecipitates contained higher levels of CK-1α but lower levels of keratins than FAM83H–FLAG immunoprecipitates (supplementary material Table S2). Western blotting of these immunoprecipitates substantiated strong interactions of FAM83H-286N–FLAG with CK-1α but only marginal interaction with keratin 8 (Fig. 5B). These analyses suggest that FAM83H interacts with CK-1α in the N-terminal region and with keratins at the C-terminal region. Additionally, forced expression of FAM83H-286N–FLAG diminished the recruitment of CK-1α to keratin filaments (Fig. 5C). FAM83H-286N–FLAG significantly decreased the value of the Pearson's correlation coefficient between CK-1α and keratin filaments (Fig. 5C). FAM83H-286N–FLAG appeared to exert a dominant-negative effect on the recruitment of CK-1α to keratins. These results suggest that FAM83H tethers CK-1α to keratins and that a FAM83H-mediated interaction between CK-1α and keratins is needed for proper keratin cytoskeleton organization.

A CK-1α binding motif of FAM83H

FAM83H has FxxxF amino acid sequences (x, any amino acid residue), known to be CK-1-binding motifs of NFAT1, PER1 and PER2 (Okamura et al., 2004), in the N-terminal region (FMWSF, 247–251 aa; FDEEFRILF, 270–278 aa). To assess the requirement of these amino acid sequences for the binding of FAM83H to CK-1α, a coimmunoprecipitation assay using alanine mutants of FAM83H (251A, FMWSA; 274A, FDEEARILF) was performed. The FLAG-tagged FAM83H alanine mutants were transfected into HCT116 cells and immunoprecipitated using anti-FLAG antibody. Compared with wild-type FAM83H and the 251A mutant, immunoprecipitates of the 274A mutant contained very low levels of CK-1α (Fig. 6A), suggesting that the FDEEFRILF sequence is the major motif of FAM83H for CK-1α binding.

We further tested whether the 274A mutation disabled the FAM83H function to control keratin filament organization. HCT116 cells were transfected with FAM83H-274A or FAM83H-251A and analyzed by immunofluorescence of keratin filaments. Overexpression of the 251A mutant, as well as wild-type FAM83H, caused disassembly of keratin filaments, whereas the 274A mutant did not affect keratin filament organization (Fig. 6B). In addition, speckle-like localization of CK-1α was not induced by overexpression of FAM83H-274A, in contrast to wild-type FAM83H or FAM83H-251A (Fig. 6C). Taken together, these results substantiate that interaction of FAM83H with CK-1α is required for keratin filament reorganization.

Disassembly of keratin filaments by overexpression of FAM83H in human colorectal cancer tissue

To examine the expression of FAM83H in colorectal cancer cells *in vivo*, colorectal cancer tissues were analyzed by quantitative PCR (qPCR) and immunostaining with anti-FAM83H antibody. *FAM83H* mRNA levels were elevated in 10 of 12 colorectal cancer tissues compared with adjacent non-tumor tissues (Fig. 7A). Increased expression of FAM83H protein was also observed in 75 of 111 specimens, as assessed by the intensity of FAM83H immunostaining in cancer cells and adjacent normal