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New tumor suppressor CXXC finger protein 4 inactivates mitogen activated protein kinase signaling

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ABSTRACT

As a well-characterized master player in epigenetic regulatory network, EZH2 is widely implicated in the development of many malignancies. We previously found that EZH2 promoted Wnt/β-catenin activation through downregulation of CXXC4 expression. In this report, we demonstrated that CXXC4 inhibited MAPK signaling through binding to ERK-1/2 and abrogating the interaction of ERK 1/2 with MEK1/2. L183, the critical residue in CXXC4 ERK D domain, was found to be essential for CXXC4–ERK 1/2 interaction and the growth inhibitory effect of CXXC4 in human cancer cells. In summary, CXXC4 directly disrupted MEK1/2–ERK 1/2 interaction to inactivate MAPK signaling. L183 site is indispensable for the binding of CXXC4 to ERK1/2 and growth inhibitory effect of CXXC4. Therefore, EZH2 can activate MAPK signaling by inhibiting CXXC4 expression.

1. Introduction

Gastric cancer is one of the most common malignancies and the second leading cause for cancer-related mortality worldwide [1,2]. Genetic and epigenetic alterations have long been considered as two essential mechanisms during the multi-step processes of carcinogenesis including gastric cancer development, such as oncogenic activation, inactivation of tumor suppressor genes and dysregulation of many signaling pathways important to cell proliferation, differentiation, cell cycle and cell fate decision [3].

The mitogen-activated protein kinase (MAPK) signaling pathway is composed of several key signaling cascades and phosphorylation events pivotal to tumorigenesis. Among them, the RAS–RAF–MAP kinase (MEK)–extracellular signal-regulated kinase (ERK)–MAPK (RAS–MAPK) cascade is one of the most frequently deregulated signaling pathways in human cancers [3]. Like many other signaling pathways, MAPK signaling is initially triggered by the ligation of receptor tyrosine kinases (RTKs) with cognate growth factors [4]. Thus, the aberrant activation of Ras/MAPK pathway could attribute to overexpression of RTKs such as Her-2 in addition to gain-of-function mutations in Ras or Raf genes. Recently, some epigenetic changes have been found to contribute to the aberrant activation of Ras/MAPK signaling in addition to genetic changes [5–8].

Enhancer of zeste homologue 2 (EZH2) is the core catalytic subunit of Polycomb Repressive Complex 2 (PRC2) and an important player in the epigenetic regulatory network. It is a highly conserved histone methyltransferase (HMTase) which functions to catalyze the lysine-27 trimethylation of histone H3 (H3K27me3) and repress the transcription of genes closely associated with cell senescence, differentiation, apoptosis and cancer development [9–11]. Importantly, overexpression of EZH2 is widely implicated in many human malignancies including breast cancer, prostate cancer and gastric cancer [12–14]. Our previous study has demonstrated that CXXC4 is a novel potential tumor suppressor directly regulated by EZH2 [15]. EZH2 promoted the activation of Wnt signaling in gastric carcinogenesis through the downregulation of CXXC4 expression. In the present study, we found that CXXC4 inactivated Ras/MAPK signaling by directly binding to ERK-1/2 to disrupt MEK1/2-ERK1/2 association.

2. Materials and methods

2.1. Cell lines and antibodies

Human gastric epithelial cell line GES-1 and human cancer cell lines MKN28 (gastric cancer cell line), SGC7901 (gastric cancer cell

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line) and HEK293 cells (Human Embryonic Kidney 293 cells) were cultured in RPMI 1640 medium or DMEM (Life technologies, Carsbad, CA, USA) supplemented with 10% fetal bovine serum and incubated at 5% CO_2, 37 °C and 95% humidity as previously described [6]. The immortalized but non-tumorigenic GES-1 cell line was established from the fetal stomach cells infected with SV40 virus [16]. Antibodies to Myc-Tag, EZH2, phosphorylated-ERK1/2, ERK1/2, phosphorylated-Elk-1, SAPK/JNK, phosphorylated-MEK1/2 and MEK1/2 were purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies to GAPDH were obtained from Epitomics (Burlingame, CA, USA).

2.2. Plasmid construction and transfection

The open reading frame (ORF) of human CXXC4 was cloned into pCMV-3Tag-7 using BamHI and XhoI restriction sites [15]. Three CXXC4 L183A mutants were constructed using QuikChange Site-Directed Mutagenesis Kit (Agilent, La Jolla, CA, USA). Primers used are listed in Table 1. X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) were used for plasmid transfection.

2.3. SiRNAs and transfection

SiRNAs for EZH2, CXXC4 and β-catenin were synthesized by GenePharma (Shanghai, China). The sequences of siRNAs are listed in Table 1. Cells were transfected with siRNA duplexes (10 nM) using Lipofectamine™ RNAiMAX transfection reagent (Life Technologies, USA), according to the manufacturer's instructions.

2.4. Western blotting analysis

Cells were scraped and lysed in Cytobuster™ Protein Extraction Reagent (Novagen, Darmstadt, Germany) and protein concentrations were determined by Bio-Rad protein assay kit II (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of cellular protein were resolved by SDS–PAGE and transferred to PVDF membrane. Proteins of interest were detected as previously described [17].

2.5. Immunoprecipitation

Cells were lysed in 1 ml of lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 20 mg of leupeptin/ml, 20 mg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 10% glycerol). Primary antibodies (normally 1–2 μg) were incubated with the pre-cleared cell lysates overnight at 4 °C. The immunocomplexes were precipitated by 30 μl Pure Proteome™ Protein G Magnetic Beads [18].

2.6. Cell viability assay

Cell viability assay was performed using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). Cell cycle distribution was determined by flow cytometry analysis of PI (propidium iodide) staining cells.

Table 1
Primers or siRNAs used in this study.

Names	Sequence
Primers	
CXXC4-L183A-F	AAAGAAAAAACCTGGCACTTCAGCAGAGAGAACACCTGTTCC
CXXC4-L183A-R	GGAACAGGTGTTCTCTCTGCTGAAGTGCCAGGTTTTTTCTTT
siRNAs	
CTNNB1	GGACACAGCAGCAAUUUGUTT
	ACAAAUUGCUGCUGUGUCCTT
EZH2	CCAUGUUUACAACUAUCAATT
	UUGAUAGUUGUAAACAUGGTT
CXXC4	CACAGACAGUGCGUUUCAATT
	UUGAAACGCACUGUCUGUGTT

2.7. Statistical analysis

Unless specifically indicated, the Student's *t* test or non-parametric Mann–Whiney test was used for a comparison between two groups. All statistical analyses were performed using GraphPad Prism software (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Potential implication of CXXC4 in MAPK signaling

Our previous study has demonstrated that CXXC4, a novel target of EZH2, serves as a tumor suppressor gene to inhibit Wnt signaling pathway in human gastric carcinogenesis [15]. To explore whether CXXC4 exerts its tumor suppressive effects through Wnt-independent signaling pathways, we analyzed CXXC4 protein structure and found a ERK D domain located adjacent to Dvl-interaction motif (KIXXXΦ motif) (http://scansite.mit.edu/) (Fig. 1A). This ERK D domain was conserved among CXXC4 from various species and other well-known ERK interaction partners such as MEK1 and Elk1 (right panel, Fig. 1A), suggesting that CXXC4 may function as an anchor of ERK-1/2. Indeed, co-immunoprecipitation results confirmed that CXXC4 interacted with ERK 1/2 in vivo (Fig. 1B). Since leucine (L183) was suggested to be the critical amino acid residue in the ERK D domain (http://scansite.mit.edu/) [19], we wondered whether the mutation of this residue could affect the interaction of CXXC4 with ERK 1/2. As shown in Fig. 1B, L183A mutant of CXXC4 failed to interact with ERK 1/2, highlighting the ERK D domain was indeed responsible for the interaction of CXXC4 with ERK 1/2. If this is true, the ERK CD domain mutant defective to bind the ERK D domain such as sevenmaker mutation should disrupt its interaction with CXXC4 [20,21]. Indeed, CXXC4 can only bind the wild type ERK 2 but not D319N mutant (Fig. 1C).

3.2. CXXC4 inhibits MAPK signaling independent of Wnt/β-catenin signaling

Taken together, these findings indicate that CXXC4 might have a role in the regulation of MAPK signaling. In consistence with such assumption, the wild-type CXXC4 but not L183A mutant suppressed the phosphorylation of ERK 1/2 in two human cancer cell lines (Fig. 2A and B). In contrast, phosphorylation of ERK-1/2 was increased after CXXC4 depletion (Fig. 2C and D). Moreover, in contrast to the wild-type CXXC4, L183A mutant failed to inhibit the viability of human cancer cells (Fig. 2E), indicating that CXXC4 can inactivate ERK-1/2 signaling to inhibit cell viability. Importantly, CXXC4 siRNA induced phosphorylation of ERK-1/2 was not attenuated by knockdown of β-catenin (Fig. 2F and G), further indicating that CXXC4 can inhibit ERK 1/2 signaling independent of Wnt/β-catenin signaling.

3.3. CXXC4 inhibits MAPK signaling by directly disrupting the interaction of MEK 1/2 with ERK 1/2

To further investigate how CXXC4 affects the phosphorylation of ERK 1/2, we analyzed the state of ERK 1/2 phosphorylation at different time-points after serum stimulation. Wild-type CXXC4 but not L183A mutant inhibited serum-induced ERK 1/2 phosphorylation (Fig. 3A), raising the possibility that CXXC4 functions to inhibit ERK 1/2 activation. However, neither wild-type CXXC4 overexpression (Fig. 3B) nor CXXC4 knockdown (Fig. 3C) affected

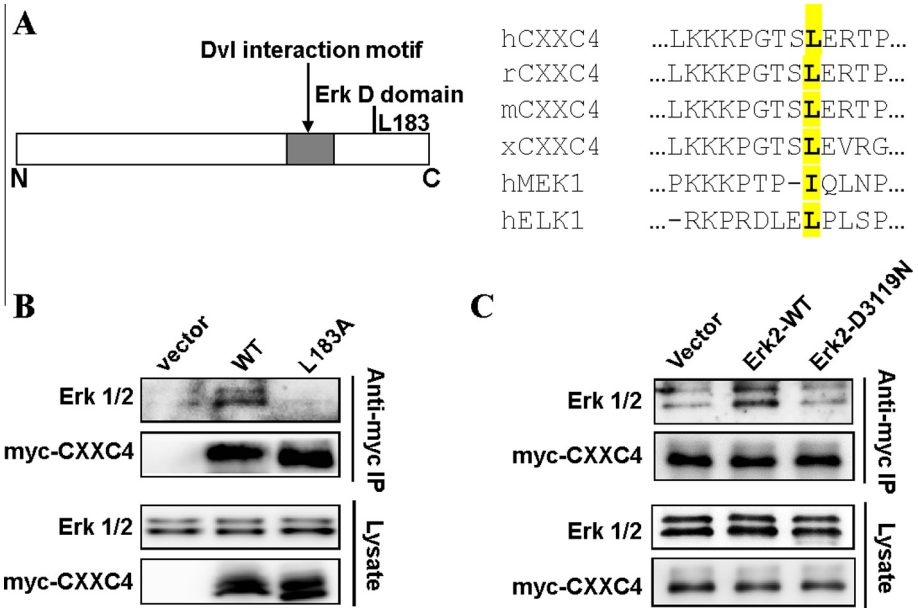

Fig. 1. Potential implication of CXXC4 in ERK signaling. (A) The structure of CXXC4 protein. The Dvl-interaction motif (KIXXXΦ motif) locates in its C-terminus and ERK Docking domain (D domain) locates adjacent to Dvl-interaction motif. The right panel shows the alignment of ERK D domain in CXXC4 from different species and well-known ERK-interacting proteins such as MEK1 and Elk1. (B) The interaction of wild type CXXC4 and L183A with ERK 1/2 were analyzed by co-immunoprecipitation. (C) The interaction of wild type CXXC4 with wild type ERK 1/2 or ERK1/2 CD mutant were analyzed by co-immunoprecipitation.

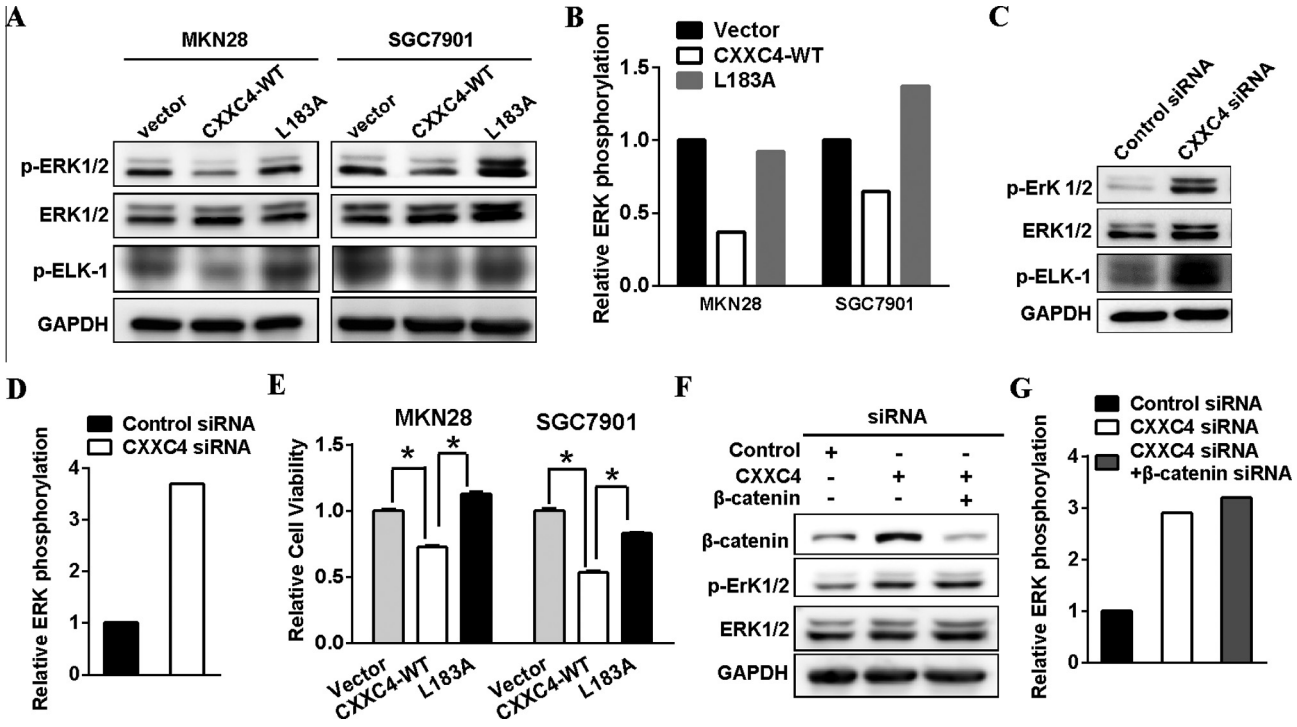

Fig. 2. CXXC4 inhibits MAPK signaling independent of Wnt/β-catenin signaling. (A) The effect of wild-type CXXC4 or L183A mutant on ERK 1/2 phosphorylation in MKN28 and SGC7901 cells were determined by Western blotting. The quantification of relative phosphorylation ERK 1/2 (phosphorylated ERK 1/2/total ERK 1/2) was shown in (B). (C) ERK 1/2 phosphorylation in GES-1 cells before and after CXXC4 depletion were determined by Western blotting. The quantification of relative phosphorylation ERK 1/2 were shown in (D). (E) Relative cell viability of MKN28 and SGC7901 cells overexpressed with wild-type CXXC4 or L183A mutant were determined by MTS assay. (F) The amount of β-catenin or phosphorylated ERK 1/2 in GES-1 cells before and after CXXC4 or β-catenin depletion were determined by Western blotting. The quantification of relative phosphorylation ERK 1/2 were shown in (G).

MEK 1/2 phosphorylation, indicating that CXXC4 may abrogate the activation of ERK 1/2 by MEK 1/2. Indeed, CXXC4 depletion promoted their association (Fig. 4D) while overexpression of CXXC4 disrupted the interaction of ERK 1/2 with MEK 1/2 (Fig. 4E).

3.4. EZH2 activates MAPK signaling through downregulating CXXC4 expression

Since CXXC4 was recently identified as a new target of EZH2 [15], we wondered whether EZH2 activated MAPK signaling

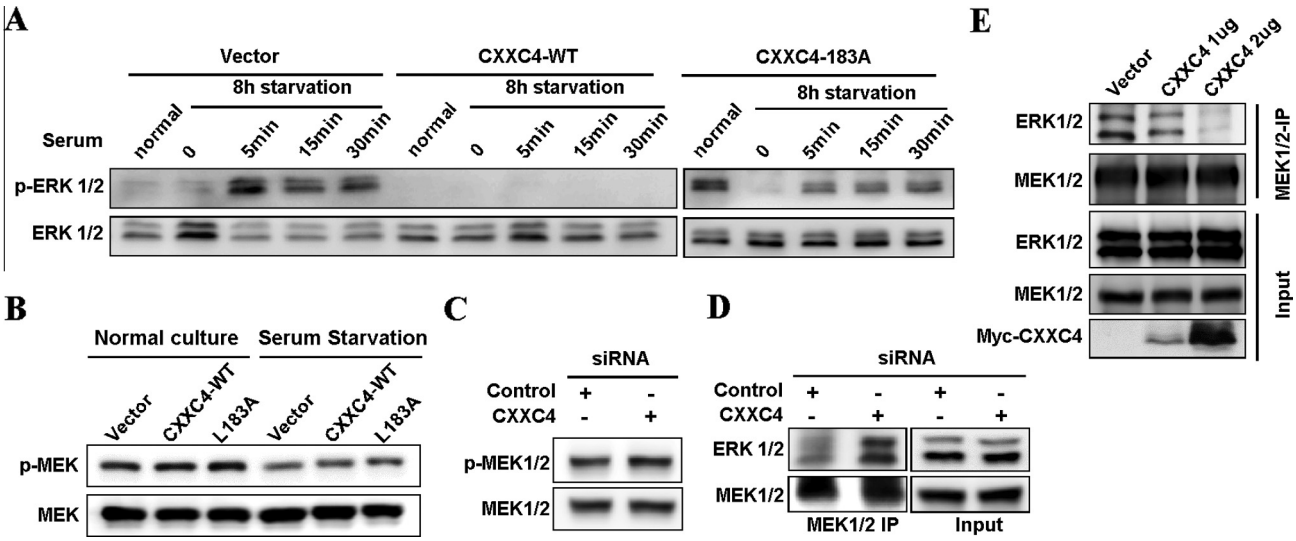

Fig. 3. CXXC4 inhibits ERK signaling by disrupting the interaction of ERK 1/2 with MEK 1/2. (A) ERK 1/2 phosphorylation before and after wild-type CXXC4 or mutated L183A overexpression were determined by Western blotting. Times mean minutes after serum stimulation. (B) The effect of wild-type CXXC4 or L183A mutant on MEK 1/2 phosphorylation in MKN28 cells cultured with or without serum were determined by Western blotting. (C) MEK1/2 phosphorylation before and after CXXC4 depletion in GES-1 cells was examined by Western blotting. (D) The interaction of MEK 1/2 with ERK 1/2 in the presence or absence of CXXC4 were analyzed by co-immunoprecipitation. (E) The interaction of MEK 1/2 with ERK 1/2 in the presence of various amounts of CXXC4 were analyzed by co-immunoprecipitation.

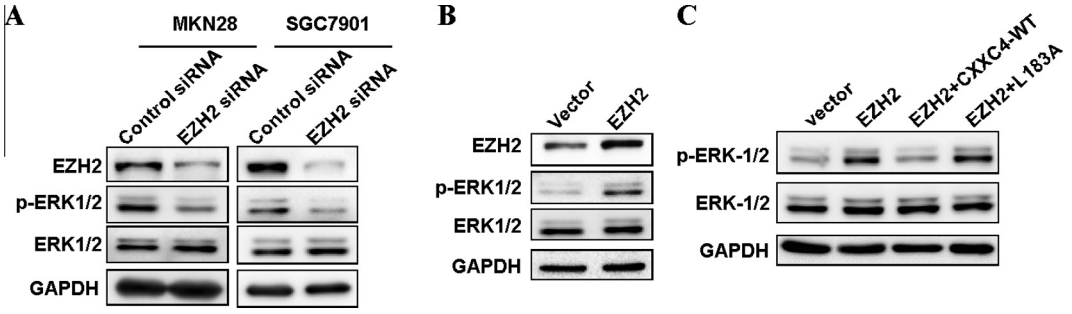

Fig. 4. EZH2 activates ERK signaling through downregulating CXXC4 expression. (A) The effect of EZH2 depletion on the phosphorylation of ERK 1/2 in MKN28 and SGC7901 cells were analyzed by Western blotting. (B) ERK 1/2 phosphorylation in GES-1 cells with ectopic EZH2 expression were determined by Western blotting. (C) The effect of ectopic EZH2 expression in the presence or absence of CXXC4 on the phosphorylation of ERK 1/2 was explored by Western blotting.

through inhibiting the expression of CXXC4. Indeed, EZH2 depletion led to decreased activation of ERK 1/2 in human cancer cells with high EZH2 expression (Fig. 4A). Consistently, EZH2 expression promoted ERK 1/2 phosphorylation in cells with low EZH2 expression (Fig. 4B). Furthermore, EZH2-increased ERK-1/2 phosphorylation were attenuated by wild type CXXC4 but not L183A mutant (Fig. 4C). These results suggest that EZH2 can promote the activation of ERK/MAPK signaling through downregulation of CXXC4 expression.

4. Discussion

As the first oncogene isolated from human carcinoma tissues, Ras gene achieved its notoriety by activating oncogenic MAPK signaling in most of human cancer cells. However, oncogenic point mutations were identified in less than 30% human cancer, indicating that alternative mechanisms might be responsible for the activation of Ras/MAPK signaling in the majority of human cancers. Indeed, epigenetic changes such as epigenetic downregulation of RASAL or microRNA-204 downregulation contribute to the aberrant activation of Ras/MAPK signaling [5–8]. In this study, we reported a new epigenetic regulation of Ras/MAPK in human cancers. EZH2, the master regulator of chromatin modification, promoted the activation of Ras/MAPK signaling through downregulating a newly characterized tumor suppressor gene CXXC4 [15].

CXXC4 was firstly identified as a negative regulator of Wnt signaling in renal cell carcinoma (RCC) [22,23]. Our previous study has clarified the underlying molecular mechanism of CXXC4-mediated inhibition of Wnt signaling [15]. CXXC4 attenuated Wnt/β-catenin signaling by competitively binding to the PDZ domain of Dvl and stabilizing the β-catenin destruction complex to promote the degradation of β-catenin. In addition, CXXC4 inhibited tumor growth both in vitro and in vivo. However, it remains to further explore whether CXXC4 exerts its tumor suppressive effects by regulating Wnt-independent oncogenic signalings. In this study, we discovered a novel function of CXXC4 through structural prediction and biochemical analyses. CXXC4 can directly interact with ERK 1/2 to abrogate ERK 1/2-MEK 1/2 association. The ERK D domain in C terminus of CXXC4 is critical to this function since L183A mutant unable to bind to ERK 1/2 lost its ability to inhibit the activation of ERK 1/2. In consistence with our findings, the planar cell polarity pathway including RhoA and Rac that function downstream of Ras/MAPK can be activated by the downregulation of CXXC4 [22].

MAPK pathways consist of a widely and evolutionarily conserved family of serine/threonine protein kinases essential for con-

verting extracellular stimuli into a series of important cellular responses [24]. Although we have demonstrated the potential influence of CXXC4 in MAPK inactivation through disturbing MEK 1/2–ERK 1/2 association directly, we cannot exclude the possibilities that CXXC4 mediates MAPK inactivation through other mechanisms. For example, several recent reports have revealed that the C-terminal region of Idax shares homology with a putative MAPK and NF-κB activating protein (Q8TB79) containing the KTXXI motif that is necessary for Idax–Dvl binding [25,26]. In addition, CXXC domain might play an essential role in modulating DNA methylation status. CXXC domain containing proteins such as TET1 (tet methylcytosine dioxygenase 1) can demethylate genomic DNA by oxidizing 5-methylcytosine [27–30]. The CXXC domain in TET1 was important to its binding to CpG-rich DNA sequences [31]. However, CXXC4 might be involved in DNA demethylation indirectly through its interaction TET2 that has dioxygenase activity but lacks CXXC domain [32].

In conclusion, our findings firstly demonstrate that EZH2 activates MAPK signaling to promote gastric carcinogenesis by suppressing CXXC4 expression. CXXC4 directly disrupts MEK 1/2–ERK 1/2 interaction to mediate MAPK inactivation and L183 in CXXC4 ERK D domain is indispensable to the binding of ERK 1/2 and inhibit gastric cancer cell proliferation. These findings may provide promising insights into developing novel cancer therapies by targeting CXXC4 to obstruct MAPK cascade activation.

Author contributions

X.W., H.L. and H.J. conceived and designed the experiments. H.L., W.J., J.S., L.F., H.L., Q.S., Y.M., F.W., J.L. and Y.Y. performed experiments. H.L., X.W. and H.J. analyzed data and wrote the manuscript.

Competing interests

We have no any conflict of interest to claim.

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