Doctoral Thesis

博士論文

The Functional Analysis of a novel ERK Substrate, MCRIP1

（新規ERK 基質分子MCRIP1 の生理機能解析）

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The Functional Analysis of a novel ERK Substrate, MCRIP1

新規 ERK 基質分子 MCRIP1 の生理機能解析

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1. Abstract

In previous study, Ichikawa *et al.* successfully identified a novel ERK substrate, MCRIP1 (MAPK-regulated Co-Repressor Interacting Protein 1), using a unique yeast-three-hybrid screening system. By analyzing its molecular function, the study suggests that MCRIP1 mediates crosstalk between ERK signaling and the transcriptional co-repressor CtBP, thereby promoting ERK-induced epigenetic silencing of E-cadherin and Epithelial-Mesenchymal Transition (EMT)[1]. However, the physiological function of MCRIP1 remained to be investigated. In this study, , I established MCRIP1-knockout (MCRIP1-KO) mouse in order to uncover the physiological role of MCRIP1 *in vivo*. From the phenotype analysis, MCRIP1-deficient (MCRIP1-KO) mice showed significantly decreased survival rate and low body weight when compared to those of MCRIP1-wildtype (MCRIP1-WT) mice. Therefore, the aim of this study is to identify the cause of observed phenotypes and the underlying mechanism.

By monitoring a pool of MCRIP1 neonates immediately after birth, I found that a large number of MCRIP1-KO neonates died within 24 hours after birth due to respiratory failure from observation. Histological analyses also showed defect in lung inflation in MCRIP1-KO mice lungs. To further investigate the cause of this phenotype, I isolated the lung tissues from both MCRIP1-WT and MCRIP1-KO neonates and examined the expressions of surfactant proteins that are essential for normal respiratory functions. As a result, both protein and mRNA expressions of surfactant protein B (SP-B) and C (SP-C) were significantly decreased in MCRIP1-KO lungs.

Alveolar epithelial cells, or AECs, are responsible for the secretion of surfactant proteins. Therefore, I investigated whether MCRIP1 regulates the differentiation of AECs by examining the differentiation marker expressions. However, the expression levels of differentiation markers, Muc1 and Abca3, were similar between MCRIP1-WT and MCRIP1-KO mice. These results indicated that MCRIP1 regulates the transcription activity of SP-B and SP-C, but not the differentiation of AECs. Next, to investigate the underlying molecular mechanism, I focused on CtBP-interacting transcription repressors Foxp1/2 that regulate the transcriptional activities of SP-B and SP-C. The results from co-immunoprecipitation experiments demonstrated that MCRIP1 competitively inhibited CtBP-FOXP1/2 interaction. Furthermore, SP-B promoter activity suppressed by CtBP was rescued by MCRIP1. Overall, a novel physiological function of MCRIP1 in lung development and the underlying mechanism was uncovered.

2. Introduction

2-1. Signal Transduction

Cell communication is essential for organisms to coordinate their behaviors. In single cell organisms, there is simple machinery that transmits signals between one cell and another so they can adapt to various conditional changes. In multicellular organisms, the communication machinery has developed into a more elaborated signal transduction system to ensure that signals are emitted and received properly. In general, the cells in multicellular animals initiate signal transductions through various kinds of signaling molecules that could be in forms of proteins, small peptides, amino acids, nucleotides or dissolved gases. The signaling cells either release or present the signaling molecules into the extracellular space, then the targeting cells with specific receptor proteins can respond to the signals through intercellular signaling. Depending on the purpose, there are mainly four different types of intercellular signaling systems: contact-dependent, paracrine signaling, synaptic signaling for neurons and endocrine signaling for hormones (Fig.1). Despite the differences between various intercellular signaling ways, the types of receptor proteins embedded in the plasma membrane of the target cells play critical roles on the activation of intracellular signal transduction[2,3].

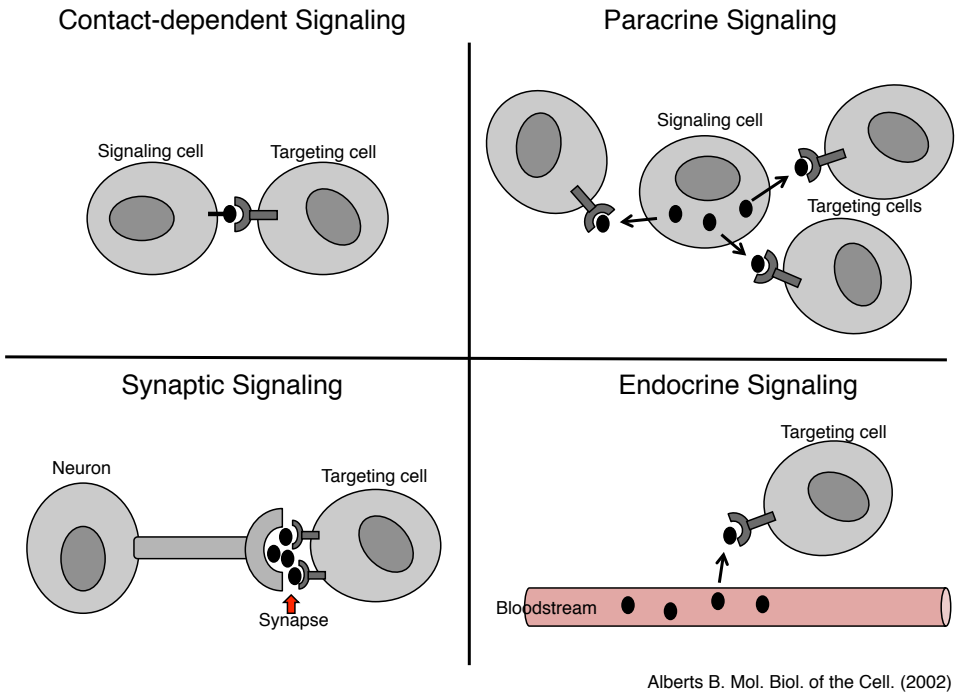


Fig.1 Four types of Intercellular Signaling

There are three large classes of cell-surface receptor proteins that respond to different types of signals, ion-channel-coupled, G-protein-coupled, and enzyme-coupled receptors. Of all, enzyme-coupled receptors, especially protein kinase-coupled ones, are involved in adverse variety of intracellular signaling pathways.

There are two major groups of protein kinases, tyrosine protein kinase and serine/threonine protein kinase. Tyrosine protein kinase includes a group of receptors, such as EGFR or ErbB2, that function as receptors for growth factors, cytokines, or hormones. Whereas serine/threonine protein kinases, such as MAPK or CDK, often contributes to the relay of the signal, thereby stimulating a cell response (Fig. 2)[3,4].

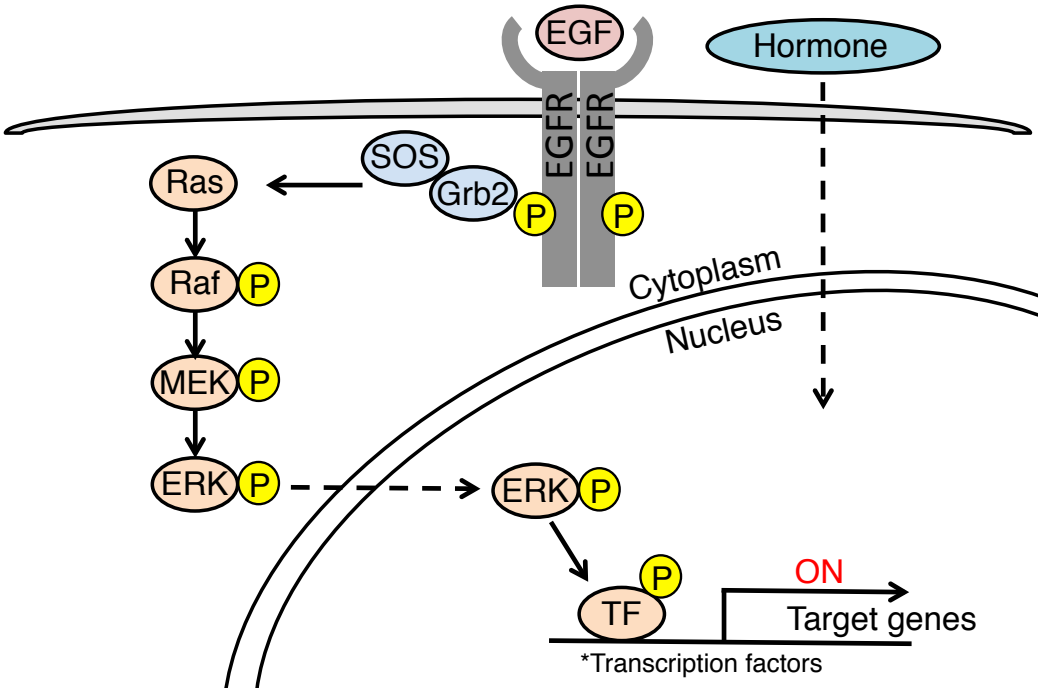


Fig.2 Examples of Intracellular Signal Transduction

2-2. MAP kinase cascades

Mitogen-activated protein kinases (MAPK) pathways are signaling transduction modules that convert a wide range of extracellular stimuli into intracellular signaling in eukaryotic cells, resulting in various biological outcomes. There are at least three main MAPKs in mammals, ERK, p38, and JNK (c-Jun amino N-terminal kinases). They can be further divided into two groups: mitogen-activated pathway (MAPK) and stress-activated pathway (SAPK). Whereas p38 and JNK SAPKs are involved in stress-activated pathways that regulate cell death and inflammation, ERK is mainly involved in mitogen-activated pathway that contributes to cell proliferation and differentiation[5–7].

MAPK pathways are consisted of three tiers of protein kinases, MAPKKK, MAPKK, and MAPK. Firstly, MAPKKKs are activated in response to extracellular stimuli, such as growth factors or various environmental stresses. MAPKKK activation leads to the activation of MAPKK and MAPK through phosphorylation. Activated MAPKs then phosphorylate their substrates to regulate downstream gene expressions (Fig. 3)[7,8].

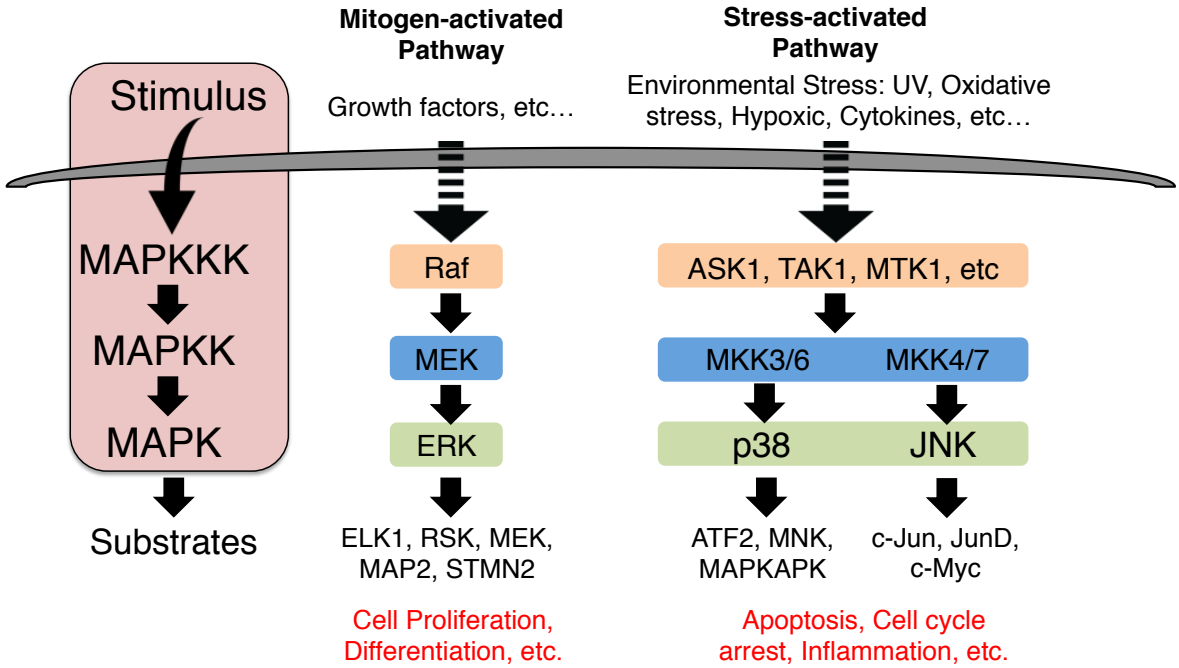


Figure 3. MAPK Pathways

2-3. ERK MAPK pathway

ERK (Extracellular signal-regulated kinase) MAPK pathway is activated by mitogen stimuli, such as growth factors. Similar to p38 and JNK MAPK pathways, ERK MAPK pathway is also consisted of three-tiered protein kinases: Raf MAPKKK, MEK MAPKK, and ERK MAPK. The activation of ERK MAPK pathway contributes to a number of cellular processes, such as cell proliferation, differentiation, metabolism and cell migration.

This signaling pathway usually begins with receptor tyrosine kinases (RTKs) that function as receptors for various growth factors (EGFR, FGFR, PDGFR, and etc.). The activated RTKs undergo trans-autophosphorylation, thereby promoting the activation of Ras small GTPase through Grb2/Sos complex. Activated Ras then interacts with and activates its effector, Raf MAPKKK, followed by the sequential activation of MEK MAPKK and ERK MAPK.

It is known that ERK MAPK activation promotes the phosphorylation of its substrates in the cytoplasm and the nucleus. By phosphorylating ERK substrates, ERK positively regulates the expression of genes involved in cell cycle, proliferation, and differentiation. In addition, it is reported that active ERK negatively regulates genes that contributes to apoptosis or cell cycle inhibition[9–11].

2-4. ERK MAPK

ERK1 and ERK2 are homolog of the protein kinase that acts as the core member of Ras-Raf-MEK-ERK signal transduction pathway. ERK1 is composed of 379 amino acids, and ERK2 is 360 amino acids. In humans, ERK1 and ERK2 are highly similar in sequence and structure with 84% homology (Fig. 4). In addition to their similar structures, they also share many, but not all, functions as both ERK1/2 contribute to positive regulation of cell proliferation and growth since most of the substrates are phosphorylated by both ERK1 and ERK2. Despite the similarity between ERK1 and ERK2, they also have distinct physiological functions according to knockout mouse analysis. It is reported that ERK1 knockout mice are viable and normal with only modest defect in thymocyte maturation. In contrast, ERK2 knockout mice are embryonic lethal due to defective mesoderm differentiation[5,12–14]. Additionally, it is also reported that ERK2 but not ERK1 induces epithelial-mesenchymal transition (EMT), a cellular process that is important in development as well as cancer metastasis[15].

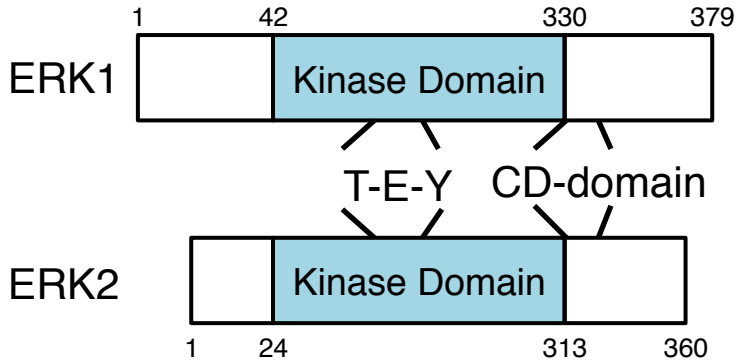


Figure 4. ERK1 & ERK2

2-5. Aberrant activation of ERK MAPK pathway in cancer

Since ERK MAPK pathway is involved in various physiological processes, ERK MAPK pathway must be properly regulated in order to maintain in a healthy state. Dysregulation of ERK MAPK pathway often leads to pathological phenomenon. In fact, aberrant activation of ERK MAPK pathway is frequently found in cancers. For example, Ras, the upstream component of ERK MAPK pathway, is often mutated to oncogenic forms in 25%-30% of human cancers. Not only Ras, Raf MAPKKK mutations that constitutively activate ERK MAPK pathway are also found in 80% of cancers[16,17]. Therefore, it is suggested that hyperactivation of ERK MAPK pathway contributes to the promotion of cancer progression.

2-6. ERK and Epithelial-Mesenchymal Transition (EMT)

Epithelial-Mesenchymal Transition (EMT) is a process by which epithelial cells lose their epithelial characteristics and transform into mesenchymal cells, which possess migratory and invasive nature (Fig. 5). In addition to the transformation of cell morphology, the loss of epithelial markers occurs while mesenchymal markers begin to express in cells (Table 1).

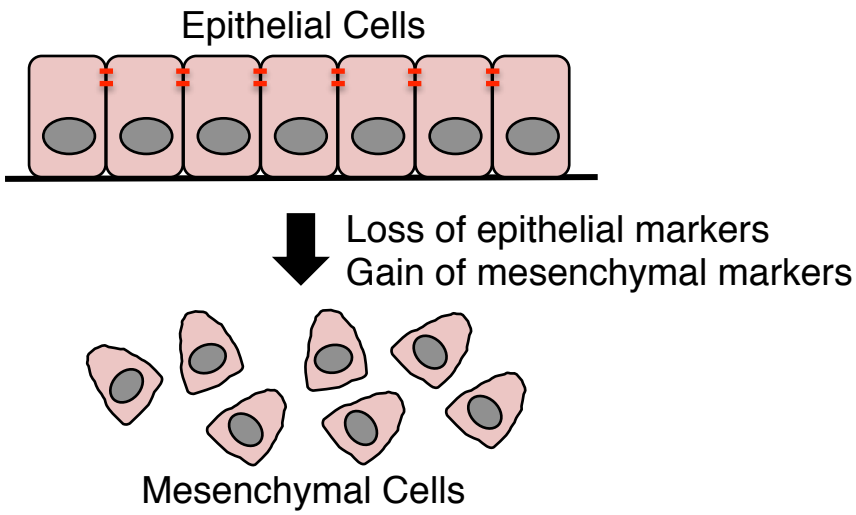


Fig.5 Epithelial-Mesenchymal Transition (EMT)

Physiologically, the process of EMT contributes to development, wound healing, as well as differentiation process, including transformation of cell properties. Pathologically, EMT also plays important roles in tissue fibrosis, tumor invasiveness,

and metastasis as tumor cells gain ability to invade and migrate[18–20].

EMT can be categorized into three different subtypes depending on the process involved. Type 1 EMT is involved in embryogenesis and organ development, type 2 EMT is associated with tissue regeneration and organ fibrosis, and type 3 contributes to the pathological states of cancer progression and metastasis[18].

Recently, evidences suggested that ERK pathway played a part in EMT induction. Also, ERK2 overexpression suppresses the expression of epithelial marker, E-cadherin, thereby inducing EMT in MCF10A human epithelial cells[15,21,22]. However, the molecular mechanism remained unclear.

Table.1 Epithelial & Mesenchymal Markers

Epithelial Markers	Mesenchymal Markers
E-cadherin	Fibronectin
Claudin	Vimentin
Desmoplakin	N-cadherin
ZO1	ZEB
	Twist

2-7. Identification of novel ERK substrate

In a previous study, Ichikawa *et al.* established a yeast-three hybrid system in order to screen for novel ERK substrates. First, The yeast-three hybrid system utilizes the polo-box domain (PBD) from PLK2 along with the LexA DNA-binding domain as the bait, in which the PBD of PLK2 is able to bind to phosphorylated Serine-Proline (SP) or Threonine-Proline (TP) sequences specifically[23,24]. In addition to the constructs of prey and bait, a constitutively active ERK2 is introduced into yeasts. In the case of potential substrates phosphorylated by ERK2, the polo-box domain would recognize and bind to the phosphorylated SP/TP, followed by the transcription of LexA in yeasts and the expression of His3 gene (Fig. 6). By using this uniquely designed system, MAPK-regulated Co-Repressor Interacting Protein 1 (MCRIP1) was successfully identified a novel ERK substrate.[1]

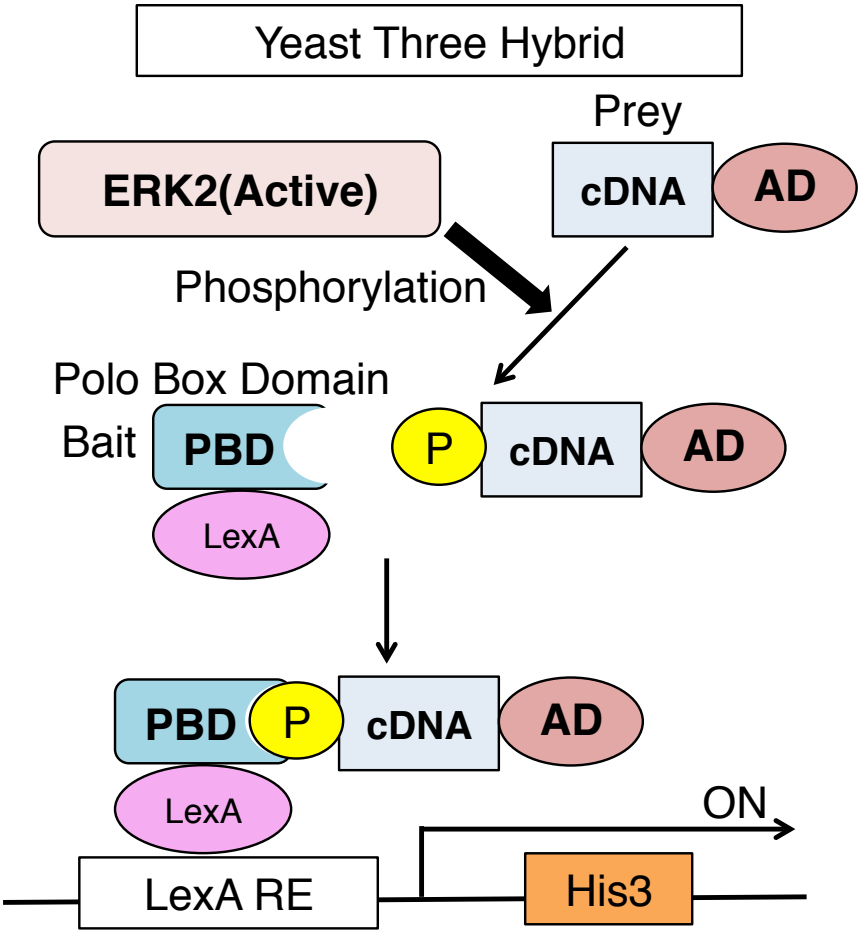


Fig.6 Yeast three hybrid screening system designed to identify novel ERK substrates

2-8. MAPK-regulated Co-Repressor Interacting Protein 1 (MCRIP1)

MCRIP1 (MAPK-regulated Co-Repressor Interacting Protein 1, or Fam195b) is consisted of 97 amino acids and highly conserved among vertebrates. Amino acid sequence analysis indicates that MCRIP1 has two phosphorylation sites and a CtBP-binding motif (PIDLS) all of which are evolutionarily conserved (Fig. 7a, Table 2). To confirm whether the predicted phosphorylation sites were truly phosphorylated, Ichikawa *et al.* co-expressed a constitutively active MEK1 (MEK1-DD) with wild type MCRIP1 or MCRIP1 phospho-mutants (S21A, T30A, and S21A/T30A) and detected the phosphorylation of MCRIP1 by ERK using anti-phospho-SP/TP antibody. When both S21 and T30 were substituted by alanine, the phosphorylation of MCRIP1 completely disappeared, suggesting that S21 and T30 are the phosphorylation sites of MCRIP1 by ERK (Fig. 7b).

In addition to 2 phospho-sites, MCRIP1 also has a potential CtBP-binding motif that is consisted of consensus amino sequence, PXDLS (Fig. 7, Table 2). Therefore, Ichikawa *et al.* next investigated whether MCRIP1 interacts with the transcriptional co-repressor CtBP by immunoprecipitation experiments. The results also confirmed that MCRIP1 interacts with CtBP1 endogenously (Fig. 7c). In addition, it is proven that mutants with abrogated PXDLS motif have lost their ability to interact with CtBP. These data demonstrated that PIDLS sequence in MCRIP1 is required for its interaction with CtBP (Fig. 8)[1].

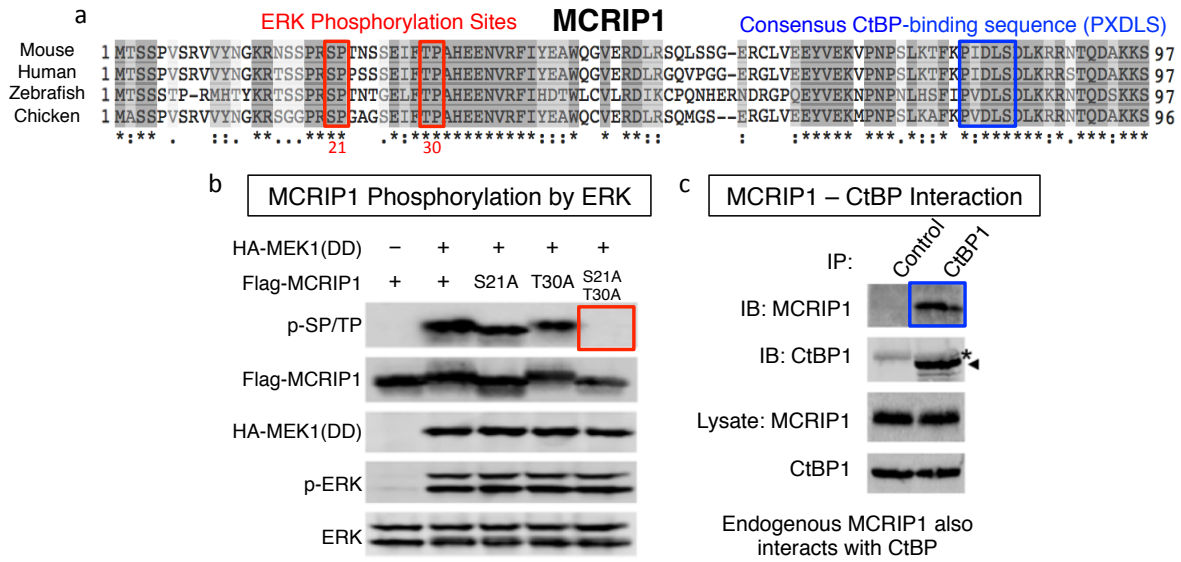


Fig.7 MCRIP1

(a) MCRIP1 amino acid sequence shows its ERK phosphorylation sites and CtBP-binding sequence are highly conserved in vertebrates.
(b) Western analysis using α-phospho-SP/TP antibody showed that he S21 and T30 are phosphorylated by ERK.
(c) Co-immunoprecipitation experiment showed that endogenous MCRIP1 binds to CtBP1.

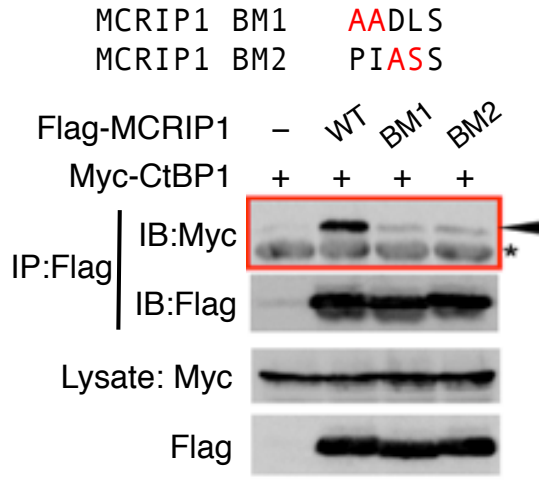


Fig.8 MCRIP1 interacts with CtBP via PIDLS motif

Immunoprecipitation experiments using MCRIP1-binding mutants, BM1 and BM2, indicates that PIDLS is required for MCRIP1-CtBP interaction.

2-9. Transcriptional co-repressor, C-terminal binding protein (CtBP)

C-terminal binding protein (CtBP) was firstly identified as a binding protein to the C-terminal region of adenovirus E1A protein[25]. It is highly conserved among plants, invertebrates, and vertebrates. In contrast to invertebrates and plants that have only one CtBP protein, vertebrates have different genes that encode two different CtBP proteins: CtBP1 and CtBP2[26]. CtBP1 and CtBP2 share similar structure and contain a NAD(H)-binding motif (Fig. 9). Evidences have also shown that CtBP1 and CtBP2 function mainly as transcriptional co-repressors through its interaction with target transcription factors that possess PXDLS motif (Table. 2)[27,28].

However, despite the shared functions, CtBP1 and CtBP2 also have distinct functions. For example, the unique N-terminal domain of CtBP2 possesses a nuclear localization sequence (NLS). It is suggested that the N-terminal domain controls the nuclear-cytoplasmic distribution of CtBP proteins[29]. Indeed, an *in vivo* study analyzing CtBP1 and CtBP2-knockout mice showed duplicative but unique roles in mouse embryo development. In comparison, CtBP1-null mice are small but viable, whereas CtBP2-null mice are embryonic lethal at E10.5. It is likely that defects in CtBP1-null mice were compensated by CtBP2 expression. However, CtBP1 cannot support the roles of CtBP2 in embryo development[30].

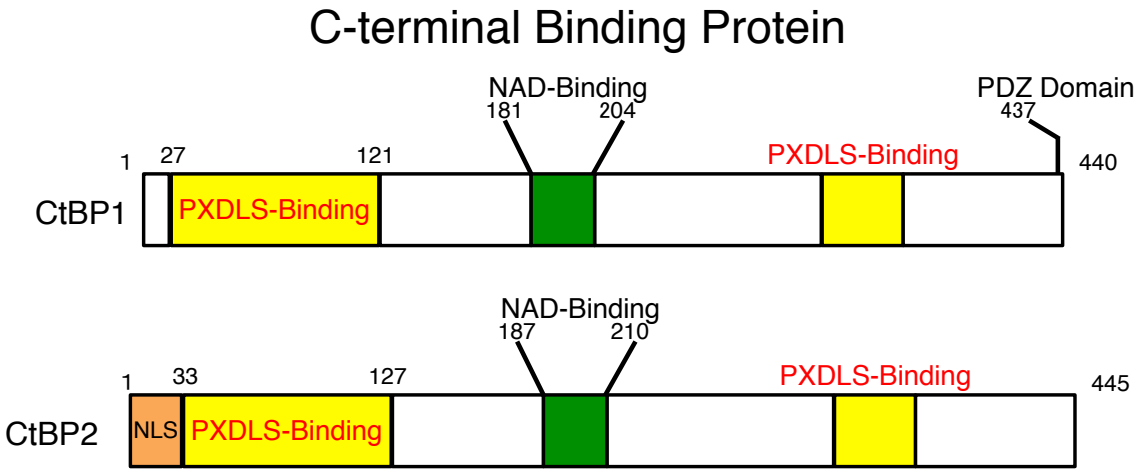


Fig.9 CtBP1 & CtBP2 structure

Table 2. Groups of CtBP-binding proteins and motif

Group	CtBP-binding protein	Motif							
		1	2	3	4	5	6	7	8
D(3)K(7)	Ad2/5 E1A	P	L	D	L	S	C	K	-
	RIP140	P	I	D	L	S	C	K	-
	Knirps	P	M	D	L	S	M	K	-
	Snail	P	Q	D	L	S	L	K	-
	Consensus	P	X	D	L	S	X	K	-
D(3)R(7)	CtIP	P	L	D	L	S	D	R	-
	Ad7	P	L	D	L	S	T	R	-
	Consensus	P	X	D	L	S	X	R	-
S(3)K(7)	xTcf-3 site 1	P	L	S	L	T	T	K	-
	xTcf-3 site 2	P	L	S	L	V	T	K	-
	hTcf-4 site 1	P	L	S	L	S	L	K	-
	hTcf-4 site 2	P	L	S	L	V	T	K	-
	Hairy	P	L	S	L	V	I	K	-
	Consensus	P	X	S	L	X	X	K	-
D(3)K(8)	BKLF	P	V	D	L	T	V	N	K
	ZEB	P	L	D	L	S	L	P	K
	MCRIP1	P	I	D	L	S	D	L	K
	Consensus	P	X	D	L	S	X	X	K

2-10. ERK phosphorylation of MCRIP1 disrupts MCRIP1-CtBP interaction

MCRIP1 is a novel ERK substrate that interacts with the transcriptional co-repressor CtBP. However, how MCRIP1 regulates cellular functions was unknown. In order to understand the molecular function of MCRIP1, the effect of ERK phosphorylation on MCRIP1 was examined by co-immunoprecipitation experiments. According to the study done by Ichikawa *et al.*, HEK293 cells were transfected as indicated (Fig. 10), and the interaction between MCRIP1 and CtBP1 was analyzed with or without ERK activation. Interestingly, MCRIP1-CtBP1 interaction was disrupted in an ERK-dependent manner. This phenomenon was cancelled when phospho-mutant MCRIP1-AA was used, or when MEK1 kinase-dead mutant was used. MCRIP1-CtBP1 interaction was also disrupted in MCRIP1-DD that mimics the phosphorylated state of MCRIP1. These results indicated that ERK activation regulates MCRIP1-CtBP1 interaction (Fig. 10)[1].

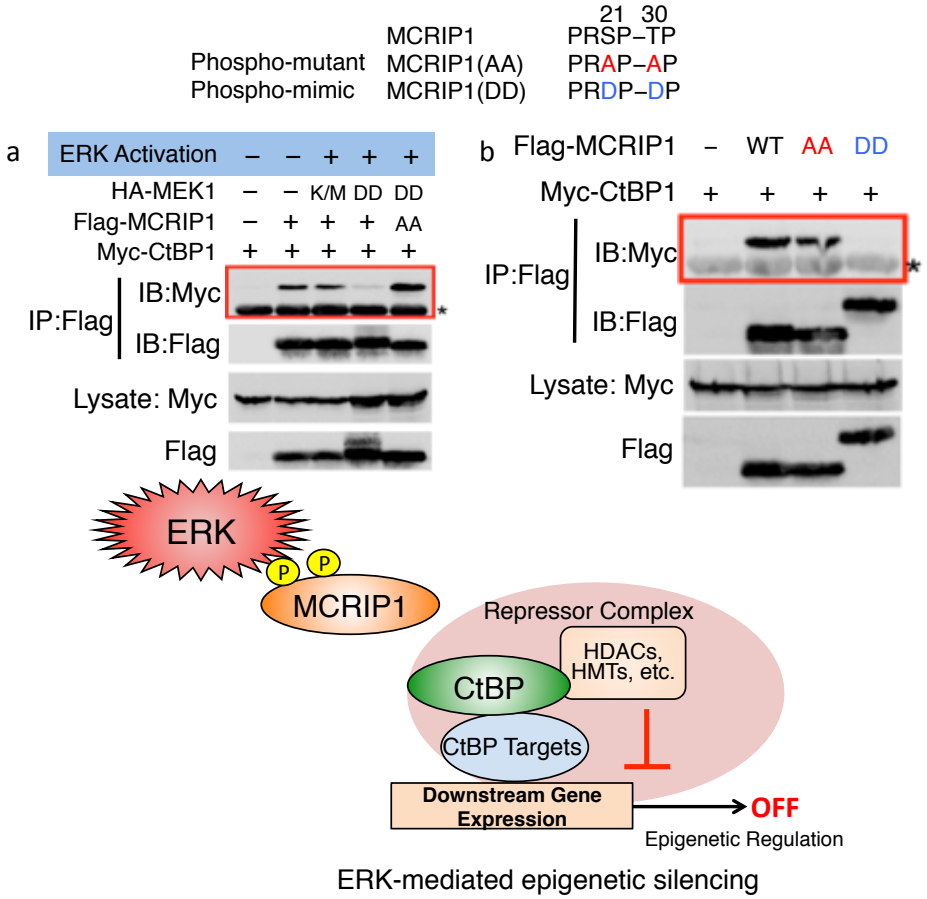


Fig.10 ERK phosphorylation disrupts MCRIP1-CtBP interaction

(a) Co-immunoprecipitation performed under ERK activation shows disrupted MCRIP1-CtBP1 interactions. The phenomenon is reversed when using phospho-mutant of MCRIP1 (AA) or kinase-dead MEK1 that blocks signal transduction of ERK pathway.

(b) Co-immunoprecipitation using phospho-mutant of MCRIP1-AA and phospho-mimic of MCRIP1-DD.

2-11. MCRIP1 competitively inhibits the interaction of CtBP to ZEB1

Based on the findings mentioned above, Ichikawa *et al.* demonstrated that MCRIP1 interacted with CtBP via the PXDLS motif, and phosphorylation of MCRIP1 by ERK disrupted MCRIP1-CtBP interaction. To gain insight into the molecular mechanism of how MCRIP1 regulates CtBP function in response to ERK activation, Ichikawa *et al.* focused on the CtBP target, ZEB1. It was reported that ZEB1 down-regulated downstream gene expression of E-cadherin through interaction with co-repressor CtBP[31]. Therefore, it is curious whether MCRIP1 affected the interaction of CtBP and ZEB1. According to the results reported by the same study, they demonstrated that MCRIP1 competitively inhibited CtBP-ZEB1 interaction in a dose-dependent manner (Fig. 11a, c). Furthermore, CtBP-ZEB1-mediated suppression of E-cadherin promoter activity was also inhibited by co-expression of MCRIP1 in cells (Fig. 11b, c)[1].

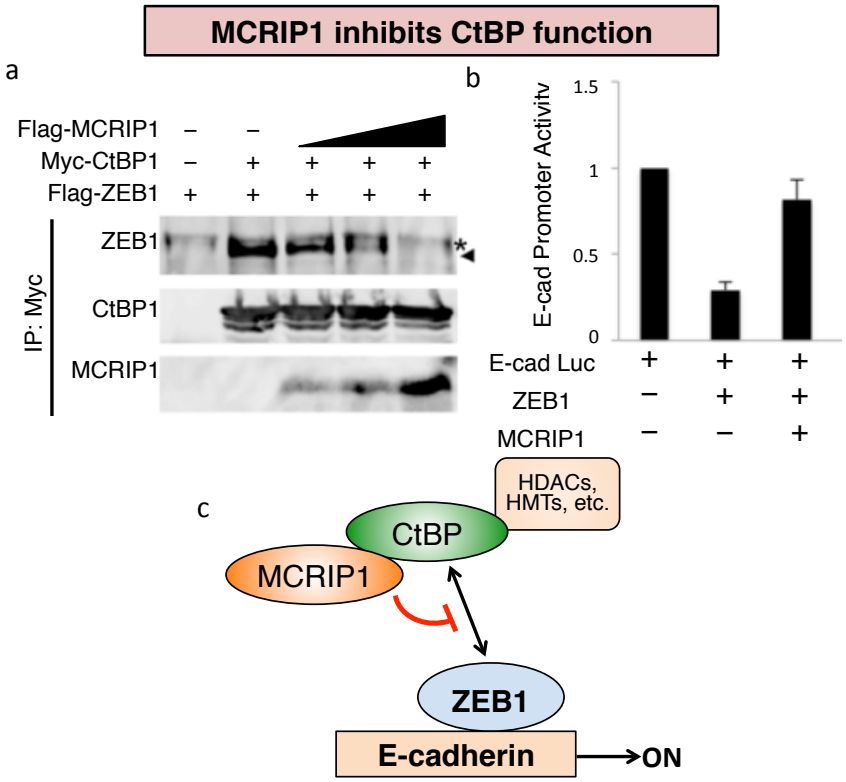


Fig.11 MCRIP1 inhibits CtBP-ZEB1 interaction

(a) Co-immunoprecipitation assay using HEK293 cells transiently expressing MCRIP1, CtBP, and ZEB, followed by western analysis, showed dose-dependent interference of MCRIP1 on CtBP-ZEB1 interaction.
(b) U2OS cells were transfected as indicated and used for luciferase assay. CtBP-ZEB1 suppressed downstream E-cadherin promoter activity recovered when co-expressing MCRIP1 in the cells.
(c) A schematic model of MCRIP1 inhibiting CtBP-ZEB1 interaction.

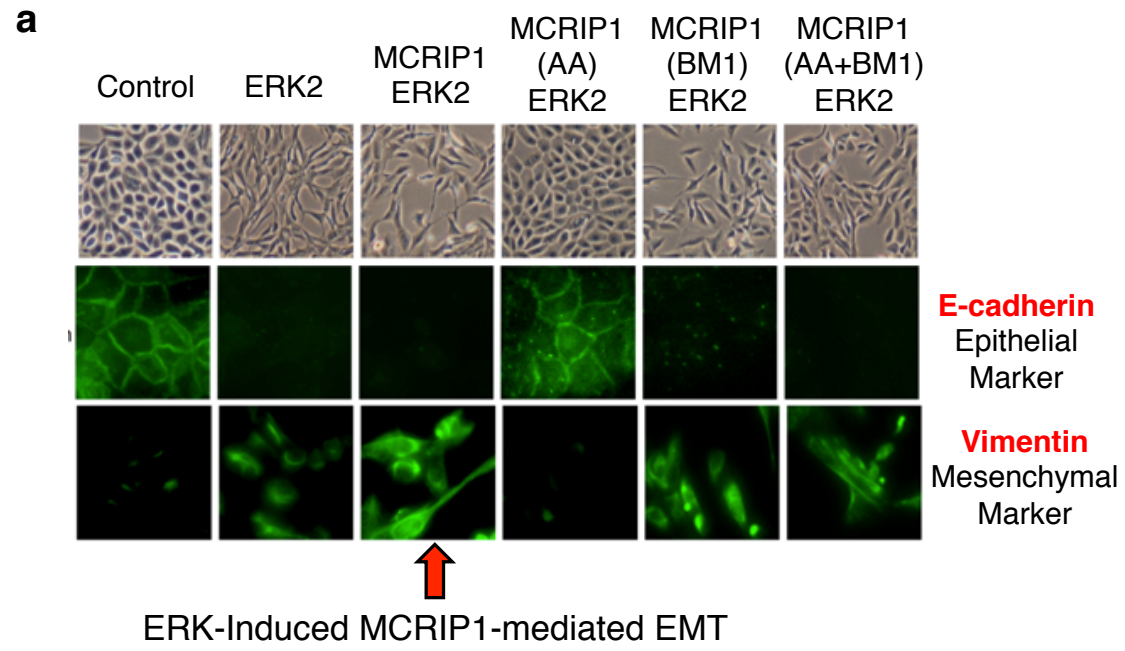
2-12. MCRIP1 mediates ERK-induced EMT

Down-regulation of E-cadherin expression is a hallmark of EMT. Both CtBP and ERK signaling were reported separately for their roles in inducing EMT. However, it was unknown whether there was any regulatory pathway connected between CtBP and ERK pathway. According to previous findings, MCRIP1 interacts with CtBP and inhibits CtBP functions in an ERK-dependent fashion. From these two properties, it is highly possible that MCRIP1 might be the key mediator that connects ERK signaling and CtBP regulation, thereby contributing to EMT induction.

To evaluate this hypothesis, Ichikawa *et al.* used MCF10A cells stably expressing ERK2 and MCRIP1, or ERK2 alone. In agreement with a previous report from Shin et al., ERK2 expression induces EMT in MCF10A cells[15]. Similarly, co-expression of MCRIP1 and ERK2 also induced EMT in MCF10A cells. However, when MCF10A cells are co-expressed with MCRIP1-AA mutant that cannot be phosphorylated by ERK, EMT induction was inhibited. Consistently, co-expression of MCRIP1-BM mutant that cannot bind to CtBP promoted EMT induction in MCF10A cells (Fig. 12a). These results proved that MCRIP1 indeed functions as a mediator of ERK-induced, CtBP-regulated, EMT.

From these results, Ichikawa *et al.* proposed the following signaling axis (Fig. 12b). Under condition of low ERK activity, MCRIP1 binds to CtBP co-repressor and inhibits the formation of CtBP-ZEB1 repressor complex and promotes E-cadherin gene expression. However, when ERK is activated, phosphorylated MCRIP1 releases CtBP and allows interaction of CtBP-ZEB1, which is required for CtBP-regulated EMT induction.

Since CtBP is involved in many aspects of biological processes, it is important to understand how MCRIP1 regulates CtBP functions overall. Therefore, the purpose of this study is to elucidate the physiological roles of MCRIP1 *in vivo.*



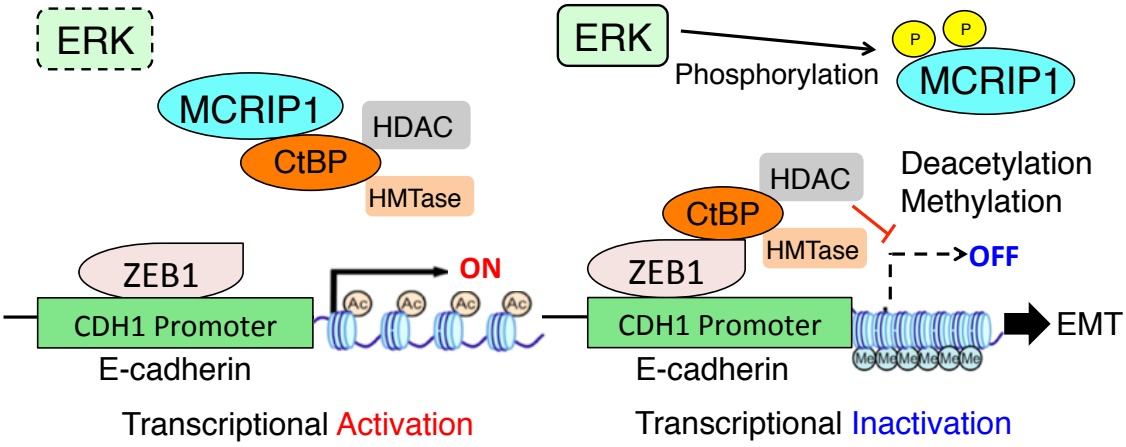


Fig.12 MCRIP1 mediates ERK-induced CtBP-regulated EMT

(a) MCR10A breast epithelial cells stably expressing MCRIP1 (Wild type, phospho-mutant AA, or CtBP-binding mutant BM) and ERK2 were used to examine ERK-induced EMT phenomenon.
The top panel indicates cell morphology assessed by phase-contrast microscopy. The bottom panel showed immunoflurescent staining of E-cadherin (epithelial marker) and Vimentin (mesenchymal marker).

(b) A schematic model of ERK-induced gene silencing mediated by MCRIP1-CtBP interaction.

2-13. Overview of lung development

The respiratory system is essential for terrestrial life to exchange oxygen with carbon dioxide. Therefore, any failure in breathing can result in severe consequences such as death. Although there are many congenital lung diseases, abnormalities and acquired lung diseases reported from the clinical perspectives, the process of lung development and pathogenesis of lung diseases had only been vaguely understood. Recently, researchers have started to gain new insights into how lungs develop and into the molecular mechanisms behind the process[32,33].

The lungs develop from the anterior foregut endoderm, which also give rise to other organs including esophagus, thyroid and liver. Normally, the specification of lung progenitor cells begins at embryonic day (E) 9.0 in mouse. By E9.5 to E12.5, formation of the trachea and lung buds start to develop. Next, by E12.5 to E16.5, also called pseudoglandular stage, the lung buds undergo branching morphogenesis process to form the tree-like airways. Following the pseudoglandular stage, the canalicular stage (E16.5 to 17.5) and saccular stage (E18.5 to postnatal day (PN) 5) allow the lung buds to continue to branch narrower and form clusters of epithelial sacs, which will develop into alveoli, where the gas exchange will take place at birth. Finally, the alveoli continue to mature during alveolarization stage at PN0 to PN14 and onward (Fig. 13)[32,33]. Since the lung develops in a timely manner to prepare the newborns for breathing, immature lung development often results to defects in pulmonary or cardiovascular diseases. In fact, it is known that immature newborns suffer from respiratory distress syndrome due to surfactant protein insufficiency resulted from immature lung development.

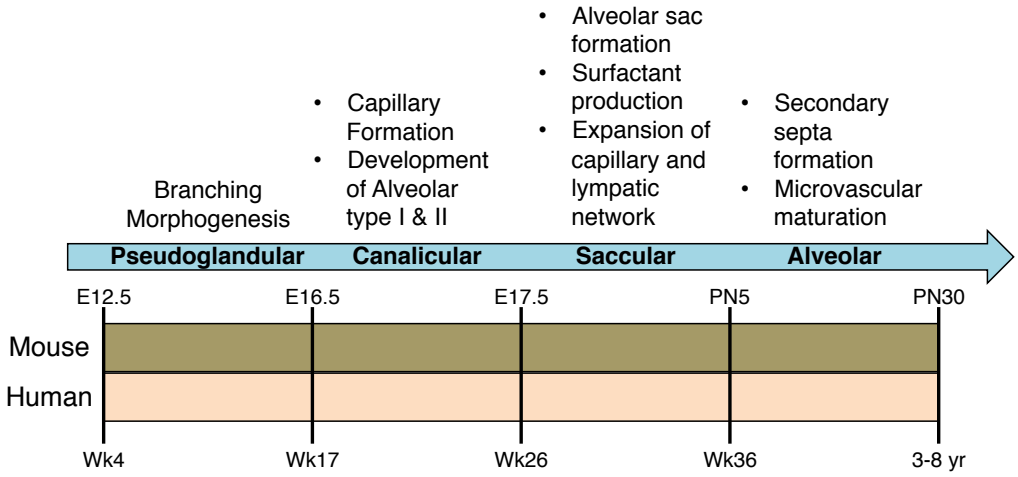


Fig.13 Overview of Lung Development

2-14. Transcription factors in lung development

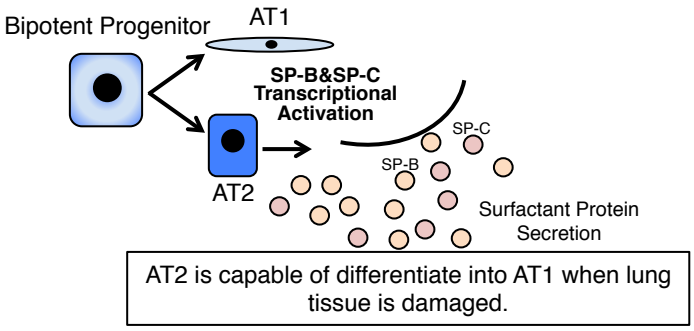
Behind the morphogenesis of the lungs, the signaling pathways and transcription factors that drive the specification and differentiation of lung cells have been extensively studied in the last decade. Expectedly, the signaling pathways involved in the regulation of lung development are complex, and include a large number of transcription factors from different families, such as homeobox factor family Nkx2.1, Gata family, forkhead box transcription factor family, and Ets transcription family[32–34].

Loss of some transcription factors, such as Gata6 or Nkx2.1, results in perinatal death and respiratory distress at birth, whereas loss of many genes does not completely result in lethal phenotypes, though defects were found in epithelial differentiation or morphogenesis[34–36].

Among all, the forkhead box (Fox) family proteins is a highly conserved transcription factor family, many members of which are involved in lung development[32,37]. For example, Foxa1 and Foxa2 gene expressions are both found in the foregut endoderm before lung formation. It was also reported that Foxa2 contributed to late epithelial differentiation[38,39]. Also, Foxp1, Foxp2, and Foxp4 gene expressions are found in both developing and postnatal lungs. Knockout mouse study showed that Foxp2-null mouse died after birth due to defective alveolarization[40]. Although loss- or gain-of-functions studies have revealed many roles of transcription factors in lung development regulation, the molecular mechanisms of how these transcription factors regulate the process of lung development *in vivo* is still largely unknown.

2-15. Differentiation of Alveolar Epithelial Cells (AECs)

During the process of lung development, alveolarization is critical for animals to gain breathing ability. Alveolar sacs, where the gas exchange occurs, are composed of alveolar epithelial cells (AECs). Therefore, the differentiation of AECs is essential for alveolar sacs to function normally. There are two types of AEC, type I AECs and type II AECs. Type I AECs provides the interface for gas exchange, whereas type II AECs are responsible for the secretion of surfactant proteins that reduce the surface tension during respiration. Although the molecular mechanism of AEC differentiation remains largely unknown, it was suggested that a bipotential progenitor cells are present in the lung that give rise to either type I or type II AECs (Fig. 14). For example, as differentiation of type II AEC from bipotent progenitor cells continues, expression of specific genes such as Muc1, Nkx2.1, and Abca3s begin to appear (Fig. 15)[41–43].



*AT1: Type I Pneumocyte
*AT2: Type II Pneumocyte

Fig.14 Differentiation of Alveolar Epithelial Cells

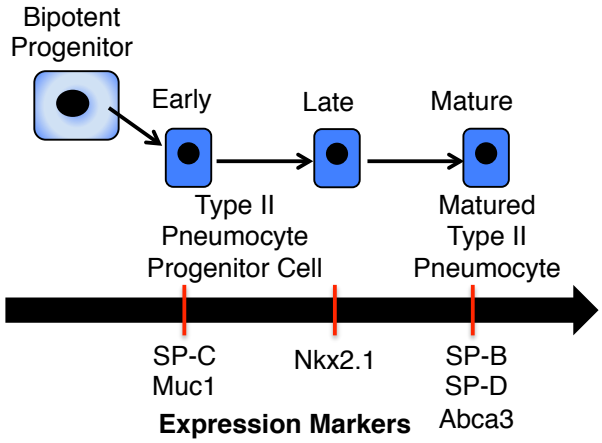


Fig.15 Specific Markers in Type II Pneumotye differentiation

2-16. Surfactant Proteins

The surface of lungs is where gas exchange takes place. Therefore, the lungs must sustain the surface tension created from air-liquid interface, as well as the risk of cells being exposed to pathogens, allergens, or pollutants. In this regard, pulmonary surfactant proteins are essential for the lungs to overcome these challenges.

There are four types of surfactant proteins, SP-A, B, C and D. They can be divided into two groups based on their hydrophobic or hydrophilic nature. SP-A and SP-D are the hydrophilic surfactant proteins that provide host defense system when pathogens and small particles invade through the airways. On the other hand, SP-B and SP-C are the hydrophobic surfactant proteins that prevent the lungs from collapse when air enters. Because of their roles in lung functions, deficiency in surfactant proteins is frequently found in pulmonary diseases. Knockout studies of surfactant proteins also demonstrated severe pulmonary defects, emphasizing their importance[41,44,45].

3. Results

3-1. Establishment of MCRIP1-deficient (MCRIP1-KO) mouse

From our previous study analyzing the molecular function of MCRIP1, MCRIP1 functions as a mediator of ERK-induced, CtBP-regulated, gene silencing, thereby promoting epithelial-mesenchymal transition (EMT). To further investigate the physiological function of MCRIP1 *in vivo*, we established MCRIP1-deficient murine model. As the targeting strategy, MCRIP1 gene was replaced with the Neo cassette and the LacZ reporter gene on chromosome 11 in C57BL/6 mouse (Fig. 16a). By using the designed primers that are able to detect both wild type and mutant alleles, I confirmed that the MCRIP1 gene was replaced by the LacZ reporter gene (Fig. 16b). Next, to examine the depletion of MCRIP1 protein, mouse embryonic fibroblasts (MEFs) were isolated at E14.5 (embryonic day 14.5) and examined by immunoblotting analysis. In contrast to the expression of MCRIP1 in wild-type MEF, MCRIP1-KO MEF showed complete depletion of MCRIP1 expression (Fig. 16c).

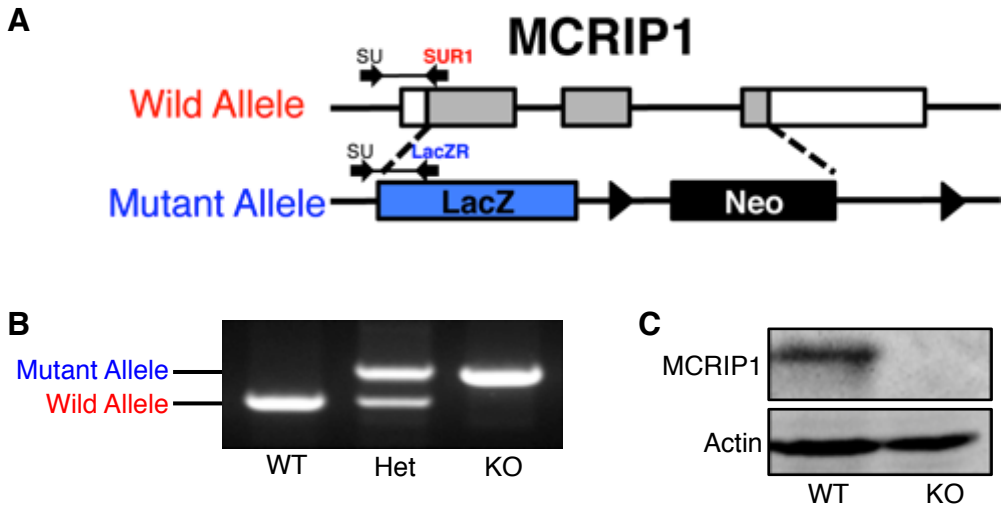


Figure16. Establishment of MCRIP1-KO mouse

(a) Targeting strategy of MCRIP1-KO mouse.

(b) Genotyping PCR confirmed the successful establishment of MCRIP1-KO mice.

(c) Western analysis of mouse embryonic fibroblasts isolated from MCRIP1-WT and MCRIP1-KO mice. MCRIP1 protein expression is depleted in MCRIP1–/– mice.

3-2. Phenotype analysis of MCRIP1-KO mouse

Next, in order to identify any defects due to the depletion of MCRIP1 expression, I performed phenotype analysis. Although the knockout of the MCRIP1 gene is viable in C57BL/6 mice, only a few MCRIP1-KO mice survived up to adult age (data not shown). Therefore, I monitored the survival rate of all mice to investigate whether MCRIP1 depletion resulted in early death. First, all mice were genotyped at age 1 week. Since the sexuality of 1-week old mice cannot be determined by observation, it is necessary to design another system for sex typing. The sex-typing system was constructed utilizing the expressions of Y chromosome-specific gene, Sry, and Myogenin as control. SRY, also known as sex determining region Y, is encoded by Sry gene found on the Y chromosome. It is known that SRY protein produced from Sry gene functions as a transcription factor and regulates particular genes that are involved in male sexual development. Due to the expression of the Sry gene, the male gonad development is promoted while the development of female reproductive system is suppressed[46]. On the other hand, myogenin protein, encoded by the Myog gene, is known as a transcriptional activator of muscle-specific target genes that promote muscle development. As myogenin protein is expressed in both male and female mice, it is used as a control gene in the sex-typing system. Therefore, by detections of Sry and Myog genes, this system functions as an indication of the sexuality (Fig. 17)[47].

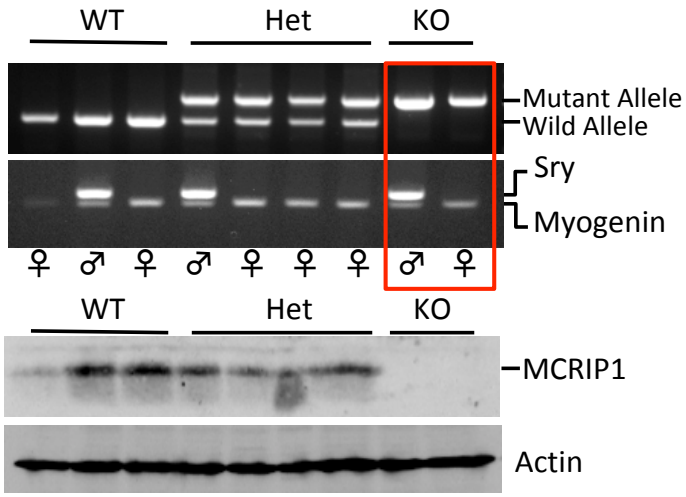


Figure17. Genotyping & Sex-typing system for MCRIP1 newborns

By both genotyping and sex-typing systems, I monitored the survival of all MCRIP1 mice. As a result, both heterozygous (MCRIP1-Het) and knockout (MCRIP1-KO) mice showed significant decrease in survival rate when compared to that of wild-type (MCRIP1-WT) mice (Fig. 18a). Furthermore, I also measured the body weight of the survived mice at age of 2 weeks. Compare to MCRIP1-WT mice, both MCRIP1-Het and MCRIP1-KO mice showed relatively low body weight (Fig. 18b). These results suggested that the depletion of MCRIP1 might be lethal during neonatal stages. However, once surpassed the neonatal stage, MCRIP1 might possess different functions that contribute to the tendency of slimness.

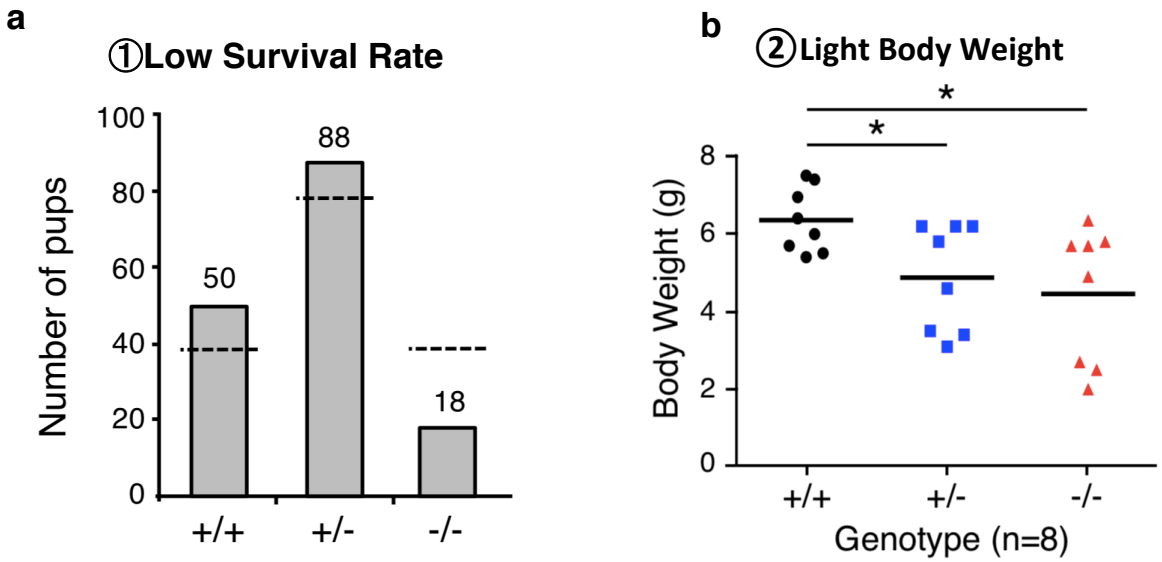


Figure18. Phenotypes of MCRIP1 mice

(a) Analysis of MCRIP1 mouse survival. Gray dashed line indicates expected survival according to Mendel's law. Both MCRIP1+/– and MCRIP1–/– mice showed decreased survival rate.

(b) Body weight of MCRIP1 mice was monitored at age 2 week. Both MCRIP1+/– and MCRIP1–/– showed tendency of lower body weight when compared to that of wild type mice. (n=8, *p-value<0.05)

From the early deaths found in MCRIP1-KO mice, my observation suggested that the expression of MCRIP1 was required for normal development. Therefore, the purpose of this study will focus on the identification of dying cause and the underlying molecular mechanism behind the phenotype of MCRIP1-KO mice

3-3. MCRIP1-deficient neonates suffer respiratory failure

In order to define the cause of early deaths in MCRIP1-KO mice, I monitored MCRIP1 mice within 24 hours after birth. As a result, MCRIP1-KO mice suffering from breathing difficulty was observed frequently in contrast to normal breathing MCRIP1-WT mice. In addition, MCRIP1-KO mice showed a symptom of cyanosis, as the skins appeared blue/purple due to lack of oxygen in circulation (Fig. 19). From this finding, we speculated that MCRIP1-KO mice might die of respiratory failure.

Figure19. MCRIP1-deficient newborn suffering respiratory failure
MCRIP1 mice were monitored within 24 hours after birth. +/+ represents MCRIP1-WT mice, whereas -/- represents MCRIP1-KO mice.

To evaluate this possibility, the whole-body sections of MCRIP1 mice was prepared and stained by hematoxylin and eosin (H&E) staining.

3-4. Whole-body sections of MCRIP1-deficient mice

In order to investigate the true cause of death in MCRIP1-KO mice, the neonates were sacrificed and collected immediately after birth for whole-body histological analysis. By using the genotyping system (Fig. 17), three MCRIP1-WT and MCRIP1-KO mice were sacrificed and subject to whole-body sections followed by hematoxylin and eosin (H&E) staining. By comparing the histology of each tissue, no significant defects were found in MCRIP1-WT and MCRIP1-KO mice. However, only the lung tissues of MCRIP1-KO mice were relatively smaller than MCRIP1-WT lungs (Fig. 20).

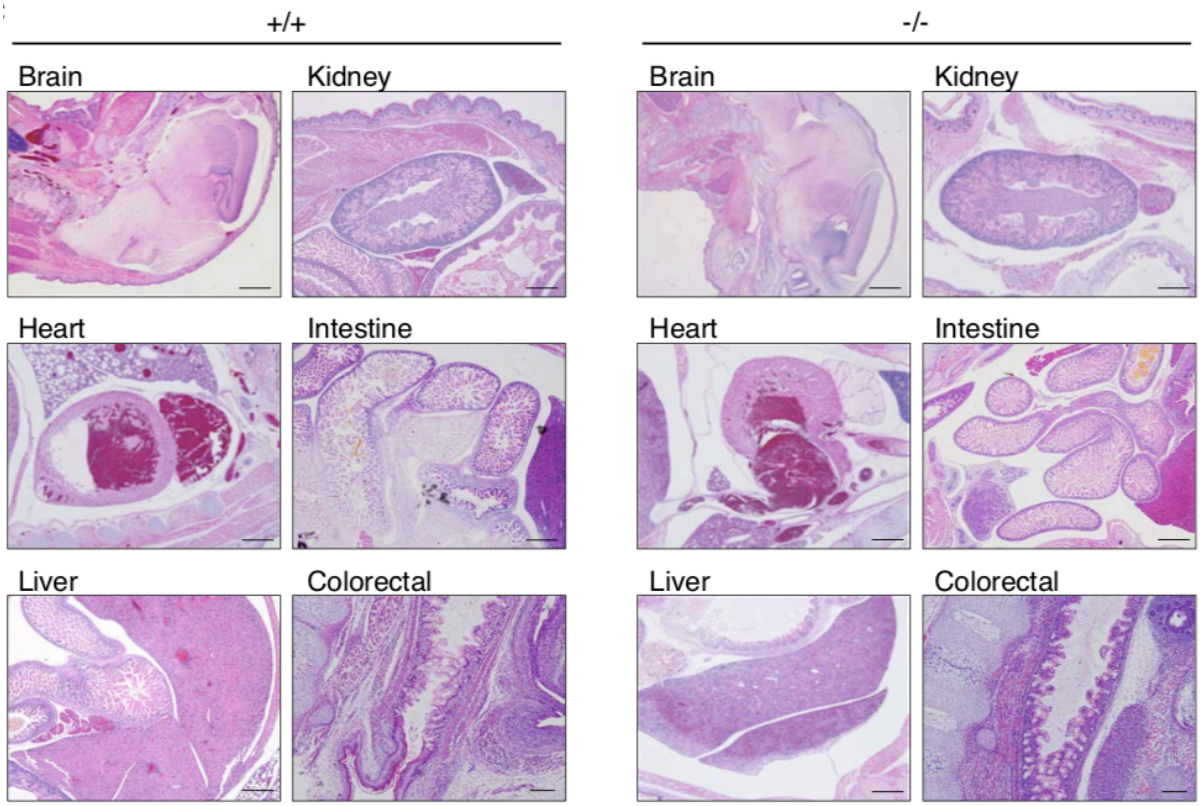


Figure20. Gross histology of tissues of MCRIP1 mice analyzed by H&E staining. Scale bars, 100 μm.

3-5. MCRIP1-deficient neonates show defect in lung inflation

For animals to obtain sufficient oxygen, gas exchange occurred in the alveolar sacs of the lungs is extremely important. However, when alveolar sacs cannot be inflated properly, insufficient exchange of oxygen can result in respiratory failure. To evaluate whether the deflated lungs in MCRIP1-KO mice are due to any defects in the alveolar sacs, I compared the lung tissues of MCRIP1-KO mice with those of MCRIP1-WT mice in magnified view. As the alveolar sac is indicated in the black bold line, the alveolar sac in MCRIP1-KO is relatively less expanded (Fig. 21a). Furthermore, I performed immunohistochemistry analysis using anti-MCRIP1 antibody to confirm MCRIP1 expression in the lung. This result suggested that MCRIP1 might function in alveolar sac expansion.

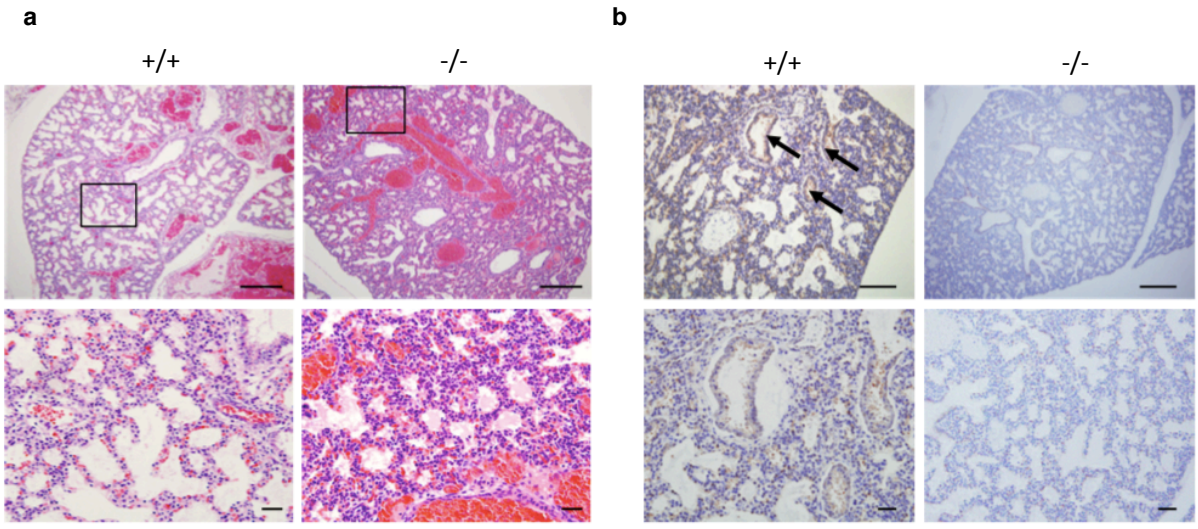


Figure21. Histology of lung tissues in MCRIP1 mice

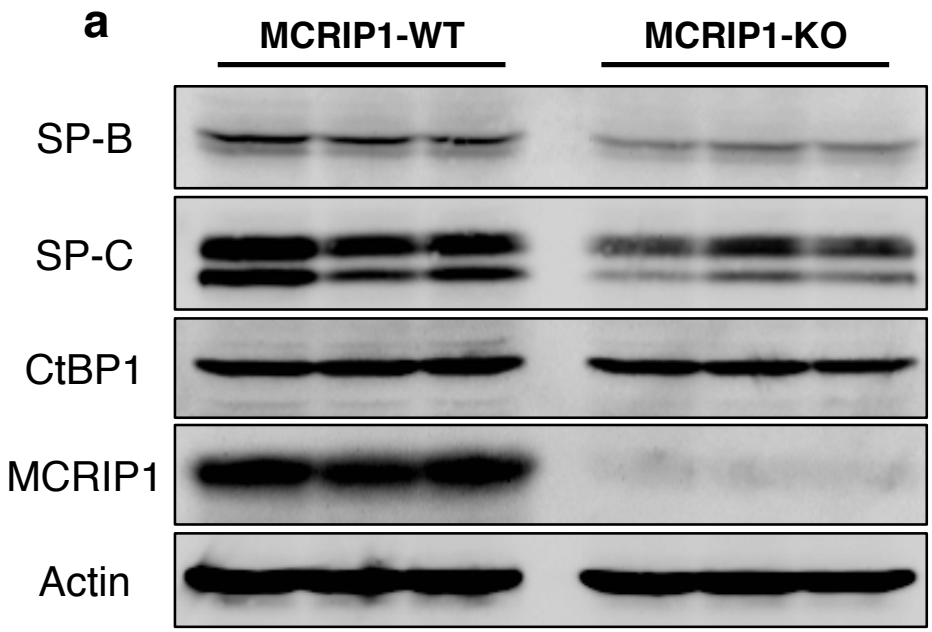
(a) H&E staining on paraffin sections in MCRIP1-WT and MCRIP1-KO mice. Gross analysis showed abnormality in the lungs, indicating possible cause of death (upper). Magnified view showed less inflated alveolar sac in MCRIP1-KO mice (bottom).

(b) Immunohistochemistry (IHC) analysis using anti-MCRIP1 antibody on frozen sections of lung tissues. MCRIP1 is expressed in the epithelial layer of lungs (arrows).

3-6. Pulmonary surfactant protein expressions in MCRIP1 mice

Secretion of pulmonary surfactant proteins in the lungs plays important roles in maintaining lung functions. There are four types of surfactant proteins, surfactant protein A (SP-A), B (SP-B), C (SP-C), and D (SP-D). As mentioned in introduction, SP-B and SP-C are hydrophobic and function in reducing the alveolar surface tension when air comes in, whereas surfactant A and D (SP-A and SP-D) are involved in the immune system of the lungs. As the distressed alveolar sacs were observed in MCRIP1-KO mice, it is suspected that insufficient secretion of pulmonary surfactant proteins might cause the collapse of alveolar sacs in knockout mice.

Therefore, it is necessary to examine the expression of surfactant proteins in MCRIP1 mice. For this purpose, I sacrificed the neonates immediately after birth and isolated the lung tissues to collect the protein lysates. The amount of SP-B and SP-C expressions were detected by western blot analysis. As expected, MCRIP1-KO mice showed significant decrease in both SP-B and SP-C expressions. In addition, RT-qPCR results also confirmed decreased mRNA expressions of SP-B and SP-C in MCRIP1-KO mice. These results suggested that MCRIP1 regulates SP-B and SP-C expressions transcriptionally (Fig. 22).



Decreased SP-B and SP-C expressions in MCRIP1-KO neonates

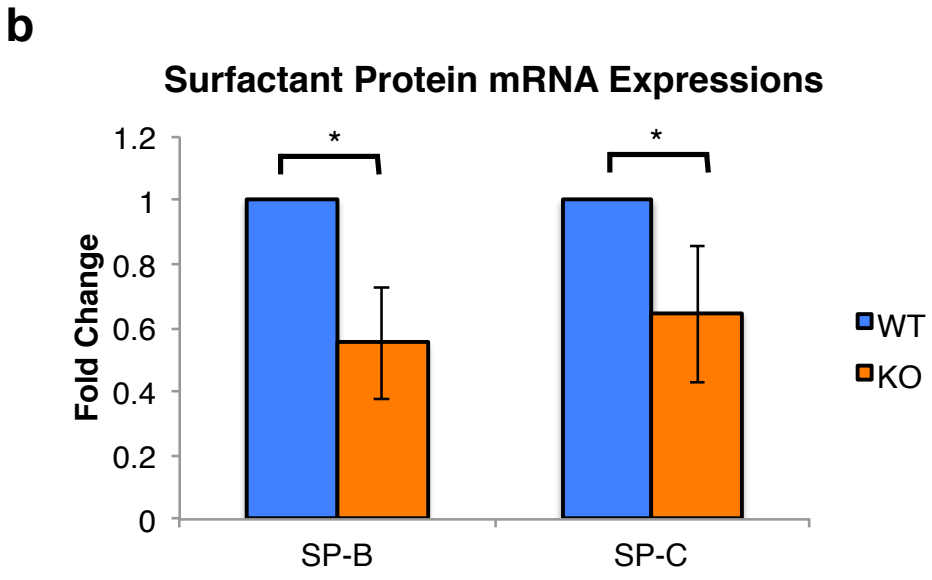


Figure22. Surfactant Protein B & C Expressions in MCRIP1 mice

(a) Western analysis of SP-B and SP-C expressions in neonatal lung lysates. MCRIP1–/– mice showed decreased SP-B and SP-C expressions.

(b) RT-qPCR of surfactant protein expressions in MCRIP1-WT and MCRIP1-KO mice. Data represent mean ± SEM obtained from three mice. *,$p<0.05$.

Furthermore, the collected neonatal lung tissues used for immunohistochemistry analysis by anti-SP-B and SP-C antibody. Similarly, both SP-B and SP-C expressions were decreased in MCRIP1-KO mice when compared to MCRIP1-WT mice (Fig. 23). These results suggest that MCRIP1-KO mice die at neonatal stage due to respiratory failure resulted from insufficient expressions of SP-B and SP-C.

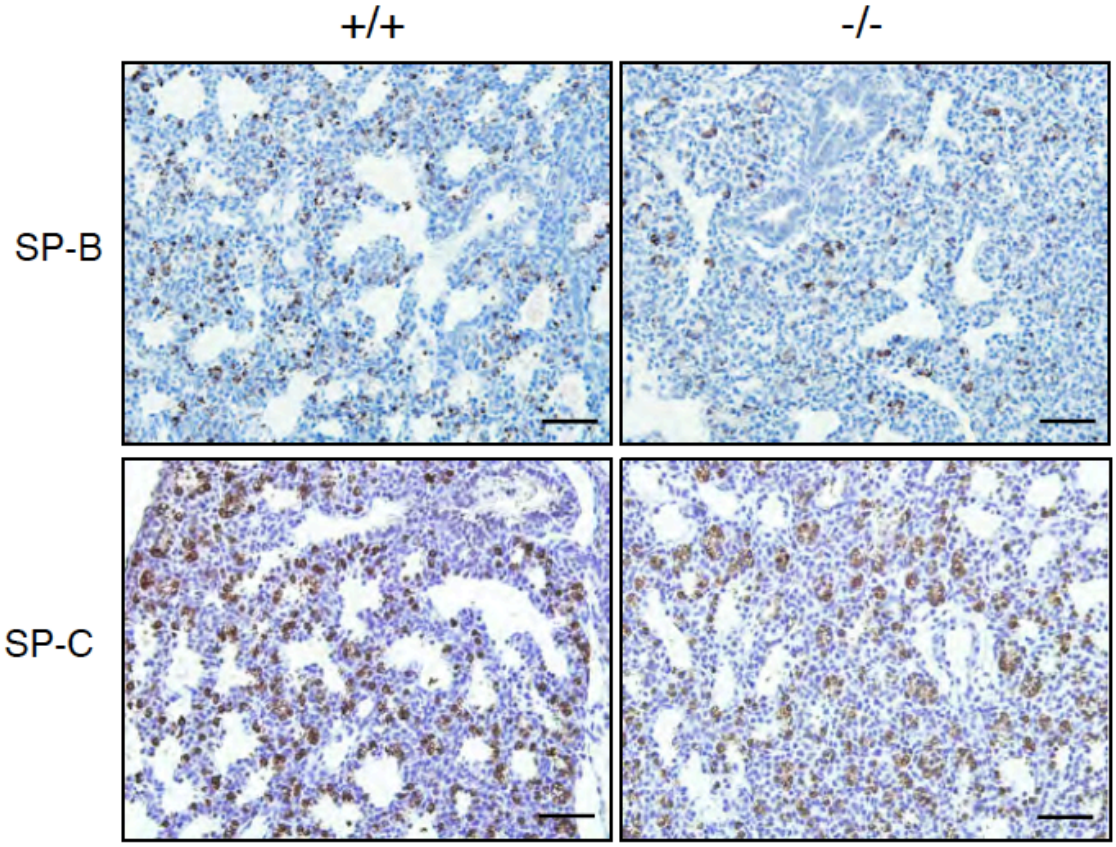


Figure23. IHC analysis on SP-B & SP-C Expression

Immunostaining of SP-B and SP-C in MCRIP1-WT and MCRIP1-KO neonatal lungs.

3-7. Type II AEC differentiation marker expressions in MCRIP1 mice

In alveoli, the alveolar epithelial cells are responsible for normal lung functions. There are two types of AECs, type I and type II. Both type I and type II AECs differentiate from bipotential progenitor cells. Along the differentiation process, specific protein expressions start to appear (Fig. 15). During early stage of AEC differentiation, early type II AEC marker, Muc1, first starts to appear. As the cells differentiate into matured type II AEC, late-stage marker, Abca3, begins to express.

As the insufficient expressions of SP-B and SP-C were observed in MCRIP1-KO mice, it is necessary to examine whether the reduced SP-B and SP-C expressions were due to defective AEC differentiation. First, I compared the morphology of lung tissues from MCRIP1-WT and MCRIP1-KO mice (Fig. 24). As a result, no significant change in morphology was found.

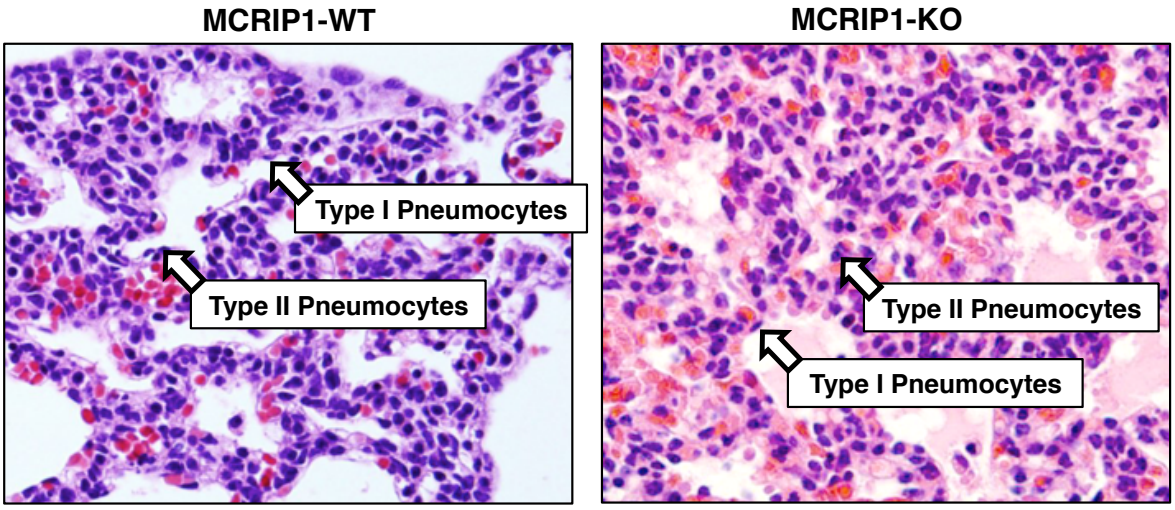


Figure24. H&E staining of lung tissues in MCRIP1 mice

Next, I examined the early and late type II AEC markers, Muc1 and Abca3 expressions in MCRIP1-WT and MCRIP1-KO mice. To do so, the lung tissues of MCRIP1-WT and MCRIP1-KO mice were isolated and used for western blotting analysis or RT-qPCR. By western blotting analysis, both MCRIP1-WT and MCRIP1-KO mice showed similar protein expression level of both early and late type II AEC markers, Muc1 and Abca3 (Fig. 25a, 26a). Complementary to the results obtained from western blotting analysis, the mRNA expressions of both Muc1 and Abca3 expressed in the lungs of MCRIP1-WT and MCRIP1-KO also showed insignificant difference (Fig. 25b, 26b). These results suggested that MCRIP1 did not affect the differentiation of type II AEC during lung development.

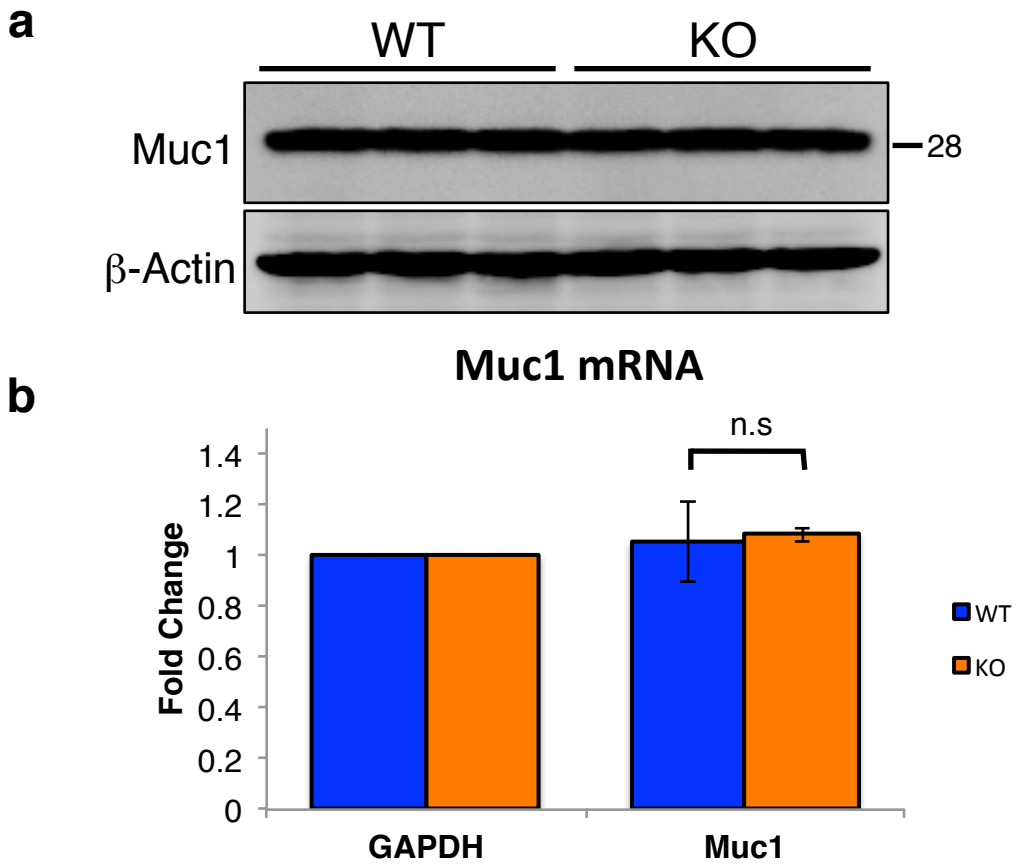


Figure25. Expression of differentiation marker, Muc1

(a) Immunoblotting analysis of the expression of Muc1 in MCRIP1 mouse lung lysates.

(b) RT-qPCR analysis of Muc1 expression in MCRIP1 mouse lung lysates. Error bars indicate mean ± SEM obtained from three mice. n.s., not significant.

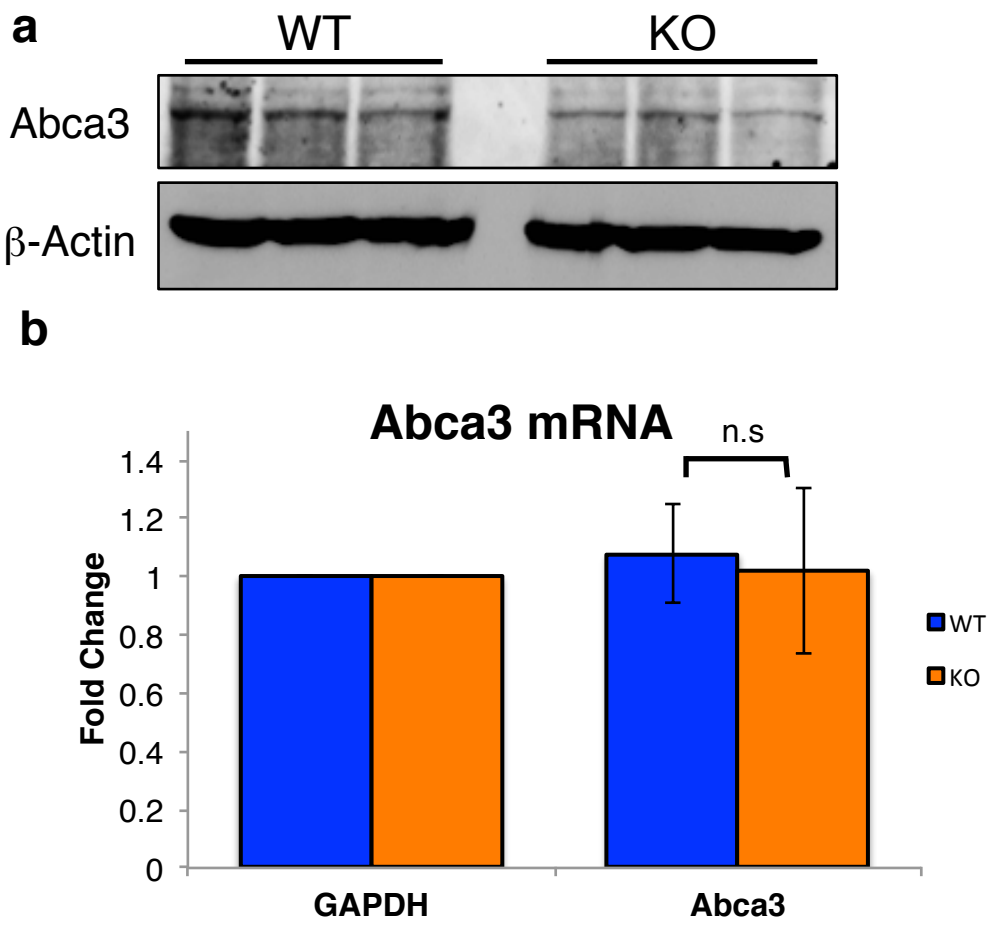


Figure26. Expression of differentiation marker, Abca3

(a) Immunoblotting analysis of the expression of Abca3 in MCRIP1 mouse lung lysates.

(b) RT-qPCR analysis of Abca3 expression in MCRIP1 mouse lung lysates. Error bars indicate mean ± SEM obtained from three mice. n.s., not significant.

3-8. Ultrastructure of MCRIP1-KO lungs demonstrates the lack of surfactant protein

In addition to examining the protein and mRNA expressions of differentiation markers, Muc1 and Abca3, I investigated the ultrastructure of type II AECs in MCRIP1-WT and MCRIP1-KO mouse lungs before (E21) and after birth (P0) using electron microscopy. Although the distribution of type II AECs was similar in the lungs of both mice, the number and the size of the cytoplasmic lamellar bodies (LBs) in individual type II AECs were markedly decreased in MCRIP1-KO mice versus those in the MCRIP1-WT mice (Fig. 27). Accordingly, the tubular myelin (TM) formation was also decreased in the alveolar extracellular space of MCRIP1-KO mice versus MCRIP1-WT mice. These phenotypes are similar to those observed in SP-B deficient mice in that fully formed LBs were not detected in type II AECs and, reflecting the lack of the major surfactant protein, the mice died shortly after birth due to respiratory distress[48]. From these findings, it suggested that MCRIP1 depletion did not significantly affect the differentiation of type II AECs, but led to insufficient synthesis of the surfactant protein B and C in type II AECs.

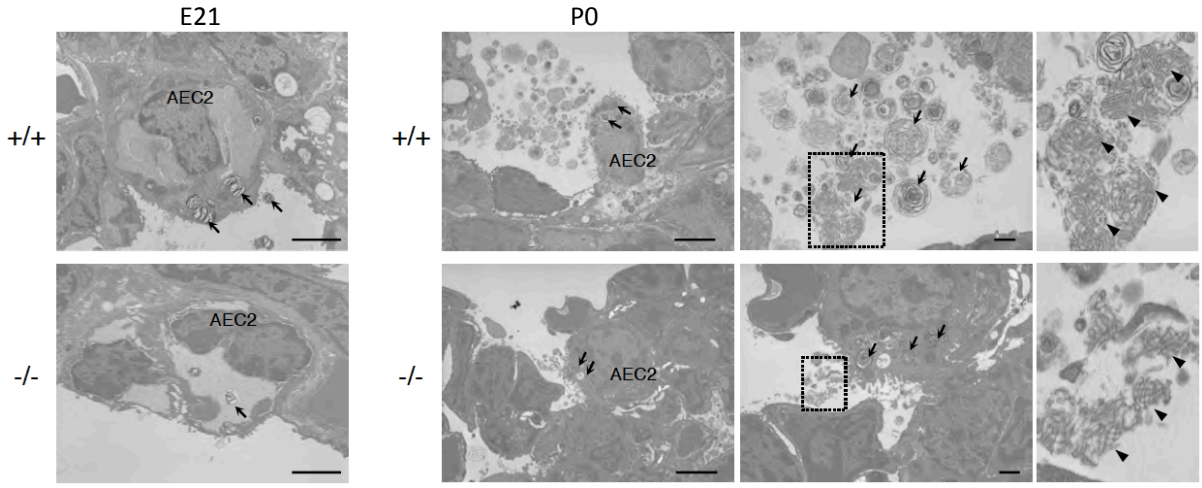


Figure 27. Ultrastructure of type II AECs in MCRIP1-WT and MCRIP1-KO lungs
Arrows indicate lamellar bodies and arrowheads indicate tubular myelin. Scale bars, 2μm in E21 lungs. Scale bars, 2 and 1μm in the left and middle panels of P0 lungs, respectively

3-9 MCRIP1is highly expressed in the alveolar epithelial layer during lung development

In previous study, Ichikawa *et al.* demonstrated that MCRIP1 binds to transcriptional co-repressor CtBP and disrupts CtBP-mediated gene silencing by competitively inhibiting interactions between CtBP and DNA-binding transcriptional repressors harboring the CtBP-binding motif[1]. Although the precise role of CtBP in the regulation of lung development remains unclear, reports have shown that the CtBP-binding transcriptional repressors, Foxp1 and Foxp2, are highly expressed in AECs and suppress the expression of SP-C and other lung-related genes[40,49–51]. Based on these facts, it is speculated that MCRIP1 might inhibit CtBP and Foxp1/2-mediated transcriptional repression of surfactant proteins in type II AECs.

To explore this possibility, firstly, the expression patterns of MCRIP1, CtBP1/2, and Foxp1/2 in lung tissues of MCRIP1-WT mice at critical embryonic stages during development were monitored. E17 represents the end of the canalicular stage (when type I and type II AECs start to emerge), E18 represents the beginning of the saccular stage (when alveolar sacs form), and E21 represents the alveolar stage. Immunohistochemistry showed that MCRIP1 was expressed in the epithelial layer of the developing lung at all stages tested, indicating that MCRIP1 is constitutively expressed in embryonic lung development (Fig. 28a). Furthermore, as reported previously[40], Foxp1 was expressed at high levels in the nucleus of lung epithelial cells and at lower levels in the developing mesenchyme, while Foxp2 expression was restricted to distal lung epithelial (Fig. 28b). CtBP1 and CtBP2 were found to be widely expressed in the nucleus of various types of cells, but particularly high in lung epithelial cells (Fig. 28c). Along with the development of alveolar epithelial cells, SP-B and SP-C expression was detected in embryonic lungs at E17 and accumulated thereafter (Fig. 28d).

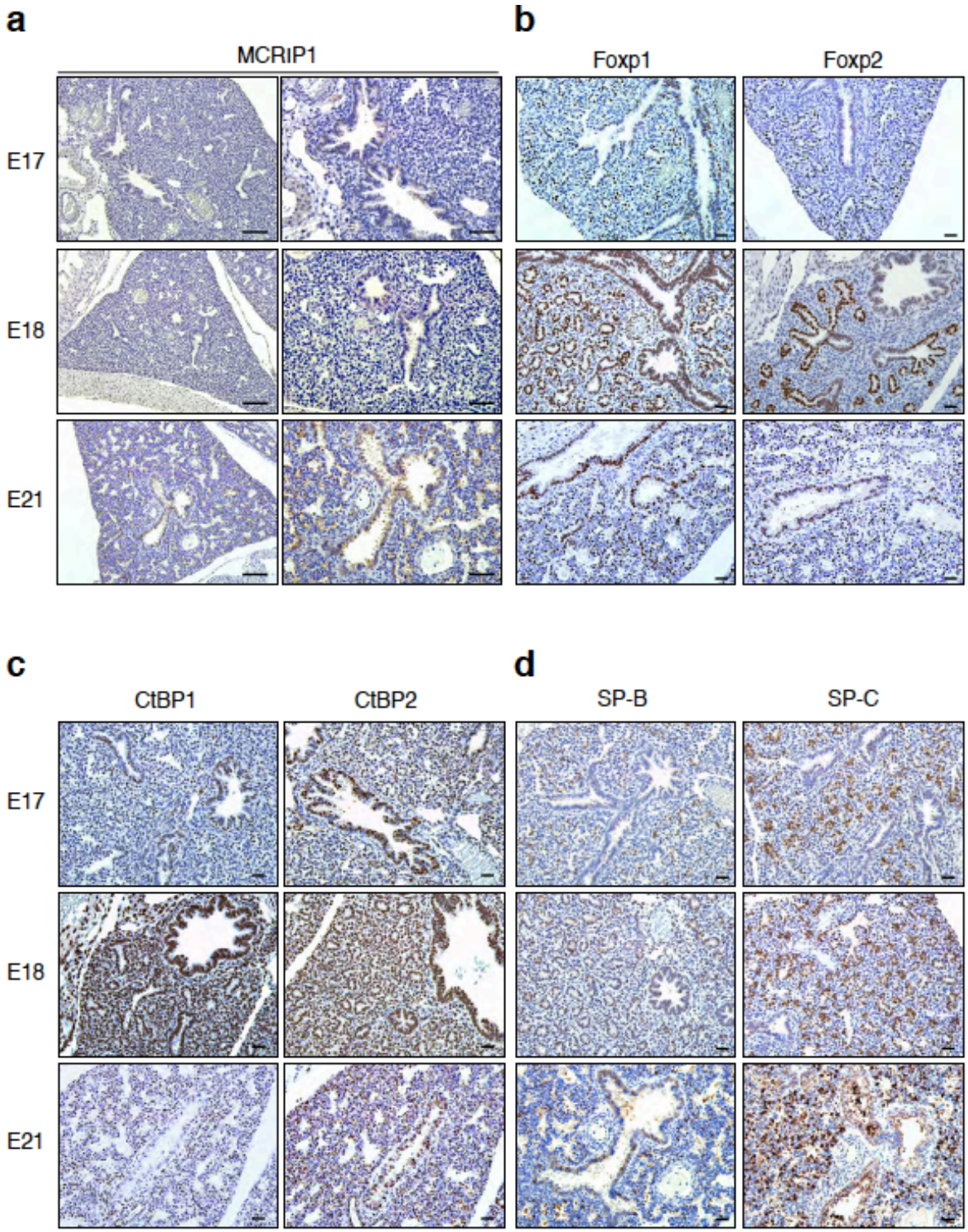


Figure28. Foxp1 and Foxp2 expressions during embryonic lung development

Immunohistochemical analysis of the expression of MCRIP1 (a), Foxp1 and Foxp2 (b), CtBP1 and CtBP2 (c), and SP-B and SP-C (d) in MCRIP1-WT lungs at the embryonic stage of E17, E18, and E21. Scale bars, 100 and 50 μm in the left and right panels of (a), respectively. Scale bar, 50 μm in (b–d).

To determine whether MCRIP1 regulates Foxp1 and Foxp2 expression, I examined these expressions in both MCRIP1-WT and MCRIP1-KO neonatal lungs (P0) by western blot analysis and immunohistochemistry (Fig. 29). The results demonstrated that MCRIP1 depletion did not affect the expression levels of Foxp1 and Foxp2 in lungs, as well as CtBP1 and CtBP2. Similar results were obtained from immunohistochemistry analysis. These findings suggested that MCRIP1 promotes the production of surfactant proteins without directly regulating the expressions of Foxp1, Foxp2, CtBP1 and CtBP2.

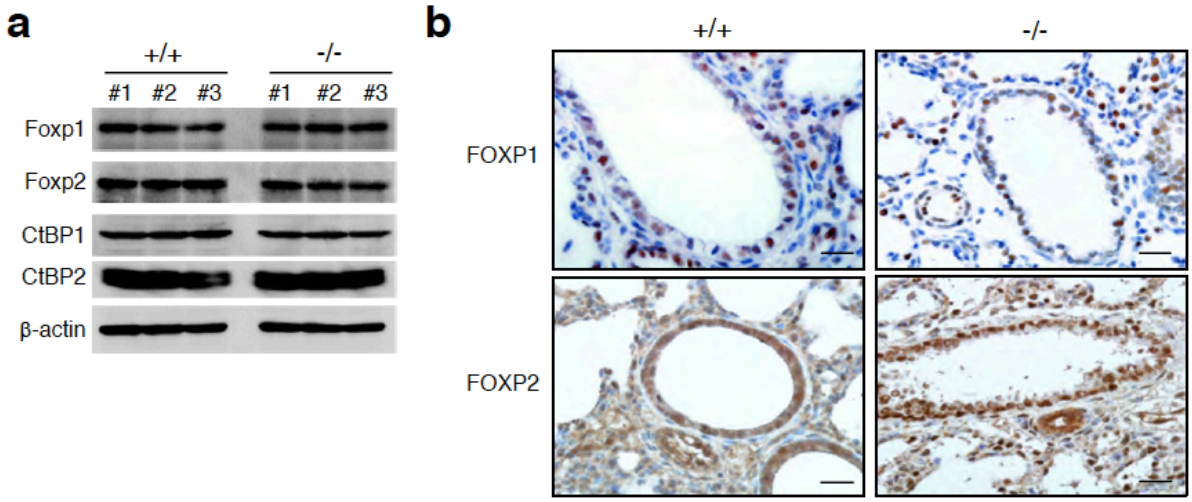


Fig. 29 Foxp1/2 and CtBP1/2 expressions in neonatal lungs

(a) Western blot analysis of whole-lung lysates isolated from MCRIP1-WT and MCRIP1-KO neonates.

(b) Immunohistochemical analysis of Foxp1 and Foxp2 in lung tissues isolated from MCRIP1-WT and MCRIP1-KO mice. Scale bars, 20 μm.

3-10. MCRIP1 competitively inhibit CtBP-FOXP2 and CtBP-FOXP1 interaction

Although the decreased SP-B and SP-C expressions were observed in MCRIP1-KO mice, the molecular mechanism remained unknown. Previously, we have reported that MCRIP1 regulates downstream E-cadherin gene expression through abrogating the interaction of transcriptional co-repressor CtBP to its target repressor, ZEB1, in an ERK-dependent manner[1]. Therefore, I hypothesized that MCRIP1 might contribute to SP-B and SP-C deficiency by regulating their transcriptional activity. To evaluate the hypothesis, it is necessary to identify potential interacting factors involved in lung development. Among various transcription factors involved in lung development, Forkhead box protein P1 and P2 (Foxp1, Foxp2) were not only highly expressed in AECs, but were also reported to interact with CtBP through CtBP-binding motif (PLNLV) and other sites[49,50,52]. Therefore, in this study, I investigated whether MCRIP1 affected the interaction between Foxp1/2 and CtBP1/2.

First, I confirmed the interactions of Foxp2 and CtBP, and whether this interaction is via CtBP-binding motif (PLNLV) by immunoprecipitation experiments. As mentioned in Table 2, CtBP interacts with its target through a conserved PXDLS-motif. Therefore, the Foxp2 mutants in PXDLS-motif (BMs) were used to determine whether CtBP-Foxp2 interaction is PXDLS-dependent. As a result, mutations in PXDLS motif of Foxp2 abrogated its interaction with CtBP (Fig. 30).

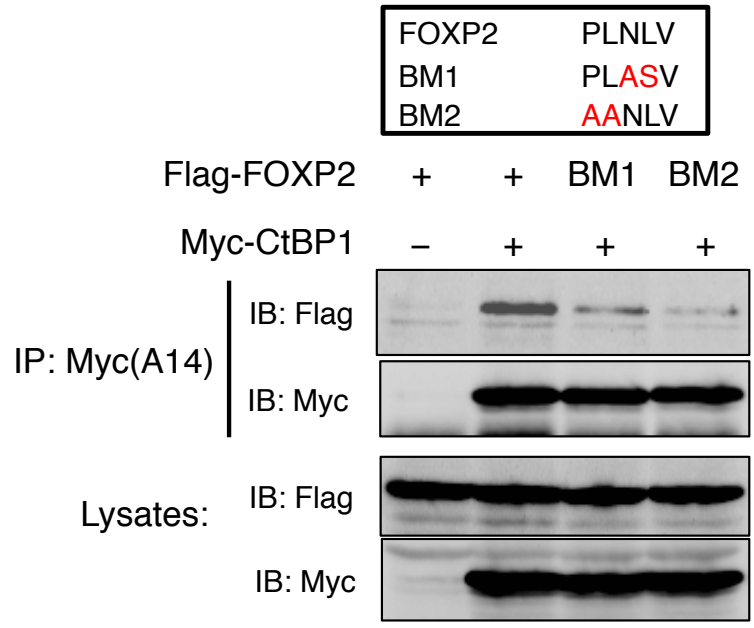


Figure 30. FOXP2 interacts with CtBP1 through PXDLS motif

Next, to investigate whether MCRIP1 competitively interfered with

CtBP-Foxp1/2 interaction, U2OS cells transiently expressing MCRIP1, CtBP, and Foxp2 or Foxp1 were used for co-immunoprecipitation experiments. As a result, introduction of MCRIP1 blocked CtBP-Foxp1 or CtBP-Foxp2 interactions (Fig.31a), and the inhibition occurred in a dosage-dependent manner (Fig. 31b).

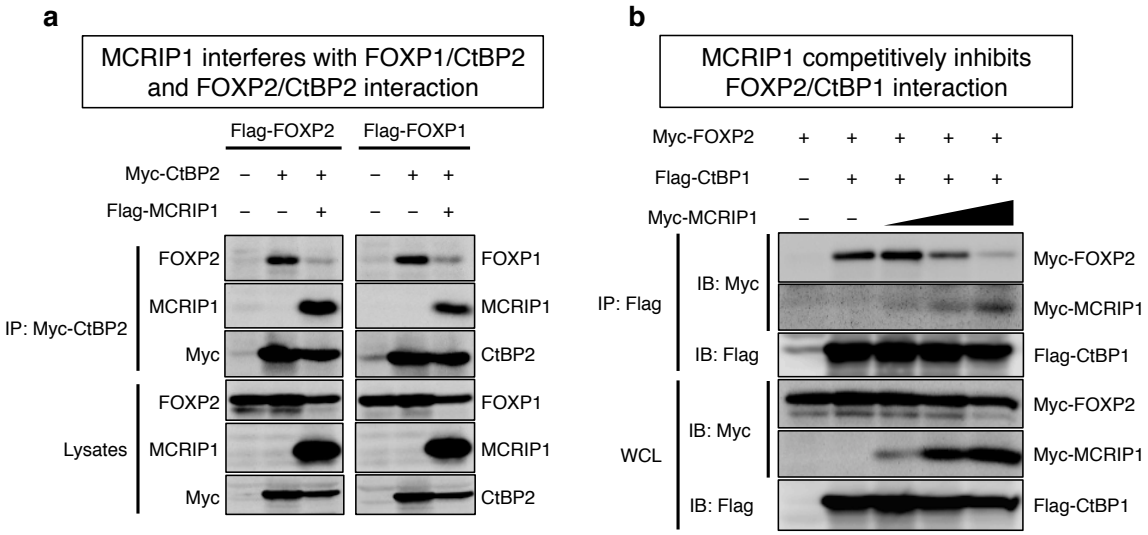


Figure 31. MCRIP1 abrogates FOXP1&FOXP2 interaction with CtBPs.
(a) U2OS cells were transiently transfected as indicated. Myc-CtBP2 was immunoprecipitated, and co-precipitated Flag-MCRIP1, Flag-FOXP2 or Flag-FOXP1 were probed with anti-Flag antibody. Transfection of MCRIP1 interferes with the interaction between FOXP2 and CtBP2 or FOXP1 and CtBP2. (b) U2OS cells were transiently transfected as indicated. Increasing amounts of Myc-MCRIP1 expression vector were used. Flag-CtBP1 was immunoprecipitated, and co-precipitated Myc-FOXP2 and Myc-MCRIP1 were probed by anti-Myc antibody. MCRIP1 competitively inhibits FOXP2-CtBP1 interaction.

3-11. MCRIP1 modulates the promoter activities of SP-B & SP-C genes

Since Foxp1/2 cooperate with CtBP to repress the transcription of target genes[50,53], next, I investigated if MCRIP1 could modulate the promoter activities of the SP-B and SP-C genes by using luciferase reporter assays. For this purpose, lung adenocarcinoma A549 cells were utilized as a model cell line for human type II AECs[54]. Overexpression of Foxp2 significantly repressed SP-B promoter activity in these cells. Co-expression of wild-type MCRIP1 reversed the Foxp2-mediated repression of SP-B reporter activity (Fig. 32a). Similarly, overexpression of CtBP2 resulted in the repression of SP-B promoter activity and the co-expression of MCRIP1 abrogated the repression (Fig. 32b). Conversely, depletion of endogenous MCRIP1 by an siRNA targeting human MCRIP1 resulted in the repression of SP-B and SP-C promoter activities in A549 cells (Fig. 32c). The knockdown of MCRIP1 in A549 was confirmed by western blotting (Fig. 32d).

Consistent with these data, MCRIP1 depletion using two siRNAs targeting different regions of mouse MCRIP1 indeed suppressed the expression of SP-B and SP-C mRNAs in MLE12 cells, a murine type II AEC cell line[55] (Fig. 33a). Furthermore, similar results were obtained in the following CRISPR/Cas9-mediated genome-editing experiments. Two independent MCRIP1-KO MLE12 cell lines (KO#1 and KO#2) were established using two different guide RNAs (Fig. 33b). In these MCRIP1-KO MLE12 cells, the expression of SP-B protein (Fig. 33b) and SP-B and SP-C mRNAs (Fig. 33c) were suppressed. These results supported previous findings that MCRIP1 is important for the robust expression of these surfactant proteins.

Altogether, these data indicate that MCRIP1 interfered with the CtBP1/2 and Foxp1/2 interactions and thereby disrupted CtBP-mediated transcriptional repression to promote the expression of SP-B and SP-C.

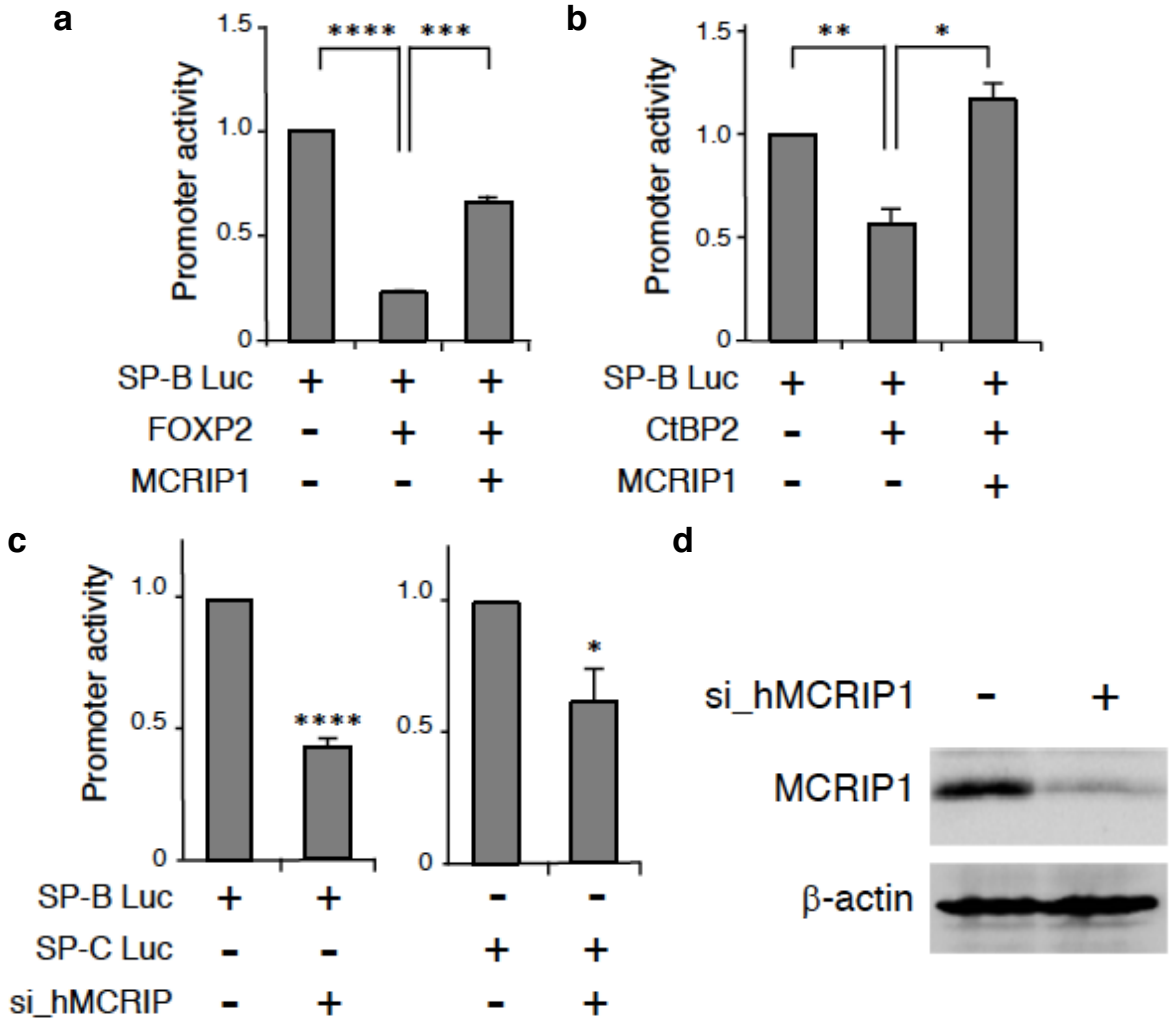


Fig. 32 MCRIP1 modulates SP-B and SP-C promoter activities

(a, b) Expression of MCRIP1 inhibits the repression of the SP-B promoter induced by Foxp2 (a) or CtBP2 (b). Human type II AECs, A549, were transfected with the SP-B-luciferase reporter plasmid (SP-B Luc) and the pMCRIP1 plasmid, together with either the pFoxp2 or the pCtBP2 expression plasmid, and SP-B promoter activity was analyzed by fold induction. (c) A549 cells were transfected with siRNA targeting human MCRIP1 gene, were cultured for 48 hours, and were then re-transfected with the luciferase reporter plasmid SP-B Luc or SP-C Luc. After an additional 48h incubation, SP-B or SP-C promoter activities were analyzed by fold induction. Data are means ± SEM from three independent experiments performed in triplicate. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

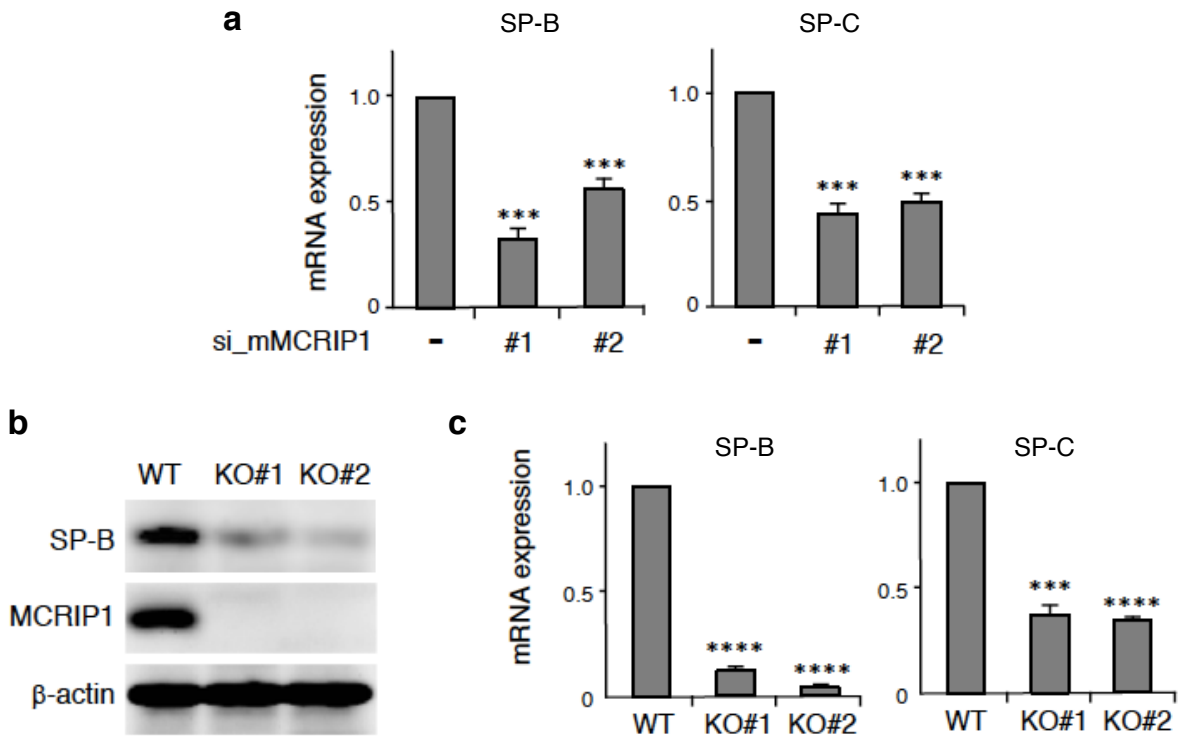


Fig. 33 MCRIP1 depletion decreases the expression of SP-B and SP-C in MLE12 murine type II AECs

(a) MLE12 murine type II AECs were transfected with siRNAs targeting mouse MCRIP1 genes (#1 or #2). 48 hours after transfection, total RNA was extracted and analyzed for endogenous SP-B or SP-C mRNA expressions using RT-qPCR. The data represent the mean ± SEM obtained from three independent experiments. ***, $p<0.001$.

(b, c) MCRIP1-KO MLE12 cells were established using the CRISPR/Cas9 approach with two different guide RNAs (KO#1 or KO#2). In (b), the expression levels of endogenous SP-B and MCRIP1 proteins were analyzed by immunoblotting. In (c), the expression levels of SP-B and SP-C mRNAs were analyzed using RT-qPCR (fold change). The data represent the mean ± SEM from three independent experiments. ***, $p<0.001$; ****, $p<0.0001$.

3-12. MCRIP1 depletion allows the recruitment of a CtBP co-repressor complex to the SP-B promoter and leads to altered histone modification

DNA-binding transcriptional repressors such as Foxp1/2 recruit CtBP and its associated chromatin-modifying enzymes including histone deacetylases (HDACs) and histone methyltransferases (HMTs) to the promoter region of their target genes, where the CtBP complex induces epigenetic gene silencing by deacetylating and methylating Lys9 of histone H3[1,56]. To investigate whether MCRIP1 affects the recruitment of CtBP to the promoter of the endogenous SP-B gene (*Sftpb*) and the consequent histone modification, chromatin-immunoprecipitation (ChIP) assays were conducted using MLE12 cells and antibodies specific to CtBP1, acetylated histone H3-Lys9 (Ac-H3K9) or methylated histone H3-Lys9 (Me-H3K9). In the wild-type control MLE12 cells, CtBP was absent from the *Sftpb* promoter, and therefore, the promoter-associated histone H3 was acetylated, but not methylated at Lys9 (Fig. 34a – c, top). In contrast, the depletion of MCRIP1 by CRISPR/Cas9-mediated gene silencing led to recruitment of CtBP1 to the promoter region of *Sftpb*, resulting in reduced acetylation of H3K9, but increased H3K9 methylation (Fig. 34a – c, middle and bottom). This phenomenon was likely to be due to the CtBP-mediated recruitment of HDACs and HMTs to the promoter, as hypothesized. In addition, the binding of Foxp1 and Foxp2 to the *Sftpb* promoter was examined. The results confirmed that both Foxp1 and Foxp2 binding to the promoter were not affected by MCRIP1 depletion in MLE12 cells (Fig. 34d).

Overall, these results indicated that MCRIP1 promoted the expression of SP-B and SP-C, which are important for robust inflation of alveolar sacs at the neonatal stage, by releasing these surfactant genes from CtBP-mediated epigenetic silencing during lung development as proposed in Fig. 35.

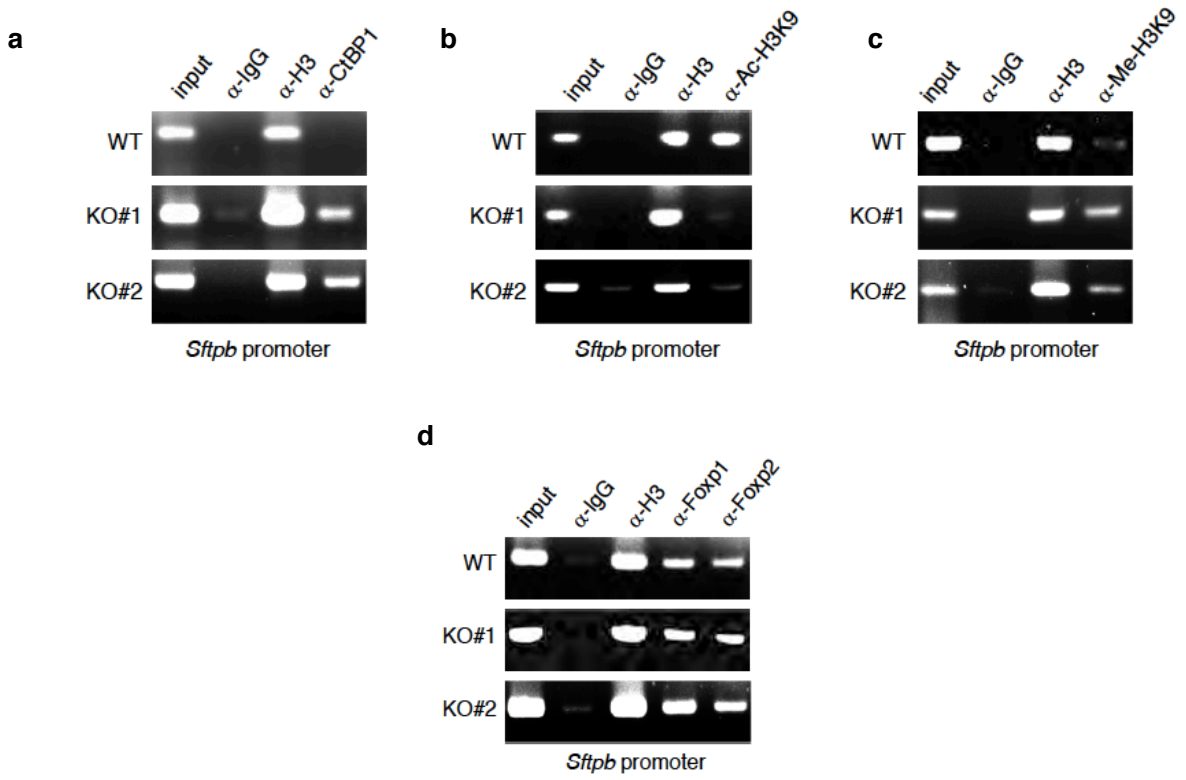


Fig. 34 MCRIP1 depletion allows the recruitment of CtBP co-repressor complex to SP-B promoter

Chromatin immunoprecipitation (ChIP) assays were performed using parental MLE12 cells (WT) and MCRIP1-KO MLE12 cells (KO#1 and KO#2).

(a – d) Cross-linked chromatin was immunoprecipitated with antibodies specific for CtBP1 (a), Ac-H3K9 (b), Me-H3K9 (c), or Foxp1/2 (d). Immmunoprecipitated DNAs were amplified with PCR primers specific for the SP-B promoter.

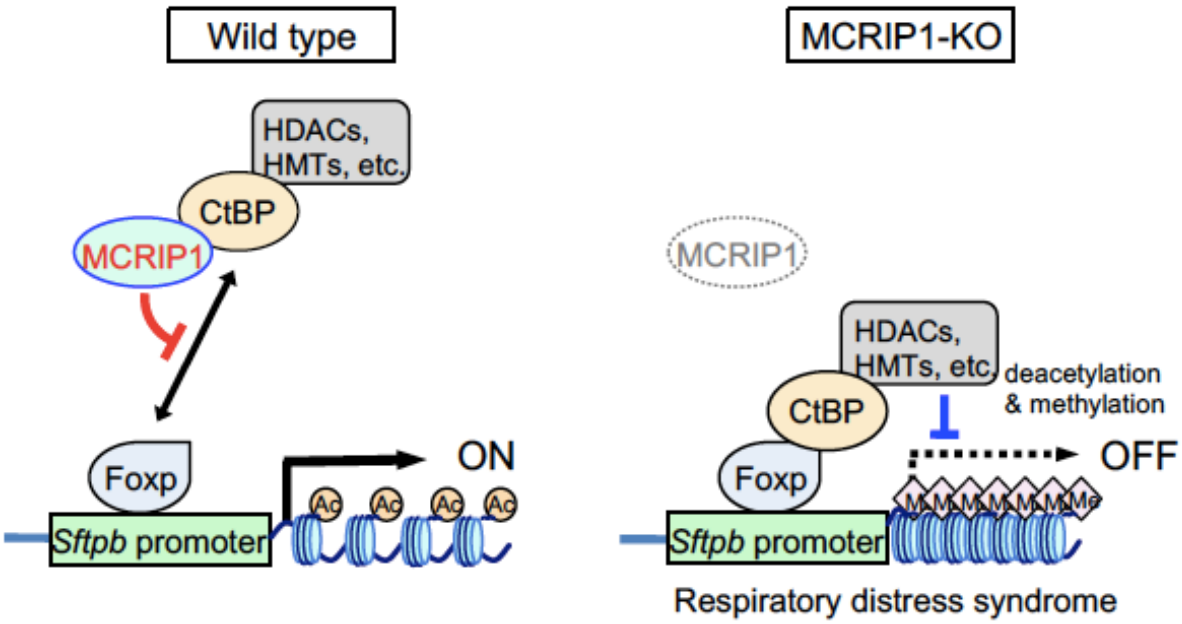


Fig. 35 A schematic model of MCRIP1 epigenetic modulation on surfactant protein activities

4. Discussion

4-1. The possibility of other transcription factors in lung development

In this study, I analyzed the physiological function of MCRIP1 by using MCRIP1-KO mouse. The results from the phenotype analysis showed decreased survival rate in MCRIP1-KO mice, possibly due to defective respiration at neonatal stage. By histological and biochemical analyses, my results suggested the cause of respiration failure is due to insufficient surfactant protein B (SP-B) and C (SP-C). In order to elucidate the molecular mechanism that contributes to the phenotypes, I examined whether MCRIP1 could regulate transcription factors through its interaction with CtBP co-repressor in lung development based on our previous study. By co-immunoprecipitation experiments, I identified potential targets, Foxp1 and Foxp2, which are regulated by MCRIP1-CtBP interaction.

However, there are a large number of transcription factors involved in lung development. In addition to Foxp1/2, Foxp4, Foxa1/2, FoxJ1, and FoxM1 from Fox protein family are also involved in lung development according to previous reports. Interestingly, a genome-wide profiling of CtBP co-repressor using breast cancer cell lines has shown that CtBP also regulates Foxa1 promoter[57]. In lung development, Foxa1 plays a role in epithelial differentiation and contributes to the downstream expressions of surfactant protein A, B, C, and D[34,58]. Indeed, MCRIP1-KO mice showed decreased mRNA expressions of SP-A, B, C and D according to my results from RT-qPCR (data not shown). Therefore, it is possible that MCRIP1 regulates transcription factors, in addition to Foxp1/2, through CtBP co-repressor during the process of lung development.

Moreover, it was reported that Foxp2 inhibits Nkx2.1-mediated transcription of SP-C[51]. Although our data indicated that MCRIP1 could inhibit CtBP-Foxp2 interaction, it is curious whether MCRIP1 also influence on its interaction with Nkx2.1, thereby contributes to suppressing expressions of surfactant proteins.

In order to fully understand to what extent MCRIP1 regulates in lung development and epithelial differentiation, a genome-wide analysis, such as RNA-seq or ChIP-seq, using lung tissues isolated from MCRIP1 mice can possibly enable us to identify more factors regulated by MCRIP1.

4-2. Foxp1/2 transcriptional repressors regulatory mechanism

Several studies have suggested that some Foxp family members including Foxp1 can function as transcriptional repressor with not only CtBP but also with other co-repressor complexes such as the nucleosome-remodeling and histone deacetylation (NuRD) chromatin-remodeling complex, and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) complex[59,60]. Furthermore, recent genome-wide analyses of Foxp1/2-target genes revealed that Foxp1/2 not only downregulate some sets of genes, but can also upregulate others by acting as transcription factors[61–64]. These findings therefore indicate that Foxp1/2 have the ability to interact with various cofactors to negatively or positively regulate their target gene expression depending on the cell type and the target promoter. Since MCRIP1 specifically binds to CtBP, it must impair the interaction of Foxp1/2 only with CtBP, and thereby it might promote the binding of Foxp1/2 to cofactors other than CtBP. Therefore, dictates the proper choice of cofactors binding to Foxp1/2 (and probably to other CtBP-binding transcriptional repressors) in developing lung epithelium in order for cells to create gene expression patterns that are necessary for normal lung development and function. Since MCRIP1 is preferentially expressed in lung epithelial cells, such MCRIP1-mediated regulation of CtBP occurs in a cell-type dependent manner. Thus, this study defines a molecular mechanism by which a single transcriptional repressor such as Foxp1/2 can exert various effects on histone modifications and the resulting gene expression depending on the cell or tissue types.

4-3. Potential compensatory effect of MCRIP2 (FAM195a)

In this study, I have identified a potential physiological function of MCRIP1 that regulates the expressions of surfactant protein B (SP-B) and surfactant protein C (SP-C) in particular. From the results of phenotype analysis, most of the MCRIP1-KO mice died of respiratory failure. Although a few number of MCRIP1-KO mice was able to survive through neonatal stage, those survived ones usually could not last until adult stage (data not shown). It is often observed in *in vivo* studies that isoforms or paralogues of specific genes share similar functions and cast compensatory effect physiologically. Indeed, MCRIP1, or Fam195b gene, also has an important paralog, MCRIP2, or Fam195a.

MCRIP1 is located on chromosome 17 in humans and chromosome 11 in mice, whereas MCRIP2 is located on chromosome 16 in humans and chromosome 17 in mice. Although the precise function of MCRIP2 is unknown, by aligning the protein

sequences, it also possess the consensus CtBP-binding motif (Fig. 36), suggesting a redundant role for these two paralogues in the regulation of CtBP. Therefore, MCRIP1 depletion might be partially compensated for by MCRIP2 in other tissues. In fact, according to the Atlas protein database, the expression level of MCRIP2 in lung is relatively low, compared with the level of MCRIP1 expression. As a future perspective of this study, the generation of MCRIP1/2 double knockout mice may deepened our understanding of MCRIP1 and MCRIP2 on their distinctive and overlapping physiological functions.

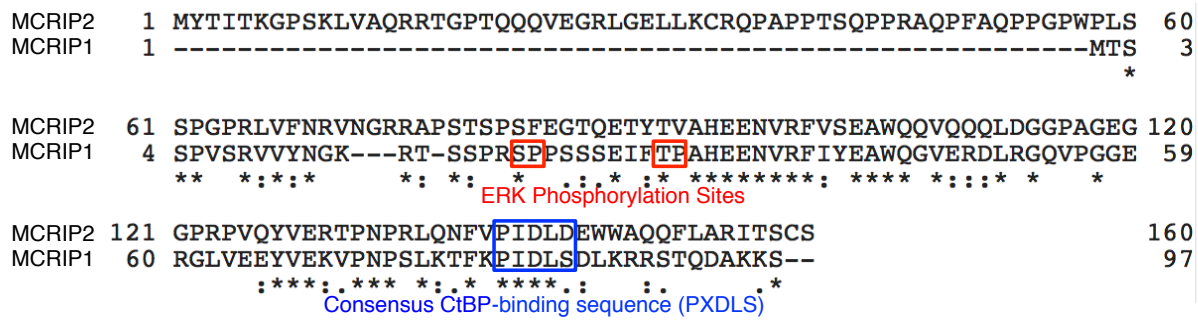


Figure 36. MCRIP1 & MCRIP2 Protein Sequence Alignment

4-4. Conclusion

In conclusion, this study demonstrated that MCRIP1 plays an important role, if not all, in promoting the production of the pulmonary surfactant proteins by regulating CtBP-Foxp1/2 interaction and chromatin remodeling at their target genes, including SP-B and SP-C. Through this regulatory mechanism, MCRIP1 contributes to the appropriate secretion of surfactant proteins for respiration. When MCRIP1 is depleted in mice, abnormal epigenetic silencing of these surfactant protein genes occurred and resulted in fatal respiratory failure at birth, which resembles human RDS. Overall, this study demonstrated a molecular mechanism by which MCRIP1 modulates specific transcriptional regulation in alveolar epithelial cells through epigenetic remodeling of gene expression during lung development.

5. Experimental Methods

5-1. Mice

The targeting strategy for generation of MCRIP1-knockout mice is indicated in Fig.16. The experiments were conducted according to the institution-approved (The Institute of Medical Science, University of Tokyo) ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments

5-2. Genotype and sextype PCR

Mouse tails are collected and the genomic DNA is extracted by using CellEase Mouse Tail (Kanto Chemical). The extracted genomic DNA is used for genotype and sextype PCR. The following PCR primers are used:

Primer	Sequence
SU f1	TTGGAGAAGCATGCACAGTC
LacZR r1	GTCTGTCCTAGCTTCCTCACTG
SUR1 r1	CATTCACCTGAGACCCACCT
Sry f1	TTGTCTAGAGAGCATGGAGGGCCATGTCAA
Sry r1	CCACTCCTCTGTGACACTTTAGCCCTCCGA
Myog f1	TTACGTCCATCGTGGACAGC
Myog r1	TGGGCTGGGTGTTAGTCTTA

For genotyping, the following PCR program is used:
95℃, 5 min → (95℃, 20 sec → 60℃, 30 sec → 72℃, 60 sec) x 35 cycles → 72℃, 5 min → 6℃, ∞
For sextyping, the following PCR program is used:
95℃, 5 min → (95℃, 20 sec → 50℃, 30 sec → 72℃, 40 sec) x 35 cycles → 72℃, 5 min → 6℃, ∞

5-3. Plasmids

MCRIP1, CtBP1, CtBP2 expression plasmids were described previously (Ref). FOXP1 and FOXP2 were sub-cloned into pcDNA3, pcDNA3-Flag, and pcDNA4-Myc expression vectors using PCR-based methods. FOXP2 mutants were constructed

using PCR-based site-directed mutagenesis. SP-B and SP-C promoter constructs were sub-cloned into pGL4-21 luciferase reporter vector (Promega).

5-4. Media and buffers

Lysis buffer used for co-immunoprecipitation experiments contained 20mM Tris-HCl (pH7.5), 10% Glycerol, 100mM NaCl, 0.5mM EDTA, 54mM b-glycerophosphate, 10mM NaF, 2mM sodium vanadate, 1mM dithiothreitol, 0.5mM phenylmethylsulphonyl fluoride (PMSF), 10mg ml^{-1} leupeptin, 10mg ml^{-1} aprotinin and 0.5% NP-40. The Lysis buffer used for mouse lung lysate extraction contained 50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 54mM b-glycerophosphate, 10mM NaF, 2mM sodium vanadate, 1mM dithiothreitol, 1mM phenylmethylsulphonyl fluoride (PMSF), 10mg ml^{-1} leupeptin, 10mg ml^{-1} aprotinin and 1.0% TritonX-100. SDS-PAGE loading buffer consisted of 65mM Tris-HCl (pH6.8), 5%(v/v) 2-mercaptoethanol, 3% SDS, 0.1% bromophenol blue and 10% glycerol.

5-5. Cell culture and transient transfection

U2OS cells were cultured in RPMI 1640 medium. A549 and MEF cells were maintained in DMEM with 4.5 g L^{-1} D-(+)-glucose. Both RPMI 1640 medium and DMEM with 4.5 g L^{-1} D-(+)-glucose medium were supplemented with 10% fetal bovine serum (FBS), L-glutamate, and penicillin-streptomycin. MLE12 murine AECII cells were maintained in DMEM/F-12 medium supplemented with 2% FBS, 10 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 30 nM sodium selenite, 10 nM β-estradiol, and 10 nM hydrocortisone, as instructed by the American Type Culture Collection (ATCC). For transient transfection, pre-seeded U2OS and A549 cells were transfected with their respective expression plasmids using the X-treme Gene 9 DNA transfection Reagent (Roche).

5-6. Electron Microscopy of Type II Alveolar Epithelial Cells (Type II AECs)

Neonatal lung tissues were isolated from *Mcrip1* mice at E21 and were cut into pieces. Cut tissues were fixed with 2.5% glutaraldehyde, 2% formaldehyde, and 0.1 M sodium phosphoate (pH 7.4) for 2 h at room temperature. After washing in 3% sucrose in 0.1 M sodium phosphate buffer, the samples were post-fixed in 1%

osmium tetroxide in 0.1 M phosphate buffer for 2 h on ice. The samples were then dehydrated in a graded series of ethanol and embedded in Epon 812 resin mixture (TAAB Laboratories Equipment Ltd., Berks, England). Samples were continuously cut with an Ultracut S ultramicrotome (Leica) into serial ultrathin sections. The resultant series of ultrathin sections were placed on a single-slot copper grid. Then, the ultrathin sections were then stained with 2% uranyl acetate in 70% ethanol for 5 min at room temperature, followed by staining in Raynold's lead for 5 min at room temperature. The stained sections were examined under a transmission electron microscope (Hitachi H-7500).

5-7. Co-immunoprecipitation assay

Cell lysates were harvested in lysis buffer described above. The appropriate antibody was added into cell lysates and incubated at 4°C for 2 hr with gentle rotation. Protein G-sepharose beads (GE) were then added and incubated at 4°C for additional 1 hr. Immunoprecipitates were collected by centrifugation and washed three times with lysis buffer. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

5-8. Immunoblotting analyses

Immunoblotting analyses were carried out as described previously.[1] Digital images were captured using the LAS-1000 Plus (Fujifilm). The following primary antibodies were used: anti-Flag M2 (Sigma), anti-Myc 9E10 (Santa Cruz), and anti-β-actin 2F3 (Wako), rabbit anti-Myc and anti-SP-B (Santa Cruz), anti-CtBP1, anti-SP-C, anti-Muc and anti-Abca3 (Abcam), anti-Foxp1 (CST), and anti-Foxp2 (Sigma). An affinity-purified anti-MCRIP1 antibody was made in-house as described previously[1]. All antibodies were used at a dilution of 1:1000 for western blotting.

5-9. Immunohistochemistry

Tissues from neonatal lungs were isolated and prepared into samples for frozen sections or paraffin sections. Sections were stained by the following primary rabbit antibodies, anti-MCRIP1 (Atlas), anti-SP-B (Santa cruz), anti-SP-C (Abcam), anti-CtBP1 and anti-CtBP2 (Abcam), anti-Foxp1 and anti-Foxp2 (CST).

5-10. RNA extraction

Mouse neonates were sacrificed according to animal experiment regulations and lung tissues were harvested. Isolated lung tissues were immediately treated with Trizol. Total RNA was isolated using the miRNeasy mini kit (QIAGEN), followed by reverse transcription using PrimeScript RT Master Mix (Perfect Real Time) (Takara). Reverse transcribed complementary DNA was used for real-time PCR quantification.

5-11. RT-qPCR analyses

Real-time PCR was performed using the following primers:

Primer	Sequence
Gapdh mus f1	CCGCATCTTCTTGTGCAGTG
Gapdh mus r1	GATGGGCTTCCCGTTGATGA
Spa f1	GAGAGCCTGGAGAAAGGGGG
Spa r1	GGATCCTTGCAAGCTGAGGA
Spb f1	CCAAGTGCTTGATGTCTACC
Spb r1	CTGGATTCTGTTCTGGCTTA
Spc f1	GTAGCAAAGAGGTCCTGATG
Spc r1	CCTACAATCACCACGACAA
Spd f1	GAGCCTGACAAACAGAGGT
Spd r1	CTGTACAAGCAAGACAAGCA
Muc1 mus f1	GTCTTCAGGAGCTCTGGTGG
Muc1 mus r1	TACCACTCCAGTCCACAGCA
Abca3 mus f1	GCATTGCCCTCATTGGAGAGCCTG
Abca3 mus r1	TCCGGCCATCCTCAGTGGTGGG

Thunderbird SYBR qPCR Mix (TOYOBO) was prepared according to instruction. Each target gene is amplified using a 2-step PCR program.

5-12. siRNA transfection experiments

A549 and MLE12 cells were transfected with siRNAs targeting human *MCRIP1* or

mouse *Mcrip1* using Lipofectamine RNAiMAX (Invitrogen). The targeting sequence was as follows:

sihMCRIP1#1, 5' – CAGAGTCGTGTACAACGGCAA – 3' (Qiagen)

simMCRIP1#1, 5' – GACCUCUGUUUCUUCGGCUTT – 3' (Sigma)

simMCRIP1#2, 5' – GUGCCUAGUGGAGGAGUAUTT – 3' (Sigma)

5-13. Dual luciferase reporter assay

Cells were seeded in a 24-well plate and transfected with the luciferase reporter plasmids: pGL4-SP-B (-3000/+450) or pGL4-SP-C (-3000/+120), pcDNA3-Foxp2, pcDNA3-CtBP2, and pcDNA3-MCRIP1 as indicated, together with Renilla luciferase plasmid as an internal control. The cells were assayed for luciferase activity 48 h after transfection, using the dual-luciferase reporter assay system (Promega).

5-14. Chromatin Immunoprecipitation (ChIP) assay

The ChIP assays were performed using the SimpleChIP Enzymatic Chromatin IP Kit (CST) according to the manufacturer's protocol. Immunoprecipitants were recovered overnight with specific antibodies or with normal rabbit IgG (CST). The purified DNA was amplified by PCR using the SP-B promoter-specific primers:

Sftpb forward, 5' – CAGACAGAAGTCATCCTTGTTGAATG – 3'

Sftpb reverse, 5' – ACATGGTACCGACTTGGCC – 3'

PCR was performed under the following conditions: 95 ^{o}C for 30 sec, 58 ^{o}C for 30 sec and 72 ^{o}C for 30 sec, and a final step of 72 ^{o}C for 5 min. The PCR products were subjected to 2% agarose gel electrophoresis.

The following rabbit antibodies were used: anti-Histone H3 (CST), anti-Pan-Methyl-Histone H3 (K9) (CST), anti-Acetyl-Histone H3 (K9) (CST), anti-CtBP1 (Abcam), anti-Foxp1 (CST), and anti-Foxp2 (Abcam).

5-15. Construction of the CRISPR/Cas9 plasmid

Target genomic DNA sequences were designed using the online design tool, CRISPRdirect (http://crispr.dbcls.jp). The following oligos were then designed according to each specific target genomic DNA sequence.

m*Mcrip1* KO#1 forward, 5' – CACCGTCTCCAGAGTTGTCTACAA – 3'

m*Mcrip1* KO#1 reverse, 5' – AAACTTGTAGACAACTCTGGAGAC – 3'
m*Mcrip1* KO#2 forward, 5' – CACCGAGTTGTCTACAACGGCAAG – 3'
m*Mcrip1* KO#2 reverse, 5' – AAACCTTGCCGTTGTAGACAACTC – 3'
The sgRNA was prepared and inserted into the pSpCas9-Puro expression plasmid according to a previously established protocol[65].

5-16. Establishment of MCRIP1-KO cell lines using the CRISPR/Cas9 system

The murine type II AEC cell line, MLE12, was transfected with a pSpCas9-puro expression vector carrying m*Mcrip1* KO#1, or KO#2 gRNA using X-treme Gene 9. Puromycin-selected cells were diluted and sub-cloned into 96-well plates in order to collect single cell clones.

5-17. Statistics

Data represent means ± SEM of at least three independent experiments. The statistical significance of the difference between mean values was tested using Student's t test, except for the distribution analysis of *Mcrip1* progeny, which was analyzed using a chi-square test.

6. Reference

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