이학석사학위논문

CORO7에 의한 Hippo 신호전달체계 조절에 관한 연구

Molecular functions of CORO7 in regulating Hippo signaling pathway

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생명과학부

박 진 아

ABSTRACT

Molecular functions of CORO7 in regulating Hippo signaling pathway

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From *Drosophila* to mammals, the Hippo pathway plays crucial roles in control of organ growth and tissue homeostasis. Through rigorous studies for the last two decades, the core kinase cassette comprised of two kinases (MST1/2 and LATS1/2) and two scaffolds (SAV1 and MOB1) has been discovered to be essential for the regulation of Hippo signaling. However, how and where its formation and activation happens are not fully understood. Here, I identify that CORO7 positively regulates the Hippo pathway through the analysis of protein interaction database and genetic screening in *Drosophila*. I demonstrate that CORO7 is necessary for Hippo signaling activation induced by serum deprivation, contact inhibition and cytoskeleton

damage. I also show that CORO7 physically interacts with Hippo core components and functions as a scaffold for the core kinase cassette. Interaction of CORO7 with SAV1 and MST1/2 is regulated by Hippo activating signals, suggesting a multi-step binding regulation of Hippo core kinase cassette by CORO7. My results provide molecular insights of Hippo signaling regulation and have implications for understanding Hippo-related diseases.

Keywords: CORO7, Hippo signaling, scaffold, protein interaction

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INTRODUCTION

The Hippo pathway functions as an essential controller of cell growth and homeostasis (Fu et al., 2017). Various upstream signals such as growth factors, mechanical stress and nutrients are identified to regulate the Hippo pathway (Meng et al., 2016; Panciera et al., 2017; Yu et al., 2015). This signaling was initially discovered in *Drosophila* to restrict organ size and its function is evolutionarily conserved in higher eukaryotes (Oh and Irvine, 2010). In mammalian system, major Hippo pathway components include two upstream kinases (MST1/2 and LATS1/2) and two scaffold proteins (SAV1 and MOB1) which form the core kinase cassette and inhibit YAP, a primary effector of the pathway (Piccolo et al., 2014; Sudol and Harvey, 2010). When the Hippo pathway is activated under unfavorable growth condition, MST1/2 binds and phosphorylates SAV1(Callus et al., 2006; Park and Lee, 2011). MST1/2 also interacts with MOB1, forming SAV1-MST1/2-MOB1 complex (Meng et al., 2016; Ni et al., 2015). LATS1/2 is then recruited to this complex and phosphorylated by MST1/2 at its hydrophobic motif (HM) (Meng et al., 2016). Although multiple mechanistic and structural studies on MOB1 revealed its function as a scaffold (Couzens et al., 2017; Kim et al., 2016a; Kulaberoglu et al., 2017; Ni et al., 2015; Vrabioiu and Struhl, 2015; Xiong et al., 2017), how SAV1 precisely regulates protein interactions of Hippo components is not fully understood. Furthermore, molecular mechanisms explaining how and where LATS1 is recruited to form

the core kinase cassette remain uncertain.

HM-phosphorylated LATS1/2 bound with MOB1 is released from MST1/2 and MOB1 induces the autophosphorylation of LATS1/2 at its activation loop, which in turn facilitates LATS activation (Manning and Harvey, 2015). Finally, LATS1/2 phosphorylates YAP, which leads its sequestration in the cytoplasm by 14-3-3 and degradation by β-TRCP E3 ligase complex (Dong et al., 2007; Zhao et al., 2010; Zhao et al., 2007). Upon dephosphorylation, YAP translocates to the nucleus and functions as a co-activator of transcription factors. YAP mainly interacts with and regulates TEAD1/2/3/4 to induce transcription of genes which promote proliferation and inhibit apoptosis (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008).

Since the Hippo pathway governs proper cell proliferation and death, deregulation of this signaling is often associated with human cancers (Harvey et al., 2013). In mouse studies, tissue-specific deletion of core Hippo pathway components or overexpression of YAP gives rise to tumors (Harvey et al., 2013). Furthermore, downregulation of Hippo components and abnormal activation of YAP have been widely observed in various human cancers (Mo et al., 2014). However, the role of the Hippo pathway in tumorigenesis could be dependent on the cellular contexts of the tumor (Lin et al., 2017). For instance, YAP has been demonstrated to have tumor-suppressive effects by inhibiting Wnt signaling in the cytoplasm (Barry et al.,

2013).

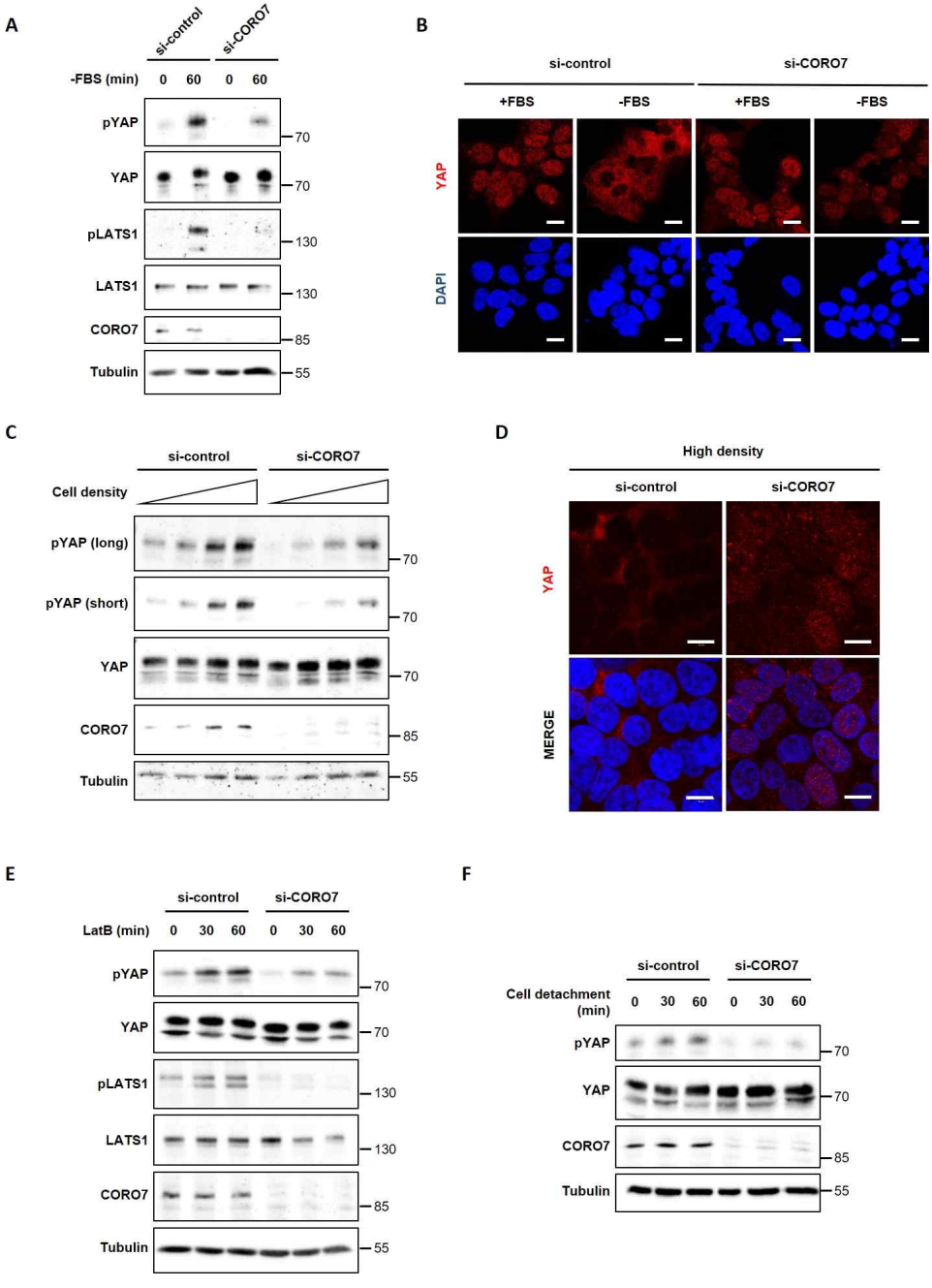
CORO7 belongs to WD40-repeat Coronin protein families (Chan et al., 2011). While other members of the family contain one, CORO7 has two WD40 domains which are generally believed to function as a scaffold to support protein-protein interaction (Xu and Min, 2011). In mammalian cells, CORO7 localizes to cytosol and trans-Golgi network (TGN) where it has roles in the maintenance of Golgi morphology and post-Golgi trafficking (Chan et al., 2011; Yuan et al., 2014). A recent study has shown that poly-ubiquitinated CORO7 is targeted to TGN and facilitate F-actin assembly and carrier biogenesis (Yuan et al., 2014). However, the function of cytosolic CORO7 and the role of its WD40 domains are largely unknown.

In this study, I identified CORO7 as a novel regulator of the Hippo pathway. Through protein interaction database analysis and RNAi screening in *Drosophila*, pod1, an ortholog of CORO7, was discovered to activate Hippo signaling. In mammalian cells, CORO7 was necessary for the activation of the Hippo pathway in response to various upstream signals. These findings indicate that CORO7 is a new member of the Hippo pathway regulating protein interactions among the core components of the signaling.

RESULTS

CORO7 is a positive regulator of the activation of the Hippo pathway in mammalian cells

To identify whether CORO7 regulates the Hippo pathway in the mammalian system, I examined the roles of CORO7 in Hippo signaling activation induced by various upstream signals. Previous research has shown that when the Hippo pathway is activated, LATS1/2 phosphorylates YAP on its Ser 127, which results in the inhibition of YAP through its sequestration in the cytoplasm (Zhao et al., 2007). Serum deprivation induced YAP-S127 phosphorylation in cells treated with control short interfering RNA (siRNA) while CORO7 knockdown significantly decreased the levels of this phosphorylation (Figure 1A). Consistent with this result, serum deprivation-induced cytoplasmic localization of YAP was also blocked by silencing of CORO7, indicating the necessity of CORO7 for repressing YAP (Figure 1B). Furthermore, CORO7 knockdown impeded Hippo signaling activation by contact inhibition, as shown by reduced YAP-S127 phosphorylation levels and nuclear YAP localization (Figure 1C and 1D).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YAP
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pYAP (short)
YAP
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130
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F
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si-CORO7
Cell detachment (min)
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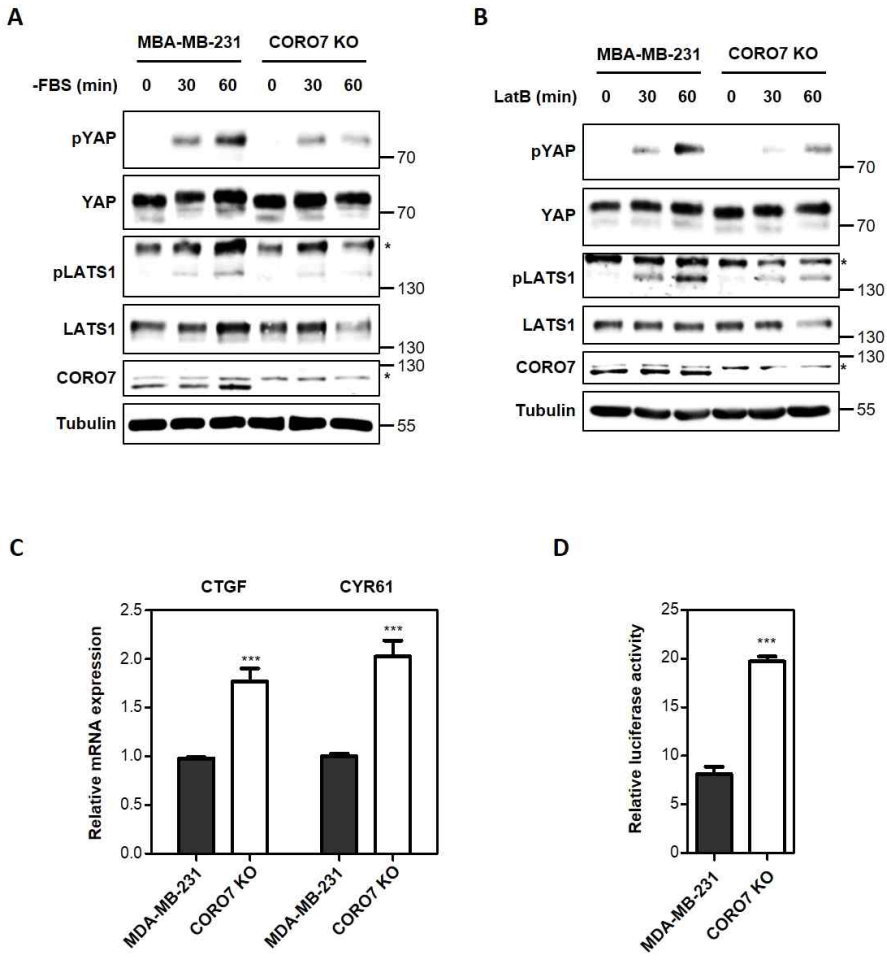
60
pYAP
YAP
CORO7
Tubulin
70
70
85
55

Figure 1. CORO7 is critical for the activation of the Hippo pathway in mammalian cells. (A) Control or CORO7-knockdown MDA-MB-231 cells were deprived of serum and collected for immunoblotting for the indicated proteins and phosphorylation. (B) Control or CORO7-knockdown HEK293T cells were cultured in media with or without serum for 1 h and fixed for immunofluorescence with YAP antibody. Cell nuclei was stained with DAPI. Scale bars, 10μm. (C) Equal numbers of control or CORO7-knockdown MDA-MB-231 cells were seeded in 24 well, 12 well, 6 well and 60mm culture plates to make the different cell density conditions 24 h before collecting and immunoblotting. (D) Control or CORO7-knockdown HEK293T cells were cultured under dense monolayer condition and subcellular localization of endogenous YAP was examined by immunofluorescence. Scale bars, 10μm. (E and F) The effect of cytoskeleton damage through Latrunculin B treatment (0.25 μg/ml) (E) or cell detachment (F) on Hippo signaling was analyzed by immunoblotting.

In recent studies, actin cytoskeleton damage was identified to activate Hippo signaling (Fernandez et al., 2011; Kim et al., 2013; Sansores-Garcia et al., 2011). As anticipated, YAP-S127 phosphorylation was induced after treatment of Latrunculin B (LatB), an actin polymerization inhibitor, or cell detachment (Figure 1E and 1F). However, loss of CORO7 diminished this phosphorylation, suggesting that CORO7 could positively regulate the Hippo pathway in response to actin cytoskeleton damage (Figure 1E and 1F). Notably, the levels of LATS1-T1027 phosphorylation induced by serum deprivation and Latrunculin B were reduced in CORO7 knockdown cells (Figure 1A and 1E). These results suggest that upon CORO7 knockdown, YAP could not be inhibited due to the inactivation of LATS1.

To further verify the role of CORO7 in proper Hippo signaling activation, I generated CORO7 knockout MDA-MB-231 cells using the CRISPR-Cas9 system. Complete deletion of CORO7 was confirmed by western blotting with CORO7-specific antibody and genomic DNA sequencing. CORO7 knockout cells showed decreased LATS1-T1027 and YAP-S127 phosphorylation levels in response to serum deprivation and treatment of Latrunculin B (Figure 2A and 2B).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YAP
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LATS1
CORO7
Tubulin
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70
130
130
130
55
C
CTGF
CYR61
Relative mRNA expression
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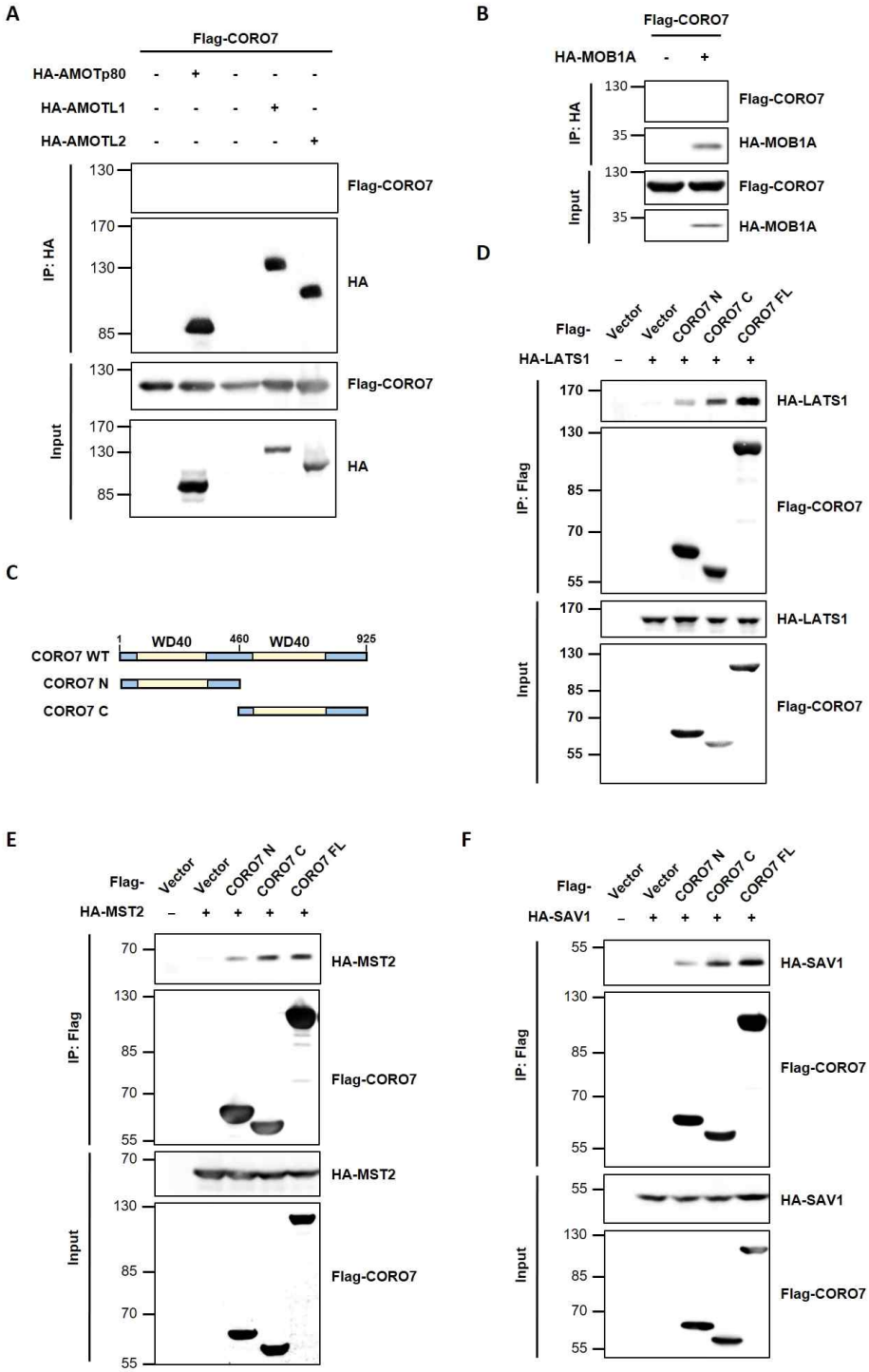
2.0
1.5
1.0
0.5
0.0
***
***
MDA-MB-231
CORO7 KO
MDA-MB-231
CORO7 KO
D
Relative luciferase activity
25
20
15
10
5
0
***
MDA-MB-231
CORO7 KO

Figure 2. Hippo signaling activation is inhibited in CORO7 KO MDA-MB-231 cells. (A and B) Immunoblots showing the indicated proteins and phosphorylation in wild-type or CORO7 knockout MDA-MB-231 cells after serum deprivation (A) or Latrunculin B treatment (B). The asterisks indicate non-specific bands. (C) mRNA levels of CTGF and CYR61 were determined by quantitative real-time PCR and normalized for alpha-actin control. The error bars represent ±s.e.m. from n=5 independent experiments. *P<0.05; **P<0.01; ***P<0.001. Student's t-test was applied. (D) Wild-type or CORO7 knockout MDA-MB-231 cells were co-transfected with pRL-TK encoding *Renilla* luciferase and 8X-TBS (8 repeats of TEAD-binding sequence) encoding firefly luciferase. 24 h after the transfection, the cells were collected and subjected to Dual-Luciferase Reporter Assay (n=3).

YAP phosphorylation results in the repression of YAP transcriptional activity. I investigated whether CORO7 affects the mRNA expression of two YAP target genes, CTGF and CYR61. As expected, their expression levels increased in CORO7 KO cells compared to wild-type cells (Figure 2C). This result was also corroborated by 8X-TBS (8 repeats of TEAD-binding sequence) luciferase reporter assay (Figure 2D). Collectively, these data demonstrate that CORO7 is necessary for the activation of the Hippo pathway under diverse conditions.

Physical interaction of CORO7 with Hippo pathway components

WD40 domain plays essential roles in several signaling pathways as a scaffold which facilitates protein-protein interactions (Xu and Min, 2011). Since CORO7 has two highly conserved WD40 domains, I tested whether it physically interacts with Hippo pathway components. Surprisingly, CORO7 co-immunoprecipitated with LATS1/2, MST1/2 and SAV1, but not with AMOT p80, AMOTL1, AMOTL2, MOB1A, indicating that CORO7 specifically interacts with a subset of Hippo pathway components (Figure 3A, 3B, 3D, 3E and 3F). To determine which WD40 domain is responsible for these interactions, I tested the N-terminal half (1-459 amino acids) and the C-terminal half (460-925 amino acids) of CORO7 (Figure 3C) using co-immunoprecipitation assay.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130
85
Flag-CORO7
HA
Input
130
170
130
85
Flag-CORO7
HA
B
Flag-CORO7
HA-MOB1A - +
IP: HA
130
35
Flag-CORO7
HA-MOB1A
Input
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35
Flag-CORO7
HA-MOB1A
C
1
WD40
460
WD40
925
CORO7 WT
CORO7 N
CORO7 C
D
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130
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HA-SAV1 – + + + +
IP: Flag
55
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HA-SAV1
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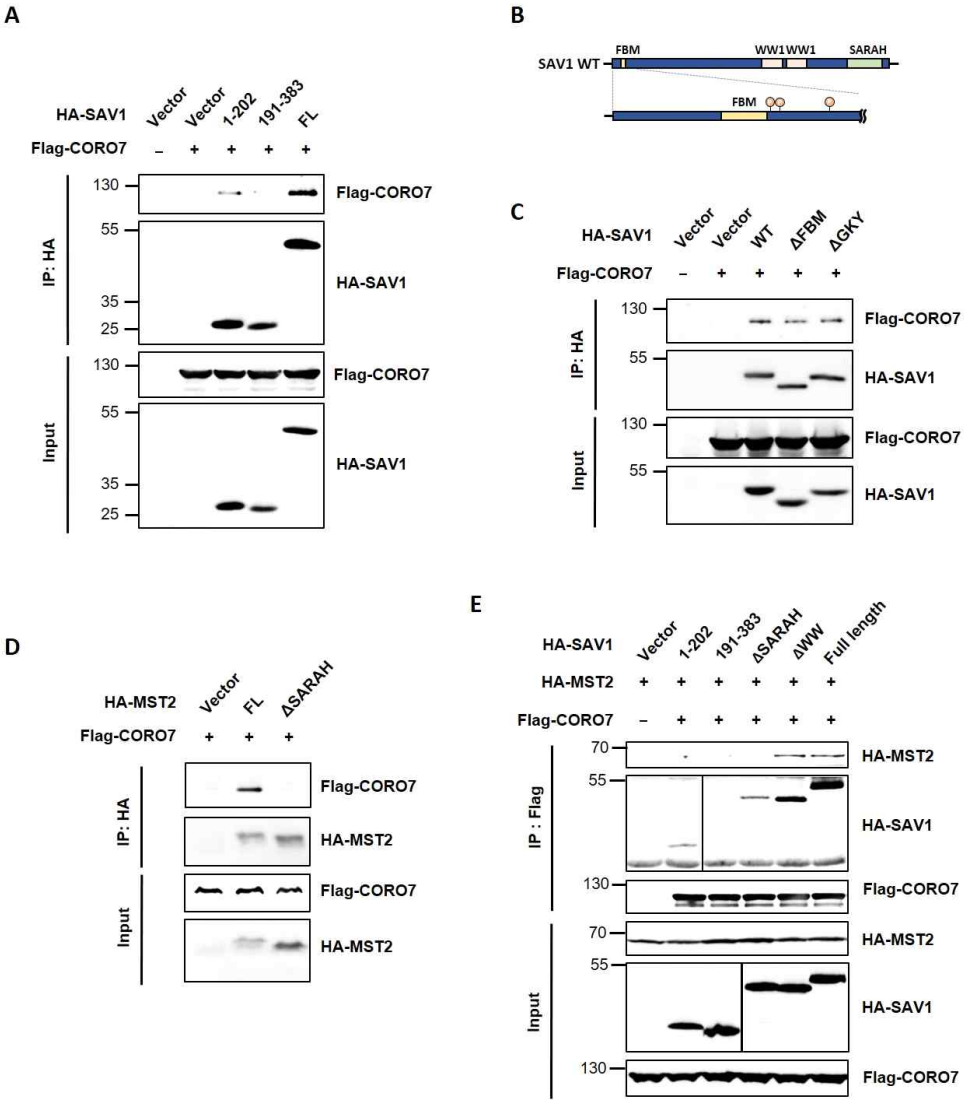
HA-SAV1
Flag-CORO7

Figure 3. CORO7 physically interacts with the components of the Hippo pathway. (A and B) Exogenously expressed AMOT p80, AMOTL1, AMOTL2 (A) and MOB1A (B) were immunoprecipitated to examine their interaction with CORO7. (C) Schematic representation of domains and truncated constructs used in co-immunoprecipitation assay (co-IP) for CORO7 (D, E and F) Overexpressed HA-LATS1 (D), HA-MST2 (E) and HA-SAV1 (F) were co-immunoprecipitated with N- or C- terminal half of Flag-CORO7 in HEK293T cells.

As shown in Figure 3D, 3E and 3F, the C-terminal half of CORO7 containing the second WD40 domain showed stronger interaction with LATS1, MST2 and SAV1 than the N-terminal half of CORO7.

To examine the domain of SAV1 involved in SAV1-CORO7 binding, I conducted co-immunoprecipitation assay with truncated forms of SAV1 and found that the N-terminal half of SAV1 (1-202 amino acids) binds to CORO7 (Figure 4A). The C-terminal half of SAV1 contains two WW domains and SARAH domain, while the N-terminal half of SAV1 involves FERM-binding motif (FBM, $SAV1^{19-25}$) which is reported to bind to the FERM domain of NF2 (Figure 4B). I observed that FBM was dispensable for SAV1-CORO7 interaction (Figure 4C). MST1/2 binds to SAV1 through its SARAH domain and phosphorylates SAV1 on several sites including Thr 26, Ser 27 and Ser 36 (Park and Lee, 2011) which are included in the CORO7-binding domain of SAV1. However, the role of this phosphorylation in the Hippo pathway has not been identified. Therefore, it is possible that SAV1 phosphorylation by MST1/2 may affect the interaction between CORO7 and SAV1. Taken together, these findings suggest that CORO7 binds to N-terminal SAV1.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WW1
WW1
SARAH
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HA-SAV1
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WT
ΔFBM
ΔGKY
D
HA-MST2
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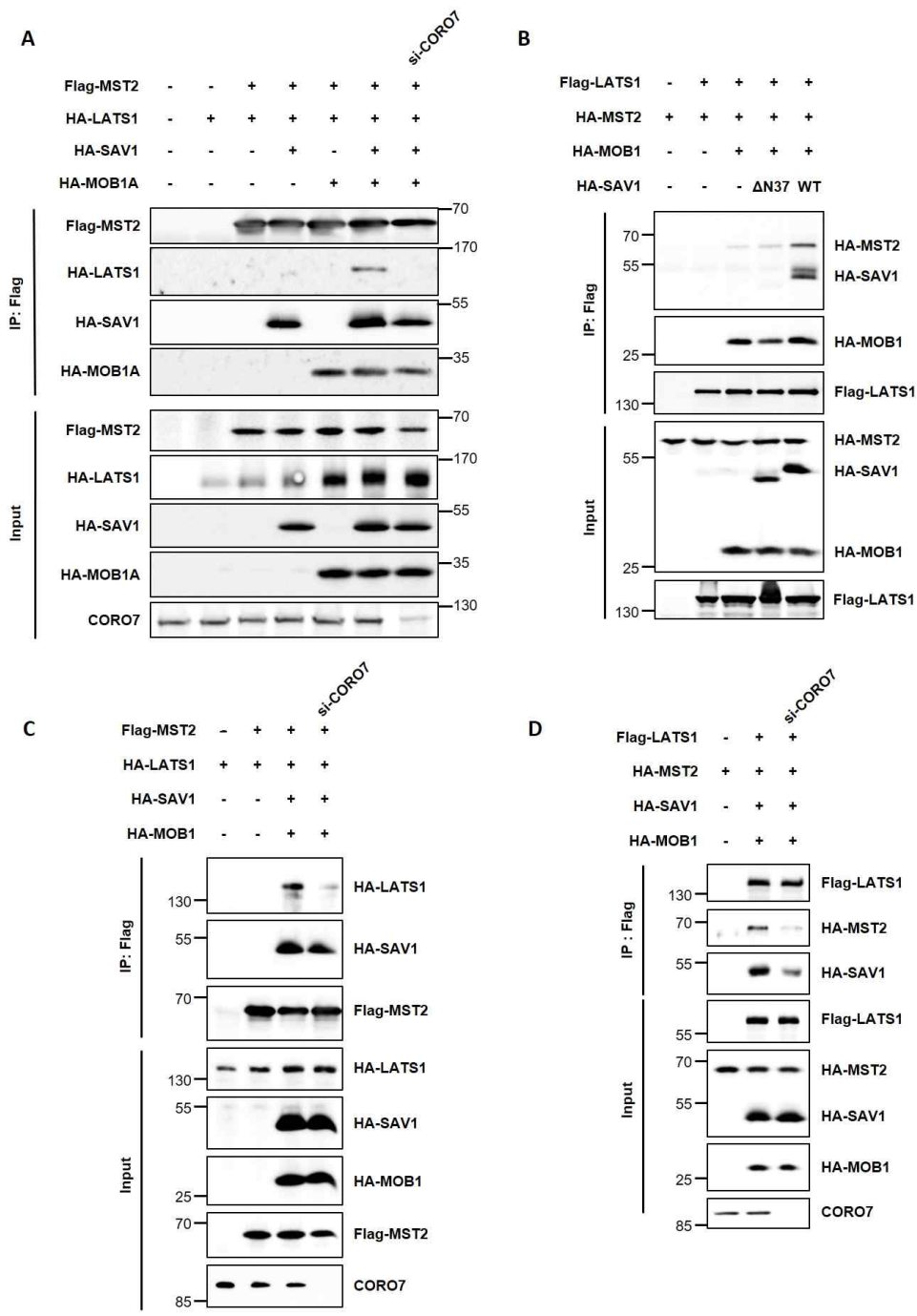
ΔSARAH
E
HA-SAV1
Vector
1-202
191-383
ΔSARAH
ΔWW
Full length
HA-MST2
IP : Flag
70
HA-MST2

Figure 4. CORO7 binds to N-terminal SAV1 and SAV1 promotes CORO7-MST2 interaction. (A) Truncated forms of SAV1 were used for co-IP assay to examine the domain responsible for CORO7 binding. (B) Schematic representation of domains and motifs of SAV1 (C) SAV1 deletion mutants of full (ΔGKYMKKD) or part (ΔGKY) of FERM-binding motif were subjected to co-IP experiments. (D) MST2 deletion mutant of SARAH domain was examined for its binding with CORO7. (E) Co-IP assay showed that co-expression of truncated forms or wild-type SAV1 affected MST2-CORO7 interaction.

As SAV1 acts as a scaffold for MST1/2, I next examined whether the formation of SAV1-MST1/2 complex affects CORO7-MST2 interaction. Indeed, SARAH domain truncated mutant of MST2 which cannot bind to SAV1 was defective in interaction with CORO7 (Figure 4D). Furthermore, co-expression of wild-type SAV1 enhanced CORO7-MST2 interaction (Figure 4E), suggesting that binding affinity of MST2 to CORO7 may increase when it forms a complex with SAV1. I also investigated which domain of SAV1 is responsible for its function as a scaffold bridging CORO7 and MST2. Co-immunoprecipitation experiment showed that the N-terminal half ($SAV1^{1-202}$) which interacts with CORO7 and SARAH domain which binds to MST2, but not WW domain, were able to increase CORO7-MST2 interaction (Figure 4E).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HA-SAV1
HA-MOB1A
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HA-SAV1
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55
HA-SAV1
HA-MOB1
25
85
CORO7

Figure 5. CORO7 is essential for the formation of the core Hippo kinase complex. (A) LATS1-MST2 interaction with or without co-expression of SAV1 or MOB1 was examined by co-immunoprecipitation assay in control or CORO7-knockdown cells. (B) HA-LATS1, HA-SAV1 and HA-MOB1 co-immunoprecipitated with Flag-MST2 were observed in wild-type or ΔN37 mutant SAV1 co-expressed HEK293T cells. (C) HA-LATS1 and HA-SAV1 co-immunoprecipitated with Flag-MST2 were observed in control or CORO7-knockdown cells. (D) HA-MST2 and HA-SAV1 co-immunoprecipitated with Flag-LATS1 were observed in control or CORO7-knockdown cells.

CORO7 is important for the formation of the Hippo core kinase cassette

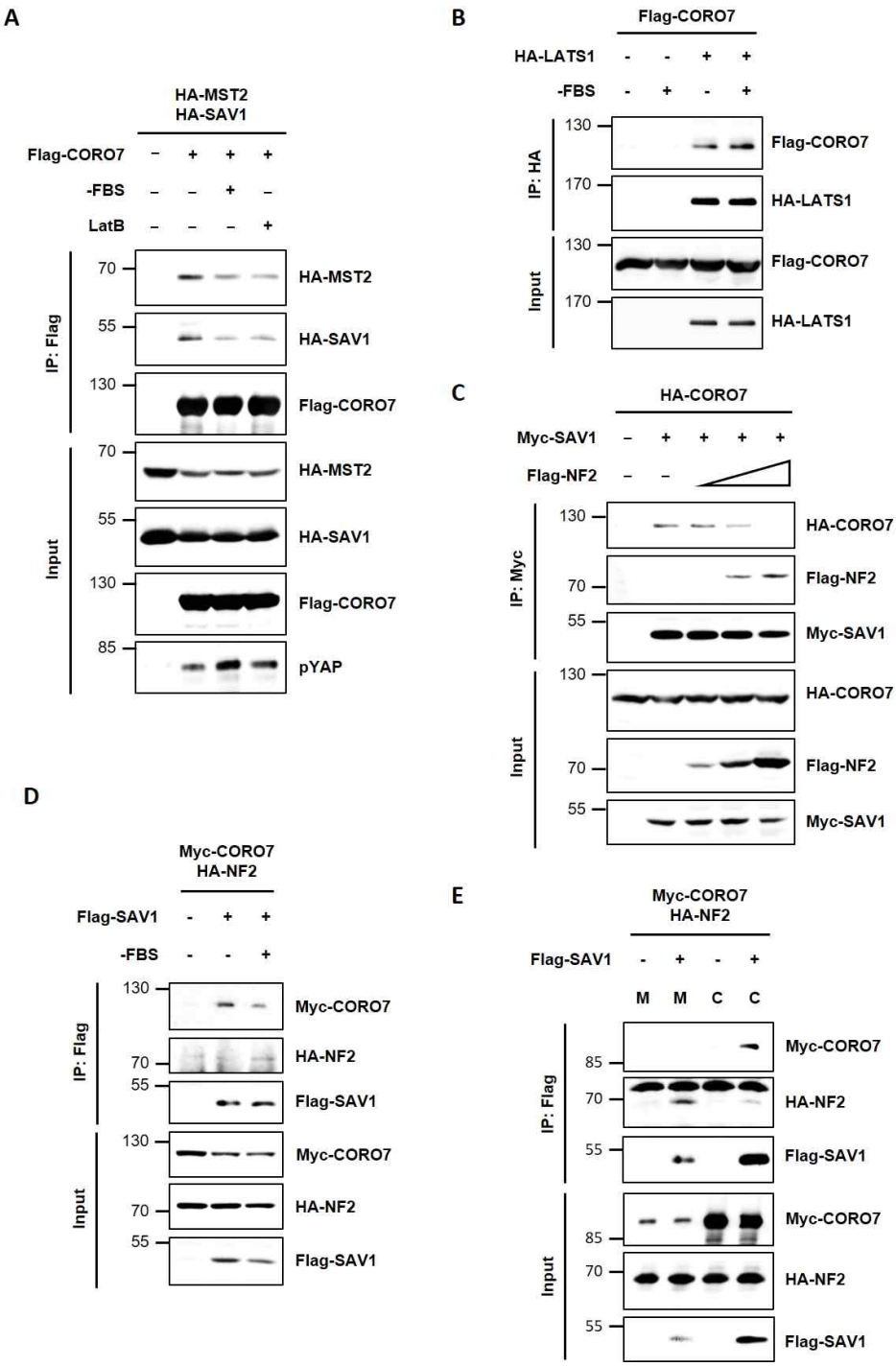
One of the most important regulatory mechanisms of the Hippo pathway is sequential phosphorylation. Hippo signaling is transduced by phosphorylation events of MST1/2-LATS1/2-YAP axis. In this process, SAV1 and MOB1 promote the formation of the Hippo core kinase cassette which is consisted of two kinases (MST1/2 and LATS1/2) and two scaffolds (SAV1 and MOB1). It has been reported that SAV1 binds to MST1/2 through its SARAH domain to activate MST1/2 (Scheel and Hofmann, 2003) and interacts with the PPXY motifs of LATS1/2 through its WW domains (Tapon et al., 2002). However, a recent structural study showed that binding between human SAV1 WW domains and a PPXY-containing peptide of LATS1 is much weaker than pMOB1-LATS1 binding (Ni et al., 2015). Furthermore, there is no direct evidence for the formation of MST1/2-SAV1-LATS1/2 ternary complex (Ni et al., 2015). Thus, I hypothesized that CORO7 could facilitate SAV1-LATS1/2 association through its binding to both SAV1 and LATS1/2. To examine this hypothesis, I performed co-immunoprecipitation assay to observe LATS1-MST2 association. As expected, LATS1 and MST2 were found to be in the same complex when both SAV1 and MOB1 were co-expressed (Figure 5A). Of note, SAV1 ΔN37 could not enhanced LATS1-MST2 interaction as wild-type SAV1, suggesting that CORO7-binding site on SAV1 is required for its scaffold function (Figure 5B). In addition, SAV1 ΔN37 could not bind to LATS1

(Figure 5B). SAV1 has been reported to bind to LATS1/2 through its WW domain, however this result indicates that N-terminal 37 amino acids on SAV1 is also necessary for SAV1-LATS1/2 interaction.

I next tested whether CORO7 is necessary for LATS1-MST2 interaction. Surprisingly, despite SAV1 and MOB1 co-expression, knockdown of CORO7 completely blocked MST2-LATS1 interaction while did not affect MST2-SAV1 interaction (Figure 5A and 5C). Furthermore, silencing of CORO7 decreased the levels of SAV1 and MST2 co-immunoprecipitated with LATS1 (Figure 5D). Taken together, these data suggest that CORO7 could act as a scaffold for LATS1 to interact with SAV1-MST2 complex.

Regulation of CORO7 interaction with Hippo components by Hippo activating signals

Serum starvation and actin depolymerization are two well-established Hippo activating signals in mammalian cells. I investigated whether these stimuli regulate the interaction of CORO7 with SAV1, MST2 and LATS1. Interestingly, when the Hippo pathway was activated by serum starvation or Latrunculin B, the levels of SAV1 and MST2 co-immunoprecipitated with CORO7 decreased (Figure 6A). In contrast, LATS1-CORO7 binding was unaffected by serum starvation (Figure 6B).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HA-SAV1
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55
HA-CORO7
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Myc-SAV1
Input
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70
55
HA-CORO7
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HA-NF2
Flag-SAV1
Input
85
70
55
Myc-CORO7
HA-NF2
Flag-SAV1

Figure 6. SAV1 changes its binding partner from CORO7 to NF2 upon the Hippo pathway activation (A) 24 h after seeded to 15% confluency, HEK293T cells were serum starved or treated with 0.25μg/ml Latrunculin B for 1 h and collected for co-immunoprecipitation assay examining CORO7-MST2 and CORO7-SAV1 interaction. (B) Similar co-IP assay was performed to determine the effect of serum starvation on CORO7-LATS1 interaction. (C) CORO7-SAV1 interaction with the increased levels of NF2 co-expression was observed by co-IP assay. (D) Similar co-IP assay as (A) and (B) was performed to determine the effect of serum starvation on SAV1-CORO7 and SAV1-NF2 interaction. (E) Cells were subjected to subcellular fractionation and immunoprecipitation to examine SAV1-CORO7 and SAV1-NF2 interaction in each fraction.

SAV1 binds to NF2 instead of CORO7 upon the Hippo pathway activation

Since CORO7-binding site on SAV1 is N-terminus which involves FBM where NF2 binds (Figure 4A), I hypothesized that CORO7 could compete with NF2 for interaction with SAV1. Indeed, binding between SAV1 and CORO7 was reduced along with the increased expression of NF2 (Figure 6C). Next, I explored the possibility that the binding partner of the N-terminal SAV1 is regulated by the Hippo pathway activation. Interestingly, SAV1 prefers to bind to NF2 over CORO7 upon the serum starvation-induced Hippo pathway activation (Figure 6D). As SAV1 translocates from the cytoplasm to the membrane in response to serum starvation or Latrunculin B treatment (Figure 7A and 7B), the binding partner of N-terminal SAV1 could be dependent on its localization. Thus, I obtained membrane and cytoplasm fractionation of cells and immunoprecipitated SAV1 from each fractionation to examine whether it binds to CORO7 or NF2. Indeed, SAV1 preferred to bind to CORO7 in the cytoplasm fraction, whereas it preferred NF2 in the membrane fraction (Figure 6E). Interestingly, the membrane association of SAV1 and LATS1 in response to Hippo pathway activation were defective in CORO7 knockout cells, indicating that CORO7 is involved in the membrane-targeting events of Hippo components (Figure 7A and 7B).

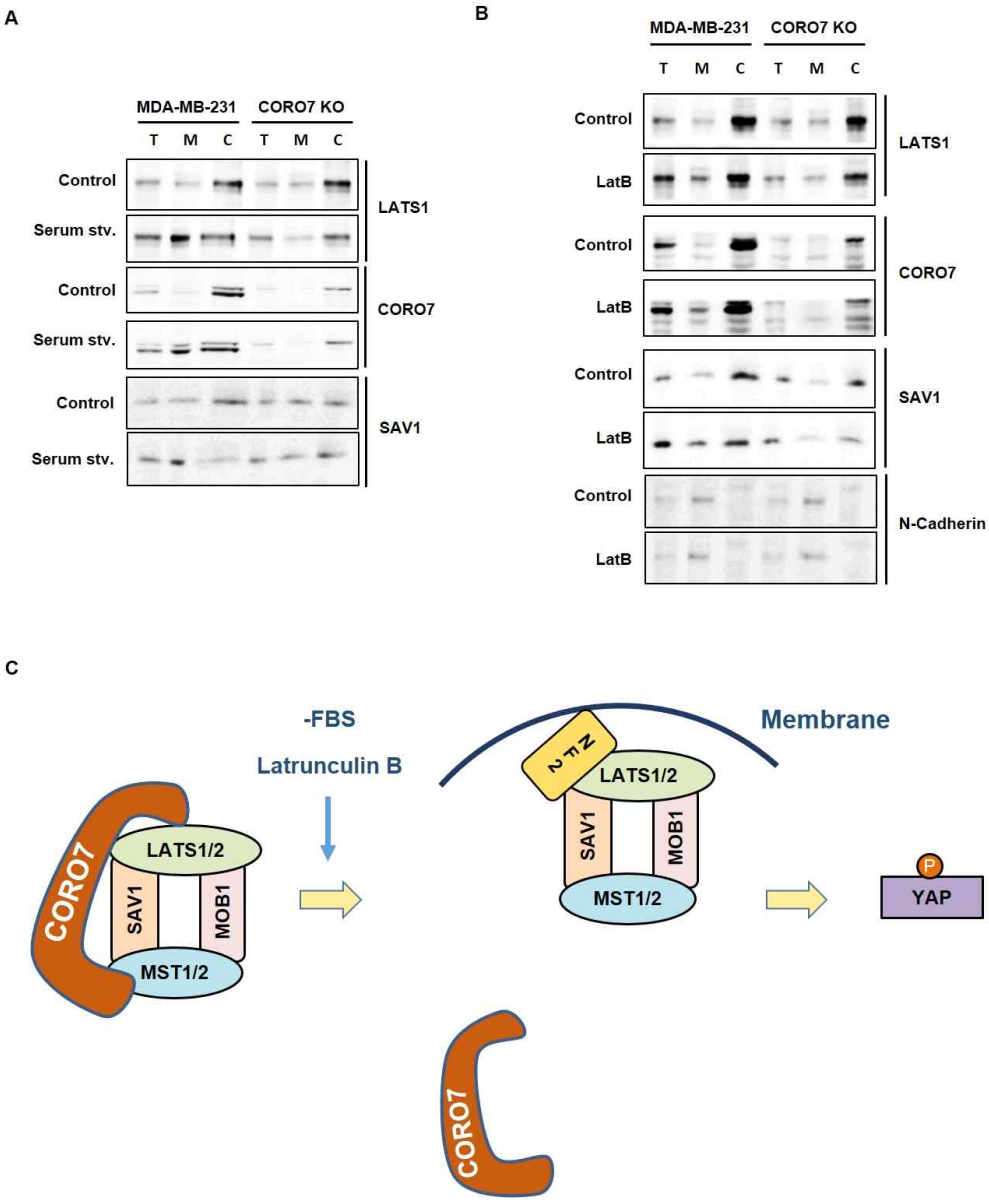
A
MDA-MB-231
CORO7 KO
T M C T M C
Control
Serum stv.
Control
Serum stv.
Control
Serum stv.
LATS1
CORO7
SAV1
B
MDA-MB-231
CORO7 KO
T M C T M C
Control
LatB
Control
LatB
Control
LatB
Control
LatB
LATS1
CORO7
SAV1
N-Cadherin
C
-FBS
Latrunculin B
CORO7
LATS1/2
SAV1
MOB1
MST1/2
Membrane
NF2
LATS1/2
SAV1
MOB1
MST1/2
P
YAP
CORO7

Figure 7. CORO7 is significant for the membrane localization of Hippo components. (A and B) Wild-type or CORO7 knockout MDA-MB-231 cells were serum starved (A) or treated with 0.25 μg/ml Latrunculin B for 1 h. Cells were subjected to subcellular fractionation and western blotting with the indicated antibodies. (C) A proposed model for protein interaction among Hippo components regulated by CORO7.

DISCUSSION

In the present study, CORO7 has been first identified as a positive regulator of the Hippo pathway. I demonstrate that CORO7 is required for the activation of Hippo signaling in mammalian cells. YAP inhibition under Hippo upstream signals is blocked upon the loss of CORO7. Through its two conserved WD40 domains, CORO7 physically interacts with SAV1, MST1/2 and LATS1/2, three major Hippo pathway components. Notably, CORO7 is required for the formation of the Hippo core kinase cassette, indicating its function as a scaffold protein.

Although CORO7 has two WD40 domains which are reported to facilitate protein interactions in several signaling pathways, the scaffold function of CORO7 was previously unknown. I propose that CORO7 activates Hippo signaling mainly through the regulation of protein interactions, which is supported by our three findings. First, CORO7 can interact with three members of the core kinase cassette. In particular, I mapped the CORO7-binding site on SAV1 where has been reported to be phosphorylated by MST1/2. Therefore, further research is necessary to investigate whether CORO7-SAV1 binding is dependent on this phosphorylation in a similar way that the WD40 domains of β-TRCP and Cdc4 recognize their substrates phosphorylated on serine and threonine (Hart et al., 1999; Orlicky et al., 2003; Winston et al., 1999).

The second evidence is that CORO7 promotes the formation of the core kinase cassette by recruiting LATS1 to SAV1-MST1/2-MOB1 complex. Without CORO7, LATS1/2 is defective in the interaction with SAV1 and MST1/2. Recently, the multi-step assembly mechanism of the core kinase cassette was proposed (Meng et al., 2016). When Hippo signaling is activated, MST1/2 makes a MOB1-docking site through the autophosphorylation at its linker region. In addition, MST1/2 also binds and phosphorylates SAV1. Next, MOB1 binds to MST1/2, which results in the open formation of MOB1 for LATS1/2. Through an unknown mechanism, LAT1/2 is recruited to MOB1-MST1/2-SAV1 complex, which forms the core kinase complex and enables the sequential phosphorylation of MST1/2-LATS1/2-YAP axis. Our findings suggest that CORO7 is the scaffold protein that is responsible for the recruitment of LATS1/2 to form the core kinase cassette through binding to both LATS1/2 and SAV1.

Finally, I proposed the binding partner switch mechanism of SAV1, which provides an additional layer of Hippo pathway regulation. Interestingly, Hippo activating signals weakens SAV1-CORO7 interaction and strengthens SAV1-NF2 interaction. Also, CORO7 and NF2 compete to bind N-terminus of SAV1. Therefore, I suggest that CORO7 regulate the Hippo pathway through two-step processes. First, CORO7 recruits LATS1/2 to SAV1-MST1/2-MOB1 complex in the cytoplasm, forming the core kinase cassette. Second, upon further Hippo activating signals, the

core kinase cassette translocates to the plasma membrane and CORO7 is released from SAV1, providing the N-terminal SAV1 binding site for NF2. Our study extends recent research that has identified the role of NF2-SAV1 interaction in Hippo signaling activation (Yin et al., 2013).

In addition to its scaffold function, CORO7 affects LATS1 stability upon Hippo activating signals. I demonstrated that when CORO7 is depleted, LATS1 levels decrease under Hippo signaling-activated conditions. LATS1 also constitutively binds with CORO7 regardless of Hippo activating signals. I note that LATS1 stability control by E3 ubiquitin ligases such as ITCH and NEDD4 is one of the key regulatory mechanisms of Hippo signaling (Ho et al., 2011; Salah et al., 2013; Salah et al., 2011). Therefore, I speculate that CORO7 may play a protecting role for LATS1 against Hippo signaling activation- induced degradation as a negative feedback mechanism. Furthermore, serum deprivation or cytoskeleton damage induced-membrane association of Hippo pathway components (SAV1 and LATS1) is blocked in CORO7 knockout cells. It has been reported that NF2 and SAV1 participate in the membrane association of LATS1/2 and MST1/2, respectively (Yin et al., 2013). Our results indicate that CORO7 is also involved in the membrane targeting of Hippo pathway components. Abnormal formation of the core kinase cassette in CORO7 knockout cells may be the cause for the defective targeting if the core kinase cassette formation is a prerequisite for the membrane association of the cassette.

Although CORO7 appears to be a critical regulator of the Hippo pathway, upstream molecules that control the function of CORO7 remain unknown. Other members of Coronin protein family bind actin and modulate actin-dependent processes (Chan et al., 2011). Furthermore, it has been reported that CORO7 regulates Golgi morphology and trafficking which are linked to the actin cytoskeleton (Yuan et al., 2014). Despite the controversy over the binding between mammalian CORO7 and actin(Chan et al., 2011), actin might be a potential regulator of CORO7 in the context of Hippo signaling. It is worth noting that CORO7 is necessary for Hippo signaling activation by serum deprivation, contact inhibition and Latrunculin B treatment which are stimuli thought to be mediated by actin cytoskeleton. Interestingly, the actin cytoskeleton dynamics is thought to be involved in Hippo signaling activation by all of these stimuli (Gaspar and Tapon, 2014; Panciera et al., 2017). Considering the wide range of Hippo upstream signals to which CORO7 can response, CORO7 may sense actin cytoskeleton remodeling to regulate Hippo signaling. Therefore, further research confirming the possibility that actin cytoskeleton is the direct upstream regulator of CORO7 is necessary for the thorough understanding of Hippo signaling regulation by CORO7.

Taken together, I demonstrate that CORO7 is an evolutionarily conserved member of the Hippo pathway. Despite extensive studies which have tried to understand Hippo signaling regulation, how and where various upstream stimuli including growth factor, mechanical

cues and cell attachment converge on Hippo core components possibly through the actin cytoskeleton remain elusive. Thus, further studies on precise integration mechanism of the Hippo pathway will be significant for the understanding of the physiological regulation of the signaling and offer insights into more effective therapeutic strategies against Hippo pathway-related diseases.

MATERIALS and METHODS

Cell culture

HEK293T and MDA-MB-231 cells were maintained in DMEM containing 10% fetal bovine serum. For serum deprivation, LatB (0.25 μg/ml) treatment and cell detachment experiments, cells were seeded to low confluency (less than 30%). Plasmids were transfected with the polyethylenimine reagent. HEK293T and MDA-MB-231 cells were kindly provided by J Blenis and DS Lim, respectively.

Expression constructs and RNAi experiments

The mammalian expression plasmids for human LATS1/2, MST1/2, SAV1, MOB1A, KIBRA, AMOT p80, AMOTL1, AMOTL2 were kind gifts from DS Lim. CORO7 plasmid was purchased from Sino Biological and cloned in pcDNA3 HA and pcDNA3.1 Flag vector. Plasmids for truncated forms of genes were generated by PCR and cloned in pcDNA3.1 HA- or pcDNA3.1 Flag expression vectors. LATS1 truncated mutants were kind gifts from NG Kim. All SAV1 and CORO7 mutants were made by site-directed mutagenesis. All of the constructs generated were confirmed by DNA sequencing. For RNAi experiments, cells were treated with indicated siRNA for 72 h using RNAiMAX reagent (Thermo Fisher Scientific) and collected for further examination. Decreased expression of the target protein was verified by the specific antibody. CORO7 siRNA sequence is 5'-CACCUUGUGUCUACUGGAU-3'

Immunoblotting

Cells were lysed in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10% glycerol and protease inhibitors). Equivalent protein quantities were subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA for 1 h at room temperature and then probed with the indicated primary antibodies, followed by the appropriate HRP-conjugated anti-mouse/rabbit secondary antibodies (Jackson ImmunoResearch). Immuno-reactive bands were visualized with ECL.

Immunoprecipitation

Cells were collected and lysed in 0.5 ml lysis buffer plus protease inhibitors (Pepstatin A, PMSF and Leupeptin) for 30 min at 4 °C. After 12,000*g* centrifugation for 15 min, the lysates were immunoprecipitated with 2 μg specific antibody overnight at 4 °C, and 30 μl A/G agarose beads were washed and then added for an additional 1 h. Thereafter, the precipitants were washed five times with lysis buffer, and the immune complexes were boiled with SDS loading buffer for 5 min and analysed by SDS-PAGE. The following antibodies were used for IP: HA (MBL life science, M132-3), Flag (MBL life science, M185-3L), myc (MBL life science, M192-3), CORO7 (Abcam, ab117446), YAP (Santa-Cruz, sc-101199), pYAP (CST, #4911), LATS1 (CST, #3477), pLATS1 (CST, #8654), Tubulin (DSHB, E7).

Immunofluorescence microscopy

Cells were seeded on coverslips. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.1% Triton X-100. After blocking in goat serum for 1 h, slides were incubated with primary antibody for 1 h at room temperature or at 4 °C overnight, washed 3 times with PBS, and then incubated with FITC- or TRITC-conjugated secondary antibodies (Invitrogen, 1:1,000) and DAPI for 1 h at room temperature. Antibodies against YAP (1:100, sc-101199) were used for immunofluorescence. The slides were then washed 3 times with PBS and mounted. Cell images were captured with a confocal microscope (Zeiss).

RNA isolation and real-time PCR

Total RNA was isolated from cultured cells using Trizol reagent. cDNA was synthesized by reverse transcription using oligo (dT) and subjected to real-time PCR with *CTGF*, *CYR61*, and *ACTB* primers in the presence of Cyber green PCR-Mix (Bioneer). Relative abundance of mRNA was calculated by normalization to *ACTB* mRNA. The following primer pairs were used to detect the mRNA levels of the following genes by RT-qPCR: *CYR61* (5′-CTCGCCTTAGTCGTCACCC-3′ and 5′-CGCCGAAGTTGCATTCCAG); *CTGF* (5′-ACCGACTGGAAGACACGTTTG-3′ and 5′-CCAGGTCAGCTTCGCAAGG-3′); *ACTB*: (5′

-CATGTACGTTGCTATCCAGGC-3′ and 5′ -CTCCTTAATGTCACGCACGAT-3′). Data were analysed from five independent experiments and are shown as the average mean ± s.e.m.

CRISPR

CRISPR genomic editing technology was used for the deletion of CORO7. The guide RNA sequences were cloned into the plasmids px459 (addgene 62988) purchased from Addgene. The constructed plasmids were transfected into MDA-MB-231 cells. 24 h after transfection, the transfected cells were enriched by 1 μg ml^{-1} puromycin selection for 2 days and then were sorted onto 96-well plates with only one cell in each well. The clones were screened by Western blot with gene-specific antibodies. The guide RNA sequences were 5'-CACCGGCTTCAGGGTGTCCAAGTTC-3'.

Luciferase assay

MDA-MB-231 cells were cultured in 6-well dishes and transfected with 0.4μg 8X-TBS luciferase, 20 ng pRL-TK using Lipofectamine3000. Reporter activities were measured using a Dual Luciferase Kit (Promega) according to the manufacturer's guides.

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국문 초록

CORO7에 의한 Hippo 신호전달체계 조절에 관한 연구

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초파리에서부터 인간에 이르기까지 Hippo 신호전달체계는 기관의 성장과 조직 항상성의 유지에 중요한 역할을 수행한다. 지난 20여 년간의 활발한 연구를 통해 두 인산화효소 (MST1/2 와 LATS1/2)와 두 scaffold (SAV1 and MOB1)로 구성된 중심 인산화효소 복합체가 Hippo 신호전달체계를 조절하는 데 필수적이라는 것이 밝혀졌다. 하지만 우리는 이 복합체가 어디에서 어떻게 형성되고 활성화되는지 충분히 이해하지 못한다. 이 연구에서 나는 단백질 상호작용 데이터베이스 분석과 초파리 유전자 스크리닝을 통해 CORO7이 Hippo 신호전달체계를 양성적으로 조절한다는 것을 밝혔다. 포유동물 세포에서 CORO7이 혈청 부족, 세포 간 접촉 그리고 세포골격 손상에 의해 Hippo 신호전달체계가 활성화되는데 필요하다는 결과를 통해 CORO7이 여러 상위 신호를 인지하여 Hippo 신호전달체계를 조절한다는 것을 규명하였다. 또한 나는 CORO7이 Hippo 신호전달체계 구성요소들과 물리적으로 결합한다는 것과 중심

인산화효소 복합체 형성을 위한 scaffold 역할을 한다는 것을 보였다. 특히, SAV1-MST2와 CORO7의 결합이 Hippo 신호전달체계의 상위 신호에 의해 조절된다는 것을 보여줌으로써 CORO7이 순차적인 중심 인산화효소 복합체 구성요소들 사이의 상호작용을 조절한다는 것을 발견했다. 이번 결과는 Hippo 신호전달체계의 조절에 대한 분자적 기작과 Hippo 신호전달체계와 관련된 인간 질병을 더욱 깊게 이해하는 데 기여할 것이다.

주요어: CORO7, Hippo 신호전달체계, scaffold, 단백질 상호작용
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