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Preliminary Study on How Tumor Suppressor Nf2 Inhibits Transcriptional Coactivators Yap/Taz in the Developing Mouse Brain

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Preliminary Study on How Tumor Suppressor Nf2 Inhibits Transcriptional Coactivators Yap/Taz in the Developing Mouse Brain

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ABSTRACT

Normal brain development requires precise coordination of neural progenitor proliferation and differentiation, the mechanism of which is not well known. Recently the tumor suppressor neurofibromatosis 2 (Nf2) was shown to regulate the balance of neural progenitor proliferation and differentiation in the developing mouse brain through the Hippo pathway effectors, transcriptional coactivators Yap/Taz. The molecular mechanism of how Nf2 regulates Yap/Taz is not understood. Here I showed that Nf2 regulated the Yap/Taz activity by decreasing the stability of Yap/Taz. The regulation was independent of Yap-S366 phosphorylation, which is required for Yap degradation. I also showed that Nf2 did not regulate Lats1/2 kinases activity. Finally I found Nf2 interacted with Yap in mouse embryonic brain and identified the domains that were required for Nf2-Yap interaction. My study suggests that Nf2 may regulate Yap/Taz independent of the canonical Hippo pathway in the developing mammalian brain.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AJ	adherens junction
GPCR	G-coupled protein receptor
IP	immunoprecipitation
Nf2	Neurofibromatosis 2
NPC	neural progenitor cell
TJ	tight junction
WT	wildtype

CHAPTER 1. INTRODUCTION

Cerebral Cortex Development

The mammalian cerebral cortex is a multilayered structure derived from the neural tube (Gao et al., 2013; Gotz and Huttner, 2005). Development of the cerebral cortex requires the precise coordination of cell proliferation, differentiation and migration (Doe, 2008). In the earliest stage, the neural tube is composed of a single layer of neuroepithelial cells that expand through symmetric divisions. A small fraction of neuroepithelial cells undergo asymmetric divisions and generate the first wave of neurons that migrate out and form the structure called preplate. As development continues, neuroepithelial cells downregulate certain features like tight junction and upregulate astroglial markers and give rise to more fate-restricted radial glial cells, which replace all neuroepithelial cells and act as the major population of neural progenitor cells (NPCs). Radial glial cells are bipolar, extending the short apical process to the ventricular lumen and the long basal process to the pial surface. They undergo symmetric divisions to expand NPC pool and asymmetric divisions to generate postmitotic neurons or intermediate progenitors (Fish et al., 2008; Gotz and Huttner, 2005; Noctor et al., 2001). Intermediate progenitors localize and divide in the subventricular zone, basal to the ventricular zone, where radial glial cells localize. Ninety percent of intermediate progenitors divide once to generate two neurons while the remainder divides twice to generate four neurons in rodents (Haubensak et al., 2004; Kowalczyk et al., 2009; Noctor et al., 2004). Newborn neurons migrate along the radial glial fiber, split the preplate into a superficial marginal zone and a deeper subplate, and reside between them creating the cortical plate, where the future cortex will form. Neurons generated later migrate past the first layer and occupy more superficial position, forming layers II-VI. Thus the cortical layers are generated in an inside-out pattern (Angevine and Sidman, 1961).

The balance of NPC expansion and differentiation is critical for normal brain development. The genetic disorder, autosomal recessive primary microcephaly (MCPH), is characterized by a reduced brain volume and mental retardation. Mutations in nine genes have been identified to cause MCPH through reducing NPC proliferation in the cortex (Noatynska et al., 2012). On the other hand, mutations in genes which cause increased NPC proliferation lead to megalencephaly, a disease with excessively big brains (Leventer et al., 2010). Thus it is important to understand the molecular mechanisms underlying the regulation of NPC expansion and differentiation.

Currently the well-characterized pathways in regulating NPC expansion and differentiation are the Wnt, Notch and Sonic Hedgehog (Shh) pathways (Doe, 2008; Lui et al., 2011). The effector in Wnt pathway is the transcription factor β-catenin. Blocking the transcriptional activity of β–catenin in mouse embryonic development by a dominant negative version of TCF4, which interferes β–catenin/TCF4 complex formation, results in premature NPC differentiation (Woodhead et al., 2006). On the contrary, transgenic mice that express stabilized β–catenin, have an expanded NPC population leading to folds resembling sulci and gyri of gyrencephalic brains (Chenn and Walsh, 2002).

Therefore, Wnt signaling promotes NPC expansion and inhibits differentiation. However, the function of Wnt signaling may be context-dependent, as some studies find Wnt signaling promotes NPC differentiation through N-myc in cortex development (Kuwahara et al., 2010; Munji et al., 2011).

Notch signaling also promotes NPC expansion and inhibits differentiation. Mutations in Notch signaling components *Notch1*, *Hes1* and *Hes5* (Notch signaling effectors) in mouse embryos all result in depletion of NPC pool and premature neuronal differentiation (de la Pompa et al., 1997; Hatakeyama et al., 2004). Conversely, activation of Notch signaling by activated *Notch1* or misexpressed *Hes1* and *Hes3* inhibits neuronal differentiation (Chambers et al., 2001; Ishibashi et al., 1994).

Shh signaling seems to maintain the identity of NPCs in cerebral cortex development as Wnt and Notch signaling. Conditional knockout of *Shh* or *Smo* in mouse cortex leads to decreased NPC proliferation due to longer cell cycle length (Komada et al., 2008). In contrast, abnormal activation of Shh signaling in *α-catenin* knockout mice causes increased proliferation and cortex hyperplasia (Lien et al., 2006).

The Hippo Signaling Pathway

Although much progress has been made to understand the molecular mechanisms regulating NPC expansion and differentiation in the mammalian cerebral cortex, we are still not fully clear about the mechanisms. In recent years, one signaling pathway, the Hippo signaling pathway, emerged to be an important pathway in regulating cell growth, proliferation and apoptosis in *Drosophila* and mammals (Zhao et al., 2010a). The Hippo pathway is involved in diverse physiological functions, such as organ growth control, stem cell function, regeneration and tumor suppression (Halder and Johnson, 2011; Pan, 2010; Zhao et al., 2011b). The pathway is deregulated at a high frequency in diverse human cancers including lung, colorectal, ovarian and liver cancer (Harvey et al., 2013).

Central to this pathway is a kinase cascade, whereby the Ste20-like kinase Hippo (Hpo) (Mst1/2 in mammals) with its cofactor Salvador (Sav) (Sav1 in mammals) phosphorylates and activates the NDR family kinase Warts (Wts) (Lat1/2 in mammals) and its cofactor Mats (Mob1 in mammals), which in turn phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki) (YAP/TAZ in mammals) by sequestering the latter in the cytoplasm. Once Yki enters the nucleus, it mainly binds to TEAD/TEF family transcription factor Scalloped (Sd) (TEAD1−4 in mammals) and activates downstream target genes involved in proliferation and anti-apoptosis (**Figure 1-1**).

The core Hippo pathway is well characterized, but the regulatory mechanisms for this pathway is less known (Yu and Guan, 2013). Three groups of upstream components have been identified to regulate the Hippo pathway in mammals: adherens junctions (AJ) and tight junctions (TJ), G-protein coupled receptors (GPCRs) and mechanical cues (**Figure 1-2**).

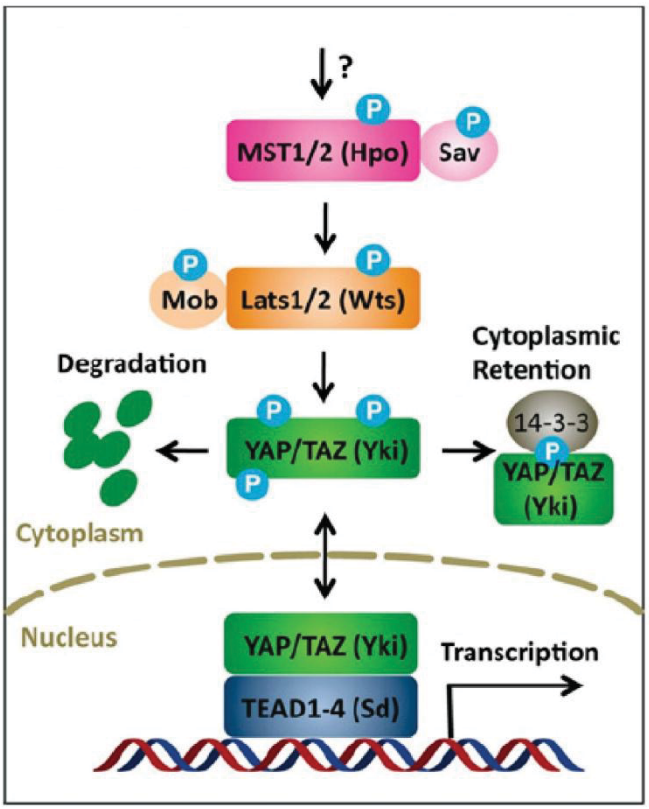

Figure 1-1. The core Hippo pathway
MST1/2 phosphorylates Sav, Lats1/2, and Mob; Lats1/2 phosphorylates YAP/TAZ; and phosphorylated YAP/TAZ interacts with 14-3-3 and results in cytoplasmic retention. Moreover, YAP/TAZ phosphorylation leads to protein degradation. When dephosphorylated, YAP/TAZ enter the nucleus and induce gene transcription by interacting with transcription factors TEAD1–4. *Drosophila* orthologs for these core components are shown in parenthesis.

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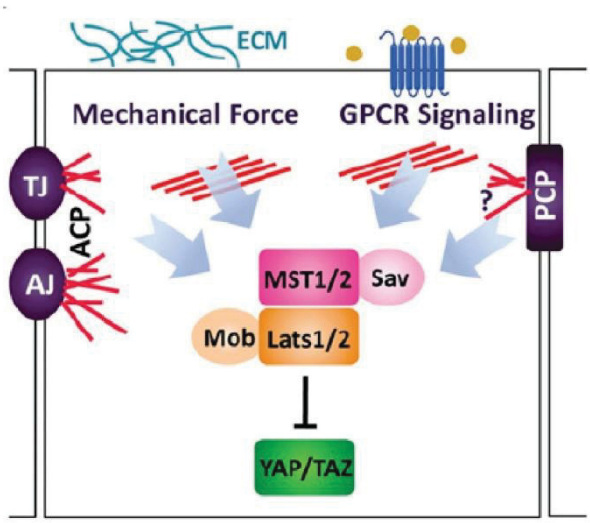

Figure 1-2. Regulatory mechanisms for the Hippo pathway
Regulation of the Hippo pathway by apical–basal polarity, GPCR signaling and mechanical cues. Red lines represent actin filaments. PCP in the figure represents planar cell polarity, which regulates Hippo pathway in *Drosophila* but not in mammals.

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Epithelial cells adhere to each other through cell-cell junctions such as AJs and TJs. Many upstream regulators of the Hippo pathway are components in AJs and TJs, such as TJ component angiomotin proteins, AJ component α-catenin. Angiomotin proteins can bind to and sequester YAP in TJs through phosphorylation-independent mechanism or bind to Lats1/2 and inhibit YAP through phosphorylation-dependent mechanism (Paramasivam et al., 2011; Zhao et al., 2011a). In keratitinocytes, α-catenin interacts with YAP through the mediator 14-3-3 and sequesters YAP in AJs and thus inhibits YAP activation (Schlegelmilch et al., 2011).

GPCRs are the largest family of plasma membrane receptors that can be activated by a wide range of ligands. One paper shows that LPA in serum binds to GPCRs and activates YAP in cultured cells through Rho GTPases (Yu et al., 2012) . GPCRs can activate or repress YAP activity: GPCRs that activate $G_{12/13}$, $G_{q/11}$, or $G_{i/o}$ activate YAP activity, while GPCRs that mainly activate G_s signaling repress YAP activity.

Cells can sense mechanical cues from the extracellular matrix (ECM), cell-cell junctions or surrounding fluids, and remodel the cytoskeleton to generate diverse responses. Several recent studies found that, in response to mechanic cues, F-actin in cultured cells remodels and leads to activation or repression of YAP/TAZ activity (Aragona et al., 2013; Dupont et al., 2011; Wada et al., 2011). In these studies, strong cellular mechanical force in conditions like stiff ECM, stretched cell shapes or cells at the

edge of multicellular sheets induces YAP activation, while weak mechanical force such as soft ECM, compact cell shapes or cells at the center of contact-inhibition multicellular sheets represses YAP activation. It seems that the regulation of YAP/TAZ by mechanical cues is independent of the core Hippo kinase cascade (Aragona et al., 2013).

Neurofibromatosis 2 (Nf2)

Although many upstream regulators for the Hippo pathway have been characterized, no study has identified the upstream regulators for the Hippo pathway during mammalian cerebral cortex development. Our lab focuses on the function of one gene called *neurofibromin 2* (*NF2* or *merlin*, encoding protein Nf2 or merlin) in mouse cerebral cortex development. Inactivating mutations in *NF2* gene cause autosomal dominant disorder Neurofibromatosis Type 2, characterized by schwannomas, meningiomas and ependymomas. It also contributes to a large proportion of sporadic schwannomas, meningiomas and a small proportion of sporadic ependymomas (Hanemann, 2008). Nf2 is related to the ERM (ezrin,radixin, moesin) family of proteins, which act as scaffolds in the cell cortex through interactions with membrane proteins and cytoskeleton (Fehon et al., 2010). Nf2 is unique for its tumor suppressor properties compared to ERM family of proteins.

The molecular function of Nf2 is highly context-dependent. It is shown to mediate contact-dependent inhibition of proliferation, formation of cell-cell junction and the redistribution of cell membrane receptors, and modulate diverse signaling pathways ranging from Rac-PAK, mTORC1, EGFR-Ras-ERK, PI3K-Akt and Hippo signaling pathway at the cell cortex to the E3 ubiquitin ligase $CRL4^{DCAF1}$ in the nucleus (Li et al., 2012). Many studies link these molecular functions to tumorigenesis but the exact mechanisms are still controversial (Benhamouche et al., 2010; James et al., 2009; Lallemand et al., 2009a; Li et al., 2010; Yi et al., 2011; Zhang et al., 2010).

In recent years, the regulation of the Hippo pathway by Nf2 was shown to be an important mechanism controlling cell proliferation in different tissues and organisms (Hamaratoglu et al., 2006; Lavado et al., 2013; Zhang et al., 2010). *Nf2* mutation in *Drosophila* promotes Yki de-phosphorylation and leads to tissue overgrowth; deletion of Nf2 in mouse liver also promotes Yap de-phosphorylation and leads to liver overgrowth and tumorigenesis (Hamaratoglu et al., 2006; Zhang et al., 2010). Moreover, the regulation of YAP by NF2 is also implicated in tumorigenesis in humans. A very recent proteomic study in large sets of human schwannomas identifys YAP as the master in controlling a signaling network including receptor tyrosine kinases (RTKs) in tumor cell proliferation, which are modulated by Nf2 as well (Boin et al., 2014). Nf2 also suppresses human meningioma cell proliferation through inhibiting YAP (Striedinger et al., 2008). Therefore, the regulation of YAP by Nf2 is critical in both physiological and pathological states.

Although Nf2 is shown to regulate YAP in different tissues and organisms, the molecular mechanisms of how Nf2 regulates YAP is not well understood. One major

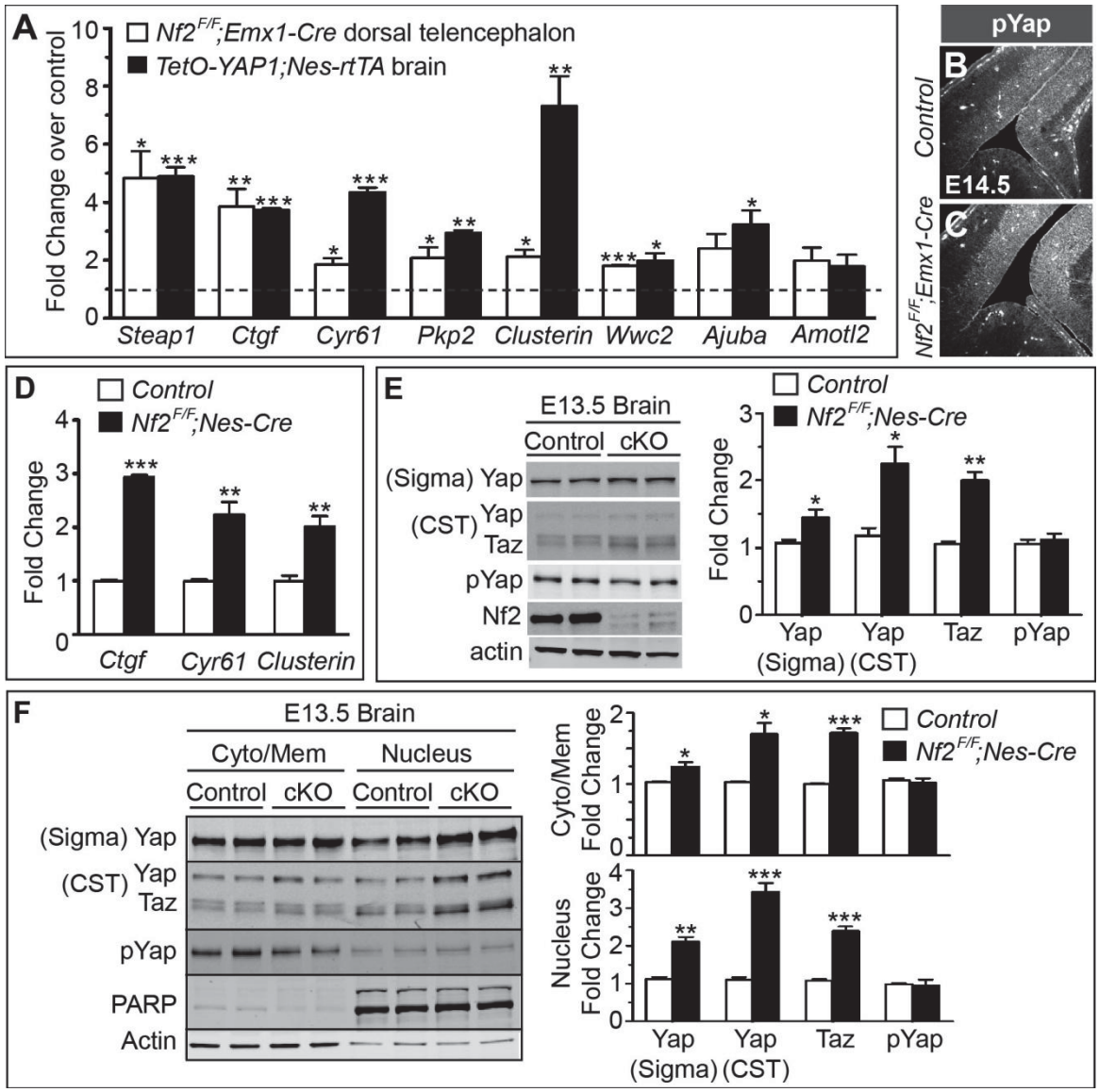
hypothesis is that Nf2 regulates YAP through the Hippo pathway. In *Drosophila* double mutations of Mer (Nf2 orthologue) and FERM protein Expanded (Ex) cause tissue overgrowth phenotype which can be rescued by lack of Hpo (Hamaratoglu et al., 2006). This places Mer upstream of Hpo genetically. Mer overexpression in *Drosophila* cells causes increased Wts phosphorylation and activity. Biochemical experiments find Mer binds to Ex. Later Kibra (a WW and C2 domain-containing protein) was found to interact with Mer and Ex, and these three proteins form a complex that cooperatively regulates Wts activity (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). As Mer-Sav, Kibra-Sav and Ex-Hpo interactions are found, it is suggested that Mer/Ex/Kibra complex may recruit Hippo pathway kinases to the plasma membrane for activation. But one recent study showed that in *Drosophila*, Mer interacts directly with Wts kinase and targetes it to cell membrane for activation by Hpo kinase without involvement of Ex or Kibra (Yin et al., 2013). The study also shows that the mechanism is conserved in mammals. It is not clear if this mechanism applies to other tissues as the study uses mouse liver as the model. Alternatively, Nf2 may regulate YAP through F-actin. Several studies shows that Nf2 can bind to actin cytoskeleton or actin-binding proteins and regulate F-actin remodeling, which is a well-characterized regulator for YAP (Cole et al., 2008; Lallemand et al., 2009b; Manchanda et al., 2005; Pelton et al., 1998).

The recent work in our lab showed that Nf2 limits the expansion of NPCs in the developing mouse brain by inhibiting Yap/Taz activity (Lavado et al., 2013). Loss of Nf2 causes increased proliferation of NPCs in the neocortical and hippocampal primordium and reduced production of Cajal-Retzius cells and hippocampal neurons, resulting in severe reduction of hippocampus size. The phenotype can be rescued by deletion of *Yap*. These results suggest transcriptional coactivators Yap/Taz are the downstream targets of Nf2 in the developing mouse brain. Interestingly, in our study although loss of Nf2 results in increased nuclear Yap/Taz protein levels (**Figure 1-3E** and **F**) and upregulation of Yap target genes (**Figure 1-3A** and **D**), the phospho-Yap -S112 (pYap-S112) level (corresponding to human YAP-S127), does not change between wildtype and *Nf2* mutant brains, different from other studies in *Drosophila* and the mouse liver (**Figure 1-3B**, **C** and **E**). Furthermore, total Yap/Taz protein levels increase in the mutant brains even though Yap/Taz mRNA levels measured by quantitative real-time PCR do not change between wildtype and mutants (**Figure 1-3E**, **F** and data not shown).

The goal of my thesis project is to investigate the biochemical mechanisms of how Nf2 regulates Yap/Taz in the developing mouse brain. I found that the increased levels of Yap/Taz protein in *Nf2* mutants was at least in part due to increased stability of Yap/Taz protein. Loss of Nf2 did not affect Yap-S366 phosphorylation level, which is required for Yap degradation. Loss of Nf2 also did not affect Lats1/2 kinase activity. I also found that Nf2 could interact with Yap in embryonic brain lysates. These suggest that Nf2 may regulate Yap/Taz independent of the canonical Hippo pathway in the developing mammalian brain.

Figure 1-3. Loss of Nf2 results in increased Yap/Taz transcriptional activity
(**A**) Quantitative RT-PCR analysis of eight genes found by microarray to be upregulated in both E13.5 *Nf2$^{F/F}$;Emx1-Cre* dorsal telencephalon and E11.5 *TetO-YAP1; Nes-rtTA* brain confirms significant upregulation of six genes in both genotypes compared with their respective controls. (**B**,**C**) No noticeable difference in phospho-S112-Yap (pYap) immunoreactivity between E14.5 control and *Nf2$^{F/F}$;Emx1-Cre* dorsal telencephalon. (**D**) Quantitative RT-PCR shows upregulation of *Ctgf*, *Cyr61* and clusterin (*Clu*) transcripts in E13.5 *Nf2$^{F/F}$;Nes-Cre* brain. (E) Quantitative western blot analysis shows increased Yap/Taz levels in E13.5 *Nf2$^{F/F}$;Nes-Cre* brain, but the amount of pYap is unchanged. Two Yap antibodies were used; that from Cell Signaling Technology (CST) recognizes both Yap and Taz. (F) Subcellular fractionation followed by quantitative western blots shows increased Yap/Taz proteins in the nuclear and, to a lesser extent, the cytosol/membrane fractions of E13.5 *Nf2$^{F/F}$;Nes-Cre* brains compared with those of control brains. Values are mean ± s.e.m. of three (**A**,**D**) or four (**E**,**F**) embryos per group.

Reprinted with permission from Lavado, A., He, Y., Pare, J., Neale, G., Olson, E.N., Giovannini, M., and Cao, X. (2013). Tumor suppressor Nf2 limits expansion of the neural progenitor pool by inhibiting Yap/Taz transcriptional coactivators. Development *140*, 3323-3334.


A
Nf2F/F;Emx1-Cre dorsal telencephalon
TetO-YAP1;Nes-rtTA brain
Fold Change over control
Steap1
Ctgf
Cyr61
Pkp2
Clusterin
Wwc2
Ajuba
Amotl2
pYap
B
Control
E14.5
C
Nf2F/F;Emx1-Cre
D
Control
Nf2F/F;Nes-Cre
Fold Change
Ctgf
Cyr61
Clusterin
E
E13.5 Brain
Control
cKO
(Sigma) Yap
(CST) Yap
Taz
pYap
Nf2
actin
Control
Nf2F/F;Nes-Cre
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Yap (Sigma)
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E13.5 Brain
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Nucleus
Control
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(CST) Yap
Taz
pYap
PARP
Actin
Cyto/Mem Fold Change
Nucleus Fold Change
Control
Nf2F/F;Nes-Cre
Yap (Sigma)
Yap (CST)
Taz
pYap

CHAPTER 2. METHODOLOGY

Animals

Animal experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee of St Jude Children's Research Hospital.

Constructs

The p2XFlagCMV2-YAP2, p2XFlagCMV2-YAPw1w2, p2XFlagCMV2-YAP S381A constructs were obtained from Addgene. p2XFlagCMV2-YAPΔC was derived from p2XFlagCMV2-YAP1-291 by PCR amplification using GAA TCG GTA CCA ATG GAT CCC GGG CAG CAG CCG CCG CCT CAA CCG and CCC ATT CTA GAC TAC TGT GGG CTC TGG GGA GCC AGG GGT GGT GG primers, digested with KpnI and XbaI and inserted into p2XFlagCMV2-YAP2 cut with the same enzymes.

p2XFlagCMV2-YAPΔTEAD was derived from p2XFlagCMV2-YAP155-488 by PCR amplification using CCA GCG GTA CCG CCC ACA GCT CAG CAT CTT CGA CAG TCT TCT and GAT CCT CTA GAC TAT AAC CAT GTA AGA AAG CTT TCT TTA TC primers, cloned with the same method as p2XFlagCMV2-YAP1-291 generation. pcDNA3.1-N-HA-Nf2 construct was generated by digesting pcDNA3-Nf2 (mouse *Nf2*) with EcoRI and XbaI and inserting it into pcDNA3.1-N-HA cut with the same enzymes. pcDNA3.1-N-HA-Nf2 ΔFERM was derived from pcDNA3.1-Myc-His-B-Nf2 342-597 by PCR amplification with GCG GCG ATA TCG AGA GAA GCA GAT GCG GGA GGA GGC CGA G and AGC TCT CTA GAC TAG AGT TCT TCA AAG AAG GCC ACT CG primers, digested with EcoRV and XbaI and inserted into pcDNA3.1-N-HA cut with the same enzymes. pcDNA3.1-N-HA-Nf2 ΔC was derived from pcDNA3.1-Myc-His-B-Nf2 1-497 using TGC AGG ATA TCC CAT GGC CGG AGC CAT CGC TTC TCG CAT GAG C and GCA ATT CTA GAC TAG CTC GGT ATG TCA GGA GGC AGT GGT GGT GG primers, cloned with the same method as pcDNA3.1-N-HA-Nf2 ΔFERM. To generate pcDNA3.1-N-HA-Nf2 Δα-helical construct, pcDNA3.1-N-HA-Nf2 1-341 was cloned first by PCR amplifying pcDNA3.1-Myc-His-B-Nf2 1-341using primers TGC AGG ATA TCC CAT GGC CGG AGC CAT CGC TTC TCG CAT GAG C and GCA TCG CGG CCG CTC GAG CCA GCC GCT GCC TTT CCA TCT G, digested with EcoRV and NotI and inserted into pcDNA3.1-N-HA cut with the same enzymes. Then pcDNA3.1-N-HA-Nf2 Δα-helical construct was derived from pcDNA3.1-Myc-His-B-Nf2 498-597 by PCR amplification using CCT GAC GCG GCC GCG TTC GAC ATT ATT GCT GAC AGC TTG TCA TTC and AGC TCT CTA GAC TAG AGT TCT TCA AAG AAG GCC ACT CG primers, digested with NotI and XbaI and inserted into pcDNA3.1-N-HA-Nf2 1-341 cut with the same enzymes.

HEK293T Cell Culture, Transfection, and Lentiviral Generation

HEK293T cells were cultured in DMEM (Invitrogen) containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin (P/S) (Invitrogen). Transfection with lipofectamine 2000 was performed according to the manufactuer's instructions (Invitrogen). Lentivirus generation was performed by transfecting lentivirus packaging vectors pCMVΔ8.9, psedutyping vector pVSV-g, along with expression vector pCDH-MSCV-MCS-EF1-GFP/Cre (obtained from Dr. Gilbertson lab) using transfection reagent Xtreme gene HP (Roche). Viral supernatant was harvest 30h, 48h and 72h after transfection, cell debris was removed by centrifugation at 3,000 x g for 15 min, and the supernatant was filtered through 0.45μm filter. The flow-through was added with Peg-It (Systems Bioscience, 1:5), and incubated at 4˚C overnight. The solution was then centrifuged at 3,000 x g for 30min, and the pellet was suspended in phosphate-buffered saline with 0.1% BSA (Sigma). After determining viral titer by counting GFP-positive cells at day 2 following infection of 293T cells, virus solution was used to transduce NPC cells.

Western Blotting and Immunoprecipitation

293T cells were washed once with ice-cold phosphate-buffered saline (PBS) and harvested with HEPES-buffered salt (HBS) (20mM HEPES pH7.4, 150mM NaCl, 10% Glycerol) containing Halt protease & phosphatase inhibitor cocktail (Thermo scientific). For western blot analysis, cells were then lysed with equal volume of HBS+4% SDS solution (20mM HEPES pH7.4, 150mM NaCl, 4% SDS). Lysates were passed through a 25G needle several times then a 28G needle several times to break DNA. Protein concentration was measured by BCA assay kit (Thermo Scientific). Lysates were diluted with 2x or 5x SDS sample buffer to the final concentration of 1μg/μl, heated at 95˚C for 5 min, fractionated by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat dry milk or 5% bovine serum albumin (BSA) in TBST (150mM NaCl, 20mM Tris-HCl pH 7.6, 0.1% Tween 20) for 2h at room temperature, followed by incubation with primary antibodies diluted in corresponding blocking solution overnight at 4˚C. Membranes were washed three times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) diluted 1:3000 in blocking solution for 2h at room temperature. Membranes were washed four times and developed using enhanced chemiluminenscence kit (Thermo scientific).

For immunoprecipitation experiments with 293T cells, the day before transfection $2x10^6$ 293T cells were plated onto 60mm plates. 1.5μg of each plasmid was used to transfect the cells. Two days after transfection, 293T cells were lysed with PLC buffer (20mM HEPES pH7.5, 150mM NaCl, 10% Glycerol, 1% IGEPAL, 1.5mM MgCl2, 1mM EGTA) containing Halt protease & phosphatase inhibitor cocktail. Lysates were extracted in PLC buffer for 10 min on the shaker at 4˚C, then centrifuged over 12,000 rpm for 10 min. Supernatants were collected and incubated with 2μg of antibody for 4h on the shaker at 4˚C, and then supplemented with 15μl protein G dynabeads (Life technology)

for another 2h shaking. Beads were washed three times with PLC buffer and eluted with 50µl of PLC buffer and 2x SDS sample buffer. Immunoprecipitation and western blotting experiments with brain samples were performed the same as 293T cells.

The following primary antibodies were used: Lats1 (Cell signaling, 3477S, 1:100), Lats2 (Bethyl Laboratories, A300-479A, 1:1000), phospho- Lats1/2 (cell signaling, 8654, 1:1000), HA-Tag (Cell signaling, 3724, 1:1000), Flag-Tag (Sigma, F1804, 1:2000), Yap (Sigma, WH0010413M1, 1:2000), phospho-S127-YAP (Cell signaling, 4911, 1:1000), Actin (Ambion, AM4302, 1:10000).

Quantitative Western Blotting

The procedure is basically the same as regular western blot. Instead of using horseradish peroxidase-conjugated secondary antibodies, the blot was incubated with IRDye 600LT-conjugated and 800CW-conjugated secondary antibodies, and detected with the ODYSSEY infrared imaging system (LI-COR). The following primary antibodies were used: Yap (Sigma, WH0010413M1, 1:200), phosphor-S127-YAP (Cell signaling, 4911, 1:100), Actin (Ambion, AM4302, 1:1000).

NPC Culture and Protein Stability Assay

The dorsal telecephalon of E13.5 embryos was dissected out, digested with papain (Worthington) in DMEM and passed through 5ml Falcon pipettes attached with a 200µl tip to get single cells. Primary NPCs were grown as neurospheres or adherent culture in DMEM supplemented with B27 (Invitogen), N2 (Invitrogen), N-acetyl cysteine (Sigma), 0.0045% BSA (Sigma), 1% P/S (Invitrogen), EGF (Miltenyi, 20ng/ml) and FGF (Miltenyi, 20ng/ml). NPC neurospheres were supplemented with fresh EGF and FGF every 3 days and passed every 6 days. For protein stability assay, $5x10^5$ NPCs were grown in 6-well ultra-low culture dish (Corning) for 60 hours and then treated with cycloheximide (100µg/ml; Fisher). Cells were harvested in six different time points, 0h, 5h, 10h, 15h, 20h, 25h, for western blot analysis.

CHAPTER 3. RESULTS

Nf2 Regulates Yap/Taz Stability

I sought to understand the mechanism of how Nf2 loss causes increased Yap/Taz proteins levels. I hypothesized that the increased Yap/Taz protein levels were the result of increased Yap/Taz protein stability. To test this hypothesis, I generated NPC lines from the dorsal telecephalon of E13.5 wildtype and mutant embryos, blocked protein synthesis with cycloheximide, and examined the protein levels of Yap/Taz at different time points. Yap/Taz proteins were significantly more stable in *Nf2* mutant NPCs than in wildtype NPCs (**Figure 3-1A**). I also detected a trend of increase for Yap/Taz protein levels in mutant NPCs (**Figure 3-1B**), which was similar to our previous results in embryonic brain lysates (**Figure 1-3E**). Therefore, loss of Nf2 increases Yap/Taz protein stability.

Nf2 Does Not Regulate Yap-S366 Phosphorylation

I next investigated how loss of Nf2 increases Yap/Taz protein stability. It is well known that the Hippo pathway phosphorylates Yap/Taz and sequesters them in the cytoplasm for retention or degradation. The mammalian Lats1/2 kinases of the Hippo pathway phosphorylate Yap mainly at five sites (Zhao et al., 2010b; Zhao et al., 2007). Of these five sites, two of them, S127 (S112 in mouse) and S381 (S366 in mouse) are the most important in the regulation of YAP activity. Blocking of both phophorylation is required to activate YAP's transforming activity. Specifically, YAP-S127 phosphorylation promotes YAP translocation to the cytoplasm, and YAP-S381 phosphorylation promotes YAP ubiquitination and degradation. Because pYap-S112 level did not change from our previous results and Yap-S366 phosphorylation is important in protein degradation, I decided to examine pYap-S366 level using an antibody against phospho-S381-YAP obtained from Lim lab (Kim et al., 2013). The Lim lab validated the specificity of the antibody by showing that samples treated with lambda-phosphatase lost the signal of pYAP-S381 compared to mock-treated samples. To further confirm the specificity, I tested whether YAP S381A mutant could be detected by the antibody. The antibody could not distinguish the pYAP-S381 protein in lysates even when YAP was overexpressed (**Figure 3-2A**). No difference in the blot between wildtype and Yap conditional knockout brain further confirmed the result (**Figure 3-2B**). However, the antibody could detect wildtype YAP but not YAP S381A protein when YAP was pulled down with immunoprecipitation (IP) experiments (**Figure 3-2A**). After validating the specificity of the antibody, I examined pYap-S366 level in E14.5 wildtype and *Nf2* mutant brains and found no significant difference in pYap-S366 level (**Figure 3-2C**). The result suggests that Nf2 does not regulate Yap-S366 phosphorylation.

Because Lats1/2 kinases are the best known kinases that phosphorylate YAP, I examined whether Lats1/2 kinases activity changed in *Nf2* mutant brains. Lats1/2 are phosphorylated by Mst1/2 at hydrophobic motif (T1079 for Lats1, T1041 for Lats2), which is required for the activation of Lats1/2 (Chan et al., 2005). Therefore, phospho-

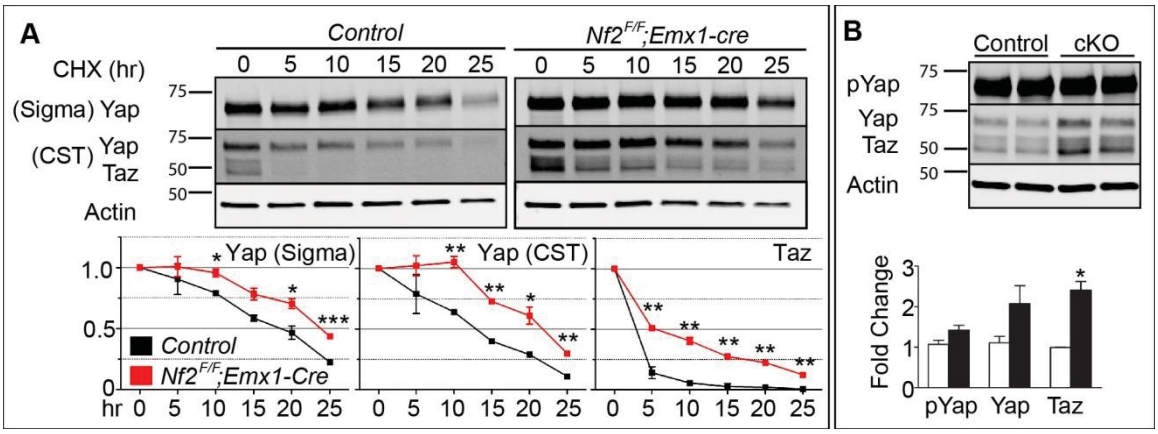

Figure 3-1. Loss of Nf2 increases the stability of Yap/Taz protein
(**A**) Quantitative western blot analysis of NPC cultures treated with cycloheximide (CHX) for the indicated time showing increased stability of Yap/Taz proteins in *Nf2$^{F/F}$;Emx1-Cre* NPCs. 20 μg protein was loaded per lane. The protein level at the zero time point is set at 1. Graphs show the mean ±s.e.m. of two control and three *Nf2$^{F/F}$;Emx1-Cre* NPC lines. (**B**) The steady-state levels of pYap do not differ between control and *Nf2$^{F/F}$;Emx1-Cre* NPCs, and those of Yap/Taz show a trend of increase. Values are mean±s.d. of two control and three *Nf2$^{F/F}$;Emx1-Cre* NPC lines. *P<0.05, **P<0.01, ***P<0.001.

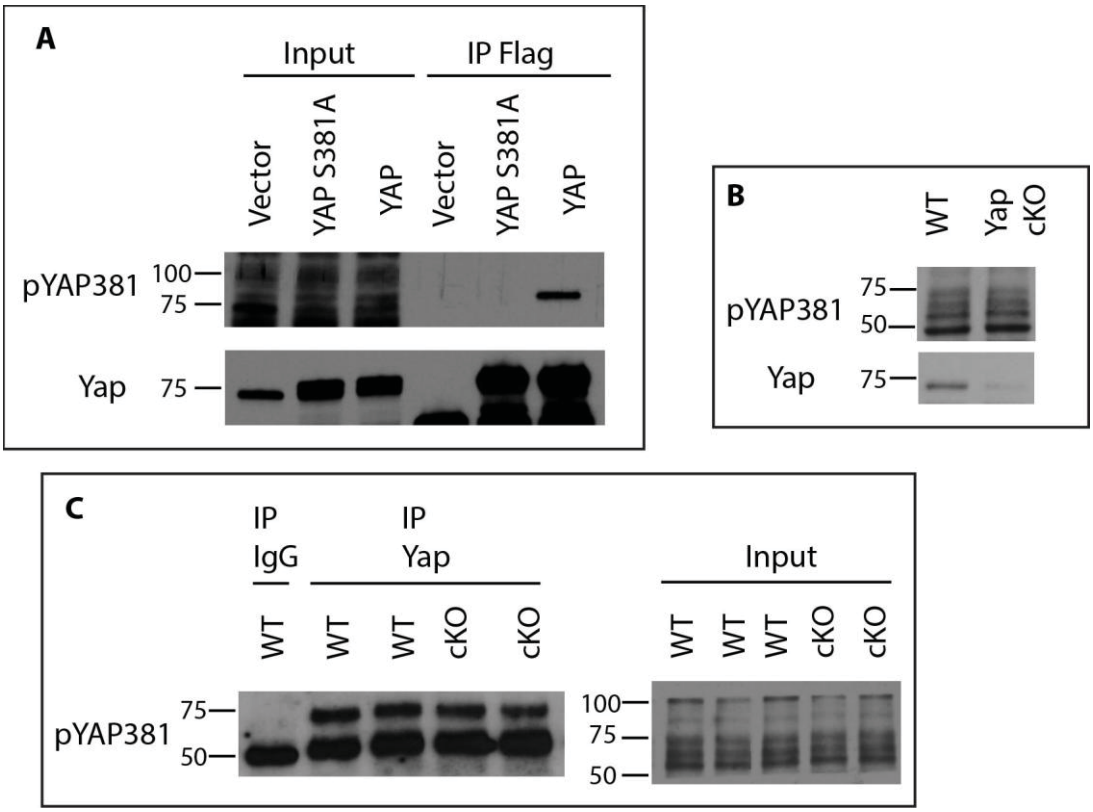

Figure 3-2. Loss of Nf2 does not affect pYap-S366 level
(**A**) pYAP-S381 antibody recognizes wildtype YAP but not YAP S381A mutant protein as shown by IP. Notice that in the input, there is no difference for the blot between YAP expression group and YAP S381A expression group. (**B**) pYAP-S381 antibody does not distinguish pYAP381 protein in mouse brain as there is no difference between E14.5 wildtype (WT) brain and *Yap*$^{F/F}$*; Nes-cr*e brain. All the bands are non-specific. (**C**) There is no difference in pYap-S366 level between E14.5 WT brain and *Nf2*$^{F/F}$*; Nes-cre* brain as shown by the IP.

Lats1/2 (pLats1/2) level can be an indicator for Lats1/2 activation. I did not detect a difference in pLats1/2 level between wildtype and mutant brains (**Figure 3-3A**). This suggests loss of Nf2 in the brain does not affect Lats1/2 activity. To further examine whether Lats1/2 were necessary for Yap phosphorylation in NPCs, I generated *Lats1*$^{-/-}$ NPCs by infecting *Lats1/2*$^{F/F}$ NPCs with lentivirus expressing Cre recombinase. Somehow *Lats2* was not deleted probably because of incorrect *LoxP* sequence which could not be recognized by Cre recombinase. There was a significant decrease of pYap-S112 level in *Lats1*$^{-/-}$ NPCs (**Figure 3-3B**), suggesting that Lats1 is required for Yap-S112 phosphorylation in NPCs.

Nf2 Interacts with Yap

The experiments above were trying to address whether Nf2 regulates Yap in the developing mouse brain through the canonical Hippo pathway. There may be alternative mechanisms. Our previous immunohistochemistry experiments showed that Nf2 was localized in the apical membrane of NPCs, and Yap/Taz were localized in the apical membrane and the cytoplasm. In addition, both Nf2 and Yap were shown to interact directly with several proteins such as angiomotin, EBP50 (Bretscher et al., 2000; Mohler et al., 1999; Yi et al., 2011; Zhao et al., 2011a). It is possible that Nf2 may sequester Yap/Taz in the apical membrane. Indeed, co-immunoprecipitation (Co-IP) experiments found that Yap co-immunoprecipitated with Nf2 in E14.5 mouse brains (**Figure 3-4A**). To map the domains that were required for Yap-Nf2 interaction, I expressed various HA-tagged mouse Nf2 deletion mutants and Flag-tagged human YAP deletion mutants in 293T cells (**Figure 3-4B**). Co-IP experiments demonstrated that the FERM domain and C-terminal domain of Nf2 contributed to Yap-Nf2 interaction, and the WW domain of Yap was required for the interaction (**Figure 3-4C** and **D**).

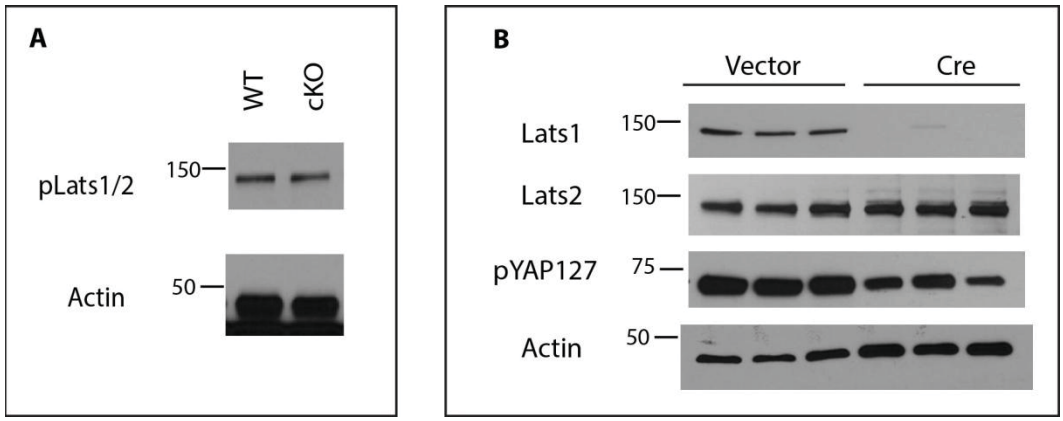

Figure 3-3. Loss of Nf2 does not affect Lats1/2 kinase activity
(**A**) The level of pLats1/2 does not change between WT and *Nf2*$^{F/F}$*; Nes-Cre* brains. (**B**) Requirement of Lats1 for Yap-S112 phosphorylation. *Lats1/2*$^{F/F}$ NPCs were transduced with lentivirus expressing empty vector or Cre recombinase.

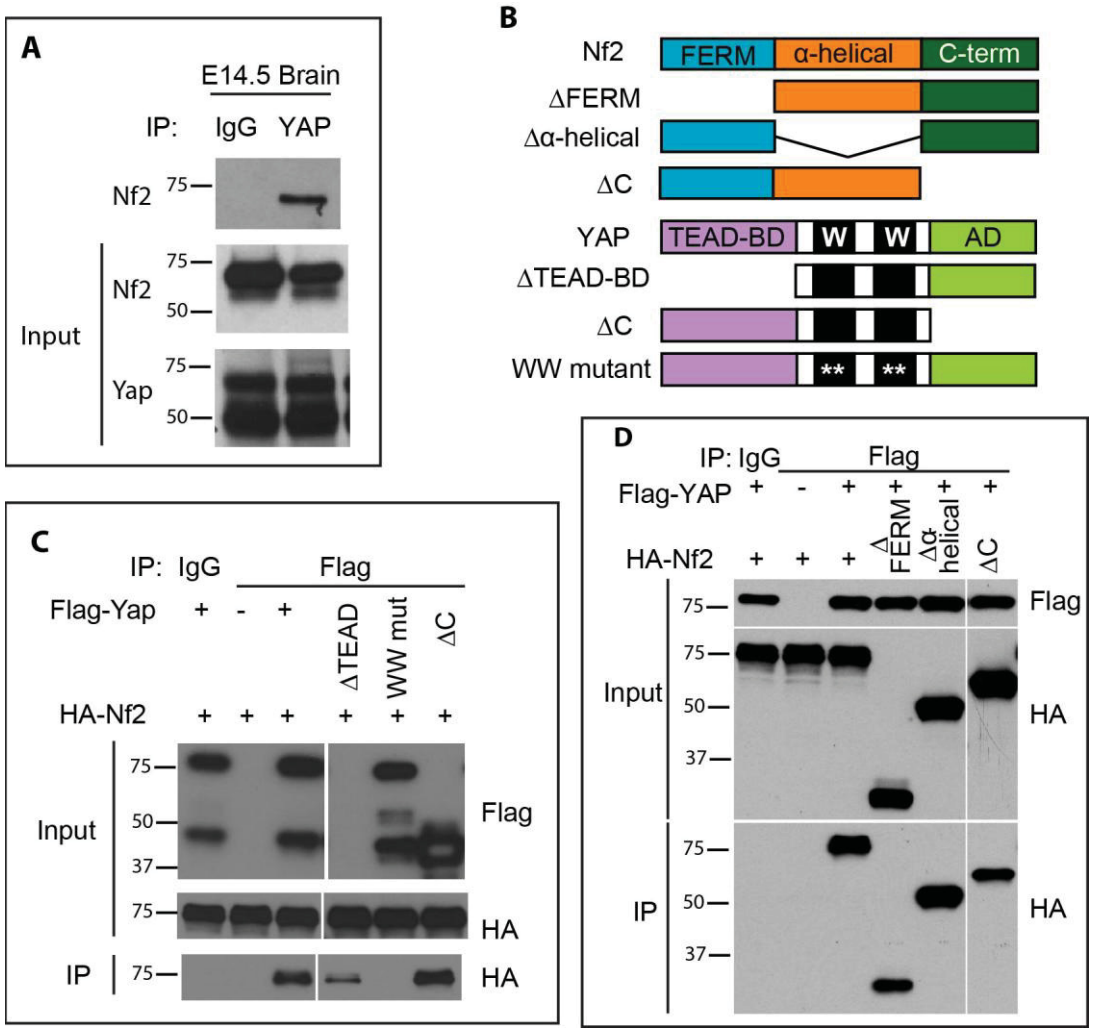

Figure 3-4. Nf2 interacts with Yap

(**A**) Nf2 Co-IP with Yap in E14.5 WT brain. (**B**) Diagram of HA-tagged full-length Nf2, Flag-tagged YAP and their deletion constructs used in (**C**) and (**D**). ** indicates the mutations in YAP WW domain. (**C**) Immunoblot analysis with HA and Flag antibodies of Flag-IP from 293T cells cotransfected with HA-Nf2 and various Flag-YAP deletion constructs. (**D**) Immunoblot analysis with HA and Flag antibodies of Flag-IP from 293T cells cotransfected with Flag-YAP and various HA-Nf2 deletion constructs.

CHAPTER 4. DISCUSSION

Here I showed that the increased levels of Yap/Taz in Nf2 mutants were at least in part due to the increased stability of Yap/Taz protein. Loss of Nf2 did not affect Yap-S366 phosphorylation, which is required for Yap degradation. Loss of Nf2 also did not affect Lats1/2 activity. I also found that Nf2 could interact with Yap in embryonic brain lysates. These suggest that Nf2 may regulate Yap/Taz independent of the canonical Hippo pathway in the developing mammalian brain.

Yap/Taz Stability

For the Yap/Taz stability experiment, I detected a significant increase of Yap/Taz protein levels in *Nf2* mutant NPCs. Compared to Yap, Taz level decreased faster in both WT and mutant NPCs. This suggests that Taz is more sensitive to degradation than Yap. The half life of Yap in NPCs was about 15h, which was much longer than a half life of 2h in 293T cells (Zhao et al., 2010b). This could be a cell type difference. Before 10h Yap protein in mutant NPCs was very stable while Yap level in WT NPCs already decreased significantly. After 10h, Yap level in both WT NPCs and mutant NPCs declined almost linearly at the same rate. Theoretically, with the addition of protein synthesis inhibitor, cycloheximide, protein level should decrease in an exponential curve (Alvarez-Castelao et al., 2012). It is difficult to distinguish whether the decrease of Yap level before 10h in WT NPCs was exponential due to big variation of Yap level at 5h. But the linear decrease of Yap level after 10h may be a secondary effect of the drug for the following evidence. I could observe that after 10h many NPC cells in both WT and mutant groups started to dissociate from neurospheres as the edges of neurospheres were loose and bulgy instead of round and smooth. Protein concentration measured by BCA assay also confirmed that after 10h protein concentration decreased more significantly than before 10h. These suggested that cells started to die after 10h, probably because of the toxic effects of cycloheximide. Therefore, the linear decline of Yap level after 10h in mutant and WT NPCs likely results from the toxic effect of cycloheximide. One alternative method to avoid this problem is to use pulse-chase experiments with radioactive amino acids to measure protein half-life, which does not exert burden on cell growth and viability (Alvarez-Castelao et al., 2012).

YAP-S381 Phosphorylation

For the pYAP-S381 phosphorylation experiment, the pYAP381 antibody can distinguish the pYAP-S381 protein in IP but not in lysates even with the overexpression of YAP. This may be because that IP concentrates the protein whose level is extremely low in lysates. The reason that pYAP-S381 level is low can be attributed to two processes: the synthesis and the degradation of pYAP-S381. It is possible that the enzymes that catalyze the generation of this protein are limited or the enzymes that catalyze the degradation of this protein are very abundant.

To prove that an antibody recognizes specific protein phosphorylation site, there are several methods. One method is to treat samples with phosphatase so that the phosphorylated protein is dephosphorylated and cannot be detected by the antibody. But this will not confirm that the antibody recognizes the specific phosphorylation site as all phophorylations in one protein disappear after the treatment. The second method is to examine whether the antibody fails to recognize the mutant protein that cannot be phosphorylated. This can confirm the specificity of the antibody. A more rigorous way is to subject the putative band for mass spectrometry analysis and determine whether a phosphate group is present in the specific site.

Nf2 Does Not Regulate Lats1/2 Activity

I also showed here that Nf2 did not regulate the activity of Lats1/2 in the brain, which is different from the study in *Drosophila* and mouse liver (Yin et al., 2013). The requirement of the Hippo core kinases for phophorylating and inactivating Yap is cell-type dependent. In mouse embryonic fibroblasts, deletion of *Mst1/2* has no effect on Yap phosphorylation in response to cell-cell contact (Zhou et al., 2009). In mouse keratinocytes, activation of Yap in the absence of α-catenin is independent of Mst1/2 and Lats1/2 (Schlegelmilch et al., 2011). One study in mouse liver shows that *Mst1/2* knockout in mouse liver does not affect Lats1/2 phosphorylation and other kinases activated by Mst1/2 are present for phosphoryalting Yap, although another study finds *Mst1/2* knockout in mouse liver decreases Lats1/2 phophorylation (Lu et al., 2010; Zhou et al., 2009). *Mst1/2* knockout in the brain also does not affect Yap phosphorylation as shown by our lab. It appears that during evolution, the core Hippo kinase cascade in mammals diversifies considerably compared to *Drosophila*.

Yap-Nf2 Interaction

For the Yap-Nf2 interaction experiment, in both brain lysates and 293T cells lysates IP of Yap can pull down Nf2, but not in reverse. Therefore, instead of using both IP of YAP and IP of Nf2 to determine the domains that are required for the interaction, I only used IP of YAP. Deletion of either FERM domain or C-terminal domain of Nf2 greatly diminished the interaction, but did not completely abolish it. It is plausible that deleting both domains is required for the complete abolishment of the interaction. The WW domain of YAP was required for YAP-Nf2 interaction as mutations in WW domain abolished the interaction. Although in the blot less Nf2 was pulled down in the group contransfected with YAP-ΔTEAD and HA-Nf2, YAP-ΔTEAD was unstable and hardly detected in the input. So the decreased Nf2 pulled down by IP may be because of decreased YAP-ΔTEAD expression, but not that TEAD domain was required for the interaction.

Although Co-IP experiments can identify the domains that are required for the interaction from, it cannot suggest whether the Yap-Nf2 interaction is direct or indirect. One experiment to show that is to express recombinant tagged proteins such as GST-

tagged or His-tagged proteins in bacteria and examine whether they interact directly using *in vitro* binding assay (Yi et al., 2011). If some GST-tagged or His-tagged proteins do not express in bacteria, we can use *in vitro* transcription/translation system to synthesize the protein and then examine the interaction of this synthesized protein with tagged protein *in vitro* (Chinnaiyan et al., 1995). An alternative method is to employ yeast-two hybrid assay in which the binding domain and activation domain of a transcription factor are split and fused to one of two proteins (Chinnaiyan et al., 1995). When the two proteins interact directly, it brings the binding domain and activation domain close enough to initiate transcription of the reporter gene.

Alternative Mechanisms for Nf2-Yap Regulation

My preliminary study does not completely reveal the mechanism of how Nf2 regulates Yap in developing mouse brain. Loss of Nf2 does not affect either YAP-S127 or YAP-S381 phosphorylation, which are the two most important phosphorylation sites in the regulation of YAP activity. It is possible that the changes in these two phosphorylation levels are too small to be detected by our methods. In addition, the other phosphorylation sites may be involved. A phos-tag-containing gel is helpful for identifying protein phosphorylation changes when dealing with proteins having multiple phosphorylation sites (Yu et al., 2012). It is also very plausible that Nf2 may regulate Yap in a phosphorylation-independent manner. Nf2 is shown to interact directly with angiomotin family proteins, which can bind to and sequester YAP in tight junctions independent of phosphorylation (Yi et al., 2011; Zhao et al., 2011a). Nf2 may also regulate Yap through F-actin, which can also regulate Yap independent of phosphorylation (Dupont et al., 2011). Since loss of Nf2 increases Yap stability, Nf2 may regulate protein-protein interactions associated with Yap and involved in Yap degradation process. A proteomic study by comparing proteins pulled down by Yap between WT and *Nf2* mutant brains will help identify the specific protein-protein interactions regulated by Nf2.

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VITA

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