BIOCHEMICAL AND GENETIC STUDIES ON CC2D1A,
A NEW NF-κB ACTIVATOR AND A REGULATOR OF SYNAPTIC FUNCTIONS

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To My Parents and My Husband

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CC2D1A is an evolutionarily conserved gene from worm to human. It belongs to a new protein family with four DM14 domains at the NH_2 terminus and a C2 domain at the COOH terminus. The function of this protein family remains largely unknown. CC2D1A has been identified as a new NF-κB activator through a large scale screen of human genes by Matsuda et al. Here I show that the conserved DM14 and C2 domains of CC2D1A are important for NF-κB activation. CC2D1A activates the IKK complex and NF-κB target genes through several key components in the canonical pathway including ubiquitin-conjugating enzyme UBC13, a RING domain ubiquitin ligase TRAF2, a protein kinase TAK1, and an essential regulator of IKK complex, NEMO. CYLD, a deubiquitination enzyme specific for Lysine-63 linked polyubiquitin chains, negatively regulates the activity of CC2D1A. These results suggest that CC2D1A activates NF-κB through the canonical IKK pathway.

In an attempt to identify the physiological function of CC2D1A, I generated CC2D1A knockout mice. The KO animals die right after birth apparently due to their inability to breathe. Histological analysis identified no significant anatomical defects. In particular, brain, heart and muscle are normal with regard to morphology. In addition, neuromuscular junction at the

diaphragm is formed in the absence of Cc2d1a. Human patients with mutations in the gene suffer from mental retardation, implying that Cc2d1a functions in the central nervous system (CNS). Here I show that Cc2d1a expression is enriched in the brain. Deletion of Cc2d1a impairs synapse maturation and function in cortical neurons. Our study may help understand the molecular basis of some human diseases such as mental retardation.

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PRIOR PUBLICATIONS

1. **Zhao, M.,** Li, X.D., and Chen, Z.J. CC2D1A activates NF-κB. *JBC. Submitted*

2. **Zhao, M.,** Raingo, J., Kavalali, E.T. and Chen, Z.J. Deletion of Cc2d1a in mice is lethal and impairs synaptic maturation. *Manuscript in preparation*

3. Chiu, Y. H., **Zhao, M.** and Chen, Z.J. Ubiquitin in NF-kappaB signaling. *Chem Rev*. 2009. 109(4): 1549-60.

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LIST OF ABBREVIATIONS

Aki: Akt kinase (PDK1)-interacting protein 1

CNS: Central Nervous System

CYLD: Cylindromatosis gene

E1: The activating enzyme

E2: The conjugating enzyme

E3: The ubiquitin-like protein ligase

Freud-1: FRE Under Dual repression binding protein-1

Lgd: Lethal(2) giant discs

mIPSC: mini Inhibitory Postsynaptic Current

NF-κB: Nuclear Factor kappa B

NEMO: NF-κB essential modulator

RRP: Readily Releasable pool

5-HT1A: Serotonin (5-HT) receptor 1A

TRAF: Tumor necrosis factor associated factor

TAK1: TGFβ activated kinase 1

IKK: IκB kinase

UBC13: ubiquitin-conjugating enzyme 13

Chapter I

General Introduction

Abstract

CC2D1A is an evolutionarily conserved gene whose function is largely unknown. It was identified as a new NF-κB activator through a large scale screen of human genes by Matsuda et al. 2003. I am interested in understanding the role of CC2D1A in NF-κB pathways, as well as its function under physiological conditions using biochemical and genetic tools. The *Drosophila* orthologue of CC2D1A is a regulator of Notch activation and endocytosis (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Human patients with mutations in the CC2D1A gene suffer from mental retardation, implying that it functions in the central nervous system (Basel-Vanagaite et al., 2006). Here I will review several key discoveries of CC2D1A as a background for my dissertation research.

Part I

Identification of CC2D1A as a Novel NF-κB Activator

A large scale screen was carried out by Matsuda et al. to identify human genes that activate NF-κB (Matsuda et al., 2003). They constructed full length cDNA libraries from human normal lung fibroblast cells cultured with or without pre-exposure to IL-4 and TNFα. On average there were 70% full length cDNAs in the libraries. Each cDNA was cloned under Sra, the promoter of large T antigen. One hundred and fifty thousand cDNAs were randomly selected to form cDNA pools. The cDNAs were introduced into HEK293 cells together with pNF-κB-Luc, a reporter containing four tandem repeats of NF-κB binding sites and positive clones were the ones that induced at least four fold activity compared to mock insert control. In total, 299 (0.2%) cDNAs were identified as “positive”.

After BLAST searches, the 299 cDNAs were clustered into 58 "Named" genes and 25 "Novel" genes. Among the "Named" genes, 30 had been reported as NF-κB activators, while the other 28 genes had not. Several control experiments were conducted. First, for all the uncharacterized genes, they repeated the luciferase assay and confirmed each gene induced reporter activity dose dependently. Second, a dominant negative mutant of IKKβ was able to suppress the induced luciferase activity. Since IKKβ is an essential kinase required by most stimuli that activate NF-κB, theses controls indicated that the genes from the screen activate NF-κB, not just the κB element in the reporter plasmid. It also shows that IKKβ acts downstream. Last, genes that are expressed abundantly but are not involved in NF-κB pathways, such as EF-1a and ribosomal protein genes showed less than 4 fold induction. These data indicated the genes identified are reliable.

Rashu Seth et al. in our lab discovered mitochondria localized protein MAVS is an essential adaptor for interferon production upon virus infection (Seth et al., 2005). The localization of MAVS is important for its function, implying that mitochondria may have anti-viral functions in addition to being a power house. Rashu found that MAVS was among the 25 "Novel" genes identified from the screen although the mitochondria localization was not annotated. Interestingly among the "Novel" genes, there are five with a mitochondria sorting signal. Therefore, we decided to characterize these genes, especially their function in anti-viral immune responses.

As shown in the results, I cloned the five genes and tested them in an interferon luciferase assay in HEK293T cells. The luciferease reporter is driven by the interferon promoter. I found none of the genes activated the interferon reporter. However, one of the genes activated NF-κB luciferase reporter strongly. The NF-κB luciferase reporter is driven by three tandem repeats of

the NF-κB binding site. The gene is named CC2D1A and contains four DM14 domains at the NH_2 terminal part and a C2 (CalB) domain at the COOH terminal end (Figure 6B). DM14 domain is unique to this protein family and its function has not been characterized. C2 (CalB) is the Ca^{2+}-binding motif present in phospholipases, protein kinases C, and several synaptic proteins. The unique domain structure has never been observed in NF-κB activators. I decided to understand how CC2D1A activates NF-κB. In the following sections, I will briefly review the NF-κB pathways as well as the role of ubiquitin in NF-κB signaling.

NF-κB pathways

Nuclear factor kappa B (NF-κB), a family of transcription factors, is involved in the regulation of immunity, inflammation, and cell survival (Hayden and Ghosh, 2008). The family members share an N-terminal Rel homology domain (RHD), which mediates dimerization, nuclear translocation, DNA binding, and association with inhibitory proteins IκBs. The NF-κB/Rel family includes RelA (p65), c-Rel, RelB, p50 and p52. At a resting state, NF-κB dimers are bound by the inhibitory proteins IκBs and sequestered in the cytosol. Thus, proteasomal degradation of IκBs is the prerequisite for NF-κB activation in most cases. P50 and p52 are generated from the precursors p105 and p100, respectively, through partial degradation of the C-terminal IκB-like ankrin repeats by the proteasome.

The NF-κB activation signaling is classified into canonical and noncanonical pathways: the canonical pathway leads to the degradation of IκBs, whereas the noncanonical pathway leads to the processing of p100 into the mature subunit p52 (Pomerantz and Baltimore, 2002). Most NF-κB stimuli activate the canonical pathway, as seen in the cases of proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β). Once ligands are bound to

their cognitive receptors, distinctive pathways are set in motion. The signaling pathways converge on a large kinase complex consisting of the catalytic subunits IKKα and IKKβ (IκB kinase α and β) and an essential regulatory subunit NEMO (NF-κB essential modulator, also known as IKKγ or IKKAP). The IKK complex phosphorylates IκBs at essential residues, and this targets IκBs for polyubiquitination and eventually degradation by the proteasome. Consequently, NF-κB dimers enter the nucleus and turn on the expression of target genes. The noncanonical pathway is triggered by several receptors in B cells, such as CD40 and B-cell activating factor receptor (BAFF-R). Similarly, signaling from these receptors leads to the activation of IKKα, which phosphorylates p100. Phosphorylation of p100 by IKKα leads to polyubiquitination. Consequently, the IκB-like autoinhibitory domain at the COOH terminus of p100 is degraded by the proteasome and the NH_2-terminal Rel homology domain is spared to form mature subunit p52. P52 binds to RelB. The dimeric complex enters the nucleus to turn on the transcription of target genes that are involved in B-cell maturation and activation.

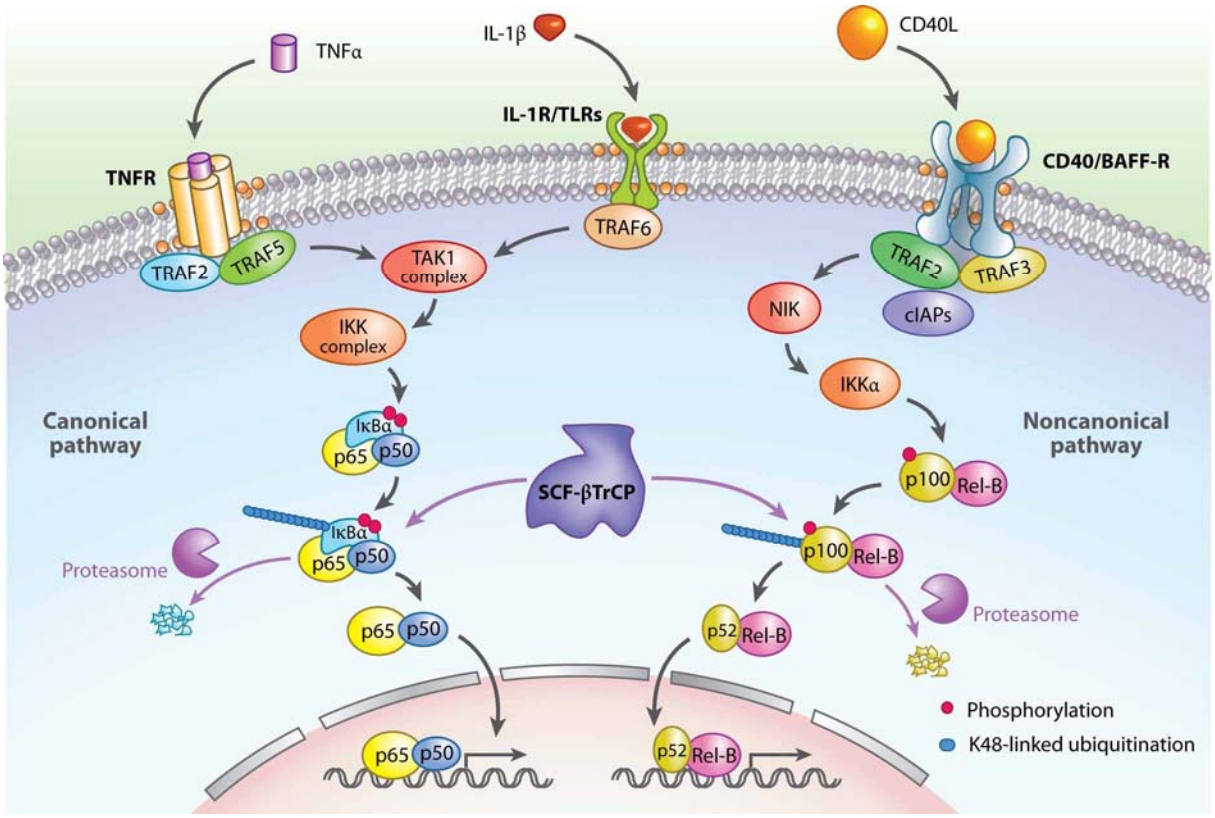


Figure 1. Canonical and Non-canonical Pathways of NF-κB Activation. In canonical pathway (*left*), stimulation of the TNF receptor (TNFR), IL-1 receptor (IL-1R), and Toll-like receptors (TLRs) leads to activation of the TAK1 complex in a TRAF proteins dependent manner. TAK1 then activates IKK, which in turn phosphorylates IκB proteins and targets them for polyubiquitination by the SCF-βTrCP E3 ligase complex. Ubiquitinated IκB is degraded by the proteasome, allowing the p50/p65 NF-κB dimer to translocate to the nucleus and activate target genes. In noncanonical pathway (*right*), stimulation of a subset of receptors, including LTβ, BAFF/Blys and CD40L in the B cells, leads to the stabilization of the kinase NIK, followed by activation of IKKα. IKKα phosphorylates p100, leading to its ubiquitination by the SCF-βTrCP complex. Ubiquitinated p100 is processed to p52 at the proteasome. The p52/REL-B dimer then translocates into the nucleus to activate gene transcription involved in development and maintenance of secondary lymphoid organs. LTβ, Lymphotoxin-β; BAFF, B-cell-activating factor belonging to the TNF family. (Adapted from Skaug et al 2009)

Role of Ubiquitin in NF-κB Signaling

In both canonical and noncanonical pathways, ubiquitination is required for the degradation of inhibitory proteins or protein domains. Moreover, IKK activation is essential to NF-κB activation. Ubiquitination and deubiquitination are important regulatory mechanism for

IKK activation (Chen, 2005; Krappmann and Scheidereit, 2005). Thus, ubiquitin plays multiple functions in NF-κB signaling.

Ubiquitin-Proteasome Pathway

Ubiquitin is a conserved 76-amino-acid protein. It is ubiquitously expressed in eukaryotic cells (Wilkinson, 2004), controlling almost all aspects of a cell's life and death through a covalent modification of its target proteins in a process named ubiquitination (Hershko, 1983; Pickart, 2004). This special post-translational modification is carried out through a series of enzymatic reactions (Figure 2). First, ubiquitin is activated by an E1 (ubiquitin-activating enzyme), in an ATP dependent manner, to form a high energy thioester bond between the C-terminus glycine of ubiquitin and the catalytic cysteine of E1. Then, the activated ubiquitin is transferred to an E2 (ubiquitin-conjugating enzyme, also known as Ubc). In the end, with the assistance of an E3 (ubiquitin-protein ligases), ubiquitin is conjugated to the target via an isopeptide bond between C-terminus glycine of ubiquitin and a lysine of the target. Target specificity is determined by E2s and E3s. Ubiquitin E3s consist of diverse members that can be divided into two categories, one containing a HECT (homology to E6AP C-terminus) domain and one with RING (really interesting new gene) domain. The HECT domain E3s contain an active site cysteine, which can accept ubiquitin from an E2 and transfer the ubiquitin to a target protein. On the contrary, the RING-domain E3s promote ubiquitination by binding to both protein targets and E2s without forming a covalent intermediate. Similar to phosphorylation, ubiquitination is a reversible modification. The reverse process is catalyzed by deubiquitination enzymes (DUBs, also known as isopeptidases).

Monoubiquitination regulates important cellular functions such as chromatin remodeling and vesicle trafficking. Ubiquitin has seven lysines, each of which can be conjugated by another ubiquitin to form a polyubiquitin chain. Linkage specificity of polyubiquitin chains determines the topology, which influences the fate of target proteins. For instance, polyubiquitin chains linked through lysine 48 (K48) of ubiquitin normally target a protein for degradation by the proteasome, whereas the function of lysin 63 (K63) polyubiquitin chains is independent of proteolysis, and is involved in protein kinase activation, DNA repair, and membrane trafficking.

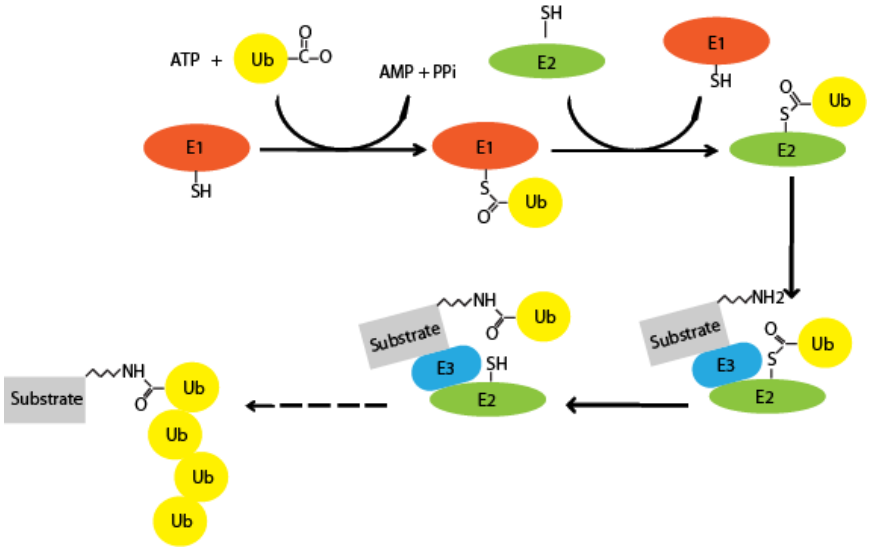


Figure 2. Ubiquitination Mechanism and Machinery. The ubiquitin cascade involves three enzymatic steps. First, ubiquitin is activated by the E1 enzyme with the hydrolysis of ATP to form thioester bond with E1. Ubiquitin is then transferred to the E2 enzyme and form E2-Ub thioester intermediate. Some E2s can directly transfer ubiquitin to a substrate, but usually an E3 enzyme is required to facilitate the transfer of ubiquitin from E2 to the substrate to form iso-peptide bond. Ubiquitination can go on to generate polyubiquitin. Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; PPi, pyrophosphate.

Ubiquitin in IκB Degradation

As mentioned before, under a resting state, NF-κB is bound and inhibited by the inhibitor of NF-κB (IκB) in the cytoplasm. IκB is a family of ankrin repeat-containing proteins, including

IκBα, IκBβ, IκBε, IκBζ, IκBNS, and Bcl-3 (B-cell lymphoma 3). IκBα, IκBβ, and IκBε bind to and sequester NF-κB in the cytoplasm, whereas IκBζ, IκBNS, and Bcl-3 are in the nucleus and cooperate with NF-κB to activate transcription.

The IκB proteins in the cytoplasm are phosphorylated, ubiquitinated and degraded upon stimulation of NF-κB signaling. Protease inhibitors can block signal-induced proteasomal degradation of IκBα and NF-κB activation, but not IκBα phosphorylation (Palombella et al., 1994). As mentioned previously, IKK phosphorylates IκBα at serine 32 and 36 in the N-terminus and plays a pivotal role in NF-κB signaling. Later on, it was shown that phosphorylation is required for IκBα ubiquitination (Chen et al., 1995). The E2 enzyme involved in ubiquitination of IκBα belongs to the Ubc4/Ubc5 family, and the E3 of the process is a protein complex containing Skp1, Cul1, Roc1 (also called Rbx1), and the F-box protein Slimb/βTrCP (SCF-βTrCP) (Maniatis, 1999). Slimb was first identified in *Drosophila* through a genetic screen as a negative regulator of the hedgehog (Hh) and Wnt/Wingless (Wg) pathways (Jiang and Struhl, 1998). βTrCP is the mammalian homologue of Slimb and it contains C-terminal WD40 repeats that recognize phosphorylated forms of substrate with a consensus sequence of DpSGXXpS, where pS represents phosphorylated serine. βTrCP also contains an NH_2 terminal F-box, which binds to Skp1, and forms a complex with Cul1 and the RING domain protein Roc1. An E2, such as Ubc5 is brought close to the phosphorylated IκBα substrate by binding to Roc1 and catalyses polyubiquitination of IκBα at K21 and K22. Therefore $\beta TrCP^{SCF}$ serves as a bridge between an E2 and its substrate and facilitates ubiquitination. Ubiquitinated IκBα still binds to NF-κB but is selectively degraded by the 26S proteasome. There are two mammalian βTrCP genes, βTrCP1 and βTrCP2. They may function redundantly in IκB degradation (Guardavaccaro et al., 2003).

Ubiquitin in the Processing of NF-κB Precursors

P100 and p105 are the precursors of p52 and p50 respectively, and they are partially processed by the proteasome to generate mature subunits (Palombella et al., 1994; Pomerantz and Baltimore, 2002). The processing of p100 to p52 is carried out in the noncanonical pathway of NF-κB activation. P100 processing requires the NF-κB inducing kinase (NIK), which is normally maintained at a very low protein level in unstimulated B cells via proteasomal degradation. The cellular inhibitors of apoptosis, cIAP1 and cIAP2, are the RING domain E3s that catalyze NIK ubiquitination in collaboration with TRAF3 (TNF receptor-associated factor 3). When noncanonical pathway is activated in B cells through a subset of receptors including CD40, BAFF-R and lymphotoxin-β receptor, TRAF3 is degraded and cIAPs fail to ubiquitinate NIK, resulting in its accumulation. NIK then phosphorylates and activates IKKα, which subsequently phosphorylates p100, resulting in its polyubiquitination by $\beta TrCP^{SCF}$. Ubiquitinated p100 is then processed by the proteasome to generate p52.

Both p50 and p105 are present in unstimulated cells, indicating that p105 is processed constitutively. However, stimulation of cells with phorbol esters can enhance the processing of p105 to p50 (Mercurio et al., 1993). Although the proteasome is believed to be important for the processing of p105 to p50, whether ubiquitination plays a role in this process is not clear yet.

In most cases, ubiquitination and proteasome recognition result in the complete degradation of a target protein. The mechanism by which NF-κB precursors p105 and p100 are only partially processed, is not well understood yet. One plausible explanation, as proposed by Rape and Jentsch, is that the proteasome is recruited to an internal unstructured sequence through binding to polyubiquitin chains (Piwko and Jentsch, 2006; Rape and Jentsch, 2004). This region

forms a hairpin-like loop and inserts into the proteolytic core of the 20s proteasome. The first cut is at the loop and proteolysis proceeds to both NH_2- and COOH- termini. Most substrates can be fully unfolded by the 19s and degraded by the 20s, while some substrates, like p105, a glycine-rich region (GRR) followed by the Rel homology domain at the NH_2 terminus forms a tightly packed structure that blocks unfolding and proteolysis, therefore p105 is only degraded at the COOH terminus.

Ubiquitin in Protein Kinase Activation by Diverse NF-κB Signaling Pathways

A prerequisite for the degradation of IκBs and the processing of p100 and p105 is the phosphorylation of these proteins by IKK, which is the converging point for almost all NF-κB pathways. Thus IKK activity must be tightly regulated. In 1996 it was reported that polyubiquitination serves a novel regulatory functions for a large protein complex which specifically phosphorylates IκBα at S32 and S36 *in vitro* (Chen et al., 1996). The large protein complex is now known as IKK (Israel, 2000). Its activation requires ubiquitin, E1 and E2 but not the proteasome. The *in vitro* activation of IKK was prevented by methylated ubiquitin, which cannot form polyubiquitin chains, suggesting that polyUb is important. However the K48R mutant of ubiquitin can still support IKK activation in the assay, implying polyubiquitination through other lysine of ubiquitin is important. Although the experiment was done *in vitro*, the finding turned out to be physiologically relevant.

Identification of TRAF6 as a RING domain ubiquitin ligase bridged the gap between upstream stimuli and ubiquitination. TRAF6 belongs to a family of seven proteins, TRAF1-7 (Chung et al., 2002). TRAF2-6 all contain NH_2 terminal RING domain and COOH terminal TRAF/MATH (meprin and TRAF homology) domain. TRAF1 misses the RING domain, and

there are seven WD40 repeats at the COOH terminal of TRAF7 instead of a TRAF domain. TRAF proteins are important players in NF-κB signaling from various receptors. TRAF6 in particular is essential for NF-κB and MAP kinase activation by interleukin-1 receptor (IL-1R) and Toll-like receptors (TLR). Interestingly TRAF6 is able to activate IKK in a cell-free system (Deng et al., 2000). Fractionation of cytosolic extracts led to the discovery of two TRAF6-regulated IKK activators, TRIKA1 and TRIKA2 (TRAF6-regulated IKK activators 1 and 2). TRIKA 1 is a dimeric ubiquitin E2-UBC13/UEV1a, whereas TRIKA2 is a protein complex containing TGFβ-activated kinase (TAK1) and the adaptor proteins TAB1 and TAB2 (TAK1 binding protein 1 and 2) (Wang et al., 2001). TRAF6 and UBC13/UEV1a catalyze the formation of a unique polyubiquitin chain linked through K63 of ubiquitin, which activates the TAK1 kinase complex independent of proteolysis. TAK1 subsequently phosphorylates IKKβ and activates IKK complex. TABs and NEMO are the receptors that bind preferentially to K63 polyubiquitin chains (Ea et al., 2006; Kanayama et al., 2004). Mutations that disrupt the binding also abrogate TAK1 and IKK activation. Therefore polyubiquitin chains may function as a scaffold to recruit the TAK1 and IKK complexes, facilitating TAK1 to phosphorylate and activate IKK (Figure 3).

Several proteins in the IL-1R/TLR pathways have been shown to be the substrates of K63 polyubiquitination by TRAF6. However, it is not clear whether ubiquitination of any of these proteins is important for TAK1 and IKK activation. Recently Xia et al. showed that free K63 polyubiquitin chains, which are not conjugated to any target protein, directly activate TAK1 by binding to TAB2 (Xia et al., 2009). Furthermore, the free ubiquitin chains activate the IKK complex. Disassembly of the polyubiquitin chains by deubiquitination enzymes prevented TAK1 and IKK activation. Therefore, it is likely that unanchored polyubiquitin chains are rapidly

synthesized as second messengers after stimulations to activate protein kinases. Specific deubiquitination enzymes disassemble polyUb chains thereby terminate the signal.

The non-proteolytic function of polyubiquitination/polyUb chains in TAK1/IKK activation is not confined to IL-1R/TLR signaling. It is a regulatory mechanism utilized in various NF-κB activation pathways. For example, RIP1 is rapidly polyubiquitinated at lysine 377 (K377) following TNFα stimulation. A point mutation at K377 abolishes RIP1 ubiquitination and its ability to recruit TAK1 and IKK to the receptor and it inhibits IKK activation (Ea et al., 2006; Li et al., 2006). cIAP1 and cIAP2 may catalyze the polyubiquitination of RIP1 (Bertrand et al., 2008). Another example involves adaptive immunity. During T cell activation, a protein complex termed CBM is formed, which consists of CARMA1 (caspase recruitment domain-containing membrane-associated guanylate kinase 1), BCL10 (B cell lymphoma 10), and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1). The CBM complex is essential for the activation of IKK through a K63 polyubiquitination dependent mechanism. MALT1 contains TRAF2 and TRAF6 binding sites. Binding to MALT1 leads to the oligomerization of TRAF6 and activation of its E3 ubiquitin ligase activity. TRAF6 together with UBC13 and UEV1a catalyze K63 polyubiquitin chains, which mediate the activation of TAK1 and IKK. BCL10, MALT1, NEMO, and TRAF6 could be the targets of ubiquitination (Oeckinghaus et al., 2007; Wu and Ashwell, 2008; Zhou et al., 2004), while unanchored polyUb may also be the activator for TAK1 and IKK.

In summary, polyubiquitin chains, especially the ones linked through K63 of ubiquitin, mediate the activation of IKK and mitogen-activated protein kinases (MAPKs) through a proteasome-independent mechanism which is used by diverse NF-κB signaling pathways.

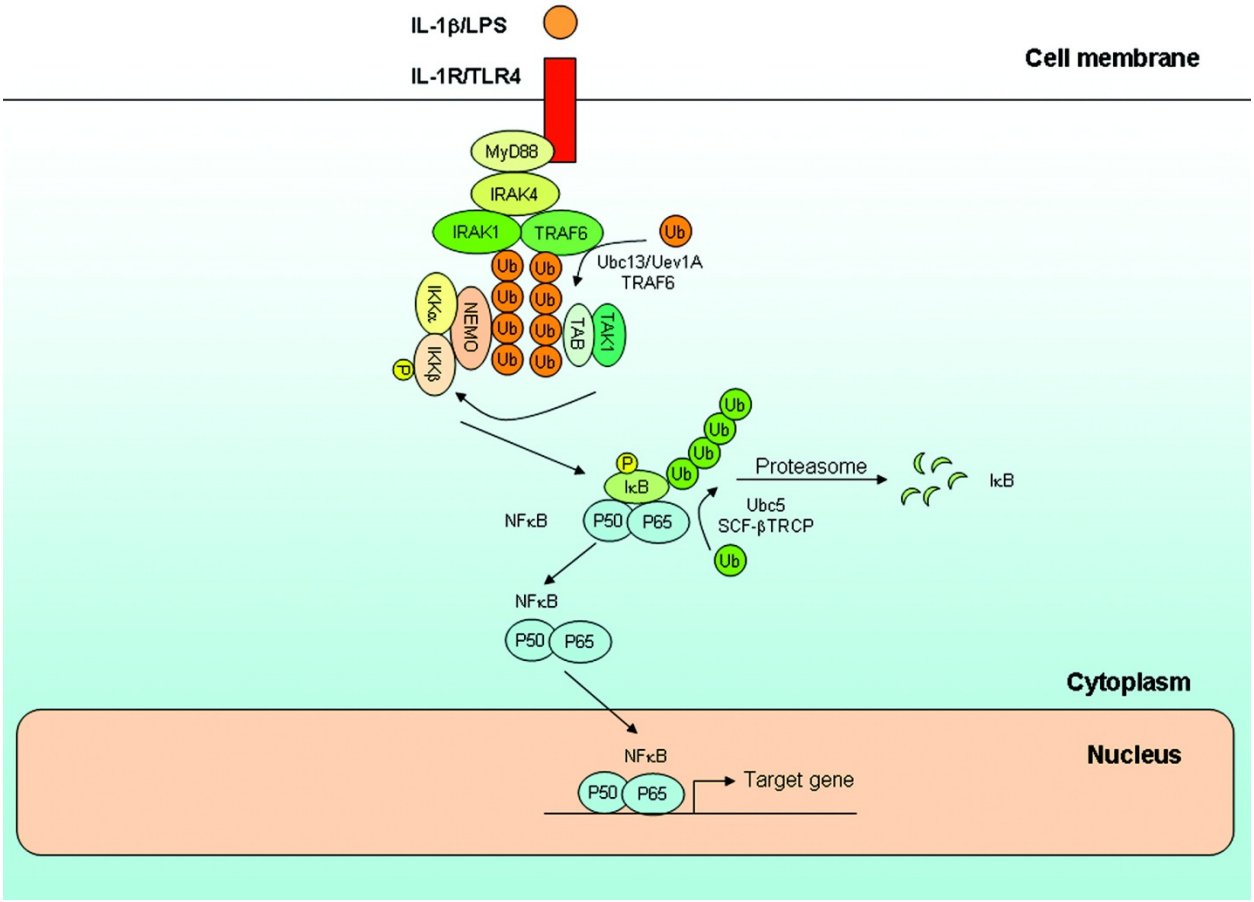


Figure 3. Roles of Ubiquitin in NF-κB Activation by IL-1 Receptor and Toll-like Receptors (IL-1R/TLR). Stimulation of IL-1R and TLRs by their ligands leads to recruitment of the adaptor protein MyD88, protein kinases IRAK4 and IRAK1, and ubiquitin ligase TRAF6. In the presence of the E2 UBC13/UEV1a, TRAF6 catalyzes K63 polyubiquitination of IRAK1 and TRAF6 itself. The polyubiquitin chains bind to TAB2 and TAB3 and activate the TAK1 kinase complex. The polyubiquitin chains also serve as a scaffold to recruit the IKK complex through NEMO, facilitating the phosphorylation of IKKβ by TAK1. IKK is activated to phosphorylate IκB proteins, which are subsequently ubiquitinated by the SCF-βTRCP ubiquitin E3 complex. The ubiquitinated IκBs are degraded by the proteasome, leading to NF-κB nuclear translocation and activation of downstream target genes.

Negative Regulation of Protein Kinases by Deubiquitination Enzymes

Like phosphorylation, ubiquitination is a reversible process and deubiquitination enzymes (DUB) are a family of proteases that catalyze the removal or trimming of ubiquitin chains from target proteins. Several DUBs have been shown to negatively regulate IKK activation in NF-κB pathways, supporting the important function of polyUb as discussed previously.

CYLD, cylindromatosis gene, encodes a tumor suppressor protein involved in the development of familial cylindromatosis, a benign skin tumor (Bignell et al., 2000). The CYLD protein contains an ubiquitin-specific protease domain (USP) domain at the C-terminus and three CAP-Gly domains (Cytoskeleton-associated proteins (CAP) glycine-rich domains) at the N-terminus. Overexpression of CYLD inhibits IKK activation, whereas reducing CYLD expression has the opposite effect (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). CYLD specifically cleaves K63-linked polyubiquitin chains from target proteins including NEMO, TRAF2, and TRAF6 (Brummelkamp et al., 2003; Kovalenko et al., 2003; Massoumi et al., 2006; Trompouki et al., 2003). A recent study by Komander et al. showed that an extended loop structure in USP domain was conserved in CYLD orthologues and it contributes to the specificity towards cleaving K63 linked polyUb chains (Komander et al., 2008). Genetic studies also reveal that CYLD removes K63-linked polyubiquitin chains from Bcl-3 and prevents it from translocating into the nucleus and forming a transactive complex with p50 or p52. In the absence of CYLD, cycline D1, one of the targets of NF-κB/Bcl-3, is turned on and the proliferation rate of keratinocytes is increased (Massoumi et al., 2006). Finally, Xia et al. has shown that CYLD is able to convert unanchored K63 polyubiquitin chains into monoUb and completely blocks the activation of TAK1 (Xia et al., 2009).

A20 is another DUB that negatively regulates NF-κB. Genetic deletion of A20 in mice causes persistent activation of NF-κB in response to TNFR and TLR stimulation, resulting in multi-organ inflammation, cachexia and neonatal lethality (Lee et al., 2000). A20 contains an NH_2 terminal OTU domain and seven zinc finger domains at the COOH terminus (Evans et al., 2003). It has been proposed that the OTU domain of A20 inhibits IKK by removing K63 polyubiquitin chains from target proteins including RIP1 and TRAF6 (Boone et al., 2004; Wertz et al., 2004). However studies on the structure of A20 OTU showed that it does not prefer K-63 chains but rather recognize specific substrates modified by ubiquitin (Komander and Barford, 2008; Lin et al., 2008a). A20 also forms complex with other ubiquitin-binding proteins such as ITCH, TAX1BP1 and ABINs, which bring substrates close to A20 to facilitate deubiquitination.

Part II

CC2D1A is Involved in Autosomal Recessive Non-Syndromic Mental Retardation

Mental retardation affects approximately 2% of the general population (McLaren and Bryson, 1987). Its defining features include an intelligence quotient (IQ) below 70, significant limitations in two or more adaptive skills and early onset from childhood (Leonard and Wen, 2002). Non-syndromic mental retardation (NSMR) is diagnosed by exclusion in mentally retarded patients who do not have any physical or neurological abnormality. Genetic basis of mental retardation has been found in nearly two thirds of cases of mental retardation (Curry et al., 1997). Various X linked genes are associated with NSMR, while only a few autosomal genes have been shown to cause autosomal recessive NSMR. The difficulties in identifying autosomal genes are heterogeneity and the absence of clinical criteria for grouping NSMR families for linkage analysis.

CC2D1A on chromosome 19 was identified from nine consanguineous families with severe autosomal recessive NSMR by homozygosity mapping (Basel-Vanagaite et al., 2006). Detailed mutation analysis revealed, in all affected patients, the deletion of 3567 nucleotides beginning in intron 13 and ending in intron 16. The mutation abolishes the last DM14 domain and C2 domain. Since the first three DM14 domains still exist in patients, the phenotype may be hypomorphic. By in situ hybridization analysis in staged mouse embryos during brain development, CC2D1A mRNA was identified at E12 throughout the ventricular zone and developing cortical plate and ganglionic eminences. By E16, it is found throughout the mouse brain, especially in the cortical plate. At postnatal day 3 (P3), the expression is still widely widespread and the strongest signals come from the cerebral cortex and hippocampus. This pattern continues in the brain into adulthood (Basel-Vanagaite et al., 2006). Given the expression patterns and the phenotype in patients, CC2D1A appears to be a regulator in the fundamental processes in the brain which control cognitive development in human.

Chemical synapses are the fundamental information processing units in the brain. In the following sections, I will review the basics of synaptic transmission as well synaptic maturation.

Structure of Central Synapses

Synapses are the intercellular junctions between presynaptic termini and postsynaptic cells. In electron microscopic pictures, synapses are asymmetric structures composed of electron dense materials, as shown in Figure 4. Presynaptic termini are filled with synaptic vesicles of ~50nm in size, clustered around the plasma membrane region called the active zone. Neurotransmitters are stored in synaptic vesicles and are released through synaptic vesicle exocytosis at the active zone (Sudhof and Malenka, 2008). Active zones are comprised of a

variety of scaffolding molecules that enable and regulate vesicle fusion and recycling (Sudhof, 2004). Postsynaptic densities are enriched in scaffolding molecules that anchor neurotransmitter receptors and transduce signals from activated receptors. The pre- and postsynaptic sides of synapses are held together by trans synaptic cell adhesion molecules and therefore pre- and postsynaptic sides of a synapse are precisely aligned and biochemically difficult to separate from each other (Lisman and Harris, 1993).

An axon is a fiber-like extension that a neuron uses to send information to its target whereas a dendrite is a-net like structure of a neuron's cell body that receives information from axons (Neurotransmitter Release, 1999, edited by Hugo Bellen). In other words, a neuron develops two sets of cellular structure, both presynaptic and postsynaptic, in order to fulfill the function in information processing and transmission. Adhesion molecules, for example, have to be differentially distributed within a neuron, and it is not clear yet what is the signal during synaptogenesis to steer this process. Nevertheless, the communication across the synaptic cleft through either adhesion molecules or soluble factors account for the coordinated assembly of the structures on both sides of a synapse.

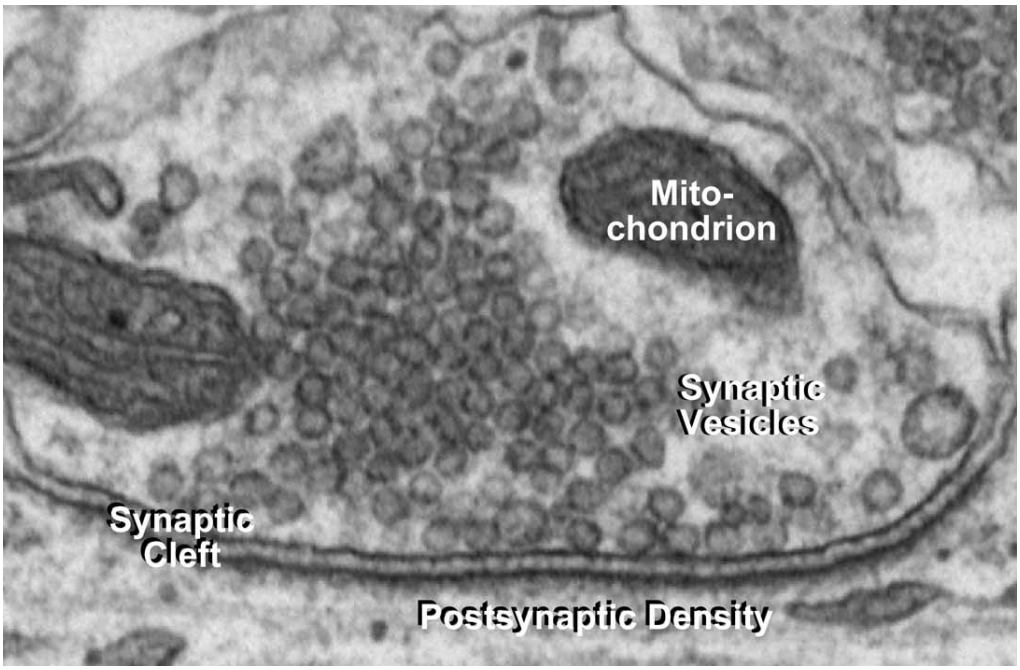


Figure 4. Electron Micrograph of A Synapse (adapted from Sudhof, 2004).

Machinery of Presynaptic Vesicle Fusion

Neurotransmitter release is mediated by a Ca^{2+} triggered synaptic vesicle fusion with plasma membrane at the active zone (Sudhof and Malenka, 2008). The fusion is catalyzed by SNARE and SM proteins. Syntaxin-1 and SNAP-25 are plasma membrane localized SNARE, whereas VAMP/synaptobrevin is synaptic vesicle-associated. Individual SNARE proteins are unfolded, and when they spontaneously assemble into an organized four helix bundle between membranes (known as "trans-SNARE complex" or "SNAREpin"), they catalyze fusion by forcing membranes closely together (Hayashi et al., 1994; Weber et al., 1998). The energy from folding is transferred into the bilayer to perturb the membrane (Cohen and Melikyan, 2004; Li et al., 2007). Once assembled, SNARE complexes are dissociated by the ATPase NSF and its adapter protein, SNAP (Sollner et al., 1993; Weber et al., 1998). Synaptic SM protein Munc18-1

binds to Syntaxin-1 (Hata et al., 1993). Exactly how SM proteins cooperate with SNARE complexes for fusion is not clear yet. It is proposed that SM proteins act as "catalysts" for SNAREs by helping them assemble into a favorable topology at the interface of two membranes (Sudhof and Rothman, 2009). Since membrane fusion is driven by a thermodynamically spontaneous process of protein folding, various regulators are necessary to prevent random fusion from occurring. Complexin is such a regulator. Synaptic SNAREs are first activated and then clamped by complexin (Giraudo et al., 2006). When calcium binds to the calcium sensor synaptotagmin, the clamp would be released allowing fusion to be completed.

Synaptic vesicles can be divided into different pools based on both the physical distance from the active zone and the functional "readiness" for release. At a mature synapse, there are a small fraction of vesicles docked at the active zone, and when calcium ions rush into the nerve terminal as a result of an arriving action potential, these vesicles are selectively released in less than a millisecond (Sabatini and Regehr, 1996). Therefore they comprise the "readily releasable" pool (RRP). It is proposed that vesicles in RRP are more kinetically advanced because their v-SNAREs have already formed a complex with t-SNAREs and complexin binding to this complex keeps the vesicle from fusion until Ca^{2+} influx (Sudhof and Rothman, 2009). There is also a secondary pool of vesicles, the reserve pool, which is spatially distant from the plasma membrane active zone and replenishes the RRP which have undergone exocytosis (Rizzoli and Betz, 2005).

Synapse Assembly Process

In the mammalian central nervous system, synapse formation is a complex process because of the enormous heterogeneity of neuron types and different timing of their development

(Goda and Davis, 2003). However, a reciprocal signaling model has been proposed as a general underlying mechanism of synapse formation in CNS (Goda and Davis, 2003). Reciprocal interactions between the pre- and postsynaptic cells can occur before the initial contact through diffusible factors whereas after the initial contact, trans-synaptic adhesion molecules trigger asymmetric signaling events to further specify the synapse assembly (Figure 5). Moreover, factors derived from glia cells may also contribute to synaptogenesis.

Filopodial extensions of neuronal processes have been suggested to induce synapse formation (Fiala et al., 1998; Jontes and Smith, 2000). The number of both motile dendritic and axonal filopodia is inversely correlated with the appearance of stable synaptic connections (Dunaevsky et al., 1999; Jontes et al., 2000; Jontes and Smith, 2000; Tashiro et al., 2003), indicating that axons and dendrites modulate synapse formation by regulating their filopodial motility. Neurotransmitters as well as several diffusible factors seem to regulate filopodial formation.

During synaptogenesis, neurotransmitters are released from exocytic “hot spots” where small clusters of synaptic vesicles reside, in response to presynaptic electrical activity (Kraszewski et al., 1995; Sun and Poo, 1987). Released glutamate enhances the motility of both axonal and dendritic filopodia through binding to the receptors (Dailey and Smith, 1996; Lendvai et al., 2000; Tashiro et al., 2003). Thus localized release of glutamate increases the possibilities of axon-dendritic contact during the early synaptogenic stage. In contrast, after the synapse forms, glutamate release has been shown to decrease filopodial motility to stabilize and promote the maturation of synaptic connection (Tashiro et al., 2003). There is also evidence that the initial contact formation does not require neuronal activity. For example, Deletion of the major synaptic SM protein Munc18-1 in mice results in a complete loss of neurotransmitter release during

development (Verhage et al., 2000). However normal brain assembly was not affected, although neurodegeneration occurred afterwards. This shows that neurotransmitters are not required for the formation of synaptic connections but are necessary for established connections. Other factors may function redundantly with neurotransmitters to steer filopodial motility and induce synapse formation.

Several members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins, have been shown to promote synapse formation (Agerman et al., 2003; Bonhoeffer, 1996; McAllister et al., 1999). Secreted Wnt proteins, including Wnt-7a, Wnt-3, have also been shown to act in synaptogenesis (Hall et al., 2000; Krylova et al., 2002). Furthermore, suppression of agrin, a key synaptogenic factor at the NMJ, in cultured hippocampal neurons severely inhibits synapse formation (Ferreira, 1999), although data from agrin knockout mice showed agrin is not essential for synapse formation *in vivo* (Li et al., 1999). Also, in the absence of glia, the number of synapses formed between retinal ganglion cells in culture is reduced and synapses are less efficient in neurotransmitter release (Pfrieger and Barres, 1997). Later on cholesterol and thrombospondins 1 and 2 were identified as glia-derived synaptogenic molecules (Christopherson et al., 2005; Pfrieger, 2003). Thus, the initial contacts between axonal and dendritic protrusions can be stimulated by secreted synaptogenic molecules and their receptors, and presumably temporal and spatial regulation of these factors help to determine the specificity of synapse formation.

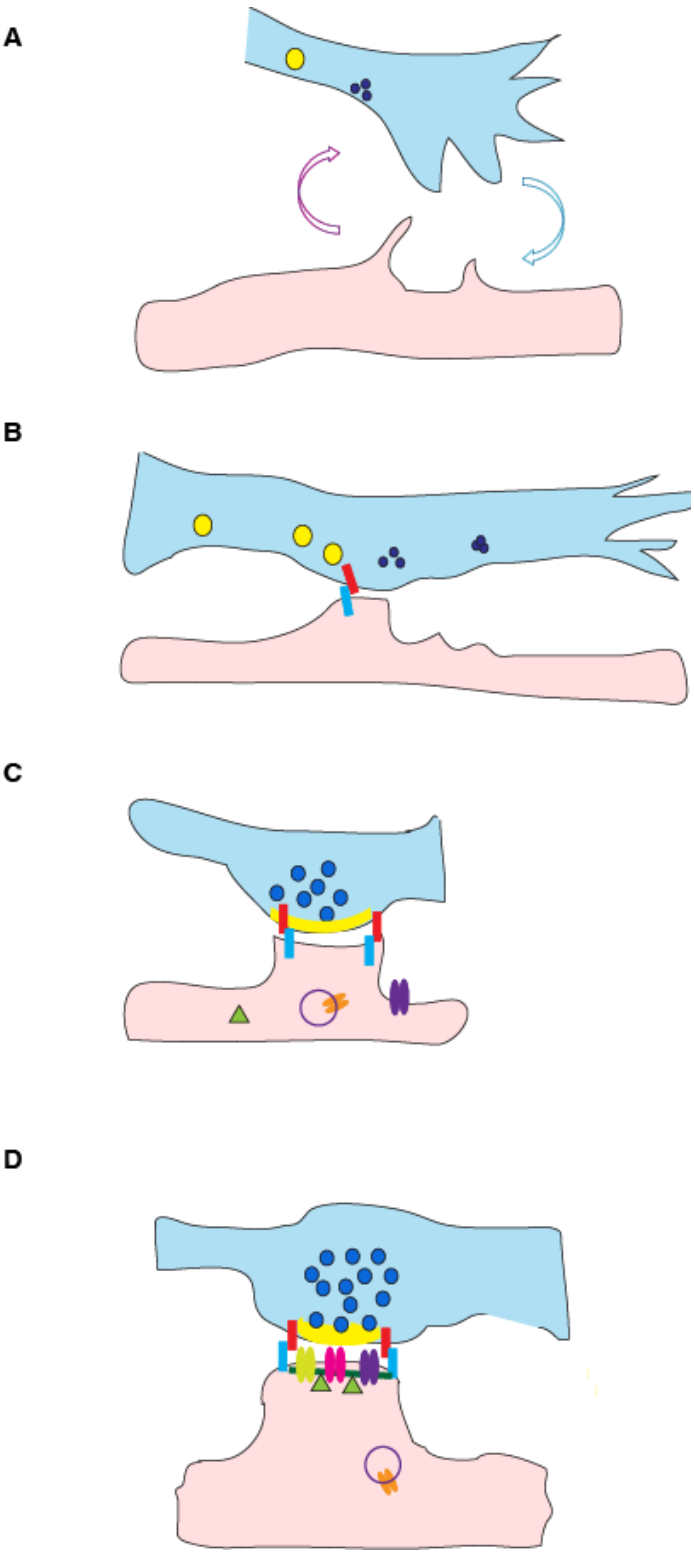


Figure 5. A Model of Excitatory Central Synapse Formation. (Adapted from Goda and Davis, 2003)

A. Before the initial contact, motile axonal and dendritic filopodia search for partners. Secreted molecules as well as neurotransmitters are released. Blue circles indicate release "hot spot" where small clusters of synaptic vesicles are located. Yellow circles stand for small packets that contain active zone elements and travel along the axon.

B. Selective contact site is strengthened by cell adhesion molecules.

C. Active zone proteins (yellow) and synaptic vesicles clustered at pre-synaptic terminal. At post-synaptic side, neurotransmitter receptors (purple) and scaffolding proteins (green) are recruited following presynaptic assembly.

D. At assembled synapses, different pools of synaptic vesicles associate with presynaptic termini whereas neurotransmitter receptors together with the scaffold proteins associate with postsynaptic termini.

Role of Synaptic Cell Adhesion Molecules in Synapse Assembly

Synaptic cell adhesion molecules mediate the physical interaction and functional communication between axonal and dendritic protrusions triggering synaptic specializations (Brose, 1999; Sanes and Yamagata, 1999; Sudhof, 2001). Extensive alternative splicing as well as post-translational modifications of these molecules creates an enormous diversity. Differential combinations of these variable molecules induce "reciprocal" signaling across synapse and create the specificity of synaptic connections in the brain (Goda and Davis, 2003). Synaptogenesis is not an event determined by a single molecule, but rather a network of protein-protein interactions, spatially and temporarily, induces, strengthens and refines the synaptic connections.

Neurexins (Nrxns) and neuroligins (Nlgns) may be the best characterized synaptic adhesion molecules. Neurexins are polymorphic synaptic receptors at the presynaptic termini, whereas neuroligins are neurexin ligands enriched in postsynaptic densities (Berninghausen et al., 2007). The trans-synaptic complex of neurexin and neuroligin interacts with PDZ-domain containing proteins on both sides. Nrxn contains a COOH terminal cytoplasmic sequence that binds to the PDZ domain of CASK and related proteins, and a membrane-proximal binding site for protein 4.1. CASK can polymerize actin on the Nrxn COOH terminal by simultaneously binding to protein 4.1 (Biederer and Sudhof, 2001) and trap traveling synaptic components. Nlgns bind to another PDZ domain protein PSD95, which in turn recruit NMDA receptors to the synapse (Irie et al., 1997; Sheng and Hoogenraad, 2007).

When expressed in a non-neuronal cell, Nlgns can induce co-cultured neurons to form presynaptic specializations onto the non-neuronal cell (Scheiffele, Fan et al. 2000). Similarly,

expressing Nrxn in a non-neuronal cell can induce formation of postsynaptic specializations in co-cultured neurons (Graf et al., 2004; Nam et al., 2005). These studies indicated that Nrxns and Nlgns may induce synapse formation. However genetic evidence from KO mice revealed that Nlgns and α-Nrxns are essential for synaptic function, but not synapse formation (Chubykin et al., 2007; Missler et al., 2003; Varoqueaux et al., 2006). It seems contradictory to the *in vitro* results. As explained in Sudhof 2008 the assay didn't measure synapse induction directly but rather measure the increase in synapse number. Thus the overexpressed proteins may only stabilize transient synapses (Sudhof, 2008). One hypothesis on how Nrxns and Nlgns function in synapses is by engaging asymmetric scaffolding proteins, they trigger bi-directional signal transduction events that modulate synaptic function and regulate plasticity. Without this regulatory mechanism, synapses can assemble but cannot function properly (Sudhof, 2008). In humans, mutations in Nrxns or Nlgns are implicated in autism and other cognitive diseases, reaffirming their function at synapses, the basic unit of information process in the brain.

Role of Neuronal Activity in Synaptic Maturation

After the initial assembly of synaptic termini, many synapses are non-functional, known as silent (Atwood and Wojtowicz, 1999). The silent synapses can become functional in response to activity. The effects of activity on synapse development are manifested through the release of neurotransmitters, which in turn binds to cognitive receptors and induce a series of signaling events in neurons. The most prominent signal is a rapid and transient rise in calcium levels, which leads to short-term or long-term synaptic alterations depending on the cellular context (Greer and Greenberg, 2008). Locally, Ca^{2+} may activate kinases such as PKCs to phosphorylate synaptic proteins and change their functions, or induce the insertion of glutamate receptors (Malinow and Malenka, 2002). In the nucleus, Ca^{2+} dependent gene transcription has been

shown to regulate dendritic growth, synapse development and neuronal plasticity (Greer and Greenberg, 2008). Over 300 genes have been shown to be regulated by neuronal activity (Lin et al., 2008b), and among them there are a large number of transcription factors, including CREB, MEF2, NFAT and NF-κB. These transcription factors control different groups of target genes both temporally and spatially to shape the proper synaptic maturation. In contrast, disturbance of the activity-dependent gene expression program, leads to various human cognitive disorders.

NF-κB in the Central Nervous System

NF-κB transcription factors are present throughout the nervous system. However the function of NF-κB in the brain is largely unknown. Since the role of NF-κB in regulating apoptosis is well characterized, it is easier to translate that knowledge into CNS in pathological settings whereas understanding the physiological function for brain NF-κB is more difficult and yet very interesting.

More than 15 years ago, NF-κB subunits were identified at the synapses of neurons (Kaltschmidt et al., 1993). Isolated synapses appear to contain the p65-p50 heterodimer selectively (Meffert et al., 2003). Glutamate can activate NF-κB transcription factors in the physiologically active synaptosomal preparation via a Ca^{2+}-dependent pathway, implying that signaling components required to activate NF-κB, including $Ca^{2+,}$ are present at synapses (Meffert and Baltimore, 2005; Meffert et al., 2003). To strengthen this argument, Meffert et al showed that intracellular Ca^{2+} chelator BAPTA but not Br_2-BAPTA or EGTA was able to block NF-κB activation as assessed by κB DNA-binding (EMSA) and κB-luciferase reporter. Comparing to BAPTA, Br_2-BAPTA has similarly rapid Ca^{2+} on-rate with a much lower equilibrium affinity (Kd 3.6uM vs. 160nM), thus, Br_2-BAPTA cannot block NF-κB activation in

the presence of uM Ca^{2+}. On the contrary, EGTA has similar high affinity for Ca^{2+} but a slower on-rate that allows Ca^{2+} to spread to within 1-2 um from the point of influx. Therefore, a micro molar level of Ca^{2+} within 1-2 um of the point of influx is sufficient to activate NF-κB and transcription in the nucleus upon synapse stimulation. In mature hippocampal neurons, the Ca^{2+} responsive signaling pathway for the activation of NF-κB involves CaMKII, which is highly enriched at synapses (Goldenring et al., 1984; Miller and Kennedy, 1985). Inhibition of CaMKII reduces depolarization-induced NF-κB activation and constitutively active CaMKII (T286D) activates NF-κB based on EMSA and κB-luciferase assay (Meffert and Baltimore, 2005; Meffert et al., 2003). The ubiquitin-proteasomal system required for IκB degradation is also present in dendrites and synapses of the CNS and is regulated by synaptic activity (Ehlers, 2003; Patrick et al., 2003). Therefore the signaling machinery for NF-κB activation exists at synapses and it is responsive to synaptic activities.

NF-κB is constitutively active in neurons (Kaltschmidt et al., 1994). It may be involved in translating short-term signals from distal synapses into long-term changes in gene expression, regulating plasticity, development and survival (Kaltschmidt et al., 2005). p65 KO mice on a TNFRI KO background showed severe learning deficit (Meffert and Baltimore, 2005). Another two mouse models expressing dominant negative IκBs in neurons (Fridmacher et al., 2003) or neurons and glia (Ben-Neriah and Schmitz, 2004) both showed a learning impairment despite some discrepancy which may be due to differential expression of DN IκBs. Moreover, behavior experiments were conducted on adult mice without any pretreatment of neurotoxic agents, all of which apparently have normal CNS development. Thus the function of NF-κB in learning and memory may not involve the effect on apoptosis.

The role of NF-κB in neuroprotection and neurodegeneration is complicated and condition-specific. Grilli and Memo have proposed that NF-κB participates in the initiation and acceleration of several neurodegenerative diseases such as Parkinson's disease (PD), Huntington's disease (HD) or Alzheimer's disease (AD), as NF-κB hyperactivation is disease promoting (Grilli and Memo, 1999). On the other hand, Kaltschmidt et al believe that NF-κB activation by low doses of Aβ protects against a high cytotoxic dose of Aβ and that the nature of apoptotic stimulus determines the pro- or anti-apoptotic outcomes of NF-κB (Kaltschmidt et al., 2005). There seems to be a NF-κB homeostasis in the CNS. Basic synaptic transmission underlies the constitutive activation of NF-κB, which is essential for neuronal survival (Bhakar et al., 2002). A perturbation of the physiological NF-κB activation may result in cell death under pathological conditions (Kaltschmidt et al., 2005).

Part III

Other Proposed Functions of CC2D1A

Drosophila Orthologue of CC2D1A Regulates Endocytosis and Notch Activation

A tumor suppressor gene Lethal (2) giant discs (Lgd) is the *Drosophila* orthologue of CC2D1A. Mutations in the gene induce strong Notch activation and hyperplastic overgrowth of *Drosophila* imaginal discs (Agrawal et al., 1995; Buratovich and Bryant, 1997; Klein, 2003). Notch pathway signaling is critically involved in many developmental processes. Notch receptors are transmembrane proteins. Upon binding to Delta, Serrate, Lag2 (DSL) ligands on adjacent cells, Notch goes through two consecutive cleavages (S2 and S3) that result in the release of the intracellular domain (Nintra) into the cytoplasm. The S2 cleavage is within the extracellular region mediated by the ADAM transmembrane metalloprotease Kuzbanian (Kuz),

creating a membrane assciated intermediate called Notch Extracellular Truncation (NEXT). The following S3 cleavage is within the transmembrane domain mediated by the γ-secretase complex, with the Presenilin protease as its core component. Finally, Nintra translocates to the nucleus, associates with CSL (CBF1, Su(H), Lag-1) transcription factors and activates expression of downstream genes (Mumm and Kopan, 2000).

Notch pathway is used in many processes during *Drosophila* development, i.e. the induction of joints in the legs, development of external sensory organs in the peripheral nervous system, and the establishment of the wing axis (Jaekel and Klein, 2006). Therefore, Notch signaling is tightly regulated and de-regulation of the pathway leads to pathological conditions. Endocytosis is an important regulatory mechanism of Notch activity. In the signal sending cells, endocytosis of DSL ligands is required for signaling probably because ligand endocytosis leads to a conformational change of the bound receptor that facilitates extracellular cleavage, or because modification made by passage through an endocytic recycling pathway is required for DSL ligands to activate Notch (Gallagher and Knoblich, 2006). In signal receiving cells, endocytic trafficking plays both positive and negative roles. S3 cleavage of Notch may require its endocytosis (Gupta-Rossi et al., 2004). In addition, non-activated Notch shuttles continuously through endosomal trafficking before being degraded in the lysosome. Several ubiquitination enzymes are required for this process, for example, E3 ligases Nedd4 and Suppressor of Deltex [Su(dx)] (Sakata et al., 2004; Wilkin et al., 2004). In the absence of these proteins, Notch is activated in a ligand independent manner. A similar phenotype was seen in the loss of function of the ESCRT components Erupted and Vps25, which block the formation of multivescular bodies (MVBs) and result in ectopic Notch activation as well (Moberg et al., 2005; Thompson et al., 2005).

In 2006, several papers were published on the role of Lethal (2) giant discs (Lgd) in the regulation of endosomal trafficking and Notch activation (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). They shared several conclusions. First, loss of Lgd activity results in ectopic activation of Notch and hyperplastic overgrowth of *Drosophila* imagical discs. Second, Lgd overexpression can lead to activation and inactivation of the Notch pathway, indicating that Notch has to be expressed at the right amount to function properly. Third, Notch and other transmembrane proteins accumulate on large endosomal vesicles. Fourth, activation of Notch in *lgd* mutants is independent of its ligands. Last, Hrs/Vps27 is epistatic to Lgd. Hrs/Vps27 is the ubiquitin binding protein involved in ESCRT-dependent endocytosis machinery (Winter and Hauser, 2006). Therefore, it is proposed that Lgd is a general regulator of protein sorting and *lgd* leads to the accumulation of Notch in a compartment where it can be more efficiently cleaved by Presenilin. Although the exact point at which the endosomal pathway is affected in *lgd* mutants remains to be determined, *hrs* mutants block Notch trafficking at an earlier step than *lgd*. In the double mutant, the early block in the trafficking pathway does not allow Notch to reach the later compartment in which it would accumulate and get activated in *lgd* mutant.

There are two orthologues of Lgd in mammals, CC2D1A and CC2D1B. Both murine orthologues are able to functionally rescue *lgd* during *Drosophila* wing development (Jaekel and Klein, 2006). Although Lgd is not conserved in yeast, it is possible that a more sophisticated mechanism is required to fulfill the task of protein sorting in multicellular organisms.

CC2D1A is a Multifunctional Gene

CC2D1A has been shown to interact with phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1) and function as a selective scaffold to activate PDK1/Akt pathway in response to epidermal growth factor signaling (Nakamura et al., 2008). It was named Akt Kinase Interacting protein 1 (Aki1). The same lab recently discovered that Aki1 also localizes at centrosome and it is required for centriole cohesion (Nakamura et al., 2009). In the absence of Aki1, multipolar spindles are formed during mitosis accompanied by centriole splitting. Interestingly, the COOH terminal deletion mutant (ΔC815-Aki1) did not localize to the centrosome and lost the ability to rescue the phenotype of Aki1 RNAi, suggesting that the COOH terminus is functionally important by targeting Aki1 to the centrosome.

CC2D1A has also been identified as a novel repressor of serotonin (5-HT) receptor 1A (5-HT1A). Serotonin and 5-HT1A are implicated in major depression, anxiety, and suicide. 5-HT1A is expressed on both presynaptic and postsynaptic sites. On presynaptic serotonergic raphe neurons, it is a somatodendritic autoreceptor which inhibits the firing of raphe neurons and serotonin neurotransmission. While on postsynaptic sites including the cortex, hippocampus, limbic system, and hypothalamus, 5-HT1A regulates mood and emotion (Tork, 1990). 5-HT1A knock-out mice display enhanced serotonergic neurotransmission, attributable to the loss of receptors on the presynaptic sites (Ase et al., 2000), and have increased anxiety-related behaviors (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Importantly, an increase in 5-HT1A autoreceptor expression is associated with major depression (Lemonde et al., 2004; Mongeau et al., 1992). Chronic antidepressant treatment for 2-3 weeks de-sensitized 5-HT1A autoreceptors and enhanced 5-HT neurotransmission and clinical efficacy, indicating that the

drugs down-regulate 5-HT1A gene transcription. However, the regulation of 5-HT1A expression is not well understood.

Upstream of the 1 KB promoter/enhancer of 5-HT1A gene, there is a strong repressor region which completely suppressed 5-HT1A gene transcription in non-neuronal cells and also reduced expression in 5-HT1A positive cells (Lemonde et al., 2004; Ou et al., 2000). The repressor (DRE, dual repressor element) is composed of FRE (5'-repressor element) and TRE (3'-repressor element). CC2D1A was identified as a DRE binding protein through a yeast one-hybrid screen of a mouse brain cDNA library (Ou et al., 2003). It was named Freud-1 (FRE Under Dual repression binding protein-1). Overexpreesion of Freud-1 down regulated 5-HT1A expression in both raphe and non neuronal cells, whereas RNAi of Freud-1 derepressed the gene only in raphe cells and increased 5-HT1A protein level. Also an intact C2 domain is required for Freud-1 mediated repression. Calcium dependent signaling impaired Freud-1-FRE interaction and derepressed 5-HT1A promoter. Inhibitors of calmodulin or CAMK blocks calcium-mediated inhibition of Freud-1 (Ou et al., 2003). In addition, in 5-HT1A expressing neurons, HDAC inhibitor trichostatin A (TSA) did not affect DRE/Freud-1 mediated repression (Lemonde et al., 2004). It remains a question whether Freud-1 plays a role in regulating 5-HT1A autoreceptors *in vivo*. Further studies are required to understand the role of Freud-1 in human depression.

Summary

Based on all these discoveries, CC2D1A seems to have multiple functions in cells ranging from signal transduction, scaffolding, centriole disengagement, endocytosis to gene transcription. These functions are correlated with its localization. CC2D1A has been shown to localize in the cytosol, centrosome, endosome and nucleus. It is likely that specific cofactors at

different subcellular regions cooperate with CC2D1A to carry out these functions. Most of the experiments were carried out in cell lines thus the physiological role of CC2D1A remains to be determined. Given the phenotype in patients, it would be interesting to understand how these proposed functions translate into the regulation of cognitive development in human. In addition since patients still have the truncated protein, the phenotype may be hypomorphic and CC2D1A could regulate a broader spectrum of processes in the central nervous system as well as other parts of the body during development.

My dissertation study was aimed at elucidating the mechanism by which CC2D1A activates NF-κB using biochemical methods. Using a luciferase reporter assay together with RNAi in HEK293T cells, I identified several components that act downstream of CC2D1A to activate NF-κB, all of which are involved in the canonical NF-κB signaling pathway. I also used yeast-two-hybrid to find the interacting partners of CC2D1A. In order to figure out the upstream signals that regulate CC2D1A as well as its physiological function, I generated Cc2d1a KO mice. The phenotype of KO led me to focus on the role in the CNS. In collaboration of Jesica Raingo, I characterized the properties of KO neurons in an *in vitro* culture system. The resulting data provide the evidence that CC2D1A not only is a potent NF-κB activator *in vitro*, it also regulates synaptic maturation and function in mice. My study could help to understand molecular mechanism for human diseases such as mental retardation.

CHAPTER II

CC2D1A ACTIVATES NF-κB

Abstract

Transcription factor NF-κB regulates diverse biological processes including immunity, inflammation and apoptosis (Hayden and Ghosh, 2008). A vast array of cellular stimuli converge on NF-κB and ubiquitination plays essential roles in regulating signal transduction from these stimuli to the activation of NF-κB. Here I show that CC2D1A is a potent activator of NF-κB. The activation of NF-κB by CC2D1A requires ubiquitin-conjugating enzyme UBC13, a RING domain ubiquitin ligase TRAF2, an ubiquitin dependent IKK kinase TAK1 and an essential regulator of IKK complex NEMO. CYLD, a deubiquitination enzyme specifically degrading Lysine-63 (K63) linked polyubiquitin chains (polyUb), negatively regulates the activity of CC2D1A, suggesting that K63 linked polyUb is also involved. Therefore, CC2D1A depends on the canonical IKK pathway to activate NF-κB.

Results

CC2D1A Activates NF-κB Luciferase Reporter.

As mentioned in Chapter one, a large scale screen of human genes was carried out by Matsuda et al. to identify NF-κB activators. They found 25 novel genes whose involvement in NF-κB pathway has never been reported (Matsuda et al., 2003). Among them, clone 031N was later on characterized as MAVS, a mitochondria localized adaptor that mediates NF-κB and IRF3 activation in response to viral infection (Seth et al., 2005). Overexpression of MAVS induces IFNβ production through activation of both NF-κB and IRF3, thus inhibiting viral replication. MAVS contains NH_2 terminal CARD like domain and COOH terminal transmembrane domain that targets the protein to mitochondria. Both domains are required for

MAVS signaling implying that mitochondria may function in innate immunity. We found five other "novel genes" from the screen have a mitochondria sorting signal and thought they might coordinate with MAVS in antiviral signaling. I obtained the five cDNAs from Openbiosystem, which are AB097000, AB097019, AB097008, AB097016, and AB097002, and subcloned the cDNAs into the mammalian expression vector pcDNA3-N-Flag so that each gene product has the NH_2 terminal Flag tag. I introduced each clone into HEK293T cells to test if it induces IFN reporter expression. IFN reporter is a luciferase gene under the control of IFN promoter and contains enhancer elements that bind to several transcription factors including NF-κB, IRF3, and ATF2. I also co-transfected the cells with pCMV-LacZ in which the beta galactosidase gene is under the CMV promoter, as an internal control for DNA transfection efficiency. I found that none of the five genes activated IFN reporter gene. This result indicated that these genes aren't able to activate all the transcription factors required to turn on IFN promoter. I then constructed an NF-κB reporter in which luciferase gene is under the control of three tandem repeats of NF-κB binding site. Again, pCMV-LacZ was used as an internal control. The fold activation of vector pcDNA3 was set as one. As shown in Figure 6A, only two genes, AB097000 and AB097002, activated NF-κB reporter. RIG-I NH_2 terminus was used as a positive control for NF-κB induction. AB097002 turned on luciferase gene expression very strongly and the fold activation was in proportion to the amount of DNA transfected.

CC2D1A is Conserved through Evolution

AB097002 is named CC2D1A (coiled coil and C2 domain containing protein 1A). It has two recognizable domains in its primary sequence: four tandem repeats of DM14 (**D**rosophila **M**elanogaster 14 domain, repeats in fly CG4713, worm Y37H9A.3) at the NH_2 terminal, whose function is unknown, and at the COOH terminal a C2 (CalB) domain, which is the Ca^{2+}-binding

motif present in phospholipases, protein kinases C, and various synaptic protein domains (Figure 6B). Five canonical aspartic acids that are typical of C2 domains (Rizo and Sudhof, 1998) are absent from C2 domain of CC2D1A, indicating it may not bind Ca^{2+}. However the *Drosophila* orthologue of CC2D1A-lethal (2) giant discs (Lgd) has been shown to bind phospholipids present on early endosomes (Gallagher and Knoblich, 2006). Therefore it is possible that CC2D1A binds phospholipids independent of Ca^{2+}. However it is not clear how CC2D1A binds to phospholipids. CC2D1A is a conserved protein from worm to human (Figure 7), especially at the C2 domain (yellow highlight) and DM14 domains (green highlight), underscoring their functional importance.

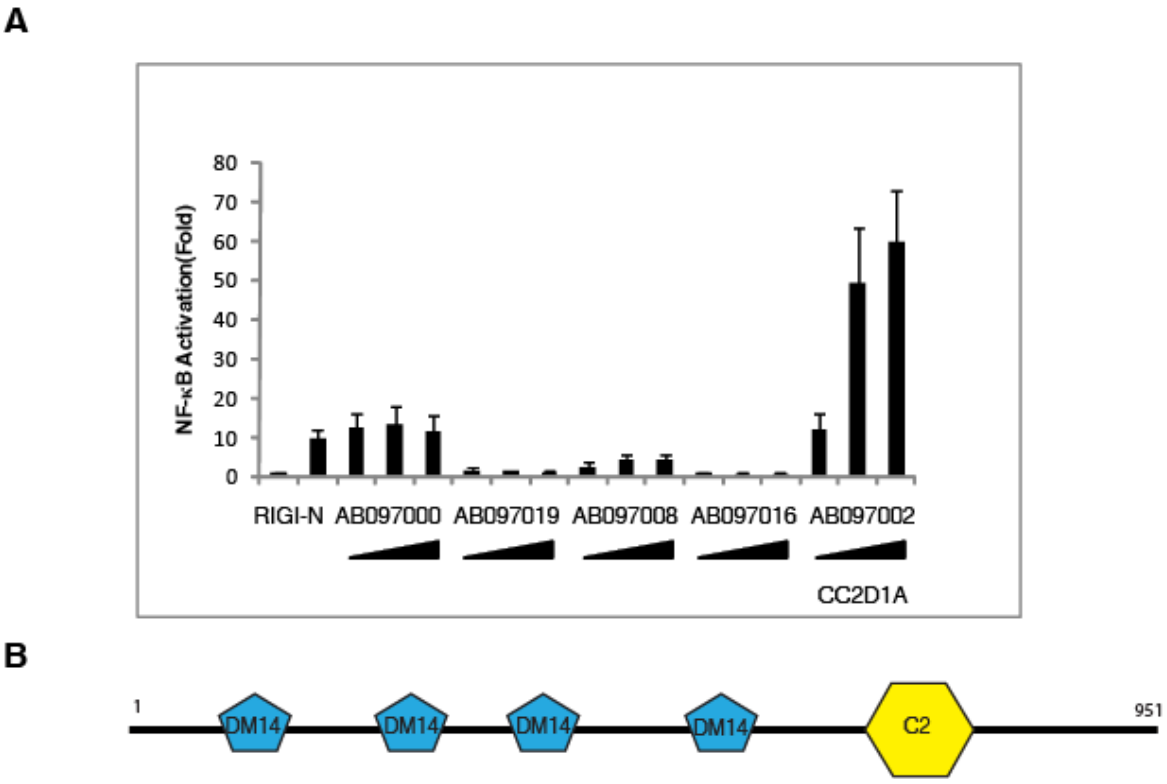
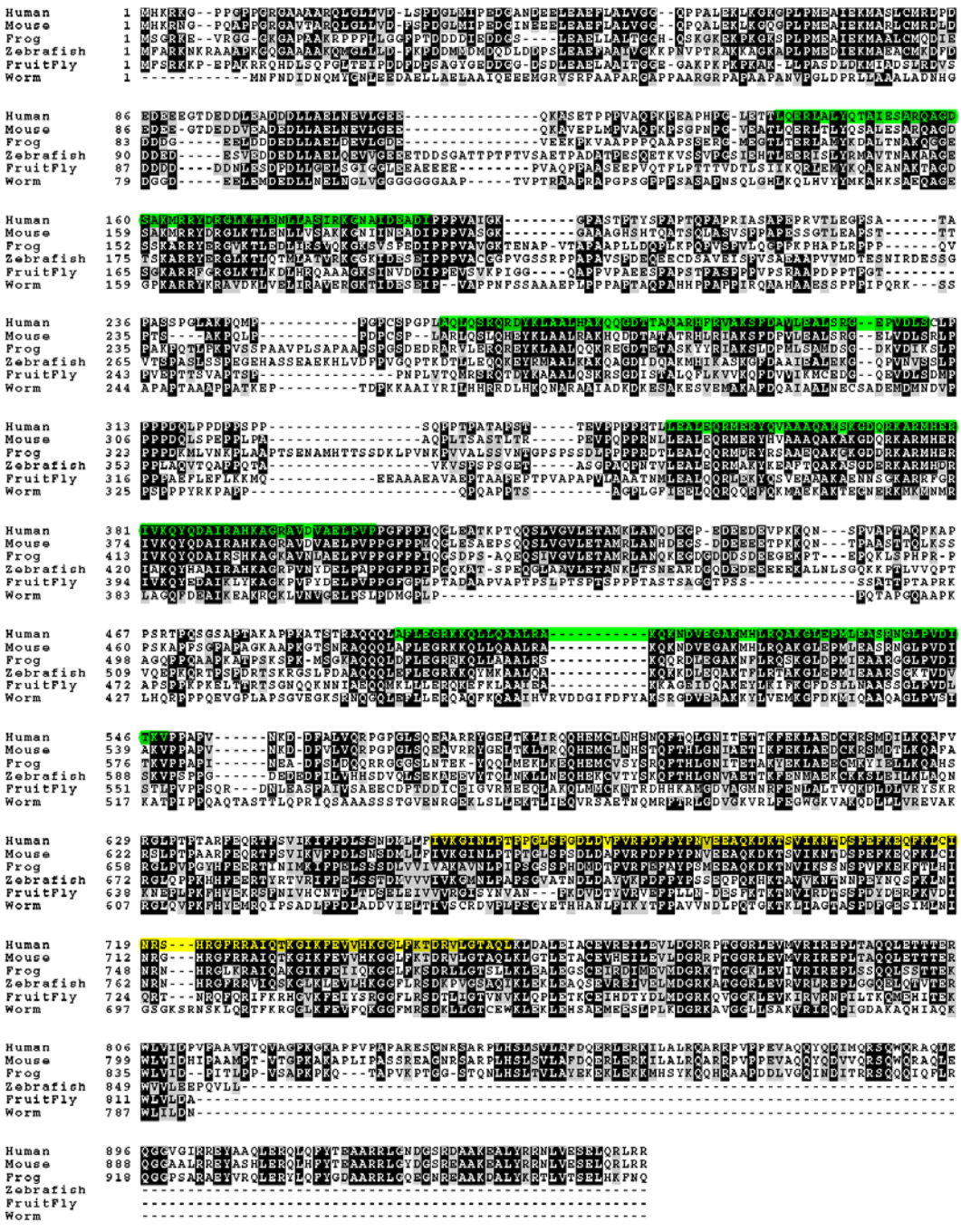


Figure 6. CC2D1A Activates NF-κB Luciferase Reporter Dose Dependently. A. Five cDNAs were cloned into mammalian expression vector pcDNA3-NFlag and introduced into HEK293T cells, together with NF-κB luciferase reporter and pCMV-LacZ. Luciferase assay was carried out as in Methods. Fold activation is normalized to vector only group. RIG-I NH_2 terminus was used as a control. **B.** Domain structure of human CC2D1A.

A.


Human
Mouse
Frog
Zebrafish
FruitFly
Worm
DM14-I
DM14-II
DM14-III
DM14-IV
C2

B.

Identity(%)	Human	Mouse	Frog	Zebra fish	Fruit fly	Worm
Human	100	83	50	45	32	27
Mouse		100	49	43	31	26
Frog			100	42	32	29
Zebra fish				100	32	30
Fruit fly					100	30
Worm						100

Figure 7. Evolutionary Conservation of CC2D1A. A. Alignment of CC2D1A protein sequences from human (*Homo sapiens,* NP_060191), mouse (*Mus musculus,* NP_666082), frog (*Xenopus tropicalis,* NP_001120385.1), zebrafish (*Danio rerio,* XP_692169.3), fruitfly (*Drosophila melanogaster,* NP_609488.1) and worm (*Caenorhabditis elegans,* NP_493412.2). DM14 domains are highlighted in green; C2 domain is highlighted in yellow. **B.** Sequence identity scores of CC2D1A proteins from different species.

CC2D1A is Inhibited by IκBα and CC2D1A Activates NF-κB Target Genes

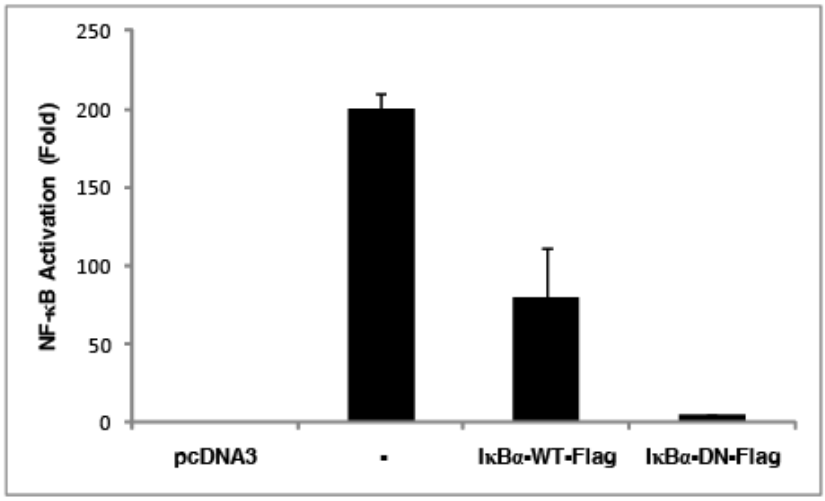
The screen mentioned previously was done using a similar NF-κB luciferase reporter gene that we used. In order to confirm that the identified genes activate NF-κB not just the κB element in the reporter plasmid, the authors introduced dominant-negative mutant of IKKβ and found in all cases, the fold induction by these genes was suppressed by the mutant IKKβ (Matsuda et al., 2003). It indicated that the identified genes are real activators of NF-κB and they function upstream of IKKβ.

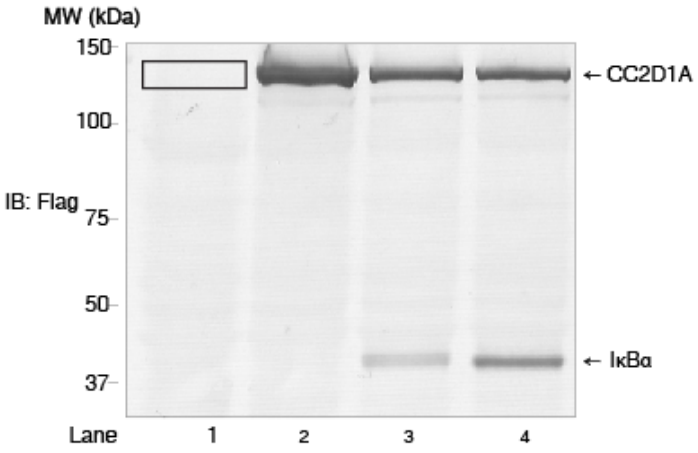
As mentioned in the introduction, after IKK is activated, IKKβ rapidly phosphorylates IκBα at serine 32 and 36 in the NH_2 terminus, which enhances the K48-linked polyubiquitination of IκBα. The ubiquitinated IκBα is then brought to the 26S proteasome for degradation. I made lentiviruses expressing either wild type or dominant negative mutant of IκBα, in which the serine 32 and 36 were mutated into alanine. (Note: The lentiviruses were made for neuronal culture, and this experiment was done to test whether IκBα-DN inhibited NF-κB activation.) I first

infected HEK293T cells with these lentiviruses, and after 24 hours I transfected CC2D1A expression vector and reporter genes. After another 48 hours, luciferase assay was carried out as before (Figure 8A). Cell lysate was examined by western blot using Flag specific antibody. CC2D1A expression was twofold lower in lentiviruses infected cells as compared to in non-infected cells and fold activation of NF-κB was normalized to CC2D1A expression level. Dominant negative IκBα abolished NF-κB activation, whereas wild type IκBα reduced the fold by about 60% (Figure 8A). It has to be taken into consideration that wild type IκBα was expressed at lower level than dominant negative mutant. Nevertheless, this data suggests that CC2D1A turns on NF-κB activation pathway and leads to the induction of luciferase reporter expression. It is less likely that CC2D1A turns on NF-κB luciferase reporter through merely binding to the kB elements.

So far, I've shown that overexpression of CC2D1A activates NF-κB reporter gene expression and IκBα proteins inhibit the activation. Next I wanted to examine if any of the known NF-κB target genes is activated by CC2D1A. NF-κB controls numerous downstream genes, including inflammatory cytokines and chemokines that are involved in immune responses. I carried out RT-PCR to analyze the mRNA expression of tumor necrosis factor alpha (TNFα) and monocyte chemoattractant protein 1 (MCP-1) after CC2D1A overexpression. As shown in Figure 8B, mRNAs of these two target genes are induced by CC2D1A. The control shows the constitutive expression of GAPDH was constant. Therefore, CC2D1A activates NF-κB signaling to turn on the downstream gene expression.

A





	CC2D1A		
	-	IκBα-WT	IκBα-DN
CC2D1A Relative Expression	1	0.56	0.51

B

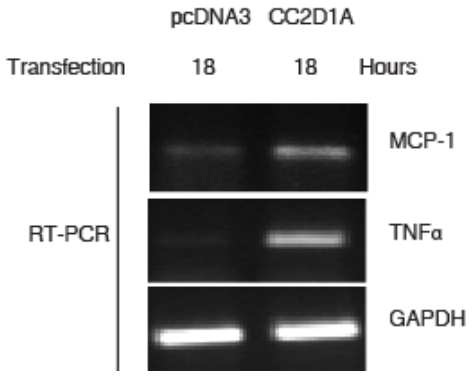


Figure 8. NF-κB Activation by CC2D1A is inhibited by IκBα proteins and CC2D1A Activates NF-κB Target Genes. **A.** HEK293T cells were infected with lentiviruses expressing either wild type or dominant negative IκBα and 24 hours later transfected with pcDNA3-Flag-CC2D1A or pcDNA3, together with reporter genes. Forty-eight hours post transfection, luciferase was carried out. Fold activation of luciferase was normalized to CC2D1A expression. Cell lysates were examined by western blot using Flag antibody. The mean value of histogram intensity was generated from Adobe Photoshop and relative expression of CC2D1A was calculated. **B.** HEK293T cells were transfected with pcDNA3-Flag-CC2D1A or pcDNA3. Eighteen hours post transfection, the induction of human TNFα and MCP-1 was measured by RT-PCR. The constitutive expression of GAPDH RNA was also measured as a control.

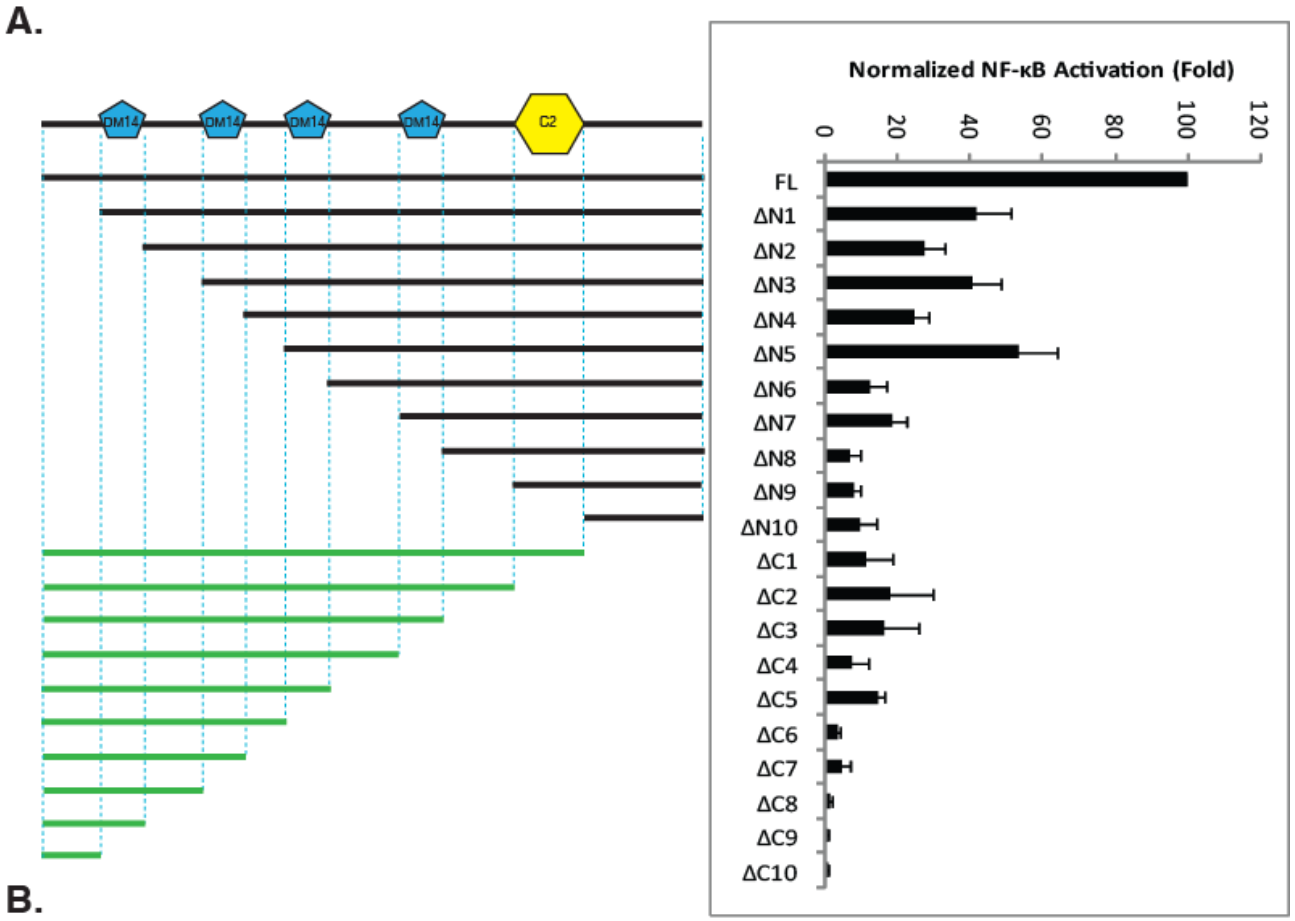
Conserved Domains of CC2D1A are Important for NF-κB Activation

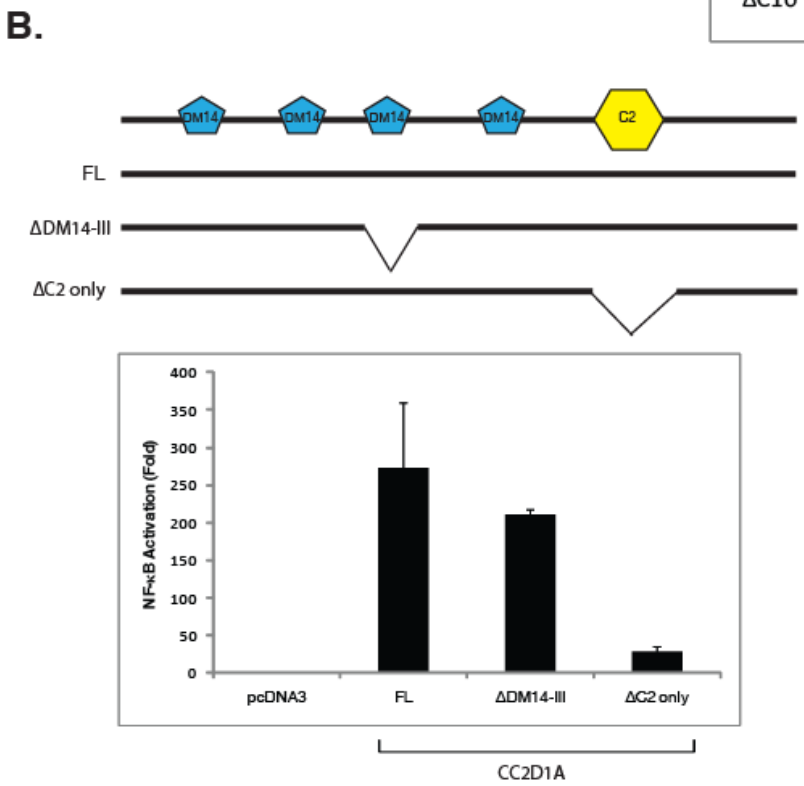
Although NF-κB can be activated by a variety of proteins in many different signaling pathways, CC2D1A is unique in that it contains conserved DM14 and C2 domains that have not been reported to mediate NF-κB activation. To determine if these conserved domains are important for NF-κB activation, I constructed a series of CC2D1A mutants with progressive truncations from the NH_2 and COOH termini and tested each construct's ability to activate NF-κB. Results in Figure 9A are based on four independent experiments. Western blot with Flag specific antibody shows the expression of each construct (Figure 10A). pCMV-LacZ was cotransfected together with reporter construct and served as a control for DNA transfection. The level of luciferase induction is normalized to β-galactosidase activity from the lysate. Fold of induction is the ratio between the level of luciferase activity from CC2D1A and empty vector transfected cells. To calculate the relative expression level of mutants compared to full length protein, I used Adobe Photoshop to quantify the bands on the western blots (Figure 10B). Fold activation of NF-κB is normalized to the relative protein expression level (Figure 9A).

Deletions from both termini greatly impaired NF-κB activation. Deletion of the very NH_2 terminal part decreased activity to about 50% (ΔN1), so does deletion of the first two DM14 domains (ΔN5). Further deletions (ΔN6-Δ10) result in less than 20% activity. The third DM14 domain could be essential since comparing ΔN5 to ΔN6, the activity decreases dramatically. Alternatively, DM14 domains may act redundantly and a minimum number of DM14 is needed for CC2D1A to function properly. To distinguish between these two possibilities, I made a single domain mutant of the third DM14 domain and tested it in luciferase assay. As shown in Figure 9B, deleting only the third DM14 domain did not have much effect, suggesting that DM14 domains have redundant roles.

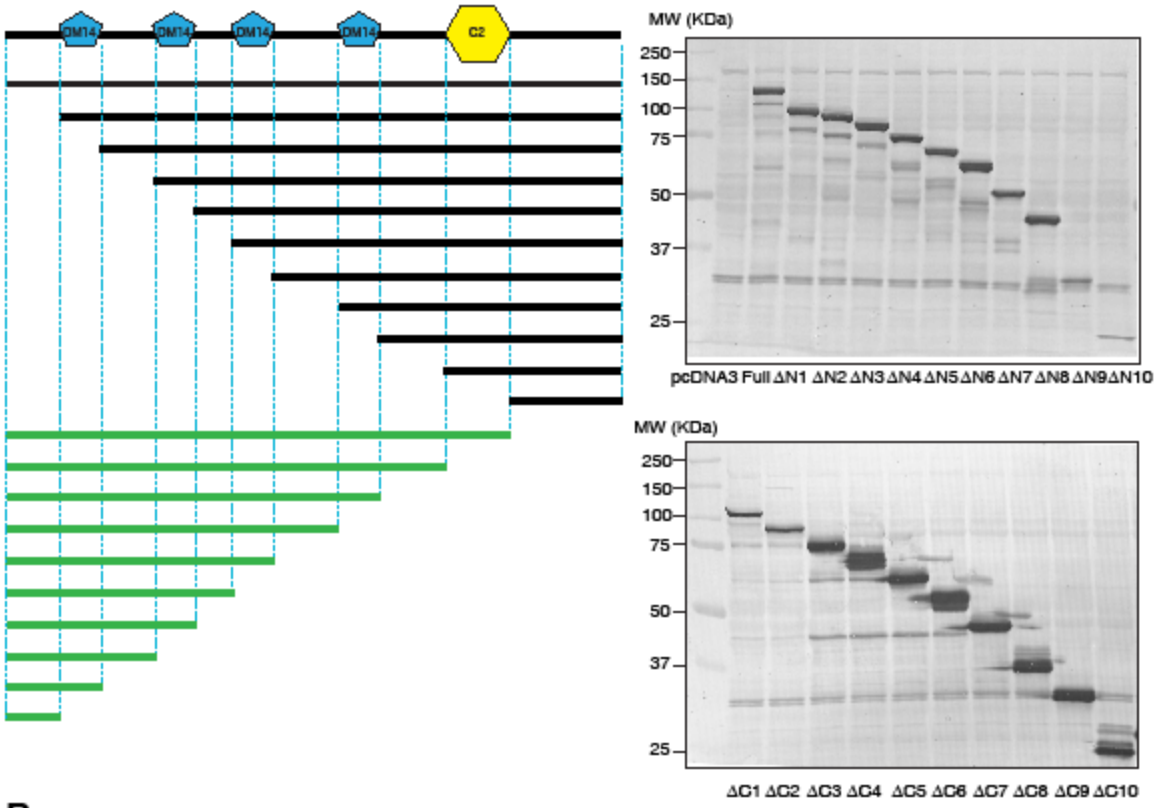
Deletions from the COOH terminal part are more detrimental. DM14 domains alone have less than 20% activity (ΔC3) (Figure 9A). When C2 domain was deleted alone (dC2), there is less than 20% activity left (Figure 9B). It shows that C2 domain and the COOH terminus are crucial, presumably for recruiting downstream components in the pathway.

Figure 9. (Next page) **Conserved Domains of CC2D1A are Important for NF-κB Activation. A.** cDNAs encoding various truncation mutants of CC2D1A protein were cloned into pcDNA3-Flag and tested in the luciferase assay as before. Domain truncations from NH_2 terminus were designated as ΔN1-ΔN10; COOH terminus as ΔC1-ΔC10. Data represent four independent experiments. Fold activation of NF-κB is normalized to protein expression shown in Figure 10. **B.** cDNAs encoding single domain deletion were cloned and tested in luciferase assay as in **A.**


A.
DM14
DM14
DM14
DM14
C2
Normalized NF-κB Activation (Fold)
0
20
40
60
80
100
120
FL
ΔN1
ΔN2
ΔN3
ΔN4
ΔN5
ΔN6
ΔN7
ΔN8
ΔN9
ΔN10
ΔC1
ΔC2
ΔC3
ΔC4
ΔC5
ΔC6
ΔC7
ΔC8
ΔC9
ΔC10


B.
DM14
DM14
DM14
DM14
C2
FL
ΔDM14-III
ΔC2 only
NF-κB Activation (Fold)
400
350
300
250
200
150
100
50
0
pcDNA3
FL
ΔDM14-III
ΔC2 only
CC2D1A

A



B

Construct				Full Length
Relative Expr				1
ΔN1	**ΔN2**	**ΔN3**	**ΔN4**	**ΔN5**
1.2	1.14	1.3	1.15	1.21
ΔN6	**ΔN7**	**ΔN8**	**ΔN9**	**ΔN10**
1.52	1.07	1.46	0.55	0.41
ΔC1	**ΔC2**	**ΔC3**	**ΔC4**	**ΔC5**
0.64	0.86	1.74	2.56	1.89
ΔC6	**ΔC7**	**ΔC8**	**ΔC9**	**ΔC10**
2.99	2.06	2.15	2.21	1.75

Figure 10. Protein Expression of CC2D1A Truncation Mutants. A. Western blot using Flag antibody. Samples are from one representative experiment in Figure 9A. **B.** Quantification of protein expression based on the bands on western blot from A.

TRAF2, TAK1 and NEMO are Required for CC2D1A to Activate NF-κB

The next question I addressed is how CC2D1A activates NF-κB. I designed siRNA targeting the coding region of NEMO and western blot using NEMO specific antibody shows that siRNA effectively knocked down endogenous NEMO expression. I found that RNAi of NEMO diminished the activity of CC2D1A as compared to a control GFPi knockdown (Figure 11A). Given that CC2D1A is inhibited by IκBα, these data indicate CC2D1A, like most NF-κB stimuli in the canonical pathway, depends on IKKα/IKKβ/NEMO complex to activate NF-κB.

The transforming growth factor (TGF)-β-activated kinase (TAK1) complex is an essential IKK kinase in diverse NF-κB signaling pathways. It phosphorylates IKK at key serine residues in the activation loop, resulting in IKK activation (Wang et al., 2001). Knocking down TAK1 expression by three different pairs of siRNA all greatly reduced the activity of CC2D1A (Figure 11B). Thus CC2D1A requires TAK1 to activate IKK and NF-κB. Biochemical studies have shown that K63 linked polyubiquitin chains mediate IKK and TAK1 activation (Deng et al., 2000; Wang et al., 2001). Could K63 polyUb also regulates CC2D1A induced TAK1 and IKK activation?

TRAF proteins are critically involved in NF-κB signaling from various cell surface and intracellular receptors. For instance, TRAF2 and TRAF5 are required by TNF receptor signaling, whereas TRAF6 is essential for NF-κB and MAP kinase activation by interleukin-1 receptor (IL-1R) and Toll-like receptors (TLR). TRAF proteins act as ubiquitin ligases (E3) to activate downstream kinases. I asked whether CC2D1A regulates TRAF. I found that RNAi of TRAF2 inhibited CC2D1A activity while RNAi of TRAF6 has no effect (Figure 11A). TRAF2 has been shown to have E3 activity *in vitro*. It is likely that TRAF2 is activated by CC2D1A to catalyze

ubiquitination, which in turn leads to TAK1 activation. These results suggest that CC2D1A activates NF-κB and IKK through a TRAF2- and TAK1-dependent pathway.

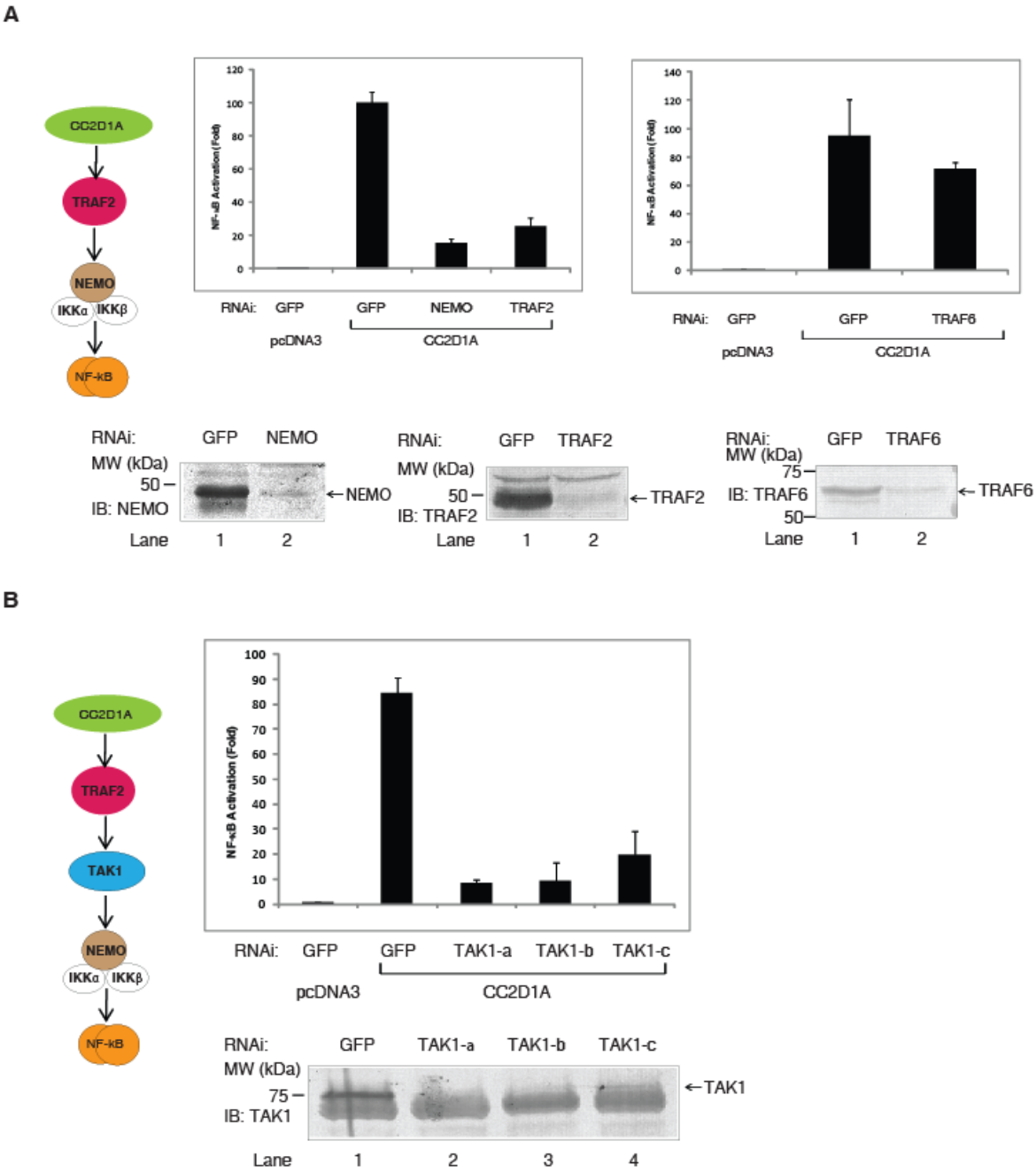


Figure 11. CC2D1A requires NEMO, TRAF2 and TAK1 to activate NF-κB. **A.** siRNAs targeting GFP, NEMO, TRAF2 or TRAF6 were transfected into HEK293T cells followed by transfection of CC2D1A expression plasmid and NF-κB luciferase reporter. **B.** Three different pairs of siRNA oligos targeting different sequences in the coding region of TAK1 gene were used to knock down TAK1. Luciferase assay was carried out as before.

Ubiquitin E2 UBC13 is an Interacting Partner of CC2D1A

To identify interacting partners of CC2D1A, I carried out a yeast-two-hybrid screen in collaboration with Dr. Xiaodong Li in the lab, using the second and third DM14 domain as bait. We identified UBC13 as a CC2D1A-binding protein. UBC13 forms an E2 ubiquitin conjugating complex with UEV1a. The dimeric complex catalyzes the synthesis of polyubiquitin chains linked through Lys-63 (K63) of ubiquitin. As mentioned previously, K63-linked polyubiquitin chains have been shown to regulate IKK and TAK1 independent of the proteasome (Deng et al., 2000; Wang et al., 2001). I first confirmed the interaction between UBC13 and CC2D1A with a GST pull-down assay. GST-UBC13 has higher binding affinity for CC2D1A than GST-UBC5 or GST alone indicating that CC2D1A binds to UBC13 (Figure 12A).

Ubiquitin E2 UBC13 is Required for CC2D1A to Activate NF-κB.

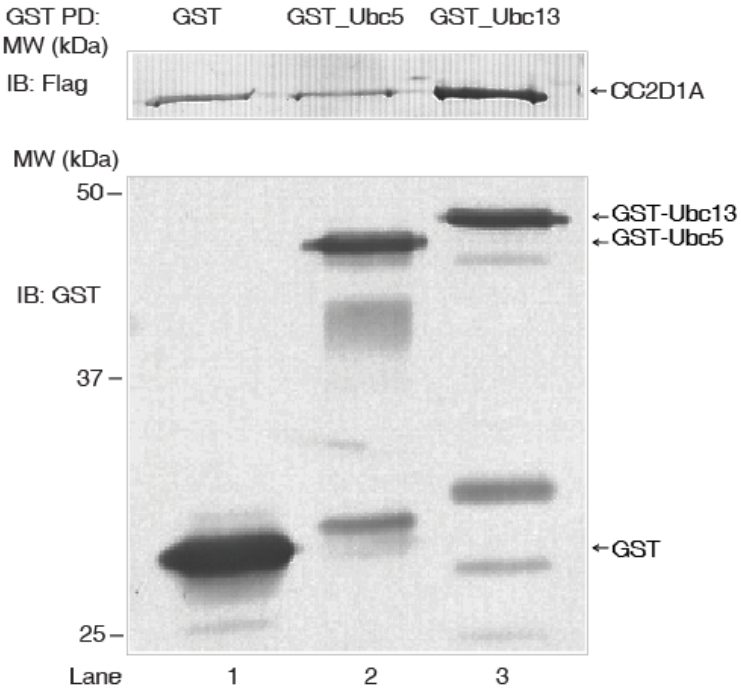
To test the functional involvement of UBC13 and CC2D1A, Dr. Ming Xu in the lab engineered the human osteosarcoma cell line U2OS to deplete endogenous UBC13 using a tetracycline-inducible short hairpin RNA (shRNA), and simultaneously replaced it with wild-type UBC13, whose expression was also controlled by a tetracycline-inducible promoter (Xu et al., 2009). After treatment in the presence or absence of tetracycline, the cells were transfected with CC2D1A expression vector or empty vector alone. Luciferase assay was then carried out as before. CC2D1A-induced NF-κB reporter expression was reduced by more than 70% compared to non-tetracycline control. Restoring UBC13 expression rescued the activity, indicating that the phenotype is specifically due to UBC13 knock down (Figure 12B). TRAF6 functions together with UBC13-UEV1a to activate IKK in response to IL-1β and TLR ligand such as LPS. Knocking down UBC13 decreased TRAF6 induced NF-κB activity whereas restoring UBC13 expression rescued the activity. On the contrary, knocking down UBC13 did not affect NIK-

induced NF-κB activity (Figure 12B). NIK functions in non-canonical pathways in B cells (see above) to activate NF-κB, independent of UBC13. Thus knocking down UBC13 using our "tet-on" system does not have general inhibitory effect on NF-κB activation. Our data shows CC2D1A requires UBC13 to activate NF-κB.

Deubiquitination Enzyme CYLD Negatively Regulates NF-κB activation by CC2D1A

CYLD is an ubiquitin specific protease with a USP domain at its COOH terminus. It negatively regulates the NF-κB pathway by deubiquitinating TRAF2, TRAF6, Nemo and Bcl-3 among others (Brummelkamp et al., 2003; Kovalenko et al., 2003; Massoumi et al., 2006; Trompouki et al., 2003). Since I have found an ubiquitin E2-UBC13 and an ubiquitin E3-TRAF2 are required for CC2D1A to activate NF-κB, I wanted to test whether deubiquitination enzyme CYLD inhibited CC2D1A induced NF-κB activation. Co-expression of wild type CYLD inhibits CC2D1A in a dose-dependent manner (Figure 13A). A catalytically inactive mutant, in which the active site Cysteine in the USP domain was substituted with Serine (C601S; CYLD mutant), abolished the inhibition, indicating the USP catalytic activity is required (Figure 13B). CYLD preferably binds K63-linked polyubiquitin chains and specifically cleaves K63 chains. In addition, UBC13 forms an ubiquitin E2 complex with UEV1a to catalyze K-63 linked ubiquitination. The data suggest that K-63 linked polyUb is required for NF-κB activation by CC2D1A.

A


GST PD:
GST
GST_Ubc5
GST_Ubc13
MW (kDa)
IB: Flag
←CC2D1A
MW (kDa)
50–
IB: GST
37–
25–
←GST-Ubc13
←GST-Ubc5
←GST
Lane
1
2
3

B

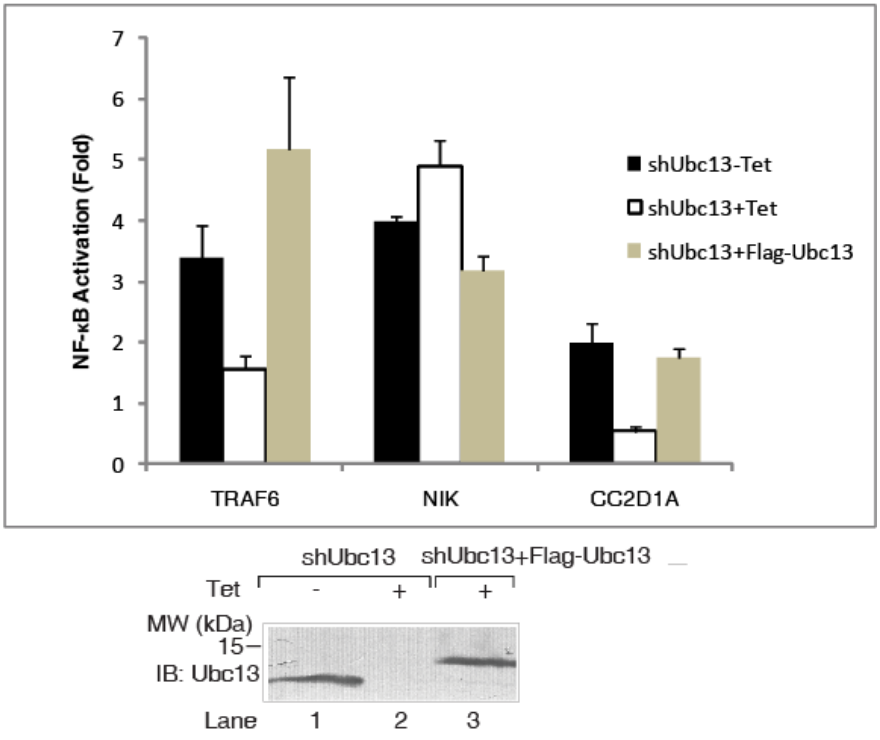

NF-κB Activation (Fold)
0
1
2
3
4
5
6
7
TRAF6
NIK
CC2D1A
shUbc13-Tet
shUbc13+Tet
shUbc13+Flag-Ubc13
shUbc13
shUbc13+Flag-Ubc13
Tet
-
+
+
MW (kDa)
15–
IB: Ubc13
Lane
1
2
3

Figure 12. CC2D1A Requires Ubiquitin E2 UBC13 to Activate NF-κB. A. Glutathione sepharose beads bound to GST, GST-Ubc5 or GST-UBC13 were then incubated with Flag tagged CC2D1A protein. After wash, immunoblotting with Flag antibody shows the amount of CC2D1A protein bound to the beads. Western blot with GST antibody shows the amount of GST tagged protein on the beads for each pull-down reaction. **B.** U2OS stable cell line expressing short-hairpin RNA in response to tetracycline was used to knock down UBC13. Wild type rescue cell line simultaneously expresses Flag tagged UBC13 after tetracycline treatment. Western blot with UBC13 antibody shows the efficiency of knock-down and rescue. NF-κB luciferase assay was carried out to test the requirement of UBC13 by TRAF6, NIK or CC2D1A.

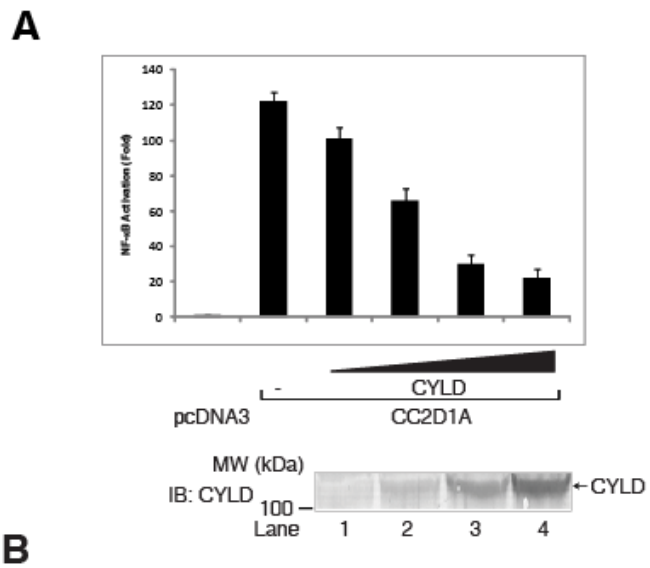

A
NF-κB Activation (Fold)
140
120
100
80
60
40
20
0
-
CYLD
pcDNA3
CC2D1A
MW (kDa)
IB: CYLD
100
CYLD
Lane
1
2
3
4
B
NF-κB Activation (Fold)
400
350
300
250
200
150
100
50
0
pcDNA3
CC2D1A
CC2D1A+CYLD WT
CC2D1A+CYLD Mut
pcDNA3
CC2D1A
CYLD WT CYLD Mut
MW (kDa)
IB: CYLD
100
CYLD
Lane
1
2
3
4
C
?
CC2D1A
TRAF2
UBC13
CYLD
K63 polyUb
TAK1
NEMO
IKK
IKK
NF-kB

Figure 13. CC2D1A is Inhibited by the De-ubiquitination enzyme CYLD. A. Increasing amount of CYLD was transfected together with CC2D1A. Inhibitory effect was measured by NF-κB luciferase assays. CYLD expression was examined by western blot. **B.** Wild type or an enzymatically dead mutant of CYLD was transfected into HEK293T cells along with CC2D1A. Inhibitory effect was examined by luciferase assay. WT/Mutant CYLD expression was examined by immunoblotting. **C.** Working model of CC2D1A activating NF-κB through the canonical pathway. CC2D1A activates NF-κB depending on the ubiquitin-conjugating enzyme UBC13, the RING domain ubiqutin ligase TRAF2, the protein kinase TAK1 and IKK complex. A de-ubiquitination enzyme CYLD negatively regulates NF-κB activation by CC2D1A. K-63 linked polyubiquitin chains may be also involved in NF-κB activation by CC2D1A.

Candidate Approach to Find Upstream Stimuli of CC2D1A

In order to rule out the possibility of non-canonical pathways (Figure 1), I've done two experiments. One was to use four different pairs of siRNA to knockdown NIK, which is a critical kinase in the noncanonical pathway. Knocking down NIK did not affect CC2D1A induced NF-κB activity (data not shown). The other experiment is to stimulate cells with CD40 ligands and monitor IκBα degradation. While RNAi of NEMO totally blocked IκBα degradation, RNAi of CC2D1A did not have any effect (data not shown).

Since all the components downstream of CC2D1A are also required by TNFα to activate NF-κB, I tested if CC2D1A functions in TNFα pathway. TNFα induced NF-κB luciferase activity was not affected by siRNAs targeting CC2D1A. Since TNFα was induced by CC2D1A (Figure 8B), it is possible that autocrine TNFα signaling leads to NF-κB activation. To test this possibility, I collected conditional medium 24 hours after transfecting CC2D1A and tested it in the NF-κB luciferase assay. Although CC2D1A overexpression resulted in NF-κB activation over 100 fold, the conditional medium did not activate NF-κB. Thus, NF-κB activation by CC2D1A is not through TNFα autocrine effect.

TRAF2 has also been shown to regulate endoplasmic reticulum stress (ER stress) induced NF-κB activation (Kaneko et al., 2003). Therefore, I tested if CC2D1A acts in the ER stress induced NF-κB activation pathway. Tunicamycin blocks the synthesis of N-linked glycoproteins and perturbs the function of ER. I treated HEK293T cells with 10ug/ml Tunicamycin (Kaneko et al., 2003) to induce ER stress. From Figure 13A we can see that the expression of Bip (HSPA5), an ER specific chaperone, was increased, indicating ER was under stress (Figure 13A). To monitor NF-κB activation, I performed electrophoretic mobility shift assay (EMSA) using a ^{32}P labeled NF-κB consensus probe. During NF-κB activation, IκBα is degraded and the DNA binding motif in NF-κB is free to interact with NF-κB consensus sequences. When the probe was mixed with extracts from 293 cells that were treated with tunicamycin for 24 hours, I observed a larger, less mobile complex consisting of ^{32}P labeled probes and interacting proteins (Figure 14B; GFPi+Tunicamycin). The "shift" was inhibited by siRNA targeting NEMO, indicating that NEMO is required for Tunicamycin to activate NF-κB (Figure 14B). On the contrary, RNAi of CC2D1A did not have inhibitory effect. Western blot using CC2D1A specific antibody shows that the endogenous CC2D1A was effectively knocked down (Figure 14A).

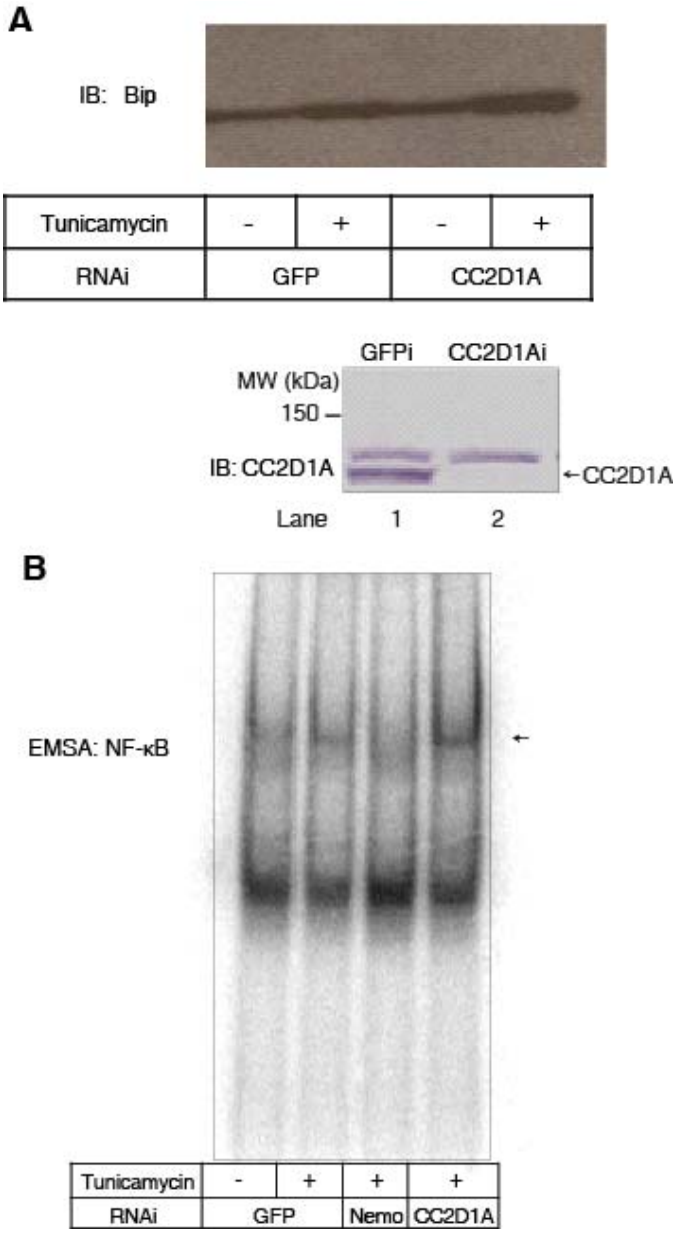


Figure 14. CC2D1A Knockdown does not Affect ER Stress Induced NF-κB Activation. A. ER stress was induced by Tunicamycin (10 ug/ml) treatment for 24 hrs in HEK293T cells, as evidenced by the induction of ER chaperon Bip (HSPA5). CC2D1A was knocked down by siRNA. Immunoblotting shows RNAi efficiency. **B.** ER stress induced NF-κB activation as examined by EMSA. Knocking down NEMO blocked NF-κB activation while RNAi of CC2D1A did not.

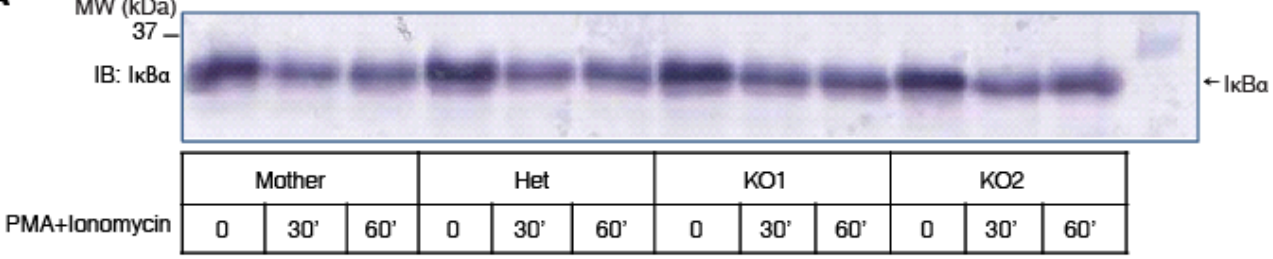
CC2D1A has unique domain structure: Four tandem repeats of DM14 domains at NH_2 terminus and C2 domain at COOH terminus. Although the function of DM14 domain is

unknown, C2 domain mediates calcium and phospholipid binding and is critically involved in protein interaction and signal transduction. As mentioned in Chapter One, C2 domain of CC2D1A does not have the conserved aspartic acids required to bind calcium (Rizo and Sudhof, 1998) but the Drosophila ortholog of CC2D1A is able to bind to phospholipid (Gallagher and Knoblich, 2006). However it is not clear how CC2D1A binds to phospholipids. PKCθ is a calcium-independent and phospholipid-dependent protein kinase that plays an essential role in the activation of NF-κB and AP-1 in T cell receptor (TCR) signaling (Rawlings et al., 2006; van Oers and Chen, 2005). Later on, I generated CC2D1A deficient mice and isolated primary thymocytes from E18.5 embryos. I stimulated thymocytes with phorbol ester (phorbol 12-myristate 13-acetate) (100ng/ml) and ionomycin (200ng/ml), which mimic the stimulation of TCR in T cells, and examined IκBα degradation. I did not observe any difference between heterozygous and knockout mice (Figure 15A). The thymocyes were from littermate embryos and for this particular experiment, there wasn't a wild type embryo, which would serve as a better control.

Drosophila ortholog of CC2D1A is named lethal (2) giant discs (Lgd). It is a negative regulator of Notch signaling and is involved in endosomal trafficking (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Several Toll-like receptors are localized at the endosome such as TLR3,TLR7/8 and TLR9. TLR3 is involved in the recognition of dsRNA generated during viral replication and a synthetic analog of dsRNA, polyinosine-deoxycytidylic acid (poly I:C) is a potent inducer of type I IFNs (Alexopoulou et al., 2001; Yamamoto et al., 2003). TLR7/8 recognize single strand RNA (ssRNA) and synthetic imidazoquinoline-like molecules that are structurally similar to ribonucleic acids (Akira and Hemmi, 2003; Diebold et al., 2004; Hemmi et al., 2002; Ito et al., 2002). TLR9 recognizes CpG

DNA motifs that are found in genomes of bacteria, protozoa and viruses (Bauer et al., 2001; Hemmi et al., 2000; Takeshita et al., 2001; Wagner, 2001). TLR9 and TLR7/8 are present in human B cells (Hornung et al., 2002). TLR7 and TLR9 are the only TLR members expressed in specific splenic DCs known as plasmacytoid DCs (pDC), which induce large amount of IFNα during viral infection (Cella et al., 1999; Siegal et al., 1999). In order to monitor NF-κB activation in response to TLR7/9 signaling, I isolated primary spleenocytes from E18.5 embryos of CC2D1A deficient mice and cultured them *in vitro*. To stimulate TLR3, TLR7 and TLR9, I used poly I:C (1ug/ml) (Figure 15C), Gardiquimod or CL087 (invivogen) (0.1ug/ml; 0.1uM) and CpG DNA (2ug/ml) (Figure 15B) respectively. Mouse IL-6 induction was measured by ELISA as a readout of NF-κB activation after three days (Figure 15B, C). Again no difference was observed between CC2D1A deficient cells and control cells in terms of NF-κB activation by TLR stimulations. It has to be taken into consideration that at E18.5, B cells and DCs have not fully developed and NF-κB activation by TLR7 and TLR9 ligands is very weak comparing to the response from mature mice (Figure 15B). It is also possible that CC2D1A is involved in TLR signaling at a different developmental stage in a different cell types.

A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IB: IκBα
←IκBα
Mother
Het
KO1
KO2
PMA+Ionomycin
0
30'
60'
0
30'
60'
0
30'
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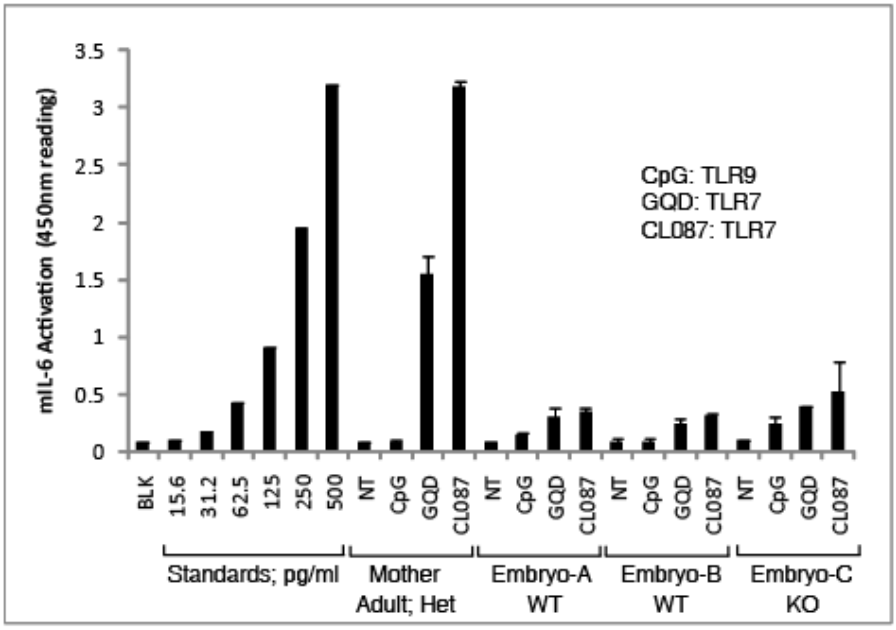
B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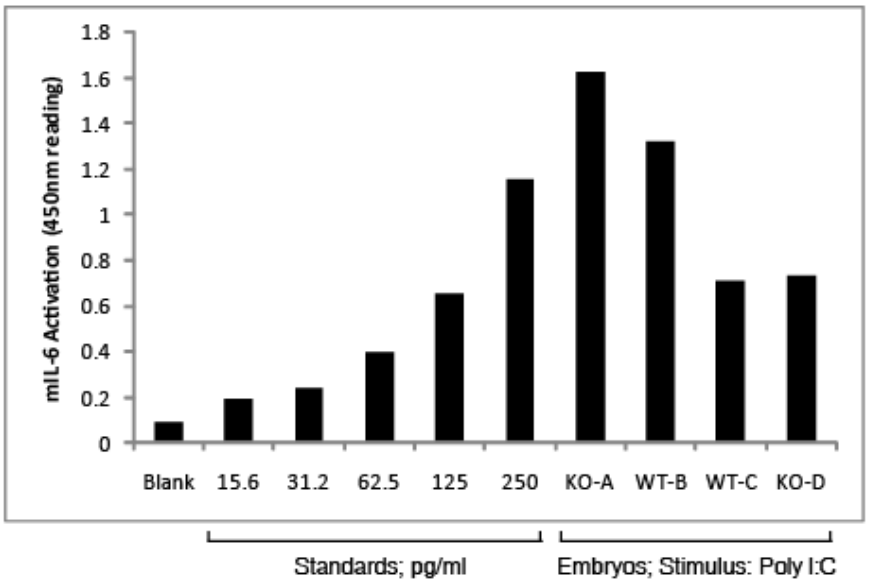
3
2.5
2
1.5
1
0.5
0
CpG: TLR9
GQD: TLR7
CL087: TLR7
BLK
15.6
31.2
62.5
125
250
500
NT
CpG
GQD
CL087
Standards; pg/ml
Mother
Adult; Het
Embryo-A
WT
Embryo-B
WT
Embryo-C
KO

C


mIL-6 Activation (450nm reading)
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Blank
15.6
31.2
62.5
125
250
KO-A
WT-B
WT-C
KO-D
Standards; pg/ml
Embryos; Stimulus: Poly I:C

Figure 15. CC2D1A Deficient Cells Response Normally to TCR and TLR Ligands. A. Primary thymocytes were isolated from E18.5 embryos and cultured *in vitro*. Phorbol ester (phorbol 12-myristate 13-acetate) (100ng/ml) and ionomycin (200ng/ml) were used to stimulate thymocytes for various times and IκBα degradation was examined. **B.C.** Primary spleenocytes were isolated from E18.5 embryos and cultured *in vitro*. To stimulate TLR3, TLR7 and TLR9, poly I:C (1ug/ml), Gardiquimod (GQD) or CL087 (Invivogen) (0.1ug/ml; 0.1uM) and CpG DNA (2ug/ml) were used respectively. Mouse IL-6 induction was measured by ELISA as a readout of NF-κB activation after three days.

Mutations in the gene encoding CC2D1A have been associated with non-syndromic mental retardation in human (Basel-Vanagaite et al., 2006). The mutation is a deletion of 3567 nucleotides from intron 13 to 16, abolishing the fourth DM14 domain and C2 domain. I have tested a similar mutation in NF-κB luciferase assay (ΔC2; Figure 9A). It has about 20% activity compared to the full length protein. Thus it is likely that the phenotype in patients is hypomorphic rather than null. Nevertheless, it indicates that CC2D1A functions in the brain. As shown in the next chapter, Cc2d1a is enriched in the mouse brain and brain specific isform of the protein product could be modified based on the motility on the western blot (Figure 19A), indicating that Cc2d1a is regulated in the brain. NF-κB activity is prominent in the central nervous system throughout development. Many brain specific factors activate NF-κB such as neurotransmitter glutamate (Guerrini et al., 1995). Genetic evidence has indicated that NF-κB is involved in learning and memory (Kaltschmidt et al., 2005; Meffert and Baltimore, 2005). It will be interesting to find out if CC2D1A regulates NF-κB in the brain under physiological conditions.

Discussion

CC2D1A was first identified by Matsuda et al as an NF-κB activator through a large scale screen of human genes (Matsuda et al., 2003). It has a unique domain structure that has never been seen in other NF-κB activators. Moreover it was predicted to be a mitochondrial protein. Given that MAVS, an essential adaptor in the antiviral signaling pathway, was localized

on mitochondria (Seth et al., 2005), we hypothesized that CC2D1A may cooperate with MAVS in mounting an antiviral defense. Several different pairs of siRNA targeting the coding region of CC2D1A were used to knock down the protein expression in HEK293T cells, but none of them inhibited Sendai virus induced NF-κB luciferase activity. As a control, MAVS knockdown by siRNA completely blocked the activity (Seth et al., 2005). Moreover, CC2D1A overexpression did not turn on IFN luciferase reporter. Therefore, it is unlikely that CC2D1A functions together with MAVS in response to virus infection. Biochemical fractionations as well as immunofluorenscence staining suggest that CC2D1A is mostly cytosolic and it is also found in the nuclear, but I did not see a specific localization on the mitochondria.

So far, I have shown that CC2D1A activates NF-κB in collaboration with several key components in the "canonical" pathway, namely TRAF2, UBC13, TAK1 and IKK. As mentioned above, K63 linked polyubiquitin chains play a role in TAK1 and IKK activation independent of the proteasome. It may function as a scaffold to bring TAK1 and IKK complex together facilitating phosphorylation and activation of IKK by TAK1. TABs and NEMO are the ubiquitin receptors in the two complexes, respectively. Since TRAF2 has ubiquitin ligase (E3) activity *in vitro* and UBC13/UEV1a complex is known to specifically catalyze K63 polyubiquitination (Deng et al., 2000; Hofmann and Pickart, 1999; VanDemark et al., 2001), it is likely that TRAF2 and UBC13 work together to synthesize K63 polyubiquitin chains. I also found that deubiquitination enzyme CYLD, which specifically degrades K63 linked polyubiquitin chains, inhibits CC2D1A. Therefore I propose that K-63 linked polyUb chains regulate NF-κB activation by CC2D1A.

TRAF2 could be a target of ubiquitination and there may be other molecules in the pathway that are conjugated by ubiquitin. Furthermore, unanchored K63 polyubiquitin chains

directly activate TAK1 by binding to the ubiquitin receptor TAB2, whereas unanchored polyubiquitin chains synthesized by TRAF6 and UBCH5C activate the IKK complex (Xia et al., 2009). Disassembly of the polyubiquitin chains by deubiquitination enzymes prevented TAK1 and IKK activation. Thus, it is also possible that free ubiquitin chains synthesized by TRAF2 and UBC13 directly activate TAK1 and IKK.

Most of the experiments shown in the results were done using a NF-κB luciferase assay. The luciferase reporter is driven by three tandem repeats of NF-κB binding sites. Overexpression of CC2D1A in HEK293T cells strongly turned on the luciferase reporter. I used RNAi to carry out the "epistasis" studies and found RNAi of TRAF2, NEMO, TAK1, UBC13 but not TRAF6, blocked CC2D1A induced luciferase activity. These data indicate that CC2D1A depends on these components in the canonical pathway to activate NF-κB. In addition, dominant negative IκBα and de-ubiquitination enzyme CYLD inhibited the luciferase activity, suggesting that canonical NF-κB activation pathway (Figure 1) is on. Based on the data and what we already known about the pathway, I propose the model as shown in Figure 13C. So far the upstream stimulus that regulates the pathway is not clear yet. Finding out the signal will help to understand how CC2D1A is regulated and what its physiological function is. An alternative model, as pointed out by my thesis committee members Drs. Nicolai Van Oers and Jane Johnson, is that CC2D1A may not directly activate the canonical pathway but rather enhance the basal NF-κB activity. Usually DNA tranfection stresses the cells and induces NF-κB activity. Thus it is possible that after transfection, the canonical pathway is turned on, and CC2D1A could act as a scaffold to bring the components together and facilitate signal transduction, stabilize the active components or act as a coactivator in the nucleus to enhance the activity of NF-κB. To circumvent the DNA transfection induced NF-κB activation, I could introduce CC2D1A using

viral infection. Proper controls like IKK kinase assay, IκBα degradation or luciferase assay can be used to test if viral infection induces basal NF-κB activation. Alternatively, I could generate CC2D1A transgenic mice and test the basal NF-κB activity in cells like MEFs. That will avoid the transient transfection but one could still argue that during ES cells electroporation, NF-κB is turned on as well. To test if CC2D1A acts in the nucleus as a co-activator, I could examine if CC2D1A binds to NF-κB. I've not done this, but as shown by Basel-Vanagaite et al. (Basel-Vanagaite et al., 2006), CC2D1A does not directly interact with neither p50 nor p65 based on immunoprecipitation. When I did immunofluorenscence staining using Flag antibody to detect overexpressed proteins, I've seen most of Flag-CC2D1A were in the cytosol, although it is possible that small amount of protein got into the nucleus. I could also test if CC2D1A binds to the NF-κB binding sites in the luciferase reporter by yeast-one hybrid or ChIP. To test if CC2D1A stabilizes or modifies any of the components, I've examined the protein expression of NEMO and TRAF2 in CC2D1A transfected cells and in pcDNA3 transfected cells and I did not see much difference. There might be unidentified partners in the pathway, which are modified by CC2D1A overexpression, and also I need a readout more sophisticated than western blot to detect protein modifications, for example mass spectrometry.

In addition to activating NF-κB, CC2D1A may have other cellular functions, such as regulating epidermal growth factor signaling (Nakamura et al., 2008) or repressing expression of serotonin-1A receptor genes and dopamine-D2 receptor genes (Ou et al., 2003; Rogaeva and Albert, 2007; Rogaeva et al., 2007a; Rogaeva et al., 2007b). Generating CC2D1A deficient mouse model will be critical to elucidate the physiological function of the gene.

Methods

Plasmids and Proteins

The Human CC2D1A cDNA sequence was amplified by PCR using the IMAGE clone: 6585236(ATCC) as a template and then subcloned into the mammalian expression vector pcDNA3(Invitrogen) in-frame with an N-terminal Flag tag. The truncated forms of CC2D1A were cloned using similar strategy. C2 domain and DM14 domain deletion mutants were generated by overlap extension PCR. pcDNA3-Flag-CYLD was described previously (Trompouki et al., 2003). Site-directed mutagenesis of the USP domain at Cys601 (C601S) and H/N was carried out using the Quick-change site-directed mutagenesis kit (Stratagene). All constructs were verified by automated DNA sequencing. Plasmids for p-κB3-TK-Luc and pCMV-LacZ were described previously (Deng et al., 2000). Expression plasmids for GST-Ubc5c and GST-UBC13 were kindly provided by Dr. Alan Weissman (NCI). Expression plasmid for TRAF6 was a gift from Dr. Jun Ichiro-Inoue (University of Tokyo).

Antibody

To generate polyclonal antisera against human CC2D1A, a recombinant protein containing residues 263–512 of human CC2D1A was expressed in *E. coli* as a His6-tagged protein and affinity purified. The CC2D1A protein was used to immunize rabbits (Rockland), and the resulting antibody was affinity purified using an antigen column. The antibodies for TAK1, TRAF2, TRAF6, Ubiquitin, and Nemo are from Santa Cruz Biotech. The antibodies for UBC13, GST (4C10), Flag (M2) and His are from Zymed, Covance, Sigma and Qiagen, respectively. Polyclonal antibody against TAB2 was generated using an N-terminal fragment (1-450) of TAB2 (Wang et al., 2001).

Cell Culture, Transfection, and Reporter Gene Assay

HEK293 cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. Transfection of HEK293 cells was carried out by calcium phosphate precipitation. For luciferase reporter assays, cells were seeded in 12-well plates at a cell density of $2X10^5$ cells per well. On the second day, cells were co-transfected with 50 ng of p-κB3-TK-Luc reporter gene, 25 ng of pCMV-LacZ as an internal control for transfection efficiency, and the indicated expression vectors. Each experiment was carried out in duplicate. Cells were harvested 48 hours after transfection and lysed in the passive lysis buffer (Promega). Luciferase activity was measured with a illuminometer (Rosys Anthos Lucy2) using luciferin as a substrate, and β-galactosidase activity was measured with a Thermo Lab systems microplate reader at the wavelength of 405 nm using o-nitrophenyl-β- D-galactopyranoside (ONPG) as a substrate.

U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. The U2OS cell line was stably incorporated with short hairpin expression vector or rescue vector under the control of tetracycline responsible promoter (Xu et al., 2009). After tetracycline treatment for five days, the cells were seeded in 12-well plates at a cell density of $2X10^5$ cells per well. On the sixth day, cells were transfected with 100 ng of p-κB3-TK-Luc reporter gene, 50 ng of pCMV-LacZ and 350 ng of the indicated expression vectors. On the eighth day, luciferase assay was carried out as mentioned above.

RT-PCR

HEK 293 cells were transfected with CC2D1A expression vector or empty vector using Lipofectamine 2000 (Invitrogen). After 18 hours, total RNA was isolated by using the Trizol LS reagent (Invitrogen) and treated with TURBO DNA-*free* (Ambion) to clear residue genomic

DNA contamination. The same amount of total RNA was used to synthesize cDNA using an iScript cDNA synthesis kit (BioRad). The sequences of the primers were as follows: hMCP-1, GCCCCAGTCACCTGCTGTTAT (forward) and TTGGCCACAATGGTCTTGAA (reverse); hTNFα, AGGCGGTGCTTGTTCCTCAA (forward) and TGGGCCAGAGGGCTGATTA (reverse); hGAPDH, AAAATCAAGTGGGGCGATGCT (forward) and GGGCAGAGATGATGACCCTTT (reverse). RT-PCR products were separated by 2% agarose gel electrophoresis.

RNA Interference

siRNA oligos at a final concentration of 20 nM were transfected into HEK293 cells using the calcium phosphate precipitation method. The transfection procedure was repeated on the next day. On the second day, cells were also transfected with expression plasmids together with siRNA. Cells were harvested on the fourth day for analysis. The sequences of the siRNA oligos are as follows (only the sense strands are shown): GFP (471–489), GCAGAAGAACGGCAUCAAG; TRAF2 (1167-1185), GAUGUGUCUGCGUAUCUACTT; NEMO (832-850), ACAGGAGGUGAUCGAUAAG; UBC13(313-331), CUGCUAUCGAUCCAGGCCU; TAB2 (1319-1337), GUCGAGCAAUAGGCAAUAA These RNA oligos were synthesized at the UT Southwestern Center for Biomedical Invention (CBI) facility. Three pairs of TAK1 siRNA were from Dharmacon: Pair 1(280-298), GAGGAAAGCGUUUAUUGUA; Pair 2(1407-1425), CCCAAUGGCUUAUCUUACA; Pair 3(1216-1234), GGACAGCCAAGACGUAGAU

GST pull down assay

GST tagged UBC13 and Ubc5 were expressed in bacteria. Resin coated with the tripeptide glutathione, strongly binds the GST fusion protein and separates it from other bacterial proteins. After washing with PBS for three times, the resin was mixed with lysate from HEK293T cells transfected with pcDNA3-Flag-CC2D1A. The pull-down reaction was carried out at 4^0C with head-to-tail rotation. Then the resin was pelleted down by centrifugation and washed three times with lysis buffer (20mM Tris-Cl pH750, 100mM NaCl, 10% Glycerol, 0.5% TritonX100 and protease inhibitor cocktail from Roche). Finally, the resin was boiled in 1X SDS sample buffer and resolved by SDS-PAGE.

Multiple Alignments

Multiple alignment (.aln) file was generated by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

The shaded alignment was carried out by BoxShade (http://www.ch.embnet.org/software/BOX_form.html)

Yeast Two-Hybrid Screen

Matchmaker™ GAL4 Two-Hybrid System (Clontech) was used to do the screen following instructions from Clontech. The bait encoding amino acid 215-405 of CC2D1A was cloned into pGBKT7 and expressed as a fusion to the GAL4 DNA binding domain (DNA-BD), while a Hela cDNA library was in pGADT7 vector and expressed as a fusion to the GAL4 activation domain (AD). AH109 yeast strain was used as the host strain and Y187 was used as a mating partner to verify protein interactions.

Electrophoretic mobility shift assay (EMSA) for NF-κB

The cells are harvested and lysed using lysis buffer (20mM Tris-Cl, pH7.5, 150mM NaCl, 10% Glycerol, 0.5% TritonX100, 20mM β-Glycerol Phosphate). After centrifugation, the supernatant is collected and the protein concentration is measured using Coomassie Plus ^{TM} protein assay reagent (Thermo, Scientific) and spectrophotometer (Pharmacia Biotech). Same amount of the cell lysate is used for the binding reaction. NF-κB consensus oligos (double stranded with sequences: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, Promega) are labeled at 5'-hydroxyl group using γ-^{32}P-ATP and T4 polynucleotide kinase (T4 PNK, from NEB) at 37^{o}C for 10min. The probe is purified using G-25 spin column (GE healthcare). EMSA Gel (5% acryl/ 0.5x TBE) is pre-run at 200V for 30min. The DNA binding reaction is carried out in the presence of 10mM Tris-Cl, pH7.5, 50mM NaCl, 4% Glycerol, 1mM MgCl2, and 0.1mM EDTA, 50ug/ml poly (dI-dC). The reaction is at room temperature for 20min and is loaded onto the gel. After electrophoresis at 200V for 30min, the gel is dried and exposed to a phosphor screen.

Chapter III

Deletion of CC2D1A in Mice is Lethal and

Impairs Synaptic Maturation

Abstract

Mutations in the gene encoding CC2D1A in humans are associated with non-syndromic mental retardation (NSMR), suggesting that CC2D1A functions in learning and memory in the brain (Basel-Vanagaite et al., 2006). Cascades of synapses, assembled into overlapping neural circuits in the brain, are the fundamental machinery of information processing. Here I show that deletion of Cc2d1a in mice is lethal and impairs the synaptic maturation and function of neurons. So far, the role of NF-κB in this synaptic maturation process is not clear. Our study may help understand the molecular basis of some human diseases such as mental retardation.

Results

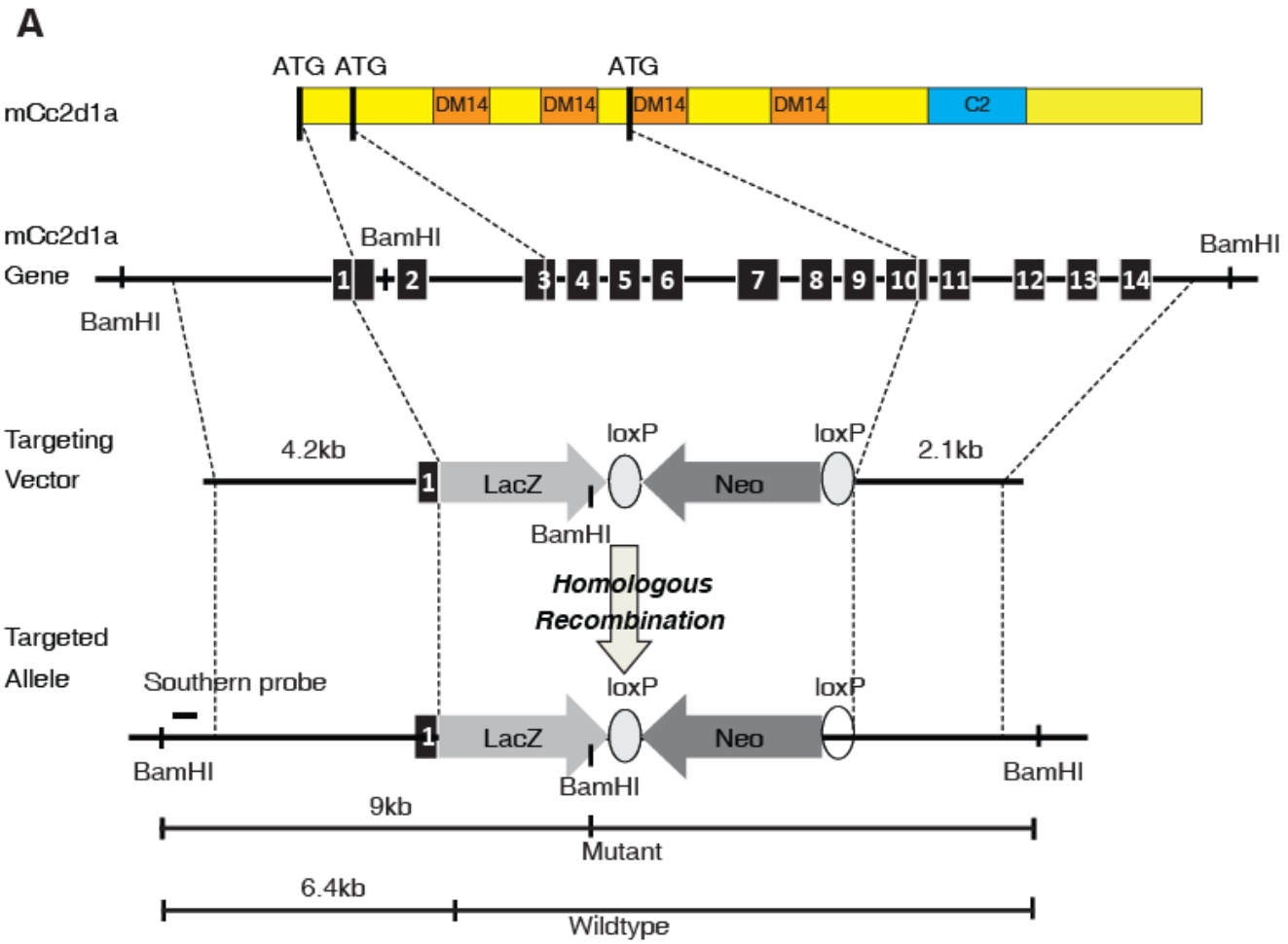
Generation of CC2D1A Knockout Mice

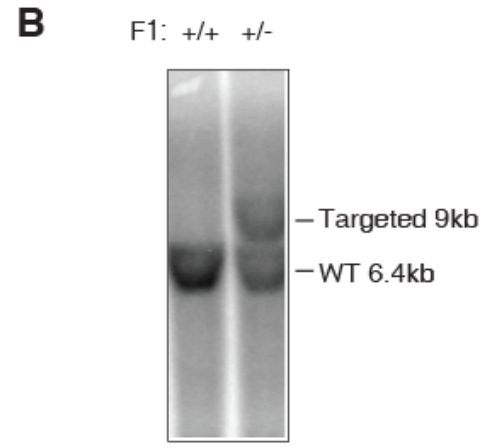
I generated a loss-of-function mutation in the mouse Cc2d1a gene by homologous recombination to investigate the function of Cc2d1a *in vivo*. The mouse Cc2d1a gene contains 29 exons and spans 14926 base pairs of DNA on chromosome 8. The targeting strategy results in the deletion of amino acid 3-349 encoded by exons 1-10 of the gene, and insertion of a LacZ reporter gene in-frame with amino acid 2 and a neomycin selection cassette flanked by loxP sites 3' to LacZ coding sequences (Figure 16A). I maintained three independent chimeric lines (1AKO-A, 1AKO-B, 1AKO-C) originating from three different ES cell subclones, and backcrossed them into a C57BL/6 background. The phenotypes of the three lines are identical, confirming that it is the result of the Cc2d1a deletion and not of another mutation introduced by ES cell manipulation. For this study, I used mice from the 1AKO-A line. Genotypes of the F1 agouti progeny, wild type or heterozygous, were confirmed by southern blot (Figure 16B). Then

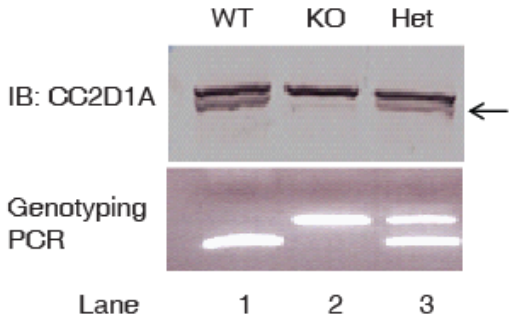
I bred heterozygous F1 male with F1 female. The deletion of Cc2d1a was verified by western blot (Figure 16C). The antibody was raised using a recombinant protein containing residues 263–512 of human CC2D1A expressed in *E. coli* as a His6-tagged protein and affinity purified. In Figure 16C, the lower band on the western blot is gone in KO and the non-specific upper bands are constant in different genotypes. The non-specific bands were also seen on western blot of HEK293T cell lysate (Figure 14A), and the lower band was knocked down by siRNA targeting CC2D1A.

Heterozygous mice are viable and fertile, whereas CC2D1A KO mice die within a few minutes after birth. Analysis of embryos, however, showed a normal Mendelian ratio of genotypes up to embryonic day (E)18.5, just before birth, indicating that lethality occurred at birth (Figure 16D). KO mice showed no spontaneous movement or breathing activity, but displayed weak and uncoordinated movements in response to mild pinches on their tails or legs, indicating the neural circuits for reflective responses were intact in these mutants. Therefore a severe postnatal respiratory failure may be a possible cause of lethality.

Figure 16. Targeting the Cc2d1a Locus. A. The strategy to generate the Cc2d1a deficient allele. The domain structure of the mouse Cc2d1a protein, first three ATG sites, corresponding exonal structure, targeting vector and targeted allele are shown. LacZ cassette and loxP-Neo-loxP replace exons 1-10. Positions of the probe used for Southern analysis are shown. **B.** Protein extracted from E14.5 MEF cells was analyzed by immunoblotting using CC2D1A specific antibody. Genomic DNA from E14.5 embryos was analyzed by genotyping PCR. **C.** The number of E18.5 embryos from the breeding of Cc2d1a $^{+/-}$. (The ES cell electroporation, clone selection and generation of chimeric mice were carried out by the Transgenic core facility at UTSW.)







D

CC2D1A	+/+	+/-	-/-
Total #	160	317	169
%	24.8	49.1	26.2

Anatomy of Cc2d1a Knockout Mice is Normal

Loss of the *Drosophila* Cc2d1a orthologue L(2)gd (Lgd) causes massive overproliferation of imaginal disc cells, extended larval life and lethality during pupae stage (Agrawal et al., 1995; Buratovich and Bryant, 1997; Klein, 2003). It raises the possibility that Cc2d1a KO mice suffer from major developmental abnormalities that cause lethality. In order to identify such abnormalities, I examined the morphology of E18.5 Cc2d1a KO embryos by H&E staining. However, I did not find any major anatomical defects in Cc2d1a-deficient mice (Figure 17). I also examined the heart, the lung and the brain in particular. These organs have no apparent abnormalities either.

Since KO mice do not breathe after birth, I asked whether the formation of neuromuscular junctions at the diaphragm was affected in the absence of Cc2d1a. The diaphragmatic muscle is innervated by phrenic nerves which branch in a characteristic pattern on reaching the muscle to form discrete NMJ synapses in the middle of the diaphragm. This pattern is controlled by synaptic activities, thus it becomes abnormal when synaptic activities are altered (Buffelli et al., 2003). I carried out immunofluorenscence staining using Syntaxin specific antibody to label phrenic nerves at the diaphragm. As shown in Figure 18A, the branching pattern was not significantly altered in Cc2d1a KO mice. I also double stained diaphragm muscles at E18.5 with synaptotagmin-2 (Syt-2) antibody (green) to label nerve termini and Texas-red conjugated α-bungarotoxin (red) to label post-synaptic AChRs (Figure 17B). High magnification views of endplates from wholemount diaphragm muscle showed that the distribution of AChRs in the wild type and Cc2d1a KO muscles are similar and the nerve termini

formed juxtaposition with AChR clusters (Figure 18B). Therefore neuromuscular junctions were established in the absence of Cc2d1a.

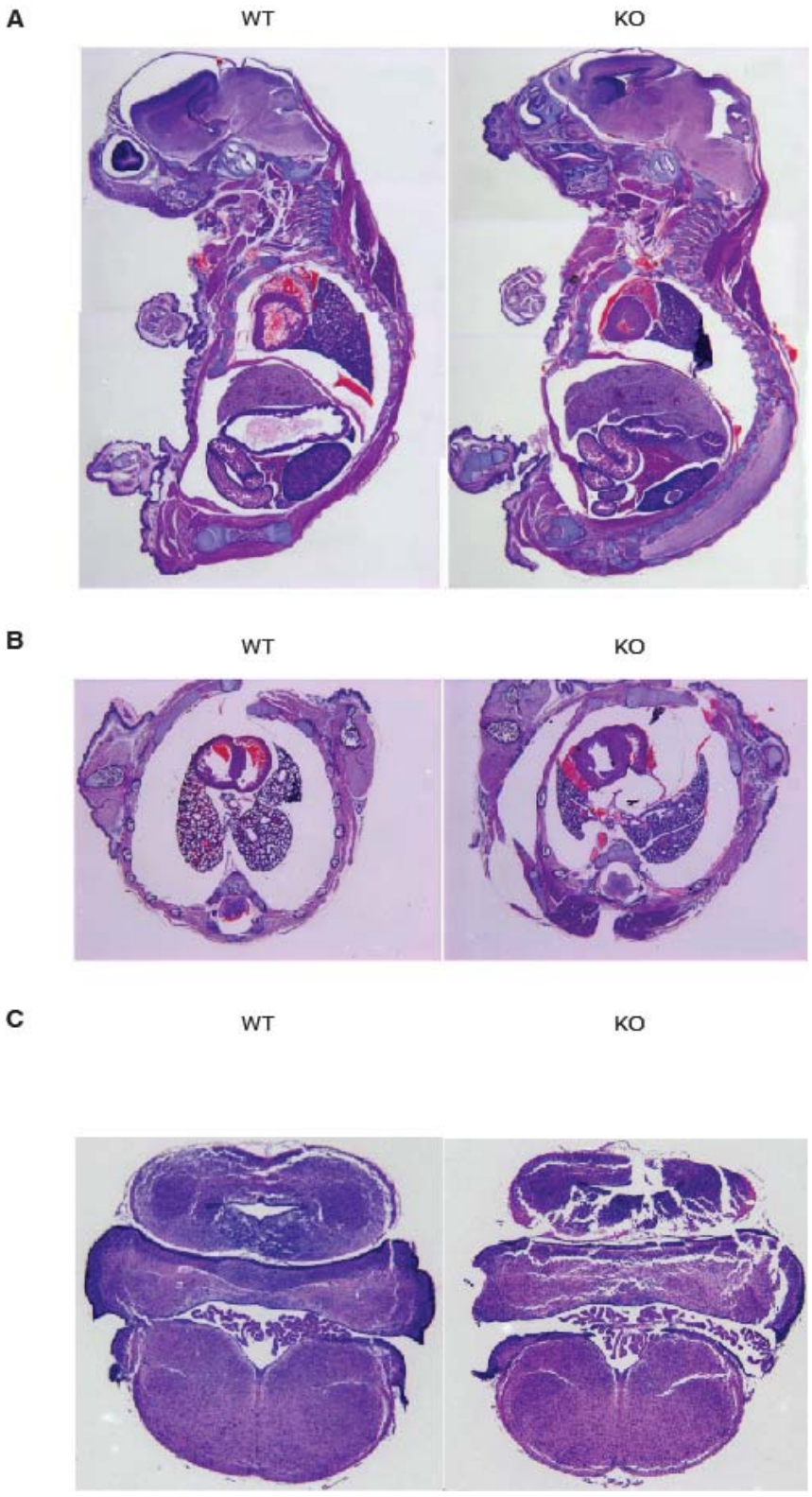


Figure 17. H&E staining of E18.5 Embryos. A. Sagittal sections of WT and Cc2d1a KO embryos at E18.5. **B.** Transverse sections through the heart and lung. **C.** Coronal sections through the brain. (The section and staining were carried out by Pathology core facility at UTSW)

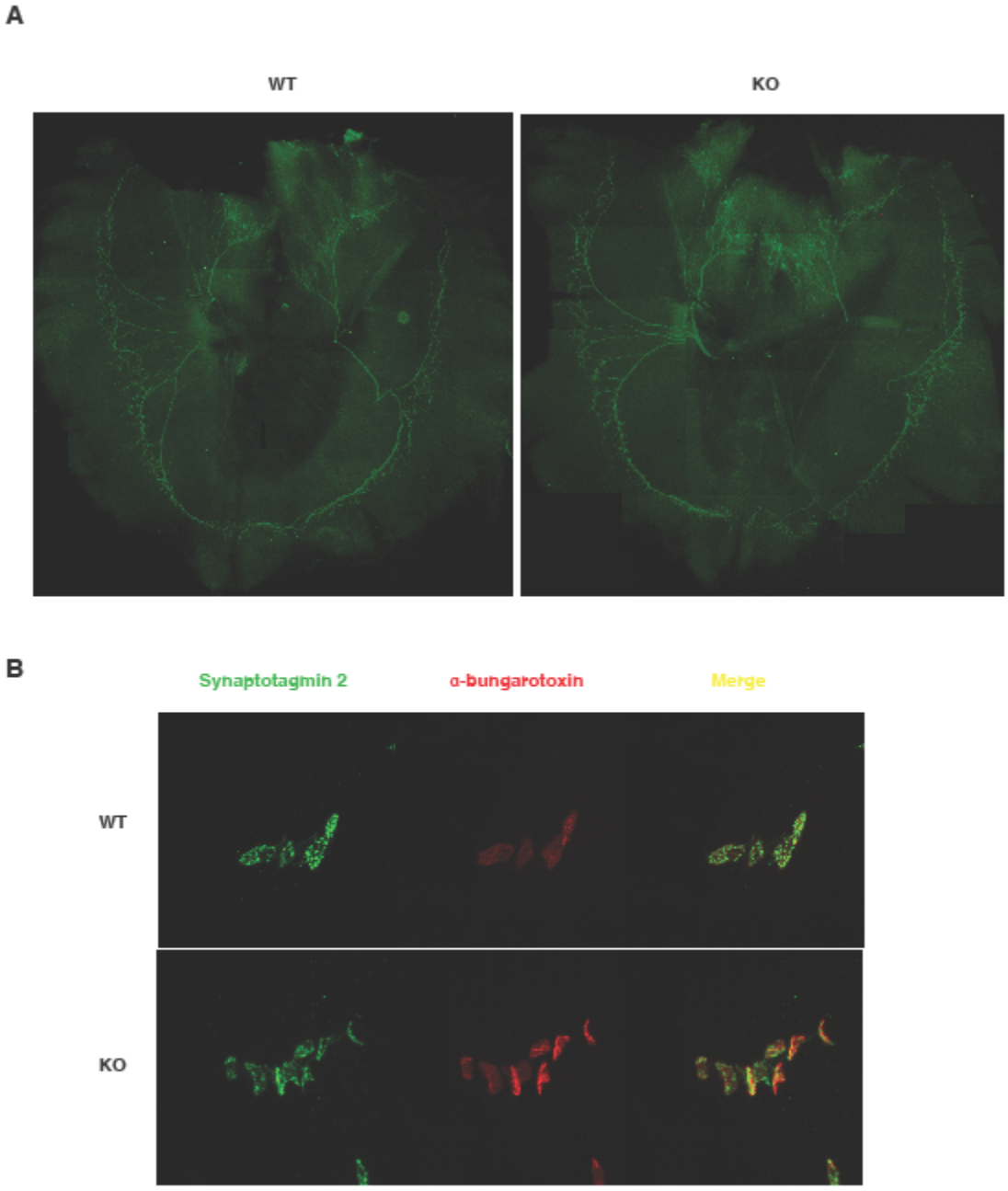


Figure 18. Developing Neuromuscular Junction in the Absence of Cc2d1a. A. Wholemount embryonic diaphragm muscles (E18.5) were immunostained with anti-Syntaxin antibody. **B.** Confocal images of single endplates from wholemount diaphragm muscle at E18.5, double-labeled with synaptotagmin-2 (Syt-2) antibody (green) and Texas-red conjugated α-bungarotoxin (red). (The antibodies and protocol were provided by Dr.Yun Liu at Dr. Weichun Lin's lab in UTSW.)

Expression of Cc2d1a is Enriched in the Brain

A previous study demonstrated that Cc2d1a is highly expressed in the developing mouse brain by in situ hybridization (Basel-Vanagaite et al., 2006). However, the tissue expression pattern of Cc2d1a has not been documented. Since a LacZ reporter gene was inserted under the control of endogenous Cc2d1a promoter in the knockout animals, I did wholemount X-gal staining to examine β- galactosidase expression at E14.5 embryos with help from David Meredith and Euiseok Kim in Dr. Jane Johnson's laboratory. Unfortunately X-gal staining did not yield any signal, although western blot confirmed the expression of Cc2d1a in wild type control. To test if the LacZ gene was transcribed, I isolated total RNA from E18.5 brain and did RT-PCR using two pairs of LacZ specific primers. PCR products were generated with expected length, indicating that LacZ was transcribed (data not shown). Thus, it is likely a stop codon at LacZ coding region is in the targeting vector.

Next I carried out immunohistochemical staining using CC2D1A specific antibody. Whole brains from E18.5 embryos were cryosectioned at 30um and the staining was performed with primary antibody rabbit anti-human CC2D1A. The brain section and staining were done in collaboration with Dr. Jane Johnson's laboratory. In theory, knockout samples should generate very low background signal and served as a negative control. However, I observed similar signals from both wild type and knockout samples. The polyclonal antibody was generated against a human antigen and it was able to recognize mouse Cc2d1a on Western blots. However it did not specifically detect endogenous protein in its native form. Although I have confirmed that Cc2d1a was expressed at E18.5 brain by western blot, the endogenous protein level in the brain may be too low to be detected by the antibody.

In order to find out the tissue expression pattern of Cc2d1a, I isolated different tissues from E18.5 wild type embryos, extracted protein using lysis buffer containing 0.5% Triton X100 and compared the expression profile of Cc2d1a among different tissues by western blot. As illustrated in Figure 19A, Cc2d1a is enriched in the brain and the brain specific isoform migrates slower on the western blot, implying Cc2d1a protein is modified in the brain. From the western blots in Figure 19A, we can see two bands. The upper bands are non-specific, while the lower bands are from endogenous Cc2d1a proteins, which are gone in KO embryos. In order to determine the spatial expression pattern of Cc2d1a in the brain, we carried out in situ hybridization in E18.5 embryos. The probe corresponds to the 5' coding sequence that is replaced in KO and the probe was ^{35}S labeled. Figure 19B shows the autoradiography results. On the upper left corner of Figure 19B, it is the negative control from a KO embryo. No signal from KO indicates that the probe specifically detects endogenous Cc2d1a mRNAs. In agreement with a previous report (Basel-Vanagaite et al., 2006), Cc2d1a mRNAs exist throughout the mouse brain. From coronal sections at the bottom of Figure 19B, it seems the signal from the olfactory bulb is the strongest and the mRNA expression is enriched in the grey matter, where neuronal cell bodies reside, including cortical plate of neocortex, hippocampus, basal ganglia and hypothalamus.

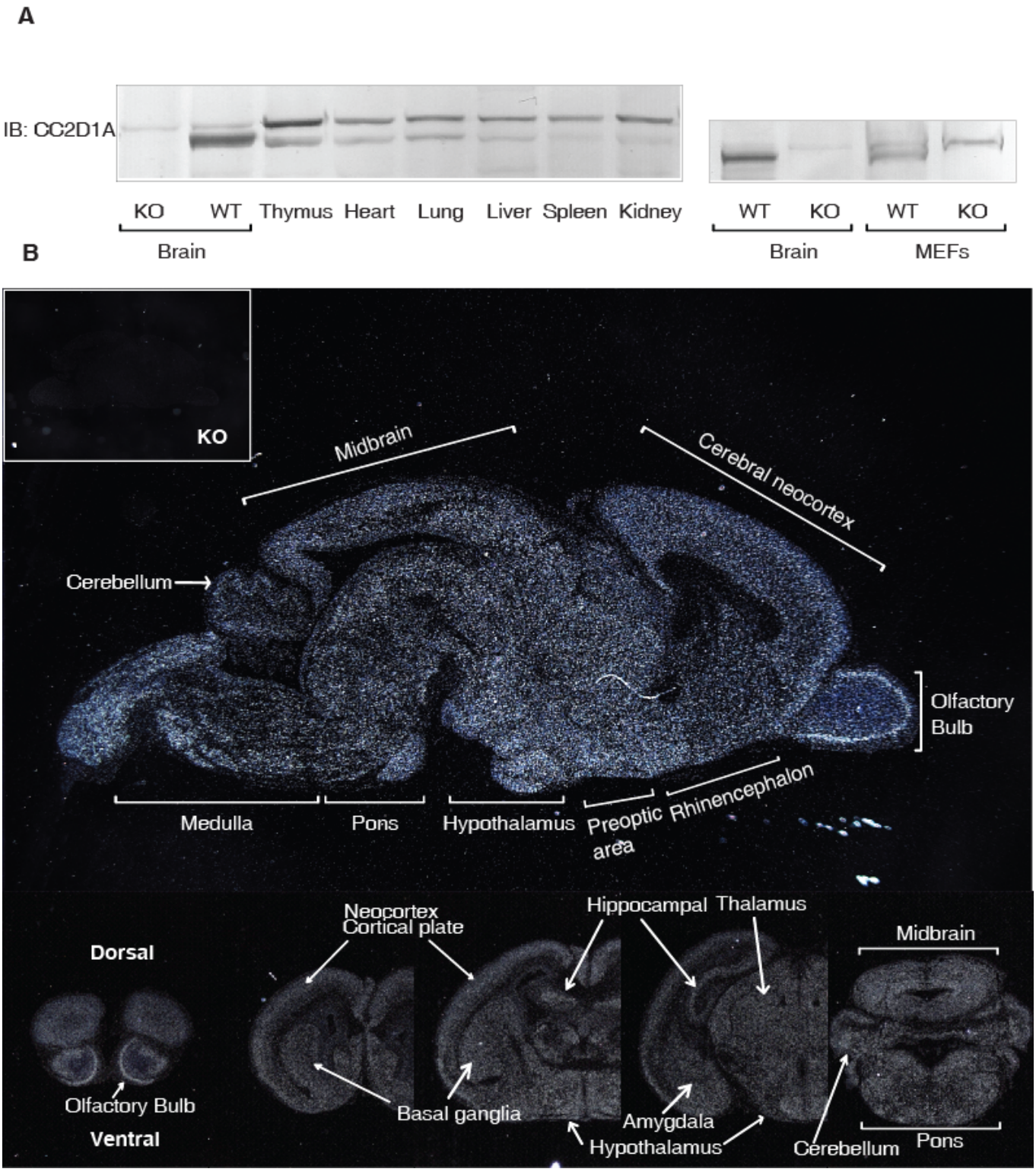


Figure 19. Expression of Cc2d1a is Enriched in the Brain. A. Protein extracted from different tissues of E18.5 embryos were examined by western blot using CC2D1A antibody. **B.** Detection of Cc2d1a transcripts by in situ hybridization. The upper left corner is a sagittal section of a KO embryo. In the center is a sagittal section of a WT embryo. At the bottom are coronal sections of a WT embryo. The in situ hybridization experiment was carried out by the Pathology core facility at UTSW.

Development and Function of Thymocytes are Normal in CC2D1A KO Mice

As mentioned previously, the drosophila orthologue of CC2D1A regulates Notch activation. Notch regulates a broad spectrum of cell lineage commitment processes, such as cardiac development, neurogenesis, gliogenesis and lymphocyte development (de la Pompa et al., 1997; Niessen and Karsan, 2008; Rothenberg and Taghon, 2005; Tanigaki and Honjo, 2007; Tanigaki et al., 2001). The absence of structural abnormalities in the heart and brain of KO mice led us to examine the hematopoietic lineages.

In the mouse, Notch1 is expressed at a particularly high level in the thymus. Without Notch1, precursors cannot develop into T cells at all. At the same time, Notch gain of function in pluripotent precursors could also enhance T cell development while blocking B cell development (Rothenberg and Taghon, 2005).

Intrathymic T cell precursors develop through several stages before becoming the peripheral mature T cells (Siebenlist et al., 2005). The most immature cells which have functional T cell receptors (β-selection) will transit from the double negative stage $CD4^-CD8^-$ into the double positive (DP) stage. DP thymocytes go through further selections before committing to single positive (SP) cells. To determine whether Cc2d1a is required for thymocyte development, I analyzed the expression of CD4 and CD8 on thymocytes by FACS. As shown in Figure 20A, at E18.5 the majority of thymocytes are $CD4^+CD8^+$ and there is no difference between WT and KO mice.

I went on to examine the functional responses of thymocytes from E18.5 littermate embryos. Thymocytes were stimulated with PBS, anti-CD3ε mAb or PMA and ionomycin. Thymocytes from both WT and KO embryos have similar responses to activation stimuli, as

indicated by the up-regulation of CD69 (Figure 20B). Therefore, I was not able to detect any impairment in the development and function of thymocytes.

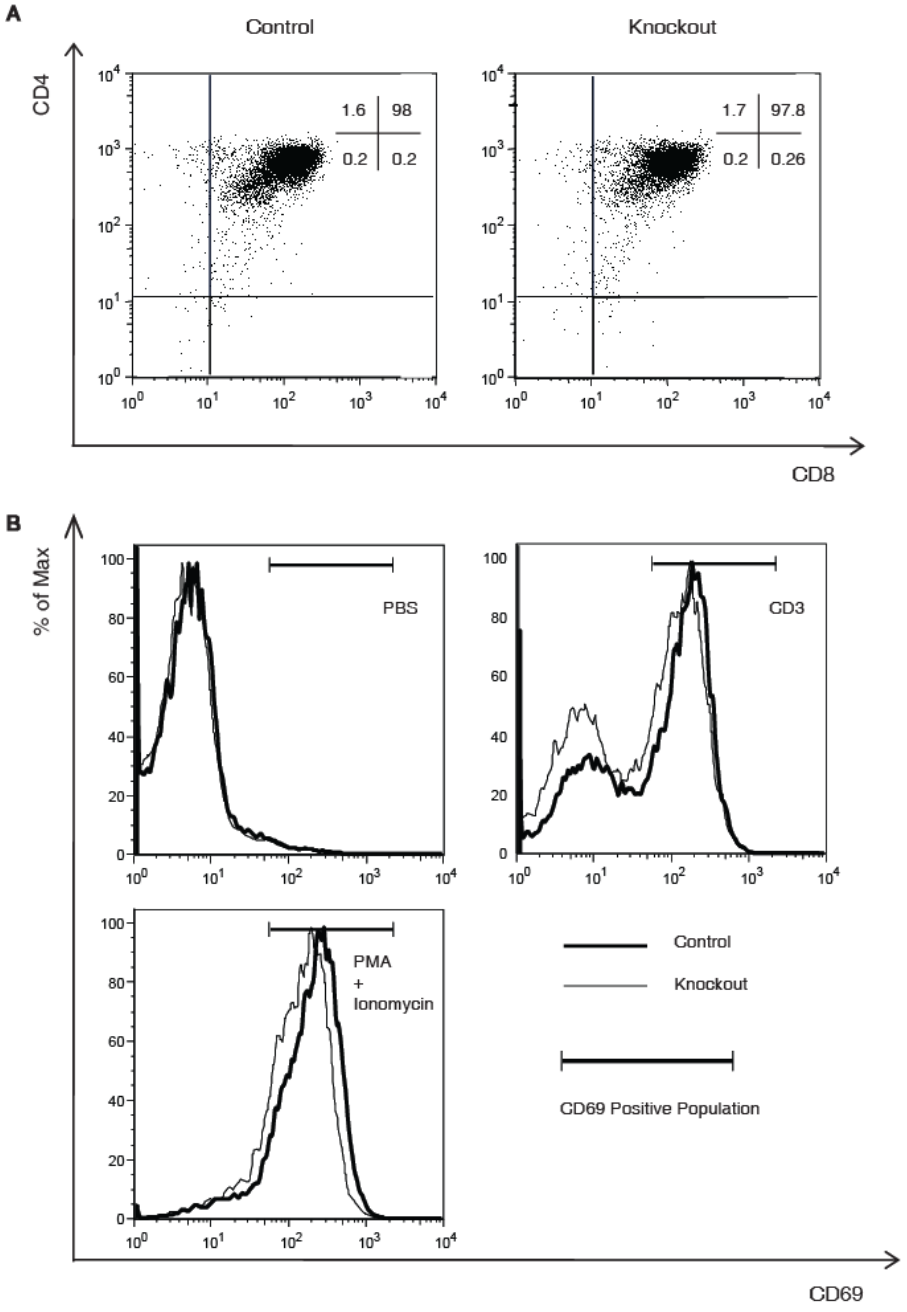


Figure 20. Development and Function of Thymocytes from Cc2d1a KO Mice. A. Suspension cells from the thymus of E18.5 embryos were analyzed by FACS using antibodies against CD4 and CD8. The percentages of thymocytes at different stages are shown in insets. **B.** Thymocytes were stimulated with PBS, anti-CD3ε mAb or PMA and ionomycin and analyzed for CD69 expression. (The anti-CD3ε mAb was provided by Dr. Nicolai Van Oers in UTSW)

Stronger Evoked Neurotransmitter Releases and Faster Synaptic Vesicle Recycling in Cc2d1a KO Synapses

Histological analysis of Cc2d1a knockout animals did not detect any defects in the peripheral respiratory apparatus, including the lung, intercostal musculature or the innervation of the diaphragm. These observations point to a central respiratory defect. Neurons that control the breathing are found in many regions of the neuraxis from the cerebral cortex through the brainstem and spinal cord (Harper et al., 1998). Given that Cc2d1a expression is enriched in the brain and *Drosophila* orthologue of Cc2d1a, Lgd, regulates endocytosis, I hypothesized that Cc2d1a is involved in neurotransmitter releases by regulating synapse exocytosis and endocytosis in the brain.

I wanted to test the basic synaptic properties of cortical neurons. However at birth when Cc2d1a KO mice die, there are very few mature synapses in the brain. In order to circumvent this problem, I cultured neurons from the neocortex of E18.5 embryos *in vitro*. In this case, neurons have sufficient time to develop and form extensive synaptic connections (Atasoy et al., 2007), allowing us to study the role of Cc2d1a in the basic synaptic functions.

To examine the properties of basic synaptic transmission in Cc2d1a KO synapses, I collaborated with Dr. Jesica Raingo in Dr. Ege Kavalali's laboratory, who performed whole-cell voltage-clamp recordings in cultured cortical pyramidal neurons. As illustrated in Figure 21A, neurons in the culture form synaptic networks with each other and one neuron receives inputs from multiple presynaptic termini. In field stimulation, all the cells in the culture receive action potentials (AP), which are electrical impulses to stimulate neurotransmitter release. A single neuron is patched and the current change in this neuron in response to all the presynaptic

neurotransmitter releases is recorded. In the experiment shown in Figure 21B, the stimulation was 50 action potentials (AP) administered at 10Hz. IPSC stands for inhibitory postsynaptic current, which is the response to inhibitory neurotransmitter release. Glutamatergic responses were inhibited by adding antagonists CNQX and AP5 in the culture. For each AP, there is one dot representing one IPSC recording, therefore there are 50 dots in each trace (Figure 21B). We examined cortical culture at 14 DIV (day *in vitro*) and found IPSC from Cc2d1a deficient synapses is much larger than control. There are several possible explanations for the phenotype. There could be more neurotransmitter releases from presynaptic termini; or more neurotransmitter receptors on the postsynaptic plasma membrane; or KO synapses are more mature at 14 DIV. Jesica found that at 14 DIV, the responses from control cortical synapses were very weak, suggesting they were not fully functional at this stage.

To monitor the fusion and retrieval of synaptic vesicles, we used Synaptophysin-pHluorin, a pH sensitive GFP fused to the synaptic vesicle protein, synaptophysin (Figure 21C). At rest, the fluorescence is quenched by the acidic pH inside vesicles. Upon stimulation (400AP at 20Hz, Figure 21D), vesicles fused with plasma membrane and intra-luminal proteins are exposed to extracellular environment and the fluorescence emission increases (Figure 20C), as seen in the rising phase in Figure 21D. F_0 is the resting fluorescence; Fmax is the peak fluorescence when all vesicles undergo exocytosis. Following stimulation, fluorescence decay is a reflection of the speed of endocytosis (Burrone et al., 2006). In Figure 21D, KO synapses manifested faster endocytosis, in other words, vesicles recycle faster at KO synapses. This is in line with our previous finding, since faster recycling results in more vesicles at the presynaptic terminal ready for the next release. However, it could also due to the fact that KO synapses are more mature than controls; therefore they use vesicles more efficiently. In Figure 21E, the amplitude of the

response to stimulation (st) corresponds to the size of "readily releasable pool" (RRP) whereas the addition of NH_4Cl unquenched all acidic vesicles that had not been released. As shown in Figure 21E, there are no major changes in the size and overall responsiveness of synaptic vesicle pools in Cc2d1a deficient synapses.

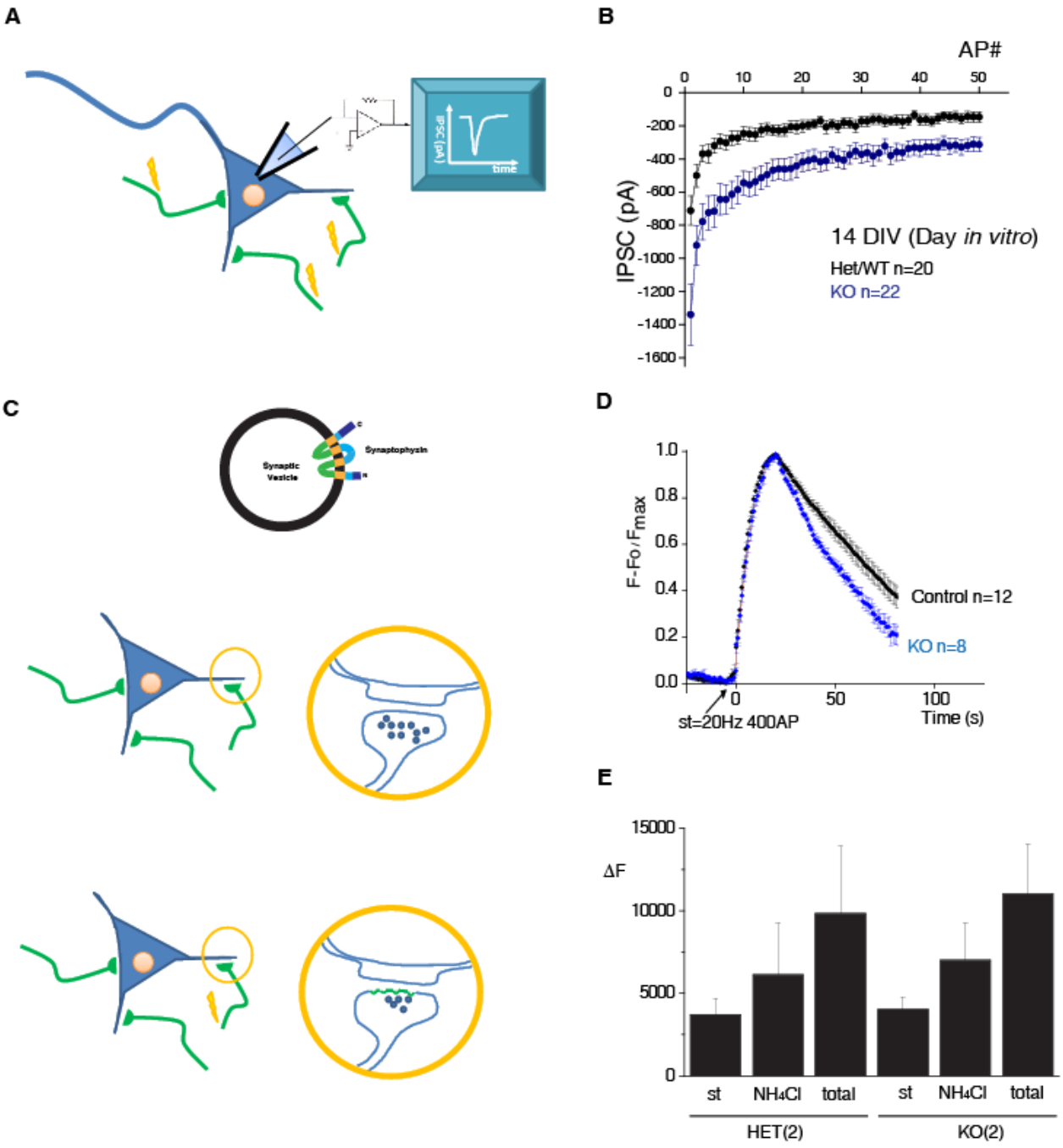


Figure 21. Stronger Evoked Neurotransmitter Releases and Faster Vesicle Recycling in Cc2d1a KO Synapses. A. A cartoon illustrates the whole-cell voltage-clamp recording in cultured neurons. **B.** Cortical neurons at 14 DIV were patched and induced by field stimulation with 50 action potentials (AP) administered at 10 Hz. The mean amplitudes of GABAergic responses (IPSC) under the inhibition of glutamatergic responses by CNQX and AP5 were measured at each action potential. **C, D, and E.** A real-time analysis of synaptic vesicle recycling using Synapto-pHluorins (spH). **C.** Cartoons illustrate the synaptic protein synaptophysin as well as the principle of Synapto-pHluorin experiment (see text for details). **D.** Fluorescence changes derived from synapses of dissociated cortical neurons expressing spH in response to the stimulation of 400 AP at 20 Hz. The magnitude of the fluorescence signal during stimulation represents the amount of spH accumulated on the synaptic surface. Fluorescence decay after stimulation represents endocytosis. $F_{0:}$ resting fluorescence; Fmax: peak fluorescence when all vesicles undergo exocytosis. **E.** Fluorescence changes during stimulation and NH_4Cl treatment, reflecting different synaptic vesicle pools. (Work in this figure was in collaboration with Dr. Jesica Raingo in Dr. Ege Kavalali's lab, UTSW, who did all the electrophysiology experiments; Cartoons in A and C except for synaptophysin are by courtesy of Dr. Jesica Raingo.)

Spontaneous and Asynchronous Releases are Abnormal in Cc2d1a Deficient Synapses

In the last section, I've described evoked neurotransmitter releases, which happen in response to action potential stimulations. There are other modes of neurotransmitter releases. For example, synaptic fusion can occur in the absence of action potentials. It is named "spontaneous release" or "miniature release", which is due to a spontaneous fusion of one synaptic vesicle at the presynaptic active zone. We examined the spontaneous release in Cc2d1a KO synapses. mIPSC stands for miniature inhibitory postsynaptic current, which is the response to spontaneous releases of inhibitory neurotransmitters. We found that the frequency of "mini" was decreased over two fold in KO synapses at 14 DIV, whereas the amplitude was unchanged (Figure 22A,B). This is an interesting observation, because less spontaneous releases suggest that the KO synapses are more tightly controlled by activity, i.e. they do not randomly release neurotransmitters but only do so in response to stimulation. Thus it also shows that KO synapses are more mature. Now the amplitude of "mini" is related to the postsynaptic receptors. If there

were more receptors, we would expect higher amplitude of "mini" as well as evoked responses. Thus our data indicate the stronger evoked neurotransmitter releases in KO are likely due to presynaptic defects.

When an action potential arrives at the nerve terminal, the incoming calcium ions trigger two distinct neurotransmitter release processes. One is "temporally precise" to action potential triggered Ca^{2+} increase and is called the synchronous release. Synchronous releases cease right after action potential. On the contrary, asynchronous releases persist even after tens of milliseconds (Hestrin and Galarreta, 2005). We found that at 14 DIV, there were three fold more asynchronous releases in control synapses as compared to KO. However at 21 DIV, the difference between the two groups was not significant any more (Figure 21C). Therefore, from 14 DIV to 21 DIV, as control synapses mature, they are able to reduce asynchronous releases, whereas KO synapses reach this mature state much earlier, at 14 DIV instead of 21 DIV.

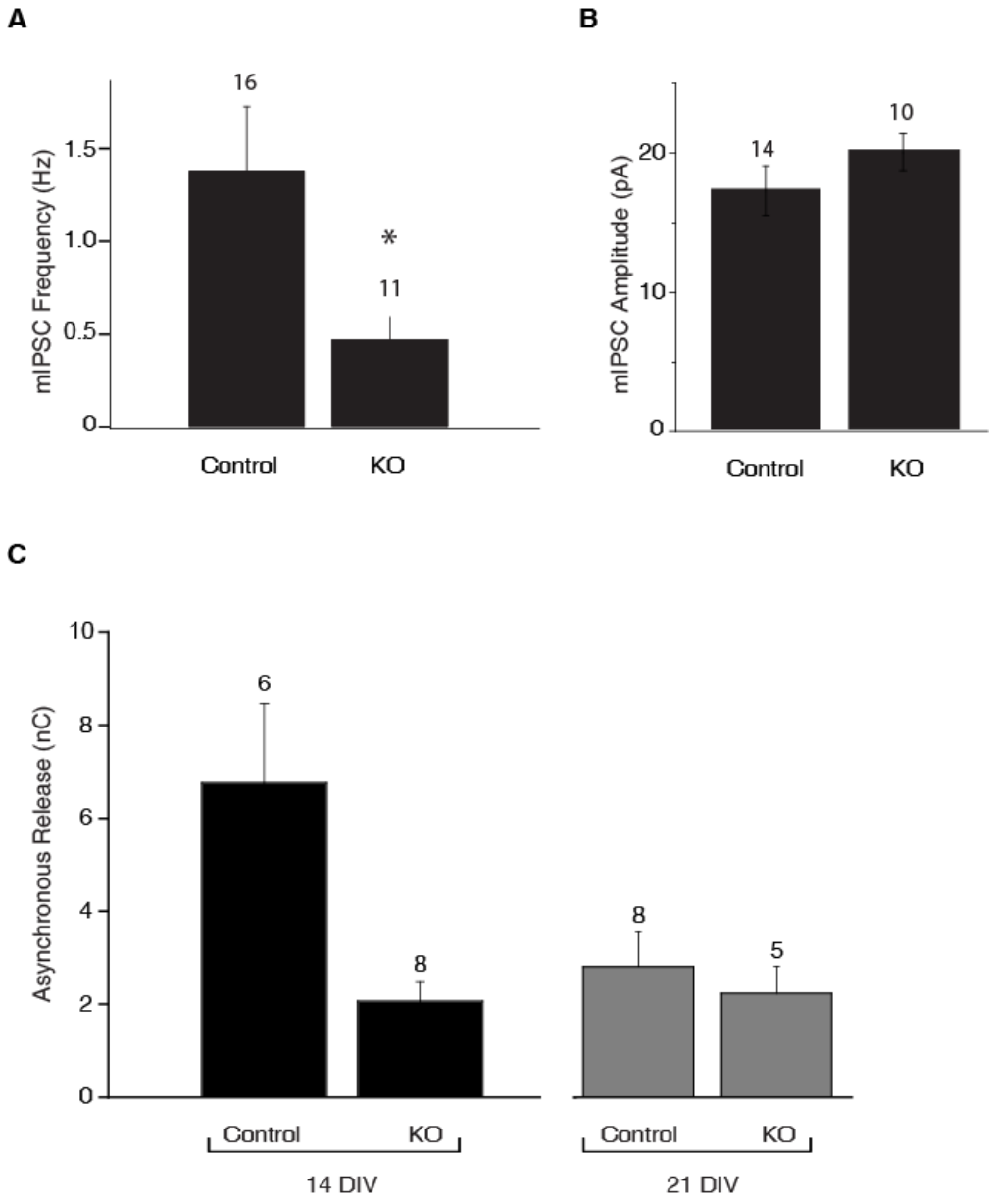


Figure 22. Spontaneous and Asynchronous Releases are Abnormal in Cc2d1a Deficient Synapses. A. A bar graph depicts the significant decrease in mIPSC frequency in Cc2d1a KO synapses compared to controls. The numbers above the bars denote the number of neurons recorded for each condition. **B.** A bar graph of mIPSC amplitude reveals no change between KO and control synapses. **C.** A bar graph of asynchronous releases from KO and control synapses at two different time points, 14 DIV and 21 DIV. (Work in this figure was in collaboration with Dr. Jesica Raingo in Dr. Ege Kavalali's lab, UTSW)

Cc2d1a Negatively Regulates Synaptic Maturation

The observation that asynchronous releases change during development and are abnormal in KO synapses led us to re-examine evoked neurotransmitter releases at different developmental stages. We monitored cultured cortical neurons at three different time points *in vitro* (at 7, 14 and 21 days). At 7 DIV, when most WT/Het synapses were non-functional, KO synapses started to exhibit significantly larger postsynaptic current in response to stimulations (Figure 22A). At 14 DIV, responses from both KO and control synapses increased, and the difference between KO and control was even larger (Figure 22A). At 21 DIV, responses from both KO and control synapses grew much stronger, as demonstrated by the value of IPSC. However the difference between KO and control was not significant any more. (Figure22A). These data suggest that endogenous Cc2d1a keeps synaptic maturation in check to make sure that the neuron develops fully functional synapses and responses to stimulations at the right time. Therefore in the absence of Cc2d1a, this maturation process is impaired.

The Cc2d1a KO Phenotype can be Partially Rescued by Cc2d1a

To investigate whether the phenotype of Cc2d1a-deficient synapses reflects a developmental change or is related to an acute action of Cc2d1a in synaptic transmission, we tested whether the phenotype could be rescued by the expression of wild-type Cc2d1a in Cc2d1a-deficient neurons. I generated several lentiviruses expressing Cc2d1a with HA or Flag tag at NH_2/COOH terminus. Although cells infected with these lentiviruses express Cc2d1a as shown by western blot, none of the viruses were able to rescue the phenotype. It is possible that putting a tag on the protein interferes with its function. To address this possibility, I generated lentiviruses expressing full length Cc2d1a without any tag. Protein expression was confirmed by

western blot (Figure 23B). Cc2d1a-deficient neurons were infected at 4 DIV with these lentiviruses and analyzed at 14 DIV.

Measurements of inhibitory synaptic responses induced by the same field stimulation of 50 APs administered at 10 Hz demonstrated that the Cc2d1a protein partially yet significantly rescued the Cc2d1a-deficiency phenotype. On the contrary, COOH terminus deletion mutant of Cc2d1a was not able to rescue the phenotype at all, indicating that C2 domain and the COOH terminus of the protein are functionally important (Figure 23D). Notably the rescue proteins were introduced into the neurons after the synapse maturation had been initiated. Although fully rescue would suggest that Cc2d1a acts acutely during neurotransmitter release, effects on the early maturation of synapses cannot be rescued. Therefore, these data are in line with our hypothesis that Cc2d1a negatively regulates synaptic maturation.

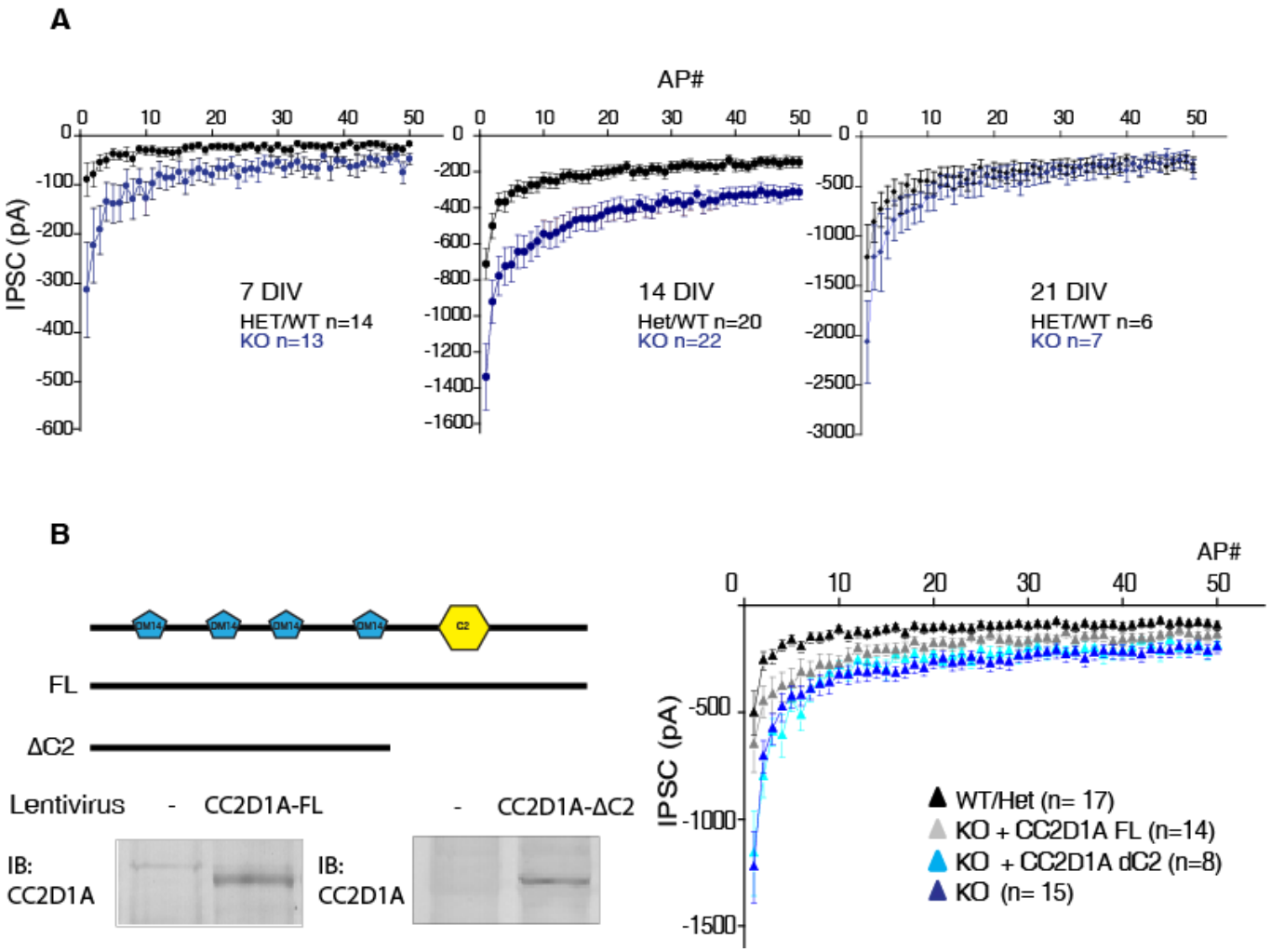


Figure 23. Cc2d1a Negatively Regulates Synaptic Maturation. A. Cortical neurons at 7, 14 and 21 DIV were patched and induced by field stimulations of 50 pulses administered at 10 Hz. The mean amplitudes of Inhibitory postsynaptic currents (IPSC) at each AP were recorded. **B.** Cortical cultures were infected at 4 DIV with lentiviruses expressing either full length or COOH terminal deletion mutant of Cc2d1a. The expression of exogenous proteins was examined by immunoblotting using CC2D1A specific antibody. IPSC was examined at 14 DIV as described previously. (Work in this figure was in collaboration with Dr. Jesica Raingo in Dr. Ege Kavalali's lab, UTSW)

Microarray Analysis of Gene Expression in Cc2d1a Knockout Cortex

To further investigate the molecular mechanism underlying the cellular phenotype we have observed, I performed microarray analysis of total RNAs isolated from the cortex of E18.5 embryos. Samples were from two litters of mice-one WT and one KO from the first litter and two WT and two KO from the second litter, so each genotype contains three samples. Gene expression analysis was done using Illumina BeadStudio Software. When Differential P value less than 0.05 and fold change greater than 2 were applied, 10 genes were upregulated and 18 genes were downregulated in Cc2d1a deficient cortex (Table 1). Real-time PCR was carried out to confirm the expression changes of two target genes, Kcnn-1 and Usp38 (Figure 24 A, B).

Fold change of Cc2d1a is 0.233 (Red box). The probe sequence for Cc2d1a is-TACTGACCCAGCTGCCCTCACCCCAGGGCCAACACAGAATGAACAGCCAA, which is at 3'-UTR right in front of the polyA tail. I used the same total RNAs mentioned above to do reverse transcription and PCR using a pair of primers at the 3'UTR. I detected a band from KO RNAs with a lower intensity compared with the one from WT (Figure 22C). I also did real-time PCR using a pair of primers in the coding sequence but downstream of the targeted region. The fold change between KO and WT was 0.18 (Figure 22D). Therefore, Cc2d1a gene downstream of the knockout region is transcribed in KO with much lower efficiency. However whether truncated proteins are produced in KO cannot be determined. An antibody raised against the COOH terminal region of Cc2d1a protein will help to address this question.

Symbol	Official Full Name	Fold change (KO/WT)
C920027J16Rik		38.5548456
Ocel1	Occludin/ELL domain containing 1	9.416416532
Neto2	PREDICTED: neuropilin (NRP) and tolloid (TLL)-like 2	5.025307902
Tmem66	Transmembrane protein 66	2.912821477
Btbd14b	BEN and BTB (POZ) domain containing	2.886480162
Zfp423	Zinc finger protein 423	2.605911343
Farsa	Phenylalanyl-tRNA synthetase, alpha subunit	2.53979456
Kcnn1	Potassium intermediate/small conductance calcium-activated channel	2.299584095
Ssbp4	Single-stranded DNA-binding protein 4	2.284664232
Plvap	Plasmalemma vesicle associated protein	2.260615641
Slc5a5	Solute carrier family 5 (sodium iodide symporter)	0.501236954
BC056474		0.472035579
LOC100047888		0.45364045
Ankrd13c	Ankyrin repeat domain 13C	0.429627489
Zfp869	Zinc finger protein 869	0.414350633
C430002D13Rik		0.351397705
Prdx2	Peroxiredoxin 2	0.270579347
Cc2d1a	Coiled-coil and C2 domain containing 1A	0.232560189
Cacna1a	Calcium channel, voltage-dependent, P/Q type	0.224910809
Tbc1d9	TBC1 domain family, member 9 (with GRAM domain)	0.221688664
Trmt1	TRM1 tRNA methyltransferase 1 homolog (S. cerevisiae)	0.212266935
Usp38	Ubiquitin specific peptidase 38	0.211770954
Tbck	TBC1 domain containing kinase	0.16081693
Morg1	Mitogen-activated protein kinase organizer 1	0.153505553
Rtbdn	Retbindin	0.141307449
Scoc	Short coiled-coil protein	0.139896886
Man2b1	Mannosidase 2, alpha B1	0.099081836
Zfp330	Zinc finger protein 330	0.004919273

Table 1. List of Deregulated Genes in Cc2d1a Deficient Cortex. RNAs isolated from cortex of E18.5 embryos were analyzed. Cc2d1a was labeled in red. (Microarray analysis was carried out by Microarray core facility at UTSW)

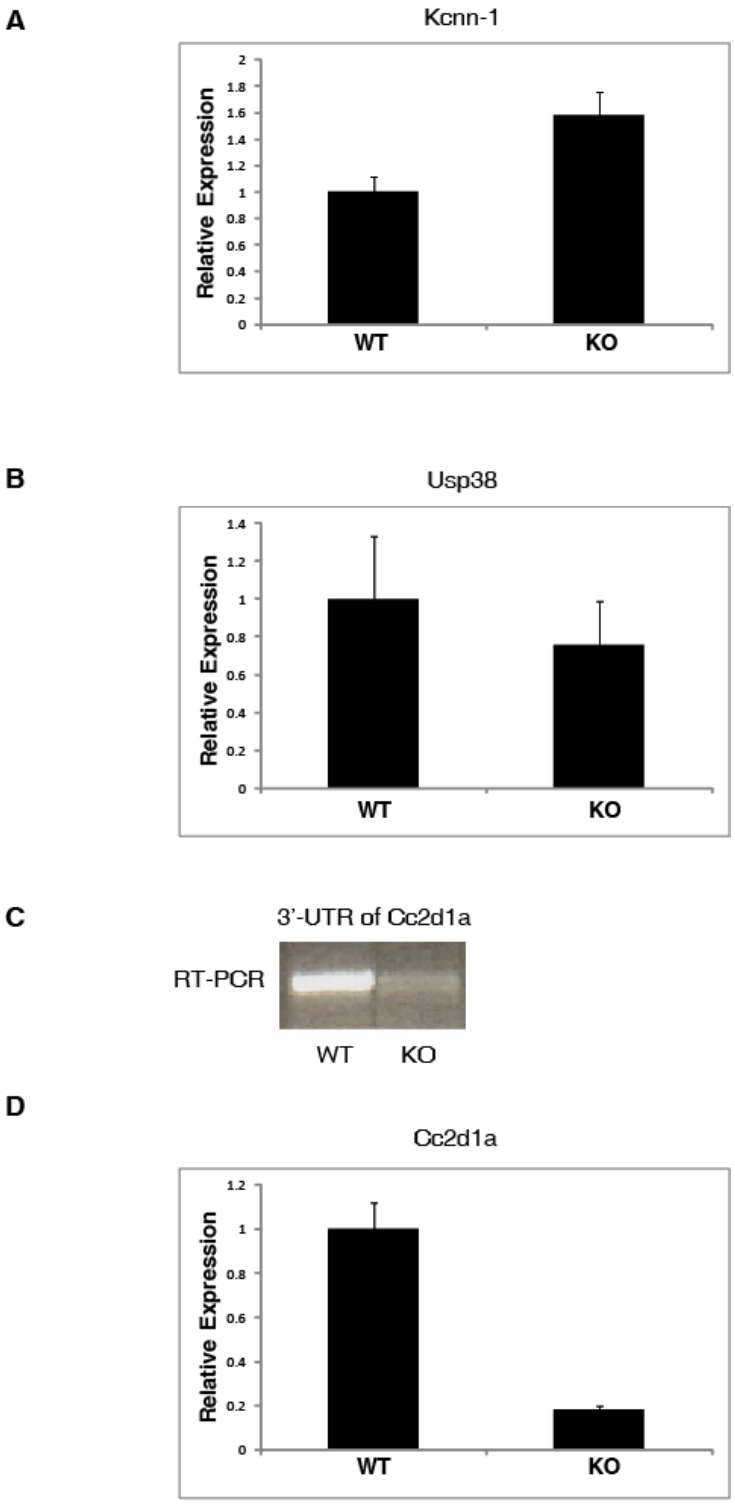


Figure 24. Deregulation of Kcnn-1 and Usp38 Expression in Cc2d1a KO cortex. A, B, D, Quantitative PCR to examine the expression of Kcnn-1 (potassium intermediate/small conductance calcium-activated channel), Usp38 (Ubiquitin specific peptidase 38) and Cc2d1a. **C.** Reverse transcription (RT)-PCR to examine the transcription of Cc2d1a 3'UTR (see text for details).

Discussion

In Chapter III, a knockout mouse model was developed to investigate the function of Cc2d1a, a C2 domain protein with unique NH_2 terminal DM14 domains. Cc2d1a belongs to a new protein family and it has another homolog Cc2d1b in mammals. Instead of four DM14 domains at NH_2 terminus, Cc2d1b (mouse) has three DM14 and shares 43% identity of Cc2d1a (mouse) based on amino acid sequences. Although CC2D1A (human) strongly activates NF-κB *in vitro*, CC2D1B (human) is a much weaker activator, based on a luciferase reporter assay (data not shown). Thus it is possible that the additional DM14 domain is crucial for NF-κB activation, presumably by recruiting additional factors, such as UBC13. Nevertheless, given that CC2D1A is the most potent NF-κB in our hands, NF-κB activation by CC2D1B could still be significant. Therefore, it is possible that Cc2d1b act as a redundant NF-κB activator *in vivo* and that could explain why I did not see deregulated NF-κB target genes in the microarray results. However, I've demonstrated that Cc2d1a is essential for survival, indicating that Cc2d1a plays other critical and non-redundant roles.

Cc2d1a KO animals have beating hearts at birth but they do not breathe on their own and soon die. In the analysis of the Cc2d1a KO mice, I focused on the brain for several reasons. First, the brain expresses the highest level of Cc2d1a among all the tissues tested (thymus, lung, heart, spleen, liver, kidney). In situ hybridization shows Cc2d1a mRNA is ubiquitous in the brain and enriched in the grey matter, including cortical plate. Second, loss-of-function mutations in CC2D1A gene in human have been associated with mental retardation, suggesting that CC2D1A functions in learning and memory. Third, perinatal lethality has been observed upon deleting many genes that regulate neurotransmitter release and synaptic functions, such as Neuroligin, αNeurexin and Munc-18. In many cases, although the targeted gene is essential for proper brain

functions, knocking out the gene does not impair normal brain development. It may be explained by the fact that only certain aspects of the presynaptic function are abolished in these mutants. In particular, spontaneous, quanta transmitter release is retained even in the most severely affected mutants. In the case of Cc2d1a KO, I did not detect any anatomical defects in the heart, lung, muscle, brain nor the innervation of diaphragm muscles. Although it is premature to claim that normal development occurs in these organs in the absence of Cc2d1a, the phenotype in KO animals suggest a functional abnormality in the CNS. We've seen the amplitude of spontaneous releases was unchanged although the frequency decreased, supporting the notion that the basal spontaneous activity in the KO brain is sufficient for structural assembly.

To circumvent early lethality in KO animals, we took advantage of an *in vitro* culture system in which embryonic neurons mature and develop extensive synaptic connections and the basal properties of Cc2d1a-deficient neurons can be studied electrophysiologically. We chose cortical neurons because the expression of Cc2d1a is enriched in the cortex based on in situ hybridization data (figure 19B). We've also tested hippocampal neurons and they did not appear to be defective in terms of evoked responses. Thus, it is not a general synapse phenotype but rather region specific. Also, we have not examined synaptic functions of neurons at the breathing center; therefore there is still a gap in understanding between the cellular phenotype we've seen and the lethality in animals.

Drosophila orthologue of Cc2d1a Lethal (2) Giant Discs (Lgd) has been shown to negatively regulate Notch activation. Notch signaling is known to regulate T cell development. I've examined the thymocyte function in Cc2d1a KO mice and did not detect any defect. Notch is also known to suppress neuronal differentiation and maintain neural stem cells by regulating the expression of bHLH transcription factors (Kageyama et al., 2005). I collaborated with David

Meredith in Dr. Jane Johnson's laboratory to examine in the early development the expression of some of the bHLH factors including Mash-1 and Ptf1a, which promote the neuronal cell type specification. We did not detect any abnormality in the expression pattern of these two factors at E10.5 spinal cord and hind brain. However, further detailed studies are necessary to determine if Cc2d1a modulates Notch activation and neuronal cell specificity during development. It is also possible that Cc2d1a regulates Notch at later stages of development and/or at different developmental processes. For example, the conditional activation of the Notch1 gene in endothelial cells shows multiple defects in vasculature (Krebs, Starling et al.) and Notch activation has been involved in lung cancer, breast cancer and leukemia. Generating a conditional knockout mouse model of Cc2d1a will be invaluable to uncover its regulatory roles in different systems with regards to Notch signaling as well as other functions. The role of Notch in the synapse maturation is unknown. It will be interesting to test if inhibiting Notch in Cc2d1a KO neurons rescues our phenotype. However, since in *Drosophila* there is only one homolog of Cc2d1a instead of two, the role of Lgd may be carried out by redundant proteins through evolution, thus in Cc2d1a KO mice, Notch activation could be normal because of the compensation.

Lgd in *Drosophila* also impairs the endosomal trafficking. I have hypothesized that Cc2d1a regulates synaptic vesicle recycling although it is a highly specialized process optimized for fast exocytosis and endocytosis. I've collaborated with Dr. Jesica Raingo from Dr. Ege Kavalali's laboratory to characterize the basic synaptic properties of primary cultured neurons. We've seen that at 14 DIV (days *in vitro*), Cc2d1a deficient synapses have stronger evoked responses to inhibitory neurotransmitters. Since we were measuring the current change in a single postsynaptic neuron, bigger current changes could be due to more neurotransmitter

releases or more receptors on the postsynaptic membranes or both. We've seen that the amplitude of spontaneous responses is unchanged, suggesting a presynaptic defect. Also, in Figure 21B, the responses become smaller and smaller, and this phenomenon is named "depression", which could be resulted from the depletion of synaptic vesicles in the presynaptic termini. We've seen the depression rate at 1Hz stimulation is slower in KO synapses (data not shown), which cannot be easily explained by the up-regulation of postsynaptic receptors. We went on to examine the vesicle pool size in two ways: activity-dependent labeling of synaptic vesicles with FM dyes (data not shown) and synaptophysin-pHluorin to monitor turn-over of synaptic vesicles, and vesicle pool size is similar in Cc2d1a deficient neurons compared to control neurons. Interestingly faster vesicle endocytosis was seen in KO neurons in synaptophysin-pHluorin experiment and it is consistent with higher IPSC and slower depression rate in KO neurons. However, the maturity difference could also explain the variations between KO and control in terms of evoked, spontaneous and asynchronous neurotransmitter releases, as well as the synaptic vesicle trafficking.

Full length Cc2d1a is able to rescue the phenotype to a certain degree whereas C2 domain deletion cannot. The C2 domain is also deleted in human patients, underscoring its functional importance. Although conserved aspartic acid residues required for calcium binding are absent, C2 domain of Cc2d1a may still bind to phospholipids independent of calcium and participates in membrane fusion reactions. In fact, I have found that CC2D1A locates not only in the cytosol and nuclear but also in membrane fractions in HEK293T cells based on fractionation experiments (data not shown). Preliminary result also shows that Cc2d1a associated synaptic plasma membranes. As mentioned in results, the lentiviral infection was done on 4 DIV when synapse maturation already took place, and that full length Cc2d1a was unable to fully rescue the

phenotype could be explained if Cc2d1a regulates maturation rather than synaptic transmission per se.

CC2D1A activates NF-κB in HEK293T cells through the canonical IKK pathway. Studies on mice deficient in NF-κB subunits have shown that these transcription factors are important for lymphocyte responses to antigens and cytokine-inducible gene expressions. In particular, RelA (p65) KO mice die from liver apoptosis due to TNFα sensitivity and embryonic lethality of RelA KO can be rescued by simultaneously knocking out TNF receptor. Another two mouse models expressing dominant negative IκBs in neurons (Fridmacher et al., 2003) or neurons and glia (Ben-Neriah and Schmitz, 2004) both showed a learning impairment, although the mice are viable. Cc2d1a KO phenotype does not resemble any of the above; it is possible that Cc2d1a only modulates NF-κB activity or Cc2d1b compensates the regulation of NF-κB. Most efforts in characterizing NF-κB subunits KO mice are focused on immune systems and potential roles of NF-κB in synapse function are not revealed yet. Unfortunately, I did not identify any known NF-κB target genes in microarray analysis and the infection of lentiviruses expressing NF-κB super repressors seem to cause cell death in cortical culture, suggesting NF-κB is required for neuron survival *in vitro*. So far, I do not have evidence that the perinatal lethality or the synaptic defects of Cc2d1a KO is due to the deregulation of NF-κB pathways.

Synaptic maturation is a complex process that is steered by multiple factors and it is likely that Cc2d1a regulates gene transcription of multiple targets through transcription factors like NF-κB. It will be interesting to compare endogenous NF-κB level in WT and KO culture. Also, Cc2d1a has been shown to bind to the cis element in serotonin receptor 1A promoter and negatively regulate 5-HT1A transcription. Thus, it is also possible that Cc2d1a directly regulate the expression of target genes. As illustrated in Figure 5, before the initial contact between

presynaptic and postsynaptic protrusions, active zone proteins are packed in vesicles and move along the developing axons. It is possible that Cc2d1a negatively regulates either the packing or transport of active zone proteins to control the formation of the active zone. Also, voltage gated calcium channels (VGCC) need recruiting to the active zone and physically coupling to synaptic vesicles as synapses mature. The molecular mechanism for this process is not known. Cc2d1a could act as a brake when VGCC and synaptic vesicles transit from loose "microdomain" coupling to tight "nanodomain" coupling. Alternatively, Cc2d1a could bind to calcium sensors on the vesicles to block the detection of calcium during action potentials; or act as a calcium buffer to curtail calcium current. Therefore, the evoked responses to action potential induced Ca^{2+} increases are stronger in KO than in control.

For other modes of neurotransmitter releases, we've observed more frequent spontaneous releases in WT synapses at 14 DIV as compared to KO. Studies have suggested that there are intrinsic differences between synaptic vesicles (Wolfel et al., 2007), be it protein or lipid composition, which determine evoked or spontaneous responses. Cc2d1a could be on the vesicles and facilitate spontaneous releases. We've also observed more asynchronous releases in WT synapses at 14 DIV as compared to KO. It has been shown that the tight coupling between vesicles and calcium channels is required for fast responses (Wadel et al., 2007). This is consistent with my hypothesis that Cc2d1a negatively regulates this coupling process, thus in WT synapses, there are more vesicles that are further away from VGCCs and it takes longer for Ca^{2+} to reach them and they are responsible for slower neurotransmitter releases, i.e. asynchronous. Less asynchronous releases in KO synapses could account for faster vesicle recycling as seen in the synaptophysin-pHluorin experiment, because in control synapses more asynchronous releases after AP contribute to the total fluorescence signals. Alternatively, there

are different modes of vesicle recycling (Sudhof, 2004), and Cc2d1a could regulate this process by favoring for example “kiss and run” instead of a fully collapsed fusion, resulting in faster recycling.

In summary, I have shown a new gene CC2D1A activates NF-κB *in vitro* through the canonical IKK pathway and mice deficient in Cc2d1a die at birth probably due to the impairment of synaptic functions in the brain. Further studies should help to understand the molecular mechanism underlying Cc2d1a regulated synaptic maturation process and how this cellular defect relates to lethal phenotype in mice.

Methods

Generation of Cc2d1a Knockout mice.

The Cc2d1a targeting construct was built using the pN-Z-TK2 vector (kindly provided by S. Kunhua), which contains a nuclear LacZ (nLacZ) cassette. The neomycin-resistance gene flanked by loxP sites were cloned from PGKneolox2DTA.2 (kindly provided by M. Tallquist) into the targeting vector downstream of nLacZ cassette. The 4.2 kb 5′ arm and 2.2 kb 3′ arm were amplified using PCR and confirmed by sequencing. The nLacZ and neomycin cassette were fused in-frame to exon 1 following the first 2 amino acids of Cc2d1a, placing the LacZ reporter gene under the control of the endogenous Cc2d1a promoter. The targeting construct was linearized and electroporated into 129 SvEv-derived ES cells. Using southern blot analysis with 5′ and 3′ probes, three Cc2d1a targeted ES clones were identified and used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 to obtain germline transmission of

the mutant allele. All results reported here were performed on littermate mice derived from heterozygous breedings.

Southern blot analysis and PCR genotyping.

DNA sequences for PCR genotyping primers are as follows: 5′-GTGCGAGGCCAGAGGCCACTTGTG -3′; 5′-GACCCTGAGAGAGCTCCTGAGAGC ′; and 5′-TTTCCCACCTCTTCTGGCCCAGAGG -3′. Southern blot probes were generated by PCR using the following primer sets: 5′ probe forward, 5′-CCA AGC ATTCAAATAGATGAGCCAACGGCAG -3', reverse, 5′-ATTCCCCCTCCCAGGCAAACAGGAGCCAAG -3′; and 3′ probe forward, 5′-AGGATCATGCCTGACTTTGAGGTGG -3′; reverse, 5′-TCTTGTCCTGGTCCACATGATCCAT -3'. In brief, tail genomic DNA was digested with BamHI and analyzed using a standard Southern blot protocol. The 20 μl PCR genotyping reaction contained 1 μl of tail DNA as template, 0.5uM of each primers and 10ul 2X GoTaqGreen mix (promega). An annealing temperature of 55°C was used and the PCR products were analyzed by electrophoresis on a 2% agarose gel.

Immunoblotting analysis

Tissues were homogenized in extraction buffer (20mM Tris-Cl pH7.50, 100mM NaCl, 10% Glycerol, 0.5% Triton X100, protease inhibitor cocktail from Roche) and subjected to centrifugation at 12,000 x *g* for 10 min at 4°C. The soluble fractions were collected, and 40 μg of proteins were loaded onto 7% SDS-PAGE gels and immunoblotting was performed according to standard protocols. The antibody against human CC2D1A was described in the last Chapter.

Histology and In Situ Hybridization

E18.5 embryos were harvested from timed mating and fixed in 4% paraformaldehyde. The skin was removed to facilitate fixation. Staining with hematoxylin and eosin was performed by Molecular Pathology Core Laboratory at UTSW by using standard procedure. Whole brain was dissected from E18.5 embryos and fixed overnight in DEPC-treated 4% paraformaldehyde. Riboprobes were labeled with ^{35}S-UTP by using the MAXIscript in vitro transcription kit (Ambion; Austin, Texas). The template was amplified by PCR using primers 5'-tagagaaactgaaaggccaag-3' and 5'-agccaactgggaagtagcttg-3' and cloned into pcDNA3 under the T7 promoter. In situ hybridization of sectioned tissues was performed by Molecular Pathology Core Laboratory at UTSW as previously described (Vega et al., 2004)**.**

Wholemount Staining of Diaphragm Muscle

Muscle samples were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.3) at 4 °C overnight, blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% BSA and 0.01% thimerosal), and then incubated with primary antibodies. The primary antibodies for Syntaxin and Synaptotagmin-2 are from Dr. Weichun Lin (UTSW). Samples were then incubated with fluorescein isothiocyanate-conjugated secondary antibody and Texas-Red conjugated α-bungarotoxin (α-bgt) (2 nM, Molecular Probes), washed with PBS and mounted in 90% glycerol, 10% Tris Buffer (pH 8.5, 10 mM) containing *n*-propyl gallate (20 mM) to reduce photobleaching (Giloh and Sedat, 1982). Fluorescence images of AChR clusters were generated from confocal images acquired with identical, sub-saturating gains.

Cortical Primary Neuronal Cultures.

The cortexes were dissected from the brains of postnatal day 1 (P1) mice, dissociated by trypsin digestion, and plated on circle glass coverslips coated with poly-lysine. The cortical neurons were maintained in MEM medium (Invitrogen) supplemented with B-27 (Invitrogen), L-glutamine, 0.5% glucose, 5% fetal bovine serum, and Arac-C (Sigma-Aldrich). The cultures were used for experiments at 13-15 DIV.

Letiviral Infection

Constructs were cotransfected with plasmids for viral enzymes and envelope proteins into HEK 293 cells using a transfection system (FuGENE6; Roche) according to the manufacturer's specifications, and lentivirus containing culture medium was harvested 3 d later and centrifugated at 1000rpm for 10 minutes. The supernatant was immediately used for infection or frozen in liquid nitrogen and stored at - 80 ° C. Cortical cultures were infected at 4DIV by adding 250 µl of viral suspension to each well.

RNA Extraction, Microarray, and Quantitative PCR

To extract RNA, tissues were first homogenized and lysed in 1 ml of TRIzol (Invitrogen). Lysate was mixed with 0.2 ml chloroform, and the aqueous phase was separated using Phase Lock Gel Heavy (5PRIME) and applied to RNeasy columns (Qiagen). Subsequent steps followed the manufacturer's recommended protocol, and RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Microarray was performed using illumina Mouse-6 V2 BeadChip, which were scanned on an Illumina BeadStation 500. The Illumina Beadstudio software was used to assess fluorescent hybridization signals. For both wild type and knockout, RNAs from three E18.5 neocortex were subjected to parallel microarray experiments. The iScript cDNA synthesis kit

(BioRad) was used to generate cDNA. Quantitative PCR was performed using Syber Green on a BioRad iCycler with the following primers: Cc2d1a (5'- TGCCAACCACGACGAAGG); (5'- TGCCAACCACGACGAAGG); Kcnn-1 (5'- AGTCACTCTGCTCTTTTG); (5'- ATTGTCCACCAAGAACAG); Usp38 (5'- GAGTACCTTACTACAGAAT); (5'- CATCTTTTACATTTGGGT)

Primers used to detect 3'UTR of Cc2d1a: 5'-ACCTGGTGGAGAGCGAGCTGC; 5'- AGCTGAGCTGGACAGCCTCAG

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