Physiological Functions of Coxsackievirus and Adenovirus Receptor (CAR): Regulation of Protein Synthesis

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Abbreviations

4E-BP	eIF4E-binding protein
5-HT	5-hydroxytryptamine, serotonin
aa	Amino acids
AA	Arachidonic acid
Ad	Adenovirus
ADAM10	A disintegrin and metalloprotease 10
AHA	L-Azidohomoalanine
Aniso	Anisomycin
ANOVA	One-way analysis of variance
arc/ arg3.1	Activity-regulated cytoskeleton-associated protein
β-gal	β-galactosidase
BDNF	Brain-derived neurotrophic factor
BT-IgSF	Brain- and testis-specific Ig superfamily
Ca^{2+}	Calcium
CAM	Cell adhesion molecule
CaMKIIα	Ca^{2+}/ calmodulin-dependent protein kinase II α
cAMP	Cyclic AMP
CAR	Coxsackievirus and adenovirus receptor
CAR-cKO	Conditional knockout of CAR
CASK	Calmodulin-dependent serine kinase
CBV	Coxsackie B virus
CHO	Chinese hamster ovary
CHX	Cycloheximide
CL	Collagen type-I
CLMP	CAR-like membrane protein
CNS	Central nervous system
Co-IF	Co-immunofluorescence
COX1	Cytochrome oxidase 1
CPEB	Cytoplasmic polyadenylation element binding protein
CREB	cAMP-responsive element-binding protein
CrPV	Cricket paralysis virus
CSF	Cerebrospinal fluid
CTX	Cortical thymocytes of Xenopus
CYTB	Cytochrome b
DAG	Diacylglycerol
DCC	Deleted in colorectal cancer
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
DsCAM	Down syndrome cell adhesion molecule
ECM	Extracellular matrix
eEF1A	Eukaryotic translation elongation factor 1A

eEF2	Eukaryotic translation elongation factor 2
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
eIF4G	Eukaryotic translation initiation factor 4G
ELH	Egg-laying hormone
En1	Engrailed 1
eRF1	Eukaryotic translation release factor 1
ERM	Ezrin/ radixin/ moesin
ERK	Extracellular signal-regulated kinase
ESAM	Endothelial-cell-selective adhesion molecule
FBS	Fetal bovine serum
FGF8	Fibroblast growth factor 8
FGFR	Fibroblast growth factor receptor
FMRP	Fragile X mental retardation protein
FN	Fibronectin
FN40	Fibronectin 40 kDa subunit
GCP	Growth cone particle
GLUT1	Glucose transporter 1
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
HBSS	Hank's balanced salt solution
HCV	Hepatitis C virus
His-	Polyhistidine-tagged proteins
HPG	L-homopropargylglycine
HPV	Human papillomavirus
ICD	Intracellular domain
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1
IgSF	Immunoglobulin super family
Impa1	Myo-inositol monophosphatase-1
IPTG	Isopropyl-β-D- thiogalactopyranoside
IQGAP1	IQ-motif containing GTPase activating protein 1
IR	Insulin receptor
IRES	Internal ribosome entry site
IRS-1	Insulin receptor substrate 1
JAM	Junctional adhesion molecule
KO	Knock out
KOR1	κ-type opioid receptor 1
LCM	Laser capture microdissection
LEC	Lymphatic endothelial cell
LNX	Ligand of Numb X
LPA	L-α-lysophosphotidic acid

LRPPRC	Leucine-rich PPR motif-containing protein
LTD	Long-term depression
LTF	Long-term facilitation
LTP	Long-term potentiation
MAG	Myelin-associated glycoprotein
MAGI1b	Membrane-associated guanylate kinase 1b
MAP1B	Microtubule-associated protein 1B
MAPK	Mitogen-activated protein kinase
MCL	Marginal cell layer
mGluR	Metabotropic glutamate receptor
MOI	Multiplicity of infection
mRNP	Messenger ribonucleoprotein
MS	Mass spectrometry
mTOR	Mechanistic (mammalian) target of rapamycin
MUPP-1	Multi-PDZ domain protein 1
NCAM	Neural cell adhesion molecule
NCAM-EC	Soluble extracellular domain of NCAM
NGF	Nerve growth factor
NHERF	Na^+/ H^+ exchanger regulatory factor
nNOS1	Neuronal nitric oxide synthase 1
hnRNPU	Heterogeneous nuclear ribonucleoprotein U
p75NTR	p75 neurotrophin receptor
PABPN1	Poly(A)-binding protein N1
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDL	Poly-D-lysine
PDZ	PSD-95, Discs-large, ZO-1
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3-kinase
PICK1	Protein interacting with C kinase 1
PLCγ	Phospholipase C gamma
PKA	Protein kinase A
PP2A	Protein phosphatase 2A
PRTE	Pyrimidine-rich translational element
PSA-NCAM	Polysialic acid-modified NCAM
PSD95	Post-synaptic density protein 95
RANBP1	RAN GTPase-binding protein 1
rER	Rough endoplasmic reticulum
RGC	Retinal ganglion cell
RGD	Arg-Gly-Asp motif
RLU	Relative light unit
RMS	Rostral migration stream

RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
Robo	Roundabout
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
S6K	Ribosomal protein S6 kinase
SEM	Standard error of the mean
SGL	Subgranular layer
SIV	Ser-Iso-Val, the last three amino acids of CAR, PDZ-binding domain
SLC1A5	Solute carrier - family 1 member 5
SNAP25	Synaptosomal-associated protein 25 kDa
SNARE	Soluble NSF-attachment factor receptor protein
SRP	Signal recognition particle
SVZ	Subventricular zone
TCA	Thalamocortical axons
TOP	Terminal oligopyrimidine tract
Tph2	Tryptophan hydroxylase 2
UPS	Ubiquitin proteasome system
UTR	Untranslated region
VAMP	Vesicle-associated membrane protein
WT	Wildtype
ZBP1	Zipcode-binding protein1
ZO-1	Zonula occludens 1

Abstract

Coxsackievirus and Adenovirus Receptor (CAR) is a transmembrane receptor for adenovirus and coxsackie B virus. During brain development, it promotes neurite outgrowth but the downstream pathways are unknown. To explore CAR's downstream pathways, we performed affinity pulldown and proteomic analysis. Interestingly, we identified many proteins functioning in mRNA translation, such as ribosomal proteins S6 and L4, and translation factors eIF4G and eEF1A. Therefore, we hypothesized that CAR may promote neurite outgrowth through regulating translation. Using GST-pulldown, ribosome centrifugation, and confocal microscopy, we showed that CAR associated with translational proteins. By *in vitro* and cell-based translation assays, we demonstrated that CAR functionally regulated translation and that CAR-regulated translation required the intact C-terminus and the extracellular D2 region. Next, we verified that FN40 (a known ligand of CAR) significantly increased neurite outgrowth and this increase was dependent on CAR and translation, respectively. Finally, we demonstrated that FN40-CAR interaction significantly increased translation using a protein metabolic labeling technique, the AHA translation assay. Taken together, our results suggest that CAR regulates translation during neurite outgrowth.

Although CAR is believed to contribute to brain development, CAR-knockout mice with central nervous system (CNS) phenotypes were hard to obtain because whole-body knockout of CAR is embryonic lethal. To overcome this challenge, we crossed CARFLOX mice with SYNCRE mice so that CAR was specifically deleted in the CNS (CAR-cKO). We performed open-field, Rota-rod, and muscle strength tests and found that CAR-cKO mice displayed poor motor coordination and might have elevated anxiety. These studies point to possible functional roles of CAR during the development of the nervous system.

Résumé

Le Récepteur de Coxsackievirus et Adénovirus (CAR) est un récepteur transmembranaire pour les adénovirus et les virus de coxsackie type B. Durant le développement du cerveau, CAR contribue à l'excroissance des neurites. Pour mieux comprendre les voies de signalisations de CAR (encore mal connues), on a utilisé la technique de précipitation par affinité suivie d'une analyse protéomique. Cette analyse s'est révélée intéressante pour identifier plusieurs protéines impliquées dans le contrôle de la traduction des ARNm messagers, telles que les protéines ribosomales S6 et L4, ainsi que les facteurs de traduction eIF4G et eEF1A. En se basant sur ces résultats, on a postulé que CAR pourrait être impliqué dans la régulation de la croissance des neurites via le control de la traduction. Aussi, en associant la précipitation par GST, la centrifugation ribosomale et la microscopie confocale, on a montré que CAR est associé aux protéines de la traduction. Parallèlement, l'utilisation des tests de la traduction *in vitro,* et aussi au niveau cellulaire*,* a révélé que CAR régule fonctionnellement la traduction, et que cette régulation nécessite que la partie C-terminal de CAR ainsi que son domaine extracellulaire D2 soient intactes. On a aussi montré que FN40 (un ligant bien connu de CAR) augmente significativement la croissance des neurites, et que cette augmentation est respectivement liée à CAR et à la traduction. Finalement, on a prouvé que l'interaction FN40-CAR augmente la traduction, par l'utilisation des techniques de marquage du métabolisme protéique (test de marquage « AHA »). De l'ensemble de ces résultats, on pourrait suggérer que CAR est impliqué dans la régulation de la traduction durant la croissance des neurites.

Le phénotype du système nerveux central (SNC) des souris double mutant pour $CAR^{-/-}$ est difficile à obtenir car la double mutation de $CAR^{-/-}$ est létale à l'état embryonnaire. Pour dépasser ce problème, on a croisé les souris CARFLOX avec les souris SYNCRE afin d'éliminer CAR spécifiquement au niveau du SNC. Les résultats préliminaires montrent que les souris $CAR^{-/-}$ souffrent de la perte de la coordination motrice et aussi d'anxiété élevée. Ces résultats ont été obtenus en utilisant les tests suivants: champ-ouvert et le test de la tige tournante, ainsi que la force musculaire. Ces études indiquent que CAR peut jouer un rôle fonctionnel durant le développement du système nerveux.

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Preface

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE:

Although CAR is known to promote neurite outgrowth, its downstream pathways were unknown; although CAR is highly expressed in many regions of the developing CNS, its role in brain development was not clear. In this study we demonstrated that:

1) CAR associates with translation machinery and regulates protein synthesis *in vitro* and in cultured cells;
2) CAR interacts with translational proteins in the nerve growth cone and regulates translation during neurite outgrowth;
3) Conditional knockout of CAR in mice leads to anxiety-like behavior and impaired motor coordination.

CONTRIBUTIONS TO DATA:

I obtained the majority of the results in Chapter 3, 4, and 5. Christine Fagotto-Kauffman helped with neuronal cultures and confocal analysis of neurites (Figure 4.4, 4.5 and 4.6). Zak Orfi cloned the His-eIF4E plasmid and purified the recombinant His-eIF4E protein (Figure 3.4). Arjuna Rajakumar prepared the ribosome fractionation using sucrose gradients (Figure 3.3). Patrick Fok performed the affinity pulldown and the proteomic analysis using mass spectrometry (Figure 3.1).

CHAPTER 1

INTRODUCTION

SIGNALING PATHWAYS OF NEURITE OUTGROWTH AND AXON GUIDANCE

Neurite Outgrowth and Axon Guidance during Development and Adult Neurogenesis

During vertebrate embryonic development, the ectoderm forms the neural tube which further develops into the brain and spinal cord (Greene and Copp, 2009). At the beginning, the vesicle-like brain contains telencephalon, diencephalon, mesencephalon, metencephalon, and medulla along the antero-posterior axis (Rice and Barone, 2000). Extensive cell proliferation and widespread neuronal migration then ensue to help develop the telencephalon into two cerebral hemispheres, diencephalon into thalamus and hypothalamus, mesencephalon into the midbrain, and metencephalon into the cerebellum and pons (Hatten, 1999). Once post-mitotic neurons migrate to their destinations, they start sprouting and extending neurites. This process is known as neurite outgrowth. Neurite outgrowth plays a key role during brain development, and dysregulation of genes related to neurite outgrowth may cause abnormal brain development and function (Vawter, 2000; Wilkinson et al., 2007).

Next, neurons further differentiate and neurites develop into one single axon and multiple dendrites. Axons find their synaptic targets with very high precision, and this process is known as axon guidance or axon pathfinding. For instance, during the development of sensory neurons in grasshopper legs, the pioneer Ti axons travel a long distance to reach their targets from the tip of the leg to the body (Keshishian and Bentley, 1983a). Along the way the Ti axons pass a few guidepost cells, and if these cells are ablated the Ti pioneers cannot reach

the targets (Keshishian and Bentley, 1983b, c). In another example, the retinal axons of *Xenopus* crawl through the optic tract to their distant destination, the tectum. When the optic tract is surgically rotated for 90°, the axons, in correspondence with the rotated optic tract, also turn 90° (Harris, 1989) suggesting that retinal axon guidance through the optic tract relies on membrane proteins, rather than diffusible factors. On the other hand, diffusible cues also regulate axon guidance: Netrin1 is a soluble protein secreted by ventral floor plate cells of the chick spinal cord (Kennedy et al., 1994) and it promotes the pathfinding of spinal commissural axons, with Netrin1 knockout mice displaying severe defects in spinal commissural axon trajectories (Serafini et al., 1996). In summary, once neurons migrate to their positions, they begin extending neurites, differentiating into axons and dendrites, guiding the axons to the targets, establishing synapses, and reaching full maturation (Arimura and Kaibuchi, 2007; Valtorta and Leoni, 1999).

In addition to embryonic development, neurite outgrowth and axon guidance are also required for adult neurogenesis and post-injury repair. During mammalian adult neurogenesis, neurite outgrowth and axon guidance are required for maintaining brain plasticity in neurons of hippocampal dentate gyrus and in neurons of olfactory bulb which migrate from the subventricular zone (Winner et al., 2011; Zhao et al., 2006). Upon injury, neurite outgrowth and axon guidance are required for spinal cord regeneration in lower vertebrates (Chernoff et al., 2003; Lee-Liu et al., 2013) but in mammals post-injury neurogenesis and regeneration are severely inhibited (Harel and Strittmatter, 2006; Rolls et al., 2009).

Both neurite outgrowth and axon guidance require an autonomous subcellular compartment – the nerve growth cone. The nerve growth cones are able to direct neurite elongation and axon guidance independent of their cell bodies. Early studies show that growth cones separated from their cell bodies continue to extend *in vitro* (Shaw and Bray, 1977) and they can navigate precisely to the synaptic target *in vivo* (Harris et al., 1987). Upon receiving extracellular cues, growth cones can regulate neurite outgrowth and axon guidance through several molecular mechanisms. These mechanisms can be generally divided into four categories: 1) cytoskeletal remodeling, 2) membrane addition, 3) cell adhesion molecule (CAM) signaling pathways, and 4) local protein synthesis. I will briefly introduce the first three pathways and then I will elaborate on the role of translation in neuronal development.

Regulation of Neurite Outgrowth and Axon Guidance by Cytoskeletal Remodeling

The nerve growth cone is enriched with cytoskeletal proteins and growth cone motility requires the regulation of cytoskeletal dynamics. In general, each growth cone has a peripheral (P) region and a central (C) region: the P region or the leading margin of growth cone mainly contains the monomeric actin (G-actin) and filamentous actin (F-actin) (Bridgman and Dailey, 1989; Forscher and Smith, 1988) whereas the C region and the neurite shaft mainly harbor tubulin proteins and the bundled microtubules (Gordon-Weeks, 2004; Lim et al., 1989). Additionally, the P region of the growth cone possesses two types of protrusions, the finger-like filopodia and the sheet-like lamellipodia, which are required for the advance and turning of growth cones (Dent and Gertler, 2003; Dent et al., 2011).

Growth cone motility requires adhesion force and traction force, both of which are mainly mediated by actin. Firstly, the transmembrane receptors can associate with F-actin and provide adhesive sites. For example, the adhesion molecule L1 and the ezrin/ radixin/ moesin (ERM) proteins anchor the F-actin to the cell membrane (Sakurai et al., 2008) and when ERM proteins are disrupted, both the adhesion and growth cone motility are greatly reduced (Marsick et al., 2012). Moreover, integrins associate with F-actin at the cell membrane through talin, vinculin, alpha-actinin, and paxillin, and disruption of this protein complex ablates growth cone turning (Myers and Gomez, 2011; Vicente-Manzanares et al., 2009). Lastly, the adhesion molecule N-Cadherin associates with actin fibers through α-catenin and β-catenin, and silencing of catenins reduces adhesion and neurite outgrowth (Bard et al., 2008). These adhesive complexes are often referred to as the "molecular clutch" and they are critical for growth cone advancing or turning.

Secondly, actin can interact with the motor proteins to generate retraction forces. For example, myosin II is a motor protein residing in growth cones where it mediates actin-based motility (Brown and Bridgman, 2004; Medeiros et al., 2006); it interacts with actin fibers and generates traction forces that pull the growth cone forward against the "molecular clutch" (Bridgman et al., 2001; Turney and Bridgman, 2005). Therefore, by continuously adhering and withdrawing the filopodia and lamellipodia, a growth cone can advance; by asymmetrically pulling the filopodia and lamellipodia with greater force on one side of a growth cone, the growth cone can turn (Gomez and Letourneau, 2014).

In addition to actin, tubulin also plays an important role in neurite outgrowth and axon guidance. It is long known that microtubules affect neurite extension (Lim et al., 1990; Tanaka and Kirschner, 1991) and many tubulin-regulating proteins and tubulin-associated proteins such as CRMP5, GSK3β, and SCG10 regulate microtubule structures and modulate neurite outgrowth (Brot et al., 2010; Byun et al., 2012; Morii et al., 2006). Additionally, emerging studies suggest that tubulin also affects growth cone turning. Microtubules are preferentially stabilized in the direction of the attractive turning of the growth cone (Sabry et al., 1991); local stabilization by taxol induces growth cone attraction whereas local destabilization by nocodazole induces repulsion (Buck and Zheng, 2002). Furthermore, the interaction between integrin and laminin directionally stabilizes microtubule assembly and affects directional axon initiation and outgrowth (Lei et al., 2012); EphB exposure in the presence of laminin and L1 does not trigger a repulsive turning but induces a redistribution of microtubules and a pause in axon outgrowth (Suh et al., 2004). These results suggest that tubulin, in addition to actin, is also a critical factor in neurite outgrowth and axon guidance.

Regulation of Neurite Outgrowth and Axon Guidance by Membrane Addition

The change in the internal cytoskeleton requires a corresponding alteration in the external membrane. It was long proposed that neurons economically incorporate vesicles into plasma membranes to promote neurite outgrowth and axonal elongation (Futerman and Banker, 1996). The SNARE (soluble NSF-attachment factor receptor) protein family regulates exocytic vesicle- membrane fusion during neurite and axon outgrowth (Tojima and

Kamiguchi, 2015). One member of this family, SNAP25 (synaptosomal-associated protein 25 kDa), regulates neurotransmitter release (Rizo and Sudhof, 2002), vesicle fusion (Jahn and Scheller, 2006), and axon outgrowth (Bloom and Morgan, 2011). Knockdown of SNAP25 expression (Osen-Sand et al., 1993) or proteolytic cleavage of SNAP25 (Morihara et al., 1999; Osen-Sand et al., 1996) inhibits neurite outgrowth *in vitro* and *in vivo*. Embryonic hippocampal neurons isolated from *SNAP25*-null mice show impaired neurite outgrowth which can be rescued by lentiviral overexpression of SNAP25 (Delgado-Martinez et al., 2007). In *Gekko japonicus* lizards, a species capable of regenerating injured spinal cord, overexpression of gecko SNAP25b promotes vesicle fusion, neurite outgrowth, and spinal cord regeneration after tail amputation (Wang et al., 2012). These results suggest that SNAP25-regulated membrane fusion is essential for neurite outgrowth.

Other members of the SNARE family, the vesicle-associated membrane proteins (VAMPs), play differential roles during neurite outgrowth. Tetanus neurotoxin-induced proteolytic cleavage of VAMP2 but not other VAMP proteins completely blocks neurite sprouting from neuronal cell bodies (Gupton and Gertler, 2010). However, after sprouting from their somata, neurites do not require VAMP2 for their elongation (Osen-Sand et al., 1996). By contrast, VAMP7, another VAMP family member, is required for both neurite sprouting (Gupton and Gertler, 2010) and for neurite elongation (Martinez-Arca et al., 2001). These results suggest that VAMP2- and VAMP7-mediated exocytosis differentially regulates the initial neuritogenesis step and the subsequent neurite extension.

In addition to neurite outgrowth, membrane fusion also plays a role in growth cone turning. VAMP2 exocytosis is essential for nerve growth factor (NGF) and myelin-associated glycoprotein (MAG)-induced attractive turning (Tojima et al., 2007) and this exocytosis is asymmetrical with more VAMP2 exocytosed towards the attractive cues (Tojima et al., 2014). On the other hand, the repulsive cue semaphorin 3A (Sema3A) causes an asymmetrical inhibition of Synaptobrevin 2-dependent exocytosis and the growth cone turns away from Sema3A (Zylbersztejn et al., 2012). Together, these results suggest that asymmetrical exocytosis is crucial for growth cone turning. Interestingly, this is similar to the *β-actin* local translation model in which asymmetrical β-actin protein synthesis induces attractive turning (Leung et al., 2006; Yao et al., 2006) while asymmetrical translation of cytoskeleton disassembling proteins triggers repulsive turning (Wu et al., 2005; Piper et al., 2006), as discussed in detail later.

Regulation of Neurite Outgrowth by Cell Adhesion Molecule Signaling Pathways

Cell adhesion molecules (CAMs) are long known as strong promoters of neurite outgrowth. Early studies suggest that CAMs such as NCAM (Hoffman et al., 1982; Thiery et al., 1977), L1 (Rathjen and Schachner, 1984), and N-Cadherin (Takeichi, 1988) significantly promote neurite outgrowth via homophilic interactions (Fischer et al., 1986; Hoffman et al., 1986) or via heterophilic interactions with extracellular matrix proteins (Bixby and Jhabvala, 1990; Bixby et al., 1987). CAMs promote neurite outgrowth through molecular mechanisms such as calcium (Ca^{2+}), tyrosine kinases, Ras-MAPK, and FGFR signaling. For example, NCAM can

induce neurite outgrowth through the Ras-MAPK (mitogen-activated protein kinase) pathway: homophilic clustering of NCAM-140 (a 140-kDa isoform of NCAM) is sufficient to activate the tyrosine kinase Fyn and recruit focal adhesion kinase (Beggs et al., 1997; Beggs et al., 1994) which triggers the Ras-MAPK signaling and activates CREB (cAMP response element binding protein)-regulated transcription (Kolkova et al., 2000; Schmid et al., 1999). On the other hand, L1-mediated neurite outgrowth requires the activation of the tyrosine kinase Src (Ignelzi et al., 1994) and the ribosomal protein S6 kinase p90RSK (Wong et al., 1996).

Neurite outgrowth induced by CAMs may also require the activation of fibroblast growth factor receptor (FGFR) (Allodi et al., 2013). Antibodies against FGFR (Williams et al., 1994), expression of a dominant negative FGFR (Saffell et al., 1997), and inhibitors of FGFR signaling (Hinsby et al., 2004) completely abolish CAM-induced, but not basal, neurite outgrowth. Different peptide fragments of FGFR1 specifically inhibit neurite outgrowth induced by L1, NCAM, and N-Cadherin, respectively (Williams et al., 1994), suggesting that FGFR-CAM interactions promote neurite outgrowth through distinct molecular mechanisms. Additionally, CAMs directly interact with FGFR: N-Cadherin associates with the first two immunoglobulin (Ig)-like domains of FGFR1 (Suyama et al., 2002) and FGFR directly interacts with the fibronectin type III (FN3) domains of NCAM and L1 to promote neurite outgrowth (Kiselyov et al., 2003; Kulahin et al., 2009). Last but not least, L1 promotes neurite outgrowth through upregulating FGF21 expression and increasing FGF21/ FGFR signaling (Huang et al., 2013).

Downstream of the CAM-FGFR interaction, the signaling pathway relays to the second messenger Ca^{2+} and then to the CREB-dependent transcription which eventually stimulates neurite outgrowth (Maness and Schachner, 2007). Activation of FGFR triggers phospholipase C gamma (PLCγ) (Carter et al., 2015; Saffell et al., 1997), generates diacylglycerol (DAG), converts DAG to arachidonic acid (AA), and increases intracellular Ca^{2+} level (Schuch et al., 1989; Sheng et al., 2013). Although DAG can promote neurite outgrowth through the MAPK pathway (Kolkova et al., 2000), the change in intracellular Ca^{2+} level can trigger neuritogenesis by inducing cAMP (cyclic AMP) or by regulating CaMKII (Ca^{2+}/ calmodulin-dependent protein kinase II) (Impey et al., 1998; Sutherland et al., 2014; Williams et al., 1995). Both cAMP and CaMKII regulate the activity of the transcription factor CREB (Johannessen and Moens, 2007) which initiates the transcription of *c-Fos* or other CREB-regulated genes, thus promoting neurite outgrowth (Lonze and Ginty, 2002; Ma et al., 2014; Tao et al., 1998).

To sum up, neurite outgrowth and axon guidance require cytoskeletal remodeling, membrane addition, and CAM signaling, and often an integral ensemble of all three components (Vitriol and Zheng, 2012). However, despite the importance of these pathways, recent evidence strongly demonstrates that local translation also plays an essential role. In the following two sections, I will first review the basics of eukaryotic mRNA translation and then introduce how local translation is regulated during neurite outgrowth and axon pathfinding.

REGULATION OF EUKARYOTIC mRNA TRANSLATION

Protein synthesis or mRNA translation is one of the most fundamental processes in cells. According to the central dogma, DNA guides mRNA synthesis through transcription and mRNA directs protein synthesis through translation (Crick, 1970; Crick, 1958). It was generally assumed that mRNA level directly correlates with protein expression. However, genome-wide studies comparing transcriptomes and proteomes of the same biological samples showed that mRNA abundance does not necessarily represent the synthesized protein level (Chen et al., 2002; Ghazalpour et al., 2011; Gygi et al., 1999) suggesting that translation of specific mRNAs is very variable and highly regulated. Here I will introduce the major steps in translation, cap- and IRES-dependent translation, and mTOR signaling pathway.

Three Major Steps of Eukaryotic Protein Synthesis

Protein synthesis can be divided into three major steps: 1) initiation, 2) elongation, and 3) termination. In eukaryotes, initiation is the rate-limiting and most regulated step (Jackson et al., 2010). Firstly, eukaryotic translation initiation factors (eIFs) eIF3, eIF1, and eIF1A bind to the 40S ribosomal small subunit (Fraser et al., 2007; Lomakin et al., 2003; Passmore et al., 2007). Meanwhile, the GTP-bound eIF2 complex carries methionine-tRNA (met-tRNA), joins with these factors and the 40S small subunit, and forms the 43S pre-initiation complex (Shin et al., 2011; Sokabe and Fraser, 2014). Next, the eIF3 complex connects the 5' cap-binding complex (will be introduced below), the mRNA and the 43S pre-initiation

complex to start 5' to 3' scanning of the mRNA (Hinnebusch, 2011; Kozak, 1989; Pestova and Kolupaeva, 2002). After recognition of the first start codon (AUG), the eIF2-bound GTP is hydrolyzed (Huang et al., 1997) and the large subunit of ribosome (60S) together with eIF5B (Pestova et al., 2000) combine with the 43S complex to become the 80S translation complex.

Then is the elongation process, which is mainly regulated by translation elongation factors (eEFs). After the first methionine-tRNA binds to AUG, the functionally active eEF1A brings the second amino acid-tRNA (aminoacyl-tRNA) to the ribosome (Fessenden and Moldave, 1963; Steitz, 2008). The other elongation factor eEF2 then catalyzes the translocation of the second aminoacyl-tRNA within the ribosome to facilitate the entry of the third aminoacyl-tRNA (Sperti et al., 1975). The process continues until the elongation process is finished.

The termination step is governed by translation releasing factors (eRFs). When the translation machinery reaches the stop codon (TAG/TAA/TGA), the eRF1-eRF3 complex binds to the ribosome (Inge-Vechtomov et al., 2003). Then eRF1 directly interacts with the stop codon and induces the hydrolysis of the bond between the last tRNA (peptidyl-tRNA) and the last amino acid of the peptide (Frolova et al., 1994), thus releasing the nascent protein chain.

Eukaryotic mRNA Structure, Cap- and IRES-Dependent Translation

Eukaryotic mRNA has a highly specialized structure. Unlike prokaryotic polycistronic mRNAs (containing coding regions for multiple peptides on one mRNA strand), eukaryotic mRNAs are monocistronic and contain only one coding region per mRNA template (Kozak, 2005). Additionally, eukaryotic mRNA contains a 5' cap added by a guanylyltransferase and methylated by a methyltransferase (Fabrega et al., 2004; Martin and Moss, 1975), a 3' poly (A) tail through polyadenylation (Colgan and Manley, 1997; Zhao et al., 1999), and between these and the coding region, the 5' and 3' untranslated regions (5' and 3' UTRs) (Pesole et al., 2001) which are essential for regulating the mRNA translation (Jackson et al., 2010).

Due to the presence of such a structure, eukaryotic mRNA translation greatly relies on the proteins in the 5' cap-binding complex. During the formation of this complex, the 5' cap firstly associates with the cap-binding protein eIF4E (Sonenberg et al., 1978; Sonenberg et al., 1979). The mRNA 5' UTR is then unwound by RNA helicases eIF4A and eIF4B so that ribosomes start scanning for the start codon (Grifo et al., 1983; Rozen et al., 1990) and the poly-adenosine binding protein (PABP) binds to the 3' poly(A) tail of the mRNA (Burd et al., 1991; Kahvejian et al., 2005). In the meantime, the scaffold protein eIF4G tethers the mRNA and proteins together to connect the mRNA head-to-tail into a circular, highly translatable conformation (Imataka et al., 1998; Yanagiya et al., 2009). This type of translation is called cap-dependent translation.

Among the proteins in the cap-binding complex, eIF4E is especially important because it directly binds to the 5' cap and connects the mRNA to the ribosome. Additionally, it has

many pathophysiological roles: overexpression of eIF4E in fibroblasts causes malignant transformation (Lazaris-Karatzas et al., 1990); fruit fly mutants with an unphosphorylated eIF4E have retarded development and smaller size (Lachance et al., 2002); mice with un-controlled eIF4E activity by knocking out 4E-binding protein 2 (4E-BP2) have autism-like behavior (Gkogkas et al., 2013). The 4E-BP protein inhibits translation. It competes with eIF4G for binding to eIF4E and dissociates eIF4E from the cap, thus suppressing translation (Haghighat et al., 1995; Mader et al., 1995). On the contrary, phosphorylation of 4E-BP leads to its dissociation from eIF4E, thus promoting translation (Gingras et al., 1999). There are three 4E-BPs (4E-BP1, 2, and 3) and 4E-BP2 is the most abundant in the mammalian brain (Costa-Mattioli and Monteggia, 2013; Klann and Dever, 2004).

Eukaryotic translation can also take place without eIF4E or other initiation factors. Various types of eukaryotic viruses have an internal ribosomal entry site (IRES). Picornaviruses such as poliovirus (Pelletier and Sonenberg, 1988) and encephalomyocarditis virus (Ghattas et al., 1991; Jang et al., 1988) contain the IRES which can initiate translation without eIF4E. Translation of hepatitis C virus (HCV) IRES relies on fewer translation factors and can take place without eIF4E, eIF4G, eIF4A, eIF4B, eIF1, and eIF1A (Ji et al., 2004; Otto and Puglisi, 2004; Tsukiyama-Kohara et al., 1992). Translation of cricket paralysis virus (CrPV) mRNA neither requires any initiation factors nor needs the initiator tRNA (Pestova and Hellen, 2003; Wilson et al., 2000). Probably, these IRES' three-dimensional conformations are mimicking the structures of initiation factors so that these factors are no longer required for translation (Svitkin et al., 2015). Taken together, this type of translation is called cap-independent or

IRES-dependent translation.

Regulation of Translation by mTOR Signaling Pathway

Mammalian target of rapamycin (mTOR), also known as mechanistic target of rapamycin, plays an essential role in controlling cap-dependent translation. mTOR is a conserved serine/ threonine kinase that regulates cell proliferation, cell survival, mRNA transcription and protein synthesis (Showkat et al., 2014). It regulates cap-dependent translation mainly through phosphorylation of 4E-BP (eIF4E-binding protein) and S6K (ribosomal protein S6 kinase). Firstly, mTOR phosphorylates 4E-BP, and, as mentioned above, 4E-BP phosphorylation allows the release of eIF4E, promoting cap-dependent translation (Gingras et al., 1999). Secondly, mTOR phosphorylates S6K, an important regulator of cell growth and cell size (Alessi et al., 1998; Pullen et al., 1998). In turn, S6K phosphorylates ribosomal protein S6 which selectively translates a class of mRNAs that have a 5' terminal oligopyrimidine tract (5' TOP) (Jefferies et al., 1997); S6K also regulates cap-dependent translation by phosphorylating a cap-binding complex protein, eIF4B (Raught et al., 2004). As to its mRNA targets, mTOR primarily controls the translation of mRNAs containing a 5'TOP or a pyrimidine-rich translational element (PRTE), and the majority of these mRNAs encodes ribosomal proteins and translational factors thus further promoting translation (Hsieh et al., 2012).

In addition, mTOR-regulated translation can be activated by a variety of extracellular signals

and its dysregulation plays a role in several neuropathologic conditions. Many extracellular conditions including change in nutrient, oxygen, and energy levels, as well as the addition of amino acids and growth factors (such as insulin and epidermal growth factor) can activate mTOR through PI3K-Akt or Ras-MAPK pathways and eventually trigger the initiation of protein synthesis (Hay and Sonenberg, 2004; Ma and Blenis, 2009). In addition to these extracellular conditions, mTOR can also be activated by axon guidance cues such as Netrin1, Slit2, and Sema3A; its activation leads to an increased global translation and growth cone turning (Campbell and Holt, 2001; Piper et al., 2006; Polleux and Snider, 2010). Moreover, dysregulation of mTOR-mediated translational control is implicated in neurological diseases such as autism due to the failure of excitatory synaptic pruning (Kelleher and Bear, 2008; Sawicka and Zukin, 2012) and Alzheimer's disease probably due to an increased translation of amyloid beta protein and tau protein (Li et al., 2005a; Oddo, 2012). mTOR-mediated translational control also regulates long-term potentiation during learning and memory through synthesis of proteins responsible for synaptic plasticity (Cammalleri et al., 2003; Sui et al., 2008). Finally, mTOR appears to play contradictory roles in adult neurogenesis after traumatic brain damage and spinal cord injury (Don et al., 2012; Kanno et al., 2012); therefore whether it is neurodegenerative or neuroprotective requires further investigation.

LOCAL TRANSLATION IN NEURITE OUTGROWTH, AXON GUIDANCE, AND SYNAPTIC PLASTICITY

Growth Cones Harbor Translation Machinery and Regulate Translation Autonomously

Local translation is essential in regulating neurite outgrowth and axon guidance during development (Jung et al., 2012) and in controlling synaptic plasticity and cognitive functions in adult brains (Buffington et al., 2014; Sutton and Schuman, 2006). The nervous system favors local translation partially because it is fast. Long ago, it was believed that axonal transport is key to axon outgrowth and synaptic maintenance, but it was later criticized that axonal transport (0.1 – 2 mm/day) is too slow for the required quick response to extracellular cues (Alvarez et al., 2000). Indeed, local translation is much faster: upon receiving extracellular signals, nerve growth cones can synthesize β-actin or cofilin-1 proteins as soon as 5 min (Leung et al., 2006; Piper et al., 2006; Yao et al., 2006).

The next step is to examine whether the translation machinery resides in nerve growth cones. Indeed it is shown that messenger RNAs (Giuditta et al., 1986), ribosomal RNAs (Giuditta et al., 1980), and actively translating ribosomes exist in squid giant axons (Giuditta et al., 1991). In mammals, ribosomes can be detected in axons of *in vitro* cultured cortical and sympathetic neurons (Bassell et al., 1998; Bunge, 1973) and clusters of ribosomes are found throughout the length of early axons in developing dorsal root neuroblasts *in vivo* (Tennyson, 1970). Recently, an extensive proteomic analysis of isolated embryonic rat nerve growth cones shows that many ribosomal proteins (35 out of 80 ribosomal proteins) and translation-regulatory proteins (such as initiation factors eIF4A and eIF4G, as well as elongation factors eEF1A and eEF2) are enriched in nerve growth cones (Estrada-Bernal et al., 2012). Together, these results suggest that growth cones harbor translation machinery and

may regulate local protein synthesis.

The next question is what mRNAs are being translated in the nerve growth cones? Using radioactive amino acid labeling, cytoskeletal proteins such as actin and tubulin are the first identified *de novo* synthesized proteins in axonal growth cones (Koenig, 1989; Koenig and Adams, 1982). Later by *in situ* hybridization, mRNAs of *β-actin* and *β-tubulin* are shown to locate in squid giant axons (Kaplan et al., 1992). Surprisingly, mRNAs encoding ribosomal proteins were also enriched in neurites as demonstrated by a screening of *Aplysia* cDNA library (Moccia et al., 2003) suggesting that even ribosome biogenesis may take place in growth cones, in addition to the canonical ribosome biogenesis site – the nucleolus.

Indeed, further research shows that mRNAs encoding translational proteins are the most abundant species in growth cones. A recent genome-wide transcriptomic study of mouse and *Xenopus* growth cones demonstrates the predominant presence of mRNAs functioning in translation (Zivraj et al., 2010). The growth cones of stage 32 *Xenopus* retinal ganglion cells (RGCs) were removed using laser capture microdissection (LCM) and the mRNAs subjected to the Affymetrix genome-wide microarray. The results showed that the majority of these mRNAs encode translational proteins (31%) including most of the ribosomal proteins (69 out of 80), translation initiation factors and elongation factors including eIF3, eIF5A, and eEF1A (Zivraj et al., 2010). Transcripts of other functions include metabolic/ glycolytic (14%), cytoskeletal proteins (9%, mostly *β-actin* and *β-tubulin*), intracellular signaling proteins (9%, such as ERK/MAPK), cell membrane and surface receptors (9%, such as L1, FGF receptor 2b, IGF-like receptor 1, Neuroligin 1, integrins and protocadherins), and other minority

transcripts (Zivraj et al., 2010). Similar results were obtained in the growth cones of mouse embryonic RGC (Zivraj et al., 2010) as well as rat embryonic and adult dorsal root ganglion (Gumy et al., 2011), in which translational protein mRNAs predominate.

To further compare the growth cone mRNA pools between the young axons (*Xenopus* stage 24, path-finding) and the older ones (stage 32, target-arriving), the RGCs during different developmental stages were isolated and the growth cones analyzed. Younger growth cones contain a much simpler mRNA composition (a total of 286 genes identified) as compared to the old ones (958 genes). Notably, growth cones from younger axons have transcripts mostly for translational proteins (52% of all genes identified), while in older growth cones the translational protein mRNAs decrease to 31% (Zivraj et al., 2010). On the other hand, ephrin mRNAs (*EphB4*, *ephrinB2*, and *ephrinA3*), which are responsible for target innervation and topographic mapping, are absent in younger growth cones but enriched in older ones (Zivraj et al., 2010). These results suggest that after reaching targets, axons synthesize receptors to establish synapses, however during axonal pathfinding growth cones synthesize more translational proteins to further promote protein synthesis. Therefore, regulation of protein synthesis appears to take a greater part in regulating neurite outgrowth and axon guidance, as compared to synapse formation.

The next question is: how do these mRNAs selectively localize to axons and dendrites? Emerging evidence suggests that the selection and transportation processes are carried out by messenger ribonucleoproteins (mRNPs), for example zipcode-binding proteins 1 (ZBP1),

also known as insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), Staufen, and hnRNPA2 (Kiebler and Bassell, 2006). ZBP1 binds to *β-actin* mRNA via a "zipcode" sequence on the mRNA's 3' UTR and transfers the mRNA to neurites and axons (Ross et al., 1997). Therefore, specific mRNAs are selected and delivered to subcellular compartments via this type of *in cis* mRNA sequence resembling the mailing "ZIP codes". Additionally, ZBP1 regulates *β-actin* translation and neurite outgrowth: the phosphorylation of ZBP1 releases *β-actin* mRNA and allows its translation thus promoting neurite outgrowth (Huttelmaier et al., 2005) as discussed later.

If both mRNAs and translational proteins locate in the growth cones, these growth cones should be able to *de novo* synthesize proteins independent of their somata. Indeed, in squid giant axons separated from their somata, proteins are *de novo* synthesized as revealed by radioactive amino acid labeling (Giuditta et al., 1968; Giuditta et al., 1986). Similarly, vertebrate axons severed from their cell bodies also synthesize new proteins (Eng et al., 1999; Koenig, 1967; Koenig and Adams, 1982). These results suggest that axonal growth cones control local protein synthesis autonomously.

Taken together, the above results suggest that the growth cones contain mRNA templates and translation machinery, and that they regulate protein synthesis independent of their cell bodies. However until recently, little was known about how local translation is regulated during neurite outgrowth and axonal elongation. In the following sections, I will discuss how local translation is regulated during neurite outgrowth and axon guidance in detail, and will

introduce its role in synaptic formation, maintenance, and plasticity regulation briefly.

Regulation of Translation during Neurite Outgrowth and Axonal Elongation

So far, local translation of *β-actin*, *β-thymosin*, *PAR3*, and *TC10* mRNAs are reported to play a role in neurite and axonal elongation. Translation of *β-actin* promotes neurite outgrowth and this can be regulated by ZBP1 (Zipcode-binding protein-1). As introduced earlier, ZBP1 binds to the 3' UTR of *β-actin* mRNA, regulates actin protein synthesis, and modulates neurite outgrowth. It inhibits *β-actin* mRNA translation: silencing of *ZBP1* increases actin protein synthesis in cultured cells, and recombinant ZBP1 proteins reduce *β-actin* translation in rabbit reticulocyte lysates (Huttelmaier et al., 2005). This ZBP1-mediated translational control depends on the Zipcode sequence because when the Zipcode is deleted, ZBP1 has no effect on *β-actin* translation (Huttelmaier et al., 2005). Further study shows that Src kinase-mediated phosphorylation of ZBP1 at Tyr396 regulates the ZBP1-mRNA binding affinity: ZBP1 phosphorylation dissociates it from the mRNA but a Tyr396 mutant (ZBP1-Y396E) fails to do so (Huttelmaier et al., 2005). In neurites, ZBP1 colocalizes with *β-actin* mRNA (Kislauskis et al., 1994; Ross et al., 1997) and functionally, phosphorylation of wildtype ZBP1 increases *β-actin* translation and promotes hippocampal neurite outgrowth while the ZBP1-Y396E cannot (Huttelmaier et al., 2005). Together, these results suggest that ZBP1 regulates *β-actin* translation to promote neurite outgrowth.

Local translation of *β-thymosin* mRNA also affects neurite outgrowth. The actin-binding

protein β-thymosin sequesters monomeric actin and prevents actin polymerization (Huff et al., 2001). Using a fresh water snail, *Lymnaea stagnalis*, it is shown that in the snail's pedal A neurons, the *β-thymosin* mRNA is five times more enriched in neurites than in somata (van Kesteren et al., 2006). Knockdown of *β-thymosin* using double-strand RNA (dsRNA) greatly promotes neurite outgrowth. Additionally, physically isolated pedal A neurites (transected from their somata) treated with *β-thymosin* dsRNA also have much longer neurites (van Kesteren et al., 2006). These results suggest that local translation of *β-thymosin* plays an important role in regulating neurite outgrowth. However, it is not known how local *β-thymosin* translation is regulated and what extracellular cues trigger its translation.

In addition to synthesizing cytoskeletal proteins and cytoskeleton-regulators, local synthesis of PAR3, a protein functioning in neuronal polarization, promotes axonal elongation as well. Netrin1 and nerve growth factor (NGF) significantly promote the rate of axon outgrowth *in vitro* and *in vivo* (Serafini et al., 1996; Tucker et al., 2001; Zhou et al., 2004). However, local application of translation inhibitors in rat embryonic dorsal root ganglia (DRG) significantly reduces Netrin1- and NGF- stimulated, but not basal, axon outgrowth (Hengst et al., 2009). These results suggest that basal axon outgrowth does not need protein synthesis but Netrin1- and NGF-stimulated axonal elongation requires the translation of certain proteins. The PAR complex regulates hippocampal neuron polarization and specialization into axons and dendrites but its role in axonal elongation was not known (Shi et al., 2003). *Par3* mRNA localizes in growth cones and PAR3 is required for axon outgrowth *in vivo*: in spinal cord explants, knockdown of *Par3* in commissural neurons greatly reduces the length of axons and

prevents the axons from crossing the midline (Hengst et al., 2009). More importantly, when NGF is added onto DRG axons severed from their cell bodies, axons still elongate and PAR3 protein level increases (Hengst et al., 2009), indicating that intra-axonal translation of *PAR3* promotes neurite extension. Together, these results suggest that NGF and Netrin-1 stimulate the local translation of *Par3* mRNA to promote axonal elongation.

In addition, local synthesis of TC10, a protein that regulates exocytosis and membrane expansion, also promotes axonal elongation. NGF increases vesicular exocytosis during axon outgrowth and this exocytotic increase is completely ablated when locally applying translation inhibitors (Gracias et al., 2014). The small Cdc42-like GTPase TC10 forms a complex with Exo70 and controls the tethering of vesicles to the membrane, followed by vesicle fusion and membrane expansion (Dupraz et al., 2009) and eventually promotes neurite outgrowth (Pommereit and Wouters, 2007). *TC10* mRNA is present in the axonal growth cones and NGF significantly increases *TC10* translation; on the other hand the addition of translation inhibitors greatly reduces NGF-stimulated TC10 translation and exocytosis (Gracias et al., 2014). Finally the local translation of *TC10* mRNA is mediated by PI3K-Rheb-mTOR pathway as revealed by pharmacological studies (Gracias et al., 2014). These results suggest that intra-axonal translation of *TC10* mRNA is required for NGF-stimulated but not basal axon outgrowth.

Regulation of Local Translation in Axon Guidance and Growth Cone Turning

In addition to regulating neurite and axon outgrowth, local translation also plays a role in growth cone turning and axon guidance. The chemo-repellant semaphorin 3A (Sema3A) causes growth cone collapse and repulsive turning (He and Tessier-Lavigne, 1997). Another extracellular cue Netrin1 normally induces attractive turning (Keino-Masu et al., 1996) but becomes repulsive when neurons are grown on laminin (Hopker et al., 1999). However, their downstream mechanisms are not completely understood. By locally applying translation inhibitors such as cycloheximide (CHX), Sema3A-induced growth cone collapse and Netrin1-mediated growth cone turning are completely blocked (Campbell and Holt, 2001). On the contrary, the effects of L-α-lysophosphotidic acid (LPA), another strong collapsing reagent signaling through G proteins (Kozma et al., 1997), do not respond to translation inhibitors suggesting that translation is only required for Sema3A/ Netrin1-induced growth cone remodeling. Additionally, soma-less neurites continue to respond to Sema3A/ Netrin1-induced growth cone turning which can be abolished by translation inhibitors; Sema3A/ Netrin1 treatment significantly increases global translation in severed neurites as revealed by radioactive amino acid labeling (Campbell and Holt, 2001). Further mechanistic study shows that both Netrin1 and Sema3A activate TOR (a *Xenopus* homolog to mammalian mTOR) pathway and increase the phosphorylation of 4E-BP1 and eIF4E (Campbell and Holt, 2001). These results suggest that Netrin1- and Sema3A-mediated growth cone turning requires local translation.

However, which mRNA transcripts are being translated was not identified in these studies. Recent studies suggest that local synthesis of cytoskeleton proteins promotes attractive

turning while local synthesis of cytoskeleton disintegrator proteins induces repulsive turning. The protein Vg1RBP, a *Xenopus* homolog of mammalian ZBP1, regulates β-actin protein synthesis and growth cone turning towards the attractive cues. Vg1RBP binds to the *β-actin* mRNA in *X. laevis* (Yisraeli, 2005). In the retinal growth cones, Vg1RBP colocalizes and interacts with *β-actin* mRNA, and upon Netrin1 treatment the Vg1RBP-*β-actin* mRNP translocates into the filopodia of growth cones as revealed by live cell imaging (Leung et al., 2006). Netrin1 also triggers a translation-dependent increase in β-actin protein level which is blocked by the translation inhibitor CHX, and knockdown of *β-actin* mRNA ablates Netrin1-induced *β-actin* translation as well as attractive turning (Leung et al., 2006). Similar to a previous study (Huttelmaier et al., 2005), Netrin1-induced local translation of *β-actin* mRNA requires an intact Zipcode sequence in the mRNA's 3' UTR (Leung et al., 2006). Finally, a gradient of Netrin1 asymetrically induces Vg1RBP-mRNA translocation, 4E-BP phosphorylation, and β-actin protein synthesis thus promoting the growth cone remodeling and attractive turning (Leung et al., 2006).

As another example, brain-derived neurotrophic factor (BDNF) also regulates Vg1RBP activity, *β-actin* translation, and growth cone turning. BDNF induces attractive turning under normal conditions but triggers repulsive turning when protein kinase A (PKA) is inhibited (Song et al., 1997). Additionally, Ca^{2+} is essential for BDNF-induced growth cone turning (Li et al., 2005b) and by intracellular local release of caged Ca^{2+}, growth cones either turn towards Ca^{2+}, or when PKA is inhibited, turn away from it (Yao et al., 2006). Both attractive and repulsive turning induced by BDNF or Ca^{2+} can be abrogated by protein synthesis

inhibitors (Yao et al., 2006). Next, extracellular BDNF or intracellular Ca^{2+} induces asymmetrical β-actin protein synthesis towards BDNF or Ca^{2+} and this asymmetry is reversed by inhibiting PKA (Yao et al., 2006). Finally, similar to the previous study (Huttelmaier et al., 2005), BDNF activates the Src kinase to phosphorylate Vg1RBP (ZBP1) and to initiate *β-actin* mRNA translation (Yao et al., 2006). Taken together, these results suggest that local protein synthesis plays an essential role in BDNF–Ca^{2+}-induced growth cone turning.

Growth cones can respond to repulsive cues by locally synthesizing RhoA. Rat dorsal root ganglia axons severed from cell bodies still respond to Sema3A and have significantly more growth cone collapse which is blocked by translation inhibitors (Wu et al., 2005). Rho family of small GTPases (such as Cdc42, Rac1, and RhoA) are known to regulate cytoskeletal dynamics and among these, RhoA is responsible for growth cone collapse and neurite retraction (Gallo and Letourneau, 2004). Only the mRNA of *RhoA*, but not *Cdc42* and *Rac1*, is present in the DRG growth cones as confirmed by *in situ* hybridization and RT-PCR, suggesting that local synthesis of RhoA protein may play a role in Sema3A-induced growth cone collapse (Wu et al., 2005). Indeed, upon Sema3A treatment, RhoA protein level is significantly increased and this increase is specific to RhoA as GAP-43 protein level is unaltered. Additionally, when RhoA is knocked down or enzymatically inactivated, the growth cones fail to respond to Sema3A-induced collapse (Wu et al., 2005). Lastly, the 3' UTR of *RhoA* mRNA, having a sequence different from *β-actin* mRNA, mediates the localization and translation of *RhoA* transcripts (Wu et al., 2005). The difference in their 3' UTRs suggests that individual mRNA templates contain unique "Zipcodes" and may require

different mechanisms and regulator proteins to control their translation.

Growth cones also respond to repulsive cues by local synthesis of an actin disintegrator, cofilin-1. Another extracellular secreted cue, Slit2, binds to the transmembrane receptor Robo (*Roundabout*) and induces repulsive turning (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). *Xenopus* retinal growth cones do not respond to Slit2 until later stages (stage 40) when *Slit* and *Robo2* mRNAs are expressed at the dorsal midline and tectum margins, indicating a role of Slit in confining RGC axons to the tectum (Piper et al., 2006). In physically severed retinal growth cones, Slit2 significantly increases global translation and induces growth cone collapse which is blocked by translation inhibitors. Next, inhibition of mTOR and MAPK pathways ablates Slit2's effects on growth cone collapse and protein synthesis and Slit2 significantly increases phosphorylation of p38, p42/p44, eIF4E and 4E-BP1 (Piper et al., 2006), suggesting that mTOR and MAPK play a crucial role. However, although Slit2 increases global translation in growth cones, it decreases the formation of filamentous actin (Piper et al., 2006). This is probably due to the translation of an actin-depolymerizing protein (Fan et al., 1993). Cofilin is an actin-depolymerizing protein homologous to vertebrate ADF (Abe et al., 1996) therefore it may play a role in Slit2-induced growth cone collapse. Indeed, *cofilin* mRNA is present in stage 40 RGC neurites and Slit2 significantly increases cofilin protein level (Piper et al., 2006). Together, these results suggest Slit2 induces repulsive turning through local translation of *cofilin* mRNA.

Additionally, local protein synthesis regulates axon guidance *in vivo* which can be carried out

by direct interactions with translation machinery (Figure 1.1). The protein DCC (deleted in colon cancer) is a transmembrane receptor for Netrin1 and it promotes axons crossing the midline floor plate *in vivo* (Keino-Masu et al., 1996). As revealed by proteomic analysis, DCC physically associates with translational proteins such as ribosomal proteins (S6 and L5), and initiation factors eIF4E, eIF3E, eIF1A, eIF2β and eIF2γ; in addition, DCC and translational proteins colocalize in fixed neurons as well as in sucrose gradients of polysome profiling (Tcherkezian et al., 2010). DCC functionally regulates translation: the addition of the cytoplasmic domain of DCC significantly decreases translation in rabbit reticulocyte lysates; in cultured cells however, DCC-Netrin1 interactions greatly promote translation but DCC with a cytoplasmic domain-deletion mutant fails to respond to Netrin1 (Tcherkezian et al., 2010). Importantly, DCC-regulated protein synthesis guides axon pathfinding *in vivo*: DCC interacts with translational proteins in the P1 region of its cytoplasmic domain, and axons transiently expressing a P1-deletion mutant of DCC failed to cross the midline floor plate, as compared to control axons (Tcherkezian et al., 2010). Together, this paper suggests a model that DCC physically sequesters translation machinery at the cell plasma membrane and stalk translation, but when Netrin-1 is added, DCC will release ribosomes into cytoplasm and start translation (Tcherkezian et al., 2010). This study also proposes a novel type of downstream signaling in addition to Src or mTOR-S6K/ 4E-BP kinase activity regulation (Figure 1.1A) – regulation of protein synthesis through physical association and dissociation with the translation machinery (Figure 1.1B).

Taken together, these studies suggest that local translation is an important regulator of axon

guidance and growth cone turning. As a model proposed by Holt and colleagues (Jung et al., 2012), upon sensing a gradient of guidance cue local translation of *β-actin* induces actin polymerization, cytoskeletal assembly and attractive turning while local translation of *cofilin* and *RhoA* triggers cytoskeletal disassembly and repulsive turning.

Local Translation in Synaptic Formation, Maintenance, and Plasticity Regulation

Local protein synthesis also plays multiple roles in the formation of synapses, synaptic maintenance and repair, and regulation of synaptic plasticity. First, local translation is required for the formation of synapses (synaptogenesis). In *Aplysia*, soma-less bag cell neurites are capable of locally translating *ELH* (egg-laying hormone) mRNAs and maintaining their electrophysiological properties (Lee et al., 2002); interaction between *Aplysia* sensory axons and motor neuron targets induces a translocation and an enrichment of *sensorin* (a peptide neurotransmitter) mRNA and proteins at the newly established synapses (Hu et al., 2002; Lyles et al., 2006). These results suggest a role of mRNA localization and translation during *Aplysia* synaptogenesis. In *Xenopus* nerve-muscle co-cultures, presynaptic axons establish contacts with a BDNF-coated bead which leads to elevated neurotransmitter secretion and potentiation (Zhang and Poo, 2002); this BDNF-induced potentiation requires local protein synthesis, even for axons severed from their cell bodies (Zhang and Poo, 2002). Additionally as introduced above, *Eph*/ *ephrin* mRNAs are abundantly expressed in older, target-arriving growth cones in *Xenopus* (Zivraj et al., 2010) and Eph/ ephrin proteins are required for retinotopic mapping and synaptogenesis (Kayser et al., 2006; Marler et al., 2008).

Although a direct link between local *Eph*/ *ephrin* translation and synaptogenesis is not yet demonstrated, these results indicate that Eph/ ephrin proteins may be locally synthesized to regulate synaptogenesis.

After axons arrive at their targets and build the synapses, local translation is required for synaptic maintenance and the post-injury repair. Local synthesis of the transcription factor CREB (cAMP-responsive element-binding protein) and its retrograde transport into the nucleus are critical for neuronal survival and axonal maintenance (Cox et al., 2008). Intra-axonal translation of *Impa1* (myo-inositol monophosphatase-1, an inositol cycle regulator) is protective against axonal degeneration (Andreassi et al., 2010) and local translation of *LaminB2* mRNA promotes axon survival by maintaining the membrane potential of the axonal mitochondria (Yoon et al., 2012). After injury, axons show increased protein synthesis *in vivo* (Hanz et al., 2003; Zheng et al., 2001) and deletion of the negative regulating genes of mTOR significantly promotes CNS axon regeneration (Park et al., 2008). The mRNAs of *STAT3* (a transcription factor), *importin-β* (a nuclear import protein), and *RANBP1* (a RAN GTPase-binding protein and a nuclear transport regulator) are translated upon nerve injury and their translation greatly promotes axonal regeneration (Ben-Yaakov et al., 2012; Hanz et al., 2003; Yudin et al., 2008).

Translational control is also required for regulating synaptic plasticity (Ho et al., 2011). Local protein synthesis is not only a privilege for axons, but also for dendrites. *In vitro*, soma-less dendrites are capable of *de novo* protein synthesis in the vicinity of the synapse (Aakalu et al.,

2001) suggesting a role of these proteins in regulating synaptic plasticity. In rat hippocampal acute slices, axons or dendrites severed from their somata maintain long-term potentiation (LTP) which can be blocked by translation inhibitors such as CHX and anisomycin (Kang and Schuman, 1996). On the other hand, long-term depression (LTD) can also be induced by local postsynaptic protein synthesis triggered by synaptic activation of metabotropic glutamate receptors (mGluR) (Huber et al., 2000). In *Aplysia*, long-term facilitation (LTF) of sensory-to-motor neuron synapses by serotonin (5-HT) also requires local protein synthesis (Martin et al., 1997; Weatherill et al., 2010). Although the change in LTP/LTD and LTF requires regulation at both the transcriptional and the translational levels (Greer and Greenberg, 2008), local protein translation is critically needed in very restricted space such as the presynaptic active zones on the axonal tips (Akins et al., 2009; Beaumont et al., 2001; Je et al., 2011) or the postsynaptic densities on the dendritic spines (Bolognani et al., 2004), rather than the whole neuron cell body.

Which mRNAs are being translated during the regulation of synaptic plasticity? Postsynaptic translation of *CAMKIIα* (calmodulin-dependent protein kinase II α) mRNA is critical in regulating plasticity and ablation of its translation reduces the hippocampal LTP and spatial memory in mice (Miller et al., 2002). Translational control of *c-Jun* mRNA also regulates synaptic plasticity: CPEB (cytoplasmic polyadenylation element binding protein), a protein usually involved in regulating poly(A) tail elongation (Hake and Richter, 1994), can bind to the 3' UTR of *c-Jun* mRNA and regulate its translation which leads to growth hormone expression and eventually affects the LTP in mouse hippocampus (Zearfoss et al., 2008).

Additionally, eIF4AIII binds to *arc/ arg3.1* (activity-regulated cytoskeleton-associated protein) mRNA and represses its translation while *eIF4AIII*-knockdown increases *arc/ arg3.1* mRNA translation and postsynaptic strength (Giorgi et al., 2007). The mRNA of *KOR1* (κ-type opioid receptor 1), encoding a membrane-bound opioid receptor, is presynaptically translated (Bi et al., 2006) and its local translation may regulate the plasticity to pain relief and sensation.

Finally, FMRP (fragile X mental retardation protein) regulates translation and synaptic plasticity, and mutations of *FMRP* lead to various neurological diseases. FMRP is an RNA-binding protein most abundantly found in the brain; it binds to many mRNAs including *arc/arg3.1*, *CAMKIIα*, *PSD95*, *GluR1/2*, and *MAP1B* (microtubule-associated protein 1B) and controls their translation in the synapse (Antar et al., 2005; Chen and Joseph, 2015). Mechanistically, FMRP represses translation by interacting with CYFIP1 (a new 4E-binding protein) and by inhibiting eIF4E-dependent initiation (Napoli et al., 2008); conversely *Fmr1*-knockout mice display a marked increase in translation of *PSD95* and *AMPAR* (AMPA receptor) mRNAs (Muddashetty et al., 2007). FMRP dynamically regulates synaptic plasticity: LTD triggered by mGluR activation is increased in the hippocampus of *Fmr1*-knockout mice and this mGluR-dependent LTD requires rapid synthesis and degradation of FMRP (Hou et al., 2006; Huber et al., 2002). Ultimately, mutations of the *FMRP* gene lead to many neurological disorders such as fragile X syndrome, mental retardation, autism, Parkinson's disease, and other cognitive deficits (Bhakar et al., 2012; Sokol et al., 2011).

Coxsackievirus and adenovirus receptor (CAR) is a novel cell adhesion molecule expressed only in vertebrates (Coyne and Bergelson, 2005). Upon binding to extracellular ligands, CAR greatly promotes neurite outgrowth (Patzke et al., 2010), but its downstream pathways are not well understood. In the next section, I will introduce its molecular biology, its extracellular and intracellular ligands, its potential role during the development of nervous system, and its physiological roles in other organs and tissues.

COXSACKIEVIRUS AND ADENOVIRUS RECEPTOR (CAR)

Molecular Biology of CAR

Coxsackievirus and adenovirus receptor (CAR, *CXADR*) was initially cloned and identified as a co-receptor for coxsackie B virus (CBV) and adenovirus serotype 2 & 5 (Ad2 & 5) and hence its name (Bergelson et al., 1997; Tomko et al., 1997). CAR is a member of the immunoglobulin superfamily CAMs (IgSF-CAMs) as shown in Figure 1.2A (Walmod, 2014; Wei and Ryu, 2012) and it belongs to a subfamily of structurally related Ig-like proteins (Schreiber et al., 2014) including CAR, CLMP (CAR-like membrane protein), JAMs (junctional adhesion molecules), BT-IgSF (brain- and testis-specific Ig superfamily), ESAMs (endothelial-cell-selective adhesion molecules) and CTX (marker for cortical thymocytes of *Xenopus*) (Chretien et al., 1996; Garrido-Urbani et al., 2014; Harada et al., 2005; Raschperger

et al., 2004; Rohatgi et al., 2009).

CAR is a single-transmembrane protein containing 365 amino acids (aa) and has an apparent molecular weight of 46 kDa by Western blot analysis (Bergelson et al., 1997; Honda et al., 2000). It has an extracellular domain (N-terminus, aa 1-222) containing two Ig-like regions (D1 & D2, Figure 1.2B): the membrane-distal D1 region is an Ig variable type (V) domain and the membrane-proximal D2 region is an Ig constant type (C2) domain (Freimuth et al., 2008). It has a short hydrophobic transmembrane domain (aa 234-258) and a long, 107-aa cytoplasmic region (C-terminus, aa 259-365) (Bergelson et al., 1997). Finally CAR has a PDZ (PSD-95/ Disc-large/ ZO-1) -binding domain, an important regulatory domain in signaling transduction and synaptic functions (Harris and Lim, 2001; Hung and Sheng, 2002; Kim and Sheng, 2004). This PDZ-binding domain is located at the last three amino acids (CAR isoform 1: SIV, CAR isoform 2: TTV) of its C-terminal region (Bergelson et al., 1997).

The N-terminus of CAR has important regulatory roles. It is shown that both the D1 and D2 domains are required for cell-cell adhesion and localization in primary airway epithelia (Excoffon et al., 2005) and that the D1 domain is required for adenovirus fiber knob binding and viral infection (Excoffon et al., 2005; Patzke et al., 2010). The D2 domain, however, interacts with various extracellular matrix proteins (Patzke et al., 2010), as introduced below. The extracellular region of CAR has two glycosylation sites (aa 106 and 201, respectively in the D1 and D2 domains) and the glycosylation is critical for CAR-mediated homophilic adhesion and for adenoviral infection (Excoffon et al., 2007). Additionally, the extracellular

domain of CAR can be cleaved by the sheddase ADAM10 (A Disintegrin And Metalloprotease 10) but the physiological impact of CAR's ectodomain shedding is not known (Houri et al., 2013).

The C-terminus of CAR is essential for its subcellular localization but not for adenoviral infection. CAR is a component of tight junctions and it is expressed exclusively in the basolateral surface of cultured cell monolayers (Cohen et al., 2001b; Walters et al., 1999). Multiple regions within CAR's cytoplasmic tail are required for basolateral sorting and deletion of CAR's C-terminus disrupts its basolateral localization (Cohen et al., 2001a). However, CAR's cytoplasmic domain has no effect on adenoviral infection (Wang and Bergelson, 1999). Additionally, two membrane-proximal cysteines (aa 259 and 260) of CAR are modified by palmitylation which is also essential for basolateral sorting but is dispensable for adenoviral infection (van't Hof and Crystal, 2002). Finally, CAR undergoes an intramembrane proteolysis by γ-secretase and releases its intracellular domain (ICD) from the plasma membrane to the nucleus (Houri et al., 2013), a process similar to the intramembrane proteolysis of Notch and Delta (Weinmaster, 2000).

Extracellular and Intracellular Ligands of CAR

The extracellular domain of CAR interacts with a variety of molecules. Above all it binds to itself through homophilic interactions to regulate cell-cell adhesion, tight-junction maintenance, and neurite outgrowth (Excoffon et al., 2007; Excoffon et al., 2005; Patzke et

al., 2010). This is similar to other adhesion molecules such as NCAM, L1 and N-cadherin (Drazba and Lemmon, 1990; Neugebauer et al., 1988; Ranheim et al., 1996). Additionally, CAR's homophilic interactions take place either *in cis* on the same cell or *in trans* between different cells (Patzke et al., 2010). However, whether there is a difference in biological functions between *in cis* and *in trans* homophilic interactions is not known.

As the primary receptor for adenovirus, CAR binds to the fiber knob of Ad2 and Ad5 through its ectodomain (Bergelson et al., 1997; Santis et al., 1999). CAR's D1 domain is necessary to bind to the fiber knob with high affinity (Bewley et al., 1999; Kirby et al., 2000). In kidney-derived epithelial cells, binding of Ad5 viral capsid to CAR activates the p38 and ERK signaling pathways leading to expression of the C-X-C chemokine IP-10 (Tibbles et al., 2002). In human respiratory epithelial cells, Ad5 fiber knob binding to CAR activates ERK and MAPK pathways, triggers NF-κB nuclear translocation, and promotes transcription of the chemokines such as interleukin-8 (Tamanini et al., 2006). These results suggest that CAR-fiber knob interaction plays a role in triggering host immune response.

The N-terminus of CAR also interacts with the IgSF member JAMs (junctional adhesion molecules) such as JAML and JAMC via heterophilic interactions. The interaction of JAML and CAR activates the PI3K pathway (Verdino et al., 2010), leads to epithelial gamma delta T cell activation, and promotes wound healing (Witherden et al., 2010). Interaction of CAR with JAML expressed on neutrophils facilitates neutrophil adhesion to endothelial cells (Luissint et al., 2008) and neutrophil migration across tight junctions (Zen et al., 2005). In

addition, CAR forms a complex with the differentiation factor JAMC in mouse testis and may play a role in mouse spermatogenesis (Mirza et al., 2006).

Finally, the ectodomain of CAR binds to extracellular matrix (ECM) proteins. Using surface plasmon resonance, it was shown that CAR directly interacts with ECM proteins such as fibronectin, laminin, tenascin-R, and agrins (Patzke et al., 2010). Among these ECM proteins, CAR binds to fibronectin through its D2 domain, and a 40-kDa fibronectin subunit (FN40) is sufficient to promote neurite outgrowth (Patzke et al., 2010). Interestingly, Ad2 fiber also greatly promotes neurite outgrowth although the physiological implications under natural conditions are not known (Patzke et al., 2010).

The C-terminus of CAR is reported to associate with many tight junction proteins such as ZO-1 (zonula occludens 1), MUPP-1 (multi-PDZ domain protein 1), PSD95 (post-synaptic density protein 95), MAGI1b (membrane-associated guanylate kinase 1b), PICK1 (protein interacting with C kinase 1), LNX (ligand of Numb X), and LNX2 (Cohen et al., 2001b; Coyne et al., 2004; Excoffon et al., 2004; Mirza et al., 2005; Sollerbrant et al., 2003). All of these proteins contain the PDZ domain and they function in the formation of cell-cell contacts or tight junctions. Additionally, CAR's cytoplasmic domain also interacts with cytoskeletal proteins such as tubulin and actin to regulate cytoskeletal dynamics, cell migration, and nerve growth cone motility (Fok et al., 2007; Huang et al., 2007).

Potential Roles of CAR in the Central Nervous System

CAR may play an essential role in the development of the central nervous system (CNS). It is most strongly expressed in the embryonic brains but its expression is very low in mature ones. During development CAR is widely distributed in the brain but in adults CAR is restricted to the locations where neural stem cells reside. However, due to embryonic lethality of CAR knockouts, CAR's role in CNS development is currently unknown.

CAR is abundantly expressed in the embryonic brain. Western blot analysis of various tissues isolated from newborn mice demonstrate the strongest CAR expression in the brain, a faint but detectable signal in the heart, and nearly no signal in kidney, lung, liver, and testis (Honda et al., 2000). Additionally, CAR expression is high in the developing brain as demonstrated by Western blotting and *in situ* hybridization but its expression is nearly undetectable in the mature ones (Honda et al., 2000), suggesting that CAR may primarily function in the wiring of neural circuits during brain development.

When taking a closer look, on embryonic day 6.5 (E6.5) during mouse development, CAR is highly expressed in the embryonic ectoderm (Hotta et al., 2003) which later develops into the neural tube and the whole central nervous system (Lee and Jessell, 1999). On E8.5, a significant amount of CAR is detected in the neural tube, including prosencephalon (the forebrain), mesencephalon (the midbrain), rhombencephalon (the hindbrain), and the somites (Hotta et al., 2003). The enriched CAR expression locates in the neural tube-derived

neuroepithelial cells spreading from the ventricular surface to the basal lamina, without anterior-posterior difference (Hotta et al., 2003). These results suggest that CAR may regulate the very early stages of CNS development and that CAR is not involved in the rostral-caudal polarization at this stage.

From E10.5 and onwards, the neural tube undergoes heterochronous development in the rostral-caudal axis; for example, the caudal mesencephalon develops earlier than the rostral telencephalon. In accordance with this, CAR expression is detected on E10.5 in the caudal mesencephalon (which will develop into the midbrain) and on E13.5 in the rostral telencephalon (which will develop into the cerebrum; Hotta et al., 2003).

Moreover, at this stage (E10.5) CAR expression also displays a ventral-dorsal difference and it may have an impact on the wiring of lower motor neurons. On E10.5, CAR is detected in the spinal cord: it is expressed highly in the commissural axons crossing the ventral (containing motor neurons) midline of the spinal cord. Notably, its expression on E10.5 is strong only in the ventral motor roots but not in the dorsal sensory roots; however at a later stage from E13.5 to E16.5, CAR's expression in the ventral motor roots decreases sharply (Hotta et al., 2003). These results suggest that CAR may contribute to the development of the lower spinal motor neurons, but not sensory neurons.

Additionally on E10.5, CAR expression is enriched in neuronal fibers and localized on cell-cell contacts of the neuroepithelial cells. Its expression is restricted to the boundary

between neuroepithelial cells but not on the apical surface, and later CAR distribution changes to basolateral cell-cell contacts (Hotta et al., 2003); this distribution pattern of CAR is similar to the epithelial cells in other organs (Raschperger et al., 2006; as discussed in the next section). Neuroepithelial cells are proliferating precursor cells which can generate glial cells and functional postmitotic neurons *in vitro* and *in vivo* (McKay, 1997; Okabe et al., 1996). CAR expression spreads from the ependymal layer of mitotic progenitor cells to the mantle layer of postmitotic neurons (Hotta et al., 2003), suggesting that CAR may regulate the functions of both proliferating progenitor cells and functional postmitotic neurons *in vivo*.

In addition to its expression in the lower motor neurons, CAR is also expressed in the upper motor neurons during development. From E13.5 to E16.5, CAR is highly expressed in the pyramidal tracts including the corticobulbar and corticospinal tracts (Hotta et al., 2003). The pyramidal tracts are aggregations of upper motor neuron fibers that originate from the cerebral cortex and terminate either at the medulla (corticobulbar) or at the spinal cord (corticospinal), and these tracts are involved in controlling motor functions (Lemon, 2008; Porter, 1985). These results, together with the above-mentioned expression patterns in the lower spinal motor neurons, suggest that CAR may contribute to the development of the CNS motor system and may regulate animal motor functions. However, whether CAR regulates motor functions is currently unknown.

From E13.5 to E16.5, during which the secondary brain vesicles are formed, CAR is expressed in telencephalon and diencephalon, and its expression is specifically enriched in

the cortico-striatal boundary, hypothalamus, hippocampal formation, and amygdaloid area (Hotta et al., 2003) suggesting that CAR may contribute to the wiring of neural circuits in these regions. In newborn (P1) mice, CAR is expressed in the association fibers in corpus callosum and anterior commissure but its expression in these fibers is sharply decreased by P21 (Hotta et al., 2003) suggesting that CAR may help establish the neural connections between the two cerebral hemispheres.

In adult mammals, CAR signal localizes and persists in the regions of adult neurogenesis. On P21, significant expression of CAR is seen in the subgranular layer (SGL) of the hippocampal dentate gyrus, the subventricular zone (SVZ) of lateral ventricles, and the rostral migration stream (RMS) to the olfactory bulb; CAR signal has a significant overlap with the proliferation marker PCNA and the astrocyte-specific marker GFAP (Hotta et al., 2003). In the adult mammalian brains, there are two main regions of adult neurogenesis: the SGL of the dentate gyrus in the hippocampus (Eriksson et al., 1998; Kempermann et al., 1997) and the SVZ in the wall of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999); the SVZ neural progenitors generate neuroblasts that migrate through the RMS to the olfactory bulb (Doetsch et al., 1999). These results suggest that CAR may contribute to the secondary neurogenesis in adult animals.

In adults, CAR is also expressed at the ependymal cells and the choroid plexus. CAR localizes at the ependymal cells, which line the brain ventricles and the spinal cord central canal (Johansson et al., 1999); these ependymal cells are involved in producing cerebrospinal

fluid (CSF) and regulating CSF circulation (Brinker et al., 2014). An immunohistochemical study of CAR expression in the adult human brain shows the most abundant staining in the choroid plexus and the pituitary gland (Persson et al., 2006). The choroid plexus consists of modified ependymal cells and is generally recognized as the source of CSF (Lehtinen et al., 2013). In addition to human brains, CAR expression is also detected in the choroid plexus in adult mouse brains (Raschperger et al., 2006). Together, these studies indicate that CAR is expressed in choroid plexus and may regulate CSF production.

Additionally, CAR is present in the stem cell niche of the pituitary gland. Immunohistochemistry of adult human brain slices shows a marked staining in the pituitary gland (Persson et al., 2006). Further study shows that CAR is also expressed in the rat pituitary gland throughout life and it is particularly localized in the marginal cell layer (MCL) of the anterior/ intermediate pituitary lobes but not posterior lobe (Chen et al., 2013). The MCL has long been indicated as a pituitary stem cell niche (Garcia-Lavandeira et al., 2009; Gremeaux et al., 2012). Additionally, CAR-positive MCL cells are actively proliferating as revealed by immunostaining against Ki67, a cell proliferation marker; CAR also co-localizes with stem/ progenitor cell markers SOX2 and E-cadherin (Chen et al., 2013). These results demonstrate that CAR is consistently expressed by the MCL cells in the pituitary gland and may regulate pituitary adult neurogenesis.

As mentioned above, CAR promotes neurite outgrowth *in vitro* upon binding to ligands such as FN40 and Ad5 fiber knob: FN40 promotes significant neurite extension in neurons isolated

from CAR+/+ and CAR+/- mice, but not from CAR-/- mice (Patzke et al., 2010). Previously in our lab, we also demonstrated that CAR-CAR homophilic interactions promoted significant neurite outgrowth and knockdown of CAR completely ablated neurite outgrowth promoted by CAR's extracellular region (Huang et al., unpublished). These results suggest that CAR is a strong neurite outgrowth promoter *in vitro*. However, the role of CAR-regulated neurite outgrowth *in vivo* requires further exploration.

Additionally, CAR plays a role in axonal transport. At the same time when CAR was cloned and characterized, it was also found that CAR is enriched in the newborn mouse nerve growth cones (Abe et al., 1997). Partial sequencing of its digested peptides reveals several homologous consensus to the dynactin complex components such as centractin and p150^{GLUED} (Abe et al., 1997), suggesting that CAR may regulate dynein for retrograde axoplasmic transport of vesicles and organelles in neurons (LaMonte et al., 2002; Martin et al., 1999). Indeed, in developing motor neurons, CAR binds to adenovirus, facilitates viral internalization, and promotes dynein-regulated retrograde transport of the virus (Salinas et al., 2009). However, whether CAR-regulated axonal transport contributes to brain development needs to be further explored.

In conclusion, although CAR is highly expressed and widely distributed in the developing brain, we do not know what CAR does to the wiring of neural circuits during CNS development. Will CAR-knockout cause deformation of the brain or abnormal behavior? How could CAR regulate neurite outgrowth *in vivo*? Unfortunately, whole body knockout of

CAR leads to embryonic death on E11.5 and E12.5 due to malformation of the heart (Asher et al., 2005; Dorner et al., 2005) and abnormal development of lymphatic vessels (Mirza et al., 2012), respectively. Therefore this embryonic lethality has impeded the research of CAR's effects on CNS development. To tackle this challenge, it will be helpful to generate CNS-specific conditional CAR-knockout mice and to study CAR's effects on brain structure and animal behavior.

Physiological Roles of CAR in Other Organs and Tissues

In addition to being expressed in the developing and mature central nervous system, CAR is also highly expressed in the developing and mature heart, in skeletal muscle, as well as in the tight junctions of epithelial and endothelial cells in the lung, intestine, pancreas, kidney, lymphatic vessels and male reproductive system in adult animals.

First, CAR is highly expressed in the developing heart (Honda et al., 2000). Like its expression pattern in the brain, CAR is expressed strongly in newborn rat hearts but at low levels in adult ones, and its expression is up-regulated in response to cardiac injury (Ito et al., 2000). Whole-body or cardiomyocyte-specific knockout of CAR led to the malformation of the heart on E10.5-11.5, including disorganized myofibrils, ill-formed intercellular junctions, and thoracic hemorrhaging which eventually led to embryonic death by E11.5-13.5 (Asher et al., 2005; Chen et al., 2006a; Dorner et al., 2005). These results suggest that CAR functions as a potent adhesion molecule during heart development.

In the adult heart, CAR is strongly expressed in the intercalated discs (Kashimura et al., 2004; Shaw et al., 2004), which are the junctions that connect heart muscle cells together. In conditional CAR-knockout mice, deletion of CAR in adult hearts reduced the electrical conductance between atrium and ventricle (Lisewski et al., 2008), which is probably due to the loss of connexin 45 localization to the cell-cell junctions in the atrioventricular node (Lim et al., 2008). On the other hand, CAR expression is increased at cardiomyocyte junctions in patients with cardiomyopathy and heart failure (Noutsias et al., 2001; Sasse et al., 2003) and targeted overexpression of CAR in the heart caused disrupted adherens junctions, cardiomyocyte disorganization and degeneration, and eventually animal death (Caruso et al., 2010). These results demonstrate that CAR plays both functional and structural roles in adult heart.

CAR is also expressed in muscle cells and may regulate muscle fiber regeneration. CAR is highly expressed in immature muscle fibers but its mRNA transcripts are barely detectable in mature muscle cells (Nalbantoglu et al., 1999). Further examination shows that CAR is expressed at the neuromuscular junctions and is confined to the post-junctional folds on the sarcolemma (similar to postsynaptic densities on neurons; Shaw et al., 2004). Transgenic mice with muscle-specific overexpression of CAR develop a severe skeletal myopathy with disturbed sarcolemmal integrity and impaired repair (Shaw et al., 2006) suggesting that CAR may regulate muscle integrity and maintenance. Additionally, CAR is abundantly co-expressed with regenerative markers in the skeletal muscle of muscular dystrophy patients

(Sinnreich et al., 2005) suggesting that CAR might be involved in muscle fiber regeneration.

The expression of CAR in other organs of the adult mice is confined to tight junctions of epithelial cells and it is detected in the cell-cell contacts in gastrointestinal tract, pancreas, liver, respiratory tract, kidney (Raschperger et al., 2006), lymphatic vessels (Vigl et al., 2009), and male reproductive system (Mirza et al., 2006; Raschperger et al., 2006). In these organs, CAR expression is correlated positively with organ integrity and inversely with tissue permeability, suggesting that CAR may have a role in regulating tissue homeostasis (Raschperger et al., 2006). On the other hand, if whole-body knockout of CAR is performed in adult mice, these KO mice have significantly enlarged intestines and dramatically smaller pancreas with an atrophy of exocrine tissue, mostly due to malformation of cell-cell contacts (Pazirandeh et al., 2011). Additionally, interaction of CAR with extracellular ligands can either induce tissue disruption or tissue recovery after wounding. During adenovirus infection, the binding of fiber knob to CAR disrupts CAR-mediated cell-cell adhesion and the tissue integrity of human airway epithelia (Walters et al., 2002) while JAML-CAR interaction *in vivo* activates epithelial gamma delta T cell to secrete cytokines, induce cell proliferation and promote wound healing (Verdino et al., 2010; Witherden et al., 2010). Taken together, these results demonstrate that in adult animals CAR plays regulatory roles in the tissue integrity and homeostasis of several organs and tissues *in vivo*.

Lastly, CAR is expressed in lymphatic vessels, but not in blood vessels, of human skin and affects lymphatic endothelial cell (LEC) functions *in vitro*, such as cell adhesion, migration,

and control of LEC monolayer permeability (Vigl et al., 2009). And if CAR is knocked out at E12.5 (approximately 24 hr later than heart malformation-induced embryonic lethality on E11.5), it causes subcutaneous edema, hemorrhage, and ultimately embryonic death (Mirza et al., 2012). In these CAR-KO mice, the lymphatic vessels are structurally disrupted at the tight junctions and the lymphatic system fails to separate erythrocytes from lymphatic cells (Mirza et al., 2012). These results suggest that CAR plays an essential role in forming the cell-cell junctions and in regulating functions of the lymphatic systems.

CAR Is Dysregulated in Various Types of Cancer

As CAR is expressed in many organs and tissues, dysregulation of CAR expression is implied in various types of cancer. It is generally regarded as a tumor suppressor. CAR expression is down-regulated in bladder cancer (Li et al., 1999; Matsumoto et al., 2005; Sachs et al., 2002), prostate cancer (Okegawa et al., 2000), head and neck squamous cell carcinoma (Jee et al., 2002), glioblastoma (Fuxe et al., 2003), endometrial carcinoma (Giaginis et al., 2008), gastric cancer (Anders et al., 2009b), and esophageal cancer (Anders et al., 2009a). Functionally, loss of CAR has been linked to reduced cell-cell adhesion, increased cell proliferation, and enhanced cancer cell migration and invasion.

On the other hand, ectopic overexpression of CAR decreased the migration and invasion of various types of cancer cells such as those of glioblastoma (Fok et al., 2007; Huang et al., 2005; Kim et al., 2003), bladder cancer (Okegawa et al., 2001; Zhang et al., 2007), colorectal

cancer (Stecker et al., 2011), oral squamous cancer (Saito et al., 2014), as well as reduced metastasis to the lung (Yamashita et al., 2007). Notably, in these cancer types, overexpression of CAR significantly reduced cell migration (Bruning and Runnebaum, 2004; Fok et al., 2007; Stecker et al., 2009) indicating that CAR plays an important role in regulating cytoskeletal dynamics, cell motility, and cellular morphological changes, a mechanism similar to nerve growth cone movement.

Conversely however, CAR is up-regulated in a few other types of cancer such as osteosarcoma (Gu et al., 2004; Kawashima et al., 2003) and breast cancer (Anders et al., 2003; Bruning et al., 2005; Martin et al., 2005). Therefore although CAR is generally regarded as a tumor suppressor, whether CAR inhibits or promotes tumor growth may depend on the cancer microenvironment, such as tissue or organ types, neighboring cells, and extracellular signal molecules around the tumor cells.

Figure 1.1

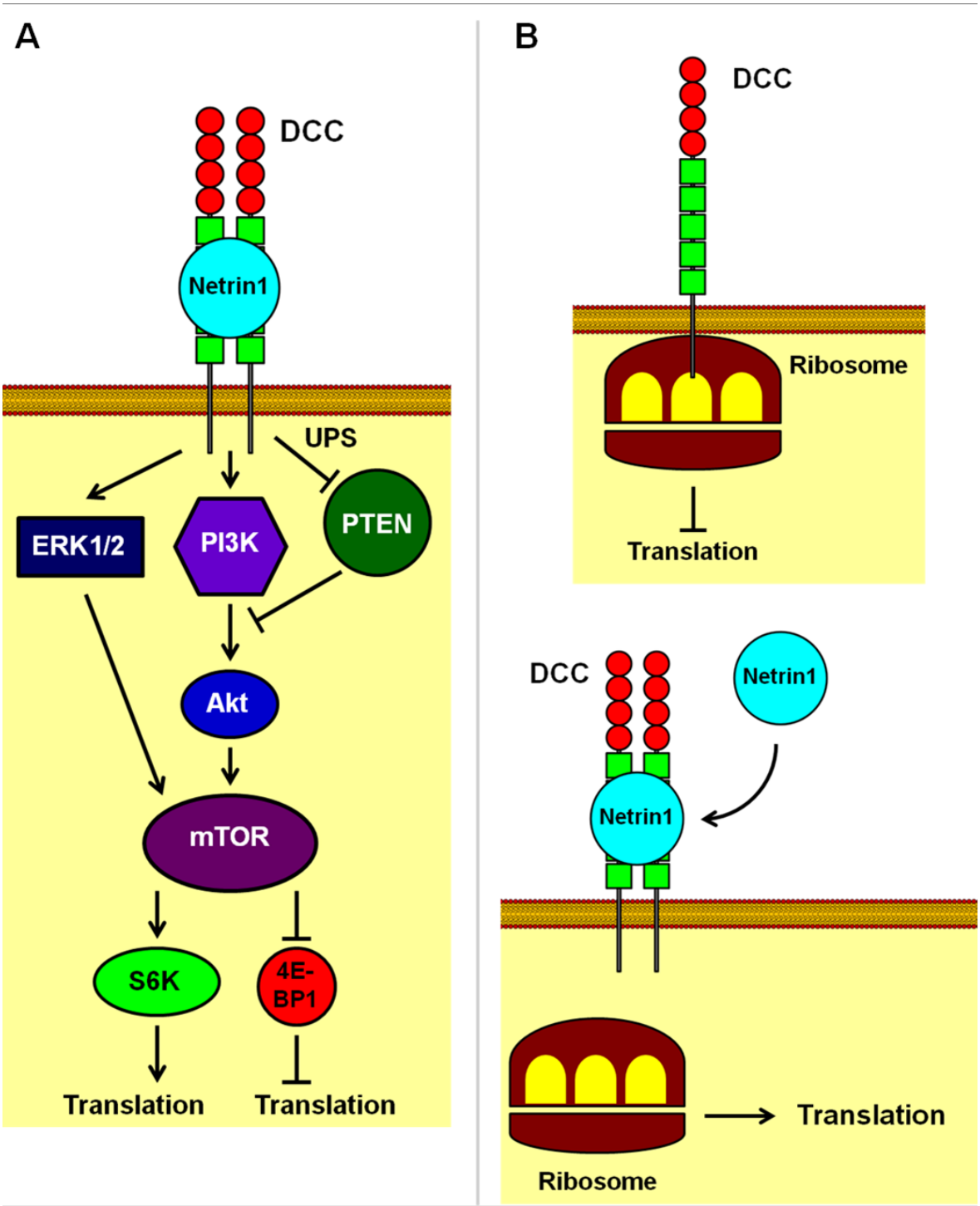

A
DCC
Netrin1
UPS
ERK1/2
PI3K
PTEN
Akt
mTOR
S6K
4E-BP1
Translation
Translation
B
DCC
Ribosome
Translation
DCC
Netrin1
Netrin1
Ribosome
Translation

Figure 1.1. **Translation in nerve growth cones can be regulated by transmembrane receptor proteins.** A) The transmembrane receptor protein DCC can trigger mTOR signaling and promote translation through activating ERK1/2 and PI3K/ Akt pathways, or through ubiquitin proteasome system (UPS) -mediated PTEN protein degradation (figure adapted from Jung et al., 2012). B) DCC can also regulate translation through direct protein-protein interactions. Without a ligand (top), DCC may sequester a ribosome at the cell surface while upon binding to Netrin1 (bottom) DCC may release the ribosome to initiate translation (figure adapted from Tcherkezian et al., 2010).

Figure 1.2

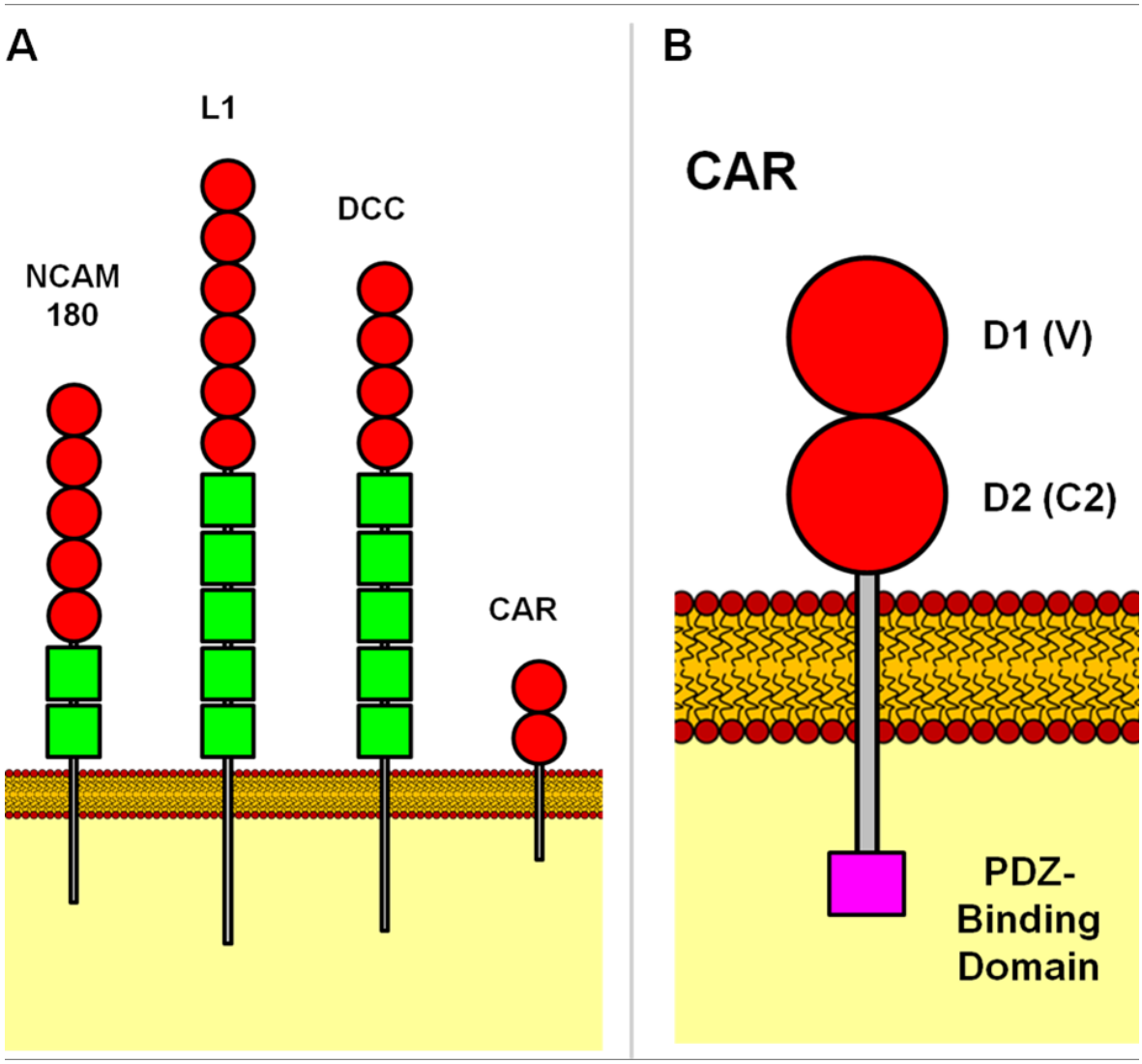


Figure 1.2. **Molecular structures of CAR and other IgSF-CAMs.** A) Molecular structures of immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs). The neural cell adhesion molecule (NCAM) contains five Ig domains (red circles) and two fibronectin type III domains (FN3, green squares); only the 180-kDa isoform of NCAM is shown here. L1-CAM and DCC also contain multiple Ig and FN3 domains (figure modified from Wei and Ryu, 2012). CAR is one of the smallest members of this superfamily and it is only expressed in vertebrates. B) The molecular structure of CAR. CAR contains two Ig domains – D1 (V) and D2 (C2) – in its extracellular region, one short hydrophobic transmembrane domain and a long cytoplasmic (C) tail (figure adapted from Coyne and Bergelson, 2005). CAR also contains a PDZ-binding domain at the very end of its C-terminus (the magenta rectangle).

RATIONALE AND HYPOTHESIS

Firstly, we performed a proteomic analysis of proteins interacting with the cytoplasmic domain of CAR and identified many ribosomal proteins and translation regulatory proteins. I hypothesize that CAR may associate with translational proteins and regulate protein synthesis. Secondly, although CAR-FN40 interaction promotes neurite outgrowth, the downstream mechanism is not clear. I hypothesize that CAR-FN40 interaction may regulate protein synthesis during neurite outgrowth. Lastly, although CAR is widely distributed in the embryonic brain and spinal cord, we do not know how CAR contributes to CNS development. I hypothesize that the CNS-conditional knockout mice may have behavioral phenotypes.

Therefore, the objectives of this research are as following:

1) To test whether CAR associates with translational proteins and regulates translation;

2) To investigate whether CAR promotes neurite outgrowth through regulation of protein synthesis;

3) To perform a preliminary behavioral analysis of CNS-conditional CAR-knockout mice.

CHAPTER 2

MATERIALS AND METHODS

Animals

Pregnant CD1 mice (Charles River Laboratories) were used for isolating growth cone particles (postnatal day 6, P6) or for culturing primary cortical neurons (embryonic day E16-E17). To generate CAR conditional knockout mice, CARFLOX mice (the exon 2 of CAR is flanked by loxP sites, a kind gift from Dr. Bergelson, University of Pennsylvania) were crossed with transgenic mice that express the Cre recombinase under the control of the Synapsin1 promoter (Zhu et al., 2001) (SYNCRE, a kind gift from Dr. Cloutier, McGill University). The mRNA and protein levels of CAR were significantly reduced in CAR-null mice as previously verified (Zheng MSc thesis). Transgenic mice at the age of 2-6 months were tested with open-field, Rota-rod, and other tests. All procedures were performed in accordance with the Animal Care and Use Program Guidelines of McGill University. All experimental protocols were approved by the Animal Care Committee of the Montreal Neurological Institute, McGill University.

Cell Lines and Cell Culture

Human glioblastoma U87MG cells expressing full length CAR (U87CAR) and control (U87LNCX) cells were generated previously (Huang et al., 2005). These U87-lineage cells and HEK293 cells expressing full length CAR fused to a V5 tag (293CARV5 cells) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/ streptomycin. Chinese Hamster Ovary (CHO)

cells were maintained in F12 medium supplemented with 10% FBS, 2 mM L-Glutamine (Gibco), and 100 U/mL penicillin/ streptomycin. Isolated primary cortical neurons were cultured in NeuroBasal medium with all supplements (see below in "*Culture of Mouse Embryonic Cortical Neurons*"). All cultured cells were maintained at 37ºC with 5% CO_2.

Cloning, Production and Purification of GST-Fusion Proteins

The plasmid for producing GST-CAR was generated previously (Fok et al., 2007). To generate plasmids for GST-ΔSIV (nucleotides (nt): 778-1089), GST-Δ26 (nt: 778-1017), GST-Δ74 (nt: 778-873), and GST-Δ98 (nt: 778-801) of CAR (GenBank accession: Y07593), PCR products were cloned into the expression vector pGEX-3X (Amersham Biosciences). Generated plasmids were verified by restriction enzyme digestion and by sequencing (Applied Biosystem 3730xl DNA Analyzer, at Genome Quebec, Montreal).

To produce GST-fusion proteins, plasmids were transformed into BL21 bacteria, grown in 500 mL culture until the OD_{600} reached 0.8, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Fisher Scientific) at 37ºC for 2 hr, pelleted and lysed in phosphate-buffered saline (PBS) containing protease inhibitors. The bacterial lysates were then sonicated, treated with 1% Triton X-100 for 15 min at 4ºC, and centrifuged to pellet the bacterial debris. Cleared lysates were incubated with glutathione-Sepharose beads (Amersham Biosciences) for 30 min at 4ºC, washed three times with PBS containing protease inhibitors, and resuspend in 50% glycerol in PBS.

Cloning, Production and Purification of His-eIF4E Proteins

To generate the plasmid for eIF4E fused with a 6xHis tag at its N-terminus (His-eIF4E), the open reading frame of eIF4E (IMAGE ID: 4282826, Thermo Scientific) was PCR amplified and cloned into a pET15b (Novagen) expression vector. The generated plasmid was verified by restriction enzyme digestion and by sequencing (Applied Biosystem 3730xl DNA Analyzer, at Genome Quebec, Montreal).

To produce recombinant His-eIF4E protein, the plasmid was transformed into BL21 bacteria, grown in 500 mL LB media until the OD_{600} reached 0.8, and induced with 0.5 mM IPTG at 26ºC for overnight. The recombinant His-eIF4E protein is usually present in the inclusion bodies (Stern et al., 1993). The inclusion body pellets were prepared by resuspending the BL21 bacteria in the lysis buffer (50 mM HEPES pH7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100), centrifuging at 27,000 x *g* for 10 min, and by repeating this resuspension and centrifugation cycle for one more time.

The inclusion body pellets were then processed by dissolving and rapid dilution (Joshi et al., 2004): the pellets were dissolved in 6M guanidine hydrochloride (0.1 M Tris-HCl pH 8.0, 6 M guanidine hydrochloride, 0.1 M dithiothreitol, 20 mM EDTA), renatured by rapid dilution (20-fold) into buffer containing 50 mM HEPES-KOH pH7.2 and 200 mM NaCl, and

incubated at 4°C for 1 hr with agitation. The renatured fusion proteins were purified by nickel affinity chromatography (Life Technologies) and eluted with the elution buffer (20 mM Tris-HCl pH8.0, 500 mM imidazole, 100 mM EDTA). The final protein elution was then concentrated using a Centricon (MWCO 10,000, EMD Millipore) and buffer-exchanged with the storage buffer (20 mM HEPES-KOH pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% v/v glycerol, and protease inhibitors; Joshi et al., 2004). The eluted proteins were stored at -80°C. The purity and integrity of the His-eIF4E protein were verified by SDS-PAGE and coomassie blue staining.

Cloning of CAR C-Terminal Truncation Plasmids

To generate plasmids for full length CAR and CAR's C-terminal truncates, pCAR (nt: 1-1098), pΔSIV (nt: 1-1089), pΔ26 (nt: 1-1017), pΔ50 (nt: 1-955), pΔ74 (nt: 1-873), and pΔ98 (nt: 1-801) were generated by cloning their respective PCR products into pcDNA3 (Invitrogen). Constructed plasmids were then verified by restriction enzyme digestion and by sequencing (Applied Biosystem 3730xl DNA Analyzer, at Genome Quebec, Montreal). These plasmids were then used to transfect cultured CHO cells. Before transfection, these plasmids were large-scale prepared and purified using Genopure Plasmid Maxi Kit (Roche).

GST Pulldown Assay

GST pulldown assays were performed with U87CAR cell lysates. Plates (10-cm) with confluent U87CAR cells were lysed in 1 mL of ice-cold HEPES-buffered saline with Triton (HBST: 20 mM HEPES pH 8, 150 mM NaCl, 1% Triton X-100, and protease inhibitors). The cell lysates were then passed though an 18-gauge needle for 5-6 times and centrifuged at 10,000 x *g* for 30 min at 4ºC to clarify the lysates. For each pulldown, 5 µg of GST-CAR or truncation fusion proteins on glutathione-Sepharose beads (Sigma) were incubated with the lysates under rotation at 4ºC for 1 hr. The beads were then rinsed three times with cold HBST buffer. Bound proteins were eluted by boiling with 2x Laemmli loading dye (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% w/v SDS, 0.02% w/v bromophenol blue, and 5% β-mercaptoethanol) and analyzed by Western blot.

GST pulldown assays were also performed with isolated growth cone particles (GCPs). After the fractionation protocol (see below) the pelleted GCPs were reconstituted in HBST buffer and the pulldown was performed with the same procedure as U87CAR cell lysates.

For direct interaction with His-eIF4E recombinant protein, 200 ng of His-eIF4E, together with 1 µg of GST, GST-CAR, or GST-ΔSIV were mixed in HBST buffer. The rest of the pulldown was performed with the same procedure as U87CAR cell lysates.

m^7GTP mRNA Cap Pulldown Assay

The eukaryotic mRNA 5'-cap pulldown was performed with U87CAR lysates and with isolated GCPs. Confluent U87CAR cells was lysed in the cap-pulldown lysis buffer (20mM Tris-HCl pH7.4, 100mM NaCl, 25mM $MgCl_2$, 0.5% NP40, and protease inhibitors) and the rest of the procedure was the same as "*GST Pulldown Assay*". For each pulldown, 25 µL of 7-methyl-GTP (m^7GTP) Sepharose 4B beads (GE Healthcare) were applied and incubated at 4ºC for 1 hr under rotation. Unconjugated Sepharose 4B beads (GE Healthcare) were incubated as a control. The beads were then washed three times with cold cap-pulldown lysis buffer. Bound proteins were boiled and subjected to Western analysis. The cap pulldown was also performed with isolated GCPs: after the fractionation protocol (see below), the pelleted GCPs were resuspended in cap-pulldown lysis buffer and the pulldown was performed with the same procedure as U87CAR cell lysates.

SDS-PAGE, Western Blot, Antibodies and Mass Spectrometry

Briefly, protein samples were separated on 8-10% SDS-PAGE gels, transferred onto nitrocellulose or PVDF membranes, and blocked in 5% BSA in Tris-buffered saline with Tween-20 (TBST: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). The membranes were then incubated with primary antibodies in 5% BSA in TBST at 4ºC overnight. Rabbit polyclonal antibody (2240) against the N-terminus of CAR was produced and purified as

previously described (Nalbantoglu et al., 1999). Polyclonal antibody (RP194) against the C-terminus of human and murine CAR-1 isoform (Sollerbrant et al., 2003) was a kind gift from Dr. Sollerbrant (Karolinska Institute, Sweden). Antibodies against translational proteins were purchased from commercial sources: S6, eIF4E, eIF4G, eEF1A (Cell Signaling), L4 (Santa Cruz), and hnRNPU (Abcam). On the next day, the membranes were incubated with secondary antibodies for 1 hr at room temperature, washed extensively with TBST, and exposed on film or detected with GeneGnome image capturing system (SynGene). Mass spectrometry was performed as previously described (Fok et al., 2007).

Confocal Immunofluorescence

Immunofluorescence was performed with HEK293 cells expressing full length CAR fused to a V5 tag (293CARV5 cells). For confocal microscopy, 1-10x10^4 cells/ well in 24-well plates were grown on 1.5-mm coverslips coated with 50 µg/mL poly-D-lysine (PDL). Forty-eight hours later, cells were fixed in 4% paraformaldehyde (PFA) for 15 min at 37ºC, permeabilized in 5% Triton X-100 for 5 min at room temperature, blocked in 5% BSA in TBST for 1 hr at room temperature, and incubated with primary antibodies in blocking buffer at 4ºC overnight (1:50 dilution for S6 antibody, Cell Signaling, and for CAR-H300 and L4 antibody, Santa Cruz). On the next day, cells were washed for 30 min in TBST, incubated with anti-mouse-AF647 and anti-rabbit-AF488 secondary antibodies (both 1:200 dilution, Molecular Probes) for 30 min, stained with 10 µg/mL Hoechst 33342 (Invitrogen) for 10 min,

and washed again for 30 min at room temperature. The coverslips were then mounted using ProLong Gold (Molecular Probes) and the co-localization analyzed using Leica TCS SP8 laser-scanning confocal microscope with a 63x/1.4 oil DIC objective (Leica Microsystems).

For immunofluorescence in cortical neurons, $2x10^4$ neurons/ well (24-well plates) were grown for 48 hr on coverslips coated with 33 µg/ mL PDL. The neurons were fixed in 4% PFA in 4% w/v sucrose in PBS at 37ºC incubator for 30 min. For co-localization between CAR and eEF1A, primary antibodies CAR-Mab.E(mh)1 (Santa Cruz) and eEF1A antibody (Cell Signaling) were diluted 1:50, and secondary antibodies anti-mouse-AF488 and anti-rabbit-AF647 (Molecular Probes) were diluted 1:200. The rest of the procedure is the same as 293CARV5 cells.

Ribosome Velocity Sedimentation in Continuous Sucrose Gradients

U87CAR cells were seeded on 150-mm culture plates until confluent. Before harvesting, cells were treated with 100 µg/ mL cycloheximide (CHX) for 5 min at 37ºC. Cells were then washed twice in ice-cold PBS containing CHX, collected in 5mL of PBS containing CHX, and centrifuged at 200 x *g* for 5 min at 4ºC. The supernatant was aspirated and the pellet resuspended in 425 µL of hypotonic buffer (5 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, 0.5% Triton X-100, 0.5% Sodium deoxycholate, 100 µg/ mL CHX, 1 mM DTT, protease inhibitors, and 100 U/ mL RNase inhibitor) and vortexed briefly. Samples were

chilled on ice for 12 min and then centrifuged at 16,000 x *g* for 8 min at 4ºC. The supernatant was collected and added onto a 5-50% sucrose gradient (200 mM HEPES pH 7.6, 1 M KCl, 50 mM $MgCl_2$, 100µg/ mL CHX, protease inhibitors, 100 U/ mL RNase inhibitor). The sucrose gradient was then centrifuged at 222,228 x *g* for 2 hours at 4ºC using a SW41Ti rotor. Following centrifugation, gradients were fractionated with a Foxy Jr. Fraction Collector (ISCO, Inc.) at a rate of 30 sec per fraction and data collected using an absorbance of 254 nm. Proteins in collected fractionations were precipitated by incubating with 14% w/v trichloroacetic acid at 4ºC overnight, centrifuged at 16,000 x *g* for 20 min at 4ºC, washed with ice-cold acetone, centrifuged at 16,000 x *g* for 10 min at 4ºC, washed with cold acetone and centrifuged again for 10 min. The pellets were air-dried, reconstituted in 50 µL of 2xLaemmli loading dye, boiled at 90ºC for 5 min, and subjected to Western blot analysis.

In Vitro Translation Assay in Rabbit Reticulocyte Lysates

Rabbit reticulocyte lysates (Promega) were used for testing CAR's effects on translation *in vitro*. For each reaction, 7 µL of rabbit reticulocyte lysates, 1-9 µg of recombinant GST-proteins (in 3 µL of *in vitro* translation buffer), 0.2 µg of dual luciferase reporter mRNA, RNaseOUT and 20 amino acids were mixed together. The recombinant GST-proteins (GST, GST-CAR, -ΔSIV, -Δ26, -Δ74, and -Δ98) were eluted in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM reduced glutathione (Sigma), dialyzed against *in vitro* translation buffer (50 mM potassium acetate, 20 mM Tris-HCl pH 7.5, 10% glycerol, 0.1 mM EDTA, 1 mM

DTT, and protease inhibitors), and quantified by SDS-PAGE with BSA standards. Reporter dual luciferase mRNA was generated by *in vitro* transcription of a plasmid pSP/$(CAG)_{33}$/FF/HCV/Ren·pA_{51} (Novac et al., 2004) using the mMESSAGEmMACHINE kit (Ambion) and quantified by O.D. reading. The *in vitro* translation mixtures were incubated at 30°C for 90 min and the firefly/ *Renilla* luciferase activity was measured using a Dual Luciferase Reporter Assay kit (Promega) in a 1250 Luminometer (BioOrbit). This assay was repeated with two independent recombinant GST-protein preparations and three independent retic lysate assays with each preparation.

Cell-Based Translation Assays in Cultured CHO Cells

To test the effects of CAR's cytoplasmic region on translation in CHO cells, the full-length CAR plasmid pcDNA3-CAR (pCAR), the C-terminal truncation plasmids (pCAR-ΔSIV, pΔ26, pΔ50, pΔ74, and pΔ98), and the empty plasmid pcDNA3 as a control were co-transfected with the dual luciferase reporter plasmid pUC18-CMV-T7-myc-R-luc-HCVIRES-myc-F-luc (Uchida et al., 2002). To test the effects of CAR's N-terminus on translation, CAR's extracellular domain deletion plasmids (pCAR-ΔD1, pΔD2, and pD0, Excoffon et al., 2005) were co-transfected into CHO cells. For each well (24-well plates), increasing amount of pCAR and its truncation plasmids (maximal amount 300 ng, the amount less than 300 ng was brought to 300 ng using pcDNA3), 100ng of dual luciferase reporter plasmid, and 1.5 µg of polyethylenimine (PEI, 25 kDa linear, Polysciences) were mixed in 25

μL of OptiMEM (Gibco) and incubated at room temperature for 10 min. The transfection mixture was then added onto CHO cells ($3x10^4$ cells/ well), and 24 hr later the cells were lysed and luciferase activity measured using a Dual Luciferase Reporter Assay kit (Promega). The lysates were also subjected to Western blot to monitor the expression of CAR and its truncation proteins.

Cell-Based Translation Assays with CAR-FN40 Ligand Engagement

For FN40-CAR ligand engagement experiment, 100 ng of pCAR, pΔD1, pΔD2, and pD0 together with 200 ng of pcDNA3 and 100ng of dual luciferase reporter plasmid were transfected with PEI as described above. As a control, 300 ng of pcDNA3 and 100ng of dual luciferase reporter plasmid were also transfected. Cells were starved with serum-free F12 medium for 2 hr to deplete residual soluble fibronectin in serum. Next, medium was aspirated, serum-free F12 (- FN40) or FN40-containing F12 (+FN40, 20 μg /mL) was added to the cells, and 15 min later the cells were lysed for measuring luciferase activity. The expression of CAR and truncates was examined by Western blot.

Cell-Based Translation Assays in U87CAR/ LNCX Cells

For transfection into U87CAR/ LNCX cells (24-well plates), 250 ng of reporter plasmid

pUC18-CMV-T7-myc-R-luc-HCVIRES-myc-F-luc, 240 ng of pcDNA3, and 10 ng of pCMV-β-Gal (as a transfection internal control) were mixed with 1.5 µL Mirus TransIT 2020 (Mirus Bio LLC) in 50 µL OptiMEM (Gibco), incubated for 20 min at room temperature, and then added onto U87-lineage cells ($4x10^4$ cells/ well). Forty-eight hours post-transfection, the cells were lysed and the firefly/ *Renilla* luciferase activity measured using a Dual Luciferase Reporter Assay kit (Promega) with a 1250 Luminometer (BioOrbit).

Western Blot for mTOR Signaling Pathway

U87CAR cells were cultured to 70-90% confluency and then incubated in serum-free DMEM for 24 hr to deplete soluble growth factors. The cells were then treated with serum-free DMEM containing 400 nM insulin, 20 µg/mL FN40, or no reagent control for 0, 15, 30, and 60 min. Cells were lysed using HBST containing protease inhibitors and phosphotase inhibitors on ice for 10 min, centrifuge at 4°C for 10 min, and the supernatant were saved. Equal amount of proteins were run on a SDS-PAGE and subjected to Western blot using antibodies (all from Cell Signaling) against 4E-BP1 total (#9644), 4E-BP1-phosphorylated (#9459), S6K total (#9202), and S6K phosphorylated (#9234).

Isolation of Neonatal Nerve Growth Cone Particles

The growth cone particles (GCPs) were isolated from postnatal day 6 (P6) CD1 mouse brains using a previously described method (Gordon-Weeks and Lockerbie, 1984) with minor modifications. Briefly, around ten P6 mouse brains (~ 2.5 g, wet weight) were homogenated in 40 mL homogenating buffer (H Buffer: 10 mM HEPES pH 7.4, 0.32 M sucrose, 1 mM $MgCl_2$, and protease inhibitors) for 10 strokes at 800 rpm. The homogenates (H, a small aliquot was saved for Western) were centrifuged at 1,000 x *g* for 8 min at 4ºC, the supernatant (S1) were saved at 4ºC and the pellet (P1) resuspended in another 40 mL of H Buffer. The new resuspension were centrifuged again at 1,000 x *g* for 8 min at 4ºC, the supernatant (S1') were saved at 4ºC and the pellet (P1') was saved for Western blot. The supernatants S1 and S1' were then combined (S, saved for Western) and centrifuged at 13,300 x *g* at 4ºC for 15 min to produce S2 and P2 fractions. The P2 fraction was resuspended in 40 mL H Buffer and centrifuged again at 13,300 x *g* at 4ºC for 15 min to produce S2' and P2' fractions. The P2' pellets were resuspended in 1 mL of H Buffer and added on the top of a discontinuous Ficoll gradient (7.5/12%, w/v in H Buffer) and centrifuged at 100,000 x *g* at 4ºC for 60 min in an ultracentrifuge (Beckman Coulter). The GCPs and synaptosomes (SYPs) were then extracted from the interfaces between the sample and 7.5% Ficoll, and between the 7.5% and 12% Ficoll, respectively. These extractions were then diluted four times with H Buffer and centrifuged at 39,800 x *g* at 4ºC for 30 min. The final pellets were resuspended either in HBST buffer for GST-pulldown assay or in cap-pulldown lysis buffer for m^7GTP-pulldown assay.

Culture of Mouse Embryonic Cortical Neurons

Cortical neurons were isolated from embryonic day 17 (E17) CD1 mouse brains as described (Beaudoin et al., 2012). The cortices were dissected out and placed in 4.5 mL of ice-cold Hank's balanced salt solution (HBSS, Gibco) supplemented with 1 mM $CaCl_2$, 1 mM $MgSO_4$, 30 mM D-glucose, 2.5 mM HEPES pH7.4, and 4 mM $NaHCO_3$. Cortices were treated with 0.5 mL of 2.5% phenol red-free trypsin (final concentration 0.25%) at 37ºC for 10 min, added with 0.5 mL of heat-inactivated FBS, and then triturated 5-6 times using a fire-polished Pasteur pipette. Dissociated single neurons were then separated from tissue debris by filtering through a 70-µm cell strainer and centrifuged at 200 x *g* for 5 min at room temperature. The supernatant was discarded and the neuronal cell pellet resuspended with NeuroBasal medium (Gibco) supplemented with B27, N2, 0.5 mM L-glutamine, and 100 U/mL Penicillin/ Streptomycin (all from Gibco). Neurons were then counted and seeded onto culture plates (for neurite outgrowth assays) or onto coverslips (for immunofluorescence) which were pre-treated with 33 µg/mL poly-D-lysine (PDL) at 4ºC overnight. For neurite outgrowth and immunofluorescence studies, $2x10^4$ neurons per well were seeded for 24-well plates. Neurons were grown at 37ºC with 5% CO_2 for 24 hr for neurite outgrowth assays and 48 hr for immunofluorescence studies.

Neurite Outgrowth Assay

When coating in 24-well plates, 33 µg/mL PDL in 500 µL of PBS as control (PDL), or 2 µg of FN40 per well together with 33 µg/mL PDL in 500 µL of PBS (FN40) was incubated at 4ºC overnight. For infection with adenoviral control vector (Ad-BFP) and CAR-antisense construct (Ad-asCAR), a multiplicity of infection (MOI) of 50 was used. Adenoviral vectors were mixed with neurons before adding to plates and infected neurons were incubated for 24 hr. For cycloheximide (CHX) treatment, the neurons were incubated for a few hours to attach, and then 1 µM CHX was added and cultured for 24 hr. Neuronal cultures were then fixed with 4% paraformaldehyde (PFA) in 4% w/v sucrose in PBS at 37ºC for 15 min, stained with coomassie blue (0.1% w/v coomassie blue R-250, 50% methanol, 10% glacial acetic acid) at room temperature for 10 min, and then rinsed with PBS. Photos were taken under a phase-contrast microscope and neurite length quantified manually with ImageJ. Only neurites that are longer than twice the diameter of the somata were quantified. For living/ dead cell staining, neurons grown on coverslips for 24 hr were incubated with 20 µg/mL propidium iodide (staining dead neurons) and 10 µg/mL Hoechst 33342 (staining all neurons) for 30 min in the 37ºC incubator, fixed with 4% PFA in 4% w/v sucrose in PBS at 37ºC for 15 min, and mounted to coverslides using ProLong Gold (Molecular Probes). Photos were then taken under a widefield fluorescent microscope.

AHA Protein Metabolic Labeling

AHA labeling was performed with cortical neurons using the Click-iT Protein Synthesis Assay Kit (Invitrogen). Neurons were seeded onto 24-well plates containing coverslips or 96-well plates (black plate, clear bottom, Corning) pre-coated with 33 μg/mL PDL (PDL), 33 μg/mL PDL plus 4 μg/mL FN40 (FN40) or PDL plus 4 μg/mL collagen type I (CL). Neurons were grown for 18 hr and then incubated with methionine-free DMEM (Gibco) for 30 min to deplete methionine, and treated with DMEM containing 50 μM L-homopropargylglycine (HPG, referred to as AHA for simplicity) for 1-6 hr to incorporate AHA in place of methionine. At this stage, treatments with DMEM containing 50 μM L-methionine (Met) and AHA-DMEM containing 40 μM CHX (CHX) were also applied as control. The neurons were then fixed with 4% PFA in 4% w/v sucrose in PBS for 30 min at 37ºC, permeabilized with 0.5% Triton X-100 for 15 min at room temperature, and subjected to Click chemical reaction as described by the manufacturer. The labeled neurons were stored in PBS at 4ºC, photos acquired with a widefield fluorescent microscope, and the relative fluorescence intensity of each neuron was quantified using ImageJ.

Mouse Behavioral Analysis with Open-Field Test

Mice in their cages were transferred from the original housing room to the test room and were kept undisturbed for 30 min. This step allowed the mice to get used to the new environment

and to avoid unnecessary anxiety. Then each mouse was placed in the open-field cage (a sterile rat cage) for 10 min to habituate and after the habituation session the open-field behavior was video-recorded for 10 min. The numbers of defecation boli were counted after this 20 min period in the open-field cage. Between each mouse, the open-field cage was cleaned using odorless detergent followed by distilled water. The time spent in self-grooming, as well as in rest and ambulatory movement were then scored and statistically analyzed.

Evaluation of Mouse Motor System using Rota-Rod Performance Test

The Rota-rod tests were performed in four consecutive days including three training days and one test day. On training day 1, the mice were placed onto the Rota-rod with a 5 rpm (revolutions per minute) fixed speed for a maximum of 300 sec; the mice were then placed onto the Rota-rod with an increasing speed of 5-8 rpm for a maximum of 300 sec. On training day 2, the mice were trained with 5-8 rpm (300 sec) and 5-12 rpm (300 sec). On training day 3, the mice were trained with 5-12 rpm (300 sec), 5-20 rpm (300 sec), and 5-20 rpm (300 sec). During training days 2 and 3, mice, especially the CAR-knockout mice, fell off frequently. When mice fell, they were placed back onto the Rota-rod immediately, and the training session was stopped if a mouse fell for 5 times. Between each training session, mice were allowed to rest for 30 min.

On the test day, one type of Rota-rod test with an accelerating speed was performed by placing the mice onto the Rota-rod with an increasing speed of 5-40 rpm for a maximum of

300 sec. Each mouse was tested for six times and between each test the mouse was allowed to rest for 30 min. The latency to fall (sec) and the rotation speed at fall (rpm) were recorded and statistically analyzed.

Another type of Rota-rod test with incremental fixed speeds was also performed. After three days of training, the mice were placed on the Rota-rod with fixed rotation speeds at 5, 10, 15, 20, and 25 rpm for a maximum of 300 sec. Each mouse was tested for three times and between each test the mouse was allowed to rest for 30 min. The latency to fall (sec) was recorded and statistically analyzed.

Measurement of Mouse Muscle Strength and Endurance

For both the strength and endurance tests, mice were brought to the experimental room 30 min before testing to ensure that they awoke properly. The muscle strength is evaluated by a weight-lifting test slightly modified from an existing procedure (Deacon, 2013b). This test only measures the strength of the forelimbs. The weight-lifting apparatus consists of a fur collector as the grip point for the mouse (core weight) and a series of chain links with increasing length (auxiliary weights). The core weight contains a big chain link which is tied to a soft plastic fur collector with fine wire mesh (with which the mouse can grip better with its small claws); this core weighs 26.4 g. The auxiliary weights are composed of much smaller chain links and each link weighs 3.7 g. Therefore, the total weight of this apparatus

can be 26.4, 30.1, 33.8, and 37.5 g and so on. The mouse was allowed to grip the fur collector and then held by its tail in the midair (1 m above the ground). If the mouse could hold the first weight (26.4 g) for more than 5 sec, then it would be tested for the next weight (30.1 g). If the mouse could not hold the weight for more than 5 sec, it would be tested again and if it failed three times, this weight as its maximal strength was recorded. Each mouse was tested for three times and mice were allowed to recover their muscular strength for 30 min between each test.

The muscle endurance was measured by an inverted cage test which is testing mouse muscle endurance with all four limbs. A mouse was put onto the center of a metal wire cage lid and the cage lid was gently inverted. The inverted metal cage lid was held in the midair at 1 m above the floor for a maximum of 300 sec (at which time most of the mice would have fallen). A clean diaper was put on the ground to protect the falling mice. The latency to fall (sec) was recorded and the test was repeated three times for each mice. As a general rule, mice were allowed to recover their muscular endurance for 30 min between each test.

Clasping Assay

Mice in cages were transferred to the test room and habituated for 30 min. Then each mouse was held by its tail in the midair (1 m above the ground) and was video-recorded for 20 sec. After 30 min, each mouse was video-recorded again to exclude clasping that may be caused

by elevated anxiety at the first time.

Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed using GraphPad Prism 3.0 by Student's *t*-test or one-way analysis of variance (ANOVA). Statistical significance was defined as: $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***.

CHAPTER 3

CAR ASSOCIATES WITH TRANSLATION MACHINERY AND REGULATES PROTEIN SYNTHESIS

Identification of Translational Proteins as CAR's Interactor Partners

To identify CAR's potential interactor partners, we performed an affinity pulldown using the recombinant cytoplasmic domain of CAR fused to a GST tag (GST-CAR). After affinity pulldown from U87CAR cell lysates, bound proteins were eluted, separated on an SDS-PAGE gel, silver-stained, and subjected to proteomic analysis as described previously (Fok et al., 2007). Mass spectrometry (MS) identified many translational proteins (Figure 3.1), such as ribosomal subunit proteins (RPS6 and RPL4), translation initiation factors (eIF4G, eIF4A, eIF3A, and eIF3C), elongation factors (eEF1A and eEF2), and mRNA-binding proteins (hnRNPU and LRPPRC). These results suggest that CAR may interact with translational proteins.

Verification of MS Results by GST Affinity Pulldown

To verify the MS results, first we performed GST-pulldown. We found that GST-CAR fusion protein indeed pulled down translational proteins from U87CAR cell lysates (Figure 3.2C). We then constructed a series of plasmids expressing GST-tagged CAR C-terminal truncates: deletion of the last three amino acids "SIV", and deletion of the last 26, 74, and 98 amino acids respectively (GST-CARΔSIV, Δ26, Δ74, and Δ98), as illustrated in Figure 3.2A. The integrity and molecular weights of these proteins were verified by Coomassie blue staining (Figure 3.2B).

By performing pulldown with these truncated proteins, we found that deletion of the last three amino acids Ser-Ile-Val (SIV) completely abolished the affinity binding of eIF4E, eEF1A, and ribosomal protein S6 (Figure 3.2C) suggesting that SIV, the PDZ-binding domain of CAR, is essential for binding to these translational proteins. However, the GST-ΔSIV and GST-Δ26 truncation proteins did not affect CAR's binding to hnRNPU, indicating that CAR may bind to other proteins through different domains within its cytoplasmic tail.

CAR Colocalizes with Translational Proteins as Revealed by Polysome Profiling

To further study physical interactions between CAR and the components of translation machinery, we performed the polysome profiling experiment which can separate the 40S subunit, 60S subunit, 80S monosome, and polysomes based on their sedimentation coefficients. The O.D. 254 reading, an indicator of ribosomal RNA (rRNA) abundance, showed three major peaks representing the 40S small subunit, 60S large subunit, and 80S monosome (Figure 3.3A); a few small peaks representing polysomes were also present (Figure 3.3A) suggesting a successful fractionation of translation machinery.

We then examined the colocalization of CAR with translational proteins in these fractions. CAR signal was detected predominantly in the translation initiation complex fractions

(colocalized with eIF4E and eIF4G, from the 40S subunit to the 80S mono-ribosome fractions) and faint signal of CAR was also present in early polysome fractions (Figure 3.3B). These results suggest that CAR may predominantly interact with the translation initiation complex and regulate translation initiation and CAR may also, to a lesser extent, bind to elongating polysomes and modulate elongation.

CAR Resides in the mRNA Cap-Binding Complex and Directly Binds to eIF4E

Since CAR appears to colocalize with eIF4E and eIF4G (both are the components of the mRNA cap-binding complex), CAR may reside in this complex. To test this hypothesis, we performed an m^7GTP cap-pulldown assay in which the m^7GTP, an analogue of the mRNA 5' cap, is covalently linked to Sepharose beads. After incubation of m^7GTP-Sepharose or control Sepharose beads in U87CAR lysates, the proteins were eluted and analyzed by Western blot. As shown in Figure 3.4A, m^7GTP captured eIF4E and eIF4G while the control beads did not, suggesting a successful pulldown of the cap-binding complex. Additionally, m^7GTP also captured CAR, indicating that CAR may indeed bind to this complex.

To evaluate whether CAR directly binds to the cap-binding protein, eIF4E, we cloned a 6xHis-tagged eIF4E (His-eIF4E) plasmid and purified the recombinant His-eIF4E protein. The molecular weight (~31 kDa) and protein integrity of His-eIF4E were examined by SDS-PAGE and coomassie blue staining (Figure 3.4B). The recombinant His-eIF4E, as well

as GST-CAR fusion proteins coupled to glutathione-beads, were mixed and incubated in GST-pulldown buffer (HBST buffer, see Materials and Methods). The samples were eluted and analyzed, and we found that GST-CAR indeed pulled down His-eIF4E (Figure 3.4C). The control GST or the GST-ΔSIV on the other hand, did not pulldown His-eIF4E (Figure 3.4C). These results suggest that CAR directly interacts with eIF4E and this interaction requires the last three amino acids – SIV.

CAR Colocalizes with Translational Proteins as Revealed by Immunofluorescence

To visualize co-localization between CAR and translational proteins in cultured cells, we performed co-immunofluorescence (co-IF) on HEK293 cells stably expressing full-length CAR with a V5 tag (293CARV5 cells). Cells grown to different confluency (at 20% confluency when cells were separate from each other, or at 90% confluency when cells grew to a monolayer) were fixed, stained with antibodies, and analyzed by a laser-scanning confocal microscopy. In individual 293CARV5 cells, CAR immunostaining partially overlapped with that of ribosomal protein L4 (Figure 3.5A) and S6 (data not shown). Notably, CAR and L4 significantly colocalized at the cell margin in the filopodia- and lamellipodia-like structures (Figure 3.5A, arrowheads). On the other hand, only L4 localized in the nucleolus (Figure 3.5A, arrows) but CAR did not.

Additionally, it is reported that when growing confluent, HEK293 cells expressing CAR can

form cell-cell contacts and CAR is highly enriched at these contacts (Sollerbrant et al., 2003). To examine whether CAR and translational proteins colocalize at these contacts, we grew 293CARV5 cells until confluency. As shown in Figure 3.5B, although considerable colocalization can be seen in the cytoplasm, very weak immunostaining of L4 was detected in the cell-cell contacts where CAR was enriched, suggesting that CAR and ribosomes do not colocalize in these contacts. One possibility is that when CAR forms the cell-cell contacts, the *in trans* homophilic interaction of CAR releases the ribosomes from these contacts.

CAR Functionally Affects In Vitro Translation in Reticulocyte Lysates

To determine whether CAR functionally regulates translation, we performed *in vitro* translation assays using rabbit reticulocyte lysates. In this system, reporter mRNA, GST-CAR proteins, and reticulocyte lysates (containing all components of translation machinery except for mRNA) were mixed together and therefore CAR's mechanistic effects on translation could be evaluated. The bicistronic translation reporter mRNA contains a firefly luciferase under the control of 5'-cap and a *Renilla* luciferase under the control of an HCV IRES (a gift from Dr. Pelletier, Novac et al., 2004), and thus CAR's effects on cap-dependent or IRES-dependent translation can be determined by the activity of the respective luciferases.

The GST-CAR fusion proteins (GST, CAR, ΔSIV, Δ26, Δ74, and Δ98) and the reporter mRNA were added into the *in vitro* translation mixture, and firefly and *Renilla* luciferase

activities measured. As shown in Figure 3.6A and B, full-length cytoplasmic domain of CAR significantly inhibited both cap-dependent and IRES-dependent translation, and the inhibitory effects were dose-dependent. Consistent with our pulldown results (Figure 3.2C), deletion of the last three amino acids SIV (ΔSIV) ablated CAR's inhibitory effects on translation, while Δ74 and Δ98 had no effect on translation (Figure 3.6A, B). Additionally, only GST-CAR significantly decreased the Cap/ IRES ratio (Figure 3.6C), suggesting that the last three amino acids SIV may differentially associate with the cap-binding complex and affect cap-dependent translation.

CAR Regulates Translation in U87CAR and U87LNCX Cell Lines

To evaluate whether CAR functionally regulates translation in living cells, we performed a cell-based translation assay by transfecting a translation reporter plasmid into cells stably expressing CAR (U87CAR) or cells stably transfected with an empty vector (U87LNCX). The reporter plasmid is also bicistronic containing a *Renilla* luciferase under 5'-cap control and a firefly luciferase under HCV-IRES control (a gift from Dr. Sonenberg; Uchida et al., 2002). To exclude the possibility that U87CAR and U87LNCX cells may have different levels of transfectability, a transfection control pCMV–β-Gal (expressing β-galactosidase) was also co-transfected with the bicistronic reporter plasmid. Beta-gal activities of U87CAR and U87LNCX cells were not significantly different suggesting that these two cell lines have no difference in transfectability (Figure 3.7C). However, reporter translation in U87CAR

cells is significantly lower than in U87LNCX cells (Figure 3.7A, B), suggesting that full-length CAR indeed regulates translation in cultured U87 cells.

CAR's Translational Control Requires An Intact C-Terminus, but Not N-Terminus

To further determine whether CAR controls translation in cultured cells, we performed another cell-based translation assay by transiently co-transfecting translation reporter plasmid and CAR plasmids into CHO cells. We first tested the effects of transfecting full-length CAR plasmid (pCAR) into CHO cells. Full-length CAR significantly decreased both cap-dependent and IRES-dependent translation in a dose-dependent manner (Figure 3.8C, D). We then constructed CAR's C-terminal truncation plasmids and examined their protein integrity by Western blot (pCAR, pΔSIV, pΔ26, pΔ50, pΔ74, pΔ98; Figure 3.8A, B). Similar to the *in vitro* translation results (Figure 3.6), the inhibitory effects of CAR were greatly reduced with SIV-deletion, and Δ26, Δ50, Δ74, Δ98 had almost no effect on translation (Figure 3.8C, D). These results suggest that the translation regulatory effects of CAR require an intact C-terminus, particularly the SIV.

To investigate whether CAR-regulated translation requires an intact N-terminus, we tested the effects of CAR's N-terminal truncates on translation. The extracellular domain of CAR contains two immunoglobulin loops, D1 and D2, respectively. We therefore tested the effects of CAR constructs lacking D1 (ΔD1), D2 (ΔD2), or both (D0) (gifts from Dr. Zabner;

Excoffon et al., 2005) on translation. The illustrative representations of these deletion mutants and the expression of these mutant proteins are shown in Figure 3.9A, B. We found that all of the truncation mutants behaved like full-length CAR and they decreased translation to a similar level (Figure 3.9C, D) suggesting that when no ligands are added, regulation of translation does not necessarily require an intact N-terminus of CAR.

CAR-FN40 Interaction Enhances Translation which Requires CAR's D2 Domain

We hypothesized that ligand-triggered translation may require an intact N-terminus of CAR. To test this hypothesis, we performed a ligand engagement assay with wildtype CAR and CAR's N-terminal truncation proteins. One of CAR's known ligands, FN40 (a 40-kDa subunit of fibronectin), directly interacts with CAR through CAR's D2 domain and its binding to CAR promotes neurite outgrowth (Patzke et al., 2010). However, the effect of FN40-CAR interaction on protein synthesis is unknown. To test whether CAR-FN40 interaction promotes translation, we first performed an optimization experiment with 0, 4, 20, and 40 μg/mL of FN40 and analyzed the cell lysates obtained at 0, 15, 30, and 60 min after adding FN40. We found that cells transfected with pCAR, but not with pDNA, responded to FN40 at a concentration as low as 4 μg/mL, and the response saturated at 20 μg/mL; this increase of translation took place as soon as 15 min (data not shown). We then tested the effect of CAR full-length and truncation plasmids on translation in cells treated with 20 μg/mL FN40 for 15 min. We found that FN40 significantly increased translation in pCAR- and

pΔD1-transfected cells (both expressing the D2 region) but not in pcDNA-, pD0, and pΔD2-transfected cells (none expressing the D2 region). These results suggest that FN40-triggered translational increase requires the D2 region of CAR.

CAR-FN40 Interaction Does Not Trigger mTOR Signaling Pathway

To determine whether this FN40-induced translational increase in CAR-expressing cells is due to mTOR signaling pathway, we tested 4E-BP1 and S6K phosphorylation in U87CAR cells. We cultured U87CAR cells to 70-90% confluency, serum-starved these cells for 24 hr, and treated the cells with serum-free medium containing 400 nM insulin, 20 μg/mL FN40, or no reagent control for 0, 15, 30, and 60 min. We then subjected the cell lysates to Western blot analysis and found that while insulin induced a significant increase in the phosphorylation of 4E-BP1 and S6K, FN40 did not elevate 4E-BP1 and S6K phosphorylation, resembling the non-treated control. These results suggest that CAR-FN40 does not regulate translation through mTOR pathways, but more likely through physical protein association/ dissociation.

Figure 3.1

Ribosomal Proteins

Protein Name	Total Score
40S Small Subunits	
RPS3	528
RPS2	142
RPS6	120
60S Large Subunits	
RPL13	210
RPL4	97, 84
RPL14	85

Translation Regulatory Proteins

Protein Name	Total Score
eIF3A	525, 293, 97
eIF3C	144, 91, 68
eIF3E	351
eIF4A1	269
eIF4G1	385, 275
PABPN1	108, 58
eEF1A1	508, 404, 274
eEF2	252

mRNA-Binding Proteins

Protein Name	Total Score
LRPPRC	1034
hnRNPU	758, 603, 537, 423

Figure 3.1. **Identification of translational proteins as CAR-interacting partners by mass spectrometry.** Mass spectrometry identification of CAR's interactor proteins after affinity pulldown using a GST-CAR fusion protein from U87CAR cell lysates. Many translational proteins were identified with high significance as shown in the Total Score (Mowse score, significance set at >32).

Figure 3.2

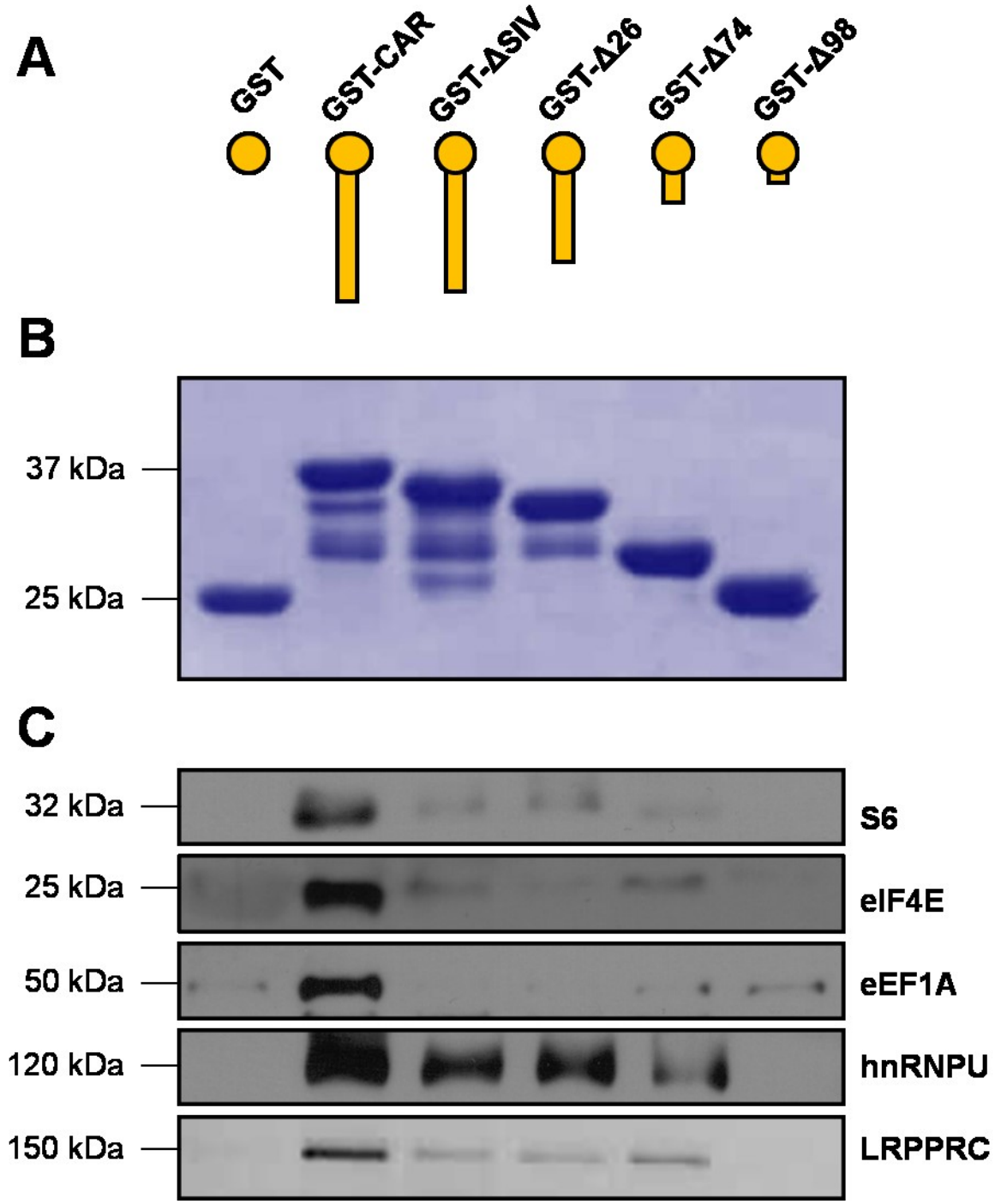

A
GST
GST-CAR
GST-ΔSIV
GST-Δ26
GST-Δ74
GST-Δ98
B
37 kDa
25 kDa
C
32 kDa
S6
25 kDa
eIF4E
50 kDa
eEF1A
120 kDa
hnRNPU
150 kDa
LRPPRC

Figure 3.2. **Physical interactions of CAR with translational proteins as confirmed by affinity GST pulldown.** (A) An illustrative representation is shown for GST-CAR and progressive truncation fusion proteins. (B) The size and integrity of these fusion proteins were verified by coomassie blue staining. (C) The pulldown samples from U87CAR cell lysates were then analyzed by Western blot using antibodies against translational proteins.

Figure 3.3

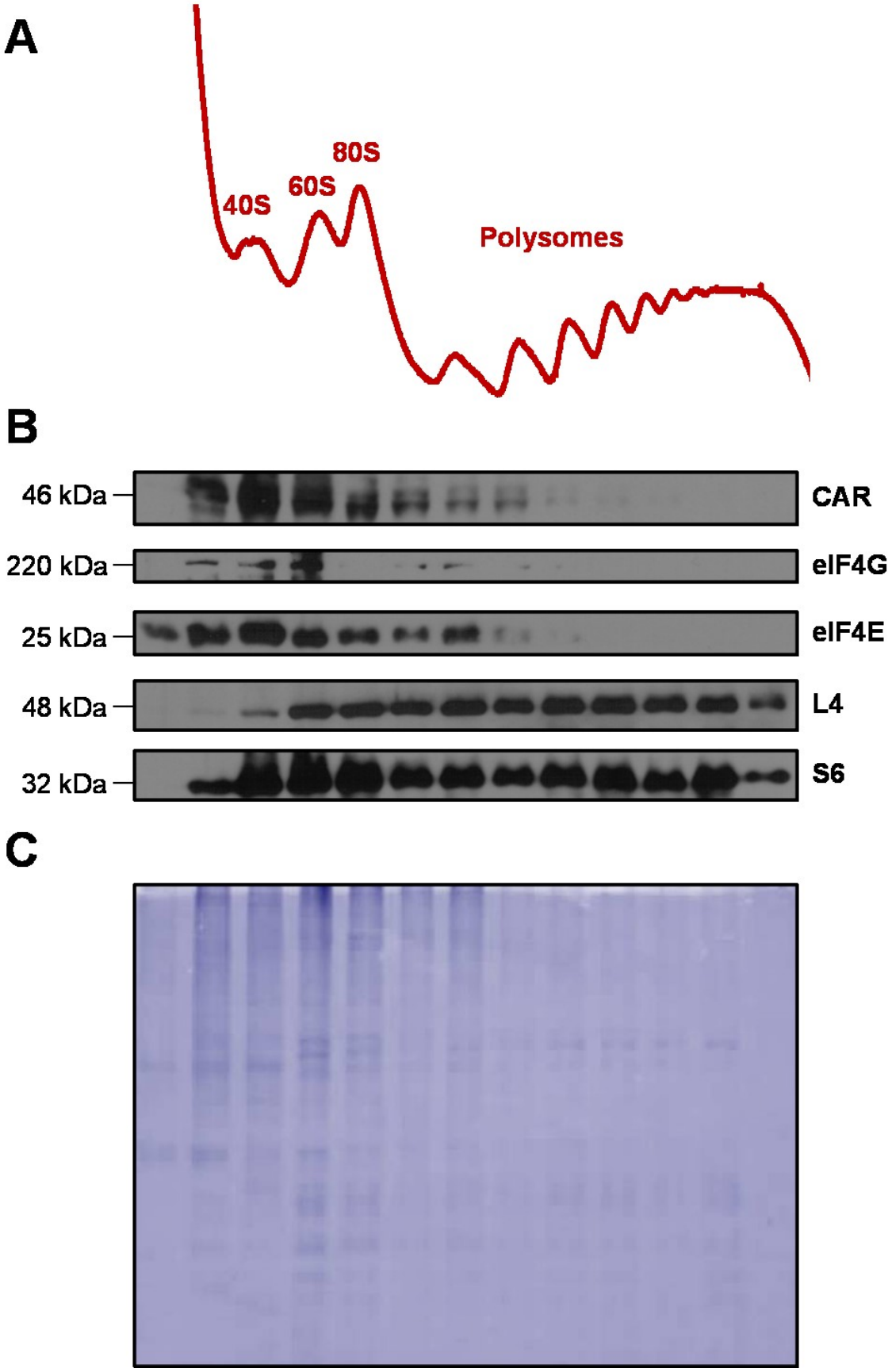

A
40S
60S
80S
Polysomes
B
46 kDa
CAR
220 kDa
eIF4G
25 kDa
eIF4E
48 kDa
L4
32 kDa
S6
C

Figure 3.3. **Colocalization of CAR with translational proteins as revealed by polysome profiling in sucrose gradients.** Ribosomal fractionations obtained from U87CAR cell lysates were subjected to continuous O.D. 260 reading for RNA abundance (A), analyzed by Western blot using antibodies against translational proteins (B) and by coomassie blue staining of the SDS-PAGE gel after transferring (C).

Figure 3.4

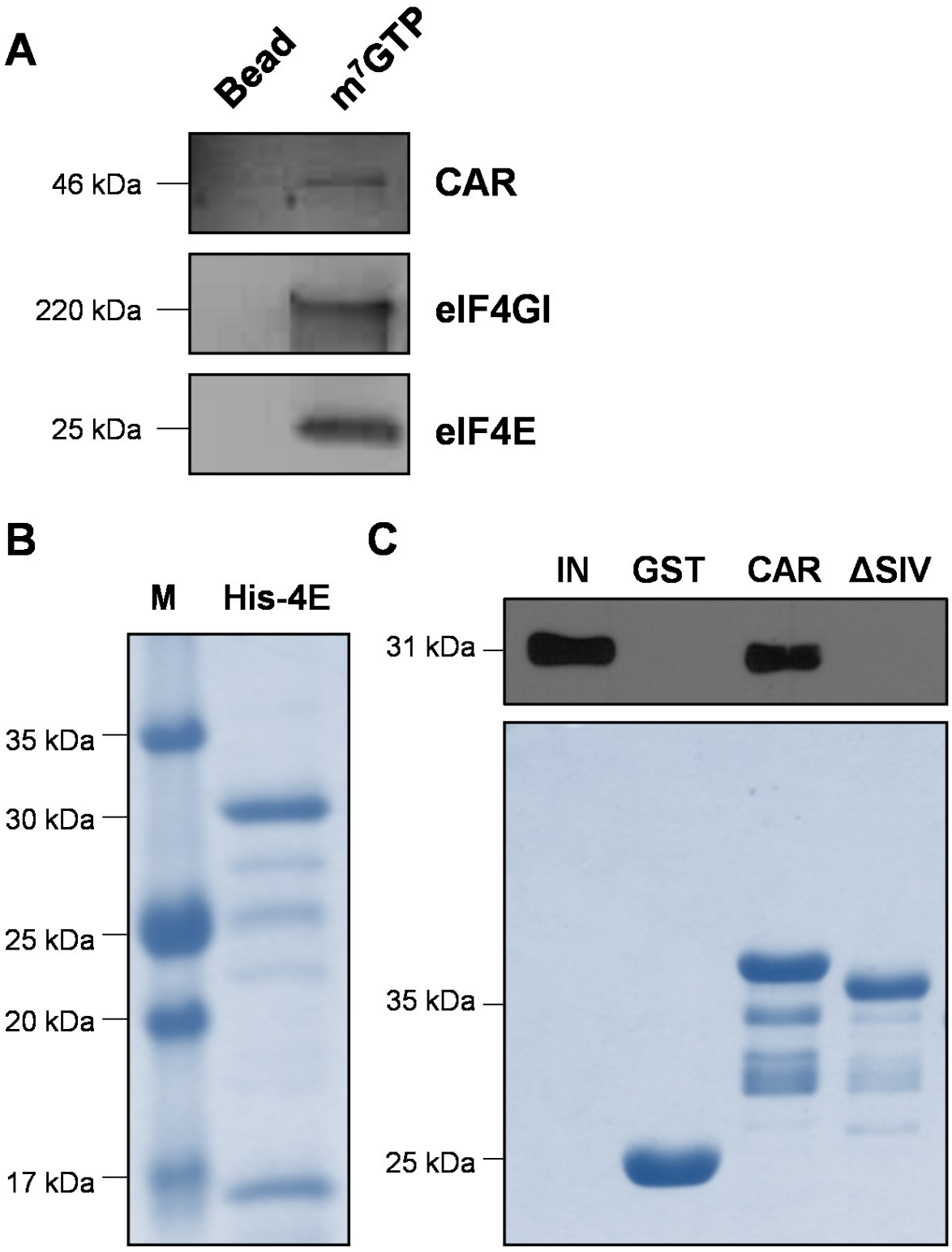

A
Bead
m7GTP
46 kDa
CAR
220 kDa
eIF4GI
25 kDa
eIF4E
B
M
His-4E
35 kDa
30 kDa
25 kDa
20 kDa
17 kDa
C
IN
GST
CAR
ΔSIV
31 kDa
35 kDa
25 kDa

Figure 3.4. **CAR resides in the cap-binding complex and directly interacts with eIF4E.** (A) m^7GTP cap-pulldown assay. Only m^7GTP, but not control beads, captured eIF4E and eIF4G, as well as CAR. (B) Coomassie blue staining of purified His-eIF4E fusion protein. His-eIF4E has an apparent molecular weight of ~ 31 kDa. (C) Direct interaction between CAR and eIF4E. Purified soluble His-eIF4E and GST-CAR coupled to glutathione beads were incubated and bound proteins were analyzed by Western blot. Only GST-CAR but not GST or GST-ΔSIV captured His-eIF4E, suggesting a direct interaction between CAR and eIF4E.

Figure 3.5

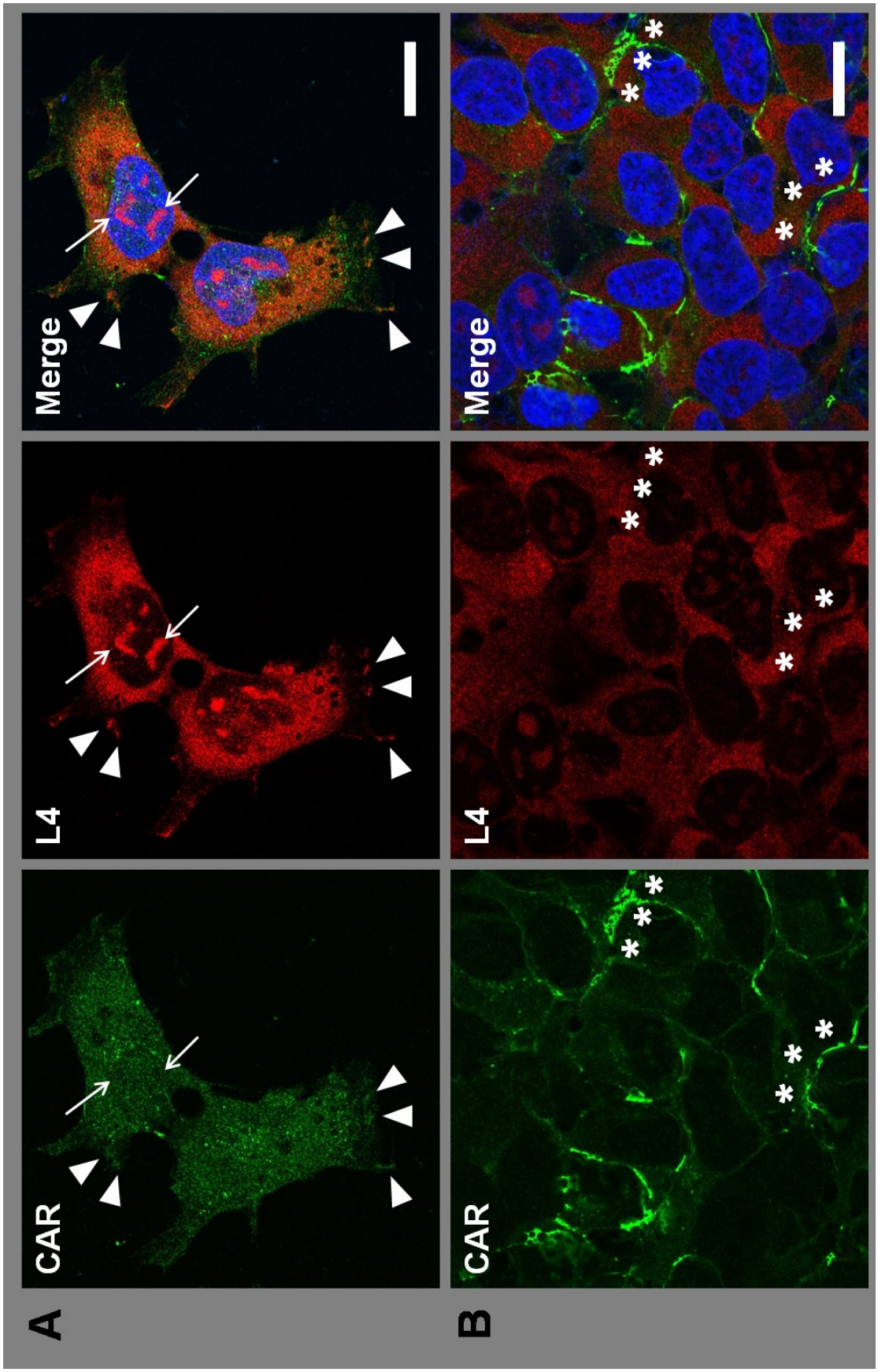


Figure 3.5. **CAR colocalizes with translational proteins as revealed by co-immunofluorescence (co-IF).** Cells were double-stained with antibodies against CAR (antibody 2240 recognizes the N-terminus of CAR) and ribosomal proteins L4 or S6 (only the results for L4 are shown) and analyzed by confocal microscopy. (A) Co-IF in single 293CARV5 cells. Superimposed, merged pictures are shown in the right panel, with yellow indicating co-localization. Partial colocalization is seen at the cell membrane and cytoplasmic region at the merged image. Notably, colocalization of CAR and L4 is detected at filopodia- and lamellipodia-like structures (arrowheads). The signal for L4, but not CAR, is detected in the nucleolus (arrows). (B) Co-IF in confluent 293CARV5 cell monolayer. Cells were double-stained with CAR and L4 or S6 antibodies. Strong CAR signal is detected at the cell-cell contacts where L4 signal is not present. Scale bar: 20 μm.

Figure 3.6

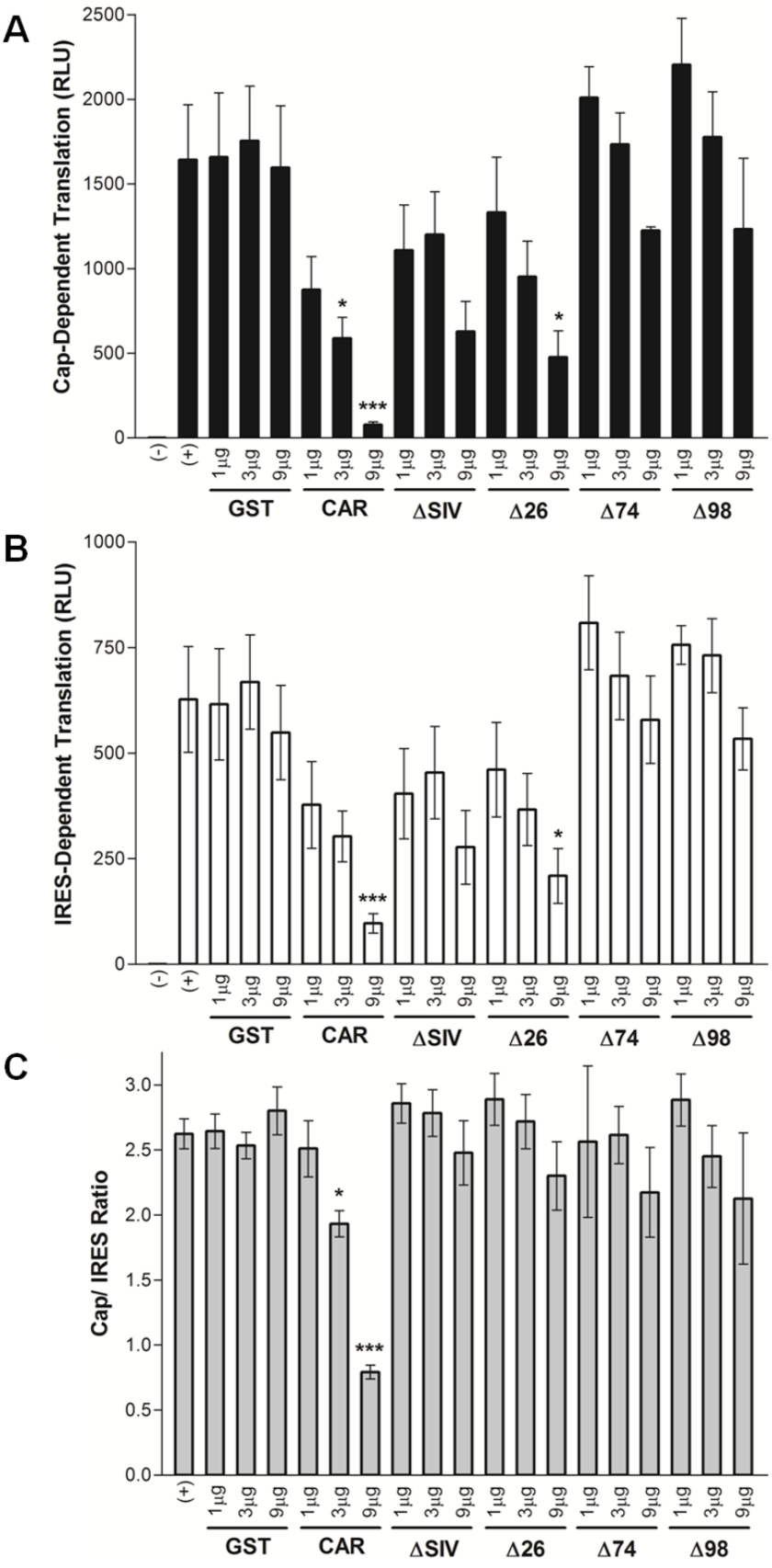

A
Cap-Dependent Translation (RLU)
2500
2000
1500
1000
500
0
*
***
*
(-)
(+)
1µg
3µg
9µg
GST
CAR
ΔSIV
Δ26
Δ74
Δ98
B
IRES-Dependent Translation (RLU)
1000
750
500
250
0
***
*
C
Cap/ IRES Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
***

Figure 3.6. **CAR functionally affects translation *in vitro* in rabbit reticulocyte lysates.** Increasing amounts (1, 3, 9 μg) of purified GST-CAR and truncation proteins were added into the retic lysates; GST was used as a control. (A) Cap-dependent translation: addition of GST-CAR significantly impaired translation but addition of up to 9 μg of GST did not have an effect. (B) HCV IRES-dependent translation: GST-CAR but not GST significantly decreased IRES-dependent translation. (C) Cap/ IRES ratio: only GST-CAR significantly affected this ratio. (-): Translation without the dual luciferase reporter mRNA. (+): Translation with the dual luciferase reporter mRNA but without fusion proteins. RLU: relative light unit. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.05$, *; $p < 0.001$, ***.

Figure 3.7

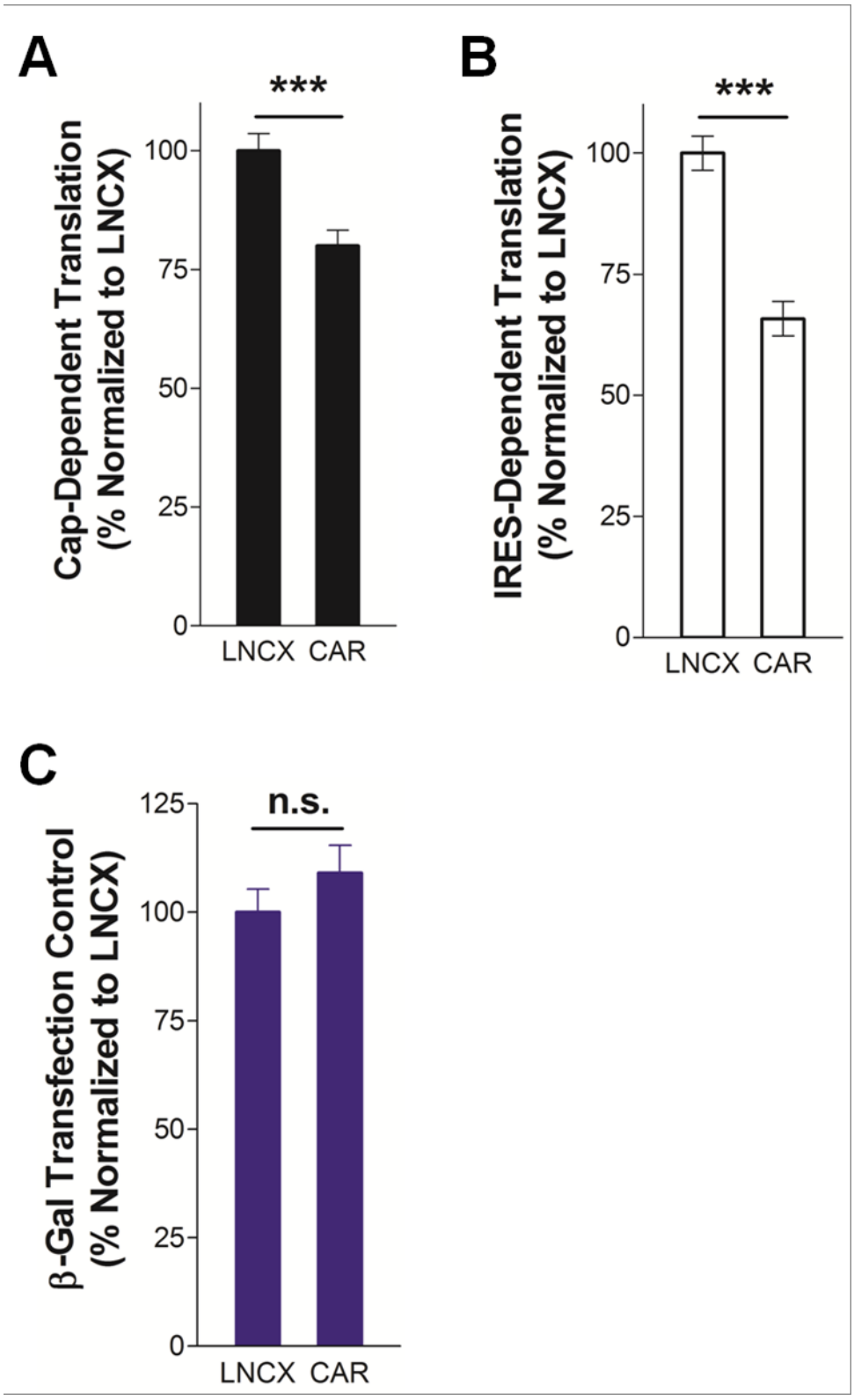

A
***
Cap-Dependent Translation
(% Normalized to LNCX)
100
75
50
25
0
LNCX CAR
B
***
IRES-Dependent Translation
(% Normalized to LNCX)
100
75
50
25
0
LNCX CAR
C
n.s.
β-Gal Transfection Control
(% Normalized to LNCX)
125
100
75
50
25
0
LNCX CAR

Figure 3.7. **CAR regulates translation in stably transfected U87 cells.** Translation assays were performed in U87 cells stably expressing full-length CAR (U87CAR) and U87 cells containing empty vector (U87LNCX). The dual luciferase reporter plasmid and a transfection control plasmid expressing β-galactosidase (β-gal) were co-transfected into U87CAR and U87LNCX cells. (A) Cap-dependent translation and (B) HCV IRES-dependent translation; luciferase readings were normalized to U87LNCX. C) U87CAR and U87LNCX cells were equally transfectable as revealed by the transfection control (β-gal readings). Beta-gal readings were normalized to U87LNCX. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.001$, ***. n.s.: not significant.

Figure 3.8

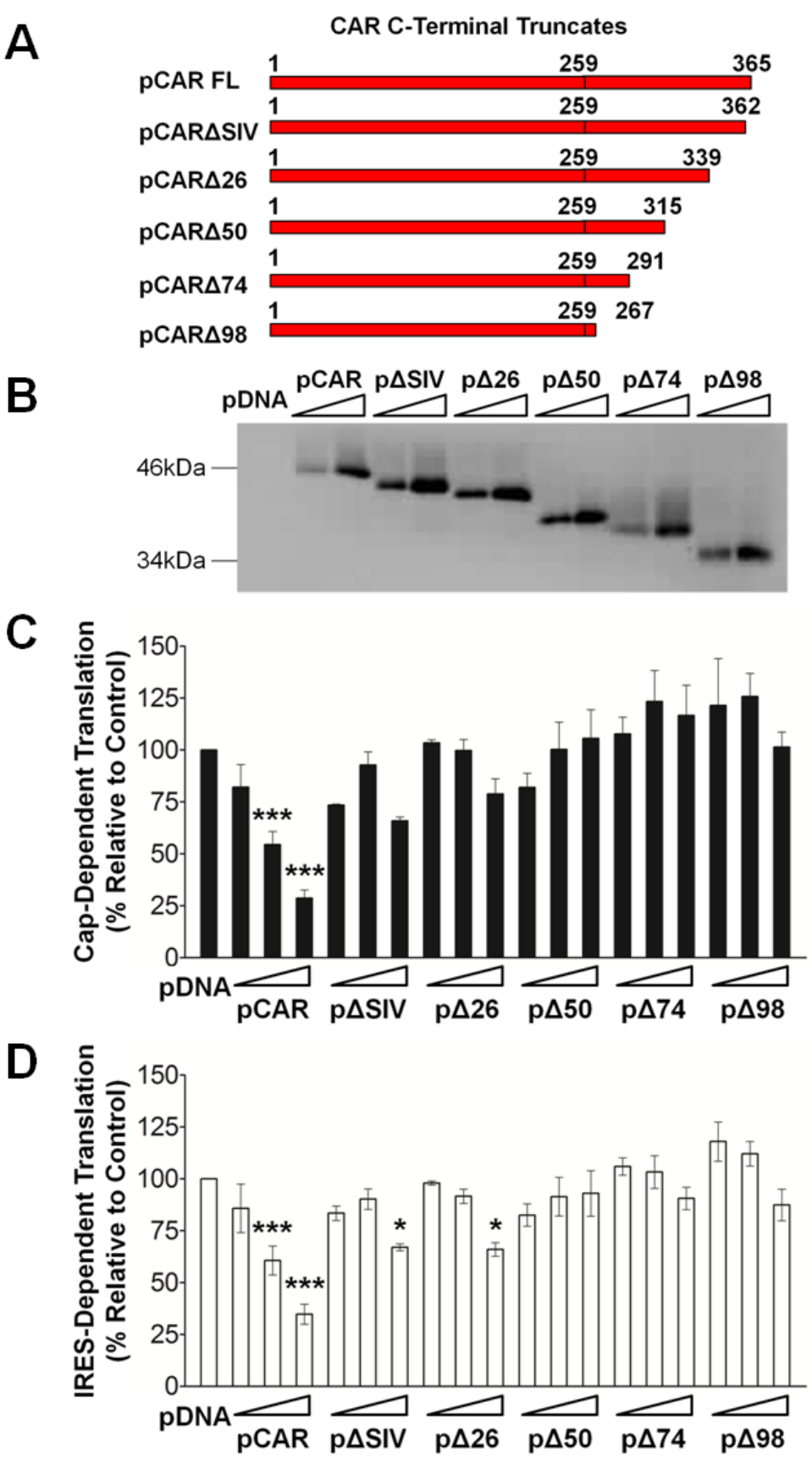

A
CAR C-Terminal Truncates
pCAR FL
1
259
365
pCARΔSIV
362
pCARΔ26
339
pCARΔ50
315
pCARΔ74
291
pCARΔ98
267
B
pDNA
pCAR
pΔSIV
pΔ26
pΔ50
pΔ74
pΔ98
46kDa
34kDa
C
Cap-Dependent Translation
(% Relative to Control)
150
125
100
75
50
25
0
***
***
pDNA
pCAR
pΔSIV
pΔ26
pΔ50
pΔ74
pΔ98
D
IRES-Dependent Translation
(% Relative to Control)
150
125
100
75
50
25
0
***
***
*
*
pDNA
pCAR
pΔSIV
pΔ26
pΔ50
pΔ74
pΔ98

Figure 3.8. **CAR's translational control in CHO cells requires an intact C-terminus.** The dual luciferase reporter plasmid and plasmids for full length and truncated CAR were transiently co-transfected into CHO cells. (A) Diagram representing CAR and its C-terminal truncation plasmids. (B) Western blotting showing the size and integrity of expressed CAR C-terminal truncation proteins. (C) Cap-dependent translation with CAR C-terminal truncates; only CAR significantly inhibited translation. (D) IRES-dependent translation with CAR C-terminal truncates. FL: full length. RLU: relative light unit. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.05$, *; $p < 0.001$, ***.

Figure 3.9

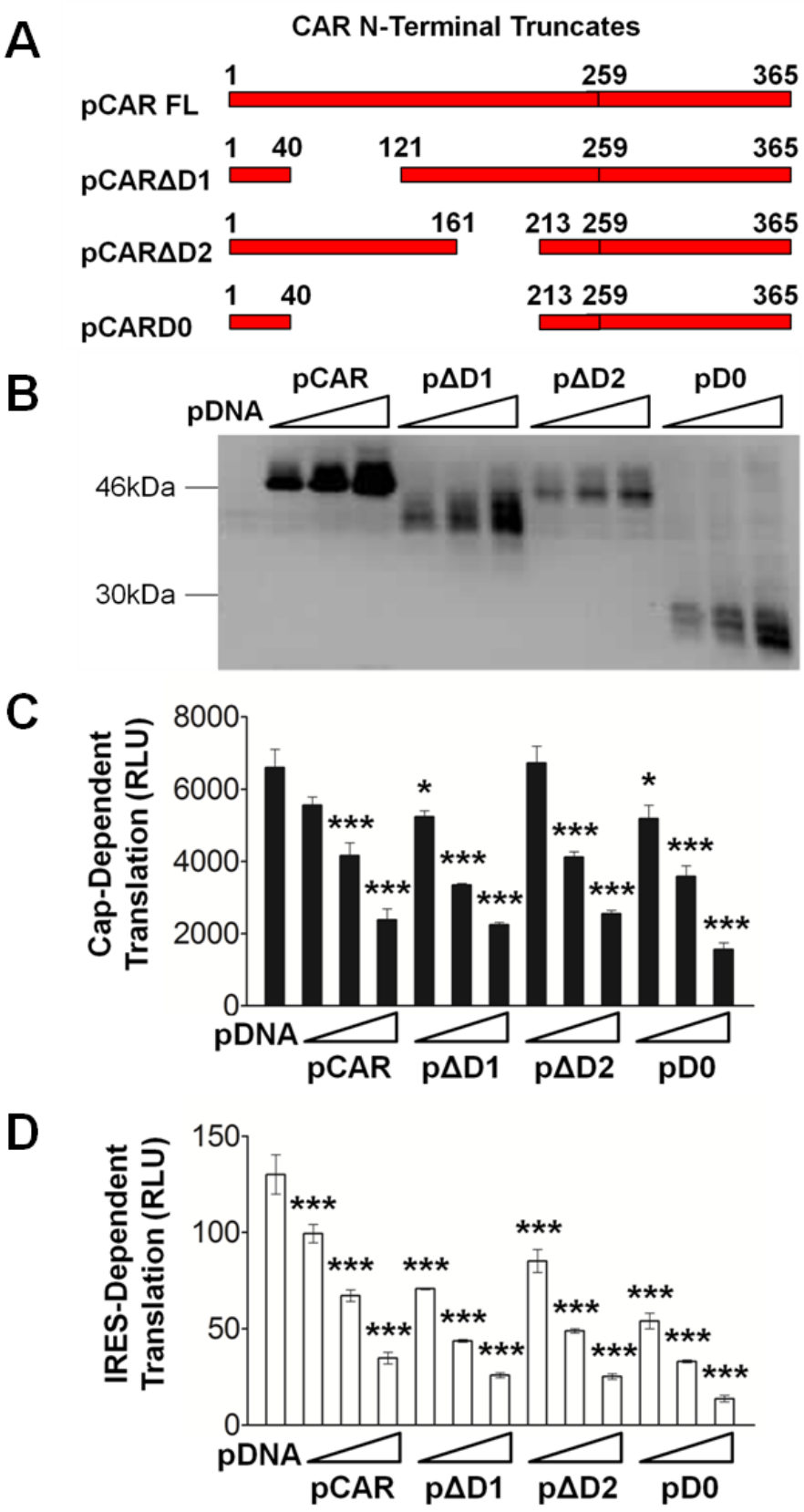

A
CAR N-Terminal Truncates
pCAR FL
1
259
365
pCARΔD1
1
40
121
259
365
pCARΔD2
1
161
213
259
365
pCARD0
1
40
213
259
365
B
pDNA
pCAR
pΔD1
pΔD2
pD0
46kDa
30kDa
C
Cap-Dependent Translation (RLU)
8000
6000
4000
2000
0
pDNA
pCAR
pΔD1
pΔD2
pD0
D
IRES-Dependent Translation (RLU)
150
100
50
0
pDNA
pCAR
pΔD1
pΔD2
pD0

Figure 3.9. **CAR's translational control in CHO cells does not require an intact N-terminus.** The dual luciferase reporter plasmid and plasmids for full length and truncated CAR were transiently co-transfected into CHO cells. (A) Diagram representing CAR and its N-terminal truncation plasmids. (B) Western blotting showing the size and integrity of expressed CAR N-terminal deletion proteins. (C) Cap-dependent translation with CAR N-terminal truncates; all N-terminal truncates impaired translation similarly to full length CAR. (D) IRES-dependent translation with CAR N-terminal truncates. FL: full length. RLU: relative light unit. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.05$, *; $p < 0.001$, ***.

Figure 3.10

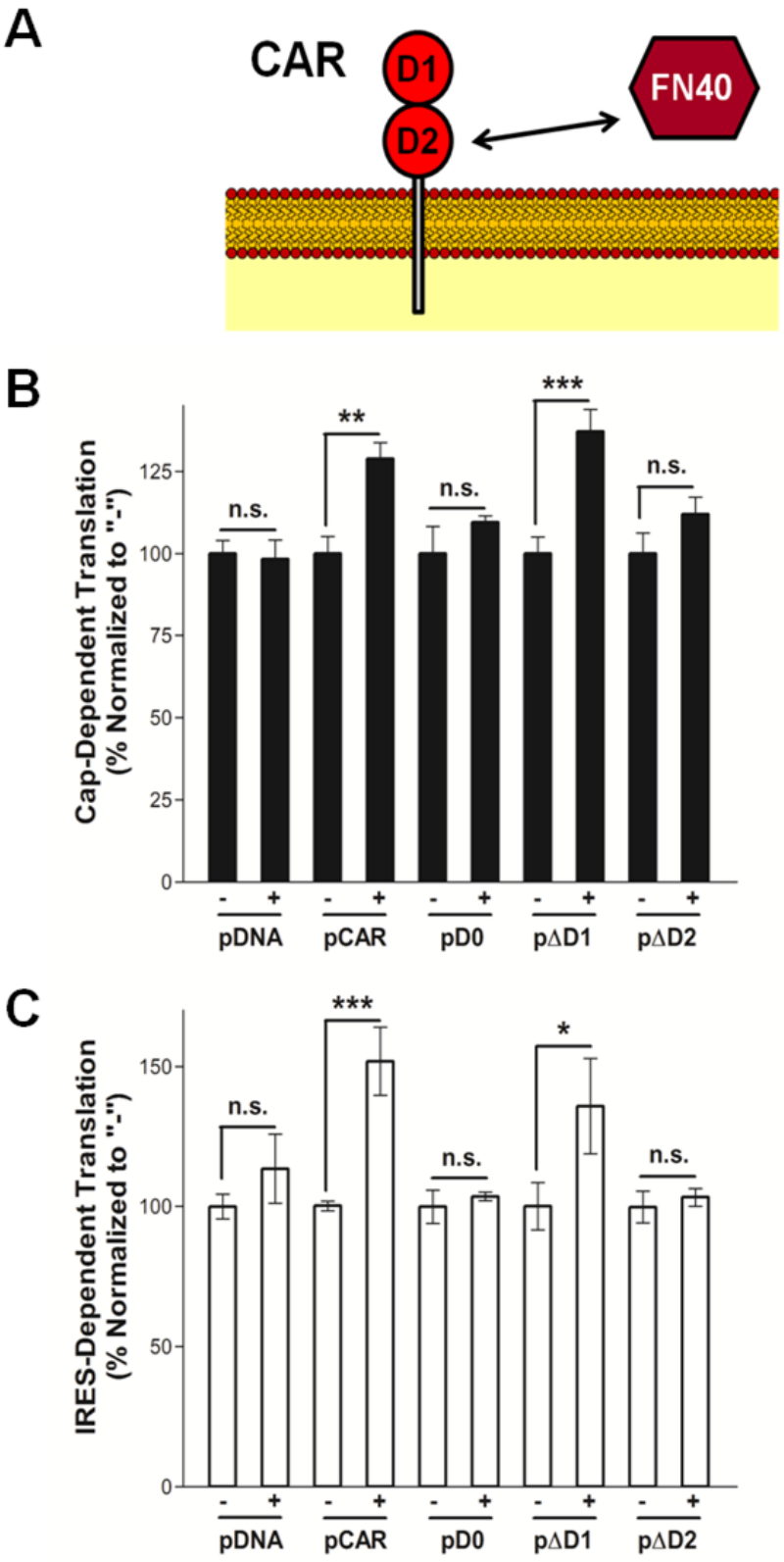

A
CAR
D1
D2
FN40
B
Cap-Dependent Translation
(% Normalized to "-")
125
100
75
50
25
0
n.s.
**
n.s.
***
n.s.
- + - + - + - + - +
pDNA
pCAR
pD0
pΔD1
pΔD2
C
IRES-Dependent Translation
(% Normalized to "-")
150
100
50
0
n.s.
***
n.s.
*
n.s.
- + - + - + - + - +
pDNA
pCAR
pD0
pΔD1
pΔD2

Figure 3.10. **CAR-FN40 interaction-induced translational increase depends on the D2 domain of CAR.** (A) A schematic representation of the interaction between CAR and FN40: CAR directly interacts with FN40 through the D2 domain of CAR (figure adapted from Patzke et al., 2010). (B) CAR-FN40 interaction increased cap-dependent translation. The addition of FN40 only promoted translation of pCAR- and pΔD1-transfected cells (both expressing CAR's D2 domain), but not pDNA-, pD0-, and pΔD2-transfected cells. (C) CAR-FN40 interaction also increased IRES-dependent translation which requires the D2 domain of CAR. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***. n.s.: not significant.

Figure 3.11

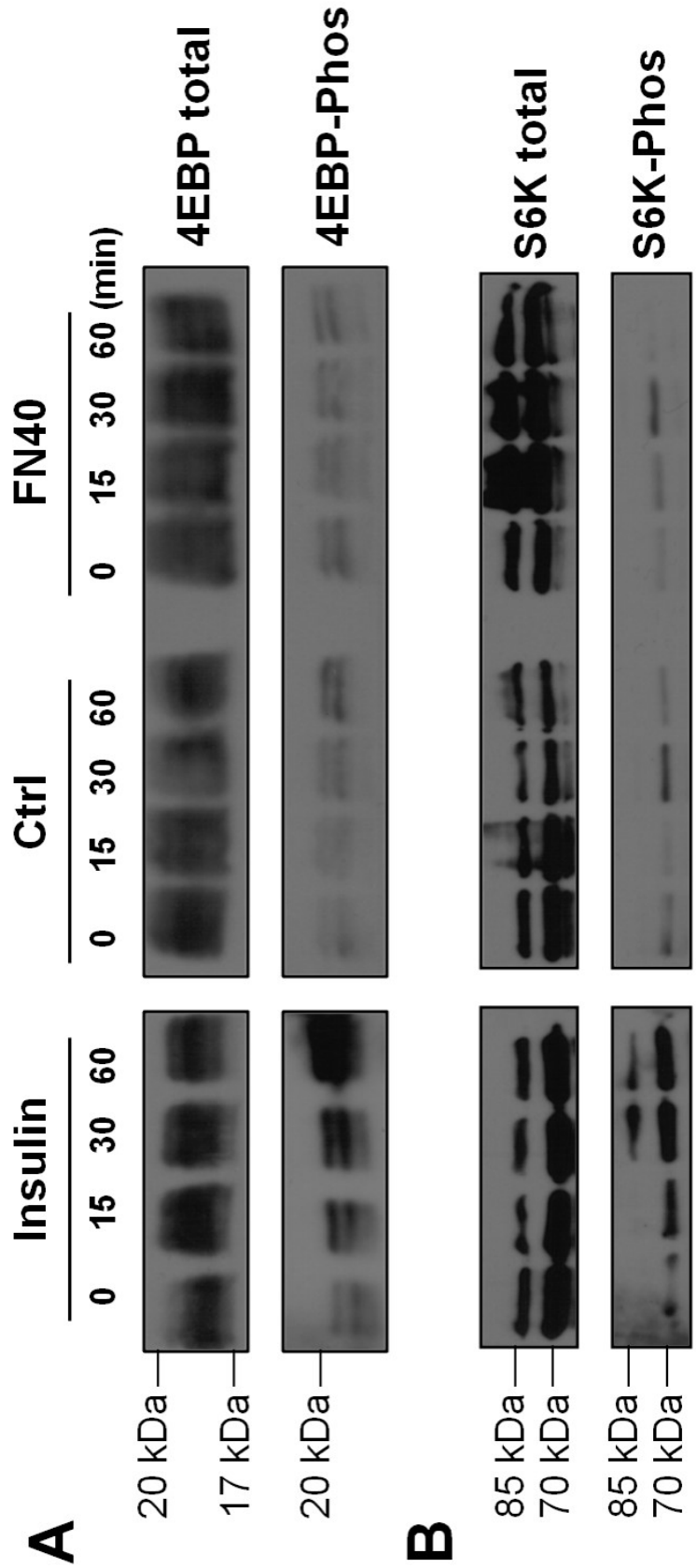

A
Insulin
0 15 30 60
Ctrl
0 15 30 60
FN40
0 15 30 60 (min)
4EBP total
4EBP-Phos
20 kDa
17 kDa
20 kDa
B
S6K total
S6K-Phos
85 kDa
70 kDa
85 kDa
70 kDa

Figure 3.11. **CAR-FN40 interaction does not affect mTOR signaling pathway.** U87CAR cells were cultured in 6-well plates to near confluency and then grown in serum-free medium for 24 hr. Serum-free media containing insulin, FN40 or nothing were added into the wells and the cells were lysed at 0, 15, 30, and 60 min. The addition of insulin significantly elevated the phosphorylation of 4E-BP1 (A) and S6K (B), but the level of phosphorylated 4E-BP1 and S6K did not change for FN40 and control. Note that although 4E-BP2 is the most abundant 4E-BP in the brain, we evaluated 4E-BP1 levels because 4E-BP1 is abundantly expressed in U87MG cell lines and can be used to test mTOR activity.

CHAPTER 4

CAR INTERACTS WITH TRANSLATIONAL PROTEINS IN NEURITES AND REGULTES TRANSLATION DURING NEURITE OUTGROWTH

CAR Associates with Translational Proteins in Growth Cone Particles

As both CAR and translational proteins are enriched in the growth cones (Abe et al., 1997; Estrada-Bernal et al., 2012), we performed GST-CAR pulldown assays in nerve growth cone particles (GCPs) isolated from homogenized postnatal day 6 (P6) mouse brain lysates. We first verified that both CAR and translational proteins locate in nerve growth cones: CAR was enriched in the GCP fractions in a pattern similar to NCAM (Figure 4.1A), suggesting a successful fractionation. A large amount of translation proteins were also present in the GCP fractions (Figure 4.1B). We then performed GST-CAR and m^7GTP (the 5'-cap of eukaryotic mRNAs) pulldown from the isolated GCPs, and found that GST-CAR could indeed pull down translational proteins (Figure 4.2) and that m^7GTP-sepharose beads could pull down CAR (Figure 4.3). These results support that CAR binds to translational proteins in nerve growth cones and it is present in the mRNA cap-binding complex.

CAR Colocalizes with Translational Proteins in Primary Cortical Neurons

To study colocalization between CAR and translational proteins in neurites and growth cones, we isolated cortical neurons from embryonic day 17 (E17) mouse brains, cultured them for 48 hours, and then fixed and immunostained with antibodies against CAR and translational proteins. At the nerve growth cones, CAR was found to colocalize with S6 (Figure 4.4A). Additionally, immunostaining of CAR also overlapped with that of elongation factor eEF1A

at the neuronal tip and the neurite shaft (Figure 4.4B). These results suggest that CAR associates with translational proteins at nerve growth cones and may regulate local translation.

CAR-FN40 Interaction Promotes Neurite Outgrowth

Since CAR promotes neurite outgrowth upon binding to its ligand FN40, a 40-kDa fragment of fibronectin (Patzke et al., 2010), we first repeated this experiment and found that FN40 indeed significantly increased the length of total neurites and longest neurite (Figure 4.5A, B); it also increased number of processes per neuron, suggesting that it also promotes neuritogenesis – the initial sprouting of the neuronal processes from the cell bodies (Figure 4.5C). To demonstrate that this neurite outgrowth promoting effect is due to CAR, we used an adenoviral vector that carries a CAR-antisense construct (Ad-asCAR) (Fok et al., 2007), and adenoviral vector carrying a BFP (Ad-BFP) was used as a control. We found that Ad-asCAR reduced FN40-stimulated neurite outgrowth to PDL control level (FN40/Ad-asCAR versus FN40/Ad-BFP) but the basal neurite growth was not affected (PDL/Ad-asCAR versus PDL/Ad-BFP), suggesting that FN40-triggered neurite outgrowth is mediated by CAR (Figure 4.5A-C).

CAR-Promoted, but Not Basal, Neurite Outgrowth is Ablated by Inhibiting Translation

To test whether protein synthesis plays a role during neurite outgrowth, we treated the neuronal cultures with 1 μM cycloheximide (CHX) upon plating the neurons. After 24 hr of treatment, the cells were not sick as revealed by a healthy morphology under phase-contrast microscope and by propidium iodide staining of the dead neurons (data not shown). However, after the treatment, FN40-stimulated neurite outgrowth was reduced to the control level while the basal neurite outgrowth was not affected (Figure 4.6A-C). This result suggests that ligand-triggered, but not basal, neurite outgrowth is dependent on protein synthesis.

CAR-FN40 Interaction Increases Translation as Revealed by AHA Labeling

However the above experiments only showed that ligand-stimulated neurite outgrowth requires both CAR and translation, respectively. To demonstrate a direct link between CAR and translation, we performed a protein metabolic labeling experiment using Click chemistry (Dieterich et al., 2010). L-Homopropargylglycine (HPG, will be referred to as AHA for convenience), an analog of methionine, will be incorporated into *de novo* synthesized proteins in place of methionine. After fixation, a fluorescence tag will be clicked to AHA and the fluorescence intensity measured to represent translation rate. We plated neurons on FN40 or PDL, after 6 hr of incubation with AHA (longer incubation caused minor cytotoxicity) neurons grown on FN40 had significantly more AHA signal (Figure 4.7A, B and Figure 4.8),

indicating that FN40-CAR interaction indeed stimulated protein synthesis. On the other hand, another extracellular matrix protein collagen (CL), which can also promote neurite outgrowth in our cortical neuron culture, did not significantly increase translation (Figure 4.8); this suggest that collagen may interact with integrins via its "RGD" motif and promote neurite outgrowth through other signaling pathways. As a control, treatments with medium containing 50 μM L-methionine (Met) and medium containing AHA and 40 μM CHX (CHX) showed only background fluorescence (Figure 4.7C, D). Although FN40 did not enhance translation as much as it promoted neurite outgrowth, this may be due to a short AHA-treatment time and a partial requirement of translation in neurite outgrowth (see Discussion).

Figure 4.1

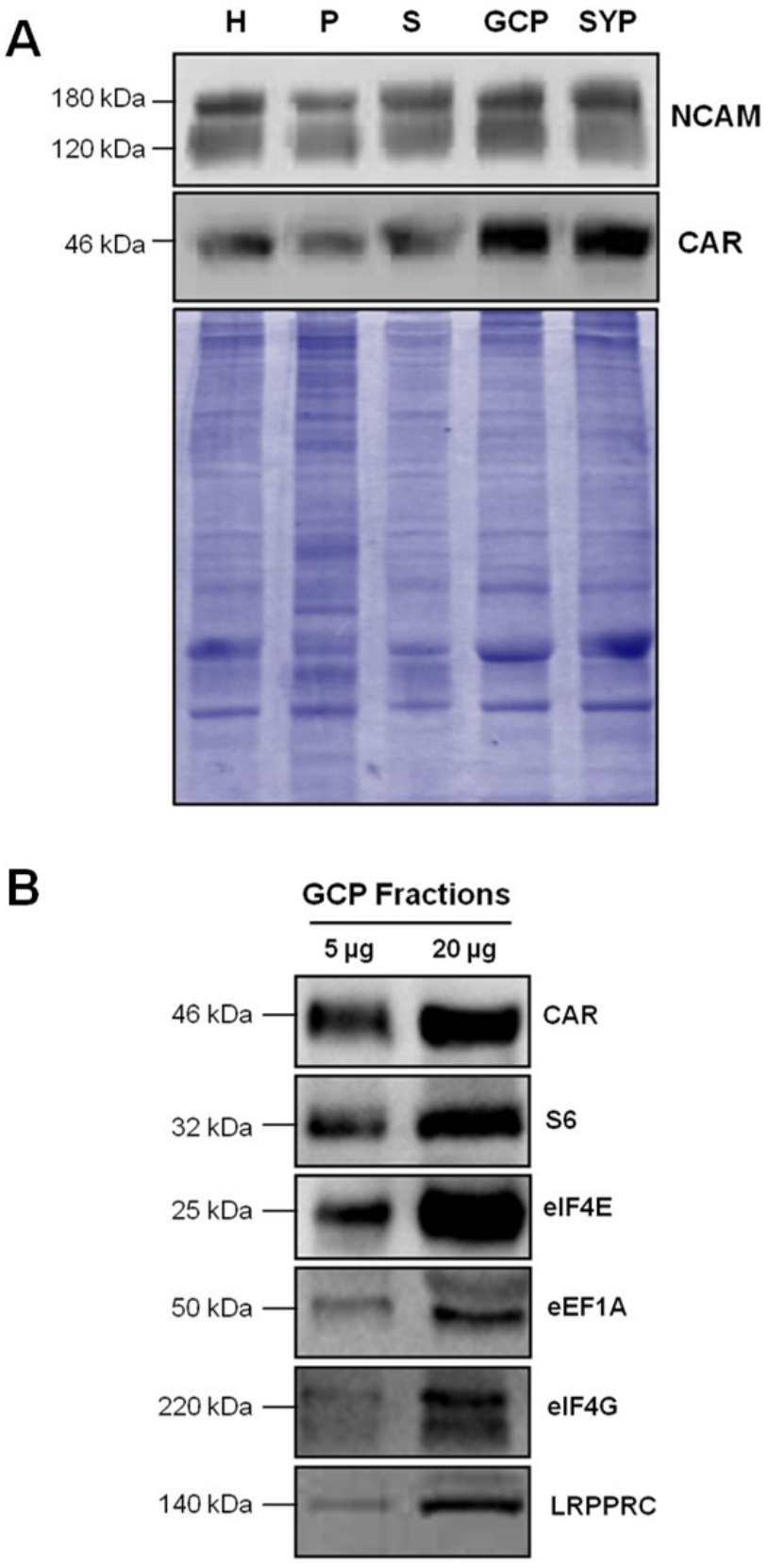

A
H
P
S
GCP
SYP
180 kDa
120 kDa
NCAM
46 kDa
CAR
B
GCP Fractions
5 µg
20 µg
46 kDa
CAR
32 kDa
S6
25 kDa
eIF4E
50 kDa
eEF1A
220 kDa
eIF4G
140 kDa
LRPPRC

Figure 4.1. **CAR and translational proteins are enriched in isolated growth cone particles (GCPs).** (A) GCPs were isolated using a fractionation protocol. CAR's distribution pattern in these fractions is similar to that of NCAM (top bands, 180 kDa; bottom bands, 120 kDa). H: homogenates; P: pellet; S: supernatant; SYP: synaptosome. (B) Western blot for translational proteins in the GCPs.

Figure 4.2

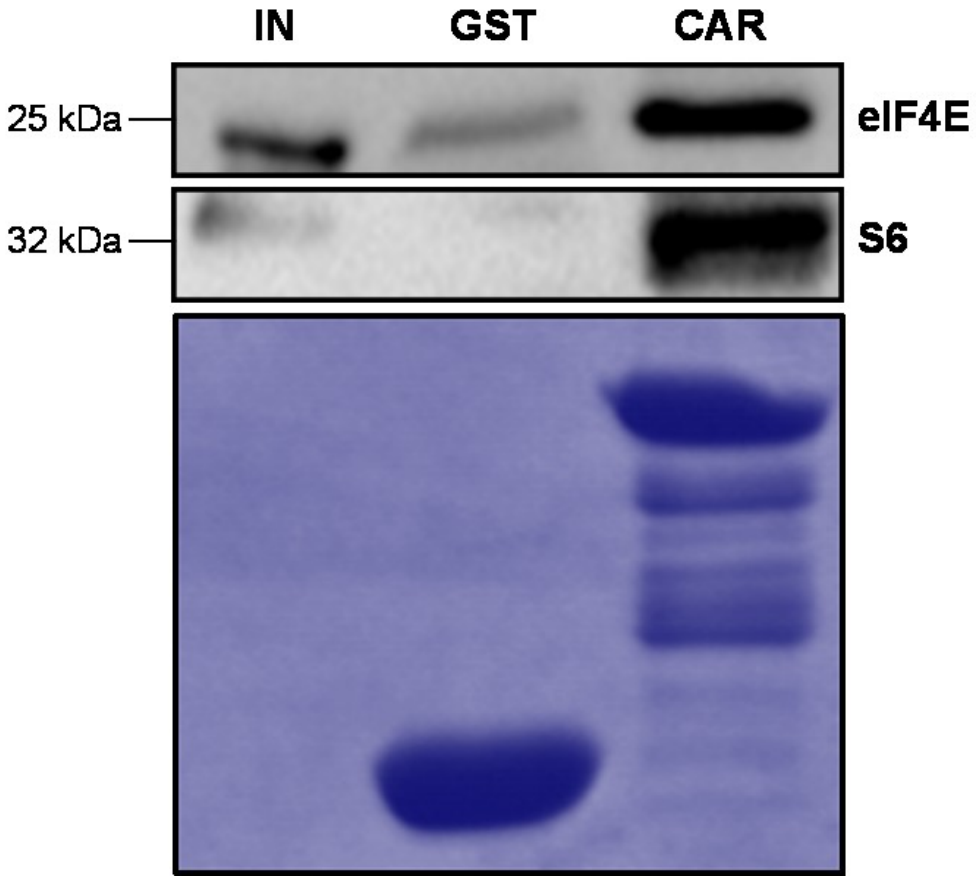


Figure 4.2. **CAR binds to translational proteins in isolated GCPs as revealed by GST pulldown.** Only GST-CAR but not GST pulled down translational proteins from the GCP fractions. Coomassie blue stain for the post-transfer SDS-PAGE gel showing that equal amount of GST/ GST-CAR was applied.

Figure 4.3

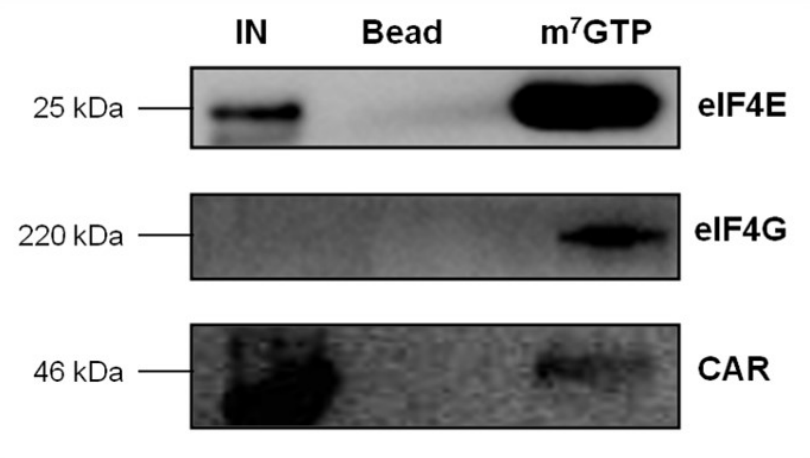


Figure 4.3. **CAR resides in the mRNA cap-binding complex in GCPs as revealed by m^7GTP cap-pulldown.** Only m^7GTP-Sepharose but not the control beads pulled down eIF4E and eIF4G, suggesting a successful pulldown of cap-binding proteins. CAR was also pulled down by m^7GTP-Sepharose, suggesting that CAR resides in the cap-binding complex in GCPs.

Figure 4.4

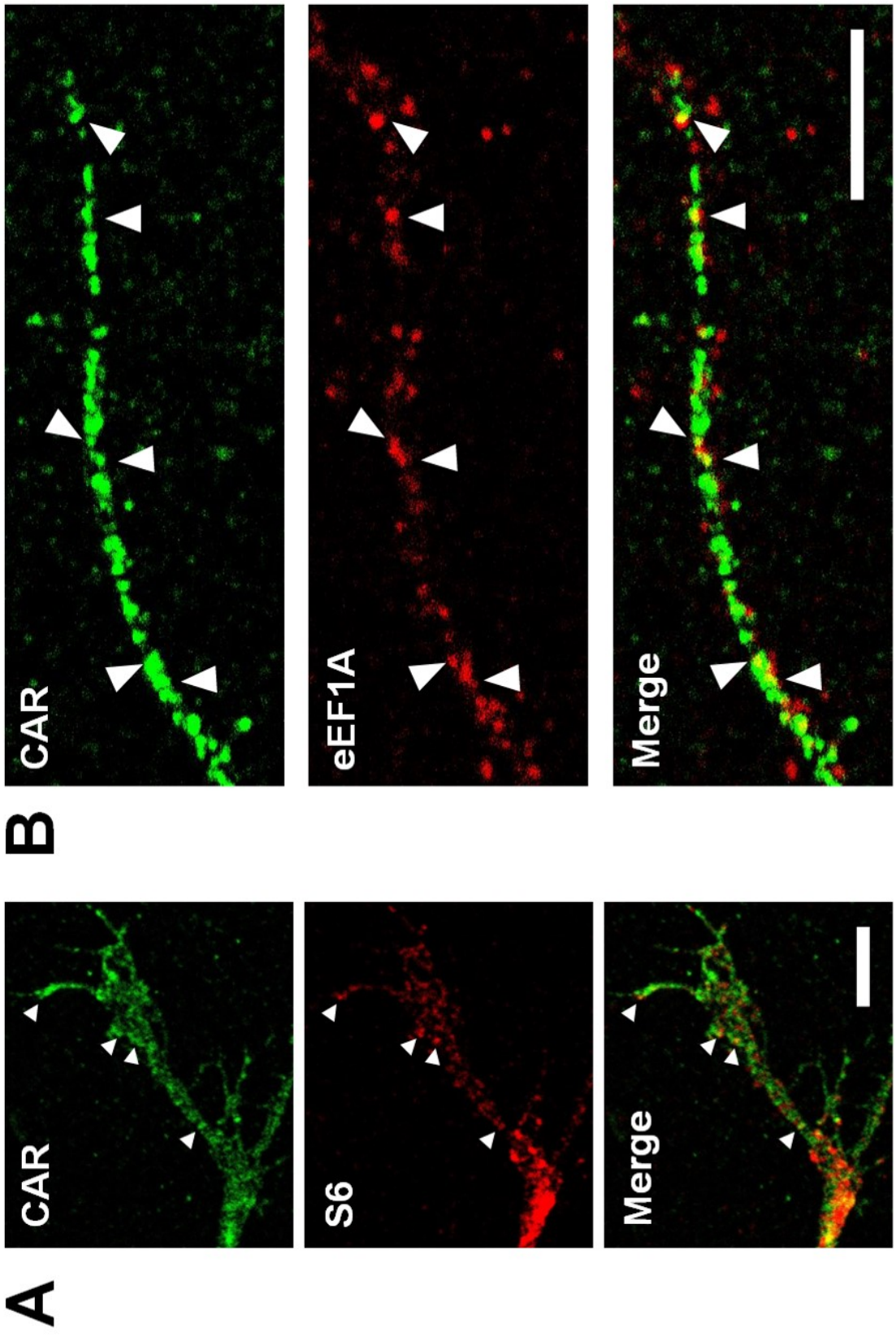


Figure 4.4. **CAR colocalizes with translational proteins in the neurite shaft and the nerve growth cone as revealed by immunofluorescence.** (A) Confocal immunofluorescence analysis of a representative nerve growth cone. CAR colocalizes with S6 at the nerve growth cone. (B) Confocal immunofluorescence analysis of a representative neurite shaft of a cortical neuron cultured *in vitro* for 2 days. CAR colocalizes with eEF1A in the neurite shaft. Scale bars: both are 5 μm.

Figure 4.5

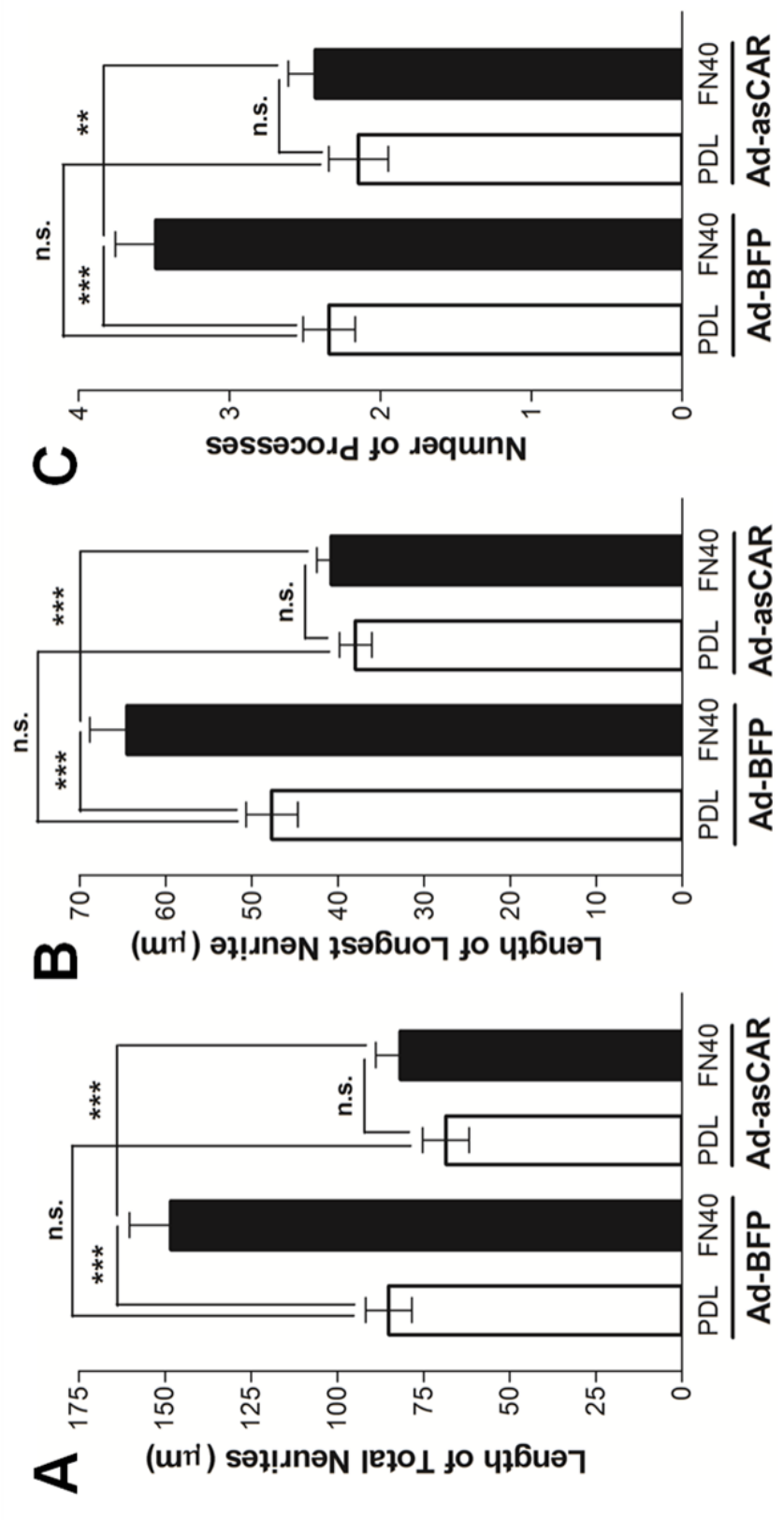

A
Length of Total Neurites (μm)
175
150
125
100
75
50
25
0
n.s.
***
***
n.s.
PDL FN40 PDL FN40
Ad-BFP
Ad-asCAR
B
Length of Longest Neurite (μm)
70
60
50
40
30
20
10
0
n.s.
***
***
n.s.
PDL FN40 PDL FN40
Ad-BFP
Ad-asCAR
C
Number of Processes
4
3
2
1
0
n.s.
***
**
n.s.
PDL FN40 PDL FN40
Ad-BFP
Ad-asCAR

Figure 4.5. **CAR-FN40 interaction promotes neurite outgrowth.** CAR promotes neurite outgrowth upon binding to FN40. FN40-stimulated neurite outgrowth is completely abolished by a CAR-antisense construct (Ad-asCAR) but not by the control construct (Ad-BFP) as shown with (A) total neurite length, (B) longest neurite length, and (C) number of processes. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.01$, **; $p < 0.001$, ***. n.s.: not significant.

Figure 4.6

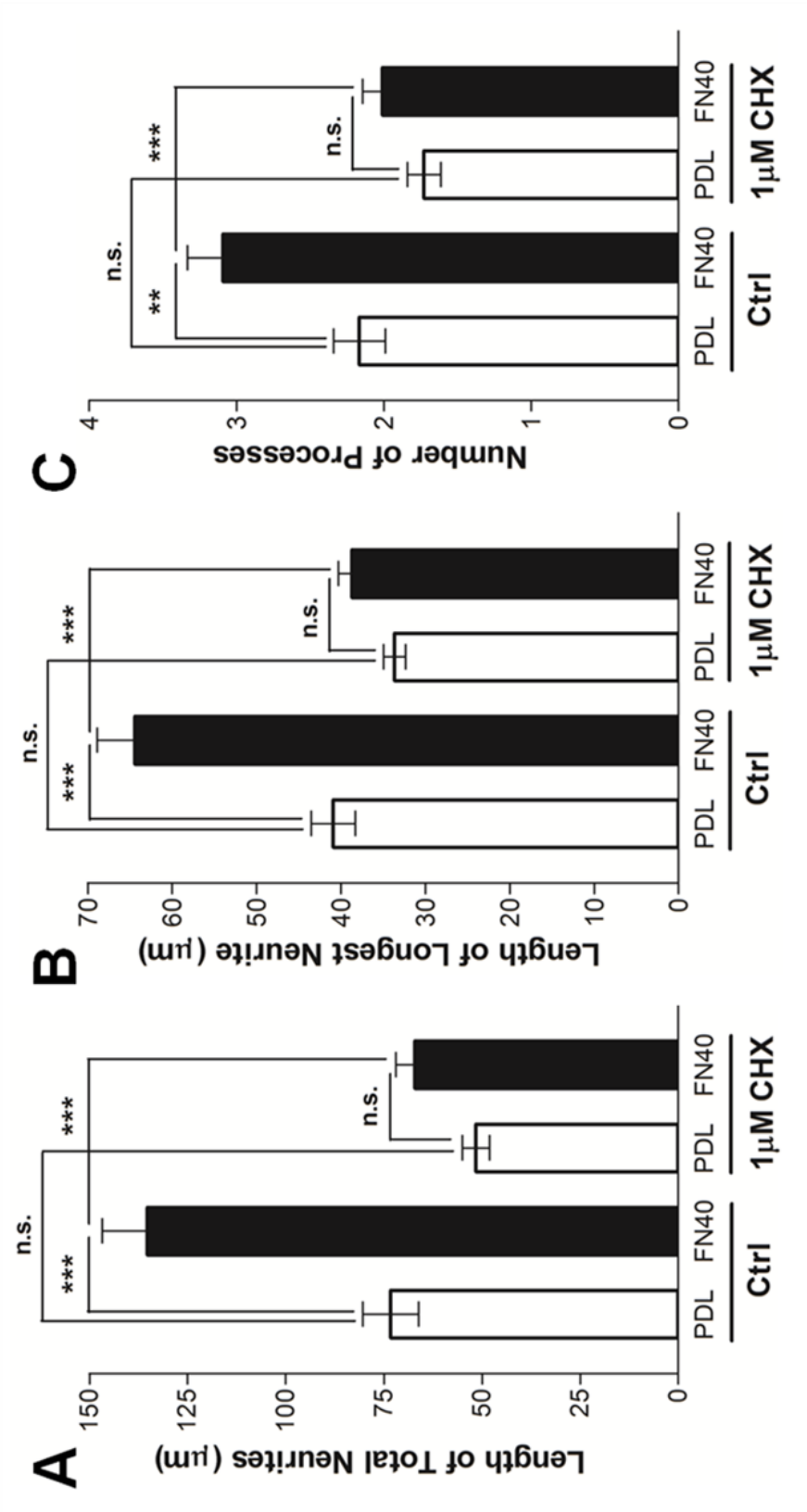

A
Length of Total Neurites (μm)
150
125
100
75
50
25
0
n.s.
***
***
n.s.
PDL
FN40
PDL
FN40
Ctrl
1μM CHX
B
Length of Longest Neurite (μm)
70
60
50
40
30
20
10
0
n.s.
***
***
n.s.
PDL
FN40
PDL
FN40
Ctrl
1μM CHX
C
Number of Processes
4
3
2
1
0
n.s.
**
***
n.s.
PDL
FN40
PDL
FN40
Ctrl
1μM CHX

Figure 4.6. **CAR-FN40-promoted neurite outgrowth requires protein synthesis.** CAR-promoted neurite outgrowth depends on protein synthesis. Overnight application of 1 μM of cycloheximide (CHX) completely abolished CAR-promoted neurite outgrowth as represented by (A) total neurite length, (B) longest neurite length, and (C) number of processes. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.01$, **; $p < 0.001$, ***. n.s.: not significant.

Figure 4.7

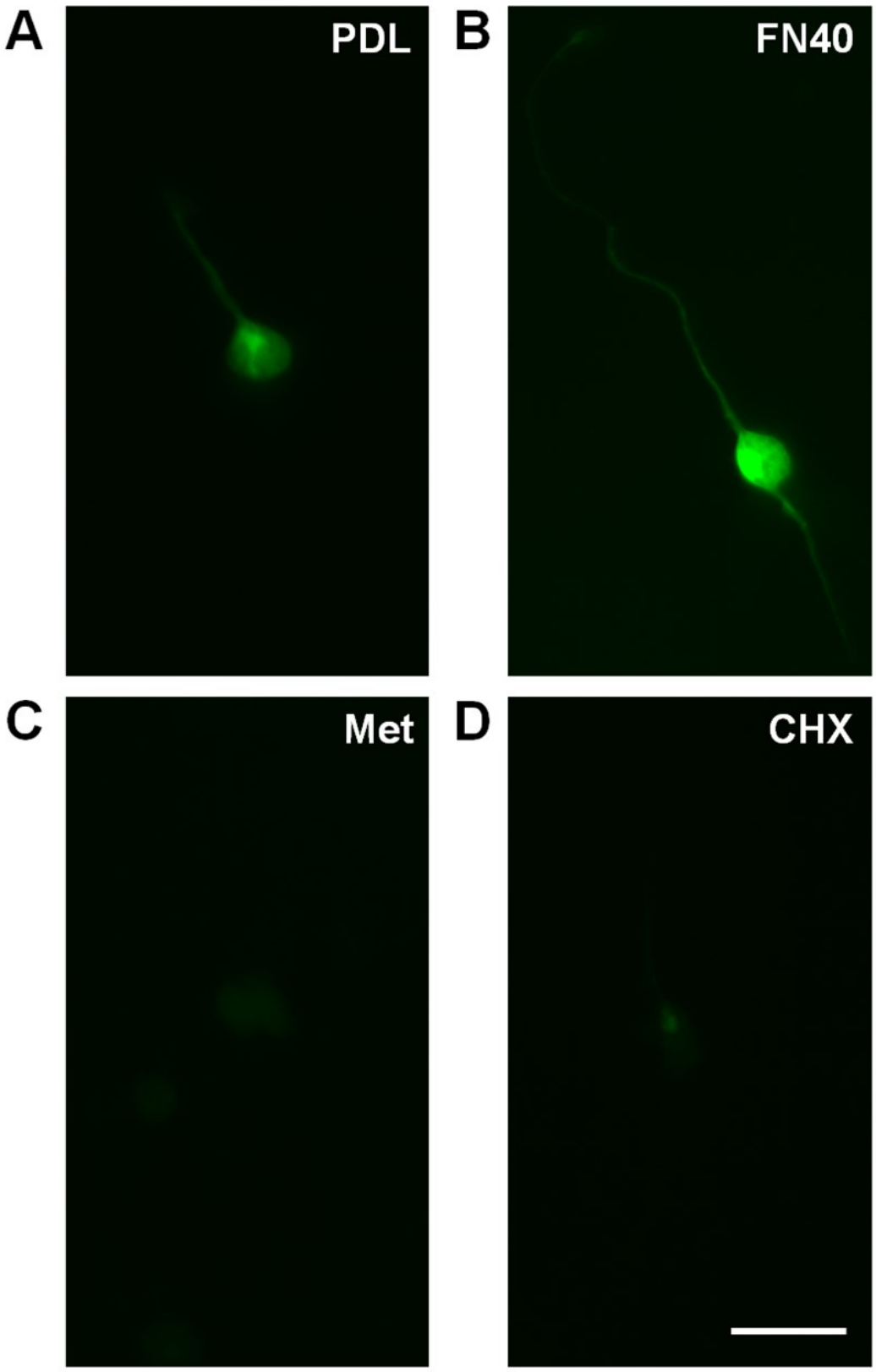


Figure 4.7. **CAR-FN40 interaction increases protein synthesis as revealed by AHA labeling.** Neurons grown on FN40 (B) had greater AHA signal and longer neurites compared to those grown on PDL control (A). (C) Met: L-Methionine was applied instead of AHA. (D) CHX: Cycloheximide (40 μM) was applied during the AHA treatment. Scale Bar: 20 μm.

Figure 4.8

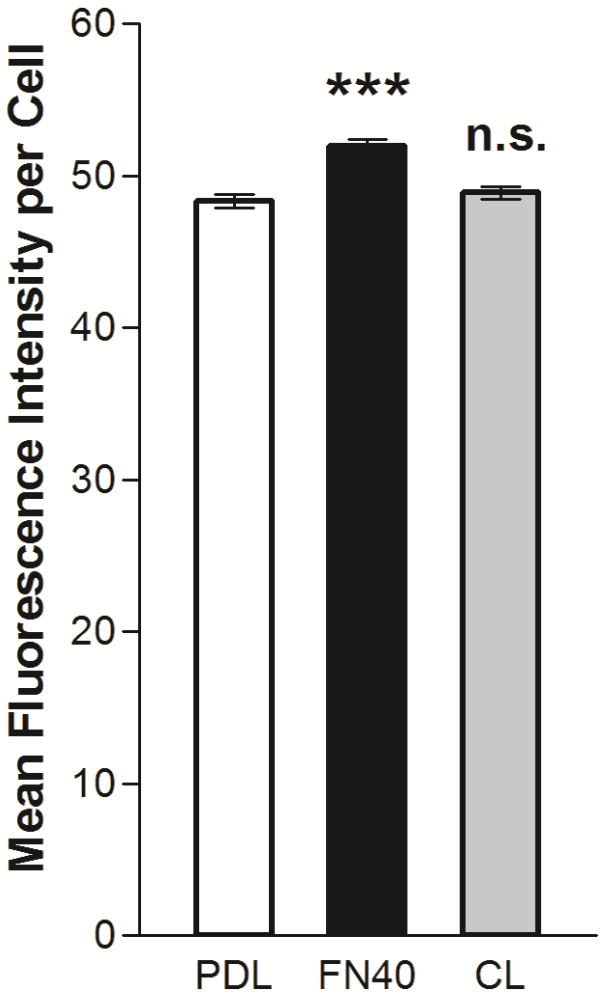

Mean Fluorescence Intensity per Cell
60
50
40
30
20
10
0
***
n.s.
PDL
FN40
CL

Figure 4.8. **Quantification of AHA-labeled *de novo* synthesized proteins.** Quantification of AHA labeling: neurons grown on FN40 had more AHA signal than PDL, suggesting more protein synthesis. On the other hand, collagen (CL) did not significantly increase translation comparing to the PDL control. The mean fluorescence intensity per neuron was quantified using ImageJ. Values represent the mean ± SEM. Statistical significance is defined as: $p <$ 0.001, ***. n.s.: not significant.

CHAPTER 5

CAR CONDITIONAL KNOCKOUT MICE DISPLAY ELEVATED ANXIETY AND IMPAIRED MOTOR COORDINATION

Strategies to Generate Conditional CAR-Knockout Mice

As introduced above, CAR mutant mice with CNS phenotypes were difficult to obtain due to the embryonic lethality caused by whole-body CAR knockout. To overcome this technical challenge, we tried a conditional knockout strategy. In the CARFLOX mice, the exon 2 of CAR is flanked by loxP sites (see Materials and Methods) so that CAR can be inactivated by the Cre recombinase expressed under a neuronal promoter. Synapsin1 is highly expressed in mammalian neurons (Hoesche et al., 1993), and its expression in mouse brain takes place as early as embryonic day E12 (Melloni and DeGennaro, 1994). Therefore, we planned to generate the conditional CAR-knockout mice by crossing the CARFLOX and the SYNCRE (Cre recombinase expressed under the Synapsin1 promoter) mice. Indeed, the mRNA and protein levels of CAR were significantly decreased in CAR-null mouse brains (Zheng MSc thesis) suggesting a successful knockout of CAR.

We chose to evaluate transgenic mice at the age of 2-6 months as an attempt to minimize the effects of age. As an overall observation, the CARFLOX(+/+)SYNCRE(+/+) CAR-conditional knockout mice (hence referred to as CAR-cKO) have regular weights/ sizes comparing to their CARFLOX(+/+)SYNCRE(-/-) wildtype littermates (hence referred to as WT). Their perceptive senses, such as vision, smell, hearing, sensation to touch, and whisker responses all appear normal. To discover the fine differences between the CAR-cKO and WT mice, I first conducted an open-field study, then a Rota-rod test, followed by the muscle strength and endurance tests, and finally a clasping assay.

CAR-cKO Mice Spend Less Time in Ambulatory Movement

In the open-field test, CAR-cKO mice displayed less ambulatory movements. First, mice in their cages were transferred from the housing room to the experimental room and they were kept undisturbed for 30 min. Before the 10 min video-recording, the mice were allowed to habituate for another 10 min in the open-field test cage. These habituation steps allowed the mice to get used to the room and cage and therefore reduced unnecessary anxiety. During the open-field test, CAR-cKO mice spent significantly less time in ambulatory movement and spent more time staying still (Figure 5.1 A and B). These observations suggest that cKO mice may have locomotor problems; however other motor tests such as Rota-rod and clasping assays are required to further verify these results.

CAR-cKO Mice May Have Elevated Anxiety

In addition, cKO mice also displayed anxiety-like behavior. The cKO mice preferred to stay at the peripheral regions of the open-field cage and they rarely traveled to the center; on the other hand, WT mice crossed the central regions more frequently (no quantitative data yet available but this can be done with an automated video-tracking system in the future). CAR-cKO mice had short, incomplete, and often interrupted self-grooming (Figure 5.2A); they also had more defecation boli comparing to WT mice (Figure 5.2B). Usually, decreased self-grooming duration and increased defecation boli indicate elevated anxiety (Fentress,

1977; Kalueff and Tuohimaa, 2005). Additionally, the movement of CAR-cKO was often more hesitant – they stood up and sat down many times without traveling in a distance (vertical rearing) and sometimes they froze (freezing); this freezing and vertical rearing is also an indication of increased anxiety (Bourin et al., 2007; Kalueff and Tuohimaa, 2005). Taken together, these observations suggest that CAR-cKO mice may be more anxious than their WT littermates.

CAR-cKO Mice Behave Poorly in Rota-Rod Test Comparing to Wildtype Littermates

Since we also discovered a possible sign of motor problem in cKO mice, next we performed a rotarod test with two types of rotation settings: 1) at incremental fixed speeds (5, 10, 15, 20, 25 rpm/min), and 2) at an accelerating speed (5-40 rpm/min); both tests have a maximal duration of 300 sec. CAR-cKO mice performed very poorly during all these tests: they fell down much earlier and more frequently compared to WT littermates.

In incremental fixed speed tests, cKO mice fell down immediately from the Rota-rod with a low rotation speed of 10 rpm whereas most WT mice stayed on the Rota-rod during the entire session (Figure 5.3A and B); knockout of CAR caused more severe motor problems in male than female (Figure 5.3B versus Figure 5.3C). In accelerating speed tests, cKO mice also fell down much earlier at ~50 sec and at low rotation speed ~10 rpm (Figure 5.4A, B, and C); similarly, female cKO mice were less affected by CAR knockout (Figure 5.4D and E).

CAR-cKO Mice Have Normal Muscular Strength and Endurance

Rotarod performance may indicate a few properties combined together, such as: 1) muscle strength, 2) muscle endurance, and 3) motor coordination. To exclude the potential influences caused by muscles, I weighed the mice (Figure 5.5A), performed a weight-lifting assay to test their muscle strength (Figure 5.5B), and carried out an inverted cage assay to test their muscle endurance (Figure 5.5C). I found no difference between cKO and WT mice in these assays. Taken together, these data suggest that cKO mice have ataxia, or poor motor coordination, which points to a cerebellar abnormality.

CAR-cKO Mice Display a Mild Clasping Phenotype

Mice with motor malfunctions normally display a clasping phenotype. In these mice, their paws often press against their bodies during tail suspension (Brooks and Dunnett, 2009; Lin et al., 2001). CAR-cKO mice displayed a mild clasping phenotype compared to WT mice (Figure 5.6A versus B) and during the 20 sec video-recording, they often used their forelimbs to grasp their hind limbs (video-recorded, data not shown), suggesting that cKO mice indeed have motor problems.

Figure 5.1

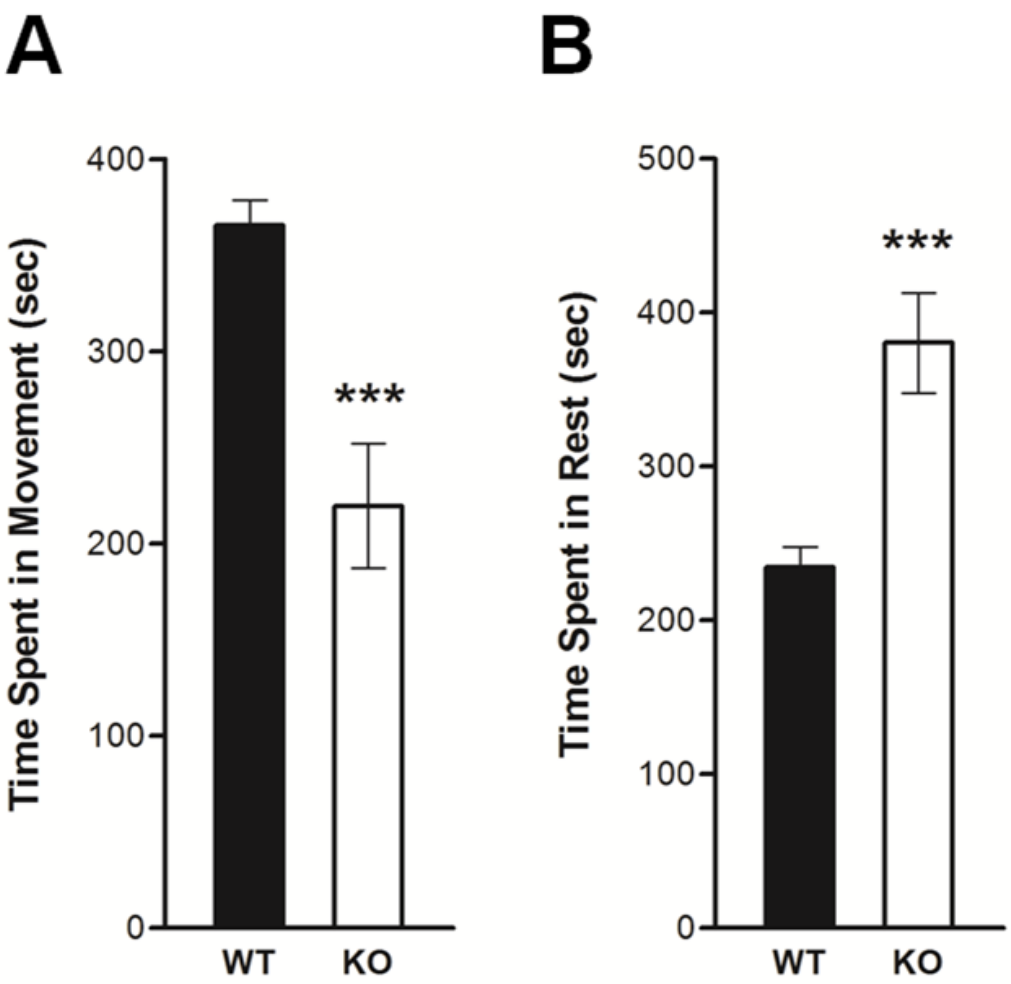

A
B
Time Spent in Movement (sec)
400
300
200
100
0
***
WT
KO
Time Spent in Rest (sec)
500
400
300
200
100
0
***
WT
KO

Figure 5.1. **CAR-cKO mice spend less time in ambulatory movement.** (A) Time spent in movement. CAR-cKO or WT mice were placed in the open-field cage and video-recorded for 10 min. The total time spent in movement was then scored and statistically analyzed. CAR-cKO mice spent significantly less time in movement comparing to WT mice. (B) Time spent in rest: cKO mice spend significantly longer time in rest. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.001$, ***.

Figure 5.2

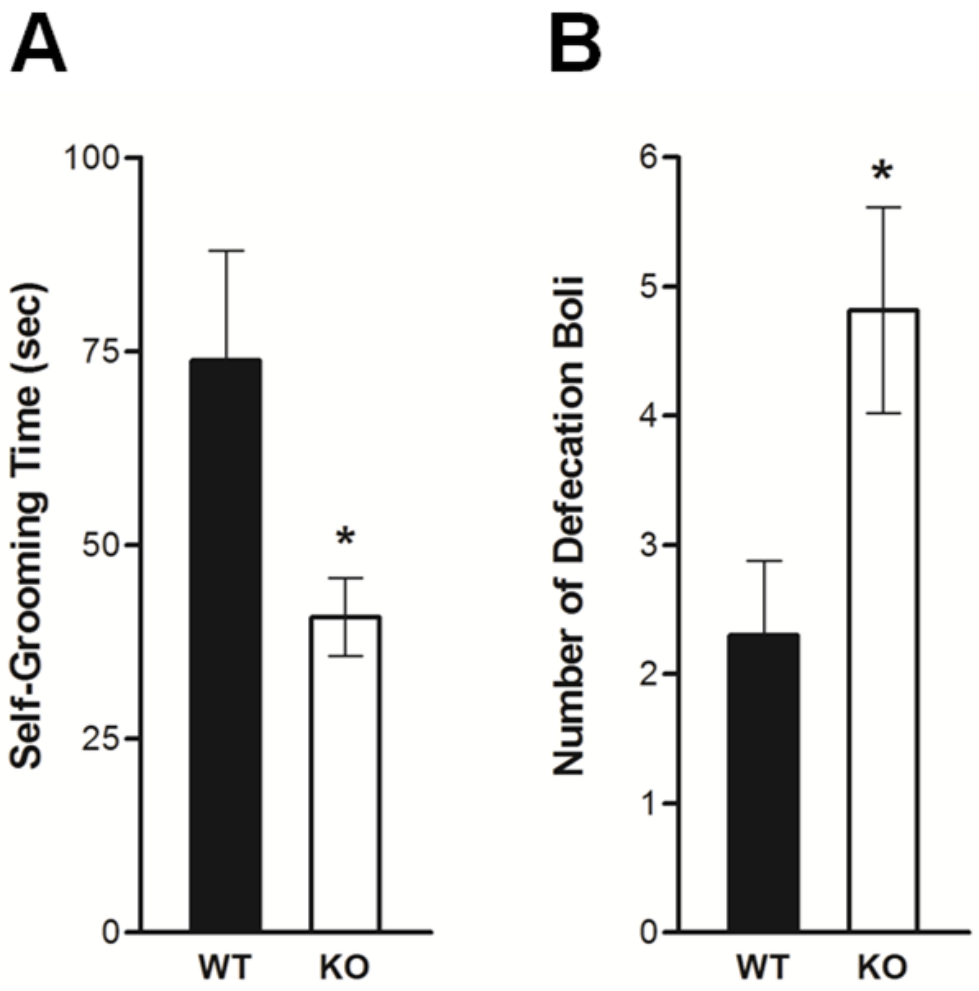

A
B
Self-Grooming Time (sec)
100
75
50
25
0
*
WT
KO
Number of Defecation Boli
6
5
4
3
2
1
0
*
WT
KO

Figure 5.2. **CAR-cKO mice have anxiety-like behavior.** (A) Self-grooming duration: the duration for self-grooming was scored for the 10 min open-field tests. CAR-cKO mice had significantly shorter self-grooming time comparing to WT mice. (B) Number of defecation boli. The defecation boli during the 10 min of habituation and the 10 min of open-field test (totally 20 min) were counted for each mouse. CAR-cKO mice had significantly more defecation boli. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.05$, *.

Figure 5.3

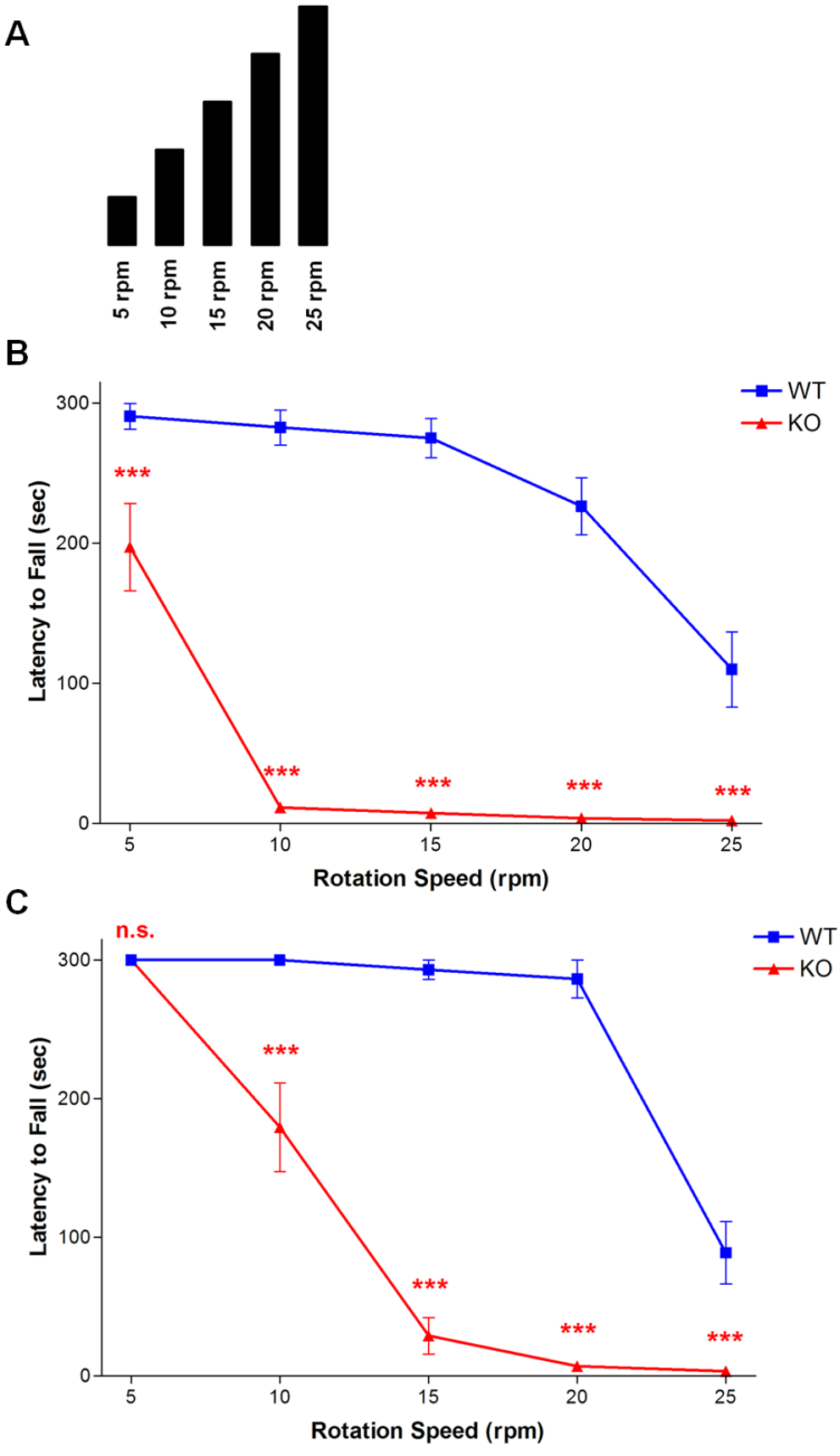
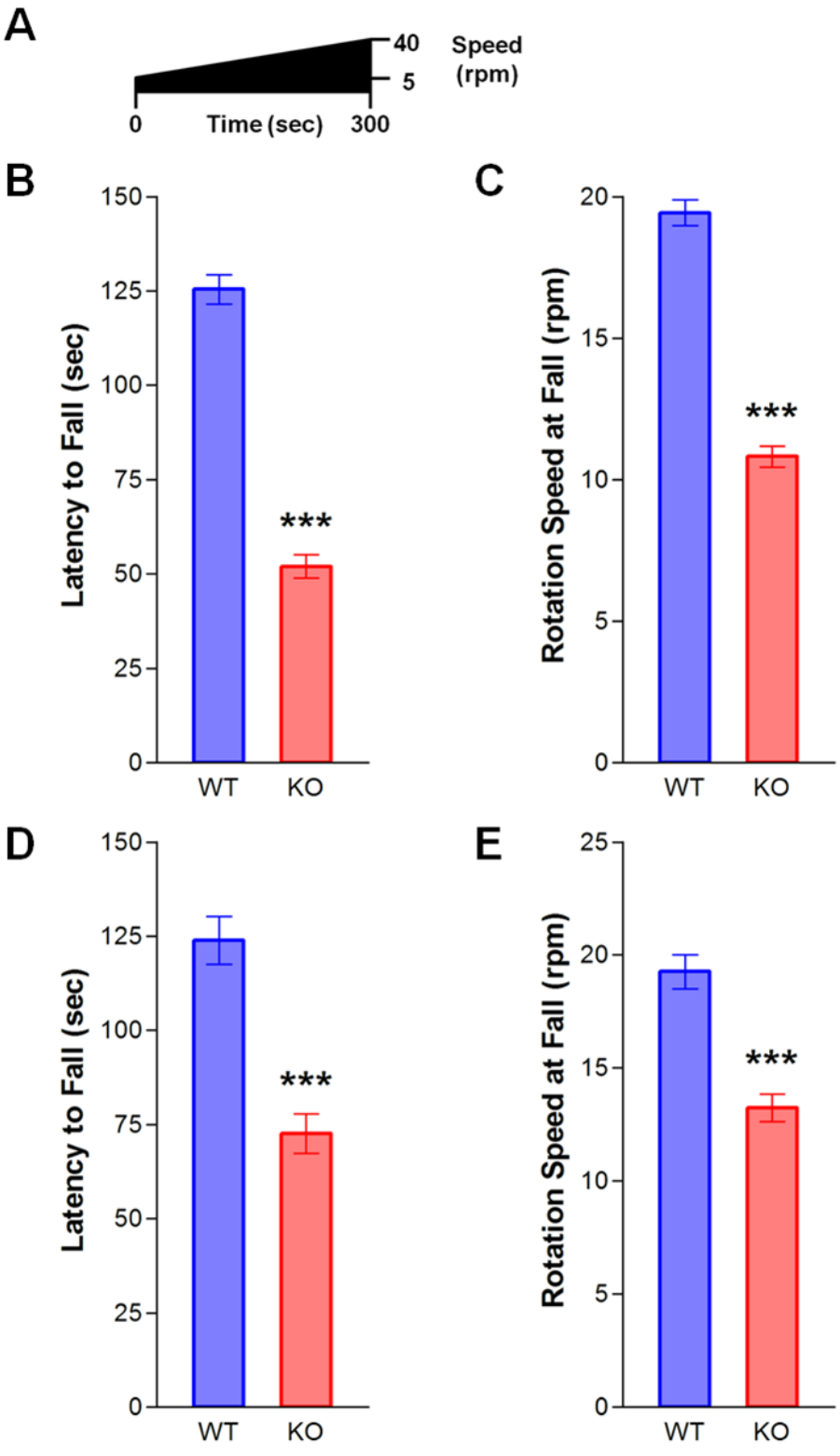

A
5 rpm
10 rpm
15 rpm
20 rpm
25 rpm
B
WT
KO
Latency to Fall (sec)
300
200
100
0
***
***
***
***
***
5
10
15
20
25
Rotation Speed (rpm)
C
WT
KO
n.s.
***
***
***
***
Latency to Fall (sec)
300
200
100
0
5
10
15
20
25
Rotation Speed (rpm)

Figure 5.3. **CAR-cKO mice perform poorly in Rota-rod tests with incremental fixed speed.** (A) The rotation settings for the Rota-rod test. The fixed speeds were set at 5, 10, 15, 20, and 25 rpm for a maximum of 300 sec. (B) Rota-rod performance of male mice. Although at 5 rpm, cKO mice performed decently, they performed much worse than WT mice at ≥ 10 rpm. (C) Rota-rod performance of female mice. Female mice were slightly less affected by CAR-knockout, however when the rotation speed is ≥ 15 rpm cKO mice performed poorly. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.001$, ***. n.s.: not significant.

Figure 5.4


A
40
5
Speed
(rpm)
0
Time (sec)
300
B
Latency to Fall (sec)
150
125
100
75
50
25
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***
WT
KO
C
Rotation Speed at Fall (rpm)
20
15
10
5
0
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WT
KO
D
Latency to Fall (sec)
150
125
100
75
50
25
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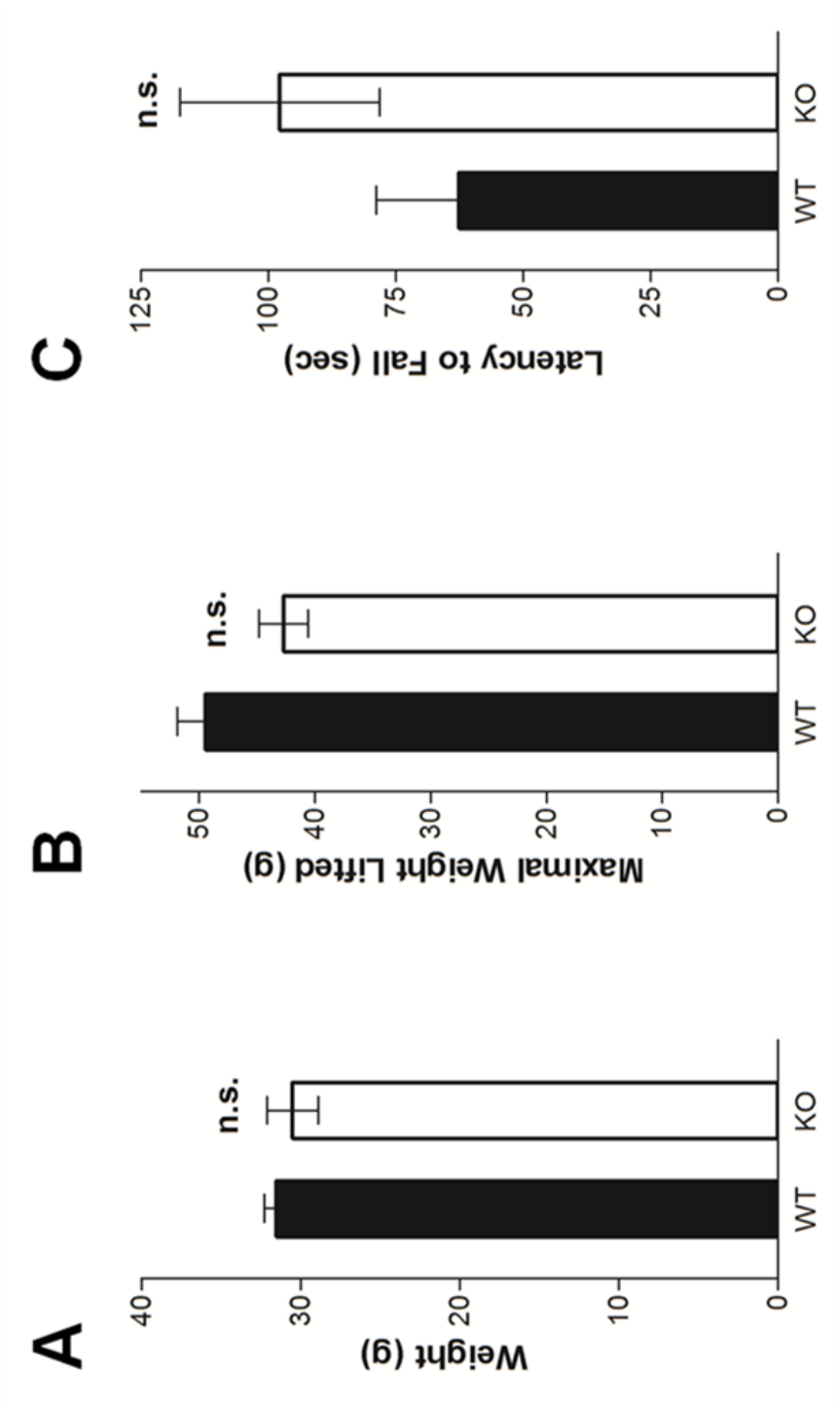
***
WT
KO
E
Rotation Speed at Fall (rpm)
25
20
15
10
5
0
***
WT
KO

Figure 5.4. **CAR-cKO mice perform poorly in Rota-rod tests with accelerating speed.** (A) The rotation settings for the Rota-rod test. The accelerating speed was set from 5 to 40 rpm within a total duration of 300 sec. (B and C) Rota-rod performance of male mice. The latency to fall (B) and the rotation speed at fall (C) were recorded. CAR-cKO mice fall within a much shorter time and at a much lower rotation speed comparing to WT mice. (D and E) Rota-rod performance of female mice. The latency to fall (D) and the rotation speed at fall (E) were recorded and statistically analyzed. Values represent the mean ± SEM. Statistical significance is defined as: $p < 0.001$, ***.

Figure 5.5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Maximal Weight Lifted (g)
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n.s.
WT
KO
C
Latency to Fall (sec)
125
100
75
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25
0
n.s.
WT
KO

Figure 5.5. **CAR-cKO mice have normal weights, muscle strength, and muscle endurance.** (A) The mouse body weights. CAR-cKO mice have similar weights comparing to WT mice. (B) The mouse muscle strength. The strength was measured by a weight-lifting assay and only the strength of the forelimbs was evaluated (see Materials and Methods). CAR-cKO mice performed equally well comparing to WT mice. (C) The mouse muscle endurance. The endurance was measured by an inverted cage assay and the endurance of all four limbs of a mouse was assessed. No significant difference was observed between cKO and WT mice. Values represent the mean ± SEM. n.s.: not significant.

Figure 5.6

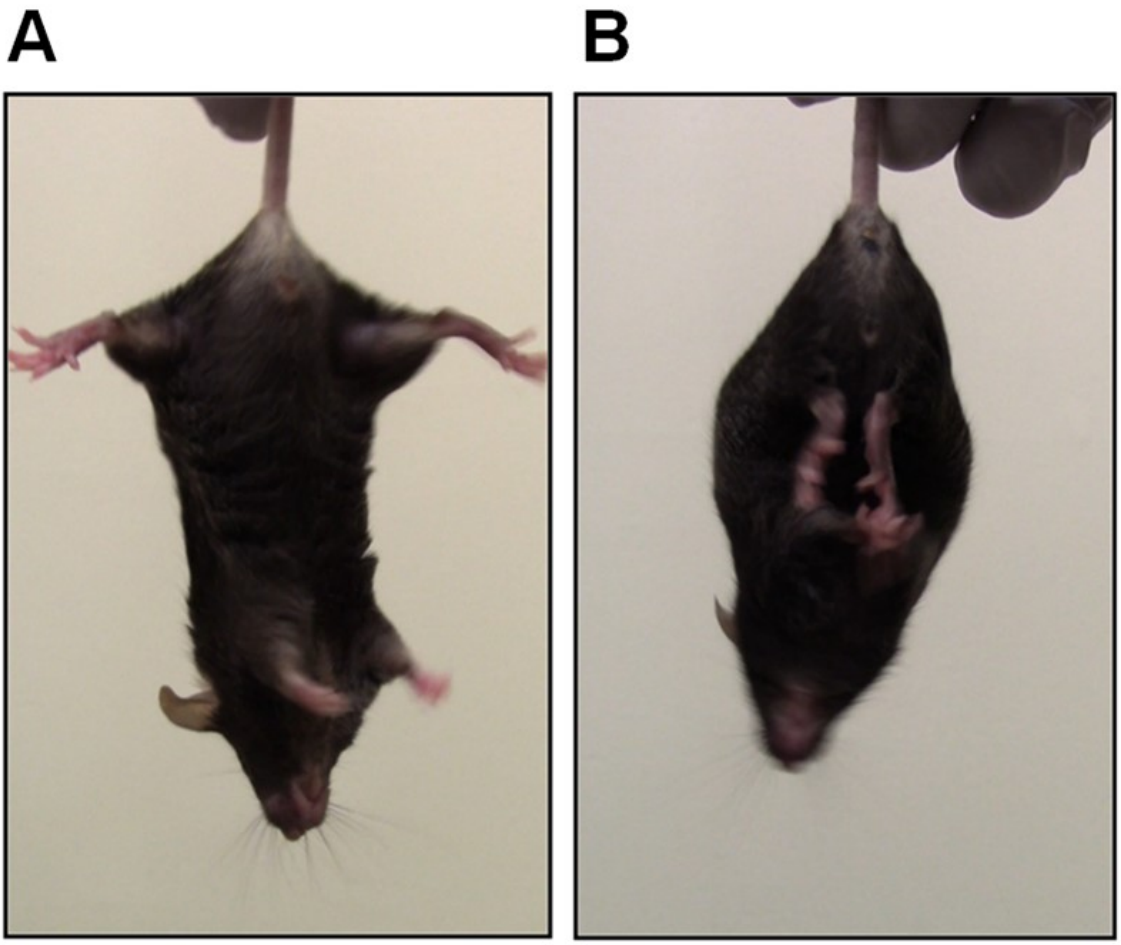

Figure 5.6. **CAR-cKO mice display a mild clasping phenotype.** Mice were suspended by their tails in midair and video-recorded for 20 sec. (A) A representative WT mouse. The mouse widely extended their forelimbs and hind limbs and did not show a sign of motor problem. (B) A representative cKO mouse. The hind limbs of the cKO mouse was hanging down during the 20 sec duration, and as an attempt to secure itself, it intermittently used its fore paws to grasp its hind paws. Therefore, cKO mice showed a mild clasping phenotype indicating a motor problem.

CHAPTER 6

DISCUSSION

Reported Transmembrane Proteins that Associate with Ribosomes on the Membrane

CAR is proposed to regulate the development of CNS (Abe et al., 1997; Honda et al., 2000; Hotta et al., 2003) but we did not know how exactly CAR does it. Although recent studies showed that CAR promotes neurite outgrowth *in vitro* upon binding to its extracellular ligands (Patzke et al., 2010), the downstream signaling pathways are poorly explored. To address this question, we applied proteomic analysis using mass spectrometry and identified many translational proteins that may bind to the cytoplasmic domain of CAR. These proteins include ribosomal subunit proteins, translation initiation factors, elongation factors, and mRNA-binding proteins (Figure 3.1), suggesting that CAR may bind to translational proteins and regulate protein synthesis.

It is interesting to hypothesize that CAR may bind to translation machinery at the cell surface membrane (plasmalemma) because such observations are scarce. Although it is known that *intracellular* transmembrane proteins can bind to ribosomes, such a binding was only reported at the inner membranes, such as the rough endoplasmic reticulum (rER) and the mitochondrial inner membrane.

It is long known that on the outer (cytosolic) surface of the rER, transmembrane proteins directly bind to ribosomes (Kalies et al., 1994; Lauring et al., 1995; Sabatini et al., 1971), and hence traditionally the ribosomes are divided into two classes: free ribosomes and membrane-bound ribosomes (Andrews and Tata, 1971a, b). Two well-studied rER

transmembrane proteins associate with ribosomes: 1) the SRP receptor and 2) the channel protein Sec61/SecY. When a polypeptide is being synthesized, the first 5-30 amino acids act as a signal which is bound by a signal recognition particle (SRP), and this SRP-ribosome complex is then bound to the rER by the transmembrane protein – the SRP receptor (Lauring et al., 1995; Zhang et al., 2012). The rER was believed to mainly produce secretory proteins and integral membrane proteins, both of which require the translocation channel proteins to cross the rER membrane (Rapoport, 2007). After binding to SRP, ribosomes are directly bound to the channel protein Sec61/SecY which regulates nascent chain translocation and protein integration into the rER membrane (Gorlich et al., 1992; Hessa et al., 2005; Kalies et al., 1994). Therefore, the SRP receptor and the channel proteins Sec61/SecY represent the transmembrane proteins that associate with ribosomes on the rER membrane.

However, many questions remain unanswered and other rER transmembrane proteins might exist to regulate rER-ribosome-mediated translation. Above all, these two transmembrane proteins mainly function in signal recognition and translocation of the nascent peptide chains (Gogala et al., 2014; Rapoport et al., 2004; Voorhees et al., 2014; Zhang and Shan, 2014); it is not clear whether they regulate translation. Additionally, after translation termination, although ribosomes can be freed into the cytosol, surprisingly a large fraction of ribosomes are still retained at the rER membrane without translating any mRNA (Seiser and Nicchitta, 2000); it is not known how the rER transmembrane proteins control the binding and release of the ribosomes. Last but not least, although rER was thought to mainly produce secretory and membrane proteins, recent transcriptome-scale studies have demonstrated that

rER-bound ribosomes also translate a large fraction of mRNAs encoding *cytosolic* proteins (Jagannathan et al., 2014; Reid and Nicchitta, 2012, 2015). Apparently, ribosomes translating the cytosolic protein mRNAs do not need to associate with these translocation channel proteins. Therefore, other ribosome-binding rER transmembrane proteins must exist and these proteins remain to be identified and their functions characterized.

In mitochondria, membrane proteins sequester the translation machinery at the mitochondrial inner membrane and regulate mitochondrial protein synthesis. First, membrane-associated translational activators bind to the mitochondrial mRNAs at the inner membrane via the 5' UTR of the mRNAs (Fox, 1996; Michaelis et al., 1991). Additionally, transmembrane proteins associate with mitochondrial ribosomes at the inner membrane: for example, the integral membrane protein Oxa1 binds to the large ribosomal subunit and probably facilitates co-translational protein insertion into the inner membrane (Jia et al., 2003; Szyrach et al., 2003). Similar to Oxa1, the membrane-associated protein Mba1 (Ott et al., 2006) and another integral membrane protein Mdm38 (Frazier et al., 2006) also bind to the mitochondrial ribosomes. Mba1 and Mdm38 proteins physically interact with mitochondrial ribosomes as revealed by GST pulldown (Bauerschmitt et al., 2010) and they regulate mitochondrial translation of *COX1* (cytochrome oxidase) and *CYTB* (cytochrome *b*) mRNAs (Bauerschmitt et al., 2010); both of these mRNAs encode proteins belonging to the mitochondrial respiratory chain complexes (Dennerlein and Rehling, 2015). In summary, these mitochondrial inner membrane proteins directly bind to ribosomes and regulate mitochondrial mRNA translation.

Only recently has the *plasmalemmal* transmembrane protein DCC, a member of the IgSF proteins, been reported to directly associate with the translation machinery at the cell plasma membrane. Colocalization of DCC and ribosomes is detected on the nerve growth cone, and PSD-95, DCC, and eIF4E colocalizing puncta can be seen along individual neurites as revealed by confocal microscopy (Tcherkezian et al., 2010). Ultrastructural studies using electron microscopy further confirm that DCC colocalizes with ribosomes at the growth cone and the filopodial tip (Tcherkezian et al., 2010). Additionally by performing the proteomic analysis of proteins co-immunoprecipitated with DCC, many translational proteins were identified such as ribosomal proteins (L5, L10a, L23, S24 and S10) and translation initiation factors (eIF2Bβ, eIF2γ, eIF3e, and eIF4E-L3); DCC also directly interacts with the ribosomal protein L5 as revealed by affinity pulldown assays (Tcherkezian et al., 2010). Together these results suggest that the transmembrane protein DCC physically binds to the ribosomes at the plasmalemma.

DCC is the first reported cell surface transmembrane protein that binds to ribosomes, indicating a potentially new category of membrane-bound ribosomes: the plasmalemma-bound ribosome (in addition to the rER-bound ribosome and the mitochondrial inner membrane-bound ribosome). Therefore, it was intriguing to investigate whether the transmembrane protein CAR also binds to ribosomes and translational proteins at the cell surface membrane and regulates translation.

The Transmembrane Receptor CAR Physically Associates with Translational Proteins

To test the hypothesis that CAR may bind to ribosomes and translational proteins, we first verified our MS results by performing GST-CAR pulldown assays with U87CAR cell lysates. Indeed, CAR associates with translational proteins such as S6, eIF4E and eEF1A (Figure 3.2A-C). Next, we tried to map the binding region of CAR using progressive truncations of CAR's C-terminus and discovered that the deletion of the last three amino acids "SIV" (GST-ΔSIV) completely abolished the binding to S6, eIF4E, and eEF1A (Figure 3.2A-C). Therefore, CAR indeed associates with translational proteins and SIV is essential for binding to these proteins. However, the GST-ΔSIV and GST-Δ26 truncation proteins did not affect CAR's binding to hnRNPU (Figure 3.2A-C), indicating that CAR may bind to other translational proteins through different regions of its cytoplasmic tail.

Since CAR binds to translational proteins, we proposed that CAR should colocalize with these proteins in ribosome subunits and in polysomes separated by sucrose gradients (polysome profiling). Indeed CAR colocalized with S6, L4, eIF4E and eIF4G across the sucrose gradients (Figure 3.3A-C). A prominent colocalization can be seen with CAR, eIF4E, and eIF4G, suggesting that CAR localizes with the 40S and 60S subunits and the mono-ribosome. Interestingly, CAR was also present in the early polysome fractions; this result is different from DCC – the signal of DCC is not present in the polysome fractions (Tcherkezian et al., 2010). It is probably because DCC does not bind to any elongation factors,

but in our MS results eEF1A and eEF2 are present with high hits. Moreover, CAR signal is faint in the polysome fractions, suggesting a weak binding to the elongating polysomes. The weak binding is not surprising because as introduced above, in addition to being an elongation factor, eEF1A is also a potent cytoskeleton-regulator: it cleaves microtubules (Shiina et al., 1994), and binds to and regulates the actin cytoskeleton (Gross and Kinzy, 2005; Yang et al., 1990). Therefore, a significant proportion of CAR may associate with the eEF1A-cytoskeleton complex and a minor fraction of CAR may bind to the elongating polysomes, partially explaining CAR's weak signal in the polysome fractions. Taken together, these results suggest that CAR strongly associates with the translation initiation complex and weakly binds to elongating ribosomes.

Additionally, the colocalization of CAR with eIF4E and eIF4G in sucrose gradients suggests that CAR may reside in the cap-binding protein complex. Both eIF4E and eIF4G locate in the mRNA cap-binding complex and they play an important role in regulating translation initiation: eIF4E directly binds to the mRNA 5' cap (Sonenberg et al., 1978; Sonenberg et al., 1979) and eIF4G is the scaffolding protein that bridges mRNA-eIF4E and ribosome together to initiate translation (Imataka et al., 1998; Yanagiya et al., 2009). To examine whether CAR is present in the cap-binding protein complex, we performed an m^7G mRNA cap-pulldown analysis. The conjugated m^7GTP-beads bound to CAR with moderate affinity suggesting that CAR indeed resides in the cap-binding complex (Figure 3.4A). To further investigate whether CAR could directly interact with the cap-binding complex proteins, we cloned and produced recombinant eIF4E protein tagged with a 6xHis tag (His-eIF4E, Figure 3.4B). Indeed, CAR

directly interacts with eIF4E as revealed by direct pulldown assay (only GST-CAR and His-eIF4E are present in the pulldown system, no other cellular proteins) and the deletion of SIV (ΔSIV) completely ablated this binding (Figure 3.4C). Taken together, these results suggest that CAR is present in the cap-binding protein complex and it directly binds to eIF4E through its PDZ-binding domain "SIV".

Since CAR contains a PDZ-binding domain at the end of its C-terminus, does it bind to other translational proteins through the PDZ domain proteins? The PDZ domain was named after the postsynaptic protein PSD-95, the *Drosophila* septate junctional protein Discs-large, and the tight junction protein ZO-1 (Cho et al., 1992; Kim et al., 1996; Woods and Bryant, 1993) and it exists in numerous proteins across species (Ivarsson, 2012; Ponting, 1997). This domain mainly functions in regulating protein-protein interactions and in assembling large protein complexes involved in signaling or subcellular transport (Harris and Lim, 2001; Hung and Sheng, 2002; Luck et al., 2012). Major PDZ domain proteins include: PSD-95, ZO-1, MUPP1, PICK1, Syntrophin, Scribble, Shank, CASK (calmodulin-dependent serine kinase), and NHERF (Na^+/ H^+ exchanger regulatory factor) (as reviewed in Harris and Lim, 2001; Ivarsson, 2012). The PDZ domain binds to ligand proteins typically through the very end of their C-terminal sequences: for example, PSD-95 can bind to the C-terminus of potassium channel, NMDA receptor subunits, Neuroligin, and CAR (Excoffon et al., 2004; Irie et al., 1997; Kim et al., 1995; Kornau et al., 1995). PDZ domains can also interact with internal peptide sequences, e.g., PSD-95 and Syntrophin interact with the central region of nNOS (neuronal nitric oxide synthase) (Brenman et al., 1996a; Brenman et al., 1996b). However, so

far the PDZ domain proteins have never been reported to associate with translational proteins.

Additionally, CAR is reported to directly associate with several PDZ-containing proteins such as PSD-95, MUPP-1, ZO-1, MAGI1b, PICK1, LNX, and LNX2 (Cohen et al., 2001b; Coyne et al., 2004; Excoffon et al., 2004; Mirza et al., 2005; Sollerbrant et al., 2003) but none of them directly associates with translational proteins or ribosomal proteins. However, one of these proteins, MUPP1, associates with viral proteins that modulate mTOR functions. MUPP1 can bind to adenovirus (Ad) E4-ORF1 and human papillomavirus (HPV) E6 oncoproteins through the PDZ-binding motifs of these proteins (Lee et al., 2000); functionally, Ad E4-ORF1 sequesters MUPP1 within the cytoplasm and HPV E6 targets MUPP1 for degradation to induce abnormal cell growth (Lee et al., 2000). Both proteins can activate the mTOR pathway in host cells. Ad E4-ORF1 mimics growth factor signaling by activating PI3-kinase, resulting in mTOR activation (O'Shea et al., 2005a; O'Shea et al., 2005b); overexpression of HPV E6 causes mTORC1 activation and an increase in cap-dependent translation (Spangle et al., 2012; Spangle and Munger, 2010). The activation of mTOR pathways may facilitate viral protein synthesis and replication. To sum up, from current literature, it seems that the binding of CAR to other translational proteins is not mediated by PDZ domain proteins.

Could CAR bind to translational proteins through other interactor proteins? As introduced above, CAR directly binds to cytoskeletal proteins such as actin and tubulin (Fok et al., 2007; Huang et al., 2007). On the other hand, cytoskeletal proteins can also directly associate with

translational proteins, for example, actin and tubulin directly interact with the elongation factor eEF1A. It was long known that eEF1A can crosslink actin (Owen et al., 1992) and sever tubulin (Shiina et al., 1994) *in vitro*. Once eEF1A binds to actin, the translational activity of eEF1A is blocked (Liu et al., 1996), and other actin crosslinkers will be excluded by eEF1A binding (Owen et al., 1992). Among the CAR-interactor candidates identified by our proteomic analysis, eEF1A is the most prevalent protein as it is present in nearly every excised protein band. Therefore, CAR may bind to eEF1A directly or indirectly through actin and tubulin. Additionally, cytoskeletal proteins also act as a dynamic scaffold to hold together other translational proteins such as cap-binding proteins (Zumbe et al., 1982), eIF4A and eIF4B (Howe and Hershey, 1984), and aminoacyl-tRNA synthase (Kim and Coulombe, 2010), and disruption of actin filaments using cytochalasin D releases mRNA from the cytoskeletal framework and inhibits protein synthesis in cultured HeLa cells (Ornelles et al., 1986). Therefore, CAR may bind to other translational proteins directly or CAR may associate with them indirectly through cytoskeletal proteins (as illustrated in Figure 6.1B).

By using immunofluorescence, we further supported that CAR colocalizes with translational proteins in cultured HEK293 cells expressing full-length CAR (293CARV5 cells). In single 293CARV5 cells, a significant overlay of immunostaining between CAR and ribosomes (as revealed by ribosomal protein L4 staining) was observed (Figure 3.5A). When 293CARV5 cells grew to a confluent monolayer, CAR was concentrated at cell-cell contacts, however very little ribosomal proteins were detected in these contacts (Figure 3.5B). This is unusual as many CAR-interacting proteins such as ZO-1, LNX, PSD-95, and MUPP1, have a strong

colocalization with CAR at tight junctions or cell-cell contacts (Cohen et al., 2001; Coyne et al., 2004; Excoffon et al., 2004; Raschperger et al., 2006; Sollerbrant et al., 2003). A possible explanation is that CAR's homophilic/ heterophilic interactions *in trans* (Patzke et al., 2010) may release the membrane-bound ribosomes into the cytoplasm. Together with the other results, our findings suggest that CAR physically interacts with translational proteins.

CAR Functionally Regulates Protein Synthesis In Vitro and in Cultured Cells

We then hypothesized that CAR may functionally affect translation. *In vitro* translation assay is the best mechanistic study as it evaluates direct interactions between CAR and translational proteins in rabbit reticulocyte lysates (Pelham and Jackson, 1976). Indeed CAR regulates *in vitro* protein synthesis: GST-CAR decreased both cap- and IRES-dependent translation, where as the control protein GST had no effect at all (Figure 3.6A and B). We also found a significant decrease in cap/ IRES ratio by GST-CAR, but not other truncation proteins (Figure 3.6C), suggesting that CAR affects cap-dependent translation to a greater extent. To examine whether CAR functionally regulates translation in living cells, we performed cell-based translation assays. We first tested the effects of CAR stable transfection on reporter translation and found that, at similar transfection efficiencies, U87CAR cells had greatly reduced protein synthesis comparing to the control U87LNCX cells (Figure 3.7). This result suggests that CAR functionally regulates mRNA translation in cultured cells.

To further confirm this result in another cell culture system, we carried out transient transfections in Chinese hamster ovary (CHO) cells which do not have endogenous CAR expression. We found that over-expression of CAR in CHO cells significantly reduced translation as compared to control transfected cells (Figure 3.8C and D). Additionally, by transiently transfecting the C-terminal progressive truncates of CAR, we confirmed that ΔSIV, Δ26, Δ50, Δ74, and Δ98 nearly had no translation inhibitory effects (Figure 3.8C and D). These results suggest that the C-terminus of CAR is essential in regulating translation and the last three amino acids SIV are necessary for this translational control. Moreover, this cell-based translation result coheres well with our *in vitro* translation results (Figure 3.6A and B) and GST-pulldown results (Figure 3.2A-C). We then explored the effects of the N-terminus of CAR on translation in cells. First, we transiently transfected CAR constructs lacking D1 (ΔD1), D2 (ΔD2), or both (D0) and found that all of the truncation mutants greatly reduced translation like full-length CAR (Figure 3.9), suggesting that CAR-regulated translation may not require an intact N-terminus. However, is it true when a ligand is present? FN40 is one of CAR's ligands and it directly interacts with CAR's D2 domain (Patzke et al., 2010). Thus we performed a ligand engagement assay and discovered that FN40 significantly increased translation in pCAR- and pΔD1-transfected cells (both expressing the D2 domain) but not in pcDNA-, pD0-, and pΔD2-transfected cells (none expressing the D2 domain; Figure 3.10). These results suggest that FN40-induced increase in translation requires the D2 region of CAR.

Many transmembrane proteins bind extracellular molecules such as insulin, growth factors,

amino acids, and glucose, and activate the mTOR signaling pathway to trigger translation. For example, insulin, insulin-like growth factor I (IGF-I), and IGF-II all bind to the insulin receptor (IR) which belongs to the large class of tyrosine kinase receptors (Belfiore et al., 2009; Ward and Lawrence, 2009). Binding of insulin to its receptor activates insulin receptor substrate (IRS-1) which in turn activates the phosphoinositide 3-kinase (PI3K)/ Akt and mTOR pathways (Corradetti and Guan, 2006; Gual et al., 2005; Shaw, 2001). Insulin-induced activation of mTOR then phosphorylates 4E-BP, facilitates the assembly of the eIF4E complex, and initiates protein synthesis (Pause et al., 1994; Shen et al., 2005). Similar to insulin, growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) also bind to their tyrosine kinase receptors (EGFR and PDGFR) and activate the Ras-ERK pathway which further activates the mTOR pathway and protein synthesis (Chang et al., 2003; McCubrey et al., 2007; Mieulet and Lamb, 2010; Perrone et al., 2009). Transmembrane proteins can also participate in uptake of amino acids and glucose to activate mTOR. For example, the extracellular amino acid L-glutamine is sensed and transported into the cytoplasm through its high affinity transporter protein SLC1A5 (solute carrier - family 1 member 5; Nicklin et al., 2009); then it will be transported out of the cells though the bidirectional transporter SLC7A5/SLC3A2 in exchange for other essential amino acids which subsequently activate mTOR (Nicklin et al., 2009). The transmembrane glucose transporter 1 (GLUT1, or SLC2A1) mediates the influx of glucose which activates mTOR and phosphorylates 4E-BP and S6K (Bell et al., 1990; Buller et al., 2011) and interestingly, the expression level of GLUT1 and GLUT4 is in turn regulated by mTOR activity (Buller et al., 2008; Taha et al., 1999).

Does the FN40-CAR interaction upregulate translation through the mTOR pathway? To answer this question, we tested 4E-BP and S6K phosphorylation upon treatment with insulin and FN40. We found that while insulin significantly increased the phosphorylation of 4E-BP and S6K, FN40 did not have a similar effect (Figure 3.11), suggesting that CAR-regulated translation does not depend on mTOR signaling but may depend on physical association/ dissociation with translational proteins. Similarly, Netrin1-DCC interaction plays a role in regulating physical interactions with translational proteins: when co-immunoprecipitating DCC without adding Netrin1, DCC binds to translational proteins; when co-immunoprecipitating DCC together with Netrin1, DCC fails to associate with these proteins (Tcherkezian et al., 2010). A potential explanation is that Netrin1 binding to DCC may change the conformation of DCC's cytoplasmic domain and release the ribosome. However, we could not perform this experiment as we do not have a CAR-antibody strong enough for co-immunoprecipitation. Taken together, these results suggest that CAR-FN40 interaction regulates translation through physical association/ dissociation with translational proteins (as summarized in Figure 6.1A), rather than mTOR signaling.

Interestingly, extracellular signals may upregulate protein synthesis by decreasing the expression of translation inhibitor proteins. Integrins bind to extracellular matrix proteins such as fibronectin (FN) and collagen type I (CL) (Akiyama et al., 1990; Buck and Horwitz, 1987) though the RGD (Arg-Gly-Asp) motif (Pierschbacher and Ruoslahti, 1984a, b). Although integrins can regulate protein synthesis through PI3K/ mTOR pathways (Chung et

al., 2002; Gorrini et al., 2005; Maeshima et al., 2002; Pabla et al., 1999), binding of β1 integrin to FN or CL results in greatly reduced 4E-BP protein levels whereas binding to laminin does not lead to an obvious 4E-BP reduction (Nho and Peterson, 2011). The interaction of β1 integrin and CL increases the activity of Src, which decreases the protein phosphatase 2A (PP2A) activity (Barisic et al., 2010; Chen et al., 1992); reduced PP2A function subsequently inhibits 4E-BP expression (Li et al., 2010; Nho and Peterson, 2011). Conversely, inhibition of Src increases 4E-BP expression via high PP2A activity (Nho and Peterson, 2011). These results suggest that the interaction between extracellular matrix proteins and transmembrane receptor proteins may increase translation through decreasing 4E-BP expression. However, in our system with the FN40 treatment for as long as 1 hr, we did not observe a significant reduction of the total level of 4E-BP proteins (Figure 3.11, 4E-BP-total). This result suggests that FN40-CAR–mediated increase of translation is not due to 4E-BP downregulation.

CAR Interacts with Translational Proteins in Neurites and Regulates Translation during Neurite Outgrowth

To test whether CAR physically associates with translational proteins in the developing nervous system, we first examined the presence of CAR and translational proteins in growth cone particles (GCPs). Indeed, a strong signal of CAR and translational proteins was detected in the fractionated growth cone particles (Figure 4.1A, B); these results agree well with the

literature that both CAR and translational proteins are enriched in GCPs (Abe et al., 1997; Estrada-Bernal et al., 2012). Additionally, we tested the interactions between CAR and translational proteins in neurons. In the isolated GCPs, CAR bound to translational proteins with high affinity as revealed by GST-pulldown assay (Figure 4.2) and CAR was present in the cap-binding complex as revealed by cap-pulldown analysis (Figure 4.3). We also detected a colocalization between CAR and translational proteins in neurites. Our confocal immunofluorescence results demonstrated that the signal of CAR and translational proteins significantly overlapped in growth cones and neurite shafts (Figure 4.4A and B). Taken together, these results suggest that CAR interacts with translational proteins in neurites.

Next we examined whether CAR regulates translation during neurite outgrowth. Since CAR is known to promote neurite outgrowth upon binding to FN40, we first tested this in our lab. Indeed, neurons grown on pre-coated FN40 had significantly more neurite outgrowth (Figure 4.5). We then showed that CAR is essential for FN40-promoted neurite outgrowth as CAR-knockdown completely abolished FN40's outgrowth-promoting effects (Figure 4.5). FN40 has been demonstrated to associate with the D2 loop of CAR's extracellular domain (Patzke et al., 2010); it does not contain a "RGD" motif so that it should not trigger the integrin signaling pathways. To verify that translation plays a role in CAR-mediated neurite outgrowth, we applied a common translation inhibitor, cycloheximide (CHX). Previous studies showed contradictory results in that translation inhibitors decreased (Jareb and Banker, 1997), maintained (Eng et al., 1999), or even greatly promoted (Louis et al., 1994) neurite/ axonal extension; these inconsistencies may be due to the neuronal cell types, culture

conditions, or treatment protocols of translation inhibitors. In our neurite outgrowth system with E16-17 cerebral cortical neurons, a 24-hr treatment of 1 μM of CHX (neurons were not sick as revealed by propidium iodide staining, data not shown) decreased ligand-triggered, but not basal, neurite outgrowth (Figure 4.6). This result suggests that neurite outgrowth promoted by CAR-FN40 interactions requires protein synthesis. Our result is similar to results obtained from an axonal elongation system, where NGF-promoted, but not basal, axon elongation is ablated by inhibiting axonal local translation (Hengst et al., 2009; Gracias et al., 2014).

Finally, we hypothesized that the FN40-CAR interaction should enhance protein synthesis and indeed, FN40 significantly promoted *de novo* translation as revealed by AHA protein metabolic labeling (Figure 4.7 and Figure 4.8). However, FN40's effect on translation is not as significant as that on neurite outgrowth, probably because: 1) treatment of AHA is only 1/6 the time of the 24-hr neurite outgrowth assays (Figure 4.5 and Figure 4.6), or 2) other mechanisms independent of translation may also play a role, such as through signaling pathways such as ERK/MAPK (Kolkova et al., 2000; Schmid et al., 1999), cytoskeletal remodeling by Cdc42/Rac1 (Govek et al., 2005; Li et al., 2002) and membrane addition by SNARE complex (Alberts et al., 2003; Kimura et al., 2003). It should be noted that we also applied AHA-labeling with type-I collagen (CL), which contains an RGD motif that can bind to integrins. Although CL promoted neurite outgrowth in our neuronal culture system (data not shown), it did not increase translation (Figure 4.8), indicating that CL may use different mechanisms other than translation, such as RGD-integrin downstream pathways.

In addition to FN40-CAR–mediated translational control, other ligand-receptor molecules also regulate translation; however, most of these molecules regulate translation through signaling pathways, rather than protein-protein association and dissociation. I have introduced above that Netrin1-DCC, Slit2-Robo, and Sema3A-semaphorin interactions increase translation through the mTOR pathway (see Introduction). In addition, the nerve growth factor (NGF), a strong neurite outgrowth promoter (Levi-Montalcini, 1987), also potently regulates translation. NGF activates the mTOR pathways, induces the assembly of eIF4F cap-binding complex, and induces a 5-fold increase of *de novo* protein synthesis within 30 min (Melemedjian et al., 2010). Together with interleukin-6 (IL-6), it induces an elevation of nociceptive responses in mice (Dina et al., 2008; Lewin et al., 1994) which can be blocked by translation inhibitors (Melemedjian et al., 2010). Since NGF's canonical receptor TrkA is a receptor tyrosine kinase (Barbacid, 1994; Lanave et al., 2007), it can induce a very fast increase in translation through activating and amplifying AKT/mTOR and MAPK signaling cascades (Kimpinski and Mearow, 2001; Melemedjian et al., 2010). Similarly, brain-derived neurotrophic factor (BDNF) also promotes neurite outgrowth (Labelle and Leclerc, 2000; Rabacchi et al., 1999); it binds to receptor tyrosine kinase TrkB and p75 neurotrophin receptor (p75NTR) and rapidly induces translation (Aakalu et al., 2001). BDNF activates the PI3K-mTOR pathway, regulates *Homer 2* and *GluR2* mRNA translation, and regulates synaptic functions (Schratt et al., 2004). Although integrins are known to activate mTOR and regulate translation in cultured cells (Chung et al., 2002; Gorrini et al., 2005; Maeshima et al., 2002; Nho and Peterson, 2011; Pabla et al., 1999), it is not known whether they do so during

neurite outgrowth. Finally, the Netrin1-DCC interaction not only regulates physical association and dissociation with translational proteins (Tcherkezian et al., 2010), but also triggers the mTOR and other signaling cascades (Ma et al., 2010; Meriane et al., 2004). However, currently it is not clear how Netrin1-DCC interaction chooses between signaling pathways and physical protein-protein interactions.

As summarized in the Introduction, neurite outgrowth is regulated by different molecular mechanisms, such as signaling pathways, cytoskeletal dynamics, and membrane addition. Does CAR promote neurite outgrowth through other pathways in addition to translational control? Yes, CAR may regulate neurite outgrowth through ERK1/2 and nNOS1. Previously in our lab, it was demonstrated that CAR can associate with Grb2 and ERK1/2 (Huang PhD thesis). ERK (extracellular signal-regulated kinase) is also known as MAPK (mitogen-activated protein kinases); Grb2 (growth factor receptor-bound protein 2) is an adaptor protein involved in signal transduction and the activation of ERK/MAPK (Kouhara et al., 1997; Schlaepfer et al., 1994). It is already known that NCAM, L1 and N-Cadherin can regulate neurite outgrowth through the MAPK pathway; similarly, CAR may do so through the MAPK pathway. Pharmacological disruption of the ERK/MAPK pathway using U0126 significantly reduced ligand (soluble CAR's ectodomain) -triggered, but not basal, neurite outgrowth (Huang PhD thesis). These results suggest that CAR regulates MAPK pathways during neurite outgrowth. Additionally, CAR interacts with the PDZ-domain protein nNOS1 as revealed by GST pulldown and co-immunoprecipitation, and pharmacological treatment with NOS inhibitors also affected ligand-triggered but not basal neurite outgrowth (Huang

PhD thesis), suggesting that CAR may regulate nNOS1 activities and modulate neurite outgrowth.

Furthermore, CAR may affect neurite outgrowth through regulating cytoskeletal dynamics. CAR directly binds to actin and tubulin, and the cytoplasmic domain of CAR (GST-CAR) plays a role in bundling actin and tubulin *in vitro* (Fok et al., 2007; Huang et al., 2007). CAR expressed in cultured cells maintains microtubule framework in the presence of a destabilizing drug nocodazole (Fok et al., 2007), suggesting that CAR may regulate microtubule dynamics in cells. Although the effects of cytoskeleton-disrupting drugs on CAR-promoted neurite outgrowth are not studied, in other systems, application of actin/tubulin stabilizing/destabilizing agents such as cytochalasin, phalloidin, paclitaxel, and nocodazole indeed affect neurite outgrowth (Esmaeli-Azad et al., 1994; Frey et al., 2000; Lankford and Letourneau, 1989; Letourneau and Ressler, 1984); therefore, it is possible that CAR may also modulate cytoskeletal dynamics during neurite outgrowth. Additionally, CAR directly binds to a cytoskeleton-regulating protein, IQGAP1 (IQ-motif containing GTPase activating protein 1; Fok PhD thesis). IQGAP1 is a scaffold protein involved in regulating actin cytoskeleton, cell migration and adhesion, as well as neurite outgrowth (Li et al., 2005c; Noritake et al., 2005; Watanabe et al., 2004). Although the role of IQGAP1 in CAR-mediated neurite outgrowth is not examined, CAR may associate with IQGAP1, regulate growth cone cytoskeleton, and modulate neurite outgrowth.

Therefore, in addition to translational control, CAR may affect neurite outgrowth through

regulating MAPK pathways and cytoskeletal dynamics. However, whether CAR regulates membrane addition during neurite outgrowth is unknown. Additionally, how CAR controls the interplay among these downstream pathways remains to be further investigated.

To sum up, our study shows that CAR promotes neurite outgrowth *in vitro* through interacting with translational proteins and regulating protein synthesis. In the future, studies could be performed to delineate how CAR interacts with eIF4E and eEF1A to regulate cap-dependent initiation and elongation. Additionally, together with the literature on DCC, our study supports a general molecular mechanism in which transmembrane receptors associate with translational proteins and regulate translation. It will be interesting to examine other IgSF-CAMs such as N-Cadherin, NCAM, and L1 to evaluate whether this is a conserved mechanism.

Does CAR-FN40 Interaction Play A Permissive or Instructive Role?

It has often been debated whether extracellular cues and transmembrane receptors play a permissive or instructive role. During axon guidance, instructive molecules *directly* trigger growth cone movement; on the other hand, permissive molecules *indirectly* regulate growth cone motility by allowing the instructive molecules to influence the growth cones. This is however, an over-simplified definition of the permissive and instructive roles; whether one molecule plays a permissive or instructive role depends on the specific biological process it is

regulating.

For example, the Slit and Robo proteins can either play an instructive role during axons crossing the floor plate midline or play a permissive role during thalamocortical axon guidance. First, during the development of the spinal cord, the Slit protein functions as an instructive (repulsive) cue. Slit are large secretory proteins that were first identified as an axonal repellent in the *Drosophila* midline and in the rat spinal cord (Brose et al., 1999; Kidd et al., 1999); there are three Slit proteins (Slit1-3) in vertebrates (Gara et al., 2015). The repulsive actions of Slit proteins are mediated by the transmembrane receptors of the Robo (roundabout) protein family (Kidd et al., 1998; Zallen et al., 1998). The Robo family receptors share a structural similarity with the Netrin receptor, DCC (Kolodkin and Tessier-Lavigne, 2011), and both receptor families belong to the immunoglobulin super family (IgSF). In mammals, there are three Robo (Robo1-3) proteins (Kolodkin and Tessier-Lavigne, 2011) and Robo3.1, a splice isoform of Robo3, inhibits the growth cone repulsion induced by Robo1 and Robo2 (Chen et al., 2008; Sabatier et al., 2004). Slit and Robo proteins function together to navigate the commissural axons crossing the floor plate midline in the spinal cord, and deletion of either Slit or Robo ablates the midline crossing (Long et al., 2004). In this scenario, Slit-Robo interaction directly regulates commissural axon guidance and therefore the Slit and Robo proteins play an instructive role.

However, during the guidance of the thalamocortical axons (TCAs), the Slit-Robo interaction can play a permissive role (Bielle et al., 2011; Dudanova and Klein, 2013). The TCA

functions in conveying sensor-motor and other perceptive information to the neocortex (Garel and Lopez-Bendito, 2014). During development, the rostral subpopulations of TCAs begin elongating from the thalamus in the diencephalon (Lopez-Bendito and Molnar, 2003), pass through a corridor of guidepost neuron cells (Lopez-Bendito et al., 2006), cross the striatum, and finally reach the neocortex in the ventral telencephalon (Garel and Rubenstein, 2004). The corridor cells strongly express gradients of Netrin1 and Slit1. When Slit1 alone was applied to the rostral thalamic explants, the TCAs were repelled; however the application of Netrin1 alone had no effect. Surprisingly, when both Slit1 and Netrin1 were applied together, the axons were attracted to Netrin1 (Bielle et al., 2011). This could be due to: 1) Slit1 mediates attraction in the presence of Netrin1, or 2) Slit1 enables Netrin1 attraction. Next, when rostral thalamic explants were cultured on COS cells expressing Netrin1, the axons did not turn; however when Slit1 were applied, the axons turned towards the Netrin1-expressing COS cells, indicating that Slit1 does not navigate the TCAs but permits them to be attracted by Netrin1 (Bielle et al., 2011). Therefore under this circumstance, Slit1 *indirectly* regulates TCA guidance though the instructive cue Netrin1; it plays a permissive role.

Now let us look at whether cell adhesion molecules (CAMs) play a permissive or instructive role. Certain CAM members such as Fasciclin II, Beat-Side, and DsCAM indeed play an instructive role during axon guidance. One of the IgSF-CAM members, Fasciclin II, directly regulates axon guidance through modulating axon fasciculation and defasciculation via homophilic interactions. During insect CNS development, the disruption of Fasciclin II homophilic interactions ablates axonal fasciculation as well as the extension and guidance of

the axons (Harrelson and Goodman, 1988; Lin and Goodman, 1994); it may do so through interacting with other proteins such as connectin and semaphorin1A (Yu et al., 2000). Another instructive IgSF member is the *Drosophila* DsCAM (Down syndrome CAM) which has over 38,000 isoforms through alternative splicing (Neves et al., 2004). Each isoform preferentially binds to itself rather than to other isoforms and, interestingly, the self-binding of DsCAM mediates homophilic repulsion, but not adhesion or attraction, so that the neurites from the same neuron do not overlap with each other (Hattori et al., 2009; Wojtowicz et al., 2007). Therefore DsCAM directly but negatively regulates axon guidance through self-avoidance (Grueber and Sagasti, 2010; Hattori et al., 2008). Lastly, the IgSF members Beaten Path (Beat) and Sidestep (Side) form a heterophilic complex which facilitates the attraction of *Drosophila* motor neuron axons (Siebert et al., 2009). Indeed, a recent study on the transcription factor network using mRNA profiling further demonstrates that Beat, Fasciclin II, and another CAM Neuroglian function in attraction, repulsion, and adhesion during *Drosophila* motor axon guidance (Zarin et al., 2014).

Despite these instructive CAMs, several traditionally well-studied CAMs including cadherin family proteins (such as N-Cadherin) and IgSF-CAM members (such as NCAM and L1) were long believed to play an instructive role; however due to the lack of strong evidence they are now believed to function mostly as permissive molecules. NCAM, L1, and N-Cadherin were known to promote neurite outgrowth through homophilic interactions or heterophilic interactions with the extracellular matrix (ECM) proteins (Appel et al., 1993; Doherty et al., 1995; Drazba and Lemmon, 1990; Neugebauer et al., 1988; Takei et al., 1999).

It was once believed that axonal guidance is regulated by the adhesion of the growth cone to substrates and that CAMs play an important regulatory role (Lockerbie, 1987; Sanes, 1989). For example, neurons attach to substrates with different adhesivity (adhesive forces: polyornithine > palladium > plastic culture dish). Neurons that were grown on the most adhesive substrate, polyornithine, had the longest neurites, while neurons grown on palladium and plastic dish had intermediate and shortest neurites, respectively (Letourneau, 1975). Therefore, the growth cones appeared to be guided by such adhesive hierarchies. However, this is not necessarily true: although the adhesiveness to poly-L-lysine is greater than that of laminin and N-Cadherin, the latter two substrates stimulate much more neurite outgrowth (Lemmon et al., 1992), suggesting that the adhesive forces mediated by CAMs only have a permissive influence on growth cone motility. Additionally, a direct role of these CAMs in growth cone turning has never been reported suggesting that they do not directly modulate axon steering. Finally, during Sema3A-regulated growth cone turning, L1 plays an indirect role as a co-receptor in the semaphorin receptor complex (Castellani et al., 2000; Castellani et al., 2002; Castellani et al., 2004). Although the expression of *Drosophila* N-Cadherin on one growth cone may help target the processes of another neuron, its exact function and mechanism are not clear (Nern et al., 2008). Therefore, these three CAMs promote neurite outgrowth to reach a place where the growth cones can be influenced by the instructive cues. Currently, they are generally recognized as permissive receptors during axon guidance.

Although NCAM, L1, and N-Cadherin play a permissive role during axon guidance, they play an instructive role during neurite outgrowth due to their direct regulation of

neuritogenesis and neurite extension. However, it must be emphasized that the effectiveness of any individual CAM depends on the cell type and the developmental stage: NCAM, L1, and N-Cadherin can all promote embryonic retinal ganglion cell (RGC) neurite outgrowth but only NCAM has effects on P10 rod cells; on the other hand, only L1 and N-Cadherin, but not NCAM, regulate postnatal RGC neurite outgrowth (Kljavin et al., 1994). From this point of view, the role of CAR is instructive during neurite outgrowth, and particularly, during the neurite outgrowth of embryonic cortical, hippocampal, and retinal neurons (based on studies in our lab and in Patzke et al., 2010). This is a very confined but more accurate description of CAR's role during neurite outgrowth. On the other hand, CAR's role during axon guidance may be permissive like other CAMs, but this needs further investigation. A good way to test CAR's role during axon guidance is to see whether applying a gradient of FN40 can induce growth cone turning in an *in vitro* system. If the growth cone turns toward the FN40 gradient then during axon guidance, CAR is an instructive receptor; if not, it is a permissive receptor.

CAR Conditional Knockout Mice May Have Elevated Anxiety

Our results show that CAR is a neurite outgrowth regulator *in vitro*, but does it affect neurite outgrowth *in vivo*? Since CAR is widely expressed in the developing brain, will the knockout of CAR have any effects on brain structure and animal behavior? To overcome the embryonic lethality caused by the whole-body knockout of CAR, we generated transgenic mice with conditional knockout of CAR (CAR-cKO) by crossing CARFLOX and SYNCRE mice. We

hypothesized that conditional knockout of CAR in the nervous system should affect brain structure and/ or animal behavior.

We first conducted an open-field test for the CAR-cKO mice and found that these mice may have elevated anxiety. During the open-field test, the cKO mice displayed reduced locomotor activity, spent less time exploring the arena (Figure 5.1A and B), had increased vertical rearing, and were inclined to stay at the peripheral region of the field. Their self-grooming was often short and interrupted (Figure 5.2A), and they had more defecation than WT mice (Figure 5.2B). We then compared our observations to the literature in animal behavior. The open-field test was originally designed by Hall to study the emotionality in rats: anxious rats had increased level of defecation and urination (Hall, 1934) and displayed less ambulatory movement (Hall, 1936); anxious animals also prefer to stay at the peripheral region rather than the central region of the open-field (Prut and Belzung, 2003). Additionally during the open-field test, elevated anxiety can also be manifested by an increased vertical rearing (Bourin et al., 2007) or short and interrupted self-grooming (Kalueff and Tuohimaa, 2005). Anxiety and fear are normal emotional responses to external threats. However, abnormally elevated anxiety is an over-reactive response when there are no threats. Compared to WT mice, CAR-cKO mice appeared to be overly anxious without any obvious threats. Taken together, these results demonstrate that CAR-cKO mice have anxiety-like behavior.

Amygdala is the emotional center and dysregulation of amygdalar activity leads to abnormal anxious feelings (LeDoux, 2000). As introduced above, during E13.5-16.5, CAR is expressed

in the amygdaloid area (Hotta et al., 2003); Synapsin1 is also expressed in the amygdala after E12 (Hoesche et al., 1993; Wemmie et al., 2004). Therefore, it is possible that CAR knockout by SYNCRE in the amygdala affects the emotionality of the cKO mice. Why does the knockout of CAR cause anxiety-like behavior in mice? What are the possible molecular mechanisms?

The neurotransmitter serotonin and the neurotrophic factor BDNF are recognized as important modulators of anxiety. Serotonin, or 5-hydroxytryptamine (5-HT), is associated with anxiety and depression. Low level of 5-HT in the brain causes generalized anxiety, depression, panic disorders, and other mood disorders in patients (Ressler and Nemeroff, 2000) and drugs that can increase 5-HT levels are widely used to treat depression (Delgado et al., 1990; Santarelli et al., 2003). In transgenic mouse models, serotonin receptor 5-HT_{1A} mutant mice display elevated anxiety in open-field and elevated zero maze (Heisler et al., 1998; Ramboz et al., 1998). Abnormal development of 5-HTergic raphe neurons caused by deleting the transcription factor *Pet1* decreases the 5-HT level and induces anxious behavior as revealed by elevated plus maze, dark-light exploration, and open-field tests (Hendricks et al., 2003; Liu et al., 2010; Nelson and Chiavegatto, 2001). Targeted ablation of the tryptophan hydroxylase gene *Tph2* leads to greatly reduced 5-HT synthesis in the brain (McKinney et al., 2005; Savelieva et al., 2008) and mice with a mutant *Tph2* (R439H) display elevated anxiety and depression-like behavior (Beaulieu et al., 2008). Several nucleotide polymorphisms in the human *Tph2* gene have been associated with anxiety and depression (Waider et al., 2011) and a mutation in the promoter region of the 5-HT transporter is associated with anxiety-related

traits in patients (Lesch et al., 1996). Additionally, BDNF also regulates anxiety. Stress and anxiety decrease the expression of *BDNF* mRNA in the hippocampus (Duman and Monteggia, 2006; Smith et al., 1995) and hippocampal BDNF infusion produces antidepressant effects (Shirayama et al., 2002; Siuciak et al., 1997). A common single-nucleotide polymorphism in the *BDNF* gene (Val66Met) causes anatomical change in hippocampus and prefrontal cortex (Bueller et al., 2006; Pezawas et al., 2004) and mutant mice with genetic knockin of BDNF $^{Met/Met}$ exhibit elevated anxiety (Chen et al., 2006b; Soliman et al., 2010).

The dysregulation of IgSF-CAMs is also reported to cause elevated anxiety. It is well known that NCAM-null mice display anxiety- and depression-like behavior (Aonurm-Helm et al., 2008; Jurgenson et al., 2010; Stork et al., 1999). For example, NCAM-KO mice spent more time in the dark compartment during the light/dark test and spent less time on the open arms of the elevated plus maze (Jurgenson et al., 2010; Stork et al., 1999), indicating increased anxiety. These anxious phenotypes can be decreased by overexpressing NCAM-180 (Stork et al., 2000) and by administrating 5-HT_{1A} receptor agonist (Stork et al., 1999), suggesting that NCAM regulates anxiety through the serotonin system. Mechanistically, the anxious behavior may be caused by abnormal NCAM homophilic interactions in the emotion circuits of the brain. Transgenic mice overexpressing soluble extracellular domain of NCAM (NCAM-EC) display a striking reduction in the number of synapses of GABAergic interneurons in the amygdala (Pillai-Nair et al., 2005) as well as in the prefrontal cortex (PFC) (Brennaman and Maness, 2008) suggesting impaired amygdala and PFC synaptic functions due to disrupted NCAM homophilic interactions. On the other hand, chronic stress decreases NCAM

expression and increases the level of polysialic acid-modified NCAM (PSA-NCAM) (Sandi et al., 2001; Tsoory et al., 2008; Venero et al., 2002); PSA-NCAM disrupts NCAM homophilic interactions and causes synaptic de-adhesion (Sandi, 2004). Interestingly, L1, another member of IgSF-CAMs, shows a response opposite to NCAM: its expression is upregulated by chronic stress (Sandi et al., 2001; Venero et al., 2002). Although the knockout of L1 does not change the level of anxiety (Sauce et al., 2015), considering that L1 expression is upregulated during chronic stress, it will be interesting to examine whether the over-expression of L1 can induce anxiety-like phenotypes.

Since NCAM and L1, the close relatives of CAR in the IgSF-CAM family, are implicated in stress and anxiety, it is intriguing to further explore the anxiety-like behavioral phenotypes of CAR-cKO mice. Generalized anxiety and agoraphobia can be tested by open-field, elevated plus maze, and dark/light exploration tests. The open-field test has been introduced above. The elevated plus maze test was originally developed in rats (Pellow et al., 1985) and soon adapted in mice (Lister, 1987). This maze has four arms: two open arms and two closed arms; due to elevated anxiety, anxious mice prefer to stay in the closed arms, but not the open ones. The light/dark exploration test (Crawley, 1981) is based on the altered exploratory activities under mild stress such as new environment and bright light, and anxious mice prefer to stay longer in the dark compartment. Additionally, preliminary depression tests such as Porsolt's forced swimming and Seligman's learned helplessness (tail-flick) tests (Porsolt et al., 1978; Seligman and Maier, 1967) can be performed to examine whether CAR-cKO mice have depressive behavior, like NCAM-KO mice. Does CAR function through the 5-HT system?

This can be studied by measuring the 5-HT level in CAR-cKO mice and by injecting drugs that increase 5-HT level or activity. Lastly, by performing immunostaining of synaptophysin on the PFC and amygdala of NCAM-null mice, it is found that the size of synaptic boutons and the number of the synaptic puncta in these areas are greatly reduced (Pillai-Nair et al., 2005; Brennaman and Maness, 2008). Therefore, we could perform synaptophysin immunostaining for CAR-cKO mice to see whether the knockout of CAR reduces synaptic connections in these brain regions.

CAR Conditional Knockout Mice Display Impaired Motor Coordination

In addition to potentially elevated anxiety, we also found that CAR-cKO mice displayed impaired motor coordination. During the open-field test, CAR-cKO mice spent significantly less time moving and spent more time staying still (Figure 5.1 A and B). Additionally, the movement of cKO mice was often more hesitant – they stood up and sat down many times without traveling in a distance, and even the ambulatory movement was with rigidity and bradykinesia (slow movement) but without apparent tremor. Although this can be interpreted as elevated anxiety, it may also be a sign of motor problems, for example, Parkinsonism-like phenotypes (Beal, 2001; Fleming et al., 2005); however, more sophisticated experiments must be done before drawing such a conclusion. To further determine whether cKO mice have motor problems, we performed Rota-rod tests and the cKO mice indeed performed very poorly during all these tests (Figure 5.3 and Figure 5.4). The poor performance on Rota-rod

may indicate: 1) weak muscle strength, 2) less muscle endurance, and 3) impaired motor coordination. We found that both the muscle strength and muscle endurance of CAR-cKO mice were normal (Figure 5.5), suggesting that these mice have very poor motor coordination. Moreover, cKO mice have a mild clasping phenotype (Figure 5.6) which also indicates a motor problem. Taken together, these results suggest that CAR-cKO mice display impaired motor coordination.

It is very likely that this impaired motor coordination, or ataxia, is due to abnormal cerebellar development. Previously in our lab, we immunostained CAR in P8 mouse cerebellar slices and found strong immunoreactivity in the neuronal populations, more specifically in the neurite-rich molecular layers; CAR was also sparsely distributed in the internal granule layer but not in the external granule layer of the cerebellum (Huang PhD thesis). This is similar to the expression pattern of Synapsin1: Synapsin1 is expressed in the cerebellum from P3 onwards and its expression is very strong in the internal granule layer (Melloni and DeGennaro, 1994), indicating that the expression of SYNCRE may cause the deletion of CAR in the cerebellum during early postnatal days. Indeed, by performing Western blot with homogenized cerebellar lysates prepared from P5 CAR-cKO and WT mice, CAR protein level in cKO mice was significantly lower (~10%) compared to WT mice (Zheng MSc thesis). Taken together, these results suggest that in the CAR-cKO mice, CAR may be deleted in the cerebellum by SYNCRE during the early postnatal period.

The expression pattern of CAR in the cerebellum is reminiscent of the patterns of L1 and

NCAM. Similar to CAR, in early postnatal (P8) mouse cerebellum, the signal of L1 is very strong in the neurite-rich molecular layer, weaker signal can be detected in the internal granule layer, and nearly no signal is detected in the external granule layer (Faissner et al., 1984; Persohn and Schachner, 1987; Rathjen and Schachner, 1984). The expression of NCAM is similar to CAR and L1 (Persohn and Schachner, 1987), but the expression of N-Cadherin is more enriched in the Purkinje cells (Redies and Takeichi, 1993). Functionally, L1 plays a regulatory role in neuronal migration during cerebellum development. During the early postnatal development of the cerebellar cortex, the granule cell neurons are migrating from the external granule layer, passing through the molecular layer, and arriving at the internal granule layer (Altman, 1972; Mecha et al., 2010). Application of antibodies against L1 in cerebellar explants significantly reduce the number of migrating cells and the cell migration speed (Lindner et al., 1983; Lindner et al., 1986), suggesting that L1 affects this migration process and may contribute to cerebellar development. However, knockout of L1 in mice does not cause abnormal cytoarchitecture of the cerebellar cortex (Cohen et al., 1998; Dahme et al., 1997; Fransen et al., 1998) but L1-KO mice do have a significant reduction in the cerebellar vermis and an enlarged fourth ventricle (Fransen et al., 1998), probably due to dysregulated neuronal migration. Interestingly, these mice display abnormal exploratory behavior in open-field tests with stereotypic peripheral circling which is similar to rats with cerebellar lesions (Fransen et al., 1998). Lastly, although no severe ataxia is observed during the Rota-rod tests, upon visual inspection, the KO mice clearly display more difficulties in keeping their balance on the rods (Fransen et al., 1998), suggesting a malfunction of the cerebellum.

In the future, we can perform more advanced behavioral tests and detailed brain structure analysis. First, we plan to perform open-field tests with automated video-tracking. In this system, we can use computer software to analyze the total distance traveled, total time spent in ambulatory movement (with a moving speed >5 cm/ sec), average traveling velocity, and time spent in the central versus peripheral regions. This system can provide more accurate measurement and more comprehensive information of CAR-cKO induced behavioral and motor changes. Next, more detailed motor coordination experiments will be performed such as tests on horizontal bars, static rods, and parallel bars (Deacon, 2013a). In addition, abnormal gaits displayed by mutant mice such as Lurcher, Reeler, Staggerer, and Scrambler mice are strong indicators of particular cerebellar malfunctions (Fatemi, 2001; Fortier et al., 1987; Sidman et al., 1962; Sweet et al., 1996); therefore we plan to test gaits of the CAR-cKO mice and compare them to the literature. Finally, we can analyze brain sections for the cerebellar cortex and the cerebellar vermis to observe whether the knockout of CAR induces cytoarchitecture changes in the brain.

Figure 6.1

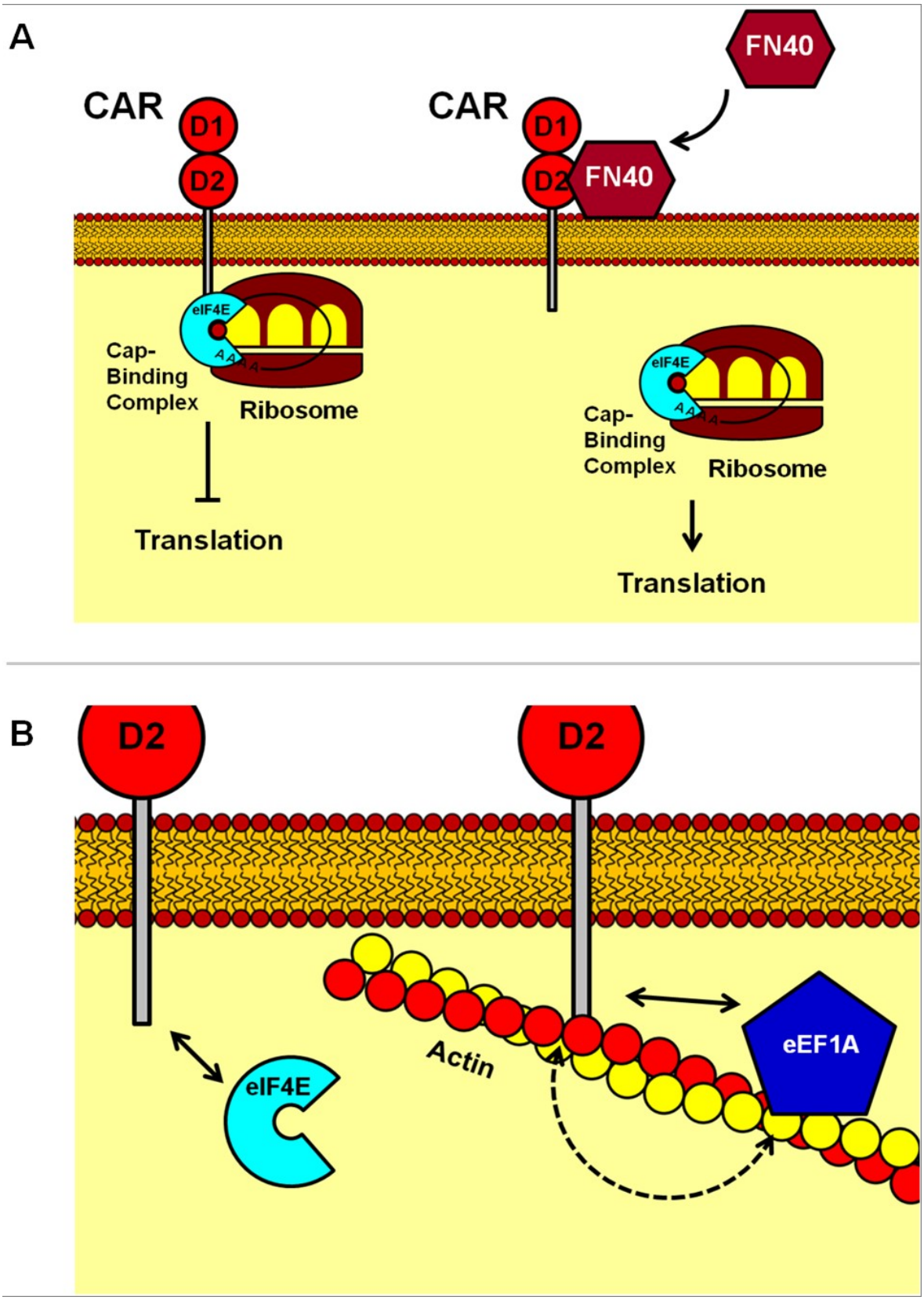
A
FN40
CAR
D1
D2
CAR
D1
D2
FN40
eIF4E
Cap-
Binding
Complex
Ribosome
Translation
eIF4E
Cap-
Binding
Complex
Ribosome
Translation
B
D2
D2
eIF4E
Actin
eEF1A

Figure 6.1. **CAR can regulate translation through direct protein-protein interactions.** A) Without a ligand (left), CAR may inhibit translation by sequestering the ribosome and the cap-binding complex (such as eIF4E) at the plasma membrane. Upon binding to FN40 (right), CAR may release the translation machinery and promote translation. B) CAR interacts with translational proteins directly or indirectly. Left, CAR associates with eIF4E via direct interactions. Right, CAR may directly bind to eEF1A or, alternatively, CAR may indirectly bind to eEF1A through binding to cytoskeletal proteins such as actin.

CONCLUSIONS

Although CAR promotes neurite outgrowth *in vitro*, the downstream molecular mechanisms remain elusive. Our proteomic analysis identified many translational proteins that may bind to the cytoplasmic domain of CAR, suggesting that CAR may associate with translational machinery and regulate protein synthesis. In this study, we showed that CAR interacts with translational proteins and regulates translation *in vitro* and in cultured cells; we also demonstrated that the interaction of CAR and FN40 significantly promoted neurite outgrowth as well as protein synthesis. Taken together, these results suggest that CAR regulates translation during neurite outgrowth. In the future, we could examine how CAR interacts with translational proteins such as eIF4E and eEF1A during neurite outgrowth and explore which mRNAs are translationally regulated by CAR.

Additionally, CAR was long proposed to regulate the development of nervous system but it was not known how CAR contributes to brain development. In this study we used a conditional knockout system to generate CAR-null mice; we showed that CAR-cKO mice displayed anxiety-like behavior and poor motor coordination. In the future, we plan to further study the behavioral phenotypes using more advanced tests and to investigate the potential molecular mechanisms that lead to the abnormal behavior in CAR-cKO mice.

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