Copine1 Enhances Neuronal Differentiation of the Hippocampal Progenitor HiB5 Cells

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Copine1 is a ubiquitously expressed protein found in various tissues including the brain, but little is known about the physiological function of this protein. Here, we showed that copine1 is involved in neuronal differentiation. Over-expression of copine1 clearly increased neurite outgrowth and expression of Tuj1, a neuronal marker protein, in HiB5 cells. In addition, endogenous copine1 was transiently increased at the early time during neuronal differentiation of HiB5 cells. When the expression of endogenous copine1 was knocked-down by its specific shRNA, PDGF-mediated neurite outgrowth was clearly decreased in HiB5 cells. Furthermore, over-expression of copine1 increased phosphorylation of Akt and copine1-specific shRNA decreased phosphorylation of Akt during neuronal differentiation of HiB5 cells. Interestingly, the phosphorylation level of PI3K, generally known as an upstream protein of Akt, was not changed by copine1 expression. These results suggest that copine1 enhances neuronal differentiation of HiB5 cells not through the PI3K-Akt pathway, but by using another Akt activated signal pathway.

INTRODUCTION

Copine proteins are a novel family of ubiquitous Ca²⁺-dependent, phospholipid-binding proteins first identified in the ciliate protozoan *Paramecium tetraurelia* (Creutz et al., 1998). Presently, nine copine genes (CPNE1-9) have been identified in humans (Maitra et al., 2003; Tomsig and Creutz, 2002). Copine proteins are highly conserved from plants to humans and widely expressed throughout the eukaryotes. Copine proteins share a common structure with two N-terminal C2 domains (C2A and C2B) and a C-terminal A domain (von Willebrand factor A domain). Generally, copine proteins are thought to be involved in membrane-trafficking phenomena because of their phospholipid-binding properties.

Among these copine proteins, copine1 is ubiquitously expressed in various tissues including brain (Perestenko et al., 2010). It was previously reported that copine1 binds with vari-

ous intracellular proteins via its A domain (Tomsig et al., 2003; 2004). Therefore, copine1 may be involved in diverse biological processes. In fact, one recent study showed that copine1 regulates the half-life of NF- κ B transcriptional responses via endo-proteolysis of the p65 protein (Ramsey et al., 2008). However, its specific roles are poorly understood and have never been studied in neuronal cells.

It is well known that Akt is activated via phosphoinositide 3-kinase (PI3K) by various extrinsic cues. Akt-mediated signaling is involved in a number of biological functions including cell proliferation, differentiation, and survival (Manning and Cantley, 2007). In neuronal precursor cells (NPCs), a number of studies have shown that the PI3K-Akt pathway is involved in NPC proliferation and/or differentiation (Read and Gorman, 2009). In addition, it was reported that active Akt promotes differentiation of NPCs into γ -aminobutyric acid-containing (GABAergic) but not glutamatergic neurons (Oishi et al., 2009).

Here, we are the first to show that copine1 enhances neuronal differentiation via activation of Akt using a cell model for neuronal differentiation, the HiB5 cell. HiB5 is a hippocampal progenitor cell line derived from the hippocampal anlagen of E16 rat. It was conditionally immortalized with a temperature-sensitive mutant allele of SV40 T antigen. HiB5 cells can differentiate into neuronal cells with neurite-like processes *in vitro* in serum-free defined N2 medium containing platelet-derived growth factor (PDGF) at a non-permissive temperature of 39°C (Chung et al., 2000).

MATERIALS AND METHODS

Plasmid constructions and copine1 specific shRNA

Full-length of copine1 cDNA (GenBank Accession No. BC001142) was obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. The full-length clone was generated with cDNA as templates, via a PCR-based gateway cloning method as described previously (Park et al., 2007). The resulting PCR products were cloned into destination vectors pDEST-AD-GFP using the gateway cloning system (Invitrogen). The shRNA vector for copine1 was

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constructed using the BLOCK-IT U6 RNAi Entry vector kit (Invitrogen). The target sequence of shRNA in copine1 was as follow: 5'-CGCTTTGGAATCTATGACATA-3'.

Cell culture and adenovirus infection

Immortalized HiB5 cells were cultured at 33°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with 0.11 mg/ml sodium pyruvate, 3.7 mg/ml NaHCO₃, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% fetal bovine serum (FBS). To induce cell differentiation, cells were shifted to the chemically defined N2 medium containing 5 µg/ml insulin, 100 µg/ml transferrin, 20 mM progesterone, 30 nM selenium, 60 µM putrescine, 0.11 mg/ml sodium pyruvate, and 2 mM glutamine at 39°C for 2 days. To promote further neuronal differentiation, 30 ng/ml of PDGF-BB (Millipore) was added to N2 media. HiB5 cells were infected with adenovirus containing the GFP-tagged gene and incubated at 33°C or 39°C for the indicated times.

Imaging analysis and quantification of neurite outgrowth

From Ad-GFP and Ad-GFP-copine1 infected cells, several randomly chosen fields were photographed using a confocal microscope (Olympus Fluoview FV-1000), and cells with processes longer than double the cell body diameter were scored as neurite-outgrowth cells. Aggregated cells were not scored and cells bearing atypical processes, such as hair-like branched processes, were not scored. Data were expressed as a percentage of neurite-outgrowth cells out of the GFP-expressing cells.

Immunoblot analysis

The immunoblot analysis was carried out as described previously (Ku et al., 2011). In brief, HiB5 cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM EDTA, 1 mM PMSF, and 1% NP-40) containing a protease-inhibitor cocktail. Whole-cell lysates were incubated on ice for 30 min and then cleared

at 20,000 g for 20 min at 4°C. Cell lysates were separated by SDS-PAGE using 8% gels and blotted onto PVDF membranes. The blots were incubated overnight at 4°C with anti-TUJ1 (1:1000; SIGMA-ALDRICH), anti-CPNE1 (1:1000; Santa Cruz Biotechnology), anti- α -tubulin (1:2000; SIGMA-ALDRICH), anti-AKT1/2/3 (1:1000; Santa Cruz Biotechnology), anti-phospho-AKT1/2/3 (S473; 1:1000; Santa Cruz Biotechnology), anti-PI3K (1:1000; Cell Signaling), or anti-phospho-PI3K (1:1000; Cell Signaling) antibodies. Blots were then washed and incubated with horseradishperoxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by washing and the detection of immunoreactivity with enhanced chemiluminescence (ECL; Santa Cruz Biotechnology).

Immunocytochemistry

HiB5 cells grown on coverslips in a 24-well plate (4×10^4 cells) were fixed in 4% paraformaldehyde for 20 min at room temperature and then permeabilized with PBS containing 0.5% NP40 for 10 min. Non-specific binding was prevented with a 30 min incubation in 10% FBS. Cells were incubated with the anti-CPNE1 (1:200; Santa Cruz Biotechnology) primary antibody for 1 h at room temperature. After washing, Cy3-labeled secondary antibody was added and cells incubated for 1 h. Cells were washed three times with PBS and then observed under a confocal microscope (Olympus, FV1000).

RESULTS

Expression pattern of endogenous copine1 during neuronal differentiation of HiB5 cells

First, we measured the expression level of endogenous copine1 during non-differentiation (33°C) or differentiation stages (39°C + PDGF) in HiB5 cells (Figs. 1A and 1B). Endogenous copine1 proteins were highly expressed at the early time points during differentiation (Fig. 1A) The expression level of endogenous

Fig. 1. Endogenous copine1 is involved in neuronal differentiation in HiB5 cells. Expression of endogenous copine1 was detected in HiB5 cells. (A) Cell differentiation was induced by N2 media in presence of PDGF-BB (30 ng/ml) for the indicated times. Expression of endogenous copine1 was detected by immunocytochemical analysis. Scale bar = 40 µm. (B) Expression levels of endogenous copine1 were measured by immunoblot analysis in non-differentiation (33°C) and differentiation (39°C) stages. (C) The histogram shows the percentage of copine1 expression cells in non-differentiation (33°C) and differentiation (39°C) stages (*P < 0.01, ANOVA). Expression level of α -tubulin was used as loading control.

Fig. 2. Ad-GFP-copine1 has time-dependent effects on neurite outgrowth in HiB5 cells. (A) HiB5 cells were infected with Ad-GFP and Ad-GFP-copine1 for the indicated times and cell morphology was imaged using confocal microscopy. Scale bar = 20 μ m. (B) The histogram shows percentages of neurite-outgrowth cells out of GFP-expressing cells (***) $P < 0.001$, ANOVA). (C) Expression levels of the neuronal marker protein, Tuj1, were analyzed for the indicated times after Ad-GFP and Ad-GFP-copine1 infection. (D) The histogram shows the percentage of Tuj1 expression level in Ad-copine1 cells versus Ad-control GFP cells (*) $P < 0.01$, ANOVA). Expression level of α -tubulin was used as loading control.

Fig. 3. Specific shRNA of copine1 blocks neuronal differentiation. (A) Confirmation of copine1 shRNA silencing. (B) Illustration of the experimental design and differentiation conditions. (C) The histogram shows percentages of neurite-outgrowth in Ad-copine1-shRNA versus Ad-control-shRNA infection (***) $P < 0.001$, ANOVA). (D) The expression level of neuronal marker protein, Tuj1, was analyzed after shRNA infection. (E) The histogram shows the percentage of Tuj1 expression in Ad-copine1 shRNA cells versus Ad-control shRNA cells (*) $P < 0.01$, ANOVA). Expression level of α -tubulin was used as loading control.

copine1 is similar at every time point during the non-differentiation stage (33°C), but is dramatically increased at early time points during differentiation (39°C + PDGF) of HiB5 cells (Fig. 1B). Neurite outgrowth cells increased approximately 2-fold after 12 h incubation in the differentiation stage (39°C + PDGF, Fig. 1C). These data indicate that endogenous copine1 is involved in neuronal differentiation of HiB5 cells.

Effect of copine1 on differentiation of the neuronal stem cell HiB5

To determine the effect of copine1 on neuronal differentiation, adenoviruses containing GFP-copine1 or GFP control genes were infected into HiB5 cells, a well-known model cell for neuronal differentiation. In a time dependent manner, we measured neurite outgrowth and morphological changes (Fig. 2A). When copine1 was over-expressed in HiB5 cells, neurite outgrowth cells increased approximately 5-fold compared with those of

GFP-control-infected cells after 72 h incubation (Figs. 2A and 2B). Over-expression of copine1 significantly increased neurite outgrowth of infected cells (Fig. 2B, $p < 0.001$).

To reconfirm the role of copine1 in neuronal differentiation, we checked the expression level of the neuronal marker Tuj1 with biochemical and imaging methods (Fig. 2C and Supplementary Fig. 1). As expected, over-expression of copine1 increased the expression level of Tuj1 in a time dependent manner (Figs. 2C and 2D and Supplementary Fig. 1). Similar to HiB5 cells, over-expression of copine1 also increased neurite outgrowth of SH-SY5Y cells, another neurogenic cell line (Supplementary Fig. 2). Together, these results demonstrate that over-expression of copine1 promotes neuronal differentiation.

Depletion of endogenous copine1 reduces neuronal differentiation of HiB5 cells

To provide insight into the biological functions of endogenous

Fig. 4. Neuronal differentiation induced by copine1 is mediated by the Akt pathway. (A) Illustration of experimental design and expression levels of PI3K, phospho-PI3K, Akt, and phospho-Akt (s473) proteins after infection of Ad-GFP-copine1 at the indicated times during the non-differentiation stage (33°C) in HiB5 cells. (B) Illustration of experimental design and expression level of Akt and phospho-Akt (s473) proteins after the Ad-control and Ad-copine1 shRNA infection at the indicated times during differentiation (39°C + PDGF) of HiB5 cells. Expression level of α -tubulin was used as loading control.

copine1 during neuronal differentiation, copine1-specific shRNA (CPNE1 shRNA) and control shRNA were developed and tested in the copine1 over-expressed HEK293T cells (Fig. 3A). When adenoviruses containing copine1 shRNA or control shRNA were infected during the neuronal differentiation of HiB5 cells as indicated in Fig. 3B, neurite outgrowth cells decreased by almost half at 48 h after PDGF treatment in copine1 shRNA-infected cells compared with control shRNA-infected cells (Fig. 3C). The expression level of Tuj1 also decreased almost 40% in Ad-CPNE1-shRNA cells versus Ad-control-shRNA cells at 48 h after PDGF treatment (Figs. 3D and 3E). These data strongly indicate that endogenous copine1 plays a pivotal role(s) in neuronal differentiation of HiB5 cells.

Copine1 is involved in activation of AKT, but not PI3K, during neuronal differentiation

Because a number of studies reported that PI3K-Akt signaling is involved in neuronal differentiation (Read and Gorman, 2009), we decided to examine whether PI3K-Akt signaling is also involved in copine1-mediated neuronal differentiation. When copine1 was over-expressed in HiB5 cells during the non-differentiation stage, phospho-Akt (s473) increased approximately 2-fold at 48 h after infection in the copine1-infected cells compared with GFP-control-infected cells (Fig. 4A). Interestingly, the level of phospho-PI3K was similar at 72 h in copine1-infected cells and control GFP-infected cells (Fig. 4A). In addition, treatment of wortmannin, a PI3K blocker, does not alter the copine1-induced phospho-Akt (s473) increase at 72 h in copine1-infected cells and control GFP-infected cells (Supplementary Fig. 3). These data indicate that over-expression of copine1 enhanced phosphorylation of Akt, but not PI3K, during the non-differentiation

stage in HiB5 cells.

Next, we examined the effect of copine1 shRNA on phosphorylation of Akt during the differentiation stage in HiB5 cells. It has been reported that treatment with PDGF at 39°C on HiB5 cells transiently increased activity of Akt (Read and Gorman, 2009). Phospho-Akt (s473) transiently peaked at 12 h after PDGF treatment at 39°C and gradually declined in a time dependent manner (Fig. 4B). However, copine1 shRNA decreased phospho-Akt (s473) levels approximately half-fold compared to control shRNA at 12 h after PDGF treatment (Fig. 4B). Because the percentage of neurite outgrowth cells and phospho-Akt (s473) levels were decreased by approximately half under the same conditions in copine1 shRNA infected cells (Figs. 3C and 4B), it appears that activation of Akt was important for neuronal differentiation. Taken together, these data suggest that endogenous copine1 is involved in Akt signaling during neuronal differentiation of HiB5 cells.

DISCUSSION

Copine1 belongs to the copine family, a novel family of ubiquitous Ca^{2+} -dependent, phospholipid-binding proteins and is ubiquitously expressed in various tissues, including the brain (Perestenko et al., 2010). Previously, we reported a screening system for genes involved in neuronal differentiation (Park et al., 2007). Using this screening system, we found that copine1 increased neurite outgrowth, a morphological change indicating neuronal differentiation, in HiB5 cells.

HiB5 cells are a multipotent hippocampal neuronal precursor cell line whose differentiation is promoted by neurotrophic factors such as PDGF and brain-derived neurotrophic factor (BDNF)

(Kim et al., 2002). Since transfection efficiency is very low and it is difficult to assess the activity of the transfected genes in these cells, we used recombinant adenoviruses containing GFP-control, GFP-copine1, control shRNA, and copine1 shRNA, to overcome the low efficiency of transfection and measure the effect of copine1 on neuronal differentiation.

It has been reported that phosphorylation of Akt at serine 473 is regulated by various kinases such as PI3K, phosphoinositide-dependent kinase-2 (PDK2), protein kinase C (PKC)- α , mammalian target of rapamycin complex-2 (mTORC2) and integrin-linked kinase (ILK) in several tissues (Ditlevsen et al., 2003; Moore et al., 2011; Persad et al., 2001; Yang et al., 2010). Recently, several studies showed that the PI3K-Akt pathway is important in neuronal differentiation (Read and Gorman, 2009; Oishi et al., 2009). Phosphorylation of Akt *via* an interaction with PKC ζ induced neuronal differentiation in HiB5 cells (Joung et al., 2005) and activation of the PI3K/Akt/mTORC1 pathway, which inhibits its downstream target p27Kip1 and induces proliferation and neurogenesis of HiB5 cells (Palazuelos et al., 2012). Our present study showed that copine1 increased phosphorylation of Akt and neuronal differentiation of HiB5 cells. Based on previous reports and our data, we suggest that copine1 enhanced neuronal differentiation of HiB5 cells *via* Akt signaling. However, we cannot yet exclude the possibility that other signaling pathways might be involved in copine1-mediated neuronal differentiation.

It is well known that the receptor tyrosine kinase (RTK)-PI3K-Akt signaling pathway is a major pathway in neurite outgrowth (Read and Gorman, 2009). Interestingly, we found that over-expression of copine1 specifically activates Akt, but not PI3K in HiB5 cells (Fig. 4A and Supplementary Fig. 3) and over-expression of copine1 was sufficient to induce neurite outgrowth of HiB5 cells under the non-differentiation stage (without PDGF treatment at 39°C; Fig. 2). These data suggest that direct activation of Akt can induce the neuronal differentiation of HiB5 cells without activation of PDGF receptor and PI3K.

There are some interesting questions revolving around the Akt activation by copine1. It has not yet been reported that copine1 has kinase activity, so it is unlikely that copine1 directly phosphorylates Akt proteins. In addition, yeast-two hybrid and immunoprecipitation assays showed that copine1 did not bind with Akt directly (data not shown). Therefore, it seems that copine1 indirectly increased Akt activity and an additional upstream kinase of Akt was needed in copine1-mediated Akt activation. Since previous reports showed that copine1 binds with various intracellular proteins *via* its A domain (Tomsig et al., 2003; 2004), we are trying to find a possible Akt-upstream kinase, whose activity can be regulated by copine1.

Copine1 also contains two N-terminal C2 domains, which has similarity with the C2 domains found in PKC, phospholipase C, synaptotagmin and rabphilin (Zhang and Aravind, 2010). In general, C2 domains are responsible for calcium-dependent phospholipid binding. Recent reports showed that translocation of copine1 to the plasma membrane was dramatically increased under the treatment of 2 mM Ca²⁺ and ionomycin, a Ca²⁺ ionophore, in copine1 expressing HEK-293 cells (Perestenko et al., 2010). Because intracellular Ca²⁺ is also important for neuronal differentiation (Jiménez and Hernández-Cruz, 2001), further investigation is needed into the roles of Ca²⁺ responsiveness and translocation of copine1 in neuronal differentiation.

Until now, copine1's role in neuronal differentiation has not been studied. Although this role needs to be studied in more depth, we are the first to report that copine1 has a pivotal role in

neuronal differentiation of HiB5 cells.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Chung, J.J., Cho, S., Kwon, Y.K., Kim, D.H., and Kim, K. (2000). Activation of retinoic acid receptor gamma induces proliferation of immortalized hippocampal progenitor cells. *Brain Res. Mol. Brain Res.* 83, 52-62.
- Creutz, C.E., Tomsig, J.L., Snyder, S.L., Gautier, M.C., Skouri, F., Beisson, J., and Cohen, J. (1998). The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from *Paramecium* to humans. *J. Biol. Chem.* 273, 1393-402.
- Ditlevsen, D.K., Köhler, L.B., Pedersen, M.V., Risell, M., Kolkova, K., Meyer, M., Berezin, V., and Bock, E. (2003) The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival. *J. Neurochem.* 84, 546-556.
- Jiménez, N., and Hernández-Cruz, A. (2001). Modifications of intracellular Ca²⁺ signalling during nerve growth factor-induced neuronal differentiation of rat adrenal chromaffin cells. *Eur. J. Neurosci.* 13, 1487-1500.
- Joung, I., Kim, H.J., and Kwon, Y.K. (2005). p62 modulates Akt activity *via* association with PKC ζ in neuronal survival and differentiation. *Biochem. Biophys. Res. Commun.* 334, 654-660.
- Kim, G., Choe, Y., Park, J., Cho, S., and Kim, K. (2002). Activation of protein kinase A induces neuronal differentiation of HiB5 hippocampal progenitor cells. *Brain Res. Mol. Brain Res.* 109, 134-145.
- Ku, B.M., Lee, Y.K., Jeong, J.Y., Ryu, J., Choi, J., Kim, J.S., Cho, Y. W., Roh, G.S., Kim, H.J., Cho, G.J., et al. (2011). Caffeine inhibits cell proliferation and regulates PKA/GSK3 β pathways in U87MG human glioma cells. *Mol. Cells* 31, 275-279.
- Maitra, R., Grigoryev, D.N., Bera, T.K., Pastan, I.H., and Lee, B. (2003). Cloning, molecular characterization, and expression analysis of Copine 8. *Biochem. Biophys. Res. Commun.* 303, 842-847.
- Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. *Cell* 129, 1261-1274.
- Moore, S.F., Hunter, R.W., and Hers, I. (2011). mTORC2 protein complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation is not required for Akt1 activity in human platelets. *J. Biol. Chem.* 286, 24553-24560.
- Oishi, K., Watatani, K., Itoh, Y., Okano, H., Guillemot, F., Nakajima, K., and Gotoh, Y. (2009). Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. *Proc. Natl. Acad. Sci. USA* 106, 13064-13069.
- Palazuelos, J., Ortega, Z., Díaz-Alonso, J., Guzmán, M., and Galve-Roperh, I. (2012). CB2 cannabinoid receptors promote neural progenitor cell proliferation *via* mTORC1 signaling. *J. Biol. Chem.* 287, 1198-1209.
- Park, J.Y., Hwang, E.M., Park, N., Kim, E., Kim, D.G., Kang, D., Han, J., Choi, W.S., Ryu, P.D., and Hong, S.G. (2007). Gateway RFP-fusion vectors for high throughput functional analysis of genes. *Mol. Cell* 23, 357-362.
- Perestenko, P.V., Pooler, A.M., Noorbakhshnia, M., Gray, A., Bauccio, C., and Jeffrey McIlhinney, R.A. (2010). Copines-1, -2, -3, -6 and -7 show different calcium-dependent intracellular membrane translocation and targeting. *FEBS J.* 277, 5174-5189.
- Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P., and Dedhar, S. (2001). Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino

- acids arginine 211 and serine 343. *J. Biol. Chem.* 276, 27462-27469.
- Ramsey, C.S., Yeung, F., Stoddard, P.B., Li, D., Creutz, C.E., and Mayo, M.W. (2008). Copine-I represses NF-kappaB transcription by endoproteolysis of p65. *Oncogene* 27, 3516-3526.
- Read, D.E., and Gorman, A.M. (2009). Involvement of Akt in neurite outgrowth. *Cell. Mol. Life Sci.* 66, 2975-2984.
- Tomsig, J.L., and Creutz, C.E. (2002). Copines: a ubiquitous family of Ca(2+)-dependent phospholipid-binding proteins. *Cell. Mol. Life Sci.* 59, 1467-1477.
- Tomsig, J.L., Snyder, S.L., and Creutz, C.E. (2003). Identification of targets for calcium signaling through the copine family of proteins. Characterization of a coiled-coil copine-binding motif. *J. Biol. Chem.* 278, 10048-10054.
- Tomsig, J.L., Sohma, H., and Creutz, C.E. (2004). Calcium-dependent regulation of tumour necrosis factor-alpha receptor signaling by copine. *Biochem. J.* 378, 1089-1094.
- Yang, L., Qiao, G., Ying, H., Zhang, J., and Yin, F. (2010). TCR-induced Akt serine 473 phosphorylation is regulated by protein kinase C-alpha. *Biochem. Biophys. Res. Commun.* 400, 16-20.
- Zhang, D., and Aravind, L. (2010). Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes. *Gene* 469, 18-30.