# Altered expression of CDC42 signaling pathway components in cortical layer 3 pyramidal cells in schizophrenia

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## Abstract

**Background**—Cognitive dysfunction in schizophrenia is associated with a lower density of dendritic spines on deep layer 3 pyramidal cells in the dorsolateral prefrontal cortex (DLPFC). These alterations appear to reflect dysregulation of the actin cytoskeleton required for spine formation and maintenance. Consistent with this idea, altered expression of genes in the CDC42 (cell division cycle 42)-CDC42 effector protein signaling pathway, a key organizer of the actin cytoskeleton, was previously reported in DLPFC gray matter from subjects with schizophrenia. Here, we examined the integrity in schizophrenia of the CDC42-PAK-LIMK signaling pathway in a layer- and cell type-specific fashion in DLPFC deep layer 3.

**Methods**—Using laser microdissection, we collected samples of DLPFC deep layer 3 from 56 matched pairs of schizophrenia and comparison subjects and measured levels of CDC42-PAK-LIMK pathway mRNAs by qPCR. These same transcripts were also quantified by microarray in samples of individually microdissected deep layer 3 pyramidal cells from a subset of the same subjects and from antipsychotic-exposed monkeys.

**Results**—Relative to comparison subjects, CDC42EP4, LIMK1, LIMK2, ARHGDIA and PAK3 mRNA levels were significantly up-regulated in schizophrenia subjects in both laminar and cellular samples. In contrast, CDC42 and PAK1 mRNA levels were significantly down-regulated specifically in deep layer 3 pyramidal cells. These differences were not attributable to psychotropic medications or other co-morbid factors.

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**Conclusions—**Findings from the present and prior studies converge on synergistic alterations in CDC42 signaling pathway that could destabilize actin dynamics and produce spine deficits preferentially in deep layer 3 pyramidal cells in schizophrenia.

## Keywords

CDC42; dendritic spine; prefrontal cortex; schizophrenia; actin cytoskeleton; mRNA

## Introduction

Cognitive deficits, such as impairments in working memory, represent a core feature of schizophrenia (1) and these impairments appear to reflect altered circuitry in the dorsolateral prefrontal cortex (DLPFC;(2)). In particular, convergent lines of evidence from postmortem (3), neuroimaging (4) and pharmacological studies (5) implicate aberrant glutamate neurotransmission in cortical dysfunction in schizophrenia (6–8). Pyramidal cells, the principal source of cortical glutamate neurotransmission, exhibit lower dendritic spine density (9–12), shorter dendritic arbors (10) and smaller somal volumes (13–16) in subjects with schizophrenia. These morphological aberrations appear to have laminar specificity as smaller pyramidal cell volumes and lower dendritic spine density were observed in deep layer 3 but not in layers 5 or 6 (15,17,18). Importantly, none of these findings appeared to be attributable to antipsychotic medications or other co-morbid factors or potential confounds (19) and therefore likely reflect the underlying disease process.

Previous postmortem studies suggest that these morphological alterations in layer 3 pyramidal cells may be the consequence of disturbed expression of genes that regulate the actin cytoskeleton (20,21), which plays a critical role in dendritic spine formation and maintenance (22–25). For example, transcript levels of the Rho GTPase CDC42 (cell division cycle 42) are lower in DLPFC gray matter in subjects with schizophrenia and are positively correlated with layer 3 spine density measures (20). The specificity of the spine density decrement on layer 3 pyramidal neurons may reflect the laminar-specific expression of certain molecules that interact with CDC42. Indeed, CDC42 effector proteins (CDC42EP) are preferentially expressed in layers 2–3 of the human DLPFC (26) and CDC42EP3 mRNA expression was reported to be up-regulated in schizophrenia (21). The combination of lower CDC42 and elevated CDC42EP3 was hypothesized to alter the integrity of the barrier formed by the septin family of proteins in the spine neck (27), rendering it less permeable to the influx of postsynaptic molecules that are necessary for spine plasticity in response to glutamate stimulation (21).

However, these previous studies did not examine CDC42-related gene expression specifically in deep layer 3 or in pyramidal cells in this location. In addition, they did not address how altered CDC42 signaling could disrupt the regulation of the assembly and disassembly of actin filaments through cofilin, a family of actin-binding proteins (Figure 1A). The activity of cofilin is downstream of a signaling pathway that involves the interaction between CDC42 and the p21-activated serine/threonine protein kinases (PAK) family of proteins (28,29). These proteins are activated upon binding of the GTP-bound forms of CDC42 and activate (among other targets) the LIM domain-containing serine/

threonine protein kinases (LIMK1 and LIMK2) (30,31) which in turn regulate the actin-depolymerizing activity of cofilin (32–34). Moreover, the activity of CDC42 is regulated by guanine nucleotide dissociation inhibitors such as ARHGDIA (Figure 1A), a class of molecules that inhibit the substitution of GDP for GTP, thereby suppressing GTPase activity (35). Therefore, multiple regulators in the CDC42-PAK-LIMK pathway appear to be crucial for the actin cytoskeleton to maintain the stability of spine structure.

To determine if alterations in the CDC42-PAK-LIMK signaling pathway could contribute to the spine deficits on deep layer 3 pyramidal cells in schizophrenia, we conducted targeted gene expression analyses at laminar and cellular levels of specificity. We used laser microdissection to collect samples of DLPFC deep layer 3 from 56 matched pairs of schizophrenia and comparison subjects, and of individual deep layer 3 pyramidal cells in a subset of these subjects, and measured the expression levels of CDC42-related mRNAs in these samples using real-time quantitative polymerase chain reaction (RT-qPCR) and microarray, respectively.

## Methods and Materials

### Human subjects

Human brain specimens (N=112) were obtained during routine autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA, USA) following consent obtained from the next-of-kin. An independent committee of research clinicians made consensus DSM-IV (36) diagnoses, or confirmed the absence of a psychiatric diagnosis, for each subject using structured interviews with family members and review of prior medical records (37). To control for experimental variance, each subject with schizophrenia (n=34) or schizoaffective disorder (n=22) was matched to one healthy comparison subject for sex and as closely as possible for age (Table S1 contains individual subject details). As in prior studies (38–42), we consider schizoaffective disorder to be a variant of schizophrenia based on the DSM-IV requirement for the class A criteria of schizophrenia to be present, in the absence of mood symptoms. Samples from both subjects in a pair were processed together throughout all stages of the study. Subject groups (Table 1) did not differ in mean age, postmortem interval (PMI), RNA integrity number (RIN), tissue storage time at −80°C or race. Brain pH significantly differed between groups (t=2.51; df=55, p=0.015), although the mean difference between groups was very small (0.1 pH unit). Every subject had RIN 7.0 indicating an excellent quality of total RNA. All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

### Laser microdissection procedure

We conducted two studies at different levels of resolution: 1) cortical layer-specific measures of gene expression in strips of tissue containing only DLPFC deep layer 3 (N=56 pairs; Table 1) and 2) cell-type specific measures of gene expression in DLPFC deep layer 3 pyramidal cells (N=36 pairs; Table 1).

For both studies, the right hemisphere of each brain was blocked coronally, frozen and stored at −80°C (43). Cryostat sections (12 μm) were cut and thaw-mounted onto glass PEN membrane slides (Leica Microsystems, Bannockburn, IL) that had been UV-treated at 254nm for 30 minutes. The sections were dried and stored at −80 °C. On the day of the microdissection, sections were stained for Nissl substance with thionin (Figure 2A). DLPFC layer 3 was identified using a Leica microdissection system (LMD 6500; ×5 objective) in portions of the section cut perpendicular to the pial surface. For the laminar microdissections, strips (~10 million $μm^2$) containing deep layer 3 (defined as the zone from the layer 3–4 border to 35% of the distance from the pial surface to the layer 6-white matter border) in DLPFC area 9 were collected from each subject using a 5× objective (Figure 2B). In nearby tissue sections, individual deep layer 3 pyramidal cells (~200 pyramidal cells per subject), identified based on their characteristic somal morphology and the presence of a prominent apical dendrite directed radially toward the pia mater (Figure 2C,D), were captured using a 40× objective as previously described (44).

## qPCR analyses

For each sample, RNA was extracted and purified using the QIAGEN RNeasy Plus Micro Kit. Total RNA was converted to cDNA using the qScript$^{TM}$ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Forward and reverse primers were designed for each target mRNA to generate PCR amplicons of 85–120 base pairs. The specificity and efficiency of qPCR amplification for each target mRNA was demonstrated by: 1) high amplification efficiency (>92%) across a wide range of cDNA dilutions (Supplemental Table S3) and 2) single products demonstrated in dissociation curve analysis.

Three internal reference transcripts (Beta-Actin [ACTB], Cyclophilin A [PPIA] and guanine nucleotide binding protein [GNAS]), selected based on their stable expression across the subjects in this cohort regardless of diagnosis (45), were used to normalize data. Transcript expression levels of CDC42-related mRNAs (*ARHGDIA, CDC42*, *CDC42EP4, PAK1, PAK2, PAK3, LIMK1* and *LIMK2*) were quantified using qPCR using Power SYBR green dye (Applied Biosystems, Foster City, CA) and ViiA$^{TM}$ 7 Real-Time PCR system (Life Technologies, Carlsbad, CA). The cDNA samples from three matched pairs of schizophrenia and control subjects were processed together on the same 384-well qPCR plate with four replicates per primer set.

## Microarray analyses

We used a microarray approach to quantify cellular expression of CDC42-related mRNAs in deep layer 3 pyramidal cells as previously described (44). For transcriptome analysis, the RNA was extracted using the QIAGEN RNeasy Plus Micro Kit. The extracted RNA was transcribed into cDNA and subjected to a single round of amplification using the Ovation Pico WTA System (Nugen Technologies, San Carlos, CA). After amplification, the cDNA was labeled using the Encore Biotin module and loaded on an Affymetric GeneChip® HT HG-U133$^+$ PM Array Plate designed to assess expression levels of transcripts in the human genome (Affymetrix, Santa Clara, CA).

## Antipsychotic-exposed monkeys

Experimentally naïve, male, macaque monkeys (*Macaca fascicularis*) received twice daily oral doses of olanzapine, haloperidol or placebo (n=6 monkeys per group) for 17–27 months (46). The doses of each antipsychotic medications produced trough serum levels in the therapeutic range for the treatment of schizophrenia (47). One monkey from each of the three groups was euthanized on the same day (48) and pyramidal cells captured (49) and subjected to microanalysis (See Supplemental Methods).

## Data analysis and statistics

**qPCR analyses**—The comparative threshold cycle (CT) method was used in which transcript levels are normalized to the geometric mean of the three reference genes (ACTB, PPIA and GNAS). The difference in cycle threshold for each transcript was assessed by deducting the mean cycle threshold for the three reference genes from the cycle threshold of the target transcript. Since the difference in cycle threshold (dCT) represents the log2-transformed expression ratio of each target transcript to the geometric mean of the three reference genes, the relative expression ratio of each target transcript is determined as $2^{-dCT}$ (37). We performed two analyses of covariance (ANCOVA) models on the expression ratio data for each transcript. Because subjects were selected and processed as pairs, the first paired ANCOVA model included mRNA level as the dependent variable, diagnostic group as the main effect, subject pair as a blocking factor, and tissue storage time, brain pH and RIN as covariates. Subject pairing may be considered an attempt to balance diagnostic groups for sex and age, and to account for the parallel processing of tissue samples from a pair, and thus to not be a true statistical paired design. Consequently, a second unpaired ANCOVA model was performed that included all covariates (i.e., age, sex, postmortem interval, storage time, RIN and pH). All statistical tests were conducted with α-level= 0.05.

We also assessed the potential influence of other factors that are frequently co-morbid with the diagnosis of schizophrenia using ANCOVA models. For these analyses, we compared subjects with schizophrenia using each variable (sex; diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death; use of antipsychotics, antidepressants, or benzodiazepines and/or sodium valproate at the time of death; or death by suicide) as the main effect and age, tissue storage time, brain pH, PMI, and RIN as covariates.

Reported ANCOVA statistics include only those covariates that were statistically significant. As a result, the reported degrees of freedom vary across analyses.

**Microarray analyses**—The probe sets were filtered and paired t-tests were performed using the Random Intercept Model with Bayesian Information Criterion variable selection (50). Differentially expressed gene discovery was conducted using meta-analysis and an adaptively weighted (AW) Fisher's method (51) was applied. Meta-analyzed p-values from AW were then adjusted by the Benjamini-Hochberg procedure for multiple comparisons to control false discovery rate (44,52).

**Antipsychotic-exposed monkey analyses—**An ANCOVA model with the level of pre-specified mRNAs as the dependent variable, treatment group as the main effect, and triad as a blocking factor was employed. For each transcript of interest, the values of all probe sets targeting that transcript were averaged within each animal.

## Results

### Expression of CDC42-related mRNAs in DLPFC deep layer 3

In subjects with schizophrenia, mean mRNA levels of ARHGDIA, a common upstream regulator of pathways related to CDC42 signaling (Figure 1B), were higher in tissue homogenates of DLPFC deep layer 3 from the 56 subject pairs (+9.6%; paired: $F_{1,55}$=6.02, p=0.017; unpaired: $F_{1,108}$=1.99, p=0.161; Figure 3A) and in deep layer 3 pyramidal cells from the 36 subject pairs (+14.5%; p=0.041; Figure 3B). Although mean CDC42 mRNA levels were not significantly lower (−3.8%; paired: $F_{1,55}$=1.43, p=0.237; unpaired: $F_{1,110}$=0.29, p=0.585; Figure 3C) in the deep layer 3 tissue homogenates, they were significantly lower (−14.7%; p<0.001; Figure 3D) in DLPFC deep layer 3 pyramidal cells. Consistent with prior studies (21) of the CDC42-CDC42EP filament pathway (Figure 1B), mean CDC42EP4 transcript levels were significantly higher in the subjects with schizophrenia in DLPFC deep layer 3 tissue homogenates (+30.2%; paired: $F_{1,54}$=7.85, p=0.007; unpaired: $F_{1,108}$=13.76, p<0.001; Figure 3E) and pyramidal cells (+39.4%; p=0.003; Figure 3F).

To interrogate the integrity of the CDC42-PAK-LIMK signaling pathway that regulates the assembly and disassembly of actin filaments through cofilin, we evaluated the expression level of several members of this pathway (Figure 1B). Mean transcript levels for PAK1 did not differ between groups in tissue homogenates of DLPFC deep layer 3 (paired: $F_{1,55}$=0.08, p=0.774; unpaired: $F_{1,110}$=0.04, p=0.841; Figure 4A) but were significantly lower in pyramidal cell samples from schizophrenia subjects (−11.6%; p=0.002, Figure 4B). In contrast, in the subjects with schizophrenia mean transcript levels were higher for PAK2 in tissue homogenates of DLPFC deep layer 3 (+21.4%; paired: $F_{1,54}$=9.14, p=0.004; unpaired: $F_{1,108}$=12.80, p=0.001; Figure 4C) but were significantly lower in deep layer 3 pyramidal cells (−21.5%; p=0.013, Figure 4D). This discrepancy between layer-specific and cell type-specific measures for PAK2 could represent opposing patterns of change in pyramidal cells relative to other cell types (i.e., interneurons and glial cells) in the illness. Furthermore, the difference in sample size between qPCR (N=56 pairs) and microarray (N=36 pairs) analyses do not account for the marked difference in expression as the mean transcript levels were higher for PAK2 (+24.2%) in tissue homogenates from the same 36 pairs used for microarray analyses. Mean transcript levels for PAK3 were significantly higher in both tissue homogenates of DLPFC deep layer 3 (+18.6%; paired: $F_{1,54}$=13.03, p=0.001; unpaired: $F_{1,107}$=7.33, p=0.008; Figure 4E) and pyramidal cells (+15.8%; p=0.0027; Figure 4F). Finally, in the subjects with schizophrenia mean transcript levels for LIMK1 were modestly higher in deep layer 3 tissue homogenates (+9.9%; paired: $F_{1,55}$=3.70, p=0.060; unpaired: $F_{1,109}$=3.63, p=0.059; Figure 4G) and were significantly higher in pyramidal cells (+22.7%; p=0.031; Figure 4H). Consistent with these changes, mean transcript levels for LIMK2 were significantly higher in both deep layer 3 tissue homogenates (+63.4%; paired:

$F_{1,55}$=29.53, p<0.001; unpaired: $F_{1,108}$=27.95, p<0.001; Figure 4I) and pyramidal cells (+22.2%; p=0.030; Figure 4J).

### Effects of psychotropic medications and other confounding variables

For the transcripts that were significantly altered in the same direction in both the deep layer 3 tissue homogenates and pyramidal cell samples from the subjects with schizophrenia, we evaluated the effect of potential confounding variables in the qPCR data. In the 56 schizophrenia subjects, levels of ARHGDIA (Figure 5A), CDC42EP4 (Figure 5B), LIMK2 (Figure 5C) mRNAs did not differ as a function of sex, diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death; use of antipsychotics, antidepressants, or benzodiazepines and/or sodium valproate at the time of death; or death by suicide (all $F_{1,48}$ 2.39; all p 0.13). Similar results (all $F_{1,48}$ 1.78; all p 0.19) were found for the other transcripts (i.e., PAK2, PAK3) that were significantly altered by RT-PCR.

In order to interrogate the potential effect of long-term exposure to typical or atypical antipsychotics, we also evaluated CDC42-related mRNAs in DLPFC deep layer 3 pyramidal cells from monkeys chronically exposed to olanzapine, haloperidol or placebo. Levels of CDC42-related mRNAs did not significantly differ among these three groups of monkeys (Table 2). Serum levels of antipsychotic medications were not significantly correlated with any gene expression measures (all lrl<0.75; all p>0.10).

## Discussion

In this study, we found that subjects with schizophrenia exhibit altered gene expression, both up- and down-regulation, for components of the CDC42-PAK-LIMK pathway in DLPFC deep layer 3 tissue homogenates and pyramidal cells (Figure 1B, right side). In addition, we confirmed and extended to the cell type-specific level, earlier findings of altered expression in the CDC42-CDC42EP pathway (Figure 1B, left side). The levels of some transcripts (i.e., CDC42EP4, ARHGDIA, PAK3, LIMK1 and LIMK2) were significantly altered in schizophrenia, with the same direction and similar magnitude of difference from comparison subjects, in both layer- and pyramidal cell samples, as measured with qPCR or microarray analyses, respectively. In contrast, the levels of other transcripts (i.e., CDC42, PAK1) were significantly altered only in deep layer 3 pyramidal cell samples, but showed the same direction of change in the layer 3 tissue homogenates, suggesting that these alterations are specific to or at least enriched in, pyramidal cells. These transcript alterations may reflect the disease process in schizophrenia as none of these changes were attributable to antipsychotic medications or other factors frequently co-morbid with schizophrenia. Our findings support the notion that altered signaling in the CDC42-PAK-LIMK pathway could perturb the regulation of the assembly and disassembly of actin filaments through cofilin, and in concert with alterations in the CDC42-CDC42EP pathway, could contribute to the lower density of dendritic spines that is most pronounced in deep layer 3 pyramidal cells in the DLPFC of subjects with schizophrenia.

## Differences in layer-specific vs. cell type-specific pathology in schizophrenia

The assessment of molecular pathology at both laminar and cellular levels of resolution revealed differences in gene expression in schizophrenia that might be cell type-specific. For example, disease-related differences in the levels of CDC42 and PAK1 mRNAs detected in DLPFC deep layer 3 pyramidal neurons, but not in tissue homogenates from the same laminar location in the same subjects, support the idea that these findings are pyramidal cell-specific. Thus, measures of gene expression in gray matter or even a specific cortical layer may obscure findings that are cell type-specific. Consistent with this idea, recent transcriptome analyses revealed substantial differential expression in schizophrenia of transcripts related to mitochondrial function in deep layer 3 pyramidal neurons and of transcripts related to the ubiquitin-proteasome system in layer 5 pyramidal cells (44), alterations that were not detected in layer-specific transcriptome studies (26). Similarly, previous studies in total gray matter tissue homogenates revealed a modest reduction (−6%) in levels of GABA$_A$ α1 subunit mRNA (53), whereas dual-label *in situ* hybridization studies showed that mean GABA$_A$ α1 subunit mRNA expression was significantly 40% lower in deep layer 3 pyramidal cells, but was not altered in interneurons in the same layer (54). Additionally, recent findings using laser microdissection to dissect neuronal populations in the thalamus from subjects with schizophrenia revealed lower expression of transcripts encoding glutamate receptor subunits and components of the postsynaptic scaffold in relay neurons but not in a mixed population of glial cells and interneurons (55). Thus, gene expression analyses at the level of individual cell types reveal distinctive alterations that are essential to understanding molecular pathology in the context of the neural circuits formed by different classes of neurons.

## Contribution of CDC42-related signaling to dendritic spine abnormalities in DLPFC layer 3 pyramidal cells

Signaling through CDC42 pathways regulates the polymerization of the actin cytoskeleton and thus is essential for the maturation of filopodia into spines and for the maintenance of a normal complement of dendritic spines. In combination, findings of the present and prior studies suggest at least three different patterns of molecular disturbances in CDC42 signaling that could contribute to spine deficits preferentially on deep layer 3 pyramidal cells in schizophrenia.

First, our findings highlight a schizophrenia-related alteration in a regulatory component of the Rho family of GTPases, including CDC42. Like other members of this family, CDC42 cycles between an active-GTP bound state and an inactive GDP-bound state (56). The activity of these GTPases is regulated by guanine nucleotide dissociation inhibitors, such as ARHGDIA, which interacts with CDC42 (57), suppresses the exchange of GDP for GTP and renders CDC42 inactive (58). Thus, our findings of higher expression levels of ARHGDIA in deep layer 3 pyramidal cells (Figure 1B) suggest that CDC42 is more likely to be held in an inactive-GDP bound state in subjects with schizophrenia. In combination with lower levels of CDC42 (Figure 1B), the capacity of both the CDC42-CDC42EP and CDC42-PAK-LIMK signaling pathways to modulate the actin filaments that form the structural framework of dendritic spines would be predicted to be impaired in deep layer 3 pyramidal neurons in schizophrenia (Figure 1C).

Second, the prominence of dendritic spine abnormalities in deep layer 3 pyramidal neurons of subjects with schizophrenia has been proposed to be the consequence of altered signaling through molecules that are expressed in a layer-specific fashion (21). For example, CDC42 effector protein (CDC42EP) mRNAs are preferentially expressed in layer 3 of the human DLPFC (26). We previously demonstrated up-regulation of CDC42EP3 in layer 3 of subjects with schizophrenia (21), and here show that CDC42EP4 is up-regulated in layer 3 pyramidal cells. According to the previously proposed model for the CDC42-CDC42EP pathway (Figure 1A), the transient activation of CDC42 that normally occurs in individual spines following glutamate stimulation disrupts the CDC42EP-mediated assembly of the septin barrier consolidated by SEPT7 in the spine neck, enabling entrance into the spine head of the postsynaptic molecules, second messengers and cytoskeleton proteins necessary for F-actin mediated growth of spines and synaptic potentiation (21). In schizophrenia, the effect of higher levels of ARHGDIA would be amplified by the combination of lower levels of CDC42 and higher levels of CDC42EPs (Figure 1B) which impairs the opening of the septin barrier in response to glutamate stimulation, and thereby inhibits the influx of molecules into the spine head required for spine growth/maintenance, contributing ultimately to spine loss (Figure 1C).

Third, in this study we identified altered expression of several genes in the CDC42-PAK-LIMK pathway. In this pathway, activated, GTP-bound CDC42 activates the PAK proteins which in turn activate LIMK proteins. This signaling cascade inhibits the cofilin family of actin depolymerizing proteins that regulate the assembly and disassembly of F-actin required for structural stability of spines (59–62) (Figure 1A). Our findings from deep layer 3 pyramidal cells indicate that schizophrenia is associated with lower expression of PAK1/2 proteins (Figure 1B) which would further reduce activity in the CDC42-PAK-LIMK pathway due to the combination of elevated expression of ARHGDIA and lower expression of CDC42 and contribute to spine deficits. Consistent with this interpretation, down-regulation of CDC42 and PAK1 proteins results in impaired long-term maintenance of spines (63), and over-expression *in vitro* of dominant negative forms of PAK1 reduces spine density (64,65). Interestingly, a recent study demonstrated that pharmacological manipulation of downstream signaling components of the actin cytoskeleton such as the PAK proteins ameliorated synaptic deficits induced by DISC1 knockdown. Although this finding suggests that PAK proteins might serve as therapeutic targets, future experiments need to delineate the pattern of alterations in multiple signaling cascades in subjects with schizophrenia that can converge on these downstream signaling components (66). In contrast, we found higher expression levels of LIMK1/2 and PAK3 in deep layer 3 pyramidal cells. We interpret this finding as a compensatory, but inadequate, response to the multiple upstream alterations that increase phosphorylation (inactivation) of cofilin family proteins and suppress actin depolymerization, resulting in F-actin destabilization and a reduction in spine number on deep layer 3 pyramidal cells (Figure 1C).

In concert, these findings are consistent with other data suggesting that alterations in deep layer 3 pyramidal cells are "upstream" in the disease process of schizophrenia (44,67) or perhaps even directly related to genetic risk for the illness. For example, recent findings highlight the CDC42-PAK-LIMK regulatory network as a pathogenetic factor in actin

cytoskeleton dysregulation in schizophrenia (68). In addition, *de novo* mutations in proteins that regulate actin filament dynamics are preferentially found in individuals with schizophrenia (69), and copy number variations at the 15q11.2 locus implicate genes such as CYFIP1 that regulate dendritic complexity and spine actin dynamics (70,71).

## Conclusion

In concert with previous findings of alterations in other mediators (e.g., RhoA, Duo, Reelin, DISC1) of spine morphogenesis in schizophrenia (20,21,72–79), the findings of our study suggest that aberrant CDC42 signaling through two different pathways might represent a molecular pathology that converges on dendritic spine deficits specifically in deep layer 3 pyramidal cells (10,17) in subjects with schizophrenia. These observations support the notion that the actin cytoskeleton is dysregulated in schizophrenia. Because both spines and the axon terminals that innervate them are rich in actin, this dysregulation could result in lower excitatory drive to DLPFC deep layer 3 pyramidal cells (67) and a reduced need for energy production in these neurons (44).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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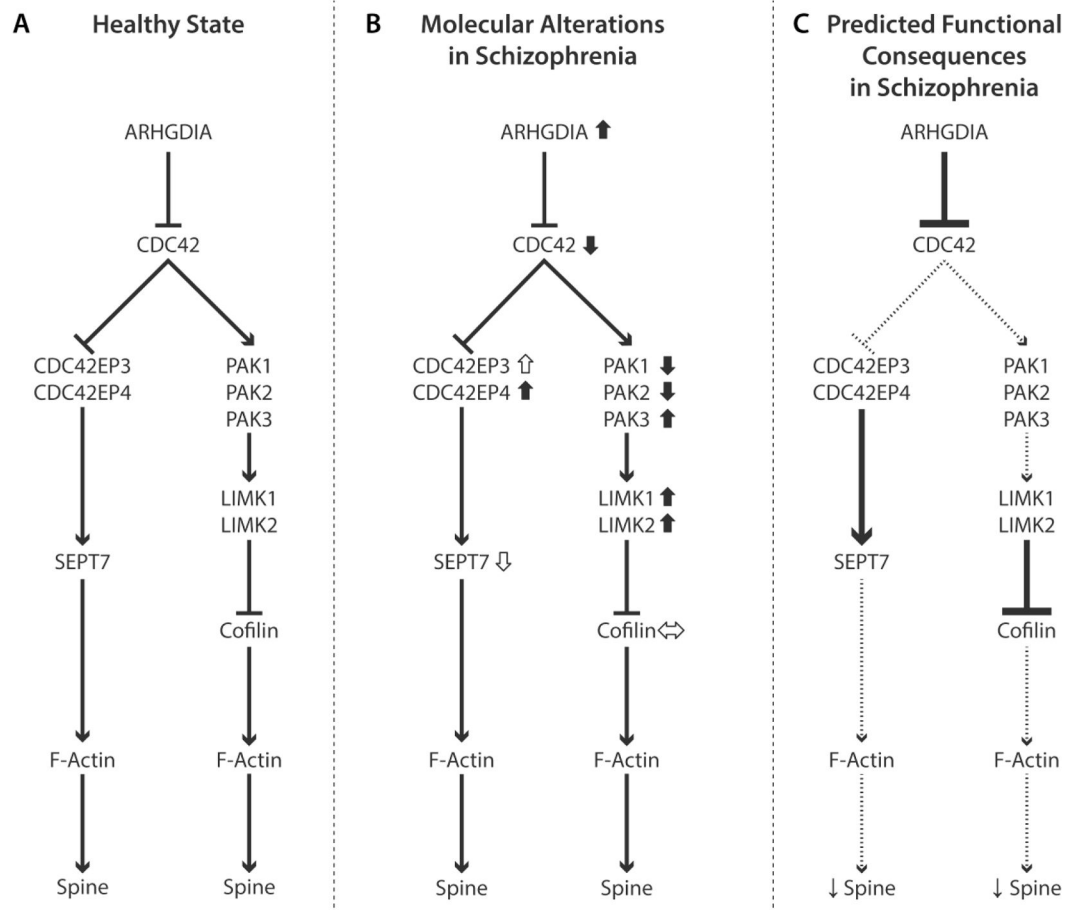
**Figure 1. Schematic diagrams of CDC42-CDC42EP and CDC42-PAK-LIMK signaling pathways and their proposed roles in spine deficits in schizophrenia**

**(A)** CDC42 signaling pathways that regulate the contribution of F-actin to dendritic spine structure. The activity of CDC42 is inhibited by ARHGDIA, a guanine nucleotide dissociation inhibitor that suppresses intrinsic GTPase activity. *CDC42-CDC42EP pathway*: Activated CDC42 inhibits CDC42 effector proteins (CDC42EP), which dissociate the complex of septin filaments consolidated by SEPT7 in the spine neck. This opening of the septin barrier permits an influx of molecules from the parent dendrite that facilitate F-actin mediated growth of spines in response to excitatory inputs. *CDC42-PAK-LIMK pathway*: CDC42 activates p21-activated serine/threonine protein kinases (PAK), which in turn activate LIM domain-containing serine/threonine protein kinases (LIMK). Activation of this cascade inhibits the cofilin family of actin depolymerizing proteins that regulate the turnover of F-actin required for structural stability of spines. Arrows indicate activation and blunted lines indicate inhibition of each target. **(B)** Subjects with schizophrenia exhibit up- or down-regulation for multiple components of the *CDC42-CDC42EP* and *CDC42-PAK-LIMK* pathways in DLPFC deep layer 3 pyramidal cells. Solid short arrows next to transcript indicates reported evidence in this paper; open short arrows indicate previously reported evidence (21, 72). **(C)** Predicted functional consequences in schizophrenia of altered CDC42 signaling pathways in DLPFC deep layer 3 pyramidal neurons. Higher levels of ARHGDIA would directly inhibit the activation of CDC42 holding it in an inactive GDP-bound state.

*CDC42-CDC42EP pathway*: The effect of higher levels of ARHGDIA would be amplified by the combination of lower levels of CDC42 and higher levels of CDC42EPs, impairing the transient opening of the septin barrier in spine necks in response to excitatory inputs, and thereby suppressing the influx of molecules into the spine head required for spine growth and maintenance (21). *CDC42-PAK-LIMK pathway*: The combination of higher levels of ARHGDIA mRNA, lower levels of CDC42 mRNAs and lower levels of PAK mRNAs (Given the much higher expression levels of PAK1 than PAK3, the down-regulation of PAK1 and PAK2 is predicted to have the dominant effect.) would all converge to increase phosphorylation (inactivation) of cofilin family proteins and suppress actin depolymerization, resulting in F-actin destabilization and spine loss. The up-regulated levels of LIMK1/2 and PAK3 may represent compensatory, but inadequate, responses to mitigate the negative impact on F-actin dynamics of the upstream molecular pathology in this pathway.
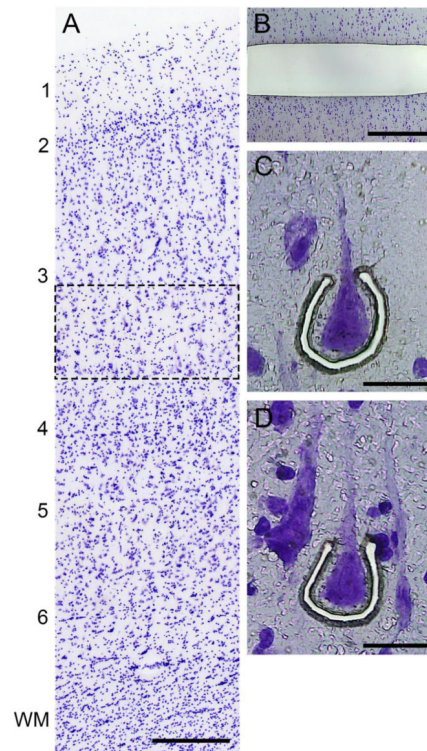
**Figure 2. Laser microdissection approach**
(A) Nissl stain of human DLPFC showing the location (dashed rectangle) of deep layer 3 which was sampled. Calibration bar equals 300 μm. (B) Nissl stained section after removal of strip of deep layer 3 by laser microdissection. Calibration bar equals 700 μm. (C and D) Representative images of individual deep layer 3 pyramidal cells being captured by laser microdissection in DLPFC deep layer 3. Calibration bar equals 30 μm. In panel A, numbers indicate cortical layers and WM indicates white matter.
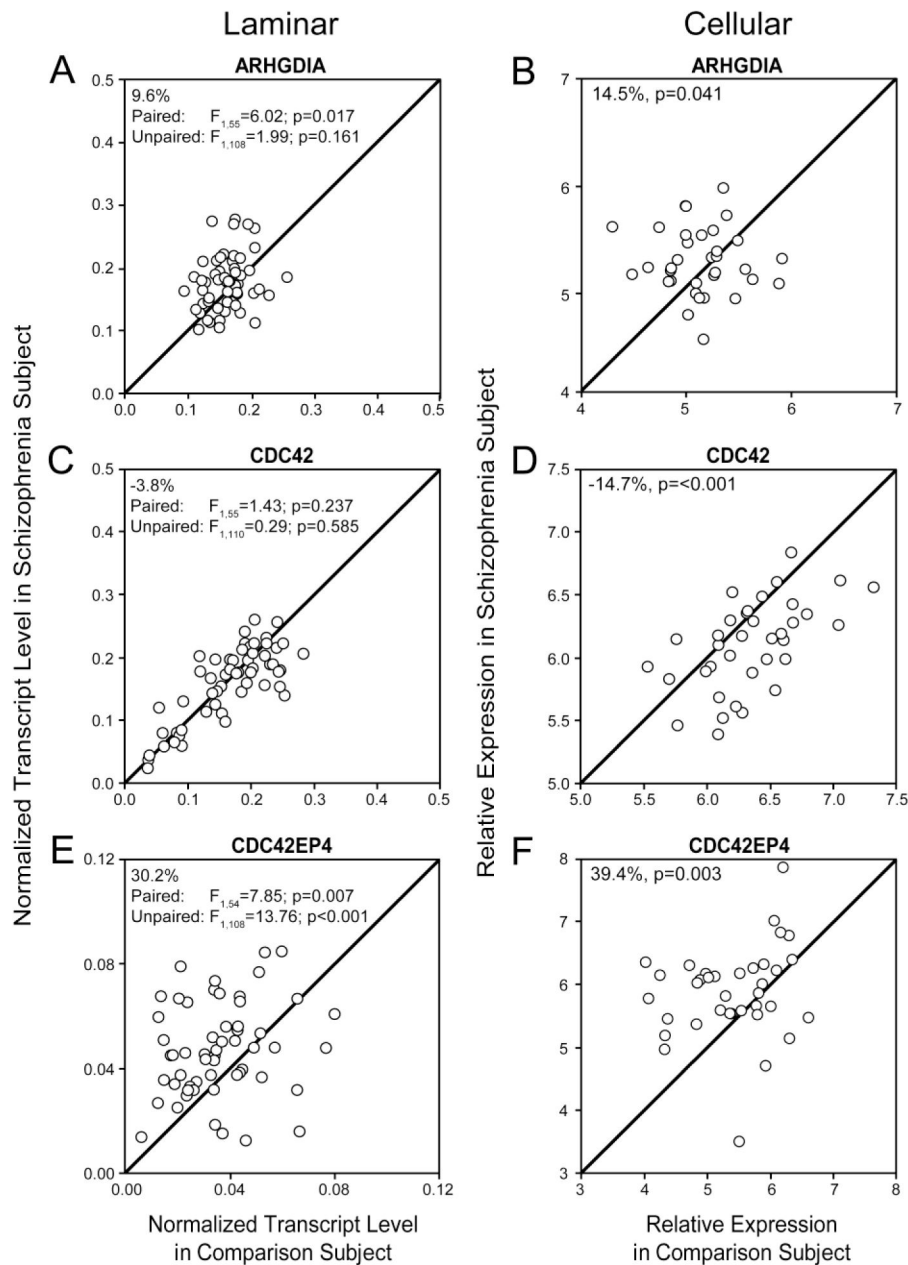
**Figure 3.**
(A,B) Rho GDP dissociation inhibitor encoded by ARHGDIA (C,D) Cell division cycle 42 (CDC42), (E,F) CDC42 Effector Protein 4 (CDC42EP4) mRNA levels in DLPFC deep layer 3 tissue homogenates (A,C,E) and DLPFC deep layer 3 pyramidal cells (B,D,F) from schizophrenia and comparison subjects. Scatter plots show the transcript levels for each matched pair of a comparison and schizophrenia subject. Values above the unity line reflect pairs in which transcript levels are higher in the schizophrenia subject relative to the comparison subject. Values below the unity line reflect pairs in which transcript levels are lower in the schizophrenia subject relative to the comparison subject.
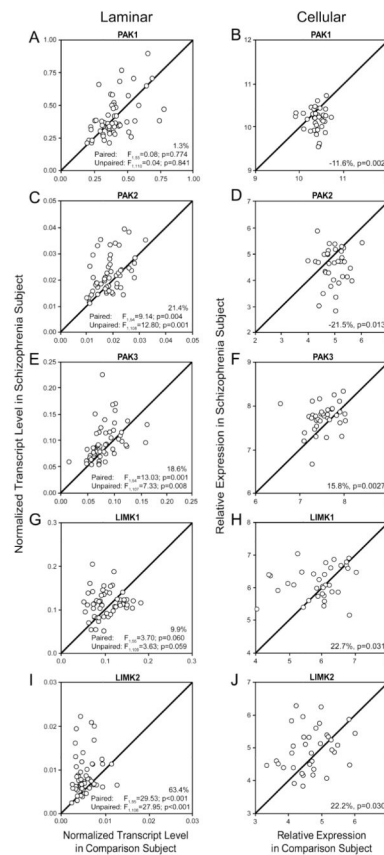
**Figure 4.**
(A,B) p21 protein (CDC42/RAC)-activated kinase 1 (PAK1), (C,D) p21 protein (CDC42/RAC)-activated kinase 2 (PAK2), (E,F) p21 protein (CDC42/RAC)-activated kinase 3 (PAK3), (G,H) LIM domain kinase 1 (LIMK1), (I,J) LIM domain kinase 2 (LIMK2) mRNA levels in DLPFC deep layer 3 tissue homogenates (A,C,E,G,I) and DLPFC deep layer 3 pyramidal cells (B,D,F,H,J) from schizophrenia and comparison subjects. Scatter plots show the transcript levels for each matched pair of a comparison and schizophrenia subject. Values above the unity line reflect pairs in which transcript levels are higher in the schizophrenia subject relative to the comparison subject. Values below the unity line reflect pairs in which transcript levels are lower in the schizophrenia subject relative to the comparison subject.
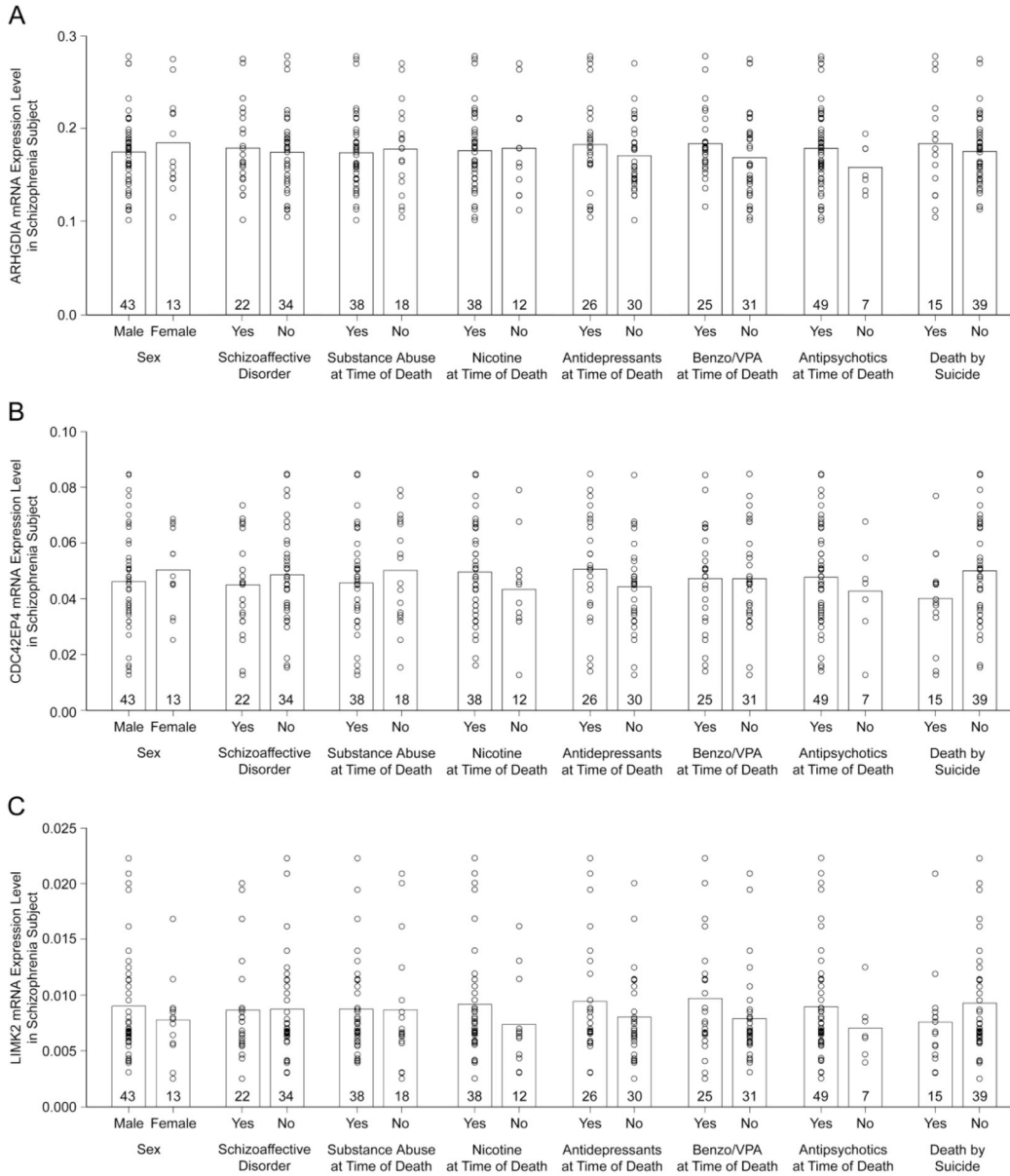
**Figure 5.**

The effect of co-morbid factors on (A) Rho GDP dissociation inhibitor encoded by ARHGDIA (B) CDC42 Effector protein 4 and (C) LIM domain kinase 2 mRNA expression levels in subjects with schizophrenia in DLPFC deep layer 3 tissue homogenates. For each panel, the circles represent mRNA expression levels for individual schizophrenia subjects by qPCR and the bars represent mean mRNA levels for the indicated group. Numbers at the bottom of bars indicate the number of subjects with schizophrenia per group. None of these confounding variables were significant for any of the transcripts (all $F_{1,48}$ 2.39, all p 0.13). Two subjects had an undetermined manner of death and were not included in the death by suicide analysis. Six subjects had unknown nicotine use at the time of death and were not included in the nicotine analysis. Benzo/VPA, benzodiazepines or valproic acid.

**Table 1**

Characteristics of human subjects

| Characteristic | Quantitative PCR | | Microarray | |
|---|---|---|---|---|
| | **Comparison** | **Schizophrenia** | **Comparison** | **Schizophrenia** |
| Number | 56 | 56 | 36 | 36 |
| Sex | 43 M, 13 F | 43 M, 13 F | 27 M, 9 F | 27 M, 9 F |
| Race | 47 W, 9 B | 37 W, 19 B | 30 W, 6 B | 24 W, 12 B |
| Age (years) | 48.8 ± 13.7 | 47.6 ± 12.5 | 48.1 ± 13.0 | 46.9 ± 12.4 |
| Postmortem Interval (hours) | 18.8 ± 5.6 | 19.3 ± 8.6 | 17.6 ± 6.1 | 18.0 ± 8.8 |
| RNA Integrity Number | 8.1 ± 0.6 | 8.1 ± 0.6 | 8.3 ± 0.6 | 8.2 ± 0.6 |
| Brain pH | 6.7 ± 0.2 | 6.6 ± 0.3 | 6.7 ± 0.2 | 6.6 ± 0.4 |
| Storage Time (months) | 116.4 ± 55.4 | 114.2 ± 60.5 | 122.2 ± 49.8 | 125.7 ± 53.1 |

| Comorbid factor | Comparison | Schizophrenia Yes | Schizophrenia No | Comparison | Schizophrenia Yes | Schizophrenia No |
|---|---|---|---|---|---|---|
| Schizoaffective Disorder | - | 22 | 34 | - | 13 | 23 |
| History of Substance Abuse | - | 38 | 18 | - | 23 | 13 |
| Nicotine ATOD | - | 38 | 12 | - | 23 | 7 |
| Antidepressants ATOD | - | 26 | 30 | - | 15 | 21 |
| Benzo/VPA ATOD | - | 25 | 31 | - | 14 | 22 |
| Antipsychotics ATOD | - | 49 | 7 | - | 31 | 5 |
| Death by Suicide | - | 15 | 39 | - | 10 | 26 |

*
Age, PMI, RIN and Brain pH are represented as mean ± SD.

M, Male; F, Female; W, White; B, Black. For the schizophrenia subject groups, the table indicates the number of subjects for the potential confounding variables such as, diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death (ATOD); use of antipsychotics, antidepressants, or Benzo/VPA ATOD; or death by suicide. Benzo/VPA, benzodiazepines or valproic acid.

**Table 2**

Summary of differences by transcript in antipsychotic-exposed monkeys

| Transcript | Mean Expression ± Standard Deviation | | | ANCOVA |
|---|---|---|---|---|
| | Placebo | Olanzapine | Haloperidol | |
| ARHGDIA | 8.2 ± 0.2 | 8.1 ± 0.2 | 8.1 ± 0.3 | $F_{2,10}=0.09$, p=0.91 |
| CDC42 | 9.9 ± 0.2 | 9.8 ± 0.2 | 10.0 ± 0.1 | $F_{2,10}=3.43$, p=0.08 |
| CDC42EP4 | 5.7 ± 0.5 | 5.2 ± 0.3 | 5.4 ± 0.4 | $F_{2,10}=3.02$, p=0.09 |
| PAK1 | 10.0 ± 0.1 | 10.2 ± 0.1 | 10.1 ± 0.1 | $F_{2,10}=2.51$, p=0.13 |
| PAK2 | 6.4 ± 0.4 | 6.4 ± 0.3 | 6.6 ± 0.3 | $F_{2,10}=0.59$, p=0.57 |
| PAK3 | 10.5 ± 0.2 | 10.7 ± 0.4 | 10.4 ± 0.2 | $F_{2,10}=3.21$, p=0.09 |
| LIMK1 | 8.9 ± 0.4 | 8.9 ± 0.3 | 8.8 ± 0.3 | $F_{2,10}=0.95$, p=0.42 |
| LIMK2 | 3.7 ± 0.2 | 3.7 ± 0.3 | 3.7 ± 0.2 | $F_{2,10}=0.03$, p=0.97 |