

Investigating the Ultrastructural Effects of F-BAR Proteins on Neuritogenesis by CLEM and Cryo-ET

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Neurons undergo a stereotyped series of morphological changes to become fully mature with differentiated axons and dendrites [1]. This begins with neurons forming lamellipodia and filopodia, from which neurites begin to elongate in a process known as neuritogenesis. The actin cytoskeleton is a critical component of such extensions, so proper control of neuritogenesis would need coordination of the plasma membrane and the actin cytoskeleton [2]. Recent work has demonstrated that F-BAR proteins (Fer/CIP4 homology [FCH] domain and Bin, Amphiphysin, RVS [BAR]), a family of proteins involved in regulating membrane and actin dynamics through their multiple domains, are directly involved in this mechanism [3-5]. In particular, overexpressing the CIP4 protein (Cdc42-interaction protein 4), from the CIP4 subfamily of F-BAR proteins, concentrated the protein to the protruding edge of early-stage cortical neurons, forming peripheral veils and increasing the density of actin bundles, which markedly decreased neurite formation [3]. Immunocytochemistry, live-cell imaging, pharmacological treatments, and co-transfection experiments showed that CIP4 localizes to the neuronal periphery by coordinating with the phospholipid content of the plasma membrane and proteins that downstream regulate the actin cytoskeleton [4]. Further studies have identified specific domains and regions of CIP4 and other proteins in the same CIP4 family that are necessary for its function [5]. Examining high-resolution images of these cells by cryo-electron tomography (cryo-ET) will provide unique insights into the membrane and cytoskeletal structure that results in the inhibition of neurite formation.

Here, we report preliminary results in sample preparation, correlative light and electron-microscopy (CLEM), and cryo-ET for CIP4-transfected mouse cortical neurons (Fig 1.). By plating on poly-D-lysine coated 200 mesh R 1.2/20 gold grids with CIP4-transfected cortical neurons from E14.5 mice, neurons successfully grew and sometimes formed the veil-like morphology that has been reported for glass cultures within 1 day *in vitro* (1 DIV). The CIP4 construct was conjugated with mScarlet, a red fluorescent protein engineered to be particularly bright and stable, which allowed easy tracking of neurons expressing CIP4 by fluorescence microscopy for CLEM (Fig 1B) [6]. This CLEM work allowed comparison studies to be made of CIP4-transfected cells and non-transfected cells from the same grid for cryo-ET targeting [7]. After plunge-freezing, it was found that the periphery of CIP4-transfected cells and non-transfected control cells are thin enough for data collection by the electron

microscope. Tilt series acquisition, reconstruction, and NAD-filtering [8] of a CIP4-transfected neuron revealed actin filaments that are aligned parallel to each other into bundles, the bundles being interspersed in random directions among other macromolecules and organelles. Such actin bundles were not observed in control neurons, being organized to either be more disperse or 'swirl' with curvature (Fig 2). Future work will include implementing montage tilt series collection to have a wider field of view [9], biophysical measurements of the actin filaments in CIP4-transfected cells vs. non-transfected cells, live-cell imaging of cells cultured on grids to understand CIP4 dynamics, pharmacological experiments, and transfecting neurons with other constructs in the CIP4 family. Such studies will provide ultrastructural insights on the process and control of neuritogenesis [10].

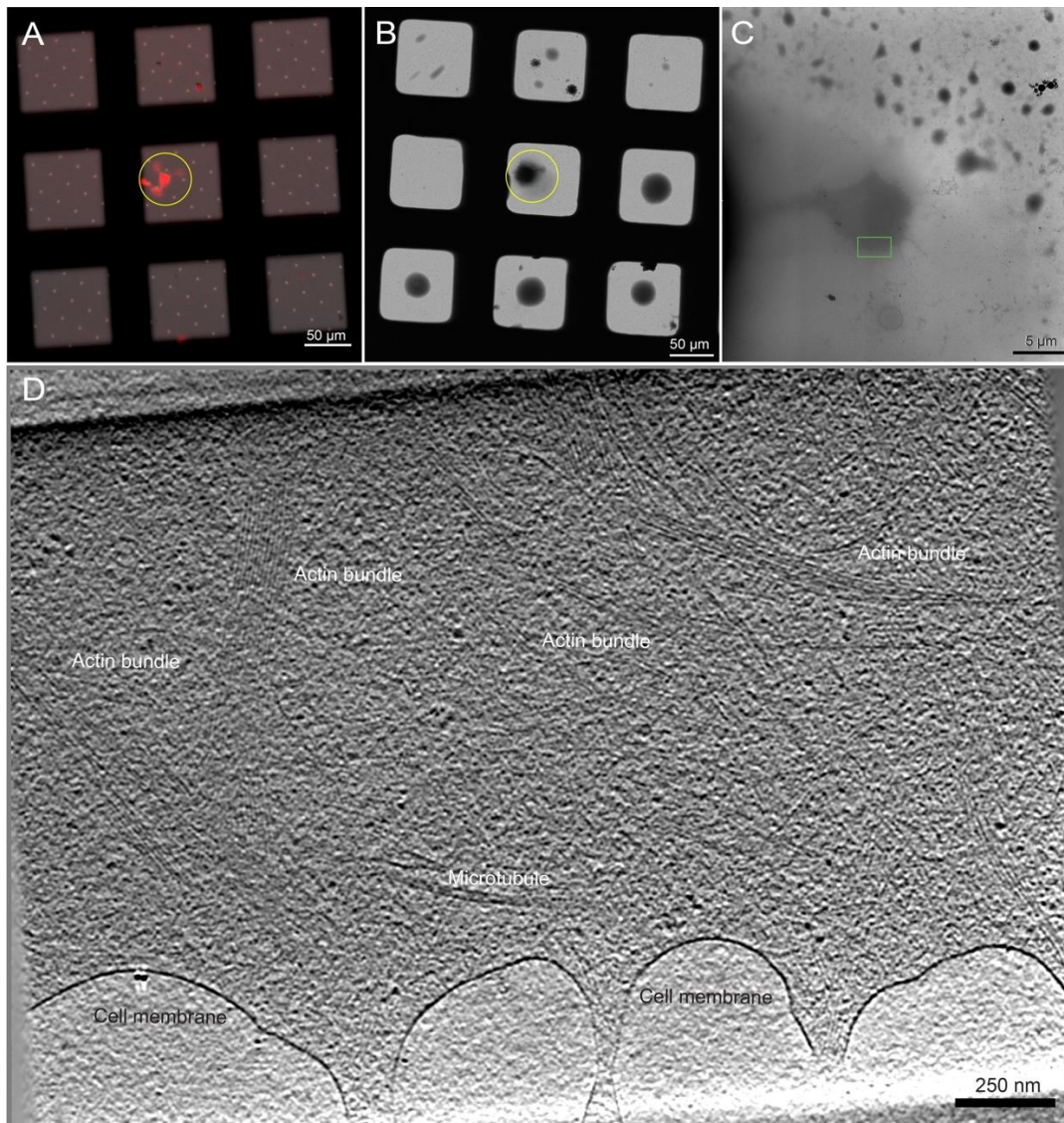


Figure 1. (A) Fluorescence microscopy image of a 1 DIV mouse cortical neuron transfected to overexpress CIP4-mScarlet (red) prior to plunge-freezing (B) Cryo-EM grid montage of the same grid in (A) after plunge-freezing. Yellow circle shows the same grid square. (C) Magnified cryo-EM montage of the yellow circle in (A) and (B) maps, revealing a small cellular extension that was thin enough for tilt-series data collection (green rectangle) (D) A 10 nm slice of a reconstructed and NAD-filtered

tomogram from data collected at the green circle in (C) [9]. This tomogram, taken at the cellular periphery, is marked by an abundance of actin bundles with other cytoskeletal elements, such as microtubules, throughout the entire tomogram. The scale bars in (A-D) are embedded in the image.

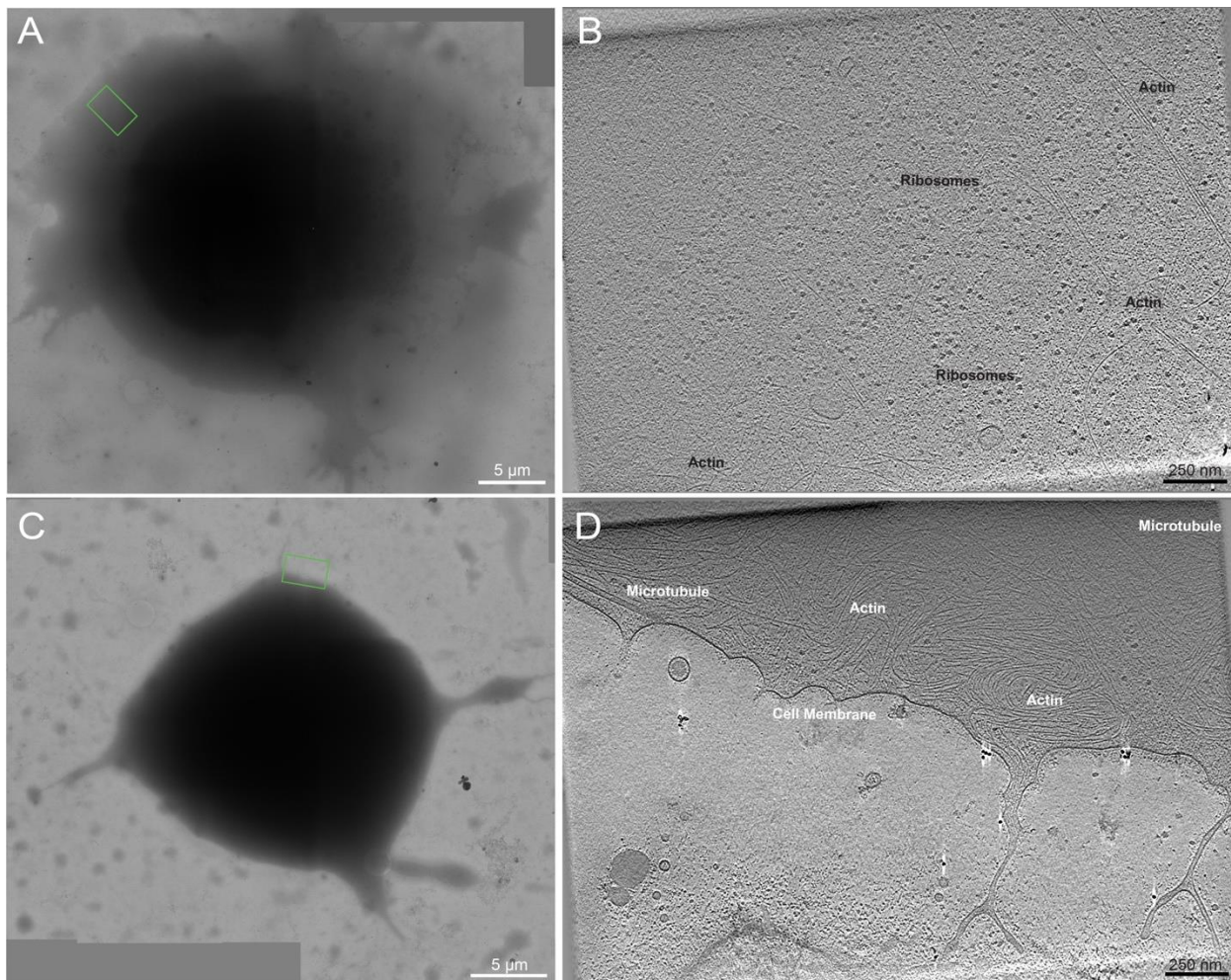


Figure 2. (A) Cryo-EM montage of a 1 DIV neuron that was not found to be fluorescent for CIP4-mScarlet. Data was collected at the region marked by the green rectangle (B) A 10 nm slice of a reconstructed and low-pass filtered tomogram from data collected at the green rectangle in (A). This tomogram is marked by what appears to be smaller bundles of long actin filaments at the upper right corner (compared to Fig 1D) and a density of shorter actin filaments in the lower left corner. (C) Cryo-EM montage of another 1 DIV neuron that was not found to be fluorescent for CIP4-mScarlet. Data was collected at the region marked by the green rectangle. (D) A 10 nm slice of a reconstructed and low-pass filtered tomogram from data collected at the green rectangle in (C). This tomogram is characterized by a high density of actin filaments that occasionally ‘swirl’. The scale bars in (A-D) are embedded in the image.

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