Rho GTPases and Neuron Morphology: Bridging the Gap with FRET Imaging

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RHO GTPASES AND NEURON MORPHOLOGY: BRIDGING THE GAP WITH FRET IMAGING

By

Nima Sharifai

A DISSERTATION

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RHO GTPASES AND NEURON MORPHOLOGY: BRIDGING THE GAP WITH FRET IMAGING

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Understanding how molecules give rise to cellular phenotypes represents a major challenge in biology. For over half a century, concerted efforts have been made to identify protein interactions in order to understand the ways in which they produce cellular physiology. As a result, a vast collection of protein-protein interaction maps (interactomes) have been produced. Although these data sets continue to make valuable contributions in our understanding of molecular biology, virtually all of them are deficient in one important respect – context. Because the technical approaches used to detect protein interactions have required that they be studied in artificial environments, they do not inform as to when and where an interaction takes place within the cells of an intact organism (i.e. its native environment). Yet, our ability to understand and manipulate the molecular logic that governs cellular events will ultimately rely on a knowledge of these spatiotemporal dynamics. Attaining this ideal requires a platform that can bridge these worlds of vastly different scale. Using Drosophila transgenics and Fluorescence Lifetime Imaging Microscopy (FLIM), this project presents strategies to capture protein interaction dynamics among a group of cytoskeletal regulators in order to understand some of the molecular events responsible for
neuron morphogenesis. Cdc42 (Cell division control protein 42 homolog) and Rac1 (Ras-related C3 botulinum toxin substrate 1) are both Rho subfamily monomeric GTPases. These proteins behave as rapid molecular switches, capable of initiating signaling pathways in response to extracellular and intracellular cues. Within neurons, both Cdc42 and Rac1 have been found to play non-redundant roles in producing the characteristic morphology of axons and dendrites. Despite numerous studies on these proteins, little light has been shed on what makes their signaling and morphological contributions unique within a given neuron, let alone how or if they coordinate in shaping neuron morphology. A clue may lie with a shared group of downstream effectors, known as CRIB proteins. Member of this cohort share a Cdc42-Rac1 Interactive Binding (CRIB) domain and have been implicated in mediating the effects initiated by Cdc42 and Rac1 on components of the cytoskeleton. The extent to which CRIB proteins interact with each GTPase in developing neurons, however, is unknown. The embryonic nervous system of the fruit fly Drosophila melanogaster offers a promising context to elucidate some of the key signaling patterns that shape neuron morphology, and may help distinguish the roles that Cdc42 and Rac1 play in this process. The results of this work demonstrate that protein-protein interactions can be visualized and quantified within neurons of intact Drosophila embryos. It reveals that Cdc42 interacts in a parallel, yet distinct, manner with two CRIB effectors (Par6 and WASp) during neurodevelopment. Despite broad co-localization of proteins within cells, these interactions were highly restricted. Within single neurons, both interactions corresponded to the time and place of
dendrite morphogenesis, where they exhibited both overlap and complementarity. These patterns help explain the dendritic defects observed once interactions are disrupted via protein knockdown. Likewise, constitutive activation of Cdc42 results in morphological abnormalities that are mirrored by a spatiotemporal expansion in both interactions. For Rac1, activation was found to take place within the aCC dendrite region, similar to Cdc42. However, while Cdc42 activation occurred almost exclusively in the dendritic region, Rac1 was activated throughout the neuron except in dendritic tips. Despite its broad activation, Rac1 signaled weakly with Par6, while not at all with WASp.

Altogether, the utilization of FLIM-FRET to study protein-protein interactions in a living organism provides the spatiotemporal context that has long been needed in biology. By characterizing the signaling dynamics and morphological contributions of the Cdc42-Rac1-CRIB protein subnetwork in Drosophila neurons, this work helps distinguish the roles of two related proteins in establishing neuron morphology. Looking forward, a dynamic in situ interactome can help clarify how molecular networks underscore normal and abnormal cellular physiology.
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<td>aCC</td>
<td>anterior Corner Cell</td>
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<tr>
<td>AEL</td>
<td>After Egg Laying</td>
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<tr>
<td>AP</td>
<td>Acceptor Photobleaching</td>
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<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>CRIB</td>
<td>Cdc42/Rac1 interactive binding</td>
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<td>FLIM</td>
<td>Fluorescence Lifetime Imaging Microscopy</td>
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<td>FRET</td>
<td>Forster Resonance Energy Transfer</td>
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<td>GDP/GTP</td>
<td>Guanosine Diphosphate / Guanosine Triphosphate</td>
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<td>mEGFP</td>
<td>monomeric Enhanced Green Fluorescent Protein</td>
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<td>PAK</td>
<td>p21 activated kinase</td>
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<td>RhoGAP</td>
<td>Ras homolog GTPase activating protein</td>
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<td>SE</td>
<td>Sensitized Emission</td>
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<td>SRM</td>
<td>Super Resolution Microscopy</td>
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<td>STORM</td>
<td>Stochastic Optical Reconstruction Microscopy</td>
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<td>VNC</td>
<td>Ventral Nerve Cord</td>
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<td>WASp</td>
<td>Wiskott - Aldrich syndrome protein</td>
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Chapter 1

Introduction

Overview

The distinction between life and the inanimate world is one of process rather than substance (McKay, 2004). While physical and chemical features can help us to classify objects within these two groups, the hallmark of life is an active coordination of chemical processes that favor self-preservation and perpetuation in the face of degenerative environmental forces (Koshland Jr., 2002). That is not to say, however, that the constituents of biology are not essential to its processes. Macromolecules have evolved over time to form highly interconnected networks within the cells of organisms. A living organism’s ability to react to its external environment, whilst maintaining its internal one, arises from the interactions between these molecules. Therefore, to understand and harness the principles responsible for life’s dynamic efficacy is to understand and manipulate its underlying molecular processes. In an effort to better appreciate one such principle, this thesis will investigate the signaling patterns and morphological contributions among a group of proteins, previously implicated in regulating cytoskeletal change, within the context of neuronal development in the fruit fly embryo (Drosophila melanogaster).
Molecular logic of the cell

Generally speaking, when one considers the size of various biological components, networks emerge at three levels: molecular, cellular, and social. Since an organism is composed of cells, which in turn are each composed of molecules, these three levels form a nested network (Fig 1.1). Social networks are generally the most accessible for study by scientists, however the various form of interaction between organisms can be difficult to identify and classify in a natural setting. Cellular networks also operate at the visible scale, although specialized tools are required to detect cell-cell communication. Of the three, molecular networks have been the most difficult to study. While we can visually observe organisms and cells, individual molecules are too small to be resolved with light. As a result, we are forced to perturb them in some way (e.g. isolation, perturbation, modification) in order to observe their behavior, which typically precludes studying them in their natural environments. The incentive to succeed in such studies, however, compensates for its difficulty.

Among the various types of molecules that form the biological world, proteins play a central role in the molecular network. Much like airport hubs that make it possible to travel from one small city to another, proteins establish paths of communication between different classes of molecules via their broad binding capabilities (Lodish et al., 2000). Owing to their functional diversity and structural flexibility, proteins play a part in virtually every aspect of cell physiology. Processes essential for cellular function and survival, which would otherwise be too slow or unlikely to take place on their own, are accelerated or realized by
these molecular machines. Unlike most other types of molecules, the three-dimensional structure of proteins can undergo conformational change in response to its immediate environment. As a result, they can quickly associate or disassociate with other molecules, drastically changing molecular events. In network terminology, the various components of a network are called nodes and the connections between them are known as links or edges (Albert and Barabasi, 2002). The dynamic nature of the molecular network is largely characterized by the formation and loss of links involving protein nodes. Given their essential role in cellular functions, protein interactions are of great importance when attempting to understand the molecular network.

**Protein interactions regulate neurodevelopment**

Characterizing the molecular network is of limited value without knowledge of the cellular context in which it manifests. Indeed, the same is true when studying any network – our intention is to ultimately decide which network states (i.e. link combinations) are responsible for specific phenomena that emerge at a higher level. The nervous system is one of the most heavily studied, yet one of the least understood, components in biological systems. Central nervous systems, such as the human brain, coordinate and execute the most complex behaviors observed in the animal kingdom. This organ and its capabilities are the manifestation of a cellular network, where up to several billion neurons connect and communicate electrochemically via synapses (Robichaux and Cowan, 2014). As such, the ability of neurons to initially form synapses is a necessary
prerequisite for nervous system formation. As one might expect, protein interactions play crucial roles in this process.

**Neuron morphology and connectivity**

The formation of a nervous system requires several developmental processes, including cell division, neurogenesis, and cell migration (Robichaux and Cowan, 2014). Establishing brain circuitry subsequently depends on the growth and precise navigation of axons to their target regions, where they will form synapses with other neurons or muscle cells (synaptogenesis). Neurons typically extend another type of structure, known as dendrites, to receive signals from axons of other neurons. Axons and dendrites (collectively referred to as neurites) serve as the pre-synaptic and post-synaptic interfaces, respectively, for neuron-to-neuron communication in organisms. Like axons, dendrites undergo a period of growth and development that is necessary for proper synapse formation. A common mechanism drives both structures in their developmental journey towards one another: binding between extracellular guidance cues and protein receptors on the cell surface, which in turn initiate signaling cascades within the neuron (Robichaux and Cowan, 2014).

The roles of different guidance molecules and their receptors have been, and continue to be, the focus of extensive study (Shen and Cowan, 2010). This thesis, however, is interested in a common intracellular response invoked by many of these extracellular signals – morphological change. Neuron morphology refers to the shape and structure of a neuron (and its neurites). A key intracellular process prior to and during synaptogenesis is remodeling of the
cytoskeleton, which is primarily composed of actin filaments and microtubules (Vitriol and Zheng, 2012). Polymerization of globular actin (G-actin) protein monomers forms filamentous actin (F-actin). The growth, branching, and contraction of these filaments are largely responsible for the dynamic changes observed at the leading edge of growing axons and dendrites, known as the growth cone (Vitriol and Zheng, 2012). Like actin, microtubules undergo cycles of assembly and disassembly from individual protein subunits, known as tubulin (Conde and Cáceres, 2009). Unlike actin, however, microtubules provide long-term stability to initially dynamic extensions of the membrane. For example, actin polymerization is responsible for thin membranous protrusions, known as filopodia, at the growth cone. Filopodia are largely unstable structures, disappearing almost as rapidly as they form (Shen and Cowan, 2010). However, the extension of microtubules into some of these nascent protrusions, and their subsequent membrane attachment in a process known as microtubule capture, is thought to produce the stable branch and terminal structures found in axons and dendrites (Conde and Cáceres, 2009).

There are undoubtedly many processes that are essential for the formation of functional synapses (e.g. postsynaptic receptor localization, presynaptic vesicle docking, etc.). Neuron morphology, however, is one of its most readily visible and measurable aspects; providing a promising context to investigate the relationship between changes at the cellular and molecular level. Moreover, the molecular players involved in regulating neuron morphology offer
an opportunity to study one of the most dynamic and coordinated protein subnetworks in biology.

The essential roles of Rho GTPases in neuronal morphogenesis

The Rho GTPases are a well-studied class of proteins with distinct signaling properties. All GTPases cycle between an active (GTP-bound) and inactive (GDP-bound) state, which is regulated by a number of upstream proteins including Guanine Nucleotide Exchange Factors (GEFs), GTPase-Activating Proteins (GAPs), and Guanosine Nucleotide Disassociation Inhibitors (GDIs). Rho GTPases behave as diverse molecular switches, interacting with and regulating signaling pathways that control cell proliferation, apoptosis, adhesion, motility and differentiation (Etienne-Manneville and Hall, 2002). Perhaps not surprising then is the fact that this family of proteins is among the most-well conserved in the animal kingdom, expressed in virtually all eukaryotic cell types (Boureux et al., 2007). Climbing the evolutionary ladder, one finds additional members in higher organisms, purportedly adding to the complexity and capacity of cell signaling.

In the realm of neuroscience, three Rho GTPases - Cdc42, Rac1, and RhoA - have been strongly implicated in shaping neuron morphology during development and plasticity. A variety of extracellular signals have been linked to local activation of Rho GTPases within neurons, where they initiate signaling pathways involved in cytoskeletal remodeling (Govek et al., 2005). Of particular intrigue are Cdc42 (Cell division control protein 42 homolog) and Rac1 (Ras-related C3 botulinum toxin substrate 1), as they appear to be remarkably similar
in their structure and function (Hakoshima et al., 2003). These two proteins share 70% of their amino acid sequence and have been found to possess similar functions in neuron morphogenesis, including promoting neurite formation (Govek et al., 2005), regulating axonal guidance and growth cone extension (Kim et al., 2002, 2003; Ng et al., 2002), and supporting growth and branching in both axons and dendrites (Ng et al., 2002; Govek et al., 2005). Indeed, Cdc42 and Rac1 are often mentioned in unison, with RhoA typically having an antagonistic role in these same processes (Li et al., 2000; Nakayama et al., 2000; Wong et al., 2000). Despite their resemblances, Cdc42 and Rac1 clearly play non-redundant roles and are distinct in some important respect. Genetic removal of either Cdc42 or Rac1 is embryonic lethal (Sugihara et al., 1998; Chen et al., 2000; Heasman and Ridley, 2008). While displaying common morphological abnormalities, loss of function experiments have shown that each plays an essential role in neuronal development (Ng et al., 2002; Garvalov et al., 2007). Altogether, the extensive similarities between Cdc42 and Rac1 have not been contrasted with notable differences. While studies have claimed discerning characteristics regarding the morphological roles of Cdc42 and Rac1, experiments in different species and cell types have led to conflicting, and sometimes contradictory, conclusions (Govek et al., 2005).

**Cdc42/Rac1 Interactive Binding (CRIB) proteins as mediators of morphological change**

Of the over 100 putative binding partners identified for Cdc42 and Rac1 in yeast and mammalian line screens, those which contain a Cdc42-Rac1 binding
(CRIB) motif have been associated with mediating effects on cytoskeletal machinery (Burbelo et al., 1995; Kessels et al., 2011). The CRIB domain, located near the proteins’ N-terminus, consists of a 16 amino acid consensus sequence I-S-x-P-x(2,4)-F-x-H-x(2)-H-V-G. Using previous literature and a bioinformatics sequence search, there are ten proteins that possess this domain: Pak1 (p21-activated kinase), Pak2, Pak3, gek (genghis khan) and slpr (slipper) are serine/threonine kinases; Ack (activated Cdc42-associated tyrosine kinase) and PR2 (Fak-like kinase) are tyrosine kinases, and WASp (Wiskott-Aldrich syndrome protein), Par6 (partitioning-defective protein 6) and Spec2 (small binding proteins for Cdc42) are scaffold proteins without any known catalytic domain.

Binding of either Cdc42 or Rac1 is purported to induce dramatic conformational changes in these effectors, altering their signaling activity (Hakoshima et al., 2003). In theory, the presence of a CRIB domain would allow both Cdc42 and Rac1 to serve as interaction partners. In reality, however, some CRIB proteins have a significantly greater affinity for one GTPase over the other. For instance, biochemical studies have found that WASp, and to a much lesser extent Par6, possess greater affinity for Cdc42 than Rac1 (Garrard et al., 2003; Tomasevic et al., 2007). Conversely, PAK1 and PAK2 have a greater affinity for Rac1 (Zhang et al., 1998).

Although not all the CRIB proteins are well studied in the context of neurodevelopment, there is considerable evidence implicating Pak, Par6 and Ack in synapse formation (Ruiz-Canada et al., 2004; Linseman and Loucks, 2008;
Kessels et al., 2011). It is unknown when, where, and to what extent Cdc42 and Rac1 may interact with this group of proteins during synaptogenesis. Answering these questions, however, may shed some light onto where Cdc42 and Rac1 diverge, and how they might coordinate in order to establish the compartments comprising synapses.

**Static vs dynamic interactomes: Methodologies for studying protein interactions**

Given the small size of proteins size and the transient nature of their interactions, identifying protein-protein interactions has historically required clever biochemical assays and snapshot measurements. A protein-protein interaction network, termed the interactome, can be gradually constructed by determining which proteins exhibit interactions. Before assessing the current interactomic landscape, it is helpful to briefly describe and compare some common methodologies for studying protein-protein interactions.

**Yeast-2-Hybrid**

Yeast-2-Hybrid (Y2H) is an *in vivo* approach for studying protein interactions. Although implemented in yeast (*Saccharomyces cerevisiae*), the technique is capable of assessing proteins derived from any organism using via genetic manipulation. The principle of Y2H is based on the idea that a eukaryotic transcription, such as the GAL4 transcriptional activator, consists of a DNA-binding domain and catalytic (activating domain). Both domains are critical to its function in promoting the transcription of genes involved in galactose
metabolism; and while these two domains are normally physically connected as one protein, they can still operate effectively if they are expressed as two separate fragments within close proximity of each other (Rao et al., 2014). Generally speaking, random encounters between the two fragments alone will not be frequent enough to promote much gene transcription. However, if the fragments are fused to two proteins which tend to interact, then the two fragments will be brought into proximity with a high enough probability to ensure gene transcription. By creating genetically engineered yeast strains that are dependent on this fragmented transcription factor (e.g. to express genes for synthesizing a nutrient that is lacking in the media), a protein interaction can be detected by the organism’s survival when expressing those fusion proteins.

Libraries of different fusion proteins have been created to assess hundreds of thousands unique pairwise protein interactions. The advantage of Y2H is that it is a high-throughput screening approach, in that hundreds of interactions can be tested and read simultaneously. Indeed, comprehensive interactomes have been created for several organisms using this approach (Uetz et al., 2000; Giot et al., 2003). A big drawback of Y2H is that co-expressing proteins that are foreign to this unicellular organism can produce false positive interactions, while a lack of endogenous co-factors can also result in false negatives. Furthermore, transient interactions may be hard to detect using this approach since the readout requires protein complexing that is stable enough to last through the transcriptional process. The inconsistency in results obtained from independent screens led one study to estimate that 25-45% of detected
interactions are stochastic false-positives, while 75-90% are false negatives arising from statistical undersampling (Huang et al., 2007).

**Coimmunoprecipitation (Co-IP)/Co-affinity purification (Co-AP) + Mass Spectrometry (MS)**

A general approach to isolating protein interactions is to extract a given protein from cells by using an antibody (immunoprecipitation) or binding substrate (affinity chromatography), and subsequently analyze all of the proteins that are bound to it using Mass Spectrometry (MS) analysis. Unlike other methods that require proteins be selected beforehand to assess potential interactions (e.g. Immunoprecipitation, Western Blotting, Y2H), MS can infer protein partners by analyzing the mass-to-charge ratio of peptide fragments bound to a specific protein of interest (Rao et al., 2014). Hence, Co-IP/Co-AP + MS approaches are unique in their ability to identify protein interactions in an unbiased manner.

A drawback of MS is that the proteins bound to the extracted protein may not be its direct partners, but rather indirectly bound and part of a larger complex. While it is still maybe useful to identify such proteins, one typically cannot distinguish between a direct and indirect interaction.

**Genetic Interactions**

Genetic interaction studies take a functional approach in studying protein interactions. When many genes are mutated or removed from an organism, this becomes reflected by a physical anomaly (phenotype) that is observable in the organism. A genetic interaction is established when the introduction of a mutation/deletion in a second gene results in an enhancement or suppression of
the phenotype that arises from the original first mutation, and vice versa. The interpretation can vary depending on how the genes are modified, but let us consider gene deletions for one illustration. If a second gene’s deletion exacerbates an original phenotype, such as causing a further loss in hair pigment (black$\rightarrow$brown$\rightarrow$blonde), it can be said that those two genes (and their protein products) act synergistically in a functional sense.

However, this may be because the two genes are involved in separate signaling pathways, with both involved in synthesizing some hair pigment. Therefore, genetic interactions are prone to connecting proteins that do not physically interact (Dixon et al., 2009; Jaimovich et al., 2010). Nevertheless, genetic interactions study proteins in their native context (a living organism), something that is not possible with most other methodologies.

**Biochemical studies**

Biochemical studies isolate proteins from their native environment in order to definitely assess whether a direct protein-protein interaction takes place. Within cells, a given protein-protein interaction may require a particular microenvironment or cofactor (e.g. intermediate or scaffolding proteins) in order to take place. Biochemical studies performed in vitro aim to delineate such factors, while also providing quantitative measurements (such as disassociation constant, $K_D$), regarding the interaction (Zhang et al., 1998; Tomasevic et al., 2007).
**Forster Resonance Energy Transfer (FRET)**

Despite the less than optimal detection sensitivities/specificities found in some of the aforementioned approaches, the data they have provided thus far has helped create frameworks for exploring various interactomes in further detail. Some details, however, cannot be filled by the use of binary assays, artificial environments, and broad detection parameters has left an important gap in our knowledge. Specifically, the vast content in protein interactomes lack context, offering only a static representation of a dynamic molecular world. Given the extensive modifications that many proteins constantly undergo within cells, these molecular networks represent biochemical possibilities more than they do the molecular circuitry needed to produce a particular cellular state. Without a consideration of space, time, and native environment, we cannot appreciate the dynamic nature of protein interactions within a living system. As such, there exists an important gap in our knowledge regarding the link between molecular and cellular events. In order to elucidate the molecular logic that produces higher-order phenomena, interactions must be studied in tandem with measurable cellular phenotypes, and in a manner that allows dynamic acquisition and correlation of both processes in space and time.

Fortunately, advances in genetics and imaging technology have made it possible to study protein interactions directly within intact animals, thanks to the phenomenon of near field fluorescence resonance energy transfer described by Förster (1948). Conventional confocal microscopy has been a mainstay in determining protein localization; and while useful in some applications, the
colocalization of proteins says little about whether or not they interact. This is because conventional microscopy is limited by the Abbe diffraction limit of a microscope (d), which is equal to:

\[ d = \frac{\lambda}{2 \times NA} \]  

where \( \lambda \) is wavelength of light being detected and NA is the numerical aperture of the objective lens. Current confocal microscopes possess a diffraction limit that is one-half to one-third of \( \lambda \). A system optimized for maximal spatial resolution (\( \lambda = 400 \) nm, \( NA = 1.6 \)) cannot resolve two fluorescent molecules as separate below 125 nanometers (Day, 2009). When dealing with proteins, typically less than 10 nm in size, spatial resolution is simply inadequate. A pixel denoting colocalization may contain two proteins that are microscopically distant without any form of interaction.

The advent of Super-Resolution Microscopy (SRM) has curtailed this problem to some extent. SRM encompasses a variety of approaches that surpass the spatial resolution set by the Abbe diffraction limit. One particular method, Stochastic Optical Reconstruction Microscopy (STORM), uses photoswitchable fluorophores to reduce the likelihood of detecting overlapping molecules. Subsequent image reconstruction can produce an image with a lateral (XY) spatial resolution of 20 nanometers (Schermelleh et al., 2010).

However, SRM methods that approach such resolutions are currently limited in their application to live cell imaging, namely because they typically require high excitation intensity (increased cell phototoxicity), prolonged exposure time...
(confounded by cellular and subcellular movement between frames) and low labeling density (excludes labeling of many signaling proteins) (Schermelleh et al., 2010).

Future developments in SRM may overcome some of these limitations, but the detection of protein-protein interactions in living cells, does not require SRM at all. Because of the physical nature of fluorescence, one can actually measure photon energy transfer between two properly selected fluorescent molecules, where the emission spectrum of one fluorophore (donor) overlaps with the excitation spectrum of the other (acceptor). This phenomenon, known as Forster Resonance Energy Transfer (FRET), can be detected by measuring a change in fluorescence intensity (donor/acceptor) or fluorescence lifetime (donor) when the two fluorescent molecules are expressed together versus when expressed alone. FRET is an ideal proxy for the physical association between a pair of proteins because it does not occur unless the fluorescent molecules are within a very short distance of one another. For Green Fluorescent Protein (eGFP) and mCherry, the Forster Radius (radius of half-maximal FRET efficiency) is 5.1 nm, with virtually no FRET activity above 8.1 nm separation (Buranachai et al, 2008; Clegg et al, 2009). Since GFP and mCherry do not normally bind each other, FRET only occurs when they are used as fluorescent labels for (i.e. fused to) two proteins that do bind (Shaner et al., 2005). Furthermore, because image acquisition is on the order of seconds, positive FRET is an indicator of two proteins that are either in a stable complex or frequently associating-disassociating with each other. Best of all, this method
does not require any chemical treatment or animal sacrifice as protein isolation is not necessary, and can therefore be performed on a living system over time.

**Linking Molecular and Cellular Events: An Experimental Platform to Bridge the Gap**

**Genetic manipulation and Neurodevelopment in Drosophila**

The *UAS-GAL4* system, a genetic expression system derived from yeast and transferred into *Drosophila*, is a tool that permits tissue and cell-specific gene expression of exogenous proteins by coupling them to endogenous tissue-specific enhancers. By restricting the expression of fluorescent proteins in specific subset of cells, this technique enhances cellular visualization and limits potentially deleterious effects from organism-wide exogenous protein expression.

The *Drosophila aCC (anterior corner cell)* neuron, also known as the pioneer motorneuron, is a well-studied and highly stereotyped CNS neuron that develops relatively early on in the embryo. It possesses a single axon, which it extends to the dorsal body wall musculature on the opposite end of the embryo. Not far from where the axon extends from the cell body, the aCC axon sprouts a dendrite that synapses with a number of other ventral cord neurons, most of which are interneurons (Tripodi *et al.*, 2008; Corty *et al.*, 2009). This is an intriguing region to study protein-protein interactions, as dendrite extension does not take long before synapses are established.

**Frequency Domain - Lifetime Imaging Microscopy (FD-FLIM)**

The most popular approach for detecting FRET has traditionally been to perform ratiometric comparisons of fluorophore intensity. Ratiometric FRET
methods include *sensitized emission* and *acceptor photobleaching*. Sensitized emission (SE) measures the amount of acceptor emission that arises from donor excitation, while acceptor photobleaching (AP) measures the amount of donor emission that is absorbed in acceptor excitation. In SE, the sample is excited at the donor excitation wavelength while photons with wavelengths in the acceptor emission spectrum are measured (Clegg, 2009). In AP, a specific region of the sample is excited at high intensity at wavelengths that will photobleach the acceptor, with donor excitation/emission images are captured before and after photobleaching. As the number of acceptors capable of fluorescing drops, less donor fluorophores are able to undergo FRET. If FRET was indeed taking place, donor intensity will be higher after photobleaching than before (a phenomenon known as donor “dequenching”).

While ratiometric approaches are perfectly legitimate methods of measuring FRET, they possess important limitations in the detection of protein-protein interactions in live samples. Compared to SE and AP, Fluorescence Lifetime Imaging Microscopy (FLIM) offers a number of advantages (Clegg et al., 2003). First, ratiometric methods must capture both donor and acceptor intensity and thus requires two lasers and two or more filter sets. FLIM, on the other hand, measures only donor lifetime and so requires only one laser and one filter set during image acquisition. Not only does this generally accelerate acquisition time, which is crucial when imaging live samples, but it also reduces the number of controls and corrections (Feige et al., 2005) needed for FRET calculation. With FLIM, FRET can be detected by observing a decrease in the fluorescent decay
(lifetime) of eGFP when in the presence of an acceptor compared to when it is absent. Furthermore, unlike fluorescence intensity, fluorescence lifetime is not dependent on fluorophore concentration. In experiments where the tagged proteins are not tethered to each other (i.e. donor:acceptor concentration ratio is variable or difficult to quantify), FLIM and AP are the only reliable methods for quantifying FRET (Clegg et al., 2003). Yet, compared to FLIM, acceptor photobleaching requires a longer time for measurement (before image \(\rightarrow\) bleaching \(\rightarrow\) after image), risks greater phototoxicity, and cannot perform continuous measurements in the photobleached region.

High quality FRET data acquisition also hinges on the selection of appropriate fluorophores. An ideal fluorescent protein pair includes a donor fluorophore whose emission spectrum overlaps with the excitation spectrum of the acceptor fluorophore, with little spectral bleed through (arising from direct acceptor excitation by the donor laser and/or a long donor emission tail that overlaps extensively with the acceptor's emission spectrum (Day, 2009). During the early adoption of FRET by biologists, the most widely used pair was Cyan Fluorescent Protein (CFP) and Yellow-Fluorescent (YFP). Over the years, however, this fluorescent pair has been found to introduce a few complications to FRET experimentation. First, CFP and YFP are relatively unstable proteins and extremely susceptible to photobleaching, making it difficult to collect enough signal (photons) to acquire multiple images over time (Tramier et al., 2006). Second, because CFP and YFP derive from the same parent fluorescent protein, there is a chance that they can oligomerize on their own and potentially create
false positives when assessing protein-protein interactions (Shaner et al., 2005).
Next, because they are blue-shifted on the visible spectrum, there is less tissue
penetration and greater background with their excitation and emission. Finally,
the use of CFP is contraindicated in when performing FLIM-FRET, since CFP
lifetime has been found to decrease as it photobleaches (Tramier et al., 2006).
To circumvent these issues, I decided to use eGFP (donor) and mCherry
(acceptor) as my FRET pair in this project, since they do not possess any of
these problems (Tramier et al., 2006).

**Research Objectives and Hypotheses**

It has become apparent that interactomics, especially in neuroscience,
requires the mapping of native networks in order to understand their link to
cellular physiology. Specifically, I am interested in molecular events that are
responsible for forming the morphological structures necessary for neuron-
neuron communication. The primary goal of this work is to characterize and
compare the signaling properties of Cdc42 and Rac1 within single neurons in
order to discriminate the contribution of each GTPase to neuronal
morphogenesis. In order to achieve this goal, an initial objective was to develop
and implement strategies to demonstrate that Frequency Domain –FLIM can
serve as a reliable tool for detecting protein-protein interactions *in vivo* (Chapter
2). Once established, it will explore the roles of Cdc42 (Chapter 3) and
Rac1(Chapter 4) within the aCC motoneuron.
Hypotheses

My central hypothesis is that Cdc42 and Rac1 possess distinct signaling properties. The spatial and temporal context of their activation and interaction with Par6 and WASp will reveal some of their distinct, yet complementary, roles during neuronal morphogenesis in the pioneer motorneuron within Drosophila embryos.

Hypothesis #1 regarding GTPase activation: Rac1 has a similar, yet distinct, activation pattern from Cdc42 in the aCC neuron during development. While this difference may be subtle, it will reflect the microenvironments containing their upstream effectors.

Rationale: Previous work done by the Chiba lab established the activation pattern of Cdc42 in the developing Drosophila aCC neuron using a similar FRET bioprobe (Kamiyama and Chiba, 2009). While Cdc42 is expressed ubiquitously throughout the aCC neuron, even at very early stages of embryonic development, it is only activated at approximately hour 13 and localized to the base of future dendrites and distal portion of the growing axon. This activation coincided with dendrogenesis, and early activation of Cdc42 resulted in premature dendrite formation. These findings reinforce the notion that precise localization and timing of GTPase activation is an important component of proper synaptic development. One would expect this to also be true for Rac1 activation in live developing neurons, but it has not yet been investigated. Previous studies demonstrated that Cdc42 and Rac1 have similar activation patterns in fibroblasts during membrane protrusion and ruffling, however Cdc42 is concentrated slightly
closer to the membrane edge with a slight temporal shift in activation (Machacek et al., 2009). While neurons exhibit a more stable and extensive form of cellular polarity compared to motile cells, I expect the signaling dynamics that underlie morphological change generally to be conserved, at least in part, for these Rho GTPases.

**Hypothesis #2 regarding CRIB proteins:** Although the localization pattern of a given CRIB protein may extend into multiple neuronal compartments, their interactions with either GTPase will be constrained to regions of neurite outgrowth. While there may be some overlap in Cdc42 and Rac1’s interaction profile with these CRIB partners, it will be to a lesser extent than seen in in their activation patterns; thus, further distinguishing their roles in the context of dendrite morphogenesis.

**Rationale:** The spatiotemporal restriction in GTPase-CRIB interactions are influenced by a number of factors, such as GTPase activation patterns, CRIB effector localization and/or the presence of required co-factors (activating proteins, docking proteins, etc.). Earlier work done in our lab demonstrated that one downstream CRIB effector, PAK, is expressed ubiquitously in the aCC neuron, while WASp appears to punctuate regions featuring dynamic membranes (unpublished). Although the acronym implies that CRIB proteins are capable of binding both GTPases, Cdc42 and Rac1 possess distinct biochemical kinetics and significantly different binding affinities for some CRIB partners (Zhang et al., 1998; Garrard et al., 2003; Tomasevic et al., 2007). It is possible
then that even highly similar activation patterns and identical partner availability can result in unique signaling dynamics between Cdc42 and Rac1.
Figure 1.1. The scale and scope of biological networks

The fruit fly (*D. melanogaster*) embryo is approximately half a millimeter in length. Its central nervous system is composed of neurons whose diameters are ~100x smaller in comparison. An additional 1000-fold drop in distance is required to reach the scale of a typical protein interaction. The diffraction limit of light restricts visual resolution of individual proteins, but the interactions between them can be probed using FRET Microscopy.
<table>
<thead>
<tr>
<th></th>
<th>Genetic Interaction</th>
<th>Y2H</th>
<th>Co-IP</th>
<th>TAP-MS</th>
<th>FRET</th>
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<td>Some</td>
<td>Yes</td>
<td>Some</td>
<td>Some</td>
<td>Yes</td>
</tr>
<tr>
<td>Native Cellular Context <em>(in vivo/ex vivo)</em></td>
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<td>No (unless yeast proteins)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Native Extracellular Environment <em>(in situ)</em></td>
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<td>No</td>
<td>Yes</td>
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<tr>
<td>Spatiotemporal context *</td>
<td>No</td>
<td>No</td>
<td>No</td>
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Table 1.1. Capabilities of different methodologies for detecting protein interactions

Most approaches to studying protein interactions have their limitations. Genetic interaction screens look for an enhancement or suppression (via a mutation in gene B) of a specific physical trait induced by a mutation in gene A. While cellular and extracellular context is preserved with this approach, the proteins in question are not necessarily signaling partners. Conversely, Yeast-2-Hybrid (Y2H) is tailored for the detection of direct interactions; however it must take place in a unicellular organism and provides only binary output (i.e. do/do not bind). Both coimmunoprecipiation (Co-IP) and affinity purification – Mass spectrometry (TAP-MS) are capable of isolating direct protein binding partners, however this is in addition to proteins bound indirectly (in complex). * Furthermore, tracking protein interactions over both space and time within the same cell (spatiotemporal context) is impractical on a large scale is not possible with any of the aforementioned methods *. Forster Resonance Energy Transfer (FRET) is unique in that it able to provide both specificity (direct binding) and context (within cells of a live animal) to the study of protein interactions.
Figure 1.2. Forster Resonance Energy Transfer (FRET) as a proxy for protein-protein association

The size of proteins, and the scale at which they interact (<10 nanometers), fall below the diffraction limit of visible light (~200 nanometers) and thus cannot be resolved with traditional confocal microscopy. However, it is possible to probe these molecular distances by exploiting the phenomenon of Forster Resonance Energy (FRET). FRET is a physical event that can take place between two light-sensitive molecules (e.g. chromophores), whereby an excited molecule (donor) emits energy at a level (electromagnetic wavelength) that is capable of exciting a complementary molecule (acceptor). FRET is radiationless energy transfer, meaning that no light is actually emitted during this process. At such short distances, virtual photons act as the energy carriers and the efficiency of energy transfer is inversely proportional to the sixth root of the fluorophores’ distance of separation. Such molecular proximities invariably occur during protein interaction events, and so FRET provides considerable specificity as a means of detecting direct protein-protein interaction.
Figure 1.3. Neurodevelopment in Drosophila melanogaster

(A) Ventral nerve cord (VNC) of a Drosophila embryo 16 hours after egg laying (AEL) at 25°C. Inset shows neuronal organization, with cell bodies clustered in the periphery of the VNC and their neurites (axons and dendrites) extending into the nerve cord center and fasciculating into a ladder-like structure. (B) The aCC (anterior corner cell, red circle) is the first motor neuron to extend its axon from the VNC to the body wall and synapse onto dorsal muscle fibers. Dendrites emerge from the proximal portion of the axon near the cell body.
Figure 1.4. Cdc42 activation is spatially restricted and precedes
dendrogenesis in the Drosophila aCC motorneuron

Using a FRET bioprobe sensor, Cdc42 activation patterns were found to occur first at the dendrite base at Hour 13 AEL (after egg laying) and then, from Hour 15 onward, within dendrite branches and at the axon terminal. Three samples at each time point are shown to illustrate the pattern’s reproducibility. Taken from Kamiyama et al., 2009.
Chapter 2

In-situ visualization of protein-protein interactions in the developing Drosophila nervous system: Strategies for implementation, optimization, and quantitative analysis

Summary

Protein interactions underlie the complexity of neuronal function. Potential interactions between specific proteins in the brain are predicted from assays based on genetic interaction and/or biochemistry. Genetic interaction reveals endogenous, but not necessarily direct, interactions between the proteins. Biochemistry-based assays, on the other hand, demonstrate direct interactions between proteins, but often outside their native environment or without a subcellular context. We aimed to achieve the best of both approaches by visualizing protein interaction directly within the brain of a live animal. Here, we show a proof-of-principle experiment in which the Cdc42 GTPase associates with its alleged partner WASp within neurons during the time and space that coincide with the newly developing CNS.

Background

Protein interactions are physical events that take place at nanometer scales. Dissociation constants indicate that the non-covalent bonds which form between interacting proteins can increase their affinity by a million-fold relative to random encounters (Kastritis and Bonvin, 2012). However, within each cell of an intact organism, the probability of protein association and hence signaling changes continually through diverse posttranslational modifications. Due to a
shortage of methods capable of uncovering its dynamics, the time and place of individual molecular signaling remains undetermined in vivo for a majority of cases. In neuroscience, decades of research progress have been hampered by the difficulties of bridging molecular explanations to cellular neurobiological phenomena (Sanes and Lichtman, 1999). The question of when and where a particular pair of interacting proteins engages in physical association is rarely investigated on the same experimental platform as the question of how it might contribute specifically to synaptogenesis or any other aspects of neuronal differentiation. To address this challenge, we sought to visualize protein interactions directly in their native environment by utilizing Förster resonance energy transfer (FRET). Such an approach, i.e., interpreting intermolecular FRET as proxy for protein-protein interaction, could circumvent the need to characterize posttranslational protein states in response to changes in immediate cellular environment. In this study, by combining transgenics with an imaging technology, we quantitate protein interactions by Cdc42 (cell division control protein 42 homolog) and its alleged signaling partner WASp (Wiskott–Aldrich Syndrome protein) within the native environment of a developing brain.

**Signaling proteins within neurons**

The GTPase Cdc42 is thought to regulate aspects of neuronal morphogenesis (Luo et al., 1994). Genetic deletion of Cdc42 in Drosophila results in both presynaptic and postsynaptic defects manifested toward the end of neurogenesis in the embryo (Fehon et al., 1997; Kamiyama and Chiba, 2009). Despite ubiquitous expression throughout the neuronal cytosol, Cdc42's function
appears to be highly restricted in time and space within neurons. Part of this is thought to reflect Cdc42’s endogenous activation being concentrated during later stages of neurogenesis (Kamiyama and Chiba, 2009), just prior to dendritic formation (beginning around Hour 13 after egg laying). Cdc42’s activation pattern within the nervous system is, nevertheless, considerably more widespread compared to where its knockout phenotype emerges. This raises the possibility that factors other than the Cdc42’s activation might further limit its signaling within the neurons. Biochemistry-based assays have isolated an array of cytoplasmic proteins as Cdc42’s potential binding partners (Hall, 1993; Van Aelst and D’Souza-Schorey, 1997). Among these, WASp is implicated in cytoskeletal and membrane dynamics within the axons and/or dendrites of neurons (Kessels et al., 2011). Essential to animal survival (Fehon et al., 1997; Ben-yaacov et al., 2001), both Cdc42 and WASp are expressed ubiquitously throughout neurogenesis. WASp receives extensive phosphorylation that potentially modulates its ability to interact with activated Cdc42. While they have been shown to bind in vitro and within cultured cells (Rohatgi et al., 1999), it is not known whether WASp binds Cdc42 in vivo, let alone when and where they might signal within neurons.

**FRET as proxy for protein interaction**

When donor and acceptor fluorophores come within a distance of approximately 9 nm from each other, the donor’s fluorescence lifetime decreases as a result of FRET (Clegg, 2009). We chose monomeric eGFP (Bierhuizen et al., 1997) and mCherry (Shaner et al., 2004) as the donor and acceptor,
respectively (Note: monomeric eGFP will be denoted simply as GFP from here on). The separate phylogenic origins of these fluorophores reduce the probability of dimerizing on their own (Shaner et al., 2005). With their transparency, anatomical compactness and genetic manipulability, Drosophila embryos offer unparalleled opportunities to visualize various cellular and molecular events within a whole organism without requiring dissection or fixation (Boulina et al., 2013). In order to express Cdc42 and WASp as fluorescently-labeled proteins at a reproducible low dosage, we designed expression vectors carrying both GAL4-responsive UAS and phiC31-dependent attp recognition sequences (Duffy, 2002; Groth et al., 2004). Under a single cell type-specific driver, the GAL4/UAS system allows for expressing two fluorescently labeled proteins within the same cells. The site-specific integration of the transgenes with phiC31 integrase further eliminates any position-dependent variability that might arise in the transgene expressivity. Crossing the stocks thus produces embryos expressing both UAS-mEGFP::Cdc42 and UAS-mCherry::WASp each at a single transgene dosage in all of neurons under elav-GAL4 driver (Fig. 2.1A). Previous study with genetic replacement confirmed that GFP tagging of Cdc42 can be considered functionally benign (Kamiyama and Chiba, 2009). Thus, we were able to monitor the interaction of the protein pair in vivo with minimal artefacts expected from expressing them as fluorescently labeled exogenous proteins (Fig 2.1 D).

There exists a fundamental difference between the intermolecular FRET, which we seek to detect, and the intra-molecular FRET that is designed into many biosensors. The latter cases have both the donor and acceptor of FRET
encoded within a single polypeptide chain. The FRET pair’s maximum distance, then, is two foreign protein domains away from each other — still a FRET-able distance. Users only need to differentiate a high-level FRET from a low-level FRET that occurs in the same molecules. In contrast, we aim to detect FRET that might or might not occur between two fluorescently-labeled proteins that are separately introduced into the cytosol. The weight of negative and positive controls in such experiments is substantial.

Measuring the donor fluorescence lifetime is a reliable way to quantify FRET within cells in vivo (Harvey et al., 2008). Therefore, we adopted 3D frequency-domain fluorescence lifetime imaging microscope (FLIM) (Buranachai et al., 2008) to measure the fluorescence lifetime of GFP (Fig 2.1). Utilizing a low-intensity light with a spinning disk confocal unit on CCD-based fluorescence lifetime imaging components, this microscope allows for efficient detection of an average donor lifetime in the entire image field. Fluorescence lifetime is a concentration-independent value, circumventing the issue of variable expression levels within neurons that can compromise ratiometric FRET calculations. Nevertheless, donor/acceptor concentration ratio is an important consideration even in FLIM experiments because too high (~10x) or too low (~0.1) of a concentration discrepancy can effectively eliminate the dynamic range of FRET detection. This could potentially lead to false negative or false positive results, respectively (Berney and Danuser, 2003). Additionally, high absolute fluorophore concentrations can lead to false positives by increasing the frequency of donor-acceptor encounters (Kastritis and Bonvin, 2012). Therefore, we set out to
validate that FRET can be detected between the fluorescently labeled Cdc42 and WASp, a well-known interaction pair, within the nervous system of the intact Drosophila embryo.

**Materials and Methods**

**Transgenic resource**

We constructed transgenic GAL4-responsive lines that label Cdc42 with GFP, the donor of FRET, and WASp with mCherry, the acceptor of FRET. GFP and mCherry together yield Förster radius of 5.4 nm (Lam et al., 2012). With a fast decay by the sixth power of the increasing distance between donor and acceptor fluorophores, this translates to FRET being detectable up to 9.1 nm. The two fluorescent proteins are among the best genetically encoded FRET pairs available. Both can mature rapidly at 25 °C, a normal temperature for Drosophila experiments. Photo-stable as imaging agents, they are also non-toxic to cells. Derived from evolutionarily discrete protein families of jellyfish and coral, respectively, the pair has no propensity to oligomerize. Because we intend to determine when and where the proteins being labeled by these fluorescent proteins associate physically, any inherent fluorophore affinities could confound our ability to determine this. Both GFP and mCherry are red-shifted as compared to, for example, CFP-YFP pair and would allow for a superior tissue penetration depth while producing less background by light. To minimize the experiment-to-experiment variables and also keep artefacts at a low level if any, we combined several transgenic technologies. Frist, the cDNA’s were sequence-
validated clones from the Berkeley Drosophila Genome Project. Second, we prepared a set of expression vectors designed to label proteins of interest (POI) at their amino terminus with either GFP or mCherry. An additional set was made to tag POIs at their C-terminus. In all cases, we used a short flexible linker sequence. Third, we adopted site-directed phiC31 integrase to insert the transgenes to specific loci in the Drosophila genome. We targeted all GFP-containing transgenes to attp40 (25C6) site and all mCherry-containing transgenes to vk01 (59D3) site. This facilitated recombining them into double-transgene stocks. Using these allowed us to express different protein pairs in a GAL4-positive cell population. With the elav-GAL4 driver, we delivered two single-copy transgenes (for example, UAS-GFP::Cdc42 and UAS-mCherry::WASp) in all neurons. Fly viability showed no significant disadvantage from larval through adult stages. Even their fertility rate turned out the same as the control, and transgene fidelity in the offspring was as expected.

**Sample preparation**

UAS-transgene males were crossed with GAL4-driver virgin females in mating cages with grape agar(+yeast) plate bottoms to collect embryos. Mating cages were placed at 25 degrees Celsius and collection plates were swapped out after an hour in order to gather embryos for a given developmental stage (hour). Embryos were manually dechorionated and developmental staging was confirmed by gut morphology (Bownes, 1975). Embryos were placed on double-sided tape on silicone-well glass slide and immersed in HL3.1 hemolymph buffer
for imaging. In some cases, devitellinization or filet dissection was performed to increase detection of GFP signal intensity.

**Imaging tool**

FLIM used in this study combines micrometer spatial resolution of fluorescence imaging with nanosecond temporal resolution of fluorescence lifetime. Whereas intensity-based FRET imaging measurements require the determination of several parameters and corrections of artefacts, FLIM does not require these and, thus, is a highly reliable way to determine the FRET values. As compared to previously described time-domain FLIM in which high-energy pulsed laser is employed to illuminate each pixel multiple times, photo-damage in our system was virtually non-existent and, furthermore, the speed of data acquisition was faster. These features were advantageous for measuring the lifetime of genetically encoded fluorescent proteins such as GFP in biological samples. FRET leads to both an energy loss in the donor and corresponding gain in the acceptor. FLIM quantifies the change in the donor’s fluorescence lifetime. In many FRET-based biosensors in which the donor and acceptor are tethered in a single polypeptide, ratiometric sensitized emission quantification methods are suitable and could even be economical. However, when the local concentration of the donor and/or the acceptor is either unknown or difficult to measure, as in most biological samples, fluorescence lifetime is considered to be the best, if not the only, reliable method for quantifying FRET in live samples (Clegg et al., 2003). FLIM collects the observed lifetime for all fluorophores present in a given pixel. High-quality confocal imaging is thus essential, yet the
data acquisition time must not be overly long. The proteins that produce FRET could translocate or the tissue might begin to deteriorate. Without dissection, we were able to collect an image of 696 x 520 pixels, capturing the mean per-pixel donor fluorescence lifetime at a single focal plane of the CNS of a Drosophila embryo. This translated to our not having to fix the samples, an effective way to eliminate a major source of artefacts.

**Image Acquisition**

Images were acquired using a custom-assembled frequency-domain upright FLIM system from Intelligent Imaging Innovations Inc. (3i). A continuous-wave laser modulated using Pockels cell electro-optic modulator, was synchronized with a CoolSnap EZ camera using a Lambert Instruments II18MD intensifier in this method of FLIM. Yokogawa CSU-X1 was used for fast image acquisition with a Zeiss W Plan-Apochromat 63x (n.a. 1.0) water-immersion objective lens. Semrock 440/521/607/700 emission filter was used with Semrock Di10 T488/568 dichroic as the emission pathway. Image intensification was maintained at 2800 units across all experiments. To calibrate the system, 1-hydroxypyrene-3,6,8-trisulfonate (HPTS) in phosphate buffer solution at pH 7.5 was used for a standard lifetime of 5.4 ns. We found HPTS to be a reliable standard and superior to fluorescein, owing to its greater stability over time and pH shifts (invariant across the physiological pH range from 5.8 to 7.9 - Ryder et al., 2001). Images were taken in a focal plane where the embryo’s CNS possessed maximal neuropil width. Exposures were set for the channels to create an intensity dynamic range ~75% for CCD capture. On average, 2.0-4.0
seconds of total exposure was needed to collect four images with different phase-shifts from a given sample.

**Compartment segmentation**

In the Ventral Nerve Cord (VNC), *longitudinal fascicles* and *axon commissures* were primarily distinguished by local contrast (GFP intensity thresholding) and the orientation of axonal fibers (longitudinals – along the anteroposterior axis, commissures – along the mediolateral axis). *Longitudinal nerves* were identified as axonal fibers extending laterally from the longitudinal fascicles into the cortical region. A high local intensity (~1.5:1 relative to the surrounding cortex) was used in conjunction with manual tracing for nerve segmentation. The remaining area in the periphery, with signal:noise > 3:1, was deemed as the *cortex*; which contains the neuronal cell bodies.

**Statistical analyses**

Statistical testing was performed using a one-tailed Welch’s t-test (p<.05) to compare lifetimes in control (UAS-GFP::Cdc42 x mCherry alone) and experimental samples. Sample sizes indicate number of embryos considered.

**Supplemental analysis and visualization with MATLAB**

16-bit channel intensities for GFP intensity, mCherry intensity, and GFP lifetime (Single Tau) were exported to MATLAB in order to create correlational pixel scatter plots, volume-compensated 3D projection images with custom Lookup Tables, and to perform detailed analysis of lifetime distributions using custom MATLAB code. In each case, GFP intensity or region-of-interest masks
were used to filter pixels for subsequent analysis. All MATLAB code used for image processing is available for download upon request.

Results

Assessing FLIM-FRET as a proxy for protein-protein interaction in vivo

When expressed alone in the entire nervous system (Hour 15 AEL), GFP exhibited a constant fluorescence lifetime of 2.56 ns, independent of its local concentration. We measured the GFP lifetime after fusing it to Cdc42 with a short flexible linker at its amino-terminus (GFP::Cdc42), and obtained the same fluorescence lifetime of 2.56 ns (Fig. 2.2B). Adding WASp, instead of Cdc42, to GFP (GFP::WASp) also resulted in the lifetime of 2.56 ns. Indeed, we found that GFP exhibited a constant fluorescence lifetime when fused to any protein we studied, irrespective of N- or –C terminal position (data not shown).

Having established this, we coexpressed GFP::Cdc42 and cytoplasmic mCherry in the nervous system as separate proteins to determine whether an interaction (FRET) occurs between mCherry and either GFP or Cdc42. While neither GFP nor Cdc42 is anticipated to bind mCherry, this is a fundamental experiment because it serves as the ‘baseline’ control for assessing the presence interaction between Cdc42 and potential partners in further experiments. If the presence of mCherry alone decreases GFP fluorescence lifetime and it is not accounted for, it could confound interpretations of FRET and potentially lead to false positive results. We found that mean GFP fluorescence lifetime was unaffected by the presence of cytoplasmic mCherry (Fig. 2.2C), therefore we define our ‘baseline’ GFP fluorescence lifetime as 2.56 ns.
We next measured the fluorescence lifetime of GFP, the FRET donor, when Cdc42 and WASp were labeled with GFP and mCherry, respectively. In this case, donor fluorescence lifetime in the nervous system fell significantly (2.443ns ± .023ns); \( t(37) = 14.743, P < .001 \) (Fig. 2.2D).

Several mutations in Cdc42 are known to disrupt its ability to bind its partners. Although biochemical evidence indicates that interaction between Cdc42 and WASp is a result of direct association (Owen et al., 2000), we utilized mutant forms of Cdc42s to test whether this was responsible for the FRET observed. One is a single amino acid substitution G17N, which retains Cdc42 at its GDP-bound inactive state (Luo et al., 1994). When wild type Cdc42 was replaced with this mutant form (GFP:Cdc42\textsuperscript{N17}), FRET did not occur with WASp-mCherry (2.558ns ± .010); \( t(22) = 0.445, P = 0.330 \) (Fig. 2.2F). Conversely, the G12V amino acid substitution prevents GTP hydrolysis and thus keeps Cdc42 in a constitutively active state. As expected, we found that GFP:Cdc42\textsuperscript{V12} exhibited a higher level of FRET with WASp-mCherry (2.391ns ± .020); \( t(10) = 29.327, P < 0.001 \) than wild-type Cdc42 (Fig. 2.2H). If a second mutation is introduced (a double point-mutation G12V-Y40C), Cdc42’s ability to bind a specific subset of its effectors, i.e. those that contain Cdc42/Rac1 interactive binding (CRIB) domain (Kim et al., 2003), is impaired despite its active state. We found that this second mutant (GFP:Cdc42\textsuperscript{V12C40}) also eliminated FRET with WASp-mCherry (2.560ns ± .010); \( t(21) = 0.399, P = 0.347 \) (Fig. 2.2G), providing evidence that direct association between Cdc42 and WASp is necessary for FRET to occur in vivo. To confirm that Cdc42\textsuperscript{V12C40} is otherwise functional in vivo, we tested
whether it affected Cdc42’s interaction with a protein not possessing the CRIB domain. We found such a protein in the GTPase-activating protein RhoGAP5A. Interaction took place between mCherry:RhoGAP5A and GFP:Cdc42 in the nervous system (2.348ns ± .020); [t(28) 26.844, P <0.001] , and was still present with the G12V-Y40C mutation in Cdc42 (2.401ns ± .030); [t(21) = 22.181, P <0.001] (Fig. 2.2I).

**Modes of quantifying, visualizing, and analysing protein interactions**

a) **FRET Efficiency (FE)**

In the presence of mCherry, a decrease in GFP lifetime from its baseline value signifies that FRET is taking place between the two fluorescent proteins. All FRET detection methods are characterized by relative measurements, meaning that they require a particular parameter (e.g. donor lifetime) to be measured before and after FRET can occur. Because lifetime varies among different donor fluorophores, and potentially even between subcellular compartments expressing the same fluorophore, a normalized value is necessary to compare FRET levels across experiments or regions within a sample. The commonly encountered measure is *FRET Efficiency* (FE), which relates the change in donor lifetime measured in the sample (τs) to the baseline donor lifetime (τb):

\[
FE = \frac{\tau_b - \tau_s}{\tau_b} \tag{2.1}
\]

b) **Probability of Association (PA)**

FRET Efficiency conveys the percentage of donor emission energy being transferred to an acceptor molecule via FRET, and hence is a normalized
measure of the energy transfer taking place. However, it does not provide quantitative information on the nature of the interaction itself, such as the proportion of donor molecules undergoing interaction FRET. This is because a FRET-inducing interaction never reaches 100% FRET Efficiency in practice, as it would require a near-zero donor-acceptor proximity. Instead, the FRET Efficiency during interaction is dependent on the conformation and orientation of the interaction in question. Unless this latter value can be isolated, it is impossible to distinguish whether a FRET Efficiency signal of, say 45%, represents all donor molecules undergoing a 45% FE or only half of donors undergoing a 90% FE.

The advantage of FD-FLIM is that the FE during interaction can be estimated using polar plot analysis. Assuming that there are two lifetime states for GFP ($\tau_b$ – ‘No FRET’ and $\tau_f$ – ‘FRET’), then the mean lifetime of a sample is a fractional combination of these two GFP populations. The polar plot provides a theoretical estimate of the lifetime when 100% of the GFP population undergoes FRET with mCherry (Figure 2.3), which in turn allows a calculation of the probability that a donor molecule (Protein X::GFP) is associating with an acceptor molecule (Protein Y::mCherry), i.e. the probability of association $P_A$, via Eqns (2.2) and (2.3).

$$P_A = \frac{d_{b->s}}{d_{b->s} + d_{s->f}}$$ (2.2)

where $\tau_s$ is the mean lifetime of the sample, and, $d_{b->s}$ and $d_{s->f}$ is the distance between the X-Y coordinates of the baseline-to-sample and sample-to-full FRET, respectively, with
Because it is possible for protein association-disassociation events to occur on a time-scale faster than image acquisition time, probability of association is a more accurate term than something like ‘donor binding fraction’. After compiling polar plots across different samples and experiments, I determined the average GFP lifetime value for the “No FRET” (base lifetime) and “FRET” (minimum lifetime) states for each protein interaction pair. A simple conversion from lifetime to an association probability value was then performed for each sample and used in subsequent analysis (Table 2.1).

c) Extent of Association (E_{A})

While donor lifetime alone can capture the proportion of donor proteins undergoing interaction, comparing the number of donor molecules that are interacting (i.e. extent of association, E_{A}) requires donor concentration to be considered. The extent of association present in any given pixel can be quantitatively expressed as a product of its association probability (P_{A}) and normalized GFP concentration:

$$E_{A} = P_{A} \times \frac{G'}{G_{max}}$$ (2.4)

where G’ and G’_{max} are the pixel GFP intensity and maximal pixel GFP intensity, respectively, after correcting for intensity(G) lost to FRET:
The extent of association captures the relative number of FRET events (interactions) occurring within a given pixel or region relative to all other pixels or regions within a given image, allowing one to identify spatial hotspots. It is worth noting that it is possible for one to quantify the absolute number of FRET events, which would be comparable across different images, if a calibration was performed with the imaging using a fixed concentration of the donor (e.g. a GFP solution). We have yet to perform this calibration because of some technical and time limitations, however we plan to do so in the near future.

Visually, PA and EA can be depicted using a 2-dimensional color scale (Fig. 2.4A-C). This image conveys both association probability (increasing from white to magenta) and concentration (increasing from black to white) for each pixel, with the extent of interaction ranging from a low (nonexistent) level in black to a high level in bright magenta. Furthermore, normalized PA and EA values for distinct biological regions can be grouped and averaged from multiple images, allowing one to compare the probability and extent of a protein interaction across space and time (see Spatiotemporal analysis).

d) **Percentage above Threshold (P_T)**

Ultimately, any measure of protein interaction must be determined with reference to an appropriate negative control and amenable to statistical testing. Because the sample lifetime of the negative control is used to determine the baseline lifetime, PA and EA are equal to zero in the control. An alternative
statistical approach is to establish a lifetime threshold (LT) in which less than 0.15% of all pixels (p<.0015) in the negative control possess a lifetime below this value. Because GFP lifetime follows a normal distribution in the absence of FRET, the baseline lifetime minus three standard deviations (M- 3SD) can serve as a suitable method of estimating LT.

Using this threshold, interactions in a potentially FRETing sample can be rendered in a binary fashion on a pixel-by-pixel basis. Interaction is considered present only in pixels with lifetimes below the threshold, and is considered absent in all other pixels. Visually, this binary FRET approach isolates and highlights only those pixels showing strong FRET within an image (Fig 2.4D). Quantitatively, the % of pixels below LT can be statistically assessed as to whether it is significantly different from the threshold percentage (0.15%) using a one-tailed Welch’s t-test.

The primary advantage of this approach, however, is in its visual depiction. Because there is a notable interpixel variance regarding GFP lifetime, an ‘association image’ may be somewhat misleading in conveying the actual number of pixels undergoing interaction. Thus, while an association image of a negative control sample will display numerous magenta-tinted pixels, a binary FRET image will be almost completely devoid of such pixels.

The drawback of binary FRET thresholding is that it is an imperfect quantitative measure when assessing whether or not FRET is taking place within a sample. This is because FRET can occur in only a relatively small proportion of donor molecules within a samples, lowering the mean GFP lifetime and changing
the general pixel lifetime distribution (e.g. positive skewing of median/mode) without significantly altering the lifetime of the lower 0.15% of pixels. Generally speaking, binary thresholding is superior for visualizing interaction hotspots within an image and association analysis is desirable for statistical testing. Each approach, however, offers a unique perspective in both the visualization (hotspots vs gradients) and quantification (spatial extent vs molecular extent) of protein interactions. Thus, both should be considered with regard to the biological insights that are desired.

The association probability and percent of pixels above threshold for different protein pairs in the Hour 15 embryonic CNS are compared in Table 2.1.

**Spatiotemporal analysis of Cdc42 interaction patterns in vivo**

In addition to providing a native environment, an important aspect of studying protein interactions *in situ* is the ability to assess whether spatiotemporal patterns exist for a given interaction. Regarding the temporal aspect, we decided to image embryos in three-hour increments in order to assess each protein interaction during key biological milestones in *Drosophila* CNS neurodevelopment. At Hour 12 AEL, neurons in the VNC have just begun extending axons, but not yet dendrites. At Hour 15 AEL, neurons are undergoing dendritic elaboration and their axons have begun fasciculating to form nerve bundles, most of which coalesce in the center of the VNC and form a ladder-like structure known as the neuropil. The neuronal cell bodies themselves surround the neuropil, and collectively form the cortex region. At Hour 18 AEL, the neuropil continues to mature and synaptic contacts between neurons begin
forming along the *longitudinal connectives* of the neuropil. Conversely, the horizontal ‘rungs’ of the neuropil, known as *commissures*, are devoid of synapses as they only contain contralateral crossing axons (see Fig 1.3). By Hour 21 AEL, the VNC has contracted, the rate of synaptogenesis has slowed, and synaptic maturation (e.g. synaptic pruning and strengthening) predominates. In summary, these four time points sequentially provide a biological context of axonal extension, dendritic elaboration, synaptogenesis, and synaptic maturation to the protein interactions under study.

**a) Spatial pixoplot**

Supplying the aforementioned biological contexts to an interaction also requires spatially isolating functionally distinct subcompartments of the CNS. As mentioned above, the cortex and neuropil are the primary subregions of the VNC, containing the soma and neurites of all neurons, respectively. The VNC is segmentally repeated in the anteroposterior direction, and therefore possess a general structural symmetry along that axis. Because the neuropil and cortex fall within a distinct lateral distance range from the VNC midline, we initially decided to plot pixels as a function of their *extent of association* and lateral distance from the VNC midline in order to visualize general spatial consistencies in the FRET pattern (Fig. 2.5). Sample images were acquired at the dorsoventral plane of maximal neuropil width, and the VNC midline was manually drawn to produce bilateral symmetry. Using a custom MATLAB script, the *extent of association* was calculated for each pixel in an image (approximately 50,000 pixels considered per image) and multiple images were combined to create a spatial pixoplot for
each interaction at a specific time point. (Fig 2.5). An ‘interaction threshold’ was established from the top 1% of EA values in negative control embryos (p<.01). To aid in visualization, pixel colors were used to indicate their assignment as either neuropil or cortex. These spatial pixoplots offered a convenient way to summarize data from multiple embryos while simultaneously elucidating the areas where a given interaction is most consistent and prominent.

Both Cdc42 and WASp were expressed in the Drosophila nervous system very early on during embryogenesis. However, the interaction between Cdc42 and WASp was found to be spatially and temporally limited (Fig 2.6). At hour 12, when the CNS was narrow, little interaction occurred as few pixels exceeded the interaction threshold. By hour 15, the CNS increased its volume as the neuropil began to form near the center of the CNS. The Cdc42-to-WASp interaction became noticeable at this point, with slightly more pixels above threshold in the neuropil than in the cortex (Fig 2.6A). At hour 21, as embryogenesis neared its completion, the spatial pattern of interaction became dramatic. Neuropil pixels within the longitudinal connectives, approximately from 5 to 15 µm on both sides of the midline, showed the largest degree of interaction. The cortex, meanwhile, exhibited a similar level of interaction as it had at Hour 15. Thus, the Cdc42-to-WASp interaction became apparent at hour 15 and increased several hours later in the neuropil, primarily in the region where axon terminals and dendritic branches intermingle.

While Cdc42 concentration was higher in the neuropil than in the cortex, the converse was true of WASp. We noted, nevertheless, that both proteins were
present in all pixels of neurons throughout the entire nervous system, yet interacted in only a fraction of the total space-time in which they were co-localized. We decided to repeat the FRET measurements under reciprocal tagging conditions, i.e., GFP::WASp and mCherry::Cdc42 (Fig 2.6B). The resulting pattern of FRET was very similar overall, although the probability of WASp associating with Cdc42 (~30%) was approximately three times larger than that of Cdc42 associating with WASp (~10%). This observation indicates that a greater percentage of the WASp pool interacts with Cdc42 than vice versa in neurons. We believe this to be due to the fact that Cdc42 is expressed at higher levels than WASP within neurons. Since their interaction is 1:1, there is approximately three times the number of Cdc42 proteins compared to WASp proteins in the Drosophila nervous system. Moreover, if only a small fraction of Cdc42 is activated (free to bind) at any one time, as has been reported (Kamiyama and Chiba, 2009), than relatively few Cdc42 proteins are capable of associating with WASp. Because Cdc42 is a signaling hub (i.e. has more interaction partners than most of its partners have themselves), its relatively high expression level may be essential for its potential to signal with multiple partners simultaneously.

b) Compartmenetal analysis

Although the spatial pixoplot offers a streamlined method of visualizing general interaction patterns in space, a more discrete form of analysis was needed to better isolate subcompartments in order to carry out rigorous statistical comparisons.
The neuropil can be subdivided into subcompartments according to morphological and physiological characteristics, namely the *longitudinal connectives* (axons, dendrites, synapses), *commissures* (crossing axons only), and *lateral nerves* (axon fascicles entering/leaving the VNC) can be readily identified from images (Fig 2.7A). Along with the *cortex*, this results in four distinct subcompartments (Sánchez-Soriano *et al.*, 2005). Segmentation was performed by manually tracing binary masks using *Slidebook* software, and pixels were subsequently grouped and analyzed according to spatial compartment, time point, and expression construct (i.e. interaction pair).

We found significant levels of interaction for Cdc42 and WASp in the neuropil and cortex beginning at Hour 15, at the same time when Cdc42-Par6 interaction became evident in the neuropil and lateral nerves (Fig 2.7B). We also included RhoGAP5A in our experiment as a control. This protein is one of dozens of proteins carrying a GTPase-activating (GAP) domain that is expected to bind Cdc42 regardless of its activation state. Indeed, unlike the two CRIB effectors, Cdc42 does not show spatiotemporal discrimination in its interaction with RhoGAP5A, being detectable from Hour 12 in every region. Interestingly, however, we did notice a decreasing trend in this interaction within the longitudinals and commissures as neurodevelopment progressed, complementing and possibly contributing to Cdc42’s increased interaction with WASp and Par6 in this region.

Interestingly, this spatiotemporal trend mirrors that of synaptic formation in the embryonic CNS. We expressed a presynaptic marker, GFP::synaptotagmin,
in the nervous system to track presynaptic vesicle accumulation during neurodevelopment. In this case, we plotted pixels according to synaptotagmin concentration (GFP intensity) and their lateral distance from the CNS midline at different stages of development (Fig 2.7C). Much like the Cdc42-WASp interaction, presynaptic vesicle began accumulating around Hour 15 in the longitudinal connectives, where they continued to increase in number up through Hour 21. We propose that Cdc42 interacts with WASp at the time and place that coincides with the development of the functional CNS at this stage — where it is most likely involved in dendrite elaboration, fine glial wrapping of axons and/or synapse maturation within the neuropil.

In summary, the majority of pixels showing colocalization between Cdc42 and WASp/Par6 in the CNS do not exhibit significant interaction. This spatiotemporal restriction is likely due to regulation of Cdc42 activation (Kamiyama and Chiba, 2009), and coincides with the place and time of synaptic formation/maturation in the nervous system. Fig 2.7B provides a quantitative visual summary of the dynamics for all three interactions during neurodevelopment.

Discussion

Networks evolve because their links change dynamically over time and space. In this work, we showed how a specific link in the protein-protein interaction network changes within the developing brain through a method that offers spatial and temporal resolution. Of all pixels that exhibited co-localization
of Cdc42 and WASp, less than half feature Cdc42 in its active state during mid-late embryogenesis (Kamiyama and Chiba, 2009). In turn, less than 20% of those pixels show Cdc42 interacting with either WASp and Par-6 (Table 2.1). This fraction, however, coincided with the formation of the synapse-enriched neuropil within the Drosophila CNS. A notable exception was the presence of interaction in the lateral nerves, a region containing only axons. We suspect that Cdc42’s interaction with WASp and Par-6 in this region may play a role in fasciculation of the efferent axons within each nerve.

Importantly, these results demonstrate that knowledge of when and where a protein interacts with its partners, rather than where they colocalize, are of most value when predicting how a knockout phenotype will manifest within the animal. Future studies could look at both downstream and upstream signaling partners of Cdc42 and WASp and, using different GAL4 drivers, characterize their pathways within a single developing neuron. Unlike biochemistry-based assays, the direct imaging described here does not compromise tissue or cellular integrity. It is a quantitative approach capable of bridging nanometer-scale molecular circuitry to the micrometer-scale neuronal connectivity within the developing brain.
A

UAS-GFP::Cdc42 / +; elav-GAL4 / +
Hour 18 Embryonic CNS

B

Lifetime

C.1

C.2

C.3

D

<table>
<thead>
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<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
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<tbody>
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<td>GAL4 stock</td>
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</tr>
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<td>Viability Experimental/Control</td>
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<td>100%</td>
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**Figure 2.1. Measuring and quantifying protein interactions through Fluorescence Lifetime**

An image of the ventral nerve cord in an Hour 18 *Drosophila* embryo expressing GFP-labeled Cdc42 under the *elav-GAL4* promoter (A) captured by a Fluorescence Imaging Lifetime Microscope (FLIM). All images are acquired at a focal plane where neuropil width is at its maximum (red bracket). In addition to GFP intensity, every pixel contains a GFP lifetime value (as calculated by the phase shift, $\Phi_F$, and demodulation, $M$, of the photons emitted), which is demonstrated in the grayscale representation (B, units - nanoseconds) of the rectangular selection in A. Mating strategy is shown in Figure C, where transgene stock flies (C.1.) carrying two GAL4-responsive transgenes (for example *UAS-GFP::Cdc42* and *UAS-mCherry::WASp*) and GAL driver stock flies (C.2.) (*elav-GAL4*) are crossed to produce experimental animals with single transgene dosages (C.3). D. The viability of experimental flies between different stages of development (A. Embryo to larva B. Larva to adult. C. Adult fertility) indicates that fusion protein expression in the nervous system slightly reduces viability, likely due to early-stage lethality. However, surviving experimental flies demonstrate fertility levels comparable to parental controls. *Viability* is the survival rate of the experimental animals as compared to both parental controls.
Figure 2.2. FD-FLIM is a reliable method for detecting to direct protein interaction *in vivo*

The lifetime of GFP (A), being expressed in neurons of the Drosophila ventral nerve cord, does not change when it used as a fluorescent tag for Cdc42 (B). Importantly, co-expression of cytoplasmic mCherry (C) also does not affect GFP lifetime, meaning that (false positive) FRET does not occur between the donor and acceptor fluorophores on their own. However, when serving as tags for Cdc42 and WASp (D, E), FRET does occur and is detected as a drop in donor fluorescence lifetime. Point mutations in Cdc42 that prevent its activation (Cdc42N17 – F) or disrupt its binding site for WASp (Cdc42V12C40 – H) abolish FRET, while a point mutation that locks Cdc42 in the activated state (Cdc42V12C40 – G) still results in a GFP lifetime drop. Each protein (pair) displays a mean lifetime (Mean ± S.D.) in n embryos at hour 15 (see Fig. 5 for scale bar).
Figure 2.3. Determining probability of association from the polar plot histogram

Every GFP-expressing pixel contains a fluorescence lifetime value as calculated by the phase shift, $\Phi_F$, and demodulation, $M$, of the photons emitted. $\Phi_F$ and $M$ values can be represented on a polar plot histogram in order to verify the presence of FRET, as well as to estimate the probability of donor protein associating with an acceptor protein. X and Y polar plot coordinates for each pixel are determined by the demodulation multiplied by the cosine and sine of the phase shift, respectively (A).

GFP can possess two possible lifetime states in the presence of an acceptor fluorophore (e.g. mCherry): its baseline lifetime ($\tau_b$, No FRET) and a reduced interaction lifetime ($\tau_f$) when FRET occurs. Because GFP has single exponential fluorescence decay, the polar plot coordinates for both states fall on the semicircle. In a GFP-expressing sample, a given pixel contains many GFP molecules and so its mean lifetime ($\tau_s$) is characterized by a mixture of non-FRETing and FRETing GFP molecules. The lifetime value of the latter state is approximated by drawing a line from the baseline lifetime point through the mean lifetime point (determined from control and experimental samples, respectively) until it crosses the polar plot semicircle (B). From this, the probability of GFP undergoing FRET with mCherry (i.e. the probability of Cdc42 associating with WASp) is calculated from Eqs. (2.2) and (2.3).
Table 2.1. Quantification of protein-protein interactions in the whole ventral nerve cord of Hour 21 Drosophila embryos

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<th>Baseline lifetime ( { T_b } )</th>
<th>Mean Lifetime ( { T_s } )</th>
<th>Interaction Lifetime ( { T_f } )</th>
<th>Probability of Association ( { P_A } )</th>
<th>% pixels above threshold ( { P_T } )</th>
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<tr>
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<td><strong>Cdc42 and RhoGAP5A</strong></td>
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<td>2.36ns</td>
<td>.60ns</td>
<td>10.2%</td>
<td>5.40%</td>
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</table>
Figure 2.4. Extent of protein association by considering donor concentration

(A) While donor lifetime alone can capture the proportion of donor proteins undergoing interaction (e.g. probability of association, $P_A$), donor concentration (B) must be considered to compare the relative number of donor molecules that are interacting (i.e. extent of association, $E_A$). The extent of association present in any given pixel is a product of its association probability and its normalized intensity (concentration), calculated using Eqn (2.3), to provide a quantitative normalized value. The probability and extent of association occurring in pixels can both be depicted visually using a 2-dimensional color scale (C), which ranges from a low level of interaction in black to a high level in magenta.
Figure 2.5. Spatial pixoplots and interaction patterns

(A) Neuropil emerges near the center of the CNS as a plasma membrane-enriched region by hour 16 (dotted line), and is surrounded by cell bodies of the cortical region. (B) Samples from a given experiment possess variability in lifetime, and are pooled together to reveal consistencies between samples. (C) Pixoplot displays individual pixels from multiple samples (n) based on their extent of association and lateral distance from the CNS midline (orange vertical line). Black color indicates pixels with neuropil assignment.
Figure 2.6. Spatiotemporal interaction dynamics reveals the peak of Cdc42-WASp association coincides with synaptic maturation

Interaction between Cdc42 and WASp before at the onset of, and after the formation of neuropil. Interaction was assessed with both Cdc42 (A) and WASp (B) acting as the FRET donor (i.e. GFP-labeled). Arrow points to significance threshold (p<.0015) determined by calculating the mean minus three standard deviations (M – 3SD) in negative control experiments.
Fig. 2.7. Compartmental summary of Cdc42 interactions during Drosophila neurodevelopment

(A) Cdc42 is co-expressed with RhoGAP5A, Par6 and WASp using pan-neuronal elav-GAL4 driver. Scale bar 2 μm. Sample size indicates the number of embryos examined. (B) Spatiotemporal interaction patterns with RhoGAP5A, Par6 and WASp. (C) Pattern of presynaptic vesicle accumulation in the developing Ventral Nerve Cord visualized with GFP::synaptotagmin.
Chapter 3

*Cdc42 interaction dynamics, dendrogenesis, and the importance of spatiotemporal restriction*

**Summary**

Here we zoom into protein interaction in aCC motoneurons of a live Drosophila embryo. We visualize when and where the Cdc42 GTPase interacts with its signaling partners. Freely dispersed in cytosol, the proteins physically associate in a fraction of the time and place they co-localize, when the neurons initiate dendrites. This restrictive interaction is consistent with knockout phenotypes and support Cdc42 coordinating a signaling program through microtubule-stabilizing Par6 and actin fiber-assembling WASp. However, such restrictions completely dissolve when mutant Cdc42 is present, resulting in a severely disrupted phenotype. Altogether, this work demonstrates the fundamental role that spatiotemporal regulation plays in the dynamic interactome.

**Background**

Efforts are underway to map endogenous proteins in the human brain (Uhlén et al., 2015). Since proteins signal through physical association, knowing the cellular locations and times of their co-expression within animals is the first step toward understanding their functions. Yet, in a given cell, co-localized proteins do not necessarily interact with each other as they often require specific posttranslational modifications as a prerequisite. Such dynamic patterns at the
local protein network level shape the ways neurons in the brain interconnect, enabling unique behaviors and the storage of long-lasting memories. Even in simple model organisms, however, the experimental determination of these nested networks in their intact form has been difficult.

In their 1999 review on long-term potentiation (LTP), Sanes and Lichtman (1999) point out that the precise contribution from over 100 implicated proteins in this form of neuronal memory formation is unclear. They argued for the need of an empirical platform that allows connecting molecular components to cellular complexity. Fortunately, advances in microscopy and transgenics have made this endeavor possible. Fluorescence resonance energy transfer detects co-localization of molecules at the scale of nanometers (Clegg, 1995), matching the size of proteins and the distance relevant for direct association. Since methods are available to transgenically label individual proteins with complementary fluorescent tags without compromising either cell integrity or animal viability, fluorescence lifetime imaging microscope enables the quantification of FRET in vivo (Sharifai et al., 2014). Furthermore, optically transparent organisms such as Drosophila embryos allow visualization of both molecular and cellular changes during the course of development. Combining these elements, we developed an imaging platform for investigating the dynamics of protein interactions in their natural context of vibrant cellular morphogenesis. Our approach enables us to visualize protein interactions without the need for any molecular or cellular amplification. As a consequence, spatiotemporal patterns of protein interactions and accompanying cellular phenotypes can be visualized simultaneously in an
intact animal, allowing us to evaluate hypotheses that previously were outside the realm of direct imaging.

Our previous results indicated that neurons produce small dendrites in Drosophila as a consequence of knocking out the ubiquitously expressed Rho GTPase Cdc42 (cell division control protein 42 homolog) (Kamiyama and Chiba, 2009). Such site-specific neuronal malformation was noticeable at 60% (hour 13:00) of embryogenesis among the first group of neurons born in the CNS, hours before the embryos themselves reach lethality. This rather subtle phenotype implied spatiotemporal restrictions on Cdc42’s interaction patterns, and that its role is specific to dendrite formation. Here, we visualize Cdc42 protein interactions in single neurons within developing Drosophila embryos. Specifically, we test the hypothesis that Cdc42 coordinates a signaling program through Par6 and WASp, two effectors linked to cytoskeletal regulatory pathways in neurons.

**Materials and Methods**

We used the frequency-domain modulated 3D FLIM and the polar plot analysis, described by Clegg and colleagues (Redford and Clegg, 2005; Buranachai *et al.*, 2008), to quantitate association probability among Cdc42 and other independently expressed proteins.

*Image data acquisition*

Frequency domain-fluorescence lifetime imaging FLIM (FD-FLIM), designed and built by Intelligent Imaging Innovations Inc. (3i), and system
calibration were performed as described previously (Chapter 2; Sharifai et al., 2014). Three-dimensional z-stack images were acquired spanning 5-8 µm at 1.5 µm intervals with the dendrite base as the central plane. Exposures times were set so as to occupy 75% of the camera’s intensity dynamic range, and lasted between 5 and 45 seconds to collect four images at variable laser modulation frequencies for a given sample. Phase-shift, demodulation, and GFP lifetime were calculated by Slidebook 5.0 Software.

For aCC morphological analysis, a Zeiss laser scanning confocal microscope (LSM 780) was used to acquire three-dimensional z-stacks of the aCC motorneurons at 70% (hour 15:00) of embryonic stage with 63x objective, pinhole size of 1 airy unit, and 0.45 µm z-step size. A dynamic 3D-image viewing software (Vaa3D) was used to manually count and quantify the number of primary dendrites (filopodia sprouting directly off axon) and terminal dendrites (tip points) in neurons expressing wild type (UAS-GFP::Cdc42/+; eveGAL4+/+) and mutant (UAS-GFP::Cdc42V12/+; eve-GAL4+/+) forms of Cdc42. Statistical significance between groups was determined using Welch’s two-tailed t-test (P < .05).

Regions and compartments

The Drosophila aCC motorneuron possesses several biologically distinct subcompartments, offering a number of cellular contexts when interpreting protein-protein association. Predefined criteria were established for manual segmentation of images for spatial analysis. Within the aCC motorneuron, 10 subcompartments were distinguished based on biologic, structural, and/or spatial considerations (See Fig 3.2D) (Thomas et al., 1984; Baines and Bate, 1998).
These included the *cell body* (A), *axonial region prior to dendrite base* (B), the *dendrite base* (C) and its subcompartments (D – shaft, E- tips), *axonial region between dendrite base and the acc-RP2 fasciculation junction* (F), the *aCC-RP2 fasciculation junction* (G), the *distal axonal region* (H), and the *axon terminal* (I).

The dendrite base and fasciculation junction were key landmarks for segmentation. The dendrite base was identified as that portion of the axon where primary dendrites emerged, with its lateral boundaries set by the shaft diameter of the primary dendrite branch. The fasciculation junction was demarcated by forming a triangle, with two sides formed (and joined) by lines that separated each axon pre/post-merging and the third line joining the edges of these lines.

Other important morphological criteria included the dendritic shaft and tip. Dendritic tips were distinguished by identifying the tip point (the most distal visible pixel along the dendritic arbor) and a point 4 pixels (~1um) proximal to the tip point along its shaft. The tip region includes the volume encompassed between these two points in the lateral and z-directions. All other dendritic volumes were considered as shaft pixels. Statistical testing was performed using a one-tailed Welch’s t test (p<.05) to compare lifetimes in control (UAS-GFP::Cdc42 x mCherry alone) and experimental samples. In compartmental analysis, separate control lifetimes were determined for each subcompartment.

Sample sizes indicate number of compartments considered.

**Supplemental analysis with MATLAB**

Sixteen-bit channel intensities for GFP intensity, mCherry intensity, and GFP lifetime (single tau) were exported to MATLAB in order to create volume-
compensated 3D projection images with custom lookup tables and to perform time-lapse analysis. In each case, GFP intensity or region-of-interest masks were used to filter pixels for subsequent analysis. For time-lapse analysis, separate masks for dendrite base, shaft, and tip regions were used to extract the mean lifetime and number of pixels for each region across time points. All MATLAB code used for image processing is available for download upon request (Email: nimasharifai@gmail.com).

Drosophila stocks

All fluorescent fusion proteins used in FRET experiments were N-terminally tagged and expressed under identical GAL4-driver fly lines. elav-GAL4 - source: Bloomington Stock # 8760) and eve-GAL4 (eve-GAL4[RN2-Gal4-E] - source: M. Fujjoka) were used for pan-neuronal and aCC/RP2/pCC fluorescent labeling, respectively. Transgenic flies carrying site-specific UAS-GFP::Cdc42 (attp40 site), UAS-mCherry::WASp (vk01 site), and UAS-mCherry::Par6 (vk01 site) genetic sequences on the 2nd chromosome were constructed as described previously (Chapter 2). UAS-GFP::Cdc42[V12][49] and UAS-mCherry::CBD (source: D. Kamiyama) were integrated on the second chromosome with P-element transformation at random sites. Fusion between the fluorescent protein and protein of interest was achieved with a short flexible linker sequence. UAS-transgene males were crossed with GAL4-driver virgin females in mating cages with grape agar plate coated with yeast to collect embryos.
Transgenics and sample preparation

To attain scalability in labelling pairs of proteins, we combined several transgenic strategies. These methods, along with sample preparation for imaging have been described previously (Chapter 2; Sharifai et al., 2014).

Dynamic 3D rendering

To automatically locate the aCC dendrites, visualize the protein interactions, and record their spatial characteristics in 3D, we used a variant of open-curve active contours (Wang et al., 2011). First, the axon was traced using the cell body and the CNS midline as reference, by evolving locally open curves based on some shape and image intensity-derived criteria (Mordohai and Medioni, 2006). Second, the dendrites were located automatically, using intensity clustering (Hartigan et al., 1979) in the vicinity of the aCC axon traces. To capture in detail of the main aCC dendrite and quantify its spatial characteristics, we used the computer vision approach (Tsechpenakis et al., 2012) and the pattern recognition method (Chang et al., 2014).

Results

Morphological regulators

During the development of the nervous system, a small set of motoneurons pioneer the motor pathway (Thomas et al., 1984). In Drosophila larvae, aCC (anterior corner cell) is the pioneer motoneuron for innervating the distal (dorsal) aspect of the body wall musculature. In each half segment, a total of thirty-eight motoneurons project their dendritic arbors to thirteen partially overlapping
neuropil domains in the CNS (Kim et al., 2009) and extend their axons to thirty separate muscle cells (Hoang and Chiba, 2001). Such precise matching between neuronal input and muscular output of the motoneurons allows for coordinated locomotion from the onset of postembryonic development. The main dendrite of the aCC motoneuron projects ventrally from the axonal shaft into the dorsal neuropil domain-1, while its axon terminal targets the dorsal most longitudinal muscle cell muscle-1 (Fig 3.1A) (Tripodi et al., 2008). Development of the aCC dendrite begins at 60% (hour 13:00) of embryogenesis, corresponding to two hours before the neuron’s axon reaches its target muscle (Fig 3.1B).

Functional analysis of a protein often relies on the knockout of its underlying gene. Previous work in our lab (Kamiyama and Chiba, 2009) suggested that Cdc42 proteins are required for normal dendrite formation in the aCC motoneuron. As a corollary, we hypothesized that Cdc42 proteins interact with cytoskeletal regulatory effectors at the time and place of dendrite formation. In particular, interactions of Cdc42 and other Rho GTPases with their binding partners are among the best characterized. GAPs (GTPase-accelerating proteins) and GEFs (guanine nucleotide exchange factors) bind and facilitate inactivation and activation, respectively, of these monomeric GTPases. A number of assays have predicted putative effectors for Cdc42, including Par6 (partitioning defective 6 homolog) and WASp (Wiskott–Aldrich Syndrome protein), that both contain a Cdc42/Rac1 interactive binding (CRIB) domain. Both Par6 and WASp are predicted to interact with Cdc42 through three separate classes of assays: yeast two hybrid, co-complexing, and protein structure-based computational prediction.
While the CRIB domain is sufficient to secure affinity to activated Cdc42 (Owen et al., 2000), the two effectors differ widely in structure outside of this domain. Par6 interacts with the PAR microtubule cell polarity complex via its PDZ domain, while WASp can associate with the Arp2/3 actin cytoskeleton complex by VCA domains (Rohatgi et al., 1999; Peterson et al., 2004). Such observations suggest that these CRIB-containing effectors may serve as parallel signaling modules in executing cytoskeletal change, being activated and coordinated by Cdc42 activity.

Genetic knockout of cdc42, par6 or wasp genes resulted in highly specific yet remarkably indistinguishable phenotypes. The development of the aCC motorneuron was assessed through single-neuron dye labelling (Furrer et al., 2003) in cdc42/Y, par6Δ226/par6Δ226 or wasp1/Df(3R)3450 at 70% (hour 15:00) of embryogenesis, a time when the neuron normally forms about 10 dendritic terminals at stereotyped positions. In all cases, the aCC motorneuron significantly reduced its total number of dendritic terminals (P < 0.01, Fig 3.1C). Although dendrite elaboration was disrupted, dendrite initiation (sprouting) was unaffected, as evidenced by a typical number of primary dendrite branches (two). Despite ubiquitous expression of Cdc42, Par6, and WASp proteins in the nervous system, morphological anomalies in the knockout mutants were limited, being visible only within the dendritic region of neurons that develop their dendrites before others. We hypothesized that Cdc42’s interaction with these two cytoskeletal effectors is similarly restricted in time and place within the neuron, i.e., during dendrogenesis, likely as a result of Cdc42’s activation pattern. These interactions may account for
similar phenotypes observed in the aCC motorneuron when each of the three proteins is mutated separately.

Interaction dynamics during dendrite morphogenesis.

When Cdc42 was co-expressed with either Par6 or WASp in all neurons in the brain of *Drosophila* embryos, we detected FRET signals (Fig 2.2). Every voxel in the brain contained fluorescent signals from the given protein pair, indicating their co-localization throughout the neuronal cytosol at a level beyond the detection threshold. However, their individual spatiotemporal expression patterns did not predict where FRET occurred. In particular, Cdc42 clearly exhibited spatiotemporal specificity of its interactions with Par6 and WASp, while FRET was observed toward the end of neurogenesis and in the neuropil (Fig 2.8B). Such interaction patterns coincided spatiotemporally with the accumulation of Synaptotagmin1-positive synaptic vesicles in the axon terminals within the neuropil (Fig 2.8C). Since both axonal and dendritic terminals intermingle at the neuropil in the CNS, we could not determine whether FRET occurred in the axon terminal or in dendritic arbors (see below). In contrast to both Par6 and WASp, RhoGAP5A, a promoter of Cdc42 inactivation, exhibited interaction signals with Cdc42 nearly uniformly throughout the aCC cytosol at all three stages studied.

To attain single-neuron resolution, we next examined Cdc42’s interactions in the aCC motorneuron (Fig 3.2A). Of about 200 neurons born in every half segment of the *Drosophila* CNS, aCC development precedes that of all the others, pioneering the outgrowth of efferent axons into the periphery. We compared three time points: at 50% embryonic stage (hour 11:00) before the initiation of both the
dendrites and axon terminal, at 60% embryonic stage (hour 13:00) immediately after dendrogenesis begins, and at 70% embryonic stage (hour 15:00) immediately after the axon terminal starts to differentiate on its target muscle while the dendrites continue to elaborate. As expected from the observation in the whole brain, Cdc42 remained unassociated with either Par6 or WASp in aCC motorneuron before dendrogenesis. When new dendrites began to form, however, the protein exhibited FRET at a probability exceeding 20% association with each of its cytoskeletal regulatory effectors (Table 1). We examined six confocal planes spanning the z-axis of about 8 µm in the CNS and three confocal planes spanning the z-axis of about 2 µm in the periphery where the aCC neuromuscular synapse forms. Approximately 1,000 voxels from a single aCC motorneuron were divided into twelve subcellular compartments (Fig. 3D). While FRET signals of Cdc42 with either Par6 or WASp were sparse at 50% embryonic stage, we detected a FRET signal at the budding dendritic base with both Par6 and WASp in the newly forming dendrite at 60% embryonic stage, the onset of Cdc42 activation in this neuron. Three hours into the dendritic elaboration Cdc42 increased its interaction with Par6 and WASp in the newly forming dendrite at 70% embryonic stage.

Zooming into the dendritic region exposed a subtle difference in the patterns of Cdc42 interaction with Par6 versus WASp. As dendrites enlarge these interactions appear to complement one another spatially. Par6 shows the strongest interaction within shaft regions, while WASp activation is seen to occur at the leading edge of growth (Fig 3.2B).
Time-lapse imaging offered us an opportunity to gauge how fast a molecular signal translates into a morphological change. On average, one new branch is added to the aCC dendrite every 10-15 minutes. Examining the aCC dendritic region every five minutes, we noted that Cdc42’s interaction with WASp increased at the dendritic tip right at the time its growth was captured. Conversely, Cdc42 interacted with Par6 at high levels within the dendritic shafts subsequent to its elaboration (Fig 3.2C). Such in-vivo observations suggest that molecular events may cause phenotypic changes in as little as five minutes.

One commonly proposed explanation for the relatively minor phenotype change we observed when ubiquitously expressed proteins are genetically removed is other proteins' ability to compensate for the loss. However, the restrictive pattern of signaling observed between Cdc42 and its effectors Par6 and WASp within wild type animals leads to a simple conclusion: their tightly regulated interactions in time and space severely restrict the biological processes they participate in. Consequently, their loss putatively will have subtle and very constrained consequences as evidenced by the disruption of dendrite formation (Fig 3.2E).

The importance of spatiotemporal restriction

The rapidly expanding knowledge base of human genome sequences has intensified interest in the potential consequences of the expression of mutant proteins in the human body. We chose one well-studied point mutation of Cdc42, where a single nucleotide change led to the substitution of glycine with valine at the twelfth residue. Such a mutant protein loses the ability to hydrolyze GTP,
remaining activated out of context. Expressing this mutant Cdc42 protein in the whole fly brain results in malformations of the neuropil within the CNS as well as truncation and/or misguidance of motorneuron axonal growth cones in the periphery (Luo et al., 1994; Kim et al., 2002; Kamiyama and Chiba, 2009).

Expressing this mutant Cdc42 protein in the aCC motorneuron, we found that all subcellular compartments of the aCC motorneuron displayed significantly abnormal morphogenesis. Ectopic filopodia appeared in large portions of axon and cell body (P < 0.01, Fig 3.3A). Many of these ectopic filopodia were pointed incorrectly, facing away from the neuropil. Localizations of the cell body, fasciculation junction as well as axon terminal were often abnormal. In fact, in no cases was the aCC axon terminal found to have reached its normal target at 70% of embryogenesis (Fig 3.3A). Both ectopic filopodial extension and misguided axonal growth cones indicate that the proper wiring of this neuron is disrupted. As a consequence, we hypothesized that mutant Cdc42 interacts with cytoskeletal regulatory effectors ectopically in the neuron to induce cytoskeletal aberrations that differ in time and place of normal dendrogenesis.

Applying FLIM-FRET to this mutant protein, we found that mutant Cdc42, unlike the wild type, interacts with Par6 outside the dendrite, i.e., in the cell body, neurite, nerve root and axon (Fig 3.3B). Similarly, mutant Cdc42 exhibited ectopic interactions with WASp. Interestingly, this ‘activated’ mutant Cdc42 protein still exhibited little FRET with Par6 in the tips of the aCC dendrite region. As a positive control, a mCherry-tagged CRIB domain from WASp showed extensive association with the constitutively activated Cdc42 similarly to the wild type
effectors. Importantly, such altered interaction dynamics are consistent with the timing and location of anomalous growth. Thus, the ability of Cdc42 to hydrolyze GTP is crucial in constraining its spatiotemporal interaction patterns with Par6 and WASp, and likely other effectors not examined in our work (Fig 3.3C).

Discussion

Why does the removal of Cdc42, Par6, or WASp lead to a highly limited phenotype that is indistinguishable from the others? A likely explanation is that all three proteins are required to create the complex morphology of elaborating dendrites. The parallel signaling pathways involving WASp and Par6 most likely converge functionally, coordinating distinct components of the cytoskeleton that are necessary in forming dendritic structures. Furthermore, while Cdc42 activation is not necessary for dendrite initiation (sprouting), it is sufficient to form such outgrowths. The key to understanding these contrasting conditions lies in the spatiotemporal interaction patterns within neurons.

While spatiotemporal characteristics of individual protein expression are well understood, we demonstrated that their interactions play a decisive role for developmental processes. Notably, we found that such proteins show highly restrictive spatiotemporal interaction patterns that putatively drive the underlying phenotype of specific neurons. Consistent with this, a mutation in Cdc42 that causes the protein to remain activated destroyed the observed spatiotemporal organization and led to a highly disrupted phenotype. The next chapter will explore whether the interaction dynamics of the Rac1 GTPase follows similar principles.
Table 3.1. Cdc42’s probabilities of association with Par6 and WASp in different compartments of the aCC motorneuron.

Cdc42’s mean interaction probability ± S.E.M. with Par6 and WASp in N aCC motorneuron samples. * indicates a statistically significant difference from the neurite background at P < 0.01 with a two-tailed t-test.

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Neurite</th>
<th>Dendritic base</th>
<th>Dendritic shaft</th>
<th>Dendritic tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% (hour 11:00)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with Par6</td>
<td>-1.5±1.9% (7)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>with WASp</td>
<td>2.3±2.6% (8)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>60% (hour 13:00)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with Par6</td>
<td>1.1±1.9% (10)</td>
<td>13.7±1.0% (10)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>with WASp</td>
<td>0.8±5.0% (14)</td>
<td>21.2±2.3% (14)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>70% (hour 15:00)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with Par6</td>
<td>2.2±2.0% (16)</td>
<td>7.3±0.2% (17)</td>
<td>12.2±0.7% (12)</td>
<td>1.6±0.8% (15)</td>
</tr>
<tr>
<td>with WASp</td>
<td>3.3±2.8% (15)</td>
<td>11.0±0.4% (15)</td>
<td>-3.5±0.6% (11)</td>
<td>21.8±0.5% (15)</td>
</tr>
</tbody>
</table>
Figure 3.1. The aCC motorneuron.

(A) The main dendrite of the aCC motorneuron develops at the dorsal neuropil domain. Dotted line indicates the midline. (B) The formation of dendrite begins at 60% of embryogenesis. (C) Knockout of Cdc42, Par6 or WASp reduces the number of terminals emerging from the dendrite. Sample size in parenthesis indicates the number of aCC motorneurons examined. Scale bar 2 µm.
Figure 3.2. Spatiotemporal characteristics of Cdc42 interactions in single neurons.

(A) Cdc42 is co-expressed with Par6 and WASp using eve-GAL4 driver and exhibits FRET within the aCC motoneuron at 70% of embryonic stage. Scale bar 2 µm. (B) Zooming into the region where new dendrites emerge, Cdc42 interacts
with Par6 and WASp at the ‘shaft’ (✩) and ‘tip’ (★) of dendritic processes, respectively. (C) Such spatial patterns of Cdc42 and its interaction partners increasingly occur during consecutive stages of filopodia growth, suggesting that molecular events may lead to phenotypic changes in as little as five minutes. (D) Voxels from single aCC motoneurons are divided into twelve subcellular compartments, allowing us to create a spatiotemporal map of Cdc42 interactions. Notably, we observed that underlying protein interaction patterns are significantly restricted to dendritic shafts and tips in late stages of embryogenesis. Sample size in parenthesis indicates the number of aCC motoneurons examined. (E) Knock-outs of Cdc42, Par6 or WASp produce an indistinguishable phenotype that is limited in time and space to dendrite formation of the neuron (left). Such phenotypic changes coincide with spatiotemporally restricted interaction patterns of Cdc42 and its partners Par6 and WASp (right).
Figure 3.3. Impact of mutated Cdc42 on spatiotemporal interaction patterns.

(A) Mutant Cdc42 protein induces filopodia development at abnormal compartments and misguide axonal growth cone at 70% of embryonic stage. In the presence of wild-type Cdc42 aCC dendritic processes occur at the expected restricted sites in the CNS while aCC axon targets muscle-1. In contrast, mutant Cdc42 produces dendritic processes throughout the CNS while aCC axon fails to target muscle-1. (B) Ectopic and extensive interactions of the mutant Cdc42 protein at 70% of embryonic stage indicate that wild-type restricted spatiotemporal interaction patterns are significantly dissolved by mutant Cdc42. Sample size in parenthesis indicates the number of aCC motorneurons examined. Scale bar 2 µm. (C) Mutant Cdc42 induces a severely disrupted phenotype (left) that coincides with unconstrained spatiotemporal characteristics of its interactions with WASp and Par6 (right).
Chapter 4

Rac1 signaling during dendrite morphogenesis

Summary

Despite decades of research on Rho GTPase family, the unique physiological roles and signaling profiles between Cdc42 and Rac1 is unclear. In an effort to compare its role during neuron morphogenesis with that of Cdc42, we investigated Rac1’s activation pattern, the morphological consequences of its constitutive action, and its signaling profile with WASp and Par6 in the aCC neuron. Here we present findings that demonstrate differences between these GTPases in all three respects.

Background

Ras-related C3 botulinum toxin substrate 1, also known as Rac1, is believed to have originated the Rho family of GTPases around 1.5 billion years ago. Being the only Rho GTPase member in slime molds and plant species, it plays broad physiological roles (e.g. cell division and cytokinesis) in comparison to its roles in fungi and early metazoans, where other members Cdc42 and RhoA first appear (likely due to Rac1 gene duplication) (Boureux et al., 2007). This marked the beginning of unique and indispensable specializations among these three GTPases. The genetic expansion of this family of molecular switches, their upstream regulators, and their downstream effectors parallels the evolution of cellular complexity seen in multicellular animals (Pirone et al., 2001). Not
coincidental, rather this arsenal of signaling hubs helped to facilitate intercellular and intracellular communication.

One such example can be found during neuronal morphogenesis. In the *Drosophila* aCC neuron, extracellular cues guide the formation of its axon and dendrites (Wong *et al.*, 2000). Cdc42 and Rac1 are two proteins responsible for integrating these signals and subsequently effecting cytoskeletal remodeling. Yet, their influences on neuron morphology and the manners in which they enact them are unclear. In order to clarify similarities and differences between these GTPases, we wanted to compare Cdc42 and Rac1 within the same neuron. Having established them for Cdc42 (Kamiyama and Chiba, 2009; Chapter 2), we set out to determine Rac1’s activation pattern, morphological effects, and signaling dynamics with WASp and Par6.

**Materials and Methods**

**Rac1 activation**

Sb/Cyo;UAS-Raichu-Rac1 flies were obtained courtesy of the Giniger and Montell labs. The Rac1 activation bioprobe in these flies are based on the original construct designs from the Matsuda group (Itoh *et al.*, 2002), which is a multimeric fusion protein expressed as CFP::Rac1::RBD::YFP. Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) are the FRET donor and acceptor, respectively. Upon Rac1 activation, the Rac1 Binding Domain (RBD, derived from the CRIB domain of PAK1) is able to bind Rac1 and thereby induces a conformational change in the fusion protein as a whole. As a result, the
distance between CFP and YFP decreases and an increase in FRET Efficiency is observed.

To express the Rac1 bioprobe in aCC neurons, UAS-Raichu-Rac1 flies were crossed to eve-GAL4 flies. Embryos were collected and prepared for imaging as mentioned previously (Chapter 2). Because CFP lifetime decreases upon photobleaching (Tramier et al., 2006), ratiometric methods are required to obtain accurate FRET measurements. Therefore, a Zeiss Laser-scanning confocal microscope (LSM 780) was used to acquire three-dimensional z-stacks of Hour 11-15 aCC neurons at high resolution with identical capture parameters (63x magnification, a fixed pinhole size of 41.6µm, 45 µm z-step size, no zoom, 850 gain, 1.0 digital gain, scanning speed of 5) for all three capture channels (Donor, Acceptor, FRET). Excitation/emission settings were as follows. Donor: excitation 458 nm (80% laser power), emission 465-500 nm. Acceptor: excitation 514 nm (15% laser power), emission 525-550 nm. FRET: excitation 458 nm (80% laser power), emission 525-550 nm. Image size was set at 1024x1024 pixels at 16-bit depth with each channel exported in RAW image format for subsequent analysis.

In order to calculate FRET values for image pixels, we utilized the ImageJ plugin, PixFRET, developed by the Gelman group (Feige et al., 2005). In addition to Raichu-Rac1, we imaged aCC neurons in embryos expressing the donor only (UAS-gap::CFP) and acceptor only (UAS-Rac1::YFP) using imaging parameters identical to that of Raichu imaging. This allowed us to subtract CFP and YFP spectral bleed through (SBT) from the FRET channel using the PixFRET plugin.
After setting an intensity threshold (4.0 times background) and a Gaussian Blur (2.0), the software produced processed/blurred images for Donor, Acceptor, and NFRET channels. NFRET denotes Normalized FRET, where pixel values correspond to their SBT corrected FRET signal divided by their Donor intensity (\(\text{FRET}_{\text{SBT}}/\text{Donor}\)).

Processed images were subsequently exported to MATLAB for further analysis. According to the original paper by Itoh et al. (2002), the FRET/CFP fluorescence ratio is 1.8x greater when Rac1 is activated (Raichu-Rac1\(^{V12}\)) relative to when it is inactive (Raichu-Rac1\(^{N17}\)). However, this value does not take SBT correction into account. Once SBT contribution is removed from the FRET channel, we were able to increase the dynamic range between inactive-active states to ~3.5x. The NFRET values for the inactive (\(F_i\)) and activated (\(F_a\)) states of Rac1 were 0.32 and 1.12, respectively. Using these values for normalization, the % of Rac1 that is activated in each pixel can be estimated as follows:

\[
\% \text{ activation} = \frac{\text{NFRET} - F_i}{F_a - F_i} \tag{2.1}
\]

Additionally, by using the standard deviation values from Itoh et al (2002), the % of pixels with activation were determined using the M+3SD (0.32 + 3 \times 0.6 = 0.50) threshold method previously discussed (Chapter 2). Finally, neuronal subcompartments were segmented using Slidebook 5.0 software as mentioned previously (Chapter 3) in order to determine % activation and % pixels with activation using a custom MATLAB program.
**Mutant morphological analysis**

Morphological analysis of wild-type and mutant neurons were conducted as before (Chapter 3).

**CRIB Protein Signaling**

Rac1 interaction dynamics with WASp and Par6 were studied using Frequency domain-Fluorescence Lifetime Imaging (FD-FLIM), similar to experiments done with Cdc42 (Chapter 2). A notable difference in these experiments, however, is that mCherry was used as the FRET donor and NiRFP (Near-infrared fluorescent protein) as the FRET acceptor. Like GFP, mCherry possesses a monoexponential lifetime decay (Seefeldt *et al.*, 2008), permitting the quantitative approaches discussed in Chapter 2. NiRFP has superior brightness and photostability relative to other far-red fluorescent proteins (Shcherbo *et al.*, 2010). Combined with their substantial emission→excitation overlap, mCherry and NiRFP make a suitable donor-acceptor pair for FRET studies.

For system calibration with the mCherry excitation/emission filter sets, we used a preparation of Rhodamine B (10⁻⁴ M in methanol) that has been demonstrated to minimize Rhodamine aggregation, which in turn reduces lifetime variability (Kristoffersen *et al.*, 2014).

Furthermore, all experiments used Rac1 as the FRET acceptor (UAS-NiRFP::Rac1) with the FRET donor fused to a CRIB protein (UAS-mCherry::WASP, UAS-mCherry::Par6). This is in contrast to earlier experiments where Cdc42 was used as the FRET donor. All NiRFP fusion proteins were designed
from the same DNA constructs as GFP and mCherry fusion proteins (Chapter 2), but inserted into the attp2 site (at 68A4) on Chromosome 3 using the phic31 integrase system. The effect of this different design is that the probability of association values will refer to the probability of WASp or Par6 associating with Rac1, rather than the probability of Rac1 associating with WASp or Par6. Thus, it is only a matter of stoichiometric perspective, however its implications in interpreting the results will be discussed in further detail (Chapter 5). Finally, baseline lifetimes for each mCherry::CRIB protein were determined when coexpressed with cytoplasmic NiRFP (UAS-NiRFP).

**Results**

*Rac1 activation occurs throughout the aCC neuron during embryogenesis*

We wondered whether differences between Cdc42 and Rac1 existed at the level of upstream activation, either in space or time. Using a ratiometric FRET activation probe, similar to that used previously to study Cdc42 activation (Kamiyama and Chiba, 2009), we assessed the extent of Rac1 activation in each aCC subcompartment in the hours leading up to and during dendrogenesis (Hours 11 through 15 AEL).

At Hour 11 AEL, we found no evidence of Rac1 activation in the aCC. By Hour 13 AEL, as dendrite sprouting began to take place, activation was evident in all subcompartments. This trend continued through Hour 15 as a larger proportion of the Rac1 protein pool became activated, with one notable exception. Activation was not seen at dendritic tips (Fig 4.1)
Constitutive Rac1 activation disrupts dendrite elaboration

We previously showed how constitutive activation of Cdc42 resulted in excessive and ectopic dendrite sprouting (primary processes) in the aCC neuron, and yet interfered with subsequent branching in these processes. In a similar fashion, we expressed a constitutively active Rac1 mutant (YFP::Rac1V12) in the aCC and assessed neuron morphology at Hour 16 AEL (Fig 4.2). Compared to control neurons (YFP::Rac1) (2.111 ± 0.333), neurons expressing Rac1V12 displayed a similar number of primary processes (2.375 ± 0.744) [t(23) = 1.711, P >0.05]. However, the number of dendritic tips (terminals) we counted was significantly less in the Rac1 mutant neurons (8.375 ± 2.560) than in controls (13.556 ± 2.186) [t(33) = 1.692, P <0.001]. Therefore, constitutively-activated Rac1 resulted in fewer branches per primary dendrite process (2.615 ± 0.975) than normal (5.556 ± 1.424) [t(30) = 1.697, P <0.001]. We also noted the presence of axonal stalling, as 60% of axons failed to reach the target muscle by Hour 16 AEL. Finally, artificial Rac1 activation did not produce primary dendritic processes outside of the dendritic region.

Rac1 signaling with CRIB proteins

To study Rac1’s interactions with WASp and Par6 in the aCC neuron, we conducted FD-FLIM experiments as before (Chapters 2 and 3), with one noteworthy difference. This time we used mCherry as the FRET donor and NiRFP (Near-infrared fluorescent protein) as the FRET acceptor. According to the handful of previous studies that have looked at the properties of mCherry and
NiRFP (Seefeldt et al., 2008; Shcherbo et al., 2010), they possess many of the characteristics of an ideal FRET pair (see Methods). All experiments were performed with Rac1 as the FRET acceptor (UAS-NiRFP::Rac1) and potential CRIB partner as the FRET donor (UAS-mCherry::WASp and UAS-mCherry::Par6).

To establish baseline lifetimes for each interaction, both UAS-mCherry::WASp and UAS-mCherry::Par6 were coexpressed with cytoplasmic NiRFP (UAS-NiRFP). We found both controls had a mean mCherry lifetime of 1.46 ns, agreeing with previous reports (Seefeldt et al., 2008), when the entire aCC neuron was considered (data not shown). However, like the experiments involving GFP (Chapters 2 and 3), there was lifetime variability between individual subcompartments. As before, we established a baseline lifetime for every combination of compartment, time point, and FRET donor.

When UAS-mCherry::WASp and UAS-NiRFP::Rac1 were coexpressed in the aCC, we did not detect a significant change in mCherry lifetime (i.e. FRET) in any neuronal subcompartment at any of the three time points tested (Hours 11, 13, and 15). When we tested UAS-mCherry::Par6 and UAS-NiRFP::Rac1, however, we were able to detect interaction in the aCC. Specifically, FRET was observed in the cell body, proximal axon, and dendrite base at Hours 13 and 15 (Fig 4.3). Additionally, we observed FRET between these proteins in dendritic shafts at Hour 15.
Discussion

A close visual examination reveals a distinguishing feature in Rac1 mutant neurons compared to Cdc42 mutant neurons. Dendritic growth cones exhibit a wide, fanned out, morphology in the presence of excessive Rac1 activation (Fig 4.2A). This is in agreement with previous studies that have found Rac1 activation to promote lamellipodial protrusion (Waterman-Storer et al., 1999; Chen et al., 2008). These lamellipodia were interdigitated with filopodial protrusions; but while some of these small protrusions may be future branching sites, filopodia arising from a lamellipodial veil were not considered as individual dendritic tips in morphological analysis, owing to their often transient nature in this context (Shen and Cowan, 2010). Nevertheless, this may be subject to alternative interpretation. Namely, that constitutive Rac1 activation does not disrupt branch formation, but rather branch extension. Artificial Cdc42 activation formed many filopodial-like projections in the aCC (Figure 3.5), however these protrusions were not joined by a lamellipodial veil. Similar findings on Cdc42V12’s effect in neurons have been reported previously (Aoki et al., 2004).

Rac1 and Cdc42 do share similarities in their temporal activation profile. Both remain inactive until just prior to dendrite sprouting, and both experience an increased level of activation as dendrogenesis progresses. Nevertheless, Rac1’s spatial activation pattern is clearly distinct from that of Cdc42. While Cdc42 activation was mostly restricted to the dendritic region (Fig 1.4), Rac1 activation occurred throughout the entire aCC neuron. Interestingly, an increasing trend %
activation and % pixels was observed the further a compartment was from the cell body. Thus, the highest levels of activation were observed in the distal axon.

The lack of interaction between Rac1 and WASp is not entirely surprising, given that the affinity of WASp for Rac1 is substantially lower than its affinity for Cdc42 (Tomasevic et al., 2007). Unlike mammalian systems which have two homologues of WASp (WASp and N-WASp), Drosophila only has one (orthologous with WASp). While there is evidence that the N-WASp protein has substantial affinity for Rac1, the same is not true of WASp. Par6, however, did interact with Rac1, although not to a great extent. Since Par6 exists at lower concentrations in the cell than Rac1, its probability of association with Rac1 is not lowered by excess donor molecules (as was likely the case with Cdc42’s probability of association with Par6, a reciprocal view). Accounting for this, Par6’s interaction with Rac1 can be considered to occur much less frequently than its interaction with Cdc42.
Figure 4.1. Rac1 displays broad activation patterns within the aCC neuron

Rac1 activation patterns at Hour during dendrite sprouting (A - Hour 13) and elaboration (B - Hour 16). Color legend indicates the ratio of activated:total Rac1 in a given pixel. (C) Spatiotemporal summary schematic of Rac1 activation. Color legend indicates the % of pixels within each compartment exhibiting FRET above activation threshold value (p < .03). Sample size (number of neurons) shown in parentheses.
Figure 4.2. Expression of a constitutively active Rac1 mutant disrupts normal dendrite elaboration in the aCC neuron

Constitutive Rac1 activation (B) results in abnormal dendrite morphology in Hour 16 aCC neurons compared to wild-type Rac1 (A). Defects are seen exclusively in higher-level branching from primary dendrites (C), as evidenced by the reduced number of dendritic terminals (tips). Mutant neurons exhibit dendritic growth cones that are unable to extend filopodial processes in order to form stable dendrite branches. Bottom and right panels of (C) serve as compartmental legends for interpreting the four neuronal subregions that were grouped during morphological analysis.
Figure 4.3. Rac1 interacts with Par6, but not WASp, during aCC dendrogenesis

Spatiotemporal summary schematic of Rac1’s interaction with Par6 (A) and WASp (B). Color legend indicates the % of mCherry-labeled Par6 or Wasp interacting with NiRFP-labeled Rac1 within each compartment. Sample size (number of neurons) shown in parentheses.
Chapter 5

Discussion

Summary
This project successfully demonstrated the ability to visualize protein-protein interaction dynamics in the living Drosophila embryo during neurodevelopment. Importantly, it presented methodological and analytical approaches for extracting and interpreting quantitative information regarding protein interactions. The results of this work revealed that Cdc42 interacts with two CRIB effectors (Par6 and WASp) in parallel in order to coordinate distinct aspects of dendrite elaboration. It further showed that the morphological abnormalities which result from a constitutively active Cdc42 mutant are accompanied by an expansion of Cdc42’s interactions, spatially and/or temporally, with these effectors. Finally, this project demonstrated a number of differences, and similarities, in the roles of Cdc42 and Rac1 within the aCC neuron. Constitutive activation of either Cdc42 or Rac1 disrupted dendrite elaboration (higher-order branch formation). However, each had a unique effect on dendrite morphology. While Cdc42 over-activation resulted in excessive and ectopic dendrite sprouting (primary branch formation), constitutive Rac1 activation was characterized by extreme lamellipodial protrusion at dendritic growth cones. Both proteins were found to be normally activated within the dendritic region during dendrogenesis, although Rac1 activation was also present in other subcompartments as well. Additionally, whereas Cdc42 exhibits significant interaction with both CRIB effectors in
dendrites, Rac1 weakly interacts with only Par6. In summary, these studies have characterized the signaling dynamics of the small GTPases Cdc42 and Rac1 within neurons, identified key differences and similarities between them in the context of dendrogenesis, and provided evidence of coordinated spatiotemporal signaling and its essential role during neuronal morphogenesis.

**Novel Insights**

*Protein Interactions can be detected in vivo using FLIM-FRET*

By implementing recent genetic and imaging technologies, this project has outlined a means to study protein interaction dynamics in their native biological context. Frequency Domain-Fluorescence Lifetime Imaging Microscopy (FD-FLIM) is ideally suited for detecting intermolecular FRET in living samples. Unlike ratiometric approaches which require fluorophore concentrations to be carefully calibrated (SE) or actively manipulated (AP), fluorescence lifetime is concentration-independent and allows for rapid and reliable FRET measurements a living (dynamic) sample (Clegg et al., 2003). The use of FRET in biology traces back over thirty years (Morris et al., 1982), and while it has been implemented within living organisms (Kamiyama and Chiba, 2009) and used to study interactions between independently-expressed proteins (Sun et al., 2011) previously, these two aspects had yet to be combined in a controlled, non-invasive manner. Our systematic approach takes advantage of *Drosophila* transgenics to achieve site-specific genome insertion of fluorescently-tagged protein constructs, which allowed us to obtain consistent protein expression
levels that are suited for sensitive and reliable FRET detection. Although lifetime is concentration independent, the level of FRET observed reflects the stoichiometry of the donor and acceptor in the sample. A previous report (Berney and Danuser, 2003) concluded that donor-acceptor concentration ratio should fall within a range of 0.1 and 10.0 to obtain accurate FRET efficiency values. Fortunately, our system does not produce excessively high levels of transgene expression or mCherry:GFP concentration ratios, both of which can result in false positive FRET. We did find, however, that a high GFP:mCherry concentration ratio can lead to false negative FRET measurements as a result of the disproportionately high number of donor molecules with a baseline lifetime (GFP::Cdc42\textsuperscript{v12} x mCherry::CBD - data not shown). Therefore, donor-acceptor levels must be controlled and/or monitored when performing intermolecular FRET experiments. This may present a technical hurdle when initially designing FRET experiments, especially in vivo; nevertheless, the effort of implementing such a system will ensure that experiments yield valuable results.

Crucially, FD-FLIM examines interactions in a manner that is quantitative and reflects the association kinetics of the proteins being investigated. Illustrating the sensitivity of the system was our ability to detect a presumably transient interaction between Cdc42 and RhoGAP5a (Figure 2.2); an interaction that might not be detected with a method that requires stable complexing, such as yeast two-hybrid (Y2H). The differential effect caused of a point mutation (Y40C) on Cdc42's ability to bind WASp and RhoGAP5A (Figure 2.2), is consistent with biochemical findings (Owen et al., 2000) and provides strong evidence that direct
binding is responsible for the FRET observed in these studies. Measurement of a single parameter, donor (GFP) lifetime, in control (e.g. GFP::Cdc42 + cytoplasmic mCherry) and experimental (e.g. GFP::Cdc42 + mCherry::WASp) samples allows for calculation of the proportion of donor molecules interacting with an acceptor molecule ('probability of association'). Including an additional measurement parameter, donor (GFP) concentration, allows one to consider the relative number of donor molecules interacting with acceptor molecules ('extent of association'). We found that the extent of association between Cdc42 and WASp is considerably higher in the neuropil relative to the cortex during late-stage embryogenesis, while the probability of association is only slightly higher (Table 1.1). We interpret this to mean that significantly more Cdc42-WASp interactions take place within neurites than in the soma of a neuron. Each value offers a unique perspective regarding a given interaction and both should be considered when attempting to compare spatially distinct regions within a sample.

The ability to study molecular dynamics in its native environment provides the spatiotemporal context that has long been missing in protein interaction studies. Future development and implementation of FRET imaging technologies will be instrumental in biology’s ability to bridge molecular circuitry and cellular physiology.
Cdc42 signals with WASp and Par6 in parallel to drive dendrite elaboration in the aCC neuron

In order to analyze spatial characteristics of protein interactions, we performed manual segmentation of neuronal compartments. While automated segmentation approaches are currently being developed (see Future Directions), I strove to minimize human error and bias by establishing specific criteria prior to segmentation. Because fluorophore lifetime is sensitive to its immediate environment (Ryder et al., 2001), it is important to establish separate donor baseline lifetimes for each subcompartment with control samples. Highlighting this point, we found mean GFP lifetimes ranging between 2.53 ns and 2.58 ns across different regions in the nervous system (data not shown), possibly due to variations in the pH, membrane/cytosol volume, and/or molecular milieu. Using the appropriate baseline lifetime, however, should account for such differences and allow one to correctly assess whether FRET is occurring.

Using the pan-neuronal driver elav-GAL4 to express our FRET constructs, we first looked at whether Cdc42 interacted with its potential partners (WASp, Par6, and RhoGAP5A) in the nervous system (Figure 2.8). The interaction between the GTPase Cdc42 and its effector WASp underwent a progressive increase from early-to-late embryogenesis (Hour 12 –21 AEL) within the developing Drosophila nervous system. This was most prominent in the longitudinal connectives of the ventral nerve cord, the region of the neuropil containing virtually all of the synapses made in the CNS. Indeed, the peak of the Cdc42-WASp interaction coincided with the time that most synapses are
functional and undergoing maturation (Hour 18-24 AEL). A possible explanation for the correlation between synaptogenesis and Cdc42-WASp interaction is that neuronal surface area increases up through synaptogenesis as neurites undergo morphogenesis. Marked structural change occurs during axonal and dendritic growth, with the leading edge of growth being an area that undergoes constant cytoskeletal remodeling (Vitriol and Zheng, 2012). As axons and dendrites elongate and branch, they create more sites for synaptic contact. In the Drosophila VNC, these sites become enriched along the longitudinal connectives. Known to mediate both membrane protrusion and branch formation (Pinyol et al., 2007; Kessels et al., 2011), interaction between Cdc42 and WASp may be related to the morphological expansion that takes place before synaptic contact. Similarly, processes that take after synapse formation (e.g. synaptic pruning, glial wrapping) depend on morphological remodeling (Shen and Cowan, 2010).

Considering that the protein WASp has classically been associated with actin dynamics and morphological change, why did we find it interacting with Cdc42 within neuronal cell bodies? Although most papers on WASp focus on its role at the cell membrane, there is evidence that Cdc42 and WASp are also involved in membrane trafficking (Matas et al., 2004) and endosomal recycling (Parsons et al., 2005) away from the cell membrane. In fibroblasts and HeLa cells lines, Cdc42, N-WASp, and Arp2/3 were all found to be localized within Golgi complexes, with Cdc42 activation causing recruitment of N-WASp and Arp2/3 to the lateral edges of cis-Golgi membranes (Matas et al., 2004). The
authors conclude that Cdc42 and WASp play a role in Golgi-associated membrane trafficking, and that actin nucleation/polymerization contributes to the budding or retrograde transport vesicles. This study, and others like it (Tzima et al., 2003), demonstrate that interaction between Cdc42-WASp interaction can extend throughout the cell. In neurons, such a mechanism could serve to facilitate turnover of new protrusions during morphological change at both the presynaptic and postsynaptic end, transport soluble proteins between neuronal subcompartments, and recycle vesicle membranes at the presynaptic end. The latter function may contribute to the increase in Cdc42-WASp FRET seen during late stage embryogenesis.

In order to explore the subcellular context in which Cdc42’s interactions with WASp and Par6 take place, we expressed the same FRET constructs in a subset of motorneurons using the eve-GAL4 driver. Focusing on the well-characterized aCC motorneuron and its stereotyped morphological development, we imaged neurons prior to dendrogenesis (Hour 11 AEL), at its onset (Hour 13 AEL), and during branch elaboration (Hour 15 AEL). Shortly after it becomes activated (Kamiyama and Chiba, 2009) in the aCC neuron, we found that Cdc42 interacts with both WASp and Par6 at the dendrite base prior to initial sprouting and continues to do so throughout dendrogenesis. As dendrogenesis progressed, both interactions extended into the growing dendrite arbor, yet with different spatial patterns. While the interaction with WASp was strong at the leading edge of growth (dendritic tips), no interaction was present there with Par6. The converse was true within dendritic shafts (Fig 3.2).
These findings indicate that the progressive increase in Cdc42-WASp interaction seen in the *Drosophila* nervous system is at least partly due to the increasing number of dendritic leading edges (tips) that form as dendrites elaborate. Regarding Par-6, multiple studies provide evidence for a role in stabilizing microtubules via centrosome recruitment (Dormoy *et al.*, 2013), which was found to be necessary for synaptic bouton formation in *Drosophila* motor axons (Ruiz-Canada *et al.*, 2004). Such a process may explain our finding that Cdc42 and Par-6 interact at the dendrite base and dendrite shaft within aCC neurons, where it can recruit centrosomes (microtubule-organizing centers) to serve as anchor points for directed microtubule growth. In such a scenario, microtubule stabilization would help to reinforce dendrite shafts after they have been formed via actin-polymerization.

One of the intriguing characteristics of GTPases is that they behave like rapid molecular switches. Once activated, they are capable of initiating signaling cascades with a multitude of partners. The CRIB proteins are a group of structurally diverse proteins that, in addition to the domain they share, are united by their roles as cytoskeletal regulators. A logical speculation is that the CRIB effectors constitute a functional module, executing different aspects of cytoskeletal remodeling that are necessary for morphological change. The hypothesis entails that Cdc42 and Rac1 are responsible for executing this complex program by coordinating CRIB protein activity in space and time. Our results, showing that Cdc42 signals with WASp and Par6 simultaneously in the
context of dendrite morphogenesis, provides now strong evidence in support of this model.

When Cdc42 is constitutively activated in the aCC, ectopic neurite sprouting occurs throughout the length of the axon. We show that the spatiotemporal restriction of these interactions is lost upon constitutive Cdc42 activation, correlating with the morphological abnormalities that are seen. Future studies that knock down individual CRIB proteins, in conjunction with Cdc42\textsuperscript{V12} expression, may be able to assess which interactions are actually responsible for the excessive sprouting phenotype.

The ectopic outgrowths arising from constitutive Cdc42 activation are likely filopodia. Filopodia are thin, highly dynamic, and often transient structures composed of actin filament (F-actin) bundles (Heasman and Ridley, 2008). Rather than becoming permanent neurite extensions, it is thought that the role of filopodia is to serve as environmental sensors that aid in neurite guidance (Koleske, 2003) Notably, although the ectopic dendrite processes induced by Cdc42\textsuperscript{V12} fail to form secondary branches, the two original primary dendrites (also found in wild-type neurons) do show some degree of higher-order branching. Therefore, constitutive Cdc42 activation does not completely disrupt higher-order branching, although it does appear to disturb the timing and stereotyped patterning of the dendrite arbor. Unbranched dendrite processes, which are likely filopodia, most likely lack the protein partners of Cdc42 that are necessary for dendrite elaboration.
The aCC neuron’s ability to form its primary dendrites in the absence of Cdc42, WASp, or Par6 indicates that another signaling mechanism is normally responsible for dendrite sprouting, or that such a mechanism compensates in this process when Cdc42/WASp/Par6 are absent. According to experiments performed in mouse cortical neurons, it is likely that Rac1 and its partner PAK1 are responsible for setting the site of dendrite sprouting and initiating primary dendrite growth (Hayashi and Ohshima, 2002).

**Distinct signaling patterns and morphological roles of Cdc42 and Rac1 during dendrogenesis**

The requirement of both Rac1 and Cdc42 for animal viability and normal neurodevelopment is a mystery given the two proteins’ similarities in structure and claimed functions. Due to conflicting reports regarding the roles of Cdc42 and Rac1 in neuron morphogenesis (Govek et al., 2005), we decided to look at the function and signaling properties of both proteins within the same neuron in order to make some direct comparisons.

The consequence if Rac1 removal on neuronal morphology has been explored in several systems, although with some conflicting results. In *Drosophila* mushroom body neurons, a triple Rac1/Rac2/Rac3 hyopmorph results in a significant reduction in dendrite number and mean dendrite length (Ng et al., 2002). Similar findings were reported with mouse, rat and Xenopus neuronal cultures after either RNAi-mediated depletion or when a dominant-negative Rac1 mutant (Rac1\textsuperscript{N17}) is expressed. Other studies, however, done in the same organisms using the same procedure, but in different cell types, found that
dominant-negative Rac1 has little to no effect of dendrite morphology (Govek et al., 2005). Unfortunately, few studies look at both Cdc42 and Rac1 in the same system using the same approach. Thus, in order to compare the morphological roles of Cd42 and Rac1 in the aCC neuron, I expressed a constitutively active Rac1 mutant, fluorescently labeled with YFP, in the aCC neuron protein in the same manner as I had with Cdc42 (Figure 4.2).

In contrast to Cdc42, I found that Rac1 over-activation did not result in excessive or ectopic dendrite sprouting as the number of primary dendrite processes were similar to that seen in wild-type neurons. There was a reduction in the number of higher-order branches per primary dendrite process, similar to what was seen with Cdc42. However, while the two original primary dendrite process were capable of some higher-order branching in Cdc42 mutant neurons, such branching was compromised with the Rac1 mutant. Finally, an additional similarity among the two GTPase mutants was the presence of axonal stalling, occurring at a similar frequency (~60%) in both mutant proteins.

Apparent from these findings is the fact that the activation of both Cdc42 and Rac1 must be tightly regulated, in space and/or time, for dendrite elaboration to progress normally. Considering the GTPases are characterized by their ability to act as rapid molecular switches, regular cycling between activation states is likely to be for their normal functioning during morphogenesis. Indeed, in one study which monitored Rho GTPase activation in the context of fibroblast motility, each GTPase possessed a distinct spatial pattern and cycling interval with respect to membrane protrusion (Machacek et al., 2009). Moreover, some
studies have found similar morphological defects in both constitutively active (V12) and inactive (N17) mutants expressing neurons (Kuhn et al., 1998), supporting the importance of proper cycling.

Another difference between phenotypes seen in aCC neurons expressing Cdc42\textsuperscript{V12} and Rac1\textsuperscript{V12} is the morphology of the dendritic growth cone. While dendritic processes in neurons expressing Cdc42\textsuperscript{V12} tended to be filopodia-like (thin and of moderate length), dendrites in Rac1\textsuperscript{V12}-expressing neurons are wide (fan-shaped) and stunted in length. Rac1 is known to promote lamellipodium formation in many cell types, including neurons (Heasman and Ridley, 2008), which likely explains the unique dendritic growth cone anomaly seen in Rac1\textsuperscript{V12} – expressing aCC neurons. Furthermore, spatially-extended Cdc42 activation resulted in sprouting of filopodial-like process throughout the aCC axon, whereas Rac1 only produced aberrant effects in the normal dendritic region despite its ubiquitous activation state. The latter finding is not surprising, however, given that we found Rac1 to be broadly activated under normal conditions.

If differences such as these do indeed exist, why have Cdc42 and Rac1 been considered to have such similar roles in the context of neuron morphogenesis? One likely reason is that processes like dendrogenesis or axon guidance are often considered as whole, so that a disruption in the activity level of either Cdc42 or Rac1 are similarly found to result in a general disruptions of the process in question (Kaufmann et al., 1998). As demonstrated in this work, however, the distinction between the morphological roles of Cdc42 and Rac1 are subtle. Indeed, there is evidence that a disturbance in either Cdc42 or Rac1’s
normal activation pattern creates an imbalance between lamellipodial/filopodial protrusion dynamics and interferes with lamellipodial formation (Kurokawa et al., 2004), indicating that coordination between Cdc42 and Rac1 coordination is necessary for executing any instance of complex morphological change.

A major finding of this thesis is that the signaling characteristics of Rac1, within a developing CNS neuron, are quite distinct from those of Cdc42. With regards to their activation, there was a similarity regarding its temporal pattern. Both Cdc42 and Rac1 first displayed significant levels of activation just prior to dendrogenesis (Hour 13 AEL) and increased in degree during dendrite elaboration (Hour 15 AEL). Spatially, however, it found that Rac1 activation in the aCC neuron extends much further than Cdc42 activation (Figure 4.1). Cdc42 activation was found to be restricted largely to the dendritic compartment, with some activation in cell bodies and late-stage axon terminals (Kamiyama and Chiba, 2009). Rac1 activation, conversely, occupied virtually every neuronal compartment by Hour 15 AEL with the highest levels in the distal axon. The one region where Rac1 was not activated, intriguingly, was at the leading edge of dendritic growth (tips). This region was the only region exclusive to Cdc42.

Further distinguishing the two GTPases were their signaling patterns with the CRIB proteins WASp and Par6. Since the CRIB proteins act as the FRET donor in the Rac1 experiments, interactions between them would be more easily detected and pronounced compared to the Cdc42-CRIB experiments. This is because GTPases are expressed at higher levels within neurons, and a 100% Fretting donor population (i.e. maximum lifetime drop) is only possible when the
rate-limiting protein (CRIB protein) is used as the donor. Nevertheless, as one might predict from biochemical studies comparing Cdc42/Rac1 affinities for WASp (Tomasevic et al., 2007) and Par6 (Garrard et al., 2003), there was minimal signaling between Rac1 and these CRIB proteins in the aCC neuron (Figure 4.3). No interaction was detected between Rac1 and WASp at any time point or spatial compartment. However, Par6 did interact with Rac1 in the cell body, proximal axon, dendrite base, and dendrite shafts during dendrogenesis.

Model

Figure 5.1 shows a working model of Cdc42/Rac1 interaction dynamics with WASp and Par6 during dendrogenesis. At Hour 11, Cdc42 and Rac1 are in the inactive state. Just before dendrite sprouting takes place (Hour 13), Cdc42 and Rac1 both become activated at the dendrite base. Once activated, Rac1 binds Par6 and Cdc42 binds both WASp and Par6. Par6’s activation by Cdc42/Rac1 allows it to recruit centrosomes (microtubule organizing centers) to the dendrite base. WASp becomes activated by Cdc42 and together, they bind the protein complex Arp2/3 which promotes actin nucleation and polymerization in the direction of dendritic growth. These actin dynamics are responsible for membrane protrusion at the leading edge of dendrite growth. As dendrogenesis progresses (Hour 15), Par6’s activation extends into dendritic shafts, where it again helps to recruit centrosomes. Centrosomes act as an origin and anchor for growing microtubules. Within dendrites, microtubules grow from the shaft toward the dendrite base and become stabilized via centrosomes. Microtubule stability is
ultimately responsible for stabilizing dendrite branches as they grow. Conversely, Cdc42 and WASp remain at the leading edge (tips), where they drive dendrite growth and branching. These two also maintain their interaction within the base, possibly aiding in retrograde endosomai transport.

Future Directions

The advent of FD-FLIM as a technique suitable for live imaging came more than 50 years after FRET was first postulated (Förster, 1948). The current progressions in technology are bringing consistent improvements in camera sensitivity, image resolution, and excitation sources. In the near future, it will be possible to capture high quality images of interaction dynamics at video frame rates with lower phototoxic effects. Such capabilities can truly enhance, and complicate, the types of questions that we can ask about the molecular world.

While these studies have been able to shed some light on the differences between Cdc42 and Rac1 and their dynamics within the CRIB protein subnetwork, several questions still remain. First, I did not demonstrate which proteins and interactions are responsible for dendrite initiation in the aCC neuron. There are indications that PAK and Rac1 (possibly Cdc42 as well) are responsible (Hayashi and Ohshima, 2002). If this is true, its evolutionary conservation in vertebrates and invertebrates may signify that this mechanism is universal. Although not included in this work, I have collected and begun analyzing data on PAK1’s interaction with both Cdc42 and Rac1. Performing a
Rac1 knockout experiment would also help clarify this question, as well as offering another point of comparison with Cdc42.

Although some novel distinctions between Cdc42 and Rac1 were made in this work, there are surely many more differences that exist. Expanding the experiments performed in this work to include all ten CRIB proteins would be a comprehensive and undoubtedly insightful approach. Aside from this, however, there may be nuances in the spatiotemporal interaction profiles with shared CRIB effectors (e.g. Par6). Ideally, one would be able to simultaneously visualize both Cdc42 and Rac1’s interaction with a mutual partner. This would allow a direct comparison of their signaling on a pixel-by-pixel basis at exactly the same time, rather than approximate developmental time points within large subcompartments. In fact, the allure of such promising data led us to develop three-way FRET experiments in order to achieve this goal. Using GFP, mCherry, and NiRFP as fluorescent labels, it is possible to carry out two FRET experiments simultaneously. In these experiments, mCherry can act as both a FRET acceptor and FRET donor. Another unique advantage of using FLIM to detect FRET is that the lifetime of the acceptor is unchanged during FRET, so there are no complicated interpretations of mCherry lifetime if both interactions are taking place. As an example, we have begun collecting three-way FRET data between GFP:Cdc42, mCherry:Par6, and NiRFP:Rac1. In this experiment, FRET is possible between Cdc42→Par6 and Par6→Rac1, but not Cdc42→Rac1.

Finally, we have long envisioned an improved and more objective approach to performing spatial analysis within neurons. Manual segmentation of
neuronal subcompartments is not only time-consuming and prone to errors, but the averaging of so many pixels during analysis has the potential to dilute an interaction that is taking place in a very precise spatial location. If so, there is a good chance that a significant difference will not be found. In collaboration with computer scientists, Gavriil Tsechpenakis, Ph.D. and his graduate student Sarun Gulyanon, we have developed a strategy to create a standardized coordinate system where every pixel in a neuron is uniquely identified according to key landmarks (initial axon segment, dendrite base, dendritic tips, and fasciculation junction). This approach will allow statistical analysis to be conducted on a pixel-by-pixel basis, which will reveal only the most consistent spatial segments in which an interaction occurs. Not only can this provide greater resolution in discerning an interaction pattern, but it may be able to provide a better indication of its biological relevance, which potentially revealing spatial segments where an interaction is most consistent and

**Signaling profiles – a means to uncover molecular logic within the cell**

My studies have shown that the context provided by studying protein-protein interactions *in situ* can help to delineate the molecular dynamics that underlie cellular processes. First, FD-FLIM was demonstrated to be a reliable tool for detecting protein interactions within developing *Drosophila embryos*. Additionally, a number of analytical approaches were introduced in order to assess different facets of a given interaction. Using these tools, we were able to show that Cdc42 interacts with its CRIB partners, WASp and Par6, in distinct spaces and times
within the aCC motoneuron in order to regulate dendrite morphogenesis. While Rac1 only weakly interacted with Par6, data collected on its normal activation pattern and the consequences of its constitutive activation, indicate that this protein does regulate normal dendrite morphology. Furthermore, by characterizing the signaling patterns of a constitutively active Cdc42 mutant, this work was able to show how pathological phenotypes can be linked to changes in the underlying molecular network.

Altogether, the utilization of FLIM-FRET to study protein interactions provides a native and dynamic context that has been missing in biology. Going forward, our ability to link molecular and cellular events will create new avenues in medical intervention and biological exploration.
At Hour 11, Cdc42 and Rac1 are in the inactive state. Just before dendrite sprouting takes place (Hour 13), Cdc42 and Rac1 both become activated at the dendrite base. Once activated, Rac1 binds Par6 and Cdc42 binds both WASp and Par6. Par6’s activation by Cdc42/Rac1 allows it to recruit centrosomes (microtubule organizing centers) to the dendrite base. WASp becomes activated by Cdc42 and together, they bind the protein complex Arp2/3 which promotes actin nucleation and polymerization in the direction of dendritic growth. These actin dynamics are responsible for membrane protrusion at the leading edge of dendrite growth. As dendrogenesis progresses (Hour 15), Par6’s activation extends into dendritic shafts, where it again helps to recruit centrosomes. Centrosomes act as an origin and anchor for growing microtubules. Within
dendrites, microtubules grow from the shaft toward the dendrite base and become stabilized via centrosomes. Microtubule stability is ultimately responsible for stabilizing dendrite branches as they grow. Conversely, Cdc42 and WASp remain at the leading edge (tips), where they drive dendrite growth and branching.
REFERENCES


