Functional analysis of the extended N-terminus for the Drosophila Raf protein and initial characterization of the Arl1 gene

Jian Ding
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/etd
Part of the Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation
Ding, Jian, "Functional analysis of the extended N-terminus for the Drosophila Raf protein and initial characterization of the Arl1 gene" (2010). Graduate Theses and Dissertations. 11815.
http://lib.dr.iastate.edu/etd/11815

This Dissertation is brought to you for free and open access by the Graduate College at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Functional analysis of the extended N-terminus for the Drosophila Raf protein and initial characterization of the Arl1 gene

by

Jian Ding

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

Program of Study Committee:
Linda Ambrosio, Major Professor
Amy Andreotti
Jack Girton
Ted Huiatt
Jo Anne Powell-Coffman

Iowa State University
Ames, Iowa

2010

Copyright © Jian Ding, 2010. All rights reserved.
TABLE of CONTENTS

ABSTRACT iii

CHAPTER 1. General Introduction 1
   Introduction 1
   Literature Review 3
   Literature Cited 11
   Figure Legends 15
   Figures 16

CHAPTER 2. Drosophila Raf’s N-terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling 18
   Abstract 18
   Introduction 19
   Materials and Methods 22
   Results 25
   Discussion 34
   Acknowledgements 40
   Literature Cited 40
   Tables 46
   Figure Legends 47
   Figures 53
   Contribution of this work 59

CHAPTER 3. Initial Characterization of Drosophila Arf-like1 60
   Abstract 60
   Introduction 61
   Materials and Methods 63
   Results 65
   Discussion 73
   Acknowledgements 77
   Literature Cited 77
   Tables 81
   Figure Legends 87
   Figures 90
   Contribution of this work 97

CHAPTER 4. General Summary 98
   Summary and Conclusions 98
   Suggestions for Future Research 103
   Literature Cited 105

APPENDIX 106

ACKNOWLEDGEMENTS 109
ABSTRACT

The Raf serine/threonine kinases play a key role(s) in receptor tyrosine kinase (RTK) signaling. However, the mechanisms that modulate Raf activity are complicated and remain elusive. Clues to Raf regulation were derived from the identification of conserved regions/motifs/sites. Drosophila Raf (DRaf) has an extended N-terminus. We show this N-terminal segment contains a novel region (CRN) that is conserved in BRaf proteins of vertebrates. The extended N-terminus can contribute to Torso RTK signaling during embryogenesis in both loss and gain-of-function genetic backgrounds. Furthermore, stronger interactions between DRaf’s RBD (Ras Binding Domain) and the small GTPase Ras1, as well as Rap1, were observed in vitro when CRN and RBD sequences were linked. Together, these studies suggest that the N-terminal segment may assist in the association of DRaf with its activators (Ras1 and Rap1) and play a positive regulatory role(s) in DRaf activation in vivo through CRN-mediated mechanisms.

We also tried to characterize the roles of DRaf’s conserved CR2 domain. In our yeast 2-hybrid screen using CR2 as the bait, a putative DRaf binding partner, small GTPase Arf-like1 (Arl1) protein, was identified. Using genetics approaches we have made an effort to understand the roles of Arl1 in Drosophila. Our studies suggest that Arl1 may function in membrane traffic.
CHAPTER 1. General Introduction

Introduction

Numerous fundamental cellular processes including differentiation, proliferation and cell survival in eukaryotes dependent on the Ras-Raf-MEK signal cassette and its regulation of extracellular signal-regulated kinase (ERK) pathways (SCHLESSINGER 2000). Binding of ligands with receptor tyrosine kinases (RTK) activates RasGEFs (Guanine-nucleotide exchange factors), which initiates GTP loading of Ras. GTP-Ras recruits Raf protein to the cell surface and triggers its activation. Once activated, Raf can phosphorylate its downstream effectors MEK (mitogen-activated protein and extracellular signal-regulated kinase kinase), subsequently leading to activation of ERK that modulates the transcriptional program of target genes (ROBBINS et al. 1994; Figure I-1). Dysregulation of ERK pathways caused by activating mutations in RTKs and components of the Ras-Raf-MEK module have been frequently found in human cancers (BEERAM et al. 2005; SRIKALA et al., 2005). Therefore, study of the regulatory mechanisms of these ERK pathway complexes, which represent the strategic targets of anti-tumor drugs, will be very essential for development of therapeutics against cancer.

Ras has been evaluated as a putative target of anti-cancer therapeutic strategies, since oncogenic mutations in Ras were linked to ~15% human cancers (WELLBROCK et al. 2004). Both the regulatory mechanisms (GTPase cycle, post-translational modification), as well as the biophysical nature of Ras and other small GTPases in general have been relatively well defined. However, the clinical results of Ras-targeting drugs, including farnesyltransferase
(FTase) inhibitors (FTIs) etc, appear disappointing (ROWINSKY et al. 1999; BEERAM et al. 2005; SRIKALA et al. 2005).

As a major effector of Ras, the Raf serine/threonine kinases play a key role in ERK signal transduction. Constitutively active forms of Raf and Ras appear to have a comparable potential to transform malignant cells, while expression of dominant negative mutants of Raf can antagonize Ras transforming activity. Furthermore, it has been discovered that BRaf is mutated at a high frequency in human cancers, particularly in 70-80% malignant melanomas (Reviewed by SRIKALA et al. 2005). These observations have led to considerable amount of attention paid to targeting Raf as an anticancer therapeutic strategy, thus highlighting the importance of Raf kinases. Therefore, understanding of the mechanisms that modulate Raf activity is an important issue with physiological significance.

Mammals have three Raf isoforms, ARaf, BRaf and CRaf, while the invertebrates Caenorhabditis elegans and Drosophila melanogaster have one Raf gene referred to as Lin-45 and DRaf, respectively. All the Raf proteins share a common primary structure consisting of three conserved regions (CR1, CR2 and CR3, Figure I-2). Clues to regulatory events of Raf were derived from the identification of motifs/sites in these conserved regions. The catalytic portion of the kinase is located in the C-terminal conserved region 3 (CR3), while CR1 and CR2 represent the regulatory half of the Raf protein. Although an auto-inhibitory role had been previously assigned to residues compromising CR1 and CR2, the N-terminal regulatory part also functions in promoting Raf activity. CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site, but its functional role is less well defined (WELLBROCK et al. 2004). In addition to CR1, CR2 and CR3, BRaf
and DRaf contain an extended N-terminal segment followed by CR1. However, little attention was given to this region thus far.

Research in our laboratory has focused on understanding the regulatory mechanisms of the DRaf protein in the model system Drosophila. Particularly, our goal is to characterize the roles of the extended N-terminal segment and CR2 region in DRaf signaling. We have found that the N-terminus of DRaf contains a novel region that is conserved in BRaf proteins of vertebrates. It may assist in Ras-DRaf interaction and can play a positive role(s) in Torso RTK signal transduction during Drosophila embryogenesis. We also discovered a putative DRaf binding partner, small GTPase Arf-like1 (Arl1) protein, which interacts with CR2 region. Using genetics approaches we have made an effort to understand the functional roles of Arl1 in Drosophila.

**Literature Review**

**Activation of Raf by Ras:** Cytosolic Raf proteins exist as an “inactive” complex with other binding partners including 14-3-3 and heat shock protein (Hsp90). Raf has two specific serine residues that act in 14-3-3 binding and lie within the CR2 (Ser259, refer to CRaf) and CR3 (Ser621, refer to CRaf) domains, respectively. Association of either site with 14-3-3 requires phosphorylation of the corresponding serine residues. Formation of the inactive complex via the interaction between 14-3-3 and both of these phospho-Ser residues may maintain the Raf molecule in a “closed” conformation and mask its catalytic region, since a Ser259 to Ala mutation that may prevent the CR2 from interacting with 14-3-3 appears to facilitate CRaf activation (CLARK et al. 1997; KUBICEK et al. 2002).
Activation of Raf is initiated by its translocation to membrane, where Raf can form a complex with different binding partners to elicit its full activation. The small GTPase Ras plays a crucial role(s) in regulation of Raf’s membrane localization and is evaluated as a major regulator of Raf, although it was reported that interaction between a basic motif in CRaf’s kinase region and phosphatidic acid (PA) can recruit Raf to the membrane in a Ras-independent way (RIZZO et al. 2000; KRAFT et al. 2008). Association with Ras, not only translocates Raf to the membrane, but may disrupt 14-3-3 binding to CR2, leaving the phospho-Ser259 residue more accessible to dephosphorylation by protein phosphatase 2A (PP2A) or other phosphatases (KUBICEK et al. 2002). However, the molecular mechanisms of Ras-Raf coupling is complicated and not completely defined.

Cellular Ras exists in two guanine nucleotide-bound states, GTP-Ras and GDP-Ras, with different molecular conformations in its switch 1 and switch 2 regions. Raf’s RBD can directly interact with GTP-Ras and is thought to be the core element for Ras-binding (NASSAR et al. 1995). Arg89 (refer to CRaf) in RBD is essential for its association with Ras and substitution of Arg 89 to Leu abolishes this interaction (FABIAN et al. 1994; LI et al. 1998). In addition, CRD functions in Ras-Raf coupling, as well, through its contact with the lipid moiety of Ras, which is independent of GTP status of Ras. It seems the hydrophobic patch within CRD is required for Ras binding, since mutation of the hydrophobic surface impairs the interaction (WILLIAMS et al. 2000; THAPAR et al. 2004). Recently, it was found that BRaf’s extended N-terminus facilitated BRaf-HRas interaction in vitro, and deletion of the N-terminal segment reduced BRaf’s binding affinity with HRas (FISCHER et al. 2007). However, the identities of residues/sites in this process were not characterized.
Phosphorylation of Raf: Phosphorylation/dephosphorylation of Raf plays crucial roles in Raf regulation. Identification of conserved phosphorylation sites/motifs has provided very important clues in understanding Raf’s regulatory mechanisms.

Phosphorylation of Ser43, Ser259 and Ser233 (refer to CRaf with Ser43 and Ser233 specific for CRaf) exhibits negative effects on Raf activation. Phosphorylation of Ser43 both in vivo and in vitro can reduce the apparent Ras binding affinity and appears to sterically prevent the interaction of CR1 with Ras (Wu et al. 1993). In CR2 region, phosphorylated Ser259 mediates the interaction with 14-3-3 (Clark et al. 1997). It was reported that phosphorylation of Ser233 provides an additional binding site for 14-3-3 (Dumaz and Marais 2003). Therefore, phosphorylated Ser259 and Ser233 may contribute together to maintain the Raf molecule in an inactive conformation. These three serine residues (Ser43, Ser259 and Ser233) seem to be phosphorylated by protein kinase A (PKA) when the cellular cAMP level is elevated (Dumaz et al. 2002). It was also found that Ser259 can be phosphorylated by AKT (also known as protein kinase B, PKB) and serum glucocorticoid-inducible kinase (SGK) (Zimmermann and Moelling 1999; Beeram et al. 2005)

The C-terminal phosphorylated Ser621 (Refer to CRaf) is another 14-3-3 binding site and seems to play dual roles, both negatively and positively, in Raf activation. 14-3-3 associates with both phospho-Ser259 and Ser621, and helps to maintain the inactive Raf complex. However, when its binding to Ser259 is disrupted, the interaction with phospho-Ser621 seems essential to attain full activation of Raf, since mutation at Ser621 abolishes Raf’s activity in cultured Cos-7 cells (Yip-Schneider et al. 2000). At the present time, it is still controversial whether phosphorylation of Ser621 is regulated during Raf activation.
The N-region (Negatively charged) preceding the catalytic kinase domain in CRaf contains two additional regulatory sites Ser388 and Tyr341. Phosphorylation of these two residues seems to relieve the inhibitory effects of the N-terminal regulatory portion of the protein on its catalytic domain and is required to achieve full activation of the kinases. Mutations at either site block CRaf activation, and maximal activity is not obtained unless both sites are phosphorylated (MASON et al. 1999). Phosphorylation of Ser338 and Tyr341 in CRaf is likely mediated by PAK and Src family kinases, respectively (KING et al. 1998; TILBROOK et al. 2001).

A few of these phosphorylation sites mentioned above are isoform specific. The two conserved residues (Thr491, Ser494, refer to CRaf) residing in the active segment within the kinase domain are probably common to all Raf members (DOUZIECH et al. 2006). Phosphorylation of these two sites seems essential for activity of Raf family members with their mutations abolishing Raf activation (CHONG et al. 2001). However, the identity of those kinases, which mediate this phosphorylation, remains unknown.

Recently, a novel phosphorylation site (Tyr510) in DRaf was identified. This Tyr residue is conserved in all Raf proteins. Phosphorylation of this Tyr510 by Src68B kinase appears to relieve autoinhibition of full-length DRaf in Drosophila (XIA et al. 2008). This regulatory mechanism may be shared by other Raf proteins, including ARaf, BRaf and CRaf.

**Regulation by scaffold proteins and molecular adaptors:** Raf’s activity is also modulated through formation of functional complexes with scaffolding adaptors, including the kinase suppressor of Ras (KSR) and connector enhancer of KSR (CNK).

KSR contains a C-terminal kinase-like domain, which interacts with MEK. However, this domain, in which the key sites essential for kinase function are substituted with other
residues. appears to be devoid of catalytic kinase activity. In stead, it has been widely depicted that KSR acts as a scaffold protein rather than kinase, and coordinates the assembly of Raf-MEK-MAPK complexes (ROY et al. 2002; ROY and THERRIEN 2002; ROIGNANT et al. 2006; RAJAKULENDRAN et al. 2008). Recently, it was found that overexpression of KSR without co-overexpression of other scaffolding partners in Drosophila S2 cells could independently trigger DRaf activation, probably by participating in heterodimerization with DRaf in a side-to-side manner. This suggests that beyond its “scaffolding” function, KSR may possess an intrinsic Raf activating potential (RAJAKULENDRAN et al. 2009).

CNK does not contain any catalytic regions/motifs. Instead, CNK has several conserved domains, which provide numerous interaction sites for its binding partners, suggesting it functions as a scaffold protein (KOLCH 2005). Drosophila CNK plays dual roles in DRaf regulation, both negatively and positively, according to RTK signal status. Its C-terminal RIR (Raf inhibitory region) interacts with DRaf and inhibits Raf activity in quiescent S2 cells. However, once RTK is activated, the N-terminal SAM (sterile α-motif) and CRIC (a conserved region in CNK) regions mediate Raf activation by Ras, and the inhibitory effects of RIR can be relieved by active Src42 (DOUZIECH et al. 2003; DOUZIECH et al. 2006). The RIR is not conserved in CNK proteins of mammals. Mammalian CNK can promote CRaf activation by simultaneously interacting with CRaf and Src, thereby facilitating the phosphorylation of Tyr341 in CRaf by Src (LANIGAN et al. 2003; KOLCH 2005).

**Isoform-specific regulation of Raf kinases:** All the Raf members have a similar primary structure, share Ras GTPase as a common regulator, MEK as the common effector, and were thought activated by similar means. However, differences in their regulatory mechanisms have recently emerged.
It has been reported that RBDs of different Raf isoforms differentially interact with active Ras. BRaf and CRaf RBD's exhibit higher binding affinity with HRas than ARaf RBD in vitro, and consistently, in HEK 293 cells, active HRas preferentially activates CRaf as compared to ARaf. The isoform specific Ras binding affinity is, at least partially, due to one residue difference in their RBD regions, Arg59 of CRaf is replaced with Lys (Lys22) in ARaf, since mutation of Lys22 to Arg in ARaf can increase its affinity to HRas in vivo and in vitro (WEBER et al., 2000).

Both BRaf and CRaf can interact with the small GTPase Rap, which shares nearly identical effector binding portions with Ras. However, association of different Raf isoforms with active Rap results in distinct effects in signaling. Rap can activate BRaf in a parallel way with Ras, but plays a negative role and functions as an antagonist of Ras in regulating CRaf activity. It was found that CRD of CRaf exhibits higher binding affinity for Rap than that of BRaf. The strong interaction between CRaf’s CRD and Rap may exclude Ras binding, therefore inhibiting activation of CRaf (OHTSUKA, et al. 1996). However, it is still unclear why CRaf and BRaf are regulated by Rap in distinct manners.

Furthermore, modulation of Raf kinases’ phosphorylation status appears to be isoform-specific, too. The Ser43 site in CRaf is not conserved in ARaf or BRaf. BRaf contains two specific phosphorylation sites (Ser428 and Thr439) that ARaf and CRaf do not possess. Phosphorylation of Ser338 in CRaf, which is critical for its activation, is responsive to upstream stimulation, while the corresponding Ser445 in BRaf is constitutively phosphorylated. In the regulatory N-region preceding the kinase domain, BRaf lacks a tyrosine site corresponding to Tyr301 of ARaf and Tyr341 of CRaf, instead, it has an acidic residue (Asp448) in the equivalent position. To obtain full activation, Tyr301 of ARaf and
Tyr 341 of CRaf need to be phosphorylated by Src. However, for BRaf, the corresponding acidic Asp448 residue mimics constitutive phosphorylation and results in a higher basal activity for Braf (MASON et al., 1999).

Endogenous Raf proteins are predominantly distributed in the cytoplasm. However, overexpressed Raf kinases exhibit isoform specific intracellular localization. CRaf was found located in mitochondria, while ARaf was colocalized with an endosomal marker (GALMICHE et al. 2008). The different cellular localization patterns suggest these Raf members play isoform specific roles, although originally they were thought to be functional redundant to some extent.

**BRaf isoform specific features:** Compared with ARaf and CRaf, BRaf has a higher basal activity and is more liable to oncogenic mutation. Somatic mutations of BRaf were frequently found in different human cancers (~83% anaplastic thyroid carcinoma, and 55-68% malignant melanoma). Particularly, a substitution of Val599 with Glu (BRaf^{V599E}) accounts for ~90% oncogenic BRaf mutations in cancers (SRIKALA et al., 2005). BRaf^{V599E} is constitutively active, and its activity appears to be independent of Ras binding. Crystal structure of BRaf kinase domain indicates that the V599E mutation may eliminate the requirement for activation segment phosphorylation. Surprisingly, some BRaf mutants with impaired kinase activity can activate CRaf and signal to ERK in cells, suggesting that BRaf may function as a scaffold protein (WAN et al. 2004). Furthermore, over-expression of wild type BRaf in malignant melanoma without NRas mutation can activate MAPK signal and promote cell growth (TANAMI et al. 2004), indicating that BRaf may have an intrinsic potential to activate CRaf (or other BRaf molecules). These studies highlight the importance and physiological significance of BRaf. Thus, understanding isoform-specific functions and
regulatory mechanisms of BRaf has been attracting more research interest.

The negative charged motif (D447-D448) preceding the kinase domain is a BRaf specific feature. As mentioned already, the corresponding sites in ARaf and CRaf must be phosphorylated by Src kinases to achieve full activation. However, the negative charged motif in BRaf mimics constitutive phosphorylation and may account for BRaf’s high basal activity (OHTSUKA, et al. 1996; MASON et al. 1999).

Different from ARaf and CRaf, BRaf has an extended N-terminus in addition to CR1, CR2 and CR3. However, studies of BRaf regulation have mainly focused on CR1, CR2 and CR3 with little attention, thus far, given to the role of this BRaf specific N-terminal region. Although Fischer et al. (2007) found this extended N-terminus facilitated BRaf’s interaction with HRas in vitro, at the present time, the molecular mechanisms are not clear and the biological implications of this BRaf specific N-terminal region in vivo are not well defined.

**Drosophila Raf:** Drosophila has one Raf gene encoding DRaf protein. As a key component of the MAP kinase signaling module, DRaf plays an essential role in numerous receptor tyrosine kinase (RTK) pathways in Drosophila development. The regulatory mechanisms of DRaf are quite similar to that of mammalian Raf proteins. Almost all the regulators, co-factors as well as effectors of DRaf are conserved in mammals. Forward genetics screen for components of Ras-Raf-MAPK pathway in Drosophila have tremendously advanced our understanding of the signaling in mammals, and study of DRaf have been providing lots of important clues regarding to regulator mechanisms of mammalian Raf proteins.

Based on the primary structures, Drosophila Raf is more similar to BRaf than either ARaf or CRaf. DRaf has 61% overall sequence similarity with BRaf. DRaf shares isoform
specific features with BRaf and is thought to be a BRaf ortholog. DRaf has the signature negative charged motif (E420-E421) that corresponds to D447-D448 within BRaf (MASON et al. 1999; MISHRA et al. 2005). DRaf also has an extended amino-terminus followed by CR1.

DRaf and BRaf also share parallels in their modes of regulation. Rap1 can activate both BRaf and DRaf, but not ARaf or CRaf (MISHRA et al. 2005; OHTSUKA, et al. 1996). Therefore, studies on DRaf using the powerful Drosophila genetic system may provide important unique insights to better understand the functional roles and regulatory mechanisms of BRaf.

**Literature Cited**


DOUZIECH, M., M. SAHMI, G. LABERGE and M. THERRIEN, 2006 A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent


Figure Legends

FIGURE I-1. Raf-mediated Receptor Tyrosine Kinase (RTK) signal pathways: The binding of ligand to a RTK receptor triggers the activation of small GTPase Ras, which recruits Raf to the plasma membrane. Once activated, Raf transfers its signal to MEK, in turn leading to activation of ERK. By regulating downstream gene expression, the RTK pathways function in numerous fundamental processes, including differentiation, proliferation, cell survival and cell fate determination.

FIGURE I-2. Schematic representations of Raf kinases: Raf family members share a common primary structure consisting of three conserved regions (CR1, CR2 and CR3). CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site. CR1 and CR2 represent the regulatory half of the Raf protein, while the catalytic portion of the kinase is located in the C-terminal conserved region 3 (CR3). In addition to CR1, CR2 and CR3, BRaf and DRaf contain an extended N-terminal segment followed by CR1.
Receptor Tyrosine Kinase (RTK) signal pathway

Cell proliferation  Cell differentiation  Cell survival  Cell fate determination
Primary structure of Raf proteins

ARaf

BRaf

CRaf

DRaf

Lin-45

RBD  CRD

Ras binding  14-3-3 binding  Kinase domain  14-3-3 binding
CHAPTER 2. Drosophila Raf's N-terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling

http://www.genetics.org/cgi/reprint/184/3/717

Jian Ding, Oren Tchaicheeyan and Linda Ambrosio*
Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, 50011
*Corresponding author (lima@iastate.edu)

Abstract

Drosophila Raf (DRaf) contains an extended N-terminus, in addition to three conserved regions (CR1-3), however, the function(s) of this N-terminal segment remains elusive. In this paper, a novel region within DRaf's N-terminus that is conserved in BRaf proteins of vertebrates was identified and termed Conserved Region N-terminal (CRN). We show that the N-terminal segment can play a positive role(s) in the Torso receptor tyrosine kinase pathway in vivo, and its contribution to signaling appears to be dependent on the activity of Torso receptor, suggesting this N-terminal segment can function in signal transmission. Circular dichroism analysis indicates that DRaf’s N-terminus (amino acids 1-117) including CRN (amino acids 19-77) is folded in vitro and has a high content of helical secondary structure as predicted by proteomics tools. In yeast two-hybrid assays, stronger interactions between DRaf’s Ras binding domain (RBD) and the small GTPase Ras1, as well as Rap1, were observed when CRN and RBD sequences were linked. Together, our studies suggest that DRaf’s extended N-terminus may assist in its association with the upstream activators (Ras1 and Rap1) through a CRN-mediated mechanism(s) in vivo.
Introduction

Evolutionarily conserved receptor tyrosine kinase (RTK) signaling pathways function in fundamental cellular processes including differentiation, proliferation and cell survival in eukaryotes (SCHLESSINGER 2000). The Raf serine/threonine kinase, as a key component of RTK signaling modules, plays a central role in transmitting upstream stimuli to the nucleus (DAUM et al. 1994). Cyclic control of Raf depends on activities of GTPases, kinases, phosphatases and scaffold proteins (CHONG et al. 2001; DHILLON et al. 2002; KOLCH 2000; MORRISON 2001; RAABE and RAPP, 2002). Clues to these regulatory events were derived from the identification of conserved regions/motifs/sites. However, the mechanisms that modulate Raf serine/threonine kinases are complicated and remain elusive.

Mammals have three Raf isoforms, ARaf, BRaf and CRaf. They share a similar primary structure consisting of three conserved regions (CR1, CR2 and CR3). Conserved region 1 (CR1), where a Ras binding domain (RBD) and a cysteine-rich domain (CRD) reside, is required for Ras-Raf interaction. CR2, a serine/threonine-rich region, contains a 14-3-3 binding site. CR1 and CR2 are embedded in the regulatory N-terminal half of Raf proteins, while CR3, including the catalytic kinase region and an additional 14-3-3 binding site, resides in the C-terminus (Reviewed by WELBROCK et al. 2004). In addition to these three conserved regions, BRaf has an extended amino-terminal segment followed by CR1 (TERAI and MATSUDA 2006; FISCHER et al. 2007). However, studies of BRaf regulation have mainly focused on CR1, CR2 and CR3 with little attention, thus far, given to the role of this N-terminal region.

Translocation of Raf proteins to the plasma membrane, a critical step in their activation, can be mediated through different mechanisms. It is reported that direct interaction between a
basic motif in CRaf’s kinase region and phosphatidic acid (PA) can recruit Raf to the membrane (RIZZO et al. 2000; KRAFT et al. 2008). This PA-binding site is conserved in ARaf and BRaf proteins. Also, association with Ras, a major regulator of Raf kinases, plays a crucial role(s) in translocation and activation of Raf. However, the molecular mechanisms of Ras-Raf coupling are not completely understood. Raf’s RBD can directly interact with the switch 1 region of GTP-Ras and is thought to be the core element for Ras binding (NASSAR et al. 1995). CRD is involved in Ras-Raf coupling, as well, through interaction between its hydrophobic patch and the lipid moiety of Ras (WILLIAMS et al. 2000; THAPAR et al. 2004). Thus, both RBD and CRD contribute to Ras-Raf interaction and the effects are likely additive. Disabling either RBD or CRD is thought to reduce but not completely eliminate Raf activity (HU et al. 1995). Recently, Fischer et al. (2007) found BRaf’s interaction with HRas was also facilitated by the extended N-terminus, in vitro. At the present time, however, the identity of residues/sites that participate in this process are unknown and the biological implications of this N-terminal region in vivo have not been defined.

Drosophila has one Raf gene first described genetically as l(1) pole hole, and later referred to as DRaf or Raf. As a member of the MAP kinase signaling module, DRaf plays an essential role in numerous receptor tyrosine kinase (RTK) pathways in Drosophila development (BRENNAN and MOSES 2000; VAN BUSKIRK and SCHÜPBACH 1999; DUFFY and PERRIMON 1994; RAABE 2000). Based on its primary structure, the DRaf protein is more similar to BRaf than either ARaf or CRaf (DHILLON and KOLCH 2002; CHONG et al. 2003; MORRISON and CUTLER 1997). DRaf and BRaf have two acidic residues (E420-E421 in DRaf; D447-D448 in BRaf) preceding the kinase region that correspond to residues Y301-Y302 in ARaf and Y340-Y341 in CRaf, respectively. These negative charged acidic residues
mimic constitutive phosphorylation and are thought to be related to the higher basal activity of B Raf (MASON et al., 1999; MISHRA et al. 2005). Both D Raf and B Raf have an extended amino-terminus, when compared to A Raf and C Raf, in addition to CR1, CR2 and CR3. D Raf and B Raf also share parallels in their modes of regulation. Rap1 can activate both B Raf and D Raf, but not A Raf or C Raf (MISHRA et al. 2005; OHTSUKA, et al. 1996). Like the Raf proteins in mammals, the activity of D Raf is regulated through phosphorylation/dephosphorylation (BAEK et al. 1996; LABERGE et al. 2005; RADKE et al. 2001; ROMMEL et al. 1997), interaction with scaffold proteins or other binding partners (DOUZIECH et al. 2003; DOUZIECH et al. 2006; ROY et al. 2002; ROY and THERRIEN 2002; ROIGNANT et al. 2006; RAJAKULENDRAN et al. 2008). These regulatory events occur within the three conserved regions (CR1-3) of D Raf, however, the role of D Raf’s N-terminal region has not been elucidated.

Development of both embryonic termini in Drosophila is dependent on D Raf-mediated Torso RTK signaling. Binding of Trunk or Torso-like with the Torso receptor initiates Ras1-D Raf-MEK signaling at the poles of early staged embryos, and in turn, triggers expression of at least two gap genes, tailless and huckebein, which specify terminal structures and help to establish segmental identities in the embryo (Reviewed by FURRIOLS and CASANOVA 2003). The domain of tailless (tll) expression in the embryonic posterior region has been used as a quantitative marker to measure the strength of the Torso RTK signal in early embryos. At the cellular blastoderm stage, embryos from wild type mothers show posterior tll expression from approximately 0-15% embryo length (EL). At a later stage embryos exhibit normal internal head structures, three thoracic segments (T1-T3), eight abdominal denticle belts (A1-A8), as well as the Filzkörper tail structure. Decreased or loss of Torso RTK pathway activity
results in a reduced posterior expression domain of \textit{tll} and consequently absence of embryonic tail structures. In contrast, gain-of-pathway activity can lead to expanded \textit{tll} expression domains at both poles, and subsequently enlarged head and tail structures, accompanied by deletion of central abdominal segments (GHIGLIONE et al. 1999; JIMÉNEZ et al. 2000).

In this study, using the Drosophila embryonic termini as both a qualitative and quantitative \textit{in vivo} assay system, we examined the role played by DRaf’s N-terminus in Torso signaling in different genetic backgrounds. We observed a subtle, but consistent, higher signaling potential for full-length DRaf proteins when compared with those lacking amino-terminal residues 1-114 (DRaf$^{\text{AN114}}$). Furthermore, a novel region within DRaf’s N-terminus that is conserved in RAF genes of most invertebrates and BRaf genes of vertebrates was identified and termed Conserved Region N-terminal (CRN). Our studies suggest that DRaf’s extended N-terminus may assist in its association with the upstream activators (Ras1 and Rap1) \textit{in vivo} and thus, potentially play a regulatory role(s) in DRaf’s activation through a CRN-mediated mechanism(s). Minor adjustment by CRN on Ras1 and Rap1 binding may help to fine-tune DRaf’s activity and consistently provide optimal signal output.

\textbf{Materials and Methods}

\textbf{Drosophila strains and genetics:} In this study, \textit{y w, Draf}^{11-29} (Draf$^-$; DRaf protein null, MELNICK et al. 1993), \textit{trunk}^{1} (trk$^-$; loss-of-function allele, lacks C-terminal 16 amino acids, SCHÜPBACH and WIESCHAU 1989, CASANOVA et al. 1995), \textit{torso}^{XR1} (tor$^+$; Torso protein null allele, \textit{tor} gene deletion, SPRENGER et al. 1989), and \textit{torso}^{RL3} (tor$^{RL3}$; gain-of-function allele, H242L amino acid replacement in the extracellular domain, SPRENGER et al. 1993)
strains were used. The “FLP-DFS” technique was utilized to generate $Draf^{11-29}$ germline clones (CHOU and PERRIMON 1996). Drosophila stocks were raised at 25° on standard cornmeal medium. To study the gain-of-function effects of the temperature-sensitive $tor^{RL3}$ allele (Figure II-3), virgin females were collected and mated with wild type males at 25° for 3-4 days and then moved into a 29° incubator. Eggs were collected at 29° during the first 1-2 days for western analysis and phenotypic characterization.

Transgene design: Full-length and truncated DNAs were amplified using wild type $Draf$ cDNA (GenBank#AY089490, obtained from Drosophila Genomics Research Center) as template, and inserted into the polylinker site of the $P$-element transformation vector pCaSpeR-HS83. The full-length cDNA sequence (FL $Draf$) encodes a $Draf$ protein with 739 residues, while the truncated cDNA sequence ($Draf^{AN114}$) corresponds to amino acid 115-739 of the FL $Draf$ protein. The constitutively active heat-shock 83 gene (HS83) promoter was used to drive the expression of $Draf$ transgenes to simplify the generation of transgenic lines with various genetic backgrounds. Transgenic lines were generated by Genetic Services (Sudbury, MA).

Multiple lines derived for each transgene were used in this study. $Draf^{AN114}$ (L1, #a and #b), FL $Draf$ (#a and #b) were used to generate germline clone bearing females (Figure II-1). Lines #1, #2, #3 of $Draf^{AN114}$ and #1, #2, #3 of FL $Draf$ were used in $tor^{RL3}$, trk$^I$ and trk$^{XR1}$ backgrounds. The $Draf^{AN114}$ line #1 is homozygous lethal, thus we generated trk$^I$/trk$^I$; $Draf^{AN114}$#1/ $Draf^{AN114}$#3 and trk$^{XR1}$/trk$^{XR1}$; $Draf^{AN114}$#1/$Draf^{AN114}$#3 lines that produce $Draf$ protein levels equivalent with other lines (Figure II-4, Table II-1).

Western analysis: To produce protein extracts, 100 eggs were collected and homogenized in 36ul lysis buffer containing 20mM Tris-Cl (pH8.0), 150mM sodium
chloride, 0.2% Triton-X 100, 0.2% Nonidet P-40, 10mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride, 0.15U/ml aprotinin and 20mM leupeptin. Insoluble material was removed by centrifugation (13000 rpm, 10 minutes) at 4 °C. Protein extracts were separated by 8% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. DRaf proteins were probed with rabbit anti-Raf antibody (70.1, SPRENGER et al. 1993) and horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Thermo Scientific). α-tubulin proteins probed with mouse antibody (Sigma) and horseradish peroxidase-coupled goat anti-mouse secondary antibody (Thermo Scientific) were used as an internal control. The membranes were developed using SuperSignal West Pico kit (Thermo Scientific). Protein level was quantified with Image J.

*In situ* hybridization: *tailless* and *engrailed* probes were generated from wild type cDNA clones (*tailless*: Genebank #BT022195; *engrailed*: Genebank #AY069448, obtained from the Drosophila Genomics Research Center) using the PCR DIG probe synthesis kit (Roche Applied Science). Whole-mount mRNA *in situ* hybridizations were performed in embryos according to the protocol of Tautz and Pfeifle (1989) with minor modifications.

**Circular dichroism (CD) spectral measurement:** DNA corresponding to amino acids 1-117 of DRaf (DRafN117) was recombined into the pGEX vector. The GST-DRafN117 fusion protein was produced by expression in *E.coli* BL21, and purified by standard affinity chromatography. Purified GST-DRafN117 protein was digested with thrombin. The DRafN117 protein (~13KD) was purified by a size-exclusive column (Amersham Biosciences), and verified by Mass spectra and N-terminal sequencing. Protein sample (0.05 mg/ml in 10 mM sodium phosphate buffer) was loaded to 0.1 cm quartz CD cuvette. CD spectra was measured by Jasco J-710 spectropolarimeter (Protein facility at Iowa State University) at room
temperature. Data were collected with 0.2 nm resolution and at a scan rate of 1.5 nm min$^{-1}$. The ellipticity value of the blank buffer at each wavelength was subtracted from each point.

**Yeast two-hybrid analysis:** The R174 to L mutation in DRaf (DRaf$^{R174L}$) was generated by PCR-based site-directed mutagenesis, and confirmed by sequencing. DNA sequences corresponding to amino acid 1-117 (N), 1-212 (NRBD), 18-212 (Δ17NRBD), 78-212 (Δ77NRBD) and 115-212 (RBD) were obtained by PCR using wild type DRaf as the template, while DNAs encoding NRBD$^{R174L}$ and RBD$^{R174L}$ were amplified from DRaf$^{R174L}$ DNA. Amplified DNAs were cloned into pGADT7 vector (Clontech). DNA sequences encoding amino acid 1-183 of Ras1 (Ras1ΔCAAX), 1-180 of Rap1 (Rap1ΔCAAX) were amplified from cDNAs of wild type Ras1 and Rap1 (Ras1: Genebank #AF186648; Rap1: NCBI Reference #NM_057509, obtained from the Drosophila Genomics Research Center) respectively, and inserted into the pGBKKT7 vector (Clontech).

Constructed pGADT7 and pGBKKT7 plasmids were transformed into yeast Y187 strain. Protein-protein interactions were tested by β-Galactosidase assays using X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma; solid-support assay) or ONPG (ortho-nitrophenyl-b-D-galactopyranoside, Sigma; liquid quantitative assay) as substrates. β-Galactosidase units in quantitative assays were calculated according to the Yeast Protocol Handbook (Clontech). All yeast two-hybrid experiments are confirmed by reciprocal bait-prey assays and repeated at least 4 times.

**Results**

To study the potential function of DRaf’s N-terminal residues (amino acids 1-114), we generated transgenic flies expressing full-length DRaf (FL DRaf) or DRaf proteins lacking
amino-terminal residues 1-114 (DRaf$^{\Delta N114}$; Figure II-1A). The constitutive heat-shock 83 (HS83) promoter was selected to drive transgene expression, to simplify the generation of complex genetic backgrounds required to test the functionality of N-terminal residues. We used the Torso pathway to test the signaling potential of these maternally expressed DRaf proteins. Since the Torso signaling system is solely dependent on activity of maternal DRaf proteins, we could readily determine and verify the quantity of DRaf proteins available for Torso signal transduction in early staged embryos by western blot analysis. Thus, at equivalent protein concentrations, we compared the signal potential of FL DRaf and DRaf$^{\Delta N114}$ proteins to characterize the role of the N-terminus in a well-defined RTK pathway in vivo.

**DRaf’s N-terminus can contribute to RTK signaling in Drosophila embryos:**

Embryos that were deficient for maternal DRaf protein (derived from Draf$^{f1-29}$/Draf$^{f1-29}$ female germ cells, see MATERIALS and METHODS) lack posterior till expression at ~2.5 hours after egg deposition and subsequently exhibit abnormal cuticle pattern with deletion of posterior structures due to loss of Torso RTK signaling (Figure II-1D ii, ii’). We generated females with germ cells homozygous mutant for the Draf$^{f1-29}$ allele (Draf$^{-/-}$) but expressing FL DRaf or DRaf$^{\Delta N114}$ proteins using the “FLP-DFS” technique (CHOU and PERRIMON, 1996). Cuticles of embryos produced by Draf$^{-/-}$; DRaf$^{\Delta N114}$ female germline clones expressing maternal truncated proteins at ≥ to endogenous wild type DRaf levels were essentially equivalent to those of wild type embryos with only one (1/245) lacking posterior Filzkörper (line L1, Figure II-1B, E). However, when DRaf$^{\Delta N114}$ was expressed at low maternal levels (~ 1/4 of endogenous DRaf level; 2 independent transgenic lines #a and #b; Figure II-1B), ~4.5% of the embryos assayed lacked posterior Filzkörper (Figure II-1D iii, E).
At such a reduced expression level (2 independent lines #a and #b, Figure II-1B), FL DRaf showed rescue of posterior pattern with Filzkörper development observed for a higher percentage of embryos (~98.5%, \( \chi^2=9.91976318, P<0.01; \) Figure II-1D, E). In agreement with the cuticle phenotype, an abnormal posterior \( tll \) expression pattern (<13% EL) was observed more often for embryos that inherited truncated \( \text{DRaf}^{\text{AN114}} \) (78.8%, \( n=52 \)) rather than full-length DRaf proteins (91%, \( n=78 \); \( \chi^2=3.9386844, P<0.05, \) Figure II-1D), suggesting that \( \text{DRaf}^{\text{AN114}} \) was less active than FL DRaf in Torso RTK signaling.

To test our protein quantification assay, a more rigorous examination was conducted using western blot analysis. Three samples representing lysates of 6, 12, and 18 eggs from each line (\( \text{Draf}^{-/-}; \text{DRaf}^{\text{AN114}} \#a \) and \( \text{Draf}^{-/-}; \text{FL DRaf} \#a \)) were loaded on to a SDS-PAGE gel. As shown in Figure II-2A, the intensity of DRaf and corresponding tubulin bands exhibits a roughly linear correlation with the number of eggs lysed (Figure II-2B). In addition, the normalized DRaf protein level was consistent among the three samples loaded for the same transgenic line (Figure II-2C), suggesting our western blots analysis was reliable. Importantly, we also addressed the question of maternal DRaf protein stability and whether deletion of the N-terminal region altered DRaf accumulation levels during Torso signal transduction. Embryonic lysates from eggs collected at 0-1, 1-2 and 2-3 hours after deposition were prepared (\( \text{Draf}^{-/-}; \text{DRaf}^{\text{AN114}} \#a \) and \( \text{Draf}^{-/-}; \text{FL DRaf} \#a \)). As shown in Figure II-2D and E, DRaf protein levels remained roughly constant, indicating both FL DRaf and \( \text{DRaf}^{\text{AN114}} \) proteins are stable throughout the 0-3 hour period when the Torso pathway is active.
Next, we genetically altered the Torso pathway to create a sensitized signaling environment and compared the potential of DRaf$^{AN114}$ and FL Raf proteins in this background. $tor^{RL3}$ is a temperature-sensitive, recessive, gain-of-function allele of the Torso receptor. At the non-permissive temperature 25-29°, $tor^{RL3}/tor^{RL3}$ mothers produce embryos that show broad $tll$ expression at both anterior and posterior ends. These embryos develop and show deletion of central abdominal segments, and do not hatch (STRECKER et al. 1989).

Eggs derived from females heterozygous for $tor^{RL3}$ ($tor^{RL3}/+$) can hatch as larvae, however, some of these larvae show a gain-of-function phenotype with deletion of an abdominal segment(s). At 29°, we found 7.4% (n=553, Figure II-3D) of the larvae from $tor^{RL3}/+$ mothers showed deletion, fusion or broken abdominal denticle bands. In this genetic background, when expressed at comparable protein levels (Figure II-3A, B), FL DRaf enhanced the $tor^{RL3}$ phenotype much more significantly than DRaf$^{AN114}$, resulting in a greater number of embryos with central abdominal defects. We found 31.8% (n=422) embryos with FL DRaf proteins showed the gain-of-function phenotype (Figure II-3C, D), while only 17.1% (n=450) of the DRaf$^{AN114}$ embryos showed such defects (Figure II-3C, D). We repeated these experiments using two additional, independently derived transgenic $FL \, DRaf$ and $DRaf^{AN114}$ lines and observed similar results ($\chi^2=51.063876$, P<0.001; Figure II-3D).

To test if the cuticle phenotypes observed were due to alterations in the embryonic fate map, we determined the mRNA accumulation pattern for the engrailed ($en$) segmentation gene in ~ stage 11 embryos. The $en$ mRNA wild type pattern is dependent on normal signaling in the Torso pathway. We found 36.1% (n=169) of the embryos from $tor^{RL3}/+$; $FL \, DRaf$ mothers had at least one deleted, fused or broken $en$ central abdominal stripe(s) (Figure II-3E iv). The segmentation defects observed were most likely due to the expansion of head
and/or tail domains and indicative of the gain-of-function phenotype. In contrast, only 33 of 141 (23.4%) embryos from tor^{RL3/+}; DRaf^{AN114} mothers had such defects ($\chi^2=6.38030206$, $P<0.02$). Consistent with the cuticular phenotypes and en expression patterns, expansion in the domain of tll expression was observed more often for embryos from tor^{RL3/+}; FL DRaf mothers (32.0%, $n=50$) compared with those from tor^{RL3/+}; Draf^{AN114} females (13.1%, $n=76$; $\chi^2=5.50220096$, $P<0.02$, Figure II-3E iii’, iv’). Together, these in vivo studies consistently indicated that deletion of the N-terminus reduces the ability of DRaf to enhance the ectopic gain-of-function effects of tor^{RL3} and that these N-terminal residues could participate in Torso RTK signaling.

The contribution of DRaf’s N-terminus to signaling appears to be dependent on the activity of the Torso receptor: Embryos lacking normal maternal Trunk (Trk) activity show little or no posterior tll expression (occasionally, trace-level tll expression was detected in 3-4 cells at the posterior embryo tip) and exhibit an abnormal expression pattern for the engrailed (en) segmentation gene. Instead of 3 thoracic and 9 abdominal en stripes as observed for embryos from wild type mothers (Figure II-4C i’), these Trk-deficient embryos have 3 thoracic and only 6-7 abdominal en stripes (Figure II-4C ii’), and exhibit terminal defects with deletion of all posterior structures (A8 denticle belt and Filzkörper; Figure II-4C ii). Interestingly, overexpression of FL DRaf partially restores the A8 denticle belt structure in embryos from trunk^{I/trunk^{I}} (trk^{-/-}; lacks the last 16 amino acids) mothers (Figure II-4C iv). This result is consistent with our unpublished findings (K. H. BAEK and L. AMBROSIO) using the trk^{I} allele (encodes the first 89 amino acids) and a different method. Rescue of posterior structures for some Trk-deficient embryos was found after injection of wild type DRaf mRNA, also suggesting that accumulation of exogenous DRaf proteins promotes signaling in
this \textit{trk}⁻ background. However, expression of the $\text{DRaf}^{\text{AN114}}$ transgene at a similar level failed to rescue the A8 denticle band defect in embryos from \textit{trk}⁻⁻ mothers (Figure II-4 A, C iii). We repeated these experiments using two additional \textit{FL DRaf} and $\text{DRaf}^{\text{AN114}}$ transgenic lines and observed similar results ($\chi^2=82.8574882$, P<0.001; Figure II-4D). Thus, \textit{FL DRaf} appears to possess greater activity compared with $\text{DRaf}^{\text{AN114}}$. To test if these cuticle phenotypes were correlated with segmentation gene expression, we examined the accumulation of $\text{en}$ mRNA in early gastrulating embryos. All embryos from \textit{trk}⁻⁻; $\text{DRaf}^{\text{AN114}}$/\text{DRaf}^{\text{AN114}} mothers had 3 thoracic and only 6-7 abdominal $\text{en}$ stripes (Figure II-4C iii’), while some embryos (12.5%, n=24) derived from \textit{trk}⁻⁻; \textit{FL DRaf}/\textit{FL DRaf} females had 3 thoracic and 8 abdominal $\text{en}$ stripes ($\chi^2=3.97058824$, P<0.05; Figure II-4C iv’). This indicated that addition of \textit{FL DRaf} but not $\text{DRaf}^{\text{AN114}}$ proteins partially restored posterior Torso RTK signaling in the \textit{trk}⁻ background. Consistent with this hypothesis, as shown in Figure II-4C iii’’ and iv’’, partial rescue of posterior $\text{tll}$ mRNA expression was detected in some cellular blastoderm embryos derived from \textit{trk}⁻⁻; \textit{FL DRaf}/\textit{FL DRaf} females (8.1%, n=37) but not for those derived from \textit{trk}⁻⁻; $\text{DRaf}^{\text{AN114}}$/\text{DRaf}^{\text{AN114}} mothers (n=52, $\chi^2=4.36329353$, P<0.05). Together, these data consistently suggest that the absence of the N-terminal segment reduces the signaling potential of DRaf and the N-terminus can contribute to Torso RTK terminal signaling in a positive manner.

Sprenger \textit{et al.} (1993) previously observed a low level of Torso receptor phosphorylation in eggs derived from \textit{trk} loss-of-function, but not \textit{tor} loss-of-function females. Therefore, a small amount of Torso signal activity may exist in our \textit{trk}⁻ background. This may be due to 1.) the presence of active Torso-like (Tsl) ligand, 2.) potential residual Trunk activity, considering the molecular lesion of the \textit{trk}¹ allele we used (lacks only the last
16 amino acids), or 3.) the intrinsic activity of the Torso receptor. This activity could allow rescue of posterior structures by FL DRaf expression. If so, the contribution of the N-terminus to terminal signaling is likely sensitive to such upstream events. Thus, we examined the consequences of DRaf expression in embryos from $tor^{XR1}/tor^{XR1}$ ($tor^{-/-}$; protein null) mothers that lacked the Torso receptor. We found that expression of FL DRaf or DRaf$^{AN114}$ failed to restore terminal structures for these embryos (Table II-1). This indicates the contribution of DRaf’s N-terminal residues to signaling is dependent on activity of the receptor.

The N-terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure: We analyzed the amino acid sequence of DRaf’s N-terminus using several bioinformatics tools, to obtain hints regarding its structure, and perhaps mechanism(s) of its functional role(s). A PROSITE motif search showed a putative protein kinase C (PKC) phosphorylation site within the “T-S-K” motif of the N-terminus (positions 60-62; SIGRIST et al. 2002). Phosphorylation site prediction by NetPhos 2.0 suggested that the Thr in this “T-S-K” motif had a high phosphorylation potential (Figure II-5A; BLOM et al. 1999). Predictions of secondary structure for the N-terminal region using GORV, PHD and Predator indicated a high $\alpha$-helical propensity (Figure II-5A; FRISHMAN and ARGOS 1996; ROST et al., 1994; GARNIER et al., 1996; COMBET et al. 2000).

A blastp search of other organisms with DRaf’s N-terminal sequence identified honeybee Raf, chick C-Rmil, and BRaf proteins of sea urchin, zebrafish, frog and human. The region containing amino acid 19 to 77 of DRaf showed homology between candidates. These sequences were aligned using ClustalW, and are shown in Figure II-5A (COMBET et al. 2000). Overall, the amino acids showed 18.6% identity and 47.5% similarity, and we term
this region CRN, Conserved Region N-terminal. Interesting features of CRN include the putative phosphorylation site, and a propensity to form two α-helical structures. This suggests that the N-terminal region of DRaf may have function(s) shared by other BRaf proteins.

The conserved structural features, including α-helical propensity, may be related to the functional role(s)/regulatory mechanism(s) of DRaf’s N-terminus. To confirm the prediction attained by bioinformactics tools, circular dichroism (CD) spectral measurement of the N-terminal part of DRaf (amino acid 1-117, DRaf\textsuperscript{N117}) was performed after its expression and purification \textit{in vitro} (see MATERIALS and METHODS). As shown in Figure II-5B, a bilobed spectrum with local minima at ~209.4 nm and at ~221.4 nm was observed, indicating the relatively high content of helical secondary structure for DRaf’s N-terminus. The estimated helix content of DRaf\textsuperscript{N117} is ~77% based on the CD spectra data analysis using DICHROWEB (WHITMORE, \textit{et al.}, 2008, \url{http://dichroweb.cryst.bbk.ac.uk/html/home.shtml}). This result bolsters the predictions by GORV, PHD and Predator.

The \textbf{N-terminus assists in association of DRaf’s RBD with small GTPases Ras1 and Rap1 in vitro:} Fischer \textit{et al.} (2007) found that association of BRaf with HRas was facilitated by N-terminal sequences, \textit{in vitro}. To examine if the presence of DRaf’s N-terminus can affect Ras1 binding, we tested interaction between Ras1ΔCAAX and DRaf’s RBD (Ras binding domain) using the yeast two-hybrid assay. A stronger interaction with Ras1ΔCAAX was detected when N-terminal residues were linked to RBD in both solid-support (data not shown) and liquid quantitative β-Galactosidase assays (P<0.05, \textit{t test}; Figure II-6B), suggesting that the N-terminus may assist in association of DRaf with Ras1. This is consistent with results obtained for BRaf (FISCHER \textit{et al.} 2007).
No direct interaction was detected between Ras1\(\Delta\)CAAX and isolated N-terminal residues of DRaf (Figure II-6B, and solid-support data not shown). Thus, the N-terminus appears to contribute to Ras1 binding, but as an isolated protein fragment cannot directly interact with Ras1. Arg174 located in DRaf’s RBD region is essential for its association with Ras1 and substitution of Arg174 to Leu in RBD (RBD\(^{R174L}\)) abolishes Ras1 binding (FABIAN et al. 1994; LI et al. 1998). We found that N-terminal residues cannot restore Ras1 interaction when linked with RBD\(^{R174L}\) (Figure II-6B, and solid-support data not shown). This indicated the effects of the N-terminus were dependent on interaction between RBD and Ras1.

Moreover, we tested the idea that the conserved CRN region (19-77) might be essential for the contribution of DRaf’s N-terminus to Ras1\(\Delta\)CAAX binding. Deletion of the first seventeen N-terminal amino acids (\(\Delta17NRBD\)) did not change Ras1\(\Delta\)CAAX binding. However, if N-terminal amino acids including CRN were removed (\(\Delta77NRBD\)), interaction with Ras1\(\Delta\)CAAX was reduced to a level similar to that observed by deletion of the entire N-terminus (amino acids 1-114; Figure II-6A, B). Together, these findings suggested the hypothesis that N-terminal residues of DRaf can assist in Ras1 interaction through a CRN-mediated mechanism(s).

The small GTPase Rap1, a close relative of Ras1, is known to interact with DRaf and play a role in Torso RTK signaling in vivo (MISHRA et al. 2005). To examine if DRaf’s N-terminus affects its association with Rap1, we tested interaction between Rap1\(\Delta\)CAAX and DRaf’s RBD (Figure II-6C). A stronger interaction with Rap1\(\Delta\)CAAX was detected when the N-terminus was linked to RBD, similar to our findings with Ras1 (P<0.05, \(t\) test). Furthermore, the conserved CRN region (19-77) seems essential for the contribution of the
N-terminus to this interaction with Rap1, suggesting a CRN-mediated mechanism(s) may be a general feature for its binding to both Ras1 or Rap1.

**Discussion**

In our study, a novel region (amino acids 19-77) within DRaf’s N-terminus, conserved for Raf genes of most invertebrates and BRaf genes of vertebrates, was identified and termed Conserved Region N-terminal (CRN). This conserved region has not been described by others, but potential roles for the extended N-terminus have been proposed in two reports. Terai and Matsuda (2006) found that in Hela cells, the N-terminus of BRaf may mediate Raf dimerization to generate BRaf-BRaf or BRaf-CRaf complexes, and play an important regulatory role in calcium-induced BRaf activation. However, Fischer *et al.* (2007) reported that deletion of BRaf’s N-terminus did not affect BRaf-CRaf dimer formation. Instead, they found that N-terminal residues appeared to facilitate interaction with HRas *in vitro*. In accordance with their data, stronger interactions between DRaf’s RBD (Ras binding domain) and the small GTPase Ras1ΔCAAX were observed when N-terminal and RBD sequences were linked in our yeast two-hybrid analysis. This suggested that the N-terminus might assist in Ras1 binding. Furthermore, the identity of specific residues in the N-terminus that might participate in Ras1 binding were mapped to the CRN region (amino acid 19-77). Two known Raf motifs, RBD and CRD (cysteine rich domain), are involved in Raf’s interaction with Ras. Our studies, and results obtained by Fischer *et al.* (2007) using BRaf, suggest that the N-terminal residues of DRaf and BRaf proteins, particularly the CRN region, might be another element that plays a role(s) in Ras-Raf coupling.
The small GTPase Rap shares with Ras nearly identical Raf binding regions that comprise switch 1 and the lipid moiety (HARIHARAN 2005). Rap functions as an antagonist of Ras in regulating CRaf activity (COOK et al. 1993), but can activate BRaf in a parallel way with Ras (OHTSUKA et al. 1996). Isoform-specific features of different Raf family members may explain their distinct responses to Rap. In flies, both Ras1 and Rap1 can interact with and activate DRaf (MISHRA et al. 2005). Thus, it was reasonable to test whether DRaf’s N-terminus including CRN might also assist in Rap1 binding. In agreement with this idea, stronger interaction between RBD and Rap1ΔCAAX was observed when DRaf’s CRN and RBD sequences were linked in vitro, further suggesting that the N-terminus may contribute to both Ras1 and Rap1 binding potentially through a CRN-mediated mechanism(s) in vivo.

What is the molecular mechanism(s): No direct interaction between Ras1 or Rap1 and the isolated DRaf N-terminal segment (amino acids 1-117) was detected, or when the N-terminus was linked with the Ras1/Rap1 binding-deficient RBDR174L. Thus, the contribution of DRaf’s N-terminal residues to Ras1 and Rap1 binding requires the presence of RBD. It is possible that the CRN-containing N-terminus may assist in Raf-Ras interaction by making RBD more accessible to Ras1 and/or in a sequential manner, subsequent to RBD-Ras1 interaction, by stabilizing the RBD-Ras1 complex. Deletion of CRN may result in conformational or structural changes that reduce Ras1 binding affinity. Structural analysis of these complexes may provide important clues and help to understand the molecular mechanism(s) by which CRN assists in Ras-Raf interaction. Our computational analysis suggested conserved CRN has the propensity to form two α-helical structures (α1 and α2; Figure II-5A) and contains a putative phosphorylation motif “T-S-K” located in α2. In agreement, DRaf’s N-terminus (amino acids 1-117) was folded in vitro and had a high
content of helical secondary structure (Figure II-5B). These findings may help to establish a basis for future determination of molecular structure.

Although no verified binding partner(s) for DRaf or BRaf’s N-terminus has been identified, it is still possible that CRN may interact with other regulatory factors in vivo, that may affect Ras or Rap binding and/or function in activation of DRaf and BRaf. If so, the conserved structural features of CRN most likely relate to these regulatory events in vivo. Site-directed mutagenesis of conserved sites/motifs could provide useful information regarding the molecular mechanism(s) of CRN’s role in the activation of DRaf and BRaf.

**Torso RTK signal is differentially elevated by overexpression of FL DRaf and DRaf\(^{\text{AN114}}\) in vivo:** We initiated our in vitro studies of DRaf’s N-terminus based on our in vivo findings using both loss- and gain-of-function genetic assays that deletion of N-terminal residues consistently reduces DRaf’s signal potential in the Torso pathway. When expressed at high levels, FL DRaf enhanced the gain-of-function effects of the \(\text{tor}^{\text{RL3}}\) allele much more significantly than DRaf\(^{\text{AN114}}\). In embryos from \(\text{trk}^-/\cdot\) mothers, addition of FL DRaf, but not DRAF\(^{\text{AN114}}\), partially restored the A8 denticle belt structure (Figure II-4). These findings indicate that the N-terminus can play a positive role(s) in Torso RTK signaling. Interestingly, the contribution of DRaf’s N-terminus in the Torso pathway appeared to be dependent on upstream receptor activity, suggesting its role in transmission of the signal. Together with our yeast two-hybrid data, as well as the results obtained by Fischer et al. (2007) for BRaf, we propose that the presence of N-terminal residues may facilitate the association of DRaf with the upstream regulators Ras1 and Rap1, thereby assisting in transmission of the RTK signal in vivo.
For instance, in the trk⁻ background, a small amount of active GTP-Ras1 and GTP-Rap1 are likely present, mostly due to activation by residual upstream Trunk activity, the presence of Torso-like ligand, and/or the intrinsic activity of the Torso receptor. The trk¹l mutation used in this analysis results in protein truncation at the last 16 amino acids. It is possible that over-expression of FL DRaf proteins in this background increases the likelihood of interaction between abundant DRaf proteins and membrane bound GTP-Ras1 or GTP-Rap1. This in turn, could elevate RTK signal and partially restore development of A8 posterior structures in some embryos. On the other hand, deletion of the N-terminus could destabilize Ras1-DRaf (or Rap1-DRaf) coupling or decrease the duration of interaction, resulting in reduced DRaf signal transmission. This may explain why expression of DRaf<sup>AN114</sup> failed to rescue the A8 denticle belt in embryos from trk⁻/⁻ mothers.

**Why are only minor differences detected in vivo between FL DRaf and DRaf<sup>AN114</sup>:**

Previously, an auto-inhibitory role had been assigned to residues compromising the first half of the DRaf protein, in addition to their functions in promoting its activity. Deletion of the N-terminal 1-272 (including the N-terminus and CR1) or 1-402 (including the N-terminus, CR1 and CR2) of DRaf at least partially relieved these negative effects (BaeK et al. 1996). Here, although removal of the N-terminal 1-114 residues did not result in constitutive DRaf<sup>AN114</sup> activity in embryos lacking the maternal Torso receptor (Table II-1), it is still possible that the N-terminus may contribute to auto-inhibitory effects. Together with CR1 and CR2, these N-terminal residues (1-114) may help maintain DRaf’s inactive conformation. If so, the N-terminus might play dual roles, both positively and negatively regulating DRaf. Therefore, its contribution to signaling may be neutralized by this auto-inhibition and consequently result in a subtle in vivo effect. If so, selective mutagenesis of the “inhibitory” motifs/sites in the N-
terminal region or removal of other co-factors involved in its negative regulation may amplify signaling differences between FL DRaf and DRaf$^{AN114}$.

Ras binding has been thought crucial to recruit Raf to the plasma membrane and promote its RTK signaling activity. However, the Drosophila Torso pathway appears tolerant of alterations in Ras1-DRaf coupling (HOU et al. 1995). Draf$^{C110}$ has a R174L point mutation in the RBD domain and likely comprised for Ras1 binding (LI et al. 1998). The RBD$^{R174L}$ is Ras binding-deficient in our yeast two-hybrid assay (Figure II-6B). However, tll expression patterns and cuticles of the embryos derived from mothers with $Draf^{C110}/Draf^{C110}$ germ cells were indistinguishable from those of wild type embryos (MELNICK et al. 1993), suggesting a mechanism(s) independent of RBD-Ras1 interaction might function in recruiting DRaf to the plasma membrane. In agreement with this model, Rizzo et al. (2000) found membrane translocation of CRaf could be mediated by its interaction with phosphatidic acid (PA), and independent of Ras binding. This PA binding site is also conserved in ARaf, BRaf, and DRaf. Thus, Draf$^{C110}$ could be recruited to the cell surface by associating with PA. Moreover, it is known that Raf’s CRD participates in Ras binding through its interaction with the lipid moiety of Ras (WILLIAMS et al. 2000; THAPAR et al. 2004). Once at the membrane, it is also possible that the interaction between Draf$^{C110}$’s CRD and Ras1 could further promote its membrane attachment and result in relatively normal Torso signal production. In this study, the presence of RBD, CRD and the potential PA binding site may be sufficient to promote DRaf’s activation in Torso signaling. This may explain why at ~ endogenous wild type protein level maternally expressed DRaf$^{AN114}$ is able to rescue the embryonic terminal defects of $Draf^{H1-29}$ mutants (Figure II-1B, C and E). Together, considering the Torso pathway’s tolerance of alterations in Ras1-DRaf coupling
and the minor role DRaf’s N-terminus plays in Ras1 binding, it is reasonable that the phenotypic consequences of removing these N-terminal residues (DRaf\textsuperscript{AN114}) are not great in Torso signaling.

The subtle phenotypic effects of DRaf’s N-terminus could also be due to compensation provided by potential autoregulatory feedback or alternative redundant processes in the \textit{in vivo} system. In our study, the expression of DRaf proteins at a low level (~1/4 endogenous wild type level) appeared to sensitize the assay system. We found deletion of the N-terminus seemed to increase the threshold of DRaf protein levels required for normal signaling. Furthermore, by adding one copy of the ectopic \textit{tor}\textsuperscript{RL3} allele or removing wild type maternal Trunk activity we apparently increased the sensitivity of the Torso pathway. These allowed the embryonic terminal system to display enhanced differences between FL DRaf and DRaf\textsuperscript{AN114} proteins.

\textbf{The biological implications of the N-terminal region:} Why is this N-terminus with its “subtle” functional effects conserved during evolution, and what is its biological relevance? There are numerous RTK pathways functioning in Drosophila cellular and developmental processes. In spite of the identical Ras-Raf-MEK signal cassette they share, these RTK pathways can lead to different biological responses. Previous studies indicated that such specificity might be due to the difference in the intensity and/or duration of the signal (Ghiglione \textit{et al.} 1999; Kerkhoff and Rapp 1998; Woods \textit{et al.} 1997; Woods \textit{et al.} 2001). This suggested that the magnitude of Raf signal could function as a critical determinant of biological responses. Participation of multiple DRaf elements in Ras1 or Rap1 binding could be a good strategy to modulate its activity. Normally, tight association with Ras1 or Rap1 through RBD and CRD regions is required and sufficient to initiate the
activation of DRaf, while minor adjustments/regulation of interaction by the CRN region could optimize signaling potential and reduce variability. Thus, the extended N-terminus including CRN may play a role(s) as one element in a multi-domain effort to promote DRaf’s interaction with Ras1 and Rap1, participating and assisting in regulation to reliably attain maximal signal output.

**Acknowledgement**

We gratefully acknowledge the gift of the Raf antibody from Deborah K Morrison, fly strains from Drosophila Stock Center (Bloomington) and cDNA clones from Drosophila Genomics Research Center. We thank Yosef Scolnik for assistance in analysis of CD spectra, Clark Coffman and Lei Li for helpful discussions.

**Literature Cited**


COOK S. J., B. RUBINFELD, I. ALBERT, F. MCCORMICK, 1993 RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. EMBO J. 12:3475-3485.


FRISHMAN D., P. ARGOS, 1996 Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence. Protein Eng. 9:133-142


HARIHARAN, I. K., 2005 Ras and Rap: are former enemies now friends? Dev Cell. 8:303-304.


ROMMEL, C., G. RADZIWIŁŁ, K. MOELLING and E. HAFEN, 1997 Negative regulation of
Raf activity by binding of 14-3-3 to the amino terminus of Raf in vivo. Mech Dev. 64:
95-104.

ROST B., C. SANDER, R. SCHNEIDER, 1994 PHD—an automatic mail server for protein

ROY, F., G. LABERGE, M. DOUZIECH, D. FERLAND-MCCOLLOUGH and M. THERRIEN,
2002 KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev.

R325–R327.


SCHÜPBACH, T. and E. WIESCHAU, 1989 Female sterile mutations on the second
chromosome of Drosophila melanogaster. I. Maternal effect mutations. Genetics. 121:
101-117.

PROSITE: a documented database using patterns and profiles as motif descriptors.
Brief Bioinform. 3: 265-274.

torso encodes a putative receptor tyrosine kinase. Nature. 338:478-483

SPRENGER, F., M. M. TROSCLAIR and D. K. MORRISON, 1993 Biochemical analysis of torso
and D-raf during Drosophila embryogenesis: implications for terminal signal

effects of hyper- and hypoactivity mutations in the Drosophila pattern gene torso.
Science 243:1062-1066.

TAUTZ, D. and C. PFEIFLE, 1989 A non-radioactive in situ hybridization method for the
localization of specific RNAs in Drosophila embryos reveals translational control of the

TERAI, K. and M. MATSUDA, 2006 The amino-terminal BRaf-specific region mediates

THAPAR, R., J. G. WILLIAMS, S. L. CAMPBELL, 2004 NMR characterization of full-length
farnesylated and non-farnesylated H-Ras and its implications for Raf activation. J Mol
Biol. 343:1391-1408.


### Tables

**TABLE 1.** Expression of FL DRaf or DRaf<sup>AN114</sup> did not result in rescue of the A8 denticle belt in embryos produced by tor<sup>XR1</sup>/tor<sup>XR1</sup> females

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>A6/A7 segment</th>
<th>A8 denticle belt</th>
<th>Total#</th>
</tr>
</thead>
<tbody>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;</td>
<td>117 (100)</td>
<td>0 (0)</td>
<td>117</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; DRaf&lt;sup&gt;AN114&lt;/sup&gt; #1/DRaf&lt;sup&gt;AN114&lt;/sup&gt; #3</td>
<td>72 (100)</td>
<td>0 (0)</td>
<td>72</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; DRaf&lt;sup&gt;AN114&lt;/sup&gt; #2/DRaf&lt;sup&gt;AN114&lt;/sup&gt; #2</td>
<td>77 (100)</td>
<td>0 (0)</td>
<td>77</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; DRaf&lt;sup&gt;AN114&lt;/sup&gt; #3/DRaf&lt;sup&gt;AN114&lt;/sup&gt; #3</td>
<td>98 (100)</td>
<td>0 (0)</td>
<td>98</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; FL Draf #1/FL Draf #1</td>
<td>61 (100)</td>
<td>0 (0)</td>
<td>61</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; FL Draf #2/FL Draf #2</td>
<td>70 (100)</td>
<td>0 (0)</td>
<td>70</td>
</tr>
<tr>
<td>tor&lt;sup&gt;XR1&lt;/sup&gt;/tor&lt;sup&gt;XR1&lt;/sup&gt;; FL Draf #3/FL Draf #3</td>
<td>81 (100)</td>
<td>0 (0)</td>
<td>81</td>
</tr>
</tbody>
</table>
Figure Legends

Figure II-1. Rescue of posterior structures in embryos derived from Draf<sup>−/−</sup> female germ cells by expression of full-length D Raf or truncated D Raf<sup>ΔN114</sup> transgenes. (A) Schematic representations of full-length DRaf (FL DRaf) with 739 amino acids and truncated DRaf<sup>ΔN114</sup> proteins. In addition to the 3 conserved regions (CR1, CR2 and CR3), FL DRaf has an extended N-terminus. (B) Western analysis of embryonic DRaf proteins from eggs produced by Draf<sup>11-29</sup>/Draf<sup>11-29</sup> (Draf<sup>−/−</sup>), wild type (WT), Draf<sup>−/−</sup>; D Raf<sup>ΔN114</sup> (3 independent lines, L1 with ~1X endogenous DRaf level, #a and #b with ~1/4 endogenous DRaf level) and Draf<sup>−/−</sup>; FL DRaf (2 independent lines, #a and #b with ~1/4 endogenous DRaf level) germline clone bearing females. Lysate was prepared from eggs at 0-3 hours after egg deposition. Full-length DRaf (~90 KD) and DRaf<sup>ΔN114</sup> (~77 KD) proteins are denoted by arrows. Lysate of eggs from Draf<sup>−/−</sup> germ cells was used as a negative control. α-tubulin (α-tub) was used as the loading control. (C) A bar graph representing relative levels of DRaf proteins normalized with α-tubulin. (D) Cuticles of mature embryos derived from wild type (WT), Draf<sup>−/−</sup>, Draf<sup>−/−</sup>; D Raf<sup>ΔN114</sup> and Draf<sup>−/−</sup>; FL DRaf female germ cells are shown in left panels. Accumulation of tll mRNA in cellular blastoderm embryos was detected by in situ hybridization (Right panels). Posterior tll expression is solely dependent on the Torso pathway and used as a marker for Torso RTK signaling. Anterior expression of tll is regulated by another pathway(s) in addition to Torso signaling, is more complex, and is used as an internal control for staining here. Wild type embryos show (i) normal Filzkörper structure (arrow), (i’) tll mRNA accumulation at the posterior (approximately 0-15% embryo length, EL), and an anterior head “stripe” (~75-85% EL). Embryos derived from Draf<sup>−/−</sup>
germ cells lack (ii) posterior structures (A8 denticle belt, Filzkörper) and (ii’) posterior tll mRNA expression. (iii) An embryonic cuticle derived from Draf\textsuperscript{−/−}; DRaf\textsuperscript{AN114} germ cell lacks the Filzkörper structure. (iii’) A reduced posterior tll expression domain is at ∼0-8% EL in an embryo from Draf\textsuperscript{−/−}; DRaf\textsuperscript{AN114} germline clone bearing mother. (iv) Filzkörper structure (arrow) and (iv’) normal expression pattern of tll mRNAs are rescued by FL DRaf expression for embryos derived from Draf\textsuperscript{−/−}; FL DRaf maternal germ cells. (E) A bar graph showing the percentage of embryos without Filzkörper (Fk) structures. When expressed at low maternal level (∼1/4 endogenous level), embryos without Fk were found more often for those that inherited truncate Draf\textsuperscript{AN114} rather than full length DRaf proteins (χ\textsuperscript{2}=9.91976318, P<0.01).

**Figure II-2. Verification of DRaf protein quantitation assays and stability of DRaf proteins in early embryos.** (A) Three samples representing lysates of 6, 12, and 18 eggs for each line (Draf\textsuperscript{−/−}; DRaf\textsuperscript{AN114} #a and Draf\textsuperscript{−/−}; FL DRaf #a) were loaded for western blot analysis. Full-length DRaf (∼90 KD) and DRaf\textsuperscript{AN114} (∼77 KD) proteins are denoted by arrows. Lysate of eggs from Draf\textsuperscript{11-29}/Draf\textsuperscript{11-29} (Draf\textsuperscript{−/−}) germ cells was used as a negative control. α-tubulin levels were probed as a loading control. (B) Bar graph showing relative intensity of DRaf (dark bar) and α-tubulin (gray bar) bands. (C) A bar graph depicting normalized DRaf protein level from (A). (D) Western analysis of embryonic DRaf proteins from eggs collected at 0-1, 1-2 and 2-3 hours after deposition and produced by Draf\textsuperscript{−/−}, Draf\textsuperscript{−/−}; DRaf\textsuperscript{AN114} (line #a) and Draf\textsuperscript{−/−}; FL DRaf (line #a) germline-bearing females. Full-length DRaf (∼90 KD) and DRaf\textsuperscript{AN114} (∼77 KD) proteins are denoted by arrows. α-tubulin was used as the loading control. (E) Normalized DRaf protein level from (D) is shown in this bar graph depicting the stable accumulation of these DRaf proteins.
Figure II-3. Gain-of-function effects of tor$^{RL3}$ are differentially enhanced by expression of FL DRaf and DRaf$^{AN114}$ transgenes. (A) Western analysis of embryonic DRaf proteins from eggs (0-3 hours) produced by tor$^{RL3/+}$, tor$^{RL3/+}$; DRaf$^{AN114}$ (3 independent lines, #1, #2, #3), or tor$^{RL3/+}$; FL DRaf (3 independent lines, #1, #2, #3) females at 29°C. Full-length DRaf (~90 KD) and DRaf$^{AN114}$ (~77 KD) proteins are denoted by arrows. α-tubulin was used as the loading control. (B) Normalized DRaf protein level from (A) is shown as a bar graph. (C) Cuticles of mature embryos are shown. (i) A wild type (WT) embryo exhibits normal cuticle pattern with 8 abdominal denticle belts. (ii) An embryonic cuticle derived from tor$^{RL3/+}$; FL DRaf mother has one broken abdominal denticle band (arrow head), and is missing one central abdominal denticle belt (arrow). (D) Percent of embryonic cuticles with gain-of-function phenotypes is shown in this bar graph. Gain-of-function effects of tor$^{RL3}$ were differentially enhanced by FL DRaf and DRaf$^{AN114}$ proteins ($\chi^2=51.063837$, P<0.001). (E) Expression of engrailed (en) at ~stage 11 (Left panels) and accumulation of tailless (tll) mRNA at cellular blastoderm stage (Right panels) in embryos from WT, tor$^{RL3/+}$, tor$^{RL3/+}$;DRaf$^{AN114}$, or tor$^{RL3/+}$;FL DRaf mothers: Examples of embryos derived from (i) WT, (ii) tor$^{RL3/+}$, and (iii) tor$^{RL3/+}$;DRaf$^{AN114}$ mothers exhibit normal en mRNA pattern with 3 thoracic (T1-3) and 9 abdominal (A1-9) expression stripes. (iv) An embryo from tor$^{RL3/+}$; FL DRaf mother with partial deletion of en stripes (arrow) in a region that gives rise to central abdominal segmental pattern is shown. Examples of embryos derived from (i’) WT and (ii) tor$^{RL3/+}$ mothers exhibiting a normal tll mRNA pattern. (iii’) An embryo from a tor$^{RL3/+}$; DRaf$^{AN114}$ mother shows slightly expanded posterior expression domain of tll. (iv’) An embryo derived from tor$^{RL3/+}$;FL DRaf females exhibits expanded domain of tll expression for both anterior and posterior regions.
Figure II-4. Effects of FL DrAf and DrAf<sup>AN114</sup> expression on posterior development in embryos derived from trk<sup>1</sup>/trk<sup>1</sup> mothers. (A) Western analysis of embryonic DrAf proteins from eggs (0-3 hours) produced by DrAf<sup>11-29/11-29</sup> (DrAf<sup>-/-</sup>), wild type (WT), trk<sup>1</sup>/trk<sup>1</sup> (trk<sup>-/-</sup>), DrAf<sup>AN114/AN114</sup> (3 lines, #1/#3, #2/#2, #3/#3) and trk<sup>-/-</sup>; FL DrAf/FL DrAf females. Full-length DrAf (~90 KD) and DrAf<sup>AN114</sup> (~77 KD) proteins are denoted by arrows. Embryonic lysate from DrAf<sup>-/-</sup> germline clone females was used as a negative control. α-tubulin was used as the loading control. (B) Normalized DrAf protein level from (A) is shown in the bar graph. (C) Representative cuticles of mature embryos derived from wild type (WT), trk<sup>-/-</sup>, trk<sup>-/-</sup>; DrAf<sup>AN114/AN114</sup>, or trk<sup>-/-</sup>; FL DrAf/FL DrAf females are shown (Left panels). Accumulation of en (Middle panels) and tll (Right panels) mRNAs were detected by in situ hybridization. (i) A wild type (WT) embryo has normal cuticle pattern with 8 abdominal denticle belts and Filzkörper structure. (i') A WT embryo at gastrula stage has 3 thoracic (T1-3) and 9 abdominal (A1-9) en stripes. (i'') A WT embryo at cellular blastoderm stage exhibits a normal posterior expression domain of tll. (ii) Cuticle of a mature embryo from a trk<sup>-/-</sup> mother is missing posterior structures (A8 segment, Filzkörper). (ii') A gastrulae embryo from a trk<sup>-/-</sup> mother has abnormal en expression pattern with only 7 abdominal stripes. (ii'') A cellular blastoderm embryo from a trk<sup>-/-</sup> mother lacks posterior tll expression. (iii) An embryonic cuticle from a trk<sup>-/-</sup>; DrAf<sup>AN114/AN114</sup> mother lacks posterior structures (A8 denticle belt, Filzkörper). (ii') A gastrula embryo from a trk<sup>-/-</sup>; DrAf<sup>AN114/AN114</sup> mother has only 7 abdominal en stripes. (ii'') A cellular blastoderm embryo from a trk<sup>-/-</sup>; DrAf<sup>AN114/AN114</sup> mother lacks posterior expression of tll mRNA. Expression of FL DRaf (iv) restores the A8 denticle belt (arrow), (iv') partially rescues the en mRNA pattern (8 abdominal stripes), and (iv'')...
posterior *tll* expression (arrow) in embryos lacking maternal Trk activity. (D) Effect of *FL DRaf* or *DRaf\(^{\Delta N114}\)* transgene expression on A8 denticle development in embryos derived from *trk\(^{-}\)* mothers (percent of embryonic cuticles with A8 denticle belt). Shown are results using transgenic *DRaf\(^{\Delta N114}\)* or *FL DRaf* lines that express DRaf protein at similar levels. Expression of exogenous FL DRaf, but not DRaf\(^{\Delta N114}\), results in partial rescue of A8 denticle belt in some embryos derived from *trk\(^{-}\)* mothers (\(\chi^2=82.8574882, P<0.001\)).

**Figure II-5. The N-terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure.** (A) Drosophila Raf (NP_525047; 739 amino acids) has in addition to its three conserved regions (CR1-3), an extensive N-terminus. A novel region (amino acids 19-77) within the N-terminus is conserved in honeybee Raf (*A. mellifera* XP_396892); frog BRaf (*X. laevis* AAU29410); chicken C-Rmil (*G. gallus* CAA47436); human BRaf (*H. sapiens* NP_004324); zebrafish BRaf (*D. rerio* BAD16728), sea urchin BRaf (*S. purpuratus* XP_781094), and termed Conserved Region N-terminal (CRN). Sequences of CRN were aligned using ClustalW (identities were denoted as “*”, strong and weak similarities were denoted as “:” and “.”, respectively in consensus line, [http://www.ebi.ac.uk/tools/clustalw/](http://www.ebi.ac.uk/tools/clustalw/)), and the conserved residues were shaded using BOXSHADE (identities in black, similarities in gray, [http://www.ch.embnet.org/software/box.form.html](http://www.ch.embnet.org/software/box.form.html)). Secondary structure prediction with GORV indicates CRN has the propensity to form two \(\alpha\)-helices (\(\alpha_1\) and \(\alpha_2\)). The putative PKC phosphorylation site DRaf’s Thr60 is framed. (B) Circular dichroism (CD) spectral measurement of DRaf’s N-terminus (amino acids 1-117) *in vitro*: The bilobed spectrum (arrows, local minima at ~209.4 nm and at ~221.4 nm) indicative of helical secondary structure is shown.
Figure II-6. Effects of the extended N-terminus of DRaf on Ras1 and Rap1 binding.

(A) Schematic representations of different DRaf constructs used for yeast two-hybrid analysis. (B) Interactions between DRaf’s RBDs and Ras1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Ras1ΔCAAX binding (P<0.05, \( t \) test). (C) Interactions between DRaf’s RBDs and Rap1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Rap1ΔCAAX binding (P<0.05, \( t \) test).
Figure II-1

A

B

C

D

E
Figure II-2
Figure II-3

A

B

C

D

E

en mRNA  tll mRNA

WT

torM2/4

torM2/4; DRaf \^ N114

torM2/4; FL DRaf

Relative Level of DRaf Proteins

% Gain in Cells with

\( \text{tor}^{M2/4} \)
\( \text{tor}^{M2/4}; \text{DRaf} \^ N114 \)
\( \text{tor}^{M2/4}; \text{FL DRaf} \)
Figure II-4
Figure II-5

A

Full-length DRaf

N 19 77 CR1 CR2 CR3

CRN

B

Mean Residue Ellipticity \([\Theta]\): [X10^-3 deg cm^2 dmol^-1]

Wavelength [nm]

-35
-30
-25
-20
-15
-10
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
65

190 200 210 220 230 240 250 260
Contribution of this work

Unless otherwise noted, the experiments in this paper were performed by Jian Ding. The circular dichroism assay in Figure II-5B was performed by Oren Tchaicheeyan. Jian Ding and Dr. Ambrosio contributed to the writing, reviewing and editing of this work.
CHAPTER 3. Initial Characterization of Drosophila Arf-like 1

Manuscript in preparation

Jian Ding¹, Yukiko Yamada², Clark Coffman², Linda Ambrosio¹*
¹ Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, 50011 and ² Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa, 50011
*Corresponding author (lima@iastate.edu)

Abstract

The gene encoding Arl1 was originally cloned from Drosophila. Studies on this small GTPase have mainly focused on mammalian and yeast Arl1 proteins and mostly were conducted in cultured cells or in vitro. Although it has been demonstrated that Arl1 can recruit a set of golgins to golgi through interaction with the GRIP domain and functions in endosome-to-TGN (trans- Golgi Network) trafficking, the roles Arl1 plays in the development of multi-cellular organisms are not well defined. In this study, we describe our strategies to characterize the function(s) of Arl1 in the developmentally tractable system of Drosophila. We found, like its homologues in mammals and yeast, epitope-tagged fly Arl1 was colocalized with Golgi markers both in vitro and in vivo. We analyzed three different arl1 mutants in Drosophila, and found that Arl1 is limitedly required during embryogenesis, although its mRNA accumulates ubiquitously in embryos. Most homozygous mutants of Arl1 died during larval-pupa stage, however, we did not detect specific phenotypes associated with lethality. Ubiquitous expression of constitutively active arl1Q70L-mCherry reduced viability of flies, but did not affect development of the eye, wing, leg or bristles. Accumulation of arl1Q70L-mCherry in nurse cells resulted in delay of border cell migration, suggesting that Arl1 may function in cellular morphogenetic movements.
Introduction

The small GTPase ADP-ribosylation factor-like (Arf-like, Arl) proteins have high sequence similarity with Arfs. Among all Arl family members, Arl1 (Arf-like 1) is the closest relative to the Arfs (Burd et al. 2004). In spite of some properties similar to ARF proteins, such as the ability to bind and hydrolyze GTP, Arl1 was found completely devoid of ADP ribosylation activity, suggesting Arl1 is not a functional homolog of ARF (Tamkun et al. 1991).

The gene encoding Arf-like 1 (Arl1), was first cloned from Drosophila (Arl72A). It appears essential for normal development of flies (Tamkun et al., 1991). Subsequently, Arl1 was identified in mammals and yeast, exhibiting 60~80% amino acid sequence identity with Drosophila Arl1 (Schurmann et al., 1994; Lee et al., 1997).

Like other G proteins, Arl1 has two guanine nucleotide binding forms, GTP-Arl1 and GDP-Arl1. The GTP-bound Arl1 is active, while GDP-Arl1 is inactive. In mammals and yeast, active Arl1 is associated with the trans-side of Golgi. It seems that association of Arl1 with Golgi is saturable and requires N-terminal glycine myristoylation. A mutant of Arl1 lacking the myristoylation motif (arl1^G2A, Gly2 Ala2) was absent from the Golgi (Lowe et al. 1996; Lee et al. 1997; Lu et al. 2001).

Active Arl1 can recruit a subset of golgins (Golgin-97, Golgin-245, Imp1p, etc.) onto Golgi membranes. These golgins have been suggested to function in maintaining the structure of the trans- Golgi network (TGN) and play a role(s) in endosome-to-TGN traffic (Wu et al. 2003; Lu et al. 2004; Yoshino et al. 2005). The active Arl1 binding site has been mapped into the specific coiled-coil GRIP domains at the C-terminus of these golgins. However, interaction between Arl1 and some of its putative effectors are still controversial.
Burguete et al. (2007) found that both Arl1 and another golgi localized small GTPase, Rab6, bound to the C-terminus of golgin GCC185 in vitro and may cooperate in recruitment of GCC185 to Golgi membrane. While in the study by Houghton et al. (2009), the interaction between Rab6 or Arl1 and GCC185 was not detected, and localization of GCC185 appeared to be independent of Rab6 and Arl1 in culture cells.

In CHO cells, exogenous expression of the GTPase-defective and constitutive active mutant arl1Q71L causes expansion of Golgi. While over-expression of arl1T31N, the dominant negative nucleotide exchange defective mutant of Arl1, leads to disassembly of Golgi apparatus (LU et al. 2001). Using a different approach, depletion of Arl1 by RNAi results in redistribution of specific TGN golgins including Goglin245, Golgin97 as well as SNARE proteins Vti1a and syntaxin 6 and 16, suggesting it functions in maintaining Golgi structures and intracellular traffic (NISHIMOTO-MORITA et al. 2009).

Overexpression of the GRIP region of golgin97, which is an effector of Arl1, abolished transport of E-cadherin from TGN to plasma membrane in Hela cells (LOCK et al. 2005). However, knockdown of Arl1 by RNAi did not change E-cadherin traffic (ZAHN et al. 2008). Although Arl1 was reported to regulate retrograde transport of Shiga toxin to TGN in cultured cells, no specific endogenous cargo was found for Arl1-mediated trafficking machinery so far.

In Drosophila, Eisman et al. (2006) identified a GRIP-domain containing protein, centrosomin’s beautiful sister (Cbs, CG4840), which might be the homolog of mammalian Golgin-97 based on their sequence similarity. This Cbs protein functions in the centrosome cycle during mitosis potentially in an Arl1-dependent manner in Drosophila embryos and may be a putative effector of Arl1. However, the role Arl1 plays in regulating Cbs, and how
Arl1 functions in development of the multi-cellular organism remains elusive.

In a genome-wide RNAi assay performed by Friedman and Perrimon (2006) on Drosophila S2 cells, Arl1 was found among the candidates, which could be involved in the Ras-Raf-MEK RTK signaling. However, such information from high-throughput screen needs to be confirmed by further analysis and validated in vivo.

In this study, genetic analysis of three different arl1 mutants suggested that Arl1 could function in cell morphogenesis. Although Arl1 was found to interact with DRaf’s CR2 region in our yeast two-hybrid assay, it seemed that Arl1 mutations did not alter DRaf signaling. While an essential gene, Arl1 plays limited roles during Drosophila embryogenesis likely due to its functional redundancy with other genes. We also propose potential strategies, which may be helpful for further characterization of Arl1’s function.

Materials and Methods

Fly stocks: In this study, Oregon R, arl14107 (Bloomington Stock Center, #4107), arl19.2BC, arl1 (obtained from Dr. Jim Kennison) lines were used. The “FLP-DFS” technique was utilized to generate arl19.2BC and arl14107 germline clones (CHOU and PERRIMON 1996). Drosophila stocks were raised at 25° on standard cornmeal medium.

Molecular Characterization of Mutations: DNAs from arl19.2BC/+ and arl14107/+ were used as templates for PCR reactions with primers P1-Forward (5’ GCGCCAGCAGACCAAAAG 3’) and P1-Reverse (5’GAAATAGCAGAATTGAGATTAG 3’) that compass the open reading frame (ORF) of the Arl1 gene. PCR products were cloned to the pGEM-T vector. For each allele, 10 colonies were picked up and sequenced.
**DNA constructs:** The YFP-Golgi construct was kindly provided by Dr. Cungui Mao. T30N and Q70L mutations in Arl1 were generated by PCR-based site-directed mutagenesis and confirmed by sequencing. EGFP-tagged Arl1s were constructed by inserting DNA encoding Arl1 or Δ17arl1 into the pEGFP-N1 vectors (Clontech). The coding sequence for Myc or mCherry epitopes were fused to the C-terminus of arl1 cDNA inserted into the pMT vector to generate Arl1-Myc or Arl1-mCherry constructs.

**Transgenic design:** DNA encoding mCherry-tagged wild type Arl1 was cloned into the polylinker site of the P-element transformation vector pCaSpeR-HS83. The constitutively active heat-shock 83 gene promoter was used to drive the expression of wild type Arl1-mCherry. The arl1Q70L-mCherry cDNA was cloned into the pUASp vector and used to generate the UASp-arl1Q70L-mCherry construct. The UASp promoter allows spatio-temporal expression when combined with different Drosophila Gal4 drivers. Transgenic lines were generated by P-element-mediated transformation.

**Transfection in S2 cells:** Drosophila S2 cells (obtained from Drosophila Genomics Research Center) were grown in Schneider’s medium (Cambrex) supplemented with 10% fetal bovine serum (Invitrogen) at 25 °C. The cultured cells were transfected using CaPi transfection kit according to the standard protocol (Invitrogen). Cells transfected with pMT constructs were treated with 50ug/ml CuSO4, 14 hours after transfection to induce the MT (Metallothionein) promoter.

**Immunostaining:** Embryos and ovaries were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH7.4) and immunostained with antibodies using standard protocols. Hoechst was used to stain DNA. Mouse-anti-FasIII (1:1000) and Rat-anti-E-Cad (1:200) were obtained from Developmental Studies Hybridoma Bank (Iowa City), and
Rabbit-anti-GFP (1:1000) was from (Torrey Pines Biolabs). Secondary antibodies conjugated with Alexa Fluor were obtained from Molecular Probes.

**In situ hybrizidation:** arl1 probes were generated from wild type cDNA clones (NM_079374) using the PCR DIG probe synthesis kit (Roche Applied Science). Whole-mount mRNA in situ hybridizations were performed in embryos according to the protocol of Tautz and Pfeifle (1989) with minor modifications.

**Yeast two-hybrid:** Cbs (NM_137031) cDNA was obtained from the Drosophila Genomics Research Center. DNA sequences corresponding to DRafCR2, Δ17Arl1, Δ17Arl1T30N, Δ17Arl1Q70L, and CbsGRIP proteins were cloned into pGADT7 or pGBKT7 vectors (Clontech). These recombinant constructs in pGADT7 and pGBKT7 plasmids were transformed into yeast Y187 strain according to standard protocol (Clontech). Protein-protein interactions were tested by β-Galactosidase assays using X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma; solid-support assay) as substrates. All yeast two-hybrid experiments are confirmed by reciprocal bait-prey assays and repeated at least 4 times.

**Results**

**Interaction between Arl1 and DRaf’s CR2 region:** In our yeast two-hybrid screen using the CR2 region of DRaf as the bait, we obtained a positive colony and subsequently the plasmid Drosophila insert was identified as the small GTPase Arl1 (Table III-1). The interaction between Arl1 and CR2 was confirmed by reciprocal bait-prey assays, suggesting Arl1 may be a binding partner of DRaf and play a role(s) in Raf signaling.

**Generation and identification of arl1 alleles:** We initiated our study on Arl1 using a classic genetic strategy to characterize Arl1 phenotypes in arl1− mutant animals. A recessive
lethal arl1 mutation was recovered by Tamkun et al. (1991), and it was used to demonstrate that arl1 is an essential gene. We obtained one loss-of-function allele of arl1, arl14107, from the Bloomington Drosophila stock center (#4107). Generation of an additional loss-of-function allele and phenotypic analysis of these different arl1 alleles is the strategy we used to characterize Arl1’s function in Drosophila development. Using arl14107, we conducted a mutagenesis screen to identify non-complementing alleles of arl1 (See Appendix 1). In our screen (~3,000 chromosomes), we isolated one new allele arl19.2BC. When placed over a deficiency for the chromosomal region that covers arl1 gene [Df(3L)th102], both arl14107 and arl19.2BC exhibited non-complementation. Both arl14107/Df(3L)th102 and arl19.2BC/Df(3L)th102 were viable when in a background that contains a duplication for the arl1 gene (Table III-2). So, it was confirmed that both arl14107 and arl19.2BC are loss-of-function alleles of arl1. However, neither arl14107/arl14107 nor arl19.2BC/arl19.2BC can be rescued by the wild type Arl1 transgene obtained from J. Kennison, indicating these two lines contained additional lethal mutations on the mutant arl1 chromosome. We sequenced the two arl1 alleles. The arl19.2BC mutation was likely a complete loss-of-function allele, as it was predicted to encode a truncated Arl1 protein of 36 amino acids (Figure III-1). The equivalent change for mammalian Arl1 would eliminate the effector and all known protein interaction domains (Wu et al. 2003). The arl14107 mutation was predicted to encode a truncated Arl1 protein of 165 amino acids, lacking the C-terminal 15 amino acids (Figure III-1), this region is known to form an α-helical (αS) structure in mammalian Arl1 (Wu et al. 2003).

Classical genetic analysis of Arl1 using arl1 mutations: Both arl14107 and arl19.2BC are recessive lethal mutations. We found most homozygous arl14107 or arl19.2BC mutants proceeded through embryogenesis and died during larval and pupal stages (Table III-3).
Cuticles of unhatched of arl1/arl1 embryos appeared to be relatively normal with 1-2% showing head involution defects (HID) (Table III-4). All arl1/arl1 larvae appeared normal in their exoskeletal pattern. Using a different allele, arf72A1 (arl1'), Eisman et al. (2006) reported that loss of Arl1 function led to mitotic defects and embryonic lethality. Subsequently, we requested and obtained the arl1 line from the Kennison’s group, who originally isolated the arl1 allele. Like arl14107 or arl19.2BC alleles, arl1 is recessive lethal. However, most eggs homozygous for the arl1 mutation hatched as larva. Embryonic cuticles of homozygous arl1 mutants appeared relatively normal (Table III-3, Table III-4). It seems that the arl1 chromosome also contains mutations in other genes, for arl1/Df(3L)th102, but not arl1/arl1 mutants, can be rescued by the wild type Arl1 transgene. Therefore, our genetics analysis using three independent alleles suggests that zygotic Arl1 may be not essential for the development of embryos.

Next we generated females with arl19.2BC/arl19.2BC or arl14107/arl14107 germ cells to understand Arl1’s maternal role. Using the FLP-FRT system (CHOU and PERRIMON 1996), arl1/arl1 germ line clones were produced and used to study early fly development in the absence of maternal Arl1 activity. When crossed with Oregon R (OR) wild type males, the arl1 containing germ line clone virgin females laid eggs, which are zygotically heterozygous for Arl1, but lacked maternal Arl1 activity. Some eggs derived from arl1/arl1 germ cells did not hatch. Most embryonic cuticles of the unhatched eggs are phenotypically normal (Table III-5, Table III-6), and all have the posterior Filzkörper structure, and normal central abdominal development suggesting the Tors RTK-Ras-Raf signaling pathway is not affected by the arl1 mutation (Loss of Tors RTK signaling results in loss of the Filzkörper, while gain of pathway activity causes reduced segmentation in the central abdominal region).
About 70% of those unhatched eggs do not have cuticles and are likely unfertilized, since most embryos (0-15 hours after egg deposition), when stained with a nucleus marker and the FasIII antibody, appeared to be phenotypically normal with only a few showing head involution defects (Table III-6, 7; Figure III-2). The genetic cross with arl1+/ males did not significantly alter the hatch rates of the eggs from the females with arl1/arl1− germ lines (Table III-5), implying removal of paternal Arl1 in half of the eggs did not affect their embryogenesis. Therefore, our study suggests both maternal and zygotic Arl1 proteins play limited roles during embryogenesis.

In our genetic assays, head involution defects were observed for some embryos, although at low penetrance. Thereby, loss of Arl1 function may lead to abnormal cellular morphogenetic movements. The HID defect may not be relevant to Ras-Raf signaling, since we did not detect a genetic interaction, when the arl1/arl1− germline-bearing females were crossed with ras1e2b/+ males (Table III-6).

The head involution defects observed could be due to the dysregulation of adhesion proteins (VANHOOK and LETSOU 2007). Adhesion molecules are thought to function in cellular morphogenesis through their dynamic presence on the cell surface, which can be regulated by endocytosis and exocytosis (EDELMAN 1985; GUMBINER 1996; SETO et al. 2002). Considering Arl1’s potential roles in trafficking, we developed the hypothesis that Arl1 might participate in intracellular transport of adhesion proteins. If so, then in eggs from arl1/arl1− germ cells when fertilized by males with a mutation in a gene that acts in adhesion, reduced viability is anticipated for transheterozygous individuals. We tested for genetic interaction of arl1 alleles with mutations in numerous genes encoding membrane-localized adhesion molecules. However, no additive or synergistic effects were seen (Table III-8, 9).
Many coiled-coil golgin tethers, containing GRIP domains, have been identified as Arl1 effectors. The Golgi localization of these golgins, likely mediated by the interactions between the GRIP domains and Arl1, appeared essential for their functions (Lu et al. 2004). However, recently these golgins have been shown to associate with numerous additional small GTPase regulators. For instances, dGolgin97, dGolgin245 and dGCC185 contain multiple binding sites for the Rab family of G proteins (Sinka et al. 2008). Therefore, Arl1 may act, together with these Rab proteins, to mediate the function of downstream golgins. This implies a degree of redundancy between the Arl1 and Rabs, which could explain only mild phenotypes observed in arl1 mutants upon removal of normal Arl1 activity. However, no genetic interaction, with the limited number of candidates (Rab6, Rab11 etc.) that we tested, was detected (Table III-10, 11). Therefore, there might be other proteins that function redundantly with Arl1. Together, our analysis implies that it is difficult to dissect Arl1’s roles using classical genetic methods to study these arl1 alleles.

An over-expression strategy to characterize Arl1’s function in Drosophila: One strategy for characterizing a gene’s functions is to study the effects of overexpressing its mutant products. In mammalian cultured cells, over-expression of constitutive active arl1Q71L abolishes traffic of VSVG and Shiga toxin (Lu et al. 2001). We generated the mCherry-tagged arl1Q70L with an equivalent point mutation. The arl1Q70L-mCherry cDNA was cloned into pUASp vector and used to generate UASp-arl1Q70L-mCherry transgenic flies. The UASp promoter allows spatio-temporal expression when combined with different Drosophila Gal4 drivers in somatic and germ-line cells (Rorth 1998). We also generated the wild type Arl1-mCherry lines as the control. The constitutively active heat-shock 83 (HS83) promoter was selected to drive the ubiquitous expression of mCherry-tagged Arl1 protein (pCaspeR-HS83-
Arl1-mCherry). Overexpression of Arl1-mCherry did not affect the viability of the flies, and the Arl-mCherry transgene rescued the lethality of \( arl^{4107}/Df(3L)th102 \) and \( arl^{9.2BC}/Df(3L)th102 \) flies, suggesting mCherry-tagged Arl1 is capable of replacing the endogenous gene.

We found ubiquitous expression of \( arl^{Q70L}-mCherry \) driven by actin-Gal4 or tubulin-Gal4 decreased the viability of flies. However, development of eyes, wings, legs and thoracic bristles of surviving adults appeared phenotypically normal. Considering the potential roles of Arl1 in traffic, we also tried to over-express \( arl^{Q70L}-mCherry \) using the salivary gland-Gal4 (SG-Gal4) driver to see if it affects secretion of glue proteins and/or the morphology of salivary gland cells. However, we did not detect any defects for \( SG-Gal4/+; arl^{Q70L}-mCherry \) larva and their salivary glands appeared normal when stained with E-cadherin and a nuclear marker. All the these larvae stick to the vial wall and form pupa as well as wild type, suggesting secretion of glue proteins was relatively normal.

**Expression of \( arl^{Q70L}-mCherry \) in ovaries:** During oogenesis, border cells migrate between nurse cells to the anterior end of oocyte. Cell surface-localized adhesion molecules mediate the border-nurse cell contacts required for this process. Their localization on plasma membranes can be regulated dynamically by exocytosis and endocytosis, so that rounds of binding and release allow the border cells to move between the nurse cells (Montell 2003; Bryant and Stow 2004). Dysregulation of the dynamic presence of adhesion proteins on the cell surface could result in border cell migration defects, which subsequently leads to a failure of micropyle structure formation and female sterility. We found over-expression of \( arl^{Q70L}-mCherry \) in germ cells driven by nanos-Gal4 reduced female fertility and ~8% of stage 10 egg chambers of these females exhibited a delay in border cell migration (Figure III-
We did not detect such migration defects upon expression of arl1\(^{Q70L}\)-mCherry driven by slbo-Gal4 in border cells. Considering Arl1’s potential functions in intracellular transport, we propose that the border cell migration defects upon over-expression of arl1\(^{Q70L}\)-mCherry might be due to dysregulation of adhesion molecules in nurse cells. However, none of the mutations in adhesion molecules we tested showed additive or synergistic effects when arl1\(^{Q70L}\)-mCherry was over-expressed in germ cells (Table III-13).

**Accumulation of Arl1 mRNA in Drosophila embryos:** The spatio-temporal expression pattern of a gene sometimes exhibits correlation with its function(s). A study of the Arl1 mRNA accumulation pattern in embryos may provide some clues about its biological roles. We performed whole mount *in situ* hybridization to explore when and in which cells the Arl1 gene is transcribed during embryogenesis. We found Arl1 mRNA was ubiquitously distributed in embryos with a higher level in certain tissues. Arl1 mRNAs were enriched underneath the cortical layer of stage 5 embryos. At later stages (stage 6-10), abundant Arl1 mRNAs were detected in the ventral, cephalic, anterior and posterior transversal furrows. After completion of gastulation, Arl mRNAs were enriched in brain lobes and central nerve system (Figure III-4).

**Epitope-tagged Arl1 is colocalized with Golgi marker:** In yeast and mammalian cultured cells, Arl1 was found localized in Golgi membrane (LOWE *et al.* 1996; LEE *et al.* 1997; LU *et al.* 2001). To study the subcellular localization of Arl1, Drosophila S2 cells expressing different epitope-tagged (mCherry, Myc and EGFP) Arl1 proteins were co-transfected with YFP-tagged Golgi marker (YFP-Golgi, containing the N-terminal 1-81 region of human β 1,4-galactosyltransferase, which is a *trans-* Golgi protein) or stained with the Golgi marker TRITC-Ceramide (LAJEUNESSE *et al.* 2004). All these epitope-tagged Arl1
proteins exhibit a punctuate pattern and show nice colocalization with Golgi markers (Figure III-5). Arl1’s localization in vivo was also tested using the Arl1-mCherry transgenic lines. We dissected the ovaries from females YFP-Golgi/Arl1-mCherry and found Arl1-mCherry is colocalized with the Golgi marker in oocytes. Consistently, Arl1-mCherry also showed a pattern of colocalization with YFP-Golgi in brain cells from the 3rd instar larva of the transgenic lines (Figure III-6).

In mammals, the N-term 1-17 region is very likely to form an amphipathic helix and contains a glycine site (G2), which can be myristoylated. The glycine myristoylation and probably the amphipathic helix structure, are important for targeting Arl1 to Golgi membranes (Lu et al., 2001; Wu et al., 2003). Deletion of the N1-17 region or mutation at G2 results in loss of golgi localization. In our study, when the N-terminal 1-17 region was removed (Δ17ar11-EGFP), Arl1 lost its fragmented pattern and instead was dispersed in the cytoplasm (Figure III-5B). These findings in Drosophila are consistent with those in yeast and mammals.

**Interaction between Arl1 and GRIP:** Previously, Eisman et al. (2006) identified a GRIP domain-containing protein, centrosomin’s beautiful sister (Cbs, CG4840). Cbs appears to be the ortholog of golgin97, and is a putative effector of Arl1. We found that Arl1 can associate with the GRIP region of Cbs in a GTP dependent manner in a yeast two-hybrid assay. The coiled-coil region (CC) preceding GRIP contains binding sites for small GTPase Rab proteins, however, we did not detect interaction between CC and Arl1 (Figure III-7). These are consistent with previous studies by others (Sinka et al. 2008).
Discussion

Is Arl1 involved in DRaf signaling: In our yeast two-hybrid screen using the CR2 region of DRaf as the bait, we identified the small GTPase Arl1 as a potential binding partner of DRaf. In yeast and mammalian cultured cells, the Golgi-localized Arl1 can interact with the GRIP domain of coiled-coil golgin proteins, and is thought to function in intracellular transport (LOWE et al. 1996; LEE et al. 1997; LU et al. 2001; LU et al. 2004). Although mammalian Raf has been found associated with Golgi, this was attributed to Ras binding (CHIU et al. 2002). The interaction between Arl1 and Raf has not been described, and it is not clear if Arl1 is relevant to Raf signaling.

Friedman and Perrimon (2006) performed a genome-wide RNAi assay in Drosophila S2 cells to identify potential signal components of RTK pathways. In their primary screen, knock down of Arl1 seemed to increase the phosphorylation level of ERK, suggesting that Arl1 may have inhibitory effects on RTK signaling. However, such information from high-throughput screen needs to be confirmed by further analysis. Particularly, genetic characterization of Arl1 in vivo is necessary for validation of its potential roles.

Previously, a golgi protein, RKTG, has been identified as a negative regulator of CRaf and BRaf in mammalian cells. Over-expression of RKTG can prevent Raf activation by recruiting cytoplasmic Raf to golgi and hidering its translocation to plasma membrane (FENG et al. 2007). If Arl1 functions in inhibiting the ERK signal, as suggested by Friedman and Perrimon’s study (2006), it is possible that it acts like RKTG to capture Raf on golgi membrane through its interaction with CR2, thus playing a negative role in Raf signaling.

We attempted to determine the function of this small GTPase Arl1 in Drosophila using classic genetic methods. However, we did not detect Raf-related phenotypical effects upon
mutation of Arl1, thus, it is still unclear whether Arl1 is relevant to ERK signal transduction
in vivo.

**Arl1 may function in cellular morphogenetic movements:** Adhesion molecules, like
cadherin proteins, play very important roles in epithelial morphogenesis, cell-cell
communications, as well as cell migration. In order to fulfill these roles, these adhesion
proteins must be delivered efficiently to the cell surface, where they are incorporated into
protein complexes with other partners (EDELMAN 1985; GUMBINER 1996). During cell
migration, their localization to the plasma membrane can be regulated dynamically by
exocytosis and endocytosis (SETO *et al.* 2002; BRYANT and STOW 2004). Dysregulation of
their dynamic presence on the cell surface could result in morphogenesis defects. In our
study, over-expression of arl1^{Q70L}-mCherry in germ cells resulted in a delay of border cell
migration in some egg chambers. We also observed, though at low penetrance, head
involution defects in embryos with Arl1 mutations. These suggest Arl1 may function in
cellular morphogenetic movements. Considering Arl1’s golgi localization and its potential
role in intracellular transport, it is possible that Arl1 may be involved in trafficking of
adhesion molecules. For instance, loss of normal Arl1 function could inhibit the endocytosis
of adhesion molecules in nurse cells. Therefore the constitutive presence of the “sticky”
adhesion proteins on the cell surface would hider the movement of border cells and result in
delay of their migration.

Dynamic rearrangement of the cytoskeleton, including actin filaments, also contributes
to cellular morphogenesis. Distorting the actin cytoskeleton in Drosophila embryos results in
head involution/dorsal closure defects (ELISABETH and MULLER 1998). Previously, it has
been reported that some small GTPases (Rac, Rho, *etc.*) can regulate the structure of the actin
cytoskeleton (SHARP et al. 2005). It is also possible that Arl1 could be involved in cellular morphogenetic movement, like these small G proteins, by regulating dynamic rearrangement of cytoskeleton.

**Challenges in genetic analysis of Arl1’s developmental roles:** The Arl1 small GTPase is thought to regulate coiled-coil golgin proteins through interaction with their GRIP domains (LU et al. 2004). The golgins may act like tethers and provide the contact sites for other golgi cisternae, arriving cargo, and intracellular vesicles including endosomes, thereby function in golgi morphology and traffic. However, these golgins contain binding sites for other regulators, in addition to Arl1. Particularly, the Golgi-localized Rab small GTPases (Rab2, Rab6, Rab19, Rab30, Rab39, etc.) can interact with numerous coiled-coil golgin tethers and may function redundantly with Arl1 (SINKA et al. 2008). This may explain why loss of Arl1 activity only resulted in mild phenotypes in Drosophila embryos.

In mammals, there are ~70 members of the Rab small GTPase family. These Rab proteins have been found localized in different intracellular organelles (ER, Golgi, early endosome, late endosome, recycling endosome, etc.) and function in membrane trafficking (STENMARK and OLKKONEN 2001). In Drosophila, 33 Rab members with high sequence similarity have been identified (ZHANG et al. 2007). The golgin proteins contain multiple binding sites in their coiled-coil regions for numerous Rab proteins, which may include, besides those localized in Golgi, some associating with other intracellular vesicles. It is possible, as proposed by SINKA et al. (2008), that golgins may be anchored to the Golgi through interactions between their C-termini and Arl1 or Rab6 etc., while the remaining portions of these molecules may project into the cytoplasm like tentacles. Numerous coiled-coil golgin proteins (Golgin97, Golgin245, etc.) share this structural feature with mutiple
binding sites for Rab members located along the tentacles (SINKA et al. 2008), so that they could act collectively by capturing arriving vesicles bearing Rabs (Rab4, Rab5, Rab9, or Rab11-bearing endosomes, etc.) to Golgi membranes. If so, the GRIP-containing golgin proteins, which are potential effectors of Arl1, could also function redundantly with each other. This could explain why, in several recent studies, functional depletion of particular golgins did not lead to severe phenotypic defects (SINKA et al. 2008). Together, the potential functional redundancy for Arl1 as well as its effectors may confound our genetic analysis on Arl1.

Potential strategies for further characterization of Arl1’s role: Drosophila has been thought to be one of the best-understood multi-cellular model organisms. However, among more than 15,000 annotated genes in flies, most (~85%), when mutated, did not show detectable phenotypes. Lack of phenotypic information regarding these genes does not mean they don’t have significant functions, instead, it is likely that most of them play important roles but act redundantly with other genes (www.flyrnai.org/TriP-HOME.html). To resolve such an issue of functional redundancy, one could disrupt the activity of all/most of the redundant genes that play a role in a particular cellular process. NIH has just begun to support TRiP (Transgenic RNAi Project), a project to develop the UAS-RNAi system in a genome-wide scale. By combining RNAi sequences of several different genes (Arl1, Rab2, Rab6, etc.), we could simultaneously deplete their functions in flies. Particularly, based on their mRNA pattern in embryos, we could knock down these genes in specific tissues or at specific developmental stages using the Gal4-UAS system, and test their functional roles with a spatial and temporal resolution.
In our study, over-expression of arl1 Q70L-mCherry in germ cells resulted in delay of border cell migration in some egg chambers. We also observed, though at low penetrance, head involution defects in embryos that lack maternal Arl1 activity. These findings suggest Arl1 may function in cellular morphogenetic movements. Considering Arl1’s golgi localization and its potential role in intracellular transport, we propose that Arl1 may be involved in trafficking of adhesion molecules, although we did not detect any additive or synergistic genetic interaction between Arl1 and numerous genes encoding adhesion proteins. Identification of the components/cargo of the Arl1 complex/traffic machinery using proteomic approaches could be a helpful approach to characterize its developmental roles. Particularly, one could co-precipitate or pull-down the proteins of Arl1 complexes from cellular lysates, and using mass spectrum analysis, identify components of the complex(es). This method may also validate Raf-Arl1 interaction in vivo. A combination of such proteomic approaches may allow the in vivo genetic analysis of Arl1 more promising.

Acknowledgement

We gratefully acknowledge the arl1/ and arl1 transgenic lines from Jim Kennison, YFP-Golgi plasmid form Cungui Mao, fly strains from Drosophila Stock Center (Bloomington) and cDNAs from Drosophila Genomics Research Center. We thank Genetic Services (Sudbury, MA) for generating the arl1 Q70L-mCherry transgenic flies.

Literature Cited


GUMBINER B.M. 1996 Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84:345-357


LU L., G. TAI and W. HONG, 2004 Autoantigen Golgin-97, an effector of Arfl GTPase, participate in traffic from the endosome to the trans- Golgi network Mol. Biol. Cell 15:4426-4443


STENMARK H. and V. M. OLKKONEN, 2001 The Rab GTPase family. Genome Biol. 2:3007.1-3007.7


### Tables

#### TABLE III-1. Interaction between Arl1 and the CR2 region of DRaf.

<table>
<thead>
<tr>
<th>Interact w/</th>
<th>N</th>
<th>CR1</th>
<th>CR2</th>
<th>CR3</th>
<th>CR1CR2</th>
<th>NCR1CR2</th>
<th>FL DRaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arl1</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

#### TABLE III-2: Both arl1^{4107} and arl1^{9.2B} alleles are loss of function alleles of arl1.

<table>
<thead>
<tr>
<th>Allele tested</th>
<th>arl1^{4107}</th>
<th>arl1^{9.2B}</th>
<th>Df(3L)th102</th>
<th>Df(3L)st-f13</th>
<th>Df(3L)brm11</th>
<th>Df(3L)th102: Dp(3;Y)131-D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>arl1^{4107}</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>V</td>
<td>L</td>
<td>V = males</td>
</tr>
<tr>
<td>arl1^{9.2B}</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>V</td>
<td>L</td>
<td>V = males</td>
</tr>
</tbody>
</table>

L = no complementation, lethal; V = viable
Breakpoints: 71F3-5;72D12
Breakpoints: 72C1-D1;73A3-4
Breakpoints: 71F1-4;72D1-10
Breakpoints: 72A2;72D10, 72A;75D4.5;Y

#### TABLE III-3. Most homozygous arl1 mutants proceeded through embryogenesis.

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Paternal</th>
<th>#Hatched (%)</th>
<th>#Unhatched* (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>arl1^{9.2B}/+</td>
<td>arl1^{9.2B}/+</td>
<td>655 (75.1)</td>
<td>217 (24.9)</td>
<td>872</td>
</tr>
<tr>
<td>arl1^{9.2B}/+</td>
<td>+/-</td>
<td>627 (87.1)</td>
<td>93 (12.9)</td>
<td>720</td>
</tr>
<tr>
<td>arl1^{4107}/+</td>
<td>arl1^{4107}/+</td>
<td>482 (79.8)</td>
<td>123 (20.2)</td>
<td>605</td>
</tr>
<tr>
<td>arl1^{4107}/+</td>
<td>+/-</td>
<td>355 (87.9)</td>
<td>49 (12.1)</td>
<td>404</td>
</tr>
<tr>
<td>arl1^{1}/+</td>
<td>arl1^{1}/+</td>
<td>201 (83.1)</td>
<td>41 (16.9)</td>
<td>242</td>
</tr>
<tr>
<td>arl1^{1}/+</td>
<td>+/-</td>
<td>177 (89.4)</td>
<td>22 (10.6)</td>
<td>198</td>
</tr>
<tr>
<td>arl1^{1}/+</td>
<td>arl1^{9.2B}/+</td>
<td>213 (84.9)</td>
<td>38 (15.1)</td>
<td>251</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>347 (86.3)</td>
<td>55 (13.7)</td>
<td>402</td>
</tr>
</tbody>
</table>

* Unhatched eggs comprise three classes: unfertilized eggs, embryos that develop poorly and do not make cuticles and embryos that produce cuticles but do not hatch.
### TABLE III-4. Embryonic cuticles of arl1/arl1' mutants are relatively normal.

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Paternal</th>
<th>Non-GFP embryos</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#Normal (%) #HID *(%)</td>
<td></td>
</tr>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/GFP</td>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/GFP</td>
<td>71 (98.6) 1 (1.4)</td>
<td>72</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/GFP</td>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/GFP</td>
<td>88 (98.8) 1 (1.2)</td>
<td>89</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;1&lt;/sup&gt;/GFP</td>
<td>arl1&lt;sup&gt;1&lt;/sup&gt;/GFP</td>
<td>60 (100) 0 (0)</td>
<td>60</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/GFP</td>
<td>25 (92.6) 2 (7.4)</td>
<td>27</td>
</tr>
</tbody>
</table>

*HID: Head involution defects

### TABLE III-5. Hatch rates of eggs from germline clone bearing females.

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Paternal</th>
<th>#Hatched (%) #Unhatched * (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/+ &amp; FRT2A/FRT2A</td>
<td>203 (60.4) 133 (39.6)</td>
<td>336</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>+/-</td>
<td>456 (63.3) 264 (36.7)</td>
<td>720</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/arl1&lt;sup&gt;4107&lt;/sup&gt;</td>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/+</td>
<td>287 (41.8) 400 (58.2)</td>
<td>687</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/arl1&lt;sup&gt;4107&lt;/sup&gt;</td>
<td>+/-</td>
<td>372 (33.8) 728 (66.2)</td>
<td>1100</td>
</tr>
<tr>
<td>FRT2A/FRT2A</td>
<td>+/-</td>
<td>146 (70.9) 60 (29.1)</td>
<td>206</td>
</tr>
</tbody>
</table>

* Unhatched eggs comprise three classes: unfertilized eggs, embryos that develop poorly and do not make cuticles and embryos that produce cuticles but do not hatch.

### TABLE III-6. Some embryonic cuticles of unhaughted eggs from germline clone bearing females have head involution defects or body hole.

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Paternal</th>
<th>#Normal (%) #HID or BD* (%)</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>+/-</td>
<td>32 (94.1) 2 (5.9)</td>
<td>34</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/+ &amp; FRT2A/FRT2A</td>
<td>29 (93.5) 2 (6.5)</td>
<td>31</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/arl1&lt;sup&gt;4107&lt;/sup&gt;</td>
<td>+/-</td>
<td>31 (96.9) 1 (3.1)</td>
<td>32</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/arl1&lt;sup&gt;4107&lt;/sup&gt;</td>
<td>arl1&lt;sup&gt;4107&lt;/sup&gt;/+</td>
<td>21 (95.5) 1 (4.5)</td>
<td>22</td>
</tr>
<tr>
<td>arl1&lt;sup&gt;9.2BC&lt;/sup&gt;/arl1&lt;sup&gt;9.2BC&lt;/sup&gt;</td>
<td>Ras&lt;sup&gt;28&lt;/sup&gt;/+</td>
<td>25 (96.2) 1 (3.8)</td>
<td>26</td>
</tr>
<tr>
<td>FRT2A/FRT2A</td>
<td>+/-</td>
<td>27 (100) 0 (0)</td>
<td>27</td>
</tr>
</tbody>
</table>

*HID or BD: head involution defects or body hole

### TABLE III-7. Some embryonic from germline clone bearing females have head involution defects (FasIII staining).

| Maternal                     | #Normal (%) #HID*(%) #Total |
|------------------------------|----------------------------|-----------------|-------|
| arl1<sup>9.2BC</sup>/arl1<sup>9.2BC</sup> | 71 (92.2) 6 (7.8) | 77    |
| arl1<sup>4107</sup>/arl1<sup>4107</sup> | 54 (94.7) 3 (5.3) | 57    |
| FRT2A/FRT2A                  | 34 (100) 0 (0)      | 34    |

*HID: head involution defects
### TABLE III-8. Genetic interaction tests between arl1 mutations and alleles of adhesion genes on the second chromosome (*Cyo: Balancer chromosome)*.

<table>
<thead>
<tr>
<th>Genetic cross</th>
<th>#CyO *</th>
<th>#Non-CyO</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X+/CyO;+/+</td>
<td>371</td>
<td>444</td>
<td>815</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X+/CyO;+/+</td>
<td>146</td>
<td>179</td>
<td>325</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X+/CyO;+/+</td>
<td>320</td>
<td>364</td>
<td>684</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X shg^{035401}/CyO;+/+</td>
<td>169</td>
<td>125</td>
<td>294</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X shg^{035401}/CyO;+/+</td>
<td>113</td>
<td>103</td>
<td>216</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X shg^{035401}/CyO;+/+</td>
<td>95</td>
<td>89</td>
<td>184</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X dystroglycan^{/CyO;+/+}</td>
<td>82</td>
<td>86</td>
<td>168</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X dystroglycan^{/CyO;+/+}</td>
<td>28</td>
<td>37</td>
<td>65</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X dystroglycan^{/CyO;+/+}</td>
<td>150</td>
<td>129</td>
<td>279</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X dg^{R184}/CyO;+/+</td>
<td>61</td>
<td>62</td>
<td>123</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X dg^{R184}/CyO;+/+</td>
<td>37</td>
<td>43</td>
<td>80</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X dg^{R184}/CyO;+/+</td>
<td>26</td>
<td>32</td>
<td>58</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X ft^frv^{/CyO;+/+}</td>
<td>63</td>
<td>102</td>
<td>165</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X ft^frv^{/CyO;+/+}</td>
<td>38</td>
<td>46</td>
<td>84</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X ft^frv^{/CyO;+/+}</td>
<td>40</td>
<td>61</td>
<td>101</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X stan^{192}/CyO;+/+</td>
<td>63</td>
<td>73</td>
<td>136</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X stan^{192}/CyO;+/+</td>
<td>77</td>
<td>79</td>
<td>156</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X stan^{192}/CyO;+/+</td>
<td>56</td>
<td>64</td>
<td>120</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X l(2)gl^{/CyO;+/+}</td>
<td>29</td>
<td>38</td>
<td>67</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X l(2)gl^{/CyO;+/+}</td>
<td>23</td>
<td>31</td>
<td>54</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X l(2)gl^{/CyO;+/+}</td>
<td>30</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X ds^{58k}/CyO;+/+</td>
<td>26</td>
<td>34</td>
<td>60</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X ds^{58k}/CyO;+/+</td>
<td>24</td>
<td>28</td>
<td>52</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X ds^{58k}/CyO;+/+</td>
<td>26</td>
<td>28</td>
<td>54</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X cadN2^{/CyO;+/+}</td>
<td>43</td>
<td>31</td>
<td>74</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X cadN2^{/CyO;+/+}</td>
<td>24</td>
<td>33</td>
<td>57</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X cadN2^{/CyO;+/+}</td>
<td>22</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X cadN^{/CyO;+/+}</td>
<td>17</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X cadN^{/CyO;+/+}</td>
<td>24</td>
<td>32</td>
<td>56</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X cadN^{/CyO;+/+}</td>
<td>19</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td>+/+; arl1^{9.2BC}/arl1^{9.2BC} X cadAM12^{/CyO;+/+}</td>
<td>44</td>
<td>31</td>
<td>75</td>
</tr>
<tr>
<td>+/+; arl1^{4107}/arl1^{4107} X cadAM12^{/CyO;+/+}</td>
<td>34</td>
<td>28</td>
<td>62</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X cadAM12^{/CyO;+/+}</td>
<td>30</td>
<td>25</td>
<td>55</td>
</tr>
</tbody>
</table>
### TABLE III-9. Genetic interaction tests between arl1 alleles and mutations in adhesion genes on the 3\(^{rd}\) chromosome (*Tm3: Balancer chromosome).

<table>
<thead>
<tr>
<th>Genetic cross</th>
<th>#Tm3*</th>
<th>#Non-Tm3</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X +/+; cad74A(^{+/+})</td>
<td>90</td>
<td>87</td>
<td>177</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X +/+; cad74A(^{+/+})</td>
<td>106</td>
<td>97</td>
<td>203</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; cad74A(^{+/+})</td>
<td>103</td>
<td>73</td>
<td>176</td>
</tr>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X +/+; fat2/Tm3</td>
<td>62</td>
<td>53</td>
<td>115</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X +/+; fat2/Tm3</td>
<td>19</td>
<td>23</td>
<td>42</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; fat2/Tm3</td>
<td>68</td>
<td>53</td>
<td>121</td>
</tr>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X +/+; scrib(^{783})/Tm3</td>
<td>63</td>
<td>53</td>
<td>116</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X +/+; scrib(^{783})/Tm3</td>
<td>23</td>
<td>21</td>
<td>44</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; scrib(^{783})/Tm3</td>
<td>124</td>
<td>131</td>
<td>255</td>
</tr>
</tbody>
</table>

### TABLE III-10. Genetic interaction test between arl1 alleles and mutations in candidate genes (2\(^{nd}\) chromosome), which may act redundantly with Arl1 (*Cyo: Balancer chromosome).

<table>
<thead>
<tr>
<th>Genetic Cross</th>
<th>#Cyo*</th>
<th>#Non-Cyo</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X +/Cyo(^{+/+})</td>
<td>371</td>
<td>444</td>
<td>815</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X +/Cyo(^{+/+})</td>
<td>146</td>
<td>179</td>
<td>325</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/Cyo(^{+/+})</td>
<td>320</td>
<td>364</td>
<td>684</td>
</tr>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X rab6(^{0232D})/Cyo(^{+/+})</td>
<td>111</td>
<td>128</td>
<td>239</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X rab6(^{0232D})/Cyo(^{+/+})</td>
<td>57</td>
<td>61</td>
<td>118</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X rab6(^{0232D})/Cyo(^{+/+})</td>
<td>34</td>
<td>35</td>
<td>69</td>
</tr>
<tr>
<td>+/+; arl1(^{9.2BC})/arl1(^{9.2BC}) X rab6(^{08323})/Cyo(^{+/+})</td>
<td>44</td>
<td>53</td>
<td>97</td>
</tr>
<tr>
<td>+/+; arl1(^{4107})/arl1(^{4107}) X rab6(^{08323})/Cyo(^{+/+})</td>
<td>32</td>
<td>39</td>
<td>71</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X rab6(^{08323})/Cyo(^{+/+})</td>
<td>53</td>
<td>60</td>
<td>113</td>
</tr>
</tbody>
</table>
TABLE III-11. Genetic interaction test between arl1 alleles and mutations in candidate genes (3rd chromosome), which may act redundantly with Arl1 or or be involved in post-translational modification of redundant gene products (*Tm3: Balancer chromosome).

<table>
<thead>
<tr>
<th>Genetic cross</th>
<th>#Tm3*</th>
<th>#Non-Tm3</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+; arl1^9.2BC/arl1^9.2BC X +/+; arf79F/Tm3</td>
<td>136</td>
<td>203</td>
<td>339</td>
</tr>
<tr>
<td>+/+; arl1^4107/arl1^4107 X +/+; arf79F/Tm3</td>
<td>51</td>
<td>74</td>
<td>125</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; arf79F/Tm3</td>
<td>66</td>
<td>95</td>
<td>161</td>
</tr>
<tr>
<td>+/+; arl1^9.2BC/arl1^9.2BC X +/+; rab11^93B/Tm3</td>
<td>154</td>
<td>225</td>
<td>379</td>
</tr>
<tr>
<td>+/+; arl1^4107/arl1^4107 X +/+; rab11^93B/Tm3</td>
<td>60</td>
<td>80</td>
<td>140</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; rab11^93B/Tm3</td>
<td>193</td>
<td>214</td>
<td>407</td>
</tr>
<tr>
<td>+/+; arl1^9.2BC/arl1^9.2BC X +/+; rab11^2d/Tm3</td>
<td>119</td>
<td>179</td>
<td>298</td>
</tr>
<tr>
<td>+/+; arl1^4107/arl1^4107 X +/+; rab11^2d/Tm3</td>
<td>65</td>
<td>93</td>
<td>158</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; rab11^2d/Tm3</td>
<td>132</td>
<td>198</td>
<td>330</td>
</tr>
<tr>
<td>+/+; arl1^9.2BC/arl1^9.2BC X +/+; rabGGT/Tm3</td>
<td>62</td>
<td>73</td>
<td>135</td>
</tr>
<tr>
<td>+/+; arl1^4107/arl1^4107 X +/+; rabGGT/Tm3</td>
<td>27</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>+/+; FRT2A/FRT2A X +/+; rabGGT/Tm3</td>
<td>83</td>
<td>85</td>
<td>168</td>
</tr>
</tbody>
</table>

TABLE III-12. Some egg chambers have border cell migration defects upon over-expression of arl1^Q70L-mCherry germ cells.

<table>
<thead>
<tr>
<th>Maternal</th>
<th># w/ Border Cell migration defects (%)</th>
<th>#Normal (%)</th>
<th>#Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arl1-mCherry</td>
<td>0 (0)</td>
<td>32 (100)</td>
<td>32</td>
</tr>
<tr>
<td>arl1^Q70L-mCherry</td>
<td>4 (7.8)</td>
<td>47 (92.2)</td>
<td>51</td>
</tr>
</tbody>
</table>
TABLE III-13. Genetic interaction tests of mutations in adhesion genes or candidate genes, which may act redundantly with Arl1 (border cell migration assay).

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Normal gene products</th>
<th>Genetics interaction with arl1^{Q70L}-mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab6^{D23D}</td>
<td>Rab6</td>
<td>No</td>
</tr>
<tr>
<td>Rab6^{D852S}</td>
<td>Rab6</td>
<td>No</td>
</tr>
<tr>
<td>arf79F^{-}</td>
<td>Arf1</td>
<td>No</td>
</tr>
<tr>
<td>rab11^{R36H}</td>
<td>Rab11</td>
<td>No</td>
</tr>
<tr>
<td>rab11^{D12d}</td>
<td>Rab11</td>
<td>No</td>
</tr>
<tr>
<td>rabGGT</td>
<td>Rab geranylgeranyl transferase</td>
<td>No</td>
</tr>
<tr>
<td>shg^{ko3401}</td>
<td>E-cadherin</td>
<td>No</td>
</tr>
<tr>
<td>shg^{R69}</td>
<td>E-cadherin</td>
<td>No</td>
</tr>
<tr>
<td>dystroglycan^{-}</td>
<td>Dystroglycan</td>
<td>No</td>
</tr>
<tr>
<td>dg^{B148}</td>
<td>Dystroglycan</td>
<td>No</td>
</tr>
<tr>
<td>ff^{g-rv}</td>
<td>Protocadherin Fat</td>
<td>No</td>
</tr>
<tr>
<td>stan^{192}</td>
<td>Atypical cadherin Flamingo</td>
<td>No</td>
</tr>
<tr>
<td>l(2)gl^{l}</td>
<td>Giant Larva (cytoskeleton protein)</td>
<td>No</td>
</tr>
<tr>
<td>ds^{88k}</td>
<td>Dachsous (Cadherin)</td>
<td>No</td>
</tr>
<tr>
<td>cadN2^{-}</td>
<td>Neural Cadherin 2</td>
<td>No</td>
</tr>
<tr>
<td>cadN^{-}</td>
<td>Neural Cadherin</td>
<td>No</td>
</tr>
<tr>
<td>cadN^{M12}</td>
<td>Neural Cadherin</td>
<td>No</td>
</tr>
<tr>
<td>cad74A^{-}</td>
<td>Cadherin74A</td>
<td>No</td>
</tr>
<tr>
<td>fat2^{-}</td>
<td>Protocadherin Fat2</td>
<td>No</td>
</tr>
<tr>
<td>scrib^{-}</td>
<td>Septate junction Scribble</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure Legends

FIGURE III-1. Molecular lesions of arl1 alleles: Schematic representations of Arl1 proteins are shown. Wild type Arl1 (180 amino acids) consists of 6α-helices and 6β-stands (Wu et al. 2003). The arl14107 mutation is predicted to encode a truncated Arl1 protein of 165 amino acids, lacking the C-terminal 15 amino acids; this region is known to form an α-helical (α5) structure in mammalian Arl1. The arl19.2BC mutation is likely a complete loss-of-function allele, as it was predicted to encode a truncated Arl1 protein of 36 amino acids. The equivalent change for mammalian Arl1 would eliminate the effector and all known protein interaction domains.

FIGURE III-2. Head involution defects in embryos of arl1 mutants: (A-C) Embryos stained with anti-FasIII antibodies are shown at 40X magnification. (A) A wild type (WT) embryo with normal epithelial pattern. (B) An embryo derived from an arl19.2BC/arl19.2BC germ cell exhibits head involution defects (arrow). (C) An embryo derived from an arl14107/arl14107 germ cell exhibits an abnormal epithelial pattern (head involution defect and body hole are denoted by arrow and square, respectively). (D) An example of normal WT embryonic cuticle is shown. (E) An embryo cuticle derived from an arl19.2BC/arl19.2BC germ cell exhibits a head involution defect (arrow). (F) An embryo cuticle derived from an arl14107/arl14107 germ cell with a head involution defect (arrow and large body hole (arrow head). (G) Square inset from (C) is shown at 100X magnification with arrow denoting body hole.

FIGURE III-3. A border cell migration defect in a stage 10 egg chamber expressing arl1Q70L-mCherry: Border cells are stained with anti-FasIII and denoted by arrows. (A) In a wild type egg chamber, border cells arrive at the anterior end of the oocyte at stage 10. (B) A
stage 10 egg chamber expressing Arl1-mCherry with normal border cell migration. (C) A stage 10 egg chamber expressing arl1<sup>Q70L</sup>-mCherry exhibits a border cell migration defect.

**FIGURE III-4. Accumulation of Arl1 mRNA in embryos:** Arl1 mRNA was ubiquitously distributed in embryos with a higher level in certain tissues. (A) A stage 5 cellularized embryo with Arl1 mRNAs enriched within the cortical layer. (B-E) During stages 6-10, abundant Arl1 mRNAs were detected in the ventral (arrow v), cephalic (arrow c), anterior and posterior transverse (arrows at and pt, respectively) furrows. (F) A late staged 11 embryo that has formed segmentation furrows shows a ubiquitous pattern of Arl1 mRNA accumulation. (G-I) After the completion of gastrulation, Arl mRNAs show enriched accumulation in the brain lobes (arrows) and central nervous system (arrow head).

**FIGURE III-5. Subcellular localization of Arl1 proteins in S2 cells:** (A) In a S2 cell that is transfected with DNA encoding Arl1-EGFP (Green) and (A’) stained with Golgi marker TRITZ-Ceramide (red), (A’’) the EGFP-tagged Arl1 is colocalized with the Golgi marker. (B) When the N-terminal 1-17 region was removed (Δ17arl1-EGFP, green), Arl1 lost its fragmented punctate pattern and instead is dispersed throughout the cytoplasm. (C) In a S2 cell transfected with DNAs for Arl1-mCherry (red) and (C’) YFP-Golgi (Golgi marker, green), (C’’) mCherry-tagged Arl1 is colocalized with the Golgi marker. (D) Myc-tagged Arl1 (red) is colocalized with the YFP-Golgi (green) in S2 cells.

**FIGURE III-6. Subcellular localization of Arl1 in oocyte and brain cells:** In an oocyte expressing (A) Golgi marker YFP-Golgi (green) and (A’) Arl1-mCherry (red), (A’’) mCherry-tagged Arl1 proteins exhibit a nice colocalization with the Golgi marker. In brain cells expressing (B) Golgi marker YFP-Golgi (green) and (B’) Arl1-mCherry (red), (B’’) the mCherry-tagged Arl1 is colocalized with the Golgi marker.
FIGURE III-7. Arl1 can interact with the GRIP domain of Cbs in yeast two-hybrid assay: Interaction of Arl1 proteins, including wild type Arl1 and mutants expected to alter its preference for guanine nucleotide binding (arl1<sup>Q70L</sup>: GTP; arl1<sup>T30N</sup>: GDP), with the GRIP domain (GRIP) or the coiled-coil region (CC) preceding the GRIP domain was tested. Arl1 binds to GRIP in a GTP dependent manner, but did not interact with the coiled-coil region preceding the GRIP domain.
FIGURE III-1

WT Arl1

Myristoylation

arl1^{4107}

arl1^{9.28C}

Nα helix α helix β strand
FIGURE III-2
FIGURE III-3
FIGURE III-5
FIGURE III-6

Oocyte

Brain cells

YFP-Golgi  Arl1-mCherry  Merge

20 μm

5 μm
Contribution of this work

Unless otherwise noted, the experiments in this paper were performed by Jian Ding. The Arl1-mCherry transgenic flies were generated by Yukiko Yamada and Dr. Clark Coffman. Jian Ding and Dr. Ambrosio contributed to the writing, reviewing and editing of this work.
CHAPTER 4. General Summary

The Raf serine/threonine kinases play a key role in ERK signal transduction. However, the mechanisms of Raf regulation are complicated and remain elusive. Clues to regulatory events of Raf were derived from the identification of motifs/sites in these conserved regions.

Raf kinases consist of three conserved regions, CR1, CR2, and CR3. The catalytic kinase portion is located in the C-terminal CR3 region, while CR1 and CR2 represent the regulatory half of the Raf proteins. CR1 contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD), and is required for recruitment of Raf to the plasma membrane. The Ser/Thr-rich region CR2 has a 14-3-3 binding site, but its functional role is less well defined (WELLBROCK et al. 2004). In addition to the three conserved regions, BRaf and DRaf contain an extended N-terminal segment. However, little attention was focused on this region thus far.

Summary and Conclusions

With the general objective of understanding the roles of DRaf’s N-terminal segment and the CR2 region: 1) we analyzed the consequences of N-terminal deletion of DRaf both in vivo and in vitro, and found that the N-terminus of DRaf contains a novel conserved region and can contribute to Toros RTK signaling, potentially by assisting in Ras1 or Rap1 binding; 2) we also performed a yeast two-hybrid screen using the CR2 region as the bait. We identified a small GTPase, Arl1, as a potential binding partner of CR2. Using genetics approaches we have made an effort to understand the functional roles of Arl1 in Drosophila. The studies in this dissertation are summarized here.
**Drosophila Raf’s N-terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling:** To study the potential function of DRaf’s N-terminal residues (amino acids 1-114), we generated transgenic flies expressing full-length DRaf (FL DRaf) or DRaf proteins lacking amino-terminal residues 1-114 (DRaf$^{AN114}$). At ≥ endogenous wild type DRaf levels, maternally expressed DRaf$^{AN114}$ was able to rescue the embryonic terminal defects of Drafl$^{11-29}$ mutants. However, when expressed at low maternal levels (~1/4 endogenous wild type DRaf level), terminal defects, as well as, abnormal posterior tll expression pattern (<13% EL) was observed more often for embryos that inherited truncated DRaf$^{A114}$ rather than full-length DRaf proteins. This finding suggested that deletion of the N-terminus reduces DRaf’s signal potential in the Torso RTK pathway. Furthermore, in the sensitized tor$^{RL3}$ background, the ectopic gain-of-function effects of tor$^{RL3}$ allele were more significantly enhanced by over-expressed FL DRaf, compared to DRaf$^{A114}$. Together, these data indicate that the extended N-terminal segment could contribute to Torso RTK signaling.

Accordingly, FL DRaf also exhibited higher signal potential than DRaf$^{A114}$ proteins in a trk$^I$ background, in which a small amount of Torso signal activity may exist. Over-expression of FL DRaf but not DRaf$^{A114}$ partially restored the posterior terminal defects (A8 denticle belt) in some embryos from trk$^I$/trk$^I$ mothers, again implying that the N-terminus can participate in Torso RTK signaling. However, in the tor$^{XR1}$/tor$^{XR1}$ background with complete loss of receptor activity, expression of neither FL DRaf nor DRaf restored terminal structures (A8 denticle belt). Thereby, the N-terminal region’s contribution to the RTK pathway appeared to be dependent on upstream receptor activity, suggesting that it may play a role(s) in transmission of the signal.
We analyzed the amino acid sequence of DRaf’s N-terminus and identified a novel region (CRN) conserved in BRaf proteins. Circular dichroism analysis indicated that DRaf’s N-terminus (amino acids 1-117) including CRN (amino acids 19-77) is folded in vitro and has a high content of helical secondary structure as predicted by proteomics tools. In yeast two-hybrid assays, stronger interactions between DRaf’s Ras binding domain (RBD) and the small GTPase Ras1, as well as Rap1, were observed when CRN and RBD sequences were linked. This is consistent with the results obtained by Fischer et al. (2007) for BRaf.

Together, our in vivo and in vitro assays suggest that DRaf’s extended N-terminus can assist in its association with the upstream activators (Ras1 and Rap1) in vivo and thus, play a regulatory role(s) in DRaf signal transmission potentially through a CRN-mediated mechanism(s).

The N-terminus of DRaf seems to participate in Ras1 and Rap1 binding, but as an isolated protein fragment cannot directly interact with Ras1 or Rap1. Furthermore, the effects of the N-terminal residues on Ras1 or Rap1 binding in vitro, as well as on Torso RTK signaling in vivo appears to be subtle. However, the minor contribution of the extended N-terminus could have important biological implications. We propose that tight association with Ras1 or Rap1 through RBD and CRD regions is required and sufficient to initiate the activation of DRaf, while minor adjustments/regulation of the interaction by CRN could optimize signaling potential and reduce variability. Thus, the extended N-terminus including CRN may play a role(s) as one element in a multi-domain effort to promote DRaf’s interaction with Ras1 and Rap1, participating and assisting in its regulation to reliably attain maximal signal output.
**Initial characterization of Drosophila Arl1:** We initiated our study on Arl1 based on the hypothesis that Arl1 may be involved in DRaf signaling, since Arl1 can interact with the CR2 region of DRaf in our Yeast two-hybrid assay. Three different arl1 alleles were analyzed using classical genetic methods. However, we did not detect DRaf relevant phenotypes for Arl1 mutants.

We found most homozygous arl1- mutants proceeded through embryogenesis and died during larval-pupa stage. Cuticles of unhatched embryos or arl1+/arl1- larvae appeared to be relatively normal. Thereby, zygotic Arl1 may not be essential for the development of embryos.

Next we generated females with arl1+/arl1- germ cells to understand Arl1’s maternal role. Most embryos lacking maternal Arl1 activity appeared to be relatively normal, although ~6% of them exhibited head involution defects. When crossed with arl1+/+ males, the hatching rates of eggs from females with arl1+/arl1- germ lines were not significantly altered, implying removal of paternal Arl1 in half of the eggs did not appear to affect their embryogenesis. It seems that both maternal and zygotic Arl1 is limitedly required during embryo development.

The head involution defects we observed, although at low penetrance, suggest Arl1 can be involved in cellular morphogenesis. Considering Arl1’s potential role(s) in intracellular transport, we tested for genetic interaction between arl1 alleles and mutations in numerous genes encoding membrane-localized adhesion molecules. These have been shown or are thought to function in cellular morphogenetic movement due to their dynamic presence on the cell surface, which is regulated by endocytosis and exocytosis (EDELMAN 1985;
However, no additive or synergistic effects were seen.

The mild phenotypes observed in arl1 mutants could be due to the functional redundancy of Arl1 with other small GTPases, including Rab proteins. However no genetic interaction, with the limited number of candidates that we tested, was detected. Therefore, there might be other proteins that function redundantly with Arl1. Together, our analysis implies that it is difficult to dissect Arl1’s roles using classical genetic methods to study these arl1 alleles.

Next, we employed an over-expression strategy to characterize Arl1’s function in Drosophila. We found over-expression of arl1Q70L-mCherry in germ cells reduced female fertility and ~8% of stage 10 egg chambers of these females exhibited delay in border cell migration. Considering Arl1’s potential functions in intracellular transport, we proposed that the border cell migration defects upon over-expression of arl1Q70L-mCherry might be due to dysregulation of the adhesion molecules in nurse cells. However, none of the mutations in adhesion molecules we tested showed additive or synergistic effects when arl1Q70L-mCherry was over-expressed in nurse cells.

We also examined Drosophila Arl1's subcellular localization. Epitope-tagged Arl1 proteins were colocalized with golgi markers both in vitro for Drosophila S2 cells and in vivo for the oocyte and brain cells. This is consistent with the studies by others in yeast and mammalian cells. We also found that the mRNA of Ar11 ubiquitously accumulates in embryos with higher levels observed for certain tissues.

Together, our genetic analysis of three different arl1 alleles suggested that Arl1 could function in cell morphogenesis. Although Arl1 was found to interact with DRaf’s CR2 region
in our yeast two-hybrid assay, it seemed that mutations in Arl1 did not alter DRaf signaling. While an essential gene, Arl1 plays limited roles during Drosophila embryogenesis, likely due to the functional redundancy with other genes. Our studies provide the basis, which may be helpful for further characterization of Arl1’s function.

**Suggestions for Future Research**

**Study of DRaf or BRaf’s N-terminus:**

1) The N-terminus of DRaf appears to participate in Ras1 binding, however, the details of this process is still unclear. Structural analysis of the complexes (i.e., NRBD-Ras etc.), may provide important clues and help to understand the molecular mechanism(s) by which CRN assists in Ras-Raf interaction.

2) The CRN region contains several conserved putative phosphorylation sites, which may play regulatory roles in DRaf or BRaf signaling. Studying the consequences of point mutation on DRaf’s signal potential or Ras binding would provide important insights into the functional roles and mechanisms of this N-terminal region.

3) Compared to ARaf and CRaf, BRaf is more liable to oncogenic mutation, likely due to its higher basal activity. The negative charged D447-D448 motif (corresponding to E420-E421 in DRaf) preceding the kinase domain in BRaf mimics the constitutive phosphorylation in this region (MASON et al., 1999). This may account for its high basal activity. Fischer et al. (2007) reported that CRaf’s activity was elevated ~25% by the addition of the first 98 amino acids of BRaf to its N-terminus, implying that the extended N-terminus may contribute to BRaf’s high basal activity, too. One could test if there is additive or synergistic interaction between the N-terminal residues and the negative charged phosphomimetic motif. Particularly,
comparing the activity of DRaf$^{\Delta N114,V420V421}$, DRaf$^{V420V421}$, DRaf$^{\Delta N114}$ and FL DRaf (BRaf$^{\Delta N98,V447V448}$, BRaf$^{\Delta N98,V447V448}$, BRaf$^{\Delta N98}$ and FL BRaf) would be helpful to extend our understanding of DRaf or BRaf’s basal activity, as well as, the liability to oncogenic mutations.

4) BRaf$^{V599E}$ accounts for ~90% oncogenic BRaf mutations in human cancers (SRIKALA et al. 2005). BRaf$^{V599E}$ is a constitutive active mutant. Although its gain-of-function effects seem independent of Ras binding, it is still possible that presence of the extended N-terminus may contribute to its constitutive activity. One could test if the deletion of the N-terminus affects the oncogenic activity of BRaf$^{V599E}$.

**Further characterization on Arl1:**

1) While an essential gene, Arl1 seems to play limited roles in Drosophila embryogenesis. Only mild defects were observed in Arl1 mutants. Lack of significant phenotypic information is likely due to its functional redundancy with other genes. We tested whether there were additive or synergistic effects between arl1 alleles and mutations of several candidate genes, which may act redundantly with Arl1. However, due to the difficulty of generating complex genetic backgrounds using classical methods, we did not resolve the issue of functional redundancy because our genetics interaction assays appeared not sensitive enough. NIH has just begun to support a TRiP (Transgenic RNAi Project) project to develop the UAS-RNAi system in a genome-wide scale. One could also combine RNAi sequences of several different genes (Arl1, Rab2, Rab6, etc.) and simultaneously deplete their functions in flies. This would be very helpful for characterization of Arl1’s functions.
2) Identification of the components/cargo of Arl1 complex/trafficking machinery using proteomic approaches could also be helpful to characterize its developmental roles. Particularly, one could co-precipitate or pull-down the proteins of Arl1 complex from cellular lysates, and using mass spectrum analysis to identify the components. A combination of these proteomics approaches could allow the in vivo genetic analysis of Arl1 more promising.

Literature Cited


GUMBINER B.M. 1996 Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84:345-357


APPENDIX

Appendix 1. Genetic Screen for arl alleles: EMS mutagenesis

Parental:

\[ \text{EMS} \]
\[ \begin{align*}
\text{♂} \quad w^Y; e/e & \times \quad \text{♀} \quad w^{1118}/w^{1118}; TM2, \ Ubx / TM6, Tb, Sb, e \\
\end{align*} \]

F1 single male mating:

\[ \begin{align*}
\text{♂} \quad w^{1118}/Y; +*, e/TM6, Tb, Sb, e & \times \quad \text{♀} \quad yw/yw; arl^{4107}/TM6, Tb, Sb,e
\end{align*} \]

Screen:

\[ \begin{align*}
1) +*, e/ arl^{4107} \\
2) +*, e/TM6, Sb,Tb, e \\
3) arl^{4107}/TM6, Sb, Tb, e \\
4) TM6, Sb, Tb, e/TM6, Sb,Tb, e \\
\end{align*} \]

Screen vials and keep those with only Tubby (Tb) pupae.
Select 5: y w /Y; +* e / TM6, Tb, Sb, e [yellow, white, ebony] males to outcross with y w y w; TM2, Ubx / TM6, Tb, Sb, e females. Repeat the outcross for several times, then generate stocks for +* e / TM6, Tb, Sb, e

Note:

Double Tm6 balancer → lethal
X chromosome markers:
\[ w = \text{white eyes, recessive} \]
\[ w^{1118} = \text{white eyes, recessive} \]
\[ y = \text{yellow wings/body, recessive} \]
3rd chromosome markers:
\[ Ubx = \text{large halters, dominant} \]
\[ Sb = \text{short bristles, dominant} \]
\[ Tb = \text{Short body, dominant} \]
\[ e = \text{dark body, recessive} \]
## Appendix 2. List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRIRaf-F</td>
<td>CGGAATTCCACCATGTCCAGGAGTGTCCTCCAC</td>
</tr>
<tr>
<td>EcoRICR1-F</td>
<td>CGGAATTCCACCATGCACCGGCAACGCATC</td>
</tr>
<tr>
<td>EcoRICR2-F</td>
<td>CGG GAA TTC ATG CTG TGC CAG CCC TTT C</td>
</tr>
<tr>
<td>EcoRICR3-F</td>
<td>CGG GAA TTC GTG TGC ATC AAC AAC ATT CG</td>
</tr>
<tr>
<td>EcoRIΔ17Raf-F</td>
<td>CGGAATTCATGGGCCAGAGCTGCAC</td>
</tr>
<tr>
<td>EcoRIΔ77Raf-F</td>
<td>CGGAATTTCATGAACTCGGAGGACCAG</td>
</tr>
<tr>
<td>BamHIRaf-R</td>
<td>CGCGGATCCGGCTGGATATTTAGTATCCAG</td>
</tr>
<tr>
<td>BamHICR1-R</td>
<td>CGGGATCC GAAAGGGTAC GCACAGCATC</td>
</tr>
<tr>
<td>BamHIRBD-R</td>
<td>CGG GAT CTT TAT TCC AGC AGC CTT AAG ATC AAG ATC</td>
</tr>
<tr>
<td>BamHICR2-R</td>
<td>CGGGATCC GGT GGT GAT TAC CACATTTGG</td>
</tr>
<tr>
<td>BamHIN-R</td>
<td>CGGGGATCTTAATGTACTCGGCAATGGCCGTGG</td>
</tr>
<tr>
<td>C110-F</td>
<td>GCC CCT GAA ACT CCT GCA ACT AAG GCC GGA TAT G</td>
</tr>
<tr>
<td>C110-R</td>
<td>CAT GAT CCG CAT TAG TTG CAG GAG TTT CAG GGC C</td>
</tr>
<tr>
<td>NTE-F</td>
<td>GAATTACGAGGTAGGATATTTAGTATCCAG</td>
</tr>
<tr>
<td>NTE-R</td>
<td>CAGTTCGAGCAGGTAGGATATTTAGTATCCAG</td>
</tr>
<tr>
<td>NTV-F</td>
<td>GAA TAC CAG GAG TTC GTA TCC AAG CTC CAC GAA CTC TAC</td>
</tr>
<tr>
<td>NTV-R</td>
<td>CAG TTC GAG CTT GGA TAC CAA CTC CAG GTA TTC TAC</td>
</tr>
<tr>
<td>EcoRIRas-F</td>
<td>CG GAA TCTAGACGGGATACAAACTGGTC</td>
</tr>
<tr>
<td>BamHIRasA-R</td>
<td>CG GGA TCC TA TCTACATTGGCTTTGTC</td>
</tr>
<tr>
<td>EcoRIRap-F</td>
<td>CGG AAT TCA TGC GTG AGT ACA AAA TCG TG</td>
</tr>
<tr>
<td>BamHIRapA-R</td>
<td>CGG GAT CCT TAT AGG GAC TTT TTC GGC TTC</td>
</tr>
<tr>
<td>EcoRI1433-F</td>
<td>CGGAATTCATAGGCGACGTGCATAGGG</td>
</tr>
<tr>
<td>BamHI1433-R</td>
<td>CG GGA TCC TGAATTGTCTTTAAGTGTGTC</td>
</tr>
<tr>
<td>ChaiKSRCA-F</td>
<td>CC AT CGA TCC AGGATGACGCAACGACAC</td>
</tr>
<tr>
<td>XholKSRCA-R</td>
<td>CCCTCGAG CTCTTATAGGCGCCTACTTCG</td>
</tr>
<tr>
<td>XholKSRCC-R</td>
<td>CC TCT GAG TTA CGC AGT ACCGCTTCCTCGC</td>
</tr>
<tr>
<td>XholKSRN-R</td>
<td>CC TCT GAG TCA GCC ACT GCT CTC CAC</td>
</tr>
<tr>
<td>HindIIIAr1-F</td>
<td>AAGCTTGCTGCTACATTGGCTGAGATAC</td>
</tr>
<tr>
<td>HindIII17arl-F</td>
<td>CG AAGCTTTA CCA TGC GCA TCT TAA TCC TGG</td>
</tr>
<tr>
<td>BamHIarl-R</td>
<td>CGG GATCC GAA AG TAT CTA CTT GCG ACT C</td>
</tr>
<tr>
<td>KpnIarl-F</td>
<td>CGG GATCC GAA AG TAT CTA CTT GCG ACT C</td>
</tr>
<tr>
<td>XbalAr1-R</td>
<td>GC TCT AGA TGA TGA CTT CCG ACT CTC C</td>
</tr>
<tr>
<td>XbalInChe-R</td>
<td>GATCAGAGCAACATTAGTGAGCAAG</td>
</tr>
<tr>
<td>AgeIInChe-R</td>
<td>TCACCGGTCTTTCTTTCTGAGGCTGTC</td>
</tr>
<tr>
<td>EcoRIarl-F</td>
<td>CGG AAT CTA CCA TGC GCA TCT TAA TCC TGG</td>
</tr>
<tr>
<td>BamHIChe-R</td>
<td>CGG GGA TCC TTCTTACTTGTACGCTCGTC</td>
</tr>
<tr>
<td>EcoRIΔ17arl-F</td>
<td>CGG AAT TCA CCA TGC GCA TCT TAA TCC TGG</td>
</tr>
<tr>
<td>BamHIIarlS-R</td>
<td>CGG GATCC GAA AG TAT CTA CTT GCG ACT C</td>
</tr>
<tr>
<td>T30N-F</td>
<td>GA CGG CGG CGG CAA GAA CAC GAT CTT CTA CAG</td>
</tr>
<tr>
<td>T30N-R</td>
<td>CTG TAG AGG ATG TTC GTC TTC CGG GCC CCG GC</td>
</tr>
<tr>
<td>NdelCbsCC-F</td>
<td>CC CAT ATG GACGAAACCATATAATGCCAANTGTG</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>BamHIbsCC-R</td>
<td>CG GGA TCC GTGTTTCAATATTGAAAATTAAC</td>
</tr>
<tr>
<td>EcoRIGrip-F</td>
<td>CG GAA TTC GTTAATTTTCAATATTGAAACAC</td>
</tr>
<tr>
<td>BamHIgrip-R</td>
<td>CG GG ATC CTA CAA TGT GTG TAG CAG C</td>
</tr>
<tr>
<td>tll-F</td>
<td>TCGGAGGGGTTCCACCCAGAC</td>
</tr>
<tr>
<td>tll-R</td>
<td>GAGATGAGGCGCACAATGG</td>
</tr>
<tr>
<td>en-F</td>
<td>AATGGCCCTGGAGGATCG</td>
</tr>
<tr>
<td>en-R</td>
<td>CCGTCAGAAGGAGTAACCC</td>
</tr>
<tr>
<td>P1-F</td>
<td>GCGCCA GCACTAGACAAAG</td>
</tr>
<tr>
<td>P1-R</td>
<td>GAAATAGCAGCAATTGAGATTAG</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First I would like to thank my major professor, Dr. Linda Ambrosio, for her guidance and support throughout my Ph.D program. Her encouragement always helped me a lot to handle frustrations, to overcome difficulties and to make progress in understanding the mystery of developmental genetics. I also deeply appreciate her insightful advices on my research that are essential for me to develop a critical thinking in pursuit of science.

I would like to thank my POS committee members, Dr. Amy Andreotti, Dr. Jack Girton, Dr. Ted Huiatt and Dr. Jo Anne Powell-Coffman. Here I want to express my sincere gratitude to them for their comments, advices, and very helpful suggestions on my thesis.

I want to thank Dr. Janice Buss, Dr. Clark Coffman, Dr. Yanhai Yin and Dr. Kristen Johansen for their assistances during my Ph.D study. I also want to thank Oren Tchaicheeyan, Virginia Lephart, Laurence Woodruff, Weili Cai, Yun Ding, Yukiko Yamada, and Buss lab members, Hui Zheng, Xing Wang, Jodi Mckay, Jessica Steenhoek, and Daniel R. Zamzow for their help on my research.

Here, I want to thank my parents and my brother. Their support always is an essential part of my life. Specially, I want to thank my wife, Lei Li. She gives me, not only family warmthness, but the courage and faith that makes my life go on and on.