The SH2 Domain-Containing Adaptor Protein SHD Reversibly Binds the CRKL-SH2 Domain and Knockdown of shdb Impairs Zebrafish Eye Development

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THE SH2 DOMAIN-CONTAINING ADAPTOR PROTEIN SHD REVERSIBLY BINDS THE CRKL-SH2 DOMAIN AND KNOCKDOWN OF SHDB IMPAIRS ZEBRAFISH EYE DEVELOPMENT

A Thesis Presented

by

Brendan Chandler

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Master of Science
Specializing in Biology

May, 2018

Defense Date: March 23, 2018
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ABSTRACT

The adaptor protein CT10-Regulator of Kinase (CRK) and the closely related CRK-Like (CRKL) are adaptor proteins that play important roles in many signaling pathways regulating cell proliferation and cell motility. A notable example is their required role in Reelin signaling during development of the laminated structures of the vertebrate central nervous system, including the cerebral cortex, cerebellum, hippocampus, and retina. As adaptors, CRK/CRKL are important in coupling phosphotyrosine signaling to G protein activity to regulate both cell proliferation and changes in the actin cytoskeleton, thereby exerting control over cell motility, and migration. While many proteins that interact with CRK/CRKL have been identified, the diverse roles of these molecules suggest that more remain to be found.

Herein is described a novel CRK/CRKL interacting protein, Src Homology 2 domain-containing protein D (SHD), which demonstrates a phosphorylation-dependent interaction with the CRK/CRKL SH2 domain in HEK 293 cells stimulated with hydrogen peroxide, which globally boosts tyrosine phosphorylation by inhibiting tyrosine phosphatases. Treatment with an inhibitor for Src family kinases (SFKs), Src-1, or an inhibitor of Abl/Arg kinases, STI571, reduces peroxide-induced binding of the CRKL-SH2 domain to SHD. We show that overexpression of Abl kinase, but not the SFK Fyn is sufficient to induce binding of the CRKL-SH2 to SHD and that this interaction requires at least one of the five tyrosines in YxxP motifs found in SHD. Using mass spectrometry, we found that Abl phosphorylates SHD on Y144, which is located in a YxxP motif. Mutation of this site to phenylalanine reduces, but does not prevent, Abl-induced binding of SHD to the CRKL-SH2 domain, suggesting that other YxxP sites also facilitate the interaction. A discussion of the cellular consequences of the interaction between SHD and CRK/CRKL is presented.

To explore the biological role of SHD, we used the zebrafish to study shdb, a putative ortholog of human SHD. The expression of shdb was unknown and so we performed in situ hybridization and determined that shdb was expressed in the developing nervous system. To study the function of this gene, we used a morpholino to knock down expression of shdb which resulted in significantly reduced eye size. Possible roles of Shdb in eye development are discussed as is future research aimed to elucidate the cellular and developmental mechanisms by which Shdb functions in the developing eye.
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CHAPTER 1: REVIEW OF THE ROLES OF CRK/CKRL IN SIGNALING

1.1. Introduction

Protein phosphorylation constitutes a major mechanism of signaling and regulation in eukaryotes which is required for many cellular processes. Enzymes known as kinases catalyze the transfer of δ-phosphate from ATP to the free hydroxyl group onto tyrosine, threonine, or serine residues of target proteins. The additions of the negatively-charged phosphate group to a protein can have two major consequences. Phosphorylation can alter the activity of proteins with enzymatic activity or create docking sites for other proteins. Actions mediated by phosphorylation are readily reversible by phosphatases, enzymes which remove phosphate groups from proteins. In addition to regulating other enzymes, phosphorylation regulates kinases and phosphatases themselves. By regulating the activity level and localization of kinases and phosphatases, the cell tightly controls protein phosphorylation both spatially and temporally to produce an appropriate response to external signals. This is an important balance for cells to maintain and many disease states result from misregulation of phosphorylation including cancers and developmental disorders [51, 107].

Protein phosphorylation was first discovered by Phoebus Levene in the early 1900s [72]. Phosphoserine was specifically discovered in the 1930s by Fritz Lipmann [76]. However, phosphorylation on tyrosine was not identified until the 1970s [52]. This is in part due to the relative abundance of phosphoamino acids in the cell. Phosphoserine accounts for as much as 90% of the phosphorylated amino acid content in the cell, with phosphothreonine accounting for another ~9%, and phosphotyrosine typically making up
~1% [51]. Despite this, 17% (90 of 540 kinases encoded in the human genome) are tyrosine kinases [82, 102]. In addition, humans have 108 tyrosine phosphatases [52], and the half-life of phosphotyrosine is brief compared to phosphoserine and phosphothreonine, sometimes lasting only a few seconds [66]. These observations highlight the importance of tyrosine phosphorylation as a particularly tightly regulated mechanism for cell signaling.

The best characterized tyrosine phosphorylation events are the actions of Receptor Tyrosine Kinases (RTKs), a group of transmembrane protein kinases that transduce many external signals influencing cell growth, migration, differentiation, division, and death [51, 107]. RTKs bind ligands such as EGF, VEGF, PDGF, and NGF, leading to dimerization of the receptor and trans-autophosphorylation of their intracellular domains [52]. The phosphorylated tyrosine residues on the intracellular domain then act as docking sites, recruiting relevant downstream proteins to the cell membrane and bringing them into proximity with other proteins necessary to propagate the signal. Non-receptor Tyrosine Kinases (TKs) have also been characterized and function in the cytoplasm to phosphorylate proteins not only at the membrane but also in the cytoplasm or nucleus [52]. Perhaps the most well studied non-receptor tyrosine kinase is Src. The Src kinase is a ubiquitously expressed protein integral to many cell functions. It was originally characterized in its viral form, v-Src from a chicken virus, where it was found to be the gene product sufficient for tumor formation by its intrinsic kinase activity [17, 73, 104]. A cellular homolog, c-Src was soon discovered and identified specifically as a tyrosine kinase [8, 18, 53].
interactions of Src kinase could fill several books, but some notable roles include cell
growth, differentiation, adhesion, and motility [8]. Indeed, an entire group of related
tyrosine kinases are known collectively as Src Family Kinases (SFKs), of which Src, Fyn,
and Yes are the most widely expressed and ubiquitous [120].

Once a tyrosine residue has been phosphorylated by a kinase, it can serve as a
binding site for proteins which contain either a Src Homology 2 (SH2) or
Phosphotyrosine Binding (PTB) domains. These interactions are not only dependent on
the phosphorylated tyrosine. PTB and SH2 domains all display preferences for specific
amino acid motifs near the phosphorylated tyrosine, allowing for high precision in the
protein-protein interactions mediated by phosphotyrosine [34, 131].

The discovery of modular protein domains such as the SH2 domain led to a new
understanding in cell biology where protein-protein binding through scaffold and adaptor
proteins often results in the formation of large signaling complexes. Scaffolds and
adaptors are proteins which typically lack enzymatic activity and function by acting as
intermediaries, binding proteins via modular protein domains. Both scaffolds and
adaptors function by controlling the localization of proteins to initiate, propagate, and
amplify signals within the cell. Although the difference between scaffold and adaptor
proteins is not well defined in the literature, and they are sometimes used
interchangeably, it has become colloquially accepted that the terms refer to distinct types
of molecules. Scaffold proteins are typically larger, associate with specific structures
within the cell, and often have multiple sites that facilitate the same interaction. The
scaffold protein CRK (chicken tumor virus CT10-Regulator of Kinase) Associated
Substrate (CAS) is an excellent example of a stereotypical scaffold protein. CAS is a relatively large (130kDa) protein which localizes to focal adhesions, becomes phosphorylated after integrin activation, and contains up 16 sites that when phosphorylated mediate the binding of CRK-SH2 [45, 67]. Adaptor proteins are generally smaller, mobile in the cell, and contain two or more modular protein domains or protein-protein binding regions. The above-mentioned protein CRK and its close relative CRKL (CRK-like) contain an SH2 domain, one or two SH3 domains, and are classic examples of adaptor proteins. They function by localizing proteins bound to their SH3 domain to substrates of their SH2 domain upon phosphorylation of the appropriate site on targets such as CAS [45, 67]. The actions of scaffolds and adaptors can be roughly described with an analogy where scaffold proteins act as the baseball stadium where the game will be played, and the adaptor acts as the bus that brings all the players to the game.

1.2. The CRK/CrkL Adaptor Proteins:

CRK and CRKL are ubiquitously expressed adaptor proteins with numerous functions and a rich history [32, 96]. CRK was first identified by Bruce Mayer as a viral oncogene in an avian sarcoma virus which shared some sequence homology with the mammalian SH2 domains of tyrosine kinases and the enzyme phospholipase C [83]. It had previously been demonstrated that this avian virus caused tumors to form when injected into chickens [56]. The transforming factor was identified and termed p47\textsuperscript{gag-crk}, later renamed v-Crk after discovery of its cellular homolog [83, 84]. v-Crk alone was
sufficient to cause transformation, and it was observed that v-Crk selectively increased phosphorylation on some proteins including endogenous kinases, despite lacking any tyrosine kinase activity itself [7, 83, 84]. This was intriguing and puzzling at the time, but we now know that fusion of CRK to the viral gag protein results in translocation of v-CRK to the cell membrane, an event that facilitates the cellular actions of CRK through activators bound to its SH3 domain [7, 84]. In normal cells, localization of c-CRK to the membrane is tightly controlled by phosphorylation events.

Our understanding of these adaptor proteins has grown a great deal since these first discoveries. In humans, CRK occurs in three isoforms: CRK-I, CRK-II, and CRK-III, while CRKL occurs as only a single splice product [7]. CRK-II is the predominant isoform in most cells and is similar in structure to CRKL. Both CRK-II and CRKL contain an SH2 domain near the N-terminus, a flexible linker region, followed by two SH3 domains [68]. The isoform CRK-I lacks the C-terminal SH3 domain, but still contains one SH2 and one SH3 domain [7]. CRK-III contains an SH2 domain, an SH3 domain, and a partial second SH3 domain [16]. The SH2 domains of CRK and CRKL have been found to bind to target proteins with phosphorylated tyrosine in a YxxP motif [131]. The SH3 domain binds to proline rich regions on proteins with the preferred consensus motif PxxPxxK [4, 16]. This structure allows CRK and CRKL to act at the heart of many signaling events, where it facilitates the formation of large signaling networks that can incorporate many, often opposing signals to produce a unified output signal and direct an appropriate cellular response.
1.3. CRK and CRKL in Cell Signaling:

The actions of CRK and CRKL seem relatively simple, and yet achieve a wide array of functions in the cell which can cause disease when aberrant. Both these molecules function by binding two other proteins. The SH3 domain binds to proline rich regions (PXXPXK) on target proteins including the guanine nucleotide exchange factors (GEFs) C3G, Dock180, and SOS, and kinases such as c-Abl and JNK. The CRK/CRKL SH2 domain targets a large and growing list of proteins including Paxillin, Signal Transducer and Activator of Transcription 5 (STAT5), Endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN), also known as Discoidin, CUB and LCCL domain containing 2 (DCBLD2), Crk Associated substrate of Src (CAS), and Disabled-1 (Dab-1) [4, 68].

CRK and CRKL were initially of interest because of the oncogenic properties of v-CRK, which in cells increases phosphorylation despite lacking any kinase activity. The role of these proteins in cancer became apparent when it was shown that CRKL is responsible for transmitting the effect of the BCR-Abl fusion protein which causes Chronic Myelogenous Leukemia (CML) [91, 92]. CML accounts for approximately 15% of Leukemia cases in adults [30]. Fortunately, it is now amongst the most treatable forms of cancer thanks to intensive research and rational drug design resulting in the production of Imatinib, an inhibitor of the Abl tyrosine kinase [59, 90]. The BCR/ABL fusion protein is the product of the Philadelphia chromosome (Ph), named for the city where it was discovered by David Hungerford and Peter Nowell in 1959 [127]. Caused by a reciprocal translocation between chromosome 9 and 22, BCR-Abl acts as a constitutively
active Abl kinase. This is caused by the BCR fusion which results in loss of auto-
inhibitory characteristics of Abl encoded by exon 1, which is lost in the translocation [90]. Abl kinase phosphorylates substrates on tyrosine and prefers a proline in the +3 position, a motif that matches the CRKL-SH2 binding sequence [116]. CRKL was identified as a constitutively phosphorylated protein in neutrophils isolated from CML patients [91, 92]. The overactive BCR-Abl protein hyper-phosphorylates both CRKL and many CRKL-SH2 binding proteins, leading to over activation of CRKL signaling pathways including those controlling cell growth and proliferation [117]. The precise mechanism of transformation by CRKL in the presence of BCR-Abl is, however, unknown. Importantly, propagation of signals from the BCR-Abl oncogene is unique to CRKL. This is due to a regulatory mechanism found in CRK where phosphorylation of Y221 on CRK results in binding of its own SH2 domain, condensing the molecule, and blocking the binding of substrates to both the SH2 and SH3 domains [16]. The analogous site on CRKL, Y207, also appears to play an inhibitory role, and loss of this site results in greater association of CRKL with its SH2 substrates and increased ability to transform cells in culture [111]. The Y221 of CRK and Y207 of CRKL are the primary sites of phosphorylation by BRC-Abl [16, 25]. In spite of this, the phosphorylation on Y207 does not appear to be sufficient to inhibitor the actions of CRKL in CML [110]. This constitutes one of the first differences observed between CRK and CRKL. While they are analogous in some settings, they are not equivalent [68].

Another key role of CRK and CRKL was found downstream of integrin receptor proteins. Integrins link the cytoskeleton of the cell to the extracellular matrix, and so are
critical for regulating growth and proliferation, cell motility, and adhesion [16, 67].

Associated with integrins on the cell surface are large protein complexes known as Focal Adhesions (FAs) which serve as the interface between the integrins and the cytoskeleton. Two major constituents of FAs are paxillin [10] and CAS [45, 98]. Interaction between integrins and the ECM induce clustering of integrins, which can directly activate Src kinase by interaction between integrin αIIbβ3-tails and the Src SH3 domain [3]. Upon CAS phosphorylation by Src, CRK/CKR is recruited to CAS and binds phosphorylated YxxP sites. Translocating with CRK and CRKL via binding to the SH3 domain are proteins such as DOCK 180, a GEF that activates primarily Rac1 [65], and C3G, which primarily activates Rap1 [124]. The close proximity of the GEF to the GTPase attached to the cell membrane results in exchange of GDP for GTP and activation of the GTPase. In the case of DOCK180, activation of Rac1 causes actin polymerization leading to extension of the membrane for cell crawling [16, 65]. Translocation of CRK and CRKL via their SH2 domain is critical to its function. This is elegantly demonstrated in an experiment where fusing a farnesylation tag sequence from the Ki-ras protein to CRKL creates a construct which is forced to associate with the membrane [54]. This was shown to enhance the activity of the GTPase Rap-1 through the guanine nucleotide exchange factor (GEF) C3G, even in constructs where the SH2 domain was deleted [54]. GTPase activity did not increase, however, when the N-terminal SH3 domain was deleted, even in the farnesylated construct [54]. Similar results were observed by fusion of a paxillin targeting LIM domain to CRKL resulting in localization of CRKL to focal adhesions [74]. This fusion protein was able to rescue motility defects in cells deficient in Src, Yes,
and Fyn kinases (SYF cells) [74]. Together, these experiments demonstrated that both the SH2 and SH3 domains of CRK/CRKL are critical to its function. The SH2 domain deletion can be rescued by targeting CRKL close to its SH2 substrates, but loss of the SH3 domain cannot be rescued as the molecule can no longer bind the activators that it normally brings into proximity with effectors.

Yet another function of CRK and CRKL was discovered downstream of Dab-1 in Reelin signaling, a critical component of development in the vertebrate brain [5, 101]. Reelin is a large extracellular protein that was found to be deleted in reeler mice, a mutation that arose spontaneously and resulted in uncoordinated animals with severe morphological defects in nervous tissues and especially the brain [21, 31, 101]. During neuronal development, progenitor cells differentiate and undergo radial migration towards the basal surface of the developing cortex. As they migrate, the cells encounter Reelin produced by Cajal-Retzius cells which binds to its receptors Very low–density lipoprotein receptor (Vldlr), and the Apolipoprotein E Receptor 2 (ApoER2) [20, 122]. Binding of Reelin to these receptors results in the activation of Src kinase and phosphorylation of the adaptor Dab-1 at four sites including Y220 and Y232, inducing the binding of CRK and CKRL [5, 80]. Once CRK/CKRL is recruited, the same downstream molecules found in integrin signaling are able to influence migration, specifically the SH3 bound C3G activate Rap-1 to stabilize actin and stop migration [5]. This causes the migrating neurons to detach from radial glia and join the corresponding layer of the developing cortex.
1.4. Evolution and Biology of Crk/Crkl:

Considering the essential functions of CRK and CRKL, it is not surprising to find that the evolutionary origin of these proteins is ancient. Comparison of genes encoding CRK/CRKL across species has found that these two exist as a single ancestral gene in invertebrates known as crka [114]. It is most likely that the genes diverged before the split between the last common ancestor of vertebrates and invertebrates, as all vertebrates have homologs of both crk and crkl [114]. Primitive crka genes can be found in many organisms, including animals as simple as Choanoflagellates and Choanozoa, and the origins of CRK/CRKL appear to be as old as the most basic elements of phosphotyrosine signaling [114].

The popular model organism C. elegans provided some of the first insight into the function of CRK and CRKL in animals. The mutant worm lines ced-2, ced-5 and ced-10 were observed to have similar defects where cell migration and engulfment of apoptotic cells were impaired [99]. The genes were found to encode proteins homologous to CRK, DOCK180, and Rac, respectively [99]. Thus, a mechanism highly similar to what is found in vertebrate integrin signaling also regulates motility of cells in the worm. In the fruit fly, a drosophila homolog, dCRK, was found and shown to interact during muscle development with Drosophila Myoblast City (MBC), a close homolog of mammalian DOCK180 [36]. Subsequent investigation in the zebrafish found that CRK and CRKL interact with Dock1 and Dock5 during myoblast fusion in Danio rerio as well [89]. It is clear that from the humble worm to humans, CRK and CRKL are fundamental molecules required for processes such as development which require coordinated cell motility.
A great deal of insight into the roles of CRK and CRKL in vertebrates has been gained from knockout models in mice. Loss of CRK or CRKL in mice results in phenotypes which, while similar in some respects, are distinct. CRK-null mice mostly die late in embryonic development, with a few surviving until shortly after birth [94]. Gross histological inspection showed several defects including cardiac defects, nasal deformation and cleft palate [94]. Similarly, most CRKL-null mice do not survive past E16.5, although a few survive past birth with minor craniofacial defects, or rarely with no observable phenotype [42]. Histology of recovered embryos revealed defects in many neural crest derived tissues including cranial ganglia, thymus, and craniofacial tissues [42, 86]. The expression of CRK and CRKL overlap in many tissues, and the inability of loss of one to compensate for loss of the other is consistent with biochemical findings that CRK and CRKL have at least some unique functions [79]. The variable and possibly incomplete phenotypic penetrance in CRKL-null mice that survived to later stages of development and the lethality of CRK loss made complete investigation of the resulting phenotypes difficult. To address this, a double knockout mouse was generated using the Cre-lox system to restrict loss to the brain [95]. This allowed for investigation of mice throughout development and into adulthood in CRK, CRKL, and CRK/CRKL-null animals. These mice were also useful because they could be compared to animals null for Reelin and Dab-1 [95]. The phenotypes of these animals all show severe defects in the development of laminar structures of the brain such as the cortex and cerebellum [5, 95]. The cellular ectopia observed is also a hallmark of the reeler mutant mouse. In normal development of the cortex, each successive wave of progenitor cells passes the
previous layer. The youngest cells form the outermost layers and the oldest cells the innermost. In reeler mice, these layers are essentially inverted, as progenitors do not receive the signal to pass the previous layer, and the younger cells accumulate beneath older layers. These observations helped to establish that the roles of CRK and CRKL in the cell largely overlap, as a conditional loss of only CRK or CRKL in the brain displayed a mild phenotype compared to double-knockout animals which had severe defects [95].

In humans, CRK and CRKL are associated with some developmental disorders caused by chromosomal deletions. Two relatively common examples are Miller-Dieker Lissencephaly Syndrome (MDLS) and deletion 22q11.2, more commonly known as DiGeorge Syndrome (DGS) [12, 85]. MDLS is characterized by congenital malformations caused by microdeletions in 17p13.3 [49]. While the primary effects appear to be caused by loss of genes encoding LIS1 and 14-3-3ε, a wide variety of deletions have been reported including loss of crk, which is associated with more severe MDLS [12]. A case study of a fetus bearing a 0.7Mb microdeletion on chromosome 17p which includes deletion of crk, and showed no abnormal morphology in ultrasound [14]. The fetus was terminated at 19 weeks, and at that time was observed to have mild fascial dismorphism [14]. It seems therefore that loss of CRK leads to somewhat similar, if less severe, phenotype in humans compared to mice. DGS presents more specifically as cardiac and craniofascial defects, typically caused by a 3Mb deletion on 22q11. Harbored within this deletion are tbx1, a transcription factor which is thought to drive most of the defects observed in the DGS phenotype, and crkl [57, 88]. Investigation in
mice has shown that CRKL null animals also display many of the defects observed in DGS [42, 88, 94]. Loss of both tbx1 and crkl in mice results in a much more penetrant phenotype than tbx1 alone, consistent with the incomplete penetrance observed in human DGS [41].

1.5. CRK/CKRL-SH2 Binding Proteins and SHD:

While many signaling pathways that CRK and CRKL participate in have been elucidated, the array of defects observed in null animals suggests that others exist as well. It therefore seems likely that the list of proteins known to interact with Crk and CrkL is not exhaustive. The Ballif lab has recently validated several novel Crk/CrkL interacting proteins by looking for proteins enriched in the Crk/CrkL SH2 binding motif YxxP followed by validation in cell culture experiments. Several candidate have been shown to bind to CRKL-SH2 including ESDN1 and ESDN2 [109]. These previously uncharacterized interactions may represent important functions of CRK/CRKL in events that require signaling to the cytoskeleton such as axonal pathfinding and cell migration during development. Indeed, it is likely that a large number of signals converge on CRK/CRKL, making identification of its interacting proteins essential.

The Src Homology domain containing protein D (SHD) is a 320 amino acid protein which contains five YxxP sites. It is a relatively uncharacterized protein suggested to be an adaptor because it contains an SH2 domain and is a relatively small protein. SHD was first identified in 1997 by a yeast two-hybrid screen looking for proteins that act as substrates for Abl tyrosine kinase, using the kinase domain of Abl as the bait [93]. It has
been reported that SHD was expressed in the brain and nervous tissue as determined by northern blot in mice [93] and by RNA-seq in humans (human protein atlas) [123]. The function of this protein is unknown. It has three closely related SH2 domain containing proteins, SHB, SHE, and SHF, of which only SHB is well represented in the literature. The subsequent chapters in this thesis will investigate the nature of the interaction between SHD and CRKL, and attempt to answer some basic questions about its biological role. We first investigate the binding of SHD to CRKL via the CRKL-SH2 domain, which requires the YxxP sites of SHD. We show that the SHD/CRKL interaction can be induced by inhibiting tyrosine phosphatases using hydrogen peroxide, or by overexpression of Abl kinase. We also show that Abl kinase phosphorylates SHD on Y144 and other sites, and that mutation of Y144 to phenylalanine reduces but does not abrogate binding to CRKL-SH2. Subsequent investigation of the related proteins SHB, SHE, and SHF reveals that overexpression of Abl kinase also results in binding of SHB and SHE to CRKL-SH2. Finally, we show that the zebrafish ortholog shdb is expressed in several areas of the nervous system during development, and knockdown of shdb results in several obvious defects including smaller eye size, which we have quantified. We therefore suggest that SHD represents an uncharacterized substrate of the CRKL-SH2 domain critical for development of some areas of the nervous system. Additionally, SHD and the closely related SHB and SHE might represent a group of CRK/CRKL binding proteins for which the outcome of this interaction is unknown. Future research will aim to answer if the effects of shdb knockdown are dependent or independent of CRK/CRKL to direct further inquiry of SHD function biochemically and in the nervous system.
CHAPTER 2: THE SH2 DOMAIN-CONTAINING ADAPTOR PROTEIN SHD
REVERSIBLY BINDS THE CRKL-SH2 DOMAIN

2.1: Introduction

The CRK and CRKL proteins are important molecules in many biological processes and function in many phosphotyrosine-dependent signaling pathways. CRK and CRKL play roles in diverse processes such as development, where these molecules are required for Reelin signaling in the development of the vertebrate brain, and cancers such as chronic myelogenous leukemia, where CRKL is a requirement for the oncogenic effects of the Philadelphia chromosome [5, 24, 91, 95, 105]. The actions of CRK and CRKL depend upon localization of the molecule to phosphorylated targets via their Src Homology 2 (SH2) domains. Upon binding, the enzymatic action of effectors which bind to their Src Homology 3 (SH3) domains can exert effects. While extensive research has identified many CRK/CRKL interacting proteins, the diversity of functions in which CRK and CRKL have been implicated in suggests that these lists are incomplete, and identification of additional interacting proteins could aid our understanding of CRK/CRKL dependent cell processes. Interaction of the SH2 domain is particularly relevant, as this will determine where in the cell the protein may act, and identification of new SH2 interacting proteins might provide insight into a novel signaling pathway which utilizes CRK and CRKL, or mechanisms of CRK/CRKL regulation not currently known. We therefore attempted to find novel binding partners for the CRK/CRKL SH2 domain, starting with an in-silico screen for human proteins enriched in the CRK/CRKL-SH2
consensus binding motif, YxxP [108]. As a next step, proteins of interest would be expressed in cell culture and assessed for their ability to bind to the CRKL-SH2 domain.

One protein of interest, the SH2 Domain-containing protein D (SHD) contains five YxxP sites which might bind the CRKL-SH2 domain. Herein we show that SHD becomes tyrosine phosphorylated by kinases endogenous to HEK 293 cells. SHD tyrosine phosphorylation was induced by treating cells with hydrogen peroxide (H$_2$O$_2$) to inhibit tyrosine phosphatase activity. When SHD was expressed in cells stimulated with H$_2$O$_2$ SHD was induced to bind to the CRKL-SH2 domain in pulldown assays. We show that treating cells with inhibitors for Src Family Kinases (SFKs) or Abl/Arg tyrosine kinases prior to H$_2$O$_2$ stimulation reduces this interaction, and that co-expression of Abl kinase with SHD is sufficient to induce binding. We go on to show that a mutant SHD with a Y/F mutation of the five YxxP sites is unable to be bind to CRKL when co-expressed with Abl. Using mass spectrometry, we identified phosphorylation on Y144 within a YxxP motif by Abl. Mutation of this site alone to phenylalanine reduced, but did not abrogate Abl-induced binding of SHD to CRKL. Finally, we show that the close relatives SHB, SHE, and possibly SHF, can also bind to CRKL-SH2 when co-expressed with Abl. These findings show that SHD and its relatives are novel CRK/CRKL interacting proteins with the potentially to affect the regulation or dimensions of CRK/CRKL signaling mechanisms.
2.2. Materials and Methods

Constructs and Plasmids:

The V5-tagged SHD construct was obtained from DNASU (DNASU Plasmid Repository, The Biodesign Institute/Arizona State University) and consists of the cDNA sequence encoding human SHD (GenBank HQ448179) cloned into the mammalian expression vector pLX304 bearing a V5-tag fused in frame with the C-terminus of SHD. Flag-tagged SHB, SHD, SHE, and SHF were obtained from Origene (Rockville, MD 20850) and consisted of the mouse cDNA sequence of SHB (NM_001033306), SHD (NM_001159523), SHE (NM_172530), or SHF (NM_001013829) cloned into the SgfI-MluI restriction sites of the mammalian expression vector pCMV6 bearing a Myc-DDK (Flag) tag in frame with the C-termini of each protein. The mouse SHD Y/F mutants were prepared at our request by BioBasic (Markham, Ontario, Canada) and were re-inserted into the pCMV6-Entry vector. The human c-Abl construct, with a C-terminal Flag-tag, was a gift from A. Howe (University of Vermont), and was originally constructed in the Kufe laboratory (Harvard Medical School) [11]. The WT human Fyn construct in pRK5 was acquired from AddGene (Cambridge, MA, U.S.A.). The bacterial expression vectors encoding the glutathione-S-transferase CrkL-SH2 fusion protein (GST-CrkL-SH2) was a gift from A. Imamoto (University of Chicago).
Cell culture and lysis, Transfections, and Cell treatments

Human Embryonic Kidney 293 cells (HEK 293) were maintained at 37 °C under 5% CO₂ in a humidified incubator. Cells were cultured in DMEM media (Mediatech, Manassas, VA) supplemented with 5% Fetal Bovine Serum (FBS) and 5% Cosmic Calf Serum (CCS) and antibiotics (sera were from Hyclone, Logan, UT). HEK 293 cells were transfected with 2-10 μg of DNA, depending on the experiment, and when cells were at ~70% confluence using calcium phosphate precipitation. 6-16 hours after transfection cells were washed with PBS and then returned to complete media for 12-24 hours.

In experiments using small molecule inhibitors, media was aspirated off cells and complete media containing inhibitors was added for the specified times. The SFK inhibitor Src-1 (EMD-Calbiochem, Billerica, MA) and the Abl kinase inhibitor Imatinib (STI571; Selleck Chemicals, Houston, TX) in DMSO were used at 2 μM and 10 μM respectively, for 25 minutes prior to H₂O₂ stimulation, or 30 minutes prior to lysis in inhibitor experiments which did not use H₂O₂ stimulation. Cells stimulated with H₂O₂ were incubated for 15 minutes with 8.8 μM H₂O₂.

To lyse cells, 10 cm plates of cells were placed on ice and media was aspirated off. Cells were then gently washed with 1X PBS and then lysed with 1mL of lysis buffer (25 mM Tris pH 7.4, 137 mMNaCl, 10% glycerol, 1% Igepal) containing protease inhibitors (5 μg/mL⁻¹ pepstatin, 10 μg/mL⁻¹ leupeptin, 1 mM PMSF), and phosphatase inhibitors (1 mM NaVO₃, 25 mM NaF, 10 mM Na₂H₂P₂O₇). Plates were scraped using a cell scraper and lysates were transferred to a 1.5 mL Eppendorf tube and briefly vortexed. Lysates
were then centrifuged at 12,000 rpm for 30 minutes at 4 °C and then transferred to a clean 1.5 mL tube leaving the insoluble pellet behind. Protein concentrations were determined using Bradford Reagent (VWR, AMRESCO Inc; Radnor, PA) and an Eppendorf BioPhotometer Plus (Eppendorf; Hamburg, Germany) with bovine serum albumin (BSA) standards. Cell lysates were then standardized by diluting with the appropriate volume of lysis buffer.

**Immunoprecipitation and Calf Intestinal Phosphatase (CIP) Assay**

Depending on the experiment, standardized lysates containing 1-2 mg of total protein was immunoprecipitated (IP) using α-V5 mAb or α-Flag Affinity Gel (10 μl of a 50% slurry in BCLB) at 4 °C overnight with rocking. In experiments using the α-V5 mAb for IP, 1.5 μg of antibody was added to 1.5 mg of total protein and incubated at 4 °C with rocking for 1 hour before adding 20 μL of a 50% bead slurry in cell lysis buffer made with 7.5 μL of protein A slurry (Rockland; Pottstown, PA, U.S.A.) and 7.5 μL protein G slurry (Biosciences, Allentown, PA, U.S.A.) washed once with PBS and eluted to 20 μL in lysis buffer. The extracts were then incubated at 4 °C overnight with rocking. The resin was washed 3X with cell lysis buffer before being prepared for SDS-PAGE or the phosphatase assay.

The Calf intestinal phosphatase (CIP) treatment was administered to immune complexes after IP. After the overnight incubation with cell lysates, protein A/G resin bound to αV5 antibodies were washed an additional time in PBS and diluted into 500 μL of CIP buffer (10 mM NaCl, 1 mM Tris-HCl pH 8, 10 mM MgCl2, 1 mM DTT) and
separated into two tubes. The buffer was aspirated off and the resin was resuspended in 50 μL of CIP buffer. 2.5μL of CIP (1 U/μL) was added to one tube, and the CIP treated and untreated tubes were incubated in a dry block at 37 °C for 1 hour. The samples were then denatured by the addition of 16.66 μL of 4X sample buffer and boiling at 95 °C for 5 minutes before being subjected to SDS-PAGE.

**GST-CrkL-SH2 Pulldown assay**

Pulldown experiments used standardized lysates containing 1-2 mg of total protein. The volume of beads added varied from 10-20 μL of 50% slurry depending on bead saturation with the GST-CRKL-SH2 construct. GST-CRKL-SH2 beads were prepared by inoculating a 50 mL Lysogeny Broth (LB) with 50 μg/mL ampicillin with *E. coli* BL21 bearing the previously described construct. The 50 mL culture was grown overnight at 37 °C with shaking and used to inoculate 500 mL of LB + ampicillin. This culture was grown for two hours to reach log phase and then induced by the addition of β–D-1-thiogalactopyranoside (IPTG) to 1 mM. The induced culture was incubated for an additional 3-4 hours, and then centrifuged (6,000 x g, 20 minutes). The bacterial pellet was resuspended in 10 mL bacterial lysis buffer (100 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, and 10 μg/mL leupeptin and pepstatin-A in PBS), and lysed by sonication in six 30 second intervals with equal intervals on ice. After sonication, 1 mL of 10% Triton-100 was added. Lysates were mixed and then centrifuged in Oakridge tubes (12,000 x g, 20 minutes). Pellets were discarded and the lysate was incubated with 300 μL of a 50% slurry of glutathione (GST) coated sepharose beads (Biosciences,
Allentown, PA) at 4°C with rocking overnight. The GST-CRKL-SH2 beads were washed 3X with bacterial lysis buffer, 3X with sterile PBS and stored at 4°C in PBS. Aliquots of the culture before and after stimulation along with GST-CRKL-SH2 beads were subjected to SDS-PAGE and coomassie stained gel to confirm induction of bacteria, the quality, and approximate concentration of protein on the beads.

**SDS-PAGE, Antibodies, Western Blotting, Enhanced Chemiluminescence**

Protein samples were denatured with protein sample buffer (1X = 150 mM Tris (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 7.8% glycerol, and 0.25 ng/ml bromophenol blue) at 95°C for 5 minutes and separated by SDS-PAGE. Whole cell extracts were diluted 3:1 with 4X sample buffer and 25 μL of 1X sample buffer was added to pulldowns and immunoprecipitations. After denaturation, 20 μL of sample was loaded into the appropriate well using a Hamilton syringe and separated on a 10% acrylamide gel (30% (w/v) and 37.5:1 acrylamide:bis-acrylamide) with a 4.2% acrylamide stacking gel. Gels were then run at 20 mA/gel for ~10 minutes until the protein reached the resolving gel, at which point the current was increased to 30 mA/gel. Gels were then either coomassie stained (0.1% coomassie brilliant blue R-250, 20% glacial acetic acid, 40% methanol) for mass spectrometry, or transferred to a nitrocellulose membrane at 0.4 A for 2-4 hours or at 0.2 A for 12-16 hours in transfer buffer (190 mM glycine, 25 mM Tris–base, and 20% methanol) using a submersible transfer unit. After transfer, a reversible Ponceau stain (0.5% Ponceau and 1% acetic acid in H₂O) was used to assess total protein levels. Membranes were then washed with deionized water and blocked.
with 5% dry milk in TBS-T (150 mM NaCl, 20 mM Tris–HCl, and 0.1% Tween 20) for 20 minutes and then incubated at 4 °C with the primary antibody diluted in TBS-T with 0.005% sodium azide for 16-24 hours. Primary antibodies and dilutions used are the following: α-phosphotyrosine 4G10 (mouse mAb; 1:1000; EMD Millipore, Billerica, MA, USA), α-V5 (rabbit mAb 1:2000), α-Flag M2 (mouse mAb, 1:2000; Cell Signaling Technologies), α-Src (rabbit mAb; 1:2000; Cell Signaling Technology), α-Src pY416 (rabbit mAb, 1:5000; Cell Signaling Technology), α-Abl (rabbit mAb; 1:1,000; Santa-Cruz), and αAbl pY412 (rabbit mAb; 1:1000; Cell Signaling Technology). Blots were washed with TBS-T and then incubated with Horseradish peroxidase (HRP) conjugated secondary antibody at RT for 2-4 hours. Secondary antibodies and dilutions used were: α-mouse-HRP (goat IgG, 1:5000; EMD Millipore), α-rabbit-HRP (goat mAb; 1:5,000; EMD Millipore).

Proteins were detected using enhanced chemiluminescence and X-ray film (ThermoFisher Scientific, Waltham, MA, USA), and film was developed using a Medical Film Processor SRX-101A (Konica Minolta Medical & Graphic, Tokyo, Japan).

Mass Spectrometry

We were unable to resolve the band containing human SHD from the heavy chain of the antibody, and so the band containing both was split into two pieces, of which the higher contained mostly SHD and less heavy chain. Both sections were diced into approximately one millimeter cubes and then prepared for mass spectrometry as described previously, but without the steps for reduction and alkylation [13]. Briefly,
proteins were digested in-gel with sequence-grade modified trypsin in 50 mM ammonium bicarbonate for 8-12 hours at 37 °C. The tryptic peptides were dried using a speed vac and then resuspended in 2.5% acetonitrile, 0.15% formic acid and separated by HPLC before MS/MS analysis on a linear ion trap-orbitrap (LTQ-Orbitrap) mass spectrometer controlled by Thermo XCALIBUR 2.1 software (Waltham, MA, USA) using the protocol described previously [109] and summarized below.

Tryptic peptides were separated on a reverse phase HPLC column packed with 5 μm C18 silica. Peptides were loaded onto the column using solvent A (2.5% Acetonitrile, 0.15% Formic acid) and eluted off the column using a gradient of solvent B (99.85% Acetonitrile, 0.15% Formic acid). Eluting peptides and solvent were electrosprayed (2.1 kV) into the instrument. The precursor scan (360-1700 m/z) was followed by 10 low-energy collision-induced dissociation (CID) tandem mass spectra. The CID spectra were acquired for the two most abundant ions in the precursor scan, followed by targeted scans for SHD (see table 2.1 and 2.2).

SEQUEST searches were performed using a forward and reverse 2011 Uniprot Human Protein database requiring tryptic peptides and permitting the following modifications: phosphorylation of serine, threonine, and tyrosine (+79.9663 Da), oxidation of methionine (+15.9949 Da), and acrylamidation of cysteine (+71.0371 Da).
2.3. Results

SHD is reversibly tyrosine phosphorylated and shows H$_2$O$_2$-dependent binding to the CRKL-SH2 domain

Having identified SHD in-silico as a protein enriched in the CRKL-SH2 binding motif, YxxP, we tested if SHD expressed in cells could become phosphorylated and bind to the CRKL-SH2 domain in pulldown assays. Reactive oxygen species (ROS) such as H$_2$O$_2$ have been shown to inhibit tyrosine phosphatases with relatively high specificity [26]. Cells can be treated with H$_2$O$_2$ for short periods to block tyrosine phosphatase activity, allowing endogenous kinases to phosphorylate targets unopposed and increase the content of phosphotyrosine in the cell many fold [26].

Stimulation of HEK 293 cells transiently transfected with human SHD with H$_2$O$_2$ induced phosphorylation on SHD as visualized by western blotting immunoprecipitated SHD with an α-phosphotyrosine antibody (Fig. 2.1). This signal is lost when extracts are treated with calf intestinal phosphatase (CIP) after the IP (Fig. 1a). A pulldown experiment using a glutathione S-transferase-CRKL-SH2 fusion protein (GST-CRKL-SH2) immobilized on glutathione agarose showed that phosphorylated SHD from H$_2$O$_2$-stimulated cells is able to bind the CRKL-SH2 domain (Fig. 2.1b).
Figure 2.1: Western blots of SHD immunoprecipitated from extracts of H₂O₂-stimulated HEK 293 cells shows reversible phosphorylation on SHD and pulldown assays show the binding of SHD from the same extracts to the CRKL-SH2 domain. (A) Western blot of HEK 293 cell lysates transfected with a V5-tagged SHD construct and treated with water or H₂O₂ (WCE) or immunoprecipitated (IP). Samples were probed on membranes with the indicated antibodies. (B) Western blots of HEK 293 cell extracts transfected with a V5-tagged SHD construct and treated with water or H₂O₂. Lysates were subjected to immunoprecipitation (IP) with (WCE), immunoprecipitated (IP) or pulldown assay with a GST-CRKL-SH2 construct and probed on membranes with the indicated antibodies. Results from (A) were generated by Jaye Grundy Weinert.
SHD is phosphorylated and induced to bind to the CRKL-SH2 domain when co-expressed in cells with Abl.

To understand the kinases responsible for the H$_2$O$_2$-dependent interaction of SHD with CRKL, we used small molecule kinase inhibitors prior to H$_2$O$_2$ stimulation. Pre-treatment with the inhibitor Src-1 for SFKs and the inhibitor STI571 for Abl/Arg kinases prior to H$_2$O$_2$ stimulation reduced the binding of SHD to CRKL-SH2 in cell extracts subjected to pulldown (Fig. 2.2). While each inhibitor dramatically reduced the binding of SHD to the CRKL-SH2 domain, both inhibitors together did not abrogate binding completely, suggesting either incomplete inhibition of SFKs and Abl/Arg by Src-1 and STI571, or the involvement of additional kinases not targeted by these inhibitors. These results argue that both SFKs and Abl kinases are involved in the H$_2$O$_2$-induced interaction of SHD and CRKL. To test if these kinases were sufficient for the interaction, the SFK Fyn and Abl was individually co-transfected with a Flag-tagged mouse SHD construct. We used a Flag-tagged construct here as in the same experiment we used a Flag-tagged positive control (DCBLD2-Flag) which we have shown to be induced to bind to the CRKL-SH2 domain by both Fyn and Abl [108]. We observed that co-expression of Abl was sufficient to induce binding to the CRKL-SH2 domain in pulldown assays, but co-expression of Fyn was not, even though both induced the binding of DCBLD2 to the CRKL-SH2 domain (Fig. 2.3).

It was interesting that the Src-1 inhibitor was able to reduce H$_2$O$_2$-induced binding, but overexpression of the SFK Fyn did not induce SHD binding to the CRKL-SH2 domain. SFKs have been shown to be upstream of Abl activation following H$_2$O$_2$ stimulation.
stimulation, therefore we attempted to inhibit cells co-expressing SHD and Abl with both the Src-1 and STI571 inhibitors and found that Src-1 was able to reduce SHD binding to CRKL, and STI571 was able to block the interaction entirely (Fig. 2.4) [108]. These results suggest a role for Fyn and other SFKs upstream of Abl rather than directly phosphorylating SHD. They also suggest that Abl-dependent phosphorylation of SHD is rapidly reversibly given that a brief treatment with the inhibitors led to a dramatic decrease in the binding reaction.
Figure 2.2: Inhibition of SFKs or Abl/Arg reduces the H$_2$O$_2$-induced binding of SHD to the CrkL-SH2 domain (n=2). HEK 293 cells were transiently transfected with SHD. Prior to lysis, cells were treated with the indicated inhibitor or DMSO for 20 minutes and then stimulated with 8.8 mM H$_2$O$_2$ for 15 minutes. Lysates were standardized and either immunoprecipitated with α-V5 (IP), subjected to pulldown with GST-CrkL-SH2 beads (PD) or run as whole cell extract (WCE). Membranes were blotted by the indicated antibodies.
Figure 2.3: Abl kinase, but not Fyn, is sufficient to induce SHD to bind CrkL-SH2. Human embryonic kidney (HEK 293) transiently transfected with the indicated constructs. Lysates were subjected to pulldown with GST-CRKL-SH2 beads (PD) or run as whole cell extract (WCE). Membranes were blotted with the indicated antibodies.
Figure 2.4: Inhibitors of SFKs and Abl/Arg reduce the Abl-induced binding of SHD to the CRKL-SH2 domain (n=2). HEK 293 cells were transiently transfected with the indicated constructs. Prior to lysis, cells were treated with inhibitors or DMSO for 30 minutes. Lysates were subjected to pulldown with GST-CRKL-SH2 beads (PD) or run as whole cell extracts (WCE). Membranes were blotted using the indicated antibodies.
The YxxP sites of SHD are required for the Abl-induced binding of SHD to the CRKL-SH2 domain.

Given the CRKL-SH2 domain binds with high-selectivity to tyrosine phosphorylated YxxP motifs, it is likely that the interaction between SHD and the CRKL-SH2 is mediated by phosphorylation at one or more of the five YxxP motifs on SHD. In order to test this hypothesis, we obtained a mutant Flag-tagged SHD with tyrosine mutated to phenylalanine at all five YxxP sites (SHD Y5F). The mutant SHD Y5F was unable to bind CRKL-SH2 domain when co-expressed with Abl (Fig. 2.5).

Abl induces the phosphorylation of SHD at Y144

We next sought to determine if Abl induced the phosphorylation of specific tyrosines in YxxP motifs. HEK 293 cells were transfected with V5-tagged SHD or co-transfected with V5-tagged SHD and Abl. Cell lysates were then immunoprecipitated with an α-V5 antibody, run on SDS-PAGE and then were analyzed by mass spectrometry to search for phosphorylation of specific YxxP sites. We found two of the four tryptic peptides which harbor the five SHD YxxP motifs (Table 2.1) and identified phosphorylation at Y144 (Fig. 2.6a and 2.6b). The expected b- and y-type ions resulting from Collision Induced Dissociation (CID) of the precursor ions harboring Y144 and pY144 are shown (Table 2). Several diagnostic ions allowed for verification of phosphorylation at Y144. In the both the unphosphorylated and phosphorylate peptide spectra, the y-22⁺² ion has an m/z close to the expected 1207.57. This ion contains all the amino acids C-terminal to Y144, and shows that no
modification is detected on these amino acids. The $y-23^{+2}$ ion has an increased $m/z$ indicating the addition of tyrosine which is close to the expected value of 1289.07 in the unphosphorylated spectra, but in the phosphorylated spectra has an $m/z$ of 1329.2. The extra mass is explained by the addition of phosphate to Y144. This is supported by the detection of several peaks which match expected values of b-ions containing the phosphotyrosine.

The spectra generated showed measurements of fragment ions that were predicted and no major peaks remained unresolved. We were unable to detect other sites of phosphorylation in this experiment, and so were uncertain if phosphorylation at this site might be the requirement for interaction with the CRKL-SH2 domain. To assess the functionality of this site, we obtained a Flag-tagged SHD Y1F construct bearing a Y-to-F mutation at Y144 (Fig. 2.7). When co-expressed with Abl, the SHD Y1F protein was able to bind to the CRKL-SH2 domain, although the interaction was reduced compared to the binding of wildtype SHD.
Figure 2.5: The SHD Y5F construct bearing Y-to-F mutations at the five YxxP motifs is unable to bind to the CRKL-SH2 domain when co-expressed with Abl (n=1). HEK 293 cells were transiently transfected with the indicated constructs. Lysates were subjected to pulldown with GST-CRKL-SH2 beads (PD) or run as whole cell extracts (WCE). Membranes were blotted with the indicated antibodies.
Table 2.1: Summary of tryptic peptides of SHD which harbor YxxP sites detected in MS. The table was generated using sequences and mass values from the Institute for Systems Biology protein digest tool. Detection of YxxP-containing peptides is indicated as detected or not, as is the tyrosine phosphorylated species. PPM was calculated from MS1 experimentally measured m/z values and the expected m/z according to the protein digest tool for the M+3H+ ions of the indicated tryptic peptides.

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<th>Mass:</th>
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Table 2.2: Predicted y-type and b-type singly and doubly-charged fragment ions from the M+3H⁺ precursor ions harboring Y144. The table was generated using values obtained from the Institute for Systems Biology fragment ion calculator tool (http://db.systemsbiology.net).

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Table 2.3: Predicted y-type and b-type singly and doubly-charged fragment ions from the M+3H\(^+\) precursor ions harboring phospho-Y144. The table was generated using values obtained from the Institute for Systems Biology fragment ion calculator tool (http://db.systemsbiology.net).

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Figure 2.6: The experimentally observed MS/MS spectra for the tryptic peptide harboring Y144 and pY144. (A) The MS/MS spectra resulting from collision induced dissociation (CID) of the M+3H+ precursor ion containing Y144 (m/z = 992.13). (B) The MS/MS spectra resulting from collision induced dissociation (CID) of the M+3H+ precursor ion containing phospho-Y144 (m/z = 1018.78). Samples came from HEK 293 cells transfected with V5-tagged SHD or co-transfected with V-5 SHD and Abl kinase. The phosphorylated precursor ion was only detected in conditions where Abl was also expressed. The b- and y-type ions have been labeled. No major peaks were unresolved.
Figure 2.7: The SHD Y144F mutant shows reduced binding to the CRKL-SH2 domain induced by Abl. HEK 293 cells transiently transfected with the indicated constructs. Lysates were subjected to pulldown with GST-CRKL-SH2 beads (PD) or run as whole cell extract (WCE). Membranes were blotted by the indicated antibodies. The doublet appearing in the Ponceau stain is due to breakdown of the GST-CRKL-SH2 beads; however, the beads were still viable as demonstrated by their ability to bind to phosphorylated SHD in lanes 3 and 5 of the α-Flag blot of the GST-CRKL-SH2 pulldown (top blot).
SHD belongs to a family of SH2-domain containing proteins which are capable of interacting with the CRKL-SH2 domain.

Having shown that H$_2$O$_2$ and Abl induce SHD to interact with the CRKL-SH2 domain, and given that the related proteins SHB, SHE, and SHF also harbor YxxP motifs, we asked if Abl might also induce these family members to bind to the CRKL-SH2 domain. To investigate this, we transfected HEK 293 cells with Flag-tagged constructs of SHB, SHD, SHE, and SHF alone or co-transfected with Abl (Fig. 2.8). We found that in addition to SHD, SHB and SHE also bind to CRKL when co-expressed with Abl. A conclusion regarding the binding of SHF to the CrkL-SH2 domain was elusive given that SHF-Flag ran at the molecular weight of GST-CRKL-SH2 and the anti-Flag antibody had significant background binding to GST-CRKL-SH2.
Figure 2.8: The SHD family members SHB and SHE also bind to CRKL-SH2 when co-transfected with Abl (n=2). HEK 293 cells were transiently transfected with the indicated constructs. Lysates were standardized and subjected to pulldown with GST-CRKL-SH2 beads (PD) or run as whole cell extracts (WCE). After SDS-PAGE gels were immunoblotted with the indicated antibodies. Experiment replicated by Anna Schmoker (not shown).
2.4. Discussion and Future Directions

Conclusions and Discussion:

There is little published data available on SHD, so no comparisons can be made between these experiments and the literature. While we have attempted to replicate as many experiments as possible, follow up will be necessary to better understand how CRKL is induced to bind SHD. We have drawn several conclusions from the data presented.

First, we have found that SHD can become phosphorylated in cell culture upon stimulation by H$_2$O$_2$ or by overexpression of Abl tyrosine kinase. It seems likely that Abl phosphorylates SHD directly, as the Abl-induced binding of SHD to the CRKL-SH2 domain can be rapidly reversed by addition of the Abl inhibitor STI-571. While this provides some insight, it is unclear under what cellular conditions this phosphorylation normally occurs, or if any specific extracellular cues might lead to this interaction. The experiments we present using small molecule inhibitors suggest that even if Abl kinase is primarily responsible for phosphorylation of SHD in cells, multiple kinases including the SFKs, might play a role in regulating this event upstream of Abl.

Observations from MS and pulldown experiments with SHD mutants indicate that SHD likely has multiple YxxP sites which can mediate binding to the CRKL-SH2 domain. Although we only detected one site of phosphorylation in the MS experiment presented, subsequent mutation of this tyrosine to phenylalanine reduced but did not eliminate the Abl-induced binding of SHD to the CRKL-SH2 domain. The SHD Y5F mutant was unable to bind CRKL-SH2, and so at least one site other than Y144 and
potentially all five sites might facilitate the binding of SHD to the CRKL-SH2 domain upon phosphorylation.

An important consideration is that SHD is a member of a family of SH2-domain-containing proteins which include SHB, SHD, SHE, and SHF (Fig. 2.9). We show that of these, murine Shb, Shd, and She bind CRKL-SH2 when co-expressed with Abl in cell culture (Fig. 2.8). Shf may also bind, but it did not resolve from the GST-CRKL-SH2 and so its binding was not observed. It is also possible that murine Shf behaves differently than human SHF, as the Shf protein is 238 amino acids in length, compared to 438 in humans. A large segment of the C-terminus is deleted, as well as another gap just C-terminal to the SH2 domain. As a result, murine Shf contains only two YxxP sites, rather than the three found in humans. However, these hypotheses remain to be tested fully.

This SH2-domain-containing (SH) family of proteins is of interest to our lab group both because of their potential interaction with CRKL and their role in phosphotyrosine signaling via their SH2 domains. Given the importance of SH2 domains in cell signaling, proteins containing SH2 domains have been the focus of many large-scale proteomics screens, some of which have included SHD and its family members [40, 50, 58, 71]. The preferred SH2-domain binding sequences as determined by OPAL (Oriented Peptide Array Library) are Y(D/E)EL for SHB, Y(D/E)N(L/Y) for SHD, Y(N/D)xM for SHE, and Y(Y/F)E(L/Y) [50]. The preference for SHB had previously been reported to be Y(R/V/I)xL by a degenerate peptide library, although both methods agree that lysine (L) is required in the +3 position, and this position is generally regarded
as the primary determinant in SH2-domain substrate specificity [60, 87, 121]. In these motifs, the first Y is the phosphorylated tyrosine, followed by the +1, +2, and +3 positions. A “x” represents any amino acid (no preference). Some amino acid preferences outside to residues 0 to +4 have also been identified, however the three amino acids following tyrosine account for most of the substrate specificity of SH2-domains [69].

This “SH” family of proteins represent an understudied group of adaptor proteins that may function to regulate CRK/CRKL. The diversity of their structures could result in a number of subsequent interactions after binding to the CRKL-SH2 domain. SHB is better studied than the other family members, and has previously been reported to bind to CRK-II [77]. It was first identified as a serum-inducible gene product in an insulin producing pancreas cell line (βTC-1) [128]. SHB contains both a proline rich region near the N-terminus, a phosphotyrosine binding (PTB) domain, and an SH2 domain near the C-terminus (Fig. 2.9). The proline rich region is known to bind v-Src [60]. The SH2 domain can bind to fibroblast growth factor receptor-1 (FGFR-1) and vascular endothelial growth factor receptor-2 (VEGFR-2) [47, 60]. The PTB domain was shown to bind Focal adhesion kinse (FAK) downstream of FGF-2 to regulate cell spreading [46]. Abl kinase is known to phosphorylate SHB, which we have shown can induce binding between SHB and the CRKL-SH2 domain [44]. SHB has been implicated in wide array of functions, and its loss leads to notable vascular and immune defects in mice [43]. In cell culture, SHB alters differentiation in PC12 neuron-like cell lines, and
induces apoptosis in 3T3 fibroblast cells [61, 77]. It is widely expressed and seems to have numerous functions.

Relatively little is known about SHD, SHE, and SHF. From the structure of SHD, we have some proposed models for how SHD might interact with CRKL, and this might inform how the other family members behave to some extent as well (Fig. 2.11). One possible consequence of an SHD interaction with CRKL is the amplification of CRKL signaling, where SHD could aggregate CRKL molecules through its multiple YxxP sites. This binding could also, however, be attenuating in nature, and by binding CRKL prevent it from localizing to CRKL-SH2 domain substrates. A third and intriguing possibility is that CRKL would then be translocated to targets of the SHD-SH2 domain, thus specifically altering CRKL signaling. These possible interactions are not necessarily mutually exclusive, and so our investigation into the YxxP sites will be necessary to resolve the potential complexity of these interactions. We have designed some experiments to begin understanding the consequences of an SHD interaction with the CRKL-SH2 domain, which are outlined in the next section.
Figure 2.9: Protein schematic of human SHB, SHD, SHE, and SHF. Schematics were generated using the IBS protein illustrator. YxxP sites are indicated above the protein, an asterisk indicates tyrosine in YxxP motifs on which phosphorylation has been detected as reported at phosphosite.org. Known domains are indicated: SH2 domains (blue) and PTB domain (green). Predicted domains are also indicated: proline-rich regions (red) and serine rich regions (yellow).
Figure 2.10: Alignment of human and murine SH family proteins. Alignments were created using the Genius program with NCBI verified or predicted sequences. YxxP sites are boxed and highlighted.
Figure 2.11: Hypothetical model for SHD molecular interaction with CRKL-SH2 domain. The major domains within these proteins are indicated: SH2 domain (blue), SH3 domain (green), Proline rich regions (red), YxxP sites (yellow diamonds) and phosphorylation (yellow circles). The negative regulatory YxxP site on CRKL is also shown. The most likely outcomes of the SHD/CRKL interaction are signal amplification, attenuation, or alteration.
Future Directions:

The function of SHD is currently unknown. At its discovery, it was overexpressed in several cell lines and no obvious phenotypes were observed [93]. This could be explained by the requirement of binding to other proteins such as CRKL, which may only occur under specific conditions. Future investigation into SHD should therefore take such considerations into account.

A query of the proteomics database phosphosite (phosphosite.org) shows that phosphorylation has previously been detected on YxxP sites at Y144 and Y181 [48]. We would like to assess the ability of a mutant SHD with Y/F mutations at both of these sites to bind CRKL-SH2 when co-transfected with Abl kinase as a continuation of the experiments already described in this chapter. Understanding the sites involved in the interaction between CRKL and SHD will inform future research into the role of SHD in cells. Specifically, as we identify how these sites become phosphorylated either by Abl or other kinases, it will be possible to better understand where and when the interaction is induced in the cell, and what the outcome of the subsequent interaction with CRKL might be. We would also like to follow up with our observation that the Y1F SHD is still able to bind CRKL-SH2 by performing an α-Flag IP to purify Y1F SHD and WT SHD from cells co-expressing Abl kinase and blot for phosphotyrosine. We would expect to see a reduction in phosphotyrosine signal corresponding to the reduced number of phosphorylated tyrosine residues. This would support that the reduced binding is due to loss of phosphorylated YxxP motifs available to become phosphorylated. We would also like to repeat the MS experiment and enrich our pool of phospho-SHD by using the
GST-CRKL-SH2 bead pulldown rather than the IP technique used in the experiment shown. This may enable detection of additional phosphorylation sites and direct future experiments with mutant SHD constructs bearing Y/F mutations at sites identified in MS experiments to assess functionality of these sites in binding the CRKL-SH2 domain, with the goal being to understand the relative contribution of each YxxP site to the binding of SHD to the CRKL-SH2 domain.

The binding of SHD to the CRKL-SH2 domain in these experiments suggest that SHD may play an important regulatory role. The nature of this interaction is not currently known, however we have some hypotheses which will guide future investigation. Based on the structure of SHD, we can think of three major mechanisms by which it could regulate CRKL cellular biochemistry. This interaction could amplify, attenuate, or alter signaling of CRKL (Fig. 2.11). The outcome of these interactions may be difficult to distinguish, but we have some experiments in mind to address this. The first question that will need to be answered is if the interaction of SHD with CRKL will have any measureable effect in the cell. To effectively investigate SHD in cell culture, there are some challenges which must be addressed.

The first major challenge for investigation of SHD in cell culture will be to ensure that cellular conditions are amenable for SHD to become phosphorylated and bind the CRKL-SH2 domain. Since we have identified Abl as a major inducer of SHD phosphorylation leading to an interaction with CRKL, this could serve as an initial mechanism of inducing the CRKL-SH2 interaction with SHD. These cells, however, would likely not provide conditions under which SHD could bind CRKL. While it would
be possible to transiently transfect Abl into cells to stimulate phosphorylation of SHD, the percentage of cells co-expression both the kinase and SHD would be likely be low, and would complicate analysis. A stable transfection might provide a better model system, but overexpression of Abl typically needs to be high to overcome cell regulatory elements and result in a noticeable increase in phosphorylation of Abl substrates [97]. Alternatively, this problem might be overcome by use of a mutant Abl, such as the BCR-Abl found in Chronic Myelogenous Leukemia (CML) derived cell lines. BCR-Abl has relatively high kinase activity in cells, primarily due to its lack of the N-terminal regulatory region where the translocation has caused fusion to BCR, and so the chimeric BCR-Abl would not be subject to many of the regulatory elements that normally inhibit c-Abl [90]. Importantly, BCR-Abl has been shown to phosphorylate SHD in cell culture previously [92]. A second challenge will be ensuring that CRK and/or CRKL are present and how to best measure their actions in the cell. Some of the most crucial experiments for understanding the cellular function of CRK and CRKL have been cell motility assays. However CRK and CRKL have other measurable effects on cells, including cell proliferation. In many cancers, CRK and CRKL contribute to rapid proliferation, as well as other factors that increase severity [35, 78, 126].

Taking these challenges into consideration, an effective experiment to understand the effect of SHD on CRK/CRKL signaling might be to start by asking how SHD affects CRK/CRKL-induced proliferation of a CML-like cell line. This can be demonstrated in Ba/F3 cells, which are interleukin-3 (IL-3) dependent. The Ba/F3 cell line also expresses CRKL, and stable transfection of BCR-Abl into Ba/F3 by retroviral
infection transforms these cells to be IL-3 independent [23, 112, 113]. The proliferation of the Ba/F3 BCR-Abl cells could be quantified and compared to cells overexpressing SHD. This could be accomplished by transfecting SHD, either transiently or stably. A stable transfection might be preferable, as it would then be possible to ensure the entire cell population expressed SHD, simplifying analysis. Since Ba/F3 cells are grown in suspension, the readout would be simple and proliferation could be measured by flow cytometry or using a hemocytometer after fixed numbers cells were allowed to grow for an allotted time. If SHD reduces CRK/CRKL signaling, we would expect to see reduced proliferation in the Ba/F3 BCR-Abl cells overexpressing SHD. If the cells show increased proliferation this would suggest signal amplification. No change in proliferation might require a different experimental approach to investigate, but could occur especially if a significant amount of CRK/CRKL is active in the nucleus and is not available to be bound by SHD, as has been reported in some cases where BCR-Abl is overexpressed [100]. Another possible readout would be dependence on growth factors. Although we would have to first determine if the IL-3 independence observed in the Ba/F3 cells requires CRK/CRKL, this could serve as another readout for CRK/CRKL signaling, and could be determined using RNAi directed against CRK and CRKL. If this prevented Ba/F3 BCR-Abl cells from becoming IL-3 independent, it would suggest we might be able to use SHD in a similar experiment. If SHD attenuates CRK/CRKL signaling, the cells might lose the ability to grow without IL-3.

If proliferation experiments prove problematic or inconclusive, another possible line of investigation could examine cell migration. For example, the monkey kidney-
derived cell line COS-7 have minimal migration behavior on collagen, but when
transfected with CRK or CRKL this migration increases 4-fold or more [67]. These
migration experiments can be performed and measured in a variety of ways. Possibly the
most widely used and comprehensive assay is the Boyden chamber [15]. This consists of
two chambers separated by a filter containing pores through which cell must migrate.
The most commonly used version measures haptotaxis, where cells migrate up a gradient
of chemoattractant in a direction form of migration dependent on adhesion to a surface
such as the ECM, rather than chemotaxis where a cell freely migrates up gradients in a
fluid. Haptotaxis describes the migration of cells which crawl along surfaces in processes
such as wound healing, axonal outgrowth, and morphogenesis to name a few. An
experiment to examine COS-7 cells would measure therefore measure haptotaxis, as
these cells require a surface on which to grow. It is unlikely that SHD would become
phosphorylated under normal COS-7 cell conditions, and so BCR-Abl could again be
stably expressed in COS-7 cells to facilitate the interaction between SHD and the CRKL-
SH2 domain. These COS-7 BCR-Abl cells would then have to be evaluated for baseline
migration behavior. Experiments could then be performed to test the effect of SHD on
CRK/CRKL signaling. In this system, we could compare COS-7 BCR-Abl cells to cells
overexpressing SHD, overexpressing CRKL, or cells co-expressing SHD and CRKL. As
was previously reported, we would expect overexpression of CRKL, but not SHD, to
increase migration of COS-7 cells. It seems likely that co-expression of SHD with
CRKL would either not result in little or no increase in migration above COS-7 BCR-Abl
cells, and would be significantly less than cells expressing CRKL. Assuming we see the
expected result, the Y5F SHD construct which cannot bind to CRKL-SH2 should not induce a significant change in proliferation compared to the cells expressing CRKL alone.

The experiments outlined here might provide insight into the nature of the molecular interaction between SHD and the CRKL-SH2 domain which will be vital to understanding the role of SHD in the cell and ultimately in animals. This will also begin to address the question of if SHD might have other roles outside of its interaction with CRKL. If one or both of these experiments provide reproducible quality results, we could use them to investigate additional questions. For example, if Ba/F3 BCR-Abl cells co-expressing SHD and CRKL are less proliferative than cell expressing only CRKL, would a SHD construct where the SHD-SH2 domain is deleted cause the same effect? It would be interesting if loss of the SHD-SH2 domain changed its actions, as this would suggest that it has functional binding partners in our cell culture model. We will have many questions to address as we better understand SHD and its interaction with CRKL.

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CHAPTER 3: THE ROLE OF SHDB IN ZEBRAFISH EYE DEVELOPMENT

3.1. Introduction

While we have begun to characterize the protein-protein interaction between CRK-Like (CRKL) and Src Homology 2 Domain-containing protein D (SHD), the biological function of SHD is unknown. To investigate SHD function in animals, we utilized the model organism Danio rerio, the Zebrafish. The zebrafish was chosen for several factors. Importantly, crk and crkl are known to be expressed in the zebrafish and presumably have a similar function as in other vertebrates, as demonstrated by their interaction with the zebrafish Dedicator of cytokinesis 1 (Dock1, formerly Dock180) in myoblast fusion [89]. Use of the zebrafish was also advantageous for our investigation as the embryos develop quickly and externally, allowing for access to the embryo during development for manipulation. Two orthologs of SHD exist in the zebrafish, Shda and Shdb. An alignment shows that Shdb has greater homology to human SHD, and contains four of the five YxxP sites found in SHD, compared to three in Shda. We therefore chose to initially characterize Shdb.

We first wished to know where shdb was expressed in the zebrafish, and so performed in situ hybridization. Expression was observed in the developing nervous system, the head, and eyes. This is roughly consistent with what is known about shd expression in mice and humans, where SHD is reported to be restricted to the nervous system [93]. The expression in and around the eye was of particular interest, as the eye develops in a stereotyped progression which is similar to other vertebrates including humans. Recently CRK and CRKL have been implicated in aspects of eye
development downstream of FGF signaling [19]. Specifically, the FGF dependent elongation of fiber cells in the developing lens of the eye requires either Crk or Crkl, and conditional loss of both results in severe defects in elongation, while their differentiation was unaffected [19]. Since we observed expression of shdb in the lens, it is possible that Shdb functions in lens fiber elongation as well.

We then sought to characterize how loss of shdb might affect the development of the fish. Embryos were injected at the single cell stage with a morpholino (MO) to target shdb pre-mRNA. These morphants displayed several defects, including cardiac edemas, hindbrain swelling, and small eyes. RT-PCR shows that the dose of MO used in these experiments knocks down shdb significantly relative to a control gene ef1a. Given the severity of the defects observed, this suggests that Shdb is important for development of specific nervous tissues. The role of this protein in these tissues is uncertain, and future research will aim to understand what this function might be.
3.2. Materials and Methods

**Zebrafish Husbandry**

All experiments were performed with approval of the University of Vermont Institutional Animal Care and use Committee (Protocol #17-031). Zebrafish were maintained and bred under standard conditions as described previously [9].

**Fish Lines and Transgenic Animals**

The zebrafish lines used in this work were the TL wild-type fish and the Spectrum of Fates (SoFa) fish, the latter of which was a generous gift from Bill Harris (Cambridge University). The SoFa fish combine three transgenic lines harboring gapRFP driven under Atoh7, cytGFP under Ptf1a, and gapCFP under Crx [1]. This results in a transgenic animal where all 6 major neurons of the retina are differentially labeled in a window of time useful for developmental research.

**PCR, RNA Probes, and in situ Hybridization**

The *shdb* probe was generated from zebrafish cDNA using a forward primer harboring a SP6 site and reverse primer harboring a T7 site as described previously and underlined in the sequences below [103]. These primers were designed to bind to *shdb* ~500 bp apart to allow generation of a RNA probe for *in situ* hybridization. The primer sequences are as follows:

FSP6: 5’ – TTTAGGTGACACTATAAGGGGGGTGGTTCCATCGCTCCAGT – 3’

RT7: 5’ – TAATACGACTCATATAGGGGAGACTCTCTCTCCCCAATCATGGAA – 3’
RNA probes were made from 100 ng of cDNA template combined with 1X transcription buffer (Promega), 10 μL of 20 mM dithiothreitol, 1X RNA labeling mix (Roche), 2 mg/mL RNA inhibitor (Invitrogen), and 2 U of DNAse (Ambion) and incubating at 37 °C for 15 minutes. The RNA was then precipitated overnight at -20 °C after the addition of 104.5 μL of 10 mM EDTA and 100 mM LiCl in 100% EtOH. RNA was spun down in an Eppendorf centrifuge (14,000 x g; 4 °C; 30 minutes), washed with 70% EtOH and centrifuged for an additional 15 minutes at the same speed, before being air dried and resuspended in 20 μL of nuclease free water. 80 μL of hybridization buffer (50% formamide, 5X saline-sodium citrate (SSC), 5 mg/mL tRNA, 0.1% Tween-20) was added and the probe was stored at -20 °C.

Embryos for in situ hybridization were collected and the chorion was removed manually. Dechorionated embryos were fixed in 4% paraformaldehyde (PFA) in egg water at 4 °C for 24 hours. The fixed embryos were then dehydrated in 100% methanol (MeOH) at 4 °C for 24 hours, and were stored in 100% MeOH at -20 °C until used. To perform in situ hybridization, embryos were rehydrated with serial washes of PBT/MeOH increasing in the ratio of PBT to MeOH in increments of 25% until in 100% PBT. The embryos were then permeabilized with 10 μg/mL proteinase K (Promega Corporation) for 20 minutes for 36 and 48 hpf embryos, or 25 minutes for embryos older than 48 hpf. Embryos were then re-fixed in 4% PFA in PBT for 15 minutes and then washed 3 times with PBT for 5 minutes and then incubated at 70 °C for 12-24 hours with the shdb probe diluted 1:100 in hybridization buffer. Following hybridization with the probe, the embryos were then equilibrated in 15 minute serial washes of hybridization buffer/2X.
SSC at 70 °C, followed by two 30 minute washes at 0.2% SSC. Embryos were rehydrated with 10 minute serial washes of 0.2% SSC/PBT into 100% PBT. Embryos were incubated with α-digoxigenin-AP (Roche Diagnostics) diluted 1:5,000 in in situ block (Phosphate-buffered saline (PBS), 5% normal sheep serum (NSS), 1% BSA) at 4 °C for 12-24 hours. The embryos were then washed with PBT and stained with 1mM nitro-blue tetrazolium/5-bromo-4-chloro-3’-indolyphosphate (NBT/BCIP) (Thermo Scientific). Staining was stopped by the addition of 4% PFA for 12-24 hours at 4 °C. Embryos were washed with PBT and stored at 4 °C until sectioned or imaged.

**Morpholino Design and Injection**

Splice inhibiting morpholino (MO) was purchased from Gene Tools (Philomath, OR, USA). The shdb MO (sequence AGAAAGGCAGCAGAAGTGCCTGT) was designed to bind across the e3i3 boundary of SHD (Fig. 3.4). Zebrafish embryos were injected at the single cell stage with 5 ng of MO as described previously [29]. Injected and uninjected control (UIC) embryos were incubated in egg water at 28.5 °C and staged according to (Kimmel et al.,1995) [64].

**Tissue Preparation and Resin Sectioning**

Embryos were reared to the desired time point and manually dechorionated, then fixed at 4 °C in 4% paraformaldehyde for 12-24 hours. Embryos were then dehydrated in 100% ethanol for a minimum of 2 hours followed by embedding using a JB-4 embedding kit (Polysciences, Inc.) as per the manufacturer’s instructions. Embryos were manually
manipulated during embedding under a Nikon SMZ800 dissecting scope so that subsequent sectioning would result in transverse slices. Embryos were sectioned on a Leica RM2265 microtome at a thickness of 7 μm. Embryos stained by \textit{in situ} were prepared using the same protocol and were cut to 20 μm thickness to allow visualization of the NBT/BCIP staining. Sections were collected in order onto slides and stored at room temperature until imaging.

\textbf{Imaging and Measurements}

All bright field whole mount images were taken using a Nikon SMZ800 dissecting scope with SPOT Insight camera operating on SPOT software (Diagnostic Instruments Inc.). Higher magnification bright field images were taken on an Olympus IX71 fluorescent microscope using the same camera and software. Image contrast and brightness was adjusted using Adobe Photoshop (Adobe systems Inc.). Measurements were made using the SPOT software calibrated to the correct magnification.

\textbf{Graphs and Statistical Analysis}

All measurements made in SPOT were exported to GraphPad Prism (V 6.0) for the generation of figures. Comparisons were made using an unpaired Student’s t-test assuming equal variance. Error bars represent the standard error of the mean. The cutoff for statistical significance was P<0.05. In the figures presented here, all P values are marked above the groups compared (all values ****: P<0.0001).
Alignments and Phylogenetic Tree:

Sequences for phylogenetic trees and alignments were obtained from the NCBI protein database. The Genius program was used to generate alignments and subsequent phylogenetic trees [62].
3.3. Results

To understand the biological function of SHD, we first asked in what organisms it can be found. We created a phylogenetic tree using NCBI predicted or verified protein sequences for Shd in 19 representative species. Shd appears to be specific to vertebrates. A duplication event has produced two genes, *shda* and *shdb*, in bony fish (Fig. 3.1). This is unsurprising as a large scale duplication in the lineage leading to zebrafish has resulted in the zebrafish bearing duplicate genes for ~20% of its genome [38]. Interestingly, the Shda found in zebrafish and in Japanese rice fish (Medaka) are more similar to each other than to Shdb of the same species, and could indicate a divergence of function between Shda and Shdb. The avian sequences were automatically assigned to the out-group, and no unexplained clusters were observed in the tree.

We then aligned human SHD with the homologs of several important vertebrate model organisms. These were mouse and frog Shd, and zebrafish Shda and Shdb. Mouse Shb shares 81.1% homology with human SHD and shares all five YxxP sites. The *Xenopus* Shd and zebrafish Shda are less similar to human, and contain only three YxxP sites. Shdb, is slightly more similar to human SHD with 62.3% sequence homology, and contains four of the five YxxP sites. This includes the site analogous to human SHD Y144, which we have shown participates in CRKL-SH2 binding. This site is not conserved in Shda, potentially making Shdb of greater interest for understanding SHD function as it relates to complexes involving CRK and CRKL.
Figure 3.1: Phylogenetic Tree of SHD in representative vertebrates. The tree was generated using Genius software with NCBI-retrieved -verified or -predicted protein sequences. Sequences were assembled using the Jukes-Cantor genetic distance model with neighbor-joining and resampled using the bootstrap method with100 replicates.
Figure 3.2: Alignment SHD homologs from human, mouse, frog, and zebrafish. The alignment was generated using Genius software (Blossum62 cost matrix; gap open penalty = 12, gap extension = 3) with NCBI-verified protein sequences. YxxP motifs have been boxed and highlighted. Conserved amino acids are highlighted with black, partially conserved (present in >50% of sequences) amino acids are highlighted in grey.
To identify where shdb mRNA is expressed in the fish, we designed primers to generate a ~500 bp RNA probe for in situ hybridization (Fig. 3.3 and 3.4). Hybridization with the digoxygenin labeled probe and staining using an α-DIG secondary conjugated to HRP allowed for visualization by staining with NBT/BCIP. The staining observed showed a variable expression pattern. Imaging of 24, 36, 48, 60, and 72 hour post fertilization (hpf) embryos stained by in situ hybridization showed above background expression in several areas of the head, eye, and developing nervous system (Fig. 3.4). At 24 hpf, expression is observed in the Floorplate and the eye, primarily in the lens (Fig. 2.4a). The expression in the eye decreases and is nearly absent by 48 hpf. By 60 hpf, shdb is detected in several areas of the brain including the midbrain-hindbrain boundary, and is also seen in the fin buds. We also see expression likely in the cerebellum in whole mount embryos starting at 48 hpf.

Sectioning of embryos revealed more detailed expression in the eye and brain. At 24 hpf, shdb is strongly expressed in the developing lens and in the eminentia thalimi. This expression is also observed at 36 hpf, but is absent by 48 hpf. Some expression is also detected in the developing tectum at early time points, just dorsal to the eminentia thalimi (Fig. 3.4 B’’). At 48 hpf, expression is detected in the cerebellum, and was detected at all subsequent time points (Fig. 3.4: C’’, D’, E’).
Figure 3.3: In situ hybridization of zebrafish embryo *shdb* mRNA expression during development. Lateral (A-E), dorsal (A’-E’), and 18 μM thick cortical sections (A’’-E’’) of zebrafish embryos stained with a digoxigenin-labeled antisense probe against *shdb* at the indicted time points. Credit goes in part to Ashley Waldron for her assistance making the *shdb* in situ probe. Areas of high expression have been annotated: Floorplate (Fp), Eminentia thalimi (EmT), Lens (Ls), Cerebellum (Ce), Finbuds (Fb), and Midbrain-hindbrain boundary (Mhb).
Zebrasfish *shdb* morphant phenotypes

We next sought to determine the effect that knockdown of *shdb* might have on the zebrafish during development. Development is a critical period that requires precise coordination both within and between cells, and disruption of cell processes by knockdown can result in quantifiable phenotypes that provide clues about the functional mechanisms of the targeted gene transcript. We observed demonstrable *shdb* expression in several areas of the nervous system, and so expected knockdown of *shdb* to present with defects in at least some of these areas.

To knockdown *shdb*, we designed a morpholino (MO) to bind across the e3i3 boundary of *shdb* pre-mRNA and disrupt splicing to form *shdb* mRNA lacking E3 (Fig. 3.4). The aberrant product introduces a stop codon in exon 5, producing a truncated 201-amino acid protein missense after exon 2. Injection of the MO into zebrafish embryos at the single cell stage resulted in a dose-dependent phenotype. The morphant phenotype included a reduction in eye size with a small or sometimes absent lens, swelling in the hindbrain, heart edemas, and defects in the body axis and a slight reduction in body size (Fig. 3.5).

The reduced eye size was somewhat surprising given that expression was not observed to any great extent after about 48 hours post fertilization. We quantified this small eye phenotype at a dose of 5 ng per embryo, as this produced fish that exhibited obvious phenotypes with a low mortality rate similar to un-injected control (UIC) animals. To assess the effect of *shdb* knockdown specifically on the eye without bias due to smaller body size, we measured eye diameter and adjusted these values for the body
length of the fish. The morphant fish had significantly smaller eyes at all time points examined, as determined by measure of eye size adjusted to body length (24 hpf, p = 3.17 x 10^{-5}; 48 hpf, p = 1.48 x 10^{-7}; 72 hpf p = 3.37 x 10^{-9}) (Fig. 3.5b).
Figure 3.4: Schematic of the shdb MO design and RT-PCR verification of shdb knockdown. (A) Schematic of the shdb MO design. The MO was designed to bind across the e3i3 boundary of shdb pre-mRNA, resulting in deletion of exon 3 during splicing and a mature mRNA lacking e3. The resulting protein is a truncated 201 amino acid protein missense after exon 2. (B) Agarose gel image of RT-PCR products. Template cDNA was made from RNA extracted from pooled uninjected control (UIC) or morpholino injected (MO) embryos at 72hpf. (C) Densitometry of bands normalized to the ef1α gene performed in Photoshop (Adobe).
Figure 3.5: Knockdown of shdb using morpholino results in reduced eye size relative to body length. Zebrafish embryos were injected with 5 ng of shdb MO at the single cell stage and allowed to develop for 72 hours. Images were taken of morphant and un-injected control animals at 24, 48, and 72 hour time points for comparison (A). (B) The size of eyes, measured by diameter adjusted for body length, is less in MO injected than in control animals were significant at all time points as determined by unpaired student’s t-test assuming equal variance (**** = P < 0.0001). Credit goes to Alica Ebert for performing the MO injections.
3.4. Discussion and Future Directions

Conclusions and Discussion

Studying *Danio rerio* Shdb is likely to be informative for understanding SHD function due to its similarity to the human protein. Our findings suggest that the four YxxP sites contained in Shdb will be sufficient to bind Crkl in the zebrafish. The outcome of this interaction is unknown, and little is known about Crk or Crkl function in the zebrafish. No currently published studies have explored how loss of Crk/Crkl affects the zebrafish. As the interaction between SHD and CRKL is expected to be critical for the biological function of SHD, the lack of understanding of Crkl in the zebrafish may complicate resolution of these mechanisms. Our collaborators in the Ebert lab are currently working to characterize *crk* and *crkl* mutant fish generated by Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR), and once published this data will inform future studies into both CRK/CRKL and Shdb.

The data presented here suggest that Shdb plays a role in the development of the eye and nervous system. Despite the reduced eye size observed in Shdb morphants, the *in situ* hybridization did not detect any expression in the neural retina, although the 24 hpf section reveals expression in and around the lens (Fig. 3.3). There are many factors which could affect the size of the eye without directly acting on the neurons within. While an argument could be made that Shdb is perhaps involved in cell proliferation or death, the question remains as to why the eye is specifically affected. Another consideration is if the mechanism of action for Shdb in the formation of the zebrafish eye is dependent or independent of Crk and Crkl, which is addressed in the next section.
The Small Eye Phenotype

It is interesting that we see such a dramatic phenotype in the eye, considering the expression observed in the in situ hybridization where shdb is only found in the eye at early time points, and is largely absent by 48 hours post fertilization. Despite this, the small eye phenotype persists and is still highly significant at 72 hpf. We will continue to investigate how this might occur and there are several processes important for eye development that might be affected.

The Ebert lab has found that defects in vascularization of the eye can result in reduced eye size [130]. However, the fact that 24 hpf shdb morphant fish already have significantly smaller eyes might preclude this possibility, as blood vessels do not begin to innervate the eye until about 48 hpf, and will not attach to the lens until 60 hpf [2]. By this point, most shdb staining is absent in the eye. Interestingly, a related family member, she, was reported to be significantly upregulated in a microarray screen using overexpression of Etsrp/Etv2 to induce genes involved in vascularization [129]. It remains an unlikely but also unresolved possible mechanism to explain the observed phenotype in shdb knockdown.

A more likely mechanism could be through disruption of lens formation. We observed that the lens in fish injected with MO appears to be reduced or even absent in many morphants (Fig. 3.4). A recent investigation into fibroblast growth factor (FGF) signaling in the eye found that Crk and Crkl are required downstream of FGF for elongation of lens fibers in mice [19]. We have also observed expression of shdb in and
around the lens at 24 and 36 hpf. The lens placode is mostly enveloped by the optic cup by 24 hpf, and begins to become visible under light microscopy around this time [22]. By 36 hpf, the lens has separated from the surface ectoderm and is completely enveloped. At this point, the cells within the lens begin to differentiate into lens fiber cells [22]. A notable difference between zebrafish lens development and other vertebrates is that no hollow vesicle is formed in the lens during its development. As development continues, the lens fibers elongate to expand the lens, and produce crytallin proteins. The fiber cells undergo apoptosis as crystalline proteins build up, leaving a clear, crystallin filled lens capable of focusing light onto the retina [22]. If Shdb is indeed expressed in the lens as our in situ hybridization results suggest, it could be involved in Crk/Crkl signaling which is necessary for fiber cell elongation. While it is possible that shdb is involved in another process such as differentiation of lens fibers, the lack of effect of loss of Crk/Crkl in mice on fiber cell differentiation make this unlikely [19]. This could be investigated by examining the developing lens using immunohistochemistry to look for crystalline, as if cells failed to differentiate they would fail to produce crytalline. Fiber cell elongation could be quantified using H&E staining of sections from wildtype and morphant fish over a similar time course of 24-72 hpf.

It is possible that other mechanisms might also be involved in the small eye phenotype of shdb morphants. This phenotype is commonly seen in knockdowns which target components necessary for migration of optic vesicles, which detach from the forebrain and migrate bilaterally out to form the eyes [38, 81]. One example is PlexinA2, a semaphorin receptor classically involved in repulsive signaling during axonal guidance.
which is also expressed in the eye fields early in development. Knockdown of PlexinA2 in zebrafish by morpholino results in a reduced eye size and was determined to occur primarily through loss of cohesion during migration of the eye fields early in development [27, 29]. Although we do not have data to support a role of Shdb in such early development, it is notable that the 24 hpf time point shows the greatest degree of expression in the eyes. It is likely that if earlier time points were examined, we might find shdb expression in early optic vesicles. Since CRK and CRKL are known to play a role in neural development in neuronal migration in humans and mice, it also is plausible that Crk and Crkl might have a function in migration of the eye fields, although to our knowledge, this has not been investigated. In mice, loss of Crk also appears to cause reduced eye size, although this was not quantified [42].

**Other shdb Morphant Phenotypes**

While the eye phenotype was initially quantified because it was so apparent, we have observed other defects in shdb morphants including hindbrain swelling and heart edemas. The edemas are interesting as CRK and CRKL are known to have important function in cardiac tissue, and heart edemas are also observed in mice null for either CRK or CRKL [88, 94]. It is important to note that edemas are commonly seen in MO which exert off target effects, and so future experiments will need to address this to confirm if the edemas observed are indeed due to shdb knockdown. The hindbrain swelling observed in morphant animals is interesting given that highly specific expression was observed in the *in situ* hybridization in the cerebellum and possibly other hindbrain
structures. Future experiments will aim to investigate this as an important potential function of Shdb in zebrafish.

**Future Directions**

There are several follow up experiments that would help to both clarify the data presented and address unanswered questions. We have shown that our *shdb* MO successfully knocks down *shdb* mRNA using RT-PCR (Fig. 3.4), however there are several control experiments that should be performed. Knockdown using MOs is notorious for off target effects, and proper controls are important to verify that untargeted mRNAs are not being affected [6, 28]. Splice-inhibiting MOs such as the *shdb* MO used in these experiments tend to have fewer off target effects as they have more unique sequence compared to ATG-targeting (start codon) MOs [6]. Despite this, it will still be necessary to repeat these knockdown experiments with a second MO to validate the specificity of the knockdown. A more immediate experiment to address the specificity of the MO will be rescue experiments. We expect that injection of human or murine *shd* mRNA will rescue the eye size phenotype observed in *shdb* knockdown. This will both help verify the effect of the *shdb* MO, and allow us to identify off target effects, as any effects not rescued by reintroduction of Shdb are likely to be off target effects. Importantly, if this rescue is successful, we can then attempt to rescue with the SHD Y5F construct. We have shown that this construct is unable to bind the CRKL-SH2 domain. Therefore, if SHD Y5F is able to rescue *shdb* morphants, this would suggest that the function of Shdb in zebrafish eye development is independent of Crk/Crkl. It seems
likely the Y5F construct will be unable to rescue shdb knockdown and that the interaction with Crk/Crkl is critical for Shdb function. Determination of the shdb morphant small eye phenotype as dependent or independent of Crk/Crkl will be essential for designing future experiments.

To continue investigation of how loss of Shdb affects the zebrafish, we will examine the histology of morphant animals. H&E stained sections from control and MO injected animals will be compared to better understand the effects of shdb knockdown. This should provide information on specific regions of the eye and nervous system affected by knockdown, and will help resolve the question of why eyes are smaller in morphants since cell layers that are affected should be possible to identify in the highly stereotypical structure that is the eye. We are interested to see if any of the areas we have observed expression are affected in morphant animals. While we quantified the small eye phenotype, we would also like to see if the cerebellum appears normal in these fish. The observed hindbrain swelling and shdb expression observed in in situ suggests that the cerebellum might have clearly observable defects in H&E stained sections. Since the cerebellum is critical for motor functions, observation of swimming behavior could be assessed in shdb morphant or mutant zebrafish. A simple experiment could record the startle response of these fish, as when disturbed they should swim away, and could look for abnormal swimming behavior or loss of coordination.

Further characterization of the shdb small eye phenotype will still be important regardless of the rescue experiment results in regards to Shdb function dependence or independence on Crk/Crkl binding. Two major factors in determining eye size are cell
proliferation and cell death [38, 75, 81]. A decrease in proliferation or increase in cell death will result in smaller eyes. It will therefore be critical to evaluate these properties in the eyes of control and morphant animals to determine if these factors are involved, particularly at early stages when shdb expression is observed most in the eye.

Proliferation can be assessed by immunostaining with antibodies for phospho-histone 3 (H3) or for Ki-67. Cell death can be detected by the popular Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNNEL) assay or an antibody against cleaved caspase-3, a terminal caspase important for both intrinsic and extrinsic cell death [39, 55].

Finally, it will be important to examine time points earlier in development as the 24 hpf in situ embryo data display strong expression in a number of areas in the eye and nervous system at this time, and morphant fish already have significantly smaller eyes. It would be interesting to see how early shdb expression can be detected. With regards to examining the effect on eyes before 24 hpf, an effective way to investigate such early periods of development is through the use of transgenic animals. The Rx3 transgenic zebrafish labels precursor cells of the forebrain and early eye fields with GFP, and was used in the previously mentioned study into PlexinA2 loss in eye development [27]. This allows visualization and determination of the early eye fields when it is challenging to use light microscopy.

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CHAPTER 4: RELATING MOLECULAR INTERACTIONS OF SHD TO BIOLOGICAL FUNCTION

4.1. SHD Functions and Interactions

A critical component for understanding the biology of SHD will be to determine how its phosphorylation by Abl and subsequent interaction with the CRKL-SH2 domain relates to its function. While we have not yet addressed this in our experiments, it is also possible that SHD has functions entirely independent of CRK and CRKL. Our findings suggest that one mechanism for SHD signaling is likely through an Abl-SHD-CRK/CRKL axis. Here we discuss what we have discovered about SHD function and how it might be explained at least in part either by function of this Abl-SHD-CRK/CRKL axis or by SHD functions independent of Abl and CRK/CRKL.

Lessons from Knockdown of Shdb

The zebrafish revealed two tissues which appear to be specifically affected by loss of shdb. This is the eye, which was quantified, and the observed hindbrain swelling which has not been investigated in detail. Since shdb expression was observed in the cerebellum, it seems likely that this is the major hindbrain structure affected. The fundamental processes which underlie both eye development and cerebellar development might therefore provide insight into the function of Shdb. This includes at least three processes including cell proliferation, apoptosis, and migration.
Cell proliferation is a critical component of nearly all developmental processes. At the stages of development we investigated, edemas such as we observed in the hindbrain can result from a hypoproliferation of normal tissue, causing fluid to accumulate. Similarly, a proliferation defect would account for reduced eye size. The opposite process, cell death, would also account for this underlying lack of tissue. Both of these could be investigated using immunohistochemistry (IHC) to probe for markers of proliferation such as phospho-Histone H3 (pHH3) or Ki67, or assays for cell death such as the cleaved caspase-3 antibody or the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNNEL) staining assay. Migration defects could also account for these phenotypes, as cells that failed to reach their targets would lead to loss of tissue in those areas. Because the eye and cerebellum are both highly stereotypically-layered structures and so it might be possible to identify gross migratory defects in H&E stained sections.

The Abl-SHD-CRK/CRKL Axis

While we have little data available to inform how SHD might function independent of CRK/CRKL, the experiments presented here suggest that SHD might have a role regulating CRK/CRKL signaling downstream of Abl kinase.

Abl and the Abl-related gene (Arg) make up the Abl family of non-receptor tyrosine kinases [70]. Like many other non-receptor tyrosine kinases, Abl and Arg contain modular protein domains to facilitate binding to other proteins, in addition to their enzymatic kinase domains. Abl and Arg contain an SH2 domain, an SH3 domain,
and several proline rich regions to bind SH3 domains of other proteins including CRK and CRKL. Abl is known to phosphorylate CRK at Y221 which negatively regulates subsequent CRK-SH2 binding [33]. The Abl family kinases were described to be unique from other non-receptor tyrosine kinases in that they contain both F-actin and G-actin binding domains near the C-terminus, which are not found in other tyrosine kinases [125].

In zebrafish, Abl is expressed throughout the developing animals and is particularly apparent in the nervous system [118]. Crk and Crkl are also observed throughout the developing nervous system [119]. The in situ hybridization we performed for shdb shows that expression is observed in the lens of the eye before 48 hpf, and is expressed in the cerebellum starting around 36-48 hpf. It seems therefore that all the components of the Abl-SHD-CRK/CRKL axis are present in the tissues most affected by loss of shdb, suggesting that the interaction of SHD with the CRKL-SH2 domain might be necessary for many, if not all, of its functions. We have also discussed the need for rescue experiments using the Y5F SHD construct, which lacks the YxxP sites critical for binding the CRKL-SH2 domain to address specificity of the morpholino and to allow for determination of phenotypes which require that SHD interacts with CRK/CRKL.

4.2. Future Directions and Conclusions

The developmental processes most likely affected by loss of shdb which might explain both the small eye phenotype and the hindbrain swelling are defects in cell proliferation, apoptosis, and migration. Here we propose some experiments which will
provide valuable information for understanding if these processes are involved in defects resulting from the loss of Shdb. This will direct future research and provide a framework for understanding the biology and molecular actions of SHD.

**SHD and Cell Proliferation**

As discussed, we would like to examine shdb morphants for changes in cell proliferation using immunohistochemistry to evaluate markers such as pHH3 and Ki67. If loss of Shdb seems to have an effect on cell proliferation, the next logical step would be to attempt to determine what proliferative pathways might be involved, starting with the canonical Ras/MAPK pathway. This is particularly relevant if Abl is involved, as this pathway is required for transformation of cells by the BCR-Abl oncogene [106]. Ras is activated by its Guanine Nucleotide Exchange Factor (GEF) Sos which is typically associated with the adaptor protein Grb-2 downstream of Receptor Tyrosine Kinases (RTK). However, the CRK/CRKL-SH3 domain also binds to Sos. It is possible that if a significant portion of the signal telling a progenitor to proliferate occurred through a non-receptor tyrosine kinase, the signal could likely reach Ras through CRK or CRKL binding Sos rather than Grb-2. Overexpression of CRKL is observed in many cancers and drives cell proliferation [78, 126]. If loss of shdb results in a reduction of proliferation, this would support a model in which SHD serves to amplify CRK/CRKL signaling. This could occur by aggregation of CRK and CRKL by Shdb via the multiple YxxP sites on Shdb. This would bring multiple copies of the effectors bound to the CRK/CRKL-SH3 domain into close proximity, increasing local activity of these effectors.
potentially many fold. Therefore, a loss of Shdb might result in a decrease in mitogenic Ras signaling, leading to reduced proliferation of precursor cells and hypodevelopment of tissue, explaining the reduced size of eyes and loss of tissue in the hindbrain.

**SHD and Cell Death**

A lack of proliferation and an increase in cell death often appear very similar when examining gross histology of a morphant or mutant animal, and both can explain the absence of cells leading to smaller eye size and the tissue loss which most likely underlies the hindbrain swelling of *shdb* morphants. In addition to proliferative markers, IHC can also be used to look for markers of apoptosis such as cleaved-caspase 3, or the TUNNEL assay. We have discussed the possibility of Shdb regulation of Ras/MAPK activity through binding of CRKL bound to Sos. While this is often a proliferative signal, under some conditions it can instead promote cell death. In the classic signaling cascade, Ras activates Raf, which in turn activates ERK1/2. At this point, ERK1/2 can translocate to the nucleus to interact with activator protein (AP-1) transcription factors such as Jun and Fos, or other mitogenic transcription factors such as Myc. These transcription factors will promote cell survival and/or cell cycle progression. However, signaling downstream of Ras is much more complicated. For example, Ras also activates opposing pathways to control the fate of the cell. Downstream of Ras, MAPK-activated Rsk and PI3K activated Akt promotes cell survival primarily by phosphorylating Bad [37, 115]. At the same time, Ras also activates RASSF1 and Nore1, which heterodimerize with Mst1, a serine/threonine kinase which increases caspase-3 activity
and promotes cell death [63]. Thus the outcome of Ras signaling depends on a complex stoichiometry of molecules that promote opposing outcomes. It seems likely that if Shdb were to affect Ras signaling, an increase in cell death and a decrease in proliferation are both possible. On the other hand, if Shdb were to attenuate CRKL-Ras-dependent cell proliferation, this would lead to decreased survival signaling and increased cell death. Assuming these hypotheses are correct, it would not be surprising to see either a lack of proliferation and increased cell death contributing to the loss of tissue underlying the reduced eye size and hindbrain swelling seen in shdb morphant fish.

**SHD and Migration**

Migration is a fundamental process critical for the development of many tissues including the nervous system. For eyes to form, early during development progenitor cells must bud off the telencephalon and migrate cohesively to reach the surface where they will contact the endothelium and invaginate, forming the optic cup which will continue developing into the adult eye. Even after the eye fields have migrated to their final locations, the neurons of the retina will be born near the apical surface and must migrate to their final positions. Similarly, the cerebellum contains three distinct layers: the granucale layer, the Perkinje cell, and molecular layer. These arise due to coordinated migration of progenitor cells. It is possible that Shdb might have a role in cell migration and this would help explain the effects we see in knockdown experiments. Migration is one mechanism where it seems particularly likely that the action of Shdb occurs through CRK and CRKL signaling, as CRK and CRKL are critical components of cell migration.
in numerous contexts. It is well established that Reelin signaling is just as important for
development of the cerebellum as it is in other areas of the brain such as the cortex [101].
The function of CRK and CRKL in the eye has not been well investigated, recently it has
been demonstrated that they are required for the elongation of lens fiber cells, and
therefore development of the lens in the developing eye [19]. The fact that loss of shdb
in the zebrafish does not affect more tissues that depend on CRK and CRKL functions
might be explained by the expression pattern of shdb. Alternatively, it is possible that
shda compensates for loss of shdb in some tissues, although this remains to be
investigated. If CRK/CRKL signaling depend on Shdb either for signal amplification, or
as a regulatory element if Shdb binding results in disruption of binding to Crk/Crkl-SH2
domain substrates, migration behavior could be altered. If the disruption is sufficient to
prevent cells from reaching their destinations, they might fail to reach subsequent signals
or growth factors that would allow them to continue to differentiate and/or proliferate.
This would result in loss of tissue and explain the shdb morphant phenotype.

Closing Remarks

Many questions remain about how SHD and Shdb functions. Either through the
Abl-SHD-CRK/CRKL axis we described or through another mechanism, further research
will be necessary to understand how knockdown of Shdb has resulted in these defects.
The research herein has laid the groundwork for future investigation. As we learn more
about SHD and Shdb, we will better understand both the function of the important
molecules CRK and CRKL, and the complex processes which govern the development of animals including humans.
BIBLIOGRAPHY


