CHAPTER 1

INTRODUCTION
Chapter 1: Introduction

1. INTRODUCTION

The actin cytoskeleton is highly dynamic and active reorganization is required during various essential cellular functions like cell adhesion, migration, phagocytosis, axonal path finding, neurite extension, wound healing and apoptosis (Papakonstanti and Staurnaras, 2008). The regulation of actin assembly and disassembly is under the control of complex signaling systems that link external signals to remodeling events, which result in altered cellular activities that adapt cell shape and/or behavior to suit new environmental conditions.

Characterization of the molecular modulators that govern the formation of the different types of F-actin structure is an area of intense research. Cells must co-order signals for actin polymerization, depolymerization, bundling, severing, capping, focal adhesion turnover and actin-myosin contraction to drive processes that require cell movement. Evidence of actin playing an important role in regulating apoptosis has emerged in the last decade, placing actin remodeling as a key player at the interface between environmental sensing mechanisms and controlling cell death decision apparatus (Franklin-Tong and Gourlay, 2008). Actin dynamics has been shown to play a role at multiple stages of apoptosis in mammalian cells. Clustering of CD95/Fas and CD44 death receptors has been shown to require actin and this action is important to elicit a full response upon stimulation of these death receptors. Treatment of mammalian cells with drugs such as Jasplakinolide and Cytochalasin D that disrupt F-actin dynamics has been shown to affect the sensitivity of a number of different cell types to apoptotic stimuli. After the initiation of apoptosis, membrane blebbing and the formation of apoptotic bodies is facilitated by the cytoskeleton, and actin is a key component in the manipulation of the plasma membrane required for eliciting these phenotypes (Franklin-Tong and Gourlay, 2008).

Among the molecular modulators involved in regulation of cytoskeletal organization, the protein tyrosine kinase c-Abl, a mediator of apoptosis has been implicated in regulating actin dynamics by phosphorylating cytoskeletal proteins and also through its ability to bind F-actin directly (Woodring et al., 2003; Hernandez et al., 2004).
1.1. The Abl family of non-receptor tyrosine kinase

1.1.1. Members

The Abl family of protein tyrosine kinases has been implicated in the regulation of cell proliferation, survival, cell adhesion, and migration. c-Abl belongs to this family of non-receptor tyrosine kinases which in mammals includes two members, c-Abl (Abl1) and its parologue Arg (Abl related gene or Abl2).

c-Abl is conserved throughout metazoan evolution. Abl homologues have been identified in Drosophila and C. elegans (Goddard et al., 1986; Hoffman-Falk et al., 1983). c-Abl (Abl 1) tyrosine kinase was originally identified as the cellular homolog of the v-Abl oncogene product of Abelson murine leukemia virus (A-MULV) (Goff et al., 1980; Wang et al., 1984). Subsequently, c-Abl was shown to be involved in human leukemias as a result of chromosomal translocation events that fuse the Bcr and c-Abl genes, producing Bcr–Abl chimeric oncogenes (Groffen et al., 1984; Melo, 1996). The Abl oncoproteins BCR-Abl and v-Abl alter normal cell proliferation and survival pathways, and induce aberrant cell adhesion and migration (Pendergast, 2002). A great deal of knowledge has been obtained regarding the signaling pathways employed by the oncogenic Abl tyrosine kinases to transform cells. In contrast, the biological roles of the c-Abl and Arg kinases are less understood, and their downstream targets remain poorly defined.

1.1.2. Domains and Structure

c-Abl is a multifunctional molecule acting through multiple domains. It consists of an SH3 domain at the N-terminus (which binds peptides with the PXXP motif), an SH2 domain (which binds peptides containing the Y(p)XXP motif) and a tyrosine kinase domain (Pendergast, 2002). The C-terminal tail contains proline-rich sequences (that function as binding sites for the SH3 domains of adapter proteins such as Crk, Grb2, and Nck), nuclear localization and nuclear export motifs, a DNA binding domain as well as G- and F-actin binding domains (Fig.1.1).
Figure 1.1: Schematic showing the domain organization of c-Abl protein. The extreme N-terminus of the c-Abl protein contains a variable region, which in some family members contains a Cap region (gray) and/or a consensus motif for N-terminal myristoylation (Myr-, in case of 1b isoform). The Src homology-3 domain (SH3, orange), Src homology-2 domain (SH2, light blue) and the catalytic domain (Kinase, red) make up the remaining N-terminal half of c-Abl. In the C-terminal half there are four PXXPXK/R sequences (pink), three nuclear localization sequences (NLS, black), one nuclear export sequence (NES, light brown). In addition, at the extreme C-terminus there is an actin-binding domain (Actin-BD), which contains a region that mediates binding to monomeric actin (G, yellow) and a consensus motif that mediates binding to filamentous actin (F, green). The regulatory Y245 and Y412 are indicated as blue triangles.
1.1.3. EXPRESSION AND SUBCELLULAR LOCALIZATION

The mammalian c-Abl protein is ubiquitous and localizes to multiple subcellular compartments, including the cytosol, nucleus, plasma membrane, endoplasmic reticulum (ER), mitochondria, actin cytoskeleton, and lipid rafts (Van Etten et al., 1989, 1994; Wetzler et al., 1993; O'Neill et al., 1997; Koleske et al., 1998; Lewis et al., 1996; Frasca et al., 2001; Westphal et al., 2000; Plattner et al., 1999; Ito et al., 2001; Zipfel et al., 2000). In fibroblasts it resides predominantly in the nucleus while in primary haematopoetic cells and neurons c-Abl is more cytoplasmic. Nuclear c-Abl has a role in transcription regulation and induction of apoptosis in response to genotoxic stress (Zhu and Wang, 2004). Cytoplasmic c-Abl regulates F-actin dependent cytoskeletal changes to affect cell adhesion, migration, pathogen infectivity, neurite outgrowth and apoptosis (Van Etten, 1999). In sharp contrast, all the transforming Abl variants are exclusively cytoplasmic. The presence of c-Abl in multiple cellular compartments suggests that the protein might move from one place to another within the cell, transducing signals in response to physiological stimuli. Alternatively, c-Abl might have distinctly different functions in different compartments.

In addition to its localization to early focal complexes (Lewis et al., 1996), c-Abl has been shown to localize to other F-actin-containing structures such as F-actin stress fibers (Van Etten et al., 1989, 1994), F-actin-containing pseudopodia and cytoplasmic protrusions at the migrating edge of polarized cells (Frasca et al., 2001), and axonal growth cones of primary cortical neurons (Zukerberg et al., 2000).

1.1.4. REGULATION

The catalytic activity of c-Abl must be very tightly controlled in the cell, owing to its potentially deleterious effects. The intrinsic kinase activity of c-Abl is strictly regulated through intra- and intermolecular interactions as well as tyrosine phosphorylation of critical residues (Smith and Mayer, 2002). A detailed illustration of regulation of c-Abl catalytic activity has been described in Fig. 1.2.

(a) Intra- and Intermolecular regulation

A major player in the intra-molecular regulation of c-Abl activity is the Abl
**Figure 1.2. Mechanism of activation of c-Abl tyrosine kinase.** (A) Abl-family kinases are held in an inactive closed conformation, in which their SH3 (3, red) and SH2 (2, blue) domains fold back on their kinase domain (green). The N-terminal cap (dotted gray line) makes additional inhibitory contacts with the SH3 and SH2 domains, and allows docking of the myristoyl group (orange) in a hydrophobic pocket in the kinase domain. (B) The Myr-Cap may be unlatched through membrane binding. (C) SH3 or SH2 ligands may unclamp the kinase domain from the SH3-SH2 regulatory domains. Proteins with SH3 domains that bind to the Abl PXXP motifs may also activate Abl. (D) Autophosphorylation of Y245 in the SH2-CAT linker and/or phosphorylation of Y412 in the activation loop by Src/Abl kinases may stabilize the active conformation. Once activated, c-Abl phosphorylates substrate proteins to regulate various F-actin-based processes, such as membrane ruffling, filopodial exploration, neurite extension and cell migration. Adapted from Bradley et al., 2009.
SH3 domain. Deletion (c-Abl ΔSH3 or ΔXB) or mutation (c-Abl P131L) of the Abl SH3 domain stimulates c-Abl kinase activity in vivo (Mayer and Baltimore, 1994). c-Abl kinase activity is regulated in vivo by intermolecular interactions with other proteins like Nck, Abi, Msp23, CrkL, CrkII, etc. resulting in its activation or inhibition. These proteins might also be substrates of c-Abl.

(b) Regulation by F-actin

c-Abl binds to F-actin through the C-terminal F-actin binding sequences which negatively regulate its activity. c-Abl protein lacking the F-actin-binding domain (∆FABD) cannot bind F-actin and shows enhanced kinase activity. F-actin inhibits the kinase activity of purified c-Abl in vitro (Woodring et al., 2001). The ∆FABD has higher kinase activity than full-length c-Abl as measured by use of immune complex kinase assays, but purified ∆FABD and c-Abl wild-type proteins have comparable catalytic activities (Woodring et al., 2001). These findings suggest that co-immunoprecipitated cellular factors other than F-actin may be responsible for modulating c-Abl kinase activity.

(c) Regulation by tyrosine phosphorylation

Full catalytic activity of the wild-type kinase requires phosphorylation of tyrosine 412 in the activation loop. Phosphorylation of Y412 of c-Abl by Src family kinases leads to increased kinase activity (Brasher and Van Etten, 2000; Dorey et al., 2001). c-Abl also undergoes auto-phosphorylation which occurs primarily at Y245 and Y412 resulting in auto-activation of the kinase.

c-Abl and Arg kinase activities are also regulated by phosphotyrosine phosphatases. Tyrosine phosphorylation of c-Abl is enhanced by treating cells with orthovanadate, an inhibitor of protein tyrosine phosphatases (Echarri and Pendergast, 2001; Dorey et al., 2001; Cong et al., 2000) suggesting that c-Abl is rapidly dephosphorylated by phosphotyrosine phosphatases in vivo. The c-Abl kinase is known to be a substrate of PEST-type PTPases (Cong et al., 2000). The finding that c-Abl tyrosine kinase activity and phosphorylation are rapidly downregulated by a closely associated PTPase may explain the difficulty in detecting significant levels of tyrosine phosphorylated, active c-Abl in cells. This mechanism for downregulating c-Abl kinase activity ensures that only low levels
of transiently activated c-Abl are present in the cells, thereby preventing the deleterious consequences of deregulated Abl kinase activity in multiple cellular signaling pathways.

**D** Regulation by ubiquitin dependent proteasome machinery

Activated c-Abl is rapidly downregulated by ubiquitin-dependent degradation (Echarri and Pendergast, 2001). Activated and tyrosine phosphorylated forms of c-Abl are more unstable than wild type and kinase inactive forms of c-Abl. Furthermore, phosphorylation of wild-type c-Abl on tyrosines 245 and 412 decreases protein stability (Echarri and Pendergast, 2001). Significantly, inhibition of the 26S proteasome leads to increased c-Abl and phospho-c-Abl levels. Tyrosine-phosphorylated c-Abl proteins are ubiquitinated *in vivo*. Inhibition of the 26S proteasome results in enhanced levels of tyrosine-phosphorylated endogenous c-Abl (Echarri and Pendergast, 2001). Downregulation of activated c-Abl by the ubiquitin-dependent proteasome machinery represents a novel mechanism for the irreversible destruction of the active c-Abl kinase *in vivo*. Thus, the reversible dephosphorylation and the irreversible degradation of active c-Abl *in vivo* effectively maintain transient levels of catalytically active c-Abl, thereby limiting Abl kinase activity available for downstream signaling.

1.1.5. Upstream activators

The catalytic activity of c-Abl is increased by multiple physiological stimuli like DNA damage, oxidative stress, entry into S-phase, integrin activation and platelet-derived growth factor stimulation (Pendergast, 2002) but the mechanism of regulation of c-Abl activity by these agents is not well understood. Various stimuli can selectively activate pools of c-Abl in different subcellular compartments. For e.g. nuclear c-Abl is activated by DNA damaging drugs while cytoplasmic c-Abl is activated upon oxidative stress and integrin activation. c-Abl kinase activity at the membrane is required for signaling from activated growth factor receptors to reorganization of the actin cytoskeleton.
1.1.6. Functions

Homozygous mutation of mouse c-Abl results in neonatal lethality, indicating its importance in development (Tybulewicz et al., 1991). Roughly 75% of abfr mice die postpartum. Some of the surviving mice develop lymphopenia, thymic atrophy and/or osteoporosis, and a lower percentage exhibit defects in eye development or spermatogenesis (Li et al., 2000; Schwartzberg et al., 1991; Tybulewicz et al., 1991). Interestingly, truncation of c-Abl by gene disruption results in similar sporadic phenotypes (Schwartzberg et al., 1991). argm mice exhibit behavioral phenotypes (Koleske et al., 1998), but unlike the abfr mice, they do not exhibit lethality. However, mice die at a much earlier developmental stage (E9-11) when both abl and arg genes are disrupted (Koleske et al., 1998). Together these data suggest functional redundancy between c-Abl and Arg during early embryonic developmental stages and non-redundant functions later in development.

1.1.6.1. Role in Cell Survival and Growth

c-Abl has antagonistic functions in cell proliferation depending on its subcellular localization. The nuclear c-Abl can induce G1-phase block of the cell cycle, whereas cytoplasmic c-Abl can promote mitogenesis (Vigneri and Wang, 2001).

(a) De-regulation in cancer:

Cytoplasmic c-Abl kinases are frequently deregulated in human leukemias, in which they drive neoplastic transformation and cancer progression (Krause and Van Etten, 2005). In chronic myeloid leukemia, deregulation is ensured by translocation of ABL1 next to the BCR gene, generating the Bcr-Abl fusion oncoprotein, which has constitutive cytoplasmic kinase activity. Recent observations indicate deregulation of c-Abl and Arg also in solid tumours. High cytoplasmic kinase activities have been detected in breast carcinomas (Srinivasan and Plattner, 2006) and non-small-cell lung cancers (Rikova et al., 2007). An increase in protein levels has been reported in breast carcinomas (Srinivasan and Plattner, 2006) and anaplastic thyroid cancers (Podtcheko et al., 2003); furthermore, Arg overexpression has been correlated with colon carcinoma progression (Chen et al., 1999). Although Abl fusion proteins have not
been detected in solid cancers, recent investigations by high-throughput genomic analyses of a large set of tumours and cancer cell lines (Greenman et al., 2007; Ruhe et al., 2007) have lead to the identification of somatic mutations and/or sequence deletions in c-abl transcripts most of which are clustered in the Cap region, the SH2 domain and the kinase domain.

(b) EMT (epithelial-to-mesenchymal transition) and cell invasion

Abl kinases play important roles in cancer cell migration and invasion by mediating EMT. Growth factors induce c-Abl activation through SFK-induced phosphorylation. c-Abl transmits EMT signals by induction of β-catenin phosphorylation and/or association with phosphorylated p68/RNA helicase. c-Abl has been shown to regulate the invasive activity of aggressive breast cancers (Srinivasan and Plattner, 2006) and some thyroid cancer cells (Rao et al., 2006).

In addition, cytoplasmic c-Abl can also have a negative effect on neoplastic cell invasion. For instance, c-Abl regulates EphB4 tumour-suppressive activity in human breast cancer (Noren et al., 2006). It has been shown that activated EphB4 utilizes an Abl–CrkII pathway to inhibit invasion (Noren et al., 2006).

1.1.6.2. ROLE IN CELL DEATH

The nuclear form of c-Abl is activated in the cellular response to genotoxic stress, which involves induction of the proapoptotic c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated protein kinase pathways. Nuclear c-Abl also contributes to DNA damage-induced apoptosis by mechanisms in part dependent on the p53 tumor suppressor and its homolog p73. In response to ionizing radiation treatment, c-Abl kinase activity is stimulated by ATM-mediated phosphorylation of Abl on Ser465 (Baskaran et al., 1997).

In the cellular response to oxidative stress, the cytoplasmic, and not the nuclear, form of c-Abl is activated. Reactive oxygen species induce cytoplasmic c-Abl activity by a mechanism dependent on protein kinase Cδ (PKCδ). c-Abl is required for reactive oxygen species-induced release of mitochondrial cytochrome c, caspase-3 activation, and apoptosis. H$_2$O$_2$-induced apoptosis is
attenuated in c-Abl-deficient cells (Sun et al., 2000a).

C-Abl is involved in signaling of apoptotic response to ER stress (Ito et al., 2001). ER stress induces mitochondrial cytochrome c release and apoptosis by a c-Abl-dependent mechanism. C-Abl is also an important component of the cell death response activated by curcumin. It mediates this response partly through activation of c-Jun N-terminal kinase (JNK).

These suggest the involvement of C-Abl in the cell death responses to genotoxic, oxidative stress and ER stress.

1.1.6.3. ROLE IN CYTOSKELETAL DYNAMICS

Abl kinases have important functions in actin cytoskeleton reorganization. Their role in morphological processes has been illustrated by the significant defect in the actin latticework of abl arg−/− mouse embryos (Koleske et al., 1998). Loss of C-Abl disrupts actin organization in epithelial cells and neural tube closure during embryonic development (Koleske et al., 1998). In tissue culture models, Abl kinases regulate F-actin organization required for lamellipodia, filopodia and neurite extension. These morphological changes mediate cell adhesion, migration and neurogenesis (Woodring et al., 2003). Upon growth factor stimulation, a fraction of active C-Abl is localized at sites of F-actin assembly where lateral ruffles are formed (Ting et al., 2001). In addition to its kinase activity, actin binding plays an important role since expression of a C-Abl truncation mutant that lacks the actin binding domain is unable to rescue phenotype of knock-out mice (Pendergast, 2002).

Through its ability to modulate F-actin dynamics, C-Abl regulates various cellular processes like decrease in cell proliferation, cell motility, cell invasion and increase in apoptosis thereby enabling cytostatic effects (Fig. 1.3A, B). Because C-Abl is involved in several different F-actin dependent processes, it is likely to collaborate with other F-actin regulators, which may include specific C-Abl substrates, to determine the dynamic biological output towards cytoskeletal effects. Multi-protein complexes containing C-Abl or its substrates may have specific subcellular localization to regulate distinct F-actin structures in various F-actin-dependent processes.
Figure 1.3: Functions of c-Abl. (A) Many functions of c-Abl involve cytoskeletal rearrangement. It regulates actin dynamics by phosphorylating cytoskeletal proteins and also through its ability to bind F-actin directly. (B) c-Abl has functions not only towards cellular transformation but also towards antagonistic pathways involved in decrease in cell proliferation, migration, invasion and increase in apoptosis, which are mediated through cytoskeletal changes. Thus c-Abl has functions in pathways leading to cancer as well as those regulating normal physiology.
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The reciprocal regulation of F-actin and c-Abl may act as a molecular rheostat for the rapid and dynamic regulation of c-Abl-dependent actin-based processes. F-actin binding domain (FABD) negatively regulates c-Abl activity and F-actin binding has been shown to inhibit c-Abl kinase activity under certain conditions (Woodring et al., 2001), but the mechanism by which it does so is currently unclear.

Characterizing the key upstream components in c-Abl cytoskeletal signaling pathways and determining precisely when, where and why the crucial c-Abl substrates are phosphorylated and how this affects the architecture of the F-actin cytoskeleton during various physiological processes form crucial platform for future investigation.

1.1.7. Substrates of c-ABL

c-Abl functions through phosphorylation of target proteins in a context dependent manner. p73, PKCδ, c-Jun, IκB, caspase-9, etc are substrates of c-Abl in apoptotic pathways and Dok-1, Crk, N-Wasp, etc are substrates that regulate signalling to actin rearrangement.

Properties of C3G, a GEF identified as a c-Abl substrate through this study are described in the following section.

1.2. C3G: A Rap1 Guanine Nucleotide Exchange Factor

Guanine nucleotide exchange factors (GEFs) regulate signalling pathways by linking cell surface receptors with intracellular GTPases that control wide variety of cellular functions. They are responsible for switching G proteins from an inactive GDP bound state to an active GTP bound form.

C3G (also known as RapGEF1) is an ubiquitously expressed GEF that targets the Ras family members Rap1, Rap2, R-Ras, and Rho family member TC-10, leading to activation of MAP kinases that play a role in cell proliferation, apoptosis and integrin-mediated signaling (Tanaka et al., 1994; Knudsen et al., 1994, Gotoh et al., 1995; Tanaka et al., 1997; Uemura and Griffin, 1999; Arai et al., 1999; Chiang et al., 2001; Ling et al., 2003; Buensuceso et al., 2000; Shivakrupa et al., 2003). C3G is involved in signaling pathways triggered by
growth factors, cytokines, G-protein-coupled receptors and adhesion receptors and, in a cell type and stimulus-dependent manner, functions as both positive or negative regulator of cell proliferation (Tanaka et al., 1997; Ling et al., 2003; Ishimaru et al., 1999; Mochizuki et al., 2000; Schmitt et al., 2002; Guerrero et al., 2004). A generalized mechanism of C3G mediated signaling has been described in Fig. 1.4.

1.2.1. DOMAINS AND STRUCTURE

The C-terminal catalytic domain of C3G is homologous to CDC25 and is responsible for target G protein activation. The N-terminal region negatively regulates C3G catalytic activity and also interacts with E-cadherin (Hogan et al., 2004). It has multiple proline-rich sequences in its central region that bind SH3 domains of Crk, Cas, c-Abl and Hck (Knudsen et al., 1994; Tanaka et al., 1994; Kirsch et al., 1998; Shivakrupa et al., 2003; Radha et al., 2007). A detailed account of different domains of C3G protein has been described in Fig. 1.5.

1.2.2. REGULATION

The catalytic activity of C3G is regulated by Crk binding and tyrosine phosphorylation at Y504 (Ichiba et al., 1999). Src family kinases, Hck and Src phosphorylate C3G and Tyr504 phosphorylated C3G localizes to the Golgi and subcortical actin cytoskeleton (Radha et al., 2004). When co-expressed in mammalian cells, interaction of Hck with C3G results in the activation of an apoptotic pathway, which is independent of the catalytic activity of C3G (Shivakrupa et al., 2003). The non-catalytic sequences of C3G have also been shown to suppress transformation induced by oncogenes (Guerrero et al., 2004).

C3G is present in the cytoplasm in a complex with members of the Crk family of small adapter molecules. In response to stimuli, this complex is recruited to the cell membrane involving association of Crk with phosphorytrosine containing proteins like receptor tyrosine kinases, p130 Cas, IRS-1 and paxillin (Ichiba et al., 1997; Kiyaokawa et al., 1997; Kirsch et al., 1998). Following translocation from cytosol to cell membrane, C3G activates downstream signaling. Its activation has been shown to lead to an activation of mitogen activated protein kinase and Jun N-terminal kinase (Nosaka et al., 1999;
Figure 1.4: Generalized mechanism of C3G mediated signalling. C3G is involved in signalling pathways triggered by growth factors, cytokines, G-protein-coupled receptors and adhesion receptors and, in a cell type and stimulus-dependent manner. It activates downstream G proteins by switching them from an inactive GDP bound state to an active GTP bound form. It targets the Ras family members Rap1, Rap2, R-Ras, and Rho family member TC-10, leading to activation of MAP kinases that play a role in cell proliferation and integrin-mediated signaling. C3G also has functions which are independent of its catalytic domain, where it behaves like an adaptor protein. Such functions include apoptosis and suppression of transformation.
Figure 1.5: Schematic showing the domain organization of C3G protein. The C-terminal catalytic domain of C3G is homologous to CDC25 and is responsible for target G protein activation. The N-terminal region negatively regulates C3G catalytic activity and also interacts with E-cadherin. It has multiple proline-rich sequences in its central region that bind SH3 domains of Crk, Cas, c-Abl and Hck. The catalytic activity of C3G is regulated by Crk binding and tyrosine phosphorylation at Y504. Src family kinases, Hck and Src phosphorylate C3G and showed that Tyr504 phosphorylated C3G localizes to the Golgi and subcortical actin cytoskeleton. Interaction of Hck with C3G when coexpressed in mammalian cells results in the activation of an apoptotic pathway, which is independent of the catalytic activity of C3G.
Studies involving overexpression of membrane targeted C3G or dominant negative forms have shown that C3G is involved in both growth suppression as well as transformation (Guerrero et al., 1998; Ishimaru et al., 1999; Schmitt et al., 2002).

Integrin-mediated cell adhesion leads to transient increase in tyrosine phosphorylation of C3G. Upon coexpression of Crkl, C3G is activated by phosphorylation of Tyr-504 (de Jong et al., 1998).

**1.2.3. INTERACTING PARTNERS:**
C3G is known to interact with a number of molecules such as: p130Cas (Kirsch et al., 1998), Crk (Knudsen et al., 1994; Tanaka et al., 1994), Crk-L (Ahmad et al., 1997), Grb2 (Tanaka et al., 1994; Smit et al., 1996), Hck (Shivakrupa et al., 2003), PDGF (Yokote et al., 1998), Cas-L (Kirsh et al., 1998), Shc (Chin et al., 1997), Rap1 (Gotoh et al., 1995), c-Abl (Radha et al., 2007), PP2A (Martin Encabo et al., 2007) and E-cadherin (Hogan et al., 2004).

**1.2.4. C3G KNOCKOUT, HYPOMORPH AND MUTATION: ROLE IN DEVELOPMENT**
C3G has been shown to play a significant role during mammalian development, be it during early embryogenesis, vascular myogenesis or cerebral cortex development, as indicated by various studies using C3G knockout or mutant hypomorphic allele.

C3G<sup>−/−</sup> homozygous mice died before embryonic day 7.5. The lethality was rescued by the expression of the human C3G transgene. Mouse embryonic fibroblasts from C3G knockout mouse embryo showed impaired cell adhesion, delayed cell spreading and accelerated cell migration. This suggested the requirement of C3G dependent activation of Rap1 for adhesion and spreading of embryonic fibroblasts and for early embryogenesis (Ohba et al., 2001).

A mouse strain carrying hypomorphic C3G allele, C3G<sup>9t</sup>, which produces less than 5% normal protein allowed survival of the C3G<sup>9t/9t</sup> mutant embryos up to embryonic day 14 (E14.5). C3G<sup>9t/9t</sup> mutant embryos die due to a blood vessel maturation defect. C3G-deficient fibroblasts responded to PDGF-BB abnormally, exhibited cell adhesion defects and lacked paxillin and integrin-β1-positive cell
adhesions. This elucidates the requirement of C3G for vascular myogenesis, cell adhesion and response to PDGF, necessary for vascular myogenesis (Voss et al., 2003).

Mice lacking C3G show overproliferation of the cortical neuroepithelium. C3G-deficient neuroepithelial cells accumulate nuclear β-catenin and fail to exit the cell cycle in vivo. C3G mutant neural precursor cells fail to activate Rap1, exhibit activation of Akt/PKB, inhibition of the β-catenin-degrading enzyme, Gsk3β and accumulation of cytosolic and nuclear β-catenin when exposed to growth factors, in vitro. This highlighted the role of C3G signalling to activate Rap1 in response to extracellular stimuli, restrict Akt/PKB activation, alleviate Gsk3β inhibition, limit cytosolic and nuclear accumulation of β-catenin, and to restrict the size of the cerebral cortex precursor population. This indicated that the size of the cortical neural precursor population is controlled by C3G-mediated inhibition of the Ras signalling pathway (Voss et al., 2006).

Mouse embryos lacking C3G exhibit a cortical neuron migration defect leading to a failure to split the preplate into marginal zone and subplate and a failure to form a cortical plate. The basement membrane is disrupted and radial glial processes are disorganised and lack attachment in C3G-deficient brains. This suggested the requirement of C3G in neuronal migration and radial glial attachment during cerebral cortex development (Voss et al., 2008).

Role of C3G in development of invertebrates is also known. During Drosophila eye and wing development, overexpression of membrane-tagged full-length DC3G phenotypically mimics overactivation of the RAS1–MAPK pathway, suggesting that DC3G is involved in MAPK activation in vivo. The effects of C3G overactivity can be suppressed by reducing the gene dose of components of the RAS–MAPK pathway and of RAP1. DC3G is likely to stimulate both RAS1 and RAP1 directly, which in turn leads to a convergent activation of the MAPK pathway (Ishimaru et al., 1999).

1.2.5. FUNCTIONS OF C3G IN VARIOUS OTHER PATHWAYS

(A) Role in suppression of transformation

Although C3G shares homology with cdc25 and sos proteins (GEFs for Ras protein), it does not function through Ras but instead employs Rap1 and R-
Ras proteins as its signaling effectors in mammalian cells (Gotoh et al., 1995, 1997). Rap1 protein was initially identified as a suppressor of Ras-mediated downstream signaling events. Rap1 is known to get activated on interacting with C3G protein (Gotoh et al., 1995; van den Berghe et al., 1997). C3G is capable of reversing the v-Ki-Ras transformation phenotype (Gotoh et al., 1995). It also shows transformation-suppression activity toward various oncogenes, which is independent of its catalytic activity (Guerrero et al., 1998). C3G suppresses Ras oncogenic transformation by a mechanism involving inhibition of ERK phosphorylation, cyclin A expression and alterations of anchorage-independent growth (Guerrero et al., 2004). This suppression mechanism is mediated by serine/threonine phosphatases of the PP2A family (Guerrero et al., 2007).

Through its ability to activate Rap1, C3G has been shown to counteract signaling through the Ras/mitogen-activated protein kinase pathway and has also been shown to transmit signals through the stress kinase c-Jun NH2-terminal kinase pathway (Mochizuki et al., 2000).

(B) Role in Cell Survival and Apoptosis
Co-expression of Hck with C3G induced a high level of apoptosis in many cell lines and this induction of apoptosis was not dependent on Tyr-504 phosphorylation or the catalytic domain of C3G but required the catalytic activity of Hck.

By negative regulation of p38α MAPK C3G plays a dual role in regulating cell death in MEFs depending on stimuli. C3G mediates cell death in response to oxidative stress, while it induces cell survival upon serum starvation. Upon serum deprivation, C3G induces survival through inhibition of p38α MAPK activity, which mediates apoptosis. Whereas, in response to oxidative stress, C3G behaves as a pro-apoptotic molecule, as its knockdown or knockout enhances survival through up-regulation of p38α activity, which plays an anti-apoptotic role under these conditions (Gutiérrez-Uzquiza et al., 2010).

(C) Role in Filopodia formation
Earlier work from our laboratory has shown that C3G plays a role in cytoskeletal reorganization and filopodia formation (Radha et al., 2007).
Knockdown of C3G protein inhibited c-Abl-induced filopodia during cell spreading on fibronectin. C3G expression induces actin cytoskeletal reorganization and promotes filopodia formation independent of its catalytic activity. It showed enrichment at filopodia tips characteristic of molecules involved in filopodia dynamics.

(D) Role in Neuronal differentiation

C3G is induced during neuronal differentiation and regulates survival and differentiation of human neuroblastoma cells (Radha et al., 2008). Human neuroblastoma cells, IMR-32 induced to differentiate by serum starvation or by treatment with nerve growth factor (NGF) or forskolin showed enhanced C3G protein levels. Transient overexpression of C3G stimulated neurite growth and also increased responsiveness to NGF and serum deprivation induced differentiation. Forskolin and NGF treatment resulted in phosphorylation of C3G at Tyr504 predominantly in the Golgi. C3G expression induced the cell cycle inhibitor p21 and C3G knockdown enhanced cell death in response to serum starvation indicating its requirement in survival signalling.

(E) Role in proliferation and oncogenesis of neuroblastoma cells

The activation of the C3G/Rap1 pathway results in neurite outgrowth of PC12 cells, which is inhibited by either overexpression of Rap1GAP or siRNA-mediated knockdown of Rap1 itself or the guanine nucleotide exchange factor C3G. This pathway appears to function in the regulation of proliferation of neuroblastoma cells such as SK-N-SH and SH-SY5Y, because abrogation of Rap1 activity by Rap1-specific siRNA or overexpression of Rap1GAP reduces cellular growth. This indicates Rap1 activity may contribute to cell proliferation and oncogenesis of neuroblastoma cells (Schonherr et al., 2010).

(F) Role in muscle integrity

C3G is an accessory component of the Drosophila musculature, essential for the proper localization of integrins at muscle-muscle and muscle-epidermis attachment sites and important for maintaining muscle integrity during larval stages (Shirinian et al., 2010).
1.2.6. **FUNCTIONS OF C3G EFFECTORS**

Rap1, the major effector of C3G activation, has been shown to regulate adhesion and motility-dependent cellular functions by controlling actin dynamics (Bos, 2005; Caron et al., 2003). Rap1 is activated by a variety of stimuli such as growth factors, adhesion, neurotransmitters and cytokines. Though its downstream effectors are not very well understood, Rap1 can activate other GTPases leading to cytoskeletal reorganization (Sato et al., 2006). Rap1 plays a key role in formation of cadherin-based cell-cell junctions. Inhibition of Rap1 generates immature adherens junctions, whereas activation of Rap1 tightens cell-cell junctions. The diverse functions of Rap1 underscore the fact that the activation and action of Rap1 are regulated by complex factors that are cell-type specific.

TC10, another substrate of C3G induces actin-rich cellular processes. TC10 generated actin-filament-containing peripheral processes when exogenously expressed in fibroblasts and induced neurite outgrowth in PC12 and N1E-115 cells (Abe et al., 2003). The activation of TC10 is essential for insulin-stimulated glucose uptake and GLUT4 translocation. The TC10 pathway functions in parallel with PI(3)K to stimulate fully GLUT4 translocation in response to insulin (Chiang et al., 2001).

1.3. **Background and objectives of the current study**

The c-Abl tyrosine kinase maintains tissue homeostasis through its ability to regulate apoptosis and actin dynamics. Quite in contrast to the hyperactive oncogenic BCR-Abl whose activity is deregulated, c-Abl activity is stringently regulated and mechanisms involved in regulation are not fully understood. Also, c-Abl phosphorylates various substrates in a context dependent manner. Therefore, it becomes essential investigating the mechanisms of regulation of c-Abl activity as well as identifying novel substrates in specific physiological conditions.

Recent investigations in our lab have shown that C3G plays a role in cytoskeletal reorganization and filopodia formation. It was found that C3G is required for c-Abl induced filopodia formation in response to integrin stimulation, indicating the association of these two molecules in a common pathway towards...
actin cytoskeletal remodelling. In the current study we explored the possibility of interaction between these two molecules and characterized the consequence of the interaction. We elucidated the possibility of C3G being a substrate of c-Abl tyrosine kinase and also characterized the physiological conditions for phosphorylation of C3G by c-Abl.

The main objectives of this work have been:

1) To determine whether C3G is an interacting partner and substrate of c-Abl kinase.
2) To analyse the mechanisms of regulation of c-Abl activity towards phosphorylation of C3G
3) To characterize the consequence of interaction and phosphorylation of C3G by c-Abl.

Chapter 1 provides information on the properties and functions of c-Abl and C3G pertinent to the current study.

Chapter 2 provides the description and source of reagents used and a detailed description of the methodologies used in this study.

Chapter 3 describes results, which identify C3G as a substrate and effector of c-Abl tyrosine kinase. C3G interacts with c-Abl both in vivo and in vitro. Ectopic expression of c-Abl induced phosphorylation of endogenous C3G on Y504 in cells undergoing apoptosis. p-C3G showed polarized distribution due to restricted c-Abl activation in regions rich in F-actin dependent on cellular F-actin dynamics. Unlike C3G or c-Abl, p-C3G was resistant to detergent extraction. Localized C3G phosphorylation, and coincidence with cells undergoing apoptosis was dependent on F-actin binding domain of c-Abl. Oxidative stress but not cisplatin activates cellular Abl to phosphorylate C3G. Inhibition of C3G expression and function using RNAi or dominant negative approaches inhibited c-Abl mediated apoptosis. These findings identify C3G as a novel target of c-Abl activated in response to physiological stimuli such as oxidative stress and during apoptosis.